Chitosan-Driven Bio Flocculation for Low-Energy Microalgae Harvesting: Optimization Using Response Surface Methodology

[1]Ashwathy Sunny, [2]David K Daniel, [3]Julia Sebastian

[1]Department of Chemistry, St. Dominics College, Kanjirappally, Kottayam, Kerala-686512, India

[2]Department of Chemical Engineering, Saintgits College of Engineering, Kottayam, Kerala-686532, India

[3]Department of Physical Sciences and Mathematics, St. Francis de Sales College (Autonomous), Bangalore - 560100, Karnataka, India

Abstract—Micro-algae are recognised as a promising renewable resource for biofuels, nutraceuticals, and wastewater treatment. However, large-scale commercialisation is hindered by the difficulty and cost of biomass harvesting due to the small size and negative charge of algal cells. This study explores chitosan - a natural, biodegradable, and non-toxic biopolymer derived from chitin - as an eco-friendly bioflocculant for efficient micro-algae recovery. The flocculation process of *Chlorella vulgaris* was optimised using a three-factor, three-level Box–Behnken design under response surface methodology (RSM). The independent variables examined were chitosan dosage (0.10-0.20 mg/L), pH (5-9), and settling time (1–5 min). Statistical analysis (ANOVA) revealed that settling time ($p = 0.002$) and the quadratic effect of chitosan dosage ($p = 0.001$) significantly influenced flocculation efficiency, while pH had minimal impact. The developed quadratic regression model showed good agreement with experimental data ($F = 11.79$, $p < 0.05$), confirming its adequacy for predicting biomass recovery. Results demonstrate that chitosan effectively promotes cell aggregation at low concentrations, minimises sludge formation, and preserves biomass integrity. Hence, chitosan-based bioflocculation presents a sustainable and energy-efficient alternative for large-scale microalgal harvesting.

Index Terms—Chitosan, Bioflocculation, Microalgal harvesting, *Chlorella vulgaris*, Response Surface Methodology

I. INTRODUCTION

Micro-algae have drawn worldwide interest as a renewable resource because of their widespread utilisation in different sectors, including food, cosmetics, nutraceuticals, wastewater treatment, high value bioproducts [1-3]. The bioactive products which they produce are effective in the prevention and treatment of many disease conditions [4-6]. Despite their broad applications, one of the major challenges towards large-scale commercialisation is the harvesting of algal biomass. Microalgal cells, being exceptionally small, have low specific gravity and carry a negative surface charge, which prevents them from settling naturally and makes their separation from the culture medium both energy-intensive and expensive [7]. Although conventional methods like centrifugation, filtration, and sedimentation recover high biomass, as they require high energy and operational expenses, they can't be used for large-scale applications. Flocculation has emerged as the most practical and cost-effective alternative to conventional methods, which include centrifugation, filtration, and coagulation [8-11]. Flocculation helps in the aggregation of individual algal cells into large clusters and catalyses the process of sedimentation, thus making the biomass harvesting efficient. The traditional chemical flocculants have been used for a long time due to their effectiveness and affordability. Though being effective, they raise health and environmental concerns [12].

Bioflocculants emerge as the viable eco-friendly substitute for harvesting micro-algae as they are safe, nontoxic, biodegradable and they don't produce harmful by-products [13,14]. Bioflocculants don't use additional chemicals and enables the recovery of the resource without causing damage to cells, and can initiate flocculation through aggregation of biomass through natural microbial interactions. It does not need high-energy-demanding equipment's to run so it reduces the energy demand and carbon footprint. As they are of natural origin, they reduce environmental impacts which contributes to the effectiveness of bioflocculants in areas like wastewater treatment and micro-algae harvesting. Collectively, these features make bioflocculants both environmentally responsible and practical for large-scale microalgae-based production systems.

Chitosan shows greater potential as a bioflocculant since it has a strong cationic nature, which helps in the neutralisation of negatively charged algal cell surfaces, thus enhancing aggregation [15,16,12]. Moreover, at relatively low concentrations, it restricts excess sludge formation and helps to maintain the integrity of the harvested biomass, which is valuable for applications in food, feed, cosmetic and pharmaceutical industries [17,18]. Modern studies suggest that the ability of chitosan to cause flocculation is influenced by the degree of deacetylation as well as the molecular weight of chitosan [19]. Nanoscale analysis carried out, which suggests that pH influences protonation of chitosan and its interaction with the algal cells [20].

In this context, chitosan, a natural biopolymer derived from chitin, becomes an eco-friendly and efficient bioflocculant for micro-algae harvesting as it is biodegradable, non-toxic and widely available from crustacean shell waste, making it a sustainable alternative to chemical flocculants. The present work focuses on the potential of chitosan as a low-cost bioflocculant for the recovery of microalgal biomass. An attempt has been made to study the effects of parameters that influence

the flocculation efficiency, namely chitosan dosage, pH, and settling time and to identify the conditions required for maximum biomass recovery. The optimisation was performed using Box–Behnken design [21] in combination with Response Surface Methodology [22].

II. MATERIALS AND METHODS

Materials

Microalgal strain for *Chlorella vulgaris* obtained from the Department of Botany, Catholicate College, Pathanamthitta, was grown in a 9L, 290mm *187mm *320mm photobioreactor. The algae were grown by adding 600ml of inoculum along with the media in FPPB. The composition of media for algal growth included NPK, Dolomite, Urea, and rice bran. Ambient conditions required to provide the growth were provided by two CFL bulbs of light intensity 30W (3000 lumens) and 9W (450 lumens). Sparging was done with a CO_2-air mixture in a 1:100 ratio using an air pump with a flow capacity of 0.051/min. From Extra pure grade chitosan (High MW), SRL (Sisco Research Laboratories Pvt. Ltd.) A chitosan stock solution was prepared by dissolving 1g of chitosan gradually in 100 mL of acetic acid solution using a laboratory stirrer until complete dissolution.

Jar test is conducted with different dosages of chitosan, 0.1-10 mg/L chitosan. To the algal culture, chitosan is added and gently stirred for 2–5 minutes at low speed (to ensure even distribution) and then allowed to stand for 10-30 minutes. Cell aggregates were seen forming and settling to the bottom.

Box Benkhen Experimental design

To optimise chitosan-mediated micro algae flocculation, a 3-factor, 3 Level Box Behnken Design (BBD) under RSM was carried out. The three independent variables used for the design were: chitosan dosage (X_1, mg L^{-1}), pH (X_2), and settling time (X_3, min). Each variable was set to three different coded levels (−1, 0, +1), standing for low, medium, and high levels. BBD generated 15 experimental runs with three centre points to improve precision and enable estimation of pure error. This design avoids extreme combinations of factors that may be impractical in biological systems, making it particularly well-suited for improving flocculation conditions [23].

In the BBD design, the relation between actual and real values of the variable is found using the equation

$$x_i = \frac{X_i - X_0}{\Delta X_i}$$

where x_i is the value of the variable, X_0 is the actual value at the centre point, and ΔX_i gives the change of variable. The second-order polynomial generated creates the response value and the lack-of-fit test, which represents the relation between input variables and biomass recovery [24,25].

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum\sum \beta_{ij} X_i X_j + \epsilon$$

III. RESULTS AND DISCUSSION

Optimisation of Factors

The effects of a single factor for algal growth with fixing levels of other factors are widely used in the conventional one factor at a time design technique [26-28]. Though the operation and analysis can be easily carried out, the insignificance lies as it is a very time-consuming process. When different factor interactions are significant, the optimal conditions found cannot be trusted. In such a situation, Response surface methodology is often found beneficial for the researchers for advancing and optimising [29-32]. Earlier Laboratory studies indicate that biomass production during the growth of *Chlorella vulgaris* is influenced significantly by Chitosan dose, pH, settling time and these three factors are regarded as the major variables that influence the growth of *C. vulgaris,* and optimisation can be done by Response Surface Methodology. In the current study, the Box Behnken Model for three variables are used. Chitosan Dosage (0.1-0.2 mg/l), pH (5-9), and settling time (1-5 min) were taken as input variables. Each variable was set to three different coded levels: low (-1), medium (0), and high (+1) levels. The design performs 15 experimental runs, which gives the biomass (mg) as the response of the design and different runs are carried out based on the design.

Model Fitting and Statistical Analysis

The experimental data obtained from the Box-Behnken Design (BBD) were analysed using Response Surface Methodology (RSM) to evaluate the effects of three operational parameters - chitosan dosage (X_1), pH (X_2), and settling time (X_3) on the flocculation efficiency of *Chlorella vulgaris* as tabulated in Table 1 and Table 2. The quadratic model was found to best describe the relationship between the independent variables and the biomass recovery response.

The ANOVA results (Table 3) revealed that the model was statistically significant, with an F-value of 11.79 and a p-value of 0.007 (< 0.05), confirming a strong correlation between the experimental and predicted values. The lack-of-fit was found to be non-significant ($p > 0.05$), indicating that the model adequately represents the experimental data. Among the factors tested, settling time ($p = 0.002$) and the quadratic term of chitosan dosage ($p = 0.001$) were the most significant contributors to flocculation efficiency. The linear effect of chitosan dosage ($p = 0.051$) was nearly significant, whereas pH ($p = 0.802$) and other interaction terms showed limited influence within the studied range.

The regression equation derived from the analysis (in coded variables) was $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$

This model indicates a strong nonlinear dependence of biomass recovery on chitosan dosage and settling time, while the influence of pH was relatively minor. The Response Surface Methodology (RSM) analysis using the Box–Behnken Design (BBD) was conducted to evaluate the influence of chitosan dosage, pH, and settling time on the flocculation efficiency of *Chlorella vulgaris*. The

experimental data is fitted to a quadratic polynomial model, which was found to be statistically significant (F = 11.79; p = 0.007). The non-significant lack-of-fit (p > 0.05) confirmed that the model was adequate for prediction within the experimental range [33].

According to the ANOVA results, settling time (p = 0.002) and the quadratic term of chitosan dosage (p = 0.001) had highly significant effects on flocculation efficiency. The linear effect of chitosan dosage (p = 0.051) was near-significant, whereas pH showed limited influence (p = 0.802). These results indicate that both the amount of bioflocculant and the sedimentation period are key determinants of biomass recovery, while the process remains stable across moderate pH variations.

The final quadratic regression equation obtained in coded form was

$$Y = 26.68 - 214.0X_1 - 2.05X_2 - 0.406X_3 + 4.50X_1X_2 + 2\ .25X_1X_3 + 0.0688X_2X_3 + 610.0X_1^2 + 0.0813X_2^2 + 0.000X_3^2$$

where X_1, X_2, and X_3 represent chitosan dosage, pH, and settling time, respectively.

Table 1 Variables and their levels tested (coded and actual values)

Factor	Variable	Unit	Levels of coded variables		
			-1(Low)	0(Centre)	1(High)
X_1	Chitosan dosage	mg L^{-1}	0.10	0.15	0.20
X_2	pH		5	7	9
X_3	Settling time	min	1	3	5

Table 2 Experimental Box-Behnken design matrix and biomass responses by Chitosan

Run	Chitosan Dosage (mg/L)	pH	Settling Time (min)	Weight of biomass (mg)
1.	0.1	5	3	6.1
2.	0.2	5	3	6.0
3.	0.1	9	3	4.6
4.	0.2	9	3	6.3
5.	0.1	7	1	4.3
6.	0.2	7	1	4.5
7.	0.1	7	5	5.9
8.	0.2	7	5	7.0
9.	0.15	5	1	3.7
10.	0.15	9	1	3.5
11.	0.15	5	5	4.4
12.	0.15	9	5	5.3
13.	0.15	7	3	3.9
14.	0.15	7	3	3.9
15.	0.15	7	3	3.9

Table 3 ANOVA for quadratic response surface model

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	17.1418	1.90465	11.79	0.007
Linear	3	6.8425	2.28083	14.12	0.007
Chitosan Dose (X_1)	1	1.0513	1.05125	6.51	0.051
pH (X_2)	1	0.0113	0.01125	0.07	0.802
Settling Time (X_3)	1	5.7800	5.78000	35.79	0.002
Square	3	9.0843	3.02811	18.75	0.004
Chitosan Dose*Chitosan Dose (X_1^2)	1	8.8708	8.87077	54.93	0.001
pH*pH(X_2^2)	1	0.3323	0.33231	2.06	0.211
Settling Time*Settling Time(X_3^2)	1	0.0023	0.00231	0.01	0.910
2-Way Interaction	3	1.2150	0.40500	2.51	0.173
Chitosan Dose*pH(X_1X_2)	1	0.8100	0.81000	5.02	0.075
Chitosan Dose*Settling Time(X_1X_3)	1	0.2025	0.20250	1.25	0.314
pH*Settling Time(X_2X_3)	1	0.2025	0.20250	1.25	0.314
Error	5	0.8075	0.16150		
Lack-of-Fit	3	0.8075	0.26917	*	*
Pure Error	2	0.0000	0.00000		
Total	14	17.9493			

IV. EFFECT OF PROCESS PARAMETERS

Effect of Chitosan Dosage

Chitosan dosage played a crucial role in determining the flocculation efficiency. At low concentrations (< 0.1 mg/L), insufficient neutralisation of the negatively charged algal cell surfaces occurred, leading to poor aggregation. As dosage increased, the positively charged amino groups of chitosan effectively neutralised surface charges, promoting inter-particle bridging and floc formation. However, beyond the optimal dosage (~0.15 mg/L), a decline in flocculation efficiency is observed, as excess chitosan caused charge reversal and re-stabilisation of algal cells. This parabolic trend explains the statistical significance of the quadratic term (p = 0.001) in the ANOVA results. Similar trends are reported [12,18], emphasising the importance of dosage optimisation to achieve maximum floc yield with minimal reagent use.

Effect of pH

The influence of pH on biomass recovery was found to be statistically insignificant (p = 0.802) within the tested range (5–9). However, RSM plots suggested a moderate improvement in flocculation near neutral pH (~7). This can be attributed to the protonation of chitosan's amino groups at lower pH, enhancing its cationic nature and promoting better interaction with the negatively charged algal cell surfaces. At higher pH levels (>8), deprotonation reduces its positive charge density, thereby decreasing flocculation efficiency. Although pH did not show a strong

individual effect, its slight interaction with chitosan dosage (p = 0.075) suggests a coupled influence under certain conditions.

Effect of Settling Time

Settling time was found to be the most critical parameter influencing the overall recovery of biomass. Increasing the settling time from 1 to 5 minutes significantly improved biomass sedimentation, as confirmed by the low p-value (0.002) in the ANOVA results. Prolonged settling allows sufficient time for flocculated aggregates to settle under gravity, leading to clearer supernatant and higher recovery efficiency. Beyond 5 minutes, no further improvement was observed, suggesting that equilibrium conditions were achieved. This finding demonstrated that maintaining the correct retention time ensured optimal floc recovery without delay or loss of energy input.

Response Surface Interaction Effects

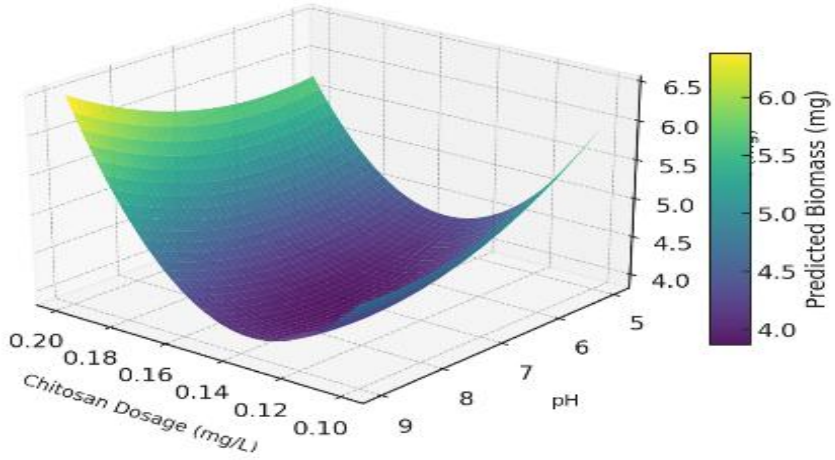
The 3D surface plots and contour diagrams illustrated that the interaction between chitosan dosage and settling time had the most pronounced influence on flocculation efficiency. Optimal biomass recovery was achieved at a chitosan dosage of ~0.15 mg/L, pH around 7, and settling time of 5 minutes. Beyond these conditions, efficiency decreased due to over-flocculation or re-dispersion effects. The interactive surface between chitosan dosage and pH indicated that at low dosages, acidic to neutral pH favoured aggregation, while higher dosages minimised the dependence on pH. These results confirm that chitosan-mediated flocculation is primarily governed by electrostatic neutralisation and polymer bridging mechanisms, both of which depend on dosage and contact time.

The 3D surface and contour plots illustrate the combined effects of the variables on biomass recovery (Figure 1, Figure 2, Figure 3).

Chitosan Dosage vs pH

Figure 1. Chitosan Dosage vs pH

Figure 1. Effect of Chitosan Dosage and pH on Biomass Recovery



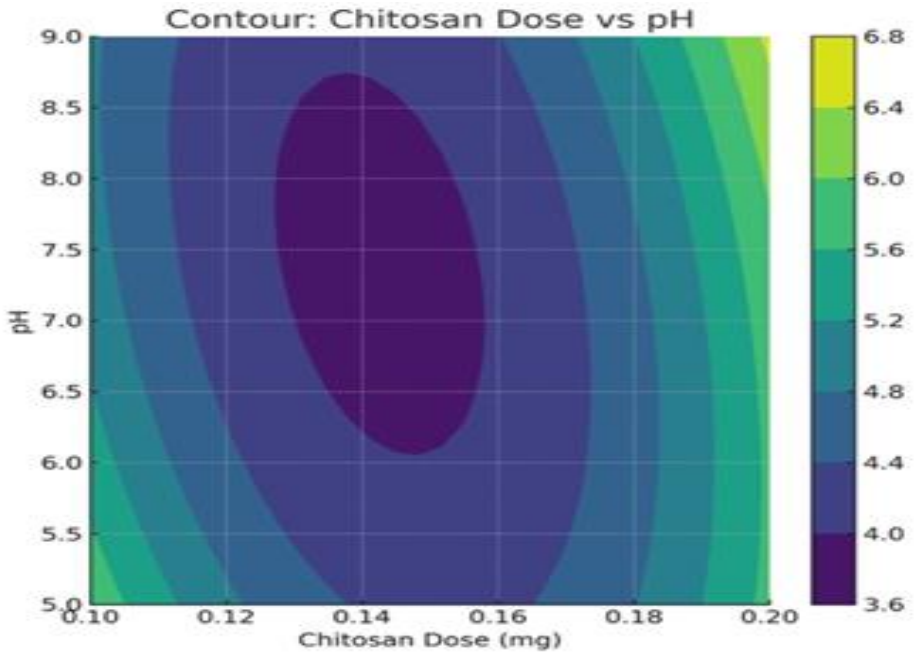


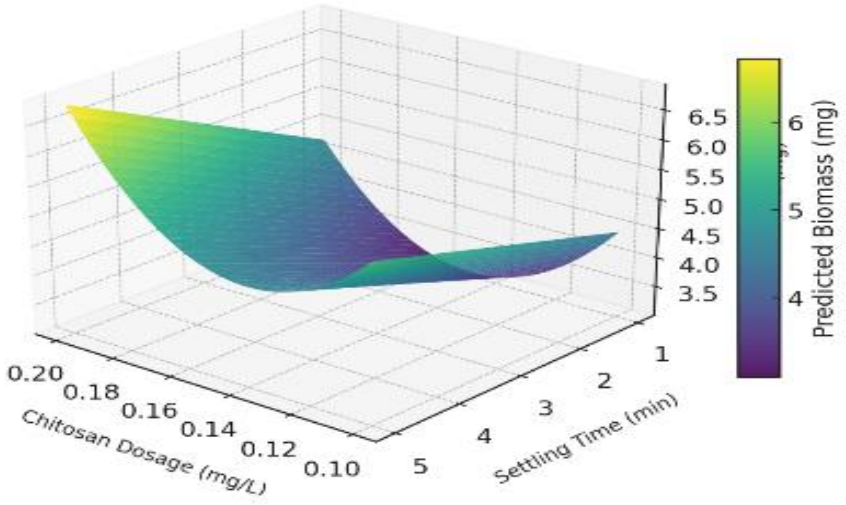
Fig.1 *Response surface and contour plot showing the combined effect of chitosan dosage and pH on biomass recovery*

At low chitosan concentrations, flocculation efficiency was highly dependent on pH, showing a moderate increase near neutral conditions. As chitosan dosage increased, the effect of pH became negligible, and high efficiencies were achieved across the pH range tested. This suggests that sufficient chitosan dosage can overcome the limitations of unfavourable pH conditions, enhancing the robustness of the process.

Chitosan dose vs Settling Time

Figure 2. Chitosan Dosage vs Settling Time

Figure 2. Effect of Chitosan Dosage and Settling Time on Biomass Recovery



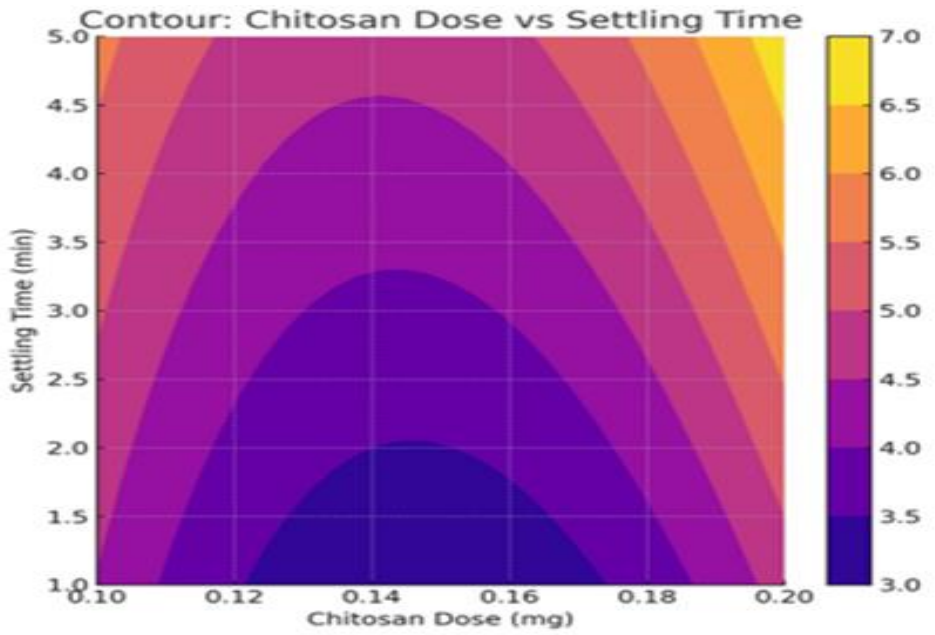


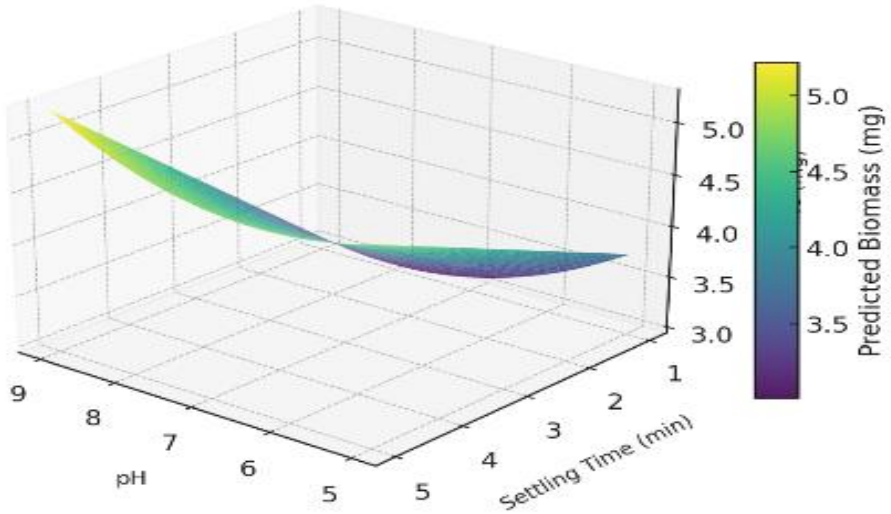
Fig. 2 *Response surface and contour plot showing the interactive effect of chitosan dosage and settling time on biomass recovery*

This interaction had the most pronounced influence on flocculation efficiency. The response surface revealed that at lower dosages, extended settling time significantly improved biomass recovery. However, at higher dosages, maximum efficiency (~7.0 mg biomass) was achieved. This synergistic interaction reflects the complementary roles of charge neutralisation (by chitosan) and gravitational settling (by time) in enhancing separation performance.

pH vs Settling Time

Figure 3. pH vs Settling Time

Figure 3. Effect of pH and Settling Time on Biomass Recovery



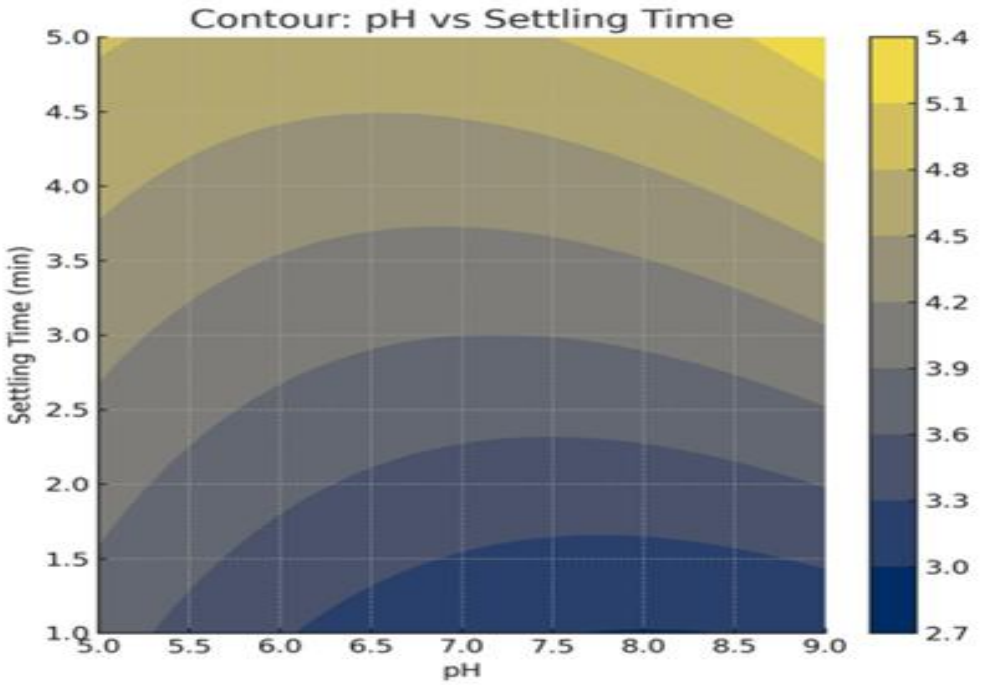


Fig. 3 *Response surface and contour plot showing the interactive effect of pH and settling time on biomass recovery*

The interaction between pH and settling time was relatively weak, consistent with ANOVA results ($p = 0.314$). Nevertheless, a slight improvement in biomass recovery was observed with increasing settling time at neutral to slightly acidic pH values. The nearly flat contour lines in this plot confirm that pH plays a secondary role compared to settling time in the overall process.

Overall, the combined statistical and graphical analysis indicates that chitosan dosage and settling time are the dominant factors affecting flocculation efficiency. The process is governed primarily by electrostatic charge neutralisation and polymer bridging, mechanisms enhanced by optimal chitosan concentration and sufficient contact time. The model's predictive accuracy and the clear response patterns in RSM plots validate the reliability of chitosan as an effective, low-cost, and environmentally benign bioflocculant for Chlorella vulgaris harvesting. The optimised conditions (chitosan dosage ≈ 0.15 mg/L, pH ≈ 7, settling time = 5 min) provide a balance between performance and economic feasibility, aligning with sustainable algae-based bioprocess design principles

V. CONCLUSION

This study has proven the significance of chitosan as an effective, eco-friendly bioflocculant that meets the industrial requirements while safeguarding our environment, which serves as a sustainable alternative to conventional chemical flocculants. Using a three-factor Box–Behnken design, the influence of chitosan dosage, pH, and settling time on flocculation efficiency was systematically evaluated.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors further declare that no conflict of interest exists regarding the publication of this manuscript.

REFERENCES

[1] L. Gouveia, A. Raymundo, A. P. Batista, I. Sousa, and J. Empis, "Chlorella vulgaris and *Haematococcus pluvialis* biomass as colouring and antioxidant in food emulsions," *Eur. Food Res. Technol.*, vol. 222, pp. 362–367, 2006.

[2] J. Moncada, J. A. Tamayo, and C. A. Cardona, "Integrating first, second, and third generation biorefineries: Incorporating microalgae into the sugarcane biorefinery," Chem. Eng. Sci., vol. 118, pp. 126–140, 2014.

[3] H. M. D. Wang, C. C. Chen, P. Huynh, and J. S. Chang, "Exploring the potential of using algae in cosmetics," Bioresour. Technol., vol. 184, pp. 355–362, 2015.

[4] D. Vandamme, I. Foubert, and K. Muylaert, "Flocculation as a low-cost method for harvesting microalgae for bulk biomass production," Trends Biotechnol., vol. 31, no. 4, pp. 233–239, 2013.

[5] S. Lordan, R. P. Ross, and C. Stanton, "Marine bioactives as functional food ingredients: Potential to reduce the incidence of chronic diseases," Mar. Drugs, vol. 9, no. 6, pp. 1056–1100, 2011.

[6] N. D. Riediger, R. A. Othman, M. Suh, and M. H. Moghadasian, "A systematic review of the roles of n-3 fatty acids in health and disease," J. Am. Diet. Assoc., vol. 109, no. 4, pp. 668–679, 2009.

[7] N. Abdel-Raouf, A. A. Al-Homaidan, and I. B. M. Ibraheem, "Microalgae and wastewater treatment," Saudi J. Biol. Sci., vol. 19, no. 3, pp. 257–275, 2012.

[8] V. Ananthi, P. Balaji, R. Sindhu, S. H. Kim, A. Pugazhendhi, and A. Arun, "A critical review on different harvesting techniques for algal based biodiesel production," Sci. Total Environ., vol. 780, Art. no. 146467, 2021.

[9] G. Goswami, R. Kumar, A. Sinha, S. K. Maiti, B. C. Dutta, H. Singh, and D. Das, "A low-cost and scalable process for harvesting microalgae using commercial-grade flocculant," RSC Adv., vol. 9, pp. 39011–39024, 2019.

[10] F. Roselet, D. Vandamme, K. Muylaert, and P. C. Abreu, "Harvesting of microalgae for biomass production," in Microalgae Biotechnology for Development of Biofuel and Wastewater Treatment. Singapore: Springer Nature, 2019, pp. 211–243.
[11] D. C. Montgomery, Design and Analysis of Experiments. New York, NY, USA: Wiley, 1997, ch. "Response Surface Methods and Other Approaches to Process Optimization."
[12] R. Divakaran and V. N. S. Pillai, "Flocculation of algae using chitosan," J. Appl. Phycol., vol. 14, no. 5, pp. 419–422, 2002.
[13] J. Nilsen-Nygaard, S. P. Strand, K. M. Vårum, K. I. Draget, and C. T. Nordgård, "Chitosan: Gels and interfacial properties," Polymers, vol. 7, no. 3, pp. 552–579, 2015.
[14] N. Aibani, R. Rai, P. Patel, G. Cuddihy, and E. K. Wasan, "Chitosan nanoparticles at the biological interface: Implications for drug delivery," Pharmaceutics, vol. 13, no. 10, Art. no. 1686, 2021.
[15] A. L. Ahmad, N. H. Mat Yasin, C. J. C. Derek, and J. K. Lim, "Optimization of microalgae coagulation process using chitosan," J. Appl. Phycol., vol. 23, no. 5, pp. 923–931, 2011.
[16] W. H. Chen, X. Zhang, J. Lin, and S. H. Ho, "A review of microalgae harvesting via coagulation–flocculation: Role of chitosan and its derivatives," Sep. Purif. Technol., vol. 254, Art. no. 117604, 2021.
[17] C. A. Machado, A. F. Esteves, and J. C. M. Pires, "Chlorella vulgaris harvesting: Chemical flocculation with chitosan, aluminum sulfate, and ferric sulfate," Appl. Sci., vol. 14, Art. no. 598, 2024.
[18] N. Duraisamy, S. Muthu, K. Thangavel, K. Krishnan, and S. Sathasivam, "A bioflocculant made from chitosan and modified shrimp waste might collect fresh and saltwater microalgae," Biosci. Biotechnol. Res. Asia, vol. 21, pp. 99–107, 2024.
[19] S. Abdul Karim, A. B. Sulong, C. H. Azhari, T. H. Lee, and M. H. Ng, "Optimization of electrospinning parameters using response surface methods to enhance fiber diameter, mechanical properties and orientation of nanofibers," J. Appl. Sci. Res., vol. 8, pp. 2510–2517, 2012.
[20] Y. Li, X. Li, W. Zhou, and R. Ruan, "Unravelling the mechanism of chitosan-driven flocculation of microalgae in seawater as a function of pH," ACS Sustain. Chem. Eng., vol. 6, no. 6, pp. 8293–8300, 2018.
[21] "Influence of chitosan properties and operating parameters on the flocculation efficiency and harvesting of microalgae (Scenedesmus sp.)," Int. J. Biol. Macromol., vol. 272, Art. no. 132894, 2024.
[22] G. E. P. Box and D. W. Behnken, "Some new three level designs for the study of quantitative variables," Technometrics, vol. 2, no. 4, pp. 455–475, 1960.
[23] S. L. C. Ferreira, R. E. Bruns, H. S. Ferreira, et al., "Box–Behnken design: An alternative for the optimization of analytical methods," Anal. Chim. Acta, vol. 597, no. 2, pp. 179–186, 2007.
[24] R. H. Myers, D. C. Montgomery, and C. M. Anderson-Cook, Response Surface Methodology: Process and Product Optimization Using Designed Experiments, 4th ed. Hoboken, NJ, USA: Wiley, 2016.

[25] F. Han, H. Y. Hu, F. M. Li, and Y. H. Wu, "Optimization of chitosan flocculation for Scenedesmus sp. using response surface methodology," Bioresour. Technol., vol. 146, pp. 159–165, 2013.

[26] Y. Liang, N. Sarkany, and Y. Cui, "Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions," Biotechnol. Lett., vol. 31, no. 7, pp. 1043–1049, 2009.

[27] J. O. Grady and J. A. Morgan, "Heterotrophic growth and lipid production of Chlorella protothecoides on glycerol," Bioprocess Biosyst. Eng., vol. 34, no. 1, pp. 121–125, 2010.

[28] X. M. Shi, H. J. Liu, X. W. Zhang, and F. Chen, "Production of biomass and lutein by Chlorella protothecoides at various glucose concentrations in heterotrophic cultures," Process Biochem., vol. 34, no. 4, pp. 341–347, 1999.

[29] G. M. Abou-Elela, N. A. El-Sersy, and S. H. Wefky, "Statistical optimization of cold adapted α-amylase production by free and immobilized cells of Nocardiopsis aegyptia," J. Appl. Sci. Res., vol. 5, pp. 286–292, 2009.

[30] H. A. Mowafy, "Response surface methodology for the development of self-nanoemulsified drug delivery system (SNEDDS) of indomethacin," J. Appl. Sci. Res., vol. 5, pp. 1772–1779, 2009.

[31] C. F. Mandenius and A. Brundin, "Bioprocess optimization using design-of-experiments methodology," Biotechnol. Prog., vol. 24, no. 6, pp. 1191–1203, 2008.

[32] P. Jamal, M. Z. Alam, U. S. Mohd Yusof, and W. M. F. Wan Nawawi, "Optimization of process conditions for extraction of biosurfactant," J. Appl. Sci. Res., vol. 7, pp. 2156–2161, 2011.

[33] M. A. Bezerra, R. E. Santelli, E. P. Oliveira, L. S. Villar, and L. A. Escaleira, "Response surface methodology (RSM) as a tool for optimization in analytical chemistry," Talanta, vol. 76, no. 5, pp. 965–977, 2008.