Biosynthesis of Gold Nanoparticles by Biotechnologically Important Active and Inactive Form of the Ascomycetes - *Pichia membranifaciens* and *Debaryomyces hansenii*

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Abstract

The two biotechnologically important Ascomycetes, *Pichia membranifaciens* and *Debaryomyces hansenii*, were tested (in active and inactive form), in the present study for efficient bio-reduction of trivalent aurum to gold nanoparticles. Strong surface plasmon resonance was observed, between 530 and 540 nm, from the gold nanoparticles recovered from the test Ascomycetes. The characterization of the gold nanoparticles thus formed was performed by HR-TEM, FE-SEM, FTIR spectroscopy. Comparing the active and inactive form of the two Ascomycetes, the inactive form showed better productivity and recovery of gold nanoparticles. It was also found that *P. membranifaciens* is more productive in terms of time, quality and quantity, than *D. hansenii*. The nanoparticles were relatively monomorphous in *P. membranifaciens* than in *D. hansenii*. The present study also presents a promising outlook towards the effective recycling of the bioactive wastes.

Keywords

Gold nanoparticles, UV-visible analysis

Introduction

Synthesis of nanoparticles of noble metals is of enormous importance due to their biomedical, pharmaceutical, optical, electronic and chemical properties [1-9]. Microbial interactions with metals have been explored for biotechnological application such as ore leaching and metal recovery [10-12]. However, only in the recent past, microbes have gained significant attention for the synthesis of metal nanoparticles. Different microorganisms such as bacteria and fungi from different habitats and nutritional modes have been employed for the synthesis of metal nanoparticles either intra- or extra-cellular [13]. Most of the research in this field has been concentrated on the synthesis of noble metal nanoparticles such as gold and silver [14, 15]. Microorganisms have been a preferred source of synthesis due to ease of culturing, manipulation and downstream processing [13]. Microbes including simple prokaryotes to complex eukaryotic have been explored for the fabrication of nanoparticles. Among them, the yeasts and fungi are more interesting for the biosynthesis of nanoparticles due to the large amounts of secreted proteins and high yields of biomass, which make them more promising for industrial AuNPs production [13-16].

The conventional processes for nanoparticle synthesis involve top-down or bottom-up approaches usually employing physical or chemical protocols. The "Top-down" approach begins with a specific structure which is decreased in size gradually. On the other hand, the "Bottom-up" approach involves formation of nanostructures through the self-assembly of atoms and molecules. However, such

processes are often energy-demanding and cost-intensive [17]. Moreover, the chemicals used in such procedures are toxic and the size of the nanoparticles is difficult to control. In view of these problems, biological systems have emerged as effective alternatives for the clean and green synthesis of nanostructures [15].

In a previous study conducted by our group, we have already explored the intracellular and extracellular gold nanoparticles synthesis ability of *Aspergillus oryzae* var. *viridis* [18]. It is an economically important fungus used for production of rice koji in Japan. Continuing the same work, here we explore the two ascomycetes, *Pichia membranifaciens* and *Debaryomyces hansenii*, known as antagonists against diverse group of phyto-pathogens infecting the grape plant and are thus used as bio-control agents [19, 20]. *Debaryomyces hansenii* has biotechnological potential and utility in diversity of areas [21, 22]. This paper describes the conditions for the synthesis of AuNPs (by Live and inactive cells) and their characterization by a variety of analytical techniques.

Materials and Methods

Materials

$\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (99%) was supplied by Sigma-Aldrich. GPY agar/ broth (Glucose Peptone Yeast extract), YPD broth (Yeast extract Peptone Dextrose Broth) were supplied by Becton, Dickinson and Company (BD), USA. *Pichia membranifaciens* (KCTC: 17999), *Debaryomyces hansenii* (KCTC: 17995) were supplied by Korean Collection for Type Cultures, Daejeon, Republic of Korea.

Yeast growth condition and biomass production

Stock culture of *P. membranifaciens* and *D. hansenii* was prepared on GPY agar plate. For production of biomass, the organisms were grown aerobically in sterile YPD broth and GPY broth, respectively. The flasks were inoculated and incubated on orbital shaker at 25 °C and 230 rpm. The culture was transferred equally to 50 mL falcon tubes (For each strain), after 24 hours of incubation. The yeast biomass was harvested by centrifugation (6000 g, 10 min at 10 °C) and the pellet was washed thrice to remove residual glucose. The washed pellets were re-suspended in 10 mL distilled water and 3 of the 6 tubes containing the live cell biomass were autoclaved (121 °C, 15 psi, 15 min).

Synthesis and recovery of gold nanoparticles

AuNPs were synthesized by incubating the live and autoclaved yeast biomass in 1 mM HAuCl_4 solution on orbital shaker at 25 °C and 250 rpm. The total volume of reaction mixture was 50 mL. After synthesis the reaction tubes were centrifuged (6000 g, 10 min at 10 °C), and the supernatant containing only AuNPs was collected and used for characterization. Visual changes and UV-visible spectra were recorded. All experiments were carried out in triplicates with positive (Only biomass suspended in water) and negative controls (Only 1 mM HAuCl_4).

Characterization of the gold nanoparticles

The reaction mixtures were monitored over a period of

time and visual observations were recorded. A Shimadzu UV-1800 spectrophotometer was used for UV-visible spectroscopy measurements. Washed yeast cells were coated onto glass pieces and thoroughly dried. XRD measurements of thin films of yeast cells exposed to HAuCl_4 were carried out under vacuum combined with energy dispersive X-ray spectroscopy (EDX) (JEOL JSM-5410LV) and X-ray diffractograms using D/Max 2005, Rigaku. FE-SEM analysis was performed on air-dried, platinum-coated cell samples using an analytical SEM (JEOL JSM-6360A). In order to determine the functional groups involved in nanoparticle synthesis, FTIR spectroscopy was carried out as described earlier [23]. For transmission electron microscope (TEM) observations, samples were coated on carbon-coated copper grids (200 $\mu\text{m} \times 200 \mu\text{m}$ mesh size) and images were obtained on an Orius SC10002 JEM-2010 electron microscope.

Results and Discussion

UV-visible analysis

The preliminary observation and analysis of AuNP formation was based on the change in color of the reaction mixtures of *P. membranifaciens* and *D. hansenii* after 12 and 24 hours, respectively [24]. A reddish-purple transformation from straw yellow could be detected in both active and inactive cell biomass of *P. membranifaciens* and *D. hansenii*, whereas in the control tubes showed no color change [Figure 1]. A more precise detection of AuNP synthesis was done by UV-visible spectroscopy, which is an important technique to investigate surface plasmon resonance (SPR). The UV-visible spectra of AuNPs synthesized by the active and inactive cell biomass of *P. membranifaciens* and *D. hansenii* are presented in Figure 2. As shown in Figure 2, the spectral peak of the AuNPs obtained from active and inactive cell biomass of *D. hansenii* was distinctively broader than that of *P. membranifaciens*. This shows that the nanoparticles from *D. hansenii* are more polymorphic compared to that of *P. membranifaciens* [25].

Figure 1: The preliminary visual observation and analysis. (A) Autoclaved (Inactive) cell biomass suspended in 50 mL distilled water. (B) 1 mM HAuCl_4 solution 50 mL. (C) Autoclaved (Inactive) cell biomass suspended in 50 mL 1 mM HAuCl_4 , imparting a straw color appearance. (D) Reaction mixture showing reddish-purple color after reduction of HAuCl_4 to gold nanoparticles by the inactive cell biomass.

The absorbance level of the nanoparticles synthesized by *P. membranifaciens* is higher than that of the nanoparticles synthesized by *D. hansenii* [Figure 2]. This may be due to the amount of nanoparticles that could be recovered from both the strains.

Figure 2: UV-visible spectra of gold nanoparticles recovered from reaction mixture containing, (A) inactive *P. membranifaciens*, (B) active *P. membranifaciens*, (C) inactive *D. hansenii*, (D) active *D. hansenii*.

TEM, SEM and XRD analysis

Transmission electron microscopic studies were used to analyze the particle size and morphology. Gold nanoparticles synthesized by *D. hansenii* show more polymorphism, with respect to shape and size, as compared to the ones synthesized by *P. membranifaciens* [Figure 3]. Figure 3B shows AuNPs of circular, triangular, pentagonal, hexagonal and irregular shaped nanoparticles [26]. Whereas, Figure 3A show TEM images of discrete gold nanoparticles of less than 50 nm size and they were mostly spherical-pentagonal indicating that it was possible to bio-synthesize AuNPs with satisfactory level of mono-disparity using *P. membranifaciens*. These results are in accordance to our findings in the UV-visible analysis. Non-biological methods have been used in the past for synthesizing highly monodispersed spherical gold nanoparticles by mixing aqueous HAuCl_4 and oleylamine followed by sonication at room temperature [27].

Figure 3: TEM images of gold nanoparticles synthesized by active and inactive (A) *P. membranifaciens* and (B) *D. hansenii*.

High-resolution transmission electron microscope (HR-TEM) is an indispensable tool in nanotechnology. HR-TEM images of the AuNPs were used to confirm the particle morphology and size. The representative HR-TEM images of AuNPs reduced by yeasts, *P. membranifaciens* and *D. hansenii*, were deposited on carbon-coated copper 200 mesh grids and the morphology of the nanoparticles were found to be pentagonal, spherical, triangular and rhombohedral, with a very narrow size distribution [Figure 4]. The particles were found to be stable for more than 4 months after freeze-drying and keeping at room temperature in dry and dark condition.

Figure 4: HR-TEM images of gold nanoparticles synthesized by active and inactive *P. membranifaciens* and *D. hansenii* showing different morphology. (A) Pentagonal (B) Spherical (C) Triangular (D) Rhombohedral.

Field emission scanning electron microscope (FE-SEM) is important technique to investigate the surface phenomenon in detail. FE-SEM has been used in this study to confirm the extracellular synthesis and also autoclaved cell mediated synthesis of AuNPs. FE-SEM analysis of the yeast cells with nanoparticles shows the maximum particle concentration outside or on the cell surface [Figure 5A and 5B]. In case of autoclaved cells, the particles could be seen on the cell

Figure 5: FE-SEM images of gold nanoparticles synthesized by yeast cells. (A) Image of yeast cells with nanoparticles on their surface. (B) Image of nanoparticles near the yeast cells. (C) Image of autoclaved yeast with nanoparticles. (D) Image of nanoparticles on the autoclaved cell debris.

debris [Figure 5C and 5D]. This extracellular and intercellular presence of nanoparticles illustrates the extracellular synthesis of gold nanoparticles. Use of inactive fungal strain of *Aspergillus oryzae* is the only report of inactive cells involved in synthesis of AuNPs [18].

The representative XRD pattern of the prepared gold nanoparticles is shown in Figure 6. The synthesized AuNPs show Bragg reflections indexed to (111), (200), (220) and (311) facets of the face-centered cubic metal gold structure.

Figure 6: X-ray diffraction spectrum of gold nanoparticles synthesized by inactive cell biomass of *P. membranifaciens*.

FTIR analysis

The FTIR profiles of gold loaded, active cell biomass (blue line) and inactive cell biomass (black line), of *P. membranifaciens* is shown in Figure 7. Except for the change in the intensity of the bands, there was no major difference in the peak pattern between the biomaterials used. The peaks represent the functional groups present in the biomaterial and thus the steepness represents the difference in quantity [28]. The two peaks at 1660 and 1530 cm^{-1} are assigned to the amide I and II bands of proteins, respectively. Being a fact that proteins can bind to gold nanoparticles and therefore stabilization of the gold nanoparticles by surface bound proteins is a possibility [29–31]. There is also a stretching vibration of the band representing NH group at 3300 cm^{-1} , C-H group at 2940 cm^{-1} and C-N group at 1054 cm^{-1} [32]. The above data suggest the involvement of C-N and amino groups in the nanoparticle synthesis.

Figure 7: FTIR spectrum of gold nanoparticles synthesized by active and inactive cell biomass of *P. membranifaciens*.

Conclusion

The present study clearly illustrates the significant role of two novel biotechnologically important Ascomycetes, in the synthesis of gold nanoparticles, in inactive and active state. Although, nanoparticles are being efficiently synthesized using chemical and physical methods, however the adverse effects of these methods sought for the discovery of novel sustainable methods. These results may open up a novel path for research communities to identify the particular component of these yeast cells responsible for nanoparticle synthesis even in inactive state. This may also lead to the industrial use of inactive or autoclaved yeast cells for production of bioactive and non-toxic gold nanoparticles.

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