Biochemical synthesis of silver nanoparticles using filamentous fungi *Penicillium decumbens* (MTCC-2494) and its efficacy against A-549 lung cancer cell line

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[ABSTRACT] Biosynthesis of silver and other metallic nanoparticles is one of the emerging research area in the field of science and technology due to their potentiality, especially in the field of nano-biotechnology and biomedical sciences in order to develop nanomedicine. In our present study, *Penicillium decumbens* (MTCC-2494) was brought from Institute of Microbial Technology (IMTECH) Chandigarh and employed for extracellular biological synthesis of silver nanoparticles. Ag-NPs formation was appeared with a dark brown color inside the conical flask. Characterization of Ag-NPs were done by UV-Spectrophotometric analysis which showed absorption peak at 430 nm determines the presence of nanoparticles, Fourier transform infrared (FT-IR) spectroscopic analysis, showed amines and amides are the possible proteins involved in the stabilization of nanoparticles as capping agent. Atomic force Microscopy (AFM) confirmed the particle are spherical, size was around 30 to 60 nm and also the roughness of nanoparticles. Field emission scanning electron microscopy (FE-SEM) showed the topology of the nanoparticles and were spherical in shape. The biosynthesis process was found fast, ecofriendly and cost effective. Nano-silver particle was found to have a broad antimicrobial activity and also it showed good enhancement of antimicrobial activity of Carbenicillin, Piperacillin, Cefixime, Amoxicillin, Ofloxacin and Sparfloxacin in a synergistic mode. These Ag-NPs showed good anti-cancer activity at 80 µg·mL⁻¹ upon 24 hours of incubation and toxicity increases upon 48 hours of incubation against A-549 human lung cancer cell line and the synergistic formulation of the antibiotic with the synthesized nanoparticles was found more effective against the pathogenic bacteria studied.

[KEY WORDS] Silver nanoparticles; *Penicillium decumbens* (MTCC-2494); Antibacterial; Anti-cancer


Introduction

Resistance developed by the pathogenic bacterial and fungal strains to commercially available antimicrobial drugs or antibiotics has become increasing alarming and has emerged as a serious menace in the recent times [1-3]. Microorganisms available in the environment are pathogenic and causes severe dysfunctions in human beings [4-5]. There is an urgent need of newer antimicrobial nanomedicine either from natural or from inorganic substances [6-8]. Silver has been employed from the ancient times to overcome infections in human as well as animals as inorganic drugs. The antibacterial efficacy of silver and its compounds has been thoroughly investigated against pathogenic bacteria and the research have shown a remarkable development in recent years [7,8]. Due to the antibiotic resistance developed by pathogenic bacteria, research in nanobiotechnology has been shifted to a new era in finding out new antimicrobial drugs to combat the multi drug resistant pathogen [9]. Nanobiotechnology is an advanced technology which aims to control matter at low molecular and atomic level. In the present century nanobiotechnology is gained much attention to the researchers due to its unique properties like small size with high efficacy to the target site. Various advanced procedures like biological, chemical and physical method are used for the biosynthesis of silver nanoparticles and the biological method offers an cost effective and ecofriendly environment[10].

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Synthesis of nanoparticles from noble metal have been studied extensively due to their magnetic, optical, catalytic, antimicrobial and electronic properties[16]. Several fungi have been used for the biosynthesis of nanoparticles since they have advantage over plants and bacteria for having contained enzyme reductase, hydrogenase or electron shuttle quinone that is responsible for the reduction of silver ions in to nano silver[11-12]. Antibacterial activity of specially formulated metal noble nanoparticles have been confirmed [15] and antimicrobial formulations based on nanoparticles could be bactericidal as an effective materials [13-15].

In the present investigation we have reported the extracellular synthesis of Silver nanoparticles from Penicillium decumbens (MTCC 2494). This was followed by characterization of nanoparticles and assay for antimicrobial activity against few MDRs. In the study, nanosilver were also evaluated for its antibacterial property against pathogenic bacteria (MDR) and also evaluate in combination with different antibiotics like Amoxicillin, Piperacillin, Cefixime, Carbencillin and Ofloxacin to assess their bactericidal activity against MDRs. These nanoparticles were further checked for its anticancer activity against A-549 lung cancer cell line and cytotoxicity effect on normal Vero cells.

Materials and Methods

Penicillium decumbens (MTCC 2494) fungus was brought from Institute of Microbial Technology (IMTECH) Chandigarh, India. The fungal culture was sub cultured on Potato Dextrose Agar (PDA) medium during this study.

Synthesis of silver nanoparticles

The biomass of *P. decumbens* (MTCC-2494) was produced aerobically in a medium containing (g·L−1): yeast extract 0.6; MgSO4.7 H2O, 0.1; glucose, 10.0; (NH4)2SO4, 1.0; KH2PO4, 7.0; K2HPO4, 2.0 for 72 h at (25 ± 3) °C. After sufficient growth of biomass after grown the media was filtered through Whatman filter paper No. 1 and thoroughly washed 3–4 times with Milli-Q water to remove debris and media components. After washing, clean and fresh biomass of fungal species was taken into the flasks containing 100 mL of deionized Milli-Q water and incubated at 25 °C with 140 r·min−1 on orbital shaker for 72 h following incubation the biomass was sonicated for ten min and filtered through Whatman filter paper and the cell free extract was further used in the experiment. The cell free extract was added to 1 mmol·L−1 of AgNO3 solution and kept in a shaker at 25 °C and 140 r·min−1 in dark condition.

Characterization of AgNPs

The change in color of the solution was observed and absorbance was measured using Cary 100 UV-visible spectrophotometer. The sample was further subjected to FT-IR analysis to determine the components for stabilization of nanoparticles. Briefly 2 mg of the sample was taken and pressed to form the thin pellet on cover slip. Sample holder was used to keep the sample and FT-IR spectra were analyzed. The sample was further characterized by AFM to check the roughness, particle size, agglomeration and shape of the nanoparticles. For AFM analysis sample was prepared by sonicating the liquid sample for 5 min followed by centrifugation at 20 000 r·min−1, made a thin film of the pellet on cover slip and subject for AFM analysis. Silver nanoparticles were further analyzed by FE-SEM analysis which is used to determine the size, shape and surface morphology of nanoparticles. For FE-SEM sample was prepared by centrifuging the liquid sample for 10 min at 20 000 r·min−1. Supernatant was discarded and pellet was dried to make it into powder form and subjected for FE-SEM analysis.

Antibacterial analysis

The multi drug resistant (MDR) pathogens were isolated and reviewed from the urine, stool and blood culture, and data were included for analysis. Then AgNPs were analyzed for its antibacterial effect using disc diffusion method[16] against various pathogenic Gram negative and Gram positive bacteria isolated from the collected sample, such as *Proteus vulgaris*, *Escherichia coli*, *Staphylococcus aureus* and *Vibrio cholera*. Infections with multi-drug resistant *Proteus vulgaris* and *E. coli*, is the leading cause of hospital-acquired and community urinary tract infections (UTIs). Twenty µg of silver nanoparticles was used along with standard antibiotic discs such as Piperacillin, Carbencillin, Cefixime, Amoxicillin and Ofloxacin. AgNPs alone were evaluated for its antibacterial property and also in combination with Carbencillin, Piperacillin, Cefixime, Amoxicillin and Ofloxacin to assess their bactericidal activity against pathogenic bacteria (MDR). Inhibition zone was measured after 24 h incubation at 37 °C. Experiment repeated three times and data were analyzed by standard deviation (SD) and standard error means (SEM).

Anticancer and cytotoxic activity of AgNPs

Cell culture

The human lung cancer cell line A-549 and Vero cell line was brought from National Centre for Cell Science (NCCS) Pune India. The A-549 cells and Vero cells were separately grown in MEM as monolayer supplied with 1% glutamine, 10% fetal bovine serum (FBS), 100 U·mL−1 penicillin and 100 U·mL−1 streptomycin at 37 °C in 5% carbon dioxide (CO2) environment.

Viability of cells

The toxicity of silver nanoparticles on human lung cancer cells (A549) and Vero cells depends on the viability of cells was evaluated by the MTT colorimetric technique. The solution of AgNPs was formed in sterile distilled H2O and just dilute it up to the certain concentrations like (20, 40, 60, 80, 100 and 120 g·mL−1) by applying the medium of cell culture. The required concentration of AgNPs (W/P) of that was added to the cultured cell in the wells to obtain AgNPs final respective concentration and then incubated for 24 and 48 h at 37 °C. Alongside cells treated without AgNPS were used as...
control. After 24 and 48 h of incubation cells were gently washed with the solution of Phosphate buffer, then 100 μL of (2,5-diphenyltetrazolium bromide (3-(4,5-dimethylthiazolyl-2)- yellow), tetrazolium MTT solution was added to each of the titer well. After that microtiter plates were kept for incubation at 37 °C for 2–3 h, so that MTT dye will be reduced by normal active cells due to the dehydrogenase enzymes present normally in the mitochondria which reduces NADH in to NADPH. The crystal formed off MTT dye was solubilized by DMSO adding 100 μL to each 96 well plate. These micro titer plates were kept for 10 to 15 min in a shaker, so that the crystals of A-549 cells get completely solubilize was observed by eluting the dye. Spectrophotometer was used to measure optical density (OD) at 595 nm. Cell viability percentage was calculated by applying the formula: (OD of sample/OD of cell control) × 100= % cell viability. Each Experiment was done in triplicate. For full cell viability to check toxicity both Sample and control cell were included in each assay.

Cell viability percentage=$\frac{OD \text{ value sample (Ag NPs)}}{OD \text{ value control}} \times 100$

Cytomorphological changes in A-549 lung cancer cells.

A-549 lung cancer cells and also normal Vero cells separately were treated with different concentrations of biologically synthesized AgNPs and incubate for 24 and 48 h at 37 °C in 5% carbon dioxide environment. After 24 and 48 h of incubation morphological change were observed in the cells under inverted phase contrast microscope (Nikon, Japan).

Result and Discussion

In our present study, P. decumbens (MTCC-2494) was first time employed for the extracellular biological synthesis of silver nanoparticles in laboratory condition. AgNPs were synthesized from Ag⁺ ions by treating the cell free extract of the P. decumbens biomass with 1 mmol·L⁻¹ AgNO₃. The appearance of dark brown color in the conical flask suggested the formation of Ag-NPs. These AgNPs showed the absorbance peak at 430 nm which is specific for silver nanoparticles due to the excitation of surface plasmon vibrations confirmed with the previous author Sankar et al[17,18] (Figs. 1−2).

Fig. 1  Bioynthesis of AgNPs from Penicillium decumbens (MTCC2494)

FT-IR spectroscopic analysis was used to determine the possible proteins, essential molecules and functional groups which mostly involved for the reduction of silver ions in to nanosilver and these proteins caps the nanoparticles and stabilizes them (Fig. 3). FT-IR analysis obtained showed the absorption peaks located at 3 414.48 cm⁻¹ (NH stretch amines), 2 923.8 cm⁻¹ (C-H stretch alkane), 1635.5 cm⁻¹ (C=O stretch of amide), 15.6 cm⁻¹ (NH bend of amines), 1380.8 cm⁻¹ (CH₃ bend alkenes), 1 103.2 cm⁻¹ (CO stretch of carboxylic acid), 617.7 cm⁻¹ (CN stretch of amines) and 547.7 cm⁻¹ (C-Br stretch alkyl halides).

Fig. 3  FTIR analysis of AgNPs synthesized from Penicillium decumbens (MTCC 2494)

The AFM image showed that nanoparticles are well dispersed, agglomerated and are spherical in shape. Whereas histogram analysis showed that nanoparticles have the average particle size in the range of 70 nm (Fig. 4).

Fig. 4  Histogram analysis of AgNPs

FE-SEM analysis showed the average topology of the nanoparticles and also showed that particles are spherical and well dispersed (Fig. 5).

Invitro antibacterial analysis of silver nanoaprticles were carried out against various clinically isolated pathogens, such as Proteus vulgaris, Staphylococcus aureus, Escherichia coli, and Vibrio cholera by disc diffusion method. These nanoparticles showed good antibacterial activity alone and in
Fig. 4  AFM analysis of AgNPs synthesized from *Penicillium decumbens* (A) 2D AFM image, (B) Histogram analysis of AgNPs 3, (C) line profiling analysis of AgNPs

Fig. 5  FESEM analysis of AgNPs synthesized from *Penicillium decumbens* (MTCC2494)

combination with different antibiotics available in the market [19] For checking the antibacterial activity each disc was impregnated with 20 µg·mL⁻¹ of AgNPs and for the checking of synergistic activity of nanoparticles with different antibiotic like Carbenicillin, Pipercillin, Cefixime, Amoxicillin, Ofloxacin, each antibiotic disc were impregnated with half of the nanoparticle concentration (10 µg·mL⁻¹). Carbenicillin, Pipercillin and Ofloxacin showed good activity whereas Cefixime and Amoxicillin showed less activity against bacterial pathogens [19] but their antibacterial activity enhances remarkably in presence of silver nanoparticles as shown in Tables 1 and 2. The results showed were remarkable and satisfactory during the present study. Each experiment was repeated three times and average was calculated by standard deviation (SD).

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Fugal filtrate</th>
<th>AgNPs 20 µg/disc</th>
<th>Carb 100 µg/disc</th>
<th>Carb 100 µg + Nps 10 µg/disc</th>
<th>Pip 100 µg/disc</th>
<th>Pip 100 µg+Nps 10 µg/disc</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vulgaris</em></td>
<td>7 ± 0.72</td>
<td>13 ± 0.66</td>
<td>26 ± 0.38</td>
<td>40 ± 0.14</td>
<td>13 ± 0.56</td>
<td>24 ± 0.88</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>7 ± 0.34</td>
<td>12 ± 0.18</td>
<td>31 ± 0.36</td>
<td>39 ± 0.12</td>
<td>12 ± 0.44</td>
<td>20 ± 0.42</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>9 ± 0.92</td>
<td>13 ± 0.33</td>
<td>33 ± 0.66</td>
<td>38 ± 0.22</td>
<td>14 ± 0.82</td>
<td>21 ± 0.62</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>8 ± 0.27</td>
<td>12 ± 0.58</td>
<td>23 ± 0.74</td>
<td>41 ± 0.16</td>
<td>12 ± 0.33</td>
<td>17 ± 0.44</td>
</tr>
</tbody>
</table>

Carb: Carbenicillin; Pip: Pipercillin; NPs: Nanaoparticles
Table 2  Zone of inhibition (mm) of Cefixime, Amoxicillin and Ofloxacin against test pathogens in presence and absence of silver nanoparticles

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Cef 10 µg/disc</th>
<th>Cef 10 µg + NPs 10 µg/disc</th>
<th>Amox 10 µg/disc</th>
<th>Amox 10 µg + NPs 10 µg/disc</th>
<th>Of 5 µg/disc</th>
<th>Of 5 µg + NPs 10 µg/disc</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vulgaris</em></td>
<td>7 ± 0.77</td>
<td>11 ± 0.86</td>
<td>8 ± 0.83</td>
<td>14 ± 0.74</td>
<td>25 ± 1.74</td>
<td>31 ± 0.59</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>7 ± 0.36</td>
<td>10 ± 0.24</td>
<td>9 ± 0.34</td>
<td>16 ± 0.36</td>
<td>24 ± 0.14</td>
<td>28 ± 0.32</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>9 ± 0.96</td>
<td>12 ± 0.33</td>
<td>11 ± 0.93</td>
<td>16 ± 0.45</td>
<td>23 ± 0.56</td>
<td>30 ± 0.62</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>7 ± 0.99</td>
<td>9 ± 1.36</td>
<td>9 ± 0.68</td>
<td>18 ± 0.76</td>
<td>23 ± 0.21</td>
<td>29 ± 0.34</td>
</tr>
</tbody>
</table>

Cef: Cefixime; Amox: Amoxicillin; Of: Ofloxacin; NPs: Nanoparticles

The in vitro cytotoxic effect of biologically synthesized silver nanoparticles from *P. decumbens* (MTCC-2494) against human alveolar lung cancer cells A-549 cell line. The use of silver nanoparticles against A549 cell line to determine the cytotoxicity by MTT assay. These nanoparticles showed good anticancer activity. Upon using the different concentration IC50 value was shown by 80 µg·mL−1 for 24 h of incubation but their toxicity increases upon further incubation showed IC50 value at 60 µg·mL−1 upon 48 h of incubation as shown in Fig. 6. Alongside, Vero cells were incubated with Silver nanoparticles at various concentrations as A549 cells. After 24 h of incubation, cells treated with AgNPs at 100 µg·mL−1 showed a 50% survival (Fig 7). There was a significant cytotoxic effect in a dose dependent manner with reduced survival rate (20%). Moreover, the cells exposed to AgNPs exhibited severe cell damage compared with the untreated cells. Additionally morphological changes associated with increase in dose such as cell rounding, shrinkage and non-adherence to the surface were observed [20]. As the Vero cells were affected only at higher concentrations (100 µg·mL−1) with respect to the dosage (80 µg·mL−1) fixed for A549 cells. The cytotoxic effect induced by AgNPs at minute concentrations determines its therapeutic potential in bringing about cell death. It was also anticipated that AgNPs increase ROS (reactive oxygen species) causes oxidative distress harming vital enzymes and DNA resulting in DNA strand breaks and necrosis at higher concentrations [21].

Conclusion

Bio synthesis of AgNPs from *P. decumbens* (MTCC 2494) was carried out first time. Silver nanoparticles showed good antimicrobial activity alone and also in combination with different antibiotics enhance their antibacterial property, hence could become a strong antibacterial material also in combination with different antibiotics studied, but needs further experimental study to check its cytotoxicity before using as a strong bactericidal agent. These silver nanoparticles also showed good anticancer activity against A-549 human lung cancer cell line and showed the IC50 value at 80 µg·mL−1 after 24 h of incubation and toxicity increase upon 48 hrs of incubation showed the IC50 value at 60 µg·mL−1, hence could be used as a strong anticancer agent and also can be used as an alternative medicine to treat various dreadful diseases caused by various resistant bacterial strains.

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