Accepted Manuscript

Title: BIOSYNTHESIS OF SILVER NANOPARTICLES
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PII: S0141-8130(17)32898-2
DOI: <https://doi.org/10.1016/j.ijbiomac.2017.12.007>
Reference: BIOMAC 8668

To appear in: *International Journal of Biological Macromolecules*

Received date: 3-8-2017
Revised date: 29-11-2017
Accepted date: 3-12-2017

Please cite this article as: J.L.Castro-Mayorga, F.Freitas, M.Reis, A.Prieto, J.M.Lagaron, BIOSYNTHESIS OF SILVER NANOPARTICLES AND POLYHYDROXYBUTYRATE NANOCOMPOSITES OF INTEREST IN ANTIMICROBIAL APPLICATIONS, International Journal of Biological Macromolecules <https://doi.org/10.1016/j.ijbiomac.2017.12.007>

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BIOSYNTHESIS OF SILVER NANOPARTICLES AND POLYHYDROXYBUTYRATE NANOCOMPOSITES OF INTEREST IN ANTIMICROBIAL APPLICATIONS

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ABSTRACT

This study deals with the optimization and scaling up of the production of poly(3-hydroxybutyrate), PHB, nanocomposites containing biosynthesized silver nanoparticles (AgNPs) to generate materials with antimicrobial performance. First, a comparative study of the chemical and biological synthesis of AgNPs during the fermentation process of *Cupriavidus necator* at shake flask-scale was carried out. These experiments demonstrated the inherent capacity of *C. necator* to reduce the silver salt and produce AgNPs without the need for adding a reducing agent and, that the method of synthesis

(with or without reducing agent) affects the dispersion of the AgNPs and their antimicrobial performance. Finally, the process was scaled-up to a 10 liters bioreactor and the relevant physical properties of the PHB-AgNPs nanocomposites pressed into films were determined. From the characterization work, the AgNPs were found to be well dispersed and distributed into the polymer matrix, having a maximum frequency of particles with average diameter of 76-95 nm. Moreover, the presence of AgNPs did not cause any effect on the thermal properties of the biopolymer, although a slight reduction in crystallinity was seen. The developed materials presented a strong antimicrobial activity against the food-borne pathogens *Salmonella enterica* and *Listeria monocytogenes*, which makes them potentially suitable for active coatings and packaging applications. Complete biodisintegration of the samples occurred during composting conditions within the first 40 days. Interestingly, the presence of the AgNPs did not impair the profile of biodegradation of the microbial polymer.

Keywords: Polyhydroxyalkanoates, metal nanoparticles, antibacterial, films.

INTRODUCTION

Given the environmental concerns related to the massive use of petroleum-based plastics and their high demand in the packaging market, which was around 40% of the global plastic's consumption in 2014 [1], a great interest to develop biodegradable polymers obtained from renewable resources has emerged in the last decades. Among the most widely studied biopolymers, polyhydroxyalkanoates (PHAs) have attracted increased

attention due to their thermoplastic, biodegradable, biocompatible and renewable nature [2].

PHAs are synthesized by many bacteria as intracellular carbon and energy storage granules. The Gram negative, facultative bacterium *Cupriavidus necator* (before called *Ralstonia eutropha* and *Wautersia eutropha*) has been intensively studied for almost 50 years [3]. *C. necator* was described by [4] as a non non-obligate bacterial predator of various soil bacteria and fungi ([5-7] and it is currently an interesting candidate for the large-scale production of polyhydroxybutyrate (PHB). It provides many of all the necessary characteristics, which are required for a biotechnological production strain. Due to its metabolic versatility, it can convert a broad range of renewable carbon resources into diverse valuable compounds [8]. It uses fragmented organic wastes (conversion of organic wastes to short chain volatile fatty acids through hydrolysis and acidogenesis) from the food and beverage industries for the PHAs production [9]. Furthermore, this bacterium is accessible to engineering of its metabolism by genetic modification approaches [10].

The PHB produced by *C. necator* can be used for applications ranging from replacement of fossil resource-based bulk plastics to high-value special purpose polymers. In the packaging field, PHB has been used to make various biodegradable products, including films, coated paper and boards, compost bags, dispensable food service-ware, and molded products such as bottles and razors [11].

Particularly, packaged foods with enhanced quality and safety are demanded by consumers. In this context, the incorporation of antimicrobials into packaging materials allows the progressive diffusion of target bactericidal or bacteriostatic compounds into

the packaging surface and from there also into the food matrix, thus reducing the use of high concentrations of antimicrobials in the food matrix to extend foods shelf-life [12].

Inorganic compounds in the nanosize range, i.e. below 100 nm, such as silver nanoparticles (AgNPs) have emerged as efficient tools to prevent microbial spoilage and microbial outbreaks in food contact plastics and surfaces. Traditionally, AgNPs are synthesized by chemical methods using different organic and inorganic reducing agents, such as sodium borohydride (NaBH_4) [13, 14], sodium citrate [15], ascorbate [16], elemental hydrogen, Tollen's reagent, N,N-dimethyl formamide (DMF) and poly(ethylene glycol) block copolymers [17]. However, most chemicals used in this kind of synthesis are toxic and lead to non-eco-friendly by-products. To solve these problems, biological synthesis of nanomaterials using living organisms such as bacteria [18], fungi [19] or plant extracts [20] are currently being developed.

So far, there is only a study reported in the literature about the effects of AgNPs on *C. necator* growth dynamic, which indicates growth stimulation of this bacteria at certain AgNPs concentrations (40 $\mu\text{g/mL}$ of AgNPs with primary particle size distribution $D_{90} < 15$ nm and added after 12 hours of cultivation), as well as varying susceptibility to nanoparticles at different growth stages [21]. However, because it has been known and accepted that the silver susceptibility depends on the medium culture composition [22], it is necessary to make an analysis of the unbalanced growth conditions where PHB is produced. On the other hand, in a recent publication, the synthesis of AgNPs was carried out by using the cell-free supernatant of *Alkaliphilus oremlandii*, strain ohILA, culture. In that case, the synthesized AgNPs were subsequently stabilized by the extracted and purified biopolymer also produced by *A. oremlandii* [23].

The main goal of this study was to report the development, optimization and scaling up of a simple bioprocess for the simultaneous biosynthesis of PHB and AgNPs with antimicrobial properties. First, a comparative study of the chemical and biological synthesis of AgNPs during the fermentation process of *C. necator* at shake flask was carried out and then optimized and scale-up to fully-automated 10 liters bioreactor. Moreover, the physical properties and the antimicrobial performance of the developed nanocomposites, was determined.

2.0 MATERIALS AND METHODS

2.1 Materials

Silver nitrate (>98%; Silma-Aldrich, Germany), Sodium borohydride (96%, Panreac, Spain), chloroform stabilized with ethanol (Technical grade, Panreac, Spain), methanol (For analysis, ACS, ISO, Panreac, Spain), hiperpuric nitric acid (69%, Panreac, Spain), sulphuric acid in methanol solution (Sigma–Aldrich, HPLC grade) and benzoic acid in chloroform (1 g/L) (Sigma–Aldrich, HPLC grade) without further purification were used throughout the experiments.

2.2 Bacterial growth and media

Cupriavidus necator strain H16 (DSM 428, Wilde 1962) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and used in this study. The culture, cryopreserved in 20% (v/v) glycerol at -80 °C, was reactivated in solid Luria Bertani (LB) medium, at 30 °C for 24 h, and subsequently used as inoculum. The cultivation medium consisted of: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.39 mg/L; K_2SO_4 , 0.45 mg/L;

FeSO₄·7H₂O, 15 mg/L; NH₄Cl, 0.01% (w/v); H₃PO₄, 1 M (12 mL/L); trace elements, 24 mL/L (CuSO₄·5H₂O, 20 mg/L; ZnSO₄·6H₂O, 100 mg/L; MnSO₄·4H₂O, 100 mg/L; CaCl₂·2H₂O, 2.6g/L) and 1% (w/v) of sodium gluconate as sole carbon source.

2.3 Microbial synthesis of PHB and AgNPs

The biosynthesis of PHB and AgNPs is based on a procedure generally described in a patent pending methodology (Spanish Patent P201630829). First, the process was analyzed at shake flask scale and then optimized and scaled-up to 10 L capacity fully-automated laboratory-scale bioreactor.

2.3.1 Synthesis at shake flask scale

Batch cultivation experiments of *C. necator* in shake flask were performed in order to investigate the AgNPs synthesis from silver nitrate. The inoculum for shake flasks experiments was prepared by inoculating a single *C. necator* colony into 200 mL of LB medium and incubation in an orbital shaker at 30° C and 200 rpm for 24 h. The cells were then harvested and resuspended in the culture medium described above (see 2.2 section) to reach an optical density of 0.3 at a wavelength of 600 nm. The cultivation was done in 2 L Erlenmeyer flask with an initial working of 800 mL, which were incubated at 30° C and 200 rpm for 24 h. Subsequently, the cultures were treated with 267 mL of silver nitrate solutions at 1 mM or 3.5 mM. The silver nitrate treatment was applied at the beginning and after 24 h of the cultivation and carried out both in presence and in absence of a reducing agent. For the experiments in the presence of the reducing agent, 8 mL of the culture broth were replaced by 8 mL a 0.1 M sodium borohydride solution to get 1 mM concentration and, after 20 min, the desirable silver nitrate solution were added dropwise

During the addition of silver nitrate and sodium borohydride and for further 16 h, the cultures were kept under agitation (200rpm) and 30° C.

2.3.2 Scaled up synthesis to lab-scale bioreactor

Bioreactor cultivation experiments were performed in a 10 L bioreactor (BioStat B-Plus, Sartorius, Germany), with an initial working volume of 7 L. The inoculum was 10% (v/v) of the initial reactor working volume. It was prepared by inoculating a *C. necator* colony into LB medium and incubation in an orbital shaker, at 30° C and 200 rpm, for 24 hours. The culture thus obtained was then transferred into culture medium described above (see 2.2 section), further incubated for 48 h at the same agitation and temperature and used for the bioreactor experiments. The bioreactor was operated with controlled temperature (30 °C) and pH (7.0). The pH was controlled by the automated addition of HCl (1 M) and NH₄OH (25% v/v, in the first 20 hours) which served also as nitrogen source, or NaOH (2 M, after 20 hours) to impose nitrogen-limitation conditions. The dissolved oxygen (DO) concentration was controlled at 30% by automatically adjusting the stirring rate (between 300 and 2000 rpm). The experiments comprised an initial batch phase of 20 h, followed by the fed-batch phase, wherein 1 L of sodium gluconate, at 1% (w/v) at 0.12 L/h were fed to stimulate the polymer accumulation. At the end of the cultivation runs, a sample of 10 mL was withdrawn from the bioreactor of broth was centrifuged at 4000 rpm for 15 min and the pellet washed with deionized water and lyophilized for the gravimetric the cell dry weight (CDW) quantification. In the bioreactor experiments the silver nitrate treatment was applied following the optimal parameters obtained in the shake flask cultivation.

2.4 Polymer extraction

The extraction of the polymer produced in the shake flask and bioreactor experiments was carried out by using organic solvents. To this end, the culture broth was centrifuged for 15 min at 4000rpm, the supernatant discarded and the biomass transferred to 300 mL a chloroform solution and left to form a suspension for a period of 20 h at 50 °C in a closed bottle with stirred with a magnetic bead. Subsequently, 300 mL of distilled water was added to the polymer rich solution. The mixture was separated in volumes of 50 mL, stirred in vortex for 1 min and centrifuged for 2 min at 2000 rpm to separate the organic phase. The resulting organic fraction was removed using glass Pasteur pipettes, and the polymer was then precipitated in ice-cold methanol (10-fold excess) with vigorous stirring. Finally, the precipitated polymer was separated by filtration and dried overnight in a vacuum oven at 60 °C. The PHB content in the biomass (% w/w) and the overall volumetric productivity (r_p , g/ L day) ~~was~~ were calculated using the equations 1 and 2, respectively following equation (Equation 1):

$$\% PHA = \frac{\text{weight of extracted polymer}}{\text{cell dry weight}} \times 100 \quad (1).$$

$$r_p = \frac{\Delta P}{\Delta t} \quad (2).$$

where ΔP (g /L) is the PHB produced during cultivation time Δt (day). The purity of the extracted polymer was determined by gas chromatography (GC) as described elsewhere [24, 25]. Briefly, 3-5 mg of the extracted polymer were hydrolyzed with 20% (v/v) sulfuric acid in methanol solution (1 mL) and benzoic acid (internal standard) in chloroform (1 mL), at 100 °C for 3.5 h. After the digestion, the methylated monomers were extracted and injected into a gas chromatograph (Varian CP-3800) coupled with a flame ionization detector (FID) (CTC Analytics, Switzerland) in a BR89342 WCOT

fused silica column (60 m × 0.53 mm), using helium as carrier gas with a flow rate of 1 mL/min. The oven temperature program was as follows: 40 °C; 20 °C/min, until 100 °C; 3 °C/min, until 175 °C and, finally, 20 °C/min, until 220 °C. The injector and detector temperature were set at 280 °C and 250 °C, respectively. Pure copolymer solution (0.25-10 mg/L) containing poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV) (Sigma–Aldrich), was used as standard.

2.5 Transmission electronic microscopy (TEM) of bacteria

The size and morphology of AgNPs obtained from the shake flask experiments were characterized using a transmission electron microscope (JEOL 1010, Hitachi) equipped with a digital Bioscan (Gatan) image acquisition system at an accelerating voltage of 80 kV. The samples for TEM analysis were prepared by fixing the cells with 2.5% glutaraldehyde and 2% osmium tetroxide for 2 h at room temperature. The pellets were then washed three times with phosphate buffer saline (PBS) and dehydrated in ethanol series (30%, 70%, 90% and 100%). The specimens were further embedded in LR-White resin and cut with an ultramicrotome (Leica UC6). Ultrathin sections were then positively stained with uranylacetate and lead citrate and then placed onto carbon-coated copper grids for microscopic examination.

2.6 Preparation and characterization of films

The polymer produced in the lab-scale reactor experiments was compression molded into films of 80 µm of thickness using a hot-plate hydraulic press (Carver 4122, USA) at 180 °C and 1.8 MPa for 5 min and used for further characterization. Pure and silver containing films were prepared for comparative purposes. The morphology and size distribution of AgNPs into the film were analyzed through observation of ultramicrotomed cross sections by TEM using the same microscope that was used above (JEOL 1010, Hitachi).

The particle diameters were estimated by means of Adobe Photoshop CS4 software from 300 particles at random.

2.6.1 Molecular weight determination

The molecular weight of the pure and the silver containing polymer were determined using Gel Permeation Chromatography (GPC) as described elsewhere [26]. Briefly, a 10- μ L aliquot of 0.5 % w/v sample solution in chloroform was injected into the system operated at 35 °C with an eluent flow rate of 1 mL/min using a Spectra-Physics 8800 solvent delivery system with a set of two Mixed C Styragel columns and a Shodex SE 61 refractive index detector. Polystyrene standards with narrow molar mass dispersity were used to generate a universal calibration curve.

2.6.2 Thermal properties

The thermal properties of the polymers were determined by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). DSC analysis were carried out using a Perkin-Elmer DSC 8000 with an intracooler (Intracooler 2 from Perkin Elmer). Samples were first heated from 0 °C to 200 °C, kept for 1 min at 200 °C and then cooled down to -50 °C. The heating and cooling rates for the runs were 10 °C/min and the typical sample weight was ~3 mg. The melting temperature (T_m), melting enthalpy (ΔH_m), crystallization temperature (T_c) and crystallization enthalpy (ΔH_c) were determined from the heating and cooling curves. The DSC instrument was calibrated with an indium standard before use. The tests were done in triplicate.

Thermal stability of the PHB and PHB-AgNPs nanocomposites was performed on a Perkin Elmer Thermobalance TGA 7 equipment operated under nitrogen atmosphere. After conditioning for 5 min at 30 °C, the sample was heated to 600 °C at a heating rate

of 10 °C/min. Derivative TGA curves (DTG) represent the weight loss rate as function of temperature and the temperature of maximum rate of degradation (T_d).

2.6.3. Wide Angle X-ray Diffraction analysis

Wide Angle X-ray Diffraction analysis (WAXD) measurements of the pure and silver containing films were recorded at room temperature using a Bruker AXSD4 Endeavour diffractometer with a Cu-K α source (wavelength = 1.54178 Å) and a generator at 40 kV and 40 mA. Peak fitting was carried out using IgorPro software package (Wavemetrics, Lake Oswego, Oregon). Gaussian function was used to fit the experimental diffracton profiles obtained. The crystallinity index (X_c) was calculated by the equation 3. (± 3).

$$X_c = \frac{\sum A_{Crystal}}{\sum A_{Total}} \times 100 (\pm 3)$$

where $\sum A_{Crystal}$ is the sum of the areas corresponding to crystalline peaks and $\sum A_{Total}$ is the sum of the areas under all the diffraction peaks in the range of $2\theta = 5^\circ$ and 40° .

2.6.4 Determination of silver content

To estimate the total silver content within the films, ~100 mg of silver containing films were subjected to digestion with 2 mL of HNO₃ at 80 °C for 16 h. The silver concentration in the resultant digestant was determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Perkin-Elmer, USA) using a silver standard solution (traceable to SRM from NIST, AgNO₃ in HNO₃ 2-3 % 1000 mg/L Ag Certipur®, Merck, Germany) for calibration. The measurements were done in triplicate.

2.7 Antimicrobial activity of PHB-AgNP films

The microorganisms used for the antimicrobial assays were the food-borne pathogens *Salmonella enterica* CECT 4300 and *Listeria monocytogenes* CECT 7467. The strains were obtained from the Spanish Type Culture Collection (CECT, Spain) and stored in phosphate buffered saline (PBS, Sigma Aldrich, U.S.) with 10 wt.% tryptic soy broth (TSB, Conda Laboratories, Spain) at -80°C until used. Before antimicrobial activity test, an aliquot of bacteria stock was transferred twice to TSB and grown at 37 °C to the mid-exponential phase. This culture served as inoculum for the susceptibility tests.

Antimicrobial activity was determined by using a modification of the Japanese industrial standard test (JIS Z 2801-ISO 22196). In brief, the test samples were placed in petri dishes and inoculated with 10⁵ CFU/mL of bacterial culture. The inoculum was covered with a Low-Density Polyethylene film and incubated for 24 h at 25 °C and >90% RH. After exposure, bacteria were then recovered with PBS, spread on TSA for plate counts after incubation at 37 °C overnight. The temperature of 25 °C was chosen as to mimic the room temperature condition of food storage and consumption. PHB films without silver were used as negative control. The bacterial reduction (R) was calculated as follows: $R = \log_{10} (N_0/N_t)$, where N_0 is the average of the bacterial counts recovered from the PHB negative control film and N_t is average of the bacterial counts recovered from the PHB films loaded with silver.

2.8 Biodisintegration of films under composting conditions

The biodesintegration of the PHB and PHB-AgNPS films under controlled composting conditions was evaluated according to the ISO 20200 standard for “The determination of the degree of disintegration of plastic materials under simulated composting conditions in a laboratory-scale test”. For the preparation of solid synthetic waste, 10% of activated mature compost (Vigorhumus H-00, purchased from Burás Profesional, S.A., Girona,

Spain) was mixed with 30 wt. % rabbit food, 10 wt. % starch, 5 wt. %. sugar, 1wt. % urea, wt. 4% corn oil and 40 wt. % sawdust. The water content of the mixture was adjusted to 55 wt. % and the aerobic conditions were guaranteed by gently mixing the compost and periodically adding water according to the standard requirements. The test samples were cut from the films (10 x 10 x 0.1 mm³) and buried inside plastic mesh bags to simplify their extraction and allow the contact of the compost with the specimens, and were incubated at 58°C. At different composting times (14, 22, 30 and 40 days) samples were recovered for analysis, washed with distilled water, dried at 40°C under vacuum for 24 h, and weighed. The disintegration degree was calculated by normalizing the sample weight, at different days of incubation, to the initial weight with Equation (2), where m_i is the initial dry mass of the test material and m_f is the dry mass of the test material recovered at different incubation stages.

$$D = \frac{m_i - m_f}{m_i} \times 100 \quad (2)$$

2.9 Statistical analysis

The statistical analysis of data was carried out by means of OriginPro 8 (OriginLab Corporation, USA) with a one-way analysis of variance (ANOVA) and using the Tukey's multiple test at the 95% confidence level. Reported values of quantitative measurements were the means of triplicate data $\pm$ standard deviation.

3. RESULTS AND DISCUSSION

3.1 Microbial synthesis of PHB-AgNPs in shake flasks

The addition of 1 mM or 3.5 mM silver nitrate solutions at the beginning and after 24 h of the *C. necator* cultivations resulted in a complete and irreversible cell growth inhibition, as no colony-forming units were detected in TSA plates (detection limit of 1.30 log CFU/mL). This result was likely expected due to the natural and well known antimicrobial effect of silver. Thus, in subsequent experiments the silver nitrate solutions were added after ca. 24 h of cultivation, when the *C. necator* achieved a concentration of cells achieved 8 log CFU-mL and an optical density of 6 at a wavelength of 600 nm.

3.1.1 Visual and microscopic observations

At the lower concentration of 1 mM, no visual changes in the colour of the reaction mixture with or without reducing agent were observed. However, a brown colour appeared in the reaction mixture incubated with the silver nitrate solution at 3.5 mM and the reducing agent (Fig. 1). These colour changes are associated with the synthesis of colloidal silver. Silver colloids show different colours due to light absorption and scattering in the visible region based on plasmon resonance, which depend on the shape and size of AgNPs and dielectric constant of the surrounding medium [27].

A typical TEM image of untreated *C. necator* cells filled with large PHB granules is shown in the Fig. 2A. Interestingly, when the bacterial culture in stationary phase was treated with 1 mM of silver nitrate, spherical electron dense nanoparticles appeared inside and outside the cellular cytoplasm both in absence (Fig. 2B) and in presence of reducing agent (Fig. 2C). This finding confirms the inherent capacity of *C. necator* to reduce the silver salt and produce AgNPs. Furthermore, noticeable differences in the dispersion and the frequency of nanoparticles inside the cells were evidenced between the two preparations. Some bigger agglomerates and a higher number of particles located outside the cytoplasm were observed in the samples prepared in the presence of the reducing

agent (Fig. 2C) as compared with those prepared without reducing agent. Incorporation of a reducing agent to the bacterial culture adds unnecessary reduction capacity to that already inherent within the biomass resulting in much stronger or in excess reduction power.

The discussed colour changes were also evident after polymer purification and compression moulding process, as deduced from the contact transparency pictures in Figure 3. In fact, the film prepared with the polymer obtained in the presence of at 1 mM silver nitrate (Fig. 3B) in the absence of the reducing agent present a very slight colour difference from the neat PHB film (Fig. 3A). However, some visual dissimilarities were found for the material prepared with PHB synthesized at 1 mM silver nitrate in the presence of reducing agent (Fig. 3C) and those prepared at 3.5 mM (Fig. 3D and 3E). In the case of materials prepared in the presence of the reducing agent, in both concentrations, small black particles were visually detected (see samples C and E of Fig. 3). It should be also noted that at 3.5 mM of silver nitrate concentration, the films showed a brown colour, which was more intense for those samples containing the reducing agent (see Fig. 3D and 3E). These findings could be explained by the excess of the reducing power and the AgNPs agglomeration discussed above, which is even more significant at the higher silver loading of 3.5 mM.

3.1.2 Antimicrobial performance

The silver-based films developed at Erlenmeyer flask scale were tested against two of the most frequent food-borne pathogens, *S. enterica* and *L. monocytogenes* (Table 1, Fig. 4). The Gram-negative bacterium *S. enterica* appeared to be more susceptible to the antimicrobial effect of silver nanoparticles than the Gram-positive bacterium *L. monocytogenes*, which can be explained by the differences on structural features of its cell envelope [28-30]. Furthermore, the active films showed dose-dependent antibacterial

activity that directly increased with the silver concentration. This dose-dependent effect was particularly noticeable in the *L. monocytogenes* tests. No viable counts of *S. enterica* were recorded for both silver nitrate concentrations used for the biosynthesis, except for the one prepared in the presence of 1 mM silver nitrate, with reducing agent, which might be due to the lower dispersion, the bigger size and the higher agglomeration degree of AgNPs as compared to its counterpart prepared without reducing agent, as it has been previously observed by TEM (see Fig. 2). On the other hand, the PHB film obtained in the presence of a higher silver concentration of 3.5 mM was more effective to reduce the bacterial population of *L. monocytogenes* (by least 3 log CFU/mL, which is the minimal value to consider that the sample presents a relevant antimicrobial activity) [31, 32].

3.2 Microbial synthesis of PHB-AgNPs in 10 liters bioreactor

3.2.1 Fermentation process

In accordance with the results of the study at shake flask scale, fed-batch fermentations under controlled cultivation conditions at the 10-liter scale were carried out to obtain higher cell densities and higher polymer contents. In these experiments, the PHB production and the biological synthesis of AgNPs by using the intrinsic reducing power of the biomass were simultaneously performed. The silver nitrate concentration used in these experiments was 3.5 mM because of its effective antimicrobial activity against the two food-borne pathogens already described in the shake flask experiments.

The addition of silver nitrate in the bioreactor experiments was carried out 40 h after de cultivation, when the culture achieved a relative constant optical density of 9-10 at a wavelength of 600 nm. At this point, a CDW of 4.17 ± 0.38 g/L was obtained, which

corresponded to a polymer production of 3.38 ± 0.65 g/L and an overall volumetric productivity of 2.03 ± 0.39 g/(L day). These values are significantly higher than the values obtained in the shake flask experiments (overall productivity 0.5 ± 0.04 g/(L day) and in previous studies on PHB production from *C. necator* fed using virgin soybean oil, rapeseed oil, olive oil or glucose under batch flask mode [33, 34], but still much lower than those reported by Heinrich et. al (2012) [35] and Cruz et al. (2015) [36] for fed-batch and batch bioreactor cultivation of *C. necator* with used sodium gluconate and cooking oils, respectively. Nevertheless, the final polymer content of 57.5 ± 4.9 wt. % obtained in the present work, was in the range of those reported in the literature [35, 37], although more studies are needed in order to gain a higher cell density and improve the productivity at large scale, but it is outside the scope of the present study.

3.2.2 Polymer characterization

As expected, the polymer produced by *C. necator* was the homopolymer poly(3-hydroxybutyrate), (PHB), which is in agreement with recent reports of PHB production from *C. necator* employing sodium gluconate as sole carbon source [35]. PHB purity after the recovery procedure was 91.8 ± 0.6 wt. %, as measured by GC, without any significant variation after silver incorporation. The purity achieved with the present procedure was within the reported values (range of values?) for PHB extraction with similar procedures [33, 35, 38].

According to GPC results, there were no changes in the molecular weight of PHB after silver addition, since the molecular weight of the PHB and PHB/AgNPs films was 1.12×10^6 g/mol. Similar values were reported for PHB synthesized by *C. necator* from used cooking oils under pulse feeding fermentation (2.0×10^5 - 2×10^6 g/mol) [39].

3.2.3 Morphological properties of PHB-AgNPs films

Figure 5A shows a typical TEM micrograph of the ultramicrotomed cross section of the PHB containing AgNPs (hereinafter called PHB-AgNPs). The TEM results indicated that the particles are small, well dispersed and spherical in shape, having a maximum frequency of particles with an average diameter at 76-95 nm (Fig. 5B). Thus, the good dispersion of AgNPs into the PHB matrix led to macroscopically homogeneous films with a good contact and background transparency (Fig. 5C).

3.2.3 Thermal properties of PHB-AgNPs films

The thermal properties of the pure PHB and the PHB-AgNPs films were analyzed by TGA and DSC. The thermal degradation curves of the two materials exhibited a single weight loss with maximum decomposition temperature (T_d) at 276 °C for PHB and 278 °C for PHB-AgNPs (Fig. 6). This result is in contrast with previous studies carried out with other PHA's materials, in which an improvement in the thermal stability of the composites were seen induced by the presence of metal nanoparticles [40-43]. Nevertheless, in the present work, the thermal stability was not seen to depend on the AgNPs loading since, in comparison with the previous studies, it was rather small to show any potential effect (i.e. 0.015 ± 0.001 wt. %, as quantified by ICP-OES).

The DSC thermograms for heating and cooling of the neat and silver containing films are shown in Figure 7 and the resulting thermal properties are gathered in Table 2. A single melting peak was observed in the heating runs of both samples. Indeed, no significant or slight differences ($p < 0.05$) were found between the melting temperature (T_m) or melting enthalpy (ΔH_m) obtained from the heating step of the neat PHB and silver containing PHB. However, a measurable decrease in the crystallization temperature (T_c) and crystallization enthalpy (ΔH_c) of PHB-AgNPs as compared to PHB was registered,

suggesting an antinucleation effect in which the nanoparticles could be hindering the PHB crystallization [40, 44].

The crystallinity of PHB and PHB AgNPs was assessed by Wide Angle X-Ray Diffraction (WAXD). Figure 8 shows the diffraction patterns and the crystallinity index calculated after curve fitting of the experimental WAXD patterns. It can be seen that when the silver is incorporated, the intensity of the sharp diffraction peaks between $2\theta=13-25^\circ$ decreases. This indicates a decrease in the crystallinity index, which can be explained by the antinucleant effect of AgNPs discussed above. It is worth mentioning that crystallinity was seen to decrease in the silver loaded sample while a heat of fusion drop was not observed by DSC. Both techniques seldom match one another, since DSC is a dynamic measurement and it is well known that PHAs develop crystallinity during the actual DSC run [41, 45, 46].

3.2.4 Antimicrobial activity of PHB-AgNPs films

The antibacterial capacity of the films developed at lab scale reactor was further confirmed (Table 3). As expected, in the PHB-AgNPs films, the bacterial population of *S. enterica* was reduced below detection limit (33 CFU/mL), while the viable cells of *L. monocytogenes* showed a reduction of 2.5 log units (99.8%) compared with the control. These results evidenced the great ability of PHB films loaded with very low amounts of silver, to exert a strong antimicrobial activity against food-borne pathogens.

3.2.5 Biodisintegration of films under composting conditions

PHA's are of great interest in many applications because on top of their interesting physical and chemical properties they readily degrade in natural soils [47] and under composting conditions [48]. Figure 9 shows the biodisintegration (as percentage of

weight loss) as a function of time for the PHB and PHB-AgNPs films. Weight loss remained practically unchanged until the 14th day of composting for the two compositions studied. Similar induction time has been already reported for PHB films obtained by solvent casting technique [48] film and polyhydroxybutyrate-co-hydroxyvalerate with a 3 mol % of valerate (PHBV3) blends obtained by thermo-compression moulded [49, 50]. Interestingly, full biodegradation occurred for both samples within 40 days, suggesting that the AgNP's do not actually impair the biodegradation process under composting and as a result do not limit the usability of the material in this regard.

CONCLUSIONS

A novel process for the biological synthesis of AgNPs and polyhydroxybutyrate (PHB) from the fermentation process with *Cupriavidus necator* was developed and scaled-up to fully automated 10 liters bioreactor. Interestingly, this work demonstrated for the first time, the inherent capacity of *C. necator* to reduce the silver salt and produce AgNPs without the need of adding a reducing agent. It was also observed that the method of AgNPs synthesis (with or without reducing agent) affected the dispersion of the nanoparticles and therefore their antimicrobial performance, being the biosynthesized ones the most effective in reducing the bacterial population of *S. enterica* and *L. monocytogenes*. Moreover, the incorporation of AgNPs by means of this method did not cause any detrimental effects on the thermal degradation, the optical properties and the biodegradability of the active polymer, although a slight reduction in crystallinity was seen. Thus, the procedure here developed allows the production of AgNPs- based polyhydroxyalkanotes, which are suitable for antimicrobial applications.

ACKNOWLEDGMENTS

The authors would like to thank to the Active and intelligent fibre-based packaging - innovation and market introduction (ActInPak) COST Action FP1405 for the funding of the Short Term Scientific Mission, to Octavio Cedenilla from the Instituto de Ciencias Agrarias-CSIC for the ICP-OES analysis and to Dr. Luis Cabedo, from Universitat Jaume I for his support with mechanical testing, WAXD analyses and biodisintegration tests. Research leading to these results has received funding from the Comunidad de Madrid (P2013/MIT2807) and the Spanish Ministerio de Economía y Competitividad, (BIO2013-44878-R and AGL2015-63855-C2-1-R). Jinneth Lorena Castro-Mayorga is supported by the Administrative Department of Science, Technology and Innovation (Colciencias) of the Colombian Government.

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FIGURE CAPTIONS

Figure 1. Visual aspect of *C. necator* cultures at shake flask-scale. (A) untreated bacteria, (B) bacteria treated with a silver nitrate solution at 3.5 mM in absence of the reducing agent, (C) bacteria treated with a silver nitrate solution at 3.5 mM in presence of the reducing agent.

Figure 2. TEM micrographs of *C. necator* at 24 h of cultivation in shake flask. (A) untreated bacteria, (B) bacteria treated with a silver nitrate solution at 1 mM in absence of the reducing agent, (C) bacteria treated with a silver nitrate solution at 1 mM in presence of the reducing agent.

Figure 3. Contact transparency pictures of the pure PHB and the PHB-AgNPs films prepared with silver nitrate solutions at different concentrations. (A) pure PHB, (B) PHB-AgNPs film prepared with 1 mM silver nitrate solution and in absence of the reducing agent, (C) PHB-AgNPs film prepared at 1 mM in presence of the reducing agent, (D) PHB-AgNPs prepared at 3.5 mM in absence of the reducing agent, (E) PHB-AgNPs prepared at 3.5 mM in presence of the reducing agent.

Figure 4. Antimicrobial activity of the PHB-AgNPs films prepared at shake flask-scale against *S. enterica* and *L. monocytogenes*. The initial inoculum size was 5 log CFU/mL and the detection limit was 1.30 log CFU/mL.

Figure 5. Morphological properties of the PHB-AgNPs films prepared at lab-scale bioreactor. (A) TEM micrograph of ultramicrotomed cross section of film, (B) Photograph of the film, (C) Particle size distribution of AgNPs into the film.

Figure 6. DTG curves of the pure PHB and PHB-AgNPs nanocomposites prepared at lab-scale bioreactor.

Figure 7. DSC thermograms of first heating and cooling run of the pure PHB and the PHB-AgNPs nanocomposites prepared at lab-scale bioreactor.

Figure 8. WAXD patterns of the pure PHB and the PHB-AgNPs nanocomposites prepared at lab-scale bioreactor. The crystallinity index (X_c) was calculated from curve fitting by using the Eq. (1).

Figure 9. Disintegration of the PHB and PHB-AgNPs films over time under composting conditions. The disintegration degree was calculated according to the Eq. (2)

TABLES

Table 1. Bacterial reduction of the PHB/AgNPs films prepared at shake flask-scale.

Sample	R	
	<i>S. enterica</i>	<i>L. monocytogenes</i>
1 mM without reducing agent	>6.59	2.21
1 mM with reducing agent	3.83	1.31
3.5 mM without reducing agent	>6.59	3.28
3.5 mM with reducing agent	>6.59	3.43

Table 2. DSC melting point (T_m), melting enthalpy (ΔH_m), crystallization temperature (T_c) and crystallization enthalpy (ΔH_c) of the pure PHB and the PHB-AgNPs nanocomposites prepared at lab-scale bioreactor.

Sample	T_m (°C)	ΔH_m (J/g)	T_c	ΔH_c (J/g)
PHB	175.3 ± 1.1^a	80.5 ± 0.7^a	103.7 ± 1.5^a	76.5 ± 1.8^a
PHB-AgNPs	175.0 ± 0.4^a	83.1 ± 0.6^b	98.8 ± 1.8^b	68.9 ± 0.6^b

Mean values with different superscript letters in the same column represent significant differences ($p < 0.05$) among the samples according to ANOVA and Tukey's multiple comparison tests

Table 3. Antimicrobial activity of the PHB-AgNPs films prepared at lab-scale bioreactor against *S. enterica* and *L. monocytogenes*. The initial inoculum size was 5 log CFU/mL and the detection limit was 1.33 log CFU/mL.

Sample	LOG (CFU/mL)		R	
	<i>S. enterica</i>	<i>L. monocytogenes</i>	<i>S. enterica</i>	<i>L. monocytogenes</i>
PHB	8.11 ± 0.23	5.55 ± 0.05	-	-
PHB-AgNPs	<1.52	3.05 ± 0.05	>6.59	2.50