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QUORUM SENSING INHIBITING ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED BY *BACILLUS ISOLATE*

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ABSTRACT

Nanoparticle research is currently an area of intense scientific interest due to a wide variety of potential applications in biomedical, optical, and electronic fields. Ability of microorganisms to synthesize nanosilver is gaining momentum in research because of their unique properties. Present paper reports the quorum sensing inhibiting activity of silver nano particles synthesized by a bacterial isolate SAJSBVC51. Molecular characterization of the isolate revealed it as Bacillus subtilis. Nanosilver particles produced by the Bacillus species was identified by measuring absorbance at UV-Visible spectroscopy, stability by FTIR analysis and size determination by Scanning Electron Microscopy . Nitrate reductase enzyme activity of culture supernantant was determined to establish extracellular nanoparticles synthesis. We report application of silver nanoparticles as Quorum sensing inhibitor agents.

KEY WORDS

Silver nano particles (AgNP's), Quorum sensing inhibiting activity (QSI), Bacillus isolate.

INTRODUCTION

Treatment of infectious diseases is becoming increasingly more difficult as bacterial resistance to antibiotics emerging and expanding at an alarming rate. Several opportunistic pathogens rely on biofilm formation and quorum sensing (QS) for controlled expression of virulence factors in the process of establishing persistent infections in humans and animal [1,2,3].

New therapeutic options are being studied as an alternative to treatments with existing antibiotics in order to avoid biofilm formation and the emergence of resistant bacteria populations.[4,5] Nanoparticle research is currently an area of intense scientific interest due to a wide variety of potential applications in biomedical, optical, and electronic fields. These particles have the capacity to attach and penetrate into bacterial cells, disrupt the bacterial membrane, and interact with chromosomal DNA. Silver nanoparticles have also been used for impregnating medical devices due to the silver antimicrobial properties [6,7].

Present paper reports the quorum sensing inhibiting activity of silver nano particles (AgNP'S) synthesized by a bacterial isolate SAJSBVC51. Wide application of silver nanoparticles in a number of fields demands a larger requirement of nano silver which can be custom synthesized[8,9]. To overcome the increasing demand of AgNP's, exploring different potentially active microorganisms and conditioning them to form desired type of nanoparticle can lead to new discoveries. A large group of microorganisms have been reported to synthesize AgNP's under various conditions [10, 11]. The extracellular synthesis of AgNP's has a greater advantage for applications [12]. Therefore it becomes necessary to explore microbial world to obtain potential nanosilver producers at conditions convenient to synthesize, quantify and easy to extract in short period.

MATERIALS AND METHODS

Isolation of Silver nanoparticle synthesizing bacteria

Soil samples were screened for AgNP's synthesizing bacteria by serial dilution and plating on to Nutrient agar plates with 1mM $AgNO_3$. Bacterial colonies obtained on these plates were grown in nutrient broth at 37^0C for 24hrs

and centrifuged at 10,000 rpm for 15 min to obtain supernatant. Extracellular AgNP's production was screened by incubating bacterial supernatant (250µl) with the filter sterilized 25 ml of 1mM $AgNO_3$ in a 100ml conical flask[13]. Flasks were incubated in presence of light at room temperature for nanoparticle synthesis against control which had only 1mM $AgNO_3$.

Characterization of the Silver nanoparticles

Silver nanoparticles production was identified initially by a colour change. Bacterial isolate which showed colour change in minimum time of incubation was selected for further characterization. Presence of AgNP's was confirmed by scanning the test solution at a wavelength range of 300-600nm in a UV-Visible spectrophotometer (SYSTRONICS 117). The reduced silver nitrate solution was concentrated in the Rotovac for 2hrs and then subjected to FTIR analysis. Spectrum was recorded in the range of 4000-500cm^{-1} using a SHIMADZU Fourier Transform Infrared Spectrophotometer. The scanning data was obtained after 10 scans. The size and shape of the AgNP's was determined by Scanning Electron Microscopy using SEM S-3700 with accelerating voltage of 30000 Volts, emission current 82000nA and a magnification of 5000. [14]

Qualitative Detetection of the Nitrate Reductase

Reduction of $AgNO_3$ resulting in the formation of nanosilver was predicted by many researchers as Nitrate reductase enzyme dependent process [15]. To establish the activity of Nitrate reductase a qualitative test for detection of the enzyme in supernatant was performed by inoculating the culture in nitrate broth. The culture was grown in anaerobic conditions by layering the surface of the media with paraffin oil and sealing tubes with parafilm. After 48 hours of incubation, enzyme was identified by addition of Sulfanilic acid reagent and alpha Naphthalamine solution to observe the colour change.

Molecular characterization of the bacterial isolate

Preliminary microbiological characterization suggested that the isolate was *Bacillus* species. However molecular characterization was carried out by 16s rRNA typing to identify the *Bacillus* strain. The 16srRNA gene was isolated from the genomic DNA extracted from bacillus colony (Indigenous biotechnology Kit) and specifically amplified using universal primers by PCR. The amplicon was purified using Calcium-silicate based DNA binding matrices. The product was analyzed using Agarose Gel Electrophoresis.

The product of PCR amplification was bi-directionally sequenced using the forward **Bac8F** (forward) and 1492R (reverse) primers at Indigenous Biotechnology. The amplified DNA was run on 1% agarose gel and size was found to be 1.5 Kb.DNA sequencing was processed using ABI 3730Xl (96 capillary) electrophoresis and analyzed for nucleotide matching from gene bank by the nucleotide blast, where the sequence was pasted in the FASTA format

Phylogenetic analysis

Nucleotide sequence was compared to those in the Gene Bank database with Basic Local Alignment Search Tool (BLAST) algorithms .Phylogenetic analysis was performed after including consensus sequence of small ribosomal subunit sequences collected from the international nucleotide sequence library GenBank. Phylogenetic and molecular evolutionary analyses were conducted using the genetic distance based neighbour-joining algorithms (http://www.ncbi.nlm.nih.gov).

Antimicrobial activity

Antimicrobial activity of AgNP's was carried out by agar well diffusion method. [16] The purpose of test was to determine the antibacterial activity of AgNP's on clinical pathogens like *Pseudomonas aeruginosa* & *Staphylococcus aureus*. The nutrient agar plates were pre-seeded with overnight grown culture of test organisms. Wells were made in the agar plate with a sterile cork borer of 6mm in diameter. Wells were loaded with 20-100 µl of crude extract of the AgNP's and incubated overnight at 37°C. The antibacterial activity was calculated by measuring diameter of clear zone of inhibition .

QSI activity on Chromobacterium *violaceum12472*

QSI activity of AgNP's was tested by violacein inhibition assay using *Chromobacterium violaceum 12472* as bio indicator organism. The nutrient agar plates were pre-seeded with overnight grown culture of *Chromobacterium violaceum 12472*. Wells were made in agar plate with a sterile cork borer of 6mm diameter. Wells were loaded with 50µl of sample to be tested and incubated overnight at 30°C. The QSI activity was calculated by measuring the diameter of colourless halos created due to inhibition of violacein pigment but not the growth [17].

RESULTS AND DISCUSSION

During the screening process of various soil samples for AgNP synthesizing bacteria, isolate SAJSBVC 51 was observed to be promising. This isolate had the potential to reduce 1mM $AgNO_3$ (100ml) within 20mins, when 1ml of culture supernatant was added to it. The synthesis of nano silver was identified by the transformation of colourless to brown colour against control.[18] (Fig. No 1).The transformation in colour change is one of the visual identification of the nanosilver synthesis which could be due to the excitation of surface Plasmon vibrations in the silver metal nanoparticles .

Colony morphology of bacterial isolate looked opaque, raised irregular, with almost undulate margins on nutrient agar (Fig. No 2).Gram staining of the isolate revealed it as Gram positive bacilli. The formation of nanosilver is confirmed by UV-Visible spectroscopy. Maximum absorption observed in the range of 390nm - 500nm (Fig. No 3) which was an identification of presence of AgNP's . [19].

The stability of the nanoparticles was attributed to the proteins interacting with the particles in the solution. The FTIR analysis has been done in order to figure out the different protein molecules that may have interacted. Fig.No.4.depicts FTIR analysis peaks at 1361.79,1384.94,1635.69,2852.81,3423.76,3444.98 signifying the interaction of the groups C-N stretch, Aromatic groups, Primary and Secondary Amines , Aromatic stretch , Carboxylic acids respectively. These interactions of proteins result in the reduction of nanosilver. It has been already reported that the biological molecules are responsible for formation and stabilization of AgNP's in the aqueous medium. [20, 23, 24]

In order to characterize the nanoparticles further, SEM images were captured and the morphological features of the particles were determined. The SEM analysis revealed presence of spherical shaped AgNP's of different sizes ranging from 46.3nm to 137nm (Fig. No.5).

Biosynthesis of AgNP's by the microorganisms was generally attributed to nitrate reductase enzyme activity. In order to figure out the role of this enzyme in AgNP's production, qualitative test was performed with the culture supernatant. This analysis revealed the presence of the enzyme as the test showed the positive reddish pink colour against the colourless control. Interestingly the biosynthesis of AgNP's was observed to be fast when culture supernatant was used rather than cell pellet confirming the role of extracellular nitrate reductase in AgNP's synthesis.

After characterizing the AgNP' s produced by the isolate, it was also essential to characterize the isolate at molecular level before planning for further exploitation. Although microbiological and biochemical characters indicate that the isolate belongs to *Bacillus* genus, molecular characterization of the isolate was carried out by 16srRNA typing.

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. The 16srRNA gene was isolated from the genomic DNA extracted from bacillus colony (Indigenous biotechnology Kit) and specifically amplified by PCR using universal primers. The amplicon was purified using Calcium-silicate based DNA binding matrices. The amplified DNA was run on 1% agarose gel and the size was found to be 1.5 Kb. (Fig. No.6). PCR amplified product was then bi-directionally sequenced using the forward **Bac8F** (forward) and **1492R** (reverse) primers at Indigenous Biotechnology . DNA sequencing was processed using ABI 3730Xl (96 capillary) electrophoresis and analyzed for nucleotide matching from Gen bank by the nucleotide blast, where the sequence was pasted in the FASTA format. Phylogenetic analysis was performed by matching with the consensus sequence of small ribosomal subunit sequences collected from the international nucleotide sequence library GenBank. Phylogenetic and molecular evolutionary analyses were conducted using the genetic distance based neighbour-joining algorithms (http://www.ncbi.nlm.nih.gov).

From (Fig. No.8)., a clear evolutionary relation between all the 16S rDNA sequences exists as this represents a highly conserved sequence. All the taxa under comparison belong to the genera *Bacillus* except for a few sequences whose species has not yet been identified. The molecular analysis of the 16srRNA gene revealed that the isolate belongs to the genus *Bacillus.* The sequence analysis in the NCBI BLAST showed 97% identity to *Bacillus subtilis* and 96% identity to *Bacillus licheniformis*. The 16srRNA sequence is

deposited at Gen Bank with accession number KU174501.Thus the present isolate was identified to be ***Bacillus subtilis*** as it share 97% similarity (Fig. No 8).

Fig. No 1: Biosynthesis of AgNP's by the culture supernatant of isolate

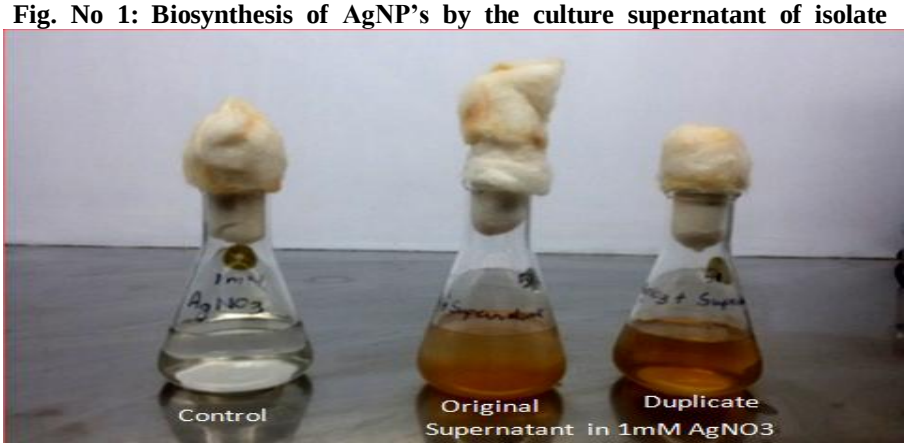


Fig: No 2. Colony morphology of isolate on Nutrient agar plate

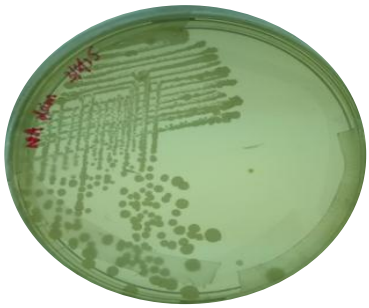


Fig: No.3. UV –Visible spectrophotometric absorbance of Silver Nanoparticles

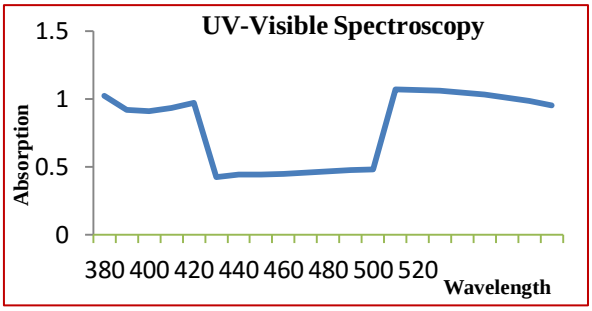


Fig: No. 4. FTIR analysis of Silver nanoparticles

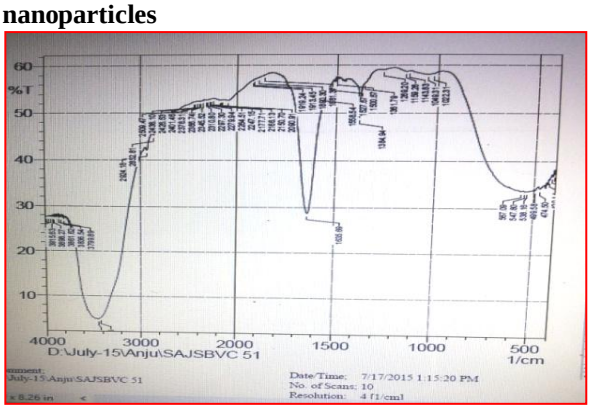


Fig: No. 5. SEM pictures of Silver nano particles

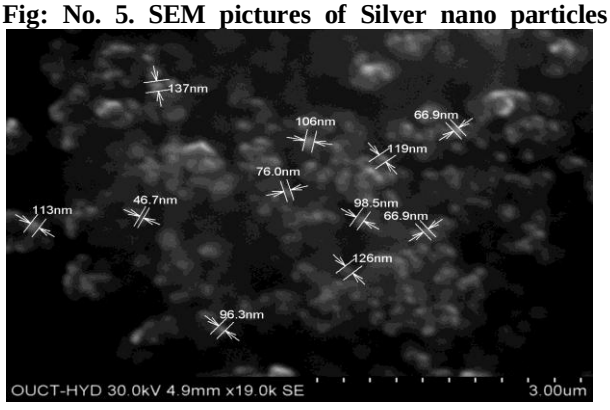


Fig:No.6 Gel picture depicting 16srRNA amplicon

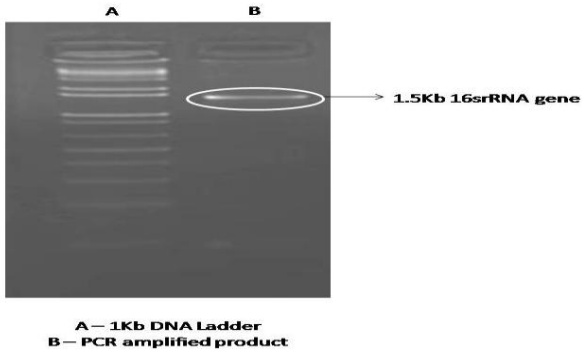
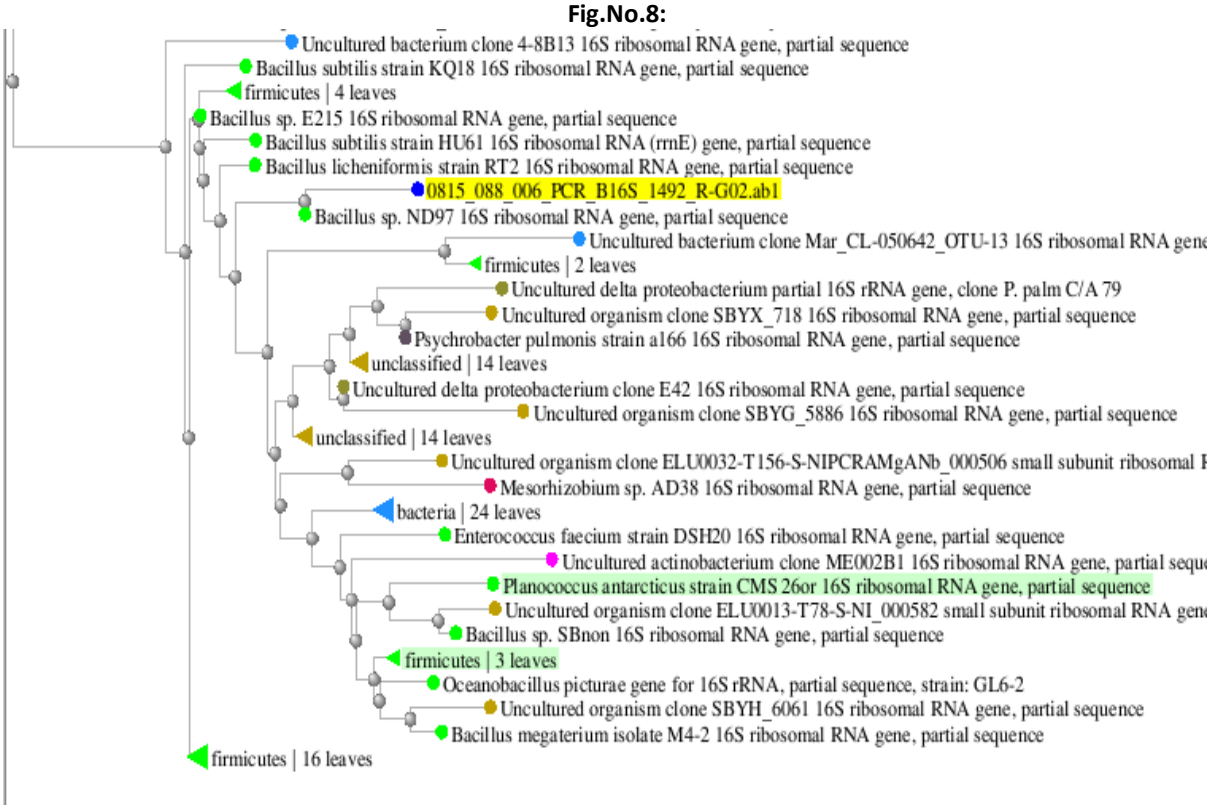


Fig No.7: Quorum sensing inhibitory activity

Fig.No.8:



AgNP's have proved to be the most effective antimicrobial agents compared to all the other types of nanomaterials. Hence, antibacterial activity of the biologically synthesized AgNPs was determined against the pathogenic strains using the agar well diffusion assay method. The test organisms used were *Pseudomonas aeruginosa* and *Staphylococcus aureus* which were known to be clinical pathogens of great concern. These strains were observed to be resistant to antibiotics i.e pencillin, chloramphenicol and tetracyclins when tested in the laboratory. In order to evaluate the antibacterial activity of AgNP's produced by the isolate , different volumes (20-100µl) of concentrated AgNP's were tested. Although both the bacterial pathogens were observed to be inhibited from Table 1, AgNP's were potent on *Pseudomonas* than *Staphylococcus.* The zone of inhibitions produced were observed to increase with the increase in volume AgNP's .Present results indicate that AgNP's could be effectively used to treat

antibiotic resistant strains of *Pseudomonas & Staphylococcus*.

TableNo1. Antimicrobial activity of AgNP's on Drug resistant Bacterial pathogens

	Diameter of inhibited zones in cm				
Test Culture	**100µl**	**80µl**	**60µl**	**40µl**	**20µl**
Staphylococcus aureus	1.9	1.4	1.2	1.0	-
Pseudomonas aerugenosa	2.2	2.2	1.9	1.7	1.5

Development of multidrug resistant pathogenic bacterial strains has become more prevalent. Novel therapeutic approaches to treat drug resistant bacterial infections are gaining momentum [21,26]. The discovery of quorum sensing systems (QS), which coordinate virulence factors during the infection process, has provided a novel target to fight bacterial infection. Compounds which are capable of interfering bacterial signalling processes known as quorum quenchers were discovered in the recent years [21]. Disruption of quorum sensing (Quorum Quenching) attenuates the pathogenicity without imposing resistance in bacteria. Recently AgNP's have also been used for impregnating medical devices due to the silver antimicrobial properties [22]. Experiments were carried out to study whether AgNP's have quorum sensing inhibiting activity by testing with standard bio indicator strain *Chromobacterium violaceium.* This bio indicator strain produces violacein pigment which is regulated by quorum sensing mechanism. Any compound or agent which can inhibit the production of violacein is reported to be a quorum sensing inhibitor, hence plate based violacein inhibition assay is thought to be the standard bioassay for screening quorum sensing activity. AgNP's tested against the bio indicator exhibited potent violacein inhibition (Fig. No.7) which proves the concept that they also have QSI activity. The inhibition zones exhibited an interesting pattern-clear zone of violacein inhibition around the sample followed by a turbid halo zone where indicator organism is not inhibited but depigmented. These results indicate that AgNP's has both bactericidal and quorum sensing activity.

The efficacy of silver nanoparticles can be attributed to the fact that their larger surface area enables them a better contact with the microorganisms. 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. Future work will be focused on understanding the role of AgNP'S in inactivating quorum sensing mechanisms.

CONCLUSION

Present paper describes the quorum sensing inhibitory activity of AgNP's produced by a bacterial isolate. AgNP's are generally known as potent bactericidal in action, however they could also exhibit QSI activity when tested with standard bio indicator *Chromobacterium violaceium.* This observation gains significance in the context of treating drug resistant bacterial infection as QSI activity of Nano silver particles could control the prevalence of multidrug resistance.

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