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# Stabilization of antimicrobial silver nanoparticles by a polyhydroxyalkanoate obtained from mixed bacterial culture

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ABSTRACT

The incorporation of antimicrobials into polymer matrices is a promising technology in the food packaging and biomedical areas. Among the most widely used antimicrobials, silver nanoparticles (AgNPs) have emerged as one of the most researched technologies to prevent microbial outbreaks. However, it is known that AgNPs are rather unstable and present patterns of agglomeration that might limit their application. In this work, AgNPs were produced by chemical reduction in suspensions of an unpurified poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) which was previously obtained from a mixed culture fermentation using a synthetic medium mimicking fermented cheese whey. The synthesis of AgNPs was carried out within the unpurified PHBV suspension (*in situ*) and by physical mixing (*mix*). The stability of crystalline and spherical nanoparticles ($7 \pm 3$ nm) obtained *in situ* was found to be stable during at least 40 days. The results suggest that the unpurified PHBV appears to be a very efficient capping agent, preventing agglomeration and, thereby, stabilizing successfully the silver nanoparticles. The *in situ* obtained AgNP–PHBV materials were also found to exhibit a strong antibacterial activity against *Salmonella enterica* at low concentration (0.1–1 ppm).



## 1. Introduction

Polyhydroxyalkanoates (PHA's) are a family of naturally occurring storage biopolyesters synthesized by more than 300 species of Gram-positive and Gram-negative bacteria [1]. Among the various biodegradable polymers, PHA's provide a good alternative to fossil-fuel based plastics as they possess thermoplastic properties similar to conventional polyolefins, such as polypropylene, with the advantage of being 100% biodegradable and compostable and being produced from renewable resources [2–5]. Production of PHA's usually involves fermentation, isolation and purification processes, which imply higher production costs as compared to polyolefins. Therefore, much efforts and improvements have been developed to reduce the costs of the fermentation and downstream processes [4,6]. As an example, the use of open mixed cultures avoids the need for sterility in the reactor and makes easier the use of low cost agricultural or industrial waste feedstock in the production of PHA's [4,7]. Gurieff and Lant [8] performed a lifecycle assessment and financial analysis and proved that PHA production by mixed cultures from renewable resources is financially and environmentally attractive. Moreover, it is a greener alternative to the pure culture processes since less $CO_2$ is produced [8].

The good biocompatibility and slow hydrolytic degradation of PHA's have prompted their implementation in packaging as well as in medical applications [9]. In the areas of food and cosmetic packaging, PHA's are already commercialized as cosmetic containers, shampoo bottles, covers, milk cartons and films, moisture barriers in nappies and sanitary towels, pens, and combs, among others (reviewed by [10]). More recently, attention has been also focused on the medical applications of PHA's, such as in bone plates, surgical sutures and blood vessel replacements [5,11], or as biodegradable carriers for long-term dosage of drugs, medicines, hormones, insecticides and herbicides [5,12].

In this sense, the incorporation of antimicrobial substances into PHA's might allow the production of biodegradable materials which could be used for the targeted release of the antimicrobials in active food packaging, food contact surfaces or medical applications.

Silver nanoparticles (AgNPs) are highly effective against a wide spectrum of bacteria, fungi and viruses, possess anti-inflammatory properties and promote epithelisation and scarring [13–16]. Owing to the growing concern about resistance to antibiotics, the

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incorporation of silver nanoparticles into different matrices has emerged as an effective way of producing materials with long-term wide spectrum antimicrobial properties either for packaging, medical or other applications [16]. One of the main challenges in producing this kind of antimicrobials is the synthesis of stable nanoparticles, as their antimicrobial effectiveness greatly depends on their size, size distribution and agglomeration state [11,17]. In this sense, several biological organisms, as well as different organic substances from natural sources have been used for the production of nanoparticles [18–20].

Some authors have shown that the fermentation residues [22,23] greatly affects the PHA's degradation. However, Serafim et al. [24] stated that the presence of the impurities did not modify the glass transition temperature (Tg) of PHA's materials produced by bacterial mixed cultures although the melting behavior (melting temperature and melting enthalpy) of the obtained materials were modified. The effect that the residual organic matter has on the PHA's degradation is unknown and it is therefore one of the objectives of this paper to ascertain its role in the stabilization of silver nanoparticles, there are no studies in the literature reporting about the use of poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) obtained from mixed cultures as capping agent to stabilize AgNPs, neither detailed information involving the nanoparticles atomic organization or the biocide effect of AgNPs synthesized within polymer suspensions. In the present paper, silver nanoparticles were produced by chemical reduction in PHBV suspensions. The stability of the nanoparticles in the polymer suspension was compared with that of AgNPs physically mixed with the polymer suspension and with aqueous AgNPs suspensions without polymer. The materials were characterized in terms of chemical composition, morphology, atomic organization, total silver content and antimicrobial performance against the food-borne pathogen *Salmonella enterica*.

## 2. Materials and methods

### 2.1. Synthesis and characterization of the PHBV

The PHBV, was obtained from a mixed bacterial culture grown under small scale laboratory conditions. For the production of the PHBV, one fed-batch test was carried out in a 5 L reactor (BioStat® B plus, Sartorius) in order to produce a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer using a mixed microbial culture enriched in PHA-accumulating microorganisms and synthetic volatile fatty acids (VFA) as precursors for PHA production. The mixed microbial culture was selected in a 20 L sequencing batch reactor (SBR) fed with a synthetic VFA mixture mimicking fermented cheese whey (% mol basis: 77 acetate, 12 propionate, 9 butyrate, and 2 valerate), supplemented with nutrients ($NH_4Cl$ and $KH_2PO_4$, at a ratio C/N/P of 100/10/1), and operated at 12 h cycles (four discrete phases: influent filling – 5 min; aeration – 675 min; settling – 30 min; and withdrawal of the exhausted effluent – 10 min), an organic loading rate (OLR) of 25.4 mM days$^{-1}$, hydraulic retention time (HRT) of 1 day, and sludge retention time (SRT) of 4 days.

The fed-batch reactor was inoculated with 2.5 L concentrated mixed liquor (12.1 g L$^{-1}$ volatile suspended solids) and pulse fed with a synthetic VFA mixture of 65.5 g VFA L$^{-1}$ (0.7 M), with a composition in % mol basis: 91 butyrate, and 9 valerate. VFA synthetic solution pH was adjusted to 7.5 with the addition of 5 M NaOH and pH was not controlled during the production assay, varying between 7.5 and 8.2. No nitrogen or phosphorus sources were supplied in order to maximize the PHBV storage response. The reactor was continuously mixed and aerated at 300 rpm and 4 L min$^{-1}$, respectively, and working at room temperature (20–23 °C). The PHBV accumulation experiments were carried by feeding the synthetic VFA mixture pulse-wise (12.2 mM), controlled by dissolved oxygen response. A new pulse of synthetic mixture was always injected when the dissolved oxygen concentration increased. This procedure was repeated until no dissolved oxygen response was observed indicating PHBV storage saturation, which took *ca.* 10 h, after the injection of 7 pulses (0.4 L of synthetic VFA mixture).

In order to recover the polymer, a quenching step (by adding *ca.* 2 M HCl until a pH of 2–3 was attained) was performed directly on the mixed liquor, followed by a 3 h reaction at room temperature (20–23 °C) with NaClO (1% $Cl_2$) at a ratio of 1 g NaClO per g cells, in order to degrade the cellular material, then the polymeric material was recovered by centrifugation (20 min × 6850 rpm), washed once with *ca.* 10 L distilled water, and lyophilized for 72 h. No further purification steps were followed to eliminate cell debris or other organic material from the polymer, this material was called unpurified PHBV throughout the manuscript.

The polymer composition, referred to the hydroxybutyrate (HB) and hydroxyvalerate (HV) content, and the purity were determined by gas chromatography (GC) using a method adapted from Serafim et al. [3]. The average molecular weight (Mw), the number average molecular weight (Mn) and the polydispersity index (PDI = Mw/Mn) were estimated using a size exclusion chromatography (SEC) apparatus (Waters) as described by Serafim et al. [24].

The unpurified PHBV was dissolved in chloroform (3%, w/v) at 60 °C and precipitated by adding it drop by drop to a 10-fold excess ice-cold methanol solution under stirring. Once all the solution was added to the cold methanol and after 1 h under stirring, the precipitated was separated by vacuum filtration using a filter of *ca.* 3 μm pore size. The filtrate was removed and the solid fraction was dried at 60 °C overnight.

The protein quantification of both purified and unpurified PHBV, was carried out by suspending a certain amount (varying between 2 and 5 mg) of the material in 1 mL of distilled water under vigorous mixing, promoting the proteins solubilization, Then the protein concentration in the solution was spectrophotometrically quantified at 750 nm by using the Folin phenol reagent [29].

### 2.2. Synthesis and characterization of the PHBV–AgNP materials

Silver nanoparticles (AgNPs) were synthesized by chemical reduction of silver nitrate (Sigma, Germany) with sodium borohydride (Panreac, Spain) according with the following reaction:

$$AgNO_3 + NaBH_4 \rightarrow Ag + \tfrac{1}{2}H_2 + \tfrac{1}{2}B_2H_6 + NaNO_3$$

Three different suspensions of unpurified PHBV and AgNPs were prepared. First, the produced unpurified PHBV was suspended (0.08 wt.%) in ultrapure MilliQ© water (Millipore Corporation Co., USA) in a 250 mL Erlenmeyer flask. The assembly was placed into an ice bath and stirred using a magnetic stirrer. Sodium borohydride was added first to the suspension to get 1 mM, 2 mM or 4 mM concentration and the volume was adjusted to 30 mL. After 20 min, 10 mL of desirable silver nitrate solution were added dropwise to the suspension to generate the nanoparticles. This suspension was labeled as PHBV–AgNP *in situ*. A silver nanoparticles colloid, labeled as "aqueous AgNPs" was prepared analogously but without polymer. The third suspension was prepared by physically mixing the AgNP colloid with the unpurified PHBV suspension (PHBV–AgNP *mix*).

The stability of the silver nanoparticles in the produced PHBV–AgNP suspensions was routinely monitored by visual inspection as well as by Ultraviolet visible (UV–VIS) spectroscopy with a UV–VIS Spectrum system 8453 (Agilent Technologies Waldbronn, Germany) from 1 h after the synthesis (as synthesized) and