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Research Article

Optimization of ZnO nanoparticles size using response surface methodology and its effect on antibacterial properties

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Abstract

The antibacterial activity of safe and sustainable zinc oxide (ZnO) nanoparticles has huge potential in tackling antibiotic-resistant bacterial infections, especially for nano-green biomedical purposes. Various chemical methods in ZnO nanoparticles synthesis require sophisticated equipment and produce toxic as by product. It is very important to eliminate the biological risks to ensure they are safe and not display unexpected side effects. In this study, ZnO nanoparticles were synthesized using a precipitating technique with the aid of a microwave heating method. Zinc nitrate had been used as salt, sodium hydroxide (NaOH) as a reducing and precipitating agent, and gum arabic as a stabilizing agent. All the parameters in synthesizing ZnO nanoparticles, which include zinc salt concentration, NaOH concentration, microwave power, and microwave irradiation time were statistically optimized to achieve the smaller size of ZnO nanoparticles; the optimization was analysed using Response Surface Methodology (RSM) based on a statistical design of experiments (DOE). The optimum ZnO nanoparticle of 66.87 nm was achieved using gum arabic of 1.01%, 0.05 M zinc nitrate, 1.46 M NaOH, 8 min of microwave irradiation time, and 275 W of microwave heating. The ZnO nanoparticles' size showed 3.8-fold higher than the values before optimization. The optimized ZnO nanoparticle performs better in killing *Staphylococcus aureus* and *Escherichia coli*.

Keywords: ZnO nanoparticles, gum arabic, response surface methodology, antibacterial

Introduction

The synthesis of nanoparticle has been presented as a crucial part of the nanotechnology sector due to its size [1]. Nanomaterials (at least in one dimension) ranging from 1 to 100 nm have a variety of physical and chemical variations including changes in optical properties such as colour, light diffraction, solubility, hardness, strength, magnetism, heat, conductivity, and surface reactivity [2]. Within metal oxide nanoparticles, zinc oxide (ZnO) has attracted great attention in both academic and technological viewpoints due to its special physical and chemical characteristics, and broad implementations [3].

However, due to the high polarity of water, ZnO nanoparticles tend to agglomerate, leading to precipitation as a sign of instability of nanoparticles in the colloidal system [4]. Agglomeration is one of the phenomena in how nanoparticles reduce their high surface energy by sorption of the surrounding molecules [5]. As a result, these nanoparticles tend to be stabilized by depressing their surface area through agglomeration [6]. In the case of short interparticle distances, adjacent nanoparticles tend to attract one another through van der Waals forces in the solution and easily coagulate in the absence of repulsive forces. Hence, the supposed size of the primary ZnO particles turns to increase to the micrometer range as the performance of ZnO nanoparticles is hindered [7].

In this study, gum arabic, which has an important multi-functional biologically active compound was used as a stabilizing agent. ZnO nanoparticles with gum arabic are expected to provide more additional functions than ZnO nanoparticles alone. Gum arabic is a complex polysaccharide extracted from branches and stem of acacia trees [8]. Gum arabic is a non-toxic, hydrophilic, biodegradable, biocompatible polymer [9]. It is a weak polyelectrolyte, consisting of charged, carboxylate and amine groups [10]. Gum arabic is composed of 10% polysaccharide and glycoprotein complex that comprise a β-galactose backbone about

arabinose and rhamnose terminated in glucuronic acid. Due to the glucuronic acid group, gum arabic is commonly negatively charged in most circumstances [11]. Based on the pH of a solution, charge-patch, steric hindrance or bridging are formed as the gum arabic is adsorbed on the surface of particles [12].

From the literature, only a few studies had reported the synthesis of ZnO nanoparticles using gum arabic as a stabilizing agent. All these studies had employed a lower temperature (below 100 °C) and long treatment synthesis duration time; however, none of them used microwave heating to synthesis ZnO nanoparticles. Ba-Abbad et al. used the sol-gel method with a solution mixture under room temperature for 4 h to synthesis 16 nm of ZnO nanoparticles with a spherical shape [13]. Likewise, Barik et al. synthesized gum arabic-ZnO nanocomposite under room temperature with 40 nm in size [12]. Preda et al. synthesized various morphology of ZnO nanoparticles using a wet chemical method through the utilization of thermostatic bath under continuous stirring with low temperature (below 100 °C) for 3 h [14]. Liu et al. used autoclave at 80 °C for 21 h to synthesis ZnO nanorod and nanoplate crystal [15]. All the reported studies did not reveal the agglomerated size of ZnO nanoparticles but only highlighted the premier size of ZnO nanoparticles, which is inadequate in comparing its real performance in colloidal conditions.

Several trials and errors have been done to find the solution to the problematic formulation by changing one or two variables with the expectation of finding a better formulation. This traditional method, called OFAT (one factor at a time), had been previously used to study the ZnO nanoparticles synthesis using gum arabic as stabilizing agent and successfully produced 200- 300 nm ZnO-gum arabic nanoparticles [16]. The considerable experience, knowledge, and expertise of the formulator are the most important factors in deciding the "optimum" formulation and understanding of the formulation characteristics. However, many problems arise during the synthesis formulation that may lead to wastage of time, energy and resources, unpredictable outcomes, or complete failure. Therefore, in this study, a new concept of systematic experimental design, such as DOE (Design of Experiment), is used in nanoparticle synthesis development to achieve an optimum formulation with fewer experimental trials. It is a very effective tool to trace any problem during formulation, better understand salt concentration and stabilizer interactions, predict the product characteristic, and provide much information needed by the formulator to achieve the best solution or optimum formulation [17]. To date, there was no report on statistical optimization using the Response Surface Methodology (RSM) study to produce the smaller size of ZnO nanoparticles using gum arabic. The smaller size of nanoparticle is expected to demonstrate a better antibacterial effect that can have huge potential in antibacterial applications.

Materials and Methods

Nanoparticles synthesis

Gum arabic was dissolved in 100 ml of distilled water and heated for 2 min in 450W microwave to fully dissolve the gum arabic. Solid $Zn(NO_3)^2.6H_2O$ zinc nitrate was added into gum arabic solution subjected to continuous stirring. Then, the obtained solution was heated for 2 min in the microwave, running at 450W. To adjust the pH, 1 M NaOH solution was dripped into the zinc nitrate and gum arabic solution, undergoing vigorous stirring until pH 10. Again, the mixture solution was exposed to microwave heating at 450 W for 4 min. The white precipitate was cleaned using distilled water before the precipitate was dried in an oven at 80 °C. The formed ZnO nanoparticles were used for the characterization process.

Nanoparticles characterization

The nanoparticle sizes of ZnO were measured using Zetasizer instrument (ZEM5002, Malvern Instrument Ltd., Malvern, UK). The optical properties were analyzed using the UV-vis spectrophotometer (U-1800 UV/VIS Spectrophotometer, Hitachi, Berkshire, United Kingdom) in the range of 250 nm to 800 nm. The chemical composition of ZnO nanoparticles was determined using the FTIR spectrometer (iD7 ATR Nicolet iS5 Spectrometer, Thermo Fisher, USA) in the range of 400 to 4000 cm^{-1}. The XRD pattern was analyzed using the Philips X'Pert MPD (Multi-Purpose Diffractometer) XRD using Cu Ka1 radiation (k = 1.5406 nm). The X-ray powder diffraction patterns were recorded at every 0.01 in the angular range of 20–80 using monochromatic X-rays. Zn ion content in the solutions was studied by inductively coupled plasma mass spectroscopy (ICP-MS) (Agilent, 7500a, USA).

Independent variables and the levels of the screening design

A 2^5 full factorial design was used to show the statistical significance of the concentration of gum arabic, the concentration of zinc salt, the concentration of NaOH, the power of microwave heating and time of microwave heating on ZnO nanoparticle size. A two-level factorial design was a statistically-based method that involved the simultaneous adjustment of experimental factors by using only two levels, which were high and low level. The levels of the variables investigated in this study were given in **Table 1**. The settings of the ranges for factors were based primarily on the investigation of single factors (screening process by OFAT method). A total of 16 sets of experiments in **Table 2** were employed in this study to

determine the significant factors affecting the size of the nanoparticles.

Design variables for the optimization process

Response surface methodology was performed to optimize the minimal size of ZnO nanoparticles. Central composite design (CCD) with five levels and two variables was applied for the optimization of minimal ZnO nanoparticles size using Design Expert 8.0.8 software. After screening 5 factors from preliminary work, the two most significant factors to be further optimized in this study were gum arabic concentration and zinc salt concentration. ZnO nanoparticles size (nm) was set as the dependent variable. A total of 20 sets of synthesis solutions, including center points were carried out. The order of the running experiments was strictly randomized to eliminate the possible bias. **Table 3** shows the experimental design of the central composite design. The validity of the model will statically be analyzed by ANOVA.

Antibacterial evaluation: Disk diffusion

The antibacterial evaluation was determined using disk diffusion tests as described by Ruparelia et al. [18]. The colonies of *E. coli* and *S. aureus* were grown in the Tryptic Soy Broth (TSB) solution at 37°C until the late mid-log phase. A total of 50 µL bacterial samples were spread onto the solid TSB agar plates. Next, 1 cm diameter of sterilized Whatman filter paper disks were placed on the inoculated plates. Subsequently, a total of 15 µL ZnO nanoparticles mixture solution was pipetted on the filter paper disk and incubated at 37°C for 24 h before the diameter of the inhibition zone was measured.

Antibacterial evaluation: Growth kinetics

The growth kinetics assay was performed using the nanoparticles concentration at the respective MIC levels. Gum arabic-ZnO nanoparticles (125, 62.5, 31.25, 15.625, 7.812 µg/mL) were added at the mid-log phase of *S. aureus* and *E. coli* culture and was incubated at 37°C in 200 rpm incubator shaker. Culture without ZnO nanoparticles was prepared as a control. Optical density (OD) measurements were taken hourly. The growth of bacteria interacting with the nanoparticles was determined from a plot of the OD versus time.

Table 1. Independent variables and the levels of the screening design

Variable	Unit	Low level	High level
The concentration of gum arabic	%	1	2
Concentration of zinc salt	M	0.05	0.15
Concentration of NaOH	M	0.5	1.5
Power of microwave heating	Watt	250	450
Time of microwave heating	Minutes	4	10

Table 2. Experimental design of 2^5 full factorial designs

Run	Factor				
	A	B	C	D	E
1	2.00	0.15	1.50	450.00	10.00
2	2.00	0.15	0.50	450.00	4.00
3	1.00	0.15	1.50	450.00	4.00
4	1.00	0.15	0.50	450.00	10.00
5	2.00	0.05	0.50	450.00	10.00
6	2.00	0.15	0.50	250.00	10.00
7	2.00	0.05	1.50	450.00	4.00
8	1.00	0.05	1.50	250.00	4.00
9	2.00	0.05	0.50	250.00	4.00
10	1.00	0.05	0.50	250.00	10.00
11	2.00	0.15	1.50	250.00	4.00
12	1.00	0.15	1.50	250.00	10.00
13	1.00	0.05	1.50	450.00	10.00
14	1.00	0.05	0.50	450.00	4.00
15	1.00	0.15	0.50	250.00	4.00
16	2.00	0.05	1.50	250.00	10.00

Antibacterial evaluation: Antibiofilm test

As followed method from Pauzi et al., the mid-log phase culture of bacteria (1×10^8 CFU/ mL) was allowed to form biofilm after incubation at 37°C for 24 h on the glass slide (2.5 cm × 7.5 cm) [19]. The slides that contained biofilm were then washed with 0.1 M phosphate buffer saline (PBS) to eliminate excess culture media. The slides were then applied with different concentrations of ZnO nanoparticles (1000, 500, 250, 125, 62.5 µg/mL) and were incubated at 37°C for 24 h. A control was performed without adding ZnO nanoparticles. The antibiofilm was estimated by crystal violet (CV) assay. The slides were washed with 0.1 M PBS and were stained with 0.5% CV solution. The slides were then again washed using 0.1 M PBS to eliminate the excess stain. The dye was extracted using a 30% glacial acetic acid solution. The OD was measured at 590 nm. The percentage of toxicity was calculated.

Results and Discussion

Two-level half factorial design

A two-level half factorial design was constructed to determine the important parameters in the synthesis of agglomerated ZnO nanoparticles. The effects of gum arabic, zinc salt, NaOH, microwave power and time of microwave heating were screened using 25 half factorial designs. The experimental design and the obtained results are shown in **Table 4**.

Analysis of variance

Analysis of variance (ANOVA) was used to determine the significant variables. The parameters in this study were tested at 95% confidence level based on their effects. As presented in **Table 5**, the regression model was found to be significant (p <0.05), which indicates that the regression model was accurate in predicting the pattern of significant parameters for the hydrodynamic size of the agglomerated ZnO nanoparticles. With P-value <0.05, the concentration of gum arabic (A), the concentration of zinc salt (B), the concentration of NaOH (C) and microwave heating (D) were significant. These variables influenced the hydrodynamic size.

Statistical analysis

The precision of a model is expressed through a coefficient of determination (R^2) and correlation coefficient (R). As stated in Table 6, the coefficient of determination (R^2) was 0.9882 for the hydrodynamic size of agglomerated ZnO nanoparticle, and this indicates that the model is capable of perceiving 98.82% of data variability. The value of R^2 higher than 0.9 is considered to have a high correlation. Also, the R^2 value indicates that only 1.18% of the total variance is not explained by the model. Moreover, the predicted R^2 is in reasonable agreement with the adjusted R^2 for the response because it shows less than 0.2 differences between predicted and adjusted R^2 (**Table 6**).

Table 3. Experimental design of the central composite design

Run	Factor	
	A	B
1	1.51	0.04
2	0.51	0.08
3	1.51	0.04
4	0.01	0.06
5	1.01	0.06
6	1.01	0.06
7	1.01	0.10
8	1.01	0.02
9	1.01	0.06
10	0.51	0.08
11	1.01	0.06
12	0.51	0.04
13	1.51	0.08
14	1.01	0.06
15	1.01	0.06
16	2.01	0.06
17	1.51	0.08
18	0.51	0.04
19	1.01	0.06
20	1.01	0.06

Table 4. Experimental design and results of full factorial design

Run	Actual Value					
	A: gum arabic (%)	**B: Zinc salt (M)**	**C: NaOH (M)**	**D: Microwave power (Watt)**	**E: Microwave heating duration**	**ZnO nanoparticle size (nm)**
1	1.00	0.15	0.50	450.00	10.00	300.0
2	1.00	0.05	1.50	450.00	10.00	180.2
3	2.00	0.15	0.50	250.00	10.00	651.0
4	1.00	0.05	1.50	250.00	4.00	109.0
5	2.00	0.15	0.50	250.00	4.00	651.0
6	1.00	0.15	0.50	250.00	4.00	312.4
7	2.00	0.15	1.50	450.00	4.00	225.5
8	1.00	0.15	1.50	250.00	10.00	210.0
9	1.00	0.15	1.50	450.00	4.00	229.1
10	2.00	0.05	1.50	250.00	10.00	90.68
11	1.00	0.05	0.50	450.00	4.00	227.2
12	1.50	0.10	1.00	350.00	7.00	441.0
13	2.00	0.05	0.50	450.00	10.00	236.0
14	2.00	0.05	0.50	250.00	4.00	177.8
15	2.00	0.15	1.50	450.00	10.00	198.2
16	1.50	0.10	1.00	350.00	7.00	453.0
17	2.00	0.05	1.50	450.00	4.00	172.7
18	1.50	0.10	1.00	350.00	7.00	412.0
19	1.00	0.05	0.50	250.00	10.00	189.9

Table 5. Regression analysis of the full factorial design for agglomerated nanoparticles size of ZnO

Factors	**Mean Square**	***F*-value**	***P*-value**
Model	43725.97	74.28	< 0.0001 (significant)
A **(**gum arabic)	26008.01	44.18	0.0002
B **(**Zinc salt)	1.21×10^5	206.24	< 0.0001
C(NaOH)	14335.27	24.35	0.0011
D **(**Microwave power)	24248.72	41.19	0.0002
E **(**Microwave heating duration)	148.35	0.25	0.6292
AB	30916.19	52.52	< 0.0001
AD	45505.42	77.30	< 0.0001
BD	78444.81	133.26	< 0.0001
CE	52523.47	89.23	< 0.0001
Curvature	77625.79	131.87	< 0.0001(significant)
Residual	588.66		
Lack of fit	636.77	1.43	0.4460(not significant)
Pure error	888.67		

Table 6. Statistical analysis for the hydrodynamic size of agglomerated ZnO nanoparticles

Regression	
R^2	0.9882
Adjusted R^2	0.9749
Predicted R^2	0.9268

Regression equation

The regression equation was achieved from ANOVA, and all terms were included in the model equation regardless of their significance (Equation 1):

$$Agglomerated\ ZnO\ nanoparticle\ size = +260.04 + 40.32A + 87.11B - 29.93C - 38.93D - 3.04\ E + 43.96\ AB - 53.33AD - 70.02BD - 57.30CE \quad \text{(Eq. 1)}$$

Therefore, the most significant parameters, including the concentration of gum arabic, and concentration of zinc salt were further optimized using response surface methodology (RSM) since these parameters affected the hydrodynamic size of agglomerated ZnO nanoparticles.

Although the concentration of NaOH (C) and microwave heating (D) were also significant variables, these two variables were excluded in the prediction of these optimum synthesis conditions using RSM. The concentration of 1.46 M NaOH and 275 W microwave heating were selected from the best conditions with the highest desirability in the two-level half factorial design. At these higher molar concentrations, the reaction completion will be ensured [20]. At this condition, the agglomerated ZnO nanoparticles synthesis phase and reflection peaks can be indexed as pure hexagonal ZnO nanoparticles.

Besides, the microwave power was excluded in the optimization using RSM because at a higher microwave power, the nanoparticle grows bigger due to Ostwald ripening and may destroy the gum arabic as well [21]. Particle coarsening also contributes to larger particle size when high power is applied to ZnO nanoparticles synthesis. Classical sintering theory predicts that large particles meet a smaller one thus the size of large particles increases [22]. Keeping the temperature as low as possible by following the condition in the highest desirability in the two-level half factorial design was the best decision. Thus, microwave power of 275 W is the best for gum arabic thermal behaviour in stabilizing the nanoparticles in optimization using RSM.

Prediction of the optimum synthesis conditions using RSM

Central composite design (CCD) was applied to predict the optimum synthesis conditions for the smallest nanoparticles size production. The experiment with a different combination of synthesis conditions involving the concentration of gum arabic (A) and concentration of zinc salt (B) was performed. The size of agglomerated ZnO nanoparticles was collected as a response. **Table 7** contains the list of range for the factors, which were selected by preliminary work of parameter screening, preferring only the 2 highest significant factors. In the experimental design, low and high factors were coded as -1 and +1, the midpoint was coded as 0, and α value was set as 2. The collected nanoparticles size ranged from a minimum value of 71 nm to a maximum value of 859 nm, depending on the experiments. As recommended by the software, a square root transformation for the model of agglomerated ZnO nanoparticles size was implemented, as shown in suplementary S1.

Analysis of Variance

Analysis of variance (ANOVA) was essential in determining the suitability and importance of the quadratic model. The P-value for the model source, each member of the model, and interactions were described in suplementary S2. The P-value less than 0.05 indicates significant model conditions with a 95% confidence interval. Furthermore, values greater than 0.1 indicate that the model conditions are not significant. Therefore, the conditions of model B, A^2, and B^2 were significant factors that influenced the size of agglomerated ZnO nanoparticles. Moreover, a P-value for the term 'lack of fit', greater than 0.05 indicates that the model for agglomerated ZnO nanoparticles was not significant. This means that the model matched the collected response data, and it was desirable for the next experiment.

Regression equation

Equation 2 illustrates the obtained model for agglomerated ZnO nanoparticles size derived from ANOVA as presented in terms of the coded factors in the equation below,

$$Sqrt\ (Agglomerated\ ZnO\ nanoparticle\ size) = +8.52 + 0.55A + 1.15B - 0.28AB + 5.12A\text{^}2 + 0.97B^2 \quad \text{(Eq. 2)}$$

Statistical analysis

The model fitness for agglomerated ZnO nanoparticle size was represented by R^2 (0.9699) as indicated in **Table 8**. This value indicates that the model can explain 96.99% of the response variability. On the other hand, the determination factor (adjusted R^2) for ZnO nanoparticle size was calculated as 0.9592, indicating that the model did not include only 4.05% of the total variation. This value indicates that the observed and predicted agglomerated ZnO nanoparticle size is in a good agreement. The resulting model was applied to predict the optimum cultural conditions that minimize the agglomerated ZnO nanoparticle size.

Table 7. The value of level for each factor in CCD

Factor	Low Level Star Point (-2)	Low-Level Factorial (-1)	Centre Point (0)	High-Level Factorial (+1)	High-Level Star Point (+2)
A: gum arabic concentration	0.31	0.51	1.01	1.51	1.71
B: Zinc salt concentration	0.02	0.04	0.06	0.08	0.10

Table 8. Statistical analysis for agglomerated ZnO nanoparticles size

Regression	
R^2	0.9699
Adjusted R^2	0.9592
Predicted R^2	0.9089

Diagnostic model

The diagnostic model is a tool used to ensure that the experimental design and resulting data align with expected statistical behaviors. In this context, it helps verify whether the synthesis of ZnO nanoparticles follows a predictable pattern based on various independent variables, such as gum arabic concentration, zinc salt concentration, NaOH concentration, microwave power, and microwave heating time.

Before adopting the model, the satisfaction of the model should be tested through applicable statistical analysis. The basic analysis is to look at the normal probability plot of the residuals, the number of standard deviations from the original values based on the predicted values. The normal probability plot showed that the residuals follow a general distribution.

Figure 1 illustrates the residuals versus the run sequence in the experiment. This graph is essential for identifying any potential time-based effects or patterns that could suggest model inadequacies. A random scatter of points without any discernible pattern would suggest that the model is appropriate and that the residuals are independent of the run sequence, meaning that time-based effects are minimal or non-existent. The goal is to confirm that the residuals, which represent the difference between observed and predicted values, are distributed randomly, indicating that the model fits well and that the assumptions of independence and constant variance are met. The relationship between actual data versus predicted values displays the real response data against the predicted responses as indicated in **Figure 1**. A linear distribution was detected that points out a suitable model. The differences between actual and predicted values are subsequent to the normal distribution.

Figure 2 displays a plot of predicted values against actual values for the size of agglomerated ZnO nanoparticles. In an ideal scenario, the points would align along a 45-degree line, suggesting that the predicted values from the model match closely with the actual experimental data. Any significant deviation from this line would indicate a discrepancy between the model's predictions and the observed results, potentially highlighting areas where the model could be improved. The plot helps assess the accuracy of the model and whether it can reliably predict nanoparticle sizes under different experimental conditions.

Response surface plot (model graphs)

After the desired goals were set up for each response, the graphical optimization tool could be employed to create an overlay contour plot of responses highlighting the desire response area. The optimum combination would be generated by employing a numerical optimization tool. This tool sorted out the possible combinations that matched goals to solutions based on the desirability scale in the range of 0 to 1.

The relationship between the response and variables was pictured through a response surface contour plot (**Figure 3**), 3D curve (**Figure 4**) and interaction plots (**Figure 5**) constructed according to the quadratic model. The elliptical arrangements of the curves display a significant relationship between the independent variables. Three-dimensional plots indicating the interactions of effective variables for both responses are depicted in achieving optimum synthesized agglomerated ZnO nanoparticles in regard to the minimum nanoparticle size.

As seen in **Figure 3**, it is observed that the higher gum arabic and zinc salt, the bigger the sizes of nanoparticles. It is obvious that the size of agglomerated nanoparticles decreases by decreasing

the concentration of zinc salt. The mean size of ZnO nanoparticles was sensitive to gum arabic concentration. At a higher concentration of gum arabic, higher aggregation of particles was observed that lead to the bigger size of nanoparticles in DLS analysis. This might be due to an increase in the layer on the surface of particles, which led to the crossing at the point of flocculation of many particles.

The steric effect of gum arabic as a stabilizer was determined through stabilizer concentration, which directly affects the size of nanoparticles. Self-association and aggregation properties of gum arabic over a large concentration range can also be the factor of an increasing size of agglomerated ZnO nanoparticles. However, for a lower concentration of gum arabic concentration (0.76% in this study), the surface of the agglomerated ZnO nanoparticles gradually coated by gum arabic decreased and made the size of agglomerated ZnO nanoparticles to be increased as well. This might be because the agglomerated ZnO nanoparticles were not enough to stabilize; causing the nanoparticles to agglomerate more and influence the size of nanoparticles. Therefore, fewer gum arabic molecules are needed to sufficiently coat the surface of the nanoparticle to provide a steric barrier against aggregation.

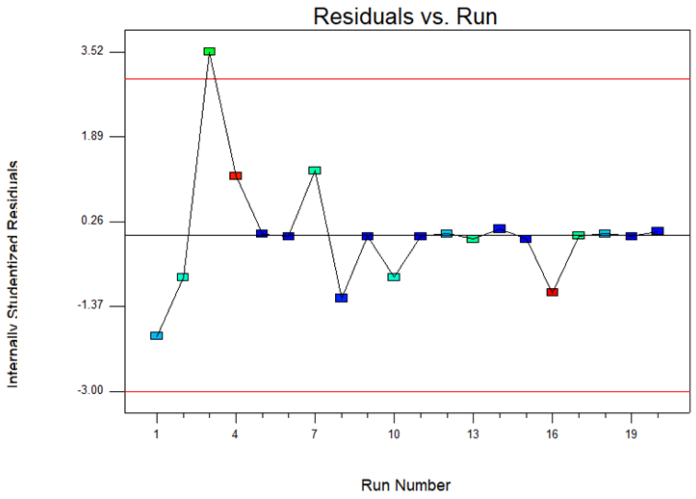

Figure 1. Residual diagnostics of a crossed model for agglomerated nanoparticles size: Residual vs run

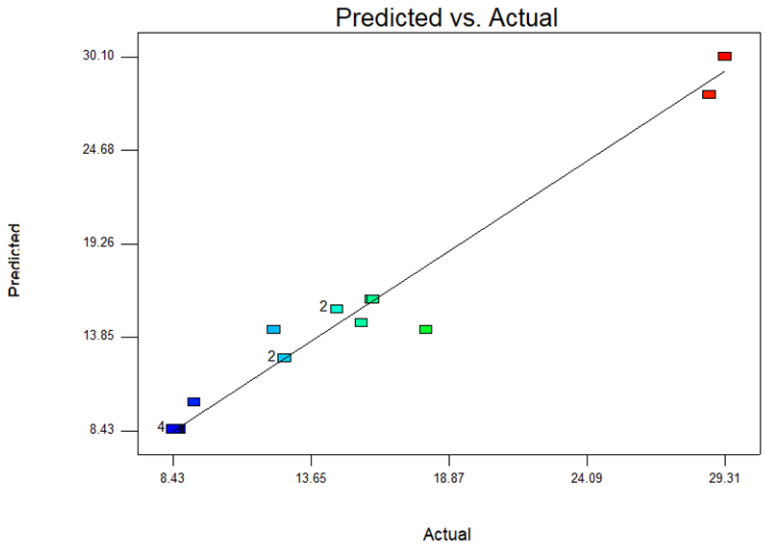

Figure 2. Residual diagnostics of a crossed model for agglomerated nanoparticles size: Predicted vs actual

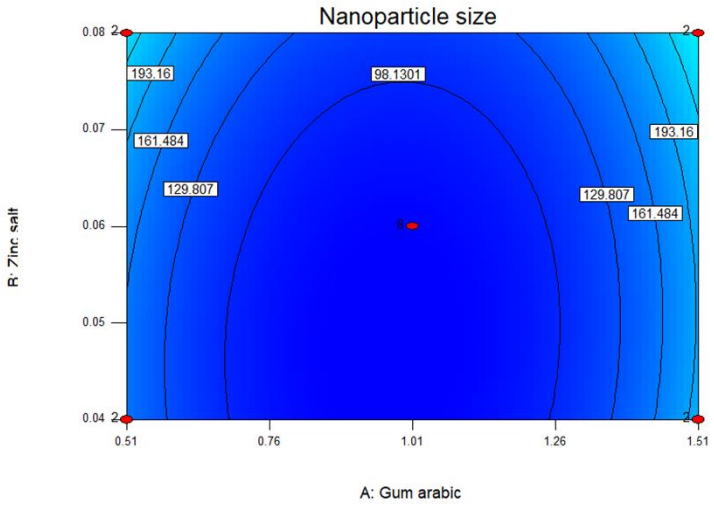

Figure 3. Optimum contour plot

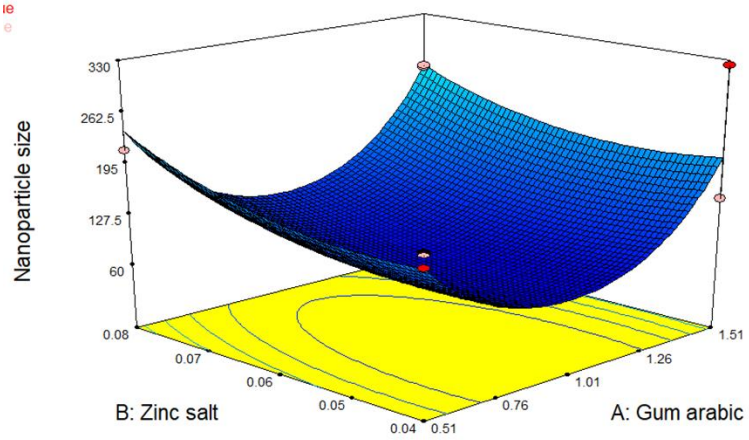

Figure 4. 3-D response surface plot showing the influence of zinc salt and gum arabic on agglomerated ZnO nanoparticle size

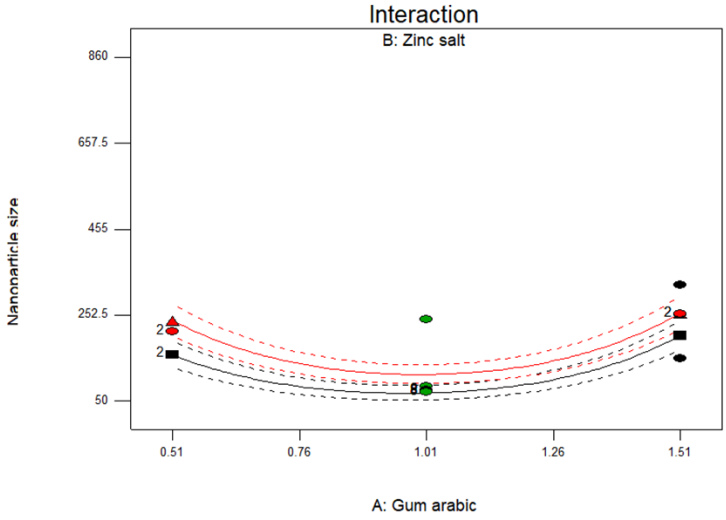

Figure 5. Interaction plot

It can be seen that the ZnO nanoparticle size increased sharply when the zinc salt concentration increased. This suggested that when nanoparticles are exposed to a solution with high salt content, agglomeration can occur. When the particles are agglomerated, they behave like larger particles and have high sedimentation rates. The optical properties of the aggregated particles are generally radically different from those of the individually dispersed particles. The increase in ionic strength caused by the high concentration of zinc salt has a significant effect on the size of ZnO nanoparticles. Therefore, for the synthesized ZnO nanoparticles of smaller size, a lower salt concentration of 0.05 M can be used.

Optimization and formulation

The last step in DOE is to confirm that the design model (RSM) was statistically acceptable and robust to predict the optimum response for the selected formulation. This was done by comparing the experimental and the predicted values with 95% of the prediction confidence interval. **Table 9** presents specific optimum conditions for the size of the defined nanoparticles of agglomerated ZnO nanoparticle aiming at minimum nanoparticle size. **Table 10** presents that all goals are united into one desirability function. The optimum formulation of 1.01% gum arabic and 0.05 M zinc salt with high desirability of 1 was used to achieve an optimum value of 66.87 nm agglomerated ZnO nanoparticles.

Validation of optimized condition

Specific information was applied in the final optimization step to compare the experimental data with the theoretical expectation's values. Additional experiments were performed under these optimal conditions to compare accurate prediction results with experimental values. **Figure 6** displays the nanoparticles size distribution of this optimized condition using the following conditions; 1.01% gum arabic, 0.05 M zinc nitrate, 1.46 M NaOH, 275 W microwave and 8 min of irradiation time. As presented in **Table 11**, the experimental results show that the experimental value of agglomerated ZnO nanoparticles size (72.24 nm) was close to the predicted value (66.87 nm).

Figure 7 shows the UV-visible characterization to prove the presence of agglomerated ZnO nanoparticles. The wavelength of bulk ZnO was 350-390 nm. **Figure 8** shows the functional groups of gum arabic-ZnO nanoparticles using FTIR analysis. The broad band at 3449 cm^{-1} matches up to O-H mode of hydroxyl groups, and a peak at 1354 cm^{-1} might be due to carboxylic acid. A peak near 1033-835 cm^{-1} might be due to NO_3 bonding that can be associated with the absorption of nitrate group on the ZnO surface. The peak in the region between 424 and 475 cm^{-1} agreed to Zn-O stretching.

The presence of ZnO, was confirmed from the XRD pattern in **Figure 9**. The diffraction peaks recorded match well with the standard ZnO hexagonal wurtzite structure. The diffraction peaks were observed at 2θ value with the peak at 31.8, 34.2, 36.2, 47.5, 56.6, 62.81, 66.31, 67.91, 69.01, 72.61 and 76.81 that correspond to (100), (002), (101), (102), (110), (103), (112), (200) and (201) reflection lines of hexagonal ZnO nanoparticles respectively. All the analyses confirm that the optimized nanoparticles were ZnO. Therefore, the model was effectively confirmed. Furthermore, **Table 11** presents a comparison between the results obtained from the experimental conditions before and after the optimization process. The ZnO nanoparticles' size showed 3.8-fold higher than the values before optimization. Therefore, the optimum synthesis condition for minimizing ZnO nanoparticles size was achieved by using the response surface methodology.

Antibacterial and antibiofilm properties of optimized ZnO nanoparticles

The optimized formulation was selected to investigate the antibacterial and antibiofilm properties of *S. aureus* and *E. coli*. **Table 12** shows the comparison of inhibition zones against *S. aureus* and *E. coli* using ZnO nanoparticle after optimization. Both strains display a larger inhibition zone after being treated with optimized ZnO nanoparticles, which indicated that the smaller ZnO nanoparticles contributed to a more effective antibacterial agent. The smaller size of ZnO nanoparticles was expected to create easier fusion in agar medium and display a larger clearance zone. Anzabi reported a larger clearance zone after using optimized ZnO nanoparticle with Berberis vulgaris extract using RSM [23].

The antibacterial properties of both strains were further investigated by monitoring the growth kinetics after the optimized ZnO nanoparticles were added to the bacteria culture at the mid-log phase. **Figures 10** and **11** show the comparison of growth pattern for ZnO nanoparticles before and after optimization on *S. aureus* and *E. coli*. OD measurements were dropped drastically when ZnO nanoparticles were added at the mid-log phase. The optimized ZnO nanoparticle displays a better effect in killing the bacteria. The higher toxicity of smaller-size nanoparticles occurs because more particles are needed to cover the bacteria surface and generate higher concentrations of ROS, which are released by ZnO nanoparticles on the surface of the cells.

However, *E. coli* seems not severely infected like *S. aureus* when smaller nanoparticles were applied. Yamamoto also found that the effect of particle size on

E. coli was less than *S. aureus* [24]. This was expected because of the difference in the structure and chemical composition of the bacteria. In *E. coli*, layers of peptidoglycan, lipid, and lipopolysaccharide are present on the cell surface, while only the peptidoglycan layer is found in *S. aureus* [25].

The antibiofilm properties of optimized ZnO nanoparticles were investigated for both *S. aureus* and *E. coli* biofilm after 24 h of treatment. CV assay highlighted a considerable inhibitory activity treatment with ZnO nanoparticles (62.5, 125, 250, 500 and 1000 µg/mL); a significant reduction in the biofilm was observed in a dose-dependent manner. The optimized ZnO nanoparticles display more than 2-fold higher antibiofilm activity at a lower concentration compared to ZnO nanoparticles before the optimization. However, for a concentration above 500 µg/mL, optimized ZnO nanoparticles displayed little increase in the percentage of antibiofilm toxicity (**Figures 12** and **13**).

Table 9. Desirability specifications of numerical optimization for crossed design

Criteria	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Gum arabic	In range	0.51	1.51	1	1	3
Zinc salt	In range	0.04	0.08	1	1	3
Agglomerated ZnO nanoparticle size	Minimize	71	859	1	1	5

Table 10. Optimization conditions, prediction and desirability of model

Gum Arabic	Zinc Salt	Agglomerated ZnO Nanoparticle Size	Desirability	Selection
1.01	0.05	66.87	1	Selected

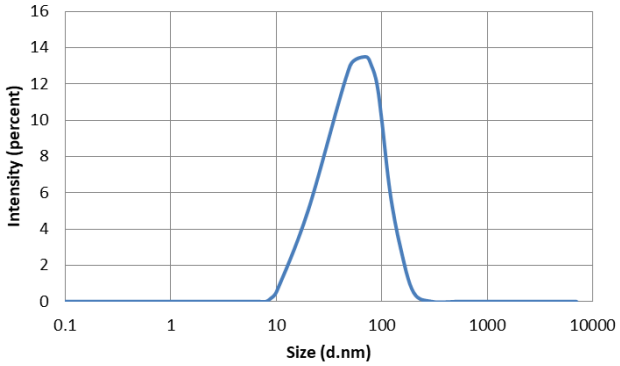

Figure 6. Size distribution of optimized condition by the intensity of ZnO nanoparticles based on DLS analysis

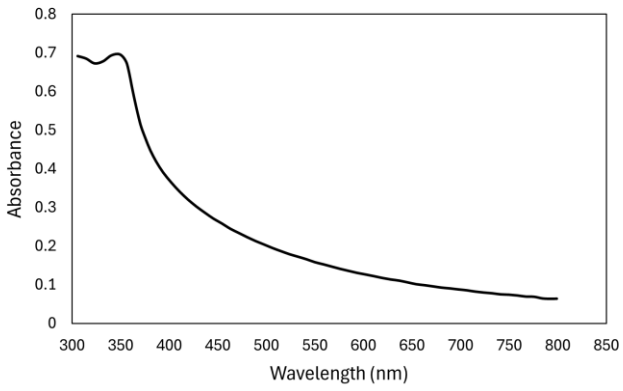

Figure 7. UV-vis spectra of ZnO nanoparticles at the optimum condition

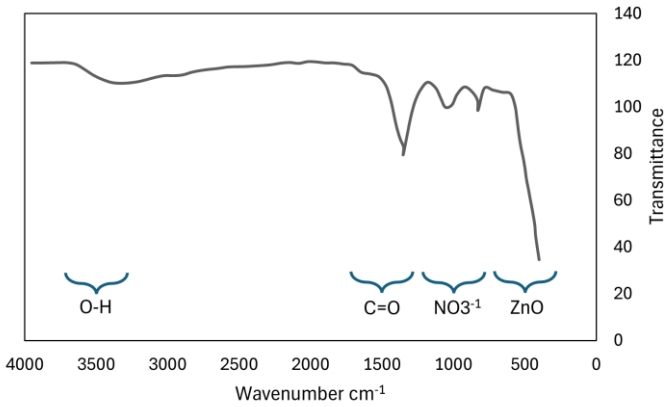

Figure 8. FTIR spectra of ZnO nanoparticle at the optimum condition

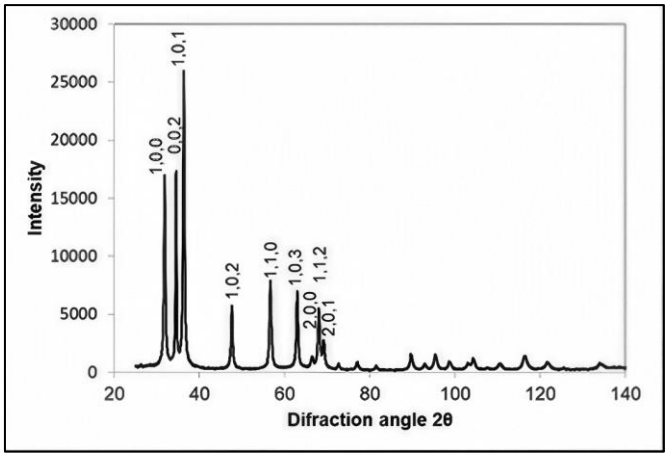

Figure 9. XRD spectra of ZnO nanoparticle at the optimum condition

Table 11. Summary of the optimized synthesis condition for minimal ZnO nanoparticles size

Parameter	Before Optimization	After Optimization
Gum arabic (%)	1.5	1.01
Zinc Salt (M)	0.1	0.05
	ZnO nanoparticle size (nm)	
Predicted		66.87
Actual	254	72.24

Table 12. Inhibition zone of optimized ZnO nanoparticle using RSM

	S. aureus	***E. coli***
Before optimization (cm)	2.6 (± 0.06)	2.0 (± 0.08)
After optimization (cm)	3.1 (± 0.08)	2.6 (± 0.04)

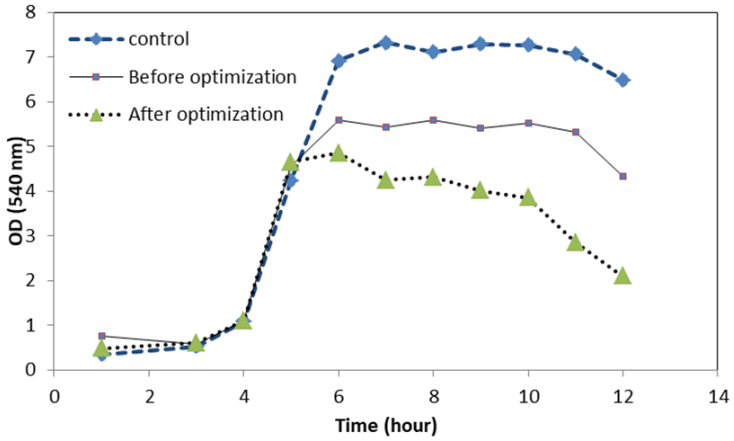

Figure 10. Comparison of growth kinetics of ZnO nanoparticle on *S. aureus* after optimization

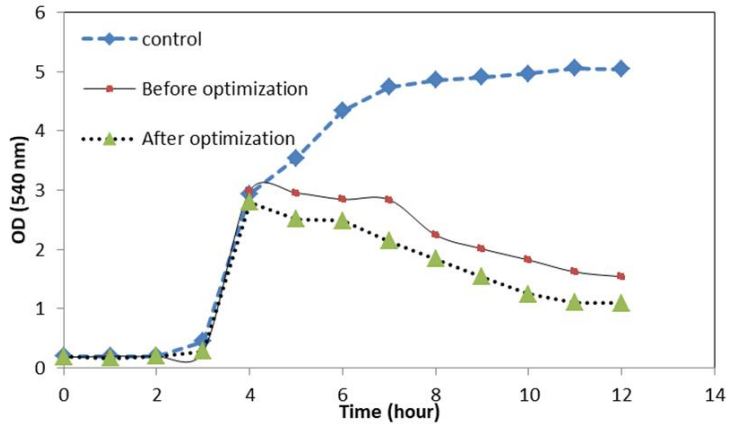

Figure 11. Comparison of growth kinetics of ZnO nanoparticle on *E. coli* after optimization

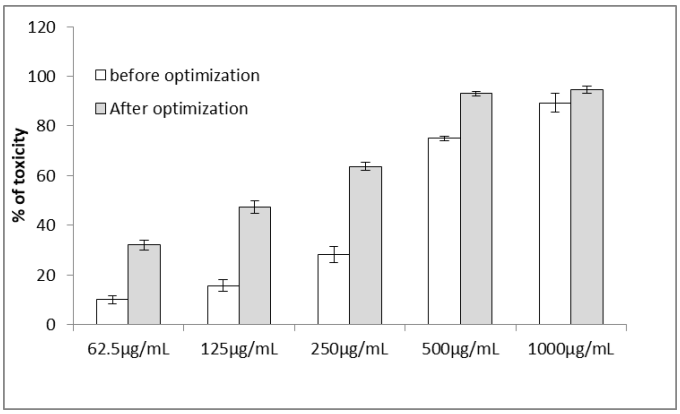

Figure 12. Comparison of agglomerated ZnO nanoparticles toxicity to *S. aureus* biofilm after optimization

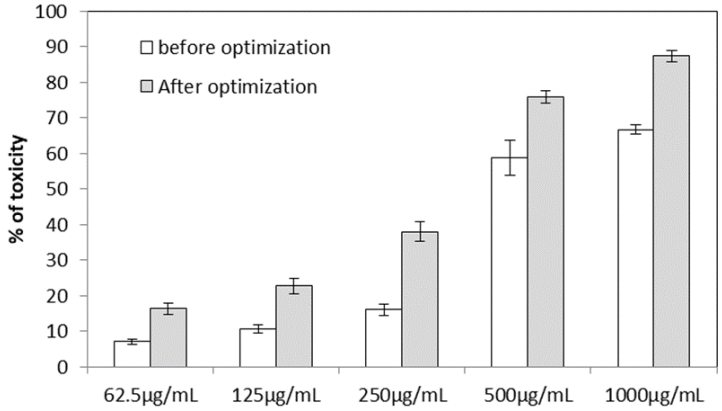

Figure 13. Comparison of agglomerated ZnO nanoparticles toxicity to *E. coli* biofilm after optimization

Conclusion

From a two-level half factorial design, the effects of significant factors were successfully studied to screen and understand their influence on nanoparticles size. Based on the result obtained from the RSM study using Central Composite Design (CCD), the optimum formulation chosen are, 1.01% gum arabic, 0.05 M zinc nitrate, 1.46 M NaOH, 8 min of irradiation time and 275 W of microwave power that produced ZnO nanoparticles of 66.87 nm. The ZnO nanoparticles' size showed 3.8-fold higher than the values before optimization. The optimized ZnO nanoparticle displays a smaller size and demonstrated a better effect in killing *S. aureus* and *E. coli*.

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