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Chlorella vulgaris Harvesting: Chemical Flocculation with Chitosan, Aluminum Sulfate, and Ferric Sulfate

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Abstract: *Chlorella vulgaris*, a widely cultivated microalgae with diverse commercial applications, faces challenges in economic viability and scalability during the crucial biomass harvesting step. This study explores chemical flocculation followed by sedimentation as a cost-effective solution. Optimization was performed for three flocculants (chitosan, aluminum sulfate, and ferric sulfate), with experiments determining optimal pH and dosage ranges (10–200 mg·L^{-1}). A 2^4-full factorial design optimized flocculant dosage, settling time, rapid mixing time, and slow mixing time, analyzing their effects on harvesting efficiency through empirical models. The optimal dosage ranges were 50–200 mg·L^{-1} for aluminum sulfate and 150–200 mg·L^{-1} for ferric sulfate at pH 9, and 10–50 mg·L^{-1} for chitosan at pH 5. Empirical models exhibited high fitting performance (R^2 > 95%) and predictive capability (predicted R^2 > 96%). All flocculants demonstrated high efficiencies (98.4–99.5%), with inorganic types requiring fast and slow mixing phases, while chitosan achieved optimal results without the need for both mixing phases, suggesting potential industrial advantages in time and energy efficiency for microalgae harvesting.

Keywords: algal biomass; flocculant optimization; harvesting efficiency; microalgae recovery; sustainable cultivation

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1. Introduction

Microalgae are garnering growing attention for their promising role as a sustainable resource for food, bioenergy, and a range of value-added products. Antioxidants, pigments, and vitamins derived from microalgae have found commercial applications across diverse industries, showcasing their versatility and economic potential [1]. With the growing concerns about food security, climate change, and the depletion of natural resources, microalgae have emerged as a promising solution to address these challenges. Among microalgal species, *Chlorella vulgaris*, a freshwater green alga, is extensively cultivated for its economic potential. These microscopic photosynthetic organisms exhibit a rapid growth rate, a rich nutritional profile, and the ability to thrive in diverse environments, making them attractive candidates for applications ranging from biofuel production to wastewater treatment [2]. However, the harvesting of microalgae remains a significant challenge that impedes the scalability and economic viability of these applications [3]. This process, constituting a significant portion of biomass production costs (up to 30%), poses a barrier to large-scale production. The economic challenge is particularly evident in the context of low-value products like biofuels, where cost reduction is imperative for competitiveness against fossil fuels. Moreover, the fragility of microalgal cells raises concerns about potential damage during harvesting, leading to the loss of intracellular products.

Microalgal harvesting presents significant challenges due to various factors. Microalgae are typically very small (2–30 μm) and are often found at low biomass concentrations, so

they do not naturally sediment easily. Typical biomass concentrations are 0.2–6 $g \cdot L^{-1}$, with an average of approximately 0.5 $g \cdot L^{-1}$ in a standard 20 cm deep raceway pond [3,4]. The negatively charged surface of microalgal cells, the presence of algogenic organic matter (AOM), and other impurities on microalgal cultures add complexity to the harvesting process, and impede easy aggregation, resulting in stable suspensions [3,5]. The negative charge is attributed to deprotonated carboxylic and amine groups on the surface. The characteristics and quantity of AOM are influenced primarily by extracellular organic matter (EOM) produced and released during microalgal growth [6]. González-Camejo et al. [7] further highlight the impact of stress factors on EOM production in *Chlorella*-dominated cultures, underlining the importance of avoiding nutrient limitations and abrupt temperature changes.

Currently, the methods most used to harvest microalgae can be: (i) mechanical (gravity sedimentation, filtration, centrifugation, flotation); (ii) physical (electroflocculation, magnetic separation); (iii) biological (bioflocculation); or (iv) chemical (chemical flocculation). Microalgal harvesting is typically a two-stage concentration procedure that involves a thickening and a dewatering phase, though both techniques can be used separately. Initially, 2–7% of total suspended solids (TSS) are concentrated from the dilute cell suspension. The slurry is then concentrated producing a cake that contains 15–25% TSS [5]. This reduces the overall cost of the process, as the thickening stage can be completed by an affordable method (flocculation), before the expensive dewatering step (centrifugation, filtration) [8].

Optimizing the energy and financial balance of microalgal harvesting is thought to be possible using a low-cost alternative technique of chemical flocculation followed by sedimentation [8,9]. Flocculation involves the application of compounds, either inorganic or organic, exhibiting flocculant activity. These compounds play a crucial role in the formation of flocs, employing processes such as adsorption and charge neutralization, adsorption and interparticle bridging, and enmeshment in a precipitate—a phenomenon referred to as sweep flocculation [10]. The typical flocculation process consists of two stages: rapid mixing (RM) and slow mixing (SM). The RM stage, conducted at 100–300 rpm for 1–5 min, initiates suspension destabilization. Subsequently, the SM stage, performed at 20–50 rpm for 9–20 min, facilitates the growth of larger agglomerates, contributing to effective flocculation [11–14].

Metallic salts based on iron or aluminum salts are the most common type of inorganic flocculants used to harvest microalgae [12,15]. Nevertheless, these non-biodegradable flocculants contribute to secondary pollution and generate toxic sludge, resulting in costly and intricate treatment processes [16]. Research indicates that the bioaccumulation of ferric ions surpasses that of ferrous ions across various microalgae, with *Chlorella* exhibiting the highest resistance [17]. In-depth investigations by other authors focused on the detrimental impacts of ferric chloride and aluminum sulfate on microalgae, revealing that over 95% of the metals were transferred to the biomass [18]. Additionally, some studies highlight the potential harm associated with human exposure to aluminum, establishing a connection with neurodegenerative diseases, notably Alzheimer's disease [19,20]. These findings underscore the need for considering the environmental and health implications of the chosen flocculants in microalgal harvesting processes. Due to environmental problems associated with the use of chemical flocculants, natural organic flocculants have emerged as alternatives considering their greener production and application [21]. These are biodegradable, non-toxic, produce smaller volumes of sludge, require smaller dosages to work efficiently, and there is no presence of metallic residues in the microalgal biomass. However, they can be more expensive. From the organic flocculants, there has been a growing interest in applying chitosan [14,22], a linear amine-based biopolymer formed by units of D-glucosamine and N-acetyl-D-glucosamine, obtained by the deacetylation of chitin. Waste from the seafood industry is currently the main natural source of chitosan [16].

While organic polymers have been documented in some studies, little is known regarding their application in *C. vulgaris* harvesting and process optimization. The main objective of this study is to evaluate and optimize the harvesting efficiency of *C. vulgaris*

by chemical flocculation, followed by sedimentation, using chitosan, aluminum sulfate and ferric sulfate for comparison. Preliminary studies aimed to determine the optimal pH for flocculation; that is, the pH at which the formation of primary flocs was achieved with the lowest flocculant dosage. Additional studies were conducted with different flocculant dosages at the optimal pH previously determined to select the optimal dosage range for flocculation. Finally, a 2^4 full factorial design experiment was conducted to investigate the impacts of flocculant dosage, settling time, and RM and SM time on harvesting efficiency. The objective was to identify optimal conditions for flocculation. Notably, when employing chitosan as a flocculant, the inclusion of two mixing phases is unnecessary. This streamlined approach not only minimizes time and energy expenditures at an industrial scale but also enhances the efficiency of microalgae harvesting through chemical flocculation.

2. Materials and Methods

2.1. Materials

2.1.1. Microalgal Culture

The microalga *C. vulgaris* CCAP 211/11B, sourced from the Culture Collection of Algae and Protozoa (CCAP, Oban, UK), was cultivated over 7 d in a 120 L channel photobioreactor featuring LED lights (photoperiod of 24:0) within the walls (baffles) and bottom spargers at 8.75 L min^{-1} were used for CO_2 supply (atmospheric air with 0.04% CO_2) and culture mixing. The growth medium was based on the Organization for Economic Co-operation and Development (OECD) culture medium [23]: 0.500 $g{\cdot}L^{-1}$ $NaHCO_3$, 0.25 $g{\cdot}L^{-1}$ $NaNO_3$, 0.45 $g{\cdot}L^{-1}$ KH_2PO_4, 0.018 $g{\cdot}L^{-1}$ $CaCl_2{\cdot}2H_2O$, 0.015 $g{\cdot}L^{-1}$ $MgSO_4{\cdot}7H_2O$, 0.012 $g{\cdot}L^{-1}$ $MgCl_2{\cdot}6H_2O$, 0.415 $mg{\cdot}L^{-1}$ $MnCl_2{\cdot}4H_2O$, 0.1 $mg{\cdot}L^{-1}$ $Na_2EDTA{\cdot}2H_2O$, 0.08 $mg{\cdot}L^{-1}$ $FeCl_3{\cdot}6H_2O$, 0.185 $mg{\cdot}L^{-1}$ H_3BO_3, 7 $\mu g{\cdot}L^{-1}$ $Na_2MoO_4{\cdot}2H_2O$, 3 $\mu g{\cdot}L^{-1}$ $ZnCl_2$, 1.5 $\mu g{\cdot}L^{-1}$ $CoCl_2{\cdot}6H_2O$, 0.01 $\mu g{\cdot}L^{-1}$ $CuCl_2{\cdot}2H_2O$. Biomass concentration, monitored via optical density at 680 nm (OD680), was correlated with biomass dry weight (DW) concentration using a calibration curve (Equation (1)). Cultures were harvested at concentrations of approximately 340 mg_{DW} L^{-1}.

$$OD_{680} = 3.784 \times \text{Biomass concentration } (g_{DW}{\cdot}L^{-1}) - 0.0016 \quad (1)$$

(R^2 = 0.9902, Limit of detection = 0.02 $g_{DW}{\cdot}L^{-1}$, Limit of quantification = 0.07 $g_{DW}{\cdot}L^{-1}$)

2.1.2. Flocculants

Inorganic flocculants, aluminum sulfate and ferric sulfate, were provided by VWR International. Solutions at concentrations of 50 $g{\cdot}L^{-1}$ and 12 $g{\cdot}L^{-1}$, respectively, were prepared using distilled water. Chitosan powder, with a deacetylation degree $\geq$75%, was sourced from Sigma–Aldrich (St. Louis, MO, USA), and a 2 $g{\cdot}L^{-1}$ solution was created following the procedure outlined by Divakaran and Pillai [24].

2.2. Methods

2.2.1. Chemical Flocculation Experiments

Flocculation assays were performed in batch mode using Jar test equipment equipped with six stirrers and a fluorescent lamp to facilitate floc formation observation. In all experiments, 800 mL beakers containing 500 mL of microalgal culture were utilized. Preliminary assays were conducted to ascertain the optimal pH for flocculation. Flocculants were incrementally added to the 500 mL microalgal culture at pH levels of 5, 6, 7, 8, and 9 until primary flocs formed. Following each addition, the solutions were stirred at 150 rpm for 3 min (RM) and 20 rpm for 15 min (SM). Visual observation assessed floc formation. The assays, performed in duplicate, proceeded with different flocculant dosages ranging from 10 to 200 $mg{\cdot}L^{-1}$ at the determined optimal pH for each flocculant. The experiments included the RM and SM stages, followed by a 15 min sedimentation period. Harvesting efficiency (η) was calculated using Equation (2), with absorbance measured at 680 nm. This wavelength was selected through wavelength scanning, indicating maximum absorbance at this value.

OD_i corresponds to the optical density of the microalgal culture before any flocculant addition, and OD_f is the optical density after the settling period. Samples for OD_f determination were taken from the supernatant approximately 2 cm below the surface. Absorbance was measured using a UV-vis spectrophotometer (UV-6300PC double beam, VWR).

$$\eta(\%) = \frac{OD_i - OD_f}{OD_i} \times 100 \quad (2)$$

2.2.2. Factorial Design of Experiments

A 2^4-full factorial experimental design was executed to assess the impacts of flocculant dosage, settling time, RM time, and SM time on harvesting efficiency. This type of design allows the identification of important factors to focus on with further experimentation. Each factor underwent testing at low (−1) and high (+1) levels in duplicate. Table 1 displays the coded values for each run, while Table 2 presents the actual values for each flocculant. A four-way ANOVA (p-value < 0.05) was conducted, leading to the establishment of empirical models characterizing flocculation for each flocculant. These models were subsequently employed to analyze the variables' effects on harvesting efficiency and predict optimal flocculation conditions. Statistical analysis and model construction utilized Minitab® Statistical Software 21.1.0.0. The optimal conditions determined by the models were then validated through experimental testing.

Table 1. Organization of the 2^4-full factorial experiments, with the coded values of each factor (dosage, settling time, rapid mixing (RM) time, and slow mixing (SM) time).

Run	Dosage ($mg \cdot L^{-1}$)	Settling Time	RM Time (min)	SM Time (min)
1	−1	−1	−1	−1
2	+1	−1	−1	−1
3	−1	+1	−1	−1
4	+1	+1	−1	−1
5	−1	−1	+1	−1
6	+1	−1	+1	−1
7	−1	+1	+1	−1
8	+1	+1	+1	−1
9	−1	−1	−1	+1
10	+1	−1	−1	+1
11	−1	+1	−1	+1
12	+1	+1	−1	+1
13	−1	−1	+1	+1
14	+1	−1	+1	+1
15	−1	+1	+1	+1
16	+1	+1	+1	+1

Table 2. Real values of the experimental factors (dosage, settling time, rapid mixing (RM) time, and slow mixing (SM) time) for each flocculant studied to harvest *C. vulgaris*.

Flocculant	Coded Value	Dosage ($mg \cdot L^{-1}$)	Settling Time	RM Time (min)	SM Time (min)
Aluminum sulfate	−1	50	5	0	0
	+1	200	15	3	15
Ferric sulfate	−1	150	5	0	0
	+1	200	15	3	15
Chitosan	−1	10	5	0	0
	+1	50	15	3	15

3. Results and Discussion

3.1. Optimal pH Determination

Preliminary studies aimed to determine the optimal pH for flocculation; that is, the pH at which the formation of primary flocs was achieved with the lowest flocculant dosage. The results are in Table 3. It was also intended to select the optimal pH for subsequent optimization tests. The optimal pH corresponded to the pH at which the minimum flocculant dosage was required to start the formation of primary flocs.

Table 3. Optimal pH for flocculation obtained for aluminum sulfate, ferric sulfate, and chitosan.

Flocculant	Initial Biomass Concentration ($mg_{DW} \cdot L^{-1}$)	Optimal pH
Aluminum sulfate	342 ± 9	9
Ferric sulfate	342 ± 9	9
Chitosan	339 ± 5	5

The optimal pH for the inorganic flocculants (aluminum sulfate and ferric sulfate) was determined to be 9. In another study, close to 100% biomass recovery efficiencies were achieved with ferrous sulfate at pH 8.47 [25]. Surendhiran and Vijay [26] also obtained higher harvesting efficiency of *Chlorella* sp. with aluminum sulfate and ferric sulfate at a basic pH. At pH 9, some charge neutralization can occur; however, this is not the primary mechanism involved in flocculation, especially since negatively charged forms of alum and iron predominate [15]. Still, optimum flocculation is expected at this pH because of the main mechanism of sweep flocculation. Aluminum and iron ions form aluminum hydroxide and ferric hydroxide precipitates. They can also bind with EOM and phosphate ions (present in the culture medium as KH_2PO_4), forming other types of precipitates. These precipitates entrap microalgal cells and allow floc formation.

Organic flocculant chitosan performed better at an acid pH, just as reported in other studies [27,28]. The mechanisms involved in the flocculation process are charge neutralization, intercell bridging, and sweep flocculation. This flocculant is a cationic polymer with high charge density at the optimal pH determined; therefore, a strong attraction between the cells and the flocculant is expected [27]. Since the pH of *C. vulgaris* cultures is normally basic, the optimal pH of 5 means that, when chitosan is used, more acid solution has to be added to the culture medium.

3.2. Optimal Dosage Range Determination

Flocculant dosages from 10 to 200 $mg \cdot L^{-1}$, at the optimal pH previously determined (Table 3), were tested to select an optimal dosage range for flocculation. The harvesting efficiencies obtained with each flocculant are presented in Table 4, and the appearance of the culture medium after flocculation and sedimentation can be observed in Figure 1.

Inorganic flocculants were very effective, with harvesting efficiencies varying from 89.8 to 96.4% and 96.7 to 97.7% for aluminum sulfate and ferric sulfate, respectively. Chitosan reached efficiencies from 91.2 to 99.3%, also proving to be capable of harvesting *C. vulgaris*. An interesting effect was observed with chitosan, especially at 50 $mg \cdot L^{-1}$. Some biomass floated instead of sediment, and gas bubbles were visible in the flocs. As the bubbles burst, the flocs quickly settled. The same phenomenon was reported by Rashid et al. [29] at chitosan dosages of 60–90 $mg \cdot L^{-1}$. According to the authors, chitosan molecules establish bonds with negatively charged cells, but others experience repulsion against themselves. As chitosan molecules have long chains, large flocs can be formed, but destabilization makes them less dense. Gas bubbles were most likely formed from the dissolved air in the medium [27]. Few flocs floated in the remaining concentrations (10, 100, 150, and 200 $mg \cdot L^{-1}$). At 10 $mg \cdot L^{-1}$, it is possible that the available binding sites of chitosan were all occupied with microalgal cells, minimizing the repulsion. In the other concentrations, Rashid et al. [29] suggested that the solution concentration increased, pushing flocs downward; thus, almost no flotation was observed.

Table 4. Harvesting efficiency of aluminum sulfate, ferric sulfate, and chitosan at 10–200 mg·L^{-1}.

Flocculant	pH	Initial Biomass Concentration ($mg_{DW}·L^{-1}$)	Dosage ($mg·L^{-1}$)	Final Biomass Concentration ($mg_{DW}·L^{-1}$)	Harvesting Efficiency (%)
Aluminum sulfate	9	335 ± 1	10	34 ± 4	89.8 ± 0.2
			50	12 ± 3	96.4 ± 0.1
			100	13 ± 3	96.2 ± 0.1
			150	14 ± 2	95.9 ± 0.1
			200	12 ± 1	96.4 ± 0.1
Ferric sulfate	9	335 ± 2	10	10 ± 1	96.9 ± 0.2
			50	10 ± 1	97.0 ± 0.1
			100	11 ± 2	96.7 ± 0.1
			150	9 ± 1	97.4 ± 0.1
			200	8 ± 1	97.7 ± 0.1
Chitosan	5	335 ± 2	10	2 ± 1	99.3 ± 0.2
			50	5 ± 3	98.4 ± 0.1
			100	13 ± 3	96.1 ± 0.1
			150	22 ± 8	93.2 ± 0.3
			200	29 ± 4	91.2 ± 0.1

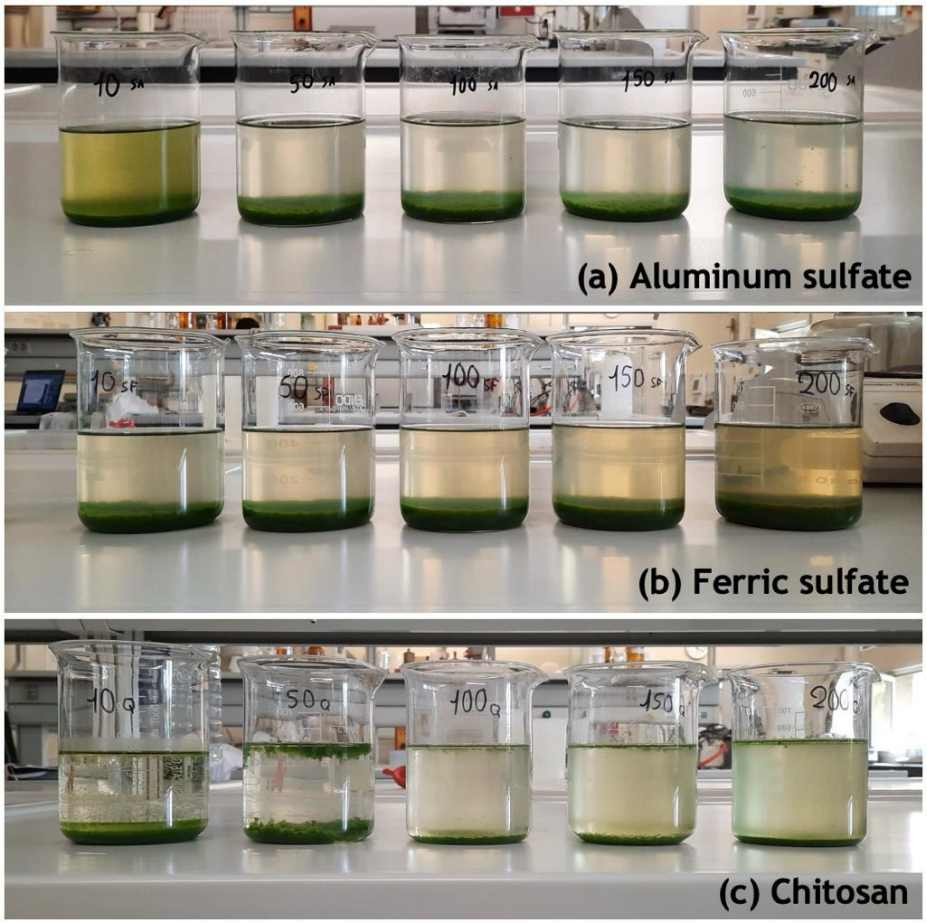

Figure 1. Appearance of the culture medium after flocculation and sedimentation with: (**a**) aluminum sulfate; (**b**) ferric sulfate; and (**c**) chitosan. Flocculant dosages from left to right: 10, 50, 100, 150, and 200 mg·L^{-1}.

Overall, high harvesting efficiencies were achieved with both inorganic and organic flocculants. However, the dosage required to achieve those efficiencies varied. The optimal dosage ranges for each flocculant are compiled in Table 5. Inorganic flocculants which

primarily act by sweep flocculation at pH 9 needed higher dosages to achieve the highest biomass recovery. The optimal dosage ranges were selected to be 50–200 mg·L^{-1} for aluminum sulfate and 150–200 mg·L^{-1} for ferric sulfate. Chitosan performed better at lower dosages. In fact, from 50 mg·L^{-1} of chitosan, the harvesting efficiency decreased. The elevated concentration of flocculant, creating an excessive positive charge around negatively charged cells, might have impeded cell aggregation, leading to the destabilization of microalgal cells due to electrostatic repulsion [30]. This means that there was an excess of chitosan molecules in the medium that did not interact with the microalgae cells and created repulsion forces. Under the conditions studied, the optimal dosage range was 10–50 mg·L^{-1} for chitosan.

Table 5. Optimal dosage range for flocculation with aluminum sulfate, ferric sulfate, and chitosan.

Flocculant	Optimal Dosage Range (mg·L^{-1})
Aluminum sulfate	50–200
Ferric sulfate	150–200
Chitosan	10–50

3.3. Optimization of Microalgal Harvesting Variables: Dosage, Settling Time, and Mixing Times

The efficiency of microalgal harvesting (response variable) obtained for the 2^4 full factorial experimental design are in Table A1 (Appendix A). The results were analyzed by ANOVA (p-value < 0.05) to quantify statistical differences among the variables. The main effects and interaction analysis between the factors (dosage, settling time, RM time, and SM time) are in Table A2 (Appendix A). The effects describe the size and direction of the relationship between a factor and the response variable; that is, they represent the predicted change in the mean response when the factor changes from the low to the high level [31]. The signs "+" and "−" of the effects indicate if the factor positively or negatively influences the harvesting efficiency, respectively. For instance, increasing the aluminum sulfate dosage from 50 to 200 mg·L^{-1} positively affected efficiency. According to the p-values, settling time was statistically not significant (p-value > 0.05) for aluminum sulfate and chitosan. The effect of dosage was also statistically not significant (p-value > 0.05) for chitosan and ferric sulfate.

The empirical models for each flocculant are in Table 6. These models describe how the different factors and their main interactions affect the harvesting efficiency. They were obtained by eliminating the not statistically significant parameters and the ones with coefficients close to zero.

The high R^2 values of each model indicate that the models appropriately fitted the data. However, it does not necessarily mean that the models predict new observations well. That information is given by the predicted R^2 values, which are close to 100%. The predicted R^2 is derived by systematically excluding each observation from the dataset, estimating the regression equation, and assessing how effectively the model predicts the omitted observation [32].

To improve the flocculation process, the regression equations were used to predict the values that the factors should have to obtain the maximum harvesting efficiency. These optimal parameters were tested to harvest *C. vulgaris*, and the results are in Table 7. All flocculants showed excellent biomass recoveries. The experimental data were very close to the predicted data, showing once again the ability of the models to predict new observations.

Table 6. Empirical models that relate the harvesting efficiency (η) with the flocculant dosage (A), settling time (B), rapid mixing time (C), and slow mixing time (D), and their main interactions.

Flocculant	Regression Equation	R^2 (%)	Predicted R^2 (%)
Aluminum sulfate	$\eta\ (\%) = -8.77 + 0.0520 \times A + 32.4 \times C + 5.86 \times D - 1.99 \times C \times D$	96.56	95.99
Ferric sulfate	$\eta\ (\%) = -0.766 + 0.105 \times B + 31.0 \times C + 6.55 \times D + 0.0113 \times B \times C - 2.11 \times C \times D$	97.00	97.00
Chitosan	$\eta\ (\%) = -0.459 + 31.6 \times C + 6.61 \times D + 0.0246 \times A \times C - 2.15 \times C \times D$	96.98	96.95

Table 7. Experimental results of *C. vulgaris* flocculation using aluminum sulfate, ferric sulfate, and chitosan with the optimal predicted parameters (the mean values of the not statistically significant (NSS) parameters were selected to determine the real harvesting efficiencies).

Flocculant	Optimal Predicted Parameters				Predicted Harvesting Efficiency (%)	Experimental Harvesting Efficiency (%)	Error (%)
	Dosage ($mg \cdot L^{-1}$)	Settling Time (min)	RM Time (min)	SM Time (min)			
Aluminum sulfate	250	NSS (5–15)	3	15	99.8	99.1 ± 0.1	0.7
Ferric sulfate	NSS (150–200)	30	3	15	99.8	98.4 ± 0.1	1.4
Chitosan	NSS (10–50)	NSS (5–15)	0	15.5	100	99.5 ± 0.1	0.5

Surendhiran and Vijay [26] reported harvesting efficiencies of 87.33% with 600 $mg \cdot L^{-1}$ ferric sulfate and 82.27% with 800 $mg \cdot L^{-1}$ of aluminum sulfate. The authors used higher dosages and only performed an RM stage. In the present study, lower flocculant dosages (150–250 $mg \cdot L^{-1}$) combined with two mixing stages allowed the achievement of better efficiencies. Gani et al. [30] also included two mixing stages and obtained biomass recoveries of 95% with only 100 $mg \cdot L^{-1}$ of aluminum sulfate. The mentioned articles studied different species of microalgae at different concentrations; therefore, the comparison is not straightforward. Nevertheless, it is still possible to perceive that the mixing stages significantly impact the flocculation process. Inorganic flocculants were most efficient when an RM and an SM phase were included.

On the contrary, chitosan reached a maximum efficiency of 99.5% with just 15.5 min of SM. When a fast mixing of the culture was provided, higher dosages of chitosan (200 $mg \cdot L^{-1}$) were needed to harvest *C. vulgaris* [22]. Koley et al. [33] used a concentration of 20 $mg \cdot L^{-1}$ and two mixing stages, but only reported 90% of flocculation efficiency. Chitosan is less charged, so the excessive fast mixing breaks the flocs. The SM stage ensures homogenization without causing floc breakage.

Table 8 contains information about the market price of each flocculant and the price of flocculant required to harvest 100 m^3 of *C. vulgaris* culture. Although chitosan is the most expensive flocculant per gram, the optimal dosage is quite low, making this flocculant the most economically viable, when comparing with aluminum and ferric sulfate. In addition, when employing chitosan as a flocculant, incorporating two mixing phases is unnecessary, potentially reducing time and energy expenses at an industrial scale, thereby facilitating the microalgae harvesting process through chemical flocculation.

Table 8. Economic analysis of the price of flocculant needed to harvest 100 m^3 of *C. vulgaris* culture.

Flocculant	Dosage ($mg \cdot L^{-1}$)	Price (€ g^{-1})	Price of Flocculant (€) to Harvest 100 m^3 of *C. vulgaris* Culture	Reference
Aluminum sulfate	250	0.20	4905	[34]
Ferric sulfate	150	0.29	4350	[35]
Chitosan	10	1.86	1856	[36]

4. Conclusions

C. vulgaris harvesting by chemical flocculation, followed by sedimentation, was successfully optimized in this work. From the preliminary assays, it was concluded that the optimal pH for flocculation with the inorganic flocculants was 9. In contrast, chitosan worked better at an acidic pH, due to their high positive charge density that neutralized the microalgal cells. Overall, both inorganic and organic flocculants were effective, reaching harvesting efficiencies from 89.8 to 96.4% (aluminum sulfate), 96.7 to 97.7% (ferric sulfate), and 91.2 to 99.3% (chitosan), with dosages from 50 to 200 $mg \cdot L^{-1}$. The rapid and slow mixing phases were found to significantly impact the flocculation process. Inorganic flocculants required both phases to reach maximum harvesting efficiencies. When chitosan was used as a flocculant, excessive fast mixing broke the flocs; therefore, a single slow mixing phase was preferable. All flocculants showed excellent biomass recoveries (98.4–99.5%) at the optimal determined conditions. The experimental data were very close to the predicted data, showing that the models could predict new observations.

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Appendix A

Table A1. Harvesting efficiency results obtained in the 2^4 full factorial design experiment for aluminum sulfate, ferric sulfate, and chitosan.

Flocculant	Run	Dosage ($mg \cdot L^{-1}$)	Settling Time (min)	RM Time (min)	SM Time (min)	Harvesting Efficiency (%)
	1	50	5	0	0	0.0 ± 0.1
	2	200	5	0	0	0.0 ± 0.1

Table A1. *Cont.*

Flocculant	Run	Dosage ($mg \cdot L^{-1}$)	Settling Time (min)	RM Time (min)	SM Time (min)	Harvesting Efficiency (%)
Aluminum sulfate	3	50	15	0	0	0.0 ± 0.1
	4	200	15	0	0	0.0 ± 0.1
	5	50	5	3	0	89.6 ± 0.4
	6	200	5	3	0	96.8 ± 0.1
	7	50	15	3	0	90.5 ± 0.4
	8	200	15	3	0	97.0 ± 0.1
	9	50	5	0	15	80.6 ± 1.2
	10	200	5	0	15	97.4 ± 1.2
	11	50	15	0	15	98.2 ± 0.4
	12	200	15	0	15	97.2 ± 1.2
	13	50	5	3	15	96.4 ± 0.8
	14	200	5	3	15	98.5 ± 0.4
	15	50	15	3	15	96.6 ± 0.8
	16	200	15	3	15	98.5 ± 0.5
Ferric sulfate	1	150	5	0	0	0.0 ± 0.1
	2	200	5	0	0	0.0 ± 0.1
	3	150	15	0	0	0.0 ± 0.1
	4	200	15	0	0	0.0 ± 0.1
	5	150	5	3	0	96.1 ± 0.1
	6	200	5	3	0	97.5 ± 0.4
	7	150	15	3	0	97.2 ± 0.1
	8	200	15	3	0	97.7 ± 0.5
	9	150	5	0	15	95.6 ± 0.4
	10	200	5	0	15	94.8 ± 0.4
	11	150	15	0	15	95.8 ± 0.1
	12	200	15	0	15	94.8 ± 0.6
	13	150	5	3	15	97.4 ± 0.9
	14	200	5	3	15	97.6 ± 0.1
	15	150	15	3	15	97.4 ± 0.4
	16	200	15	3	15	97.8 ± 0.1
Chitosan	1	10	5	0	0	0.0 ± 0.1
	2	50	5	0	0	0.0 ± 0.1
	3	10	15	0	0	0.0 ± 0.1
	4	50	15	0	0	0.0 ± 0.1
	5	10	5	3	0	95.6 ± 1.4
	6	50	5	3	0	98.7 ± 2.1
	7	10	15	3	0	98.7 ± 0.9
	8	50	15	3	0	98.1 ± 1.3
	9	10	5	0	15	99.1 ± 0.1
	10	50	5	0	15	95.6 ± 0.9
	11	10	15	0	15	99.4 ± 0.3
	12	50	15	0	15	95.7 ± 0.2
	13	10	5	3	15	97.7 ± 0.4
	14	50	5	3	15	99.0 ± 0.4
	15	10	15	3	15	98.4 ± 1.3
	16	50	15	3	15	99.1 ± 0.1

Table A2. Effects and factor interaction analysis for *C. vulgaris* flocculation with aluminum sulfate, ferric sulfate, and chitosan.

		Dosage (A)	Settling Time (B)	RM Time (C)	SM Time (D)	A × B	A × C	A × D	B × C	B × D	C × D
Aluminum sulfate	Effect	4.181	2.356	48.806	48.681	−2.319	0.256	0.756	−2.019	2.106	−44.669
	p-value	0.002	0.061	0.000	0.000	0.064	0.831	0.531	0.104	0.091	0.000
Ferric sulfate	Effect	0.069	0.206	49.719	47.831	−0.131	0.556	−0.406	0.169	−0.094	−47.406
	p-value	0.264	0.002	0.000	0.000	0.040	0.000	0.000	0.010	0.133	0.000
Chitosan	Effect	−0.337	0.463	49.450	49.100	−0.562	1.475	−0.950	0.350	−0.150	−48.337
	p-value	0.214	0.093	0.000	0.000	0.044	0.000	0.002	0.198	0.575	0.000

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