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Physiological and biocidal perspectives of Silver Nanoparticles: Bacteria-mediated green synthesis and biochemical characterization

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ABSTRACT
This study is targeted towards the synthesis of AgNPs from bacterial cultures of Salmonella Typhimurium and Bacillus Subtilis under different physical parameters (AgCH3COO concentration, pH, and incubation time) and characterization of the synthesized particles by UV- Visible Spectrophotometric analysis. The AgNPs that were synthesized under the different physical parameters were ofa similar size range ie., 20-40 nm. In the case of Salmonella Typhimurium, an increase in AgCH3COO concentration, pH, and incubation time favored AgNP synthesis but that wasn't the case for Bacillus Subtilis.
KEYWORDS-*Silver Nanoparticles, Green synthesis, Silver Acetate, UV- Visible Spectroscopy, Salmonella sp., Bacillus sp.*

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INTRODUCTION

Nanoscience and nanotechnology have attracted global attention in recent years due to its potential impact on a wide variety of scientific fields, including energy, medicine, diagnosis, pharmaceuticals, space industry, food industries, etc. [1, 2, 3, 4]. However, their importance in the medical field is increasing sharply with time, in fact, their capability to kill microbes has made them a hopeful target for researchers to treat antibiotic-resistant microbes. Antibiotic resistance in pathogenic bacteria is a critical concern in biomedical and pharmaceutical research [5,6,7]. Metallic NPs are the most promising because of their enormous surface area to volume ratio, which gives them good antibacterial characteristics [8,9,10,11]. Silver has long been recognized as a threat to a variety of microorganisms, with almost 116 silver- sensitive microbes being identified till now [12]. Though the Silver ions have a well-known bactericidal action the exact mode of their action is still partially understood. It's been hypothesized that ionic silver interacts significantly with the thiol groups of essential enzymes, rendering them inactive [13,14,15]. Other research has shown indications of structural alterations in cellular membranes [16]. Initially, silver nanoparticles (AgNP) were synthesized by either physical or chemical methods. Some of the widely used methods include chemical reduction, thermal synthesis; electrochemical reduction, vaporization, and photochemical reduction [17,18,19]. However, even though these procedures produce pure and defined NPs, they have significant drawbacks, such as being costly and having environmental hazards. So, there is an increasing demand for efficient, safe, cheap and, eco-friendly methods for the production of NPs. As a result, researchers have directed their attention towards the biological synthesis of AgNPs. These methods are expected to offer an alternate approach for resolving the problems associated with physical or chemical synthesis [20,21,22,23,24]. To date, several reports are depicting the synthesis of AgNPs by bacteria, plants, fungi, algae, and yeasts [2,25,26,27]. Microorganisms may produce inorganic NP via either extracellular or intracellular processes [28,29,30,31,32].However, if the synthesis of nanoparticles is intracellular it requires additional steps to be recovered from the cells for commercial purposes. Some of these methods may involve the use of appropriate detergents or ultrasonic treatment. However, extracellular synthesis does not involve any cell lysis method and hence is more cost-effective than intracellular biosynthesis [33,34]. Based on the information available regarding nanoparticle synthesis by microbes, we have directed our attention towards the synthesis of AgNPs by soil isolated strains of *Salmonella* sp. and *Bacillus* sp.

Though there are reports of *Salmonella* sp. being involved in the synthesis of AgNPs [35,36,37] but there are few reports related to *Bacillus* sp. mediated AgNP synthesis [38]. Hence, the present study attempts to check the capability of the isolated strains to synthesize AgNPs under different physical parameters. Physical parameters play a crucial role in the size and properties of nanoparticles. We have tried to focus on the effect of different concentrations of AgCH3COO, different pH, and duration of incubation period on the synthesis of AgNPs. Though synthesis is a crucial aspect of the study, determining the effectiveness of the produced nanoparticles based on their antibacterial activity is equally important.

MATERIAL AND METHODS

Sub culturing and Maintenance of Bacterial Culture:

Salmonella sp. and *Bacillus* sp. cultures were obtained from the Department of Biotechnology repository. The cultures were sub-cultured in Nutrient broth and then on LB Agar media. They were incubated at 37 °C for 24 hrs. These cultures were further subjected to biochemical tests and staining to reconfirm their genus.

Biochemical Characterization of Bacterial Culture:

For confirmation of *Salmonella* sp. Kings Agar media was used as a selective and differential media. The combination of *Salmonella* agar F (King's media B) and *Salmonella* agar P allows for the traditional phenotypic test to distinguish *Salmonella Typhimurium* from other *Salmonella* species [39]. The *Salmonella* culture was streaked on this media and incubated for 24 hrs to observe if there is a yellow to greenish-yellow region around the colonies which is due to the synthesis of fluorescein, which is a signature of *Salmonella Typhimurium*. MSA is a selective and differential medium for *Bacillus* sp. The high salt concentration in the medium allows the selective growth of *Bacillus* sp. only. The *Bacillus* culture was streaked on Mannitol Salt Agar (MSA) media. Simmons Citrate Agar was used to distinguish between enteric species bacteria based on their ability to ferment citrate as a primary carbon source [40]. The change in color of bromophenol blue from green to blue indicates the positive result for citrate utilization test. Simmons citrate agar slants were made and both the cultures were streaked using aseptic techniques over these slants. The change in the colour of the medium was observed after 24 hrs of incubation at 37 °C [41,42].

Acclimatization and synthesis of Silver Nanoparticles:

Before using the organisms for AgNP synthesis they were grown in increasing concentrations of AgCH3COO (0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, 1 mM) containing medium. *Salmonella Typhimurium* and *Bacillus Subtilis* were inoculated in 10 ml of Nutrient broth containing 0.01 mM, 0.05 mM, 0.1 mM AgCH3COO and grown for 24 hrs. From the 0.1 mM culture, further inoculation was done in a broth containing a higher concentration of AgCH3COO (0.5, and 1mM). Nutrient agar plates with the same concentrations of AgCH3COO were also maintained and stored as master plates.

Synthesis of NP was carried out with cell-free extract of *Salmonella* sp. and *Bacillus* sp. Briefly, the bacterial cultures were grown in 100 ml nutrient broth and incubated overnight ina shaker incubator at 37 ^{0}C and 150 rpm. The cultures were then transferred to centrifuge tubes and centrifuged at 5000 rpm for 10 minutes. The cell-free extract (CFE) was transferred to a fresh 250 ml Erlenmeyer flask and AgCH3COO solution was added from a stock of 100 mM solution to make up the final concentration to 1 mM. This was then incubated in a shaker incubator for72 hours at 37 ^{0}C and 150 rpm [43]. The synthesized NP were characterized by UV-Vis Spectrophotometric study.

Effect of two physical parameters on the synthesis of silver nanoparticles

In this study, the physical parameters considered were concentration of AgCH3COO and effect of pH.

Effect of AgCH3COO concentration:

To study the effect of AgCH3COO concentration on the silver nanoparticle synthesis, different concentrations of AgCH3COO (0.1 mM, 0.5 mM, 1.0 mM, and 2.0 mM) were added to cell-free extract of 24 hrs old cultures of *Salmonella* sp. and *Bacillus* sp. These tubes were then incubated at 37 ^{0}C for 72 hrs under dark conditions.

Effect of pH:

To evaluate the effect of pH on the synthesis of AgNPs, the optimum concentration of AgCH3COO obtained from the previous set of experiments (1 mM) was added to the cell-free extract of both the cultures at different pH conditions (6,7 and 9) and incubated at 37 ^{0}C for 72 hrs under dark conditions. After synthesis, the AgNPs were analyzed by UV-Vis Spectrophotometer.

Effect of time:

To evaluate the effect of time on the synthesis of AgNPs, 1 mM AgCH3COO was added to the cell- free extract of both the cultures and were incubated for different time periods (3 days, 4 days and 5 days). The tubes

were kept at 37 ^{0}C under dark conditions for the synthesis. After the incubation periods change in colour of the tubes were observed and UV-Vis spectrophotometric analysis was carried out.

Characterization of silver nanoparticles using UV-Visible Spectrophotometer

Color shift showed the reduction of Ag^+ ions to AgNPs, which was later confirmed by UV- Visible spectroscopy (Systronics UV-Visible Spectrophotometer 117). The nanoparticle suspension was taken in a quartz cuvette. The blank used for this experiment was fresh uninoculated broth. The absorbance of the suspension was checked under a wavelength range between 350 nm– 450 nm.

The effect of each operational parameter was investigated while keeping the other two parameters constant.

RESULTS AND DISCUSSION

The *Salmonella* sp. and *Bacillus* sp. that were streaked onto LB agar plates, from their glycerol stocks, showed growth within 24 hours of incubation. The *Salmonella* sp. colonies were large, smooth, opaque, and light tan in color on clear amber-colored LB agar. The *Bacillus* sp. colonies were white-golden, opaque, and smooth on clear amber-colored LB agar. The gram staining of the pure culture of *Bacillus* sp. *showed* gram-positive coccus and *Salmonella* sp*. exhibited* gram-negative bacilli and under 100X magnification. (Figure 1A & B).

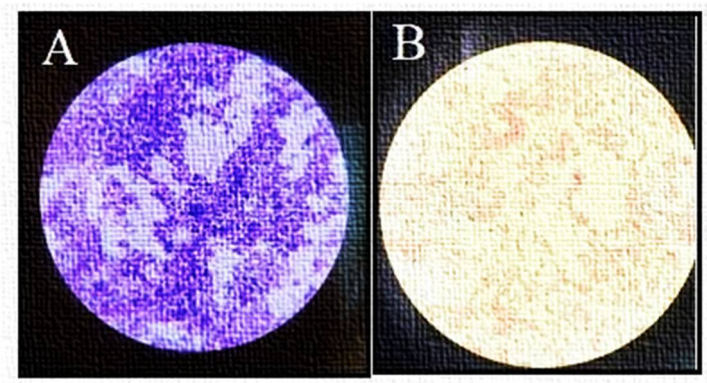


Figure 1: Gram staining of bacterial cultures. A) *Bacillus* sp. B) *Salmonella* sp.

King's Agar for *Salmonella Typhimurium* confirmation

The *Salmonella* culture on King's Agar media showed yellow-coloured colonies. This is a biochemical confirmation of the strain to be *Salmonella Typhimurium.*

MSA Agar for *Bacillus Subtilis* confirmation:

The *Bacillus* culture on MSA media produced yellow-coloured colonies, confirming the culture to be *Bacillus Subtilis.*

Citrate utilization test:

The Simmon citrate test for both the organisms confirmed that they were capable of utilizing citrate as a carbon source. Both *Salmonella Typhimurium* and *Bacillus Subtilis* exhibited citrate positive character. The change in colour of the medium from green to deep Prussian blue is a confirmation of the citrate utilization by the organisms. Citrate catabolism produces alkaline carbonates and bicarbonates, which increase the pH of the medium above 7.6, causing the bromothymol blue to change from the original green colour to blue.

Acclimatization of organisms:

Initially, growth was observed for both *Salmonella Typhimurium* and *Bacillus Subtilis* in nutrient broth containing 0.01 mM, 0.05 mM, 0.1 mM AgCH3COO. Upon subculturing from the 0.1 mM AgCH3COO to a higher concentration of AgCH3COO (0.5 and 1 mM), growth was observed at these concentrations also (Figure 2 A & B). These acclimatized cultures were further used for the synthesis of AgNP.

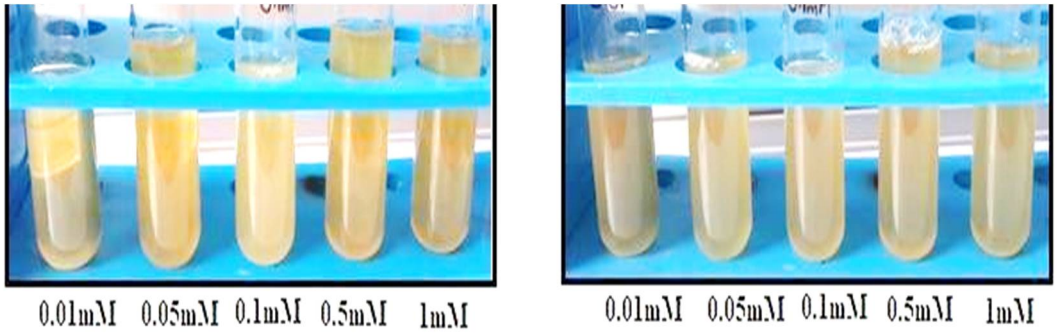


Figure 2. Acclimatization of microbial cultures in increasing AgCH3COOconcentration. A. Acclimatization of *Salmonella Typhimurium* in increasing concentration of silver acetate 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM and 1.0 mM. B. Acclimatization of *Bacillus Subtilis* in increasing concentration of silver acetate 0.01 mM, 0.05 mM, 0.1 mM,0.5 mM and 1.0 mM.

Synthesis of Silver Nanoparticles:

Synthesis of AgNPs was carried out using cell-free extract of 24 hours old culture. After the incubation of the cell-free extract with the AgCH3COO solution for 72 hours, there was a significant change in colour of the media for both the microorganisms (Figure 3). The control in the figure shows the 24 hrs old culture before removing the cells from the media. The appearance of the brown color after indicates the biotransformationof silver ions to silver atom indicating synthesis of AgNP. The change to brown colour is due to the excitation of surface plasmon vibrations (SPV) in the particles. This is a preliminary indication that the bacterial strains are able to synthesize AgNPs under the experimental conditions provided. By the acclimatization process, the organisms were adapted to an environment containing silver acetate prior to the synthesis process.

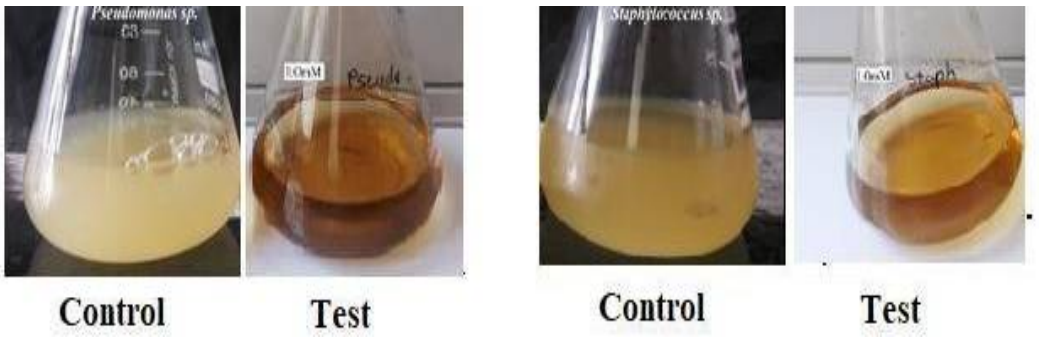


Figure 3. Synthesis of Silver Nanoparticles.

A) Synthesis with *Salmonella Typhimurium.*
B) Synthesis with *Bacillus Subtilis*

Effect of three operational parameters on the synthesis of silver nanoparticles

Effect of different AgCH3COO concentrations:

With the increase in concentration of AgCH3COO (0.1 mM, 0.5 mM, 1.0 mM, and 2.0 mM) after 72 hrs of incubation there was a clear increase in the intensity of the brown colour in the tubes containing cell-free extract of *Salmonella Typhimurium* (Figure 4A). However, *Bacillus Subtilis* under the same synthesis condition exhibits a different pattern (Figure 4B). The pictures were taken on Day 0 (immediately after the mixing of CFE and AgCH3COO solution) and Day 3 after 72 hrs of incubation to depict the visual change in the colour. This observation was further assessed by UV- Visible spectrophotometric analysis.

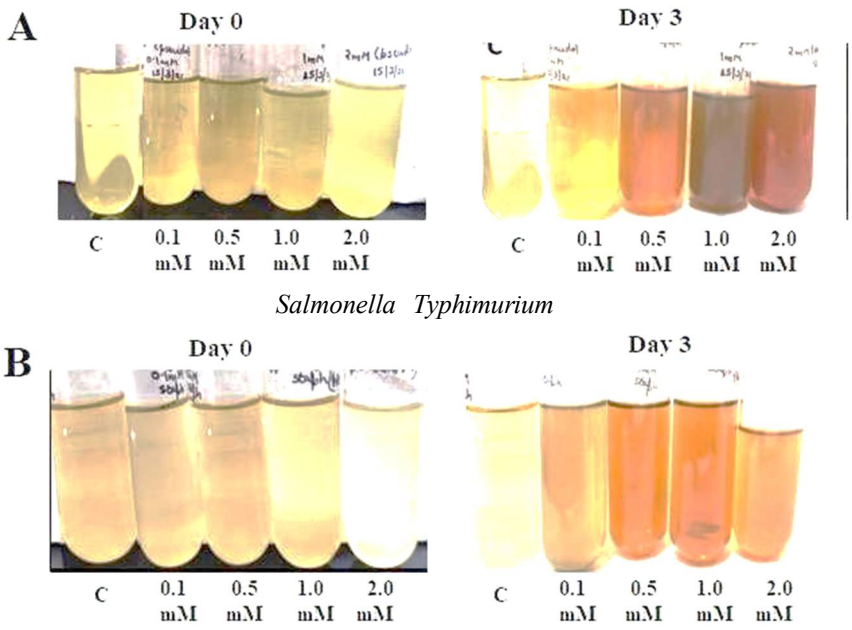


Figure 4. Effect of different AgCH3COO concentrations on NP synthesis.

Salmonella Typhimurium cell-free extract incubated with different concentrations of AgCH3COO at Day 0 and Day 3.

Bacillus Subtilis cell-free extract incubated with different concentrations of AgCH3COO at Day 0 and Day 3.

The appearance of brown colour is an indication of the formation of AgNP. The colour change was due to the surface plasmon resonance and reduction of silver ions. The wavelength, at which the solution exhibits absorbance, helps in determining the size of the NP too. Hence, the formation of AgNP was confirmed by using a UV-Visible Spectrophotometer. The UV- Visible spectrophotometric analysis of the NP synthesized by *Salmonella Typhimurium showed* peaks around 410 nm, 410 nm, 420 nm, and 410 nm for 0.1 mM, 0.5 mM, 1 mM, and 2 mM of AgCH3COO concentration respectively (Table – 1; Figure 5). This indicates that the particles synthesized are in the range of 10-30 nm. The data shown is an average of three independent experiments.

Table 1: Absorbance of NP synthesized at different concentrations of AgCH3COO (0.1 mM, 0.5 mM, 1 mM and 2 mM) with *Salmonella Typhimurium* after 72 hours incubation.

Wavelength (in nm)	0.1 mM	0.5 mM	1 mM	2 mM
360	0.481	1.290	1.838	1.927
370	0.532	1.339	1.895	2.050
380	0.587	1.393	1.965	2.150
390	0.640	1.475	2.097	2.330
400	0.683	1.570	2.187	2.504
410	0.720	1.654	2.310	2.663
420	0.640	1.586	2.223	2.595
430	0.555	1.450	2.077	2.462
440	0.483	1.319	1.936	2.336
450	0.393	1.186	1.790	2.143
460	0.283	1.028	1.612	1.890
470	0.213	0.872	1.420	1.630
480	0.172	0.724	1.218	1.373

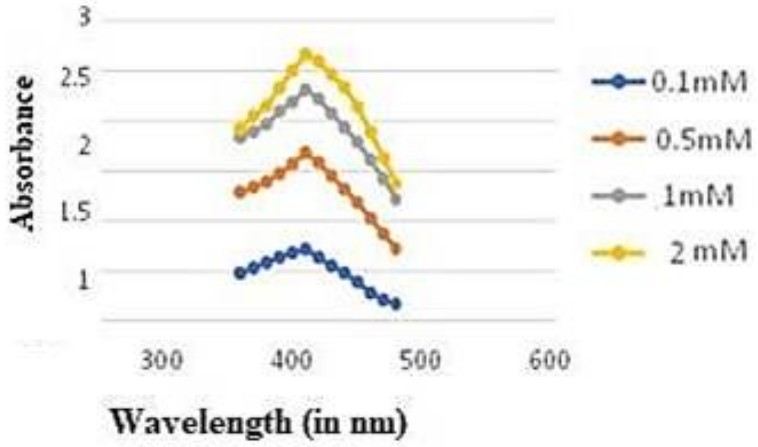


Figure 5: Graphical representation of Absorbance of AgNPs synthesized at different concentrations ofAgCH3COO by *Salmonella Typhimurium*

Whereas for *Bacillus Subtilis* the AgNP synthesized at different AgCH3COO concentration (0.1 mM, 0.5 mM, 1 mM and 2 mM) showed peaks at 410 nm, 420 nm, 420 nm and 420 nm respectively (Table 2; Figure6). Hence, the size of the nanoparticle in this case also ranges between 20- 40 nm. The data shown is an average of three independent experiments.

Table 2: Absorbance of NP synthesized at different concentrations of AgCH3COO (0.1 mM, 0.5 mM, 1 mMand 2 mM) with *Bacillus Subtilis* after 72 hours incubation.

Wavelength (in nm)	0.1 mM	0.5 mM	1 mM	2 mM
360	1.280	0.599	1.140	0.830
370	1.327	0.657	1.198	0.914
380	1.390	0.709	1.275	0.994
390	1.437	0.768	1.341	1.086
400	1.492	0.830	1.407	1.179
410	1.577	0.904	1.459	1.294
420	1.532	0.975	1.550	1.394
430	1.474	0.921	1.440	1.304
440	1.378	0.875	1.381	1.201
450	1.321	0.832	1.280	1.129
460	1.278	0.810	1.200	1.057
470	1.214	0.791	1.140	0.978
480	1.175	0.769	1.084	0.901

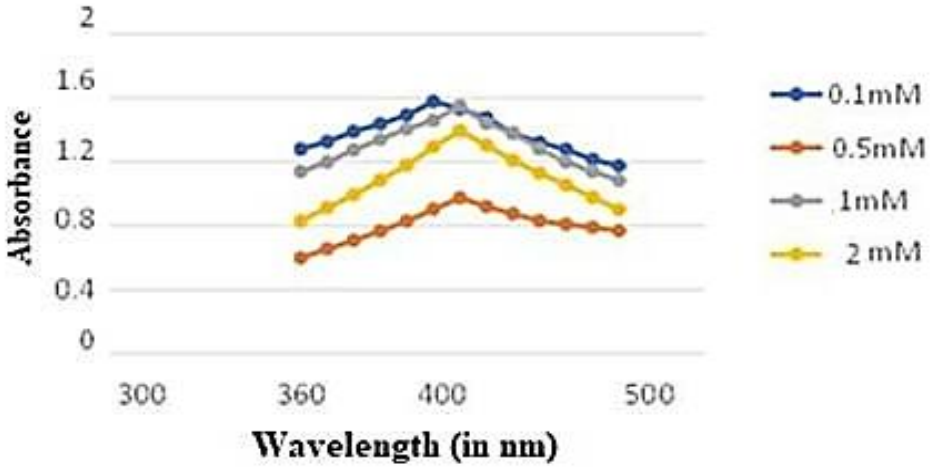


Figure 6: Graphical representation of Absorbance of AgNPs synthesized at different concentrations of AgCH3COO by *Bacillus Subtilis*

The absorbance value of AgNP synthesis with 2 mM AgCH3COO was found to be higher at 410 nm, in the case of *Salmonella Typhimurium.* Thus, an increase in AgCH3COO concentration from 1 mM to 2 mM, (Table 1 & Figure 5) led to the increase in the amount of silver nanoparticle synthesis. When a higher concentration solution is used, the plasmon resonance absorbance increases, meaning that there is more silver ion available for reduction. However, for *Bacillus Subtilis i*ncrease in the concentration of AgCH3COO is not leading to an increased synthesis of NP as the absorbance for 0.1 mM is highest (Table 2 & Figure 6). This indicates that increasing the concentration of AgCH3COO is causing aggregation of AgNP synthesized by *Bacillus Subtilis.*

Effect of different pH:

The effect of pH on the synthesis of AgNP was investigated for different pH conditions 6, 7, and 9. There was a visual change in the colour of the media for all the pH conditions. For *P. Typhimurium* there was an increase in the intensity of the brown colour with an increase in pH (Figure 7A). However, for *S. Subtilis* there was no noticeable increase in intensity of the brown colour. For all the three pH conditions the color appeared to be almost the same (Figure 7B). The colour change was further analyzed by UV-Spectrophotometer. AgNP synthesized by *Salmonella Typhimurium* under different pH (6, 7, and 9) showed peaks at 410 nm, 410 nm, and 420 nm respectively (Table 3; Figure 8A). This indicates that the particles synthesized are of mostly a 20-40 nm size range [44]. The data shown is an average of three independent experiments. Though there is an increase in absorbance with the change in pH, there is no shift in the wavelength range, this indicates that the NP synthesized at pH 6, 7, and 9 are almost of a similar size range. However, at pH 9, the absorbance was higher at 420 nm compared to pH 6 & 7 (Table 3; Figure 8A). The absorbance indicates that the amount of AgNPs formed at pH 9 was higher than pH 6 & 7. Hence for Salmonella Typhimurium, pH 9 seems to be a better choice for NP synthesis with 1 mM AgCH3COO than pH 6.

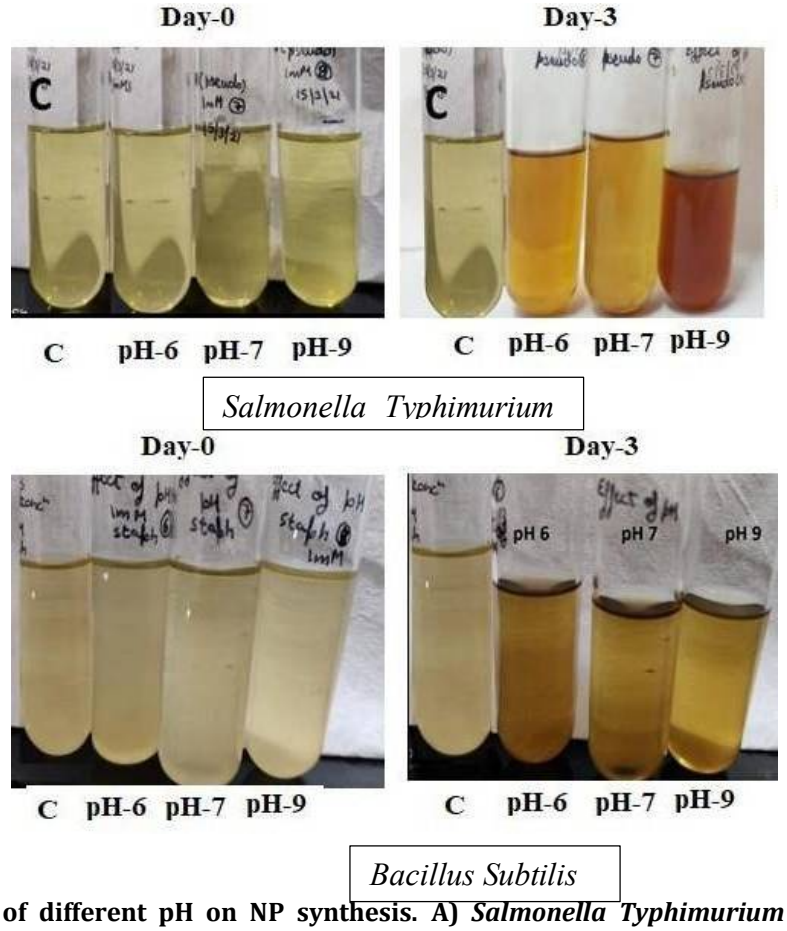
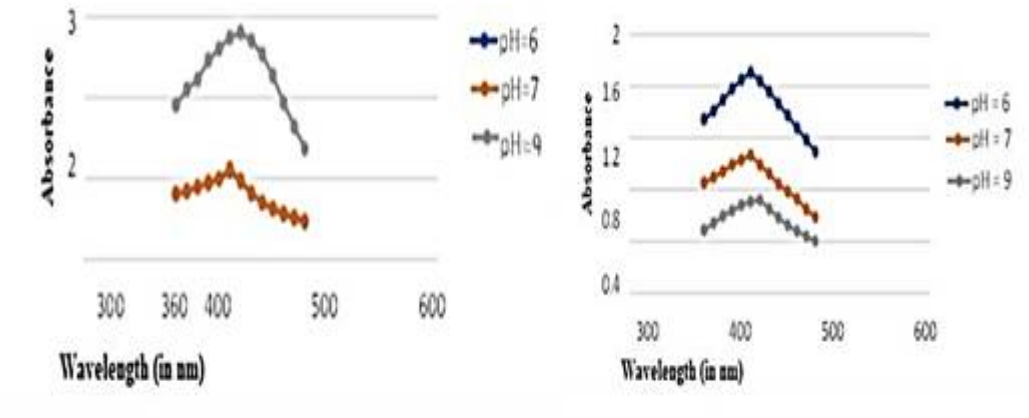


Figure 7: Effect of different pH on NP synthesis. A) *Salmonella Typhimurium* cell-free extract incubated with AgCH3COO at pH 6, 7 and 9 at Day 0 and Day 3. B) *Bacillus Subtilis* cell-free extract incubated with AgCH3COO at pH6, 7 and 9 at Day 0 and Day 3

Table 3: Absorbance of NP synthesized under different pH conditions (pH-6, 7 & 9) with *SalmonellaTyphimurium* cell-free extract and *Bacillus Subtilis* cell-free extract. AB

Wavelength (in nm)	Salmonella Typhimurium cell-free extract			Bacillus Subtilis cell-free extract		
	pH 6	pH 7	pH 9	pH6	pH 7	pH 9
360	0.810	0.817	1.915	1.346	0.854	0.488
370	0.842	0.852	2.109	1.410	0.894	0.542
380	0.897	0.892	2.230	1.496	0.941	0.597
390	0.943	0.957	2.471	1.582	0.994	0.638
400	0.994	1.004	2.612	1.652	1.031	0.682
410	1.101	1.125	2.752	1.709	1.067	0.708
420	0.966	0.983	2.814	1.642	0.993	0.721
430	0.814	0.828	2.712	1.559	0.925	0.652
440	0.700	0.707	2.543	1.467	0.843	0.583
450	0.625	0.634	2.276	1.376	0.786	0.526
460	0.561	0.574	1.942	1.278	0.730	0.480
470	0.510	0.525	1.643	1.187	0.648	0.439
480	0.461	0.481	1.371	1.094	0.587	0.403



A **B**

Figure 8: Graphical representation of absorbance of NP synthesized under different pH conditions (pH-6,7 & 9) with A) *Salmonella Typhimurium* cell-free extract B) *Bacillus Subtilis* cell-free extract

For NP synthesized by *Bacillus Subtilis,* CFE peak was observed at 410-420 nm (Table 3; Figure8B) very similar to that of NP synthesized by *Salmonella Typhimurium.* Hence, the size of the particle, in this case, is also mostly 20-40 nm. But for *Bacillus Subtilis,* the highest absorbance was observed at pH 6 compared to pH 9 for S*. Typhimurium.* Though *Salmonella Typhimurium* exhibits almost similar absorbance pattern at pH 6 & 7, for *Bacillus Subtilis* the absorbance pattern for all three pH conditions is different (Figure 8B).

Hence, the results indicate that the two microorganisms, even though they synthesis NP of similar size, they show different preferences of pH for nanoparticle synthesis.

Effect of different incubation periods on NP synthesis.

In the initial part of the experiment, a colour change was observed in the cell-free extract after incubation of72 hrs, which indicates the synthesis of NP. Further investigation was carried out to study the effect of increased incubation period on the synthesis of NP. For *P. Typhimurium* the spectrophotometric data exhibited a peak at 410 nm for all three days. However, there is hardly any remarkable change between days 3 and 4, which indicates that there is no change in the synthesized NP size or amount. But then there is an increase in absorbance at Day 5 (Figure 9A; Table 4)

Wavelength (in nm)	*S. Typhimurium*			*B. Subtilis*		
	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
360	1.818	1.796	1.862	1.128	0.542	0.337
370	1.895	1.846	2.023	1.164	0.579	0.368
380	1.965	1.927	2.169	1.194	0.611	0.399
390	2.097	2.059	2.325	1.237	0.638	0.439
400	2.187	2.194	2.514	1.284	0.684	0.495
410	2.310	2.318	2.682	1.308	0.734	0.543
420	2.223	2.219	2.662	1.336	0.807	0.589
430	2.077	2.091	2.572	1.259	0.730	0.629
440	1.936	1.960	2.398	1.184	0.658	0.562
450	1.790	1.819	2.156	1.120	0.593	0.508
460	1.612	1.638	1.837	1.063	0.533	0.459
470	1.420	1.436	1.535	1.014	0.481	0.419
480	1.218	1.220	1.250	0.969	0.434	0.383

Table 4: Absorbance of NP synthesized under different incubation period (Day- 3, 4 &5) with *Salmonella Typhimurium and Bacillus Subtilis* cell-free extract.

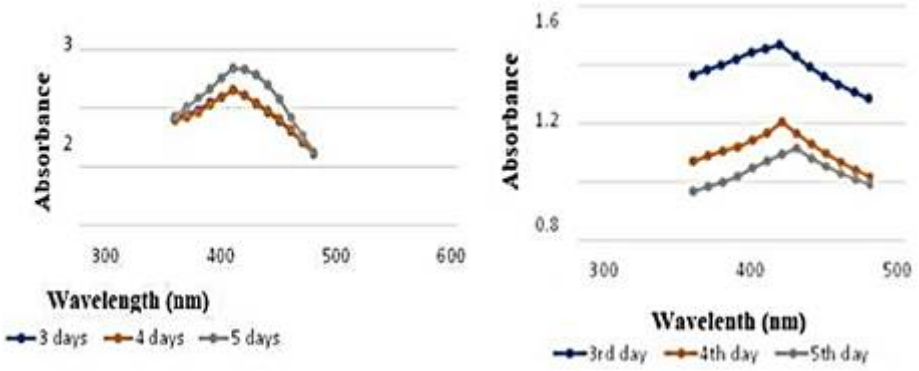


Figure 9: Graphical representation of absorbance of AgNPs synthesized under different incubation periods (Day- 3, 4 &5) with A) *Salmonella Typhimurium* cell-free extract B) *Bacillus Subtilis* cell-free extract.

For *Bacillus Subtilis,* the spectrophotometric data exhibited a peak at 420 nm. But compared to *P. Typhimurium it* exhibited a decrease in absorbance with an increase in the incubation period, which indicates that increasing the incubation for *Bacillus Subtilis* is not suitable for the synthesis of the NPs (Table4; Figure 9B).

The AgNPs synthesized from *Salmonella Typhimurium* and *Bacillus Subtilis* gave a peak at 410 nm and 420 nm which corresponds to the silver NPs in the size range of 20-40 nm, approximately. For *P.Typhimurium* increasing the concentration of AgCH3COO and increase in pH favored the synthesis of AgNPs. Evenwith the increase in the incubation period, there was an increase in the NP synthesis. However, *Bacillus Subtilis* exhibits a different pattern. The data indicates that for *Bacillus Subtilis* ideal concentration of AgCH3COO is 0.1 mM. Higher pH conditions or incubation time are also not favorable.

CONCLUSION

There has been a growing interest in the field of nanoparticle synthesis, especially green synthesis. Green synthesis can be performed from various biological sources like plant extracts, fungi, bacteria, and viruses. Though there are several papers on Silver nanoparticle synthesis from *Salmonella Typhimurium,* there are very few papers on Silver nanoparticle synthesis from *Bacillus Subtilis.* The standard synthesis condition with microbial CFE is suitable for both the organisms, but both of them shows different pattern of synthesis with alteration of physical conditions like concentration of AgCH3COO, pH, and incubation period, The UV-visible spectrophotometric analysis depicts that the synthesized NPs are in the size range of 20-40 nm, which needs to be further verified by experiments like DLS (Dynamic Light Scattering) and FTIR (Fourier Transform Infrared Spectroscopy) for more convincing results. The present study illustrates that synthesis of NPs is possible from both *Salmonella Typhimurium* and *Bacillus Subtilis* under proper experimental

conditions. Another important aspect of the study can be related to the antimicrobial activity of these NPs. However, the data needs to be validated on a large scale to confirm the economic feasibility of the synthesis process and the quality of NPs.

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