Bacteria and nanosilver: the quest for optimal production

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Abstract

Silver nanoparticles (AgNPs) have potential uses in many applications, but current chemical production methods are challenged by scalability, limited particle stability, and the use of hazardous chemicals. The biological processes present in bacteria to mitigate metallic contaminants in their environment present a potential solution to these challenges. Before commercial exploitation of this technology can be achieved, the quality of bacteriogenic AgNPs needs to be improved for certain applications. While the colloidal and morphological stabilities of biogenic AgNPs are widely regarded as superior to chemogenic particles, little control over the synthesis of particle morphologies has been achieved in biological systems. This article reviews a range of biosynthetic reaction conditions and how they affect AgNP formation in bacteria to understand which are most influential. While there remains uncertainty, some general trends are emerging: higher Ag^+ concentrations result in higher AgNP production, up to a point at which the toxic effects begin to dominate; the optimal temperature appears to be heavily species-dependent and linked to the optimal growth temperature of the organism. However, hotter conditions generally favour higher production rates, while colder environments typically give greater shape diversity. Little attention has been paid to other potentially important growth conditions including halide concentrations, oxygen exposure, and irradiation with light. To fully exploit biosynthetic production routes as alternatives to chemical methods, hurdles remain with controlling particle morphologies and require further work to elucidate and harness. By better understanding the factors influencing AgNP production a foundation can be laid from which shape-controlled production can be achieved.

Keywords: Biosynthesis, bacteria, silver nanoparticles, mechanism, reaction conditions

Contents

1 Introduction

Metallic nanoparticles have come to the forefront of research in recent years due to their unique properties which make them appealing in a wide range of applications. The broad potential uses of these nanomaterials include catalysts, anti-cancer therapeutics, antimicrobials, diagnostics, water treatment, and energy-saving glazing, among others.(1-9)

Of these particles, silver nanoparticles (AgNPs) have received much attention for their physical and chemical properties, as well as the range of production methods available. The material characteristics which lend themselves to these uses come from two key features of AgNPs: very high surface-area-to-volume ratios and surface plasmons.

Figure 1. *Chemically produced silver nanoprisms with different optical properties. Adapted and reprinted with permission from Harber and Sokolov (2017). Copyright 2017 American Chemical Society.*

The surface-area-to-volume ratio of a nanoparticle is much higher than in bulk materials due to its nanoscopic dimensions (10^6 times greater for 10 nm compared to 1 cm sized particles). As a result, the chemical activity of the particle is increased to the extent that substances, which are typically inert at the macroscopic scale, can be used as effective catalysts at the nanoscale; one such example is gold nanoparticles.(5, 10, 11) Moreover, having nanometric dimensions, the interaction between electromagnetic radiations with bigger wavelength than the nanoparticles' size can affect the nanoparticles' behaviour. The small size of nanoparticles also

affects how they interact with electromagnetic radiation. The alternating electric fields of incident light lead to oscillations of the conduction electrons in the lattice structure of metallic nanoparticles.(12, 13) When these oscillations reach a resonance frequency, known as the localised surface plasmon resonance (LSPR), the absorption of light is increased. It is this property that gives colloidal AgNP solutions their vivid colours, as demonstrated in Figure 1.

The LSPR phenomenon is dependent on the composition, dielectric environment, size, and shape of the nanoparticles.(12, 13) Synthesis methods must therefore be able to produce high quality monodispersed nanoparticles tuneable to the desired application when exploiting these properties. While this can be achieved to some degree with existing technologies, current nanoparticle production routes are limited by their dependence on hazardous chemicals, high energy demands from heating or light sources, and challenges with particle agglomeration and production scalability.(14, 15) In recent years, synthetic processes derived from biology have been demonstrated as a possible alternative production route. The biosynthesis of metallic nanoparticles has been described in a range of organisms spanning plants, fungi, and bacteria, often through metal-toxicity resistance mechanisms.(16-19) Whilst the processes for many of these remain poorly understood, they hold great potential for more environmentally friendly and up-scalable production of AgNP than chemical synthesis.

A number of review articles have been authored which give useful overviews of bacteriogenic metallic nanoparticle synthesis.(14, 15, 17-22) However, to date, no in-depth summary of findings has been published relating to the reaction conditions under which AgNPs are synthesised and the impact that these conditions have on the products. Therefore, this article aims to bring together such results and discuss how biosynthetic reaction parameters can be used to optimise AgNP production with a focus on shape and size control. It begins by reviewing the proposed mechanisms for AgNP biosynthesis in bacteria, which is then followed

by examining reaction parameters which have been varied in the quest for optimising AgNP production.

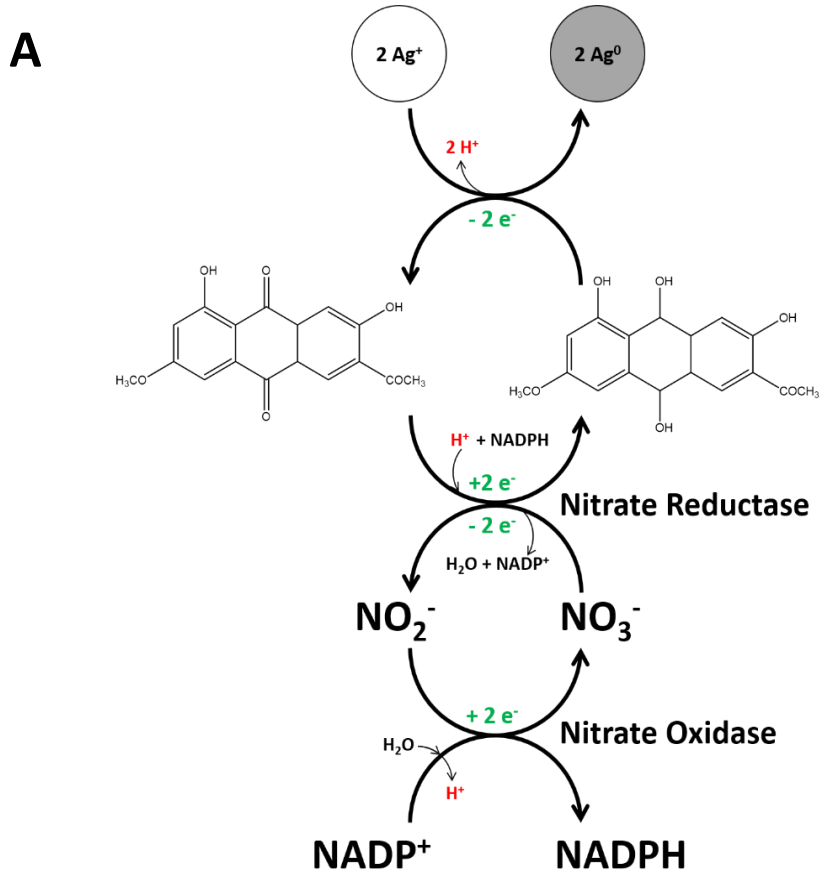
2 Silver Toxicity, Resistance, and Nanoparticle Synthesis

Following the discovery of biogenic metallic nanoparticles by Klaus et al. in 1999,(23) the possibility of controllable biosynthesis has been an ultimate yet elusive goal of the field. However, the mechanistic understanding of the underpinning biology remains limited. Especially for AgNP production, there is a relative paucity of detailed investigations. Despite this, efforts continue to identify the pathways involved in the reduction of soluble Ag^{+} and accumulation of zero valent Ag^{0}.

2.1 Silver Toxicity to Cells

To understand how bacteria produce nanoparticles, it is first prudent to understand why bacteria make nanoparticles. It has long been known that silver is either toxic to bacteria or bacteriostatic.(24) This toxicity stems from the high affinity with which Ag^{+} binds to a range of biological macromolecules, particularly those with electron rich sulphur hydryl (including thiol) and amine groups. This results in the side chains of cysteine residues in proteins often being targeted by Ag^{+} which disrupts disulphide bonds leading to the denaturation of the tertiary structure of proteins, and thus their functions.(25) Additionally, Ag^{+} ions complex with the heterocyclic amines in DNA bases, causing disruption to the transcription and replication of genetic material.(26) This activity results in substantial inhibition of major biological processes including DNA, RNA, protein, and peptidoglycan syntheses at levels comparable with commonly used antibiotics.(27) Moreover, complete cell lysis is not induced by Ag^{+}, but membrane disruption has been evidenced.(27, 28) These effects, compounded with damage to the cell membrane, have been demonstrated to have detrimental outcomes for the bacterium. Furthermore, high concentrations of Ag^{+} are thought to induce apoptosis in bacterial cells, as

demonstrated by the shrinking of cells sizes and fragmentation of DNA, a behaviour more commonly attributed to eukaryotes.(29, 30)



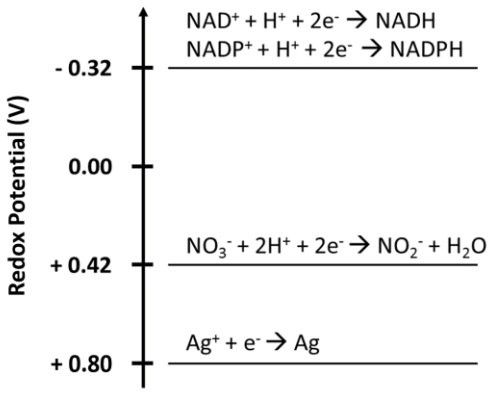


Figure 2. a) *Proposed mechanism of reduction of Ag^+ in the formation of nanoparticles utilising the reduction of nitrate to nitrite in the fungus Fusarium oxysporum. The electrons*

are shuttled from the reductase to Ag^+ by an electron shuttle, in this case 4-hydroxyquinoline. Adapted from Durán et al.(31) originally published by BioMed Central. **b)** *Reduction potentials of reactions thought to be involved in biogenic AgNP production.(32, 33)*

This induction of cell "suicide" is hypothesised to result from the increase in the concentration of reactive oxygen species in the cell, attributed to Ag^+ interfering with NADPH dehydrogenase II activity.(29)

The toxicity of Ag^+ exhibits stress on bacteria which has stimulated the evolution and prevalence of resistant mechanisms. A bacterium capable of detoxifying or removing Ag^+ holds a potential survival advantage in environments where Ag^+ is present. The processes that allow Ag^+ resistance are believed to be important for AgNP bioproduction.

2.2 Resistance of Cells

Reflecting the toxicity of Ag^+ in bacteria, two mechanistically different resistance mechanisms have evolved and are concurrently represented in resistant organisms:

The first mechanism is an efflux system. This functions to sequester Ag^+ ions from the intracellular environment and eject them via a P-type ATPase (SilP) and an efflux transporter (SilCBA).(34, 35) Homologous to the Cus copper-efflux system, which acts to sequester and remove Cu^+ from the intracellular environment,(35-37), *sil* genes have been found in Ag-resistant organisms from both environmental and clinical settings.(34, 38) These *sil* genes were identified from their sequence homology with known Cus genes, and their functions inferred.(35, 39) While both systems have a Ag^+ and Cu^+ sequestering protein, SilF and CusF, respectively, which are thought to support the efflux process, the Sil system also has the SilE protein. The SilE protein is a highly specific periplasmic Ag^+ sequestering protein which can bind up to 10 Ag^+ ions per peptide.(34) Homologues of *sil* genes have been found in organisms capable of producing AgNP and are thought to be involved, due to their ability to sequester

Ag^+, in the production process.(40) However, the mechanism of protein involvement in the process remains to be determined. The ability to sequester ions only provides the bacterium with short-term protection. For a long-term solution to the toxcitiy of Ag^+, the cell must use alternative methods.

The second mechanism of Ag resistance suppresses the hazard by utilising the reduction of Ag^+ to Ag^0. Ag in its metallic form is less toxic to the cell, so by reducing solubilised Ag^+ to insoluble Ag^0 the bacterium can reduce the chemical stress.(41) Upon creation, Ag^0 atoms undergo nucleation and continue to grow into AgNP. Like chemical reduction synthesis, this process follows a bottom-up approach building nanoparticles via multistep self-assembly (as opposed to removing material from larger materials in a top-down route), but instead of reducing agents such as sodium borohydride, less hazardous biological components are used. Exactly how this process occurs remains unclear, but two main theories have been postulated as detailed below.

2.3 Mechanisms of Silver Nanoparticle Biosynthesis

Of the two theories proposed, the first hypothesises that simple biochemicals act as reducing agents for Ag^+ reduction. Aldehyde groups, like those in sugars, have been suggested as key reduction sites and electron donors. For example, in a number of *Lactobacillus* species which demonstrated the ability to rapidly produce AgNP, both intra- and extracellularly.(42) However, reducing sugars are produced by all bacteria, AgNPs are not. This hypothesis does not account for the distinct species-dependency reported in the literature. Instead, it is likely that an enzymatic component to the mechanism is involved.

The second theory regarding the reducing agent in biological systems has led to NADH or NADPH-dependent nitrate reductase enzymes (Figure 2a), which have been identified in a number of species as having crucial roles in the detoxification and reduction of Ag^+.(43, 44)

NAD(P)H-dependent nitrate reductases are part of the molybdenum-containing dimethylsulphoxide reductase (DMSOR) enzyme family.(45) Based on the locations of DSMORs within the cell, three main classes have been identified. Nas and Nar type enzymes are cytoplasmic and respiratory membrane-bound types, respectively. As many studies have indicated that AgNP formation is located at the cell surface or in the periplasmic space,(23) of particular interest here are the dissimilatory periplasmic nitrate reductases, or Nap species.

The enzymes, as their name suggests, utilise the reductive power of NAD(P)H to reduce nitrate to nitrite. To achieve this, NAD(P)H donates two electrons via a hydride ion (H^-) to the NAD(P)H binding site of the reductase. The electrons are passed via iron-sulphur clusters to the Mo active site where the nitrate, which covalently binds to the Mo, then undergoes reduction of nitrate in the presence of H^+ to form nitrite (NO_2^-) and H_2O.(45)

α-NADPH-dependent nitrate reductase isolated from the fungus *Fusarium oxysporum* was able to produce AgNPs in a reaction which required 4-hydroxyquinoline as an electron shuttle, as shown in Figure 2a.(44, 46-48) The requirement of an electron shuttle indicated that the enzyme was not directly reducing the Ag^+ and the NO_3^- in parallel, but instead reduced the nitrate and the generated electron was transferred to the Ag^+ which was subsequently reduced in a series fashion. Additionally, the electrochemistry of the involved species favoured the electron transfer to Ag^+ from NO_3^-, as can be seen in Figure 2b. There is, however, little evidence to support the direct generalisation of this idea to bacteria.

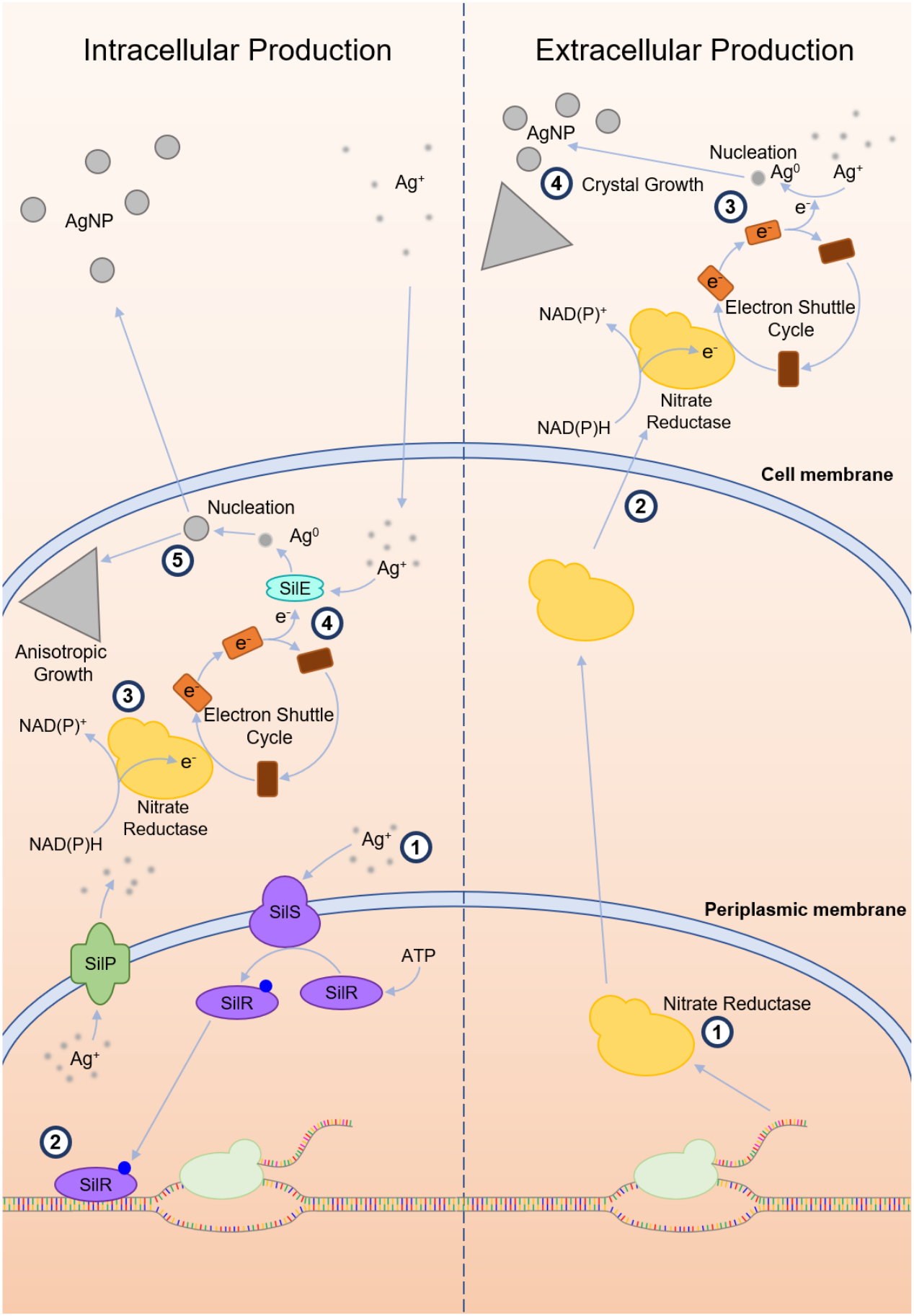
From the relatively limited amount of work presented on the bacterial mechanism, there is some support for the involvement of NAD(P)H-reductases with nitrate reductase activity having been detected in the cell-free extract (CFE) of *Bacillus subtilis* and is thought to be the catalyst for AgNP formation.(49) Moreover, partially purified nitrate reductase from *Pseudomonas aeruginosa* CFE demonstrated Ag^+-reduction activity,(50) and the presence of

piperidones (a natural inhibiter of nitro-reduction in Enterobacteriaceae) has been shown to partially inhibit AgNP synthesis, further supporting this hypothesis.(51) It should be stressed, however, that making the jump from a fungal to a bacterial mechanism needs further investigation to identify the commonalities and differences.

It has been postulated that it is the aforementioned SilE protein that chelates and then presents Ag^+ to a reductase,(15, 52) as supported by the discovery that a number of bacteria, which are able to produce nanoparticles, carry the *sil* genes.(38, 53) A summary of this proposed theory is outlined in Figure 3. By examining nitrate reductase during AgNP formation in the archaeon *Halococcus salifodinae*, Srivastava et al.(54) suggested that Ag^+ became the favoured electron donor over nitrates, as evidenced by a reduction in the concentration of nitrite in an Ag^+ dose-dependent manner. Indeed, following the conclusion of AgNP production, nitrite concentrations rose to levels above Ag^+-free controls, suggesting the presence of Ag^+ had resulted in increased expression of nitrate reductase. Additionally, by comparing nitrate reductase activity following exposure to KNO_3 and $AgNO_3$ it was possible to attribute the increase to Ag^+ and not the NO_3^-, suggesting that the nitrate reductase was involved in Ag resistance. Furthermore, heat inactivation of *Lactobacillus casei* was shown to inhibit AgNP production, supporting the theory that the process was enzyme mediated.(55) Following their formation in cells, the particles were either ejected from the cell or grew in the periplasmic space (Figure 3). Larger non-spherical particles have been observed inside cells, with smaller spherical particles frequently observed in the external environment.

Other enzymes, including amylase, laccases, peroxidase and lysozyme, have been shown to be involved in AgNP formation, but have received little attention. Further information can be found in Durán's mini-review on the catalytic roles of traditional enzymes in metallic nanoparticle formation.(56)

These mechanisms remain to be understood before they can be fully exploited. Moreover, the molecular biology influencing crystal growth has seen little research despite the species dependent nature with which nanoparticles with different geometries are made. Nonetheless, the studies discussed below have investigated the physiochemical impact of various growth conditions on the formation of the nanoparticles.


Intracellular Production
Extracellular Production
AgNP
Ag+
Nucleation
Ag0
Crystal Growth
e-
Electron Shuttle Cycle
NAD(P)+
NAD(P)H
Nitrate Reductase
Cell membrane
Anisotropic Growth
SilE
SilS
SilP
SilR
ATP
Periplasmic membrane

1

Figure 3. *Proposed mechanisms for intracellular (left) and extracellular (right) silver nanoparticle (AgNP) production in bacteria.* **Intracellular** *AgNP production using the SIL proteins is thought to be initiated by the sensor kinase SilS protein which phosphorylates the derepresser SilR (**1**). SilR phosphorylation results in expression of the Sil operon which includes the SilCBA anti-porter complex, a P-type ATPase (SilP), and SilE, which sequesters* Ag^{+} *ions (**2**). An NAD(P)H-dependent nitrate reductase is thought to reduce an electron shuttle via the oxidation of NAD(P)H to* $NAD(P)^{+}$ *(**3**). The electron shuttle facilitates the electron transfer from the reductase to SilE (**4**), though the electron exchange does not need SilE to occur. Following reduction,* Ag^{0} *atoms undergo nucleation to form seed particles (**5**).* **Extracellular** *production of AgNP, such as when using cell-free extract, through an enzymatic route likely results from the innate expression of an NAD(P)H-dependent nitrate reductase (**1**), which is secreted or leaked to the extracellular environment (**2**). Here, the electron shuttle cycling occurs in a similar way to intracellular production (**3**), and AgNP growth can then occur (**4**). However, smaller spherical particles are more often observed in extracellular production. Non-enzymatic reducing processes may also occur in both mechanisms.*

3 Biosynthetic Reaction Conditions

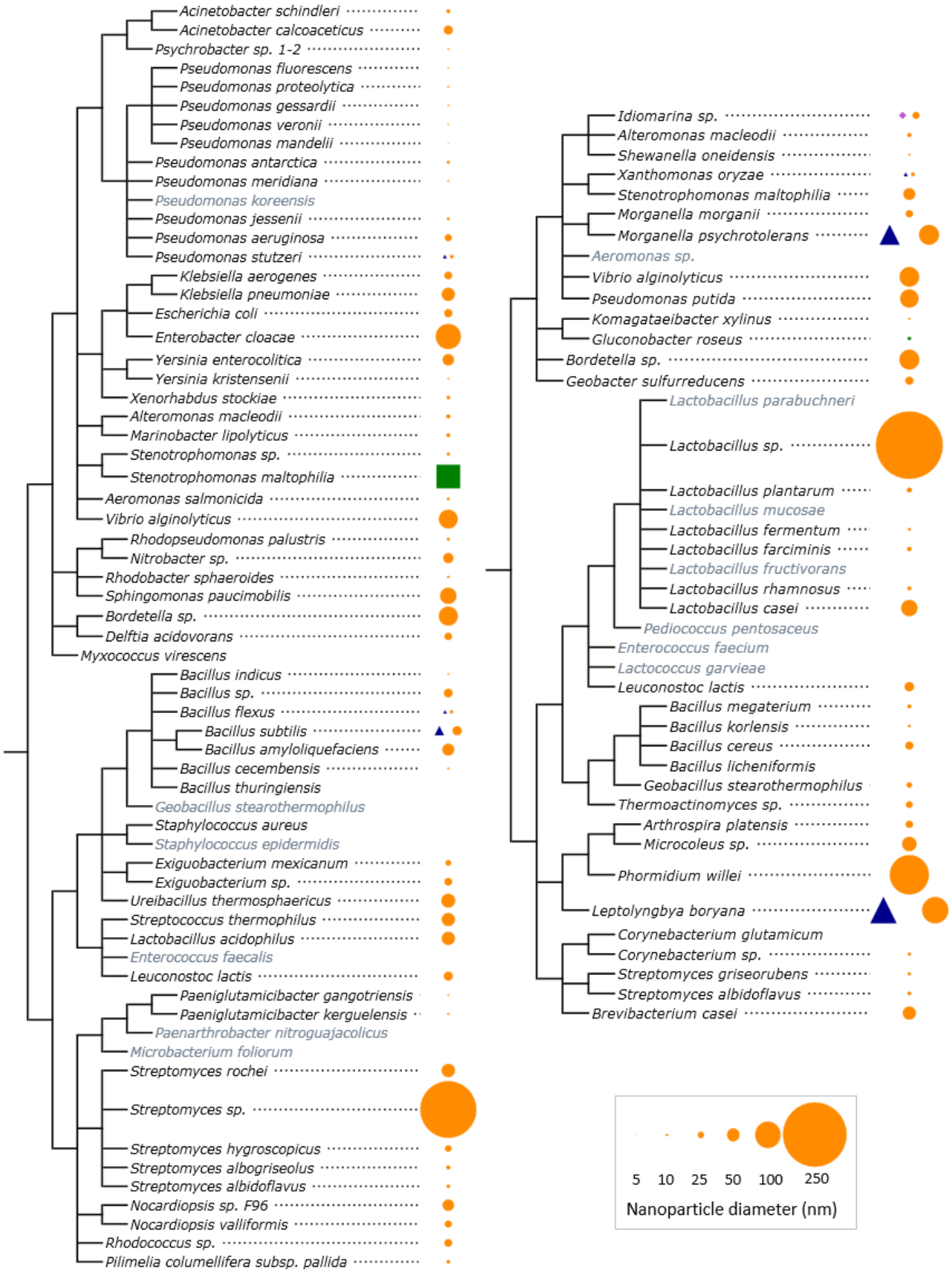
Isolated from a silver mine, *Pseudomonas stutzeri* AG259 was the first bacterium found to demonstrate the ability to produce AgNP.(23) After this discovery at the turn of the millennium, many more species capable of such activity have been identified (Figure 4). Nonetheless, the biosynthesis of AgNPs can be influenced by chemical and physical factors, such as thermodynamics, reaction and enzyme kinetics, and photo/radio-catalysis, as well as the underlying biology of the host organism. The complex nature of AgNP production means that these factors are connected in ways which are not yet fully understood. However, work has been performed in numerous studies to investigate how the reaction conditions affect AgNP biosynthesis.

3.1 Incubation Time

By far, the most investigated variable influencing the biosynthesis of AgNP is reaction duration, typically in the range of minutes (51, 57) to days (38, 58, 59). As expected, the longer the duration, the more and potentially larger particles are produced. However, the vast majority of findings report no change in the LSPR peaks throughout the duration of experiments, indicating no change in the morphologies of the AgNP being produced over 20 minutes to 120 hours.(60-62)

The previous reports notwithstanding, a small number of studies have observed changes in particles morphologies over time. For example, when using *Comamonas acidovorans* CFE, a slight blue shift and broadening of the LSPR peak over 72 hours was observed(63): this is likely the result of smaller particles forming and an increase in polydispersity.(13) Conversely, the AgNPs produced by *Aeromonas sp.* SH10 over 1 or 6 days showed a slight red shift in the LSPR peak position over 6 days, a phenomenon not observed in the shorter timescale. This

slight bathochromic-shift indicates particles with larger sizes being formed.(64, 65) These slight changes in LSPR peak position reflect subtle changes in particle morphologies and geometries, and also the surface coating of the particles. The occurrence of these changes is not understood.


Acinetobacter schindleri
Acinetobacter calcoaceticus
Psychrobacter sp. 1-2
Pseudomonas fluorescens
Pseudomonas proteolytica
Pseudomonas gessardii
Pseudomonas veronii
Pseudomonas mandelii
Pseudomonas antarctica
Pseudomonas meridiana
Pseudomonas koreensis
Pseudomonas jessenii
Pseudomonas aeruginosa
Pseudomonas stutzeri
Klebsiella aerogenes
Klebsiella pneumoniae
Escherichia coli
Enterobacter cloacae
Yersinia enterocolitica
Yersinia kristensenii
Xenorhabdus stockiae
Alteromonas macleodii
Marinobacter lipolyticus
Stenotrophomonas sp.
Stenotrophomonas maltophilia
Aeromonas salmonicida
Vibrio alginolyticus
Rhodopseudomonas palustris
Nitrobacter sp.
Rhodobacter sphaeroides
Sphingomonas paucimobilis
Bordetella sp.
Delftia acidovorans
Myxococcus virescens
Bacillus indicus
Bacillus sp.
Bacillus flexus
Bacillus subtilis
Bacillus amyloliquefaciens
Bacillus cecembensis
Bacillus thuringiensis
Geobacillus stearothermophilus
Staphylococcus aureus
Staphylococcus epidermidis
Exiguobacterium mexicanum
Exiguobacterium sp.
Ureibacillus thermosphaericus
Streptococcus thermophilus
Lactobacillus acidophilus
Enterococcus faecalis
Leuconostoc lactis
Paeniglutamicibacter gangotriensis
Paeniglutamicibacter kerguelensis
Paenarthrobacter nitroguajacolicus
Microbacterium foliorum
Streptomyces rochei
Streptomyces sp.
Streptomyces hygroscopicus
Streptomyces albogriseolus
Streptomyces albidoflavus
Nocardiopsis sp. F96
Nocardiopsis valliformis
Rhodococcus sp.
Pilimelia columellifera subsp. pallida
Idiomarina sp.
Alteromonas macleodii
Shewanella oneidensis
Xanthomonas oryzae
Stenotrophomonas maltophilia
Morganella morganii
Morganella psychrotolerans
Aeromonas sp.
Vibrio alginolyticus
Pseudomonas putida
Komagataeibacter xylinus
Gluconobacter roseus
Bordetella sp.
Geobacter sulfurreducens
Lactobacillus parabuchneri
Lactobacillus sp.
Lactobacillus plantarum
Lactobacillus mucosae
Lactobacillus fermentum
Lactobacillus farciminis
Lactobacillus fructivorans
Lactobacillus rhamnosus
Lactobacillus casei
Pediococcus pentosaceus
Enterococcus faecium
Lactococcus garvieae
Leuconostoc lactis
Bacillus megaterium
Bacillus korlensis
Bacillus cereus
Bacillus licheniformis
Geobacillus stearothermophilus
Thermoactinomyces sp.
Arthrospira platensis
Microcoleus sp.
Phormidium willei
Leptolyngbya boryana
Corynebacterium glutamicum
Corynebacterium sp.
Streptomyces griseorubens
Streptomyces albidoflavus
Brevibacterium casei
5
10
25
50
100
250
Nanoparticle diameter (nm)

1

Figure 4. *Bacteria known to produce AgNP. Both cell-free extract (***left***) and whole-cell cultures or biomass (***right***) have been investigated.(23, 38, 42, 50, 51, 55, 57-61, 63-132) The shape and relative size of the nanoparticles produced are shown. If more than one report exists for a given organism, the average has been used. Names in grey did not report particle morphologies. Taxonomy data were collected from the NCBI Taxonomy database and the figure was generated using the ETE3 tool kit.(133)*

More dramatic changes in particle shape were observed with the formation of a second LSPR peak between 650-950 nm from AgNPs produced using *Morganella psychrotolerans* over 20 hours.(38) This is likely due to be the result of non-spherical particles forming, but may also reflect aggregation of nanoparticles.

Based on the above findings, the duration of incubation appears to have minimal effect on the geometries of nanoparticles produced, but does seem to be important for yield. It is consistently evident that the longer cultures are exposed to Ag sources, the more AgNPs are being produced. The relatively slow reactions of biological production routes are a potential challenge which remains to be overcome when competing with much faster chemical synthesis routes (in the order of seconds or minutes).(134, 135)

3.2 Temperature

Temperature has a critical impact on biological systems by affecting the thermodynamics of biochemical reactions and enzyme activity, as well as physiological alterations to gene and protein expression. This is no different in the case of biogenic AgNP synthesis. As with most biochemical reactions, the rate of reduction of Ag^{+} to Ag^{0} is faster at higher temperatures. Generally, AgNP formation in CFE favours higher temperature conditions than whole cell cultures, but production will decrease if the temperature becomes too high, presumably due to thermal denaturation of the reducing enzymes.(66, 73, 89, 96, 110) Reactions using biomass

have also been reported at high temperatures; *Aeromonas* sp. *and Corynebacterium sp.* showed optimal production at 60 °C.(65, 86) In some cases (*Escherichia coli, Bacillus cereus, Staphylococcus aureus, and Yersinia enterocolitica),* higher temperatures are required to observe any AgNP production.(73) This suggests that the reaction is a chemical process with little, if any, enzymatic involvement. Despite the evidence for higher production rates at higher temperatures, it should be noted that this is not universal. For example, the temperature at which *Xanthobacter kerguelensis* CFE was incubated had insignificant effect on AgNP production.(72)

For species with optimal growth temperatures of 37 °C, the optimal temperature for AgNP production was often higher when using CFE.(73, 96) Similarly, psychrotolerant bacteria have shown greater AgNP production at temperatures above those considered optimal for growth.(38, 58) The mechanism has not been explored.

Temperature appears to be a strong effector of particle morphologies with studies reporting different geometries from different temperatures. Higher temperatures have been associated with smaller spherical AgNPs from *E. coli* and *Acinetobacter calcoaceticus*.(66, 70, 89) In contrast, at lower temperatures (15 °C and 5 °C), while production rates are markedly lower, an increased frequency of larger non-spherical particles, including hexagonal plates and triangular prisms has been reported.(38) However, there is conflicting evidence to support this as the nanoparticles produced by psychrophilic bacteria at 4 °C were typically smaller and more monodispersed compared with those produced at 30 °C, but were produced at a slower rate.(58) Moreover, a slight (+10 nm) red shift was observed at higher temperatures when Ag^{+} was reduced to nanospheres by *P. stutzeri* CFE,(110) indicating the presence of larger particles.

Caution should be taken when using high temperatures in biological systems. Although at 100 °C the CFE of *Plectonema boryanum* produced octahedral and triangular prisms more

frequently than at lower temperatures,(100) the boiling of the solution ultimately renders any biological or enzymatic involvement moot.

The usability of a colloid relies on its stability, that is, the ability of the particles to remain in suspension. Particles produced at lower temperatures have been reported to be more stable with aggregation occurring at higher temperatures.(38, 72, 102) The superior colloidal stability of biogenic AgNPs compared with chemogenic particles is thought to stem from protein corona which coat the surface of the particles. At high temperatures, the constituent proteins denature, and the corona become compromised, reducing its stabilising effects. This may explain the increase in aggregation observed.

Although changes in the reaction and enzyme kinetics, as well as reagent, product, and corona stabilities likely play important roles, the mechanism concersning what controls the shape of AgNP produced in biological systems still remains poorly understood. .

To summarise, likely through its influence on thermodynamics and enzymatics, the temperature of incubation appears to have strong effects on nanoparticle formation in biological systems. Whilst the thermodynamic factors impact directly on crystal growth, temperature is a well-known effector of enzymatic function and activity. Separating these influences in the complex environment of a bacterial cell or CFE requires further investigation. Non-spherical AgNP production, for instance nanoprisms, appears to be favoured by colder conditions. However, there is a trade-off between achieving the desired particle geometry and the production rate.

3.3 Silver Substrate

Silver nitrate ($AgNO_3$) is the predominant source of Ag^+ used experimentally for bacterial AgNP production. This is principally due to its high solubility in water compared to other Ag

salts allowing for sufficiently high bioavailability to be achieved. Many studies have been performed in which the concentration of $AgNO_3$ has been varied and the consequences on nanoparticle synthesis examined.

In most cases, the production of AgNP is higher when more $AgNO_3$ is used, as expected.(110, 128, 136) While 1 mM $AgNO_3$ is typical,(58, 67, 80, 90, 111, 130) concentrations of 9 mM or higher have been used.(23, 59, 68) However, as the reduction of Ag^+ is thought to be a method of detoxifying Ag, it is logical to presume that once the reduction system is saturated any excess Ag^+ will have toxic effects on the cell, that is, there must be an upper limit that the systems can handle. Such toxicity would be detrimental to the enzymatic activity of the mechanism leading to a decrease in reduction activity, and will likely be species dependent. Indeed, enzyme saturation has been suggested to explain why higher concentrations do not always result in greater production; numerous reports have shown a decrease in production with more Ag^+ present.(53, 66, 74, 89)

There have been few reports about the effect of $AgNO_3$ concentration on the characteristics of nanoparticles produced beyond the change in production rate. However, the smallest particles produced using *E. coli* CFE were observed at the optimal concentration of $AgNO_3$ for yield, while larger particles were above and below optimal.(89) A similar pattern was seen in LSPR peak intensity suggesting that a greater number of smaller particles were being produced with the optimal concentration compared with fewer larger particles under suboptimal conditions, though this was not thoroughly examined.

The stoichiometric ratio of the reagents is also important. Biomass harvested from *Lactobacillus casei* subsp. Casei cultures demonstrated that a lower $AgNO_3$ to biomass ratio yielded higher AgNP production.(55) In a similar way, the volume ratio of $AgNO_3$ to CFE of *Pseudomonas mandelii* was investigated by Mageswari et al.(102) A volume ratio of 1:99

proved most effective for AgNP production. The ratio reflects the stoichiometry of the reaction with the biomass supplying the reducing agent and the capping agents, suggesting that the limiting reagent in the reaction is the reducing agent (i.e. NAD(P)H) in the CFE.

A paucity of published literature exists on the use of other Ag salts than $AgNO_3$ in the bacteriosynthesis of nanoparticles. Nonetheless, AgCl is an intermediate formed during the bioreduction of $AgNO_3$ by *Klebsiella pneumoniae*. Using AgCl as the primary Ag^+ substrate, very small nanoparticles were formed in a light-assisted process,(62) though questions have been raised about the true usefulness of AgCl because of its very poor solubility in water.(58) AgCl, like most Ag salts such as $AgCO_3$ and Ag_2O, is very insoluble in water making reaching the required concentrations a challenge. However, more soluble Ag_2SO_4 has been found to produce larger spherical nanoparticles (diameters of 50-150 nm) using *Salmonella enterica* serovar *Typhimurium* CFE.(137)

Moreover, complexed Ag in the form of diamine Ag ($[Ag(NH_3)_2]^+$) was also utilized for AgNP production. When diamine Ag was added to the biomass of *Aeromonas* sp. and *Corynebacterium* sp., particles with diameters of 20 nm and 10-15 nm formed, respectively.(65, 86) $[Ag(NH_3)_2]^+$ is resistant to forming AgOH under basic conditions. AgOH is an insoluble compound which forms from hydroxide ions in water and precipitates out of solution when the pH rises above ≈8.0 under permitting conditions.

In summary, $AgNO_3$ remains the most frequently used source for introducing Ag^+ ions to reaction media for NP production. The concentration of the substrate appears to have little effect on the geometry of particles produced, but production amounts depend on the amount of Ag^+ available. In most cases, there appears to be an optimal concentration, above which the system becomes saturated, and the toxic effects likely have negative impacts on the reduction mechanism.

3.4 pH

The typically narrow optimal range of pH conditions processed by enzymes means that they are highly sensitive to conditional changes. As there have been very few extremophiles investigated for AgNP production, it is not surprising that many reports investigating how pH affects AgNP bioproduction show that near-neutral conditions (pH 6-8) are favoured.(76, 96, 124, 132) Nevertheless, slight to extreme acidic environments are optimal for biomass of *Corynebacterium* sp. and *Pseudomonas putida,* respectively.(86, 132) Such conditions have also yielded larger particles, though production rates were lower than under basic conditions.(89)

Most studies on optimal pH conditions have suggested basic environments appear desirable for production. In a whole cell culture of *Bacillus megaterium*, pH 8.1 was determined to be best for production.(76) However, extremely basic conditions of pH 9 or 10 have frequently been reported as optimal,(89, 110, 138) whilst production was not observed under acidic conditions.(131) Moreover, through systematically assessing AgNP formation under pH conditions ranging from 4 to 12 in sterile culture media, it was demonstrated that nanoparticles formed at the highest rate and with higher degrees of uniformity under very basic conditions.(139) As the pH of the reaction environment can change the reduction-oxidation process where its exact effect also depends on the reactants involved, it appears that low H^+ concentrations are favoured to drive the reduction of Ag^+ in an excess of electron donors (Figure 2a).

While pH (pH = 5, 7, and 10) had little effect on AgNP production in *Pseudomonas antarctica*, and similarly in *A. kerguelensis*, it was noted that particles were least stable in pH 7 conditions.(72) This may be due to the protein corona having a more neutral charge, so, is less effective at repelling other particles. Similarly, particle aggregation was observed at a pH of 5

or below, suggesting the usually high stability of biogenic AgNPs being compromised.(102) Additionally, in *Lactobacillus fermentum* under more basic conditions reduction rates were reduced, however recovery was increased.(42) Denaturation of the proteins may also play a role in this process, but has not been explored in detail.

While it depends on the exact concentrations of reagents used, Ag^+ can complex with hydroxide ions (OH^-) which exist in alkaline conditions to form AgOH, a water insoluble compound observed as a precipitate, typically at pH levels above 8. Additionally, silver oxide is spontaneously formed in solution which is also very poorly soluble in water. The outcome of the formation of these precipitates is the decrease in Ag^+ concentrations and their availability to reductase enzymes for reduction. As discussed above, this challenge can be overcome by selecting alternative Ag-amine sources.

3.5 Halides

Halides, especially chlorides, have high binding affinities with Ag^+; when both Cl^- and Ag^+ are present in solution, water insoluble AgCl forms spontaneously. Combined with the common occurrence of NaCl in bacterial growth media, the concentrations of halides are consequently important considerations when exploring nanoparticle formation by bacteria.

Of the dearth of reports on the matter, the presence of Cl^- appears deleterious for AgNP production,(58) while indeed many investigations use media without the addition of NaCl.(38, 61, 89) The production of insoluble AgCl reduces the bioavailability of the Ag^+ for nanoparticle production.

In a cell-free process using extracts of *Bacillus amyloliquefaciens*, 2 mM NaCl was observed to be optimal for photo-assisted AgNP production.(140) However, by adding the NaCl directly to the $AgNO_3$ the researchers produced an AgCl precipitate, whereas by adding the NaCl to the

media first the precipitation was avoided. The bioavailability of the Ag^+ was therefore maintained with over 98% of the Ag added reduced to nanoparticles. While these findings were not elaborated in detail, the effect may be due to the formation of an intermediate species. Indeed, AgCl has been suggested to be an intermediate in the production of AgNP in sterile growth media via a photo-catalysed reaction.(139)

The presence of Cl^- ions in the growth medium has sbeen thought to contaminate AgNPs to form AgCl nanoparticles.(129) Durán et al. have discussed the bioproduction of AgCl nanoparticles and stressed the importance of distinguishing, through X-ray diffraction analysis, the different compositions of biogenic nanoparticles. (141) They explained that nanoparticles reported as Ag were frequently not fully characterised or misidentified from AgCl particles or AgCl contaminants. In many applications, knowing the exact composition of the particles is crucial.

Due to the high binding affinity between Ag^+ and Cl^-, considerations into the presence of chlorides in growth media must be made. Conflicting reports have been published on the effects Cl^- ions may have on nanoparticles with limited resources relating to biological systems. There have been limited reports of other halides (Br^- and I^-) in bacterial systems.

3.6 Media Composition

Related to both pH and halide concentrations is the composition of the growth media in which the nanoparticle producing species are investigated. There has been little direct investigation into how growth media composition affects AgNP production. However, in a comparison between LB growth medium and brewery effluent, the size of AgNPs produced by *B. subtilis* CFE were larger in LB and had a greater tendency to form aggregates.(142) A more minimal media was preferred when investigating the composition of growth media used for culturing *Streptomyces rochei* for AgNP production.(124) Interestingly, it was observed that the lowest

concentration of KNO_3 was favoured. This conflicts with the observation that AgNP biosynthesis by *E. coli* DH5-α CFE was higher in nitrate broth compared to LB.(89) The involvement of nitrate reductases in the reduction of Ag^+ may explain this as their expression may be upregulated in a nitrate rich environment. Nevertheless, further investigations into the complexity and composition of growth media are necessary to better understand this process.

3.7 Aerobicity

An interesting confliction has arisen around bacterially produced AgNPs under different aerobic conditions. On one hand, most cultures are reportedly shaken (usually at 200 rpm) during incubation under aerobic conditions, regardless of whether they are aerobes, or obligate or facultative anaerobes.(38, 42, 61, 66, 70) On the other hand, some reports have indicated that nanoparticle formation is most efficient under anaerobic conditions.(125, 136)

Whilst agitation is used to improve the dissolution of oxygen (among other reasons discussed below), there appears to be no previous investigation in which dissolved oxygen has been measured. A study by Lin et al. showed that AgNP formation in *E. coli* favoured anaerobic conditions, suggesting the enzymes involved to be anaerobically-induced.(136) This is supported by evidence from other researchers that suggest a nitrate-reductase plays a key role in the reduction of Ag^+.(43, 44) Nitrate-reductases are typically, though not exclusively, expressed under anaerobic conditions. However, there has been no comparison between cultures of an organism grown aerobically and anaerobically to further investigate this.

3.8 Mixing

The mixing or shaking of bacterial suspensions is routinely used to maintain a homogenous solution of cells and nutrients, as well as to promote gas exchange with the ambient atmosphere, be it aerobic or anaerobic. In the case of AgNP biosynthesis, mixing impacts on the relative

local concentrations of reagents and acts to disperse any nanoparticles in the extracellular environment. How the mixing process affects AgNP production has received little attention. Such considerations will be crucial if the scale of production methods is increased.

From the paucity of reports on the topic, mixing during incubation appears to delay nanoparticle formation,(62) and may therefore be a potential method for controlling the rate of production. If the commercial application of AgNPs are to be fully realised, larger scale production methods are required. Consequently, it is necessary to investigate the effects of different bioreactor setups, aeration techniques, and flow dynamics.

3.9 Visible Light

Silver compounds are notoriously sensitive to light, and this fact can be exploited in AgNP production. Many bacteria have been shown to produce AgNPs when exposed to light, and some require light to occur.(68, 70, 72, 78, 108, 111, 113)

Wei et al.(140) have demonstrated that solar radiation intensity influenced the biosynthesis of nanoparticles in CFE. Sunlight caused the reaction rate to increase in an intensity-dependent manner. Sunlight is a free and renewable resource which is beneficial when considering large-scale production. However, energy efficient artificial light sources are more favourable due to the limited reliability and ability to control solar radiation. Yet, no in-depth investigation into this mechanism or which wavelengths of light are responsible for this phenomenon has been published.

In some chemical production routes, light at specific wavelengths is used to influence the specific the geometries of particles;(143) the effects of different colours of light have on the bacterial synthesis of AgNPs have not been explored in detail to date. It is well documented that irradiation of AgNPs can cause polymorphic shifts from spheres to nanoprisms in chemical

production.(144) While this does not appear to have been attempted in bacteria, exposure to light altered the size and thus the optical properties of nanoparticles produced in *K. pneumoniae*.(62)

In summary, exposing bacterial cultures to varying intensities and wavelengths of light may influence, and therefore may be ultimately used to control bacterial AgNP formation. However, which wavelengths of light should be used and at which stage of the production process would be most effective still remain to be identified.

3.10 Ionising or Electromagnetic Radiation

Additional conditions have also been investigated alongside those already discussed, though not to the same degree. For instance, γ-ray radiation has been employed to aid in the production of AgNPs in CFE from *Bacillus stearothermophilus* cultures.(79) The γ-rays were thought to cause radiolytic reduction of Ag^+ ions. Higher doses of radiation were associated with increased formation of AgNPs, however, too high doses proved detrimental. Similarly, microwave radiation has also been used with similar effects. Both CFE from *E. coli*(87) and *B. subtilis*(49) irradiated with microwaves yielded AgNPs. While the *E. coli* produced spherical particles, *B. subtilis* produced both spherical and triangular morphologies. Interestingly, the LSPR peak produced in the latter was sharper and more symmetrical than that without irradiation, indicating a higher monodispersity via the radiolytic process.(49) This may be attributed to the more homogeneous thermal field within the culture media as a result of microwave volumetric heating.

Despite the improved speeds of production and monodispersity, using ionising radiation has several obvious disadvantages, particularly if used on large scale. The risk to human health, higher energy requirements and additional costs of specialist equipment all can limit its application.

4 Concluding and Future Remarks

Bacterial biosynthesis of AgNPs is a prime candidate for overcoming shortcomings associated with physical and chemical production methods. Currently, the underpinning biological mechanism remains to be fully resolved, though the involvement of nitrate reductase and Sil proteins have been suggested. While direct molecular biological investigations into the mechanism are sparse, it is clear that the process is heavily influenced by the reaction conditions under which the AgNPs are formed. Gaining a better understanding of this process is undoubtedly valuable for further optimising production.

It is evident from the body of work investigating the effects of reaction conditions discussed in this article that most factors impact on the yield of AgNPs; a summary is show in Table 1. In particular, a longer incubation time, higher substrate concentration, higher temperature, and anaerobic conditions resulted in more AgNPs being formed. In contrast, lower temperatures were associated with a decrease in producing nanospheres, but also with an increase in non-spherical particles. Temperature appears to be the biggest influencer for particle morphology.

Most work has been performed using CFE instead of whole cell culture. This has allowed for more extreme conditions to be investigated, for example pH and temperature. While, contradicting results have been obtained between the two states, there have been few direct comparisons made. Exploring these differences may be useful in future applications.

Challenges remain for controlling the morphologies of the AgNPs produced biologically. While manipulating the temperature of reactions may provide some control, a reliable method of producing shaped non-spherical particles with a high degree of monodispersity remains a distant aspiration. The ability to produce monodispersed nanospheres is likely to be more achievable in the near future with continued efforts.

It is clear from Figure 4 that many species of bacteria or extracts thereof which can reduce Ag^{+} to Ag^{0}. Focus should now be directed to developing a better understanding of the mechanism involved, both in Ag resistant whole cell cultures, and CFE. Understanding how altered reaction conditions affect, not only the product, but also the components of the mechanism, is critical. Moreover, knowing how nanocrystals form and grow in biological environments is useful for future applications which require specific morphologies of AgNPs.

Table 1. *Summary of the effects reaction parameters have on bacterial AgNP biosynthesis.*

Reaction condition	Effect on silver nanoparticle production	
	Whole cell	**Cell-free extract**
Temperature	↑ Temp. = ↑ production, ↑ production rate, ↑ yield ↓ Temp. = ↓ production, ↑ non-spherical, ↓ particle sizes, ↑ monodispersed	↑ Temp. = ↑ production ↓ Temp. = ↑ stability
pH	↑ pH = ↓ production rate, ↑ recovery	↑ pH ≠ change in production rate
Incubation Time	↑ time = ↑ number of particles, non-spherical formations	↑ time = more particles
Silver Substrate	↑ $[AgNO_3]$ = ↑ production	↑ $[AgNO_3]$ = ↓ number of particles AgCl = ↓
Halide Concentration	↑ $[Cl^-]$ = ↓ production	↑ $[Cl^-]$ = ↑ production
Media Composition	-	↑ $[NO_3^-]$ =/≠ ↑ production
Mixing and Aerobicity	Anaerobic = ↑ production	Mixing = delay in start of reaction
Visible Light	-	↑ Dose = ↑ Production Light = faster production
Ionising Radiation	-	↑ Dose = ↑ Production Radiation = ↑ Monodispersity

↑ *Increase,* ↓ *Decrease, = Results in,* ≠ *Does not result in*

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6 Disclosure of interest

The authors report no conflict of interest.

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