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Microbial Synthesis and characterization of silver nanoparticles using the Endophytic bacterium Bacillus cereus A novel source in the benign synthesis

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Received: 7 January 2012 Accepted: 2 February 2012 Published: 13 February 2012

Abstract

The influx of nanoparticles into the living systems especially for medical purposes has opened up a new challenge of synthesizing them in a benign fashion. Green synthesis of nanoparticles is looked upon as an alternative to the existing physical and chemical methods of syntheses as they are associated with undeniable disadvantages. This initiated the biogenic synthesis of nanoparticles by using various microorganisms and plants. In this study we report the use of endophytic bacterium Bacillus cereus isolated from the Adhatoda beddomei to synthesize the silver nanoparticles (AgNPs). The AgNPs were synthesized by reduction of silver nitrate (AgNO3) solution by the endophytic bacterium after incubation for 3 days at room temperature. The synthesis was initially observed by colour change from pale white to brown which was further confirmed by UV - Vis spectroscopy. The AgNPs were characterized using FTIR, SEM â?? EDAX and TEM. The synthesized nanoparticles were found to be spherical and uniformly distributed with the size in the range of 11-16 nm. The energy-dispersive spectroscopy of the nanoparticle dispersion confirmed the presence of elemental silver. The AgNPs were found to have reasonable antibacterial activity against a few pathogenic bacteria like Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus. Determining the minimum inhibitory concentration leading to inhibition of bacterial growth is still under way.

Index terms— Endophytic bacteria, Bacillus cereus, silver nanoparticles, UV -Vis Spectra, TEM, antibacterial activity.

he last decade had witnessed an enormous focus on nanoparticles and nanomaterials because of their unique size dependent physical and chemical properties. Their widespread uses in various fields had made their study more challenging. Nanoparticles are of great scientific interest as they bridge the gap between bulk materials and atomic or molecular structures as they deal with materials at nanoscale levels (Saifuddin 2009). Some of the physical properties exhibited by nanomaterials are due to large surface atom, large surface energy and spatial confinement and reduced imperfections.

The applications of nanoparticles are innumerable ranging from fluorescent biological labels (Bruchez 1998;Chan 1998;Wang 2002) to drug and gene delivery (Mah 2000;Panatarotto 2003), bio detection of pathogens (Edelstein 2000), detection of proteins (Nam 2003), probing of DNA structure **??**Mahtab 1995), tissue engineering **??**Ma 2003;De La 2003), tumor destruction via heating (hyperthermia) (Yoshida 1999), separation and purification of biological molecules and cells (Molday 1982), MRI contrast enhancement (Weissleder1990), phagokinetic studies (Parak 2002) makes their synthesis an important area of research.

Owing to the growing usability of nanoparticles in biological systems especially as drug delivery vehicles into the cellular world, questions concerning the development of rapid, reliable and nature friendly experimental protocols is on the rise. A wide variety of physical and chemical methods to synthesize nanoparticles are in practice but

their inherent flaws that include contamination from precursor chemicals, use of toxic solvents and generation of hazardous by-products (Thakkar 2010) that makes their use inappropriate in biological systems. These disadvantages demanded the development of nanoparticles using novel and well refined methods in experimental processes. This paved the way to explore for new benign -green? routes for synthesizing high-yielding, low cost, non-toxic and environment friendly nanoparticles. Nature by itself has offered an answer by being a store house of diverse biological species including plant and plant products, algae, fungi, yeast, bacteria and viruses that could be employed in the biosynthesis of nanoparticles. This has been earlier confirmed by various reports that advocate the production of intra-cellular or extracellular organic material by unicellular and multicellular organisms (Mann 1996).

The biosynthesis of nanoparticles emerging as an intersection between nanotechnology and biotechnology has been receiving increasing attention in the recent past. First evidence of biosynthesis was reported using Pseudomonas stulzeri (Klaus 1999) where the nanoparticles were deposited on the cell T

1 Global Journal of

2 Medical Research

membrane. This study was followed by various other reports that demonstrated the use of different microorganisms that include Bacillus licheniformis (Kalimuthu 2008), Lactobacillus strains (Nair 2002), Bacillus subtilis (Saifuddin 2009), Cornybacterium sp. (Zhang 2005), E. coli (Gurunathan 2009a;Gurunathan 2009b) in the extra and intracellular synthesis of nanoparticles. Minaeian and coworkers have reported the synthesis of silver nanoparticles in the size range 50-100 nm Klebsiella pheumoniae, Escherichia coli, Enterobacterdoacae (Minaeian 2008).

Certain investigations also revealed that formation of nanoparticles using fungi like Fusarium oxysporium and Aspergillus fumigatus (Bhainsa 2006).

While a number of reports are available on the biological synthesis of silver nanoparticles, the potential of endophytic microorganisms -microbes that colonize living internal tissues of plants without causing any immediate, overt negative effects (Bacon 2000) has not yet been tapped. Very few reports are available where in endophytic fungi were used for the synthesis of nanoparticles. One such study employed an endophytic fungus (Colletotrichum sp.) isolated from geranium leaves (Pelargonium graveolens) for the extracellular synthesis of gold nanoparticles (Shiv Shankar 2003).

Another study revealed the use of Aspergillus clavatus (AzS-275), an endophytic fungus isolated from sterilized stem tissues of Azadirachta indica and reported about the antibacterial effect of silver nanoparticles synthesised by it (Vijay C Verma 2010).

To the best of our knowledge, there were no reports on the synthesis of silver nanoparticles using endophytic bacteria. The present investigation was carried out to synthesize silver nanoparticles from endophytic bacterium that is identified as Bacillus cereus isolated from Adathoda beddomei. The potential antibacterial activity of the nanoparticles has also been evaluated.

The medicinal plant Adhatoda beddomei, under study, was obtained from Siddha Institute, Chennai, India. This is an evergreen herb used in Ayurveda where the leaves, seeds and the roots are administered for treatment of cough and asthma. Silver nitrate was obtained from SISCO Research Laboratories, India. The test organisms used for antibacterial assay were Escherichia coli ATCC 35218, Pseudomonas aeruginosa ATCC 27853 and Staphylococcus aureus ATCC 25923.

3 a) Isolation of Endophytic Bacteria

Leaf samples of Adhatoda beddomei were cleaned under running tap water to remove debris and then air dried and processed within 5 hrs of collection.

From each leaf sample, 4 segments of 1 cm length were separated and treated as replicates. Surface sterilization was carried out by submerging them in 75% ethanol for 2 min. The explants were further sterilized sequentially in 5.3% sodium hypochlorite (NaOCl) solution for 5 min and 75% ethanol for 0.5 min (Ravi Raja 2006). Samples were allowed to dry on paper towel in a laminar air flow chamber. Four segments per plant were placed horizontally on separate Petri dishes containing Nutrient Agar. After incubation at 32°C for three days, the endophytic bacteria was collected and placed onto nutrient agar and incubated for 3 days and checked for culture purity. Eventually, pure cultures were transferred to nutrient agar slant tubes and subcultured regularly.

4 b) Molecular Characterization of Endophytic Bacteria

The sequence of the 16s rRNA gene has been widely used as a phylogenetic marker to study genetic relationships between different strains of bacteria. The analysis of this gene can therefore be considered as a standard method for the identification of bacteria at the family, genus and species levels (Woese 1987;Weisburg 1991), and has infact been included in the latest edition of Bergey's Manual of Systematic Bacteriology (Garrity 2005). Genomic DNA was isolated from the pure culture pellet and the approximately 1.4 kb fragments corresponding to 16s rRNA was amplified using universal primers, high -fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward, reverse primers. This sequence was compared with the 16s rDNA sequence

data from strains available at the public databases (Genbank, EMBL and DDBJ) using BLASTN sequence match routines (Procópio 2009). The sequences are aligned using CLUSTALW2 program and phylogenetic and molecular evolutionary analysis were conducted.

5 c) Culture Conditions

The endophytic bacterial culture was maintained on nutrient agar slants by subculturing at monthly intervals. 100 mL of Luria Broth medium was prepared, sterilized and inoculated with 12 hr old cultures of the endophytic bacterium. The culture flasks were incubated for 36 hrs at 37°C with shaking at 150 rpm. After incubation period, the bacterial cell pellet was collected by centrifugation at 10,000 rpm for 10 min. This was used as the starting material for the synthesis of nanoparticles.

6 d) Synthesis of Silver Nanoparticles

After 36 hrs of incubation, the biomass is separated from the medium by centrifugation and was washed three times in sterile distilled water to remove any adhering nutrient media that might interact with the silver ions. The bacterial biomass obtained, about 1g wet weight was then resuspended into 20 mL of 1mM Silver nitrate solution and incubated for 72 -120 hrs at room temperature (Shiying 2007).

7 e) Characterization Techniques

The formation of AgNPs was followed by visual observation of color change from pale white to brown and was further confirmed by the sharp peaks given by the AgNPs in the visible region from UV -vis spectrum of the reacting solution using Perkin-Elmer Lamda-45 spectrophotometer, in a 1cm path quartz cell at a resolution of 1 nm from 250 to 800 nm. The studies on morphology, size, composition and the distribution of nanoparticles were performed by Transmission Electron Microscopic (TEM) analysis using a TEM, JEM-1200EX, JEOL Ltd., Japan, Scanning Electron Microscope (SEM) using Hitachi S-4500 SEM and energy dispersive spectroscopy (EDAX) as an attachment on SEM. The probable biomolecules involved in the synthesis and stabilization of nanoparticles was recorded by FTIR spectrum using FTIR Nicolet Avatar 660 (Nicolet, USA).

8 f) Antibacterial Screening

Though different types of nanomaterials have come up, silver nanoparticles have proved to be the most effective antimicrobial agents. Hence the potential of the synthesized silver nanoparticles was determined, using the agar well diffusion assay method (Perez 1990). The test organisms used were gram negative bacteria Escherichia coli ATCC 35218, Pseudomonas aeruginosa ATCC 27853 and gram positive bacteria Staphylococcus aureus ATCC 25923. Two replicas of respective test organisms were prepared spreading 100 ?L of revived culture on the nutrient agar plate. Wells were cut with the help of a sterilized stainless steel cork borer into which 100 ?L of AgNP solution was loaded and incubated at 37 o C. The plates were examined for evidence of zones of inhibition, which appear as a clear area around the wells. The diameter of such zones of inhibition was measured for each organism and expressed in millimeter.

The intersection of nanotechnology and biology referred to as nanobiotechnology is a recently emerging field. The applications of this merger have spread across widely, extending its arms into the biological world at a rapid pace. This technical approach to biology allows the scientists to imagine and create systems that can be used for biological research. Nanoparticles that form the crux of nanotechnology have the innate ability to penetrate into the living systems owing to their size and properties. This insists a need to develop a greener route of synthesizing them thus making the process facile and ecofriendly. Biological systems possess unique ability to be selforganized and to synthesize molecules that have highly selective properties. This opened a new possibility of using microorganisms as the nanoparticle factories. This study demonstrates the capability of endophytic bacteria to synthesize silver nanoparticles in a more environment friendly manner.

9 a) Isolation and Molecular Characterization of Endophytic Bacteria

One endophytic bacterium was isolated from surfaced sterilized leaf fragments of Adhatoda beddomei after 24 hrs of incubation and appreciable growth was noticed after 48 hrs. Basic microbiological and biochemical characteristics of the endophytic bacteria identified the organism to be gram positive Bacilli sp. This was further confirmed by 16s rDNA analysis.

16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification (Procópio 2009).

The endophytic bacterial DNA was isolated and the 16s rDNA sequence was amplified and sequenced and has been deposited in GENBANK with the accession number HM998898.1.

The 16s rDNA sequence of the endophytic bacterium obtained was compared with the non-redundant BLAST database to obtain the sequences that displayed maximum similarity.

9 A) ISOLATION AND MOLECULAR CHARACTERIZATION OF ENDOPHYTIC BACTERIA

All the sequences reported by BLAST revealed that the endophytic bacterial species showed a very high percentage of similarity (99%) with the sequence of Bacillus cereus, with a reasonably high score and e-value being zero. The sequences showing the maximum similarity were used for alignment using CLUSTAL W2 to arrive at phylogentic relationship represented by a phylogenetic tree (Fig. 1) showing the evolutionary relationship that was constructed from the alignment using the neighbor-joining algorithm. There exists a clear evolutionary relation between all the 16s rDNA sequences as this is a highly conserved sequence. The tree derived by distance based, neighbor joining method is an unrooted tree inferring that the sequences do not come from a common ancestor.

But they exhibit cladistic relationship which could be due to the similarities within the sequences. All the taxa under comparison belong to the genera Bacillus and species cereus except for a few This bioreduction of Silver nitrate ions was followed by UV-vis spectroscopy. The spectrum showed a strong surface plasmon absorption band at around 425 nm (Fig. 2B) indicating the presence of spherical or roughly spherical AgNPs that remained the same throughout the reaction period, suggesting that the particles are dispersed in the aqueous solution with no evidence for aggregation (Saifuddin 2009). Observation of this sharp clear peak, assigned to a surface plasmon, was well documented for various metal nanoparticles with sizes ranging from 2 to 100 nm (Kowshik 2003;Henglein 1993). A long tailing on the large-wavelength side may be due to small amount of particle aggregation (Minaeian 2008). The same process when repeated with culture supernatant was unable to show any color change that states that the bacterial biomass was responsible for the bioreduction of AgNO 3 .

Controls (organism and reagent) showed no change in color when incubated under the same conditions indicating the role of the bacteria in the reduction of silver (Saifuddin 2009). When tested for stability, the silver nanoparticle solution was stable for two months which is evident from UV -Vis spectra after which the particles started to show aggregation (data not shown).

The stability of the AgNPs may be conferred by the proteins that may be involved in their synthesis. This is evident from the FTIR spectrum of AgNPs (Fig. 3) which gave peaks at 3442 cm -1 corresponding to the OH stretch of carboxylic acid and the peak at 2350 cm -1 corresponding to aldehydic C-H stretching and 1641 cm -1 corresponding to N-H bending of primary amines amide I bonds of proteins that may arise due to carboxyl stretch and N-H deformation vibrations (Sathyavati 2010;Mann 1996). The proteins function as capping agents as the carbonyl group from the aminoacid residues show stronger ability to bind to metals (Sathyavati 2010). It has already been reported that the biological molecules perform dual functions of formation and stabilization of silver nanoparticles in the aqueous medium (Mallikarjuna 2011).

The morphology, size and the distribution of nanoparticles was observed through the SEM and TEM micrographs (Fig. 4A & B). The SEM micrographs recorded showed comparatively spherical of roughly spherical nanoparticles which were observed to be uniformly distributed. This was further confirmed by the representative TEM images recorded from the dropcoated film of the silver nanoparticles that exposed spherical silver nanoparticles that were distributed on the surface and were uniformly dispersed without much traces of aggregation.

The size of the silver nanoparticles ranged from 11 -16 nm. The presence of elemental silver in the biologically synthesised nanoparticle solution was confirmed by EDX analysis (Fig. 5) where strong optical absorption peaks were observed approximately at 3 keV, which is typical for the absorption of metallic silver nanocrystallites (28%) due to surface plasmon resonance (Mouxing 2006). Few weaker signals from C, O and N were also recorded which may be due to X-ray emissions from the organism (Mouxing 2006). The bactericidal activity of AgNPs was studied using the pathogenic strains of bacteria namely gram negative Escherichia coli ATCC 35218, Pseudomonas aeruginosa ATCC 27853 and gram positive bacteria Staphylococcus aureus ATCC 25923 using agar well diffusion method. After the incubation time, clear zones were observed against all the test organisms by AgNPs and were recorded in millimetres (Table 1 The efficacy of silver nanoparticles can be attributed to the fact that their larger surface area enables them a better contact with the microorganisms. This is further supported by the revelation that size dependent interaction of silver nanoparticles with bacteria leads to its antibacterial activity (Pal 2007).

The toxicity of silver ions, though not very clearly understood, could be by their adhesion to the cell membrane and further penetration inside or by interaction with phosphorus containing compounds like DNA disturbing the replication process or preferably by their attack on the respiratory chain. It has also been suggested that a strong reaction takes place between the silver ions and thiol groups of vital enzymes thus inactivating them. Some studies reported that the attachment of the nanoparticles on to the surface of the cell membrane disturbs the permeability and respiration functions of the cell. Experimental evidence advocated the loss of replication ability by the DNA when treated with silver ions (Mahendra Rai 2009). The effect of the silver nanoparticles was observed to be more in gram negative bacteria than gram positive bacteria which are attributed to the fact that the relative abundance of negative charges on gram negative bacteria facilitated the interaction between the nanoparticles and the cell wall (Siddhartha 2007).

A novel approach for the green synthesis of silver nanoparticles was carried out using the endophytic bacteria, bacillus cereus isolated from adathoda beddomei. the ability of the bacteria to reduce ag+ to ag0 was harnessed with the size of nanoparticles ranging between 11-16 nm. they were found to be spherical and uniformly distributed and extracellularly synthesised. additionally the agnps were found to have antibacterial activity against the test organisms, more profound against gram negative

1

Figure 1: Fig. 1 .

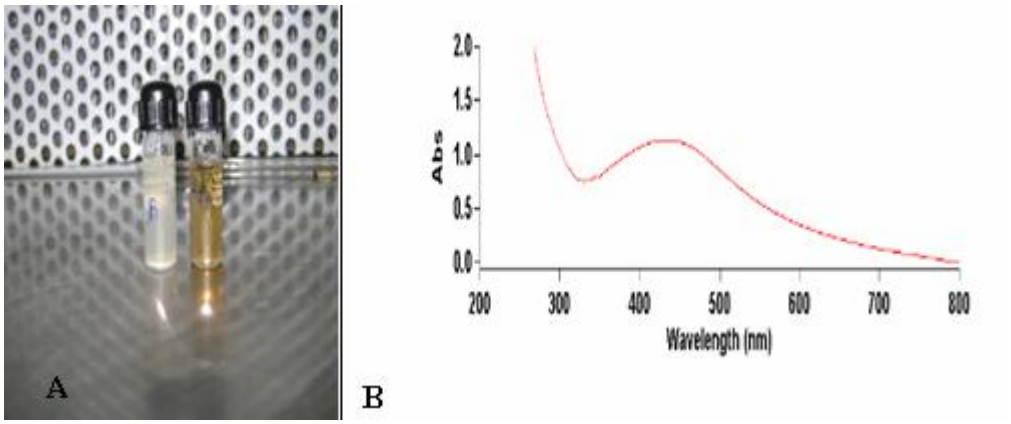

Figure 2:

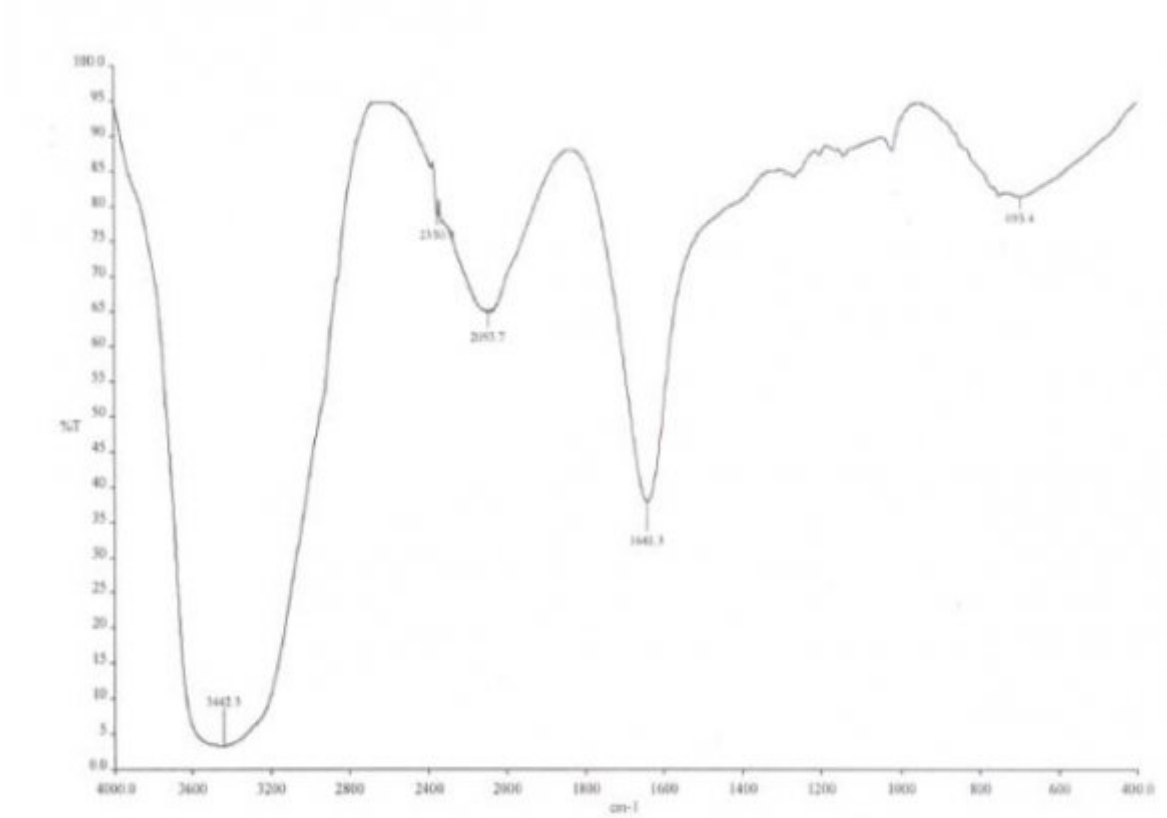

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Figure 3: Fig 2 .

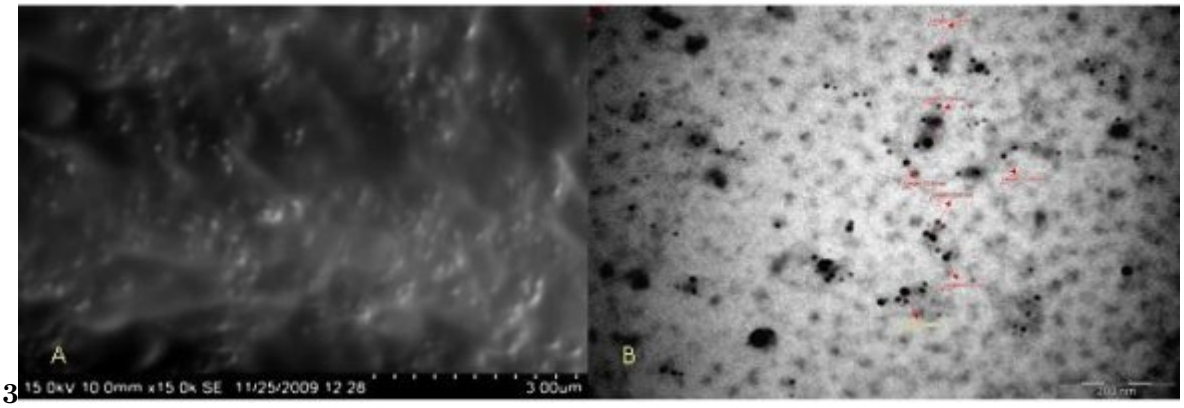

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Figure 4: Fig. 3 .

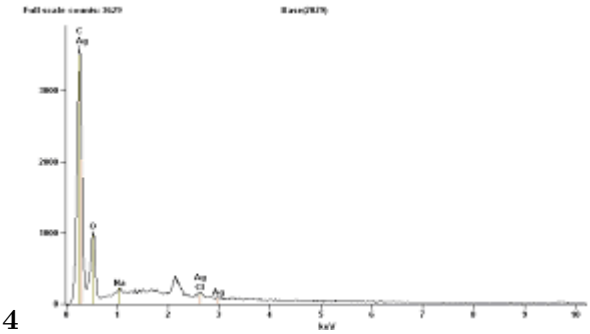

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Figure 5: Fig. 4 .

1

Test Organism	Zone of Inhibition (mm)	
	Sample	Ofloxacin
Pseudomonas aeruginosa	17	12
Escherichia coli	15	13
Staphylococcus aureus	12	15

Figure 6: Table 1 :

9 A) ISOLATION AND MOLECULAR CHARACTERIZATION OF ENDOPHYTIC BACTERIA

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