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Research Article

Box-Behnken Design-Based Optimization of Treatment Parameters for Soluble Reactive Phosphorus Removal of Synthetic Wastewater using Immobilized *Spirulina platensis* Beads

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ABSTRACT

Soluble reactive phosphorus (SRP), a bioavailable phosphorus form, contributes to over-eutrophication by stimulating uncontrolled algal growth. This study aims to determine the optimum treatment parameters for the SRP removal from synthetic wastewater using the alginate-immobilized cyanobacteria *Spirulina platensis*. *S. platensis* was immobilized in alginate beads with varying alginate concentrations (2.5%, 3%, and 3.5% w/v), and subjected to varying operation time (1, 2, and 3 days), and bead dosage (1.5, 2, and 2.5 beads/mL) for SRP removal using Box-Behnken experimental design. Resulting model indicated a strong predictive relationship with R2 = 0.9253 and p = 0.0212. Main effects of bead dosage (p = 0.01372), its quadratic effect (p = 0.01643), and its interaction with alginate concentration (p = 0.00465) were found to be statistically significant. Predicted optimum parameters (2.5% w/v alginate, 3 days, and 1.5 beads/mL) were validated and resulted in a lower SRP removal of 92.80 ± 0.73% with a percent error of 5.22% relative to a predicted SRP removal of 97.91%. Extrapolation of the prediction model to 100% outside the experimental region was verified resulting in SRP removal of 97.39 ± 0.08% with a percent error of 2.61% was achieved by adjusting the operation time to 3.4 days. The study shows promising potential of immobilized *S. platensis* beads in addressing over-eutrophication through significant phosphorus reduction.

Keywords: *Alginate, Immobilization, Soluble reactive phosphorus, Spirulina platensis, Wastewater treatment*

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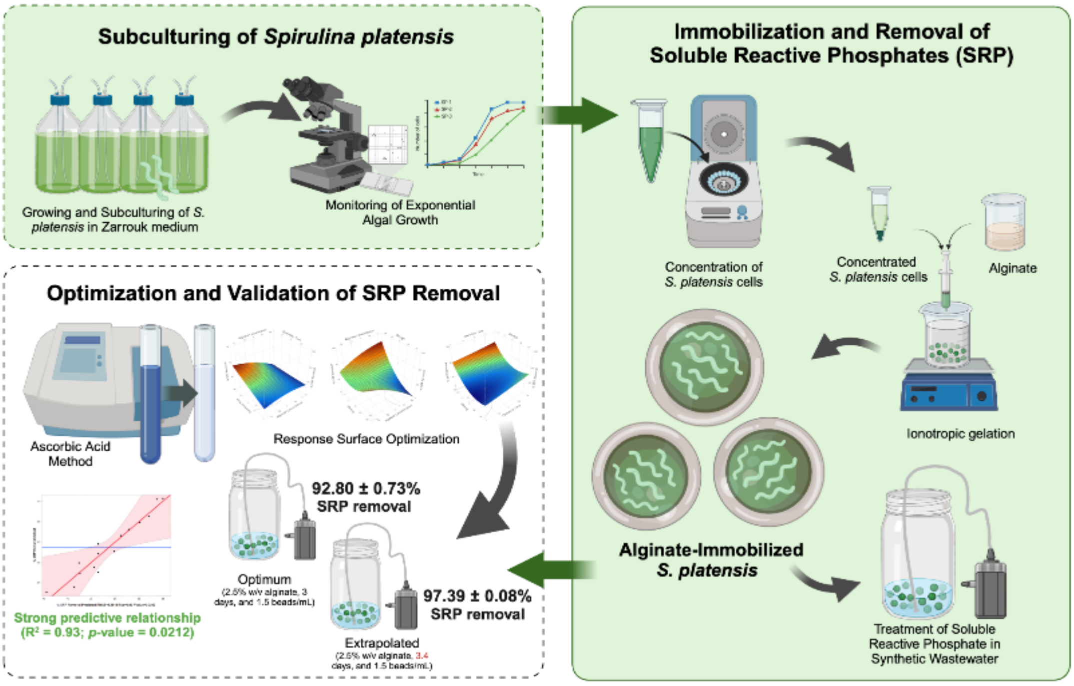


Introduction

Polluted water bodies containing excessive amounts of nutrients, such as nitrates and phosphates, can disrupt aquatic ecosystems by fueling an overabundance of algal blooms, which leads to hypoxia and "dead zones" for aquatic organisms (Malone & Newton, 2020). Soluble reactive phosphorus (SRP), such as orthophosphates, comprise a fraction of phosphorus directly taken in by algae, establishing it as a relevant indicator of potential algal growth as well as eutrophication. Though nitrates contribute significantly to eutrophication, SRP is often more prioritized due to its role as a limiting nutrient in freshwater systems, with its concentration directly proportional to algal growth (Paerl, 2009). In addition, nitrates can be converted to nitrogen gas to be released into the atmosphere through a process called denitrification (Huno, 2018), whereas some orthophosphates such as SRP do not have such natural mitigation mechanisms (Velusamy et al., 2021), emphasizing the need for efficient mitigation mechanisms.

In the Philippine context, the legally accepted amount of phosphorus in coastal waters classified as Class SB for "Phosphate as Phosphorus" is 2 mg/L (Department of Environment and Natural Resources, 2021, Sec. 5.3). However, elevated SRP levels above 0.1 mg/L P already indicate severe eutrophication (Calvo-Lopcaez et al., 2021). Thus, there is a critical need to control and reduce SRP in wastewater to prevent further water quality degradation and mitigate the risks of eutrophication in aquatic ecosystems (Karydis, 2009).

Existing SRP removal methods, such as sediment dredging and chemical precipitation, often result in ecological disturbances due to the release of trapped nutrients. Although sediment dredging is effective in nutrient removal, it is consequentially problematic due to the large volume of disposable sludge it deposits into marine-vulnerable areas (Oldenborg & Steinman, 2018) and due to its high usage of chemicals (Domini & Bertanza, 2022), which is also similar to the case for chemical precipitation in which chemical precipitating agents used in converting dissolved substances into solid particles leave residues that affect water quality (Mazur et al., 2018).

Alternative methods, such as biological nutrient removal (BNR) have incorporated microalgae in biofilters due to their capability in bioaccumulation and bioremediation, processes in which nutrients are absorbed and converted into biomass and are supplemented by their photosynthetic capabilities (Brandão et al., 2023), and due to their renewable characteristics through subculturing (Chai et al., 2021). Among various saltwater microalgae species, *Spirulina platensis* exhibits a high affinity for phosphorus through bioremediation, the process in which living organisms remove pollutants from their surrounding environment (Osman et al., 2011), adaptability for varying environmental conditions, a fast growth rate, biomass activity, and robustness towards various contaminants, ensuring efficiency in nutrient removal despite polluted environments (Ghaeni & Roomiani, 2016)—making it suitable for SRP removal. Although the commonly used biofilters fall short in its usage of free microalgae, aerated, and trickling methods resulting in microalgae instability due to its susceptibility to environmental fluctuations and sensitivity to clogs and clumps (Molinuevo-Salces et al., 2019), sustainability due to its difficulty in recovery and operational costs (Gichana et al., 2020), and lastly, targeted precision attributed to uncontrolled algal growth (Shpigel & Amir, 2007). To mitigate the issue, immobilization is typically used to ensure the microorganism remains in the biofilter (de-Bashan & Bashan, 2010), control growth rate, and improve removal efficiency due to the stability provided upon immobilization, reducing its fluctuation vulnerabilities due to environmental changes (Li et al., 2024).

The immobilization of *S. platensis* offers several advantages in the abatement of SRP in wastewater, such as cost effectivity in the efficient usage of organic carbon from wastewaters supplementing microalgal photosynthesis; enhanced stability that leads to consistent and sustained SRP uptake as opposed to free microalgae (Eroglu, et al., 2015); and a reduced need for heavy chemical treatments and energy-intensive processes that further contribute to carbon footprints (Xu et al., 2024). In this study, the immobilization matrix to be used determines the efficiency of nutrient removal due to the mechanical factors associated, such as porosity, stability, and nutrient uptake. Thus, for this research, sodium alginate is utilized for its non-toxicity and biological compatibility with *S. platensis* (Purev et al., 2023).

As previous studies mainly focused on nutrient removal done by different species of algae, whether it be immobilized or free-suspension, there appears to be a lack of studies maximizing the removal efficiency and potential of immobilized *S. platensis* in terms of parameters such as alginate concentration, bead dosage, and operation time. Consequently, the researchers aim to optimize the treatment parameters for SRP. To achieve the general research objective, the following specific objectives have been prepared:

- Subculture *S. platensis* in Zarrouk's medium and plot the growth curve;
- Immobilize *S. platensis* in alginate beads using ionotropic gelation with varying alginate concentrations;
- Determine the effects of alginate bead concentration (2.5, 3.0, 3.5 %w/v), operation time (1, 2, 3 days), bead dosage (1.5, 2.0, 2.5 beads/mL), and their interactions in SRP removal using response surface methodology; and
- Validate the predicted optimum and extrapolated treatment parameters for SRP removal with actual experiments.

Scope and Delimitations

Within the study, *S. platensis* was the only species utilized for treating wastewater. *S. platensis* was grown within Zarrouk's medium, which was adapted from Rajasekaran et al. (2015), in a closed system. Within these systems, other growth parameters such as temperature, pH level, and humidity were not adjusted for analysis. Further, only subcultures within the exponential phase of growth were subject for immobilization, due to the higher metabolic activity and nutrient uptake exhibited by the microalgae during this phase, adapted from Khatoon et al. (2021). In immobilization, three parameters for alginate beads—alginate concentration, operation time, and bead dosage—and their interactions are the sole factors to be modified, tested, and evaluated for SRP removal. The effects of bead size, temperature, pH level, humidity, and other unmentioned microalgal growth and environmental factors on SRP removal served as extraneous variables and were not measured and evaluated. SRP concentration is measured using the ascorbic acid method from Khatoon et al. (2021). Meanwhile, the preparation and composition of the dedicated synthetic wastewater for treatment is based on Halim and Wan Haron (2021). In the context of this research, the Box-Behnken design was utilized in identifying the optimized parameters, as used in the study of Hossain et al. (2018) that optimized Chlorella vulgaris growth using three factors, allowing more specificity in optimization factors to use in maximizing SRP removal by accounting for multiple variables relevant to the experiment.

Significance of the Study

In identifying the optimal conditions in the process of SRP removal, future processes related to water treatment of nutrient removal can be made more efficient and cost-effective, reducing the need for excessive amounts of resources and taking into consideration the resources to be used. Establishing optimized parameters can augment the sustainability and efficiency of alginate-immobilized *S. platensis*. With the known optimal conditions, it is possible for future initiatives to design the process that allows more effective SRP removal of immobilized *S. platensis* in wastewater treatment.

Moreover, numerous groups may also put the findings to good use, including, but not limited to, biofiltration system manufacturers, other communities prone to wastewater contamination, environmentalists, governmental and non-governmental organizations on environmental conservation, waterworks management, and climate change mitigation.

Methods

Research Design

Under Box and Wilson's response surface methodology (RSM), the researchers utilized the JMP software to create a Box-Behnken design of experiment with 15 experimental runs and three center points. This design was generated to optimize alginate concentration, operation time, and bead dosage towards optimum percentage SRP removal.

This design was notably used by multiple water treatment studies, optimizing their respective parameters for the most advantageous treatment output. For instance, Chaieb et al. (2023) utilized the Box-Behnken design as they optimized the use of natural bio-sorbent and S. algae B29 in CI Reactive Red 66 removal from artificial seawater using seven, three-leveled factors: dyes concentration, salinity, peptone, pH, algae C, cuttlebone and agitation. Another study, by You et al. (2025), sought to optimize the treatment of mariculture wastewater with Phaeodactylum tricornutum using three parameters, light intensity, illumination time, and temperature. From their Box-Behnken design, they concluded that only illumination time had a significant impact on COD and NH4+-N removal, whereas light intensity and temperature significantly affected microalgal cultivation. From their R2 value of 0.98, experimental data and predicted values showed a high correlation, with experimental data resulting in 70% COD removal, 96% NH4+-N removal, and 90% TP removal under optimum conditions. Lastly, Hossain et al. (2021) used the Box-Behnken design to optimize three treatment variables, temperature, light-dark cycle, and nitrogen-to-phosphate ratio, for the removal of nitrogen and phosphorus from tertiary municipal wastewater using Chlorella kessleri, yielding optimized removal of 99.7% and 93.48% for nitrogen and phosphorus respectively.

In line with the cited literature, the researchers employed the Box-Behnken design to statistically assess the interactions between alginate concentration, operation time, and bead dosage, and how they impact percentage SRP removal through the generation of response surface plots. In the study, the researchers optimized the aforementioned parameters for their best percentage SRP removal using alginate-immobilized *Spirulina platensis*. The three parameters and their low, middle, and high values are seen in Table 1.

Table 1. Box-Behnken optimization parameters with low, middle, and high values

Parameter	Low (-1)	Middle (0)	High (+1)
Alginate Concentration (% w/v)	2.5	3	3.5
Operation Time (days)	1	2	3
Bead Dosage (beads/mL)	1.5	2	2.5

The researchers used the design to test the effect of three factors: alginate concentration (2.5%, 3.0%, and 3.5% w/v), operation time (1, 2, and 3 days), and bead dosage (1.5, 2.0, and 2.5 beads/mL), on the response variable, percentage SRP removal (%). The alginate concentration values were modified from Banerjee et al. (2019), who tested for the optimal alginate concentration for municipal secondary effluent wastewater nutrient removal using 1.5, 2, 3, 4, and 4.5% concentrations of alginate-immobilized Chlorella vulgaris. Alginate concentration was optimized due to its effect on nutrient adsorption rate. Meanwhile, the low, middle, and high values for operation time and bead dosage were based on Khatoon et al. (2021), who highlighted the effects of bead concentration and retention time on nutrient removal. Their results graphically depict differences in percentage SRP removal across durations of 1, 2, and 3 days, and Tetraselmis sp. bead dosages of 0.5, 1.0, 1.5, 2.0, and 2.5 beads/mL.

Alginate concentration was a considered factor due to its potential effects on nutrient uptake (Banerjee et al., 2019), whereas operation time, which focused on the duration of treatment, and bead dosage, involving the number of beads used for treatment, was considered for its potential impact on percentage SRP removal efficiency (Khatoon et al. 2021; Cruz et al., 2013).

In this study, the researchers immobilized *S. platensis* in varying alginate concentrations to treat synthetic wastewater at different bead dosages and durations. This set-up differs from the aforementioned literatures that utilized alginate-immobilized Chlorella vulgaris for municipal secondary effluent wastewater nutrient removal (Banerjee et al., 2019), Tetraselmis sp. for artificial and aquaculture wastewater treatment (Khatoon et al., 2021), and jointly immobilized Chlorella vulgaris and Azospirillum brasilense for secondary municipal wastewater treatment (Cruz et al., 2013).

Experimental Phases

The methodology of the study is divided into five phases, beginning with the growth and subculturing of *S. platensis*, which involves monitoring the *S. platensis* subcultures for eventual immobilization once the ideal cell concentration is reached. Following this, the immobilization of *S. platensis* in alginate beads proceeds the growth phase, wherein *S. platensis* samples are subjected to immobilization within alginate beads at varying alginate concentrations. Next, synthetic wastewater setups were treated with alginate beads at varying operation times and bead dosages. Afterward, this phase was followed by the determination of the percentage SRP removal of each run. Finally, concluding the phases is the optimization of the parameters using response surface methodology in order to determine the optimum parameters for maximized percentage SRP removal. A flowchart of the methodology phases of the research is seen in Figure 1.

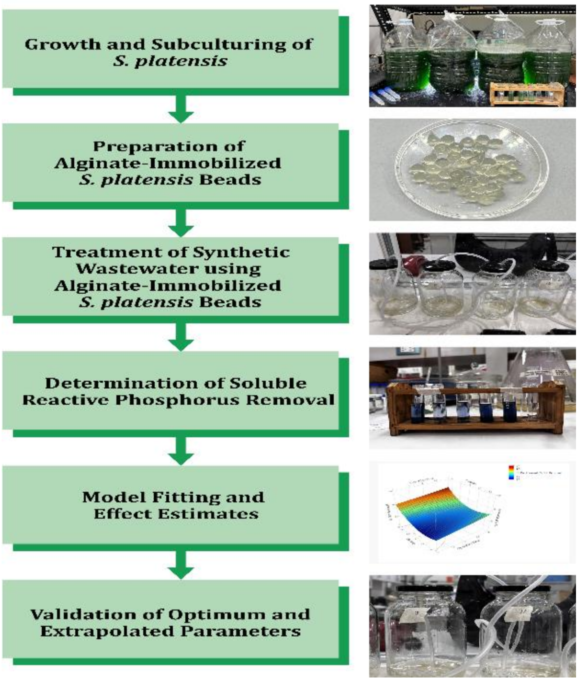


Figure 1. Experimental phases for treatment parameter optimization for SRP removal using immobilized S. platensis

Growth and Subculturing of S. platensis

The researchers subcultured the *S. platensis* culture in Zarrouk's medium (Z Cy67), obtained from the University of the Philippines Los Baños Microbial and Algal Culture Collection (Los Baños, Laguna, Philippines), into four empty 10 L distilled water bottles using Zarrouk's medium, consisting of the following chemicals based on the study of Rajasekaran et al. (2015): sodium bicarbonate (Dalkem Corporation, Philippines), sodium nitrate (Merck KGaA, Germany), sodium chloride (RCI Labscan Ltd., Thailand), potassium dihydrogen phosphate (Univar Solutions, USA), sodium sulfate (Ajax Finechem, Australia), magnesium sulfate heptahydrate (Suvchem Laboratory Chemicals, India), calcium chloride dihydrate (Duksan Pure Chemicals, South Korea), ferrous sulfate heptahydrate (Loba Chemie Pvt. Ltd., India), ethylenediaminetetraacetic (EDTA) acid salt dihydrate (Qualikems Lifesciences Pvt., Ltd., India), and B6 trace elements solution. The researchers measured the said chemicals with a Shimadzu UniBloc analytical balance (Shimadzu Corporation, Japan) and magnetically stirred on a Torrey Pines Scientific stirring hotplate (Torrey Pines Scientific, Inc., USA). The composition of the Zarrouk's medium used by the researchers was adopted from Rajasekaran et al. (2016) and is seen in Table 2.

Table 2. Chemical composition of Zarrouk's medium

Chemical Name	Chemical Formula	Concentration
Sodium bicarbonate	$NaHCO_3$	16.80 g/L
Sodium nitrate	$NaNO_3$	2.50 g/L
Sodium chloride	NaCl	1.00 g/L
Potassium dihydrogen phosphate	KH_2PO_4	0.41 g/L
Potassium sulfate	K_2SO_4	1.00 g/L
Magnesium sulfate heptahydrate	$MgSO_4{\cdot}7H_2O$	0.20 g/L
Calcium chloride dihydrate	$CaCl_2{\cdot}2H_2O$	0.04 g/L
Ferrous sulfate heptahydrate	$FeSO_4{\cdot}7H_2O$	0.01 g/L
Ethylenediaminetetraacetic acid disodium salt	$C_{10}H_{14}N_2Na_2O_8$	0.08 g/L
B_6 trace element solution		1.0 mL

The researchers subjected the four subcultures to frequent dilution with Zarrouk's medium in order to provide the microalgae with ample nutrients for continuous growth. The experimental setup of the *S. platensis* subculture vessels with the aeration pump and lighting is illustrated at Figure 2.

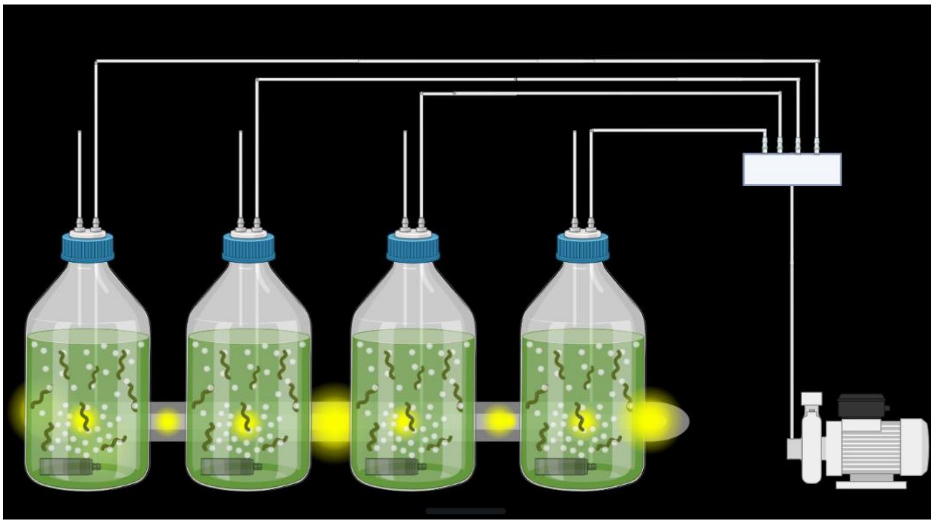


Figure 2. Experimental setup of the S. platensis subculture vessels

The experimental setup consisting of the four *S. platensis* subculture vessels was continuously aerated through an air pump airstone, a fine bubble aeration system, to ensure evenly distributed oxygen to enhance cell growth (Lin & Tanaka, 2006). Yang et al. (2018) stressed the importance of a subculture bubble aeration system for proper nutrient mixing and even carbon dioxide distribution. Additionally, it prevents drastic pH changes, oxygen overaccumulation, and cell damage. A white LED light was used to provide the subcultures with ample light for photosynthetic activity. Meanwhile, the ventilation of the subcultures consisted of silicone tubings and an air pump with a flow rate of 25 liters per minute.

Samples of each subculture were dropped into a Marienfield Superior Neubauer counting chamber (Marienfield Superior, Germany) and then observed under an Olympus B201 microscope (Olympus Corporation, Japan) to determine its cell concentration via cell counting. The hemocytometer used for cell counting, as well as drawings of the hemocytometer and its dimensions, are seen in Figure 3.

(a) (b)

Figure 3. (a) Actual hemocytometer and (b) orthographic and isometric drawings of the hemocytometer

The researchers used Equation 1, derived from the hemocytometer dimensions, to determine the number of cells present in a *S. platensis* subculture per milliliter of subculture, where cell count is the average number of cells counted in each grid of the hemocytometer counting chamber, similar to the method used by Halim and Wan Haron (2021) to measure the cell concentration of Chlorella vulgaris.

$$C_{SP}\ (\text{cells/mL}) = \frac{n_{SP}}{9 \times 10^{-4}\ \text{mL}} \quad (\text{Eq. 1})$$

Wherein:
C_SP is the cell concentration
n_SP is the number of cells

The cell count in the equation is obtained by counting the number of *S. platensis* cells per 1 mm by 1 mm grid and finding the average number of cells of the nine grids. Meanwhile, the denominator of the equation is the volume of the chamber with an area of 9 mm2 and a depth of 0.1 mm.

Preparation of Alginate-Immobilized S. platensis Beads

The alginate immobilization techniques used in this study were adopted from Halim & Wan Haron (2021). The researchers subjected ten milliliters of *S. platensis* samples in the exponential phase to 15-minute centrifugation at 4000 rpm with a Vision Scientific LTD VS-4000i centrifuge (Vision Scientific Co., USA), after which the microalgae were removed from the residual medium. The concentrated *S. platensis* cells are seen in Figure 4.

Figure 4. Concentrated S. platensis cells after centrifugation

After, the researchers prepared 20 milliliters of alginate solutions at 2.5, 3.0, and 3.5 % w/v, and were then mixed with the centrifuged *S. platensis*. Then, the solution was aspirated into a Indoplas 10 cc syringe (Indoplas Philippines Inc., Philippines) for extrusion into a consistently stirring 250 mL 2 M calcium chloride ionic crosslinking solution for bead creation (Patnaik et al., 2001). The beads were left submerged and stirred in the calcium chloride solution for an additional 30 minutes before they were filtered in a wire mesh and rinsed with distilled water. Lastly, the researchers stored the beads in a refrigerator at 4°C for synthetic wastewater treatment. An image of the alginate-immobilized *S. platensis* beads in the calcium chloride solution is seen in Figure 5.

Figure 5. Preparation of immobilized S. platensis beads

Treatment of Synthetic Wastewater using Alginate-Immobilized S. platensis

The chemicals used for the synthetic wastewater preparation, as cited in Halim & Wan Haron (2021), include glucose (HiMedia Laboratories Pvt., Ltd., India), ammonium chloride (Unichem Specialty Chemicals, LLC., USA), and potassium dihydrogen phosphate (Univar Solutions, USA).

Table 3. Chemical composition of synthetic wastewater (Halim & Wan Haron, 2021)

Chemical Name	Chemical Formula	Amount (mg/L)
Glucose	$C_6H_{12}O_6$	256.41
Ammonium chloride	NH_4Cl	35.33
Potassium dihydrogen phosphate	KH_2PO_4	43.80

The researchers prepared one hundred milliliters of synthetic wastewater for each experimental run in the Box-Behnken design. The prepared beads with adjusted alginate concentration, operation time, and bead dosage depending on the run were incorporated in synthetic wastewater for SRP removal. The experiment setup involves 100 mL of wastewater within a glass container for each run, continuously being aerated with an air pump. An image of the synthetic wastewater treatment setup is seen in Figure 6.

Figure 6. Synthetic wastewater setups with immobilized S. platensis beads

Determination of Soluble Reactive Phosphorus Removal

The determination of the percentage SRP removal of the alginate-immobilized *S. platensis* was done through the ascorbic acid method for phosphate determination as described in Khatoon et al. (2021). The process begins with the centrifugation of 10 mL synthetic wastewater samples for 15 minutes at 4000 rpm to separate the insoluble precipitates from the wastewater.

The following chemicals were used for the preparation of the reagents for the ascorbic acid method, as cited in Khatoon et al. (2021): analytical reagent grade ammonium paramolybdate, sulfuric acid (J.T. Baker, New Jersey, United States), ascorbic acid (Dalkem Corporation, Philippines), and potassium antimonyl tartrate. The composition of the reagent used for the ascorbic acid method is summarized in Table 4.

Table 4. Solution compositions for the ascorbic acid method

Chemical Name	Chemical Formula	Concentration
Ammonium paramolybdate	$(NH_4)_6Mo_7O_{24}$	15 g/500 mL
Sulfuric acid	H_2SO_4	140 mL/900 mL
Ascorbic acid	$C_6H_8O_6$	27 g/500 mL
Potassium antimonyl-tartrate	$C_8H_{10}K_2O_{15}Sb_2$	0.34 g/250 mL

The researchers mixed the reagents for the ascorbic acid method to create the phosphate indicator reagent. To make 500 mL of reagent that is usable for fifty 100 mL wastewater samples, the following solutions were stirred in a dark area: 100 mL of ammonium molybdate solution, 250 mL of sulfuric acid solution, 100 mL of potassium antimonyl-tartrate (tartar emetic) solution, and 50 mL of ascorbic acid solution. The reagent is freshly prepared prior to analysis.

Ten milliliters of reagent were dropped for every 100 mL of synthetic wastewater sample, and five minutes were allotted for the completion of the reaction. Once the reaction duration elapsed, the absorbances of the reacted wastewater samples were read using a UV-Vis spectrophotometer at 885 nm. Wastewater samples were tested in triplicates following the Box Behnken design of experiment. The synthetic wastewater samples after the reaction, as well as their absorbance readings, are seen in Figure 7.

Figure 7. Synthetic wastewater samples after the reaction

To calculate the percentage SRP removal of the treated wastewater samples given absorbance, the researchers constructed a calibration curve (Parsons et al., 1984, as cited in Khatoon et al., 2021) by analyzing the average of triplicate samples of ten phosphorus solutions (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg/L) obtained from an 8.788 mg/200 mL KH2PO4 stock solution. After subjecting the samples to the ascorbic acid method, their absorbance values were read by a spectrophotometer, and a graph was made depicting absorbance as a function of phosphorus concentration (mg/L).

Once the percentage SRP removal of each experimental run was obtained, the researchers used JMP Software to create the response surface plots depicting the most suitable values of alginate concentration, operation time, and bead dosage for optimal percentage SRP removal.

Model Fitting and Effects of Treatment Parameters on Soluble Reactive Phosphorus Removal

From the treated wastewater samples from the fifteen runs, the actual percentage of SRP removal is plotted against the predicted percentage of SRP removal with the JMP software. The coefficients of the prediction expression are to be fitted and estimated using the quadratic model shown in Equation 2. The coded variables for the three parameters in the equation are the following: Y = SRP Removal (%), X1 = Alginate Concentration, X2 = Operation Time (days), and X3 = Bead Dosage.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{13}x_1x_3 + b_{12}x_1x_2 + b_{23}x_3x_2 \quad \text{(Eq. 2)}$$

The coefficients of the equation were represented by b0 for the constant term; b1, b2, and b3, referring to the linear effects of alginate concentration, operation time, and bead dosage, respectively; b11, b22, and b33 for their quadratic effects; and b12, b13, and b23 for the interaction effects between alginate concentration and operation time, alginate concentration and bead dosage, and operation time and bead dosage, respectively. Furthermore, the R2 value, adjusted R2 value, root mean square error (RMSE), and p-value ($p \leq 0.05$) are to be determined. These dictate the statistical significance, precision of the prediction model, and the predictive relationship from the parameter effects.

Validation of Optimum and Extrapolated Parameters

After the generation of the response surface plots using the Box-Behnken design, the researchers obtained the optimum factor parameters with the maximum percentage SRP removal. Additionally, the predicted extrapolated parameters, parameters that are outside of the low, middle, and high values, but are predicted to yield a 100% percentage SRP removal, were also obtained within the JMP software. Validation of these parameters and their predicted percentage SRP removal was conducted through two sets of synthetic wastewater treatment runs at triplicates: one set using the optimum parameters and another set for the extrapolated parameters. The average of the resulting percentage of SRP removal from the three trials of both runs is to be obtained, and the percent error of the actual percentage of SRP removal relative to the predicted percentage of SRP removal will also be reported to verify the accuracy of the prediction.

Ethics Statement

Throughout the research, the researchers observed proper ethical standards and considerations to the fullest extent. Certifications were acquired for the authorized use of facilities, equipment, laboratories, and chemicals. The researchers regularly wore personal protective equipment and conducted laboratory activities under the supervision of professionals and pursuant to standardized laboratory regulations. Agendas were carried out with respect to sustainability and environmental stewardship; activities were done with the utmost consideration for proper waste mitigation, management, and disposal. Data acquired throughout the study were collected authentically, and no modifications were made to the results obtained. These protocols were consistently followed for the conduction of ethical research endeavors.

Results and Discussion

Growth and Subculturing of Spirulina platensis

The researchers set up the four 10-L distilled water bottles containing the cultures of *S. platensis* as shown in Figure 8. All subcultures show periods of growth and decline in rising and falling concentrations, reflective of environmental factors such as light intensity, access to nutrients, or temperature.

The cell concentration in cells/mL of the four *S. platensis* subcultures measured on 13 separate days from Day 1 to Day 67 is depicted in Figure 9.

Figure 8. S. platensis vessel setups with subculture samples

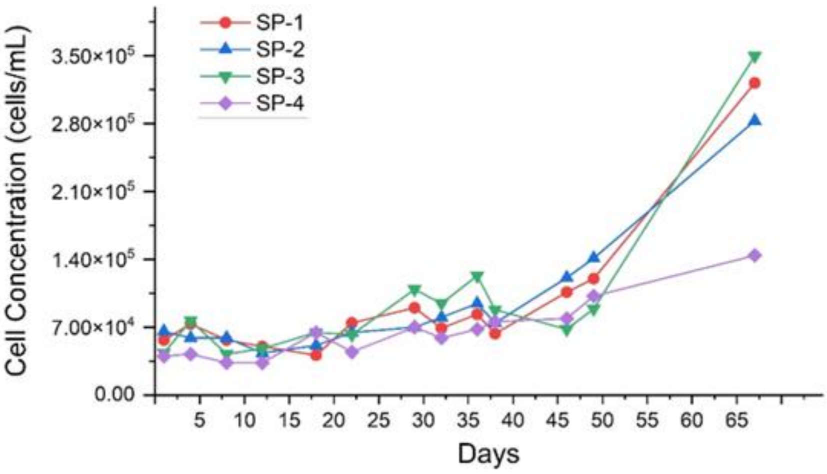


Figure 9. Cell concentration growth of S. platensis subcultures for 67 days

The four subcultures were prepared 20 days before the labeled Day 0, with the cell concentrations at 2.33 × 104 cells/mL, 4.67 × 104 cells/mL, 6.11 × 104 cells/mL, and 1.56 × 104 cells/mL, respectively, from subcultures 1 to 4.

The cell concentrations of the four subcultures lie between 3.33 × 104 cells/mL and 1.29 × 105 cells/mL range in the latter stage of subculturing from Days 30 to 40, similar to El-Sheekh et al. (2020) with their Zarrouk's medium-grown *S. platensis* cell concentration sitting between the 5.0 × 104 cells/mL and 1.5 × 105 cells/mL range from Days 28 to 42.

The period in which the graph showed a general decline in growth could be attributed to the depletion of nutrients, given the extended period before the subcultures were replenished with Zarrouk's medium, as highlighted by Vonshak (1997), emphasizing nutrient imbalance attributed to the utilized media. Other reasons attributed to the troughs in growth can be attributed to human error in counting the number of *S. platensis* cells and power outages due to natural calamities, affecting the subcultures' aerator and light source. Moreover, increases in cell concentration imply higher biomass accumulation, possibly hindering the subcultures' exposure to light and subsequent photosynthetic activity due to self-shading. As such, higher cell concentrations may decrease in the following days due to reduced photosynthetic activity (Maali et al., 2024). Meanwhile, the nonlinear, curvy trend of the subculture graphs is comparable to Taqiyyah et al. (2022) and the cell density of their *S. platensis* subcultured in aquaculture wastewater with Zarrouk's medium for eight days. In their graph, all subcultures with varying medium concentrations were found to overlap and fluctuate while following a gradual increase in cell density, similar to the fluctuating yet gradually increasing cell concentration of the *S. platensis* subcultures of the study. The images of the *S. platensis* subcultures were viewed under a microscope at 40x, 100x, 200x, and 400x magnifications, as shown in Figure 10.

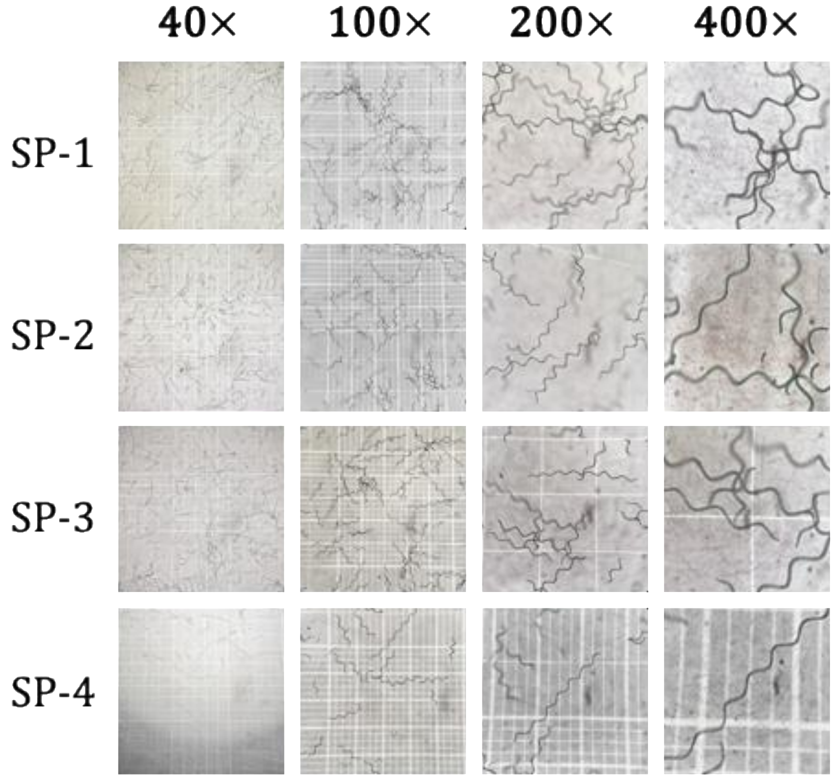


Figure 10. Microscopic images of four S. platensis subcultures

Preparation of Alginate-Immobilized S. platensis Beads

The average bead sizes and shapes of the alginate-immobilized *S. platensis* beads are summarized in Table 5. As the concentration increases, the shape of the beads upon immobilization varies from spherical to teardrop shaped. Shape variations, such as spherical to tear-drop shaped are determined from the viscosity of the alginate concentrations, which increases as more alginate is used in the solution. The macroscopic images of the alginate-immobilized *S. platensis* beads with different concentrations are shown in Figure 11.

The average bead sizes and observed shapes of the alginate-immobilized *S. platensis* beads are summarized in Table 5.

According to Klokk and Melvik (2002), alginate concentration and viscosity are directly proportional to bead diameter. However, in contrast, an inversely proportional relationship between bead diameter and alginate concentration was stated by Lee et al. (2013), due to less surface tension with higher viscosities and concentrations. The researchers observed similar findings, wherein bead size decreased with the increase in alginate concentration, as shown in the results. In contrast, although the study of Lee et al. (2013) accurately explains the results in terms of size, the results had a contradicting trend for bead shape, wherein bead deformations occurred at lower concentrations. The bead deformations and inconsistencies in bead shape are possibly due to the differences in collecting distance, which is said to affect bead shapes (Lee et al. 2013) when dripping the alginate solution into the calcium chloride solution, as this was not strictly controlled by the researchers.

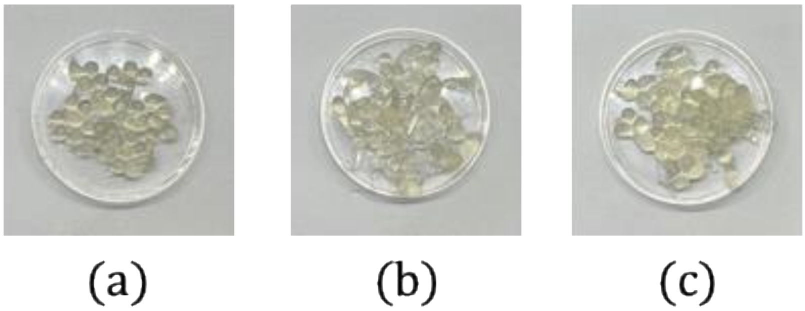


Figure 11. Beads with (a) 2.5%, (b) 3.0%, and (c) 3.5% alginate concentrations

Table 5. Average sizes and shapes of the immobilized S. platensis beads at different alginate concentrations (%)

Alginate Concentration (%)	Bead Size (mm)	Bead Shape
2.5	3.49 ± 0.19	Mostly spherical
3.0	3.39 ± 0.16	Spherical to teardrop-shaped
3.5	3.18 ± 0.33	Spherical to teardrop-shaped

Moreover, smaller beads are found to be more efficient in nutrient removal, as seen in Run 10, which had a percentage SRP removal of 95.63% using 3.5% alginate beads that bore the smallest size. whilst larger beads allow for better algal growth (Porkka, 2021); however, upon experimentation, a significant difference in the consistency of SRP removal as per alginate concentration is observable.

Treatment of Synthetic Wastewater using Alginate-Immobilized S. platensis

The calibration curve of phosphorus concentration was prepared in accordance with the methods outlined by Parsons et al. (1984), as cited in Khatoon et al. (2021). The researchers experienced errors and limitations in creating the calibration curve, such as slight mismeasurements in the dilution of the phosphorus solutions, as well as excess reaction time to the phosphate indicator reagent due to the lengthy process of UV-Vis spectrophotometry. These conditions may have contributed to the deviations of the absorbance values from the trendline. In Figure 12, the plotted calibration curve for phosphorus concentration is shown.

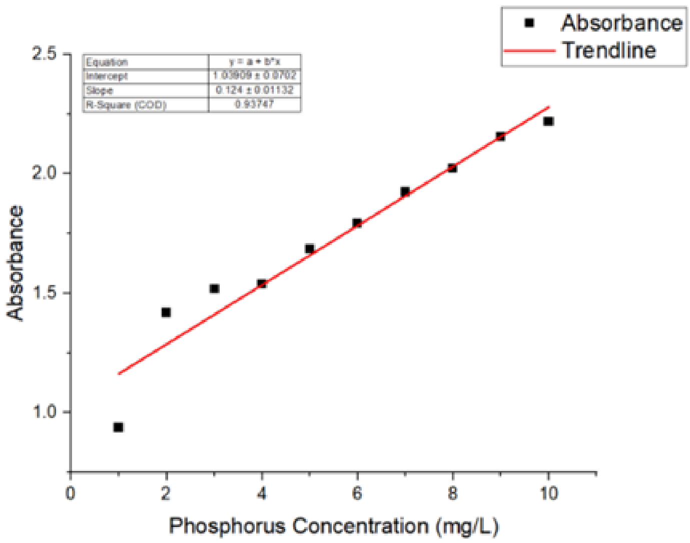


Figure 12. Calibration Curve for phosphorus concentration

From the scatter plot, the equation of the trendline of the graph of absorbance (y) as a function of phosphorus concentration in mg/L (x) is denoted by Equation 3. Given the absorbance, the equation was used to determine the phosphorus concentration (mg/L) of the wastewater samples from the experimental runs.

$$A_{885} = 0.124 \times C_{SRP}(mg/L) + 1.04 \qquad \text{(Eq. 3)}$$

Wherein:
A_885 is absorbance at 885 nm
C_SRP is SRP concentration

The researchers obtained percentage SRP removal (%) by acquiring the average phosphorus concentration (mg/L) of three untreated synthetic wastewater samples. This yielded an average phosphorus concentration of 10.2661 mg/L, which was used for the formula for percentage SRP removal (%) seen in Equation 4.

$$\%SRP = \frac{10.2661 - C_{SRP}\,(mg/L)}{10.2661} \times 100 \qquad \text{(Eq. 4)}$$

Wherein:
%SRP is percentage SRP removal

After the calibration curve was made and the necessary equations calculated, the researchers conducted the fifteen experimental runs from the Box-Behnken Design. The corresponding percentage (%) SRP removal of the fifteen runs with their set parameters from the Box-Behnken design is seen in Table 6.

Table 6. SRP removal of immobilized S. platensis beads at varying treatment parameters

Run	Alginate Concentration, %w/v (X_1)	Operation Time, days (X_2)	Bead Dosage, beads/mL (X_3)	SRP Removal, % (Y)
1	2.5	2	2.5	82.71
2	3	3	2.5	95.45
3	2.5	3	2	88.06
4	3	2	2	82.16
5	3	2	2	84.52
6	3	2	2	77.58
7	3	1	1.5	78.79
8	3.5	3	2	73.97
9	3	1	2.5	89.77
10	3.5	2	2.5	95.63
11	2.5	1	2	79.85
12	2.5	2	1.5	91.34
13	3	3	1.5	86.54
14	3.5	2	1.5	72.69
15	3.5	1	2	77.33

Based on the experimental data from the 15 runs, the range of SRP removal has a minimum of 72.69% and a maximum of 95.63%. Run 10 with an alginate concentration of 3.5%, operation time of 2 days, and bead dosage of 2.5 beads/mL shows a maximum SRP removal of 95.63%. In contrast, a minimum SRP removal of 72.69% was achieved with Run 14 with an alginate concentration of 3.5%, operation time of 2 days, and bead dosage of 1.5 beads/mL. Noticeably, the only difference between Run 10 and Run 14 is the bead dosage, indicating that separately changing the bead dosage results in the most significant effect in SRP removal compared to individual changes in alginate concentration or operation time. This is to be expounded on in the next subsection.

Some limitations were noted when using synthetic wastewater compared to real wastewater. While the researchers created synthetic wastewater with a defined composition, real wastewater varies with climate, indicating periodic changes in trace element, mineral, vitamin, precipitate and organic substance concentrations (Chen et al., 2022; Wang et al., 2023). As such, SRP removal may differ when used in real wastewater applications. Moreover, the *S. platensis* beads may not be entirely responsible for SRP removal because the presence of other microorganisms in real wastewater results in competitive nutrient uptake (Sajid et al., 2022).

Additionally, prior to application of the phosphorus indicator reagent, the researchers centrifuged the treated synthetic wastewater samples to separate the insoluble phosphates. These precipitates can be further be repurposed for other uses, such as fertilizer production from struvite, a phosphate mineral with high fertilizer value and low environmental risk (Santos et al., 2024). However, further characterization must be conducted on the study's accumulated precipitates to determine their type and eventual application.

Effect of Alginate Concentration, Operation Time, and Bead Dosage on SRP Removal

Within this section of the paper, the significance and interactions of the three parameters were analyzed. Additionally, the precision of the regression analysis and the equation for percentage of SRP removal are stated. In Figure 13, the plot of the actual percentage of SRP removal versus the predicted percentage of SRP removal can be seen. The model fits a quadratic model in relation to alginate concentration, bead dosage, and operation time, with an R2 value of 0.9253 and an adjusted R2 value of 0.799883. The R2 value indicates that the prediction of SRP removal from the model is precise, and its p-value of 0.0212 ($p \leq 0.05$) indicates its statistical significance, signifying a strong predictive relationship of SRP removal with treatment parameters. The RMSE is also indicated to be 3.251835, implying potential variations in the percentage of SRP removal from the prediction model.

The parameter estimates of the bead parameters and their linear, quadratic, and interaction effects is located in Table 7.

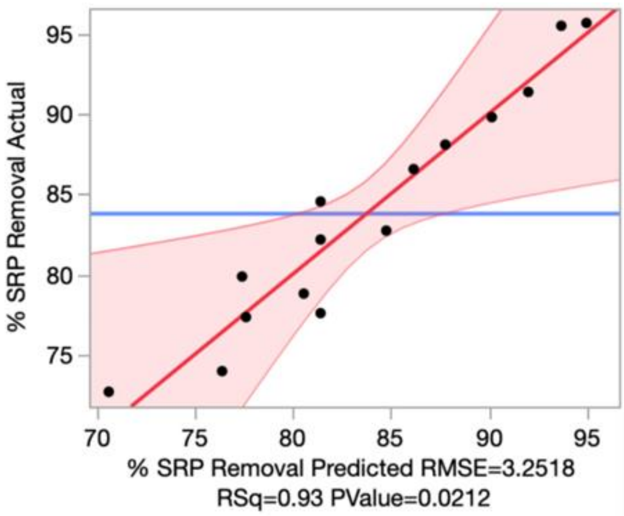


Figure 13. Regression analysis plot of actual percentage of SRP removal vs. predicted percentage of SRP removal

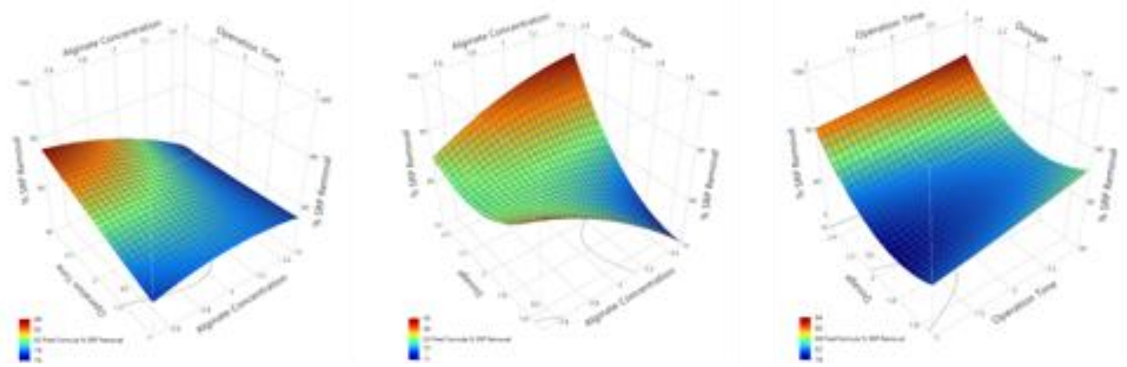
Table 7. Estimates of bead parameters and their parameter effects

Parameter		Uncoded Estimate	***p*-value**
Intercept			
	b_0	258.16	< 0.0001
Linear			
	b_1	-13.24	0.05939
	b_2	20.84	0.10361
	b_3	-180.17	0.01372

Parameter	Uncoded Estimate	*p*-value
Quadratic		
b_{11}	-7.32	0.32887
b_{22}	0.22	0.90311
b_{33}	24.01	0.01643
Interaction		
b_{12}	-5.79	0.13529
b_{13}	31.58	0.00465
b_{23}	-1.03	0.76446

Among the three parameters, bead dosage is the only statistically significant parameter out of the three, which had the least p-value of 0.01372, while the alginate concentration ($p = 0.05939$) and operation time ($p = 0.10361$) are both statistically insignificant. Despite this, the p-value of alginate concentration indicated that it is almost statistically significant. In addition, alginate concentration significantly affects SRP removal when interacting with bead dosage, and the interactions of these two parameters produce the lowest p-value of 0.00465 consequently—and are thus statistically significant. Based on the analysis, bead dosage, independently, and the interaction between this parameter and alginate concentration produce enough evidence to indicate that they affect the percentage of SRP removal. Bead dosage also has a significant quadratic effect, with a p-value of 0.01643.

The interactions between alginate concentration, operation time, and bead dosage are further explored in the succeeding paragraphs with the analysis of their response surface plots. In Figure 14, the response surface plots of the parameters from the Box-Behnken design can be seen. The interactions between two parameters are observed in these plots.



(a) (b) (c)

Figure 14. Response surface plots of (a) operation time vs. alginate concentration, (b) bead dosage vs. alginate concentration, and (c) bead dosage vs. operation time

In the surface plot of operation time vs. alginate concentration (Figure 14a), the percentage of SRP removal is maximum at 88% when the alginate concentration is 2.5% and the operation time is 3 days. However, regardless of the operation time, the trend of the plot implies that SRP removal below 80% remains with the alginate concentration at 3.5%, demonstrating that lower alginate concentrations are more ideal for augmenting the percentage of SRP removal. Moreover, the minimum percentage of SRP removal of 76% is attained at an alginate concentration of 3.5% and an operation time of 3 days.

The surface plot of bead dosage vs. alginate concentration (Figure 14b) indicates that high bead dosage (2.5 beads/mL) and high alginate concentration (3.5%) reduces that amount of SRP the most, with a percentage of SRP removal of 95%. However, decreasing bead dosage with a constant alginate concentration of 3.5% has more effect on SRP removal than reducing the alginate concentration with a constant bead dosage of 2.5 beads/mL, and high alginate concentration (3.5%) with low bead dosage (1.5 beads/mL) has the least percentage of SRP removal of all parameter configurations, which falls below 71% SRP removal, regardless of the operation time. Further, low alginate concentration and bead dosage still produces a fairly high percentage of SRP removal of above 90%—and accompanying such interaction with a high operation time is predicted to produce the most optimal percentage of SRP removal within the Box-Behnken design, which will be discussed more within the next subsection of the paper.

For the surface plot of operation time and bead dosage (Figure 14c), the highest percentage of SRP removal of 94% is obtained when both parameters are set to high (3 days and 2.5 beads/mL), while the lowest percentage of SRP removal is at 1 day of operation time and approximately 1.8 beads/mL of dosage. Adjusting operation time does not produce a notable effect in improving SRP removal compared to altering the bead dosage.

Alginate concentration and operation time merely influence bead formation as opposed to nutrient removal efficiency (Halim & Haron, 2021). These two parameters are considered statistically insignificant but still have trends that affect the SRP removal. The increase in bead dosage significantly improved the removal of SRP due to its greater algal surface area and adsorption capacity (Hameed, 2007), which is demonstrated by its statistical significance. As stated by the study of Khatoon et al. (2021), wherein alginate-immobilized Tetraselmis sp. was used in treating artificial wastewater, SRP concentration was reduced by 100.0% for 2.5 beads/mL (98.7% and 99.4% at 1.5 and 2.0 beads/mL, respectively) after two days. After three days, SRP concentration was reduced by 100.0% for 1.5, 2.0, and 2.5 beads/mL. The results from Khatoon et al.'s (2021) study suggest that higher bead dosage results in better nutrient removal, which is similar to the results gathered from this experiment wherein the maximum SRP removal of 94.97% at 2 days is obtained at a concentration of 3.5% and 2.5 beads/mL. However, the effect of adjusting alginate concentration was not further explored within the cited study.

Similarly, in a study by Tam and Wong (2000) that used sodium alginate beads with multiple rounds, it was found that a concentration of 11.89ml-1 was optimal for phosphate removal in wastewater. It effectively removed 94.3% of phosphate in the first 8 hours and leveled off to 93.9% after 24 hours. The key difference is that the study used Chlorella vulgaris as their species. Their study yields similar results to this experiment as the phosphate removal levels are only differentiated by 1.4% and that higher bead dosage leads to higher phosphate removal due to their highest run having the highest bead concentration. The study did not explore treatment times longer than 24 hours and had no further explanations on the effects of alginate concentrations in wastewater treatment.

As stated by Mollamohammada (2020), exposure over an extensive period of time can lead to the degradation of the beads and nutrient release. This was similarly deduced in the study of Banerjee et al. (2019). Within the experiments of said study, the alginate-immobilized microalgae bead systems (AIMS) were subject to an operation time of 15 days, which resulted in bead leakages for lesser alginate concentrations. The AIMS of Chlorella vulgaris were indicated to have an optimal alginate concentration for uptake rate and productivity at 3% concentration, different from the study's results where 2.5% concentration resulted as the most optimal. It was indicated that alginate concentrations higher than 3% had an integrity that lasted throughout the whole trial and had less cell leakage in return. However, upon further analysis, alginate concentration also directly impacts efficiency, as extremely high levels of concentration tend to reduce nutrient diffusion into the beads, thereby affecting its removal efficiency.

The tradeoff from minimizing cell leakage by increasing alginate concentration is less nutrient adsorption, which consequently decreases nutrient removal. Since there is a higher risk of cell leakage with higher operation times, marrying lower alginate concentrations with relatively lesser operation times (compared to the mentioned study) may be utilized to achieve a higher and more optimal SRP removal without running the risk of cell leakages. Further, this validates the trend in which high operation time enhances SRP removal when alginate concentration is set to low. This also supports the findings that high alginate concentration and operation time produce the minimal percentage of SRP removal, which may be caused by less nutrient adsorption due to more concentration.

Validation of Optimized Treatment Parameters

Within the Box-Behnken design, a maximum predicted SRP removal of 97.91% is achieved by the optimum run with parameters set at 2.5% alginate concentration, 3 days of operation time, and 1.5 beads/mL. An SRP removal of 100% is predicted to be achieved by an extrapolated run with the same parameters except with an operation time of 3.4 days. To verify the accuracy of these predictions, the researchers set up two runs: one for the maximum SRP removal with optimum parameters and one for extrapolated parameters. The results of these runs are to be found in Table 8.

Table 8. Actual and predicted SRP removal of optimum and extrapolated parameter runs

Alginate Concentration (%w/v)	Operation Time (in days)	Bead Dosage (beads/mL)	Predicted SRP Removal (%)	Actual SRP Removal (%)	Percent Error (%)
2.5	3	1.5	97.91	92.80 ± 0.73	5.22
2.5	3.4	1.5	100	97.39 ± 0.08	2.61

Based on the results from the optimum parameter runs, an average of 92.80 ± 0.73 percentage SRP removal from 2.5% alginate concentration, 3 days of operation time, and 1.5 beads/mL of bead dosage was achieved, while an average of 97.39 ± 0.08 percentage SRP removal resulted from the extrapolated parameter run with the same parameters except with an adjusted operation time of 3.4 days. The optimum parameter runs did not reach their respective predicted percentage of SRP removal. However, it is worth noting that the extrapolated run with an operation time beyond the experimental region of 3.4 days managed to achieve the highest SRP removal of all combined runs from the experiment and outperformed the run with a maximum SRP removal of 95.63% in the original 15 runs from the Box-Behnken design. Further, due to the nature of the Box-Behnken design and RSM, the extrapolation of parameters beyond the experimental design region may produce errors in the predictions, resulting in higher or lower values than expected, as demonstrated from the results.

The disparities in values can be attributed to environmental variables such as pH level, nutrient concentration, and temperature, similar to a study by Bouabidi et al. (2018), wherein the cells' adsorption is influenced by varying electrokinetic potential. In a study by Hossain et al. (2022), three parameters: temperature, light-dark cycle, and nitrate-to-phosphate ratio, were tested for nitrogen and phosphorus removal efficiency in treating municipal wastewater. Utilizing different soft computing approaches, RSM resulted in relatively high percent errors compared to Artificial Neural Network (ANN) and Support Vector Regression (SVR) after confirming the prediction accuracies of such models for nitrogen and phosphorus removal with experimental data. Notably, the phosphorus removal model from RSM resulted in an R2 value of 0.8293, indicating that a higher accuracy for prediction in the results of this study was achieved, possibly due to the differences in operational parameters. Although RSM was considered to still be a reliable prediction model, the researchers suggest that further research on other prediction models are explored as these may potentially result in more accurate predictions about SRP removal, especially with the better overall performance of SVR within Hossain et al's (2022) study.

Conclusion

To summarize, the researchers grew subcultures of *Spirulina platensis* with constant aeration and light exposure, resulting in expected fluctuations in cell concentrations due to nutrient depletion and imbalance, human errors, and biomass accumulation. These microalgae were subjected to immobilization with alginate beads of adjusted alginate concentration, operation time, and bead dosage, and were incorporated into an experimental setup for synthetic wastewater treatment afterwards, resulting in the reduction of SRP. Upon analysis, bead dosage resulted to be statistically significant, as well as the interaction effects of alginate concentration and bead dosage and the quadratic effect of bead dosage. However, alginate concentration and operation time were statistically insignificant, including other quadratic and interaction effects, but still possessed trends in affecting percentage of SRP removal. Observable effects were due to increased algal surface area with higher bead dosages, bead instability and cell leakage with lower alginate concentrations accompanied with lengthy operation times, and higher nutrient diffusion for lower alginate concentrations. Upon validating the optimum and extrapolated runs for maximized percentage of SRP removal, the predicted SRP removal rates were not met and had a percent error of 5.22% and 2.61%, which may be due to unconsidered extraneous parameters, systematic errors within the experiments, or estimation inaccuracies from the prediction model.

Additional research may be done when it comes to investigating extrapolation predictions in RSM and the usage of other prediction models like ANN and SVR for higher predictive precision. Moreover, the researchers suggest that the cultivation of *S. platensis*, as well as other microalgae species like Chlorella sp. and Scenedesmus sp., with other controlled parameters, such as varying photoperiod, light wavelength, and pH levels, is further explored, as these are expected to differ in algal growth. In immobilization, other immobilization matrices, whether natural or synthetic, may also be utilized in immobilizing microalgae. Further, the treatment of actual wastewater sampled from natural bodies of water instead of synthetic wastewater, along with increased treatment time, may additionally be researched since these may possibly produce different removal efficiency due to the presence of other organisms that may compete with the SRP removal of microalgae in a natural setting. Lastly, the precipitate from the centrifuged synthetic wastewater of the experiments within the study, especially insoluble phosphates or phosphorus, may be characterized deeper and explored for their potential in being repurposed into useful chemicals like fertilizers.

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