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Biogenic Synthesis of Silver Nanoparticles Using Phyllosphere Associated Bacterial Strain - *Pseudomonas fluorescens*

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A B S T R A C T

Biogenic synthesis of silver nanoparticles using microorganisms such as actinomycetes, fungi and bacteria has attained great interest and importance because of their potential to synthesize nanoparticles of various morphologies. This study focused on the biosynthesis of silver nanoparticles using the culture supernatant of bacterial strain BRTSI-2 isolated from the Phyllosphere. Molecular identification of the isolate BRTSI-2 by16S rDNA sequencing identified the strain as *Pseudomonas fluorescens.* The culture condition was optimized for maximizing the bacterial biomass and was found to be effective at 30 ^{o}C at neutral pH. On treating the bacterial supernatant with 1mM final concentration of $AgNO_3$ silver nanoparticles were formed, which was visually confirmed by the color change and using UV absorbance between 400-450 nm. FTIR analysis also supported the formation of silver nanoparticles from the bacterial supernatant.

Introduction

Bionanotechnology is a rapidly growing field which is an integration of biology and nanotechnology for developing ecofriendly nanoparticles. There is an increasing demand for the metal nanoparticles due to its greater availability in electronics, catalysis, textiles, degradation etc. Numerous metals such as Gold, silver, zinc, titanium, copper, iron and magnesium have been reported as nanoparticles (Schabes-Retchkiman *et al.*, 2006). Silver nanoparticle has its significant applications in biomedical engineering, bioremediation and biosensors (Singh *et al.*, 2017). Additionally, silver nanoparticles also possess antimicrobial activity against bacteria, fungi and other microbes (Gong *et al.*, 2007). Silver at nano range gained the attention of the researchers due to its antimicrobial, anti-biofilm formation, anti-cancer and anti-inflammatory ability (Singh *et al.*, 2016). Nanoparticle formation is broadly divided into two methods namely, physical and chemical methods which is considered to be a hazardous due to the release of heavy metal by products during the conversion process, it also suffers many disadvantages during reduction

of metals into ions in aqueous phase, hence to overcome such problems, researchers developed clean and nontoxic biological method for the synthesis of nanoparticles (Singh *et al.,* 2017). Among microbes, bacteria mediated synthesis of nanoparticles has received excess attention due to its availability, easy handling and successful growing rate.

Bacterial supernatant consist of reductase which mediate the reduction of metal into its ions. Several works has been carried using various culture supernatant for synthesis of silver nanoparticles. Fungal supernatant obtained from *Penicillium* sps. was used for the synthesis of silver nanoparticles (Shareef *et al.,* 2017). According to Moustafa, (2017) silver nanoparticles synthesized from fungal strains were used to remove pathogenic bacterial strains from waste water. Work done by Pugazhendhi *et al.,* (2017) proved that silver nanoparticle synthesized from algal supernatant was found to have antibacterial activity against pathogenic bacterial strains. The present study deal with the biogenic approach to synthesize silver nanoparticles from bacterial strain isolated from phyllosphere of *Psidium guajava*. The bacterial isolate was characterized by biochemical and molecular analysis. Culture conditions such as pH and temperature were optimized for maximizing bacterial biomass. The synthesized nanoparticles were characterized using UV-VIS spectrophotometry and FTIR analysis.

Materials and Methods

Sample collection

Healthy leaf samples of *Psidium guajava* were collected from the garden of Thiruvalluvar University campus, Serkkadu, Vellore, India. The leaf samples were excised from the plant using autoclaved cutter and collected in a sterile polythene bag and transported to BRT lab.

Isolation of Phyllosphere associated bacterial strains

The leaf samples of *Psidium guajava* were carefully placed over the surface of nutrient agar plate using sterile forceps and pressed gently to acquire phyllosphere microbiota. Both abaxial and adaxial surface of the leaf sample were pressed over the surface of nutrient agar plate, left undisturbed for 5 min and then removed carefully and discarded. Plates were incubated at 30 ^{o}C for 24 h (Aneja, 2003). Among various morphologically distinct colonies, strain BRTSI-2 was selected for further studies.

16 S rRNA analysis of BRTSI-2 strain

Strain BRTSI-2 was identified using conventional biochemical tests and 16 s r DNA sequencing. Genomic DNA was isolated from the pure culture of BRTSI-2. Approximately 1.5 kb rDNA fragment was amplified using high fidelity PCR polymerase. The PCR product was sequenced bi-directionally using universal primers (Forward and Reverse). The sequenced data was analyzed for its closest neighbors. The purified PCR product was directly sequenced using Big Dye Terminator version 3.1 cycle sequencing kit. The nucleotide sequence analysis was done at BLAST-n site at NCBI server www.ncbi.nlm.nih.gov/BLAST. The alignment of the sequences was done using CLUSTAL W program VI.82 at European Bioinformatics site (www.ebi.ac.uk/clustalw). The analysis of 16S rDNA gene sequence was done at Ribosomal Data Base Project (RDP) II (http://rdp.cme.msu.edu). The phylogenetic tree was constructed using the aligned sequences by the neighbour joining method using kimura-2 parameter distances in MEGA 2.1 software.

Optimization of culture conditions

100 ml of nutrient broth was inoculated with loopful culture of BRTSI-2 in different conical flasks. All the five flasks were incubated at different pH (4, 5, 6, 7, 8 and 9) and different temperature ranges (20, 25, 30, 35, 40, 45 and 50 ^{o}C) for 24 h. Following incubation, the bacterial growth was monitored in above mentioned flasks to check the optimum pH and temperature for maximizing bacterial biomass. The optimum culture condition where maximum growth was observed was maintained for further studies.

Biogenic synthesis of Ag NP' S using *Pseudomonas fluorescens*

Strain BRTSI-2 was inoculated in 100 ml nutrient broth and incubated for 24 h at 30 ^{o}C in shaker. The bacterial supernatant was collected after centrifugation at 4000 rpm for 15 mins. 1mM final concentration of filter sterilized $AgNO_3$ was mixed with equal volume of the culture supernatant and heated at 80 ^{o}C in water bath.

Biosynthesis of Ag nanoparticles was confirmed by the change in the color of culture broth. The synthesized nanoparticles were air dried and stored in sterile vial in powder form (Zaki and Husain, 2016).

Characterization of Ag NP' S by UV-VIS spectrophotometry and FTIR analysis

Synthesis of silver nanoparticles (bioreduction of Ag^{+} ions) was confirmed using UV-VIS spectrophotometry (JASCO V-730) between 350 to 450 nm. The FTIR analysis provides brief knowledge about the functional groups present in the compound and to analyze the biomolecules involved in the reduction of metal into nanoparticle. The spectrum was recorded in JASCO spectrometer in the range 400-4000cm^{-1}

Results and Discussion

Isolation and identification of Phyllosphere bacteria

Among the numerous phyllosphere associated bacterial strains, Yellow colored strain BRTSI-2 was selected for the biogenic synthesis of silver nanoparticles. 16S rDNA sequence was determined from total of 840 nucleotide base pair sequence.

The strain BRTSI-2 possessed 100 % similarity to 16S ribosomal RNA genome of *Pseudomonas fluorescens* (JF00468.1). 16S rDNA sequencing of the bacterial isolate resulted in identification of the strain as *Pseudomonas fluorescens* (Fig. 1).

Phylogenetic tree constructed was shown in Fig. 2. The 16S rDNA sequence of the bacterial isolate was submitted to GenBank with an accession number (MH412807).

Effect of pH and temperature on the biomass of BRTSI-2

Biomass of Phyllosphere associated bacterial strain BRTSI-2 gradually increased with increase in pH levels. The optimum growth was recorded at pH 7.0 after which, growth rate of *Pseudomonas fluorescens* BRTSI-2 declined gradually (Fig. 3).

The effect of temperature on bacterial biomass of *Pseudomonas fluorescens* was monitored at various temperature levels. Incubation at 30^{o}C yielded maximum bacterial biomass. However temperature ranges above or below 30^{o}C yielded comparatively reduced bacterial biomass (Fig. 4).

Biogenic synthesis of silver nanoparticle

Bacterial supernatant was used for reducing Silver nitrate from metal form to Ag^{+} ions.

Fig.1 PCR amplified 16S r RNA sequence of the isolate BRTSI-2

>Contig_C

ACACATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGA
GTAATGCCTA
GGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCT
ACGGGAGAA
AGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG
GGGTAATGG
CTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAG
ACACGGTCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCC
ATGCCGCGTG
TGTGAAGAAGGTCTTCAGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAAT
ACTCTGCAA
TTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACA
GAGGGTGCA
AGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGA
AATCCCCGG
GCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAA
TTTCCTGTGT
AGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTA
ATACTGACAC
TGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCAAC
TAGCCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG
GAGTACGGC

Fig.2 Phylogenetic tree of the strain BRTSI-2

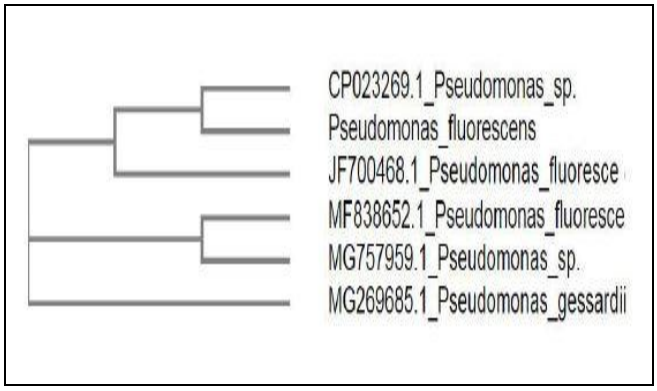


Fig.3 Influence of pH on bacterial biomass of BRTSI-2

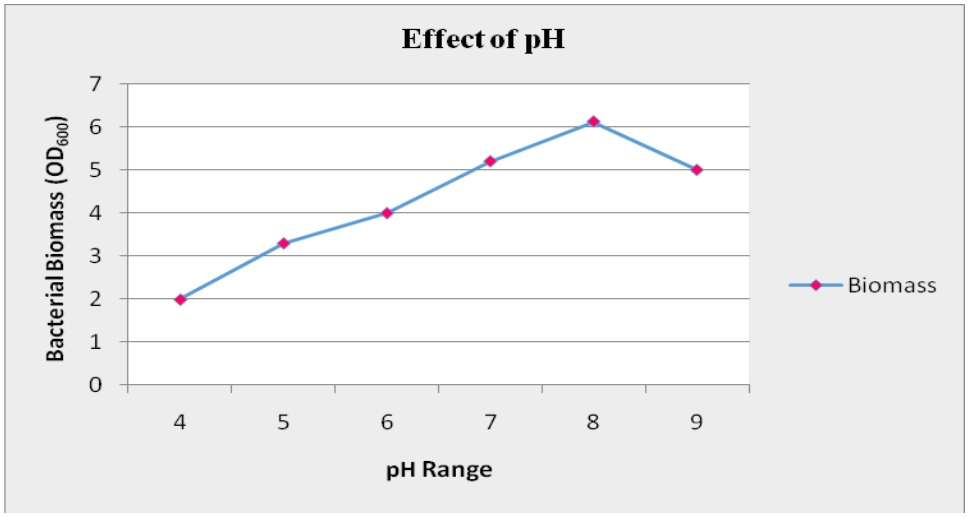


Fig.4 Effect of temperature on bacterial biomass of BRTSI-2

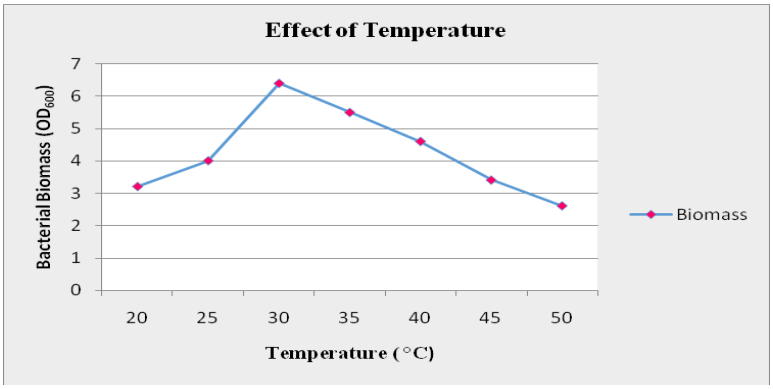


Fig.5 UV-VIS spectrophotometric analysis of synthesized Ag nanoparticles

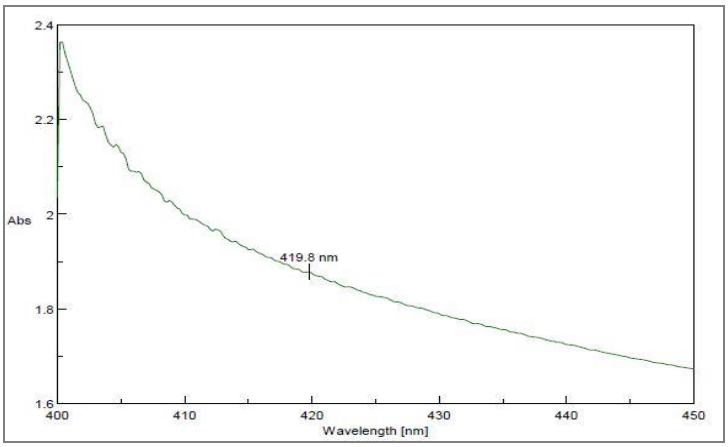
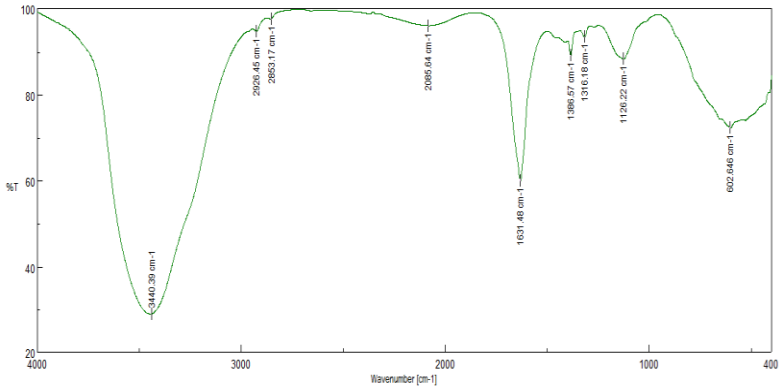


Fig.6 FTIR analysis of synthesized silver nanoparticles



Transformation of bacterial supernatant from pale white to grey color after boiling at 80^{o}C confirmed the synthesis of silver nanoparticles.

FTIR analysis of synthesized silver nanoparticles

The IR spectrum of synthesized nanoparticles explains the molecular and bio molecular

environment of the nanoparticles (Fig. 7). In the present study, IR analysis reveals the presence of carboxylic acid group at 3440 cm^{-1} (O-H stretch).Peak at 2085 cm^{-1} indicated the presence of alkynes stretch**.** The IR analysis also confirmed the presence of alkenes at 1631 cm^{-1} (C=C). Presence of alkanes and alkyls were confirmed by bond at 1386cm^{-1}. Stretch at 1386 cm^{-1} indicated the presence of alkyl halides (C-F). The above functional groups were characteristic of silver nanoparticles.

Nanobiotechnology is an emerging field of science that utilizes nanobased systems for various biotechnological and biomedical applications. Biogenic synthesis of nanoparticles has attracted scientific attention, as they have high surface area and high fraction of atoms which is responsible for their fascinating properties such as antimicrobial, magnetic, electronic and catalytic activity. The "green" route for nanoparticle synthesis proved to be a simple, cost effective, time saving and environmental friendly synthetic method gives a potential avenue for various applications. is of great interest due to eco-friendliness, economic prospects, feasibility and wide range of applications in nanomedicine, catalysis medicine, nano-optoelectronics, etc.

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