Proceedings of Science and Mathematics
Faculty of science, universiti teknologi malaysia
https://science.utm.my/procscimath
Volume 1 (2025) 33-36

Evaluation of Tetracycline Removal Efficiency by *Chlorella sorokiniana* using Box Behnken Design

Nadira Azra Kurnia Zaiful Zukry[a], Nor Azimah Mohd Zain[a*], Mohd Farizal Ahmad Kamaroddin[a], Huszalina Hussin[a]
[a]Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia

*Corresponding author: norazimah@utm.my

Abstract
The widespread use of tetracycline (TC) in agriculture, aquaculture, and human medicine has led to its persistent presence in aquatic environments, raising concerns about antibiotic resistance and ecosystem disruption. This study evaluates the degradation efficiency of tetracycline using the green microalgae *Chlorella sorokiniana* under various environmental conditions, employing Box-Behnken Design (BBD) as part of Response Surface Methodology (RSM) to optimize key parameters. The experiment investigated the influence of aeration (0.5, 1.0, 1.5 L/min), initial TC concentration (2, 5, 8 mg/L), and exposure duration (12, 30, 48 hours) on tetracycline removal efficiency and pigment content (chlorophyll a, chlorophyll b, and carotenoids) as physiological stress indicators. Results indicated that aeration and time were the most significant factors affecting tetracycline degradation, with optimal conditions (1.5 L/min aeration and 48 hours of exposure) achieving up to 90% removal. While pigment content showed some response to tetracycline stress, statistical analysis revealed limited significance for most interactions. This research demonstrates the potential of C. sorokiniana as an eco-friendly bioremediation agent and highlights the utility of statistical optimization methods, such as BBD, in designing effective wastewater treatment strategies.
Keywords: Tetracycline, antibiotic, microalgae, removal, degradation, Box-Behnken Design (BBD)

Introduction

Tetracyclines (TCs) are extensively utilized in aquaculture, livestock management, and human healthcare. TCs rank as the second most used antibiotics in Malaysia. It is frequently administered in animal feed or directly dissolved in water (Thiang et al., 2021). The potential negative impacts of improper disposal of pharmaceutical chemicals have led to growing environmental concerns regarding water contamination, which affects both aquatic ecosystems and human health. The presence of tetracycline in waterways can lead to the emergence of antibiotic-resistant bacteria, posing a substantial risk to public health and necessitating the implementation of effective remediation methods. A considerable amount of residual antibiotics is released into the aquatic environment as a result of conventional wastewater treatment methods, which are not only expensive but also inefficient in maintaining the required degree of water quality control in the face of high antibiotic effluent concentrations (Chu et al., 2023).

Chlorella sorokiniana is a microalgae species that has been extensively researched for its potential to remediate antibiotic-contaminated wastewater. It demonstrates exceptional removal effectiveness for tetracycline, with nearly 100% clearance across a diverse range of concentrations, indicating a robust biodegradation capability. The study by Chu et al. (2023) on tetracycline (TET) degradation by *Chlorella sorokiniana* showed that the microalga exhibited remarkable removal efficiencies for TET, especially at values between 1 and 50 mg/L. The maximum removal efficiency reached 93% by the second day, due to the photolysis process.

Materials and methods

A falcon tube filled with 50 ml of the microalgae strain (*Chlorella sorokiniana*) was retrieved from Nanomaterial Lab 2, T02 UTM. The sample is stored in the refrigerator (~1.7 °C to 3.3°C) until needed for laboratory testing. Direct liquid cultivation was performed using a 10% microalgae inoculum in a 1 L total working volume of BG11 medium. OD680 was taken every day for 15 days. The microalgae were grown in the following conditions: Aeration (1.0 L/min), pH (7.1), Temperature (room temperature) (Usman et al., 2024).

A total working volume of 500 mL of *Chlorella sorokiniana* was mixed with three different concentrations of Tetracycline (2.5, 5, and 8 mg/L). The number of replicates depends on the number of experimental runs generated by DesignExpert. Tetracycline degradation is assessed at various time frames: 12, 30, and 48 hours. Using a Falcon tube, 12 mL of microalgae culture was collected after the specific time frames. The sample was then centrifuged at 6,000 rpm for 10 minutes. After centrifuging, the sample's supernatant was filtered using a 0.45 µm syringe filter for HPLC analysis. Samples were chilled in the refrigerator for preservation before HPLC analysis at Block C19, UTM.

Results and discussion

This study investigated the impact of three variables—aeration, concentration, and exposure duration—on tetracycline (TC) levels using a factorial experimental design. The parameters were manipulated at various degrees, and the TC concentration was assessed as the response variable. The results were examined using Design Expert software, specifically the Box-Behnken Design, which revealed some significant trends and interactions. Table 4.1 shows the ANOVA Table for the 2FI Model, which was retrieved from DesignExpert.

Table 1 ANOVA Table for 2FI Model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	12.97	6	2.16	70.67	0.0026	significant
A-Aeration	1.19	1	1.19	39.04	0.0083	
B-Concentration	0.0719	1	0.0719	2.35	0.2227	
C-Time	5.33	1	5.33	174.14	0.0009	
AB	0.7629	1	0.7629	24.94	0.0154	
AC	1.19	1	1.19	39.04	0.0083	
BC	0.0853	1	0.0853	2.79	0.1936	
Residual	0.0918	3	0.0306			
Lack of Fit	0.0857	2	0.0429	7.08	0.2568	not significant
Pure Error	0.006	1	0.006			
Cor Total	13.06	9				

Tetracycline Concentration (After Degradation) = 17.1569 + -9.11578A + -0.927556B + 0.252741C + 0.697111AB + 0.117296AC + 0.00512963BC

The ANOVA table assesses the relevance of the Box-Behnken design in the investigation of tetracycline breakdown using Chlorella sorokiniana. The model's p-value (0.0026) is much below the 0.05 threshold, validating its efficacy. Aeration (A) and time (C) are key parameters that significantly influence tetracycline degradation, making them essential for process optimization. The concentration (B) factor has a p-value exceeding 0.05, indicating it does not significantly affect tetracycline breakdown. The interaction effects between aeration and time (AC) and aeration and concentration (AB) are statistically significant, suggesting their importance in process optimization. These data emphasize the efficacy of the Box-Behnken design in investigating tetracycline breakdown.

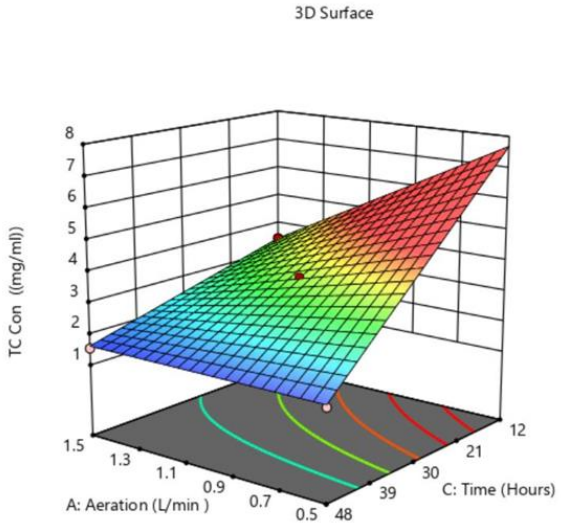

Figure 1 3D surface plot graph for AC (Aeration and Time)

This plot demonstrates that increased aeration and duration result in a reduction of TC concentration, suggesting that enhanced aeration and extended time enhance tetracycline breakdown. The colour gradient (from red to blue) illustrates the decline in TC concentration, and the pronounced slope verifies that minor variations in aeration and duration result in significant drops in tetracycline concentration.

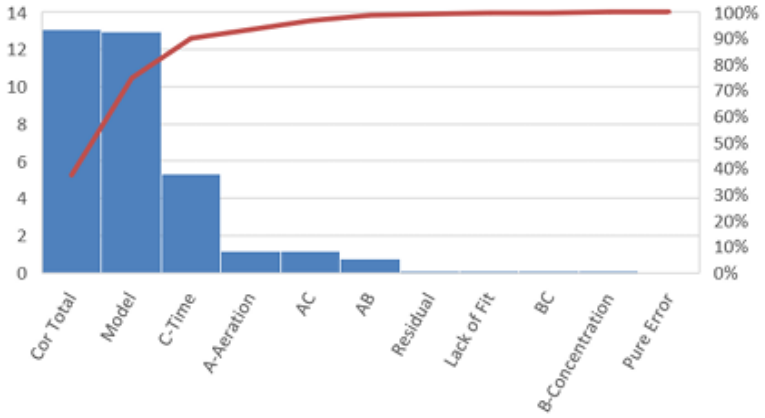

Figure 3 Pareto Chart of Factors Affecting TC Concentration

The Pareto chart visually illustrates the significance of each factor in influencing tetracycline concentration. The data distinctly indicate that model and time are the primary factors influencing tetracycline degradation, followed by aeration. The concentration factor and interaction terms exert a comparatively minor influence, as evidenced by their placement at the lower end of the chart. This confirms the previous observation that aeration and duration are the primary determinants of degrading efficiency, whereas concentration assumes a supporting function in this process.

Conclusion

In brief, this research effectively demonstrated the efficacy of Chlorella sorokiniana in the biodegradation of tetracycline (TC) in aquatic ecosystems. The Box Behnken Design (BBD) was employed to optimize experimental parameters, including aeration, tetracycline concentration, and exposure duration, in order to enhance degradation efficiency. The findings indicated that aeration (X1) and time exposure (X3) were key variables, with aeration attaining a tetracycline elimination effectiveness of almost 85% at ideal conditions. Concentration (X2) exerted a negligible influence on degradation efficiency, with clearance rates stabilizing at levels over 20 mg/L. The statistical analysis and response surface methodology (RSM) indicated that optimal degradation conditions were achieved with elevated aeration (1.5 L/min) and prolonged exposure times (48 hours), resulting in a 90% decrease in tetracycline concentration. The physiological responses of *C. sorokiniana*, specifically alterations in chlorophyll content, demonstrated that the algae could tolerate and degrade tetracycline with no effect on growth, despite observed reductions in chlorophyll a (approximately 15-20%) and b (approximately 10-15%). This study highlights the efficacy of *C. sorokiniana* as a bioremediation agent for tetracycline in wastewater treatment, emphasizing the importance of adjusting environmental conditions to enhance degradation efficiency.

Acknowledgement

We would like to express our sincere gratitude to the Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, for providing the research facilities and support needed to complete this study.

References

Chu, Y., Li, S., Xie, P., Chen, X., Li, X., & Ho, S.-H. (2023). New insight into the concentration-dependent removal of multiple antibiotics by *Chlorella sorokiniana*. *Bioresource Technology, 384*, 129409.

Usman, H. M., Kamaroddin, M. F., Sani, M. H., Malek, N. A. N. N., Omoregie, A. I., & Zainal, A. (2024). A comparative analysis assessing growth dynamics of locally isolated *Chlorella sorokiniana* and *Chlorella vulgaris* for biomass and lipid production with biodiesel potential. *Bioresource Technology, 403*, 130868.

Thiang, E. L., Lee, C. W., Takada, H., Seki, K., Takei, A., Suzuki, S., Wang, A., & Bong, C. W. (2021). Antibiotic residues from aquaculture farms and their ecological risks in Southeast Asia: A case study from Malaysia. *Ecosystem Health and Sustainability, 7*(1), 1926337.