Effect of physicochemical factors on extracellular fungal pigment-mediated biofabrication of silver nanoparticles

Sharad Bhatnagar\textsuperscript{a,b}, Christiana N. Ogbonna\textsuperscript{c}, James C. Ogbonna\textsuperscript{d} and Hideki Aoyagi\textsuperscript{a,b,e}

\textsuperscript{a}Life Sciences and Bioengineering, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan; \textsuperscript{b}Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan; \textsuperscript{c}Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Nigeria; \textsuperscript{d}Department of Microbiology, University of Nigeria, Nsukka, Nigeria; \textsuperscript{e}Microbiology Research Centre for Sustainability (MiCS), University of Tsukuba, Tsukuba, Japan

ABSTRACT

The extracellular pigment produced by \textit{Talaromyces purpurogenus} was used as a reducing agent in the production of silver nanoparticles (AgNPs), and the effect of physicochemical parameters (pH and light) on the synthesis were examined. To evaluate the effect of pigment constituents on AgNP synthesis, fractions were obtained by thin layer chromatography. Ultraviolet–visible spectroscopy, transmission electron microscopy, dynamic light scattering (DLS), and Fourier transform infrared spectroscopy were used to characterize the biofabricated AgNPs. The synthesis of AgNPs was enhanced under alkaline conditions, emphasizing the role of sodium hydroxide (NaOH) as an accelerator, and the effect of light on yield could be clearly perceived up to pH 10. As the pH value was increased further, the light dependency of the reaction decreased, although the DLS data suggested variation in the size of AgNPs obtained in the presence and absence of light. The AgNPs produced at optimum pH in the presence of light were nearly spherical with a size distribution of 5–40 nm. The AgNP production process was pH-dependent and light-mediated, indicating that AgNPs with different properties could be generated by controlling these physicochemical factors.

ARTICLE HISTORY

Received 21 January 2021
Accepted 26 January 2022

KEYWORDS

Extracellular pigment; green nanotechnology; physicochemical factors; silver nanoparticles; \textit{Talaromyces purpurogenus}

Introduction

In the last few years, silver nanoparticles (AgNPs) have attracted increasing attention owing to their distinct electric, physical, optoelectrical, chemical, and biological properties (1). As nanoscale particles (1–100 nm), the physicochemical properties of AgNPs are distinctly different from those of the bulk material, including the size, shape, surface area, aspect ratio, and surface properties. This allows for their possible applications in various avenues such as the pharmaceutical industry, agricultural industry, environmental safety, biosensors, drug delivery systems, and molecular labeling along with a multitude of other applications (1,2). The worldwide consumption of nanoparticles (NPs) is expected to rise in the near future, with the healthcare, electronics, textile, and food industry sectors being the most
significant contributors (2). AgNPs have been shown to exhibit various biological properties such as antibacterial (3), antifungal (4), anticancer (5,6), wound healing (7), antioxidant, anti-diabetic, anticoagulant, and thrombolytic activities (8–10). Biosynthesized AgNPs have also been used as a potential dental material (11,12) and for inhibition of biofilm growth (13). Size, shape, size distribution, morphology, surface charge, surface chemistry, and capping molecules present on the metal NP surface are known to drastically affect their biological activity (14–17). Other applications such as detection of pesticides (18,19), wastewater treatment, and dye degradation have also been studied (20).

In light of such versatile applications and future prospects, it is imperative to explore economical methods of AgNPs synthesis. This rising need for AgNPs has attracted interest in the research and development of various new synthesis approaches. Traditional chemical and physical methods employed in the synthesis of nanomaterials are either energy-consuming or generate exceeding amounts of hazardous chemical waste (21). In contrast to these methods, green chemistry or sustainable chemistry relies upon the creation of processes that minimize the application or production of toxic compounds. The biofabrication of NPs employing microbial and plant sources are examples of such techniques that can resolve the issues faced by traditional techniques by using environmentally friendly components with cost-effective processes, thereby contributing to green nanotechnology (22). Such green biosynthesis of AgNPs can be achieved either intracellularly, in which the precursor molecule is taken up by the cell and converted to an NP within the cell, or extracellularly using enzymes and other biological molecules secreted outside the cell in the reduction and capping of the precursor molecule (23). Intracellular production possibly occurs when the cell tries to resist and convert the toxic metal ions into a less toxic form. This type of production process requires an extra step for the extraction of NPs, which has led to the development of extracellular biosynthesis processes (24). Bacteria, yeasts, fungi, plants, algae, lichens, cyanobacteria, and actinomycetes have all been used for this purpose (4,25).

Fungi are considered particularly good candidates for NP production owing to their ability to produce high amount of proteins, and given their ease of growth at both the laboratory and industrial scales. Fungal cells and their extracellular products, including species such as Fusarium oxysporum, Aspergillus fumigatus, Aspergillus flavus, Neurospora crassa, and Penicillium purpurogenum, have been extensively used to produce various NPs to date (26–32). These biofabrication techniques follow the principles of green chemistry focusing on the use of safer solvents, biocatalysts, energy efficiency, and less toxic waste generation, among others.

This type of biofabrication of AgNPs can be further subdivided into three types: cell biomass-mediated synthesis, supernatant-mediated synthesis, and biomolecules-mediated synthesis. The former two processes are routinely used for the synthesis of AgNPs, whereas the latter process remains underexplored. Inexpensive biomolecules such as pigments produced by microorganisms have been shown to have excellent reducing properties for AgNP generation. Moreover, changes in physicochemical factors such as pH and light have been shown to have an effect on the morphology, size, and size distribution of the product, providing an opportunity to tailor the NPs to specific requirements. For example, Oscillatoria limnetica cell extract containing a light-harvesting pigment, proteins, and soluble sugars has been used to generate AgNPs under different light intensities (33). Moreover, the effect of the initial pH level, among other factors, was found to be significant for the yield of AgNPs from the O. limnetica cell extract. Similarly, the extract of the cyanobacteria Deser tifilum has also been used to generate AgNPs in a light-mediated process (34). Although the effect of individual factors on yield has been discussed previously, the concerted effect of these factors on the size and size distribution of AgNPs has not been explored sufficiently.

Recently, Monascus ruber (35), Monascus purpureus (36), and Nostoc linckia (37) have been used in AgNP production; however, their pigments generally either require extraction with organic solvents or the production technology must be adapted to achieve extracellular pigment production. In contrast, Talaromyces purpurogenus, an ascomycota, can produce high amounts of an extracellular water-soluble Monascus-like pigment. This provides the opportunity to develop a more economic process with less extraction steps and easier scale-up owing to the high extracellular pigment yield. The AgNPs produced using this pigment and their applications were described in our previous work (38). In the present study, we explored the effect of physicochemical factors (pH and light) and pigment constituents on the synthesis of AgNPs using T. purpurogenus pigment as a unique reducing agent. The pigment was obtained from a fungal culture and the produced NPs were also characterized.

Materials and methods
Materials
Sucrose, Bacto peptone (Difco, Becton Dickinson, Tokyo, Japan), yeast extract (Nihon Seiyaku, Tokyo, Japan),
magnesium sulfate heptahydrate (MgSO₄·7H₂O), dipotassium hydrogen phosphate (K₂HPO₄), sodium nitrate (NaNO₃), potassium chloride (KCl), ferrous sulfate heptahydrate (FeSO₄·7H₂O), ethanol (99%, special grade), acetonitrile, and silver nitrate (AgNO₃) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Double-distilled, deionized water was used in all experiments.

**Production of extracellular pigment from T. purpurogenus**

A *T. purpurogenus* strain was obtained from the Cell Cultivation Laboratory (Faculty of Life and Environmental Sciences, University of Tsukuba, Japan) to produce the extracellular pigment. The inoculum for pigment production was developed in a 500-mL Erlenmeyer flask containing 200 mL of inoculum development medium (in g/L): sucrose 30, yeast extract 5, K₂HPO₄ 1, NaNO₃ 0.3, KCl 0.05, MgSO₄·7H₂O 0.05, and FeSO₄·7H₂O 0.001. A spore suspension (10 mL of 3 × 10⁶ spores/mL) prepared in sterilized water was used to inoculate the medium and the flask was incubated at 30°C (150 rpm, 24 h) in the dark. Pigment production was performed in 200 mL of production medium (in g/L): sucrose 50, peptone 25, K₂HPO₄ 2, MgSO₄·7H₂O 2, NaNO₃ 1, KCl 0.05, and FeSO₄·7H₂O 0.001. Pigment production was initiated with 5% (v/v) of the starting inoculum culture. The flasks containing production media were incubated at 150 rpm and 30°C for 10 days in the dark. The pH of both media was adjusted to 5 by the addition of 6 N HCl. Pigment production was observed visually and spectrophotometrically (V-550, JASCO, Tokyo, Japan) at 500 nm.

After pigment production, the biomass was removed by centrifugation at 6,700 × g and 4°C for 20 min, followed by filtration via a 0.45 μm filter, and the clarified broth was mixed with 70% ethanol (1:1 ratio) for 3 h. The aim of partial purification of the pigment was to reduce the presence of other extracellular biomolecules in the broth. The mixture was centrifuged again at 6,700 × g and 4°C for 20 min to remove impurities, concentrated using a rotary vacuum evaporator (N-1000 series, Eyela, Tokyo, Japan), and finally dissolved in 70% ethanol. The concentrate was centrifuged again at 6,700 × g and 4°C for 20 min, and the supernatant was filtered (0.45 μm filter) and stored at 4°C (38).

**Synthesis of AgNPs from the extracellular pigment**

Initially, a reaction mixture (5 mL) was prepared with 0.5 mg/mL of extracted pigment at pH 11 along with 2 mM AgNO₃. This mixture was briefly agitated and incubated at 28°C (2,000 lux light, 48 h). Pigment (0.5 mg/mL) and 2 mM AgNO₃ in water were used as controls.

Thin layer chromatography (TLC) was performed to separate the composite pigment into its components to determine their impact on the generation of NPs. For TLC, 100 μL of the previously obtained pigment sample was loaded onto an F₂₅₄ silica plate (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), and a mixture of acetonitrile and water (7:3 ratio) was used as the mobile phase. The separated pigments were scraped off the TLC plate and dissolved in 70% ethanol for extraction. The extracts (100 μL) were then used in a reaction mixture of 1 mL at pH 11 with 0.5 mM AgNO₃ for the production of NPs to determine the synthesis abilities of the individual components.

To determine the effect of pigment pH on the synthesis process, before adding AgNO₃, the pH of the composite pigment was adjusted to 2, 4, 7, 9, 10, 11, 12, and 13 using HCl or NaOH. This mixture was briefly agitated and incubated at 28°C (2,000 lux light, 48 h). Preliminary experiments showed that 2 mM AgNO₃ was the maximum concentration that can be reduced by the pigment under all pH conditions. Moreover, owing to the difference in the reaction rate at different pH, an incubation time of 48 h was chosen as the endpoint to provide an ample amount of time for completion of the reaction for the entire pH range. The results were analyzed by calculating the absorption maxima (A_max; yield), wavelength maxima (λ_max; size), and full-width half maxima (FWHM; size polydispersity) for the ultraviolet–visible (UV-Vis) spectra (V-550, JASCO, Tokyo, Japan) of various samples. FWHM values were calculated from the normalized spectrum. The effect of pH on AgNPs synthesis was analyzed in triplicate, and data are expressed as mean ± standard deviation.

To study the effect of light on NP synthesis, the experiments were performed in the presence and absence of light. The presence of NPs was confirmed by UV-Vis spectroscopy. A part of the produced AgNPs was lyophilized, and both the freeze-dried particles and those in as-obtained condition were stored at 4°C prior to their characterization.

**AgNP characterization**

Transmission electron microscopy (TEM; H-7650, Hitachi, Tokyo, Japan) and dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Panalytical, Worcestershire, UK) were applied to estimate the size of the synthesized AgNPs. For TEM analysis, the NP suspension was dropped onto a carbon-coated formvar copper grid (mesh size: 300) and dried for 30 min before imaging.
at 80 kV. The TEM images were analyzed for particle size using ImageJ 2.0.1 (National Institutes of Health, Bethesda, MD, USA), and the histogram was constructed by Microsoft Excel 16 (Microsoft, Washington, USA). Fourier transform infrared spectroscopy (FTIR; JASCO-FT/IR-6800, Tokyo, Japan) analysis was utilized to identify the functional groups responsible for the reduction and stabilization of bulk silver. The FTIR spectra of the lyophilized AgNPs and pigment in KBr pellets were measured in the range of 400–4,000 cm⁻¹.

Results

Effect of pigment components on AgNPs synthesis

Initial synthesis for the composite pigment was accomplished at pH 11 in the presence of light for 48 h, and the UV-Vis spectra of produced AgNPs, AgNO₃, and the pigment were compared (Fig. S1, Supplementary Information). The biofabrication of AgNPs was verified by the color of the reaction mixture, which became progressively darker over time. The color changed from reddish to darker brown after 48 h, indicating the generation of AgNPs (Fig. S1a, b). The produced AgNPs exhibited a characteristic yellow color (Fig. S1c) after dilution (1:10). Distinctive surface plasmon resonance (SPR), the resonant oscillation occurring when the conduction electron interacts with incident light, was observed around 410 nm, indicating the presence of silver NPs and demonstrating that the pigment reduced the precursor silver salt to Ag⁰. This interaction is dependent upon nature of the dispersion media, and more importantly on the size and shape of the metal NPs. No such SPR or color change was detected either for the AgNO₃ control or the pigment.

To ensure that the composite pigment components were responsible for AgNP generation, partial purification was carried out by TLC, and the obtained fractions were employed for the biosynthesis of AgNPs. Different pigment components showed different reduction capacities (Figure 1b). Fraction 1 (Figure 1a) showed the lowest reducing power, with Amax around 420 nm, followed by fraction 6, demonstrating a faint yellow band on top of the TLC plate, and fraction 2, with Amax around 420 and 415 nm, respectively, in the UV-Vis spectrum obtained for AgNPs. In contrast, fractions 4 and 5 exhibited the best reduction abilities, with Amax values around 410 nm and narrower spectra. Fraction 3 exhibited a broad spectrum along with an Amax value also around 410 nm, indicating good reduction ability but also polydispersity. The presence of the SPR signature for the AgNPs showed that the pigment fractions possess sufficient reduction ability to produce AgNPs, and therefore the composite pigment is an active agent during the reduction process. Previous attempts to synthesize NPs using Monascus azaphilone pigments used isolated fractions with confirmed reducing abilities (36). Since the separation of pigments into their components to isolate the most potent reducing fraction could be a costly exercise, this experiment demonstrates that use of the composite pigment could enable a cheaper synthesis protocol. It is possible that further optimization of the conditions for each component will

Figure 1. (a) Pigment fragments obtained by thin layer chromatography (TLC). The numbers correspond to pigment fractions obtained. (b) UV-Vis spectra of AgNPs obtained from various TLC fractions. F-1 to F-6 refer to the fraction numbers. Samples were diluted 10 times.

Figure 2. Ultraviolet-visible (UV-Vis) spectrum of AgNPs synthesized by the extracellular pigment from T. purpurogenus at different pigment pH values. The samples were diluted 30 times.
provide different results in terms of their reduction capability.

**Effect of pH on AgNP generation**

The UV-Vis spectrum of AgNPs produced at various pH demonstrated that the reducing ability of the pigment and the AgNP yield increased as the pigment pH increased (Figure 2). From the UV-Vis spectrum data, generation of AgNPs begins at pH 9 ($A_{\text{max}} = 0.4$, $\lambda_{\text{max}} = 429.67$ nm) and continues to increase with a corresponding increase in the pH value. This trend maximizes at pH 12, when the $A_{\text{max}}$ reaches 0.85 at $\lambda_{\text{max}}$ 411 nm, which was designated as the optimal pH for synthesis, and diminishes subsequently, as observed at pH 13, with $A_{\text{max}}$ reaching 0.73 and $\lambda_{\text{max}}$ of 412 nm. Since better reduction was achieved in the alkaline condition, the addition of NaOH was considered to act as an accelerating and a size-controlling agent during AgNP generation. The results are further represented in terms of $A_{\text{max}}$, $\lambda_{\text{max}}$, and FWHM in Table 1. The $\lambda_{\text{max}}$ values around 400 nm imply smaller NPs, whereas a low FWHM implies a lower polydispersity degree (39). The change in yield parameters was corroborated by the increase in $A_{\text{max}}$ values and the decrease in $\lambda_{\text{max}}$ and FWHM values (Table 1), along with a hypsochromic shift when the pH values increased.

**Effects of light on AgNP generation**

To understand the effects of light on the synthesis of NPs, pigment solutions at pH 10, 11, and 12 were exposed to two different conditions: exposure to 2,000 lux of light or darkness. After 48 h, the UV-Vis spectra of reaction mixtures incubated under light and dark conditions were compared. A definitive effect of light was detected at pH 10, in which the spectrum of the illuminated samples showed a much higher peak ($A_{\text{max}}$ of ~0.46 around 420 nm) than that of samples incubated in the dark ($A_{\text{max}}$ of ~0.16) (Figure 3a). A shoulder peak was observed in samples incubated in the dark at approximately 500 nm, depicting the presence of unreacted pigment. In contrast, the illuminated samples exhibited no such peak. Interestingly, as the pigment pH was increased to 11, the reaction mixture was able to produce AgNPs in both light and dark conditions (Figure 3c), although the spectrum exhibited a slightly higher yield in darkness when compared to light ($A_{\text{max}}$, dark of ~0.85, $A_{\text{max}}$, light of ~0.79), along with a narrower curve. At pH 12, this difference was found to be marginal, with $A_{\text{max}}$ in the dark and light condition of 0.86 and 0.84, respectively (Figure 3e).

The changes in the UV-Vis spectra properties might hint at the possible differences in the size and polydispersity of AgNPs produced with and without illumination. The differences in NP sizes were monitored by DLS intensity distribution data, which indicated that AgNPs produced under dark conditions had a smaller size on average when compared with those produced under illumination (Figure 3b, d, f). At pH 10, there was a clear difference in the yield obtained under light as compared to dark conditions from the UV-Vis spectrum, whereas the size distribution was not very different (Figure 3a, b). For AgNPs obtained at pH 11 and 12, there was a noticeable shift toward a smaller particle size (Figure 3d, f). Collectively, these data suggest that both light and pH are important factors in the synthesis of AgNPs from the extracellular pigment obtained from *T. purpurogenus*. The effect of light on reduction capability of the pigment was verifiably observed up to pH 10. At higher pH, the effect of NaOH dominated and controlled the reaction rate; nonetheless, a size disparity was observed in the presence and absence of light.

**Characterization of the produced AgNPs**

The characterization of AgNPs synthesized at pH 12 in the presence of light using TEM for size estimation revealed nearly spherical particles with a core particle size of 5–40 nm and a mean average diameter of 10–20 nm (Figure 4a, b). In total, 201 particles were counted in the images via particle analysis by ImageJ. Hydrodynamic size measurement by DLS showed that most particles were around 60 nm in size. DLS size estimation accounts for the presence of stabilizing groups surrounding the core particle, which may explain the variations from the TEM estimate (Figure 5a). In our previous study, electron probe microanalysis-wavelength dispersive spectroscopy (EPMA-WDS) data were used to characterize the AgNPs produced with a similar process, and the presence of silver was observed at 3–4 keV (Figure S2a) compared with background interference (Fig. S2b) (38).

FTIR analysis of the pigment at pH 12 revealed major peaks at 3432.67, 2931.27, 1774.19, 1658.48, 1450.21, 1348.17, and 1038.07 cm$^{-1}$, which may correspond to the vibrations of **Hydrogen bonded O-H**, **C-H stretch** (symmetric), and **C-O stretch** (aldehydes), respectively. **Hydrogen bonded O-H** can be seen at 3332.71 cm$^{-1}$, which may correspond to the vibrations of **Hydrogen bonded O-H**. **C-H stretch** (symmetric) can be seen at 2931.27 cm$^{-1}$, which may correspond to the vibrations of **C-H stretch** (symmetric). **C-O stretch** (aldehydes) can be seen at 1774.19 cm$^{-1}$, which may correspond to the vibrations of **C-O stretch** (aldehydes). **Hydrogen bonded O-H** can be seen at 1658.48 cm$^{-1}$, which may correspond to the vibrations of **Hydrogen bonded O-H**. **C-H stretch** (symmetric) can be seen at 1450.21 cm$^{-1}$, which may correspond to the vibrations of **C-H stretch** (symmetric). **Hydrogen bonded O-H** can be seen at 1348.17 cm$^{-1}$, which may correspond to the vibrations of **Hydrogen bonded O-H**. **C-H stretch** (symmetric) can be seen at 1038.07 cm$^{-1}$, which may correspond to the vibrations of **C-H stretch** (symmetric).
Figure 3. Effect of 2,000-lux light vs. dark conditions. UV-Vis spectrum and dynamic light scattering (DLS) intensity distribution at (a, b) pH 10, (c, d) pH 11, and (e, f) pH 12. Dashed and solid lines represent light and dark conditions, respectively.

Figure 4. Morphology and size distribution of AgNPs. (a) Transmission electron microscopy (TEM) micrograph. (b) Size distribution histogram generated from the TEM image.
1072.23, and 879.38 cm$^{-1}$, representing the presence of H-bonded hydroxyl (O-H) bond stretching for phenols/alcohols, $\equiv$CH$_2$ methylene group asymmetric stretching, C = O carbonyl stretching, C = O group in amide or carbonyl groups, methylene group C–H bending, C–O stretching in primary alcohols, and C–H vibrations, respectively (Figure 5b). The AgNPs exhibited major peaks at 3424.86, 1604.48, 1442.49, 1388.50, and 879.38 cm$^{-1}$, which are characteristic of hydroxyl (O-H) bond stretching, C = O group in amides, C–H bending, phenol/O-H bending with the presence of residual nitrate ions, and C–H vibrations, respectively (Figure 5c). The presence of broad, shifting peaks of OH groups in both the pigment and the NP spectra point to the involvement of alcohol, phenol, carbonyl, along with other functional groups, and amide groups acting as reducing and capping agents.

Discussion

Some previous studies have explored the possibility of producing AgNPs using biologically obtained pigments from various sources as the reducing and stabilizing agent. El-Naggar et al. (37) used a cyanobacterium-derived pigment, phycocyanin, for the synthesis of AgNPs in the presence of light, with a mean size of 9.39–25.89 nm as determined by TEM. The FTIR spectrum of phycocyanin showed the presence of O-H stretching vibrations of phenol and alcohol, along with the presence of C = O stretch found in primary amides II or carbonyl groups, among other functional groups. These results were similar to the FTIR data obtained in this study. Koli et al. (36) utilized the pigment extracted from *M. purpureus* to produce AgNPs in the presence of sunlight. The FTIR spectrum of this pigment extract showed the presence of C-NH-C groups, C–H, and C = O groups, along with a lactone ring, which disappeared in the AgNPs FTIR spectrum, indicating that opening of the lactone ring and subsequent formation of carboxylic acid leads to the peak at 1646.46 cm$^{-1}$. Another study used the pigment obtained from *M. ruber* to synthesize AgNPs under exposure to xenon lamp light, obtaining particles in the 5–35 nm size range with the presence of C-NH-C, C–H, and C = O groups, along with a lactone ring, similar to the findings of other *Monascus* pigment studies. In addition, the peak near 1388 cm$^{-1}$ in the AgNP spectrum was ascribed to the presence of NO$_3$ in the residual solution (35). The peaks detected in the *T. purpurogenus* pigment in the present study were ascribed to the presence of alcohol, phenol, carbonyl, and amide groups, that were regarded as active reducing and capping agents. Similar FTIR findings were reported by Osibe and Aoyagi (40). Jena et al. (41) used fucoxanthin pigment obtained from the diatom *Amphora* to synthesize AgNPs in the presence
of sunlight. Overall, the data obtained from this study and the comparison to other pigment-mediated synthesis methods in the literature indicate that naturally produced pigments have the capacity to reduce and stabilize the precursor molecules needed to generate AgNPs.

The results also indicated the concerted effects of pH and light in the generation of AgNPs using a *T. purpureogenus* extracellular pigment. Previous studies using *Azadirachta indica* at pH 13 ([42]) and a *Lysinibacillus sphaericus* MR-1 cell-free extract at pH 12 ([43]) suggested that an increase in the reduction capability at higher pH values could be due to an increase in the availability of biofunctional groups. Based on our results, we hypothesize that in alkaline pH, the nature of the pigment extract could have been altered, leading to the same size that in alkaline pH, the nature of the pigment functional groups could have been destabilized or destroyed, consequently decreasing AgNP generation.

Alternatively, to explain the role of pH using NaOH, in presence of light, the following reaction system is proposed:

\[ 2\text{OH}^- + 2\text{Ag}^+ \rightarrow \text{Ag}_2\text{O} + \text{H}_2\text{O} \]

\[ \text{Ag}_2\text{O} + \text{Pigment}_{\text{Light}} \rightarrow \text{Pigment}_{\text{oxidized}} + 2\text{Ag}^0 \]

As the pH increases to 13, the rate of the AgNO$_3$ to Ag$_2$O reaction is faster than that of reduction to Ag$^0$ by the pigment, which leads to aggregation, diminished production, and Ag$_2$O precipitation. Therefore, the first step in this pathway becomes the rate-limiting step, which explains the role of NaOH as an accelerator and a size-controlling agent of AgNP synthesis, in line with previously published data ([44],[45]). A similar mechanism employing NaOH as an accelerator and glucose as the reducing agent was explored by Singh et al. ([45]). Several previously reported green synthesis processes have employed an alkaline pH to increase the rate of synthesis and achieve the monodispersity of AgNPs. Du et al. ([46]) used a cell extract from *P. oxalicum* in the presence of light to synthesize AgNPs, and also found that the synthesis was faster and the average particle size was smaller at pH 12. The researchers reasoned that this effect could be due to the significant enhancement in the reducing power of the bioreductor. Similarly, a bioreduction process employing *Lactobacillus fermentum* LMG 8900 showed the rapid formation of AgNPs and consequent plasmon resonance band within 1 min at pH 11.5 ([47]). Recently, Riaz et al. ([48]) used green tea extract at various pH values to synthesize AgNPs, and found that as the pH increased to 11, the SPR peak became sharper and exhibited a red shift in the UV-Vis spectrum, indicating the presence of small-sized, regular spherical particles. In contrast, *E. coli* supernatant-mediated synthesis showed the possibility of the synthesis of AgNPs at alkaline pH (8.0–12.0); however, the mean diameter of the particles started to increase after pH 10 ([49]).

In addition, according to the results regarding the effects of light, it is conceivable that the process could be a light-mediated, pH-dependent reaction, where the light dependency of the NP production process could clearly be seen up to around pH 10, and thereafter the effect of pH controls the reduction efficiency. This could possibly be due to the high concentration of NaOH acting as an accelerator, thereby increasing the reaction rate to the extent that the effect of light on the NP yield could not be perceived. Possibly, to visualize the effect of light on the AgNP yield at higher pH, exposure to higher light intensity might be required. The DLS data showed a clear trend of variation in particle size distribution in the presence and absence of light. However, it is important to consider that DLS data for polydispersed samples can be clouded by the presence of larger particles in the solution, and UV-Vis spectroscopy has been shown to be insufficiently sensitive to account for variation in particle size ([50]). Previous studies have hypothesized that metal NP synthesis methods aided by light relied on the reduction of the M$^{n+}$ cation to M$^0$ by the direct or indirect action of light. Following the absorption of light by chromophores, the molecules were excited from their ground state to an electronic state; consequently, in the presence of light, the electrons jumping between energy levels should be responsible for the reduction of AgNO$_3$ ([37],[51]).

Earlier studies have shown that the presence of light affects the final NP product. Popov et al. ([52]) reported that photostimulation during NP synthesis changed the size and shape of the final product, and this effect was dependent on the light source used and the method employed for initial reduction and stabilization prior to irradiation. More recently, Shabnam et al. ([53]) demonstrated that chloroplasts/thylakoids isolated from the leaves of *Spinacea oleracea* could produce AgNPs in the presence of light but with limited synthesis in dark conditions. Moreover, they showed that this generation was also dependent on light intensity as well as on the duration of exposure. Bao et al. ([54]) used the cell extract of the *Neochloris oleoabundans* microalga with 5,000 lux of fluorescent light for AgNP synthesis, showing the possibility of AgNPs biosynthesis under white, blue, or purple light illumination, whereas there was no product formation under orange or red light, as well as in the dark. Another study demonstrated a
change in the toxicity of AgNPs against Tetrahymena pyriformis in the presence of light: illumination at 12,000 lux for 24 h decreased the toxicity of small AgNPs by almost 32%, whereas the corresponding decrease for large AgNPs was found to be around 10.6% \(^{(55)}\). Mittelman et al. \(^{(56)}\) demonstrated that a three-day exposure to UVA and UVB radiation led to increases in the mean diameter, zeta potential, bathochromic shift in SPR, and Ag\(^+\) release. The results available in the literature and the data obtained in this study collectively indicate that the presence of light along with the reduction method employed for production of AgNPs indubitably affect the properties of the final product. The characterization of our produced AgNPs indicate that these NPs might be useful in biological applications, since biological functional groups are involved in reduction and capping. In practice, the AgNP synthesis process was found to have higher yield under high pH conditions, but production can also be achieved using a relatively low pH in conjunction with light. The combination of data obtained from this study and the related literature implies that pigment is the major reducing and stabilizing agent in this process, with NaOH and light playing the role of accelerator and mediator, respectively.

Although the use of NaOH in this process is slightly antithetical to the green synthesis principle, the amount used in the study, and the flexibility it provides in control of the bioreduction process in terms of the reaction rate and possible variance in the size distribution makes it an integral part of this biosynthesis process. We decided to use ethanol in this study to partially purify the pigment so as to minimize the interference from other biological molecules present in the broth, which helped to ensure that the pigment remains the major bioreductant in the process. This further ensures that the process remains one of biomolecule-mediated synthesis rather than becoming a supernatant-mediated synthesis process. The water-soluble extracellular pigment can also be directly used for reduction, which might help to reduce the extraction step required for the conventional pigment-mediated AgNP generation process. Combined with the presence or absence of light, the choice of operating conditions might also provide some flexibility to obtain a desired size distribution. Therefore, the choice of operating conditions can be modified according to the needs of the researcher. In essence, the process is limited by a trade-off between yield and being environmentally friendly.

Green synthesis of NPs has been explored considerably in the recent years and a multitude of biological reducing and capping agents have been discovered, but still more research is required for controlling the size and aspect ratio via biological method of NP production. This report shows that physicochemical factors such as pH and light can modulate the size and distribution of AgNPs, and possibly, in future, more studies can target the various factors involved in green synthesis to obtain a better control over yield, size, shape, and size distribution of metal NPs.

**Conclusions**

AgNPs were successfully synthesized using an extracellular pigment produced by *T. purpurogenus*. As the required pigment concentration was only 0.5 g/L, the process can be cost-effectively scaled up. The composite pigment was found to possess reducing and stabilizing properties, and optimizing the pH of the extract along with exposure to light facilitated pigment-mediated synthesis. NaOH was found to accelerate AgNP production, suggesting its function as a size-controlling agent. The results demonstrated that light acted as a catalyst in the reaction at pigment pH values up to 10. At higher pH, the effect of NaOH dominated the reaction process. Our previous study used alkaline conditions and the presence of light for the synthesis of AgNPs, and their biomedical applications \(^{(38)}\), whereas the present study explored the specific effects of these physicochemical factors. Interestingly, while studying the effect of light and pH in detail, we found that a comparable yield of AgNPs can be generated at a pH above 10 even in the absence of light, although a change in the average particle size was noticed with exposure to light. Hence, the process was found to be light-mediated and pH-dependent in nature. The variations observed in the UV-Vis spectra and DLS measurements with different combinations of physicochemical factors revealed the possibility to easily control the properties of synthesized particles. To our knowledge, this is the first report to describe the influence of light and pH on the generation of biofunctionalized AgNPs using an extracellular pigment produced by *T. purpurogenus*. Additional studies are needed in this direction to determine if there is a significant disparity in the properties of NPs synthesized at various regimes of pH, light intensity, and time. In addition to FTIR data, standards (sugar, peptides, or polyphenols) may be used to compare the data acquired from TLC fractions, in order to obtain a better idea about the molecules present in the fractions of pigment. Possibly, other advanced techniques of analysis such as X-ray photoelectron spectroscopy, inductively coupled plasma–optical emission spectrometry, or thermogravimetric analysis might be able to provide some more insights into the surface chemistry, elemental makeup, amount of organic and inorganic residues,
and other relevant data about the reduction and capping process. Further research on the structure and properties of components of the composite pigment might provide a more complete picture of the reaction process responsible for AgNPs generation by the pigment.

**Declarations**

**Conflicts of interest:** The authors declare no conflict of interest.

**Ethics approval:** Not applicable.

**Consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**Authors’ contributions:** S. B. and H. A. designed the research. H. A. supervised the research. S. B. and H. A. designed the experiments and S. B. performed them. Christiana N. Ogbonna and James C. Ogbonna helped in cell cultivation and pigment production. S. B. and H. A. analyzed and interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

S. B. was supported by a scholarship grant from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (The Global Food Security Partnership Program, University of Tsukuba). We would like to thank Editage (www.editage.com) for English language editing.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This work was supported by Hirose Foundation, Japan (grant to S. B.); JSPS: [Grant Number Grant-in Aid for Scientific Research B (19H03086)]; Sumitomo Electric Industries Group Corporate Social Responsibility Foundation; Sumitomo Foundation: [Grant Number Grant for Environmental Research Project] (grant to H. A.).

**Data availability**

The data that support the findings of this study are available from the corresponding author [H.A], upon reasonable request.

**References**

[1] Zhang, X.-F.; Liu, Z.-G.; Shen, W.; Gurunathan, S. Int. J. Mol. Sci. 2016, 17, 1534.

[2] Yaqoob, A.A.; Umar, K.; Ibrahim, M.N.M. Appl. Nanosci. 2020, 10, 1369–1378.

[3] Bonilla-Gameros, L.; Chevallier, P.; Sarkissian, A.; Mantovani, D. Nanomedicine (N. Y., NY, U. S.) 2020, 24, 102142.

[4] Roy, A.; Bulut, O.; Some, S.; Mandal, A.K.; Yilmaz, M.D. RSC Adv. 2019, 9, 2673–2702.

[5] Barabadi, H.; Damavandi Kamali, K.; Jazayeri Shoushtari, F.; Tajani, B.; Mahjoub, M.A.; Alizadeh, A.; Saravanan, M. J. Cluster Sci. 2019, 30, 1375–1382.

[6] Dey Bhoumik, A.; Bandyopadhyay, A.; Chattopadhyay, A. The Nucleus 2019, 62, 277–285.

[7] Batool, S.; Hussain, Z.; Niazi, M.B.K.; Liaqat, U.; Afzal, M. J. Drug. Deliv. Sci. Technol. 2019, 52, 403–414.

[8] Badmus, J.A.; Oyemomi, S.A.; Adedosu, O.T.; Yekeen, T.A.; Azeez, M.A.; Adebayo, E.A.; Lateef, A.; Baddeggi, U.M.; Botha, S., Hussein, A.A., et al. Heliyon 2020, 6, e05413.

[9] Elegbede, J.A.; Lateef, A.; Azeez, M.A.; Asafa, T.B.; Yekeen, T.A.; Oladipo, I.C.; Aina, D.A.; Beukes, L.S.; Gueguim-Kana, E.B. Waste. Biomass. Valorization. 2020, 11, 781–791.

[10] Lateef, A.; Ojo, S.A.; Elegbede, J.A.; Azeez, M.A.; Yekeen, T.A.; Akinboro, A. J. Cluster Sci. 2017, 28, 1379–1392.

[11] Fakhruddin, K.S.; Egusa, H.; Ngo, H.C.; Panduvavala, C.; Pesee, S.; Samaranayake, L. BMC. Oral. Health. 2020, 20, 160.

[12] Rodrigues, M.C.; Rolim, W.R.; Viana, M.M.; Souza, T.R.; Gonçalves, F.; Tanaka, C.J.; Bueno-Silva, B.; Seabra, A.B.J. Dent. 2020, 96, 103327.

[13] Lara, H.H.; Ixtepan-Turrent, L.; Jose Yacaman, M.; Lopez-Ribot, J. ACS Appl. Mater. Interfaces 2020, 12, 21183–21191.

[14] Barabadi, H.; Vahidi, H.; Damavandi Kamali, K.; Hosseini, O.; Mahjoub, M.A.; Rashedi, M.; Jazayeri Shoushtari, F.; Saravanan, M. J. Cluster Sci. 2020, 31, 323–330.

[15] Barabadi, H.; Vahidi, H.; Mahjoub, M.A.; Kosar, Z.; Damavandi Kamali, K.; Ponmurugan, K.; Hosseini, O.; Rashedi, M.; Saravanan, M. J. Cluster Sci. 2020, 31, 1173–1184.

[16] Khattua, A.; Prasad, A.; Priyadarshini, E.; Patel, A.K.; Naik, A.; Saravanan, M.; Barabadi, H.; Ghosh, I.; Paul, B.; Paulraj, R. J. Cluster Sci. 2020, 31, 1329–1340.

[17] Barabadi, H.; Vahidi, H.; Damavandi Kamali, K.; Rashedi, M.; Hosseini, O.; Golnaraghi Ghomi, A.R.; Saravanan, M. J. Cluster Sci. 2020, 31, 311–321.

[18] Che Sulaiman, I.S.; Chiang, B.W.; Osman, M.J.; Ong, K.K.; Rashid, J.I.A.; Wan Yunus, W.M.Z.; Noor, S.A.M.; Kasim, N.A.M.; Halim, N.A.; Mohamed, A. Microchim. Acta. 2020, 187, 131.

[19] Singh, R.; Thakur, P.; Thakur, A.; Kumar, H.; Chawla, P.; Rohit V. J.; Kaushik, R.; Kumar, N. Int. J. Environ. Anal. Chem. 2020, 101 (15), 1–17.

[20] Marimuthu, S.; Antonisamy, A.J.; Malayandi, S.; Rajendran, K.; Tsai, P.-C.; Pugazhendhi, A.; Ponnumusamy, V.K. J. Photochem. Photobiol., B 2020, 205, 111823.

[21] Saratale, R.G.; Saratale, G.D.; Shin, H.S.; Jacob, J.M.; Pugazhendhi, A.; Bhaisare, M.; Kumar, G. Environmental Science and Pollution Research 2018, 25, 10164–10183.

[22] Siddiqi, K.S.; Husen, A.; Rao, R.A.K. J. Nanobiotechnol. 2018, 16, 14.
