

# Lactic acid bacteria as reducing and capping agent for the fast and efficient production of silver nanoparticles

Liesje Sintubin · Wim De Windt · Jan Dick · Jan Mast · David van der Ha · Willy Verstraete · Nico Boon

Received: 19 February 2009 / Revised: 30 April 2009 / Accepted: 1 May 2009 / Published online: 2 June 2009  
© Springer-Verlag 2009

**Abstract** There is a growing demand for silver-based biocides, including both ionic silver forms and metallic nanosilver. The use of metallic nanosilver, typically chemically produced, faces challenges including particle agglomeration, high costs, and upscaling difficulties. Additionally, there exists a need for the development of a more eco-friendly production of nanosilver. In this study, Gram-positive and Gram-negative bacteria were utilized in the non-enzymatic production of silver nanoparticles via the interaction of silver ions and organic compounds present on the bacterial cell. Only lactic acid bacteria, *Lactobacillus* spp., *Pediococcus pentosaceus*, *Enterococcus faecium*, and *Lactococcus garvieae*, were able to reduce silver. The nanoparticles of the five best producing *Lactobacillus* spp. were examined more into detail with transmission electron microscopy. Particle localization inside the cell, the mean particle size, and size distribution were species dependent, with *Lactobacillus fermentum* having the smallest mean particle size of 11.2 nm, the most narrow size

distribution, and most nanoparticles associated with the outside of the cells. Furthermore, influence of pH on the reduction process was investigated. With increasing pH, silver recovery increased as well as the reduction rate as indicated by UV–VIS analyses. This study demonstrated that *Lactobacillus* spp. can be used for a rapid and efficient production of silver nanoparticles.

**Keywords** Metallic silver · Green chemistry · Cell wall · Antimicrobial · Biological synthesis · Probiotic

## Introduction

Silver has been used for its antimicrobial properties since ancient times: Alexander the Great refused to drink water which had not been stored in silver vessels, and Paracelsus claimed the beneficial properties of silver towards health (Silver et al. 2006). When penicillin was discovered and the era of the antibiotics began, the use of silver for its antimicrobial properties decreased (Klasen 2000a). Since biocide-resistant strains emerged, the interest to use silver as an antimicrobial agent is rising again (Klasen 2000b). However, antimicrobial agents based on ionic silver (e.g., silver nitrate) have one major drawback: they are easily inactivated by complexation and precipitation and thus have a limited usefulness (Atiyeh et al. 2007). Zerovalent silver nanoparticles are considered as a valuable alternative for ionic silver. Due to their large specific surface-to-volume ratio, nanoparticles have different properties than bulk material (Rao and Cheetham 2001; Tilaki et al. 2006). It has been shown that silver nanoparticles are antimicrobial towards a broad spectrum of Gram-negative and Gram-positive bacteria (Kim et al. 2007; Panacek et al. 2006). Furthermore, silver nanoparticles show antifungal (Kim et al. 2008) and antiviral

**Electronic supplementary material** The online version of this article (doi:10.1007/s00253-009-2032-6) contains supplementary material, which is available to authorized users.

L. Sintubin · D. van der Ha · W. Verstraete · N. Boon (✉)  
Laboratory of Microbial Ecology and Technology (LabMET),  
Ghent University,  
Coupure Links 653,  
9000 Gent, Belgium  
e-mail: Nico.Boon@UGent.be  
URL: [Labmet.Ugent.be](http://Labmet.Ugent.be)

W. De Windt · J. Dick  
Avecom, Industrieweg 122P,  
9032 Wondelgem, Belgium

J. Mast  
EM-unit of CODA-CERVA,  
Groeselenberg 99,  
1180 Brussel, Belgium

activity (Elechiguerra et al. 2005; Lu et al. 2008). Besides their antimicrobial properties, silver nanoparticles can be used for their catalytic, conductive, and optical features (Medina et al. 2005; Fuller et al. 2002; Kamat 2002).

Many methods exist to synthesize nanoparticles. Most chemical methods use a reducing agent (e.g., sodium borohydride) to reduce  $\text{Ag}^+$  to  $\text{Ag}^0$  and a stabilizer (e.g., polyvinylpyrrolidone) to control particle growth and prevent aggregation (Ledwith et al. 2007). However, these chemical fabrication methods often have problems with particle stability and are difficult to scale up (Tavakoli et al. 2007). In addition, there is a demand for more environment-friendly production methods. An alternative production method is based on biological production.

Some bacteria are known to sorb heavy metals from contaminated wastewaters. Some microorganisms not only have a strong biosorptive capacity but also can reduce and precipitate them in their metallic form. Fungi, such as *Verticillium*, produce silver nanoparticles intracellularly (Sastry et al. 2003), while *Fusarium oxysporum* and *Aspergillus niger* have shown to produce silver nanoparticles extracellularly (Ahmad et al. 2003; Duran et al. 2005; Sadowski et al. 2008; Sastry et al. 2003). It is presumed that the reduction of silver ions is catalyzed by enzymes, as described for *Klebsiella pneumoniae* and *Pseudomonas stutzeri* AG259 (Klaus et al. 1999; Shahverdi et al. 2007). Another frequently reported mechanism for the bacterial reduction of silver is the non-enzymatic interaction of  $\text{Ag}^+$  with cell wall functional groups. This mechanism was observed for *Lactobacillus* A09, *Corynebacterium* sp. SH09, and *Aeromonas* SH10 (Fu et al. 2006; Lin et al. 2005; Zhang et al. 2005, 2007). Non-enzymatic reduction makes it possible to apply special conditions such as high or low pH and temperature, potentially accelerating the silver reduction. Furthermore, high concentrations of  $\text{Ag}^+$  that are toxic for bacteria can be used (Zhang et al. 2007).

In this paper, we describe the synthesis of silver nanoparticles using bacteria as reducing and capping agent. The capacity to precipitate silver was evaluated for Gram-positive bacteria *Lactobacillus* spp., *Enterococcus faecium*, *Lactococcus garvieae*, *Pediococcus pentosaceus*, and *Staphylococcus aureus* and the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*. A high pH was thereby providing reduction rates similar to chemical processes and much faster than other biological methods.

## Materials and methods

### Bacterial strains and growth conditions

*Lactobacillus farciminis* LMG 9189, *Lactobacillus parabuchneri* LMG 11772, *Lactobacillus plantarum* LMG

24830, *Lactobacillus fermentum* LMG 8900, *Lactobacillus brevis* LMG 11437, *Lactobacillus rhamnosus* LMG 18243, *L. plantarum* LMG 24832, *E. faecium* LMG 24831, *L. garvieae* LMG 8162, and *P. pentosaceus* LMG 9445 (LMG culture collection, Ghent University, Ghent, Belgium) were grown for 48 h in Man–Rogosa–Sharpe (MRS) broth (Oxoid, UK) at 30°C. *P. aeruginosa* LMG 9009 and *S. aureus* LMG 8064 were grown in tryptic soy broth (Difco, USA), and *E. coli* LMG 10266 was grown in Luria Bertani broth (Difco, USA) for 24 h at 37°C. All strains were grown with agitation (120 rpm).

### Reagents

As ionic silver potentially forms oxide precipitates at high pH, silver was added as a diamine complex for all experiments. To prepare the diamine silver complex, ammonium hydroxide (Acros Organics, Belgium) was added to  $\text{AgNO}_3$  (Sigma-Aldrich, USA) until the original brown precipitate disappeared again. Subsequently, the concentration was diluted with MilliQ water to 26 mg/mL  $\text{AgNO}_3$ .

### Ag(I) bioreduction assay

Biomass was harvested by centrifugation in a Sorvall RC5C centrifuge (USA) at 5,000×g and washed two times with MilliQ water. Subsequently, biomass was resuspended in MilliQ, and the OD was adjusted to obtain 5 g/L cell dry weight (CDW). Diamine silver complex was added to the alkalinized biomass (0.025 N NaOH) in a concentration of 1:5 Ag/CDW, and the suspension was shaken at a rotation rate of 100 rpm at 28°C. After 24 h incubation, the supernatant was separated from biomass by centrifugation at 10,000×g for 15 min. Biomass was washed three times with MilliQ to remove all ionic silver that was not irreversibly sorbed or not reduced on the biomass.

### Influence of pH on the Ag(I) bioreduction

The reduction of silver was performed at pH 2, 4, 6, 7, 8, 9, and 11.5 with *L. fermentum* LMG 8900. The washed biomass and diamine silver complex were adapted separately to the desired pH. One, 3, 5, and 10 min and 2 h after adding the silver complex to the biomass, UV–VIS analysis was performed with a Uvikon 932 spectrometer (Kontron Instruments, Switzerland). Wavelength scans were taken in a scan range of 200–650 nm. The data range was 1 nm, and scan speed was 200 nm/min.

### Ag analysis by atomic absorption spectrometry

To analyze the concentration of silver precipitated on the cells and in the supernatant, biomass and supernatant were

boiled for 2 h with 65% HNO<sub>3</sub> (Normapur, VWR, Belgium) and 30% H<sub>2</sub>O<sub>2</sub> (Merck, Germany) until the sample was clear. Subsequently, the silver concentration was measured using a Shimadzu AA-6300 atomic absorption spectroscope (Japan).

#### X-ray diffraction analysis

X-ray diffraction (XRD) of the silver on the biomass, dried at 30°C, was performed with a BRUKER D8 Discover. X-rays were generated by a copper X-ray tube with power 1.6 kW (40 kV, 40 mA). The wavelength CuK $\alpha$  corresponded to 1.54 Å. Measurements were made between 25° and 90° 2-theta with a step time of 153.6 s and a step size of 0.02° 2-theta.

#### Transmission electron microscopy

To perform transmission electron microscopy (TEM), ultrathin sections of approximately 50 nm were prepared with the Ultracut microtome (Reichert-Jung A.G., Vienna, Austria) according to a previously published procedure (Mast et al. 2005), omitting OsO<sub>4</sub> fixation, uranyl acetate incubation, and Reynold's lead citrate staining to avoid background staining. Ultrathin sections made were placed on polioform- and carbon-coated copper grids and were examined using a Technai Spirit TEM (from FEI, Eindhoven, the Netherlands) operated at 120 kV. Digital micrographs were made using a bottom-mounted 4×4 k Eagle camera (Fei) at various magnifications. Micrographs were analyzed using AnalySIS Solution of the iTEM software (Olympus Soft Imaging Solutions). Particles were detected in a pre-defined region of interest excluding border particles and particles of less than 5 pixels. Particles were analyzed in ten sections of bacteria using different micrographs. The mean diameter was determined for each particle. Also, the area of each bacterium-containing particle was measured. To determine the particle size distribution, particles were counted in size ranges of 1 nm. The counts per range were plotted in a graph, and the cumulative frequency distribution was calculated.

#### Statistical analyses

SPSS for Windows version 16.0 was used for statistical analyses. Results were considered differently at an  $\alpha$  two-tailed level of 0.05. Tests for normality of the data and equality of the variances were performed using the Kolmogorov–Smirnov and Levene's test, respectively. Comparison of normal distributed data was performed with one-way analysis of variance. The nonparametric Kruskal–Wallis and Mann–Whitney *U* test were used to compare non-normal distributed data.

## Results

### Production of silver nanoparticles by different bacterial species

The ability of nine different *Lactobacillus* species to accumulate and subsequently reduce Ag<sup>+</sup> was compared to *P. pentosaceus*, *E. faecium*, *L. garvieae*, *S. aureus*, *E. coli*, and *P. aeruginosa*. The test was performed under equal conditions for all species. For the different *Lactobacillus* species, the amount of silver associated with the biomass varied between 110 and 166 mg Ag/g cell dry weight (Table 1). Since 1,000 mg/L Ag<sup>+</sup> was added as a diamine complex and the Ag/CDW ratio was 1:5, 200 mg/g was the theoretical maximum amount of silver that could be reduced. Consequently 83%, 75%, and 73% of the added silver was associated with the biomass of *L. fermentum*, *L. farciminis*, and *Lactobacillus fructivorans*, respectively. For the other lactic acid bacteria, *P. pentosaceus*, *E. faecium*, and *L. garvieae*, there was 141 mg/g CDW (70 %), 116 mg/g CDW (58%), and 146 mg/g CDW (73%) of biomass-associated silver, respectively. *S. aureus* demonstrated a recovery of 115 mg/g CDW (57%), while *P. aeruginosa* and *E. coli* had the lowest recovery of all with 107 mg/g (53%) and 52 mg/g CDW (26%), respectively.

X-ray diffraction analysis was performed to evaluate the oxidation state of the silver associated with the biomass.

**Table 1** The amount of silver associated with the biomass starting with an initial silver concentration of 200 mg/g CDW

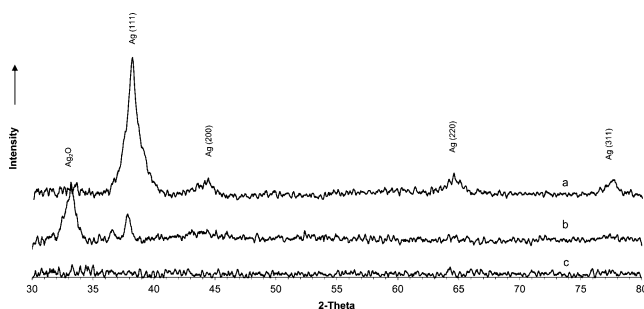
Species	Ag (mg/g CDW)	XRD
<i>Lactobacillus fermentum</i>	166±19	Ag(0)
<i>Lactobacillus farciminis</i>	151±17	Ag(0)
<i>Lactobacillus fructivorans</i>	146±29	Ag(0), Ag <sub>2</sub> O
<i>Lactococcus garvieae</i>	146±19	Ag(0)
<i>Pediococcus pentosaceus</i>	141±42	Ag(0), Ag <sub>2</sub> O
<i>Lactobacillus brevis</i>	137±10	Ag(0)
<i>Lactobacillus parabuchneri</i>	137±2	Ag(0)
<i>Lactobacillus rhamnosus</i>	130±8	Ag(0)
<i>Lactobacillus plantarum</i> LMG 24830	128±39	Ag(0)
<i>Lactobacillus mucosae</i>	116±16	Ag(0)
<i>Staphylococcus aureus</i>	115±5	No Ag(0)
<i>Lactobacillus plantarum</i> LMG 24832	110±15	Ag(0)
<i>Pseudomonas aeruginosa</i>	107±37	No Ag(0)
<i>Escherichia coli</i>	52±13	No Ag(0)

Presence of Ag(0), determined by XRD, confirmed if the species was able to reduce silver

Figure 1 shows the diffractogram for *L. fermentum* and *S. aureus*. In case of *L. fermentum*, typical peaks were present at  $2\theta=38.24^\circ$ ,  $44.46^\circ$ ,  $64.21^\circ$ , and  $77.75^\circ$  which is a typical diffraction pattern for metallic silver. The most intense peak corresponds to metallic silver (111) diffraction ( $2\theta=38.24^\circ$ ), while the less intensive peaks correspond to (200) diffraction ( $2\theta=44.46^\circ$ ), (220) diffraction ( $2\theta=64.21^\circ$ ), and (311) diffraction ( $2\theta=77.75^\circ$ ). All *Lactobacillus* spp., *P. pentosaceus*, *E. faecium*, and *L. garvieae* showed this typical diffraction pattern (data not shown). The diffractograms of *L. fructivorans* and *P. pentosaceus* indicated an additional peak that corresponded to the strongest  $\text{Ag}_2\text{O}$  reflection ( $2\theta=33.33^\circ$ ). The diffractogram of *S. aureus*, *P. aeruginosa*, and *E. coli* did not show any typical silver peak nor the  $\text{Ag}_2\text{O}$  reflection. Hence, only the lactic acid bacteria were confirmed to have the ability to produce  $\text{Ag}^0$ .

#### Particle size determined by transmission electron microscopy

TEM was performed on five of the best recovering and fast growing *Lactobacillus* spp.: *L. fermentum*, *L. plantarum* LMG 24830, *L. plantarum* LMG 24832, *L. farciminis*, and *L. rhamnosus*. TEM pictures showed that both particle localization and distribution inside the cell were dependent on *Lactobacillus* species (Fig. 2). *L. fermentum* produced two fractions of the silver nanoparticles. The most prevalent fraction consisted of small particles (5–15 nm) situated in the outer layers of the cell and on the cell wall. In contrast, the less prevalent fraction of silver nanoparticles, ranging from 15 to 40 nm, was intracellular and evenly distributed in the cytoplasm. Notably, the intracellular large nanoparticles included a few outliers between 60 and 80 nm. The nanoparticles produced by *L. plantarum* LMG 24830, *L. plantarum* LMG 24832, *L. farciminis*, and *L. rhamnosus* were situated more frequently inside the cytoplasm. Furthermore, the particles produced by these bacteria were



**Fig. 1** XRD analysis of *a* *L. fermentum* with typical metallic silver diffraction peaks, *b* *L. fructivorans* with  $\text{Ag}_2\text{O}$  and  $\text{Ag}(111)$  peak and *c* *S. aureus* without typical silver peaks ( $2\theta$ -theta values typical for silver are mentioned between brackets)

somewhat larger compared to the particles produced by *L. fermentum*. In addition, outliers were more common than observed for *L. fermentum*, especially in case of the two *L. plantarum* strains. Finally, the nanoparticles appeared more clustered in the cytoplasm of *L. fermentum*.

Based on the pictures taken by TEM (Fig. 2), nanoparticle size and the number of nanoparticles per section were determined for each of the five *Lactobacillus* species. Ten sections were analyzed for every species. Based on the frequency and cumulative frequency (Electronic supplementary material), 95% confidence interval (CI), mean particle size, mode, the number of particles per cell surface, the number of particles per cell volume, the number of particles per entire cell, and the concentration of  $\text{Ag}^0$  per cell were calculated (Table 2). Nanoparticles produced by *L. fermentum* were substantially different from those of the other *Lactobacilli*. While *L. fermentum* produced significantly more nanoparticles per surface area ( $280\pm 96$  particles/ $\mu\text{m}^2$ ), volume ( $5,600\pm 1,922$  particles/ $\mu\text{m}^3$ ), and cell ( $3,320$  particles/cell) compared to the other species, the mean particle size (11.2 nm) was significantly smaller. The silver nanoparticles produced by *L. fermentum* showed the most narrow distribution as well, as reflected by the 95% CI (10.3 nm–12.1 nm). The concentration of  $\text{Ag}^0$  per cell ( $2.44\pm 0.45\times 10^{-11}$  g/cell) was equivalent with most other *Lactobacillus* species and was only significantly lower than that of *L. plantarum* LMG 24832 ( $6.77\pm 3.13\times 10^{-11}$  g/cell). Between the other *Lactobacillus* spp., the mean particle size, the number of particles per cell surface area, cell volume, and entire cell were comparable, with only *L. rhamnosus* having a smaller mean particle size (15.7 nm) and *L. plantarum* LMG 24832 producing significantly more particles per cell ( $1,565\pm 562$ ). The mode of the particle size of silver nanoparticles produced by *L. fermentum* was between 6.0 and 8.0 nm. Similarly, the mode of the particle size produced by *L. farciminis* was between 6.0 and 7.0 nm. In contrast, the mode of particle size of *L. plantarum* LMG 24832 and *L. rhamnosus* was higher than that of *L. fermentum*, ranging between 9.0 and 10.0 nm

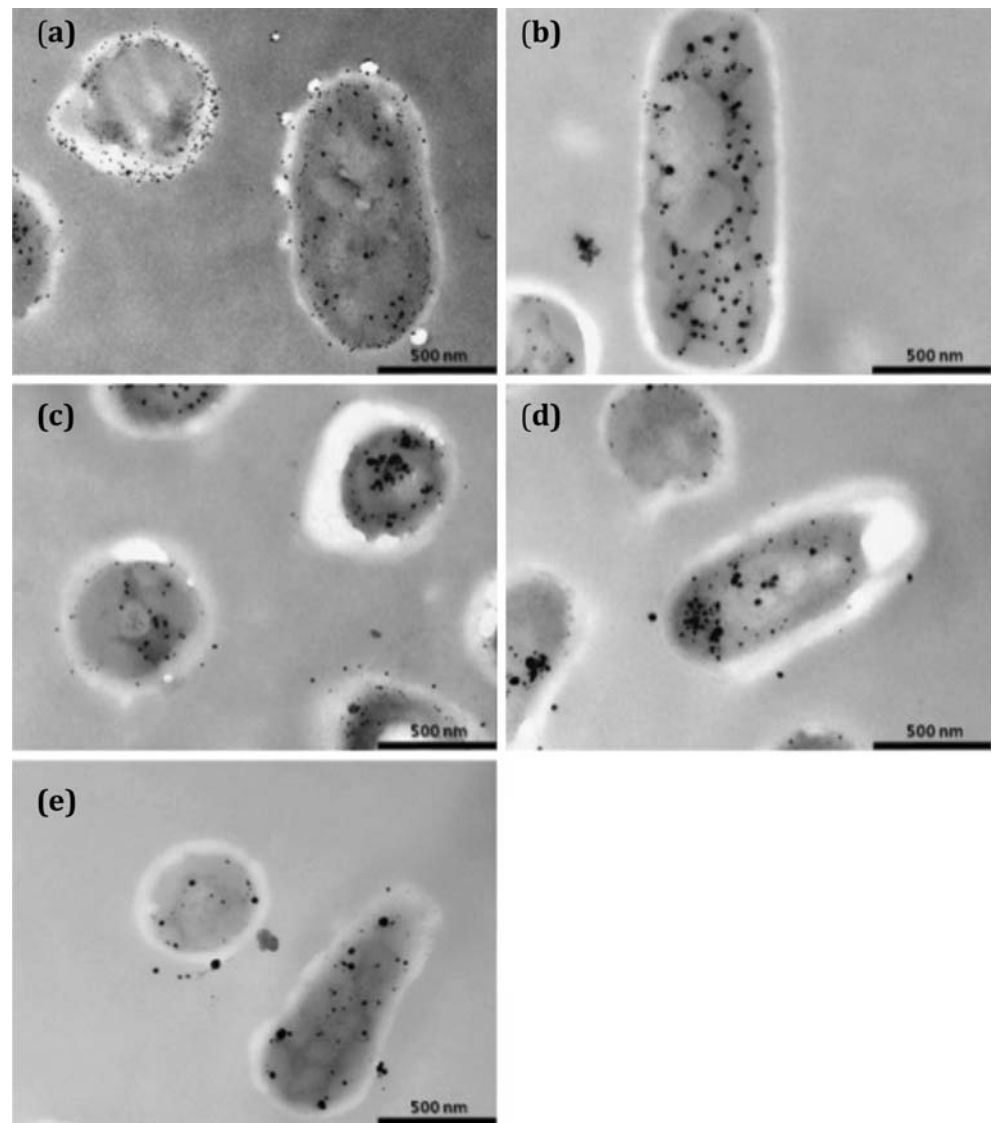
#### Influence of the pH on the silver reduction

The silver reduction in function of the pH was performed with *L. fermentum* (Fig. 3). There was a clear influence of the pH on the amount of silver associated with the biomass. When the pH increased, more silver was recovered; however, between pH 6 and 8, there was a slight decrease in the amount of silver associated with the biomass. The most alkaline pH tested, pH 11.5, gave the greatest recovery of silver.

Additionally, pH influenced the rate of the reduction reaction. The suspension turned brown when silver was reduced, and the suspension coloring accelerated when



**Fig. 2** TEM images of **a** *L. fermentum*, **b** *L. farciminis*, **c** *L. plantarum* LMG 24830, **d** *L. plantarum* LMG 24832, **e** *L. rhamnosus* after reduction of silver. The intense black spots are nanoparticles

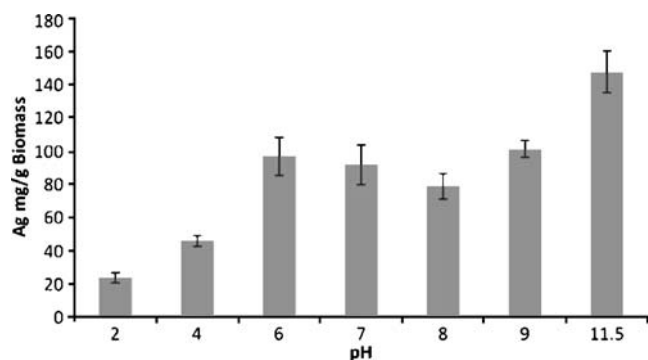


**Table 2** Particle size and distribution for the particles on the cells were evaluated using TEM for five *Lactobacillus* species: mean particles size (95% CI), mode, particles/cell surface (standard deviation), particles/cell volume (standard deviation), and  $\text{Ag}^0$  concentration/cell (standard deviation) are given

Species	Mean (95% CI) (nm)	Mode (nm)	Particles/cell surface ( $n/\mu\text{m}^2$ )	Particles/cell volume ( $n/\mu\text{m}^3$ )	Particles/cell ( $n/\text{cell}$ )	$\text{Ag}^0/\text{cell}$ ( $10^{-11}\text{g}/\text{cell}$ )
<i>Lactobacillus fermentum</i>	11.2 (10.3–12.1) a	6.0–8.0	280±96 a	5,600±1,922 a	3,320±1,139 a	2.44±0.45 a
<i>Lactobacillus rhamnosus</i>	15.7 (13.6–17.8) b	9.0–10.0	95±41 b	1,909±814 b	970±414 b	2.49±1.95 a
<i>Lactobacillus farciminis</i>	17.0 (14.3–19.7) bc	6.0–7.0	96±35 b	1,928±700 b	1,174±420 b	4.05±2.22 a
<i>Lactobacillus plantarum</i> LMG 24830	19.4 (16.8–21.9) c	7.0–8.0	105±41 b	1,878±877 b	1,167±545 b	5.00±3.22 ab
<i>Lactobacillus plantarum</i> LMG 24832	19.7 (17.7–21.7) c	9.0–10.0	90±32 b	1,807±648 b	1,565±562 c	6.77±3.13 b

Values which are not significantly different are marked with the same letter ( $P < 0.05$ )

$n$  number of particles



**Fig. 3** The amount of silver associated with the biomass in function of the pH. Ag mg/g CDW increases with the pH. All assays were done in triplicate

increasing pH. At pH 11.5, the suspension colored dark brown within a minute. These observations were supported by UV–VIS analysis (Fig. 4). In case of pH 11.5, a broad absorption band appeared after 1 min. This plasmon resonance band was centered around 425 nm, and the intensity increased with time which indicated the rapid formation of silver nanoparticles. At lower pH, no such plasmon resonance bands were observed (results not shown).

## Discussion

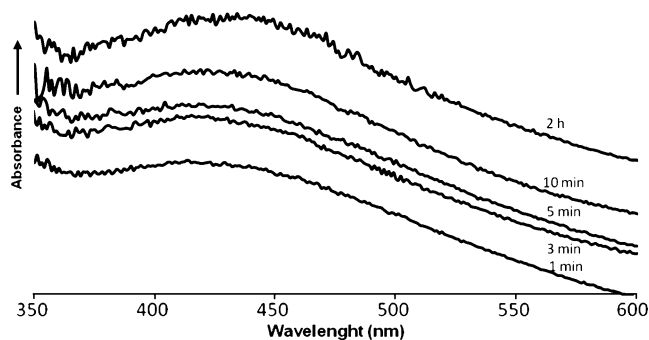
This paper describes the development of a method to produce silver nanoparticles using the biomatrix of bacteria both as a reducing and capping agent since the cell wall controls the growth of the nanoparticles and prevents their agglomeration. Several Gram-positive and Gram-negative bacteria were tested to reduce silver in a non-enzymatic way after application of a high  $\text{Ag}^+$  concentration in alkaline conditions. Only *Lactobacillus* spp. and the other lactic acid bacteria, *P. pentosaceus*, *E. faecium*, and *L. garvieae*, were able to reduce silver. TEM demonstrated that the localization of the nanoparticles inside the cell, the mean particle size, and the size distribution were species dependent (Fig. 2). The silver nanoparticles produced by *L. fermentum* had the smallest mean particle size and differed significantly of the nanoparticles produced by the other *Lactobacillus* species. Increasing pH enhanced the silver recovery and the reduction rate, creating a process which is fast compared to most other biological production methods (Nair and Pradeep 2002; Kalimuthu et al. 2008; Vigneshwaran et al. 2007). Moreover, the association of the nanoparticles with the bacterial biomass facilitates the concentration of the nanoparticles by centrifugation.

The non-enzymatic production of silver nanoparticles by *Lactobacillus* spp. allowed the application of high silver concentrations potentially toxic for the cell. We applied a

very concentrated  $\text{Ag}^+$  solution (1 g/L), which is much higher compared to enzymatic processes. Enzyme-catalyzed silver reduction is restricted to lower silver concentrations ranging between 10 and 100 mg/L  $\text{Ag}^+$  (Kalimuthu et al. 2008; Kowshik et al. 2003; Shahverdi et al. 2007; Vigneshwaran et al. 2007). The amount of silver recovered depended on the species used. *L. fructivorans* recovered 73% and *L. farciminis* 75%, while *L. fermentum* even recovered 83%. These silver recoveries are comparable if not greater than the silver recovery of non-enzymatic processes using *Corynebacterium* SH09 (50%) and *Aeromonas* SH10 (67%) to produce silver nanoparticles (Zhang et al. 2005; Zhang et al. 2007).

The recovery of silver and the reduction rate were pH dependent. When pH increased, both recovery and the reduction rate increased (Figs. 3 and 4). After adding the  $\text{AgNO}_3$  solution to the biomass at pH 11.5, the suspension started to color brown within a minute. This is an indication that  $\text{Ag}^+$  was reduced to  $\text{Ag}^0$  and nanoparticles were formed, which was confirmed by UV–VIS analysis. The silver reduction was rapid when compared to other biological precipitation processes. For example, *Bacillus licheniformis* required 6 h before silver was reduced (Kalimuthu et al. 2008), whereas 12 and 72 h, respectively, were required before silver reduction by *Aeromonas* spp. SH10 (Nair and Pradeep 2002) and *Aspergillus flavus* (Vigneshwaran et al. 2007) was observed. Only *Enterobacteria* could synthesize silver nanoparticles rapidly: *K. pneumoniae* reduced silver in less than 5 min (Shahverdi et al. 2007). Since such rapid silver reduction rates are typically only achievable using chemical processes, our method combines the eco-friendliness of biological silver nanoparticle production with the speed of chemical processes.

Only the *Lactobacillus* species, *P. pentosaceus*, *E. faecium*, and *L. garvieae* were able to reduce silver as indicated by XRD (Table 1). *S. aureus*, however, and the Gram-negative bacteria *P. aeruginosa* and *E. coli* did not

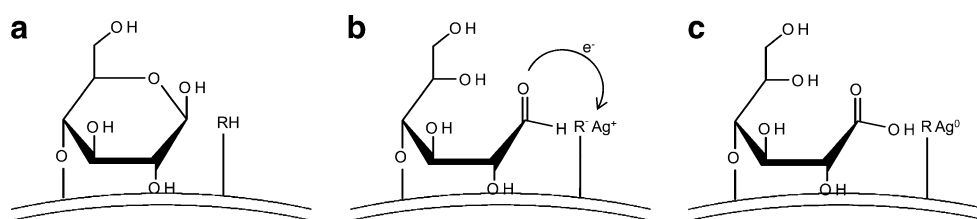


**Fig. 4** UV–VIS spectra recorded as a function of time of the reaction of *L. fermentum* biomass with silver in alkaline conditions. A plasmon absorbance band centered at 425 nm increased with time

reduce silver using this method. The amount of silver recovered with the biomass was species dependent as well. Some lactic acid bacteria recovered more than 70%, and *L. fermentum* even recovered 83%. The Gram-negative bacteria on the other hand showed a lower recovery. *P. aeruginosa* recovered 53.5% and *E. coli* 26%. The species dependency of silver recovery is potentially due to differences in the specific composition of the biomass between species. Gram-positive bacteria possess a cell wall that consists of a thick layer of peptidoglycan, teichoic acids, lipoteichoic acids, proteins, and polysaccharides. Therefore, these bacteria have many anionic surface groups which may act as sites for the biosorption and subsequent reduction of silver cations (van Hullebusch et al. 2003). In contrast, Gram-positive *S. aureus* retained lower concentrations of silver on its biomass and, as demonstrated by XRD, did not reduce silver. Lactic acid bacteria are known to produce exopolysaccharides (EPSs) (Aslim et al. 2006; Boonaert and Rouxhet 2000; van Hullebusch et al. 2003). These EPSs may serve in the protection of the bacterial cells against toxic compounds such as metal ions (Aslim et al. 2006). Furthermore, EPSs, which provide additional sites for biosorption of silver ions, consist of repeating sugar units, mainly glucose, galactose, and rhamnose in different ratios (Jolly et al. 2002). Lin et al. (2005) suggested that reducing sugars of bacterial cells may function as the electron donor in the reduction of  $\text{Ag}^+$  to its metallic form  $\text{Ag}^0$ . A suggested reduction mechanism for our production method is given in Fig. 5. Whenever pH increases, more competition occurs between protons and metal ions for negatively charged binding sites. Lin et al. (2005) discovered a slight drop in pH after biosorption of  $\text{Ag}^+$  on the biomass of *Lactobacillus* A09, which indicated the competition between protons and metal ions. High pH catalyzes the ring opening of monosaccharides such as glucose to their open-chain aldehyde (Fig. 5b). It is the aldehyde which delivers the reducing power. When metal ions are present, the aldehyde will be oxidized to the corresponding carboxylic acid, and at the same time, the metal ions will be reduced (Fig. 5c).

There are two kinds of EPSs: homopolymeric EPSs, which are synthesized by excreted enzymes, and heteropolymeric EPSs, which are synthesized in the cytoplasm and are subsequently polymerized on the outer cell prior to secretion (Jolly et al. 2002). Heteropolymeric EPSs may explain why the localization of the nanoparticles varies between the *Lactobacillus* species. Some *Lactobacillus* species produce an S-layer as well (Boonaert and Rouxhet 2000; Delcour et al. 1999). S-layers form a highly porous protein meshwork; therefore, they potentially act as a template for the nucleation of nanocrystals. (Merroun et al. 2007) used the S-layers of *Bacillus sphaericus* JG-A12 to produce gold nanoparticles. The silver nanoparticles produced by *L. fermentum* were distributed mainly in the outer layers of the cell and have a more narrow size distribution than the nanoparticles produced by the other *Lactobacillus* species. However, more research is necessary to clarify which cellular structures are truly responsible for the biosorption and reduction of silver.

The mean diameter of the silver nanoparticles produced by our method varied with the *Lactobacillus* spp. used. The smallest nanoparticles were produced by *L. fermentum* and had a diameter of 11.2 nm. This is smaller than the silver nanoparticles produced by the bacteria *K. pneumoniae* (52.5 nm), *Aeromonas* sp. SH10 (20 nm), and *B. licheniformis* (50 nm) (Kalimuthu et al. 2008; Sharverdi et al. 2007; Zhang et al. 2007). In contrast, Zhang et al. (2005) reported the bacterial production of silver nanoparticles with a diameter of 9.9 nm produced by *Corynebacterium* SH09. The fungi *Phoma* sp.3.2883 and *Verticillium* produced silver nanoparticles with a mean particle diameter of 71.06 and 25 nm, respectively (Chen et al. 2003; Mukherjee et al. 2001), which is larger than the nanoparticles produced by our *L. fermentum*. *A. flavus*, however, produced smaller silver nanoparticles (8.92 nm) (Vigneshwaran et al. 2007). Of course, it is not just the mean size of the nanoparticle that is important but the size distribution as well. Of all the *Lactobacillus* species, *L. fermentum* had the most narrow size distribution, with a 95% confidence interval between 10.3 and 12.1 nm.



**Fig. 5** Suggested bacterial reduction of  $\text{Ag}^+$  to  $\text{Ag}^0$  at the cell surface. **a** Bacterial cell with reducing sugars such as glucose and protonated anionic functional groups ( $-\text{RH}$ ). **b** When pH increases, protons dissociate and create negatively charged adsorption sites for  $\text{Ag}^+$ . The

reducing sugars turn into their open-ring structure and are now able to reduce  $\text{Ag}^+$ . **c** The aldehyde function of the reducing sugar is oxidized to its carboxylic acid, while  $\text{Ag}^+$  is reduced to  $\text{Ag}^0$

Silver nanoparticles can be applied in many areas; however, the most important field of application is likely the use of nanosilver for disinfection. Also, our silver nanoparticles showed antimicrobial properties (data not shown). Silver is used commonly in wound dressings for the treatment of burns (Atiyeh et al. 2007). Because silver ions are easily inactivated upon reaction with complexing agents in biofluids, they are often replaced by silver nanoparticles. Silver nanoparticles can be used for their catalytic, conductive, and optical properties as well (Medina et al. 2005; Fuller et al. 2002; Kamat 2002). Until now, most research was performed using chemically produced nanoparticles. As the characteristics of nanoparticles such as size, stability, shape, and therefore the activity depend on the production method, it will be important to investigate the activity of biologically produced nanoparticles in the future. Therefore, the spectrum of the antimicrobial activity towards different microorganisms will be examined more thoroughly in future research.

**Acknowledgments** This work was supported by the project grant 71333 of IWT, the Institute for the Promotion of Innovation by Science and Technology in Flanders, and by the “Industrieel Onderzoeksfonds” of Ghent University (IOF07/VAL/006). We thank Niels D’ Haese and Greet Van De Velde for their assistance in the lab; Kim Verbeken for using the AAS; Olivier Janssens of the department of Solid State Sciences at Ghent University for the XRD analyses; Bart Declercq for his help with the statistics; and Brian Laird, Pieter Van De Caveye, Pieter Verhagen, and Tom Hennebel for critically reading the manuscript.

## References

- Ahmad A, Mukherjee P, Senapati S, Mandal D, Khan MI, Kumar R, Sastry M (2003) Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum*. *Colloid Surf B-Biointerfaces* 28:313–318
- Aslim B, Beyatli Y, Yuksekdogan ZN (2006) Productions and monomer compositions of exopolysaccharides by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains isolated from traditional home-made yoghurts and raw milk. *Int J Food Sci Technol* 41:973–979
- Atiyeh BS, Costagliola M, Hayek SN, Dibo SA (2007) Effect of silver on burn wound infection control and healing: review of the literature. *Burns* 33(2):139–148
- Boonaert CJP, Rouxhet PG (2000) Surface of lactic acid bacteria: relationships between chemical composition and physicochemical properties. *Appl Environ Microbiol* 66:2548–2554
- Chen JC, Lin ZH, Ma XX (2003) Evidence of the production of silver nanoparticles via pretreatment of *Phoma* sp. 3.2283 with silver nitrate. *Lett Appl Microbiol* 37:105–108
- Delcour J, Ferain T, Deghorain M, Palumbo E, Hols P (1999) The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie Van Leeuwenhoek* 76:159–184
- Duran N, Marcato PD, Alves OL, Souza GI, Esposito E (2005) Mechanistic aspects of biosynthesis of silver nanoparticles by several *Fusarium oxysporum* strains. *J Nanobiotechnol* 3:8
- Elechiguerra JL, Burt JL, Morones JR, Camacho-Bragado A, Gao X, Lara HH, Yacaman MJ (2005) Interaction of silver nanoparticles with HIV-1. *J Nanobiotechnol* 3:6
- Fu MX, Li QB, Sun DH, Lu YH, He N, Deng X, Wang HX, Huang JL (2006) Rapid preparation process of silver nanoparticles by bioreduction and their characterizations. *Chin J Chem Eng* 14:114–117
- Fuller SB, Wilhelm EJ, Jacobson JA (2002) Ink-jet printed nanoparticle microelectromechanical systems. *J Microelectromech Syst* 11:54–60
- Jolly L, Vincent SJF, Duboc P, Nesser JR (2002) Exploiting exopolysaccharides from lactic acid bacteria. *Antonie Van Leeuwenhoek* 82:367–374
- Kalimuthu K, Babu RS, Venkataraman D, Bilal M, Gurunathan S (2008) Biosynthesis of silver nanocrystals by *Bacillus licheniformis*. *Colloid Surf B-Biointerfaces* 65:150–153
- Kamat PV (2002) Photophysical, photochemical and photocatalytic aspects of metal nanoparticles. *J Phys Chem B* 106:7729–7744
- Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ, Kim SH, Park YK, Park YH, Hwang CY, Kim YK, Lee YS, Jeong DH, Cho MH (2007) Antimicrobial effects of silver nanoparticles. *Nanomedicine* 3:95–101
- Kim KJ, Sung WS, Moon SK, Choi JS, Kim JG, Lee DG (2008) Antifungal effect of silver nanoparticles on dermatophytes. *J Microbiol Biotechnol* 18:1482–1484
- Klasen HJ (2000a) Historical review of the use of silver in the treatment of burns. I. Early uses. *Burns* 26:117–130
- Klasen HJ (2000b) A historical review of the use of silver in the treatment of burns. II. Renewed interest for silver. *Burns* 26:131–138
- Klaus T, Joerger R, Olsson E, Granqvist CG (1999) Silver-based crystalline nanoparticles, microbially fabricated. *Proc Natl Acad Sci U S A* 96:13611–13614
- Kowshik M, Ashtaputre S, Kharrazi S, Vogel W, Urban J, Kulkarni SK, Paknikar KM (2003) Extracellular synthesis of silver nanoparticles by a silver-tolerant yeast strain MKY3. *Nanotechnology* 14:95–100
- Ledwith DM, Whelan AM, Kelly JM (2007) A rapid, straight-forward method for controlling the morphology of stable silver nanoparticles. *J Mater Chem* 17:2459–2464
- Lin ZY, Zhou CH, Wu JM, Zhou JZ, Wang L (2005) A further insight into the mechanism of Ag<sup>+</sup>-biosorption by *Lactobacillus* sp strain A09. *Spectrochim Acta Part A-Mol Biomol Spectrosc* 61:1195–1200
- Lu L, Sun RWY, Chen R, Hui CK, Ho CM, Luk JM, Lau GKK, Che CM (2008) Silver nanoparticles inhibit hepatitis B virus replication. *Antivir Ther* 13:253–262
- Mast J, Nanbru C, van den Berg T, Meulemans G (2005) Ultrastructural changes of the tracheal epithelium after vaccination of day-old chickens with the La Sota strain of Newcastle disease virus. *Vet Pathol* 42:559–565
- Medina F, Chimentao RJ, Kirm I, Rodriguez X, Cesteros Y, Salagre P, Sueiras JE, Fierro JLG (2005) Sensitivity of styrene oxidation reaction to the catalyst structure of silver nanoparticles. *Appl Surf Sci* 252:793–800
- Merroun M, Rossberg A, Hennig C, Scheinost AC, Selenska-Pobell S (2007) Spectroscopic characterization of gold nanoparticles formed by cells and S-layer protein of *Bacillus sphaericus* JG-A12. *Mater Sci Eng C-Biomim Supramol Syst* 27:188–192
- Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Parishcha R, Ajaykumar PV, Alam M, Kumar R, Sastry M (2001) Fungus-mediated synthesis of silver nanoparticles and their immobilization in the lycelial matrix: a novel biological approach to nanoparticle synthesis. *Nanoletters* 1:515–519
- Nair B, Pradeep T (2002) Coalescence of nanoclusters and formation of submicron crystallites assisted by *Lactobacillus* strains. *Cryst Growth Des* 2:293–298
- Panacek A, Kvitek L, Prucek R, Kolar M, Vecerova R, Pizurova N, Sharma VK, Nevecna T, Zboril R (2006) Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity. *J Phys Chem B* 110:16248–16253



- Rao CNR, Cheetham AK (2001) Science and technology of nanomaterials: current status and future prospects. *J Mater Chem* 11:2887–2894
- Sadowski Z, Maliszewska I, Polowczyk I, Kozlecki T, Grochowalska B (2008) Biosynthesis of colloidal-silver particles using microorganisms. *Pol J Chem* 82:377–382
- Sastry M, Ahmad A, Khan MI, Kumar R (2003) Biosynthesis of metal nanoparticles using fungi and actinomycete. *Curr Sci* 85:162–170
- Shahverdi AR, Minaeian S, Shahverdi HR, Jamalifar H, Nohi AA (2007) Rapid synthesis of silver nanoparticles using culture supernatants of Enterobacteria: a novel biological approach. *Process Biochem* 42:919–923
- Silver S, Phung LT, Silver G (2006) Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. *J Ind Microbiol Biotechnol* 33:627–634
- Tavakoli A, Sohrabi M, Kargari A (2007) A review of methods for synthesis of nanostructured metals with emphasis on iron compounds. *Chem Pap* 61:151–170
- Tilaki RM, Zad AI, Mahdavi SM (2006) Stability, size and optical properties of silver nanoparticles prepared by laser ablation in different carrier media. *Appl Phys A-Mater Sci Process* 84:215–219
- van Hullebusch E, Zandvoort M, Lens P (2003) Metal immobilisation by biofilms: mechanisms and analytical tools. *Rev Environ Sci Biotechnol* 2:9–33
- Vigneshwaran N, Ashtaputre NM, Varadarajan PV, Nachane RP, Paralikar KM, Balasubramanya RH (2007) Biological synthesis of silver nanoparticles using the fungus *Aspergillus flavus*. *Mater Lett* 61:1413–1418
- Zhang HR, Li QB, Lu YH, Sun DH, Lin XP, Deng X, He N, Zheng SZ (2005) Biosorption and bioreduction of diamine silver complex by *Corynebacterium*. *J Chem Technol Biotechnol* 80:285–290
- Zhang HR, Li QB, Wang HX, Sun DH, Lu YH, He N (2007) Accumulation of silver(I) ion and diamine silver complex by *Aeromonas SH10* biomass. *Appl Biochem Biotechnol* 143:54–62