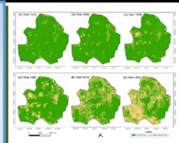
BESSM, 2023, Vol 7, No 2, 40-48
https://doi.org/10.54987/bessm.v7i2.916

HIBISCUS PUBLISHER

BULLETIN OF ENVIRONMENTAL SCIENCE & SUSTAINABLE MANAGEMENT

Website: http://journal.hibiscuspublisher.com/index.php/BESSM/index



BESSM VOL 7 NO 2 2023
Land degradation

Response Surface Method for the Optimization of Glyphosate Degradation by *Bacillus* sp. Isolated from Soils Near Lake Maninjau

Rusnam[1]*, Fachri Ibrahim Nasution[1] and Umar Abubakar Muhammad[2]

[1]Department of Agricultural Engineering, Faculty of Agricultural Technology, Andalas University, Padang, 25163, Indonesia.
[2]Department of Biological Sciences, Faculty of Science, Gombe State University, P.M.B 127, Tudun Wada, Gombe, Gombe State, Nigeria.

*Corresponding author:
Prof Dr. Rusnam,
Department of Agricultural Engineering,
Faculty of Agricultural Technology,
Andalas University,
Padang, 25163,
Indonesia.
Email: rusnam_ms@yahoo.com

HISTORY

Received: 12th Aug 2023
Received in revised form: 4th Dec 2023
Accepted: 30th Dec 2023

KEYWORDS

Glyphosate-degrading bacterium
Bacillus sp.
Bioremediation
RSM
Box-Behnken

ABSTRACT

The study utilized a Box-Behnken experimental design to assess the impact of three parameters on the percentage of glyphosate biodegradation by a bacterium. The variables examined were incubation period (measured in days), glyphosate concentration (measured in grams per liter), and pH, each assessed at three different levels. The quadratic model, which has squared terms, interaction products, linear terms, and an intercept, most accurately characterizes the connection between the variables and the response. The findings demonstrated that glyphosate concentration exerted the most pronounced impact on glyphosate degradation, with pH ranking second, as supported by substantial F-values and low p-values. The incubation period had no discernible effect. The ANOVA analysis validated the dependability of the model, as evidenced by an R^2 value of 0.9602 and an adjusted R^2 value of 0.9091. These values indicate that the model accounts for 90% of the variability observed in the response data. The contour and response surface plots demonstrated substantial interactions among the variables, specifically between pH and glyphosate concentration and between the incubation period and glyphosate concentration. The model's anticipated optimal conditions were experimentally tested, demonstrating no significant deviation from the projected values. The predicted maximum biodegradation of 90.097% closely matched the experimentally observed value of 92.505% ($p>0.05$). The predicted combination to give the desired maximum response was at pH 6.81, glyphosate concentration of 0.692 g/L and an incubation period of 3.092 days. On the other hand, the predicted combination to give the desired maximum response is based on the requirement for the conditions where biodegradation is at the highest possible glyphosate concentration of 0.844 g/L and an incubation period of 3.112 days. A higher response of about 5.779% degradation was achieved through RSM. This study showcases the efficacy of the Box-Behnken design in optimizing biodegradation processes, offering a solid statistical basis for further investigations on glyphosate degradation.

INTRODUCTION

Glyphosate, a broad-spectrum herbicide, is commonly used to control various plants. Its extensive usage has raised health worries regarding detecting glyphosate byproducts in various products. While animal studies have shown the risks of glyphosate to organs, reproduction, and the nervous system, there is evidence connecting it to cancer in humans. Environmental tests frequently find glyphosate in water samples due to its use in farming. With increasing evidence of glyphosate's effects on living beings, efforts are being made to prevent its buildup in soil and water sources and develop methods for its removal after contamination [1–8]. Globally, herbicide use is estimated at 125,000 to 130,000 tons annually. Concerns over its toxicity and health effects have led more than 20 countries to ban glyphosate for purposes. Despite these bans, farmers in some nations like Indonesia and Malaysia continue using glyphosate without measures, leading to significant health hazards. Experts suggest bioremediation of glyphosate as an eco alternative to degradation methods using chemicals or physical means. Bioremediation, a potentially safer method, uses microbes' metabolic processes to break down glyphosate into less harmful substances, providing a sustainable solution to glyphosate contamination in agricultural settings [9–15].

Glyphosate works as an herbicide by blocking the enzyme 5 enolpyruvyl shikimate 3 EPSP) synthase, a part of the shikimate pathway found in various organisms, like plants, bacteria, algae, and fungi. This pathway is essential for creating amino acids (tryptophan, phenylalanine, and tyrosine) and vitamins (folic acid and menaquinone). EPSP synthase aids in converting acid 3 phosphate (S3P) and phosphoenolpyruvate (PEP) into EPSP. By stopping synthase function, glyphosate disrupts the production of these compounds, hindering biodegradation and development in affected species [16–19]. With the harmful effects of glyphosate on living organisms becoming more evident, concerns about managing and reducing its impact are growing. This includes finding ways to prevent its buildup in the environment and practical techniques for removing it from contaminated soils and water sources. These actions are crucial for dealing with glyphosate and its potential health and ecosystem risks.

In fundamental research, experiment planning is often "intuitive". Biologists have long done "one factor at a time" experiments. This strategy keeps all components and variables the same except for the thing being researched, whose output is assessed. This technique may reveal "major effects" in biological research but will provide incorrect words due to component interactions. Because the process is complex, regulating several input elements is necessary for optimal results. DOE's core issue structure considers several factors that may affect process output. A model is proposed to characterize the system's output based on influential factors. These "response surface" models use continuous inputs and are usually first-order (linear) or second-order (quadratic) polynomials. When multiple factors affect a reaction or design, the response surface technique helps.

The response surface method (RSM) is a statistical technique used to choose a design for an experiment, identify the most effective levels or optimal points for several independent parameters, predict and verify model equations, and generate contour plots and response surfaces [20]. RSM has been used effectively to enhance biodegradation, biotransformation, and bioremediation processes such as the degradation of cyanide [21], phenol degradation [22], caffeine degradation [23], hexavalent chromium and molybdenum reduction to a less toxic form [24]. RSM optimizes yield within a process range computed using mathematical and statistical software like Design Expert® or MATLAB®. RSM strives for optimal performance with few resources. 2-D and 3-D contour plots show the ideal response and the effects of two components and interactions by setting optimal concentrations for other parameters. Visualize optimal replie. [25]. Two popular optimization methods are Box Behnken (BB) and Central Composite Design (CCD) [26,27]. In this study, the Box-Behnken approach will be selected for the optimization of glyphosate degradation by a previously-isolated glyphosate-degrading bacterium.

MATERIALS AND METHODS

Growth and maintenance of glyphosate-degrading bacterium

The bacterium, previously isolated from the soil near Lake Maninjau in West Sumatra, Indonesia (Rusnam et al., 2023), was characterized for its ability to degrade glyphosate using a minimal salts medium (MSM) supplemented solely with glyphosate as the phosphorus source.

The bacterium was previously stored in glycerol stock and was revived from a 16% glycerol stock by growing the pure culture overnight in 10 mL of nutrient broth. From this culture, 0.1 mL was transferred into 45 mL of glyphosate enrichment medium in a 100 mL volumetric flask, and the culture was incubated at 150 rpm for 48 hours at 25°C on an incubator shaker (Certomat R, USA). The minimal salts medium (MSM) used for growth contained the following components per liter: 0.5 g of NaCl, 0.5 g of KCl, 2 g of NH_4SO_4, 0.2 g of $MgSO_4.7H_2O$, 0.01 g of $CaCl_2$, 0.001 g of $FeSO_4$ [28]. To ascertain the bacterial count, one-milliliter samples were consecutively diluted in sterile tap water and then cultured on nutrient agar plates. Glyphosate underwent filter sterilization using PTFE syringe filters with a pore size of 0.45 microns.

Determination of glyphosate using HPLC

The glyphosate breakdown was measured using a High Performance Liquid Chromatography (HPLC) technique [29]. This study utilized an isocratic gradient elution technique. The equipment consisted of a Water's 600 series HPLC with Waters 600 Quat Pump; Waters 600 Controller(LCD) and a Waters 996 PDA Detector. Separating different components was accomplished through chromatography utilizing a Thermo Scientific™ BioBasic™ reversed-phase C18 HPLC column. The mobile phase consisted of a solution of 6.2 millimolar (mM) potassium dihydrogen phosphate (KH_2PO_4) in 4% (v/v) methanol. The pH of the solution was adjusted to 2.1 using 85% phosphoric acid. The flow rate was consistently maintained at 1 mL/min, while the detection wavelength was specifically fixed at 195 nm.

Optimization study using RSM

Response Surface Methodology (RSM) is a statistical technique employed to enhance and refine the optimization process in order to attain the most optimal response. This work utilized BB as a response surface methodology (RSM), which involves three sequential steps: designing and setting up the experiment, doing response surface modeling using regression, and finally, optimization. A second-order polynomial equation was used to establish the link and interrelationship between the input variables and the experimental response variable. The equation is presented in the subsequent format:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_{ii}^2 + \sum_{i=1}^{k-1} \sum_{j>1}^{k} \beta_{ij} x_i x_j + \text{error}$$

The equation depicts a regression model, where y represents the estimated response variable. β0 is the regression constant, βi is the linear regression coefficient, βii is the quadratic regression coefficient, and βij is the bilinear regression coefficient. This study employed a three-level, three-factor Box-Behnken design (BBD), as described in **Table 1**. The design incorporated significant components identified in a two-level factorial experiment, the results of which will be published separately.

The outcome was the measurement of glyphosate degradation, represented as a percentage of degradation. The Box-Behnken design (BBD) consisted of 17 randomized experimental runs (as shown in **Table 2**) in order to minimize the impact of uncontrolled extraneous factors on the observed results. The experimental runs included 12 factorial points and five center points, which were utilized to evaluate the effect of curvature in the experimental areas.

https://doi.org/10.54987/bessm.v7i2.916

Table 1. Coded and uncoded levels of the independent variables.

Factor	Name	Units	Type	SubType	Mini-mum	Maxi-mum	Coded Low	Coded High	Mean	Std. Dev.
A	Glyph-osate	g/L	Num-eric	Cont-inuous	0.3	1.0	-1 ↔ 0.3	+1 ↔ 1.0	0.65	0.2475
B	Incub-ation	Days	Num-eric	Cont-inuous	2.00	4.0	-1 ↔ 2.0	+1 ↔ 4.0	3.00	0.7071
C	pH		Num-eric	Cont-inuous	6.50	7.5	-1 ↔ 6.5	+1 ↔ 7.5	7.00	0.3536

Table 2. Experimental design and results of Box-Behnken for the biodegradation of glyphosate.

Run	Factor 1 A:Glyphosate g/L	Factor 2 B:Incubation Days	Factor 3 C:pH	Response 1 Degradation %
1	0.3	3	7.5	31.74
2	0.65	3	7	82.16
3	0.3	3	6.5	35.87
4	0.3	4	7	30.14
5	0.65	2	7.5	48.17
6	1	3	7.5	37.57
7	0.65	3	7	85.36
8	0.65	4	7.5	75.73
9	0.65	2	6.5	73.88
10	0.65	3	7	93.36
11	0.3	2	7	38.63
12	0.65	4	6.5	81.67
13	1	3	6.5	58.73
14	1	4	7	48.09
15	1	2	7	49.28
16	0.65	3	7	91.32
17	0.65	3	7	88.68

The trials were conducted thrice, and the average values are presented below. The data were analyzed using the Design Expert 11.0 software from Stat-Ease, Inc (trial version). The analysis included the use of ANOVA to determine the significant factors among the variables.

Statistical Analysis

The values are shown as the mean ± standard deviation, with each experiment being repeated three times. Group comparisons were conducted using either a one-way analysis of variance (ANOVA) followed by post hoc analysis using Tukey's test, or a Student's t-test. A p-value less than 0.05 was deemed to be statistically significant.

RESULTS

A Box-Behnken experimental design was used to study the impact of three parameters on the percentage bacterial biodegradation of glyphosate. The factors included the incubation duration (measured in days), glyphosate concentration (measured in grams per liter), and pH. Each component was tested at three distinct levels: low, medium, and high. The conducted experimental runs formed the basis for a sequence of tests that were performed. The Design-Expert application was utilized to evaluate mathematical models, such as linear, two-factor interaction, and quadratic, to assess their capacity to accurately fit the data.

The objective was to see if there was a link between the different components and the responses. Alternatively, it is recommended that BB be expressed using a quadratic equation, which incorporates squared terms, products of two components, linear terms, and an intercept [30], and this will be used in this study. The design scheme of variables with their corresponding actual values is depicted in **Table 3**, together with the experimental and predicted values of the response and the residuals.

Table 3. Design a scheme of variables with experimental and predicted values of response and residuals.

Run	A:Glyphosate g/L	B:Incubation Days	C:pH	Degradation %	Predicted Value %	Residual
1	0.3	3	7.5	31.74	30.96	0.78
2	0.65	3	7	82.16	88.18	-6.02
3	0.3	3	6.5	35.87	36.68	-0.81
4	0.3	4	7	30.14	35.76	-5.62
5	0.65	2	7.5	48.17	54.59	-6.42
6	1	3	7.5	37.57	36.76	0.81
7	0.65	3	7	85.36	88.18	-2.82
8	0.65	4	7.5	75.73	70.89	4.83
9	0.65	2	6.5	73.88	78.71	-4.83
10	0.65	3	7	93.36	88.18	5.19
11	0.3	2	7	38.63	32.99	5.64
12	0.65	4	6.5	81.67	75.25	6.42
13	1	3	6.5	58.73	59.52	-0.78
14	1	4	7	48.09	53.73	-5.64
15	1	2	7	49.28	43.67	5.62
16	0.65	3	7	91.32	88.18	3.14
17	0.65	3	7	88.68	88.18	0.51

The F-test is employed to ascertain the statistical significance of the model. **Table 4** presents the outcomes of the analysis of variance (ANOVA) and the P-value for a particular factor. The results demonstrate that the statistical model is very significant, as indicated by the F value of 18.77 and a low P-value of 0.004. The lack of fit p-value did not reach statistical significance, suggesting that the model fits well. All components in the model have statistical significance. The calculation of the correlation coefficient (R^2: 0.9602, close to 1) and the adjusted correlation coefficient (Adj R^2: 0.9091), as shown in **Table 4**, validates the model's reliability. The sum of these two coefficients indicates that the model accounts for 90 percent of the total variation in the response data. The Predicted R^2 and the Adjusted R^2 exhibited a high level of concordance, with a discrepancy of less than 0.2 between them.

Adeq Precision, scientifically speaking, is the measure of the signal-to-noise ratio in an experiment. It is more desirable to have a ratio that exceeds 4. An adequate signal was acquired with a value of 10.7107. By employing this framework, individuals may easily navigate the design area. The Lack of Fit p-value greater than 0.05 indicates that it lacks statistical significance compared to the pure error. A small lack of fit is deemed desirable since it indicates that the model is correct. As the outcome, the anticipated increase can be derived based on the coded factors outlined in **Table 5** and the calculation involving the actual components. The glyphosate concentration was shown to be the most influential factor in degradation, as indicated by the F-values. The pH also had a significant effect, but the incubation period did not show significance based on the p-value. This is also reflected in the results in **Table 5**, in the final equation represented in terms of coded and actual factors.

https://doi.org/10.54987/bessm.v7i2.916

Table 4. ANOVA analysis of the fitted Box-Behnken design.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	8197.20	9	910.80	18.77	0.0004	significant
A-Glyphosate	410.34	1	410.34	8.46	0.0227	
B-Incubation	82.37	1	82.37	1.70	0.2338	
C-pH	405.36	1	405.36	8.35	0.0233	
AB	13.28	1	13.28	0.2737	0.6170	
AC	72.51	1	72.51	1.49	0.2611	
BC	97.73	1	97.73	2.01	0.1988	
A^2	6004.32	1	6004.32	123.75	< 0.0001	
B^2	331.98	1	331.98	6.84	0.0346	
C^2	374.92	1	374.92	7.73	0.0273	
Residual	339.65	7	48.52			
Lack of Fit	258.41	3	86.14	4.24	0.0983	not significant
Pure Error	81.24	4	20.31			
Cor Total	8536.85	16				
Std. Dev.	6.97		R^2	0.9602		
Mean	61.79		Adjusted R^2	0.9091		
C.V. %	11.27		Predicted R^2	0.5008		
			Adeq Precision	10.7107		

Table 5. Final equation in terms of coded and actual factors.

Coded biodegradation equation	=	Actual Biodegradation equation	=
88.18		-1787.71	
7.16	A	575.90	Glyphosate
3.21	B	-16.10	Incubation
-7.12	C	500.35	pH
1.82	AB	5.21	Glyphosate * Incubation
-4.26	AC	-24.33	Glyphosate * pH
4.94	BC	9.89	Incubation * pH
-37.76	A^2	-308.27	Glyphosate²
-8.88	B^2	-8.88	Incubation ²
-9.44	C^2	-37.75	pH²

The factors evaluated using the OFAT (One-Factor-At-A-Time) methodology were crucial for understanding the growth of this bacterium on glyphosate. Detailed findings from this investigation are given in a separate article. The trials employed glyphosate concentrations that were comfortably within the reported tolerance range for the majority of glyphosate-degrading bacteria. Microorganisms are often harmed by concentrations of glyphosate that are higher than 1000 mg/L. This is mainly because glyphosate can disrupt the shikimate pathway, which is responsible for its toxic effects [16–19]. Extended incubation periods promote enhanced biodegradation, with the most favorable growth of glyphosate-degrading bacteria often occurring between two and five days of incubation. Therefore, it is crucial to precisely anticipate the consequences of incubation period. The majority of microorganisms that break down glyphosate thrive in settings that are close to neutral, which supports the results of our investigation and confirms the trends documented in existing literature.

The perturbation plots (**Fig. 1**) provide a way to interpret the influence of the independent factors on the responses. These plots demonstrate that a factor with a steep slope or curvature suggests that the reaction is very responsive to changes in that component, whereas a relatively flat line indicates that the response is not significantly affected by alterations in that specific factor. The plot clearly shows that component A (glyphosate concentration) has the most pronounced curvature. The perturbation plot also reveals the existence of two-factor interactions, indicating synergistic effects. Furthermore, all quadratic effects, denoted as (A^2), (B^2), and (C^2), demonstrated significant adverse synergistic effects with a p-value of less than 0.0001. The negative contributions observed suggest that an increase in these parameters has a harmful impact on the response. This outcome is not surprising, considering that the pH effect is extremely specific within a limited range, and larger concentrations of glyphosate severely hinder biodegradation.

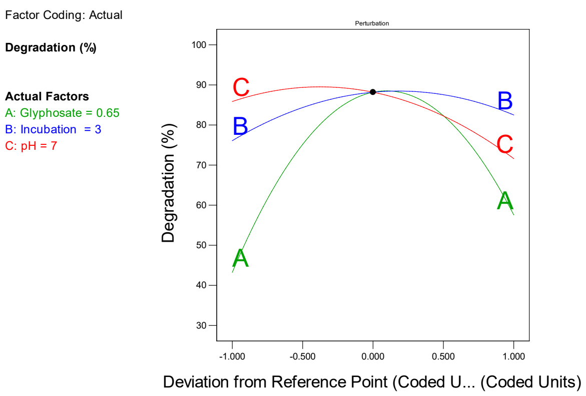


Fig. 1. Perturbation plot of operational parameters.

To verify the expectation of normality, a half-normal probability plot of the residuals (**Fig. 2**) was created and examined. The values for the internally studentized residual were found within a range of 2 and were evenly distributed along a straight line, suggesting that there was no requirement for a transformation of the response variable. The graph depicting the comparison between the experimental data and the anticipated values of the model demonstrates a strong correspondence, so providing additional evidence of the model's precision.

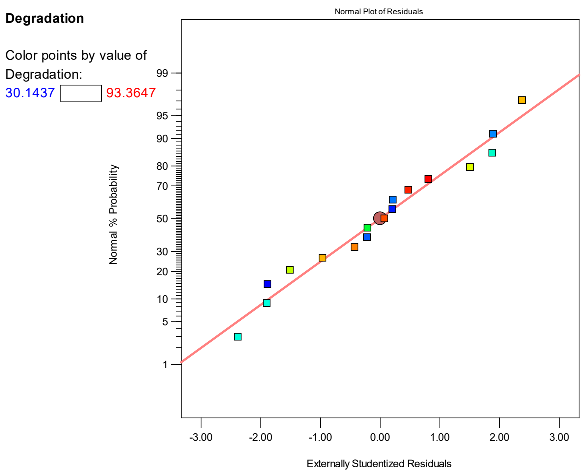


Fig. 2. Half-normal probability plot of the residuals.

Fig. 3 displays the Box-Cox plot, which is crucial for determining the suitable power law transformation according to the lambda (λ) value. Given that the 95% confidence interval encompasses the value of 1, which is consistent with the model's intended structure, it is not recommended to modify the observed response purely for the purpose of improving the model's fit. [31] suggest the following transformations: $\lambda = 0$ (natural log), $\lambda = 1$ (no transformation), $\lambda = 0.5$ (square root), $\lambda = -1$ (inverse) and $\lambda = -0.5$ (inverse square root).

BESSM, 2023, Vol 7, No 2, 40-48
https://doi.org/10.54987/bessm.v7i2.916

The diagnostic graph illustrating the relationship between predicted and actual values for the Box-Behnken optimization studies (**Fig. 4**) is a crucial tool for validating the model's predictive accuracy, as it provides a visual representation of how well the model's predictions align with the experimental results, where the x-axis typically represents the predicted values generated by the model and the y-axis denotes the actual values obtained from the experiments, ideally showing data points that closely follow a 45-degree line, indicating a perfect correlation between predicted and actual values, which suggests that the model has a high degree of accurateness in predicting outcomes based on the input variables.

Several key points can be derived from analyzing this diagnostic graph, including the strength of the correlation indicated by the proximity of the data points to the 45-degree line, which signifies a strong predictive capability of the model, while significant deviations suggest areas where the model may not be as accurate, serving as a validation tool that confirms whether the model's predictions are reliable, and if the data points consistently deviate from the 45-degree line, it may indicate issues with the model such as incorrect assumptions, overlooked variables, or the need for additional data points, while also helping to identify outliers—data points that significantly deviate from the expected trend, which may indicate experimental errors, data recording issues, or unique conditions not accounted for by the model, and addressing these outliers can improve the model's accuracy, and by examining areas where the predicted values do not match the actual values, researchers can refine their model by adjusting parameters, incorporating additional variables, or using more sophisticated modeling techniques to capture the underlying complexities of the data, thus making the diagnostic graph comparing predicted and actual values for the Box-Behnken optimization studies a vital tool for evaluating and enhancing the model's accuracy, helping to validate the model, identify potential outliers, and guide further refinements to ensure robust and reliable predictive capabilities [31].

The leverage versus run number plot (**Fig. 5**), with degradation values color-coded for each point, used to assess the influence of individual data points on the overall model in a Box-Behnken optimization study, and the key observations include leverage values ranging from approximately 0.05 to just under 0.10, indicating that no single data point has an excessively high influence on the model, as typically leverage values close to 1 would be a concern due to high influence, and the x-axis represents the run number, ranging from 1 to 17, with the distribution of leverage values appearing fairly uniform across different runs, suggesting consistent influence from each experimental run, while the color coding represents the degradation values, with a range from blue for lower degradation values to red for higher degradation values, and the points are spread across the color spectrum, indicating a variety of degradation values were observed throughout the runs, and since all leverage values are well below the critical value of 1, this indicates no single run unduly influences the model, suggesting good model stability and robustness, and the spread of degradation values and uniform leverage distribution across runs indicate that the experimental design effectively captures a broad range of conditions without any outlier runs disproportionately affecting the model parameters.

Thus the leverage versus run number plot demonstrates that the model is stable and reliable, with no single data point exerting undue influence, and the degradation values are well-distributed across the runs, further supporting the robustness of the experimental design, suggesting that the model's predictions are likely to be accurate and not overly sensitive to individual experimental runs [31]. The Cook's distance plot, depicted in **Fig. 6**, aids in evaluating the influence of individual data points on the model. Cook's distance values that are higher suggest observations that have a greater influence, and these values are always positive. A given observation is deemed significant if its Cook's D value exceeds three times the mean of the dataset [31]. The findings show that there are no outliers, as all Cook's distances fall within the range of 1 (**Fig. 6**). Furthermore, the residuals vs. run plot (**Fig. 7**) indicates the absence of serial correlation, implying that the data characteristics are random. This study affirms the accuracy of the experimental data, strengthening the model's resilience and dependability .

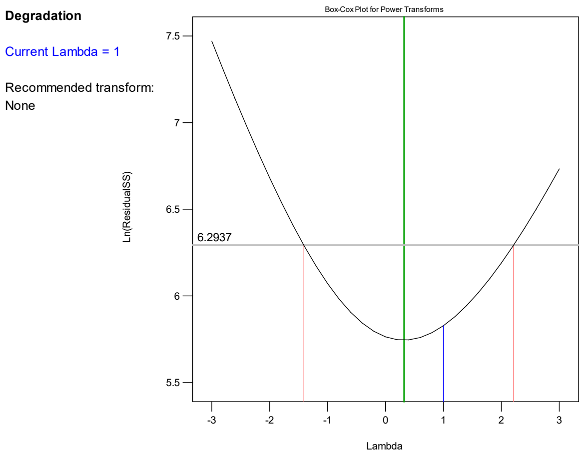


Fig. 3. Diagnostic's plot in the form of Box-Cox plot.

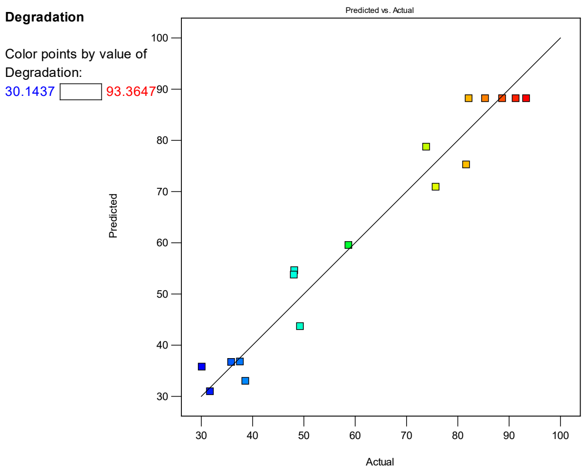


Fig. 4. Graph representing diagnostic data for the Box-Behnken optimization studies showing predicted values compared to actual values.

https://doi.org/10.54987/bessm.v7i2.916

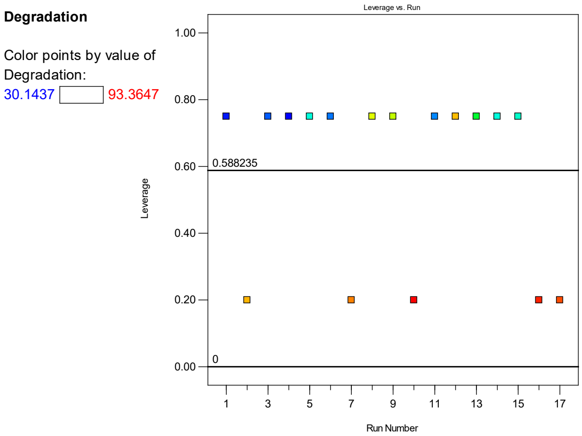


Fig. 5. For the Box-Behnken optimization experiments, the diagnostic is presented as a plot of leverage versus runs.

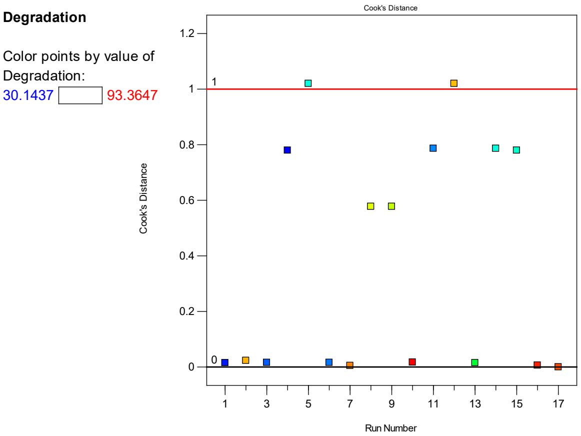


Fig. 6. Visual representation of the diagnostic data for the Box-Behnken optimization studies as a function of runs and Cook's distance.

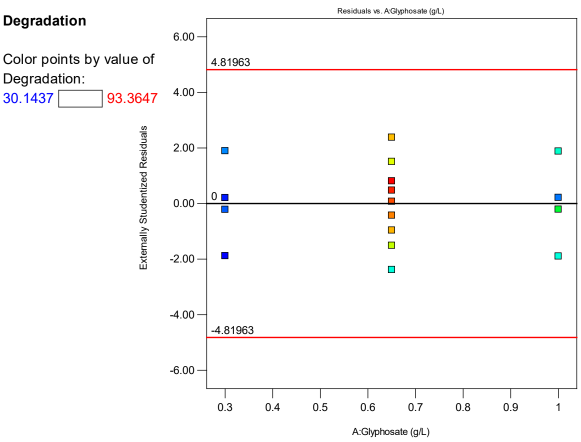
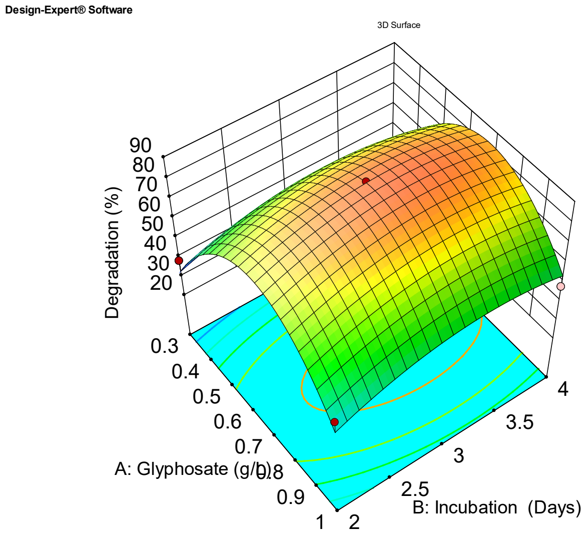


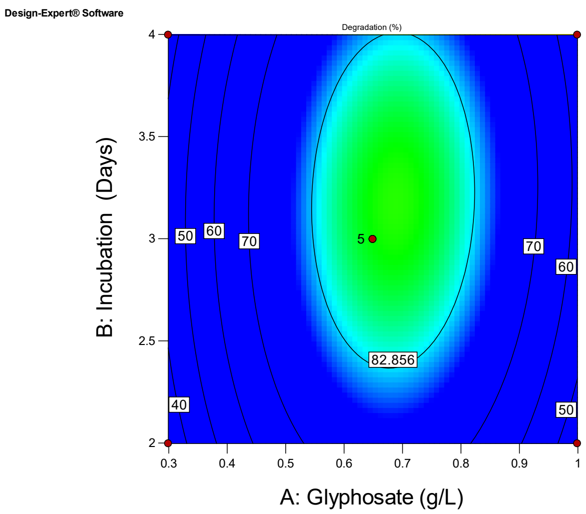
Fig. 7. Visual representation of diagnostic data for Box-Behnken optimization studies as a function of runs and residuals.

Interactive variable effect visualization using contour plots

To further understand how the parameters interacted with the response variables, we developed second-order equations and three-dimensional response surface plots. Each graph displays the reaction as a function of two independent variables within the ranges of those variables, with all other parameters held constant. Interaction effects between the variables were shown by the contour plot shapes. Plots that are deformed or elliptical suggest that the interactions between variables are significant, while plots that are circular show that the interactions are minor [33. The following sections only include the significant interactions for each answer variable. With the pH maintained at 7.0, adjusting the incubation period and glyphosate concentration factors produced an elliptical profile, suggesting a synergistic interaction (**Fig. 8a**). The studied region, spanning from 0.54 to 0.82 g/L in predicted glyphosate concentrations and 0.67 to 4 days in predicted incubation periods, had the highest response rate of 90.097% degradation (95% confidence interval from 82.856 to 97.337). The response surface model's elliptical 3D wired frame and contour plot show that the separate factors interacted with one another [31,32]. There was no statistically significant difference ($p>0.05$) inside this bordering region (**Fig. 8b**) since the 95% confidence interval of the maximum responses overlapped [33].



(a)



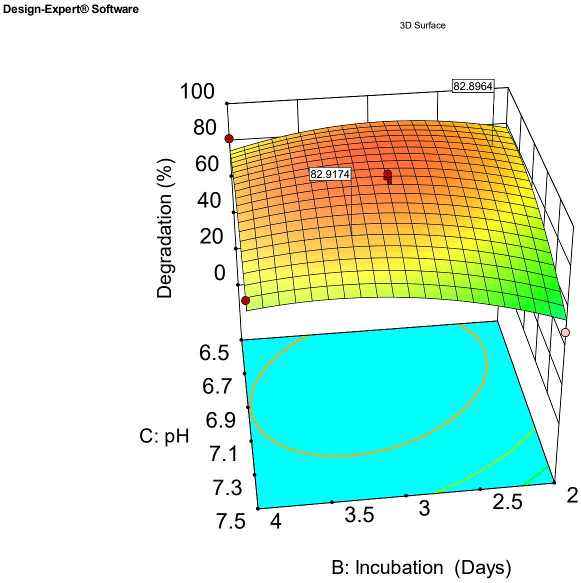
(b)

Fig. 8. The 2D- (b) and 3D- (c) contour plots show the ideal zone, while the 3D response surface plots show the relationship between the incubation factor and glyphosate concentration (a).

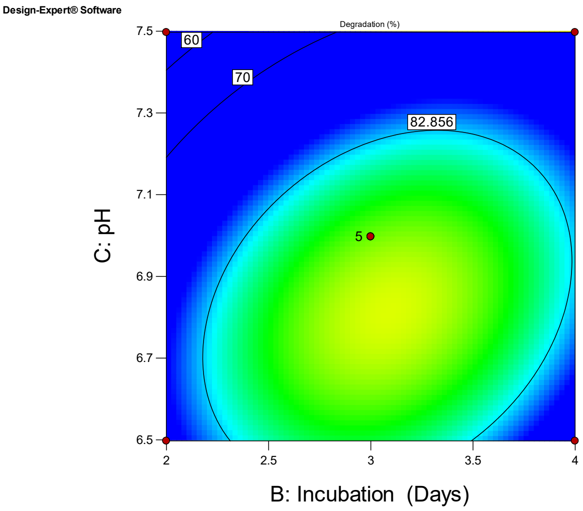
An elliptical profile indicating a relationship of synergistic interaction was observed when the glyphosate concentration was held at 0.65 g/L and the incubation period and pH were varied. The highest response, as previously observed, occurred at the predicted pH range of 6.5 to 7.25 and the longest period of 4 days (**Fig. 9a**). The 95% confidence interval of the maximum responses overlapped inside this bordering region (**Fig. 9b**),

https://doi.org/10.54987/bessm.v7i2.916

which was considered to be statistically insignificant (p>0.05). [33].



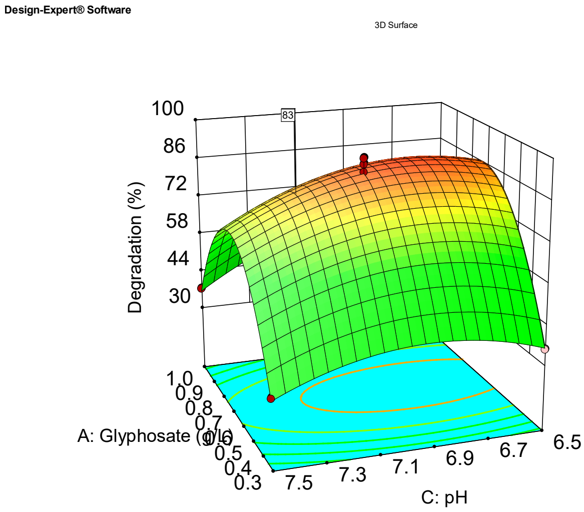
(a)



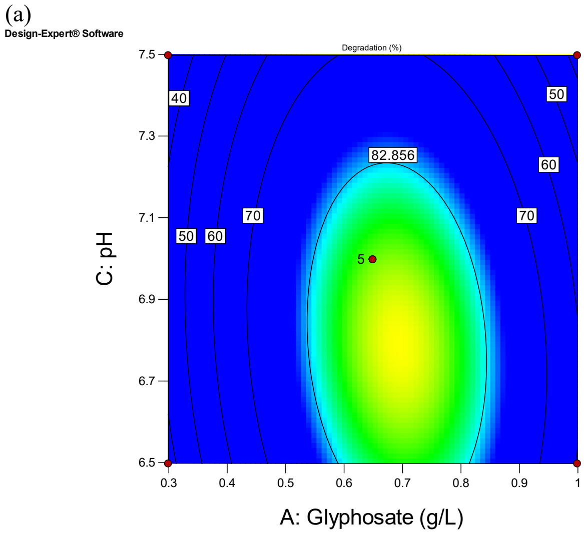
(b)

Fig. 9. The 2D- (b) and 3D- (c) contour plots show the ideal region, and the 3D response surface plots show the relationship between the incubation period (a) and the factor pH.

During the third day of incubation, changing the pH and glyphosate concentration factors resulted in a spherical profile showing significant interaction. The best response was observed at pH values between 6.35 and 7.25 and predicted glyphosate concentrations of 0.54 and 0.84 g/L (**Fig. 10a**). It seemed that the degradation was severely hindered at high glyphosate concentrations, and the perturbation plot corroborated this observation by showing that the degradation was unaffected by changes in pH from 6.5 to 7.5. There was no statistically significant difference (p>0.05) within this bordering region (**Fig. 10b**) since the 95% confidence interval of the maximum responses overlapped [33].



(a)



(b)

Fig. 10. The 3D response surface charts show the relationship between pH and the glyphosate factor (a), as well as the 2D- (b) and 3D- (c) contour plots that represent the ideal region with a 95% confidence interval.

Verification of BB experimental design of RSM for bacterial biodegradation of glyphosate

The Box-Behnken experimental design was found to be a successful tool for assessing the influence of incubation length, glyphosate concentration, and pH on the biodegradation of glyphosate by bacteria. This study determined that the pH and concentration of glyphosate are the primary factors that have a substantial impact on the process of biodegradation. Although the incubation duration has been thoroughly investigated, it did not demonstrate a statistically significant influence on the rates of biodegradation. The contour and response surface plots demonstrated notable interactions among variables, specifically between pH and glyphosate concentration, as well as between incubation period and glyphosate concentration. These interactions played a vital role in comprehending the collective impacts of the factors on glyphosate degradation. The model's predictions on the ideal conditions for achieving the highest level of biodegradation were confirmed by experimental validation.

The anticipated maximum biodegradation was found to be 90.097%, whereas the actual observed biodegradation was 92.505% (**Table 6**). The strong correlation between the predicted and experimental results ($p>0.05$) highlights the reliability of the model. The results of this study highlight the efficacy of the Box-Behnken design in optimizing biodegradation processes. This research offers a thorough foundation for future studies focused on improving glyphosate degradation by identifying and analyzing essential components and their interactions. The work described in this study provides a dependable basis for environmental scientists and engineers who are addressing comparable biodegradation difficulties, thanks to its statistical rigor and thorough model validation.

In conclusion, our work aids in the overall objective of creating sustainable and effective approaches to reduce glyphosate contamination in different ecosystems. In order to attain the highest level of biodegradation, the ideal circumstances, as projected in **Table 7**, include a pH of 6.81, a glyphosate content of 0.692 g/L, and an incubation duration of 3.092 days. In contrast, according to the information provided in **Table 8**, the most favorable conditions for the maximum glyphosate concentration were determined to be a pH of 6.785, a glyphosate concentration of 0.844 g/L, and an incubation duration of 3.112 days. A comparison between the findings obtained via OFAT (reported in another source) and RSM demonstrated that the optimization using RSM resulted in a biodegradation rate of 5.779% higher.

Table 6. The Box-Behnken design based parameter suggestions and projected responses for each variable to achieve maximum glyphosate degradation.

Name	Goal	Level	
A:Glyphosate	is in range	0.692	
B:Incubation	is in range	3.092	
C:pH	is in range	6.810	
Degradation	maximize		
Solution 1 of 1	Predicted	95% CI low	95% CI high
Degradation	90.097	82.856	97.337

Table 7. Suggested parameter and predicted response for each variable for maximum glyphosate concentration tolerable based on the Box-Behnken design.

Name	Goal	Level	
A:Glyphosate	maximize	0.844	
B:Incubation	is in range	3.112	
C:pH	is in range	6.785	
Degradation	maximize		
Solution 1 of 1	Predicted	95% CI low	95% CI high
Degradation	83.003	75.511	90.496

Comparison of optimisation parameters between OFAT and RSM

In comparison, results from OFAT (published elsewhere) and RSM were gathered and compared to each other (**Table 9**). A higher response of about 5.779% degradation was achieved through RSM optimisation.

Table 9. Comparison of optimum conditions and results obtained between OFAT and RSM for optimum % degradation of glyphosate.

	OFAT		RSM	
Factors	Optimum value	Max degradation (%)	Optimum value	Max degradation (%)
pH	7.0	86.729	6.81	92.505
Incubation period (d)	3	(83.922 to	3.09	(88.679 to
Glyphosate (g/L)	0.5	89.536)	0.692	96.331)

Table 8. Verification results between experiments and predicted response.

RSM target solution	Desira-bility	Predicted % degradation (95%, C.I.)	Experimental verification (95%, C.I.)	Statistical significant
All studied factors are within range, Maximum Degradation as a response	0.869	90.097 (82.856 to 97.337)	92.505, (88.679 to 96.331)	No significant Difference ($p>0.05$)
Only glyphosate concentration maximum. Other factors within range, Maximum Degradation as a response	0.844	83.003 (75.511 to 90.496	79.725 (75.552 to 83.898)	No significant Difference ($p>0.05$)

CONCLUSION

The Box-Behnken experimental design was found to be a successful tool for assessing the influence of incubation length, glyphosate concentration, and pH on the biodegradation of glyphosate by bacteria. The study determined that pH and glyphosate concentration are the primary factors that substantially impact the biodegradation process. Among these factors, glyphosate concentration has the greatest influence, as indicated by the perturbation plot, F-ratio, and p-value. Although the incubation duration has been thoroughly researched, it did not demonstrate a statistically significant influence on the rates of biodegradation. The quadratic model employed to assess the experimental data yielded a precise fit, as evidenced by a substantial R^2 value of 0.9602 and an adjusted R^2 of 0.9091, elucidating 90% of the variation in the response data. The Adeq Precision score of 10.7107 confirmed the model's trustworthiness, indicating a satisfactory signal-to-noise ratio. The contour and response surface plots showed notable interactions among the variables, specifically between pH and glyphosate concentration and between the incubation period and glyphosate concentration. These interactions played a vital role in comprehending the collective impact of the variables on glyphosate degradation. The model's predictions on the ideal conditions for achieving the highest level of biodegradation were confirmed by experimental validation. The model predicted a maximum biodegradation rate of 90.097%, whereas the actual observed biodegradation rate was 92.505%. The strong concordance between projected and empirical values ($p>0.05$) highlights the model's resilience. This study's results highlight the Box-Behnken design's efficacy in optimizing biodegradation processes. This research offers a thorough foundation for future studies focused on improving glyphosate degradation by identifying and analyzing essential components and their interactions. The meticulous statistical analysis and thorough model validation given in this study provide a dependable basis for environmental scientists and engineers engaged in comparable biodegradation tasks. In conclusion, our work aids in the overall objective of creating sustainable and effective techniques to reduce glyphosate contamination in different ecosystems.

REFERENCES

1. Zheng T, Jia R, Cao L, Du J, Gu Z, He Q, et al. Effects of chronic glyphosate exposure on antioxdative status, metabolism and immune response in tilapia (GIFT, Oreochromis niloticus). Comp Biochem Physiol Part - C Toxicol Pharmacol. 2021;239.
2. Pochron S, Simon L, Mirza A, Littleton A, Sahebzada F, Yudell M. Glyphosate but not Roundup® harms earthworms (*Eisenia fetida*). Chemosphere. 2020 Feb;241:125017.

3. Nwonumara GN, Okogwu OI. Oxidative and biochemical responses as indicators of chemical stress in *Clarias gariepinus* (Burchell, 1822) juvenile exposed to glyphosate. Sci Afr. 2020;8.
4. Moraes JS, da Silva Nornberg BF, Castro MRD, Vaz BDS, Mizuschima CW, Marins LFF, et al. Zebrafish (*Danio rerio*) ability to activate ABCC transporters after exposure to glyphosate and its formulation Roundup Transorb®. Chemosphere. 2020;248.
5. Carvalho WF, Ruiz de Arcaute C, Torres L, de Melo e Silva D, Soloneski S, Larramendy ML. Genotoxicity of mixtures of glyphosate with 2,4-dichlorophenoxyacetic acid chemical forms towards *Cnesterodon decemmaculatus* (Pisces, Poeciliidae). Environ Sci Pollut Res. 2020;27(6):6515–25.
6. Weeks Santos S, Gonzalez P, Cormier B, Mazzella N, Bonnaud B, Morin S, et al. A glyphosate-based herbicide induces sub-lethal effects in early life stages and liver cell line of rainbow trout, *Oncorhynchus mykiss*. Aquat Toxicol. 2019;216.
7. Yusof S, Ismail A, Alias MS. Effect of glyphosate-based herbicide on early life stages of Java medaka (*Oryzias javanicus*): a potential tropical test fish. Mar Pollut Bull. 2014 Aug 30;85(2):494–8.
8. Kittle RP, McDermid KJ. Glyphosate herbicide toxicity to native Hawaiian macroalgal and seagrass species. J Appl Phycol. 2016 Aug 1;28(4):2597–604.
9. Tiimub B, Zhenchao Z, Zhu L, Liu Y, Shuai XY, Xu L, et al. Characteristics of bacterial community and ARGs profile in engineered goldfish tanks with stresses of sulfanilamide and copper. Environ Sci Pollut Res. 2021 Mar 19;1–12.
10. Karimi-Avargani M, Bazooyar F, Biria D, Zamani A, Skrifvars M. The special effect of the Aspergillus flavus and its enzymes on biological degradation of the intact polylactic acid (PLA) and PLA-Jute composite. Polym Degrad Stab. 2020 Sep 1;179.
11. Khan N, Islam MdS. State the organic pollution level in rain fed ponds, Noakhali, Bangladesh. 2019 Aug 5;438–41.
12. Khan N, Tisha N. Freshwater algal tolerance to organic pollution: a review. Pollut Res. 2020 Dec 1;39:1297–301.
13. Omotayo AO, Olagunju KO, Omotoso AB, Ogunniyi AI, Otekunrin OA, Daud AS. Clean water, sanitation and under-five children diarrhea incidence: Empirical evidence from the South Africa's General Household Survey. Environ Sci Pollut Res Int. 2021 Jul 5;
14. Riser-Roberts E. Remediation of petroleum contaminated soils: biological, physical, and chemical processes. CRC press; 2020.
15. Guo G, Li X, Tian F, Liu T, Yang F, Ding K, et al. Azo dye decolorization by a halotolerant consortium under microaerophilic conditions. Chemosphere [Internet]. 2020;244. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85076057919&doi=10.1016%2fj.chemosphere.2019.125510&partnerID=40&md5=03084a46ed5d7d42bc674520dbd5f922
16. Wilson DJ, Patton S, Florova G, Hale V, Reynolds KA. The shikimic acid pathway and polyketide biosynthesis. J Ind Microbiol Biotechnol. 1998 May 1;20(5):299–303.
17. Amrhein N, Johänning D, Smart CC. A Glyphosate-Tolerant Plant Tissue Culture. In: Neumann KH, Barz W, Reinhard E, editors. Primary and Secondary Metabolism of Plant Cell Cultures. Berlin, Heidelberg: Springer; 1985. p. 356–61. (Proceedings in Life Sciences).
18. Hertel R, Gibhardt J, Martienssen M, Kuhn R, Commichau FM. Molecular mechanisms underlying glyphosate resistance in bacteria. Environ Microbiol. 2021;23(6):2891–905.
19. Schulz A, Krüper A, Amrhein N. Differential sensitivity of bacterial 5-enolpyruvylshikimate-3-phosphate synthases to the herbicide glyphosate. FEMS Microbiol Lett. 1985 Jul 1;28(3):297–301.
20. Khuri IA, Mukhopadhyay S. Response surface methodology. Adv Rev WIREs Comput Stat John Wiley Sons Inc. 2010;2:128–49.
21. Karamba KI, Ahmad SA, Zulkharnain A, Syed MA, Khalil KA, Shamaan NA, et al. Optimisation of biodegradation conditions for cyanide removal by *Serratia marcescens* strain AQ07 using one-factor-at-a-time technique and response surface methodology. Rendiconti Lincei. 2016 Sep;27(3):533–45.
22. Annadurai G, Ling LY, Lee J fwu. Statistical optimization of medium components and growth conditions by response surface methodology to enhance phenol degradation by *Pseudomonas putida*. J Hazard Mater. 2008;151:171–8.
23. Ibrahim S, Shukor MY, Khalil KA, Halmi MIE, Syed MA, Ahmad SA. Application of response surface methodology for optimising caffeine-degrading parameters by *Leifsonia* sp. strain SIU. J Environ Biol. 2015 Sep;36(5):1215–21.
24. Ahmad WA, Zakaria ZA, Zakaria Z, Surif S. Hexavalent Chromium Reduction at Different Growth Phases of Acinetobacter haemolyticus. 2009;26(7):1275–8.
25. Anderson MJ, Whitcomb PJ. RSM simplified: optimizing processes using response surface methods for design of experiments. 2nd ed. Boca Raton, FL, USA.: Productivity Press; 2016.
26. Halmi MIE bin, Abdullah SRS, Wasoh H, Johari WLW, Ali MS bin M, Shaharuddin NA, et al. Optimization and maximization of hexavalent molybdenum reduction to Mo-blue by *Serratia* sp. strain MIE2 using response surface methodology. Rendiconti Lincei. 2016 Dec 1;27(4):697–709.
27. Yakasai MH. A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Malaysia. Universiti Putra Malaysia; 2017.
28. Rusnam, Rahman MF, Khayat ME, Nasution FI, Umar AM, Yakasai HM. Characterisation of a Bacillus sp. Isolated from Soils Near Lake Maninjau Capable of Degrading Glyphosate. J Environ Microbiol Toxicol. 2023 Jul 31;11(1):69–76.
29. Pongraveevongsa P, Khobjai W, Wunnapuk K, Sribanditmongkol P. High-performance liquid chromatography/uv detection for determination of glyphosate in serum and gastric content. Chiang Mai Med J. 2008;47(4):155–62.
30. Box GEP, Behnken DW. Some New Three Level Designs for the Study of Quantitative Variables. Technometrics. 1960 Nov 1;2(4):455–75.
31. Whitcomb PJ, Anderson MJ. RSM Simplified: Optimizing Processes Using Response Surface Methods for Design of Experiments. 2nd ed. New York, New York: Productivity Press; 2016. 304 p.
32. Rodrigues RCLB, Kenealy WR, Dietrich D, Jeffries TW. Response surface methodology (RSM) to evaluate moisture effects on corn stover in recovering xylose by DEO hydrolysis. Bioresour Technol. 2012;108:134–9.
33. Schenker N, Gentleman JF. On judging the significance of differences by examining the overlap between confidence intervals. Am Stat. 2001;55(3):182–6.