Extracellular Synthesis of Silver Nanoparticles using *Bacillus spps* soil isolates.

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Abstract: Eight different bacterial soil isolates were primarily screened for their potential for synthesizing AgNPs extracellularly, based on nitratase activity. The Culture supernatants of these isolates was successfully used for AgNP synthesis where the need for downstream processing was not required. Synthesized AgNPs were characterized via UV spectrophotometry and TEM, revealing plasmon resonance band in the range of 410- 420 nm, with spherical nanoparticles in the size range of 2 -50 nm respectively. The soil isolates were further screened secondarily based on their antibacterial activity against the test organisms *E. coli* NCTC 9022 and *S. aureus* ATCC 6538P and the better performing isolates were selected as A1, GB1, GD1 and B5 which were all identified by VITEK 2BCL to belong to different *Bacillus spps.* The synthesized AgNPs from these isolates were stable even after storage for 6 months owing to the capping effects derived from the culture supernatant. Amongst all the isolates AgNPs synthesized from CS of A1 showed maximum antibacterial activity and A1 isolate was used for further optimization of biosynthesis. Different parameters like addition of 1N NaOH to CS, Growth culture density, ratio of CS:1mM $AgNO_3$, and microwave induced biosynthesis were optimized and it was found that efficient synthesis of AgNPs using A1 CS could be achieved rapidly within 40 secs. The A1 isolate was identified by 16srRNA sequencing as *Bacillus cereus* strain BHAVANI with an Accession No. MH598411.1. Thus, this paves the path for rapid synthesis of AgNPs that has the potential for commercial exploitation using an ecofriendly, simple, cheap and highly reproducible method of biosynthesis of AgNPs.

***Index Terms - Bacillus spps*, Culture supernatant, Extracellular, AgNPs, Rapid Biosynthesis, Optimization**

I. Introduction

The increasing applications of Silver nanoparticles (AgNPs) across multiple fields like health care, cosmetics, biomedical, drug-gene delivery, food and feed, environment, electronics, chemical & space industries etc. has led to rapid evolution of different methods for AgNPs synthesis. (Ahmed S, 2016). The challenges posed by physical methods like high energy requirements, use of hazardous chemicals and formation of non-ecofriendly byproducts etc. has led to an increasing demand of methods for biosynthesis of AgNPs (Bilal M, 2017). Biosynthesis of AgNPs employs the use of biological systems like plants, bacteria, or fungi. Plant extract mediated synthesis involves the use of different plant parts like root, stem, leaves, flowers, whole fruit, fruit peels etc. and though its rapid compared to other methods, it leads to production of polydisperse AgNPs due to involvement of multiple reducing agents like flavonoids, terpenoids, and polyphenols (Ghosh *et al*. 2012; Salunkhe *et al*. 2014) and is also affected by geographical and seasonal variations (Singh *et al*. 2013).

Microbial synthesis is not susceptible to such variations but requires regular inoculum maintenance (Salunkhe et.al 2014). This synthesis of metal nanoparticles can take place either intracellularly or extracellularly (Ahmad *et al*. 2003, 2007; Jain *et al*. 2011; Kalishwaralal *et al*. 2010; Saifuddin *et al*. 2009). Intracellular synthesis of nanoparticles requires additional steps such as ultrasound treatment or reactions with suitable detergents to release the synthesized nanoparticles (Babu *et al*. 2009; Kalimuthu *et al*. 2008). At the same time extracellular biosynthesis is cheap and it requires simpler downstream processing. This favors large-scale production of silver nanoparticles to explore its potential applications. Extracellular synthesis utilizes cell mass, cell free extracts or culture supernatant and the synthesized AgNPs are harvested by centrifugation at speeds between 10000-20000 rpm followed by resuspension in desired solvent. (Singh et.al 2014). Extracellular methods of synthesis are preferred due to the ease of recovery of AgNPs after synthesis.
The current paper describes AgNPs synthesis and its optimization using culture supernatant (CS) from soil bacterial isolates obtained from different environmental conditions, screening of the most promising isolate for the optimization of biosynthesis, characterization of the synthesized AgNPs, testing of antibacterial activity of synthesized AgNPs and biochemical as well as 16S rRNA identification of selected isolates.

II. Materials and Methods

2.1 Isolation and screening of Bacterial Isolates for AgNPs synthesis.

Bacterial isolates were obtained by streaking saline suspensions of soil samples on St Nutrient agar (NA) plates. These samples were procured from locales in Mumbai including different gardens a welder's shop and a waste dumping ground. The selected isolates [on basis of predominance and frequency of occurrence] were purified and maintained on St. NA slants.

These isolates were further screened for their ability to produce nitratase as a prelude for using them for synthesis of AgNPs. The ability of microorganisms to produce and secrete enzymes like Nitrate, Sulphate and other reductases have been documented to be essential for AgNPs synthesis (Kumar. S.A *et al* 2007, Singh. H *et al* 2018, Mohammad Hassan G. S. *et al* 2014).

2.2 Synthesis of AgNPs from Bacterial CS.

The obtained individual bacterial isolates were each inoculated in Malt Extract Glucose Yeast Extract Peptone [MGYP] medium and incubated at RT for 24 hrs. Post incubation the medium was centrifuged at 6000 rpm for 20 mins. The obtained supernatant was taken as CS and the cell pellet was discarded. 1 ml of CS was challenged with 10 ml of 1 mM aqueous $AgNO_3$ solution prepared in Double distilled water (DDW) (1:10 ratio of CS: $AgNO_3$) and the tubes were incubated at RT in dark till the color of the solution was changed to brown which is indicative of AgNPs synthesis and further confirmed by UV-Visible spectrophotometry. (Systronics). For all the spectroscopic analysis the 50 µl of AgNP suspension was diluted with 2.0 ml of DDW.

2.3. Antibacterial activity by Agar cup method

Agar Cup method was used to determine the antibacterial activity of AgNPs synthesized using CS from different isolates. Test organisms (*E. coli* NCTC 9022 and *S. aureus* ATCC 6538) [$O.D._{530nm}$ 0.1] were individually seeded in the molten and cooled NA butts and cups of 8 mm in diameter were made in the plates using a St. cork borer. 100 µl of the AgNPs solution along with negative control (DDW) was added in the wells. The plates were kept for pre-diffusion of AgNPs solution into the medium at 4^0 C for 2 hrs followed by incubation at 37^0 C for 24 hrs. (Hussain.R, 2008). Post incubation the zone of inhibition for the AgNPs solution against the test isolate was measured in mm and recorded as an indicator of extent of its antibacterial activity.
The isolates giving the best activity were chosen for further optimization of AgNPs synthesis.

2.4 Characterization of synthesized AgNPs by TEM analysis

TEM analysis at 120kV and a magnification of 80000X and resolution of 10 nm was carried out for the AgNPs synthesized using the selected isolates and to determine the size, shape and distribution of the AgNPs as well. (TEM-TECNAI) The size distribution of the AgNPs was also analyzed.

2.5 Optimization of AgNP synthesis using the most potential bacterial isolate

2.5.1 Effect of addition of 1N NaOH to CS for Biosynthesis

Increasing volumes of 1N NaOH was added to fixed volume of harvested CS which was then added to the reaction mixture comprising of 1 ml of CS and 10 ml of 1 mM aqueous $AgNO_3$ solution (in the fixed ratio of 1:10 of CS:1mM $AgNO_3$) for biosynthesis of AgNPs which was confirmed by uv-spectrophotometry.

2.5.2 Optimization of Culture growth Density on Biosynthesis

The most promising bacterial culture was grown in MGYP medium taken in side arm flasks for four increasing time periods of incubation (i.e. 8,12,18,24 hrs.) on shaker at ambient temperature (28^0 C) and the O. D_{530nm} of the culture broth was measured at the end of the incubation period. Culture supernatants were prepared for each flask and used for synthesis of AgNPs in the ratio of 1:10 as above and synthesis confirmed by uv-spectrometry.

2.5.3 Effect of Ratio of Volume of CS (pH adjusted): 1mM $AgNO_3$

CS adjusted to fixed alkaline pH values by the addition of 1N NaOH was used for AgNP synthesis in varying proportions for addition to 1mM $AgNO_3$ solution to form the reaction mixture and the optimum ratio of Volume of CS (pH adjusted): 1mM $AgNO_3$ was decided on the basis of the intensity of the brown color formed after incubating the reaction mixture in the dark and determining the absorbance by UV-spectrophotometry.

2.5.4. Effect of microwave assisted induction on biosynthesis

The reaction mixture of CS: 1 mM $AgNO_3$ solution was subjected to increasing temperature employing microwave radiation in order to achieve even and rapid heating for different exposure time periods. The effect of microwave radiation on the rate of reaction and hence the time required for the process of AgNPs synthesis was evaluated.

2.6 16S rRNA sequencing of isolates

Molecular characterization of the selected strains was done by standard method of DNA isolation, DNA purification and PCR amplification of 16S rRNA gene followed by sequencing. A similarity search for the nucleotide sequence of 16S rRNA of the isolate was carried out online at http://www.ncbi.nlm.nih.gov using the BLAST search program for the nucleotide database maintained in GenBank.

III. Results and Discussions

3.1 Isolation of Bacteria and Primary screening.

Bacterial soil isolates obtained from different sites across Mumbai were purified and maintained on St. NA slants and all of them were found to be Gram positive bacilli (Fig 1). Nitratase enzyme activity was checked for all the fifteen isolates and eight of these giving positive results were selected for further AgNP synthesis. (Fig 2)

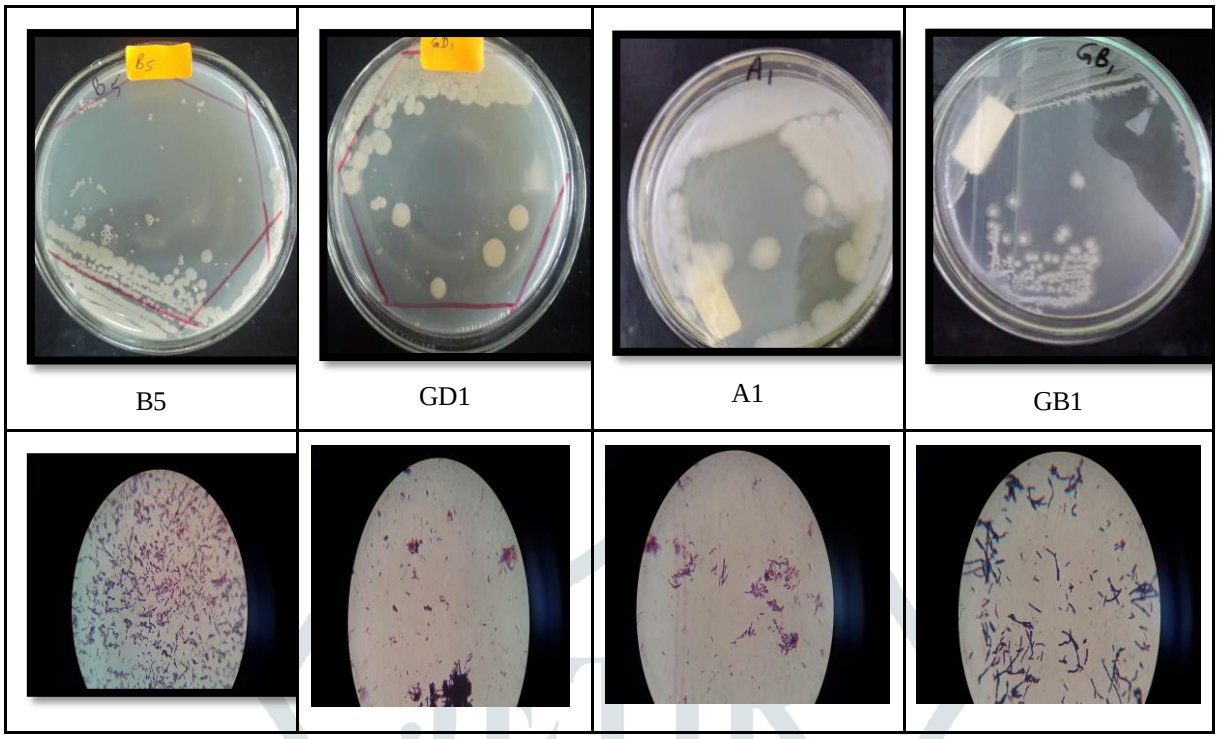


Fig 1. Isolation and Gram staining of bacteria soil isolates from different sources.

From L to R- Control, A1, GB1, GD1, B5, N1, N2 & LC

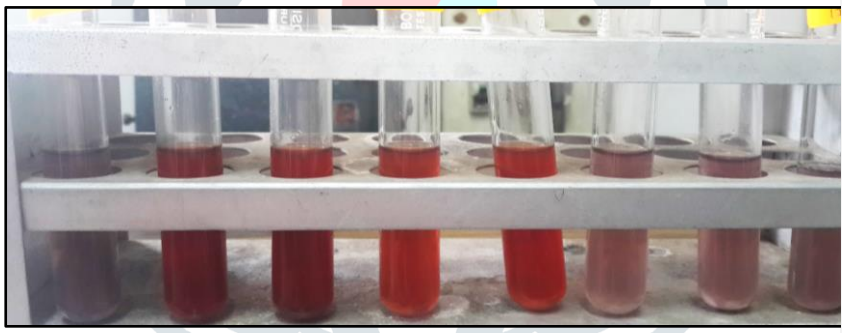


Fig 2. Nitratase enzyme test for different isolates

3.2 Synthesis of AgNPs from bacterial CS

The supernatant harvested from the MGYP culture broth of each individual bacterial isolate was taken as CS and the cell pellet was discarded. (Fig 3) 1.0 ml this CS was then challenged with 10 ml of 1mM $AgNO_3$ and incubated in dark conditions. After incubation of 10 hrs. the change in color to brown was recorded as indicator of AgNP synthesis. The synthesized AgNPs were immediately characterized by UV-visible spectrophotometry. (Table 1) 20 µl of AgNPs suspension was diluted in 2 ml of DDW for measuring the absorption spectra using DDW as blank. The negative controls used of only CS and $AgNO_3$ solution did not show absorbance in the range of 300 -500 nm. All the isolates synthesized AgNPs with an absorption peak in the range of 410 nm - 422 nm. (Fig 4) confirming synthesis of AgNPs. The use of Bacterial CS for biosynthesis of AgNPs thus has the advantage of surpassing the need for Downstream processing.

Source of CS
(from L to R): A1, A2, B5, GB1, GD1, LC, N1 and N2

Fig 3. CS obtained from different isolates.

Table 1. UV Absorption Spectra analysis of the synthesized AgNPs from CS

Isolate	A1	A2	B5	GB1	GD1	LC	N1	N2
Peak	415.2	422.0	421.1	415.2	415.2	411.2	414.8	414.8
Abs	0.483	0.015	0.290	0.175	0.139	0.124	0.113	0.032

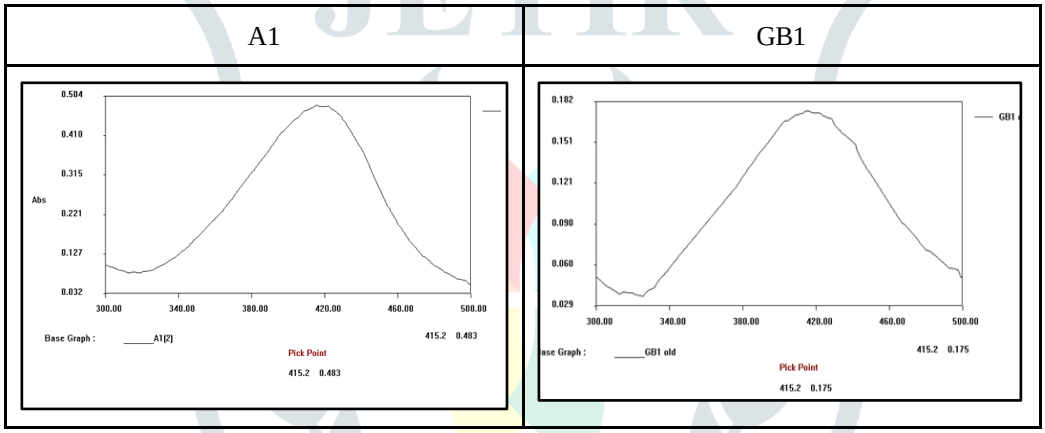


Fig 4: Absorption spectra analysis of AgNPs synthesized using isolates A1 and GB1.

3.3 Agar cup method

Antibacterial activity against E. coli NCTC 9022 and S aureus ATCC 6538 was tested for AgNPs synthesized from the following CS obtained from GB1, LC, N1, A1, N2, GD1 and B5. It was found that AgNPs synthesized using CS of A1 and GB1 were most effective against the test isolates. The values for zone of inhibition (mm) obtained are as in Table 2. The order of the isolates capable of synthesizing the most effective AgNPs was determined as A1➢GB1 ➢B5 ➢ GD1➢ N1 ➢ N2 ➢LC.

Table 2 Antibacterial activity of synthesized AgNPs

Source of AgNPs	GB1	LC	N1	A1	N2	GD1	B5
	Zone of Inhibition (mm)						
E. coli NCTC 9022	25	18	13	26	14	21	18
S. aureus ATCC 6538	19	0	17	27	15	14	19
MEAN	22	9	15	26.5	14.5	17.5	18.5
SD	7.07	12.73	3.89	0.71	0.71	4.95	0.71

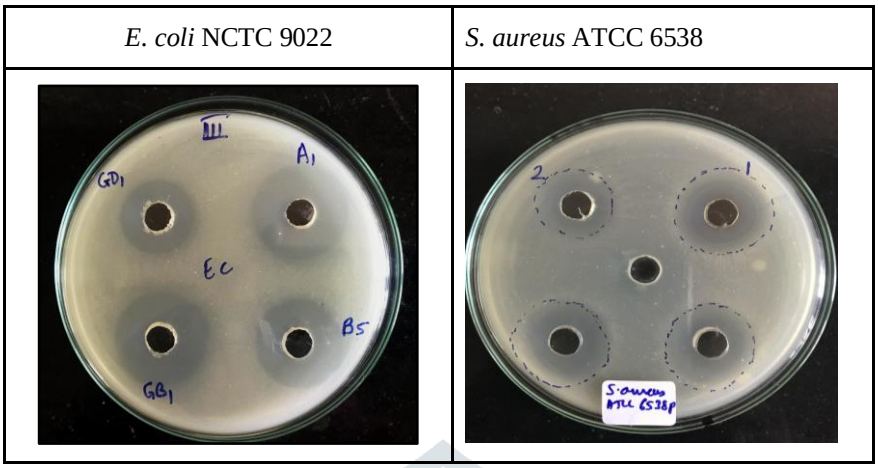


Fig 5. Antibacterial activity of AgNPs against test isolates

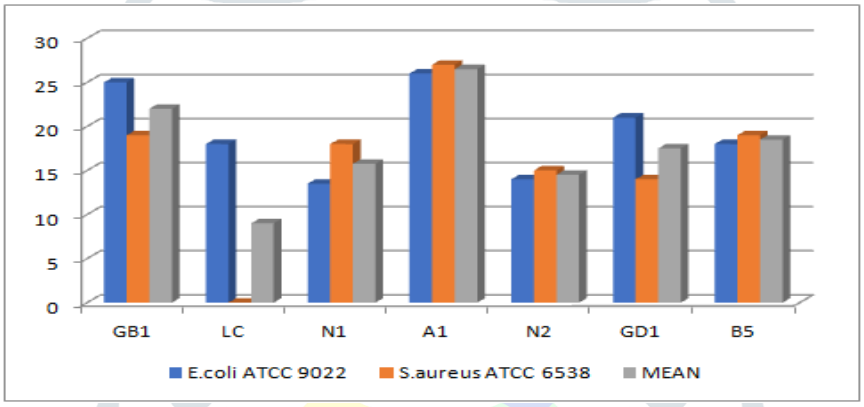


Fig 6: Comparative Antibacterial Activity of Different AgNPs

After primary screening for potency of AgNPs from all the isolates, it was found that the CS obtained after cultivating isolate A1 in MGYP medium for 24 hrs. is the most optimum for effective AgNPs synthesis and the same was used for further studies.

3.4 Characterization of synthesized AgNPs

Characterization of the synthesized AgNPs obtained from four of the isolates was determined via TEM analysis. Spherical nanoparticles in the size range of predominantly 6-14 nms. were obtained (Fig 7). The TEM micrographs were also analyzed for particle size distribution with more than 40% of the nanoparticles synthesized in the 6-8 nms. range majorly (Fig 8). These results are similar to those reported by Shekhar Agnihotri et.al,2014 who also found that nanoparticles less than 10 nm to be most effective against bacterial test organisms.

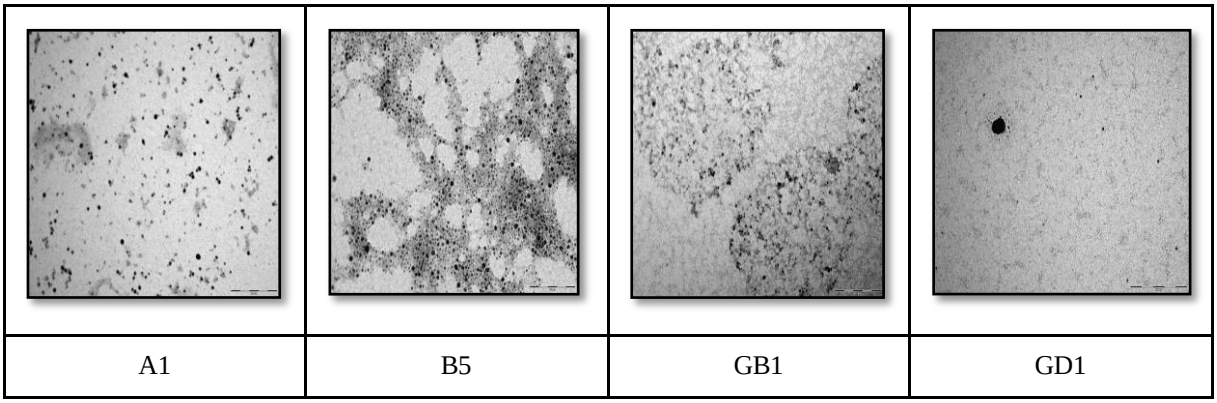


Fig 7. TEM images of AgNPs synthesized by four bacterial isolates revealing spherical nanoparticles in the range of 6-14nms predominantly

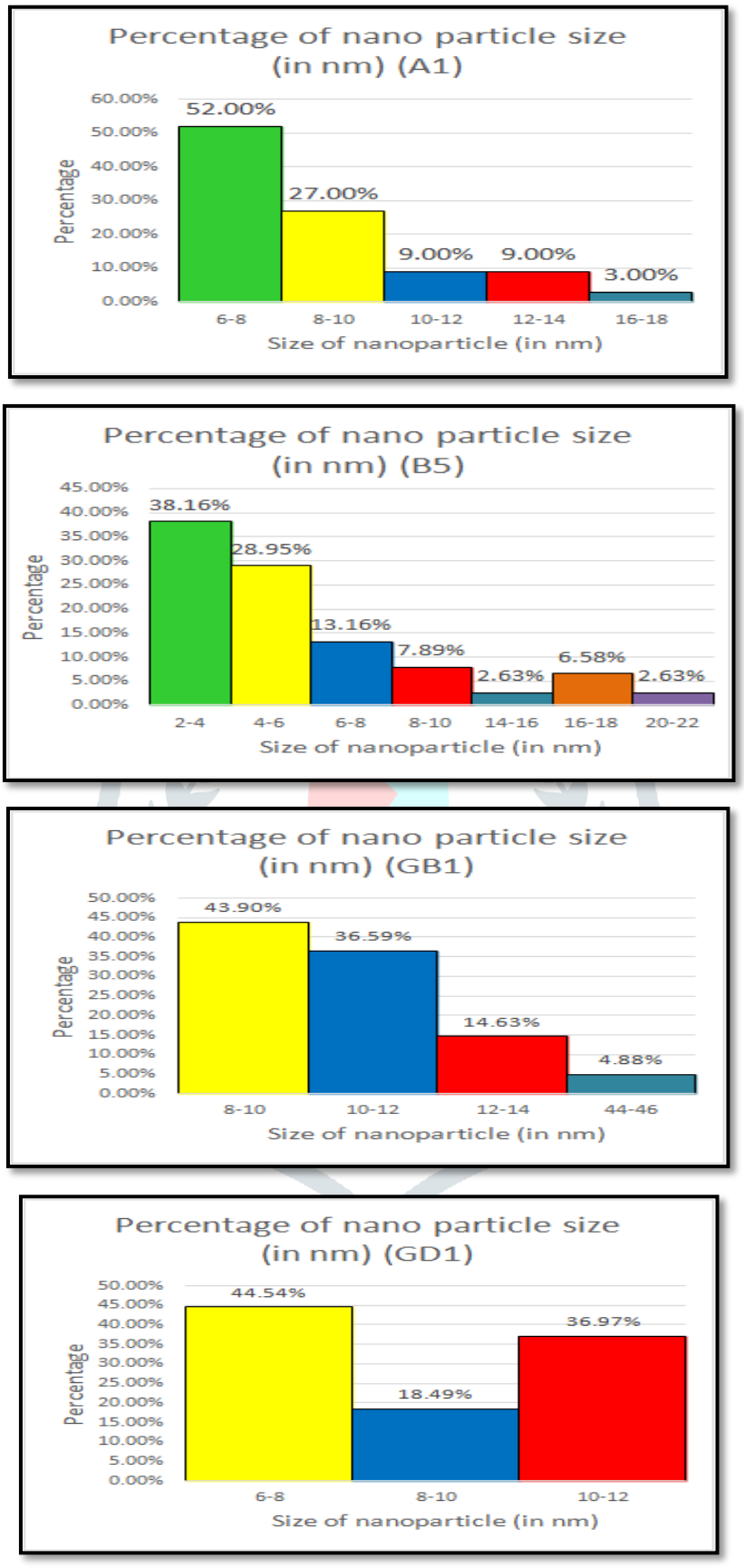


Fig 8 Size distribution analysis (%) of the synthesized AgNPs.

3.5 Optimization of synthesis using CS from isolate A1.

CS from isolate A1 was selected for further AgNP synthesis and the process was optimized for different parameters.

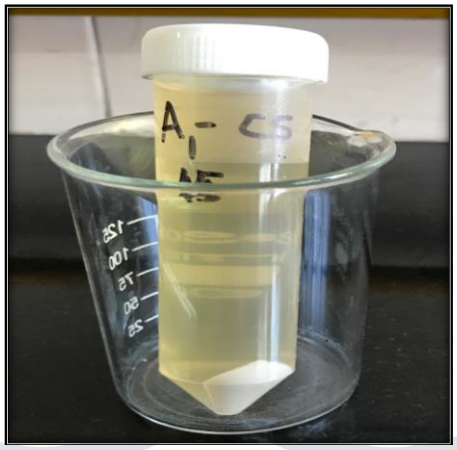


Fig 9. CS obtained from isolate A1

3.5.1 Effect of addition of 1N NaOH to A1 CS (pH adjusted) for Biosynthesis

The pH of A1 CS was modified from original values of around 6.5 via addition of 1N NaOH. The effect of addition of increasing amounts of 1N NaOH to fixed 10 ml volumes of CS on the Absorption spectra is shown in Table 3. The ratio of the volume of CS: 1mM $AgNO_3$ used was maintained at 1:10.

Table 3. Effect of Volume of 1N NaOH added to A1 CS on Biosynthesis

Sr. No.	Volume of CS (ml)	Volume of 1N NaOH(ml)	λ max (nm)	O. D
1	10	1.0	415.2	0.115
2	10	1.5	408	0.149
3	10	2.0	408.8	0.235

Suitable negative controls were set up to prove that synthesis was independent of the addition of NaOH or of the sterile medium MGYP to 1mM $AgNO_3$ individually. As shown in Fig 10, there was no brown color observed in any of the four tubes on incubation up to 1 week proving the absence of AgNP synthesis. This indicates that the Bacterial CS itself is responsible for the biosynthesis of AgNPs when added to 1mM $AgNO_3$.

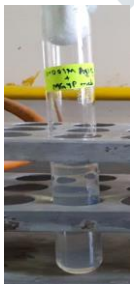
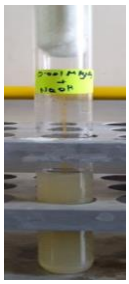
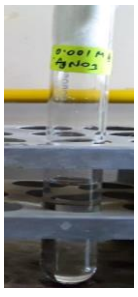
St. MGYP + NaOH	1mM $AgNO_3$ + MGYP	1mM $AgNO_3$ + NaOH	1mM $AgNO_3$
			

Fig 10 Negative Controls for AgNP synthesis.

From the observations of Table 3, it can be interpreted that addition of 1N NaOH to CS has enhanced the synthesis of AgNPs. The surface plasmon resonance (SPR) peak shifts from 415.2nm toward the short wavelength region of 408.8nm when the pH value increases which can indicate a decrease in the size of AgNPs. (M.K. Alqadi,2014). Similar findings have also been reported by Majid Darroudi ,2010. This study describes NaOH as an accelerator reagent in the green synthesis of AgNPs, wherein greater volumes of NaOH added to the reaction mixture increased the absorbance due to increases in silver concentration,

resulting in a stronger blue-shift in λmax to 407 nm from 424 nm. This blue-shift is related to a decrease in the particle size of AgNPs because the SPR band in metal nanoparticles displays the blue-shift based on the decreased size of particles. (Heath J.R,1989)

3.5.2 Optimization of A1 Culture growth Density on Biosynthesis

A1 Culture was grown in MGYP medium in side arm flasks for increasing incubation periods and the O.D. $_{530nm}$ of the culture at the end of each incubation period was measured as reported in Table 4.

The CS was then prepared from each of the flasks and treated identically with 1N NaOH and used for biosynthesis in the ratio of volume of CS (pH adjusted): 1mM $AgNO_3$ of 1:10.

Table 4 Effect of Culture growth Density on AgNP synthesis

Sr. No.	O.D of Culture Broth of A1 at 530 nm	λ max (nm)	O. D
1	0.73	415.2	0.119
2	1.47	415.2	0.096
3	1.88	415.2	0.041
4	1.96	415.2	0.038

From the findings, it can be stated that the biosynthesis of AgNPs occurred optimally at the lower culture density, indicating that the reducing and capping agents (enzymes like nitratase) that maybe responsible for synthesis present in the CS are released extracellularly maximally during the active logarithmic phase of growth (idiophase). The idiophase or the growth dependent phase is known to support the production of enzymes required for active growth and metabolism of the bacteria.

As the O.D of the culture broth increased towards 1.96, the CS obtained and used for synthesis after addition of NaOH, produced AgNPs as confirmed by uv -spectrometry λ max (nm) of 415.2. However, the intensity of the brown color formed decreased as was seen by the O.D values in decreasing order with increase in the culture density indicating that the efficiency of biosynthesis of AgNPs decreases. This could be attributed to the cells entering the stationary phase or the growth independent phase where the cells are preparing to enter the death phase due to production of toxic metabolites.

3.5.3 Effect of Ratio of Volume of A1 CS (pH adjusted): 1mM $AgNO_3$

A1 CS adjusted to fixed alkaline pH values by the addition of 1N NaOH was used for AgNP synthesis in varying proportions for addition to 1mM $AgNO_3$ solution.

Table 5 Effect of Ratio of Volume of CS (pH adjusted): 1mM $AgNO_3$

Sr. No.	Volume of CS (ml)	Volume of 1N NaOH(ml)	Ratio of CS:1mM $AgNO_3$	λ max (nm)	O.D
Set I	10	0.65	0.5:10	415.2	0.176
	10	0.65	1.0:10	415.2	0.057
Set II	10	0.65	0.5:10	415.2	0.319
	10	0.65	1.0:10	410.4	0.242

Biosynthesis of AgNPs is confirmed as is seen from the λ max (nm) values of 415.2, indicative of the reproducibility of the synthesis. In both the sets, the optimum ratio of Volume of CS (pH adjusted): 1mM $AgNO_3$ was decided on the basis of the intensity of the brown color formed after incubating the reaction mixture in the dark as well as the O.D values obtained by uv spectrophotometry. The optimum ratio of volume of CS (pH adjusted):1mM $AgNO_3$ is determined as 0.5ml: 10ml.

3.5.4. Effect of microwave assisted induction on biosynthesis by A1

The reaction mixture of A1 CS after pH optimization and 1mM $AgNO_3$ (total volume 10 ml) was exposed to microwave radiation for different time intervals increasing by 10 seconds. The effect of microwave radiation on the rate of reaction and the time required for the process of AgNP synthesis was evaluated.

It was found that synthesis started within 20 seconds of exposure to microwave and rapid synthesis was observed after an exposure of 40 seconds. Further increase in exposure time did not lead to a significant increase in the rate of synthesis. (Fig 11). The O.D at the λ max (nm) of 415.2nm kept on increasing with increase in time of exposure till 40 secs. (Table 6)

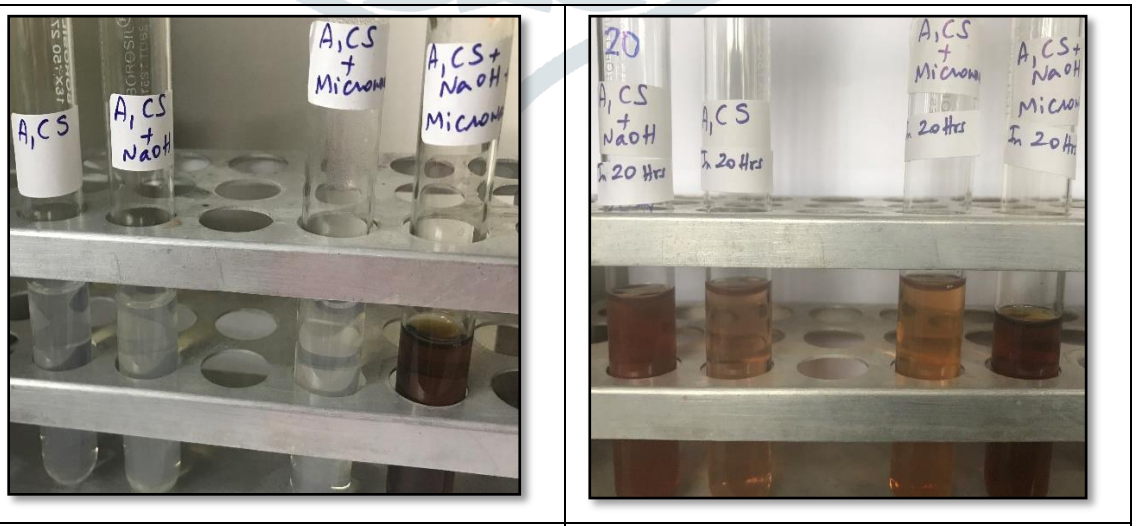
Fig 11 Effect of Microwave assisted induction on biosynthesis of AgNP using A1CS.

Table 6. UV spectrometry values for effect of Microwave induction on biosynthesis of AgNPs

Time of Exposure of reaction mixture to Microwave	λ max (nm)	O. D
10 secs	415.2	0.043
20 secs	415.2	0.057
30 secs	415.2	0.166
40 secs	415.2	0.213
50 secs	415.2	0.177

Similar findings have also been reported by N. Saifuddin,2010, describing a novel combinatorial synthesis approach by using a combination of culture supernatant of *Bacillus subtilis* and microwave (MW) irradiation in water. It was found that exposure of culture supernatant of *Bacillus subtilis* and microwave irradiation to silver ion lead to the rapid formation of silver nanoparticles within 180 secs. However, this paper reports synthesis almost instantly within an exposure to microwave at 40 secs.

Finally, the optimized parameters were also tested individually and in combination to determine the effect of each optimization on the rate of synthesis. The effect of various reaction mixtures CS and CS + NaOH, along with 1mM $AgNO_3$ both in the presence and absence of exposure to microwave irradiation was tested. The rate of synthesis was found to increase with the addition of each optimization step. The rate of synthesis increased from 20 hrs to 10 hrs to instantaneously for CS, CS + NaOH and CS + NaOH + Microwave (40 seconds). It is also noteworthy that the intensity of the brown color developed (which acts as an indicator of quantity of AgNP synthesis) also increases with each optimization and is maximum for the combination of CS + NaOH and CS + NaOH + Microwave.



At 40 secs of reaction- **After 20 Hrs of reaction**

Fig 12 -Comparison of synthesis with A1CS, A1CS+NaOH, A1CS+Microwave, A1CS +NaOH+Microwave at 0 Hrs. (LHS) and at 20Hrs. (RHS)

The stability of the synthesized AgNPs was verified both visually as well as by uv-spectrophotometric analysis and it was found to be stable even after six months of storage in dark. This could be attributed to the presence of Capping agents in the CS that prevents the AgNPs from agglomeration and thereby retain their characteristics and properties.

3.6 Biochemical identification and 16s rRNA sequencing of isolates

The bacterial isolates were identified by Metropolis Laboratories using **VITEK2 BCL card** for identification of Bacillus species and other aerobic endospore formers as follows:(Table 7)

Further, the best performing 2 isolates A1 and GB1 were subjected to 16s rRNA by Sanger's dideoxy sequencing and the obtained sequences of 1294 and 1023 base pairs were subjected to BLAST analysis and the result showed its maximum identity of 99 % to various Bacillus sp. mainly *Bacillus cereus* for A1 and *Bacillus subtilis* for GB1. (Table 7). The 16S rDNA sequences of the isolates were submitted to NCBI under the accession numbers MH598411.1 and MH665601.1. Isolate A1 was named as *Bacillus cereus* strain BHAVANI and isolate GB1 was named as *Bacillus subtilis* strain VESASC.

Table 7. Identification of the four Bacterial isolates

Isolate	Identification using VITEK 2BCL card	Identification using 16S rRNA sequencing	NCBI Accession No.
A1	*Bacillus firmus*	*Bacillus cereus* strain BHAVANI	**MH598411.1`**
GB1	*Bacillus subtilis*	*Bacillus subtilis* strain VESASC	**MH665601.1**.
B5	*Bacillus cereus*	-	-
GD1	*Bacillus subtilis*	-	-

In a study on evaluating VITEK2 BCL card for identification of Bacillus species and other aerobic endospore formers, the findings reported that 93% of the strains were identified correctly to species level, however 6% were incorrectly identified, with 1% unidentified. (Halket G ,2010).

V. Conclusion

Thus, this study revealed the advantages of extracellular synthesis of AgNPs using the CS from eight bacterial soil isolates. It was found that the AgNPs in the size range of 6-8 nm as revealed by TEM images gave maximum antibacterial activity. Antibacterial activity was also dependent on the conc of AgNPs in the solution in which the correlation of concentration was made with respect to the visual brown color development as also the O.D values obtained at the λ max (nm) of 415 nm. The better performing isolates screened as A1, GB1, GD1 and B5 were all biochemically identified using VITEK 2 BCL to belong the genera *Bacillus* spps. This paper reveals for the first-time process optimization of AgNP synthesis using soil bacterial isolate A1, identified by 16srRNA sequencing as *Bacillus cereus* strain BHAVANI with an Accession No. MH598411.1. The dependent parameters that affected biosynthesis were revealed namely, addition of 1N NaOH to CS, Growth culture density, ratio of CS and 1mM $AgNO_3$, and microwave assisted induction and it was found that efficient synthesis of AgNPs could be achieved rapidly within 40 secs. However, the method requires expertise in maintaining and handling bacterial cultures. Thus, this study describes for the first time a rapid, ecofriendly, simple and highly reproducible method of biosynthesis of AgNPs that has the advantage of avoiding downstream processing and yet retaining the stability and activity over a long period of six months and maybe beyond.

VI. Acknowledgment

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