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Biological synthesis of gold nanoparticles by *Bacillus subtilis* and evaluation of increased antimicrobial activity against clinical isolates

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Abstract−Biological sources of microorganisms and plants are playing a major role in the reduction of metallic nanoparticles such as silver and gold, as it emerges as an eco-friendly and exciting approach in nanotechnology. We report on the biological synthesis of gold nanoparticles using the culture supernatant of *Bacillus subtilis* and its effect on increased antibacterial and antifungal activities against clinically isolated organism. When the supernatant of *Bacillus subtilis* was added to $HAuCl_4$ aqueous solution, $HAuCl_4$ was reduced as Au^+ ions, which confirmed the presence of nanoparticles by the color change of pale yellow to purple. The minimum and maximum peaks were observed at 24th and 120th hours by UV-Visible spectroscopy. The combined antibacterial and antifungal activities with various antibiotics were observed against clinical isolates.

Key words: AuNP's, *Bacillus subtilis*, UV-visible Spectroscopy, Antimicrobial Activity, Antibiotics

INTRODUCTION

Gold nanoparticles have been used for more than 400 years for the treatment of certain diseases and the staining of glass and enamels [1]. In recent researches, the synthesis of nanoparticles using biological sources has been used and become very popular in the field of nanotechnology. Nanosized particles of noble metals, especially gold nanoparticles (AuNPs), have received great interest due to their attractive electronic, optical, and thermal properties as well as catalytic properties and potential applications in the fields of physics, chemistry, biology, medicine, and material science [2]. Therefore, the synthesis and characterization of gold nanoparticles have a significant role in the field of nanotechnology. Metal-microbe interactions have an important role in several biotechnological applications including the fields of bioremediation, biomineralization, bioleaching and microbial corrosion [3] and in the recent scenario, microorganisms considered as potential biofactory for the synthesis of metallic nanoparticles such as silver, gold and cadmium sulfide.

Nanoparticles can be synthesized mainly by three methods, with the chemical method being the most popular. However, some chemical methods cannot avoid the use of toxic chemicals in the synthesis protocol. Since noble metal nanoparticles such as gold, silver and platinum nanoparticles are widely applied to human contacting areas, there is a growing need to develop environmentally friendly processes of nanoparticle synthesis that do not use toxic chemicals [4]. Extracellular and intracellular preparations of metallic nanoparticles, including both silver and gold have previously been studied [5,6]. Plants have also been studied previously to produce metal nanoparticles, including gold, silver etc [7-9].

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An earlier study found that *Bacillus subtilis* 168 was able to reduce Au^{3+} ions to gold nanoparticles with a size range of 5-25 nm inside the cell walls [10]. Recent studies also reported on the biological synthesis of gold and silver nanoparticles mediated by the bacteria *Bacillus subtilis* [11], and Balagurunathan et al. [12] studied the synthesis of gold nanoparticles by *Streptomyces viridogens* strain HM10 and its antimicrobial activity [12]. Similarly, Sowrah et al., 2011 also reported on the antimicrobial activities of gold nanoparticles against food-borne pathogens, and drugs coated with nanoparticles were highly effective against tested isolates so that Au nanoparticles can minimize treatment durations and side effects of drugs [13]. Asharani et al.'s [14] report reveals the comparative toxicity on silver (Ag-NP, 5-35 nm), gold (Au-NP, 15-35 nm) and platinum (Pt-NP, 3-10 nm) nanoparticles using zebra fish embryo. Among the nanoparticles studied, Ag-NP's were found to be the most toxic and Au-NP's are the non-toxic [14].

For the past decades, gold nanoparticles (NPs) have attracted significant interest as a novel platform for various applications such as nanobiotechnology and biomedicine because of convenient surface bioconjugation with molecular probes and remarkable plasmon-resonant optical properties [15].

Conjugation of gold NPs with antibiotics and antibodies also has been used for selective photothermal killing of protozoa and bacteria [16-18]. With respect to antibacterial activity, the study showed that gold NPs do not affect bacterial growth or functional activity [19], whereas conjugates of vancomycin to gold NPs decrease the number of growing bacterial cells [17]. The synthesized stable gold NP's covered with vancomycin showed significant enhancement of antibacterial activity for this conjugate, in comparison with the activity of the free antibiotic [20].

There are many reports on the synthesis and antimicrobial activity of silver nanoparticles [21-24], but in the case of gold nanopar-

ticles very few studies have been conducted, and it also has been reported as non-toxic compared to other metallic nanoparticles such as silver and platinum nanoparticles [14]. Therefore, in the present study *Bacillus subtilis* was used, as it is easily isolatable and available in all the microbiology laboratories. Supernatant of the organism was used as reducing agent to produce gold nanoparticles, and these nanoparticles were confirmed by color changes and characterized by UV-Visible spectroscopy. Also, the synthesized nanoparticles were used for the evaluation of increased antifungal and antibacterial activity of different antibiotics against clinically isolated organism.

METHODS AND MATERIALS

1. Bacterial Strain and Growth Condition

The soil isolate was biochemically characterized as *Bacillus subtilis*, maintained on nutrient agar at 34 ºC and it is sub cultured weekly once for further use. For the preparation of supernatant, the slant culture was inoculated in 100 ml of nutrient broth medium in a flask. The flask was then incubated on a shaker at 34 ºC for 24 hours. After incubation period, 20 ml of culture was transferred into centrifuge tubes and centrifuged at 10,000 rpm for 5 minutes. The culture supernatant thus obtained was used for the synthesis of gold nanoparticles.

2. Synthesis of Gold Nanoparticles

For the biosynthesis of Au-NPs (Gold nanoparticles), two boiling test tubes were taken, one containing 10 ml of 1 mM Hydrogen tetra chloro aurate (Himedia, Mumbai) solution as control and the second tube containing 9 ml of 1 mM Hydrogen tetra chloro aurate solution and 1 ml supernatant of *Bacillus subtilis* as test solution were incubated on shaker at room temperature for 24 hours. After 24 hours, the cell free supernatant of gold nanoparticle solution thus obtained was purified by repeated centrifugation at 15,000 rpm for 20 minutes. Supernatant was discarded and the pellet was dissolved in deionized water.

3. Characterization

The gold nanoparticles were confirmed by color changes and characterized by UV-visible spectrophotometer on a Perkin Elmer (Lamda 25).

4. Antimicrobial Activity

The antimicrobial activities of gold nanoparticles were investigated by Kirby-Bauer disc diffusion method. For antibacterial activity, clinical isolates of *Shigella sonnie* from KMCH, Coimbatore (India) were used as test strain. A loop of single colony of each test strain was grown in a flask containing nutrient broth on a shaker at 35 ºC for overnight. For antifungal activity, clinical isolates of *Candida albicans* from KMCH, Coimbatore (India) were used as test strains. A single colony of each test strain was grown in a flask containing nutrient broth for 24 hrs at 34 ºC and potato dextrose broth for 48 hrs at 30 ºC in a shaker.

5. Antibacterial Activity

To determine the combined effects of each standard antibiotic disk was further impregnated with 10 µl of freshly prepared gold nanoparticle solution at a final content of 10 µg/disk. Sterilized petri plates were taken, poured sterilized Muller Hinton agar and divided into two halves using a marker. Ten petri plates were swabbed with *Shigella sonnie*. On one half of the petri plate that was swabbed with *Shigella sonnie*, standard antibiotic disk, that is, Gentamycin was placed and on the other half, the antibiotic disk that was impregnated with gold nanoparticle solution was placed.

The above-mentioned procedure was repeated with the remaining antibiotic disks for *Shigella sonnie*. The Petri plates were incubated at room temperature. After incubation at 35 ºC for 24 hours, various zones of inhibition were measured. The resulting zones of inhibition will be uniformly circular. The diameter of the zones of complete inhibition was measured, including the diameter of the disk. The zones were measured to the nearest whole diameter with a ruler.

6. Antifungal Activity

The above-mentioned procedure was repeated on the potato dextrose agar at 30 ºC for 48 hours against clinical isolate of *Candida albicans.*

RESULTS AND DISCUSSION

1. Biological Synthesis and Characterization

Based on the report of Beveridge and Murray [10], Reddy [11] *Bacillus subtilis* organism was used to reduce the aqueous chloroaurate ions into gold nanoparticles. The nanoparticle formation was confirmed from the appearance of purple color from the pale yellow (Fig. 1). Control experiments without supernatant addition stayed

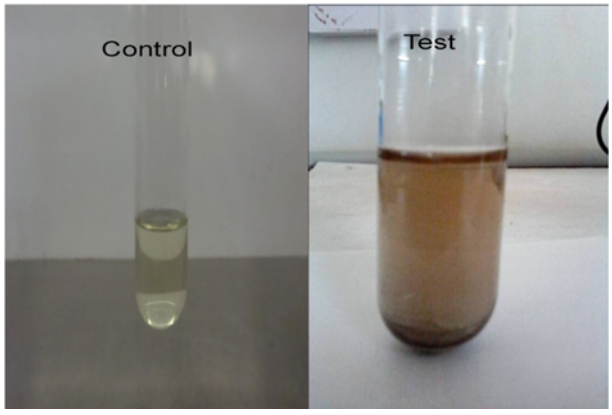


Fig. 1. Picture of boiling test tubes without supernatant containing $HAuCl_4$ solution (control). After exposure of supernatant of the *Bacillus subtilis* in aqueous solution of 10^{-3} $HAuCl_4$ (test).

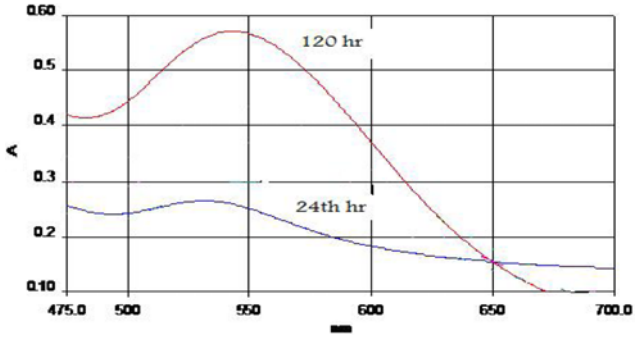
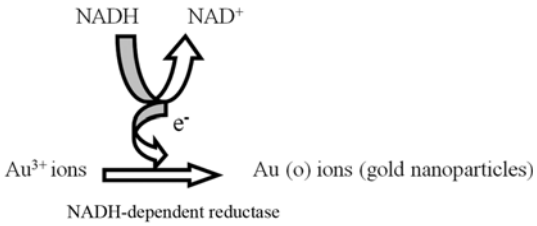


Fig. 2. UV - visible spectra recorded at minimum and maximum intervals. The minimum synthesis was observed after 24 hrs and maximum synthesis was observed after 120 hrs between the range of 530 nm and 540 nm.

pale yellow, indicating that the synthesis of gold nanoparticles was obtained by the reduction of microorganisms indeed. The UV-visible spectra showed a strong Plasmon resonance centered approximately minimum at about 530 nm after 24 hours (Curve 1 of Fig. 2) and maximum at about 540 nm after 120 hours (Curve 2 of Fig. 2). Observation of this strong broad plasmon peak has been well documented for various Me-NPs, with sizes ranging all the way from 2 to 100 nm [15].

Previous studies [12-15] clearly indicated that NADH-and NADH-dependent enzymes are important factors in the biosynthesis of metal nanoparticles. Therefore, *Bacillus subtilis* is also known to secrete cofactor NADH- and NADH dependent enzymes that may be responsible for the bioreduction of $Au^{(3+)}$ ions to $Au^{(0)}$ ions and the subsequent formation of gold nanoparticles. The reduction seems to be initiated by electron transfer from the NADH by NADH-dependent reductase as electron carrier. Then the gold ions obtain electrons and are reduced to Au (0).



Equation shows the possible mechanism of Gold ions (nanoparticles) formation by microorganism.

The combined effect of increased antibacterial (Fig. 3, Graph 1) and antifungal (Fig. 4, Graph 2) activity of different antibiotics in the presence and absence of synthesized gold nanoparticles was studied.

2. Antibacterial Activity

Fig. 3 and Graph 1 show the antibacterial activities of different antibiotics. Among which Norfloxacin showed the increased activity in the presence of gold nanoparticles, and also increased effect was observed with Ciprofloxacin, Nitrofurantoin and Chloramphenicol only in the presence of nanoparticles, but no effect was observed with these three antibiotics alone against *Shigella sonnie*. There was not much effect with other antibiotics except these four antibiotics. No effect was observed on control (AuNP's alone).

3. Antifungal Activity

Antifungal activities of Gentamycin, Norfloxacin and Ciprofloxacin were increased in the presence of gold nanoparticles (Fig. 4, Graph 2). No increased effect was observed with Chloramphenicol and Kanamycin, but its activity was increased in the presence of gold nanoparticles. No effect was observed on control (AuNP's alone) against *Candida albicans*. There was no increased effect in the remaining antibiotics.

According to Williams et al. [19] and Huang et al. [17], the gold NP's do not affect bacterial growth or functional activity, whereas conjugates of vancomycin to gold NP's decrease the number of growing bacterial cells. Nirmala Grace and Pandian [25] reports reveal that the antibacterial properties of aminoglycosidic drugs coated gold nanoparticles were higher than compared with the pure drugs [25]. Gold nanoparticles act as a good anchor carrying more amounts

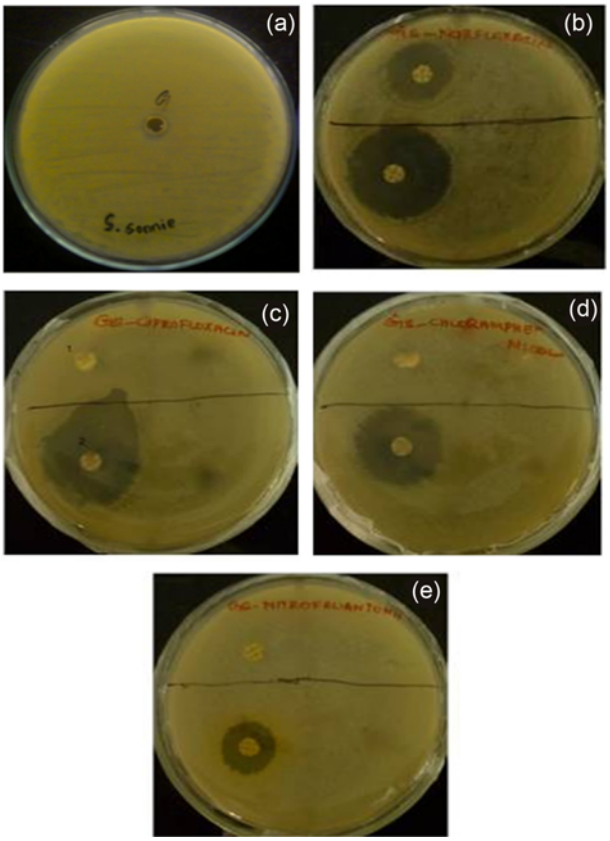
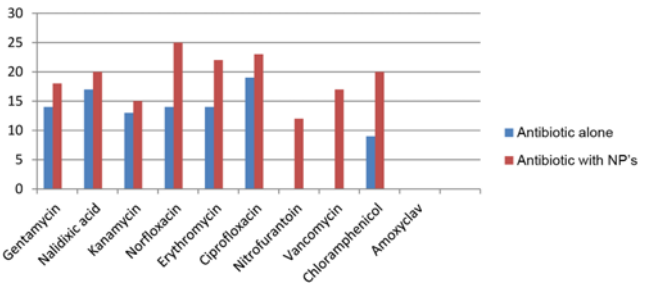


Fig. 3. The top well is impregnated with different antibiotics and the bottom well is impregnated with antibiotics with synthesized gold nanoparticles against *Shigella sonnie*. (a) Control (AuNP's), (b) Norfloxacin, (c) Ciprofloxacin, (d) Chloramphenicol, (e) Nitrofurontain.



Graph 1. Shows the increased antibacterial activity in the presence of antibiotic with AuNP's than antibiotic alone against *Shigella sonnie*.

of drugs on the surface via electrostatic attraction between the amine groups of drugs and nanoparticles which give a better activity [13].

Similar to the Williams et al. [19] and Huang et al. [17] report, our synthesized gold nanoparticles also do not affect the bacterial growth or functional activity in the absence of gold nanoparticles (Figs. 3(a), 4(a)), whereas for conjugates of various antibiotics with gold nanoparticles the activity was increased.

The small size of Au(0) having large surface area and high penetrating power might be the reason for the enhanced activity, and hence such nanoparticles could effectively bind to the substrates on the outer membrane and cell membranes of organisms. The drugs

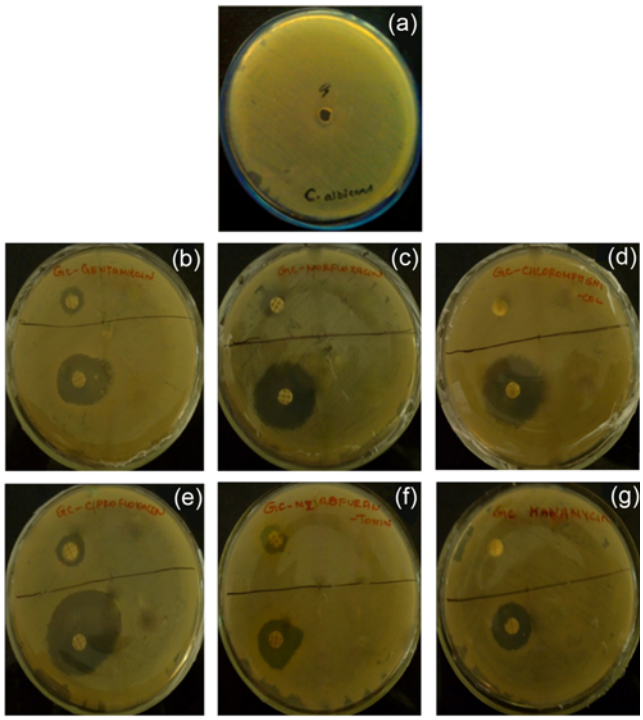
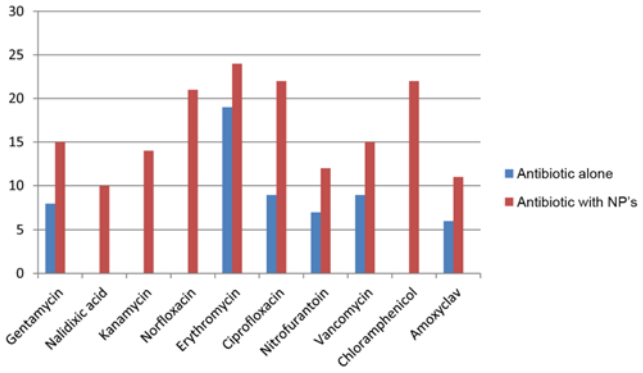


Fig. 4. The top well is impregnated with different antibiotics and the bottom well is impregnated with antibiotics with synthesized gold nanoparticles against *Candida albicans*. (a) Control, (b) Centamycin, (c) Norfloxacin, (d) Chloramphenicol, (e) Ciprofloxacin, (f) Nitrofurantoin, (g) Kanamycin.



Graph 2. Shows the increased antifungal activity in the presence of antibiotic with AuNP's than antibiotic alone against *Cantida albicans*.

get adsorbed on the surface of gold nanoparticles by chelation because aminoglycosidic drugs contain active groups that can easily react with nanogold, and hence the antimicrobial groups come in close proximity with a nanogold core surrounded by aminoglycosidic drug moieties. A single gold nanoparticle is surrounded by a number of drug moieties; hence these drug capped gold particles act as a single group against the microbial organisms [25].

Similarly, it is believed that nanomaterials release ions, which react with the thiol group (-SH) of the proteins present on the bacterial surface. Such proteins protrude through the bacterial cell membrane, allowing the transport of nutrients through the cell wall. Nanomaterials inactivate the proteins, decreasing the membrane permeability and eventually causing the cellular death [26].

We also believe that the above-mentioned points might be the reasons for enhanced antimicrobial activity of the synthesized nanoparticles along with various antibiotics against both clinical isolates. Further study is needed in this direction.

CONCLUSION

Gold nanoparticles were synthesized by using *Bacillus subtilis* supernatant as a reducing agent, and its effect on increased antibacterial and antifungal activities with various antibiotics was investigated: increased activities were observed against both the test organisms. We conclude that this is also one of the reports concerning the combined effect of increasing the antifungal and antibacterial activities. This kind of study may also make a possible platform in future for preparing nanomedicines for bacterial- and fungal-related diseases. In our future studies, the synthesized nanoparticles will be applied with food preservative agents in order to prepare the antimicrobial packaging system to extend the product shelf life and maintain food safety by reducing the growth rate of microorganisms.

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