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Zinc Oxide (ZnO) Nanoparticles from *Eichhornia Crassipes* for Inactivating Pathogenic Bacteria in Greywater

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Greywater, pathogenic bacteria, *Eichhornia crassipes*, ZnO nanoparticle, Response Surface Methodology (RSM)

Abstract

Pathogenic bacteria are dangerous bacteria found in greywater produced from household activities. *E. Coli* and *S. aureus* are among bacteria that have become an environmental issue and cause a negative impact on the ecosystem. The ZnO nanoparticles (NPs) have been found to be good deactivating agents for bacteria in greywater. This research examines how pathogenic bacteria in greywater can be neutralised by utilising ZnO NPs derived from the aquatic grass, *Eichhornia crassipes*. *E. Coli* and *S. aureus* were made using the serial dilution technique by successive resuspension in predetermined amounts of liquid diluent. *E. Coli* and *S. aureus* were matured by culturing on agar plates. The pathogenic inactivation efficiency of ZnO NPs was optimised by varying ZnO NPs dosage and irradiation time using Response Surface Methodology (RSM). The microstructural analysis demonstrated that the ZnO consisted of bigger and smaller particles with sizes in the scale of 51.9 nm to 31.0 nm. The greatest inactivation efficiency of ZnO-*E. crassipes* for bacteria in greywater was 3.498 log for *E. Coli* and 3.368 log for *S. aureus*. The ZnO-*E. crassipes* has the potential to inactivate pathogenic bacteria.

1. Introduction

Greywater is wastewater that is produced from household activities, excluding toilet waste. It has a diverse composition that is prompted by the standard of living, equipment, and weather circumstances [1]. Greywater comprises approximately 75% of the total household wastewater volume, a figure that can escalate to around 90% with the implementation of dry toilets [2]. Greywater is a significant alternative source of freshwater, particularly in Middle Eastern nations facing arid or semi-arid climates [3]. In Malaysia, greywater comes from suburban or individual houses disconnected from the sewerage line and commonly discharged directly to the drainage [2]. It can cause environmental degradation, such as high bacterial loads, nutrient discharge, and salinity impacts from untreated greywater [4]. It can deteriorate the water quality and public health. One of the main concerns is the existence of microbial multiplicity, mainly in kitchen greywater. The greywater contains a significant number of microorganisms, especially from the bathroom and kitchen. The dangerous bacteria can grow and form biofilm in kitchen and bathroom sinks [5]. These microorganisms severely attack the immune deficient people and make them more susceptible to illness [6]. Among the investigated biofilm pathogens, *Escherichia coli* (*E. Coli*) has the highest count in all biofilm samples in the bathroom and kitchen. *E. Coli* and *S.*

aureus are found in kitchen greywater with high concentrations (3.8×10^2 to 8.0×10^7 CFU/10 cm^2 and from 2.1×10^2 to 9.2×10^5 CFU/10 cm^2, respectively [7]. The high concentration of *E. Coli* and *S. aureus* in kitchen greywater has led to the growth of pathogenic bacteria. The organic food wastes are discharged without proper treatment, which could be a cause of non-pathogenic and pathogenic bacteria. Sink drains in homes have been identified as a likely source of outbreaks in several investigations [3]. In addition, harmful bacteria have been discovered to be transmitted through the kitchens and toilets of households. Many microorganisms can cause infections because they can persist on the humid surfaces of kitchens and bathrooms for several hours to weeks [8].

Zinc oxide (ZnO) is the most capable contender among the numerous metal oxide nanomaterials due to its air endurance, nontoxicity, and ability to aggregate due to its high surface energy. ZnO has been recognised as an efficient antibacterial agent in the photodegradation of bacteria in greywater [9], [10]. The antibacterial properties of zinc oxide (ZnO), graphene oxide (GO), and ZnO complex against harmful bacteria have been qualitatively assessed using a modified agar disc diffusion method, with removal efficiency up to 98.4% [11]. The ZnO NPs have been synthesised from various sources such as Cocos nucifera [10], Passiflora caerulea [12], and Pongamia pinnata [13]. The ZnO NPs from *Eichhornia Crassipes* (*E. crassipes*) for inactivating pathogenic bacteria have been reported [14]. However, its performance optimisation from kitchen greywater has not been conveyed in the literature. This work investigates the performance optimisation of ZnO NPs in activating pathogenic bacteria.

This research comprises two phases: first, the creation of zinc oxide nanoparticles from *E. crassipes*, and second, enhancing their effectiveness in deactivating E. coli and S. aureus by employing Face Centered Central Composite Design (FCCD) within the framework of Response Surface Methodology (RSM). The ZnO NPs dosage and irradiation time were optimised for deactivation of *E. Coli* and *S. aureus*.

2. Materials and Methods

2.1 Materials

E. crassipes was obtained from a plant shop in Parit Raja, Batu Pahat, Johor, Malaysia. The analytical reagent grade zinc acetate ($Zn(CH_3CO_2)_2$) and sodium hydroxide (NaOH) were obtained from Sigma Aldrich.

2.2 Methods

The research methodology encompasses several key steps in the investigation. Initially, ZnO NPs were synthesised, followed by a thorough characterisation to understand their physical and chemical properties. Subsequently, a specific culture media was prepared to facilitate bacterial growth. The synthesised ZnO NPs were then employed to evaluate their effectiveness in inactivating bacterial activity. The statistical analysis will include the use of Analysis of Variance (ANOVA) to measure the implications of observed differences. Additionally, the Box-Cox plot was utilised for data transformation and normalisation, enhancing the reliability of the findings. This comprehensive approach ensures a systematic exploration of the antimicrobial properties of ZnO NPs and contributes to a robust understanding of their potential applications in combating bacterial infections.

2.3 Synthesis of ZnO NPs

The foreign materials and defective parts were removed from the *E. crassipes* sample. Fresh leaves of *E. crassipes* were dried in sunlight and then cut into small pieces and put in a beaker containing 400 mL of distilled water and 1.6 grams of zinc acetate. The NaOH was added into 8 mL of plant extract drop by drop to obtain a pH of 12. The solution was stirred at 200 rpm until it turned into a white solution, and it was centrifuged at 4000 rpm for 25 minutes. Then, the mixture was first washed out with distilled water and then with ethanol. The precipitate was desiccated at 100°C overnight in an electric oven before being calcined at 550°C in a furnace for 3 hours [15].

2.4 Characterisation of ZnO NPs

The synthesised ZnO-*E. crassipes* was categorised using Field Emission Scanning Electron Microscopy (FESEM) (Zeiss, GeminiSem and Sigma FE-SEM). A voltage of 5 kV was used in FESEM to acquire the electron images.

2.5 Culture Media for Bacteria Growth

The agar diffusion process was consumed to establish the antibacterial activity as shown in Fig. 1. The interruption of bacteria was strengthened in the nutrient broth instrument. Trial bacteria were spread around the external of agar plates. A small volume of sample was smoothly pressed over the centre of the nutrient agar plate, immunised with bacterial cells from the guarded interaction of the sample [13]. Firstly, the plates were protected at the ideal temperature of 37 °C for growing the selected bacteria after the growth medium in the petri dish had been inoculated with the desired bacteria. Agar plates can be maintained upside down in the refrigerator for an

Int. Journal of Integrated Engineering Vol. 17 No. 3 (2025) p. 184-193

extended period after the necessary amount of growth has been attained to keep the bacteria for future experiments. Refrigeration slows down the growth and reproduction of bacteria but does not necessarily kill them. Most bacteria can survive for some time in a refrigerator, especially if the temperature is not consistently low. Refrigerators are typically set to temperatures around 4 °C (39°F) or lower, which slows down bacterial growth significantly.

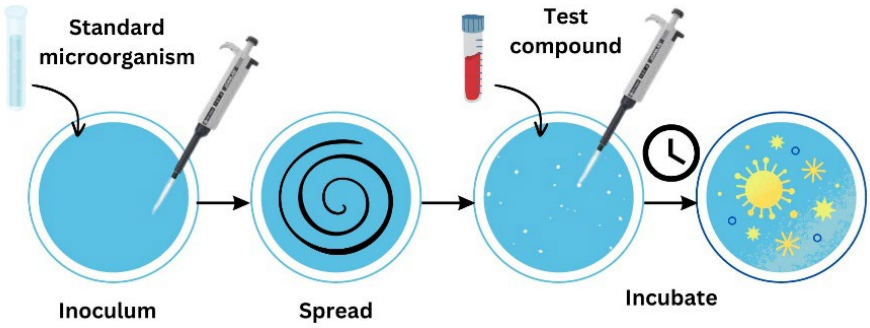

Fig. 1 *Antibacterial activity using agar diffusion method*

2.6 Inactivation of Bacterial Activity using ZnO NPs

The autoclave was used to sterilise all the lab equipment for microorganism procedures. Fig. 2 shows that 50 mL of greywater from the kitchen was used in this experiment. The samples were designed using response surface methodology (RSM). The parameters of ZnO NPs dosage and irradiation time were improved using Face Centered Central Composite Design (FCCD) of RSM. The values of the parameters are shown in Table 1, and the obtained design is shown in Table 2. The serial dilution procedure was used to learn the antibacterial activity of ZnO NPs on *E. Coli* and *S. aureus* in greywater. The serial dilution method was applied to calculate and measure the effectiveness of ZnO-NPs in pathogenic bacteria, and 5 ml mini centrifuge tubes were used for serial dilution. The serial dilution needs 4 ml of distilled water that has already been sterilised and 1 ml of the bacteria mixed and cultured on an agar plate. The bacteria culture on agar plates was incubated at 37 °C for 2-3 days. ZnO NPs will diffuse into the agar and inactivate the expansion of the *E. Coli* and *S. aureus* and growth colonies.

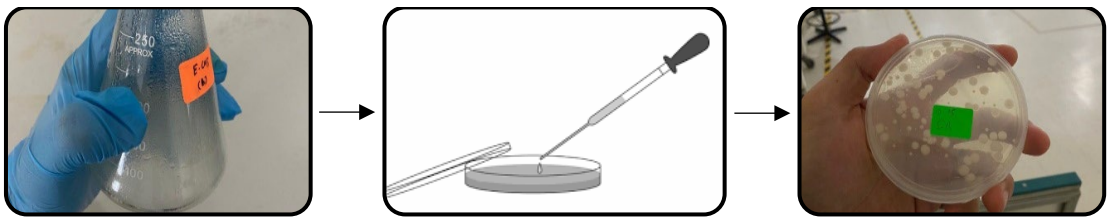

Fig. 2 *Inactivation of bacterial activity using ZnO NPs*

Table 1 *The parameters and values used for RSM design*

Factor	Lowest	Highest
ZnO NPs dosage (g)	0.01	0.1
Reaction time (min)	10	60

Table 2 *RSM design of samples*

Experiment No.	ZnO NPs dosage (g)	Reaction time (min)
1	0.055	60
2	0.055	35
3	0.01	10
4	0.055	35
5	0.055	35
6	0.055	35
7	0.055	10
8	0.1	10
9	0.01	60
10	0.01	35
11	0.1	60
12	0.1	35
13	0.055	35

3. Results and Discussion

3.1 Classification of ZnO-*E. crassipes*

The microstructural analysis results of ZnO prepared from *E. crassipes* are shown in Fig. 3. The smaller crystallites self-assemble to produce larger grains with a hexagonal shape. Particles with diameters in the range of 31.9 nm and 51.9 can be seen in Fig. 3. The fundamental constituents of zinc and oxygen showed that the concentration of surface chemisorbed oxygen species was higher than the concentration of zinc. The characteristics of ZnO NPs synthesised from *E. crassipes* are compared with ZnO NPs prepared using other plant sources in Table 3. The common shape of ZnO NPs is hexagonal and spherical [16]. The ZnO NPs with proportions in between 50 to 500 nm have the same effect on suppression of bacterial growth. The spherical NPs perform very well throughout antibacterial activity due to their capability to penetrate the cell barrier of pathogens. The results show that the ZnO NPs synthesised using *E. crassipes* show similar characteristics to those of ZnO NPs produced using other plant sources in terms of their size, composition, and adsorption peak.

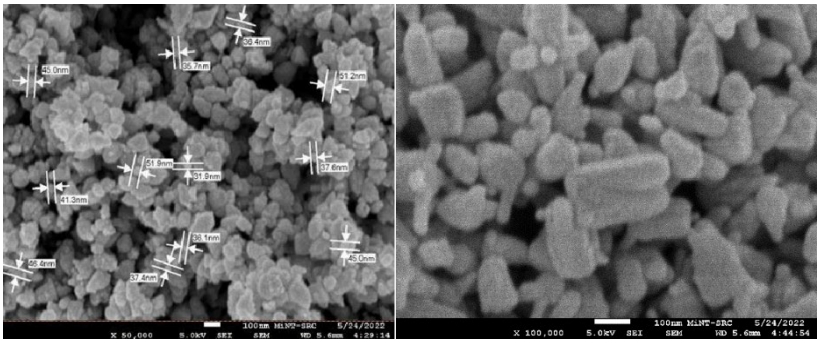

Fig. 3 *SEM images of synthesised E. crassipes-ZnO NPs*

Table 3 *The parameters and their values used for RSM design*

Plant	Shapes	Size of Nanoparticles (nm)	Reference
Cocos nucifera (L)	Hexagonal	11.01	[10]
Cissus quadrangularis	Hexagonal	27.3	[17]
Sonchus Oleraceus	Hexagonal	11	[11]
Melia azadarach	Spherical	2.72	[18]
Ocimum basilicum	Spherical	2.72	
Eichhornia Crassipes	Hexagonal	31.9	This study

Int. Journal of Integrated Engineering Vol. 17 No. 3 (2025) p. 184-193

3.2 Antibacterial Activity of ZnO NPs

The antibacterial performance of ZnO NPs for the inactivation of *E. Coli* and *S. aureus* is exhibited in Table 4. The highest inactivation is 3.498log and 3.368log for *E. coli* and *S. aureus* at 0.1 g of ZnO NPs dosage, irradiation time of 60 minutes, and speed of 150 (rpm). The lowest efficiency of ZnO-NPs is 3.977 log and 3.826 log for *E. coli* and *S. aureus* at a dosage of 0.01 g, irradiation time of 35 minutes and speed of 150 rpm. The variance in how well ZnO NPs work against E. coli versus S. aureus stems from the discrete chemical compositions of their cell walls [16]. E. Coli is a type of bacteria that is classified as gram-negative, occupying extreme levels of lipopolysaccharides. On the other hand, S. aureus is a gram-optimistic bacteria exemplified by an abundant cell wall, predominantly due to improved levels of saccharides and glutamic acid. These components possibly assist as shielding measures against the adverse impacts of nanoparticles on bacterial cells [19]. At the high dosage of 0.1g of ZnO-NPs and irradiation time of 60 min, fewer bacterial colonies grow in the agar plate, which shows the effectiveness of the ZnO-NPs in deactivating pathogenic bacteria, while at the low dosage of 0.01g of ZnO-NPs and irradiation time of 35 min, the more bacterial colonies grow. The low dosage of ZnO NPs and low irradiation time result in the low removal of bacteria. Based on a previous study, the antimycotic activity of ZnO-NPs is highly determined on the concentration of ZnO-NPs used. The ZnO NPs alongside the test fungus varied from 35 to 85 mg/mL, with the maximum MIC of 85 mg/mL recorded against Penicillium expansum and the bottommost MIC of 35 mg/mL reported alongside Aspergillus niger [20].

Table 4 *Antibacterial performance of ZnO NPs beside E. coli and S. aureus*

Experiment No	ZnO-NPs dosage(g)	Reaction time (min)	Treated *E. Coli* (log)	Treated *S. aureus* (log)
1	0.055	60	3.857	3.806
2	0.055	35	3.74	3.785
3	0.01	10	3.69	3.672
4	0.055	35	3.74	3.785
5	0.055	35	3.74	3.785
6	0.055	35	3.74	3.785
7	0.055	10	3.623	3.544
8	0.1	10	3.505	3.397
9	0.01	60	3.892	3.763
10	0.01	35	3.977	3.826
11	0.1	60	3.498	3.368
12	0.1	35	3.536	3.516
13	0.055	35	3.74	3.785

The ANOVA results for antibacterial execution of ZnO NPs beside *E. coli* are shown in Table 5. The coefficient of determination (R^2) is 0.7985. The RSM showed an important ($p \leq 0.05$) response, which matched the parameters that were considered, as well as a great R^2. Likewise, the anticipated and examined amounts demonstrated no significant ($p > 0.05$) variances. The expected R^2 of 0.5068 is not close to the Adjusted R^2 of 0.7582, and the variance is more than 0.2. This happened because serial dilution is only done twice, and the bacteria colonies that grow cannot be counted. The satisfactory accuracy of 14.0125 showed an acceptable indicator. Hence, this model can be used to traverse the design outer space. The model F-value of 19.82 shows that the model is significant comparative to the noise. There is a 0.03% probability that the large F-value is due to noise. On the other hand, the model p-value is 0.0003. The p-values larger than 0.1000 imply that the model terms are not momentous. If there are many irrelevant model terms, the p-values less than 0.0500 show that the model terms are substantial. In this case, the concentration of pathogenic bacteria is a significant model term.

Table 5 *ANOVA for quadratic model for E. Coli*

Source	Sum of Squares	Degree of freedom f	Mean Square	F-value	p-value	
Model	0.2041	2	0.1020	19.82	0.0003	significant
A-ZnO-NPs dosage	0.1734	1	0.1734	33.68	0.0002	
B-time	0.0307	1	0.0307	5.96	0.0348	
Residual	0.0515	10	0.0051			
Lack of Fit	0.0515	6	0.0086			
Pure Error	0.0000	4	0.0000			
Cor Total	0.2556	12				
Standard Deviation	0.0718					
Mean	3.71					
R^2	0.7985					
Adjusted R^2	0.7582					
Predicted R^2	0.5068					
Adeq Precision	14.0125					
C.V. %	1.93					

The ANOVA results of ZnO NPs for inactivating the *S. aureus* bacteria are presented in Table 6. The model's F-value of 23.54 indicates that it lacks significance compared to the background noise. There is a 0.03% probability that the great F-value is due to due to noise. On the other hand, the model p-value is 0.0003, which shows that it is significant. The Predicted R^2 is 0.4293, which is not close to the adjusted R^2 of 0.9038, and the variance is more than 0.2. The high values of R^2 confirm that the model can be used to predict *S. aureus* inactivation within the experimental range. This occurred due to the same reason explained earlier. The adequate precision of 13.4966 shows a passable indication, and the proportion above 4 is necessary. Hence, this model can be applied to traverse the design space.

Table 6 *ANOVA for linear model for S. aureus*

Source	Sum of Squares	Degree of freedom f	Mean Square	F-value	p-value	
Model	0.3040	5	0.0608	23.54	0.0003	significant
A-ZnO-NPs dosage	0.1601	1	0.1601	61.98	0.0001	
B-time	0.0175	1	0.0175	6.77	0.0353	
AB	0.0036	1	0.0036	1.39	0.2763	
A^2	0.0393	1	0.0393	15.22	0.0059	
B^2	0.0367	1	0.0367	14.22	0.0070	
Residual	0.0181	7	0.0026			
Lack of Fit	0.0181	3	0.0060			
Pure Error	0.0000	4	0.0000			
Cor Total	0.3221	12				
Standard Deviation	0.0508					
Mean	3.68					
C.V. %	1.38					
R^2	0.9439					
Adjusted R^2	0.9038					
Predicted R^2	0.4293					
Adeq Precision	13.4966					
C.V. %	1.38					

The 3D graph of antibacterial growth of *E. Coli* is illustrated in Fig. 4. The performance of ZnO NPs at the varying ZnO NPs dosage and time ranges between 3.498 to 3.977. The desired design point for ZnO NPs dosage

Int. Journal of Integrated Engineering Vol. 17 No. 3 (2025) p. 184-193

was from 0.01 g and above, while for irradiation time, the range was from 50 to 60 minutes. The results are consistent with Asadi and Moeinpour's optimisation search of *E. Coli* deactivation showed that raising the silver-covered magnetic nanocomposite concentration from 2 to 10 mg mL-1 resulted in a superior deactivation scale of *E. Coli* in a short time (30 min). The gap involving the dispersed spots and the average line implies whether the decrease was underestimated or exaggerated. The models become more accurate if the confidence bounds are narrowed. Three variable arrangements within the original factorial scales were used to explore the established models to validate them.

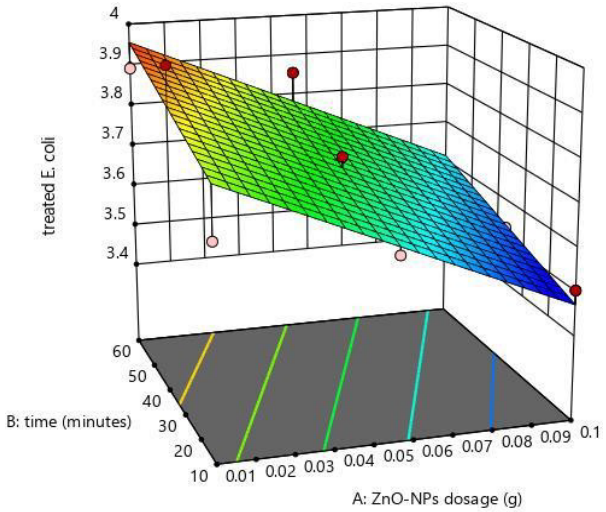

Fig. 4 *3D graph of the effect of ZnO NPs dosage against irradiation time for E. coli pathogenic bacteria*

The box-cox plot is shown in Fig. 5. It is used to identify the optimal power transmission. The graph shows the least lambda values as well as lambdas with a 95% confidence level. The current lambda is 1, and the best lambda is -0.31. The confidence level (CI) for lambda is (-11.44, 10.81), and the recommended transform is nine (lambda = 1). The maximum value of the correlation coefficient is at lambda = -0.3. The plot also shows the present power shift, which is important in this case. The graph shows the minimum lambda values as well as lambdas at 95% CI low is -11.44, while the 95% CI high is 10.81. The plot also shows the current power revolution.

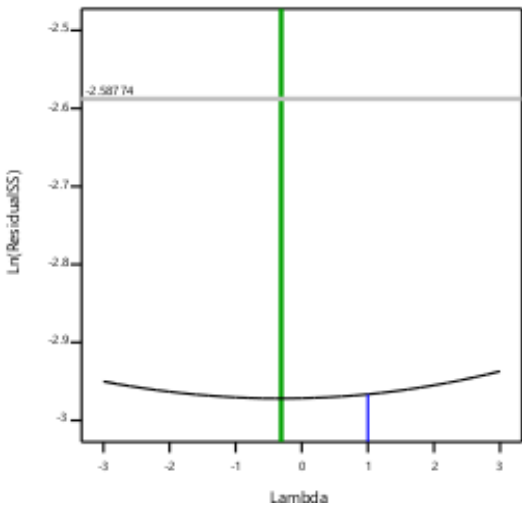

Fig. 5 *Box-Cox plot for power transform*

The 3D graph of the influence of ZnO NPs dosage and irradiation time on the antibacterial growth of *S. aureus* is shown in Fig. 6. The significant response can be observed on ZnO NPs dosage from 0.01 g and irradiation time in the range from 50 to 60 minutes, which shows that it could reach to higher value to become the significant peak. The increase in ZnO-NPs dosage considerably increased the inactivation efficiency of *S. aureus*. These results show that the major efficient factors for deactivation of pathogenic bacteria are NPs dosage and irradiation time.

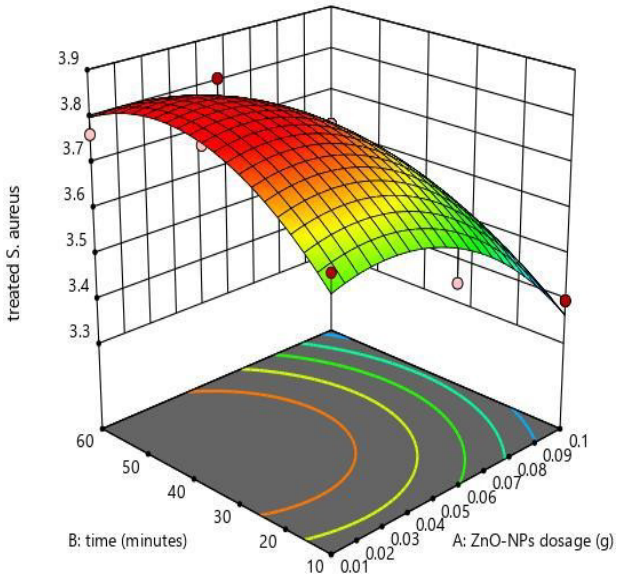

Fig. 6 *3D model graph of the effect of ZnO NPs dosage and irradiation time on the deactivation of S. aureus*

The box-cox graph for deactivation of *S. aureus* is shown in Fig. 7. The graph shows that the current transform is none, lambda is 1, and the best lambda is 3. The 95 per cent confidence interval may not be displayed since it is outside the +/-3 lambda limits.

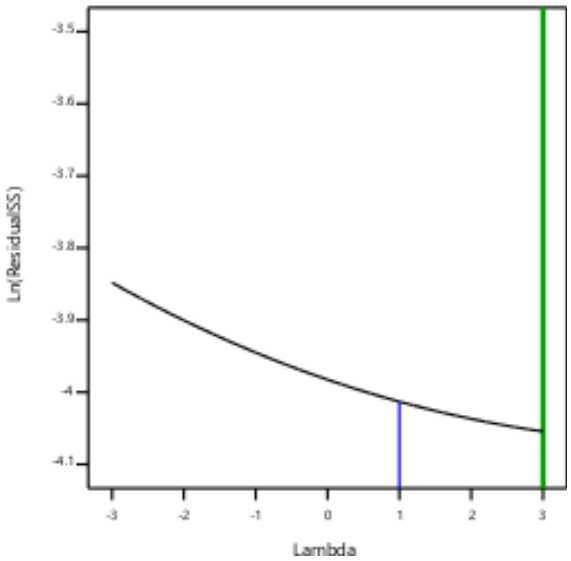

Fig. 7 *Box-Cox plot for power transform*

4. Conclusion

The ZnO NPs were magnificently produced from *E. crassipes* extract through a green synthesis process. The microstructural analysis exposed that the ZnO NPs particles were in the range of 51.9 nm and 31.9 nm. The *E. Coli* and *S. aureus* were successfully grown using the serial dilution process. Bacteria grow in countable colonies. The highest treated *E. Coli* and *S. aureus* are 3.498 log and 3.368 log with ZnO Nps. Dosage of 0.1g, 60 min and 150 rpm.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of the paper.

Author Contribution

The authors confirm contribution to the paper as follows: ***Original draft:*** *Muhammad Eizwan Safuan;* ***Review and editing:*** *Nurul Atikah Heshammuddin & Ahmer Ali Siyal;* ***Research supervision:*** *Radin Maya Saphira Radin Mohamed;* ***Resources:*** *Mohd Hairul Khamidun. All authors reviewed the results and approved the final version of the manuscript.*

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