RESEARCH PAPER

Response Surface Methodology for Optimizing Laccase-Mediated Drug Degradation.

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ABSTRACT
Background: The persistence of pharmaceuticals like amoxicillin in the environment poses risks to human health, wildlife, and the food supply. Traditional disposal methods are often inadequate, highlighting the need for sustainable solutions.
Aim: This research evaluates the effectiveness of laccase enzymes in degrading amoxicillin and identifies optimal conditions to enhance this process.
Method: Using a Box-Behnken Design (BBD), the study examines how varying pH, temperature, and amoxicillin concentration affect laccase-mediated degradation of amoxicillin.
Results: The study highlighted temperature as the most influential factor, while the effects of pH and substrate concentration were found to be insignificant. The synergistic effect of temperature and pH was determined to be the most significant interaction factor. ANOVA and Pareto analysis confirm that temperature is crucial and having the greatest effect. The highest enzymatic degradation of amoxicillin was achieved at 67%. The BBD identifies the most effective degradation conditions achieved at pH 5, 50°C, and 0.007 mg mL^{-1} amoxicillin. At 50°C, the obtained V_{max} value was calculated at 0.381 mg mL^{-1} h^{-1} while the K_m value was 0.951 mg mL^{-1}.
Conclusion: By determining optimal degradation conditions and evaluating enzyme efficiency, the research aimed to contribute to sustainable strategies for pharmaceutical removal. This work highlights the potential of laccase enzymes in degrading pharmaceutical contaminants, promoting more sustainable practices in environmental management.
Keywords: RSM, drug degradation, amoxicillin, laccase, fungi, enzyme
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1. INTRODUCTION

The presence of pharmaceutical pollutants, especially in aquatic ecosystems, is a critical issue with serious implications for human health and environmental sustainability (Rivera-Utrilla *et al.,* 2013). Improper disposal and inadequate degradation of drugs exacerbate this issue,threatening public health by fostering antibiotic-resistant microorganisms. Recent studies have reported alarming levels of antibiotics in rivers globally. This widespread contamination,particularly in regions like India, China, and parts of Africa, significantly disrupts aquatic ecosystems and accelerates antimicrobial resistance (AMR) (Pramod Barathe *et al.,* 2024; Iskandar *et al.,* 2020). For instance, two-thirds of 711 samples from rivers in 72 countries contained antibiotics, often exceeding safety thresholds set by the AMR Industry Alliance (Rayan, 2023). Massive quantities of pharmaceutical pollutants are introduced into wastewater treatment plants

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Up to the present, the process of eliminating drugs and other micropollutants in wastewater relies on physical (primary) and biological treatment (secondary) (Tiwari *et al.*,2017). Recently, high-technology (e.g. oxidation, activated carbon) has been implemented in a small number of chosen waste water treatment plants. Nonetheless, most of the wastewater treatment plants are not efficient in removing the pharmaceutical pollutants (Lin *et al.,* 2009). As a result, there is a steady release into the surface water, groundwater and it could possibly be found in the drinking water. Based on it, wastewater treatment plants contribute to the environmental presence of antibiotics and antibiotic resistance genes (Zieliński *et al.,* 2021).

Laccase, a copper-containing enzyme, is pivotal in advancing research methods focused on drug degradation, offering an eco-friendly approach to pharmaceutical studies and production. This enzyme efficiently oxidizes a wide range of medicinal compounds, including aromatic amines and polyphenols (Chauhan *et al.,* 2017; Abd Razak *et al*., 2024). Laccase is synthesised by a diverse range of species, such as fungi, plants, bacteria, and insects. However, it is mostly present in white-rot fungi, which use this enzyme to break down lignin in wood. Laccase is capable for oxidizing various organic and inorganic substances, primarily converting molecular oxygen into water (Madhavi and Lele, 2009). The enzyme's unique structure includes multiple copper atoms, arranged in three distinct catalytic sites: Type 1 (T1), Type 2 (T2), and Type 3 (T3) copper centers (Kosman, 2009). Laccase has demonstrated effective degradation of several antibiotics, including tetracycline (Abejón *et al.,* 2015; De Cazes *et al.*, 2015; Llorca *et al.,* 2015), levofloxacin (Mathur *et al.*, 2021), sulfamethoxazole (Becker *et al.,* 2016; Gao *et al.,* 2018), ciprofloxacin (Cupry *et al.,* 2018), and ampicillin (Ghariani *et al.,* 2024).

Amoxicillin, an FDA-approved antibiotic, is commonly used and has a wide range of effectiveness against many types of bacteria (Akhavan *et al.,* 2022). It is derived from penicillin and has been modified to improve its effectiveness and ability to fight different types of bacteria. Structurally, it is distinguished by the existence of a β-lactam ring, which is essential for its mode of action. The presence of this ring in amoxicillin allows it to effectively hinder the production of peptidoglycan, a vital element of bacterial cell walls. As a result, the bacteria undergo lysis and perish. Amoxicillin's acid stability enables efficient oral administration with high bioavailability (75%-90%), ensuring effective concentrations in critical areas like the lungs and urine (Veeraraghavan *et al.,* 2020). It is metabolized in the liver and excreted via the kidneys, with a short half-life of 1 to 1.5 hours, requiring multiple daily doses to maintain therapeutic levels. As a widely effective and affordable antibiotic, amoxicillin is a first-line treatment for various bacterial infections, including respiratory and urinary tract infections. However, its extensive use raises concerns about antibiotic resistance, particularly due to its degradation by β- lactamase enzymes from resistant strains (Muteeb *et al.,* 2023). To address this, amoxicillin is often combined with clavulanic acid, a β-lactamase inhibitor, enhancing its efficacy against resistant bacteria (Huttner *et al.,* 2020). Additionally, improper disposal of amoxicillin poses environmental risks, contributing to pollution and the global issue of antimicrobial resistance (Veena *et al.,* 2015). However, despite increasing studies on enzymatic degradation of pollutants, limited research has focused on the systematic

optimization of amoxicillindegradation using laccase, particularly through statistical experimental designs that evaluate the interaction effects of operational parameters.

Response Surface Methodology (RSM) has become an essential tool for optimising intricate experimental conditions, particularly in multifactorial studies (Bezerra *et al.*, 2008). The efficient analysis of multiple variables and their interactions is facilitated by RSM, which employs designs such as Central Composite Design (CCD) or Box-Behnken Design (BBD) (Szpisják-Gulyás *et al.*, 2023). Both modelling designs establish a correlation between the input and output factors which make them useful for analyzing and optimizing the behavior of any system (Mertler, Vannatta and LaVenia, 2021). These techniques are convenient, reduce time and money as they cut off the required number of tests considering the domain of the experimental set up. This approach maximises information while minimising trials, thereby enhancing research efficiency. Therefore, this study employed a BBD to systematically investigate the influence of key variables on the degradation of amoxicillin by laccase.

The specific objectives of this study were 1) To determine the effect of selected variables, namely temperature, pH, and substrate concentration, on the degradation of amoxicillin by laccase 2) To evaluate the interaction effects among these variables on the degradation process 3) To predict and determine the optimum degradation conditions using the BBD.

2. MATERIALS AND METHODS

2.1 Materials

Potassium phosphate dibasic (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4), amoxicillin, laccase, syringaldazine and absolute ethanol were purchased from Sigma Aldrich (Sigma Aldrich, USA).

2.2 Preparation of preliminary experiments

The preparation commenced with the determination of the necessary quantities of potassium phosphate dibasic (K_2HPO_4) and potassium phosphate monobasic (KH_2PO_4) for each desired pH level using the Henderson-Hasselbalch equation. The required ratio of [K_2HPO_4] to [KH_2PO_4] was determined in accordance with the target pH values of 5, 6, and 7. The buffer concentration was established at 0.1 M for all three solutions. Commercial laccase from *Trametes versicolor* (Sigma-Aldrich) was used in this experiment without further purification. 1 mg mL^{-1} of laccase was prepared by dissolving the enzyme in 0.1 M phosphate buffer, pre-prepared at pH levels of 5, 6, and 7. The mixture was gently stirred until the laccase fully dissolved, resulting in a clear, uniformly distributed solution. The laccase stock solutions were then transferred into amber glass vials to protect the enzyme from light exposure, which could cause degradation.Enzyme solutions were prepared at concentrations of 0.1, 0.5, and 1 mg mL^{-1}. 0.30 mM of syringaldazine was prepared by dissolving it in 50 % ethanol. Three independent replicates were made for every experiments conducted.

2.3 Laccase activity determination

Laccase activity was measured by monitoring the rate of oxidation of syringaldazine by the enzyme at 25±1 °C. Of the laccase solution, 0.2 mL was mixed with 3.0 ml of 0.1 M phosphate buffer (pH 5) in a cuvette. Of the syringaldazine, 0.2 mL of 0.1 mM was added and gently mixed, and the absorbance was measured immediately at 525 nm for 10 min using UV VIS

scanning spectrophotometer Genesys UV-Vis (Thermo Fisher Scientific,USA). Total reaction volume was 3.4 mL. Laccase activity was calculated as shown in Eq. 1

$$U\,L^{-1} = \frac{\Delta Abs_{525}}{\Delta time} \times \frac{1}{l} \times \frac{1}{\varepsilon_{525}} \times \frac{V_T}{V_S} \quad \text{(Eq. 1)}$$

where ΔAbs is the change of absorbance at 525 nm per minutes, l is the light path length (1 cm) and ε is the extinction coefficient for syringaldazine (ε525 = 65,000 M^{-1} cm^{-1}). V_T denotes the total volume while Vs represents the volume of the sample. One-unit activity is defined as the amount of laccase that oxidizes 1 μmol of syringaldazine per minute.

2.4 Amoxicillin degradation assay

1 mg mL^{-1} amoxicillin stock solution was prepared by dissolving 50 mg of the amoxicillin in 50 mL of 0.1 M of phosphate buffer (pH 5, 6 and 7). The phosphate buffer was chosen for its compatibility with amoxicillin and its ability to maintain a stable pH during the process. The mixture was thoroughly stirred with a glass rod to ensure complete dissolution, resulting in a homogeneous solution. Stirring continued until no visible particles remained, confirming the amoxicillin had fully dissolved. The stock solution was then transferred into amber glass bottles, labeled accordingly. Amber glass was selected to protect the solution from light exposure, which could degrade the amoxicillin. The spectrophotometer was set to scan the wavelength range from 190 to 700 nm, allowing for a comprehensive analysis of the amoxicillin solutions.

Drug degradation process was initiated by adding laccase to 5 mL amoxicillin solution in vials, which contained different concentrations of amoxicillin (0.0125 - 0.25 mg mL^{-1}). Initial laccase activity was determined at room temperature (25±1 °C) to ensure that all vials had similar level of enzyme activities. The vials were incubated in water bath and were monitored at intervals time for 72 h at different temperatures (30°C, 40°C, and 50°C). Control experiments with heat-denatured enzyme (100 °C, 30 min) were also conducted in parallel. The assays were done in triplicates. Amoxicillin concentration was routinely measured using spectrophotometer at 220 nm.

2.5 Optimization of Drug degradation using RSM

In this study, RSM was used to optimize the degradation of amoxicillin by laccase. RSM is a powerful statistical and mathematical approach that helps optimize processes by examining the relationships between one or more response variables and multiple explanatory variables.

Here, the response variable was the percentage of amoxicillin degradation. The BBD, a well-regarded design within RSM, was chosen for this experiment. This design allows for the efficient fitting of a second-order (quadratic) model without requiring all possible combinations of factor levels to be tested. It is particularly useful for experiments involving three or more factors, as it minimizes the number of trials needed while still providing sufficient data to explore the interactions between variables. Three independent variables were selected for the experiment which as shown in Table 1.

Table 1: Summary of the variable levels in the reactions

Label	Variables	Level (coded)		
		(-1)	(0)	(+1)
A	pH	5	6	7
B	Temperature (°C)	30	40	50
C	Amoxicillin concentration (mg mL^{-1})	0.005	0.0125	0.02

To study both linear and quadratic effects, as well as interaction effects across variables, the experiment was designed to systematically change these three variables using the BBD (Table 2). The quadratic model (Eq. 2) below illustrates the correlation between the independent variables and the anticipated response (percentage of degradation):

Where Y is the predicted response (percentage of degradation), β_0 is the intercept, β_1, β_2, β_3 are the linear coefficients, β_{11}, β_{22}, β_{33} are the quadratic coefficients, β_{12}, β_{13}, β_{23} are the interaction coefficients and A, B, C, A^2, B^2, C^2, AB, AC, BC are the independent variables corresponding to temperature, pH, and amoxicillin concentration, respectively.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{12} AC + \beta_{23} BC \quad \text{(Eq. 2)}$$

Table 2: Reaction condition of Box Behnken Design (BBD)

Run Order	A = pH (coded)	B = Temperature (coded)	C = Amoxicillin (coded)
1	6 (0)	40 (0)	0.0125 (0)
2	5 (-1)	40 (0)	0.02 (+1)
3	7 (+1)	30 (-1)	0.0125 (0)
4	5 (-1)	50 (+1)	0.0125 (0)
5	6 (0)	50 (+1)	0.02 (+1)
6	7 (+1)	50 (+1)	0.0125 (0)
7	6 (0)	30 (-1)	0.02 (+1)
8	5 (-1)	40 (0)	0.005 (-1)
9	6 (0)	40 (0)	0.0125 (0)
10	7 (+1)	40 (0)	0.02 (+1)
11	6 (0)	30 (-1)	0.005 (-1)
12	6 (0)	50 (+1)	0.005 (-1)
13	5 (-1)	30 (-1)	0.0125 (0)
14	7 (+1)	40 (0)	0.005 (-1)
15	6 (0)	40 (0)	0.0125 (0)

2.6 Data analysis

Using the established standard curve, the concentration of amoxicillin can be determined. *Y* is the predicted response (percentage of amoxicillin degradation), which is used as the dependent variable was estimated based on the proportion of amoxicillin consumed in a reaction, as outlined in Eq. 3;

$$Y = \frac{AMX_0 - AMX}{AXM_0} \times 100\% \quad \text{(Eq. 3)}$$

where AMX_0 is the initial concentration of amoxicillin (mg mL^{-1}) and AMX is concentration of amoxicillin (mg mL^{-1}).

Within the RSM framework, BBD was established to optimize key reaction variables. An orthogonal array design was generated using MINITAB®18. The calculation of descriptive statistics, including means and standard deviations, and the application of model fitting techniques using MATLAB's curve fitting tools to identify relationships between variables were the key steps. The statistical results and response surfaces were generated using the design software. The analysis of variance (ANOVA) was used for the various statistical evaluation of regression model. This model enabled the identification of optimal conditions for maximizing amoxicillin degradation by analysing the effects of each variable, both individually and in combination.

Rate of reaction of amoxicillin degradation (v, in mg mL^{-1} per hour) was calculated using Eq. 4,

$$v = \frac{\Delta[\mathrm{AMX}]_d}{\Delta t} \qquad \text{(Eq. 4)}$$

where $\Delta[\mathrm{AMX}]_d$ (in mg mL^{-}) is the change in amoxicillin concentration over the time interval Δt (in hours).

To calculate k_1', the rate of reaction amoxicillin degradation (Eq. 4) was plotted against different initial amoxicillin concentrations for each temperature tested. The rate constant k_1' was obtained from the slope of the resulting linear plot. The activation energy (Ea, in joule per mole) of degradation process was calculated using the linearized Arrhenius equation as shown in Eq. 5,

$$\ln k' = \ln A - \frac{Ea}{RT} \qquad \text{(Eq. 5)}$$

where A is the frequency factor, R is the gas constant (8.3145 J mol^{-1} K^{-1}), and T is the absolute temperature (in Kelvin).

In order to determine the maximal degradation rate (V_{max}) and Michaelis-Menten constant (K_m), the assays were carried out at the temperature of 50 ºC at pH 5. To determine K_m and V_{max} values, the Michaelis-Menten equation (Eq. 6) was linearized by the Lineweaver Burk method (Eq. 7),

$$v = \frac{V_{max}[\mathrm{AMX}]}{K_m + [\mathrm{AMX}]} \qquad \text{(Eq. 6)}$$

$$\frac{1}{v} = \frac{K_{max}}{V_{max}[\mathrm{AMX}]} + \frac{1}{V_{max}} \qquad \text{(Eq. 7)}$$

3. RESULTS AND DISCUSSION

3.1 Experimental data of the RSM

A linear relationship was observed between the initial reaction rate and enzyme concentration within the range of 0.309-1.118 U L^{-1}. The percentage of substrate (drug) converted to product was determined within the first 10 seconds of the assay, ensuring that no more than 5% of the drug was degraded during this period. This approach ensured that the substrate remained in excess at all tested enzyme concentrations, allowing for an accurate estimation of the initial reaction rate. Based on the linear trend obtained, a laccase concentration of 1.118 U L^{-1} was selected as the optimum concentration for the degradation process.

The responses from a Box-Behnken design experiment are presented in Table 3, showcasing the effects of three key variables namely, pH, temperature, and amoxicillin concentration on the laccase enzyme-mediated degradation process. A three-factor three level BBD methodology was used for designing the optimisation process. BBD helps identify the optimal settings for a system's variables to achieve the best possible result, considering both individual factors and their combined effects.

For the degradation of amoxicillin (Table 3), it could be seen that, the maximum degradation was achieved at Y = 64.4%. This greater capacity was obtained in the conditions of the following operations: pH=5, temperature = 50ºC, and initial amoxicillin concentration = 0.0125 mg mL^{-1}. All of them were analysed by ANOVA and regression coefficient, residual sum of squares and lack-of-fit (*p*-value).

An analysis of variance (ANOVA) was conducted to assess the effects of temperature, pH, and concentration on amoxicillin degradation, as shown in Table 4. The ANOVA table provides a comprehensive understanding of each factor's influence, both individually and in combination, on the degradation process. ANOVA table shows the following information for the model: linear, square (without interactions) and 2-way interaction (full quadratic model including interactions). Statistically significant effects are indicated by *p*-values lower than 0.05, whereas *p*-values exceeding 0.05 suggest a lack of statistical significance.

Table 3: Responses of Box Behnken design on the selected operating variables.

RunOrder	A = pH	B = Temperature	C = Amoxicillin	Y = Amoxicillin degradation (%)
1	0	0	0	55.1
2	-1	0	+1	56.1
3	+1	-1	0	32.4
4	-1	+1	0	64.4
5	0	+1	+1	62.5
6	+1	+1	0	56.4
7	0	-1	+1	24.2
8	-1	0	-1	59.3
9	0	0	0	53.2
10	+1	0	+1	50.4
11	0	-1	-1	28.4
12	0	+1	-1	62.5
13	-1	-1	0	26.3
14	+1	0	-1	50.7
15	0	0	0	55.3

Among the linear effects, the statistical analysis revealed significant effect of temperature (B) on degradation, as indicated by the *p*-value of 0. Amoxicillin concentration (C) had no substantial impact on degradation, with a p-value of 0.352, while pH (A) showed a lesser but still insignificant influence (*p*-value = 0.083). Both amoxicillin concentration and pH had an insignificant influence on degradation in the linear model, but their effects became more noticeable in the quadratic terms. For the square effects, the terms A×A and C×C demonstrated a higher level of insignificance, as

indicated by the *p*-values of 0.982 and 0.762, respectively. Table 4 shows that the interaction effects (full quadratic) between A×B yielded the highest *F*-value of 49.22 with significant different between these factors. The interaction between pH of the solution and the concentration of amoxicillin (A×C) and between the temperature and the amoxicllin concentration (B×C) were statistically insignificant. Furthermore, the lack-of-fit test is a crucial tool in model evaluation. A high p-value generally indicates a good model fit ($p>0.05$), while a low *p*-value suggests that the model may not adequately capture the underlying relationship in the data. In this experiment, the high lack-of-fit value ($p = 0.112$) further suggests that the model fits the data reasonably well.

In order to fit a second order polynomial regression model in (Eq. 2), the data for conversion, Y was subjected to multiple regression analysis using least squares regression. Estimated regression coefficients of the fitted model for conversion for each coded value are presented in Table 5. The positive sign of the coefficients implies a synergistic effect and an antagonistic effect on the response is indicated by the negative sign (Table 5). The constant 54.53 was evidently independent from any factor. Meanwhile, the linear terms B and second-order terms, A×A, and the interactive factors (A×C and B×C) had a positive effect, which meant that if the magnitude of these factors increased, the amoxicillin would be increased as well. Conversely, A, C, B×B, C×C and A×A had a negative impact on conversation.

Table 4: ANOVA analysis to determine the impact of temperature, pH, and concentration on amoxicillin degradation.

Source	DF	Adj SS	Adj MS	*F*-Value	*P*-Value	Remark
Model	9	2707.01	300.78	42.69	0.000	Significant
Linear	3	2301.50	767.17	108.88	0.000	Significant
A	1	32.80	32.80	4.66	0.083	Insignificant
B	1	2261.28	2261.28	320.94	0.000	Significant
C	1	7.41	7.41	1.05	0.352	Insignificant
Square	3	349.30	116.43	16.52	0.005	Significant
A × A	1	0.00	0.00	0.00	0.982	Insignificant
B × B	1	346.81	346.81	49.22	0.001	Significant
C × C	1	0.72	0.72	0.10	0.762	Insignificant
2-Way Interaction	3	56.21	18.74	2.66	0.160	Insignificant
A × B	1	49.70	49.70	7.05	0.045	Significant
A × C	1	2.10	2.10	0.30	0.608	Insignificant
B × C	1	4.41	4.41	0.63	0.465	Insignificant
Error	5	35.23	7.05			
Lack-of-Fit	3	32.54	10.85	8.08	0.112	Fits well
Pure Error	2	2.69	1.34			
Total	14	2742.24				

DF = Degrees of freedom; Adj SS = Adjusted sum of squares; Adj MS = Adjusted mean of squares; F: *F*-statistic; P: *P*-statistic

Table 5: Values of model coefficients of conversion

Term	Coefficients	Value	Standard error Coefficients
Constant	β_0	54.53	1.53
A	β_1	-2.025	0.938
B	β_2	16.813	0.938
C	β_3	-0.962	0.938
A×A	β_{11}	0.03	1.38
B × B	β_{22}	-9.69	1.38
C × C	β_{33}	-0.44	1.38
A × B	β_{12}	-3.52	1.33
A × C	β_{13}	0.72	1.33
B × C	β_{23}	1.05	1.33

The coefficients obtained in Table 5 were substituted into (Eq. 2) to construct a quantitative model equation (Eq. 5) for the percentage of amoxicillin degradation which given as;

Response variability was determined at 98.72%, indicated by the multiple determination coefficient value, R^2 of 0.9872.

The Pareto chart of the standardized effects plot analysis displays the same result as the variance analysis The Pareto chart is a valuable tool for visualizing the standardized effects of temperature (A), pH (B), and concentration (C) on the percentage degradation of amoxicillin, along with their interactions. Each bar represents the magnitude of an effect, with longer bars indicating greater impact. The red vertical line at 2.57 marks the threshold for statistical significance at an alpha level of 0.05. Fig. 1 shows the standardized effects (represented by the bar charts) arranged from the largest effect on the right to the smallest effect on the left, with a reference line determined at 2.57. The bars of factors B and BB, followed by interaction AB exceed the reference line (2.57), indicate these effects are statistically significant.

$$Y = 54.53 - 2.025A + 16.813B - 0.962C + 0.03A^2 - 9.69B^2 - 0.44C^2 - 3.52AB + 0.72AC + 1.05BC \quad \text{(Eq. 5)}$$

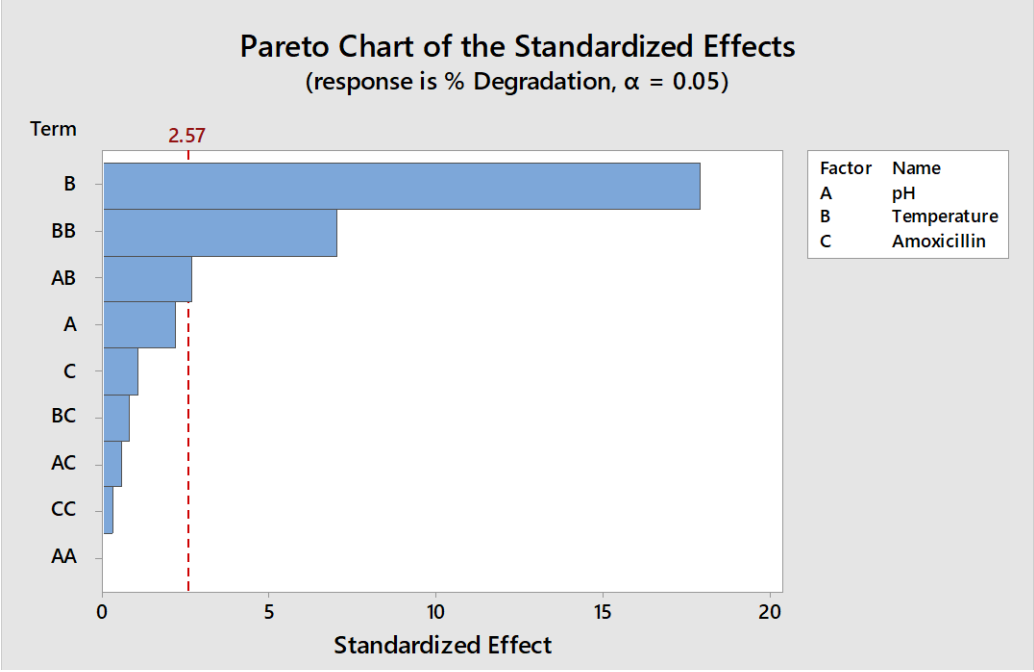

Figure 1: Pareto Chart of the standardized effects.

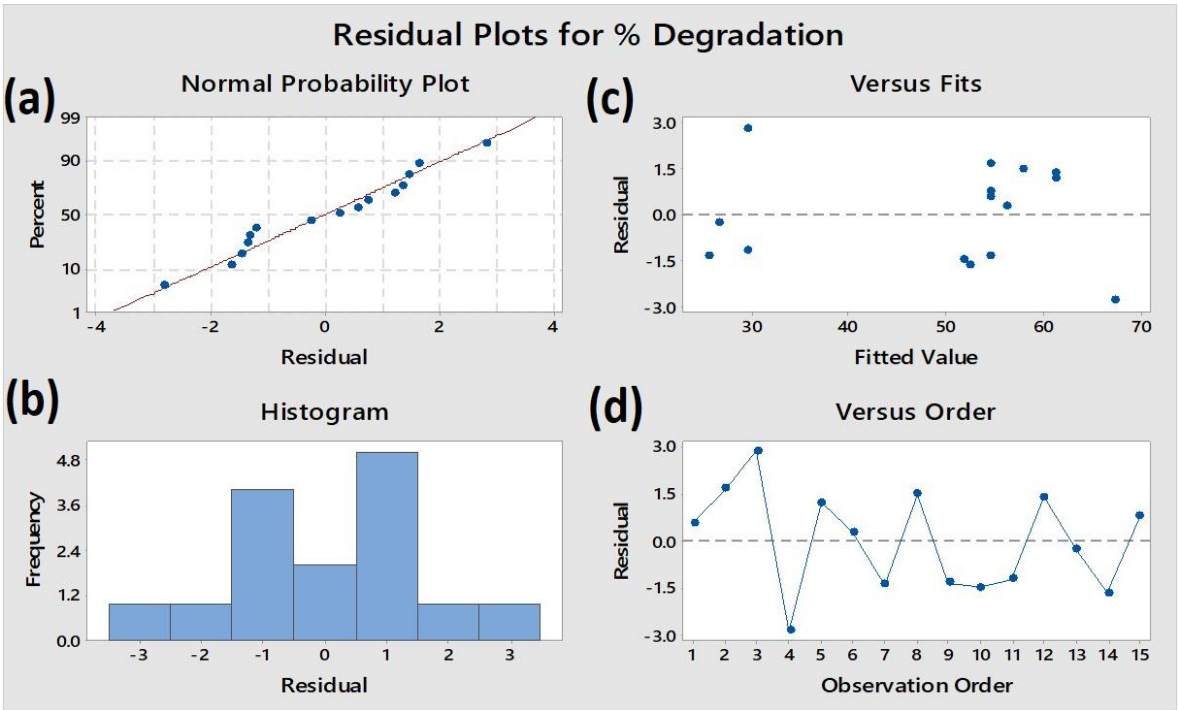

Figure 2: Residual plots for amoxicillin degradation (%) (a) normality probability plot, (b) histogram plot, (c) standardized residual vs fitted value, and (d) standardized residual vs observation order.

Validation of the applied regression model was further performed through examination of residual analyses. Residual analyses assess how well a model fits the data and characteristically shown by four different graphs (Fig. 2). The normal probability plot (Fig. 2a) illustrated that as the number of runs is less than 50, the residuals were normally distributed shown by the plots come close to a straight-line. The histogram displayed a bell-shape curve, (Fig. 2b) which further validated the normal probability assumption. An outlier can be seen from the bar indicated by slightly outside from the ± 2 range (Fig. 2b). This error is known as random error which produced from the collected experimental data. Fig. 2c (residuals against fitted value) showed that was no strong evidence of systematic error as shown by a random pattern of residuals. Lack of pattern observed in Fig. 2d, indicating that the order in which data is collected has no impact on the data collected (Abd Razak *et al.*, 2021; Yaakub *et al.*, 2024; Razak & Annuar, 2024).

In general, the residual data pattern was normally distributed and having a lower likelihood to be influenced by non-random and systematic errors.

The surface plots in Fig. 3 depict how the percentage of amoxicillin degradation is influenced by the interactions between pH, temperature, and amoxicillin concentration. This analysis enhances understanding by providing a clear visualization of how changes in these independent variables affect the response. The significant effect of curvature within the model is represented in the surface plot which also showed the optimized region. Fig. 3(a) demonstrates the significant mutual interaction between pH (A) and temperature (B) for amoxicillin degradation (%) as a response at a constant amoxicillin concentration (0.0125 mg mL^{-1}). The amoxicillin degradation decreased with increasing pH. In contracts, it was noted that as the temperature increased from 30 to 50 ^{o}C, amoxicillin degradation (%) rose up due to the enhanced kinetic energy of the molecules, which accelerated the degradation process. This indicates

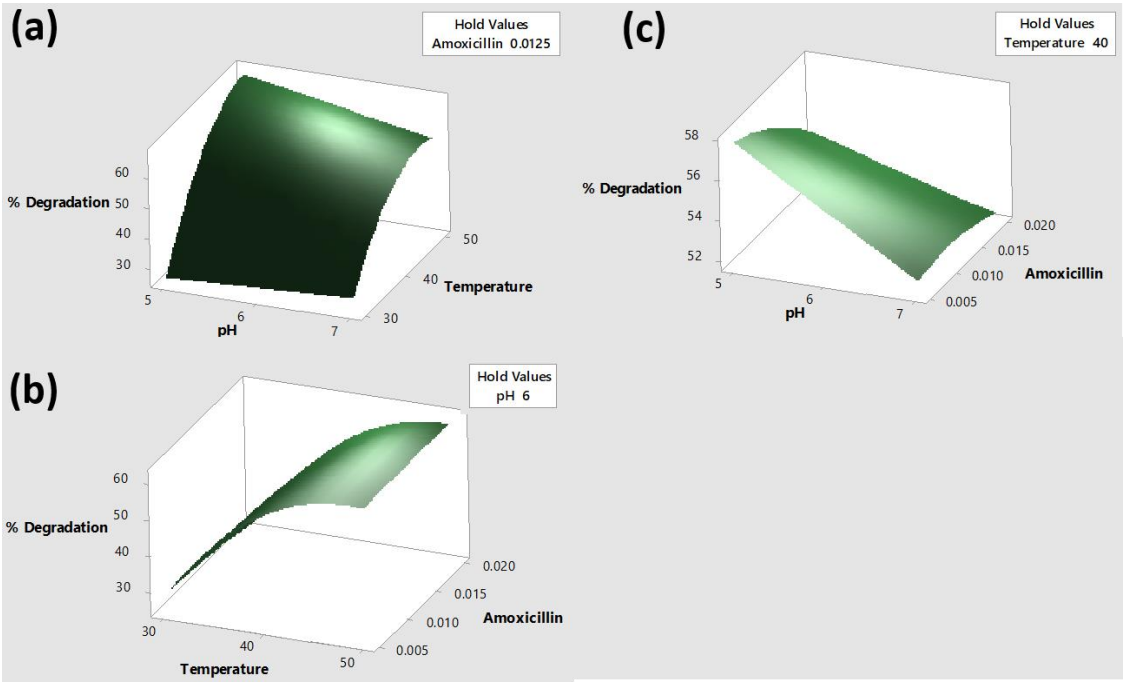

Figure 3: Surface plots of amoxicillin degradation at a constant value of (a) amoxicillin concentration, (a) pH and (c) temperature.

that the minimum pH level and the maximum temperature results in higher degradation. Therefore, it is clear that the amoxicillin degradation (%) exhibited positive synergistic effects when combined with pH and temperature. These data also align with the ANOVA analysis (Table 4), demonstrating the influence of the factors when analyzed collectively. Fig. 3(b) shows the significant interactive effect of temperature (B) and amoxicillin concentration (C) on the response (Y) at constant pH value (A) of 6. The highest amoxicillin degradation (%) can be achieved at high temperatures, irrespective of the amoxicillin concentration. It is evident that only pH had a positive effect on amoxicillin degradation (%). Fig. 3(c) shows the effect of pH (A) and amoxicillin concentration (C) and their reciprocal interaction according to the amoxicillin degradation (%) at constant temperature 30 ºC. It was not possible to detect any interaction between the factors, as no curvature was observed for amoxicillin concentration (C). These data are consistent to ANOVA analysis (Table 4).

The response, Y was then subjected to optimization variables which was done using Response Optimizer function (Fig. 4). The goal for optimization was to maximize the percentage of considering the amoxicillin degradation (%) presented in Table 3. Optimal conditions for optimization were given as (A, B, C,) = (-1, +1, 0.00712) corresponding to actual values of pH 5, 50°C and 0.07121 mg mL^{-1} of amoxicillin concentration. In these conditions, maximum predicted conversion was 67 % (see curve; Figure 4). However, the value was lower than the obtained value from the study by Shokoohi *et al.* (2018). They investigated the removal of amoxicillin from aqueous solutions through enzymatic oxidation, utilizing BBD model, and achieved an impressive removal rate of 91.5%. The presence of hydroxybenzotriazole (HBT) as a mediator in their study significantly contributed to the observed higher percentage of degradation. HBT acts as a redox mediator, facilitating electron transfer between the laccase and the amoxicillin. HBT serves as an intermediate, amplifying the oxidation of substrates

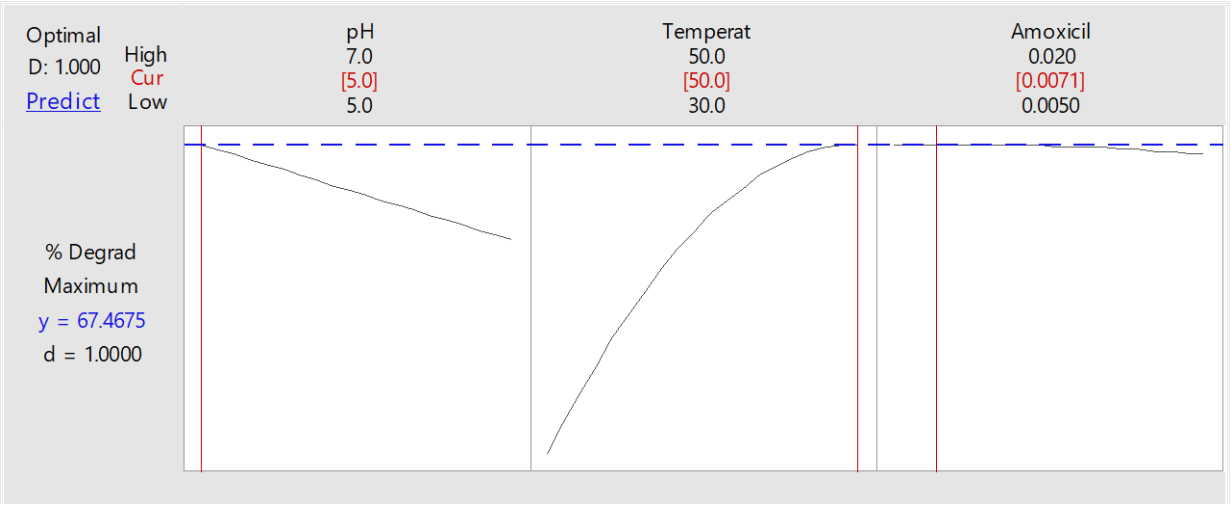

Figure 4: Response Optimizer results

that may not directly interact efficiently with the enzyme.

3.2 Kinetics of degradation

The graph of the amount of drug degraded over 72 h of incubation at various drug concentrations was plotted for each temperature tested (data not shown). The rate of reaction of drug degradation was then determined from the slope of the graph using Eq. 4. It was observed that the degradation rate is directly proportional to the initial amoxicillin concentration (0.0625-0.25 mg mL^{-1}) at any given temperature (303-323K). For the free laccase system studied, the rate of reaction for every initial drug concentration tested showed a linear increase with temperature.

Based on the *k*' values obtained at different temperatures (Table 6), the apparent activation energy (E_a) for the degradation process was estimated using an Arrhenius plot (Eq. 5). A plot of ln k' versus the reciprocal of temperature produced a linear relationship with a regression coefficient of 0.9913. From the slope of the line, the *E*a value was determined to be 34 kJ mol^{-1}, which falls within the typical range reported for enzyme-catalyzed reactions which is 16-84 kJ mol^{-1} (Dixon & Webb, 1960).

A kinetic model was fitted to the experimental data using Line Weaver Burk linearization which presented fitted plots with R^2 of 0.996 (Fig. 5). At 50°C, the obtained V_{max} value was calculated at 0.381 mg mL^{-1} h^{-1} while the K_m value was 0.951 mg mL^{-1}.

Table 6: Rate of reaction at different initial concentrations of amoxicillin

Temp (°C)	Temp (K)	Rate of reaction (× 10^{-2} mg mL^{-1} h^{-1}) at different initial concentrations of amoxicillin (mg mL^{-1})			Rate constant, *k*' (h^{-1})
		0.0625	0.125	0.25	
30	303	1.89	4.48	6.45	0.231
40	313	2.36	4.55	7.21	0.252
50	323	2.33	4.65	7.50	0.287

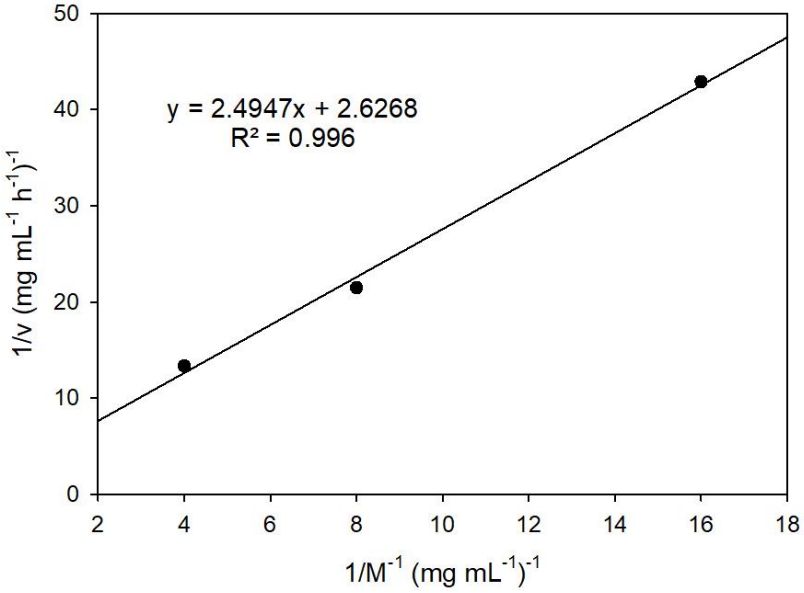

Figure 5: Line Weaver Burk linearization

The purpose of a Line Weaver Burk linearization plot is to predict the K_m and V_{max} values. V_{max} describes when all the active sites in the enzyme solution are filled by the substrate, it's the maximum rate of reaction. While the Michaelis-Menten constant, K_m represents a direct relationship of the formation and disappearing of the enzyme-substrate complex, indicating the affinity of the enzyme-substrate.

4. Conclusion

A RSM was successfully applied in this study to optimize drug degradation. An orthogonal array was generated using the BBD with three levels and three factors: pH, temperature, and amoxicillin concentration. RSM and ANOVA were used to determine the optimal conditions for maximizing the drug degradation efficiency. Temperature was identified as the primary factor influencing the degradation process with pH and substrate concentration having negligible effects. The interaction between temperature and pH was identified as the most critical combined factor.

Temperature influences the kinetic energy of molecules, accelerating reaction rates up to an optimal point, beyond which the enzyme may denature. Similarly, pH impacts the ionization of active site residues and the enzyme's overall charge, which can either enhance or inhibit substrate binding. The combined effect of these two factors often determines the reaction's success and optimal conditions for maximum enzyme activity. Optimal conditions for degradation were predicted using BBD which resulted in a 67% degradation rate. This highlights the potential of laccase as an eco-friendly solution for the reduction of pharmaceutical pollutants, which has the potential to be applied in public health and environmental management.

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6. Conflict of Interests

All authors declare no conflict of interest.

7. References

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