Microbial Production of Gold Nanoparticles

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Abstract

The development of techniques for the synthesis of nanoparticles of well-defined size, shape and composition is a challenge and an important area of research in nanotechnology. Many microorganisms have the ability to produce inorganic nanostructures and metal nanoparticles with properties similar to chemically-synthesised materials, while exercising control over the size, shape and composition of the particles. This alternative approach to chemical synthesis procedures uses microbial systems for the production of nanosized materials. Intracellular synthesis of gold nanoparticles, as well as extracellular formation of nanoparticles in the presence of fungal cell extract has been successfully demonstrated. The possibility to manipulate the size and shape of gold nanoparticles by altering key growth parameters was investigated and the results have provided some understanding as to which parameters may have an effect on the formation of gold nanoparticles.

Introduction

Metal nanoparticles exhibit unique electronic, magnetic, catalytic and optical properties that are different from those of bulk metals (1,2). Gold nanoparticles, in particular, are of interest, mainly due to their stability under atmospheric conditions, resistance to oxidation and biocompatibility (3-6). These unique properties can potentially be exploited in a diverse range of industrial applications using their optical and electronic properties in optics, electronics, medical diagnostics and treatments, sensors and coatings (3,4,7).

The development of techniques for the controlled synthesis of gold nanoparticles of well-defined size and shape is a big challenge and numerous chemical methods, aimed at controlling the physical properties of the particles, are reported in the literature (7-9). Most of these methods are still in the development stage and problems are often experienced with stability of the nanoparticle preparations, control of the crystal growth, and aggregation of the particles (2,6,8,10).

The use of microbial cells for the synthesis of nanosized materials has recently emerged as a novel approach for the synthesis of metal nanoparticles. Although the efforts directed towards the biosynthesis of nanomaterials are recent, the interactions between micro-organisms and metals have been well documented (11-14) and the ability of micro-organisms to extract and/or accumulate metals is employed in commercial biotechnological processes such as bioleaching and bioremediation.

Many microbes are known to produce inorganic nanostructures and metallic nanoparticles with properties similar to chemically-synthesised materials, while exercising strict control over size, shape and composition of the particles. Examples include the formation of magnetic nanoparticles by magnetotactic bacteria (15), the production of silver nanoparticles by *Pseudomonas stutzeri* (16), synthesis of nano-scale, semi-conducting CdS crystals in the yeast *Schizosaccharomyces pombe* (17), and the formation of palladium nanoparticles using sulphate reducing bacteria in the presence of an exogenous electron donor (18). The ability of bacteria, fungi, actinomycetes (19), yeast (20), algae (21) and plants (22) to accumulate gold ions from solution has been reported and the synthesis of gold nanoparticles has been successfully demonstrated in a range of organisms including *Bacillus* sp. (23), fungal species such as *Verticillium* and *Fusarium* (24,25), actinomycete such as *Rhodococcus* (26) and *Thermomonospora* (27) and lactic acid bacteria (28).

The interest also extends to the synthesis of nanostructures such as nanowires and the assembly of nanoparticles using biological templates such as DNA, proteins, S-layers and viruses (29,30).

The objective of this paper is to review the progress that has been made at Mintek on the biosynthesis of gold nanoparticles. A variety of microorganisms have been screened for their ability to accumulate and reduce gold ions

to form nanoparticles. In addition, the possibility to manipulate the size and shape of gold nanoparticles by altering key growth parameters was investigated.

Experimental methods

Selection and isolation of cultures

Pseudomonas stutzeri NCIMB 13420, *Bacillus subtilis* DSM 10, *Pseudomonas putida* DSM 291, *Schizosaccharomyces pombe* DSM 2791, *Schizosaccharomyces pombe* DSM 70576, *Pichia jadinii* UOFS Y-0156, *Pichia jadinii* UOFS Y-0520, *Verticillium dahliae* DSM 63083, *Verticillium luteoalbum* DSM 63545 and *Fusarium oxysporum* DSM 2723 were obtained from The National Collections of Industrial and Marine Bacteria (NCIMB), German National Resource Centre for Biological Material (DSM) and the yeast culture collection of the University of the Free State, South Africa (UOFS). These cultures were selected based on their ability to produce metal nanoparticles (16,17,24) and accumulate gold from solution (13,19,32).

In addition, a variety of bacterial, fungal and yeast cultures, isolated from soil and metal-rich dump samples, were included in the screening programme. Enrichment was performed in shaker incubators at 28°C in Erlenmeyer flasks containing universal yeast medium (YM) (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 0.15% agar) or nutrient broth. After 48h growth, the cell suspensions were plated on YM, nutrient agar and potato dextrose agar plates. Individual colonies were picked and further purified by sub-culturing on agar plates. A variety of bacterial, fungal and yeast cultures isolated were included in the screening programme, but it should be noted that no attempt was made to identify the individual cultures.

Screening of cultures for gold accumulation and nanoparticle synthesis

Cultures were grown up in test tubes containing 10ml nutrient medium in shaker incubators at 28°C. After 24 to 48h incubation the biomass was separated from the medium by centrifugation (7500 rpm, 10 min) and washed three times in sterile distilled water to remove any nutrient media that might interact with the gold ions. The biomass was resuspended in 10 ml distilled water and the pH adjusted to between 5 and 6 with 0.2 M NaOH. $HAuCl_4$ was added to give an overall Au-concentration of 250 mg/l. The mixture was left for a further 24 to 72h in a shaker incubator at 28°C. The accumulation and reduction of gold were followed by visual observation of the biomass turning purple, an indication of the formation of gold nanoparticles (Experiment 1).

Effect of growth parameters on nanoparticle production

Cultures were grown for 24, 48 or 72 h as described above. The biomass was separated from the medium by centrifugation (7500 rpm, 10 min) and washed three times in sterile distilled water before exposure to $HAuCl_4$ at a pH level of 5 (Experiment 2).

The effect of pH on nanoparticle formation was evaluated by exposing 100 mg of washed biomass in 10 ml distilled water to $HAuCl_4$ at an overall Au concentration of 250 mg/l. The pH levels were adjusted to 3, 5, 7 and 9 respectively, followed by incubation at 28°C (Experiment 3).

Gold-containing cell suspensions (100 mg wet mass/10 ml solution) were incubated in shaker incubators at 25°C, 35°C and 50°C to evaluate the effect of temperature on nanoparticle formation. In all tests, the accumulation and the reduction of gold were followed by visual observation of the biomass turning purple (Experiment 4).

Synthesis of nanoparticles in the presence of cell-free extract

Verticillium luteoalbum biomass (100 mg wet mass/10 ml solution) grown in nutrient medium as described above, was separated from the medium by centrifugation (7500 rpm, 10 min), washed twice in distilled water and the biomass re-suspended in 10 ml distilled water. The washed cells were transferred to a test tube and an equal volume of glass beads added, before the suspension was agitated at maximum speed using a vortex mixer. The cell suspension was examined microscopically until most of the cells were broken. Broken cells and those remaining intact were removed by centrifugation at 10 000 rpm for 10 min. $HAuCl_4$ (250 mg/l) was added to the cell-free extract and the pH adjusted to 5 by addition of 0.2M NaOH. The mixture was incubated at 35°C and the accumulation and reduction of the metals were followed by observation of the suspension changing colour (Experiment 5).

Characterisation of the nanoparticles

Samples were prepared for TEM analysis by separating the biomass from the liquor by centrifugation and washed twice in sterile distilled water. The samples were fixed for 1 hour in 2.5% gluteraldehyde in 0.075 M phosphate buffer (pH7.4), followed by 3 washes in 0.075 M phosphate buffer. After a second fixation step of 1 h in 1% osmium tetroxide, the cells were washed in distilled water. The cell pellet was subjected to dehydration with 30, 50, 70% ethanol, followed by three dehydration steps in 100% ethanol. Infiltration of the resin was carried out by placing the pellet in 30% Quetol in ethanol for 1 hour, followed by 1 hour in 60% Quetol. After centrifugation, the pellet was resuspended in 100% Quetol for 4h before polymerisation at 65°C for 24 hours. Ultrathin sections were not stained prior to analysis, due to possible interference of the stain with the gold particles. TEM analyses were done on a Philips 301 transmission electron microscope.

For TEM analysis of the cell-free extract, a drop of the sample was placed onto a carbon-coated copper grid. After about a minute, the extra solution was removed using blotting paper and the grid air-dried before analysis.

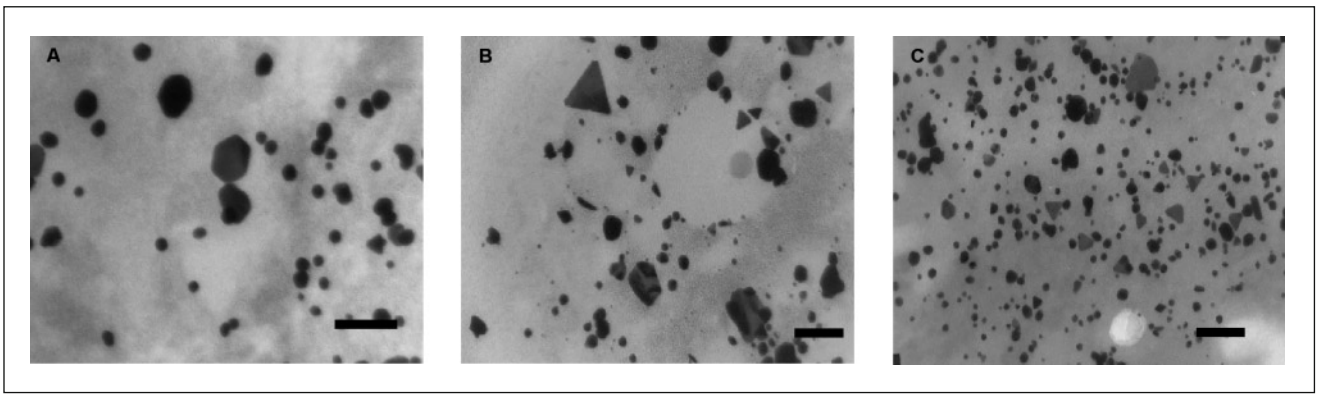

Figure 1
TEM images illustrating the formation of gold nanoparticles by (A) P. jadinii, (B) V. luteoalbum and (C) Isolate 6-3 after 24h exposure to $HAuCl_4$ (Scale bar = 100nm).

Elemental analysis on single particles was carried out on an air-dried, carbon coated sample using an energy dispersive spectroscopy (EDS) attachment on a Jeol 5800 LV scanning electron microscope using the following instrument conditions: accelerating voltage of 20 keV and counting time of 100 s.

Results

Screening of microbes for their ability to produce gold nanoparticles

The production of the nanoparticles after exposure of the cultures to gold was evaluated based on the size and shape of the particles, number of particles produced per cell and the occurrence of the particles in the cells as determined by transmission electron microscopy (TEM) – Experiment 1.

The most promising results were obtained with the yeast, *P. jadinii* (formerly *Candida utilis*) and the fungal cultures, Isolate 6-3 (isolated from a metal-rich dump) and *V. luteoalbum* (Figure 1). The biomass turned dark purple within a few hours after exposure to $HAuCl_4$, while the solution remained colourless, an indication of intracellular nanoparticle synthesis.

In all three cases, the particles did not occur in preferential areas and were deposited throughout the cells. As in the case of previous results obtained with fungi (24,25) various particle morphologies, which included spherical, triangular, hexagonal and other shapes were present in all three cultures (Figures 1 and 2). No clear relationship between the morphology of the nanoparticles and type of fungal biomass was observed. Large variations in particle size were observed and these varied from a few to approximately 100 nm in diameter. The images suggested that the spherical particles tended to be smaller than the hexagonal and triangular shaped particles.

Control of the size and shape of intracellularly produced gold nanoparticles

Although the initial screening results were promising and a variety of nanoparticles with interesting shapes were synthesised, it is realised that for most applications nanoparticles of well-defined size and shapes are required. In addition, for a biological process to successfully compete with chemical nanoparticle synthesis, very strict control over average particle size in a specific size range and uniform particle morphology is required. In an attempt to achieve better size and shape control, the effect of growth parameters such as growth stage of the cells, pH and temperature on the process was investigated using the two fungal cultures *V. luteoalbum* and Isolate 6-3.

Biomass grown for 24, 48 and 72 h respectively was exposed to $HAuCl_4$ for 24 h to evaluate the effect of the age of the culture on its ability to accumulate and reduce gold

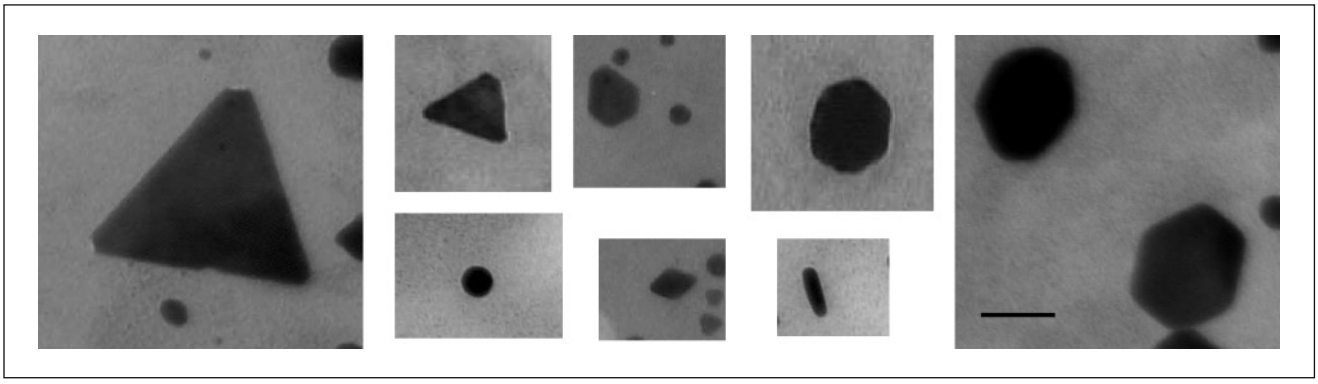

Figure 2
TEM images of a selection of different nanoparticles formed by reduction of $HAuCl_4$ (Scale bar = 50 nm)

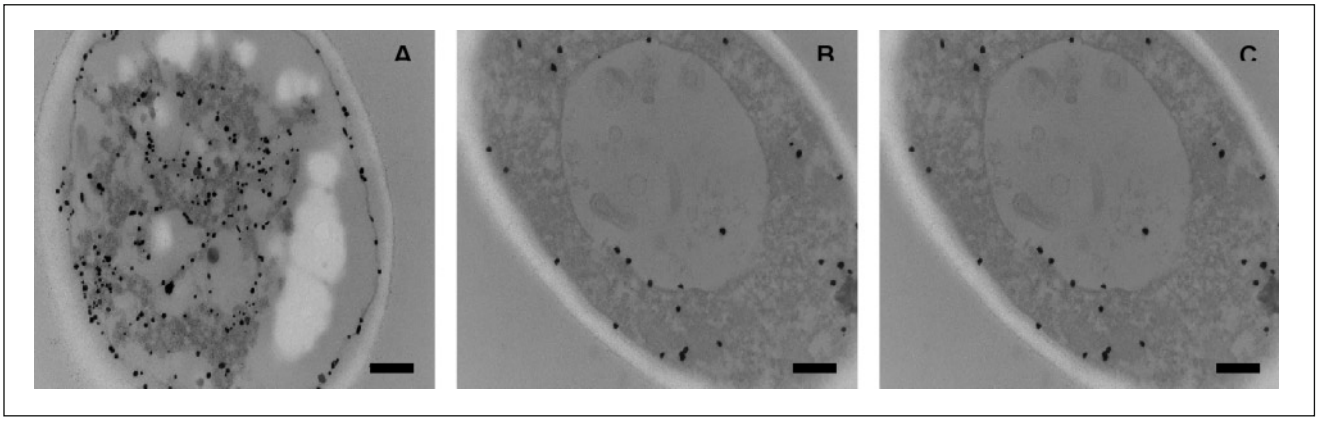

Figure 3

The effect of growth stage of the cells on the extent and appearance of nanoparticle formation in Isolate 6-3 at pH 5. (A) 24 h growth, (B) 48 h growth, (C) 72 h growth. (Scale bar = 200 nm)

ions (Experiment 2). It was shown that the age of the cells at the time of exposure to gold did not have an effect on the shape of the accumulated gold nanoparticles. However, with both *V. luteoalbum* and Isolate 6-3, a decrease in the number of particles per cell was observed when older cells, harvested later during the growth cycle were exposed to gold (Figure 3). A possible explanation might be that cells in early exponential phase of growth produce very high concentrations of enzymes and proteins that are actively involved in accumulation and reduction of the gold ions.

The pH was found to be an important parameter affecting gold nanoparticle synthesis in both cultures (Experiment 3). Variations in pH during exposure to Au-ions had an impact on the size, shape and number of particles produced per cell. Figure 4 shows representative TEM micrographs of nanoparticles produced in *V. luteoalbum* after 24 h exposure to $HAuCl_4$ at pH levels of 3, 5, 7, and 9 respectively. Similar results were obtained with Isolate 6-3.

Particles formed at pH 3 were predominantly spherical in shape, relatively uniform in size, with the majority of the particles having less than 10 nm in diameter. Nanoparticles synthesised at pH 5 included small spherical particles, similar to those dominating at pH 3. In addition a large number of bigger particles with well-defined shapes, including triangles, hexagons, spheres and rods also occurred at this pH. The shapes of the particles formed at pH 7 were similar to those formed at pH 9 and included small spherical particles as well as bigger particles with irregular, undefined shapes. These results are supported by previous studies suggesting that optimum gold accumulation by microbial cells normally occurs in the pH range of 2 to 6 (19) and test work performed with *Lactobacillus* showed that changes in the pH could have an effect on the size distribution of gold nanoparticles (28).

The variety in the shapes of particles formed at the different pH levels indicates that changes in this parameter would play an important role during optimisation of a process controlling particle morphology.

The behaviour of nanoparticle synthesis over a range of temperatures was determined by exposing *V. luteoalbum* and Isolate 6-3 biomass to $HAuCl_4$ solutions at temperatures of 25, 35 and 50°C, respectively (Experiment 4). Samples for TEM analysis were collected after 1 and 24 h incubation and the trends in nanoparticle formation in *V. luteoalbum* at 25 and 50°C are illustrated in Figure 5

The rate of formation of the nanoparticles was related to the incubation temperature and increased temperature levels allowed particle growth at a faster rate. At the lower temperatures, the majority of nanoparticles formed after 1 h exposure to the gold solution were spherical with an average diameter of less than 10 nm. Further incubation for 24 h led to the number of smaller particles decreasing, whereas the number of larger particles, exhibiting well-defined shapes, increased. At 50°C, no difference could be detected in the

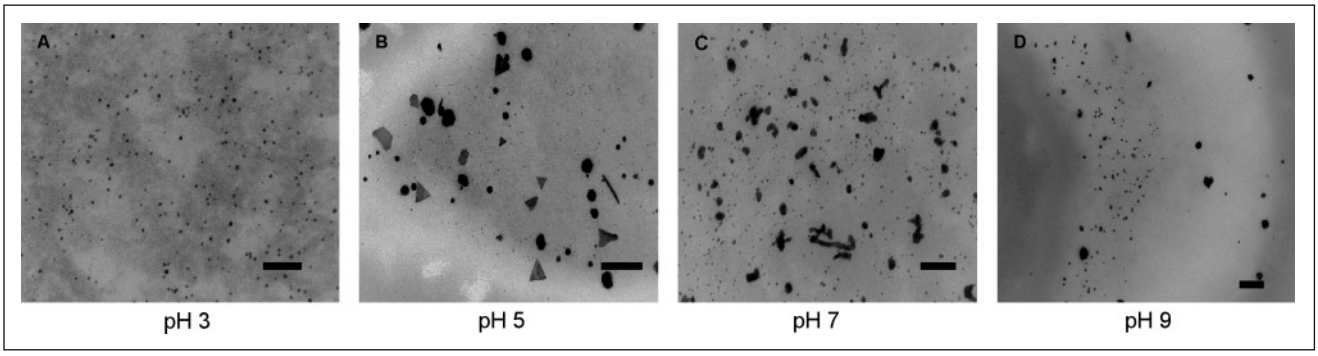

Figure 4

TEM images showing the effect of pH on nanoparticle formation in V. luteoalbum (Scale bar = 100nm)

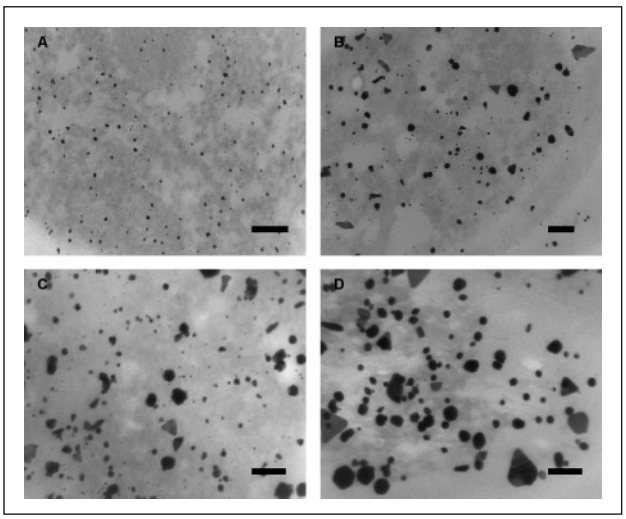

Figure 5
TEM images illustrating the effect of temperature on nanoparticle production in V. luteoalbum cells. (A) 1h exposure to $HAuCl_4$ at 25°C, (B) 24h exposure to $HAuCl_4$ at 25°C, (C) 1h exposure to $HAuCl_4$ at 50°C, (D) 24 h exposure to $HAuCl_4$ at 50°C (Scale bar = 100 nm)

size and morphology of particles produced after 1 and 24 h exposure to gold and very few small spherical particles were present. The effect of the rate of reduction on the shape of gold particles was previously described for the chemical synthesis of gold colloids in the presence of solvents (33). Formation of spherical particles was favoured at low reduction rates, whereas high reduction rates resulted in the formation of particles exhibiting nanorod and platelet-like morphologies.

The above results provide indications that the size of the nanoparticles can, to a large extent, be controlled by operating at low temperatures, which would allow particle formation at a slower rate.

Production of gold nanoparticles using cell extracts

The cultures used in this study produced gold nanoparticles intracellularly. From a process and applications point of view it would, however, be useful if the nanoparticles could form extracellularly, since it would eliminate the need to harvest the nanoparticles formed within the cells.

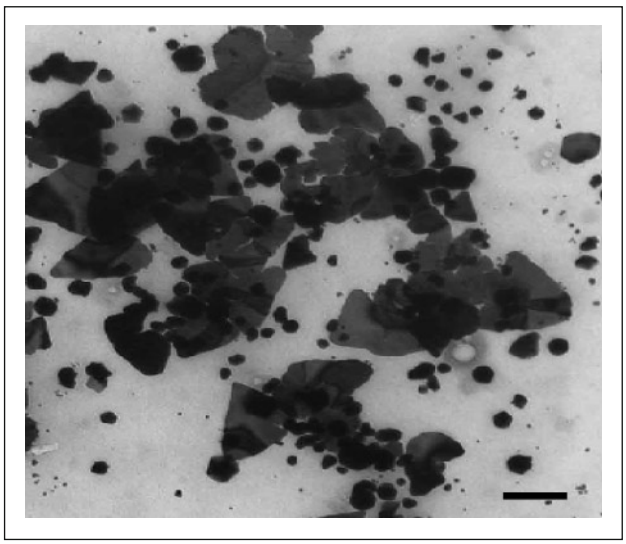

Figure 6
TEM image of gold particles formed after exposing cell-free extract from Verticillium to $HAuCl_4$. (Scale bar = 200nm)

It is speculated that gold ions are reduced by enzymes and proteins present in either the cell walls or cytoplasm of the organisms leading to the aggregation of the gold atoms and formation of nanoparticles (24). These assumptions are supported by research performed by Brown and colleagues (34) who used the crystallisation of gold as a model system to show that polypeptides could control the morphology of the resulting gold crystals. Identification of the active reducing proteins or enzymes involved in the process could potentially allow for a process in a cell-free environment, where the size and shape of the particles can be controlled.

A test, exposing cell-free extract to gold ions was performed to obtain an indication of whether reducing compounds are present in the cell extract and to assess if cell structures have a role to play in the formation of nanoparticles exhibiting specific sizes and shapes (Experiment 5).

Verticillium cells were broken using glass beads and the resultant extract exposed to gold ions. The morphologies of the particles were similar to those formed intracellularly (Figure 6), but included a large number of large thin plate-like structures, which were predominantly triangular or

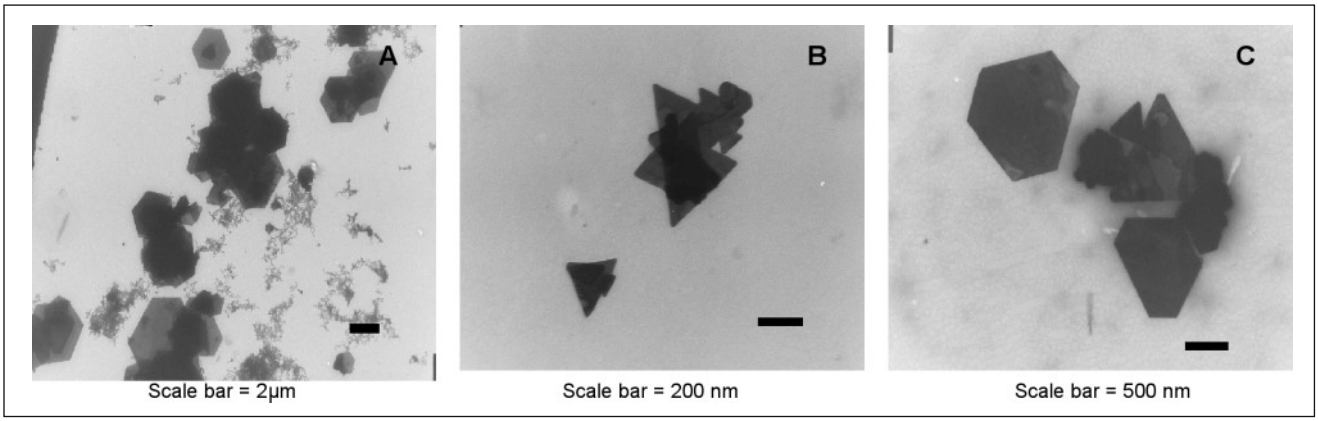

Figure 7
TEM images of a selection of gold nanoflakes synthesised in the presence of cell-free extract obtained from Verticillium

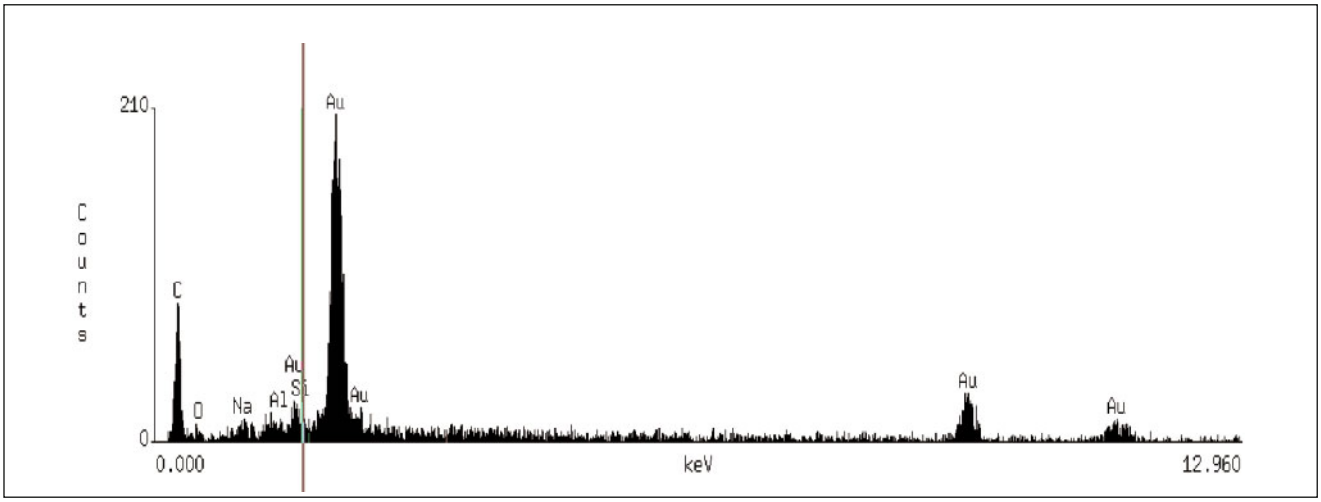

Figure 8
Spot-profile EDS spectrum for a gold nanoparticle formed after exposure to $HAuCl_4$.

hexagonal in shape (Figure 7). Similar behaviour was observed during the synthesis of gold particles using broth prepared from Neem tree leaves (35). It is possible that without the constraints of a cell wall, bigger particles could form, but further test work is needed to confirm this. Since it is known that the size and shape of metal nanoparticles can change their optical and electronic properties (36), these gold plates can potentially be used in interesting applications in the areas of optics and sensor technology.

A spot profile EDS spectrum derived from one of the nanoparticles indicated that it was mainly composed of Au with trace amounts of C, O, Na, Si and Al (Figure 8). It is not clear whether these trace elements were associated with the nanoparticles or if they were present in the vicinity of the particles.

The results imply that exposure of whole cells to the gold solution during nanoparticle formation is not necessary. Identification of the active reducing proteins or enzymes involved in the process could potentially allow for a process in a cell-free environment, where the size and shape of the particles can be precisely controlled.

Conclusions

The intracellular synthesis of gold nanoparticles of various morphologies and sizes in two fungal cultures, *V. luteoalbum* and Isolate 6-3, has been investigated. The rate of particle formation and therefore the size of the nanoparticles could, to an extent, be manipulated by controlling parameters such as the pH and temperature. The extracellular formation of gold nanoparticles after exposure of cell-free extract to gold ions was demonstrated. Extracellular formation of gold nanoparticles would be advantageous from a process point of view, since it would eliminate the need to recover the particles formed within the cells.

The development of chemical procedures to control the morphology of nanoparticles is an ongoing area of research. A biological process with the ability to strictly control the shape of the particles produced would therefore be an exciting prospect. However, the cellular mechanism leading to the biosynthesis of gold nanoparticles is not yet fully understood. Further research will therefore focus on the development of a fundamental understanding of the process mechanism on a cellular and molecular level, including isolation and identification of the compounds responsible for the reduction of gold ions.

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