

ECO-FRIENDLY SYNTHESIS OF NANOSTRUCTURED ZnO PARTICLES FROM FREE-RANGE CHICKEN EGGS AND THEIR ANTIBACTERIAL POTENTIAL

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Abstract. This study investigates an ovalbumin-assisted route to produce nanostructured ZnO powders using free-range chicken egg white and zinc acetate solutions at 1 M and 2 M. The synthesized samples, denoted ZnO_1 and ZnO_2, were calcined at 550 °C for 2 h and characterized by ATR-FTIR spectroscopy, X-ray diffraction (XRD), and scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (SEM–EDS). XRD analysis confirmed the formation of single-phase hexagonal wurtzite ZnO and indicated crystallite sizes of 11.9 ± 0.2 nm for ZnO_1 and 23.7 ± 0.2 nm for ZnO_2. SEM observations revealed agglomerated particles with irregular polyhedral morphologies and micrometric dimensions ranging from 0.244 to 0.657 μm for ZnO_1 and from 0.148 to 0.609 μm for ZnO_2. The estimated production costs were approximately EUR 1.10 g⁻¹ and EUR 1.13 g⁻¹, respectively. Antibacterial activity was evaluated against *Escherichia coli* and *Enterococcus faecalis* at a ZnO concentration of 0.2 mg mL⁻¹. After 30 min, ZnO_1 and ZnO_2 reduced *E. coli* counts by 97.7% and 99.2%, respectively, while no detectable colonies remained after 4 h. For *E. faecalis*, reductions of 52.4% and 64.8% were obtained after 4 h, increasing to 90.3% and 82.1% after 24 h. These findings demonstrate that zinc acetate concentration affects the structural and morphological characteristics of the resulting ZnO powders. The time-dependent antibacterial responses suggest that crystallite size and particle aggregation may influence activity; however, further replicated and mechanistic studies are needed to establish this relationship.

Keywords: ovalbumin; biogenic zinc oxide; structural and morphological characterization; antibacterial activity.

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1. INTRODUCTION

Due to its remarkable versatility, zinc oxide is emerging as an essential inorganic compound in a wide range of industries, from electronics and technology to medicine and cosmetics, and can be obtained through various synthesis methods, among which the biogenic approach stands out by using ovalbumin as a natural and sustainable agent, thus offering an ecological, cost-effective, and stable alternative to hazardous chemical precursors [1-11]. Biogenic synthesis of zinc oxide nanoparticles, also known as "green synthesis", represents an environmentally friendly, cost-effective, and sustainable alternative to conventional chemical and physical methods [12]. This method uses natural extracts from plants, fungi, bacteria, or algae to act as reducing and stabilizing agents [13]. Also, recent research has used ovalbumin as a natural precursor for the development of nanostructured ZnO particles. The use of ovalbumin, which is the main protein in egg white, in the synthesis of zinc oxide particles is a popular approach in green chemistry, playing several essential roles that influence the quality and stability of the final product [14]. Ovalbumin is a complex globular protein that possesses reactive functional groups (numerous amino, carboxyl, hydroxyl, and sulfhydryl groups), has a versatile 3D structure, acts as a natural template, and is non-toxic and biodegradable [15]. Ovalbumin transforms a polluting chemical process into a biological one, perfectly controlling the particle size of ZnO through its natural chemical groups. Researchers are using ovalbumin for ZnO because it is a biomimetic method that offers surprising control over particle size.

Compared to other oxides, difficulties in controlling pH and calcination temperatures make this method less attractive than classical chemical methods. The preparation of ZnO using free-range egg albumin precursor and zinc acetate reveals the ability to obtain nanostructured particles with controlled sizes and morphologies. The use of these precursors directly influences the characteristics of the prepared powders. The nanostructured particles thus obtained exhibit good antibacterial activity, demonstrated using the reference materials *Escherichia Coli* and *Enterococcus Faecalis*.

Unlike the research conducted by Schiopu et al. [16], which compared MgO and ZnO powders (synthesized using free-range chicken egg ovalbumin and zinc acetate) in terms of their morphological, structural, compositional, and antibacterial properties, the novelty of the present study lies in highlighting the properties and characteristics of ZnO nanostructured particles synthesized using free-range chicken egg ovalbumin and zinc acetate at concentrations of 1.0 M and 2.0 M. The research demonstrates that using different amounts of zinc acetate results in nanostructured particles with varying sizes, crystallite dimensions, and irregular polyhedral shapes. Another novel aspect is the presentation of particle size distribution histograms and the calculation of the actual cost per gram of ZnO powder produced using free-range chicken egg ovalbumin, thereby demonstrating that the use of a biogenic precursor leads to lower production costs.

2. MATERIALS AND METHODS

2.1. PROCEDURE FOR OBTAINING NANOSTRUCTURED ZnO PARTICLES

Nanostructured ZnO particles were prepared by biogenic synthesis through the following steps: (i) *Choice of precursors*: $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 6\text{H}_2\text{O}$ concentration 1.0 M/2.0 M

(9.174 g/18.348 g), 50 mL demineralized water, 50 mL free-range chicken egg white (about 2 egg whites). (ii) *Foam preparation*: Beat the 50 mL of egg white until foam is obtained. (iii) *Titration*: Add the zinc salt solution (1.0 mL each) to the foam, under intense magnetic stirring (1500 rpm). (iv) *pH monitoring*: Measure the pH after each milliliter added to identify the equivalence point. In the experiment using 1.0 M zinc acetate, the egg white pH is 8.18, the initial $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 6\text{H}_2\text{O}$ pH is 6.10, and the final pH is 6.25. In the experiment using 2.0 M zinc acetate, the egg white pH is 8.18, the initial $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 6\text{H}_2\text{O}$ pH is 5.72, and the final pH is 5.96. It was observed that upon adding the entire amount of solution, the pH remains acidic and the final solution is viscous. (v) *Ultrasonic treatment*: Place the egg white-Zn mixture in an ultrasonic bath (CD-4800 ultrasonic bath at a frequency of 42 kHz and a power of 60 W) for 10 minutes to disperse the particles. (vi) *Dehydration*: Dry in an oven at 120 °C for 4 hours until a yellow powder is obtained (the foam is placed in the Biobase Bioland BOV-T25F oven, Jinan, Shandong, China, for dehydration). (vii) *Purification*: Wash the powder with demineralized water and ethyl alcohol, followed by drying again. (viii) *Final processing*: Mortaring and calcination in the Mikrotest MKF-05 calcination furnace, Ankara, Turkey, at 550 °C for 2 hours to obtain the final powder. Calcination at 550 °C was performed to obtain impurity-free final powders, as the presence of proteins was still observed in the ZnO after the four-hour dehydration of the samples [1,5,16].

After the development of the two types of biogenic ZnO using free-range chicken egg ovalbumin and zinc acetate of concentration 1.0 M (ZnO_1) and free-range chicken egg ovalbumin and zinc acetate of concentration 2.0 M (ZnO_2), the production cost for the two powders obtained was calculated, taking into account the cost of the precursors used and the cost of electricity used for drying, ultrasonic treatment, and calcination. Thus, the production cost of the two types of nanostructured ZnO particles is presented in Table 1.

Table 1. Production cost.

Sample code and quantity obtained	Free-range egg ovalbumin cost [EURO]	Zinc acetate cost [EURO]	Distilled water cost	Filter papers cost	Electricity cost	Total cost	Total cost/gram
ZnO_1 3.757 g	0.58	1.65	0.32	0.20	1.41	4.16	1.10
ZnO_2 5.155 g	0.58	3.30	0.32	0.20	1.41	5.81	1.12

It is observed that for the production of one gram of ZnO obtained using ovalbumin from free-range chicken eggs and zinc acetate at a concentration of 1.0 M, 1.10 EURO is needed, and for the production of one gram of ZnO obtained using ovalbumin from free-range chicken eggs and zinc acetate at a concentration of 2.0 M, a cost of 1.12 EURO is needed. The calculation of the production cost reveals that the presented biogenic synthesis of ZnO requires a low production cost.

2.2. ANALYTICAL METHODS

Compositional analysis of samples was performed by Attenuated Total Reflectance Fourier-Transform Infrared spectroscopy (ATR-FTIR) using a Bruker Tensor 27 (Bruker Optik GmbH, Ettlingen, Germany), with a spectral range of 4,000 to 350 cm^{-1} covering the standard mid-infrared (Mid-IR) region, ideal for identifying functional groups and analyzing organic compounds, and a resolution setting of 4 cm^{-1} . This approach allowed the identification of the time interval required for dehydration and deproteinization of the samples.

To perform the X-ray diffraction analysis, an Ultima IV diffractometer (Rigaku, Tokyo, Japan), equipped with a D/teX Ultra detector, a graphite monochromator for $k\beta$

radiation, and a vertical goniometer, was used. X-ray diffractograms were recorded using CuK α radiation in the 2 θ measurement range with a step of 0.020°. The scanning speed was 1.5°/min, and the scanning range was 22.00-85.00°.

Morphological analysis was performed by Scanning Electron Microscopy - Energy-Dispersive X-ray Spectroscopy SEM-EDS) using a Hitachi SU 5000 electron microscope (Hitachi, Tokyo, Japan), with secondary electron detectors and coupled to the X-ray fluorescence spectrometry module. ImageJ software was used to measure particle dimensions.

2.3. ANTIBACTERIAL INVESTIGATION

The bacteriological properties of the synthesized powders (ZnO_1 and ZnO_2) were evaluated using the membrane filtration method with standardized reference strains: Gram-negative *Escherichia coli* (WDCM 00013) and Gram-positive *Enterococcus faecalis* (WDCM 00009). Initially, 2.0 mg samples of each ZnO powder were sterilized under UV light for 15 minutes in a microbiological safety cabinet. Testing involved immersing the powders in test tubes containing 10 mL of an artificial sample (8.0 mL of sterile distilled water inoculated with 1.0 mL of each bacterial suspension), resulting in a working ZnO concentration of 0.2 mg/mL. The laboratory-calculated limit of detection is 1 CFU/mL. Validation controls were prepared in parallel: a negative control (M-) to verify sterility and a positive control (M+, consisting of 8.0 mL of sterile water and 1.0 mL of each bacterial suspension, without ZnO) with an initial load estimated at ~250 CFU/mL for *E. coli* and ~150 CFU/mL for *E. faecalis*. After homogenization using a vortex mixer, the samples were kept in the dark at 25 °C for the selected contact times (30 minutes, 4 hours, and 24 hours).

At the end of each contact interval, a one-milliliter aliquot of the sample was filtered through sterile cellulose membranes with 0.45 μ m pores; the membranes were then transferred onto specific selective media: Chromocult Coliform Agar (CCA) for *E. coli* and Slanetz and Bartley Agar (SB) for *E. faecalis*. The Petri dishes were incubated at 37 °C for 24 hours (for *E. coli*) and 48 hours (for the more resistant strain, *E. faecalis*), respectively. Due to the total bactericidal inhibition (0 CFU/mL) observed for *E. coli* after 4 hours of contact in the presence of the oxides, monitoring at the 24-hour interval was performed exclusively for the resistant species *E. faecalis*.

3. RESULTS AND DISCUSSION

3.1. COMPOSITIONAL CHARACTERIZATION

Figs. 1 and 2 show the ATR-FTIR spectra of the hydrolysis compounds zinc acetate at 1.0 M and 2.0 M concentrations, respectively, and ovalbumin from free-range chicken eggs at 120°C, hourly, for 4 hours. The O-H group in the 3200-3500 cm⁻¹ region represents the stretching vibrations of the hydroxyl groups. The presence of this broad band indicates the presence of water in the sample and of OH groups resulting from the hydrolysis process. It is observed for both spectra that the intensity varies with time, which suggests the consumption of water molecules during the reaction.

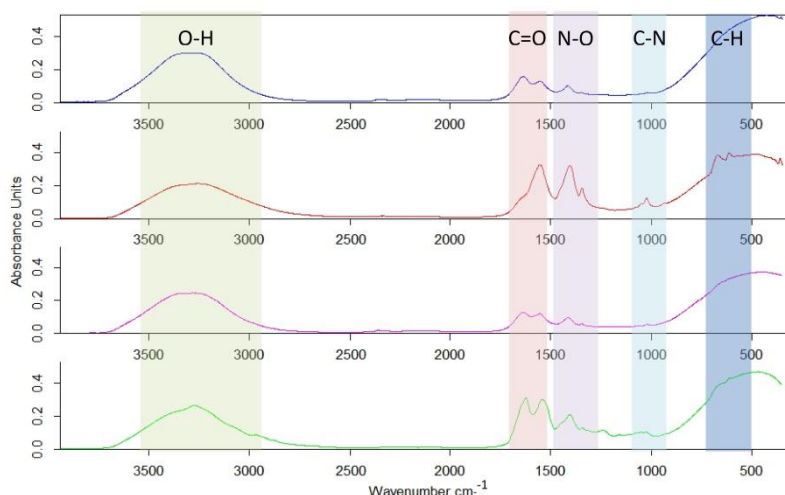


Figure 1. ATR-FTIR spectrum of the hydrolysis compound zinc acetate 1.0 M with free-range chicken egg albumin at 120 °C after: 1 hour (blue), 2 hours (red), 3 hours (purple), 4 hours (green).

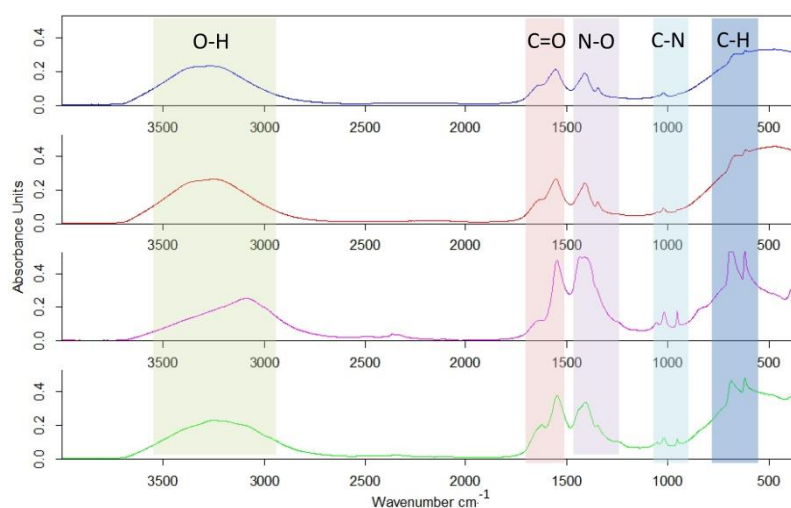


Figure 2. ATR-FTIR spectrum of the hydrolysis compound zinc acetate 2.0 M with free-range chicken egg albumin at 120 °C after 1 hour (blue), 2 hours (red), 3 hours (purple), and 4 hours (green).

The C=O region corresponding to 1600–1700 cm^{-1} indicates the presence of egg albumin, and the N-O and C-N region corresponding to 1200–1500 cm^{-1} shows vibrations specific to carbon-nitrogen bonds, suggesting strong interactions between zinc ions. The protein-assigned band, specifically the C=O region associated with the Amide I band, persists throughout the hydrolysis process, suggesting the preservation of the structural characteristics of the ovalbumin proteins, although no quantitative changes in intensity are indicated.

The overall maintenance of the spectral profile and the main bands within the 1–4 hour timeframe suggests relative stability of the zinc acetate–ovalbumin complexes under the conditions investigated. Comparing the two spectra, it is observed that in the ATR-FTIR spectrum of the hydrolysis of the compound formed by 2.0 M zinc acetate and ovalbumin from free-range chicken eggs, the vibrations are better defined, which indicates a more intense reaction and the formation of more stable complexes between zinc and ovalbumin at a higher concentration of zinc salt. After 4 hours, both spectra still show the presence of proteins, and to eliminate them, the samples are washed again, filtered, ground, and calcined at 550 °C for 2 hours.

Fig. 3 shows the ATR-FTIR spectrum of the hydrolysis compound zinc acetate at 1.0 M and 2.0 M concentrations with ovalbumin from free-range chicken eggs.

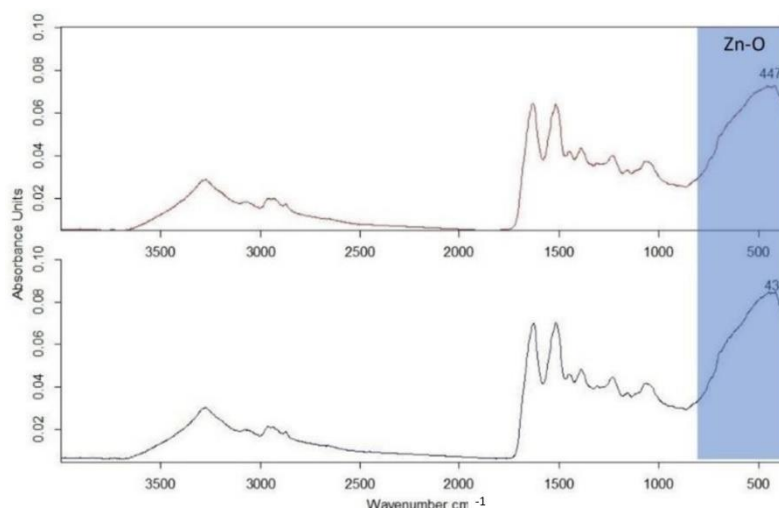


Figure 3. ATR-FTIR spectrum of the hydrolysis compound zinc acetate at 1.0 M concentration with ovalbumin from free-range hen's egg (red), and zinc acetate at 2.0 M concentration (black).

The absorption band at 447 cm^{-1} corresponds to Zn-O from hydrolysis with free-range egg albumin and 1.0 M zinc acetate, while the absorption band at 438 cm^{-1} corresponds to Zn-O from hydrolysis with free-range egg albumin and 2.0 M zinc acetate, so both samples show the presence of ZnO. The results are similar to those of other studies using ovalbumin to obtain zinc oxide. For instance, Vijan et al. demonstrated the presence of Zn-O bonds formed via hydrolysis involving egg albumin and a zinc salt, using ATR-FTIR, observing an absorption band in the $358\text{--}395\text{ cm}^{-1}$ range [1].

3.2. X-RAY DIFFRACTION ANALYSIS OF NANOSTRUCTURED ZNO PARTICLES FROM FREE-RANGE CHICKEN EGGS

In the case of XRD analysis of samples obtained by biogenic synthesis of 1.0 M and 2.0 M zinc acetate, using ovalbumin from free-range chicken eggs, the spectra in Fig. 4 were obtained. The spectrum shows only diffraction lines associated with the hexagonal ZnO phase, without impurities, in agreement with the ATR-FTIR analysis, thus demonstrating the formation of the processed ZnO particles.

Determination of crystallite size using the Williamson–Hall method. To evaluate the structural changes in nanoparticles obtained via biological synthesis using ovalbumin, the average crystallite size D (nm) was calculated using the Williamson–Hall equation:

$$\beta \cos \theta = K\lambda/D + 4\epsilon \sin \theta \quad (1)$$

where β is the full-width-half-maximum (FWHM) of the prominent peaks in X-ray diffraction; K is the shape factor; λ is the X-ray wavelength ($\lambda = 0.15418\text{ nm}$); D is the particle size ($D_{\text{W-H}}$); and ϵ is the lattice strain of ZnO nanostructured particles. The lattice parameters are presented in Table 2.

Table 2. The lattice parameters.

Sample code	a [Å]	b [Å]	c [Å]	V [Å ³]
ZnO_1	3.2555	3.2555	5.2158	47.873
ZnO_2	3.2591	3.2591	5.2218	48.033

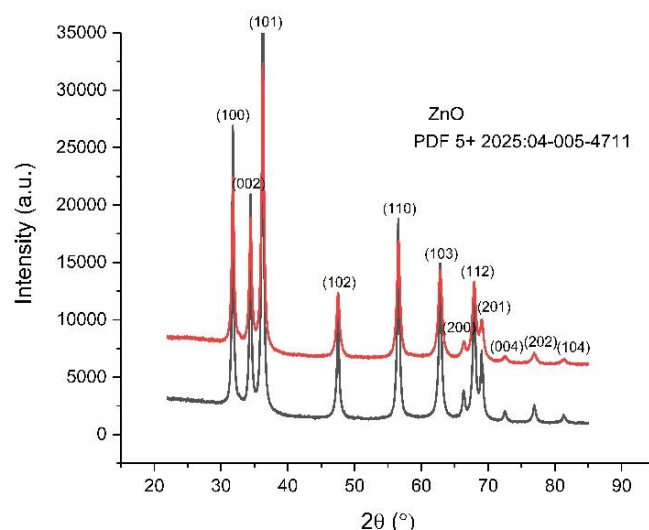


Figure 4. XRD spectra of ZnO_1 (red) and ZnO_2 (black).

ZnO adopts the hexagonal wurtzite structure, in which both Zn^{2+} and O^{2-} ions exhibit tetrahedral coordination. For an ideal wurtzite lattice, the lattice parameters are related by a theoretical c/a ratio of approximately 1.602. The lattice parameters determined for the investigated ZnO samples are consistent with those characteristic of the hexagonal wurtzite phase. In particular, the c/a ratios obtained for both samples show very similar values and remain close to the theoretical ratio of 1.602. Such agreement with the ideal structural parameter suggests that the ZnO crystallites possess a well-developed hexagonal arrangement and that the deviation from the equilibrium wurtzite geometry is relatively small.

The structural characteristics determined by XRD are presented in Table 3.

Table 3. Structural characteristics determined by XRD.

Sample code	Name phase	Chemical formula	PDF5+2025-DB	Crystallite size [nm] $\pm$ DS
ZnO_1	Zincite	ZnO	04-005-4711	11.9 $\pm$ 0.2
ZnO_2				23.7 $\pm$ 0.2

According to the structural characteristics, it is observed that the crystallite size of zinc oxide using 1.0 M zinc acetate and free-range egg ovalbumin is smaller than the crystallite size of zinc oxide using 2.0 M zinc acetate and free-range egg ovalbumin. So the crystallite size obtained increases with increasing zinc acetate concentration. XRD structural analysis demonstrates that the zinc acetate concentration is closely related to the crystallite size obtained.

The differences between the obtained crystallite size values and those reported in the literature, 12.0 nm, 12.5 nm [16], versus 11.9 nm, 23.7 nm, are attributed to experimental variations inherent to the synthesis process, as well as differences in ovalbumin composition and crystallization conditions, which influence the size of the ZnO crystallites. Additionally, other investigated ZnO crystallite sizes range from 28 to 35 nm [8] and 15.8 nm [13].

3.3. MORPHOLOGICAL ANALYSIS

The analysis of the morphology of particles developed by biogenic synthesis using zinc acetate at 1.0 M and 2.0 M concentrations and ovalbumin from free-range chicken eggs was performed using the SEM technique, and micrographs are shown in Fig. 5. The SEM micrographs were acquired at different magnifications because the selected magnifications

provided the best image clarity and allowed the morphology of the particles to be clearly resolved for each ZnO sample. Histograms showing the particle size distribution for each type of ZnO powder are presented in Fig. 6.

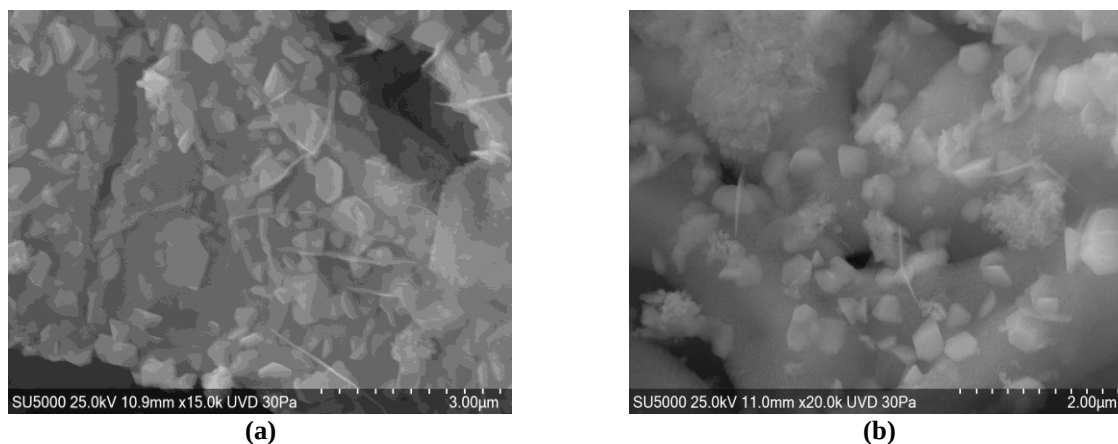


Figure 5. (a) SEM micrograph of ZnO_1 sample at 15.0k magnification, (b) SEM micrograph of ZnO_2 sample at 20.0k magnification.

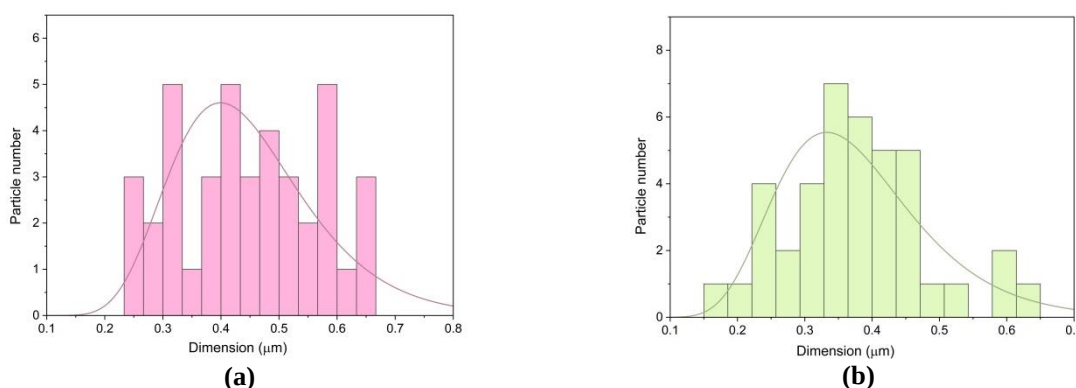


Figure 6. Particle size distribution histogram for irregular polyhedral particles for (a) sample ZnO_1 and (b) sample ZnO_2

The micrographs reveal both types of biogenic ZnO nanostructured particles of irregular polyhedral shape (Fig. 5a-b). To determine the average particle size and standard deviation of the nanostructured biogenic ZnO particles, 40 particles from each sample were measured. For the ZnO_1 sample, the histogram shows a typical bimodal distribution, the particle size varying from 0.244 μm to 0.657 μm with the following distribution: between 0.2-0.3 μm there are 5 particles, between 0.3-0.4 μm there are 9 particles, between 0.4-0.5 μm there are 12 particles, between 0.5-0.6 μm there are 10 particles, and between 0.6-0.7 μm there are 4 particles (Fig. 6a). The mean particle size is 0.447, and the standard deviation is 0.118. The ZnO_2 sample shows a typical bimodal, inhomogeneous histogram, and the particle size varies between 0.148 and 0.609 μm , as follows: between 0.1-0.2 μm there are 2 particles, between 0.2-0.3 μm there are 8 particles, between 0.3-0.4 μm there are 15 particles, between 0.4-0.5 μm there are 11 particles, between 0.5-0.6 μm there are 2 particles, and between 0.6-0.7 μm there are 2 particles (Fig. 6b). The mean particle size is 0.378, and the standard deviation is 0.106. The particles do not show homogeneity because they have quite varied sizes, show compact agglomerations, and irregular shapes.

Fig. 7 shows the EDS spectra of ZnO particles prepared at a concentration of 1.0 M zinc acetate, 2.0 M, and ovalbumin from free-range chicken eggs.

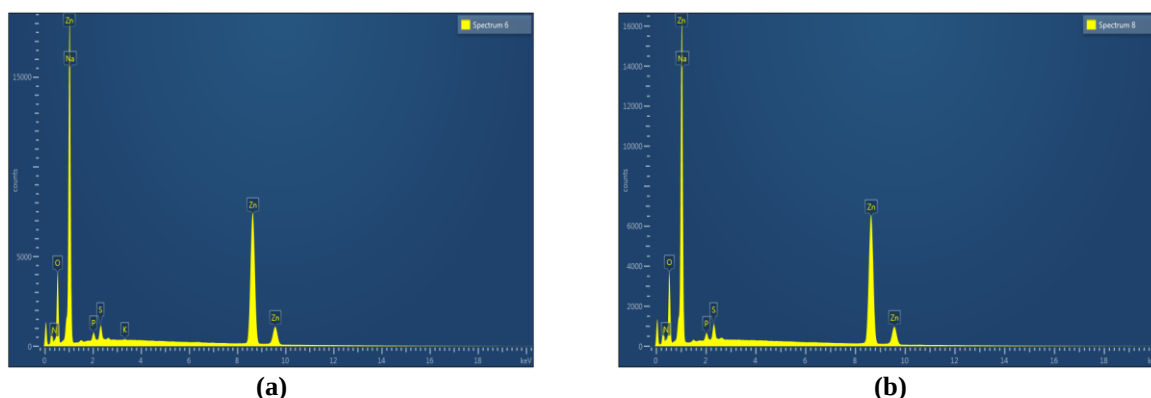


Figure 7. (a) EDS spectrum of the sample ZnO_1, (b) EDS spectrum of the sample ZnO_2.

The spectra confirm the presence of zinc oxide through the intensity maxima specific to the elements Zn and O. The existence of impurities is signaled by additional maxima, being in full agreement with the compositional data presented in Table 4.

Table 4. Elemental composition of the elaborated ZnO powders.

Result type	W [%]	±SD	W [%]	± SD
	ZnO_1		ZnO_2	
N	1.90	±0.24	2.79	±0.26
O	17.46	±0.16	17.82	±0.18
Na	7.79	±0.33	7.67	±0.35
P	1.09	±0.04	1.02	±0.04
S	1.77	±0.04	1.85	±0.05
K	0.07	±0.02	-	-
Zn	69.93	±0.34	68.85	±0.35
Total	100.00		100.00	

The composition of the powders highlights the zinc oxide phase, but also traces of residual elements (N, Na, P, S, K) from the birds' diet. Their presence is justified by their fundamental biological role in metabolism and physiological growth processes [17].

3.4. ANTIBACTERIAL ACTIVITY

Fig. 8 shows the results for the positive control *E. coli* colonies after 30 minutes and 4 hours of incubation at 25 °C, respectively, while Fig. 9 shows *E. faecalis* colonies after 30 minutes, 4 hours, and 24 hours.

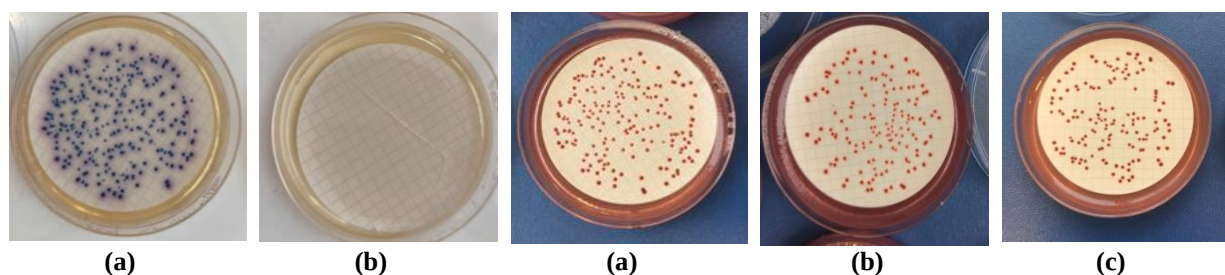


Figure 8. (a) M+ *E.coli* colonies after 30 minutes, (b) M- *E.coli* colonies after 30 minutes.

Figure 9. (a) M+ *E.faecalis* colonies after 30 minutes, (b) M+ *E.faecalis* colonies after 4 hours, (c) M+ *E.faecalis* after 24 hours of contact.

In Fig. 10, the positive control (M+) maintained a viable cell count of 95 CFU/mL, confirming that the bactericidal effect is attributable solely to the synthesized nanostructured

particles; no colonies developed in the M- sample. Testing continued for the *E. faecalis* samples, as *E. coli* showed complete inhibition four hours after contact with the ZnO powder. After 24 hours of contact with *E. faecalis*, M+ showed a decrease in the number of colonies, whereas M- showed no *E. faecalis* colonies at any time point. Fig. 11 shows that the negative control exhibited no colony growth on the medium.

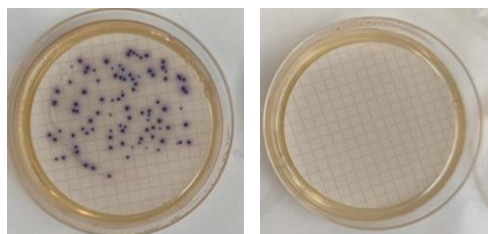


Figure 10. (a) M+ *E.coli* colonies after 4 hours, (b) M- *E.coli* colonies after 4 hours.

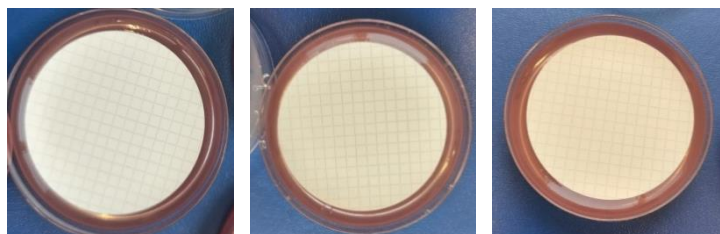


Figure 11. (a) M- *E.faecalis* colonies after 30 minutes, (b) M- *E.faecalis* colonies after 4 hours, (c) M- *E.faecalis* after 24 hours of contact.

Fig. 12 shows the ZnO_1 sample after 30 minutes of contact with *E. coli* and after 4 hours of contact. Fig. 13 shows the ZnO_1 sample after 30 minutes of contact with *E.faecalis*, after 4 hours, and after 24 hours. Fig. 14 shows the ZnO_2 sample after 30 minutes of contact with *E.coli* and after 4 hours of contact. Fig. 15 shows the ZnO_2 sample after 30 minutes of contact with *E.faecalis*, after 4 hours, and after 24 hours.

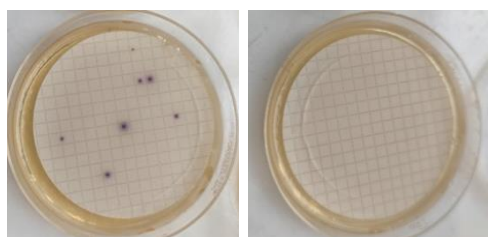


Figure 12. (a) ZnO_1 sample - *E.coli* colonies after 30 minutes, (b) ZnO_1 sample - *E.coli* colonies after 4 hours.

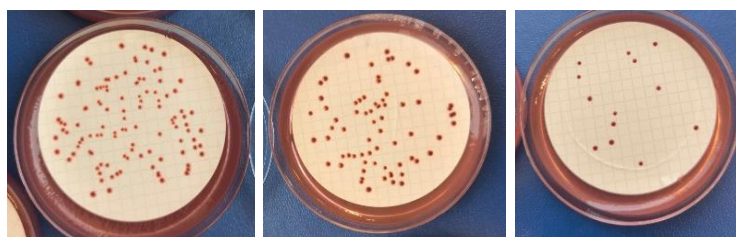


Figure 13. (a) ZnO_1 sample - *E.faecalis* colonies after 30 minutes, (b) ZnO_1 sample - *E.faecalis* colonies after 4 hours, (c) ZnO_1 sample - *E.faecalis* after 24 hours of contact.

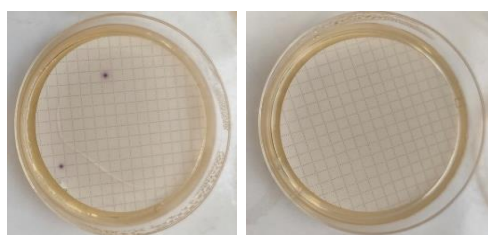


Figure 14. (a) ZnO_2 sample - *E.coli* colonies after 30 minutes, (b) ZnO_2 sample - *E.coli* colonies after 4 hours.

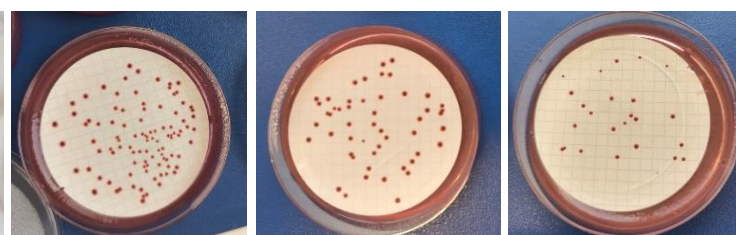


Figure 15. (a) ZnO_2 sample - *E.faecalis* colonies after 30 minutes, (b) ZnO_2 sample - *E.faecalis* colonies after 4 hours, (c) ZnO_2 sample - *E.faecalis* after 24 hours of contact.

It is observed that after 4 hours of contact, *E.coli* shows total inhibition of the ZnO_1 and ZnO_2 samples. For both types of processed ZnO, the number of *E.faecalis* colony-forming units surviving after the 3 contact times decreases with increasing contact time. After 24 hours of contact with *E.faecalis*, the ZnO_1 sample shows fewer colony-forming units than the ZnO_2 sample. In accordance with the structural analysis, which demonstrated that the ZnO_1 sample has a smaller crystallite size, it is observed that it has better antibacterial activity.

Table 5 presents the values obtained after interpreting the results.

Table 5. Results of antibacterial test.

Tested samples	<i>Escherichia coli</i> CFU/1.0 mL		<i>Enterococcus faecalis</i> CFU/1.0 mL		
	Contact time 30 min	Contact time 4 hours	Contact time 30 min	Contact time 4 hours	Contact time 24 hours
M+	~265	95	178	145	134
M-	0	0	0	0	0
ZnO_1	6	0	105	69	13
ZnO_2	2	0	99	51	24

CFU = colony-forming units

Interpretation of the results demonstrates that the sample using free-range egg ovalbumin and 1.0 M zinc acetate exhibits better antibacterial activity against *E. faecalis* and that both samples are effective against *E. coli* after a contact time of 4 hours. Using the positive control (M+) at the same organism and contact time as the reference, the antibacterial effect can be expressed as:

$$\text{Percentage reduction} = (CFU_{\text{control}} - CFU_{\text{sample}}) / CFU_{\text{control}} \times 100 \quad (2)$$

$$\text{Log reduction} = \log_{10}(CFU_{\text{control}} / CFU_{\text{sample}}) \quad (3)$$

The results are shown in Table 6:

Table 6. Antibacterial activity relative to M+.

Organism	Contact time	Positive control [CFU/mL]	Sample	CFU/mL	Reduction [%]	Log reduction
<i>E. coli</i>	30 min	~265	ZnO_1	6	97.7	1.64
<i>E. coli</i>	30 min	~265	ZnO_2	2	99.2	2.12
<i>E. coli</i>	4 h	95	ZnO_1	0	100	-
<i>E. coli</i>	4 h	95	ZnO_2	0	100	-
<i>E. faecalis</i>	30 min	178	ZnO_1	105	41	0.23
<i>E. faecalis</i>	30 min	178	ZnO_2	99	44.4	0.25
<i>E. faecalis</i>	4 h	145	ZnO_1	69	52.4	0.32
<i>E. faecalis</i>	4 h	145	ZnO_2	51	64.8	0.45
<i>E. faecalis</i>	24 h	134	ZnO_1	13	90.3	1.01
<i>E. faecalis</i>	24 h	134	ZnO_2	24	82.1	0.75

Relative to the positive control, ZnO_1 and ZnO_2 produced substantial reductions in *E. coli* counts after 30 min, corresponding to 97.7% (1.64 log) and 99.2% (2.12 log) reductions, respectively. After 4 h, no detectable *E. coli* colonies remained for either ZnO treatment, corresponding to a 100% reduction. For *E. faecalis*, the reductions after 30 min were 41.0% (0.23 log) for ZnO_1 and 44.4% (0.25 log) for ZnO_2. After 4 h, reductions increased to 52.4% (0.32 log) and 64.8% (0.45 log), respectively. At 24 h, ZnO_1 achieved a 90.3% (1.01 log) reduction, while ZnO_2 achieved an 82.1% (0.75 log) reduction.

7. CONCLUSIONS

This study investigated the biogenic synthesis of ZnO nanoparticles using free-range egg ovalbumin as a natural precursor, with the aim of evaluating the influence of synthesis parameters on structural, morphological, and antibacterial properties. The results

demonstrated that the proposed synthesis route represents a cost-effective and environmentally friendly alternative to conventional methods.

ATR-FTIR analysis confirmed the progressive elimination of residual water and organic components, indicating that properly optimized processing conditions lead to the formation of ZnO as the dominant phase. Structural characterization by XRD revealed that lower concentrations of zinc acetate favor the formation of nanostructured ZnO with smaller crystallite sizes, highlighting the critical role of precursor concentration in controlling crystal growth. In contrast, morphological analysis showed that increasing the zinc acetate concentration leads to the formation of powders with reduced particle sizes, suggesting a complex relationship between crystallite size and particle aggregation behavior. EDS analysis confirmed the presence of ZnO as the main component in all samples, while also indicating the presence of minor impurities associated with the biogenic origin of the precursor, particularly those related to bird feed composition.

The antibacterial assessment demonstrated that both synthesized ZnO powders exhibit significant activity against *Escherichia coli*, achieving effective bacterial inhibition after 4 hours of contact. In the case of *Enterococcus faecalis*, the sample characterized by smaller crystallite size (ZnO_1) showed superior antibacterial performance compared to the sample obtained at higher precursor concentration, indicating that crystallite size plays a key role in determining antibacterial efficiency. Crystallite size directly influences the kinetics of antibacterial activity through two fundamental mechanisms: the release of reactive oxygen species and Zn^{2+} ions, and the specific contact surface area.

For the ZnO_2 sample, which exhibits superior efficiency at intermediate time points (30 min - 4 h), the larger crystallites and aggregated structure facilitate local accumulation or a faster initial mechanical/kinetic effect during the first few hours (at 30 min and 4 h, the CFU count for *E. coli* and *E. faecalis* is lower for ZnO_2 compared to ZnO_1). This can be attributed to the easier initial solubilization of surface ions or to immediate electrostatic interactions favored by surfaces that are less crystallographically rearranged.

For the ZnO_1 sample, which shows maximum efficiency over the long term (24 h), the smaller crystallite size (nearly half that of the other sample) provides a much larger specific surface area. This enables a sustained and progressive long-term release of reactive oxygen species and Zn^{2+} ions. With extended contact time (24 hours against *E. faecalis*), ZnO_1 achieves a greater reduction in bacterial count (13 CFU/mL versus 24 CFU/mL for ZnO_2), as the accumulated dose of reactive oxygen species penetrates and destroys the cell membrane.

Overall, the study highlights that the physicochemical properties and functional performance of biogenically synthesized ZnO can be effectively tuned through the control of synthesis parameters. These findings support the potential of egg-derived biomaterials as viable precursors for the development of sustainable nanomaterials with promising applications in antibacterial systems and environmental technologies.

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