separations

Article

Microalgae Biomass Harvesting Using Chitosan Flocculant: Optimization of Operating Parameters by Response Surface Methodology

Harun Elcik [1,2,*], Dogan Karadag [1], Ayse Irem Kara [1] and Mehmet Cakmakci [1]

[1] Department of Environmental Engineering, Yildiz Technical University, Istanbul 34220, Turkey
[2] Water Desalination and Reuse Center (WDRC), King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Saudi Arabia
* Correspondence: helcik2010@gmail.com

Abstract: Bioflocculants can be used for cost-effective harvesting of microalgae biomass on an industrial scale. This study investigates the flocculation-based harvesting approach to recovering *Chlorella vulgaris* microalgae biomass using chitosan biopolymer. Response surface methodology (RSM) was used to design the experiments and optimize the critical operating parameters. Box-Behnken Design (BBD) was employed at three levels, and 17 experimental runs were conducted to determine the optimal conditions and the relationship between operating parameters. The highest biomass recovery of 99.10% was achieved at the following optimized conditions: pH of 5, flocculation time of 45 min, and chitosan concentration of 10 mg/L. Both experimental results and model outputs indicated that pH significantly impacts microalgae harvesting and that process performance is less dependent on chitosan concentration and flocculation time. The quadratic model has shown the best fit with the experimental results. The results could be applied to large-scale microalgae harvesting applications to promote microalgae biomass recovery and reduce operating costs.

Keywords: microalgae; sustainability; biotechnology; harvesting; biomass; process optimization

check for updates

Citation: Elcik, H.; Karadag, D.; Kara, A.I.; Cakmakci, M. Microalgae Biomass Harvesting Using Chitosan Flocculant: Optimization of Operating Parameters by Response Surface Methodology. *Separations* **2023**, *10*, 507. https://doi.org/10.3390/separations10090507

Academic Editor: Anastasios Zouboulis

Received: 17 August 2023
Revised: 13 September 2023
Accepted: 14 September 2023
Published: 15 September 2023

1. Introduction

Microalgae are microscopic photosynthetic organisms that can convert sunlight, nutrients, and CO_2 into cells rich in proteins, carbohydrates, and lipids [1]. Under favorable environmental conditions, the high photosynthetic rate of microalgae (50 times higher than terrestrial plants) enables higher microalgae biomass yields [2]. Worldwide interest has been extensively directed toward microalgae biomass production since it has significant potential to be the feedstock for producing biofuels, cosmetics, pharmaceuticals, and diets for humans and animals [3]. In addition to that, numerous reports have been released on successful bioremediation applications of microalgae to remove metal pollutants, nitrates, ammonia, and phosphates [4–6].

Microalgae are exposed to various downstream processing stages, including harvesting/dewatering and extraction/fractionation/separation/purification of the desired components [7]. The biomass concentration in the culture medium is known to be low [8]. Therefore, microalgal biomass needs to be harvested before use in further applications [9]. Harvesting is a major challenge in large-scale microalgae production due to its intensive energy requirement, and it can account for up to 60% of the total production cost [10]. In recent years, comprehensive investigations have been performed to find a more efficient and cost-effective harvesting method. Thus far, microalgae harvesting has been performed by several methods, including coagulation/flocculation [11], centrifugation [12], flotation [13], gravitational sedimentation [14], and membrane filtration [15]. These methods have shown high microalgae biomass recoveries and enable the harvest of microalgae biomass without

contaminating it for extracting high-value products [16]. However, long operating durations, high operational costs, and excessive energy consumption limit their widespread use [17–20].

From a practical point of view, flocculation is considered a promising method as it is simple and relatively cheaper when operating under optimal flocculant dose, culture pH, and settling time [21]. The flocculation process can be applied to harvesting a wide variety of microalgae species [22]. Hence, it is suitable for large-scale microalgae harvesting. Inorganic and organic flocculants are used to pre-concentrate microalgae harvesting, followed by sedimentation or flotation to reduce harvesting costs. Metal salts such as aluminum sulfate ($Al_2(SO_4)_3$) and ferric chloride ($FeCl_3$) are commonly used as inorganic flocculants for microalgae biomass harvesting [23]. However, they may pose health risks in some applications, such as in pharmacology. Organic flocculants, such as polyacrylamides, are generally preferred because of their lower dosage requirements compared to inorganic ones [24]. However, they may include acrylamide residues considered carcinogenic or potentially toxic to aquatic organisms. Alternatively, natural flocculants, such as starch, tannin, and chitosan, have recently gained interest due to their renewability, biodegradability, nontoxicity, and relative cost-effectiveness [25]. Among these flocculants, chitosan is of particular interest because it has a high cationic charge density and a long polymer chain [26]. The strong absorption between cationic-charged chitosan and negatively charged cells neutralizes the charge and enables strong aggregations [27]. Furthermore, chitosan is safe to handle, can be regenerated in many applications, and does not damage the cells [28]. This advantage also allows the reuse of microalgae culture medium for the next cultivation process [29]. Yang et al. [30] reported that the optimum 5 mg/L of chitosan concentration yielded 0.59 g of *Chlorella vulgaris* microalgae biomass per liter. They also stated that chitosan has a considerably low harvesting efficiency when the microalgae culture pH is above 7 due to its insolubility in alkaline conditions [30]. In another study, Ahmad et al. [28] highlighted that chitosan concentrations above 10 mg/L lead to the destabilization of microalgae suspensions and reduce harvesting efficiency. Mixing speed also influences flocculation efficiency, as high mixing speeds may break the floc structure, leading to the redispersion of the biomass into the medium. Pan et al. [31] underlined that increased mixing speeds could reduce the required amount of chitosan to some extent. In addition, mixing time is found to be critical, which plays a role in colloid destabilization. A prolonged mixing time enables more adsorption of microalgae cells onto chitosan, yielding a higher efficiency. This phenomenon is attributed to the reduced diameter of microalgae cells broken down with a longer mixing time, which results in a larger interfacial area that can facilitate better interaction between the cells and chitosan. The findings conclude that critical process parameters that directly affect flocculation efficiency need to be optimized to make the process economically viable at an industrial scale.

Response surface methodology (RSM) has been successfully applied to modeling and optimizing several flocculation processes [32]. RSM determines the relationship between multiple independent factors, reducing variability, operating time, and costs. Moreover, it analyzes the effects of experimental factors on responses at desired levels [33]. Box-Behnken Design (BBD) is one of the well-known optimization designs widely applied successfully in flocculation processes [34]. Zhang et al. [35] optimized the self-flocculation conditions of *Desmodesmus* sp. by a three-level BBD method and noted that the flocculation efficiency increases with prolonged biomass settling time. Singh et al. [36] successfully applied a BBD method to optimize the flocculation process efficiency and evaluated four process parameters for harvesting *Chlorella pyrenoidosa* with $CaCl_2$. Wang et al. [37] evaluated the flocculation performance of amphoteric flocculant in harvesting Coccomyxa sp. using the BBD with three independent variables. Perez et al. [38] investigated the effect of the critical operating parameters on a combined flocculation process with an organic polymer and inorganic flocculant to reduce the high inorganic flocculant demand that can be harmful to marine culture. Previous RSM reports on microalgae biomass flocculation remained limited as they modeled data using only one specific mathematical model. Therefore, further

investigation of the process parameters of the microalgae flocculation process by evaluating different mathematical models is necessary to identify the best-optimized conditions.

Accordingly, this study aims to contribute to the success of the flocculation process in microalgae biomass harvesting using response surface methodology. The novelty of this study lies in the comprehensive optimization of the most critical operating parameters, such as pH (5–11), chitosan concentration (10–100 mg/L), and flocculation time (15–45 min). Chitosan, a natural organic polymer with a cationic charge, was used as a flocculant agent. The optimal combination of the abovementioned parameters for maximum efficiency was determined based on BBD. Finally, a feasibility assessment and cost analysis of chitosan were performed. The results reported in this study are believed to provide significant insight into the microalgae biotechnology community's efforts to develop innovative flocculation technologies for microalgae harvesting on an industrial scale.

2. Materials and Methods

2.1. Microalgae and Culture Conditions

Single-cell green microalgae (*Chlorella vulgaris)* with an average diameter of 5 µm were used in the experiments. *Chlorella vulgaris* contains a high content of proteins, carbohydrates, and lipids; thus, it is widely used in biofuel production and other applications [39,40]. The microalgae cells were cultured in Bold's Basal Medium (BBM) at pH 7.5 ± 1.0. The pH of the culture medium was adjusted with either 0.1 N HCl or 0.1 N NaOH. The microalgae were grown in a 6-L volumetric flask by aeration at a fixed pressure of 0.6 L/min and under illumination at a light intensity of 70 µmol m^{-2} s^{-1} (MIC Light meter, Model 98209, Mic Co. Ltd, Wuxi, China). The light/dark ratio was kept constant at 24:0, and the cultivation temperature was 25 ± 2 °C for 15 days.

2.2. Experimental Procedure and Analytical Methods

A scheme illustrating the experimental procedure is given in Figure 1. Flocculation experiments were conducted using a natural organic polymer, chitosan. Chitosan (CAS Number: 9012-76-4) was procured from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). The flocculant solution was prepared by solubilizing chitosan at desired concentrations in 10 mL of 1% HCl at 100 rpm for 10 min. The pH level of the flocculant solution was adjusted with 0.1 N HCl or 0.1 N NaOH. Batch flocculation tests were performed in six steel-paddled Jar equipment (Velp Scientifica, FC6S, Usmate Velate, Italy) with cylinder beakers to determine the effects of operating parameters on the flocculation process. Each flocculation experiment was conducted with a 500-milliliter of solution. All tests were performed at 25 ± 2 °C, and formed flocs were allowed to settle for 15 min. After the flocculation process, the supernatant sample was taken to determine the biomass recovery rate. All the experiments were performed with a 0.373 ± 0.087 g/L initial biomass concentration. The biomass weight was estimated by measuring the optical density of microalgae cultures at 680 nm using a spectrophotometer (WTW PhotoLab 6600 UV-vis Spectrophotometer, Weilheim, Germany). The details of measuring biomass concentration can be found in our former study [41]. Biomass recovery efficiency was calculated using the following equation:

$$\text{Biomass recovery}(\%) = \left(1 - \left(\frac{\text{Sample absorbance}}{\text{Initial absorbance}}\right)\right) \times 100 \quad (1)$$

Mixing Speed

Considering extensive literature reports on the value of mixing speed, rapid mixing for 1 min at 100 rpm and then flocculation at 30 rpm for varying times were applied in our experiments [42–46].

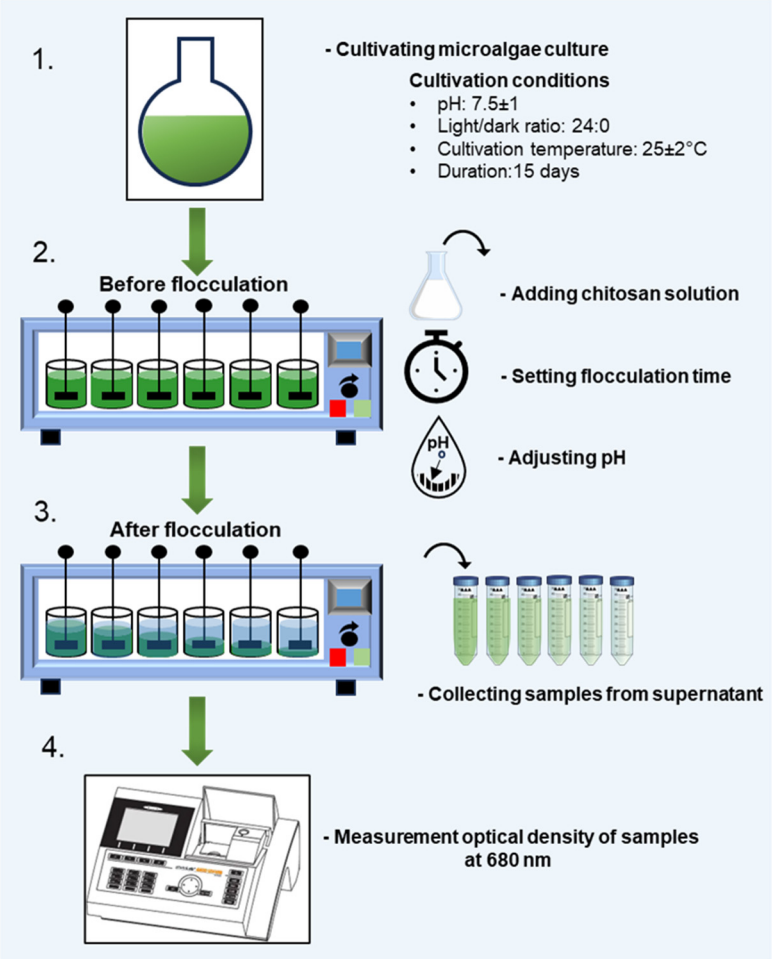

Figure 1. The scheme illustrates the experimental steps.

2.3. Experimental Design and Data Analysis

3-Level Box Behnken Design (BBD) was used to optimize the relationships among experimental factors. A version of the Design Expert 7.0.0 software was used for regression analysis of the experimental data. Response surface plots were prepared using Sigma Plot 11.0 software. Experimental factors of chitosan concentration (X_1), flocculation time (X_2), and pH (X_3) were chosen as explanatory factors at three different levels of −1, 0, and +1, presenting low, medium, and high values (Table 1).

Table 1. Ranges and levels of the Box-Behnken Design for microalgae flocculation.

Explanatory Variables	Unit	Symbol	Levels		
			−1	**0**	**1**
Chitosan concentration	mg/L	X_1	10	55	100
Flocculation time	minute	X_2	15	30	45
pH	-	X_3	5	8	11

The medium values for the experimental design were a pH of 8, a chitosan amount of 55 mg/L, and a flocculation time of 30 min in uncoded form. The range of factors was chosen based on previous reports on microalgae flocculation by chitosan. The design consisted of 17 experimental runs (Table 2). RSM was used to identify the relative significance levels of experimental factors. The relation between variables and responses was determined by fitting a model in Equation (2) to experimental data. According to Equation (2), Y is the

predicted response surface function (% biomass recovery), b_0 is the constant, b_1–b_3 are the linear coefficients, b_{12}, b_{13}, and b_{23} are the interaction coefficients, and b_{11}, b_{22}, and b_{33} are the quadratic coefficients.

$$y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (2)$$

Table 2. Comparison of observed and predicted values of the Box-Behnken Design in microalgae flocculation.

Run	Chitosan Concentration (mg/L)	Flocculation Time (min)	pH	Biomass Recovery (%)	
				Observed Values	Predicted Values
1	55	30	8	70.89	70.46
2	55	15	11	80.59	78.74
3	55	30	8	74.21	70.46
4	55	30	8	70.67	70.46
5	100	30	5	95.13	93.04
6	10	30	5	97.10	97.04
7	10	30	11	76.70	78.79
8	100	45	8	71.21	71.44
9	100	15	8	67.58	70.46
10	55	45	5	94.65	96.50
11	55	15	5	93.92	94.21
12	55	30	8	68.96	70.46
13	55	45	11	84.21	83.92
14	10	45	8	74.69	72.89
15	55	30	8	67.58	69.38
16	100	30	11	83.18	83.24
17	10	15	8	67.71	67.48

The model fitting was evaluated by the coefficient of determination (R^2) and the R^2 adjusted value. The significance of the variables in the model was verified with an analysis of variance (ANOVA). The Fisher F-test was used to check for statistical significance. Computations were performed at a 95% confidence level, and *p*-values less than 0.05 indicated that the computations were statistically significant.

2.4. A Brief Feasibility Assessment and Cost Estimate of Chitosan

The feasibility assessment was conducted based on the flocculants reported in the literature for *Chlorella vulgaris* microalgae harvesting. It highlights the disadvantages of using inorganic metal salts as flocculants, such as their unsuitability for marine microalgae harvesting, contamination issues, and the generation of toxic sludge. The feasibility assessment emphasizes the desired characteristics of flocculants used in full-scale applications, including being inexpensive, environmentally friendly, and easy to apply. It refers to specific studies that have reported the optimal chitosan concentration and flocculation efficiency for microalgae recovery. This part also presents optimization experiments that demonstrate the high biomass recovery rate achieved with chitosan for a specific microalgae species, *Chlorella vulgaris*, at specific pH and chitosan concentration conditions. A comparison of the biomass recovery rates of chitosan with other flocculants under different experimental conditions is given in a table.

The cost analysis part explains the importance of considering the cost of flocculants in the biomass recovery phase of microalgae production. It highlights that the high cost of flocculants can make the process economically infeasible and limit their applicability in full-scale processes. In this part, a cost comparison was reported between inorganic and organic flocculants based on the required flocculant dose to harvest per ton of *Chlorella vulgaris* biomass. The recommended dosages or concentrations of chitosan needed for effectively harvesting *Chlorella vulgaris* biomass were obtained from previous studies.

3. Results and Discussion

3.1. Evaluation of Operating Parameters

Flocculation-based microalgae harvesting by chitosan was investigated with the optimization of pH, chitosan concentration, and flocculation time. Relative importance among variables and responses was investigated using RSM according to BBD. Harvesting efficiencies from 17 experimental runs and predicted values by response function are shown in Table 2. The results of BBD runs showed that the best harvesting efficiency was 97.10% at Run 6, while the lowest was 67.58% at Runs 9 and 15. The comparison data in Table 2 indicated that harvesting efficiencies were mainly affected by the changes in pH values. Harvesting efficiencies were higher than 93% at pH 5, which slightly increased with the increased chitosan concentration and flocculation time. Demir et al. [47] reported that the chitosan-induced interaction mechanism with microalgae cells is contingent upon the pH value. At pH 6, chitosan inclines to interact with *Chlorella vulgaris* cell walls through a biological binding/interaction mechanism that occurs through biomolecules such as polysaccharides at the cell surface [47]. In addition, it is worth mentioning that chitosan is positively charged at lower pH due to its amine groups, thus electrostatically interacting with negatively charged microalgae cells. Electrostatic interaction mechanisms include charge neutralization, patching, and bridging; a comprehensive discussion can be found elsewhere [48]. At pH 8, the harvesting efficiencies were significantly low (67.58–74.69%), and the impact of chitosan concentration and flocculation time on the flocculation performance remained relatively limited. At higher pH values, electroneutral chitosan molecules are induced to precipitate by hydroxide ions OH^{-} that mechanically trap the cells into the chitosan floc structures via a sweeping mechanism [49,50].

The observed results suggested that pH plays a critical role in flocculation efficiency, and a slightly acidic pH is more favorable for efficient microalgae flocculation by chitosan.

3.2. Evaluation of RSM Models

Four response models—linear, interactive (2FI), quadratic, and cubic—were evaluated to correlate the experimental results and create the regression equation. The tests of the sequential model sum of squares, lack of fit tests, and model summary statistics were performed to validate the accuracy of model outputs with experimental data. Table 3 summarizes the comparison of statistics for each test and response function. R^2 is commonly used in statistical studies to determine the fitting degree of the model and experimental data. A higher R^2 value shows a higher fitting degree, while Joglekar and May [51] indicated that the R^2 value should be at least 0.80 for satisfactory fitting of a model with experimental data. Table 3 indicates that the Quadratic model has higher R^2, adjusted R^2, and predicted R^2 with the lowest p values. The lack of fit F-values for Linear, FI, and Quadratic models indicates the significance of the response model for harvesting efficiency. The quadratic model was the most suitable for harvesting microalgae by flocculation on chitosan. The determination coefficient R^2 was 0.9743, indicating that the model could explain 97.43% of the variability in harvesting efficiency. Moreover, the adjusted R^2 value of 0.9412 confirmed that the model was highly significant for harvesting. Values of $p > F$ less than 0.05 indicate that the Quadratic model is significant, while values greater than 0.1 indicate that the Linear, 2F, and Cubic models are insignificant. Moreover, the high F-value and the small p-value correspond to significant variables [52]. These results indicate that the model was significant, and the regression equation describes most of the variation in the response.

Table 3. Evaluation of models and response functions for microalgae flocculation.

Source	Sum of Squares	df	Mean Square	F	$p > F$
		Sequential model sum of squares			
Mean	1.055×10^5	1	1.055×10^5		
Linear	421.76	3	140.59	1.29	0.3178
2FI	22.74	3	7.58	0.055	0.9822
Quadratic	1341.58	3	447.19	66.35	<0.0001
Cubic	22.34	3	7.45	1.20	0.4168
Residual	24.84	4	6.21	-	-
Total	1.073×10^5	17	6311.53	-	-
		Lack of fit tests			
Linear	1386.67	9	154.07	24.81	0.0037
2FI	1363.93	6	227.32	36.61	0.0019
Quadratic	22.34	3	7.45	1.20	0.4168
Cubic	0.000	0	-	-	-
Pure error	24.84	4	6.21	-	-
Source		**Model summary statistics**			
	Std. dev.	R^2	Adjusted R^2	Predicted R^2	Press
Linear	10.42	0.2301	0.0524	−0.4174	2598.40
2FI	11.78	0.2425	−0.2121	−2.0842	5654.22
Quadratic	2.60	0.9743	0.9412	0.7838	396.28
Cubic	2.49	0.9865	0.9458	-	-

3.3. Statistical Analysis of Factors

The statistical significance of the quadratic model was evaluated by analysis of variance (ANOVA), and the results are presented in Table 4. A model F value of 29.45 indicates that the model predicted the response significantly, and there is only a 0.01% chance that this F-value could occur due to noise. The significance of the regression coefficients was evaluated by their corresponding *p* values. *P* values less than 0.050 indicate the model terms are significant, whereas *p* values greater than 0.1000 are insignificant [53]. The values in Table 4 imply that X_3 and the quadratic term of pH (X_3^2) were significant for harvesting efficiency, while X_1 and X_2, the quadratic terms of chitosan concentration (X_1^2) and flocculation time (X_2^2), and all mix product terms of X_1X_2, X_1X_3, and X_2X_3 were not significant.

Table 4. ANOVA analysis of the quadratic model for microalgae flocculation.

Source	Sum of Squares	df	Mean Square	F Value	*p*-Value
Model	1786.09	9	198.45	29.45	<0.0001 *
X_1	0.10	1	0.10	0.015	0.9059
X_2	27.98	1	27.98	4.15	0.0810
X_3	393.68	1	393.68	58.41	0.0001 *
X_1X_2	2.81	1	2.81	0.42	0.5394
X_1X_3	17.85	1	17.85	2.65	0.1477
X_2X_3	2.09	1	2.09	0.31	0.5951
X_1^2	0.24	1	0.24	0.036	0.8551
X_2^2	0.024	1	0.024	3.538×10^{-3}	0.9542
X_3^2	1334.85	1	1334.85	198.06	<0.0001 *
Residual	47.18	7	6.74		

R^2 = 0.9743, adjusted R^2 = 0.9412, R = 0.9870; adequate precision = 14.848 (>4). * Significant variable.

On the other hand, the *p*-value of X_2 (flocculation time) is slightly higher than 0.05, indicating its much lower impact on the harvesting process than that of pH. The *p*-value of X_1 (chitosan amount) is greater than 0.1 and remains insignificant. The observed results indicate that microalgal culture harvesting with chitosan mainly depends on pH, while

the process performance is less dependent on chitosan concentration. According to the analysis, the model for microalgal culture harvesting on chitosan can be written as follows:

$$y = 220.540 + 0.0444X_2 - 35.334X_3 + 1.9784X_3^2 \quad (3)$$

3.4. Analysis of Variables

The three-dimensional response surface plots for pair variables on harvesting efficiency are shown in Figure 2. The graphical representations of the regression equation present more information on the interaction between variables and responses. Figure 2a depicts the effects of flocculation time and chitosan concentration on harvesting efficiency. Increasing the flocculation time from 15 min to 35 min improved harvesting efficiency from 65% to around 80%, whereas the efficiency slightly declined with the increase in flocculation time to 45 min. Flocculation time is critical until chitosan and microalgae cells reach their maximum interaction level and produce flocs. A similar effect was observed with the changes in chitosan concentration. The concentration of chitosan is critical as it provides a surface area for microalgae cells to accumulate onto chitosan particles via different interaction mechanisms. The highest efficiency was about 80% at 60 mg/L and decreased to 70% at the lowest and highest chitosan concentrations. It is clearly evident in Figure 2b that slight changes in pH considerably enhanced the harvesting efficiency. A decrease in pH had opposite effects on response. Harvesting efficiency moderately declined with the decreasing pH from 11 to 9, while further lowering the pH sharply increased harvesting efficiency, which was the highest at pH 5.

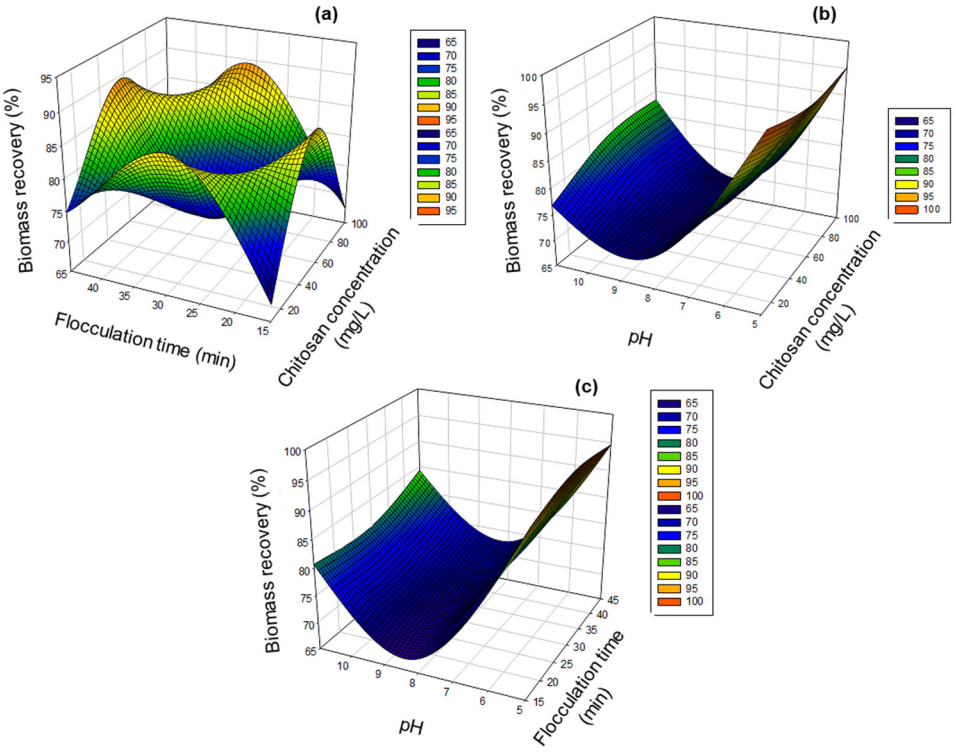

Figure 2. Three-dimensional response surface plots for microalgae flocculation illustrate the interaction effects of (**a**) flocculation time-chitosan concentration, (**b**) pH-chitosan concentration, and (**c**) pH-flocculation time on microalgae biomass recovery.

On the other hand, changes in chitosan concentration have little impact on harvesting efficiency, as an increase in chitosan concentration from 5 to 100 mg/L improved harvesting efficiency from 80% to 95%. In addition, the variation of harvesting efficiency with pH and flocculation time was analyzed in Figure 2c. Their effect was similar to the effect of pH and chitosan concentration, and the efficiency was the highest at 90% at a flocculation

time of 45 min. Analyzing the plots reveals that pH is the most important variable in harvesting performance, and the efficiency was higher at acidic pH levels. According to surface analyses, the minimum harvesting efficiency was 66.17% at a pH of 9, flocculation time of 15 min, and chitosan concentration of 10 mg/L.

In comparison, the maximum efficiency was predicted to be 99.10% at a pH of 5, flocculation time of 45 min, and chitosan concentration of 10 mg/L. The optimization analysis result for maximum efficiency was then validated with experimental runs. The deviation between the actual and predicted values was <5%.

3.5. Feasibility Assessment and Cost Analysis of Chitosan

The flocculation process of microalgae cells has been applied using various flocculants. Among these flocculants, metal salts such as $FeCl_3$, $Al_2(SO_4)_3$, and ferric sulfate ($Fe_2(SO_4)_3$) are widely used for microalgal biomass recovery [54]. However, these inorganic metal salts have some disadvantages. For example, they are unsuitable for marine microalgae harvesting [24] and pose a contamination problem for further processing of microalgal biomass, which limits its usability as food, feed, etc. [55]. Moreover, large volumes of toxic sludge produced from the flocculation process are required to be safely removed [56,57]. Most of the researchers have reported in the literature that flocculants used in full-scale applications should be inexpensive [58], environmentally friendly [56], and easily applicable [54].

Compared to inorganic flocculants, organic flocculants, including cationic and anionic polyelectrolytes, nonionic polymers, amphoteric and hydrophobically modified polymers, and naturally occurring flocculants, offer several advantages [56]. They are applicable for the purpose of high biomass recovery efficiency for a wide variety of microalgae species. In addition, these flocculants are non-toxic [58], biodegradable [55], renewable, and ecologically acceptable [54]. Moreover, they require a much lower dosage than inorganic metal salts, which reduces operational costs [58] and offers high microalgal biomass recovery efficiency with low dosage demand [54].

Recent investigations have proven that chitosan, a cationic polyelectrolyte derived from chitin, is quite an effective flocculant for microalgae recovery [28,54,59]. Beach et al. [59] reported that the optimum chitosan concentration for a 95% maximum microalgae recovery rate was 100 mg/L at 350 rpm in a 1-minute fast mixing condition. Gupta et al. [60] reported a 91% flocculation efficiency at a dose of 80 mg/L of chitosan. In this study, optimization experiments showed that 99.10% biomass recovery for *Chlorella vulgaris* is acquired at pH 5 and a chitosan concentration of 10 mg/L. A detailed comparison of the biomass recovery rates of chitosan used in this study and other flocculants under different experimental conditions for *Chlorella vulgaris* is presented in Table 5.

Table 5. Flocculation efficiency of *Chlorella vulgaris* using different flocculants.

Flocculant	Experimental Set-Up	Efficiency (%)	Ref.
Nano-aminoclays (Mg-APTES)	BC: 1 g/L; FD: 1 g/L; pH: 5.0–12.0	>90%	[61]
Mg-sericite	BC: 2.13 ± 0.21 g/L; FD: 1–30 mg/L; sericite and $MgCl_2$ ratio (S/M ratio): 40; mixing time: 5 min; mixing rate: 100–150 rpm; settling time: 5 min; pH: 9.0–11.0	99 ± 0.3	[62]
Magnetic chitosan	BC: 0.8 OD540 nm; FD: 216 mg/L; pH: 9.0–11.0	94	[45]
Poly-γ-glutamic acid	BC: 0.57 g/L; FD: 22.03 mg/L; Salinity: 11.56 g/L; PT: 2 h; pH: 7.5	91	[63]
Actipol-FB1	BC: 1 g/L; FD: 3 mg/L; pH: 8	94	[64]
Chitosan	BC: 0.59 g/L; FD: 5 mg/L; PT: 50 min	98.9	[30]
Chitosan	BC: 0.373 ± 0.087 g/L; FD: 10 mg/L; PT: 46 min. (mixing) + 15 min. (settling); pH: 5	99.1	This study

BC: biomass concentration; FD: flocculant dose; PT: process time (including mixing and settling).

Flocculant cost is another important consideration since the biomass recovery phase could contribute up to 60% of the total production cost [58]. The high cost of flocculants is economically infeasible, which, in turn, limits their applicability for full-scale processes. A cost comparison for some inorganic and organic flocculants based on the required doses and recovery efficiencies is given in Table 6. Compared to inorganic flocculants such as $Al_2(SO_4)_3$ and $Ca(OH)_2$, organic flocculants such as Flopam and Zetag (except chitosan) are quite costly in terms of microalgal biomass recovery. Previous studies have reported chitosan flocculant costs for microalgae recovery. For example, Gupta et al. [60] have calculated that the cost of the chitosan (recovery efficiency: 91.0%) for harvesting 1 kg of biomass would be 51.02 USD. In another study, the cost analysis was reported by considering retail prices of chitosan (~20 USD/kg), and the harvesting cost of chitosan would be ~200 USD/ton of biomass [54]. Chitosan is produced commercially by the deacetylation of chitin, known as the second most abundant biopolymer in the world [65]. Recently, prices have dropped to 1 USD/kg depending on the use of chitosan in many applications (Cleanwater Chemicals Co., Ltd., Yixing, China, www.cleanwat.com). In this study, the chitosan cost for 1 ton of biomass recovery is calculated at 21–35 USD. Consequently, the results suggest that chitosan, an economical, effective, and environmentally friendly flocculant, is promising for microalgae biomass recovery.

In addition to flocculant costs, reagent costs to keep the microalgae solution at a desired pH need to be considered. Especially if the solution has an alkaline characteristic, the consumption of reagents for pH adjustment would play a role in the harvesting cost of microalgae biomass [23]. In a study, acetic acid consumption to harvest 1 ton of microalgae biomass in the flocculation process by nano-chitosan was estimated to be 2.1 USD [66].

The flocculation method offers several advantages over conventional harvesting techniques that consume much energy, including lower operational costs, reduced energy demand, versatility, excellent effectiveness, and the ability to be easily incorporated into large-scale harvesting systems. Chitosan flocculant has been investigated for its effectiveness in harvesting microalgae, demonstrating encouraging outcomes in small-scale laboratory settings. However, the limited research on the performance evaluation of chitosan flocculant at larger scales, such as pilot and industrial scales, and in outdoor microalgae biomass cultivation systems is a significant gap in the current understanding of its effectiveness. A limited number of pilot-scale flocculation studies, primarily utilizing chitosan biopolymer, have indicated that the flocculation performance observed in bench-scale jar tests can significantly differ from that in outdoor cultivation systems. This discrepancy can be attributed to variations in the physicochemical conditions of the microalgae culture, such as pH, solar radiation, temperature, and oxygen saturation. In order to maintain uniformity in the performance of chitosan during the flocculation process on a larger scale, it is crucial to test chitosan under conditions that mimic the actual harvesting environment, thus ensuring the compatibility of the flocculation process in outdoor settings. Overcoming significant techno-economic barriers is still necessary for industrial-scale harvesting and utilizing microalgal biomass. Extensive research and development efforts and investments are needed to make the current harvesting processes economically feasible. Presently, no single technique is identified as the optimal procedure for biomass harvesting from numerous microalgae strains, indicating the need for future research and development initiatives to explore combinations of multiple techniques to optimize the harvesting process regarding technical and economic feasibility, scalability, energy effectiveness, and sustainability.

The flocculation process by chitosan has exhibited superior efficiency, reliability, cost-effectiveness, versatility, and scalability compared to physical and biological harvesting methods for microalgae biomass. Chitosan biopolymer has been studied in laboratory-scale investigations to harvest various microalgae strains efficiently. However, there is a shortage of research and investigations demonstrating the high effectiveness of this flocculant under industrial-scale conditions.

Table 6. Cost analysis comparison of *Chlorella vulgaris* harvesting with different flocculants.

Flocculant	Biomass (mg/L)	Flocculant Dose (mg/L)	Flocculant Efficiency (%)	Required Flocculant Dose (ton ton^{-1} biomass)	Flocculant Cost (US$ ton^{-1})	Required Flocculant Cost (US$ ton^{-1} biomass)	Ref.
$Al_2(SO_4)_3$	250	20	85	0.094	300	28	[67]
$Ca(OH)_2$	500	n.a.	n.a.	0.120	150	18	[68]
NaOH	500	n.a.	n.a.	0.120	350	42	[69]
Flopam	260	5	98	0.020	8000	157	[58]
Zetag	260	5	100	0.019	8000	154	[58]
Chitosan	590	5	98.9	0.0084	20,984	176.81	[30]
Chitosan	373 ± 87	10	99	0.021–0.035	1000	21–35	This study

4. Conclusions

This study focused on the optimization of the most critical operating parameters of the flocculation-based microalgae harvesting process, including pH, chitosan concentration, and flocculation time. Response surface methodology (RSM) allowed us to determine the influence of these parameters on the flocculation process. A Box-Behnken Design with the quadratic model was found to be the most representative of the process parameters based on the regression coefficient and *p* values. Statistical and surface analyses indicated that microalgal culture harvesting with chitosan mainly depended on pH. In contrast, the influence of chitosan concentration and flocculation time on the process's performance remained limited at the studied ranges. The optimum parameters were predicted at a pH of 5, flocculation time of 45 min, and chitosan concentration of 10 mg/L, where the maximum harvesting efficiency was recorded as 99.10%. In this process, the chitosan cost for 1 ton biomass was estimated to be 21–35 USD. The results demonstrate that chitosan flocculant can be advantageously used to harvest microalgae with a high biomass recovery rate and a low flocculant cost.

Author Contributions: H.E.: conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing—original draft preparation, and writing—review and editing. D.K.: methodology, software, validation, writing—original draft preparation, and writing—review and editing. A.I.K.: conceptualization, methodology, investigation, and writing—review and editing. M.C.: methodology, investigation, resources, supervision, funding acquisition, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rodrigues, J.R.d.S.; Santos, R.d.S.; Matos, R.A.; Pires, J.C.; Salgado, E.M. Sustainable Microalgal Harvesting Process Applying Opuntia cochenillifera: Process Parameters Optimization. *Appl. Sci.* **2023**, *13*, 1203. [CrossRef]
2. Singh, B.; Guldhe, A.; Rawat, I.; Bux, F. Towards a sustainable approach for development of biodiesel from plant and microalgae. *Renew. Sustain. Energy Rev.* **2014**, *29*, 216–245.
3. Moncada, J.; Tamayo, J.A.; Cardona, C.A. Integrating first, second, and third generation biorefineries: Incorporating microalgae into the sugarcane biorefinery. *Chem. Eng. Sci.* **2014**, *118*, 126–140.
4. Lananan, F.; Hamid, S.H.A.; Din, W.N.S.; Khatoon, H.; Jusoh, A.; Endut, A. Symbiotic bioremediation of aquaculture wastewater in reducing ammonia and phosphorus utilizing Effective Microorganism (EM-1) and microalgae (*Chlorella* sp.). *Int. Biodeterior. Biodegrad.* **2014**, *95*, 127–134. [CrossRef]
5. Prandini, J.M.; Da Silva, M.L.B.; Mezzari, M.P.; Pirolli, M.; Michelon, W.; Soares, H.M. Enhancement of nutrient removal from swine wastewater digestate coupled to biogas purification by microalgae Scenedesmus spp. *Bioresour. Technol.* **2016**, *202*, 67–75. [CrossRef]
6. Li, T.; Hu, J.; Zhu, L. Self-flocculation as an efficient method to harvest microalgae: A mini-review. *Water* **2021**, *13*, 2585. [CrossRef]
7. Nitsos, C.; Filali, R.; Taidi, B.; Lemaire, J. Current and novel approaches to downstream processing of microalgae: A review. *Biotechnol. Adv.* **2020**, *45*, 107650.

8. Elisabeth, B.; Rayen, F.; Behnam, T. Microalgae culture quality indicators: A review. *Crit. Rev. Biotechnol.* **2021**, *41*, 457–473. [CrossRef]
9. Estime, B.; Ren, D.; Sureshkumar, R. Cultivation and energy efficient harvesting of microalgae using thermoreversible sol-gel transition. *Sci. Rep.* **2017**, *7*, 40725. [CrossRef]
10. Grima, E.M.; Belarbi, E.-H.; Fernández, F.A.; Medina, A.R.; Chisti, Y. Recovery of microalgal biomass and metabolites: Process options and economics. *Biotechnol. Adv.* **2003**, *20*, 491–515. [CrossRef]
11. Seo, Y.H.; Park, D.; Oh, Y.-K.; Yoon, S.; Han, J.-I. Harvesting of microalgae cell using oxidized dye wastewater. *Bioresour. Technol.* **2015**, *192*, 802–806. [CrossRef] [PubMed]
12. Najjar, Y.S.; Abu-Shamleh, A. Harvesting of microalgae by centrifugation for biodiesel production: A review. *Algal Res.* **2020**, *51*, 102046.
13. Barros, A.I.; Gonçalves, A.L.; Simões, M.; Pires, J.C. Harvesting techniques applied to microalgae: A review. *Renew. Sustain. Energy Rev.* **2015**, *41*, 1489–1500.
14. Ferreira, J.; de Assis, L.R.; de Sousa Oliveira, A.P.; de Siqueira Castro, J.; Calijuri, M.L. Innovative microalgae biomass harvesting methods: Technical feasibility and life cycle analysis. *Sci. Total Environ.* **2020**, *746*, 140939. [PubMed]
15. Elcik, H.; Cakmakci, M. Harvesting of microalgae via submerged membranes: Flux, fouling and its reversibility. *Membr. Water Treat.* **2017**, *8*, 499–515.
16. Shaikh, S.M.; Hassan, M.K.; Nasser, M.S.; Sayadi, S.; Ayesh, A.I.; Vasagar, V. A comprehensive review on harvesting of microalgae using Polyacrylamide-Based Flocculants: Potentials and challenges. *Sep. Purif. Technol.* **2021**, *277*, 119508.
17. Ananthi, V.; Balaji, P.; Sindhu, R.; Kim, S.-H.; Pugazhendhi, A.; Arun, A. A critical review on different harvesting techniques for algal based biodiesel production. *Sci. Total Environ.* **2021**, *780*, 146467.
18. Goswami, G.; Kumar, R.; Sinha, A.; Maiti, S.K.; Dutta, B.C.; Singh, H.; Das, D. A low-cost and scalable process for harvesting microalgae using commercial-grade flocculant. *RSC Adv.* **2019**, *9*, 39011–39024.
19. Roselet, F.; Vandamme, D.; Muylaert, K.; Abreu, P.C. Harvesting of microalgae for biomass production. In *Microalgae Biotechnology for Development of Biofuel and Wastewater Treatment*; Springer Nature Singapore Pte Ltd.: Singapore, 2019; pp. 211–243.
20. Vasistha, S.; Khanra, A.; Clifford, M.; Rai, M.P. Current advances in microalgae harvesting and lipid extraction processes for improved biodiesel production: A review. *Renew. Sustain. Energy Rev.* **2021**, *137*, 110498.
21. Rashid, N.; Rehman, S.U.; Han, J.-I. Rapid harvesting of freshwater microalgae using chitosan. *Process Biochem.* **2013**, *48*, 1107–1110.
22. Mubarak, M.; Shaija, A.; Suchithra, T. Flocculation: An effective way to harvest microalgae for biodiesel production. *J. Environ. Chem. Eng.* **2019**, *7*, 103221.
23. Branyikova, I.; Prochazkova, G.; Potocar, T.; Jezkova, Z.; Branyik, T. Harvesting of microalgae by flocculation. *Fermentation* **2018**, *4*, 93. [CrossRef]
24. Vermuë, M.; Olivieri, G.; van den Broek, L.; Barbosa, M.; Eppink, M.; Wijffels, R.; Kleinegris, D. Cationic polymers for successful flocculation of marine microalgae. *Bioresour. Technol.* **2014**, *169*, 804–807.
25. Niemi, C.; Gentili, F.G. The use of natural organic flocculants for harvesting microalgae grown in municipal wastewater at different culture densities. *Physiol. Plant.* **2021**, *173*, 536–542. [PubMed]
26. Nilsen-Nygaard, J.; Strand, S.P.; Vårum, K.M.; Draget, K.I.; Nordgård, C.T. Chitosan: Gels and interfacial properties. *Polymers* **2015**, *7*, 552–579. [CrossRef]
27. Aibani, N.; Rai, R.; Patel, P.; Cuddihy, G.; Wasan, E.K. Chitosan nanoparticles at the biological interface: Implications for drug delivery. *Pharmaceutics* **2021**, *13*, 1686. [PubMed]
28. Ahmad, A.; Yasin, N.M.; Derek, C.; Lim, J. Optimization of microalgae coagulation process using chitosan. *Chem. Eng. J.* **2011**, *173*, 879–882.
29. Yin, Z.; Hu, D.; Li, X.; Zhong, Y.; Zhu, L. Shell-derived chitosan as a green flocculant to harvest microalgae for biofuel production. *Biofuels Bioprod. Biorefin.* **2021**, *15*, 637–645.
30. Yang, Z.; Hou, J.; Miao, L. Harvesting freshwater microalgae with natural polymer flocculants. *Algal Res.* **2021**, *57*, 102358. [CrossRef]
31. Pan, J.R.; Huang, C.; Chen, S.; Chung, Y.-C. Evaluation of a modified chitosan biopolymer for coagulation of colloidal particles. *Colloids Surf. A Physicochem. Eng. Asp.* **1999**, *147*, 359–364.
32. Adesina, O.A.; Abdulkareem, F.; Yusuff, A.S.; Lala, M.; Okewale, A. Response surface methodology approach to optimization of process parameter for coagulation process of surface water using Moringa oleifera seed. *S. Afr. J. Chem. Eng.* **2019**, *28*, 46–51. [CrossRef]
33. Das, D.; Vimala, R.; Das, N. Biosorption of Zn (II) onto Pleurotus platypus: 5-Level Box–Behnken design, equilibrium, kinetic and regeneration studies. *Ecol. Eng.* **2014**, *64*, 136–141. [CrossRef]
34. Fu, H.-Y.; Xu, P.-C.; Huang, G.-H.; Chai, T.; Hou, M.; Gao, P.-F. Effects of aeration parameters on effluent quality and membrane fouling in a submerged membrane bioreactor using Box–Behnken response surface methodology. *Desalination* **2012**, *302*, 33–42. [CrossRef]
35. Zhang, B.; Liu, L.; Lin, X.; Xu, Z.; Luo, W.; Luo, L. Response surface methodology to optimize self-flocculation harvesting of microalgae Desmodesmus sp. CHX1. *Environ. Technol.* **2022**, *43*, 2647–2655. [CrossRef] [PubMed]

36. Singh, H.M.; Tyagi, V.; Ahmad, S.; Kothari, R. Optimization of flocculation efficiency of *Chlorella pyrenoidosa* with $CaCl_2$ using the Box-Behnken design of response surface methodology: A cost effective statistical investigation. *Biomass Convers. Biorefin.* **2022**, 1–13. [CrossRef]
37. Wang, Q.; Oshita, K.; Takaoka, M. Evaluation of flocculation performance of amphoteric flocculant when harvesting microalgae Coccomyxa sp. KJ by response surface methodology. *J. Environ. Manag.* **2021**, *277*, 111449. [CrossRef]
38. Pérez, L.; Salgueiro, J.L.; Maceiras, R.; Cancela, Á.; Sánchez, Á. Study of influence of pH and salinity on combined flocculation of Chaetoceros gracilis microalgae. *Chem. Eng. J.* **2016**, *286*, 106–113. [CrossRef]
39. Silva, D.A.; Cardoso, L.G.; de Jesus Silva, J.S.; de Souza, C.O.; Lemos, P.V.F.; de Almeida, P.F.; de Souza Ferreira, E.; Lombardi, A.T.; Druzian, J.I. Strategy for the cultivation of *Chlorella vulgaris* with high biomass production and biofuel potential in wastewater from the oil industry. *Environ. Technol. Innov.* **2022**, *25*, 102204. [CrossRef]
40. Elcik, H.; Cakmakci, M.; Ozkaya, B. Preparation and characterisation of novel polysulfone membranes modified with Pluronic F-127 for reducing microalgal fouling. *Chem. Pap.* **2017**, *71*, 1271–1290. [CrossRef]
41. Elcik, H.; Cakmakci, M.; Ozkaya, B. The fouling effects of microalgal cells on crossflow membrane filtration. *J. Membr. Sci.* **2016**, *499*, 116–125. [CrossRef]
42. Karnena, M.K.; Dwarapureddi, B.K.; Saritha, V. Alum, Chitin and Sago as coagulants for the optimization of process parameters focussing on coagulant dose and mixing speed. *Watershed Ecol. Environ.* **2022**, *4*, 112–124. [CrossRef]
43. Ma, W.; Feng, C.; Guan, F.; Ma, D.; Cai, J. Effective *Chlorella vulgaris* Biomass Harvesting through Sulfate and Chloride Flocculants. *J. Mar. Sci. Eng.* **2023**, *11*, 47. [CrossRef]
44. Loganathan, K.; Saththasivam, J.; Sarp, S. Removal of microalgae from seawater using chitosan-alum/ferric chloride dual coagulations. *Desalination* **2018**, *433*, 25–32. [CrossRef]
45. Barekati-Goudarzi, M.; Reza Mehrnia, M.; Pourasgharian Roudsari, F.; Boldor, D. Rapid separation of microalga *Chlorella vulgaris* using magnetic chitosan: Process optimization using response surface methodology. *Part. Sci. Technol.* **2016**, *34*, 165–172.
46. Tran, D.-T.; Le, B.-H.; Lee, D.-J.; Chen, C.-L.; Wang, H.-Y.; Chang, J.-S. Microalgae harvesting and subsequent biodiesel conversion. *Bioresour. Technol.* **2013**, *140*, 179–186.
47. Demir, I.; Blockx, J.; Dague, E.; Guiraud, P.; Thielemans, W.; Muylaert, K.; Formosa-Dague, C. Nanoscale evidence unravels microalgae flocculation mechanism induced by chitosan. *ACS Appl. Bio Mater.* **2020**, *3*, 8446–8459. [CrossRef]
48. Demir, I.; Besson, A.; Guiraud, P.; Formosa-Dague, C. Towards a better understanding of microalgae natural flocculation mechanisms to enhance flotation harvesting efficiency. *Water Sci. Technol.* **2020**, *82*, 1009–1024. [CrossRef]
49. Blockx, J.; Verfaillie, A.; Thielemans, W.; Muylaert, K. Unravelling the mechanism of chitosan-driven flocculation of microalgae in seawater as a function of pH. *ACS Sustain. Chem. Eng.* **2018**, *6*, 11273–11279. [CrossRef]
50. Miranda, R.; Nicu, R.; Latour, I.; Lupei, M.; Bobu, E.; Blanco, A. Efficiency of chitosans for the treatment of papermaking process water by dissolved air flotation. *Chem. Eng. J.* **2013**, *231*, 304–313.
51. Joglekar, A.; May, A. Product excellence through design of experiments. *Cereal Foods World* **1987**, *32*, 857.
52. Banerjee, A.; Sarkar, P.; Banerjee, S. Application of statistical design of experiments for optimization of As (V) biosorption by immobilized bacterial biomass. *Ecol. Eng.* **2016**, *86*, 13–23.
53. Körbahti, B.K. Response surface optimization of electrochemical treatment of textile dye wastewater. *J. Hazard. Mater.* **2007**, *145*, 277–286. [CrossRef]
54. Xu, Y.; Purton, S.; Baganz, F. Chitosan flocculation to aid the harvesting of the microalga *Chlorella sorokiniana*. *Bioresour. Technol.* **2013**, *129*, 296–301. [CrossRef] [PubMed]
55. Banerjee, C.; Ghosh, S.; Sen, G.; Mishra, S.; Shukla, P.; Bandopadhyay, R. Study of algal biomass harvesting through cationic cassia gum, a natural plant based biopolymer. *Bioresour. Technol.* **2014**, *151*, 6–11. [CrossRef] [PubMed]
56. Renault, F.; Sancey, B.; Badot, P.-M.; Crini, G. Chitosan for coagulation/flocculation processes—An eco-friendly approach. *Eur. Polym. J.* **2009**, *45*, 1337–1348.
57. El-Qanni, A.; Alsayed, M.; Alsurakji, I.H.; Najjar, M.; Odeh, D.; Najjar, S.; Hmoudah, M.; Zubair, M.; Russo, V.; Di Serio, M. A technoeconomic assessment of biological sludge dewatering using a thermal rotary dryer: A case study of design applicability, economics, and managerial feasibility. *Biomass Convers. Biorefin.* **2022**, 1–15. [CrossRef]
58. Roselet, F.; Vandamme, D.; Roselet, M.; Muylaert, K.; Abreu, P.C. Screening of commercial natural and synthetic cationic polymers for flocculation of freshwater and marine microalgae and effects of molecular weight and charge density. *Algal Res.* **2015**, *10*, 183–188.
59. Beach, E.S.; Eckelman, M.J.; Cui, Z.; Brentner, L.; Zimmerman, J.B. Preferential technological and life cycle environmental performance of chitosan flocculation for harvesting of the green algae Neochloris oleoabundans. *Bioresour. Technol.* **2012**, *121*, 445–449. [CrossRef]
60. Gupta, S.; Kumar, M.; Guldhe, A.; Ansari, F.; Rawat, I.; Kanney, K.; Bux, F. Design and development of polyamine polymer for harvesting microalgae for biofuels production. *Energy Convers. Manag.* **2014**, *85*, 537–544. [CrossRef]
61. Farooq, W.; Lee, Y.-C.; Han, J.-I.; Darpito, C.H.; Choi, M.; Yang, J.-W. Efficient microalgae harvesting by organo-building blocks of nanoclays. *Green Chem.* **2013**, *15*, 749–755. [CrossRef]
62. Lee, S.-M.; Choi, H.-J. Harvesting of microalgae species using Mg–sericite flocculant. *Bioprocess Biosyst. Eng.* **2015**, *38*, 2323–2330. [CrossRef] [PubMed]

63. Zheng, H.; Gao, Z.; Yin, J.; Tang, X.; Ji, X.; Huang, H. Harvesting of microalgae by flocculation with poly (γ-glutamic acid). *Bioresour. Technol.* **2012**, *112*, 212–220. [CrossRef] [PubMed]
64. Granados, M.; Acién, F.; Gómez, C.; Fernández-Sevilla, J.; Grima, E.M. Evaluation of flocculants for the recovery of freshwater microalgae. *Bioresour. Technol.* **2012**, *118*, 102–110. [CrossRef] [PubMed]
65. Xu, K.; Zou, X.; Mouradov, A.; Spangenberg, G.; Chang, W.; Li, Y. Efficient bioflocculation of *Chlorella vulgaris* with a chitosan and walnut protein extract. *Biology* **2021**, *10*, 352. [CrossRef] [PubMed]
66. Farid, M.S.; Shariati, A.; Badakhshan, A.; Anvaripour, B. Using nano-chitosan for harvesting microalga *Nannochloropsis* sp. *Bioresour. Technol.* **2013**, *131*, 555–559. [CrossRef] [PubMed]
67. Vandamme, D.; Foubert, I.; Fraeye, I.; Meesschaert, B.; Muylaert, K. Flocculation of *Chlorella vulgaris* induced by high pH: Role of magnesium and calcium and practical implications. *Bioresour. Technol.* **2012**, *105*, 114–119. [CrossRef]
68. Vandamme, D.; Foubert, I.; Fraeye, I.; Muylaert, K. Influence of organic matter generated by *Chlorella vulgaris* on five different modes of flocculation. *Bioresour. Technol.* **2012**, *124*, 508–511. [CrossRef]
69. Vandamme, D.; Beuckels, A.; Markou, G.; Foubert, I.; Muylaert, K. Reversible flocculation of microalgae using magnesium hydroxide. *BioEnergy Res.* **2015**, *8*, 716–725. [CrossRef]