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ANTIBACTERIAL ACTIVITY OF ZINC OXIDE NANOPARTICLES SYNTHESIZED BY SOLOCHEMICAL PROCESS

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Abstract - ZnO-NPs can be obtained through various methods, resulting in nanoparticles with different size and morphology, which directly influences their antimicrobial potential. The objective of this work was to evaluate the antibacterial activity of ZnO-NPs obtained by a solochemical process against important human foodborne pathogens: *Staphylococcus aureus*, *Salmonella* Typhimurium, *Bacillus cereus* and *Pseudomonas aeruginosa.* ZnO-NPs were identified as nanorods with the length between 90.1 and 100 nm (10.5 % frequency), the diameter between 80.1 and 90 nm (21 % frequency), and wurtzite type crystalline structure. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were equal to 0.05 mg mL^{-1} and 0.5 mg mL^{-1} for *S. aureus* and *S.* Typhimurium, respectively, lower than previous results related in the literature. ZnO-NPs produced by solochemical method had a superior antibacterial activity. For instance, they can be incorporated in packaging materials for increasing microbial safety and food shelf-life by inhibiting bacterial growth.
Keywords: Foodborne pathogens; *Bacillus cereus*; *Staphylococcus aureus*; *Salmonella Typhimurium*; *Pseudomonas aeruginosa*.

INTRODUCTION

The increasing concern about resistant microorganisms stimulates the study of new and more effective antimicrobial agents (Raghunath and Perumal, 2017; Tang and Lv, 2014). New agents can be obtained from biological sources, such as bacteriocins and essential oils, or synthesized organic/inorganic compounds (Han, 2005; Medeiros et al., 2017; Medeiros et al., 2018). Good antimicrobial effects have been obtained from metal oxide nanoparticles (Raghunath and Perumal, 2017), such as MgO (Tang et al., 2012), Cu_2O, CuO, ZnO, TiO_2, and WO_3 (Duffy et al., 2018; Vargas-Reus et al., 2012), which present distinct behavior and properties from micrometric or millimetric particles (Azeredo, 2013; Martinez-Gutierrez et al., 2010; Morais and Durán, 2006).

Zinc oxide nanoparticles (ZnO-NPs) have been reported as an antimicrobial agent against both pathogenic and spoilage microorganisms (Ann et al., 2014; Duffy et al., 2018; Pasquet et al., 2014; Raghunath and Perumal, 2017; Raghupathi et al., 2011; Savi et al., 2013; Vargas-Reus et al., 2012; Xie et al., 2011). ZnO-NPs application as antimicrobial agent stands out in comparison to other metallic nanoparticles (Jones et al., 2008). Their main antimicrobial mechanisms have been attributed to the induction of oxidative stress due to the formation of reactive oxygen species, membrane

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disruption due to the accumulation of ZnO-NPs therein, and internalization of nanoparticles followed by the release of antimicrobial ions (Zn^{+2}) (Raghunath and Perumal, 2017; Sirelkhatim et al., 2015). In addition to its unique antibacterial properties, ZnO is classified as a Generally Recognized as Safe (GRAS) compound by the U.S. Food and Drug Administration (FDA, 2016).

ZnO-NPs can be synthesized through various methods by controlling the synthesis conditions, such as sol-gel (Kolekar et al., 2011), hydrothermal (Hu and Chen, 2008), co-precipitation (Zhong Matijević, 1996) and solochemical (Vaezi, 2008) methods. The solochemical process produces nanostructures of ZnO through the reaction between a precursor solution containing zinc and an alkaline solution. This method has significant advantages, such as synthesis under low temperatures, no addition of a stabilizing agent, short reaction time, low cost, and nanoparticles with controlled morphology and size (Gusatti et al., 2010). The synthesis method directly influences the morphology and size of NPs. In turn, functional activities (chemical, catalytic and biological) of NPs are significantly affected by the combination of their size, morphology, surface area, electronic states and surface charge (Jones et al., 2008; Pasquet et al., 2014; Ramani et al., 2014). Therefore, the synthesis method must be selected to produce NPs with optimum functional activities for the desired application (Fan and Lu, 2005; Gusatti et al., 2010; Sirelkhatim et al., 2015).

Controlling the growth of pathogenic microorganisms is of foremost importance for food safety and public health. Among others, some foodborne pathogenic bacteria have been receiving particular attention in the last decades. *Pseudomonas aeruginosa* is an ubiquitous environmental bacterial that is the major cause of opportunistic human infections. It is a common soil and water bacteria, widely distributed among fresh foods (Jay et al., 2005; Stover et al., 2000). *Bacillus cereus* is a pathogenic bacterium commonly isolated from soil and easily spread in the environment (Granum and Lindbäck, 2013). Humans are the primary reservoir of *S. aureus*, and food contaminated during its preparation is the most significant source of staphylococcal food poisoning (Seo and Bohach, 2013). *Salmonella* spp. is a resilient bacterium capable of adapting to temperature, pH, and water activity beyond their normal growth range, posing high risks to safety (Li et al., 2013).

The present study aimed to characterize ZnO-NPs synthesized via a solochemical technique and to evaluate their antibacterial activity against the above mentioned foodborne Gram-positive and Gram-negative pathogenic bacteria: *S. aureus*, *B. cereus*, *S.* Typhimurium and *P. aeruginosa*, and to obtain both minimum bactericidal and inhibitory concentrations for each of them by a proposed broth dilution method.

MATERIAL AND METHODS

ZnO-NPs

ZnO-NPs were synthesized the by the solochemical method as described by Gusatti et al. (2010) and kindly provided by Kher Nanotecnologia Química Ltda. (Santa Catarina, Brazil). Immediately before each test, ZnO-NPs suspensions in different concentrations were prepared by dispersing the ZnO-NPs in Milli-Q water using an ultrasonic bath (1650A, Unique) for 30 min followed by vortex mixing (AP56, Phoenix) for 5 s.

Characterization of ZnO-NPs

ZnO-NPs were characterized by transmission electron micrographs (TEM) (JEM-1011 TEM, 100kV) and X-ray diffraction (XRD) (X´Pert, Philips, The Netherlands). TEM analyses used an aqueous ZnO-NPs dispersion with 1.0 mg mL^{-1} placed on a grid and kept at room temperature until complete solvent evaporation. A TEM image with 200 whole ZnO-NPs was used to estimate the particle's length and diameter with the help of specific software (ImageJ1.48v, Wayne Rasband, USA) for digital measurements. XRD analyses were done at 40 kV and 30 mA with CuK at 1.5406 Å wavelengths. The samples were analyzed in an interval of 2θ between 20° and 80° with increments of 0.05°/s.

Antibacterial activity

The antibacterial activity of ZnO-NPs was tested against Gram-positive bacteria, *Bacillus cereus* (ATCC 11778) and *Staphylococcus aureus* (ATCC 25923), and Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella* Typhimurium (ATCC 1428). Stock cultures were prepared by inoculating the strains in Tryptone Soya Agar (TSA) (Himedia, India), followed by incubation at 35 °C for 24 h. These cultures were kept at 4 °C until the preparation of the working cultures. Working cultures were obtained by transferring a loopful from the stock culture into 5 mL of Brain Heart Infusion broth (BHI) (Oxoid, England) and incubating at 35 °C for 24 h.

Diffusion methods

Disk diffusion and agar well diffusion methods were accomplished aiming at a qualitative screening for bacteria susceptibility and to select the ZnO-NPs concentration for the broth dilution method. Disc diffusion tests started by swabbing the working cultures on the agar surface. Then, sterile discs of filter paper (9 mm diameter) were impregnated with 10 μL of sterile ZnO-NPs suspensions and placed onto the inoculated agar surface. For the well diffusion, the working cultures were pour plated. After the agar solidification, wells (5 mm diameter) were aseptically made and filled with 32 μL of sterile ZnO-NPs suspensions.

For both methods, Müeller-Hinton Agar (Kasvi, Italy) was used. Initial cell concentration was around 10^9 CFU mL^{-1}, and aqueous ZnO-NPs suspensions ranged between 0.01 and 100 mg mL^{-1}. Positive and negative control tests were ciprofloxacin (0.02 mg mL^{-1}) and Milli-Q water, respectively. After incubation at 35 °C for 48 h, the presence or absence of inhibition zones around the discs and wells were observed.

Broth dilution

Quantitative tests were performed in tubes with Nutrient Broth (NB) (Acumedia, USA) (5 mL) and ZnO-NPs at final concentrations of 0.05, 0.5, 1.0, and 2.0 mg mL^{-1}. Positive and negative control tests were ciprofloxacin (0.02 mg mL^{-1}) and Milli-Q water, respectively. The test tubes were inoculated with the working culture to reach an initial cell concentration around to 10^5 CFU mL^{-1} and then incubated under shaking conditions (TECNAL, TE820, Brazil) at 35 °C. After 0, 24 and 48 h of incubation, cell concentration was determined by serial dilution in peptone water (0.1 %) followed by spread plating in Plate Count Agar (PCA) (Kasvi, Italy). Plates were incubated at 35 °C for 24 h. The Minimum Bactericidal Concentration (MBC) was defined as the lowest concentration at which no bacterial colonies were detected in the 10^{-1} dilution after 48 h incubation, while the Minimum Inhibitory Concentration (MIC) was the concentration at which the bacterial concentration after 48 h incubation was equal to the initial cell concentration.

Microbial growth curve

The bacterial growth curve of each strain was determined in media containing the respective MIC and MBC of ZnO-NPs suspensions. Sterile bottles with 50 mL of NB were inoculated with a loopful of the working culture and incubated at 35 °C for 1.5 h to reach an initial cell concentration of 10^5 CFU mL^{-1}. Then, ZnO-NPs dispersions were added to the bottles at levels equal to the MIC and MBC of each bacterial strain. Positive and negative control tests were ciprofloxacin (0.02 mg mL^{-1}) and Milli-Q water, respectively. The bottles were incubated under agitation at 35 °C. After pre-determined incubation periods, cell concentration was determined by serial dilution in peptone water (0.1%), followed by spread plating in Plate Count Agar (PCA) (Kasvi, Italy) and incubation at 35°C for 24 h.

RESULTS

ZnO-NPs characterization

XRD diffraction results (Figure 1) demonstrated a pattern that matched with the standard of ZnO provided by the Joint Committee on Powder Diffraction Standards (JCPDS). This result showed the hexagonal

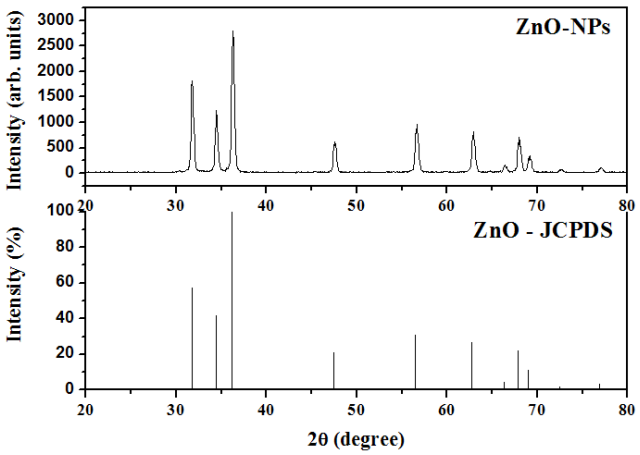

Figure 1. XRD patterns of present ZnO-NPs (top) and for standard ZnO (bottom) provided by Joint Committee on Powder Diffraction Standards (JCPDS), International Centre for Diffraction Data, card number 01-089-1397.

wurtzite structure of the studied ZnO-NPs, with spatial group $P6_3mc$ and network parameters a = 3.25 Å and c = 5.21, as specified in the card number. The nanoparticles showed a high purity level, as no peaks of any other phase were detected.

ZnO-NPs were predominantly rod-like shaped as depicted by the TEM image (Figure 2a), with varying

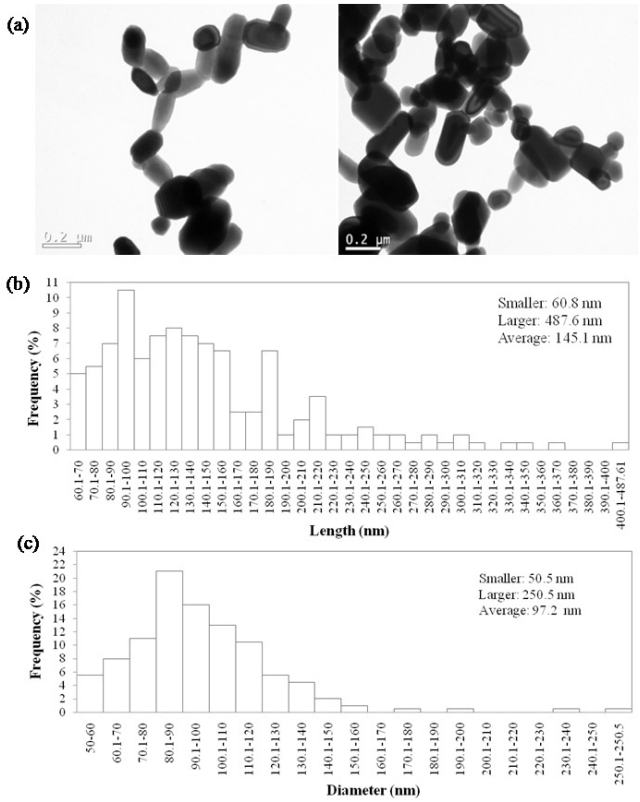

Figure 2. Characterization of the ZnO-NPs: (a) TEM images and frequency distribution histograms of the (b) length and (c) diameter.

length and diameter according to the frequency distribution histograms (Figures 2b and 2c). The nanoparticle average length and diameter were 145.1 and 97.2 nm, respectively. The histograms show that, for the established ranges, the nanoparticles were higher from 90.1 to 100 nm in length (10.5 % frequency) and between 80.1 and 90 nm in diameter (21 % frequency).

Antibacterial activity

Diffusion in agar

For *S. aureus*, the inhibition halo was present in concentrations equal to and higher than 0.1 and 2.5 mg mL^{-1} for the disk and the agar well diffusion methods, respectively. For *S.* Typhimurium, the inhibition halo was present in concentrations equal to and higher than 2.5 mg mL^{-1} for both methods. For *P. aeruginosa,* the inhibition halo was observed in concentrations equal to and higher than 1 mg mL^{-1} for the disk diffusion, and no evident inhibition was found in the agar well method, for which only some spots with an absence of growth were observed in concentrations equal to and higher than 0.1 mg mL^{-1}. For *B. cereus*, no inhibition zone was observed, even for the highest tested concentration of 100 mg mL^{-1}. This result suggests that either ZnO-NPs had no antibacterial effect against this bacterium or these methods are not suitable to detect the antibacterial effect of ZnO-NPs against *B. cereus*. The following analyses in broth media did not include tests for *B. cereus*. For all strains, negative controls showed no antibacterial effect, while positive controls had a clear inhibition zone.

Broth dilution

ZnO-NPs MIC was equal to 0.05 mg mL^{-1} (0.6 mM) for *S. aureus* (Table 1). A minor bactericidal effect was observed at the same concentration for *S.* Typhimurium. Despite this effect, 0.05 mg mL^{-1} was considered as the MIC for *S.* Typhimurium since lower ZnO-NPs concentrations were not tested. The MBC for both *S.* Typhimurium and *S. aureus* was equal to 0.5 mg mL^{-1} (6.1 mM) as no viable cells were detected after 48 h.

Table 1. Log (*N*)/Log (N_0)* for broth dilution tests at different ZnO-NPs concentrations after 48h incubation at 35°C. Positive control and negative controls were ciprofloxacin (0.02 mg mL^{-1}) and Milli-Q water, respectively.

	ZnO-NPs (mg mL^{-1})				Positive	Negative
	0.05	0.5	1.0	2.0	control	control
S. aureus	1.0	0.0	**	**	0.0	1.8
S. Typhimurium	0.83	0.0	**	**	0.0	2.0
P. aeruginosa	**	1.6	1.6	1.7	0.0	1.7

*N_0 is the initial cell concentration (CFU/mL), and *N* is the cell concentration after 48 h incubation.
**Concentrations not tested.

P. aeruginosa growth was not affected by the maximum tested concentration of 2 mg mL^{-1} (24.6 mM), and the bacterial concentration increased similarly to the negative control for all tested ZnO-NPs concentrations. Concentrations higher than 2 mg mL^{-1} were not tested as much ZnO precipitation was observed, hampering the results.

Microbial growth curve

After 24h, no viable cells of *S. aureus* (Figure 3) and *S.* Typhimurium (Figure 4) were detected when at MBC, while the negative control reached the maximum cell concentration of approximately 10^9 CFU mL^{-1}. After 6h of incubation, ZnO-NPs at MBC reduced bacterial count in 38 and 61 % when comparing to the negative control for *S. aureus* and *S.* Typhimurium, respectively. It indicates that ZnO-NPs can be more effective against *S.* Typhimurium compared to *S. aureus*. The growth curves at MIC presented a decrease in the cell concentration in the first 24 h, followed by

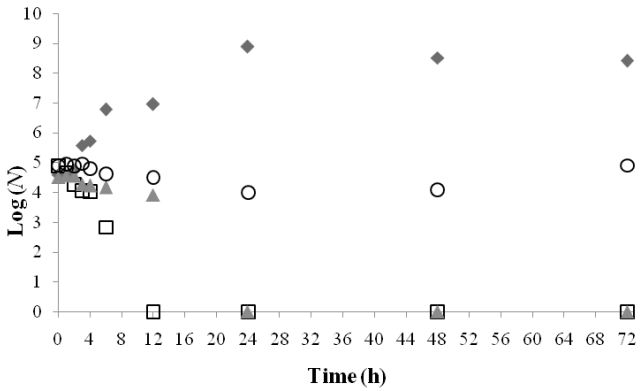

Figure 3. *S. aureus* cell concentration [Log (*N*)] over time for (○) ZnO-NPs MIC (0.05 mg mL^{-1}), (▲) ZnO-NPs MBC (0.5 mg mL^{-1}), (♦) negative control (Milli-Q water) and (□) positive control (ciprofloxacin, 0.02 mg mL^{-1}). *N* is the cell concentration in CFU mL^{-1}.

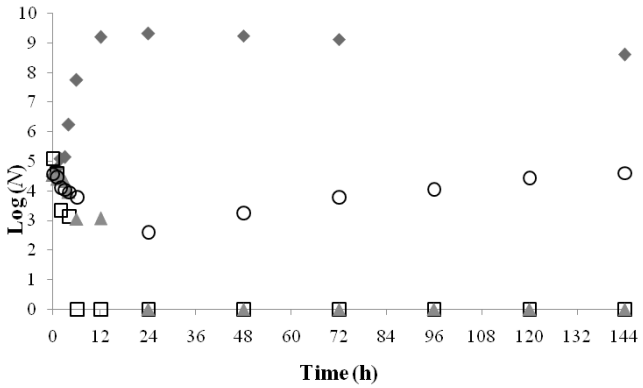

Figure 4. *S.* Typhimurium cell concentration [Log (*N*)] over time for (○) ZnO-NPs MIC (0.05 mg mL^{-1}), (▲) ZnO-NPs MBC (0.5 mg mL^{-1}), (♦) negative control (Milli-Q water) and (□) positive control (ciprofloxacin, 0.02 mg mL^{-1}). *N* is the cell concentration in CFU mL^{-1}.

an increase, reaching concentrations close to the initial cell concentration for both microorganisms. This decrease in cell concentration was also observed for *S.* Typhimurium during the MIC estimation (Table 1).

P. aeruginosa growth was not inhibited in ZnO-NPs concentrations up to 2 mg mL^{-1} (Figure 5). A minor effect of the ZnO-NPs can be observed at 48h as bacterial cell concentrations were lower than the negative control for all time points. After 216 h, the cultures containing ZnO-NPs reached a cell concentration approximately 3 logs higher than the initial concentration, while the negative control was about 3.8 times higher.

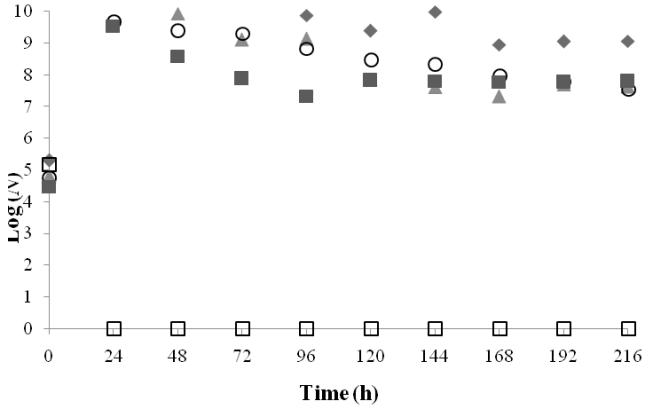

Figure 5. *P. aeruginosa* cell concentration [Log (*N*)] over time for ZnO-NPs at (▲) 0.5 mg mL^{-1} (○) 1.0 mg mL^{-1} and (■) 2.0 mg mL^{-1}, (♦) negative control (Milli-Q water) and (□) positive control (ciprofloxacin, 0.02 mg mL^{-1}). *N* is the cell concentration in CFU mL^{-1}.

DISCUSSION

Agar diffusion tests were performed as a qualitative test to observe and predict the ZnO-NPs antibacterial behavior. These methods have many advantages over other methods, such as simplicity, low cost, the ability to test a high number of microorganisms and antimicrobial agents. However, it is not able to determine the MIC or MBC, as it is impossible to determine the diffusion of the antimicrobial agent in the agar (Balouiri et al., 2016). The broth media assay can be considered as confirmative and more accurate than the agar diffusion assay as the chances of nanoparticle-bacteria interactions are higher in the liquid phase (Negi et al., 2012). It is essential for ZnO particles to contact or penetrate into microbial cells to express the antibacterial activity (Mirhosseini and Firouzabadi, 2013). Therefore, MIC and MBC values were accurately estimated by the broth dilution methods, which were equal to 0.05 mg mL^{-1} (0.6 mM) and 0.5 mg mL^{-1} (6.1 mM) for both *S. aureus* and *S.* Typhimurium, respectively, while no significant bactericidal effect was observed for *P. aeruginosa* in concentrations up to 2 mg mL^{-1} (24.6 mM) (Table 1). Agar diffusion tests revealed that *B. cereus* was highly resistant to ZnO-NPs, the reason why broth media tests were not done for this bacterium.

Many studies have reported that ZnO-NPs antimicrobial activity is significantly affected by different particle morphologies (Stanković et al., 2013; Talebian et al., 2013). This shape-dependent activity can be explained regarding the percent of active facets on the NPs. Thus, NPs research has been motivated to achieve selective nanostructured ZnO for antibacterial tests (Sirelkhatim et al., 2015). Particle size and concentration also have an essential influence on the antimicrobial activity. Studies have revealed that the smaller the NP size, the higher their toxic effect on microorganisms (Nair et al., 2009; Yamamoto, 2001). Smaller nanoparticles have relatively large interfacial area and can easily penetrate bacterial membranes, increasing their antibacterial effectiveness (Ramani et al., 2014). The ZnO-NPs used in the present work were synthesized by the solochemical process and presented a nanorod shape with a wurtzite crystalline structure with average length and diameter of 145.1 and 97.2 nm, respectively.

The results of the antibacterial effect of the ZnO-NPs synthesized via the solochemical method evaluated in the present study indicate that the MIC and MBC were smaller than the values from previous studies with ZnO-NPs synthesized by different methods, even for smaller particle sizes, or evaluated by other methodologies. For instance, ZnO-NPs with an average size of 50 nm had MIC values for *P. aeruginosa*, *S.* Typhimurium and *S. aureus* of 26, 22, and 10 mM, respectively (Tayel et al., 2011), which are much bigger than the values observed in the present work. A comparative scheme is presented in Table 2, showing literature results for the MIC and MBC of ZnO obtained from different methods against *Salmonella* and *S. aureus*.

ZnO-NPs did not affect the growth of *P. aeruginosa* in the range evaluated in the present study. This result corroborates Jan et al. (2013), who observed an antibacterial effect of ZnO-NPs more effective against *S. aureus* than *P. aeruginosa*. Lee et al. (2014) found that ZnO-NPs (< 50 nm) at 10 mM only slightly decrease the growth of *P. aeruginosa* planktonic cells, while successfully inhibiting biofilm formation. These authors suggested a MIC of about 300 mM against the planktonic cells.

A remarkable result was observed by following bacterial growth up to 9 days when the MIC concentration was applied to *S. aureus* (Figure 3) and *S.* Typhimurium (Figure 4). In both, an initial decrease was observed followed by the latest increase of the microbial population under this non-lethal condition. Initially, a population sensitive to ZnO-NPs dies, consequently decreasing the total concentration. Then, a resistant population can persist for a long time or even

Table 2. Literature results for *S.* Typhimurium and *S. aureus* inhibition, and MIC and MBC values of aqueous ZnO-NPs suspensions.

Method of synthesis (reference)	Size	Bacteria evaluated and observations	MIC	MBC
Solochemical (Present work)	97 nm	Great inhibition effect against *S.* Typhimurium and *S. aureus*	0.05 mg/mL (0.6 mM)	0.5 mg/mL (6.1 mM)
Commercial sample from Sigma-Aldrich, USA (Tayel et al., 2011)	50 nm	Inhibition effect against *S.* Typhimurium and *S. aureus*	22 and 10 mM, respectively	-
Commercial sample from Teconan, Spain. (Mirhosseini and Firouzabadi, 2013)	20 nm	2 mM do not inhibit the growth of *S. aureus* after 24h (5 mM showed 33.9% of growth reduction)	Not found	10 mM
Commercial sample from Sigma-Aldrich, USA (Jones et al., 2008)	8 nm and 50-70 nm	0.5 mM (8 nm) do not inhibit the S. *aureus* growth. 5 mM (50-70 nm) presented 40-50% of growth inhibition.	1 mM	-
Commercial sample from Inframat Advanced Materials LLC, USA. (Xie et al., 2011)	30 nm	Inhibition effect against *Salmonella enterica* serovar Enteritidis 10 mg/ml led to 1- or 2-log reduction in viable cells after an 8 h exposure.	0.4 mg/mL	-
Nosaka method (Emami-Karvani and Chehrazi, 2011)	3 nm	Inhibition effect against *S. aureus*	0.5 mg/mL	8 mg/mL
Kawano method (Ramani et al., 2013)	78 nm	0.045 mg/mL partial growth inhibition effect against *S. aureus* and *Salmonella typhimurium*.	-	-
Solochemical (Sornalatha et al., 2015)	37 nm	Presented antimicrobial index of 40% to 05 mg/mL, 50% to 0.750 mg/mL and 66% to 1.000 mg/mL against *S. aureus*.	-	-
Commercial from Sigma, Australia. (Duffy et al., 2018)	< 50 nm	Inhibition effect against *Salmonella* Typhimurium	0.313 mg/mL	> 5 mg/mL
Solvothermal synthesis (Raghupathi, Koodali and Manna, 2011)	12 nm	*Salmonella* Typhimurium growth was inhibited by about 50% with 10 mM.	-	-

start to grow. This behavior occurs due to phenotypic heterogeneity in the microbial population, resulting in distinct subpopulations. Microbial populations benefit from the creation of variant subpopulations that have the potential to be better prepared to persist during stress conditions (Avery, 2006), such as the presence of ZnO. This bacterial behavior reveals the time dependency of the MIC methodology; experiments with longer incubation times will most probably result in different MIC results. This fact, among many others, should be taken into account when designing products containing new antimicrobial compounds.

CONCLUSIONS

ZnO-NPs obtained by the solochemical method showed a strong antimicrobial effect against both Gram-negative *S. aureus* and *S.* Typhimurium. On the other hand, the effect was minor against *P. aeruginosa* for the tested concentrations, whereas no apparent effect was observed for *B. cereus*. The antibacterial activity observed was superior to nanoparticles obtained by other processes, even when the latter presented smaller particle sizes. It can result from both nanoparticle properties and the evaluation methodology. From any of them, the result impacts the amount of required ZnO-NPs, showing potential cost savings for reaching similar antibacterial effect. Importantly, the solochemical process has many advantages over other methods of nanoparticle synthesis, such as low cost and synthesis under low temperatures. The growth and survival curves obtained in this study enhance our understating of the ZnO-NPs antibacterial action over time, often neglected in the literature. Finally, the results obtained in this study suggest that the use of ZnO-NPs as an antibacterial agent in food systems can successfully inhibit some of the most dangerous and frequent foodborne pathogens.

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