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BIOFILM QUENCHING ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED USING *Bacillus subtilis*

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ABSTRACT

The green synthesis of silver nanoparticles is a basic need in the field of nanotechnology. In present study, the silver nanoparticles were synthesized using *Bacillus subtilis* supernatant alone and *Bacillus subtilis* supernatant in presence of glucose. UV-visible spectrophotometer analysis was carried out to assess the synthesis of silver nanoparticles. The synthesized silver nanoparticles were further characterized by using Nanoparticle Tracking Analyzer (NTA), Transmission Electron Microscope (TEM) and Energy Dispersive X-ray Spectra (EDX). Silver nanoparticles with controlled size and shape were observed under TEM micrograph. These silver nanoparticles showed enhanced quorum quenching activity against *Staphylococcus aureus* biofilm which was observed under inverted microscope. In near future, silver nanoparticles synthesized using green methods may be used in treatment of infections caused by highly antibiotic resistant biofilm.

KEYWORDS: ***Bacillus subtilis*; Biofilm; Quorum quenching; Silver nanoparticles**

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INTRODUCTION

The term biofilm was introduced to designate the thin layered condensations of microbes that may occur on various surface structures in nature. In dental contexts, a well-known and extensively studied biofilm structure is established during the attachment of bacteria to teeth to form dental plaque [1]. The excretion of adhesive substances (polysaccharides and proteins) is crucial for the initial attachment of organisms as well as for holding the biofilm bacteria together. The structure *per se* will then provide protection and may allow a better resistance to adverse external influences for the organisms incorporated as compared with the planktonic state [2]. In addition, a growing body of knowledge suggests that organisms in biofilm assume a stronger pathogenic potential than those in a planktonic state. From these aspects, the formation of biofilm carries particular clinical significance because not only host defense mechanisms, but also therapeutic efforts including chemical and mechanical anti-microbial treatment measures, have a most difficult task to deal with organisms that are gathered in a biofilm [3].

Although various physical and chemical methods are extensively used to produce silver nanoparticles, the stability and the use of toxic chemicals is the subject of paramount concern. The use of toxic chemicals on the surface of nanoparticles and non-polar solvents in the synthesis procedure limits their applications in clinical fields. Therefore, development of clean, biocompatible and eco-friendly methods for nanoparticles synthesis deserves merit. Biological methods [4-9] are regarded as safe, cost-effective, sustainable and environment friendly processes [10]. In present study, silver nanoparticles are synthesized using *B.subtilis* supernatant. The study deals with the *S.aureus* biofilm quenching using silver nanoparticles. Diseases such as endocarditis, osteomyelitis and medical-device related infections are caused by *S.aureus* biofilms and are not readily treatable with antibiotics. In fact, biofilms are resistant to antibiotic levels 10- to 1,000-fold higher than planktonic, or free-floating, bacteria [11]. Thus, researchers are focusing on silver nanoparticles for the treatment of infections caused by biofilms.

MATERIALS AND METHODS

1. Collection of B.subtilis supernatant

Bacillus subtilis (NCIM 2045) was grown on sterile Luria Bertani (LB) agar (Casein enzymatic hydrolysate 10 gm/L, Yeast extract 5 gm/ L, Sodium chloride 10 gm/L, Agar 25 gm/L, pH 7.5 ± 0.2) plate at 37º C for 24 hrs. The single isolated colony was inoculated in sterile LB broth and incubated at 37º C at 200 rpm in rotary shaker for 24 hours. After incubation, culture was centrifuged at 4500 rpm for 10 minutes, the supernatant was collected.

2. Synthesis of silver nanoparticles using culture supernatant of B.subtilis

Bacterial supernatant was mixed with 1 mM silver nitrate in 1:4 proportions. The pH of solution was adjusted to 9.0-9.5 [12] and then resultant solutions were kept in rotary shaker (200 rpm) at 37° C till the change in the colour of the solution was observed.

3. Synthesis of silver nanoparticles using culture supernatant of B.subtilis along with 100 mM glucose

Bacterial supernatant : 1mM silver nitrate : 100 mM glucose were mixed in 1:4:1 proportion. The pH of solution was adjusted to 9.0.-9.5. The resultant solution was kept in rotary shaker (200 rpm) at 37° C.

4. UV-visible spectrophotometer analysis

After observing colour change, the sample was subjected to mild sonication for 10 minutes. The bioreduction of silver ions in aqueous solution was monitored by UV-Vis spectra of the solution between 200 nm – 600 nm using Thermo- Biomate 3 UV-visible

spectrophotometer. Distilled water was taken to adjust the baseline.

5. Nanoparticle Tracking Analyzer (NTA) measurements

NTA analysis was carried out by using Nanosight-LM20 instrument. 0.3 ml samples were introduced to the viewing unit using a disposable syringe and enhanced by a near perfect black background; particles appear individually as point-scatterers moving under brownian motion.

6. Transmission Electron Microscopy (TEM) and Energy Dispersive X-ray Spetra (EDX) analyses

Transmission electron microscopy (TEM) analysis of the sample was done using PHILIPS- CM 200 instrument operated at an accelerating voltage of 200 kV with resolution of 0.23 nm. A drop of solution was placed on carbon coated copper grid and later exposed to infrared light (45 minutes) for solvent evaporation. The EDX analysis was carried out using JEOL JSM 7600F.

7. In vitro synthesis of Staphylococcus aureus biofilm

The biofilm related studies were performed with modification of protocol suggested by Kaplan-Ragunath-Ramasubbu-Daniel[13] and King-Tatum[14]. Tryptone soy broth (TSB-Pancreatic digest of casein 17 gm/L, Papaic digest of Soyabean meal 3 gm/L, Sodium chloride 5 gm/L, Dipotassium hydrogen phosphate 2.5 gm/L, Dextrose 2.5 gm/L, pH 7.3 ± 2) with 1% of glucose was inoculated with single isolated colony of *S.aureus* (NCIM 5022) and was incubated at 37ºC for 24 hrs. After incubation culture was diluted with fresh TSB with 1% glucose in 5:100 proportion, then 200 µl of diluted culture was added in 96 well micro titer plate and incubated at 37ºC for 48 hrs [13-14].

8. Addition of quenching agent in Staphylococcus aureus biofilm

First column of wells was served as positive control in which 25 µl of 20 % sodium dodecyl sulphate was added, second column of wells was served as negative control (untreated biofilm). In the third column of wells, 50 µl of concentrated silver nanoparticles synthesized using *B.subtilis* supernatant were added. About 50 µl of different antibiotics solution, Gentamicin (10 µg/ml) and Chloramphenicol (20µg/ml) were added in fourth and fifth column of wells respectively. In sixth column of wells 50 µl of 1mM $AgNO_3$ was added. The plate was incubated at 37ºC for overnight. After incubation, plate was washed with 200 µl of phosphate buffer saline (pH-7.2) to remove floating bacteria. Micro titer plate was stained with 100 µl of 0.1 % crystal violet for 2 minutes then washed with distilled water. Then 200 µl of 33 % acetic acid was added and incubated for 5 minutes. Then Plate was properly dried in laminar air flow cabinet. Plate was analysed under inverted microscope (40 X) to record the results.

RESULTS AND DISCUSSION

1. Synthesis of silver nanoparticles using B.subtilis culture supernatant

Colour change was observed upon mixing the *B.subtilis* culture supernatant with aqueous solution of 1 mM silver nitrate in 1:4 (pH 9.0-9.5), which was incubated at 37º C for 24 hours (Figure 1). Flask containing culture supernatant of *B.subtilis* along with 100 mM glucose and silver nitrate showed intense colour change than the method of synthesizing silver nanoparticles using culture supernatant alone. Intense colour change suggested that the synthesis of silver nanoparticles may be more in the case of this method.

2. UV-visible spectrophotometer analysis

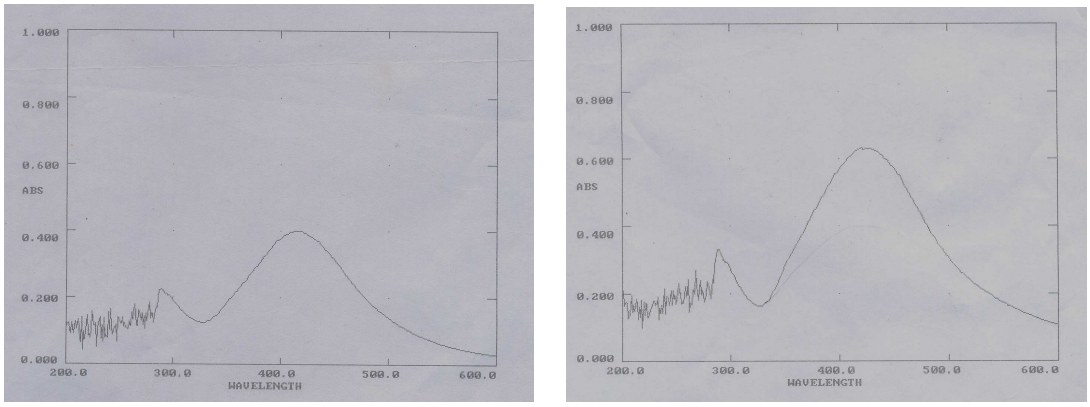
The synthesis of silver nanoparticles by reduction of aqueous metal ions during exposure of *B.subtilis* supernatant can be easily monitored by using UV-visible spectrophotometer. Figure 2 illustrates the absorbance spectra of reaction mixture containing aqueous solution of 1 mM silver nitrate and *B.subtilis* culture supernatant after incubation. Reaction mixture showed an absorbance peak at around 425 nm, which is

characteristic of silver nanoparticles, due to its surface plasmon resonance absorption band [15]. In case of synthesis mediated by *B.subtilis* supernatant in presence of 100 mM glucose, the absorbance peak was obtained at around 425 nm.

(a) (b) (c)

Figure 1

(a) Initial reaction mixture containing extract of B.subtilis supernatant and 1 mM silver nitrate in 1:4 ratio (pH 9-9.5); (b) Colour change of reaction mixture bacterial supernatant and 1 mM silver nitrate (c) Colour change in the reaction mixture containing bacterial supernatant, 100 mM glucose and 1 mM silver nitrate after 24 hours.



(a) (b)

Figure 2

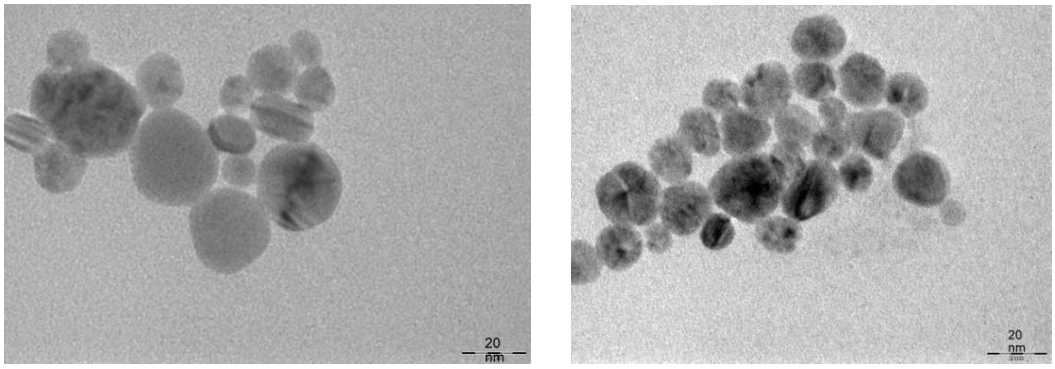
UV- visible spectrophotometer analysis (a) B.subtilis supernatant mediated silver nanoparticles synthesis, (b) B.subtilis supernatant along with 100 mM glucose mediated silver nanoparticles synthesis.

3. NTA measurements

NTA measurements revealed that the mean size of synthesized silver nanoparticles was found to be 47 nm with concentration of 6.3 × 10^{10} particles/ml in case of Bacterial supernatant mediated synthesis. The mean size of silver nanoparticles synthesized using bacterial supernatant in presence of glucose was found to be 39 nm with concentration of 9.2 ×10^{10} particles/ml.

4. TEM and EDX analyses

TEM analysis revealed that the silver nanoparticles are prominently spherical (Figure 3). The silver nanoparticles were found to be well dispersed from each other. The EDX analysis revealed that the silver is present in the solution (Figure 4).



(a) **(b)**

Figure 3

TEM micrograph of silver nanoparticles (a) B.subtilis supernatant mediated silver nanoparticles synthesis, (b) B.subtilis supernatant along with 100 mM glucose mediated silver nanoparticles synthesis.

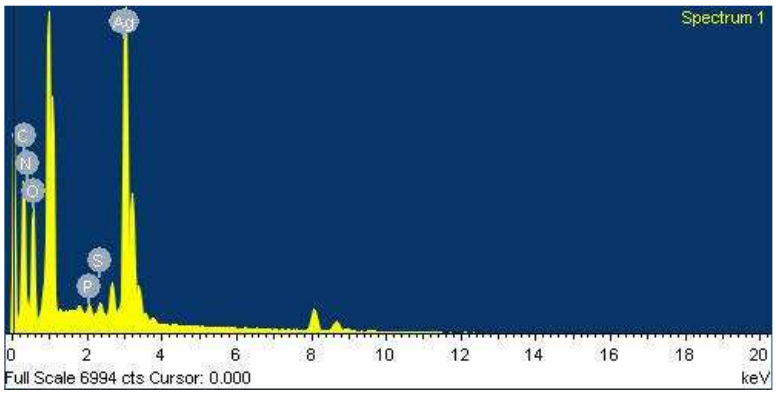


Figure 4.

EDX spectra of silver nanoparticles solution.

5. Quorum quenching effect of silver nanoparticles on Staphylococcus aureus biofilm:

Silver nanoparticles showed quenching of biofilm which can be compared with controls (Figure 5). Antibiotics failed to show biofilm quenching alone which can be compared with negative control. Silver nitrate (1 mM) also did not show any quenching of biofilm.

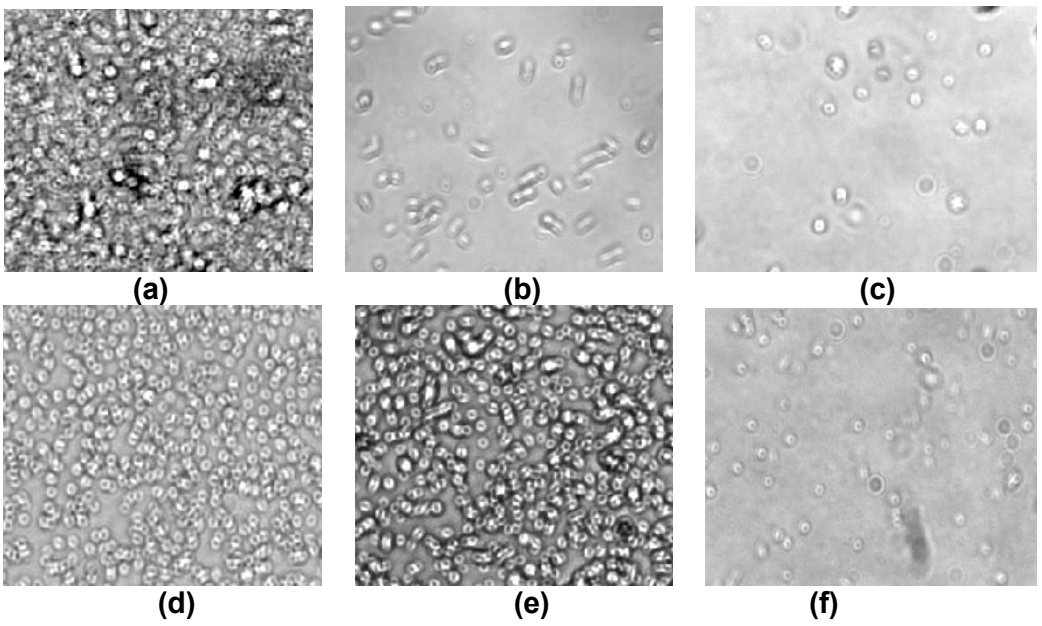


Figure 5

Effect of silver nanoparticles in biofilm quenching and prevention of S.aureus biofim formation viewed under inverted microscope (40X) (a): Negative control (intact biofilm); (b): Positive control (Biofilm Quenched using 20% SDS); (c): Silver nanoparticles; (d): Gentamicin (10 µg/ml); (e): Chloramphenicol (20 µg/ml); (f): 1mM silver nitrate.

CONCLUSION

The green synthesis of silver nanoparticles is a basic need in the field of nanotechnology. In present study, the silver nanoparticles were synthesized using *B.subtilis* supernatant alone and *B.subtilis* supernatant in presence of glucose. The more synthesis of silver nanoparticles was achieved in case of *B.subtilis* supernatant in presence of glucose. The exact role of glucose in enhancing the rate of reaction of synthesis of silver nanoparticles will be the field of research interest. Previously silver nanoparticles were synthesized using *B.subtilis* [16-17]. But time required for the synthesis of silver nanoparticles was more[16]. Secondly, microwave irradiation mediated synthesis of silver nanoparticles[17] can be problematic because it involves large amount of energy utilization. The present study deals with relatively rapid, eco-friendly and cost effective synthesis of silver nanoparticles with its utilization in anti-biofilm studies.

Biofilms are difficult to break due to their tough extracellular polysaccharide matrix. In present study, effect of silver nanoparticles (synthesized using green, eco-friendly method) in biofilm quenching has been demonstrated. From results it can be concluded that silver nanoparticles can involve in biofilm quenching. But, antibiotics failed to show any quenching of bacterial biofilm. The exact mechanism of action of silver nanoparticles in biofilm related studies is yet to be demonstrated. It is known that the excretion of adhesive substances (polysaccharides and proteins) is crucial for the initial attachment of organisms as well as for holding the biofilm bacteria together [2]. The silver nanoparticles might be involved in neutralizing these adhesive substances, thus preventing further biofilm formation. The bacterial biofilms are highly resistant to antibiotics, but in near future, the silver nanoparticles may play major role in the treatment of infections caused due to biofilm.

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REFERENCES

1. Bowden G.H. and Hamilton I.R., Survival of oral bacteria. Crit Rev Oral Biol Med, 9: 54–84 (1998)
2. Lewis K., The riddle of biofilm resistance. Antimicrob Agents Chemother, 45: 999–1007 (2001)
3. Costerton J.W., Stewart P., and Greenberg E.P., Bacterial biofilms: a common cause of persistent infections. Science, 284: 1318–1322 (1999)
4. Ahmad A., Mukherjee P., Senapati S., Mandal D, Khan M.I., and Kumar R., Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum*. Colloids Surf B., 28: 313-318 (2003)
5. Kathiresan K., Manivannan S., Nabeel M.A., and Dhivya B., Studies on silver nanoparticles synthesized by a marine fungus *Penicillium fellutanum* isolated from coastal mangrove sediment. Colloids Surf B., 71 (1): 133-137 (2009)
6. Raut R., Lakkakula J.R., Kolekar N., Mendhulkar V.D., and Kashid, S.B., Extracellular synthesis of silver nanoparticles using dried leaves of *Pongamia pinnata* (L) pierre. Nano-Micro Lett., 2 (2): 106-113 (2009)
7. Masurkar S.A., Chaudhari P.R., Shidore V.B., and Kamble S.P., Rapid biosynthesis of silver nanoparticles using *Cymbopogan citratus* (Lemongrass) and its antimicrobial activity. Nano-Micro Lett., 3 (3): 189-194 (2011)
8. Kalimuthu K., Babu R.S., Venkataraman D., Mohd B., and Gurunathan S, Biosynthesis of silver nanocrystals by *Bacillus licheniformis*. Colloids Surf B., 65: 150-153 (2008)
9. Jain D., Kachhwaha S., Jain R., Srivastava G., and Kothari S.L., Novel microbial route to synthesize silver nanoparticles using spore crystal mixture of *Bacillus thuringiensis*. Ind J Exp Biol., 48 (11): 1152-1156 (2012)
10. Schmid G, Large clusters and colloids. Chemical Reviews, 92(8): 1709-1727 (1992)
11. Monroe D., Looking for Chinks in the Armor of Bacterial Biofilms. PloS Biol, 5 (11): 2458-2461 (2007)
12. Darroudi M., Ahmad M.B., Abdlahul A.H., Ibrahim N.A., and Shameli K., Effect of Accelerator in Green Synthesis of Silver Nanoparticles. Int J Mol Sci., 11(10): 3898-3905 (2010)
13. Kaplan J.B., Ragunath C., Ramasubbu N. and Daniel H., Fine Detachment of *Actinobacillus actinomycetemcomitans* Biofilm Cells by an Endogenous Hexosaminidase Activity. J Bacteriol, 185 (16): 4693–4698 (2003)
14. King E.O., and Tatum H.W., *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. J Infect Dis., 111: 85–94 (1962)
15. Akanna, S., Prasad, K.V., Elumalai, E.K. and Savithramma, N., Production of biogenic silver nanoparticles using *Boswellia ovalifoliolata* stem bark. Digest J. Nanomat. Biostruct., 5 (2): 369-372 (2010)
16. Reddy A., Chen C.Y., Chen C.C., Jean J.J., Chen H.H, Tseng M.J, Fan C.W, Wang J.C., Biological Synthesis of Gold and Silver Nanoparticles Mediated by the Bacteria *Bacillus subtilis*. J Nanosci and Nanotech., 10 (12), 6567-6574 (2010)
17. Saifuddin N., Wong C.W., Nur yasumira A.A., Rapid biosynthesis of silver nanoparticles using culture supernatant of bacteria with microwave irradiation. E J Chem., 6 (1), 61-70 (2009)