Inactivation disinfection property of *Moringa Oleifera* seed extract: optimization and kinetic studies

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Abstract. This paper presents the statistical optimization study of disinfection inactivation parameters of defatted *Moringa oleifera* seed extract on *Pseudomonas aeruginosa* bacterial cells. Three level factorial design was used to estimate the optimum range and the kinetics of the inactivation process was also carried. The inactivation process involved comparing different disinfection models of Chicks-Watson, Collins-Selleck and Homs models. The results from analysis of variance (ANOVA) of the statistical optimization process revealed that only contact time was significant. The optimum disinfection range of the seed extract was 125 mg/L, 30 minutes and 120rpm agitation. At the optimum dose, the inactivation kinetics followed the Collin-Selleck model with coefficient of determination (R^2) of 0.6320. This study is the first of its kind in determining the inactivation kinetics of *pseudomonas aeruginosa* using the defatted seed extract.

1. Introduction

Moringa oleifera seed is a biomaterial commonly utilized for drinking water treatment in various part of the developing countries (specifically in Africa). The seed extract has been harnessed for water treatment and purification, documented in various researches [1]. Comparing *Moringa oleifera* with conventional chemical coagulants reveals it advantages such as cost effectiveness, ready availability, production of bio-degradable sludge, lower sludge volume, and production of less hazardous, non-corrosive by-product. As a result of these advantages, the seed extract is eco-friendly and cheap which proves to be a good alternative to chemical coagulants [2], [3]. The seed extract contains bioactive agents with excellent coagulation properties [4] and some previous researches reveal that the coagulation efficiency of *Moringa oleifera* seeds can be greatly enhanced by extracting active agents with salt solution having one valence electron such as NaCl, KCl etc [5]. The salt solution increased the coagulation capacity performance to about 7.4 times better than that when using distilled water. This improved performance according to the research was envisaged to be a protein-protein dissociation caused by the salt solution. Few other researchers report that a polypeptide “flo” present in the seed function as coagulant and also exert antibacterial effect on harmful bacterial strains. 4 αL-rhamnosyloxy-benzyl isothiocyanate has been identified as the bioactive antimicrobial agent which is common to both defatted and normal seed extracts. This antimicrobial agent is about 8-10% of the seed chemical composition [6], [7]. Most studies have focused on the antibacterial effect of the seed extract on different microbes such as *Escherichia coli, salmonella typhi*, however, its application as a disinfectant for water treatment is an emerging area that is worth exploring. Although few researches on the disinfectant property of the seed extract have be done with focus on *E.coli* as the target microbe in water. However, Both *Pseudomonas aeruginosa* and *E.coli* are both gram negative

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microbes, predominantly present in the environment whose natural habitat is water surface. *E.coli* is identified as a microbial indicator for fecal contamination while *Pseudomonas aeruginosa* is as an opportunistic pathogen whose transmission is through water [8], [9], [10], [11]. This means removal of *pseudomonas aeruginosa* in water is also crucial to maintain the aesthetic state of drinking water making it fit for consumption. Few studies on using *moringa oleifera* seed extract as a disinfectant in water treatment was conducted lately in a research carried out [12] the disinfection process were 31 minutes, 85rpm mixing speed and 3.25 mg/L dosage was concluded as the optimum disinfection conditions. In another similar study, the optimized conditions of the seed extract was utilized against E.coli microbes where the inactivation kinetics of the seed extract was explored. In the research, it was concluded that the seed extract followed a first order reaction and was well fitted with the Chicks-Watson disinfection kinetics model [13]. The optimization of the disinfection process conditions of *Moringa oleifera* seed extract as disinfectant is an emerging area with few researches conducted. Hence, it is essential to study the process condition of the seed extract on other bacterial strains such as *P.aeruginosa* for comparative purposes. It is also crucial to understand the inactivation kinetics of the seed extract during disinfection process which provides an insight into how the microorganisms behave during disinfection process over a period of time. This requires using various disinfection models such as the Chick-Watson, Collin-Selleck and Hom disinfection models to understand the inactivation mechanism of the seed extract. The Chicks-Watson model is limited in various disinfection process because the rate of kill is not constant. Rather, it decreases with time that depends on the form of disinfectant, type of organism to be inactivated and other operating conditions [14]. The Collins-Selleck model developed addressed deceleration rate (like a convex curve) of disinfection [8] which describes the declining rate of inactivation by observing lag in real system otherwise known as tailing. The tailing phenomenon occurs as a result of an ineffective inactivation of the subpopulation of the microbial population due to the disinfectant exposure [15]. The objectives of this study are to determine the optimum inactivation disinfection process conditions of defatted seed extract using three level factorial design under the response surface methodology, to determine the order of reaction and the inactivation kinetics of the seed extract by exploring the different disinfection inactivation models on *Pseudomonas aeruginosa* bacterial strain.

2. Materials and methodology

The following materials and methods were used in this study.

2.1. Sample preparation

Dry *Moringa oleifera* seed were de-husked and two grams of defatted *Moringa oleifera* seed powder were weighed into a liter of distilled water. Mixing rate was maintained at 6000rpm in a centrifuge for 10 minutes. The resulting solution was filtered and stock solution of about 1000mg/L was made [16].

2.2. Water sample

A thousand bacterial cells of *Pseudomonas aeruginosa* were spiked inside distilled water to make the synthetic water which was used throughout this research.

2.3. Inoculum preparation

P.aeruginosa cells obtained from laboratory stock solution at the Department of Biotechnology Engineering (IIUM) were inoculated into 10mL of LB broth with agitation overnight at 37oC. Bacterial cells inoculum density were determined with haemocytometer and about1000cells/mL inoculum density was measured.

2.4. Total viable count (TVC)

The measure of microorganism activity in water is determined by changes in the bacterial population by a process called TVC. The procedure involves taking 1 mL of the sample into a petri dish and molten LB agar was poured. The agar solidifies and the dish incubated at 37 0C for 24 hours. The concentration of the bacteria present are determined by the presence of colonies.

2.5. Three level factorial Design

The process conditions were determined using the three level factorial design. The optimum concentration of the factors were determined and the experimental region was illustrated for the highly significant factors. The three important factors during disinfection process are dosage, agitation and time and these factors were studied at low, medium and high levels. The factorial design using design expert software for three factors is 32 experimental runs with 6 centre points as shown in Table 1.

Table 1. Experimental design using three level factorial design in response surface methodology and the corresponding output of bacterial count

Run	Dosage (mg/L)	Time (minutes)	Agitation (rpm)	TVC (CFU/mL)
1	100	30	100	29
2	125	30	100	15
3	150	30	100	30
4	100	60	100	28
5	125	60	100	12
6	150	60	100	28
7	100	90	100	31
8	125	90	100	14
9	150	90	100	34
10	100	30	120	27
11	125	30	120	14
12	150	30	120	30
13	100	60	120	30
14	125	60	120	13
15	150	60	120	28
16	100	90	120	32
17	125	90	120	15
18	150	90	120	32
19	100	30	140	29
20	125	30	140	12
21	150	30	140	31
22	100	60	140	25
23	125	60	140	13
24	150	60	140	29
25	100	90	140	34
26	125	90	140	15
27	150	90	140	33
28	125	60	120	12
29	125	60	120	15
30	125	60	120	12
31	125	60	120	12
32	125	60	120	14

The second order polynomial model was used to analyse the observation.

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X^2_3 \quad (1)$$

where *Y* is the dependent factor (Bacterial Count); X_1, X_2, X_3 are independent factors (dosage, contact time and agitation); β_0 is the intercept term; β_1, β_2, β_3 are linear coefficients; β_{12}, β_{13}, β_{23} are the interaction coefficients, β_{11}, β_{22}, β_{33} are the quadratic coefficients.
The model was evaluated by analysing regression coefficient values, ANOVA (analysis of variance), *p*-values and *F*-values that reveals the goodness of fit of the model and coefficient of determination, R^2. The Design Expert 8.0.7.1 trial version (Stat Ease Inc., Minneapolis, USA) a statistical software was used to determine the regression model in predicting parameters that have significant effects on the disinfectant ability of seed extracts.

2.6. Validation of experimental model

The point prediction feature of the statistical software Design Expert 8.0.7.1 trial version predicted the combinations which were used to validate the model. Experimental combinations of four different runs were executed and both experimental and predicted results were compared.

2.7. Inactivation kinetic models

The inactivation models were determined with residual colonies estimated at a given time intervals, optimum dosage and optimum agitation. The data collected from the experimental procedure was used to fit the different disinfection inactivation models such as the Chicks, Watson, Collin-Selleck, Homs models using the following equations.

$$\ln\left(\frac{N}{N_0}\right) = -\Lambda C^n t \tag{2}$$

$$\ln\left(\frac{N}{N_0}\right) = -\Lambda_{CS}\left[\ln(Ct) - \ln(b)\right] \tag{3}$$

$$\ln\left(\frac{N}{N_0}\right) = -\kappa C^n t^m \tag{4}$$

Where equation 2 is the chicks-watson model, equation 3 is the Collin-sellecks model and equation 4 is Homs model. Where N is total of organisms present at time t, N_0 is the total of organism present at time 0, t is time, k is the rate constant, Λ is coefficient of specific lethality signifying the relative potencies of disinfectants, *n* is Hom dilution coefficient unit less, and *m* is Hom time exponent unit less. Multiple regression analysis plotting of Hom's model gives values for k, n and m. The model with the highest coefficient of determination R^2 result was selected as the best fitted. Also, multivariate regression statistical analysis with respect to the seed extract was examined.The t-test was used to assess the significance level of the regression coefficient [17].

3. Results and discussion

3.1. Disinfection parameters

The disinfection parameters range used in this optimization research was based on finding from earlier research [12]. In their research, the use of one factor at a time was used to establish the optimum range of the parameters where the disinfectant property of the seed extract was harnessed. The range of *Moringa oleifera* dosage according to the study was between 100mg/L to 200mg/L. The research also concluded that the seed extracts inhibitory action is due to the presence protein in the seed. These proteins are believed to be lipophilic and cationic in nature, acting like a molecular knife that can disrupt the microbial cell wall electrostatically and binds inside the cytoplasmic membrane of the bacterial cell [19], [20], [21] and as a result, for this study the range was fixed at 100-200mg/L. The contact time is also an important factor in the disinfection process. The effect of contact time was also investigated in several articles and most researchers concluded that the seed extract is mostly bactericidal within 30 minutes to 90 minutes. Although there is quite some discrepancies about efficient timing of the extract. In a study by [12], their reports revealed that the longer the contact time, the greater the rate of kill and in some other study, and the rate of kill of the seed extract against

microbes was within 60 minutes. In this optimization study, 30 minutes to 90 minutes was selected and for the mixing rate100 rpm to 140 rpm were selected based on reports from earlier studies showing mixing rate increases the rate of kill in higher magnitudes [14] and from previous research, significant reductions in the bacterial cells were achieved were within 100-140 rpm agitation rate [18].

3.2. Optimization of process conditions using three level factorial design

3.2.1. Statistical analysis of results

A second order regression equation was developed that indicates the dependence of contact time, dosage and mixing rate on the disinfectant property of the seed extract. The multiple regression analysis of the second order model equation reveals the empirical relationship between the process parameters and response, shown in the equation below and the analysis of variance results are reported in Table 2.

$$Y=12.56+0.56A+1.28B+0.000C+16.09A^2+2.26B^2-0.24C^2-0.33AB+0.083AC+0.42BC \quad (2)$$

where the total viable count for *P.aeruginosa* is the response (Y) and A, B and C are dosage, contact time and agitation, respectively.

Table 2. Analysis of variance (ANOVA)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	2237.77	9	248.64	118.32	< 0.0001	
A	5.56	1	5.56	2.64	0.1182	
B	29.39	1	29.39	13.98	0.0011	
C	0	1	0	0	1	
A^2	1852.37	1	1852.37	881.45	< 0.0001	
B^2	36.48	1	36.48	17.36	0.0004	
C^2	0.42	1	0.42	0.2	0.6597	
AB	1.33	1	1.33	0.63	0.4342	
AC	0.083	1	0.083	0.04	0.844	
BC	2.08	1	2.08	0.99	0.3302	
Residual	46.23	22	2.1			
Lack of Fit	38.23	17	2.25	1.41	0.3768	non-significant
Pure Error	8	5	1.6			
Cor Total	2284	31				

Analysis of variance (ANOVA) was utilized to check for the adequacy of the model and the results are reported in Table 2. The F value of 118.32 and p-value of <0.0001 suggest that the model is significant. The not significant lack of fit suggests that the calculated experimental responses satisfactorily fit with the model. The higher values of R^2 (0.9798) and adjusted R^2 (0.9715) also revealed the efficacy of the model. [22]. Based on this, R squared value for the model was significant and much fitted. Adequate precision value of 25.11 shows that the model is able navigate the design space. Coefficient of variation (CV) of about 6.37 indicates a good degree of precision and a low CV shows that the experiment is reliable. The regression equation shows the coefficient values listed in Table 2. The significance of the coefficient is indicated by the p-values revealing the interaction between independent variables. The p-values reveals the significant corresponding coefficient and the responses shows that only the linear coefficients C (time), and one quadratic coefficient were significant ($p<0.05$).

3.2.2. Three dimensional (3D) surface plot

The three dimension plots shown in Figure 1 were based on the function of two variables with the

other variable being at its optimum level. The saddle or elliptical nature of surface contour plots shows the significant interactions among variables [23].

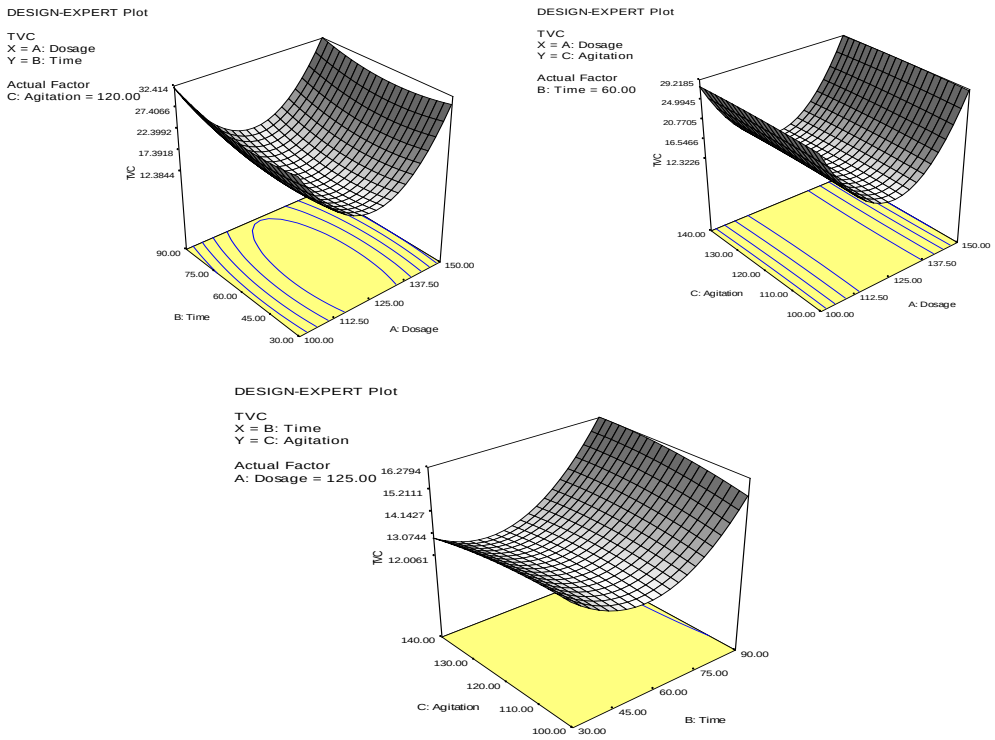


Figure 1. 3D graphical representation of surface plot

The interaction between dosage and contact time is illustrated in Figure 1a. The response surface curves showed an elliptical contour plots shape between the contact time and dosage revealing a significant interaction between these two variables. The predictive optimum range is between 120 mg/L to 125 mg/L for the dosage at a low contact time. In Figure 1b, the elliptical contour plot shows a well define optimum operating conditions between agitations in the range of 116rpm to 120rpm indicating that the effects of interaction between the two factors was significant. In case of dosage and mixing rate (Figure 1c), the response plot was elliptical depicting interaction between them. A good interaction was observed between these two variables and the predictive optimum range is between 30 minutes to 60 minutes for the contact time, 116 rpm to 120 rpm for the mixing rate.

3.3. Validation of experimental model

The second-order regression model was validated with some sets of experiments replicated thrice and were performed according to the point prediction of Design Expert presented in Table 3. The result shows that the optimum disinfection process occurred at 125mg/L, contact time of 30 minutes and 120 rpm mixing rate.

Table 3. Validation of the optimum values

Run order	Dosage (mg/l)	Time (minutes)	Mixing rate (rpm)	TVC (experimental)	TVC (predicted)	Error
1	100	60	120	27	20.07	0.260
2	140	62	100	18	19.01	0.056
3	130	33	120	18	17	0.056
4	125	30	120	17	18.07	0.063

3.4. Disinfection kinetic models

The plot of log survival of the *P.aeruginosa* bacterial colonies against time showed a deceleration of the process called "tail". The tailing as shown in Figure 2 may be explained by a vitalistic hypothesis in which individual bacteria in a population are not identical, and their inherent resistance is distributed in a permanent (time-independent) manner [24]. Also, the presence of tailing revealed in Figure 2 indicates ineffective inactivation of the microbial population when exposed to the seed extract disinfection process [15]. Another possible explanation might be as a result of decrease in the germicidal properties of defatted *Moringa oleifera* extract with time that is observed from the plot. The bacteria population were gradually building up over a period of time which might be as a result of natural heterogeneity in resistance among the microorganisms [8], [15].

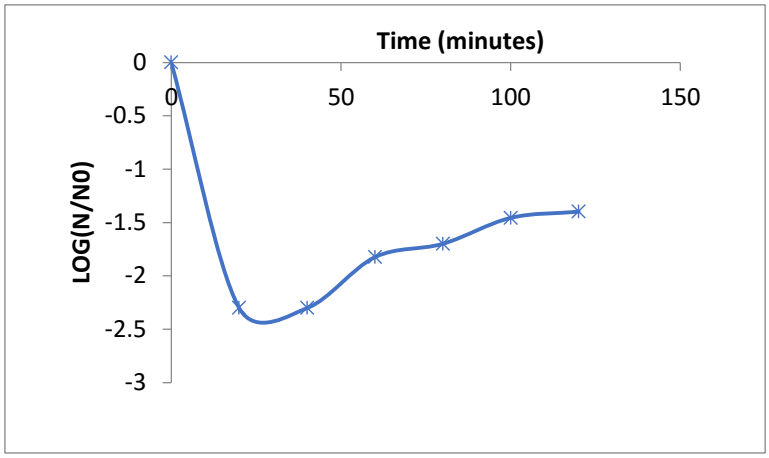


Figure 2. Plot of log survival of *P.aeruginosa* bacterial cells against time

It is also hypothesized that the tailing behaviour indicate that bacterial populations generally referred to as homogeneous are heterogeneous in nature as they comprise of mixtures of cells in varying states such as physiology etc. Hence, from the plot, it is clear that the inactivation kinetics did not follow a first order reaction which shows the deviation from the Chick-Watson model. Other disinfection models such as Hom model and Collins-Selleck empirical model are used in this research to account for deviations from the first order kinetics of the Chicks-Watson model of inactivation kinetics. The three disinfection models (Chick-Watson, Collins, Hom) were determined using linear multivariate regression analysis and results compared for *Pseudomonas aeruginosa* bacterial strain are summarized in Table 4. The coefficient of determination determined showed that the Collins-Selleck model best describe the data with R2 of 0.632 for *Pseudomonas aeruginosa*.

Table 4. Coefficient determination for different disinfection kinetic models for *P.aeruginosa*

Disinfection kinetic model	R^2	Adjusted R^2
Chick-Watson	0.035	-0.158
Collin-Selleck	0.632	0.558
Hom	0.160	-0.008

The results from Table 4 shows the Collin-Selleck model having the highest coefficient of determination of 0.632 and the T-test for the estimated Λ_{CS} shows significance value of 0.033 and standard error of 0.142. This indicates that both the dosage and the contact time are very significant for the inactivation kinetics of *Moringa oleifera* seed extract. The findings in this research is contrary to earlier research conducted by [13] whose finding revealed that the inactivation of the seed extract followed the Chick-Watson model indicating a first order reaction. The possible explanation for the differences in the results obtained even though *Moringa oleifera* seed extract was used as the disinfectant is probably due to different dosage used. In the research conducted by Bichi et al., (2012

a), the seed defatted extract was prepared using distilled water during the extraction at the dosage of 3.25 mg/L while in this study, the defatted seed extract dosage was 125mg/L. Also the bacterial indicator used in [21] was *E.coli* while the bacterial indicator used in this research was *P.aeruginosa*. Hence, it can be concluded that *Moringa oleifera* seed extract inactivates bacteria strains differently and the based on different dosage of the seed used in the preparation of the extract also significantly affects the way bacteria strains are being inhibited [20].

4. Conclusion

A second order polynomial regression model was developed for the optimization process of the seed extract using the response surface methodology. The model generated reveals that dosage, contact time and agitation were highly significant when using the seed extract to treat water. The predicted values obtained from the generated model were compared with the results from the experimental runs. The optimum parameter values for the seed extract as disinfectant for water treatment are dosage of 125mg/L at contact time of 30 minutes and mixing rate of 120 rpm. The inactivation kinetics of the seed extract shows that the Collin-Selleck model fits the experimental data with 0.632 thus, the inactivation kinetics of the seed extract is not first order.

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