

## Article

# Modeling of Green Synthesis of ZnO Nanoparticles for Water Treatment

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## Abstract

Green synthesis of zinc oxide nanoparticles (ZnO NPs) represents a sustainable alternative to conventional chemical synthesis by employing biocatalytic systems under environmentally benign conditions. This study presents a systematic modeling approach based on Design of Experiments (DoEs) to optimize the green synthesis of ZnO nanoparticles intended for water treatment applications. Fractional factorial design was first applied to identify the most influential synthesis parameters, including precursor concentration, bio-reductant ratio, pH, temperature, and reaction time. Subsequently, response surface methodology using a D-optimal experimental design was employed to establish predictive mathematical models describing the relationships between process variables and nanoparticle size. The developed models enabled identification of optimal synthesis conditions and prediction of parameter combinations required to produce nanoparticles with targeted dimensions suitable for enhanced photocatalytic activity, antimicrobial performance, and colloidal stability. The proposed data-driven optimization strategy provides a robust, reproducible, and scalable protocol for green ZnO nanoparticle synthesis while minimizing reagent consumption and environmental impact. These findings contribute to the development of sustainable nanomaterials as the first step toward the future application of these ZnO nanoparticles as sustainable photocatalysts for water treatment.

**Keywords:** Design of Experiments; response surface methodology; green synthesis; zinc oxide nanoparticles; biocatalytic synthesis; process modeling; water treatment

## 1. Introduction

The increasing contamination of water resources by organic pollutants, pharmaceutical residues, pathogenic microorganisms, pesticides, dyes, and toxic heavy metals has become one of the most pressing environmental challenges worldwide [1]. Rapid industrialization, urbanization, and agricultural activities have resulted in the continuous release of emerging contaminants into aquatic ecosystems, often exceeding the treatment capacities of conventional wastewater treatment plants [2]. Many persistent organic pollutants are resistant to biological degradation and remain in surface and groundwater for extended periods, posing risks to both environmental ecosystems and human health. Therefore, the development of efficient, environmentally sustainable, and economically viable water treatment technologies has become a major research priority.

Among advanced materials investigated for environmental remediation, zinc oxide nanoparticles (ZnO NPs) have attracted considerable scientific interest because of



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their multifunctional properties [3]. ZnO is a wide-band-gap semiconductor (3.2–3.4 eV) characterized by high exciton binding energy, excellent photocatalytic activity, strong antimicrobial performance, chemical stability, low production cost, and relatively low toxicity compared with many other metal oxide nanomaterials. Under ultraviolet and, after suitable modification, visible-light irradiation, ZnO nanoparticles generate reactive oxygen species capable of degrading a broad spectrum of organic contaminants, including dyes, pharmaceuticals, pesticides, and endocrine-disrupting compounds [4]. Simultaneously, these reactive oxygen species damage microbial cell membranes, proteins, and nucleic acids, providing effective antibacterial and antifungal activity. Owing to these characteristics, ZnO NPs are considered promising multifunctional materials capable of combining photocatalytic degradation, disinfection, and adsorption within a single water treatment platform [5].

The functional performance of ZnO NPs is closely related to their physicochemical characteristics. Particle size, morphology, crystallinity, surface defects, specific surface area, and surface chemistry all influence photocatalytic efficiency, antimicrobial activity, adsorption capacity, and colloidal stability [4]. In general, reducing particle size increases the available surface area and enhances catalytic performance. However, excessively small nanoparticles may exhibit increased aggregation, reduced stability, or enhanced  $\text{Zn}^{2+}$  ion release, which may raise concerns regarding ecotoxicity [6]. Therefore, controlling nanoparticle properties during synthesis is essential for achieving an optimal balance between catalytic efficiency, stability, and environmental safety.

Numerous synthesis methods have been reported for ZnO NPs, including precipitation, hydrothermal synthesis, solvothermal processing, chemical vapor deposition, microwave-assisted synthesis, and sol–gel techniques [7–9]. Although these methods can produce highly crystalline nanoparticles with controlled morphology, they often require elevated temperatures, high pressures, hazardous reducing agents, organic solvents, or extensive post-synthesis treatments [10]. Such procedures increase production costs and energy consumption while generating hazardous chemical waste, contradicting the principles of green chemistry and sustainable manufacturing [11,12]. These limitations have stimulated intensive research into environmentally benign synthesis routes capable of reducing both environmental impact and production costs.

Green synthesis has emerged as an attractive alternative to conventional nanoparticle production by replacing toxic chemicals with naturally occurring reducing and stabilizing agents. Various biological systems have been successfully employed for ZnO nanoparticle synthesis, including plant extracts rich in polyphenols and flavonoids, microorganisms such as bacteria, fungi and algae, natural polysaccharides including cellulose, starch, alginate and chitosan, and enzyme-based catalytic systems [13–15]. Compared with traditional chemical synthesis, these approaches generally operate under ambient pressure, moderate temperatures, and aqueous media, resulting in lower energy consumption and reduced environmental burden [16]. Moreover, naturally derived capping agents often improve nanoparticle dispersion stability and biocompatibility while minimizing hazardous by-products.

Among various green synthesis strategies, enzymatic synthesis has received comparatively limited attention despite several important advantages. Enzymes are highly selective biological catalysts capable of promoting chemical reactions under mild operating conditions while maintaining excellent catalytic efficiency [17]. Because enzymes are biodegradable and function under near-neutral pH and room temperature, enzymatic synthesis represents one of the greenest available routes for nanoparticle production. Furthermore, their substrate specificity minimizes undesired side reactions and improves reaction selectivity [18]. Nevertheless, enzyme-assisted nanoparticle synthesis remains

challenging because enzyme activity is highly sensitive to reaction conditions such as pH, temperature, substrate concentration, and reaction time. Small variations in these parameters may significantly influence nucleation kinetics, crystal growth, particle aggregation, and ultimately the physicochemical properties of the synthesized nanoparticles [19]. Therefore, achieving reproducible nanoparticle characteristics remains one of the major obstacles preventing wider industrial implementation of enzymatic green synthesis.

Most published studies investigating green synthesis optimize reaction conditions using a conventional one-factor-at-a-time (OFAT) approach, in which individual process variables are varied independently while all remaining parameters are held constant. Although straightforward to implement, this methodology ignores possible interactions among variables, requires many experiments, and often fails to identify global optimum conditions [20]. Since nanoparticle formation is governed by complex interactions among precursor concentration, enzyme concentration, pH, temperature, reaction time, and mixing conditions, simultaneous optimization of multiple parameters is required to fully understand and control the synthesis process [21].

Design of Experiments (DoEs) provides a powerful statistical framework for systematic process optimization by simultaneously evaluating multiple experimental factors and their interactions while substantially reducing the number of required experiments [17,22]. Compared with traditional optimization methods, DoE improves experimental efficiency, enhances reproducibility, and provides quantitative mathematical models describing the influence of individual variables on process responses [21]. Factorial experimental designs are particularly suitable for identifying statistically significant synthesis parameters during initial screening, whereas response surface methodology (RSM) enables detailed investigation of nonlinear relationships and interactions between variables. D-optimal experimental design allows the development of predictive regression models that can accurately estimate optimum process conditions and predict responses throughout the experimental design space [23]. Such approaches are increasingly employed in chemical engineering, pharmaceutical manufacturing, biotechnology, and materials science because they significantly reduce development time while improving process robustness and scalability.

Although Design of Experiments has been successfully applied to optimize the synthesis of numerous nanomaterials, relatively few studies have investigated its application to green synthesis of ZnO NPs, and reports focusing specifically on enzyme-mediated synthesis remain scarce [17]. Moreover, many optimization studies concentrate exclusively on maximizing synthesis yield without establishing quantitative relationships between synthesis parameters and nanoparticle properties that ultimately determine water treatment performance. Developing predictive statistical models capable of producing nanoparticles with predefined dimensions and physicochemical characteristics therefore represents an important research challenge.

The application of DoE to enzymatic ZnO synthesis offers several important advantages. Besides identifying the most influential process parameters, statistical modeling enables prediction of nanoparticle size under previously untested experimental conditions, facilitating rational process design rather than empirical optimization. Such predictive capability is particularly valuable for future scale-up, where reproducibility and process robustness become increasingly important. Furthermore, minimizing unnecessary experimental runs reduces reagent consumption, energy use, laboratory waste generation, and overall environmental impact, thereby further supporting the principles of sustainable manufacturing.

In this study, a comprehensive Design of Experiments methodology was employed to develop and optimize the green enzymatic synthesis of ZnO NPs intended for water treatment applications. As the application in water treatment depends on the size of the

nanoparticle, the main aim of this work was to model the process of synthesis and guide the process towards production of minimal and maximal ZnO NPs.

The key innovation of this manuscript lies in the development of an optimized, robust, and scalable green synthesis strategy for ZnO NPs that addresses the major limitations of conventional fabrication methods. The proposed protocol enables efficient nanoparticle production while reducing reagent consumption and minimizing environmental impact, in accordance with the principles of sustainable nanotechnology. In addition to providing an environmentally benign synthesis route, this study establishes a reproducible framework for the large-scale preparation of ZnO nanomaterials, enhancing their potential for practical applications in next-generation water treatment technologies. By integrating sustainability, scalability, and process efficiency, this work represents a significant advancement toward the development of eco-friendly nanomaterials for environmental remediation.

A fractional factorial experimental design was initially used to identify the green enzymatic synthesis parameters with the greatest influence on nanoparticle size, followed by response surface methodology based on a D-optimal experimental design to establish predictive mathematical models describing the process. The resulting statistical models were used to determine the optimum combination of precursor concentration, enzyme concentration, pH, temperature, and reaction time required to produce nanoparticles with tailored dimensions suitable for future photocatalytic and antimicrobial applications. The proposed methodology provides a reproducible, scalable, and environmentally sustainable strategy for green ZnO nanoparticle synthesis and establishes a foundation for the subsequent development of immobilized ZnO-based materials for practical water treatment.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

All chemical reagents (urea, zinc nitrate ( $\text{Zn}(\text{NO}_3)_2 \times 6 \text{H}_2\text{O}$ ) and sodium nitrate ( $\text{NaNO}_3$ )) were obtained from MERCK (MERCK, Sigma-Aldrich Chemicals, St. Louis, MO, USA) and were of pro analysis purity. Enzyme urease was obtained from Sigma Aldrich, (Burlington, MA, USA). Zinc oxide (ZnO) nanopowder (<100 nm, Sigma-Aldrich Chemicals, St. Louis, MO, USA) was used as a reference material for validation of the characterization methods and comparison with the green-synthesized ZnO nanoparticles.

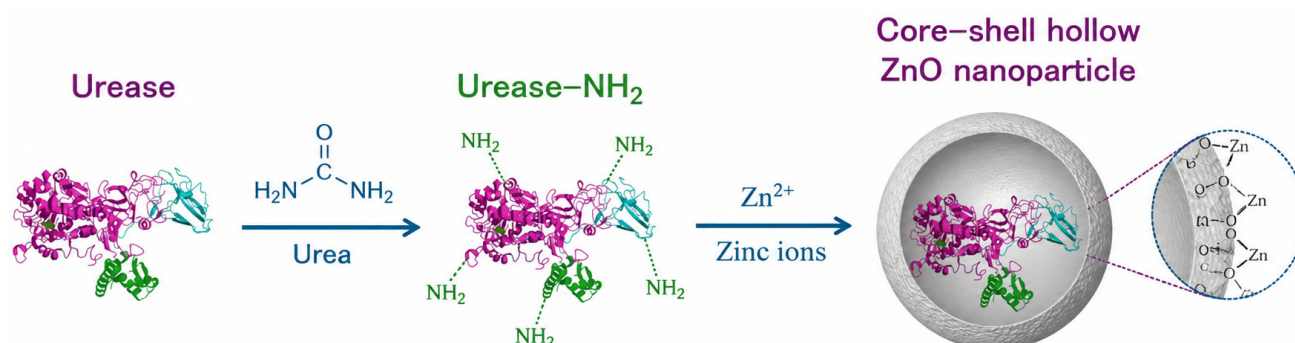
### 2.2. Enzymatic Synthesis of ZnO NPs with Enzyme Urease

ZnO nanoparticles with a urease core were synthesized using zinc nitrate hexahydrate ( $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , Merck, Sigma-Aldrich Chemicals, St. Louis, MO, USA), urea (Sigma-Aldrich Chemicals, St. Louis, MO, USA), and the enzyme *urease* as the biocatalyst. All reactions were performed in a 0.1 M sodium nitrate solution ( $\text{NaNO}_3$ , Kemika, Zagreb, Croatia), according to the experimental design shown in Figure 1.

The synthesis was carried out in 1 mL reaction vessels containing predetermined volumes of urea and urease stock solutions. All experiments were performed at 25 °C under controlled orbital shaking (150 rpm). A constant initial urease concentration of  $1 \text{ mg} \cdot \text{cm}^{-3}$  was maintained in all reactions. Briefly, 5 mg of urease was dissolved in  $4.5 \text{ cm}^3$  of 0.1 M  $\text{NaNO}_3$  solution, followed by the addition of a defined concentration of urea. The mixture was incubated for 60 s to allow urease-catalyzed urea hydrolysis, resulting in the formation of ammonium and hydroxide ions. The generated hydroxide ions increased the local pH in the vicinity of the enzyme, providing the conditions required for ZnO precipitation.

Because  $\text{Zn}^{2+}$  ions strongly inhibit urease activity, zinc nitrate hexahydrate was added only after the initial hydrolysis period. The addition of zinc ions was considered the starting point of ZnO nanoparticle formation. The appropriate volume of zinc nitrate stock solution

(prepared in 0.1 M NaNO<sub>3</sub>) was added to the reaction mixture, resulting in the formation of a ZnO shell around the urease core. The precipitation process was promoted by the localized alkaline environment generated by urease activity. Following zinc addition, the reaction was allowed to proceed for 86,400 s.



**Figure 1.** Graphical representation of the enzymatically catalyzed green synthesis of ZnO NPs with a core containing enzyme urease. All reactions were performed using the same initial urease concentration (1 mg·cm<sup>−3</sup>). The mixture was stirred for 60 s to allow the formation of hydroxide ions, which are responsible for ZnO formation, as urease-catalyzed urea hydrolysis generates ammonium and hydroxide ions. After 60 s, a specific concentration of zinc nitrate hexahydrate dissolved in 0.5 cm<sup>3</sup> of 0.1 M sodium nitrate solution was added to the reactor. The addition of zinc ions resulted in the formation of a zinc oxide shell, promoted by the localized high-pH environment in the vicinity of the enzyme. Since zinc ions significantly inhibit urease activity, their addition effectively terminated the urea hydrolysis reaction.

Preliminary experiments were performed using different initial concentrations of zinc nitrate hexahydrate and urea while maintaining the same urease concentration. Three synthesis conditions were investigated, as summarized in Table 1.

**Table 1.** Experimental conditions in which zinc nanoparticle synthesis was performed.

| Temperature [°C] | Medium                  | Urea Concentration [mM] | Zn(NO <sub>3</sub> ) <sub>2</sub> Concentration [mM] |
|------------------|-------------------------|-------------------------|------------------------------------------------------|
| 25               | 0.1 M NaNO <sub>3</sub> | 3.33                    | 1.00                                                 |
|                  |                         | 3.33                    | 2.00                                                 |
|                  |                         | 10.0                    | 1.00                                                 |
|                  |                         | 10.0                    | 2.00                                                 |
|                  |                         | 10.0                    | 3.36                                                 |

One experiment was conducted using a lower initial urea concentration (3.33 mM), whereas three experiments were performed with an initial urea concentration of 10 mM and varying zinc concentrations. During the 60 s pre-hydrolysis period, approximately 10% of the initial urea was hydrolyzed, corresponding to ammonium ion concentrations of approximately 0.33 mM and 1 mM for the 3.33 mM and 10 mM urea conditions, respectively. The inhibition of urease activity by zinc ions effectively terminated further urea hydrolysis after zinc addition, enabling control over the ZnO nanoparticle formation process.

### 2.3. Design of Experiment

The Design of Experiments (DoEs) approach was applied to identify and optimize the key process parameters influencing ZnO nanoparticle size. The investigated factors included the initial urea concentration (A), zinc nitrate concentration (B), reaction temperature (C), and stirring rate (D). The particle size of ZnO was selected as the response variable to evaluate the combined effects of these synthesis parameters.

While all four process variables which were selected for the optimization of the biocatalytic synthesis of zinc oxide nanoparticles (temperature, shaking speed, and the initial concentrations of urea and zinc nitrate) were varied thorough the investigation, the urease concentration was kept constant at  $1 \text{ mg}\cdot\text{mL}^{-1}$ . The investigated parameter ranges were 25–40 °C for temperature, 50–300 rpm for shaking speed, 1–10 mM for urea concentration, and 1–5 mM for zinc nitrate concentration. These parameter ranges were entered into the Design-Expert® software (State-Ease Inc., version 10, P.O. Box 18338 Minneapolis, MN 55418, USA), and a D-optimal experimental design was selected. Based on this design, the software generated an experimental matrix consisting of 25 runs, which were performed to develop a predictive process model and determine the optimal synthesis conditions for obtaining zinc oxide nanoparticles with the minimum particle size.

Analysis of variance (ANOVA) was performed to evaluate the statistical significance of the investigated process variables and their effects on the measured responses. Based on the predefined experimental design and the corresponding response data, ANOVA was applied to determine the contribution of individual factors and identify significant interactions between variables. The analysis was also used to assess the adequacy of the developed mathematical models describing the relationship between the independent process parameters and the experimental responses. Model significance was evaluated using statistical parameters, including the *F*-value and *p*-value, with a confidence level of 95% ( $p < 0.05$ ). The selected model was considered appropriate when it provided a statistically significant representation of the experimental data and accurately described the influence of process variables on the response behavior.

#### 2.4. Nanoparticle Characterization

The synthesized ZnO NPs were characterized by nanoparticle tracking analysis (NTA) using a Malvern NanoSight LM10 (Malvern Panalytical, Spectris, London, UK) instrument to determine particle size and concentration.

Nanoparticle tracking analysis (NTA) is based on tracking individual nanoparticles illuminated by a laser beam. The particles undergo Brownian motion and scatter light, with the scattering intensity depending on the particle size and refractive index. The scattered light is detected using an optical microscope coupled with a highly sensitive charge-coupled device (CCD) camera, which records the two-dimensional movement of individual nanoparticles in real time.

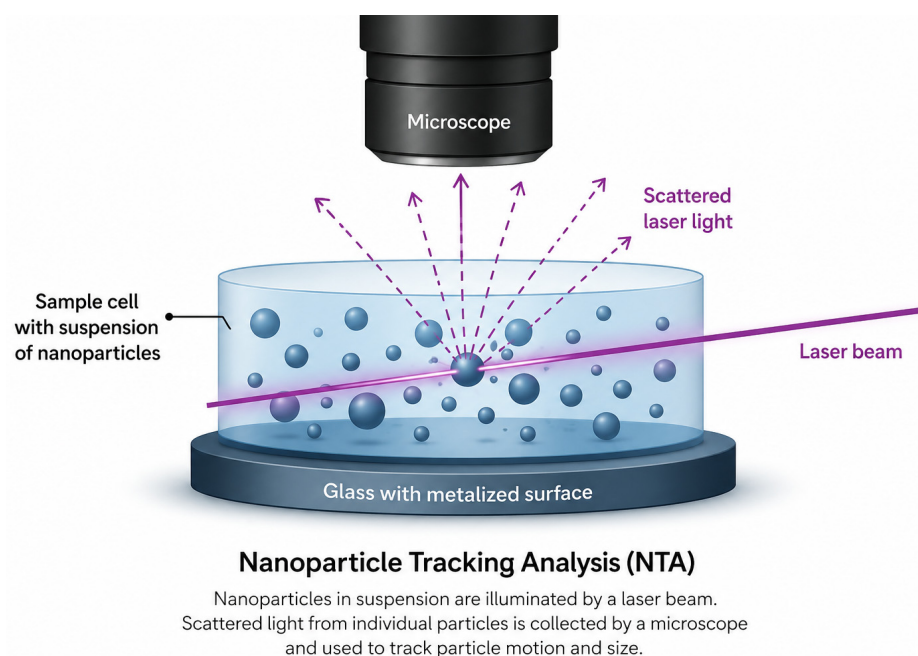
NTA is a relatively recent analytical technique that is particularly suitable for the characterization of nanoparticles in complex matrices. In this study, NTA was employed to determine the size, size distribution, and concentration of zinc oxide (ZnO) nanoparticles obtained by enzymatic reaction. Initially, certified reference materials (ZnO NPs, 100 nm; Sigma-Aldrich) were analyzed to evaluate the precision and measurement uncertainty of the method. Subsequently, the synthesized nanoparticle samples were analyzed using the same procedure.

A NanoSight NS300 instrument (Malvern Panalytical, UK) was used to determine nanoparticle concentration and particle size distribution in suspension. The instrument is equipped with a video camera that records the Brownian motion of nanoparticles in real time as the sample flows through a microfluidic channel. Data were collected from three independent measurements for each sample (Figure 2). The recorded Brownian motion was quantified by calculating the mean square displacement over a defined time interval (Equation (1)), and the hydrodynamic particle diameter was subsequently determined using the Stokes–Einstein equation (Equation (2)).

$$Dt = \frac{\langle \overline{x, y^2} \rangle}{4} \quad (1)$$

$$D = \frac{k_B T}{6\pi\eta r} \quad (2)$$

where  $D$  is the diffusion coefficient,  $\langle \bar{x}, \bar{y}^2 \rangle$  is the mean square displacement,  $k_B$  is the Boltzmann constant ( $k_B = 1.380649 \times 10^{-23} \text{ J}\cdot\text{K}^{-1}$ ),  $T$  [K] is the absolute temperature,  $\eta$  [ $\text{kg}\cdot\text{m}^{-1}\cdot\text{s}^{-1}$ ] is the dynamic viscosity of the medium, and  $r$  [nm] is the hydrodynamic radius of the nanoparticle.



**Figure 2.** Graphical presentation of the principle of nanoparticle tracking analysis for detection of nanoparticle size, concentration and size distribution in a sample suspension.

The NTA method enabled monitoring ZnO nanoparticle size, size distribution and concentration, with a variability of 5.05%.

The NTA measurements were performed at approximately 26.1 °C using a Blue 488 nm laser (Malvern Panalytical, UK) and an sCMOS camera (Malvern Panalytical, UK) with identical acquisition settings across all three replicates. Samples were diluted 1000-fold, and 1498 frames were analyzed per measurement. The average particle concentration was  $8.83 \times 10^{11} \text{ particles mL}^{-1}$ , with a standard error of  $1.60 \times 10^{10} \text{ particles mL}^{-1}$ , indicating good reproducibility among the three measurements.

Nanoparticle tracking analysis (NTA) was performed on samples diluted 1000-fold. The measurements were conducted at temperatures of 26.067, 26.095, and 26.149 °C, corresponding to viscosities of 0.867, 0.867, and 0.866 cP, respectively. An sCMOS camera and a Blue 488 nm laser were used for all measurements. The camera settings were kept constant across all replicates, with a camera level of 7, slider shutter of 165, slider gain of 15, and shutter time of 4.13 ms. The camera histogram limits were set from 0 to 16,380, and videos were acquired at a frame rate of 24.9825 fps. Samples were introduced into the measurement chamber using a syringe pump operating at 50 AU.

Data analysis was performed using identical processing parameters for all three replicates. A detection threshold of 13 was applied, while the maximum jump mode, blur, and minimum track length were all set to Auto. The maximum jump distances were 16.9929, 17.2356, and 17.2470, respectively. Analysis began from frame 0, and 1498 frames were analyzed for each replicate. No exclusion regions were applied during the analysis.

The morphology and elemental composition of the ZnO NPs were characterized using a scanning electron microscope equipped with an energy-dispersive X-ray spectroscopy

detector (SEM–EDX, TESCAN VEGA TS5136LS, Brno, Czech Republik, Europe). Measurements were performed at an accelerating voltage of 20 kV with a working distance of 25 mm. Surface morphology was examined at magnifications ranging from 2000 $\times$  to 7000 $\times$  using both secondary electron (SE) and backscattered electron (BSE) imaging modes. Initially, SEM micrographs were recorded to evaluate the morphology, size, and distribution of the synthesized ZnO NPs. Subsequently, elemental composition was analyzed by EDX spectroscopy with an acquisition time of 30 s per measurement, confirming the chemical composition and surface characteristics of the nanoparticles.

### 3. Results

The present study was focused on optimizing the enzymatic green synthesis of ZnO nanoparticles using a mathematical modeling approach and did not include an evaluation of their photocatalytic performance. Although ZnO nanoparticles are well known for their excellent photocatalytic properties in water treatment applications [24–26], assessment of these properties requires comprehensive physicochemical characterization and dedicated photocatalytic experiments. Therefore, investigation of the photocatalytic efficiency of the green-synthesized ZnO nanoparticles will be the subject of future work. The optimized synthesis protocol developed in this study provides a robust basis for the preparation of ZnO nanoparticles with tailored properties and represents an important first step toward their application as efficient and environmentally friendly photocatalysts for water treatment.

The zinc oxide nanoparticles synthesized under the experimental conditions generated by the Design-Expert<sup>®</sup> software (State-Ease Inc., version 10, P.O. Box 18338 Minneapolis, MN 55418, USA) were characterized using nanoparticle tracking analysis (NTA) on a Malvern NanoSight LM10 instrument (Malvern Panalytical, UK). Particle size and nanoparticle concentration were determined for each experimental run (Table 2), and the results obtained were entered into the corresponding columns of the Design-Expert<sup>®</sup> experimental matrix. Based on the predefined experimental design and the associated response data, the software applied analysis of variance (ANOVA) to evaluate the significance of the investigated factors and to identify the mathematical model that best described the relationship between the process variables and the measured responses. For the biocatalytic synthesis of ZnO NPs investigated in this study, the model presented in Equation (3) was identified as the most appropriate according to the ANOVA criteria. Using this model, Design-Expert<sup>®</sup> generated a comprehensive statistical analysis, from which the most relevant results are presented in this work.

**Table 2.** Precision results of determination of ZnO NP size, with standard deviation.

| Experiment | Size of Nanoparticle [nm] | Standard Deviation, $\sigma$ | Coefficient of Variation, CV [%] |
|------------|---------------------------|------------------------------|----------------------------------|
| 1          | 174.5                     | 8.2                          | 5.1                              |
| 2          | 153.8                     |                              |                                  |
| 3          | 166.4                     |                              |                                  |
| 4          | 158.8                     |                              |                                  |
| 5          | 157.9                     |                              |                                  |

NTA is based on the visualization and tracking of individual nanoparticles suspended in a liquid under laser illumination. The particles undergo Brownian motion and scatter light according to their size and refractive index. The scattered light is recorded in real time using a microscope and camera, enabling two-dimensional tracking of individual particles. The hydrodynamic diameter of the nanoparticles is subsequently calculated from the mean square displacement of each particle using the Stokes–Einstein equation [10]. In addition to particle size distribution, the NTA provides the nanoparticle concentration (number of

particles per milliliter of suspension). To evaluate measurement precision, five consecutive measurements were performed on the same sample (Table 2).

For performing experiments required to collect data required for D-optimal experimental design, 25 experiments with variation in process parameters were designed by State Ease software ((State-Ease Inc., version 10, P.O. Box 18338 Minneapolis, MN 55418, USA). The results obtained are presented in Table 3.

**Table 3.** Results of analysis of green-synthesized ZnO NPs, determined by NTA method.

| Experiment | <i>c</i> (Urea) [mM] | <i>c</i> (Zn(NO <sub>3</sub> ) <sub>2</sub> ) [mM] | <i>T</i> [°C] | Stirring Speed [rpm] | ZnO Concentration [Part mL <sup>−1</sup> ] | ZnO Size [nm] |
|------------|----------------------|----------------------------------------------------|---------------|----------------------|--------------------------------------------|---------------|
| 1          | 10.0                 | 1.0                                                | 25.0          | 117.5                | $2.04 \times 10^{10}$                      | 316.2         |
| 2          | 10.0                 | 4.3                                                | 30.4          | 147.5                | $2.97 \times 10^{10}$                      | 273.2         |
| 3          | 5.9                  | 2.9                                                | 40.0          | 193.8                | $3.37 \times 10^{10}$                      | 251.9         |
| 4          | 10.0                 | 4.3                                                | 30.4          | 147.5                | $1.90 \times 10^{10}$                      | 267.6         |
| 5          | 1.0                  | 4.6                                                | 34.8          | 257.5                | $1.90 \times 10^{10}$                      | 203.8         |
| 6          | 4.2                  | 1.0                                                | 32.3          | 163.8                | $7.48 \times 10^{10}$                      | 264.0         |
| 7          | 10                   | 5.0                                                | 40.0          | 300                  | $6.59 \times 10^{10}$                      | 202.5         |
| 8          | 5.8                  | 2.8                                                | 29.2          | 50.0                 | $1.17 \times 10^{10}$                      | 264.8         |
| 9          | 4.2                  | 1.0                                                | 32.3          | 163.8                | $1.17 \times 10^{10}$                      | 197.3         |
| 10         | 1.0                  | 1.0                                                | 40.0          | 300                  | $1.24 \times 10^{10}$                      | 279.5         |
| 11         | 6.5                  | 2.8                                                | 25.0          | 203.4                | $1.03 \times 10^{10}$                      | 282.1         |
| 12         | 1.0                  | 3.2                                                | 33.0          | 118.6                | $4.56 \times 10^{10}$                      | 203.9         |
| 13         | 1.0                  | 2.2                                                | 25.0          | 265                  | $4.63 \times 10^{10}$                      | 221.7         |
| 14         | 9.8                  | 1.0                                                | 40.0          | 208.8                | $5.75 \times 10^{10}$                      | 249.2         |
| 15         | 1.0                  | 5.0                                                | 25.0          | 86.25                | $3.22 \times 10^{10}$                      | 190.2         |
| 16         | 10                   | 4.4                                                | 39.8          | 148.8                | $5.23 \times 10^{10}$                      | 231.8         |
| 17         | 9.8                  | 1.8                                                | 32.7          | 300                  | $7.90 \times 10^{10}$                      | 229.4         |
| 18         | 5.9                  | 5.0                                                | 40.0          | 50.0                 | $3.19 \times 10^{10}$                      | 214.1         |
| 19         | 5.8                  | 2.8                                                | 29.1          | 50.0                 | $4.71 \times 10^{10}$                      | 219.6         |
| 20         | 5.9                  | 2.9                                                | 40.0          | 193.8                | $8.63 \times 10^{10}$                      | 205.0         |
| 21         | 6.3                  | 5.0                                                | 25.0          | 300                  | $5.56 \times 10^{10}$                      | 212.1         |
| 22         | 1.0                  | 1.7                                                | 40.0          | 50.0                 | $1.97 \times 10^{10}$                      | 218.9         |
| 23         | 9.8                  | 1.8                                                | 32.7          | 300                  | $2.58 \times 10^{10}$                      | 211.4         |
| 24         | 10.0                 | 1.0                                                | 40.0          | 50.0                 | $8.74 \times 10^{10}$                      | 242.8         |
| 25         | 4.9                  | 5.0                                                | 32.1          | 162.5                | $5.01 \times 10^{10}$                      | 213.8         |

The experimental data presented in Table 3, obtained for the size of bio-catalytically synthesized ZnO NPs, were analyzed using a two-factor interaction (2FI) response surface model. Analysis of variance (ANOVA) demonstrated that the model was statistically significant ( $F = 3.50$ ,  $p = 0.0164$ ), indicating that the selected process variables adequately explained the observed variation in nanoparticle size. The probability that such an  $F$ -value resulted from random experimental noise was only 1.64%.

Among the factors investigated, the initial urea concentration (factor  $A$ ) had a statistically significant effect on nanoparticle size ( $p = 0.0043$ ). In addition, two interaction terms, namely the interaction between urea concentration and temperature ( $AC$ ,  $p = 0.0353$ ) and the interaction between urea concentration and stirring speed ( $AD$ ,  $p = 0.0153$ ), were also significant.

In contrast, the individual effects of zinc nitrate concentration, temperature, and stirring speed, as well as the remaining interaction terms, were not statistically significant ( $p > 0.05$ ). These results indicate that urea concentration is the dominant process variable

governing ZnO nanoparticle formation, while its influence depends strongly on both the reaction temperature and the mixing conditions.

The estimated regression coefficients further support these observations. The positive coefficient for urea concentration (+22.08 in coded units) indicates that increasing the urea concentration alone tends to increase the particle size. Conversely, the negative coefficients for the significant interaction terms AC (−19.32) and AD (−22.98) suggest that higher temperatures and increased stirring partially counteract the size increase associated with elevated urea concentrations, resulting in smaller nanoparticles under suitable combinations of process parameters. Although zinc nitrate concentration, temperature, and stirring speed exhibited negative regression coefficients, their individual effects were not statistically significant within the experimental range investigated.

The fitted model explained 71.4% of the total variability in nanoparticle size ( $R^2 = 0.7144$ ), indicating a satisfactory overall fit. However, the adjusted coefficient of determination (adjusted  $R^2 = 0.5105$ ) and the predicted coefficient of determination (predicted  $R^2 = 0.2994$ ) were considerably lower, with a difference exceeding the recommended value of 0.2. This discrepancy suggests limited predictive capability of the model and may indicate the presence of experimental variability, model overfitting, or additional influential factors not included in the current experimental design. Consequently, the predictive performance of the model should be verified by confirmation experiments.

Although the developed model shows statistically significant relationships between the investigated factors and responses, the difference between the adjusted  $R^2$  and predicted  $R^2$  values ( $>0.2$ ), together with the relatively low predicted  $R^2$  value, indicates limited predictive capability of the model. The model provided here is a tool for describing factor–response relationships, identifying trends, and evaluating the relative effects of the studied variables within the investigated experimental range rather than as a highly accurate quantitative prediction model.

Therefore, due to the relatively low predicted  $R^2$  value, the developed model can be considered suitable for describing factor–response trends and optimization within the investigated experimental region, but its use for absolute prediction outside this range should be interpreted with caution.

The numerical optimization procedure resulted in a desirability value of 1.000; however, this value reflects the mathematical optimization of the response within the defined design space and does not represent perfect predictive accuracy. Consequently, the predicted optimal conditions should be considered as theoretical optimum conditions and require experimental validation. Additional experimental runs, expansion of the design space, or evaluation of alternative model structures may be required in future studies to improve the predictive reliability of the model.

The adequacy of the fitted model was further confirmed by the lack-of-fit test, which was not statistically significant ( $F = 0.34$ ,  $p = 0.9229$ ). A non-significant lack of fit indicates that the model adequately represents the experimental data and that the remaining variation can be attributed primarily to random experimental error rather than systematic deviations. The coefficient of variation ( $CV = 9.74\%$ ) demonstrates acceptable experimental precision, while the adequate precision ratio of 7.915, which is well above the recommended minimum value of four, confirms that the model provides a sufficient signal-to-noise ratio and can be reliably used to navigate the experimental design space.

The final regression equation describing the relationship between the process variables and ZnO nanoparticle size in terms of coded factors is presented in Equation (3).

Final Equation in Terms of Actual Factors:

$$\begin{aligned} \text{ZnO size} = & +188.43568 + 27.88651 \times c(\text{urea}) - 2.64303 \times c(\text{Zn(NO}_3)_2) + \\ & 0.40708 \times \text{Temperature} - 0.12466 \times \text{Stirring} + 0.92392 \times c(\text{urea}) \times c(\text{Zn(NO}_3)_2) \\ & - 0.57237 \times c(\text{urea}) \times \text{Temperature} - 0.040850 \times c(\text{urea}) \times \text{Stirring} - 0.16583 \\ & \rightarrow \times c(\text{Zn(NO}_3)_2) \times \text{Temperature} - 0.018610 \times c(\text{Zn(NO}_3)_2) \times \text{Stirring} \\ & + 0.010902 \times \text{Temperature} \times \text{Stirring} \end{aligned} \quad (3)$$

This equation was subsequently used to predict nanoparticle size throughout the investigated design space and to determine the optimal synthesis conditions. Graphical comparison of predicted and experimentally obtained values is presented in Figure 3.

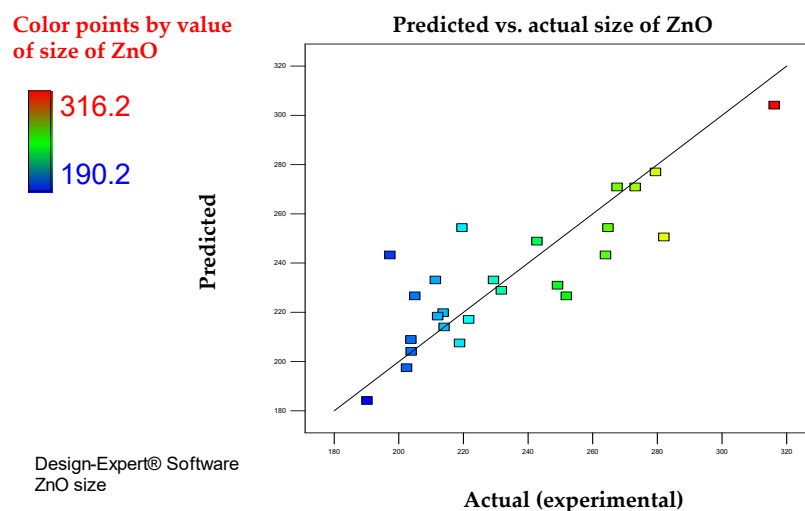


Figure 3. Graphical comparison of predicted and experimentally obtained values.

The influence of different process parameters on the size of ZnO NPs is presented in Figures 4–6.

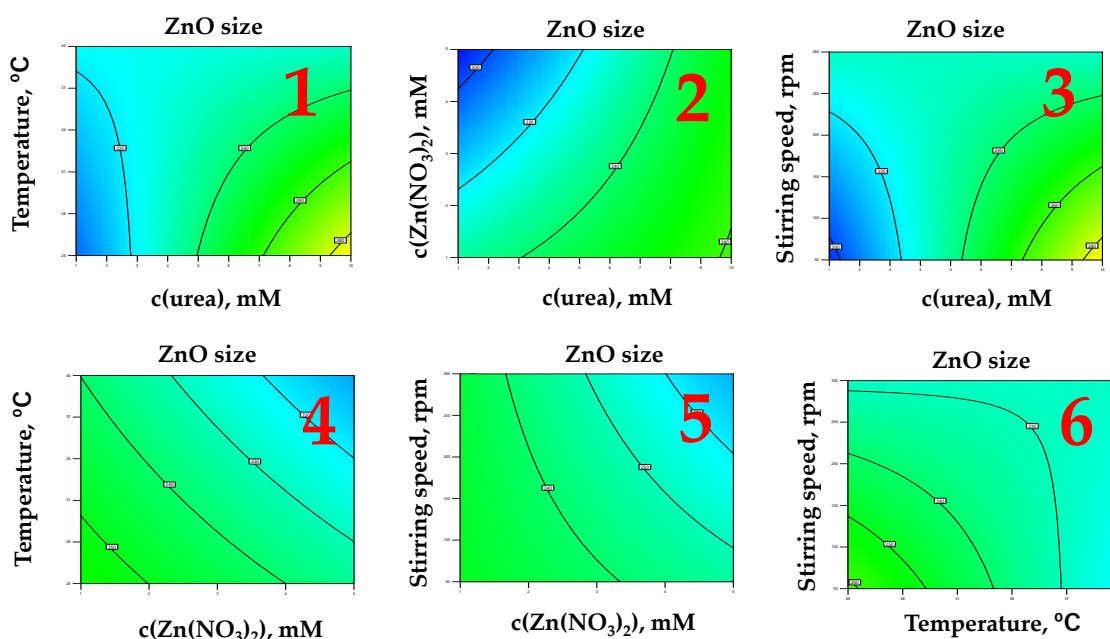
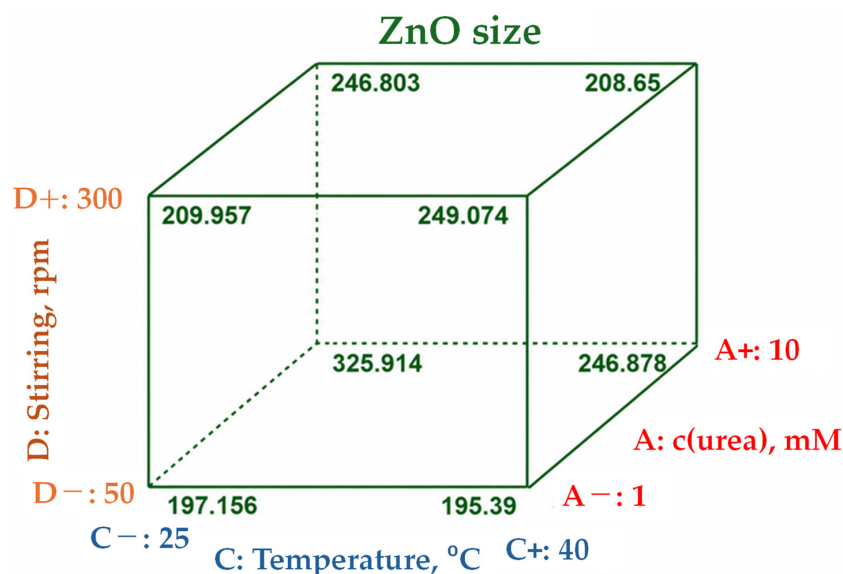
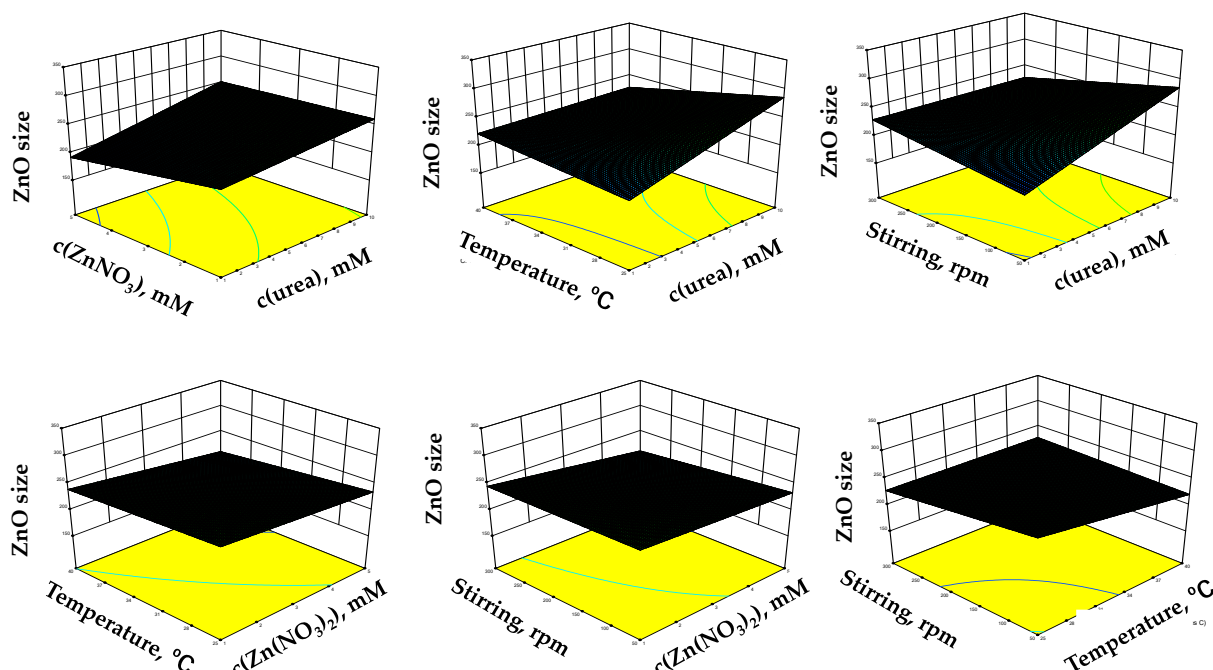


Figure 4. Graphical presentation of interaction of process parameters on desired and optimized size of ZnO NPs: (1) temperature and c(urea), (2) c(urea) and c(Zn(NO<sub>3</sub>)<sub>2</sub>), (3) stirring and c(urea), (4) temperature and c(Zn(NO<sub>3</sub>)<sub>2</sub>), (5) stirring c(Zn(NO<sub>3</sub>)<sub>2</sub>), and (6) stirring and temperature.



**Figure 5.** Graphical presentation of the interaction between all process parameters investigated (concentration of reagents, speed of stirring and temperature) on the size of ZnO NPs.



**Figure 6.** Graphical presentation of 3D interaction of process parameters on desired and optimized size of ZnO NPs.

The graphical representation of the response surface model is shown in Figures 5 and 6. Figure 5 illustrates the combined effect of the initial urea concentration, temperature and stirring speed on the size of the synthesized ZnO NPs. The response surface demonstrates that smaller nanoparticles are obtained at lower initial urea concentrations and higher zinc nitrate concentrations. This finding is consistent with the statistical analysis, which identified the initial urea concentration as the most influential process variable affecting nanoparticle size.

A graphical presentation of the interactions of parameters in 3D graphics is presented in Figure 6, which presents a three-dimensional cube plot showing the combined effects of the initial urea concentration, zinc nitrate concentration, and reaction temperature on nanoparticle size. The predicted nanoparticle size is displayed at each vertex of the cube,

while the axes represent the investigated factor levels. This graphical representation allows the prediction of nanoparticle size for different combinations of the three process variables.

The smallest ZnO NPs are predicted to be produced at the lowest initial urea concentration, the highest zinc nitrate concentration, and higher reaction temperatures within the investigated experimental range.

The normal probability plot of the residuals (Figure 7) showed no substantial deviations from normality and no discernible pattern in the residual distribution, indicating that the assumptions of the regression model were satisfied and that the applied data transformation was appropriate.

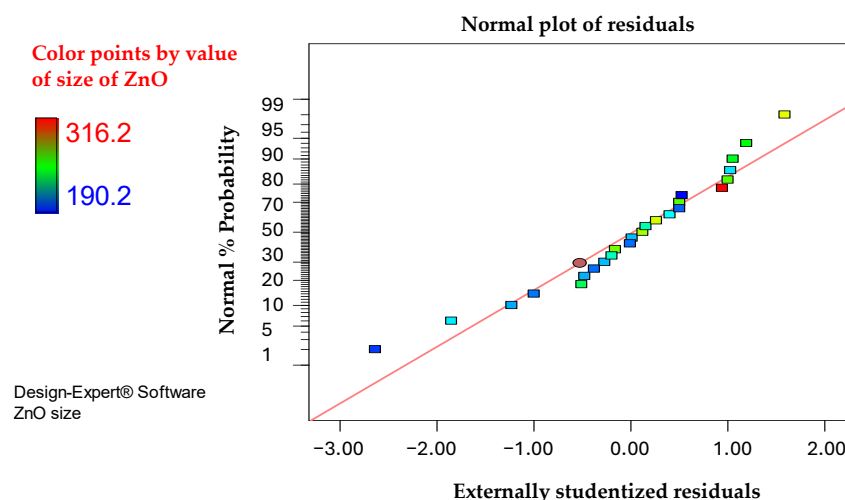


Figure 7. Normal probability plot of the residuals.

As the model was appropriate, optimization of maximal ZnO size was performed by response surface methodology.

Moreover, ANOVA analysis revealed interactions between process parameters and their hidden relations, showing a significant impact of interaction between concentration of urea and temperature (Figure 8). One of the most important advantages of optimization and statistical modeling of complex processes by Design of Experiment is inside information on the process interactions, which can be hidden.

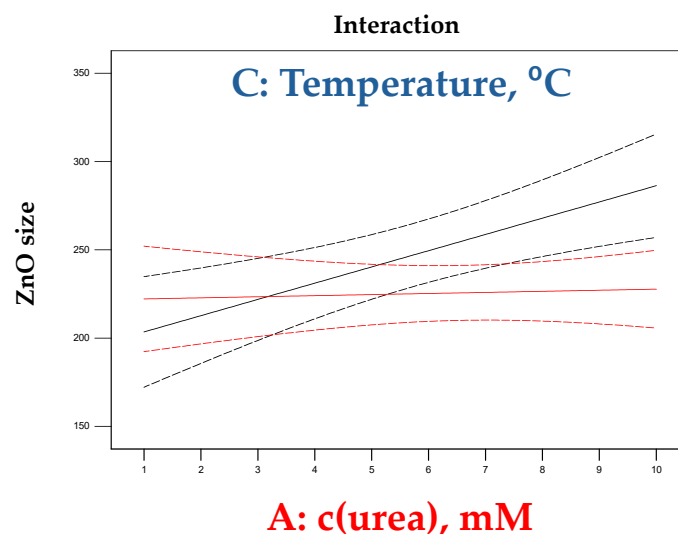


Figure 8. Interaction between parameters A (concentration of urea) and C (temperature of the process).

In the statistical software Design-Expert® (State-Ease, Inc.), interactions and interdependencies between selected parameters can be graphically represented as model outputs. As an example, the combined effect of temperature and stirrer speed on nanoparticle size is shown (Figure 9), where black lines represent the minimum stirrer speed (50 rpm), while red lines represent the maximum shaker speed (300 rpm). Based on this representation, it can be concluded that the smallest nanoparticles are formed at lower speeds combined with higher temperatures, and conversely, that higher shaker speeds favor the formation of smaller nanoparticles at lower temperatures.

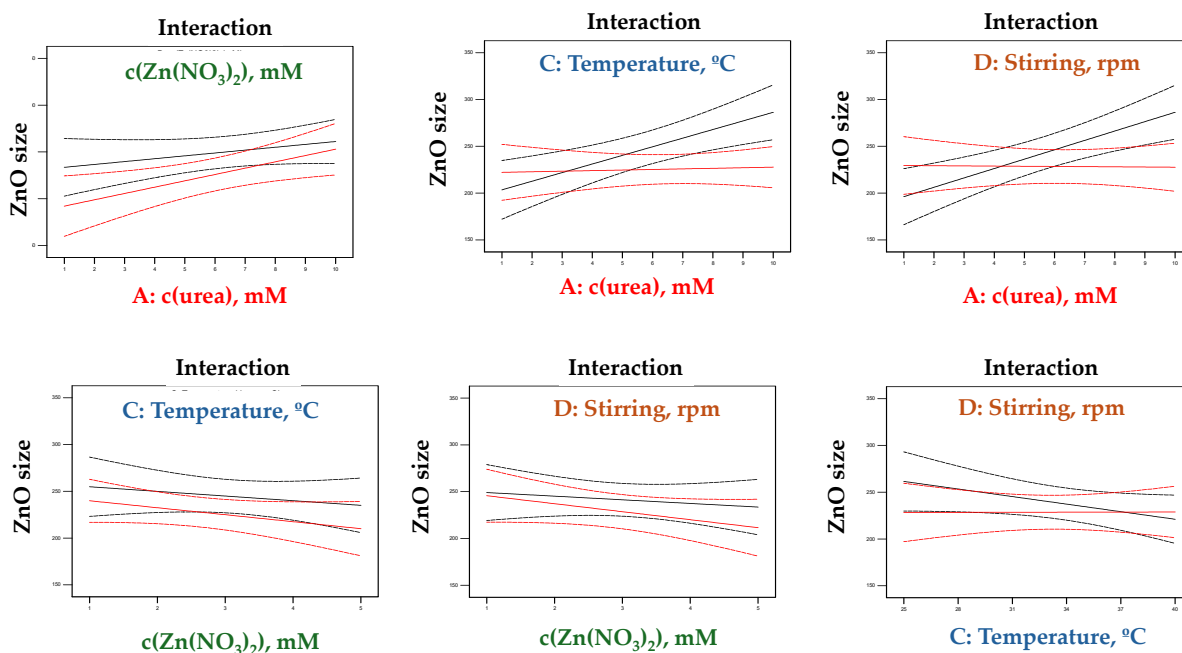


Figure 9. Interactions between process parameters.

The interaction between all parameters is shown in Figure 9.

Numerical optimization of the biocatalytic synthesis of zinc oxide nanoparticles was performed using Design-Expert® with the aim of minimizing nanoparticle size. The investigated process parameters were varied within the following ranges: initial urea concentration  $c(\text{urea}) = 1\text{--}10$  mM, initial zinc nitrate concentration  $c(\text{Zn}(\text{NO}_3)_2) = 1\text{--}5$  mM, temperature  $T = 25\text{--}40$  °C, and shaker speed 50–300 rpm.

Based on the obtained results, and the valid model, optimization and prediction of ZnO size was performed, with the goal of predicting the process parameters for obtaining the following: (a) minimal size of ZnO, (b) 200 nm predetermined value, and (c) maximal ZnO NPs.

The conditions for obtaining minimal ZnO NPs are presented in Table 4.

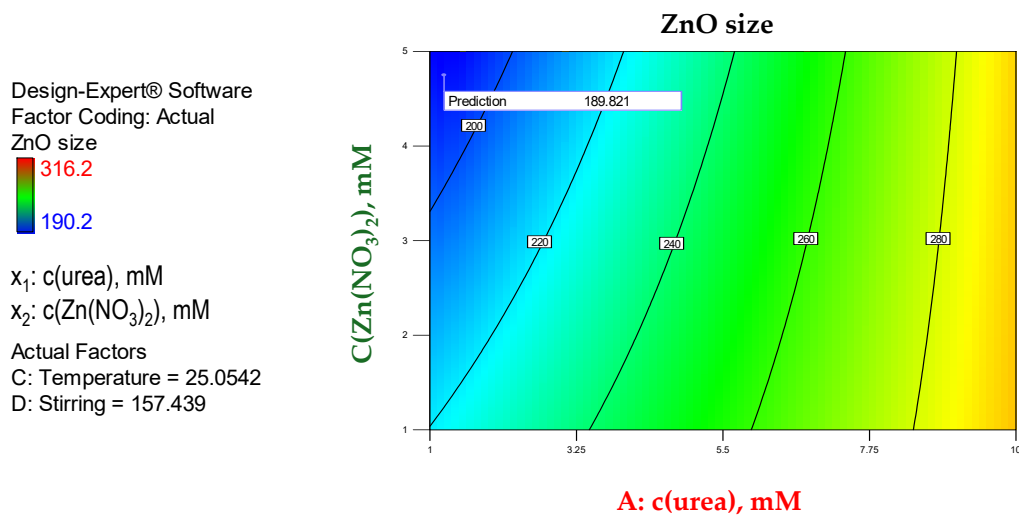
Table 4. Conditions for obtaining minimal ZnO NPs.

| Name                             | Goal        | Lower Limit | Upper Limit | Lower Weight | Upper Weight | Importance |
|----------------------------------|-------------|-------------|-------------|--------------|--------------|------------|
| A: $c(\text{urea})$              | is in range | 1           | 10          | 1            | 1            | 3          |
| B: $c(\text{Zn}(\text{NO}_3)_2)$ | is in range | 1           | 5           | 1            | 1            | 3          |
| C: Temperature                   | is in range | 25          | 40          | 1            | 1            | 3          |
| D: Stirring                      | is in range | 50          | 300         | 1            | 1            | 3          |
| ZnO size                         | minimize    | 190.2       | 316.2       | 1            | 1            | 3          |

The obtained solutions are presented in Table 5 and in Figure 10.

**Table 5.** Five different combinations of process parameters that result in minimal ZnO NPs.

| Solutions |          |                                        |             |          |          |              |
|-----------|----------|----------------------------------------|-------------|----------|----------|--------------|
| Number    | c (Urea) | c (Zn(NO <sub>3</sub> ) <sub>2</sub> ) | Temperature | Stirring | ZnO Size | Desirability |
| 1         | 1.2      | 4.8                                    | 25.1        | 157.4    | 189.8    | 1.0          |
| 2         | 2.1      | 5.0                                    | 39.1        | 68.1     | 188.9    | 1.0          |
| 3         | 1.0      | 3.8                                    | 35.9        | 50.1     | 189.6    | 1.0          |
| 4         | 1.3      | 5.0                                    | 29.1        | 135.0    | 189.8    | 1.0          |
| 5         | 1.2      | 4.7                                    | 34.0        | 68.3     | 185.9    | 1.0          |

**Figure 10.** Graphical presentation of process parameters for minimal ZnO.

(2) The conditions for obtaining the predetermined size of 200 nm ZnO NPs are presented in Table 6, and the solutions in Table 7 and Figure 11.

**Table 6.** Process conditions in which the size of green-synthesized nanoparticles will be 200 nm ZnO.

| Name                                     | Goal            | Lower Limit | Upper Limit | Lower Weight | Upper Weight | Importance |
|------------------------------------------|-----------------|-------------|-------------|--------------|--------------|------------|
| A: c(urea)                               | is in range     | 1           | 10          | 1            | 1            | 3          |
| B: c(Zn(NO <sub>3</sub> ) <sub>2</sub> ) | is in range     | 1           | 5           | 1            | 1            | 3          |
| C: Temperature                           | is in range     | 25          | 40          | 1            | 1            | 3          |
| D: Stirring                              | is in range     | 50          | 300         | 1            | 1            | 3          |
| ZnO size                                 | is target = 200 | 190.2       | 316.2       | 1            | 1            | 3          |

**Table 7.** Solutions for obtaining nanoparticles of desired size, namely 200 nm.

| Number | c (Urea) | c (Zn(NO <sub>3</sub> ) <sub>2</sub> ) | Temperature | Stirring | ZnO Size | Desirability |
|--------|----------|----------------------------------------|-------------|----------|----------|--------------|
| 1      | 1.2      | 3.2                                    | 30.4        | 76.0     | 200      | 1.0          |
| 2      | 9.1      | 5.0                                    | 40.0        | 299.0    | 200      | 1.0          |
| 3      | 1.5      | 4.8                                    | 29.5        | 257.2    | 200      | 1.0          |
| 4      | 1.1      | 2.6                                    | 36.6        | 51.2     | 200      | 1.0          |
| 5      | 1.3      | 4.0                                    | 29.1        | 153.4    | 200      | 1.0          |

Lastly, the conditions for obtaining maximal size ZnO NPs are presented in Table 8 and the obtained results in Table 9 and Figure 12.

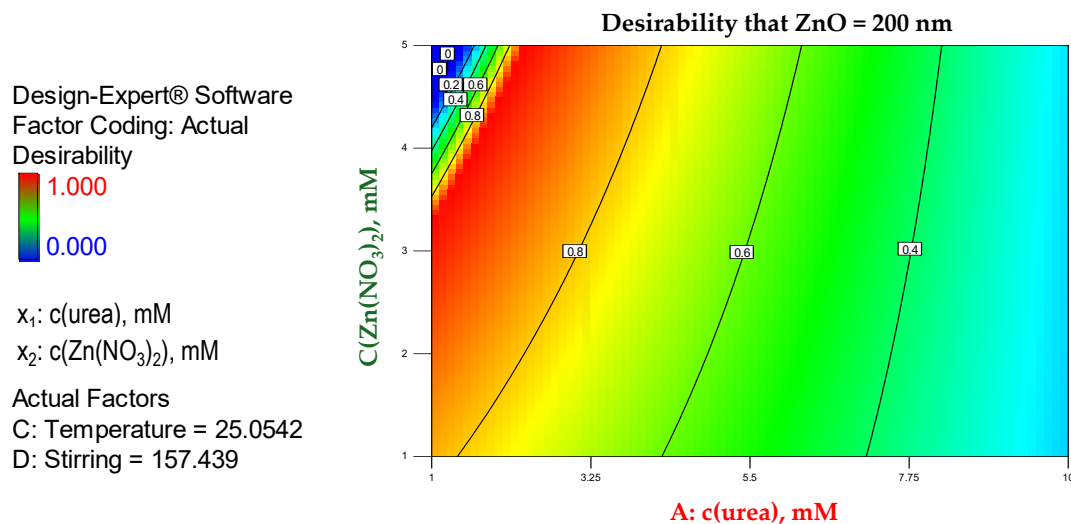


Figure 11. Graphical presentation of process parameters for obtaining ZnO NPs that are 200 nm in size.

Table 8. Conditions for obtaining maximal ZnO NPs.

| Name                                     | Goal        | Lower Limit | Upper Limit | Lower Weight | Upper Weight | Importance |
|------------------------------------------|-------------|-------------|-------------|--------------|--------------|------------|
| A: c(urea)                               | is in range | 1           | 10          | 1            | 1            | 3          |
| B: c(Zn(NO <sub>3</sub> ) <sub>2</sub> ) | is in range | 1           | 5           | 1            | 1            | 3          |
| C: Temperature                           | is in range | 25          | 40          | 1            | 1            | 3          |
| D: Stirring                              | is in range | 50          | 300         | 1            | 1            | 3          |
| ZnO size                                 | maximize    | 190.2       | 316.2       | 1            | 1            | 3          |

Table 9. Solutions for obtaining maximally large ZnO NPs.

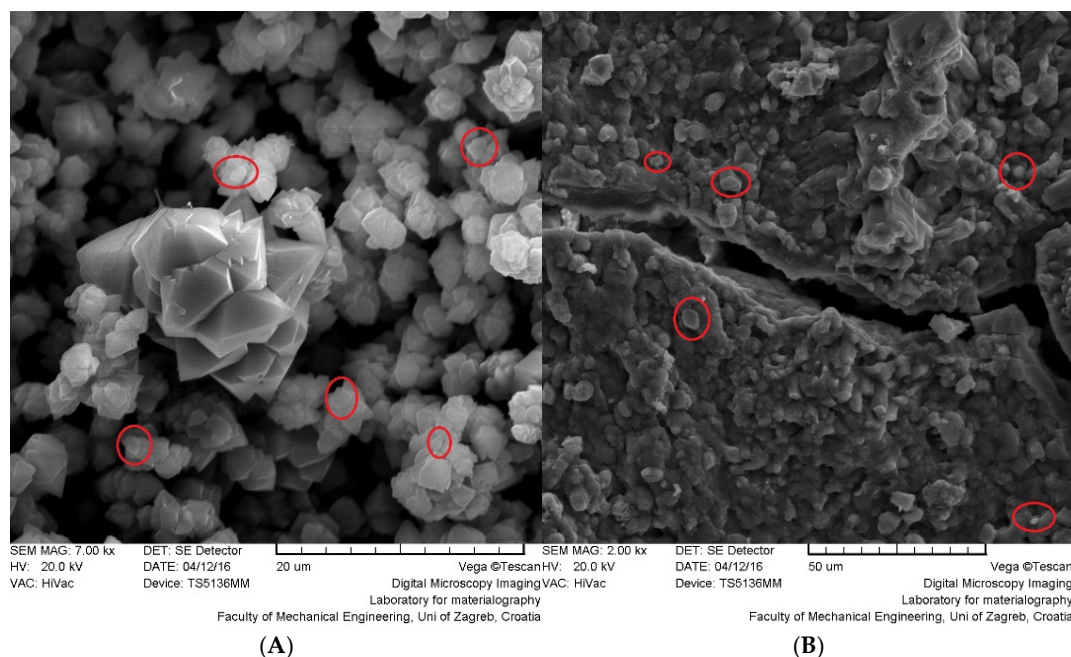
| Solutions |          |                                        |             |          |          |              |
|-----------|----------|----------------------------------------|-------------|----------|----------|--------------|
| Number    | c (Urea) | c (Zn(NO <sub>3</sub> ) <sub>2</sub> ) | Temperature | Stirring | ZnO Size | Desirability |
| 1         | 9.8      | 1.3                                    | 25.2        | 50.6     | 319.5    | 1.0          |
| 2         | 9.9      | 4.5                                    | 25.2        | 52.0     | 324.6    | 1.0          |
| 3         | 10.0     | 4.4                                    | 26.5        | 51.4     | 318.4    | 1.0          |
| 4         | 10.0     | 3.9                                    | 25.8        | 65.5     | 317.8    | 1.0          |
| 5         | 9.9      | 1.9                                    | 25.4        | 53.7     | 319.8    | 1.0          |

According to the developed model, optimal synthesis conditions for producing nanoparticles with minimal size include lower initial urea concentration, higher initial zinc nitrate concentration, higher temperatures combined with lower shaker speeds, or alternatively lower temperatures combined with higher shaker speed.

For the confirmation of the morphology and the chemical composition of obtained ZnO, SEM and SEM-EDX investigation was performed (Figure 12 and Table 10). The particle size of the synthesized Zn nanoparticles was determined from the SEM images presented in Figure 12. The average particle sizes are summarized in Table 10.

Table 10. Average size of the synthesized Zn nanoparticles obtained by SEM-EDX.

| Temperature (°C) | Medium            | Urea Concentration [mM] | Particle Size Range [μm] | Average Particle Size [μm] |
|------------------|-------------------|-------------------------|--------------------------|----------------------------|
| 25               | Redistilled water | 3.33                    | 1.383–1.556              | 1.404                      |
|                  |                   | 10.0                    | 2.310–5.582              | 4.046                      |



**Figure 12.** SEM micrographs of ZnO NPs synthesized in redistilled water at  $T = 25\text{ }^{\circ}\text{C}$ ,  $c(\text{urease}) = 1\text{ mg cm}^{-3}$ ,  $c(\text{NaNO}_3) = 0.1\text{ M}$ ,  $c(\text{Zn}(\text{NO}_3)_2) = 1\text{ mM}$ , and urea concentrations of (A) 3.33 mM and (B) 10 mM.

The results presented in Table 10 clearly demonstrate that the particle size of the synthesized zinc particles is strongly influenced by the concentration of urea used during the synthesis process. At the lower urea concentration of 3.33 mM, relatively smaller particles were obtained, with an average particle size of  $1.404\text{ }\mu\text{m}$ . In contrast, increasing the urea concentration to 10 mM resulted in the formation of significantly larger particles, with an average size of  $4.0455\text{ }\mu\text{m}$ . These findings indicate that urea concentration plays a crucial role in controlling the nucleation and crystal growth processes during particle synthesis, which is aligned with the proposed model of the process obtained by DoE methodology. A higher concentration of urea likely promotes a faster release of hydroxide ions through urea hydrolysis, thereby affecting the precipitation kinetics and favoring the growth and aggregation of zinc-containing particles. Consequently, particle size increases with increasing urea concentration under otherwise identical experimental conditions.

The SEM micrographs further reveal that, in addition to the larger micron-sized particles, smaller particles with dimensions in the nanometer range are also present at the higher urea concentration (marked with red circles). This observation suggests that the synthesis conditions promote the simultaneous formation of both micro- and nanoparticles, resulting in a heterogeneous particle size distribution. Therefore, it can be concluded that urea concentration is a key synthesis parameter governing not only the average particle size but also the overall morphology and size distribution of the synthesized zinc particles. ZnO NP concentration in suspension prior to drying and SEM-EDX measurement were determined by NTA (Table 11).

It should be noted that the particle sizes obtained by NTA and SEM are not directly comparable because the two techniques characterize the samples under different physical states. NTA determines the hydrodynamic diameter of individual ZnO nanoparticles dispersed in suspension immediately after enzymatic synthesis, yielding particle sizes in the range of approximately 190–320 nm. In contrast, SEM analysis is performed on dried samples, during which solvent evaporation promotes particle aggregation and agglomeration. As a result, SEM images reveal micron-sized structures ( $1.4\text{--}4.0\text{ }\mu\text{m}$ ) formed by the assembly of numerous primary nanoparticles into larger lattice-like agglomerates. This

morphology is clearly visible in the SEM micrographs, where the larger structures are composed of much smaller nanoscale units. Therefore, the larger particle sizes determined by SEM reflect the dimensions of the agglomerates rather than those of the individual ZnO nanoparticles.

**Table 11.** NTA instrumental setup and determined average size of ZnO NPs.

| Parameter                             | Replicate 1           | Replicate 2           | Replicate 3           |                       |                       |
|---------------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Detection Threshold                   | 13                    | 13                    | 13                    |                       |                       |
| Max Jump Mode                         | Auto                  | Auto                  | Auto                  |                       |                       |
| Max Jump Distance                     | 16.9929               | 17.2356               | 17.2470               |                       |                       |
| Blur                                  | Auto                  | Auto                  | Auto                  |                       |                       |
| Min Track Length                      | Auto                  | Auto                  | Auto                  |                       |                       |
| First Frame                           | 0                     | 0                     | 0                     |                       |                       |
| Total Frames Analyzed                 | 1498                  | 1498                  | 1498                  |                       |                       |
| Exclusion Regions                     | None                  | None                  | None                  |                       |                       |
| Parameter                             | Replicate 1           | Replicate 2           | Replicate 3           | Mean                  | Standard Error        |
| Dilution Factor                       | $1.00 \times 10^3$    | $1.00 \times 10^3$    | $1.00 \times 10^3$    | —                     | —                     |
| Particle Concentration (particles/mL) | $8.52 \times 10^{11}$ | $8.90 \times 10^{11}$ | $9.06 \times 10^{11}$ | $8.83 \times 10^{11}$ | $1.60 \times 10^{11}$ |

NTA measurements require careful optimization and documentation of both acquisition and analysis parameters, as these settings can significantly influence the obtained results. In the present study, all measurements were performed under controlled and identical conditions, including the use of a sCMOS camera, Blue 488 nm laser, fixed camera settings, and a consistent frame acquisition protocol (Table 11). The analysis parameters, including detection threshold, maximum jump distance, blur, and minimum track length, were selected and reported to ensure reproducibility between measurements (Table 2). Since NTA results are highly dependent on instrument settings and operator-defined parameters, detailed reporting of these conditions is essential for reliable interpretation and comparison of nanoparticle concentration data. Under the optimized measurement conditions applied in this study, measurements showed good reproducibility, with an average particle concentration of  $8.83 \times 10^{11}$  particles/mL and a low standard error ( $1.60 \times 10^{10}$  particles/mL). These results demonstrate the suitability of NTA for quantitative nanoparticle characterization when appropriate measurement and analysis parameters are carefully controlled.

Although SEM–EDX analysis confirmed the morphology and elemental composition of the synthesized ZnO nanoparticles, future studies should also include additional methods such as X-ray diffraction (XRD) or transmission electron microscopy (TEM) analyses. XRD would enable confirmation of the crystalline wurtzite phase and the exclusion of possible impurity phases, while TEM would provide detailed information on the morphology and size of the primary nanoparticles. Since the primary objective of this work was to investigate the enzymatic synthesis process and develop its mathematical model, these analyses were beyond the scope of the present study. Therefore, comprehensive structural characterization using XRD and TEM is planned for future investigations to further elucidate the crystallographic properties and morphology of the synthesized ZnO nanoparticles.

Although the developed response surface model provides valuable insight into the effects of the investigated process parameters on ZnO nanoparticle synthesis, its predictive capability should be interpreted with caution, as indicated by the relatively low predicted  $R^2$  value. Furthermore, the experimental response exhibited considerable variability, particularly for the NTA-derived particle concentration measurements (Table 11), which contributed to the observed prediction uncertainty. Therefore, experimental vali-

dation of the predicted optimal synthesis conditions was beyond the scope of the present study. Future work will focus on performing confirmation experiments under the predicted optimum conditions, following further optimization of the synthesis protocol and analytical methodology, and will include additional characterization techniques and statistical evaluation to further assess and improve the predictive performance of the model.

#### 4. Conclusions

A Design of Experiments (DoEs) approach combined with response surface methodology successfully identified the key process variables influencing the biocatalytic synthesis of ZnO NPs. ANOVA demonstrated that the developed two-factor interaction (2FI) model was statistically significant ( $p = 0.0164$ ), with the initial urea concentration identified as the primary factor affecting nanoparticle size. Significant interaction effects between urea concentration and temperature, as well as between urea concentration and stirring speed, further indicated that these parameters act synergistically during nanoparticle formation rather than independently.

The response surface analysis revealed that the synthesis of smaller ZnO NPs is favored by lower initial urea concentrations and higher zinc nitrate concentrations. Model optimization further indicated that nanoparticle size can be minimized under conditions of higher reaction temperature. In addition, comparable particle sizes can be achieved either at lower shaking speeds or at higher shaking speeds combined with lower temperatures, confirming a coupled interaction between temperature and agitation rate. These findings emphasize the importance of simultaneously optimizing multiple process parameters to achieve the desired nanoparticle characteristics.

Nanoparticle characterization by nanoparticle tracking analysis (NTA) proved to be suitable for determining both particle size and concentration, with an experimental variability of only 5.05%, demonstrating good measurement precision. Although the developed model showed satisfactory statistical significance and an adequate signal-to-noise ratio for process optimization, its predictive capability should be further improved and verified. Overall, the experimentally optimized conditions showed good agreement with the model predictions. Future work will focus on additional confirmation experiments and further model refinement to strengthen the correlation between predicted and experimentally obtained nanoparticle characteristics. Moreover, additional methods of investigation (TEM, AFM, XRF), as well as photocatalytic tests, are foreseen as the step toward the future application of these ZnO nanoparticles as sustainable photocatalysts for water treatment.

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