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Scenedesmus sp. Harvesting by Using Natural Coagulant after Phycoremediation of Heavy Metals in Different Concentrations of Wet Market Wastewater for Potential Fish Feeds

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Abstract: The high level of organic elements and nutrients in wet market wastewater (WMW) has raised public concerns. The phycoremediation method, which utilizes microalgae, can be further valorized by converting it into various valuable potential bioproducts. The production of *Scenedesmus* sp. in WMW was investigated as an ingredient for fish feeds in this study. The potential of two natural plant-based coagulants, *Cajanus cajan* (pigeon pea) and *Cicer arietinum* (chickpea), for harvesting microalgae *Scenedesmus* sp. were compared. Statistical analysis and response surface methodology were used to investigate the flocculant dosage and pH effect on harvesting efficiency. It was found that *Cajanus cajan* (CC) and *Cicer arietinum* (CA) both had a harvesting efficiency of 89.29% and 88.56%, respectively. The optimal dosage and pH for CC were 178.75 mg/L and 11.72, and for CA, they were 137.77 mg/L and 9.15. This study indicated that *Scenedesmus* sp. can remove heavy metals cadmium (87.24%), chromium (85.55%), and ferum (90.35%), respectively. The level of heavy metals content (µg/kg in ppb) in dry biomass was found ultimately low and did not exceed the maximum concentration set up by the European Commission Regulation. The Fourier Transform Infrared (FTIR) analysis of microalgae biomass displayed O-H, N-H and C-H functional groups. The protein–lipid for the potential application as fish feed in the sample was 45.8–43.6% and 15–13%. Moreover, the biomass contained 53% to 40% oleic acid, which is high concentration of fatty acid methyl ester (FAME). As a result, there is high potential of *Scenedesmus* sp. in wastewater treatment; both natural coagulants give the possibilities for efficient microalgae biomass recovery as fish feed and are applicable for improving the quality of *Scenedesmus* sp. cultivated in WMW.

Keywords: microalgae; natural coagulants; harvesting; response surface methodology; flocculation

1. Introduction

Microalgae have been widely commercialized because of their substantial carbohydrate, lipid, protein content, and significant environmental targeted metabolites [1,2]. Microalgae also have a well-established use in aquaculture hatcheries as animal feed supplements due to their natural source of pigments, antioxidants, and other bioactive compounds with functional properties added to their basic nutritional value [3]. Microalgae have the potential to become a substitute for fish feed due to their ease of cultivation and faster growth rates than terrestrial crops. It has been demonstrated that dried biomass of *Scenedesmus* sp., *Spirulina* spp., *Chlorella vulgaris*, *Isochryris* sp., and *Chlorella* spp. is a substantial source of protein for omnivorous and carnivorous freshwater prawns and fish during the postlarvae stage [4–7]. The current work is motivated by the need for dietary alternatives to a fish meal or supplement diets for aquaculture. In this work, microalgae are used to evaluate the value of *Scenedesmus* sp. biomass following heavy metal bioaccumulation from wet market wastewater (WMW). In addition, many researchers produced microalgae biomass by using wastewater as a production medium to maintain our environmental diversity and reduce the cost of aquafeeds, nonconventional sources of protein, which are now high in demand [8]. Moreover, *Scenedesmus* sp. used in the present study was locally collected and has been known to have high metabolic pathways and to be tolerant with the local climate.

Wastewater contaminants may lead to environmental pollution and a threat to us in the future. Rapid industrialization and urbanization are the primary causes of environmental pollution, particularly in rivers and lakes, where water is primarily derived from untreated wastewater that can produce heavy metal pollution [9]. WMW has been linked to severe dangers to human health due to pathogenic bacteria or triggering allergic reactions. When cattle, poultry, and fisheries operations come into direct contact with humans, the danger to the environment increases. WMW is also routinely dumped into the environment without being treated, which has a harmful influence on aquaculture environments as well as on human health. Lead (Pb), zinc (Zn), cadmium (Cd), mercury (Hg), and chromium (Cr) are the most commonly found heavy metals in various types of wastewaters. All of these metals are harmful to aquatic life and human where water and seafood are the aquatic ecosystem food chain. The discharge of heavy metals from wastewater into aquatic ecosystems such as rivers, lakes, and seas may cause fish and vegetables to absorb pollutants due to their high solubility in aquatic conditions [10]. Heavy metals are not biodegradable and can linger in the body for an extended amount of time after they have been consumed. For instance, an excessive Zn may result in health issues such as skin irritations, vomiting, and stomach pains [10,11].

There are numerous conventional methods to remove heavy metals such as membrane technology, chemical precipitation, and electrochemical deposition. However, the majority of these technologies require a significant amount of reagent and energy but have limited tolerance to pH changes [12,13]. Green approaches by biotechnology methods such as phycoremediation are needed to overcome the limitation of chemical usage; thus, these methods are effective, inexpensive, and can reduce the heavy metal concentration to abide by the lowest environmental standards [14]. The ability of microalgae species to adapt phosphorus and nitrogen from wastewater has increased the production of valuable microalgae biomass [15]. Many countries have implemented guidelines for their disposal to protect the environment from polluted wastewater and maintain proper wet market facilities. A number of well-known issues could be solved by combining WMW with the phycoremediation process, including a reduction in the amount of chemicals and freshwater required for cultured nutrients. Rather than discarding nutrients, WMW might be used to feed microalgae, resulting in a complete loop recycling system. Sustainability and environmentally friendly are the advantages of phycoremediation using algae to eliminate pollution in wastewater. Compared to conventional treatment methods, the phycoremediation process consumes more CO_2 and produces more O_2 during photosynthesis, resulting in fewer greenhouse gases [16]. Therefore, microalgae biotechnology is an effective technique

that can remove nutrients or pollutants and improve the management of natural resources with great bioproduct potential.

Microalgae biomass can be harvested in various ways, including flocculation, sedimentation, flotation, centrifugation, or a combination of these approaches. The cost-effectiveness of meeting commercial or industrial demands is primarily determined by the type of harvesting used [17]. It is still a big challenge to extract microalgae from microalgae-based wastewater treatment, making it difficult to separate algae from water since the size of algae is too small [18,19]. The size of microalgae cells, species of microalgae cells, cell density, and culture conditions are the most important factors to consider when selecting an effective harvesting process [20]. One of the most successful strategies for separating algae particles from suspension is flocculation with natural coagulants. Alternatively, natural coagulants derived from plants such as *Cicer arietinum*, *Cajanus cajan*, okra, red bean, sugar cane, corn, *Cactus latifera*, and *Moringa oleifera* have been studied for application in water treatment [21–23]. Natural coagulants have several advantages over chemical coagulants, including ease of implementation and inexpensive raw material costs. According to Hamid et al. [24], bioflocculation using *Moringa oleifera* is 95% efficient for extracting *Chlorella* sp. The application of this coagulant-flocculation technology can improve the protein composition of harvested microalgae as well as the protein extraction process. Natural coagulant flocculation is also cost-effective and environmentally friendly [19]. In order to maximize production, the harvesting process should achieve a desirable quality while keeping operational and maintenance costs low.

In algae biotechnology, response surface methodology (RSM) has been successfully utilized to optimize a range of parameters and processes. The RSM method was used for factor prediction in order to construct a geometrical model and individually analyze each factor and its interaction with the proposed model. This was proven by the highly significant statistical analysis achieved through the use of RSM. This method was chosen because it could give bulk harvesting of biomass for bioproduct application. Microalgae biomass recovery required undivided focus due to its size, density, application, and value product. Flocculants are considered effective when it is inexpensive, nontoxic, efficient, and recyclable [20]. Microalgae *Scenedesmus* sp. in this study was intended to be a fish feed replacement; thus, the requirement of using an edible organic coagulant was important. This study investigated the effect of flocculation dosage and pH on biomass recovery of suspended microalgae *Scenedesmus* sp. with *Cicer arietinum* and *Cajanus cajan* powder derivatives after WMW phycoremediation of heavy metals in a laboratory setting. Hence, the usage of both coagulants might enhance the quality of the harvested biomass and would have a value biomass production in agro feedstocks and the medical industry. These coagulants were considered for microalgae harvesting due to the possibility of the desired product becoming fish feed potential or another value-added bioproduct. This is the first time both natural coagulants are evaluated on their efficiency toward microalgae biomass recovery from phycoremediation of WMW. The potential usefulness of microalgae biomass composition analysis on downstream processing for fish feed potential is suggested accordingly.

2. Materials and Methods

2.1. Wet Market Wastewater Sampling

The wastewater sample for this study was taken from a wet market in the southern part of Peninsular Malaysia that generally contains all the scraps from seafood, poultry, and meat slaughtering, which includes animal entrails and blood. It was collected using acid-washed bottles that were straightaway stored in a chiller at temperatures below 4 °C in a laboratory. A 0.45 um pore membrane filter (Whatman) was used to filter the WMW sample in order to remove any suspended solids and other microorganisms. Table 1 shows the chemical and physical analyses of water sampling.

Table 1. Raw wastewater samples from the wet market.

Parameter	Unit	Value
Turbidity	NTU	112
pH	-	6.8
Chemical oxygen demand	(mg/L)	3506
Biochemical oxygen demand	(mg/L)	1784
Dissolved oxygen	(mg/L)	3.72
Total suspended solid	(mg/L)	225
Ferum, Fe	(ppb)	5050
Cadmium, Cd	(ppb)	17
Chromium, Cr	(ppb)	194

2.2. Microalgae Scenedesmus sp. Preparation

This study used tropical rainforest freshwater microalgae from the southern part of Malaysia: *Scenedesmus* sp. It was identified in a previous study as detailed by Apandi et al. [21]. Microalgae *Scenedesmus* sp. were prepared in Bold Basal Medium (BBM) as an inoculum solution.

2.3. Phycoremediation Experiments

The current study was an ongoing experiment of phycoremediation for heavy metal removal based on Apandi et al. [21]. WMW was cultivated with five different concentrations in 100 mL Erlenmeyer flasks. Deionized water was added to dilute the working volume of the culture broth in 10, 25, 50, 75, and 100% of WMW. The initial microalgae concentrations in each WMW ratio were calculated using a hemocytometer with four different initial counts of 10^4, 10^5, 10^6, and 10^7 cells/mL. In a control group, medium broth without microalgae was used to compare the elimination of heavy metals among cultivated microalgae and the medium itself. The phycoremediation experiment was carried out for 18 days under outdoor sunlight with the light intensity ranged from 2.7 to 244 μmol/m^2/s and temperature range of 28 and 39 °C, The experiment flasks were shaken twice daily for homogenization. A set volume of 10 mL was taken out under sterilized conditions and filtered through a 0.45 um pore size filter before adding deionized water to a final volume of 50 mL. The heavy metal concentration was investigated using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The removal efficiency was then calculated using Equation (1), where *Ci* is the initial concentration and *Ce* is the final concentration.

$$\text{Heavy metals removal, } \% = \frac{Ci - Ce}{Ci} \times 100 \quad (1)$$

2.4. Statistical Analysis

One-way analysis of variance (ANOVA) was used to examine the heavy metal removal data, and the Tukey test was utilized to determine if there were any statistically significant changes between treatments ($p < 0.05$).

2.5. Microalgae Harvesting by Centrifugation

Scenedesmus sp. cultivated on WMW was extracted by centrifugation for 5 min at 3500 rpm. The biomass quality of centrifuged microalgae biomass was compared to that of natural coagulant-flocculated microalgae biomass harvested by *Cicer arietinum*; CA (chickpea) and *Cajanus cajan*; CC (pigeon pea).

2.6. Flocculation Procedure Optimization

After the last day of phycoremediation, the flocculation operation was carried out. *Cicer arietinum* (chickpea) and *Cajanus cajan* (pigeon pea) were utilized as coagulants in this investigation. CA and CC solutions were created independently for coagulant solution production and ground into a powder by size passing through a 0.4 mm sieve.

The optimization of the flocculation of microalgae was investigated using the Janke–Kunkel jar test. In this study, the response surface methodology (RSM) was used to investigate the flocculation method. The requirement of using an edible organic coagulant was important for microalgae harvesting in the present study due to the desired product being intended to be a fish feed replacement. It took approximately 30 g of both powders to completely dissolve in 1000 mL of distilled water with the aid of a magnetic stirrer, which was used to ensure thorough dissolution. When tested in a 1 L experiment beaker, each 1 mL stock solution yielded 30 mg/L. Table 2 shows that the sample was added at various coagulant doses and pH (using 1 M HCL and NaOH) in accordance with the range and level.

Table 2. Factors of experimentation and their levels of setup.

Variables	Range and Level		
	−1 (Min)	**0 (Medium)**	**+1 (Max)**
pH	5	8.5	12
Coagulant dosage (mg/L)	30	105	180

The tested beaker that contains the material with coagulant was rapidly swirled for 30 min at the speed of 80 rpm [22]. After 20 min of rest, the sample was pipetted out at a height of 2/3 from the bottom of the beaker and for 10 mL at OD 650 nm. Equation (2) below was used to calculate the harvesting efficiency. OD after flocculation is represented by A, and B represents OD before flocculation.

$$\text{Harvesting efficiency percentage} = (1 - \text{A/B}) \times 100 \quad (2)$$

The statistical design and data analysis for flocculation efficiency were performed in the Design Expert V7.0 software, a face-centered central composite design (FCCCD) utilizing the response surface method (RSM). The purpose of this design was to determine the link between the variables coded in Equation (3), where *i*th is the independent variable represented by *Xi*, the studied area's point is represented by *Xo*, and the step change value is δx.

$$Xi = \frac{Xi - Xo}{\delta x} \quad (3)$$

A quadratic equation was used to assess the significance of the components' effect. Hence, this study used a second-order model of the quadratic polynomial equation to fit the response. The interaction effects of the independent variable on the dependent variable were then visualized using 3D response surface and contour plots. The experiment was repeated more than two times to ensure the statistical analysis validity. The percentage of harvesting efficiency is shown in Table 3. There are two actual factors values (pH and coagulant dosage) with a total of 13 experiment runs for each biocoagulant (*Cajanus cajan* and *Cicer arietinum*).

2.7. Microalgae Biomass as Fish Feeds Potential

The total carbohydrates, lipids, protein, and heavy metals content of microalgae biomass was determined after natural coagulant (CA and CC) flocculation harvesting. After the 18th day of the phycoremediation experiment, biomass was characterized using GC–MS and FTIR.

2.7.1. Heavy Metals Content in Dry Biomass

Microalgae biomass was dried at 105 °C, roughly crushed, and homogenized to its constant weight. In 10 mL HNO_3 (nitric acid) solution, a homogenized sample of 0.5 g was digested at 160 °C. The Inductively Coupled Plasma Mass Spectrometry (ICP-S) was used to test the solution for heavy metal concentrations after being diluted to 50 mL with deionized water for ferum (Fe), chromium (Cr), cadmium (Cd), and arsenic (As), and a Mercury Analyzer was used to test for mercury (Hg).

Table 3. *Scenedesmus* sp. factor and response values in wet market wastewater.

Run	Actual Factor Values		Response Values (Harvesting Efficiency)			
			Cajanus cajan (CC)		*Cicer arietinum* (CA)	
	pH	Coagulant Dosage (mg/L)	Actual (Experiment)	Predicted (RSM Model)	Actual (Experiment)	Predicted (RSM Model)
1	5	30	44.56	45.22	34.78	34.52
2	12	30	70.21	69.54	45	44.72
3	5	180	72.33	73.99	56.9	58.19
4	12	180	88.9	89.23	71.23	72.5
5	5	105	65.21	62.89	62.33	61.3
6	12	105	82.33	82.67	74.54	73.55
7	8.5	30	62.11	62.12	56.83	57.37
8	8.5	180	88.34	86.35	85.65	83.09
9	8.5	105	76.77	77.52	83.43	85.18
10	8.5	105	78.2	77.52	80.23	85.18
11	8.5	105	75.43	77.52	82.21	85.18
12	8.5	105	77.21	77.52	85.69	85.18
13	8.5	105	78.2	77.52	92.33	85.18

2.7.2. Total Carbohydrate, Lipid and Protein

A 6 mL chloroform:methanol (2:1) was used to extract and weigh 50 mg of dried microalgae biomass. As detailed by Yaakob et al. [23], the sample was carried out accordingly to determine the total carbohydrate and protein. In addition, the content of lipid was identified using modified methods from Bligh and Dyer [24] and Folch et al. [25].

2.7.3. FTIR and GC–MS Analysis of Microalgae *Scenedesmus* sp.

A Perkin Elmer Spectrum 100 FTIR spectrometer operating in the wavelength range 600–4000 cm^{-1} was used to record the functional groups of Fourier transform infrared (FTIR) for microalgae biomass. The analyses of gas chromatography–mass spectrometry (GC–MS) was carried out the same as in Apandi et al.'s [26] work.

3. Results and Discussion

3.1. Heavy Metals Removal by Phycoremediation

Three heavy metals were analyzed in this study: ferum (Fe), cadmium (Cd), and chromium (Cr). The graphs of removal are shown in Figures 1–6. The present study found *Scenedesmus* sp. could remove heavy metals from WMW with different initial cell concentrations, and total removal after 18 days was presented in different concentration cells of *Scenedesmus* sp. A considerable reduction in daily intake of the selected heavy metals was observed in each concentration for all percentages of WMW. After a 3- to 9-day gap, the *Scenedesmus* sp. accumulated Fe, Cd, and Cr effectively from WMW and continuously declined as shown in Figures 1, 3 and 5. The lag period of the microalgae growth can be referred to in the previous work by Apandi et al. [21]. There were some fluctuated readings recorded during the phycoremediation. It may be due to some factors such as environmental factors and pH changes in the samples: neutral to alkaline state range from 6.0–8.0 [20].

Differences in WMW concentration and microalgae initial cell culture conditions showed an interesting trend in each heavy metal's removal efficiency. The ability of *Scenedesmus* sp. to remove certain heavy metals was as follows: Cd after 18 days was successfully reduced with elimination rates going from 40.52% to 87.24% in 10%, 50%, 75%, and 100 % of WMW, respectively (Figure 1). The highest reduction occurred at 10^6 cells/mL in 25% WMW concentration on day 8 up to 87.24% removal from 6.04 ppb to 0.77 ppb. This situation occurs due to excellent growth, as described in the previous work with a maximum growth rate and biomass productivity of 0.27 μmax/day and 48.10 mg/L/d [22]. Moreover, this amount of removal was slightly higher than in the

50% WMW and 75% WMW, with the removal of about 84.76% and 82.59% (Figure 2). Meanwhile, in 1×10^7 cells/mL concentrations with removal rates ranged from 65.81% (100% WMW) to 81.22% (10% WMW). Both algae cell concentrations were successfully reducing the heavy metals of Cd presence in WMW. However, the removal of Cd in 100% WMW was ultimately low with an initial microalgae cell concentration of 1×10^4 (40.52%) and 1×10^5 cells/mL (47.15%), which indicated that *Scenedesmus* sp. could not grow effectively in 100% WMW, as mentioned by Apandi et al. [21]. The high concentration of heavy metals and nutrients (total nitrogen and phosphorus) in WMW may modify the circumstances, reducing microalgae accumulation in the sample. However, removal of Cd in 100% WM with the initial cell of 10^6 and 10^7 was effective with removal rates of 68.9% and 69.2%, respectively, indicating that this situation is dependent on initial cell concentrations and nutrients, as described by Apandi et al. [21].

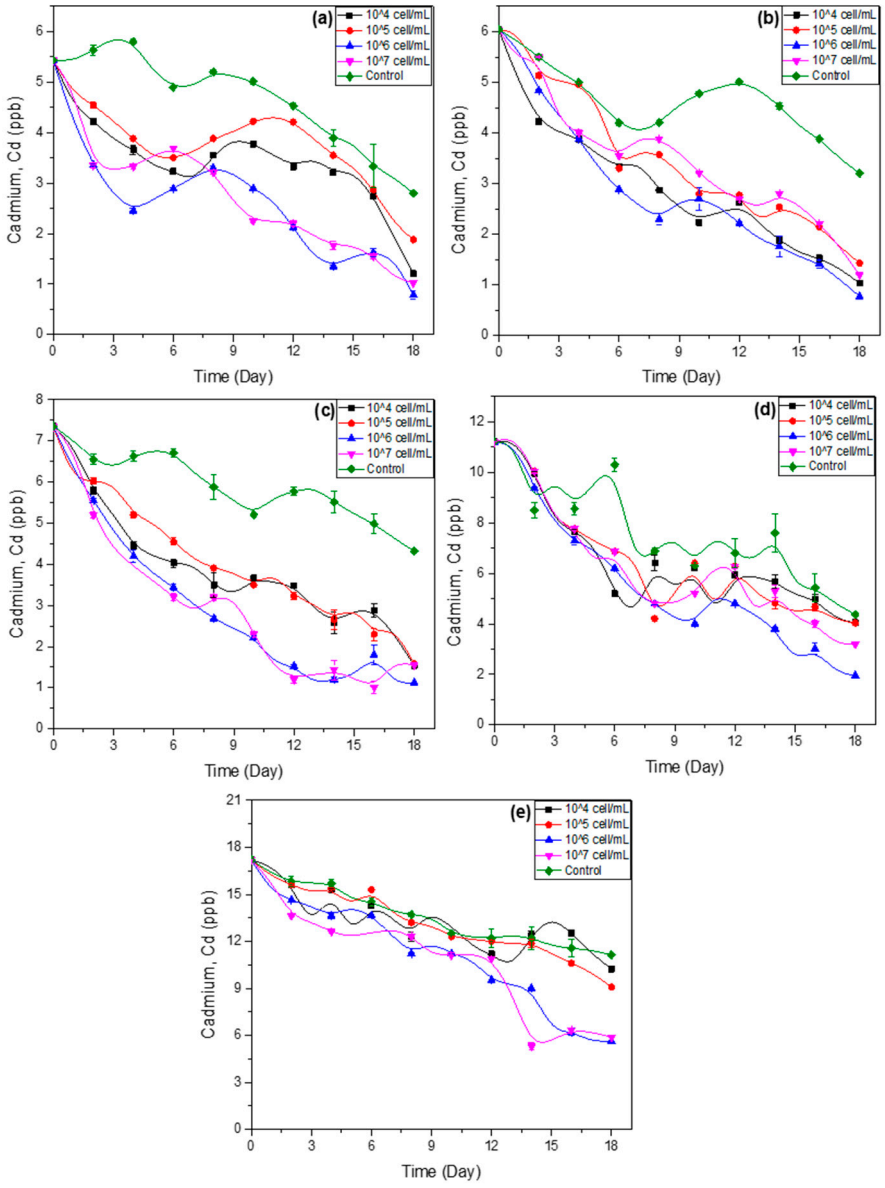


Figure 1. Graph depicting cadmium (Cd) removal from wet market wastewater (**a**) at a concentration of 10% WMW, (**b**) at a concentration of 25% WMW, (**c**) at a concentration of 50% WMW, (**d**) at a concentration of 75% WMW, and (**e**) at a concentration of 100% WMW during phycoremediation.

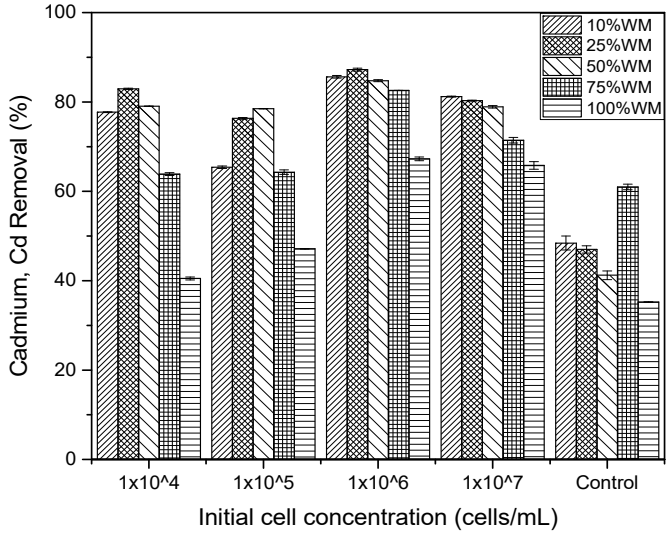


Figure 2. Removal of cadmium (Cd) for 18 days of phycoremediation.

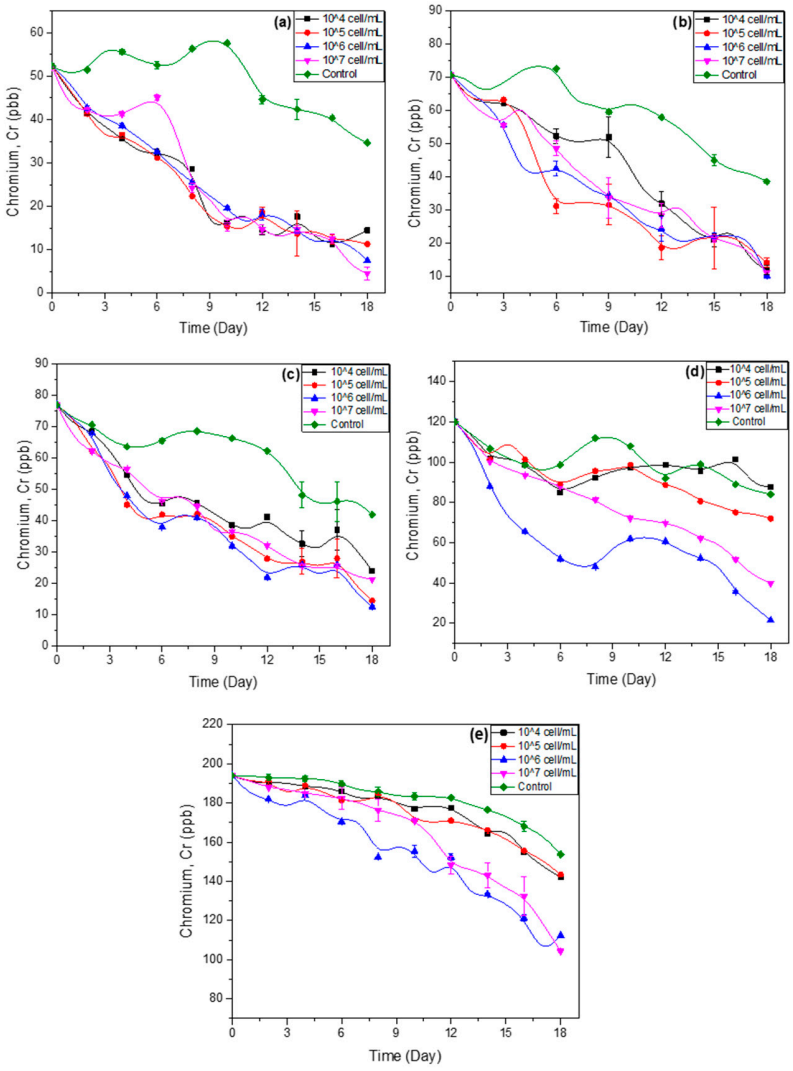


Figure 3. Graph depicting chromium (Cr) removal from wet market wastewater (**a**) at a concentration of 10% WMW, (**b**) at a concentration of 25% WMW, (**c**) at a concentration of 50% WMW, (**d**) at a concentration of 75% WMW, and (**e**) at a concentration of 100% WMW during phycoremediation.

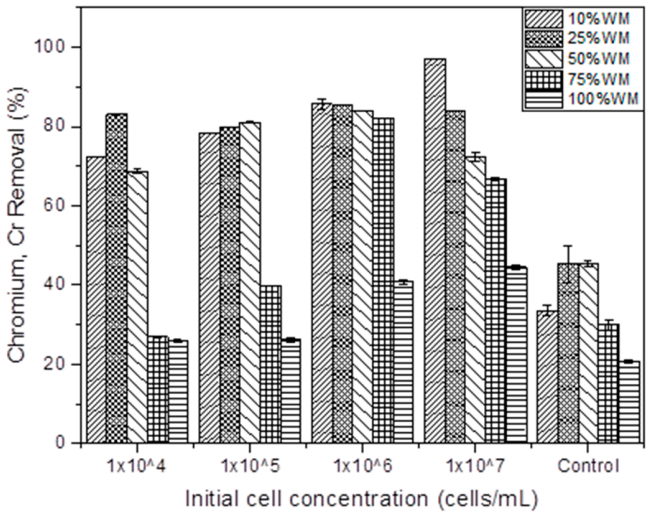


Figure 4. Removal of chromium (Cr) for 18 days of phycoremediation.

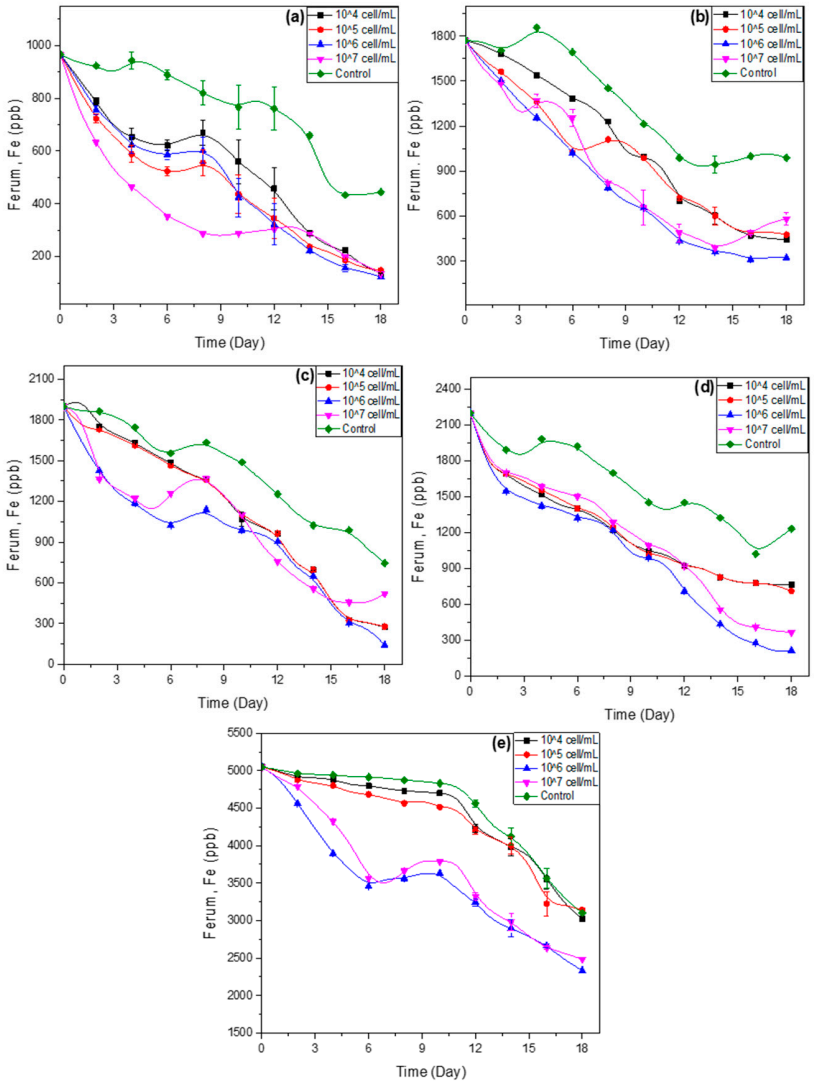


Figure 5. Graph depicting ferum (Fe) removal from wet market wastewater (**a**) at a concentration of 10% WMW, (**b**) at a concentration of 25% WMW, (**c**) at a concentration of 50% WMW, (**d**) at a concentration of 75% WMW, and (**e**) at a concentration of 100% WMW during phycoremediation.

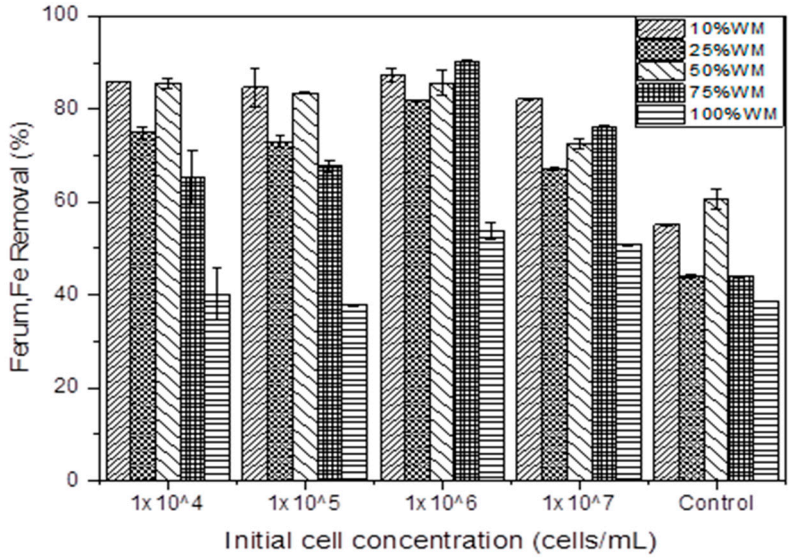


Figure 6. Removal of ferum (Fe) for 18 days of phycoremediation.

In spite of the fact that these microalgae are among the most beneficial microorganisms due to their anabolic and metabolic autotrophic organism, Apandi et al. [21] state that excess nitrogen, phosphorus, and heavy metals might negatively impact the phycoremediation process. The results show that diluting WMW slows the growth rate of *Scenedesmus* sp., improving removal efficiency.

Scenedesmus sp. cultivation in WMW resulted in the constant removal of Cr in all WMW concentrations. Most algae concentrations showed the same removal trend except for 10^4 and 10^5 cells/mL in 75% WMW. As expected, small algae cell concentrations might not be able to remove well in higher WMW concentrations. We noted that 75% WMW in the control sample had a slightly higher removal than 75% WMW with the initial cell of 10^4 cells/mL (Figure 4). This condition might have been related to the dead algae cell exposed to the stress condition and disrupted the continuous metabolic processes and the Cr reduction. A natural bioremediation process involving microorganisms or bacteria in WMW may be possible for the lower Cr concentration in the control sample. The trend removal of Cr shown in Figure 3 was such that *Scenedesmus* sp. in WMW removal was the highest on day 18, which is 10% WMW at 107 cells/mL. Cr content in 10% WMW was effectively reduced from 52.3 ppb to 4.52 ppb, equivalent to 91.4% removal (Figure 4). Meanwhile, the other condition of WMW also had an efficient removal for 25% to 100% WMW with removal rates ranging from 46.23% to 85.55% with an initial cell concentration of 1×10^7 cells/mL. This result exhibited that microalgae *Scenedesmus* sp. could remove Cr effectively at the initial cells of 10^6 and 10^7 cells/mL in all concentrations of WMW. To assess the effect of varying initial cell concentrations on all heavy metal's removal in WMW culture condition with the control, a one-way between-subject ANOVA was performed. The elimination of Cr from WMW was similarly found to have no statistically significant difference ($p > 0.05$). This condition is comparable to that described in a recent work by Ajayan et al. [27], who discovered that the microalgae *Scenedesmus* sp. were capable of removing Cr at all concentrations. (10%, 25%, 50%, 75%, and 100%) of tannery wastewater with total removal of 97%, 87%, 70%, 60%, and 57%, respectively.

Figures 5 and 6 reveal that Fe has the highest heavy metal content (5000 ppb to 900 ppb) in WMW concentrations when compared to other heavy metals. This condition might be due to the slaughtering of poultry and meat cuttings that contained blood and seafood residues that had a high level of iron (Fe) [28,29]. The majority of algal concentrations demonstrated constant Fe removal throughout all culture conditions except for 100% WMW. However, the highest removal of Fe was found in the concentration of 10^6 cells/mL in 75% of WMW from 2200 pbb to 228 pbb after 18 days of phycoremediation. This result was slightly higher than Gani et al. [30], who used *Botryococcus* sp. of 10^6 cells/mL, causing

Fe reduction of about 53.29%. Meanwhile, the removals in other conditions (10%, 25%, 50%, and 100% WMW) were also effective with the removal rates of 87.3%, 81.73%, 85.6%, and 53.83%, respectively, in the same algae concentration. Overall, the total removal of Fe throughout the study for 18 days indicated that 1×10^6 cells/mL was the best removal of Fe. Based on daily Fe elimination during the treatment, a one-way ANOVA indicated no statistically significant difference ($p > 0.05$) between the quantities studied.

Overall, the use of microalgae *Scenedesmus* sp. for phycoremediation in the removal of heavy metals (Cd, Cr, and Fe) in WMW was successfully achieved. However, the result recorded that *Scenedesmus* sp. efficiently removed heavy metals only from the diluted samples and that heavy metals cannot be removed in 100% WMW. Sengar, Singh, and Singh [31] found that on the 20th day of treatment, 100% of Cu in WMW can be removed by microalgae *Scenedesmus* sp. If the treatment day is extended, the heavy metals may be completely removed. *Scenedesmus* sp. could also reduce Zn, Cu, and Fe concentrations (76.63%, 60%, and 88.22%, respectively) in food stall wastewater. Hence, *Scenedesmus* sp. appears to be capable of removing heavy metals from WMW. The findings were consistent with the majority of earlier studies, which discovered that various species of microalgae can accrue variances in metal assimilation. Some heavy metal reductions by microalgae culture were dependent on water parameters such as pH, salinity, and hardness [32].

3.2. Microalgae Harvesting Efficiency

In this study, coagulant dosage and pH were chosen as essential variables that greatly influence microalgae harvesting efficiency in wastewater [33]. The effect of coagulation-flocculation on microalgae biomass recovery was examined by a face-centered central composite (FCCD) experimental design. These biocoagulants were chosen for response surface methodology (RSM) studies for biomass recovery. These were the cheapest source and did not contaminate recovered biomass during the production process. The products could be used directly in feed potential other than the biodiesel production industry. Moreover, natural coagulants are rich in antioxidants, active protein components, vitamins, amino acids, and complex carbohydrates, which many fish species require [34].

According to the results presented in Table 4, the response surface model for harvesting efficiency and both biocoagulant experimental models was significant at a 95% confidence level, which supported the validity of this quadratic model. The analysis of the variance table for harvesting efficiency using CC indicated an F-value of 118.31, indicating that this model obtained a significant ($p < 0.05$). Furthermore, noise has a 0.01% chance of causing a model F-value to occur, and it was found to be insignificant, indicating that the model variables presented were statistically significant for the considered answer. Apandi et al. [34] stated that a decent fit model should have an R^2 of at least 0.8. This model had an R^2 of 0.9883, an adjusted R^2 of 0.9800, and a forecasted R^2 of 0.9206, indicating that it was fit. As a result, the adjusted and predicted determination coefficients closely matched R^2. According to the *p*-value of this model, both factors contributed significantly to the response of harvesting efficiency.

The plot of residuals in Figure 7a,b were designed using Design Expert 7.0 software. This is to make sure that the model chosen for the experiment is a good fit for the data. Accordingly, the normal probability of CC and CA coagulants showed that the data values were located on a straight line, which points out that the data were normally distributed.

Table 4. ANOVA for the response surface quadratic model of harvesting efficiency of *Scenedesmus* sp. in WMW for CC coagulant.

Type of Coagulant	Source	Sum of Squares	Df	Mean Square	F Value	*p*-Value
Cajanus cajan	Model	1633.76	5	326.75	118.31	<0.0001
	A—pH	586.87	1	586.87	212.49	<0.0001
	B—Coagulant dosage	880.64	1	880.64	318.86	<0.0001
	AB	20.61	1	20.61	7.46	0.0293
	A^2	62	1	62	22.45	0.0021
	B^2	29.77	1	29.77	10.78	0.0134
	Residual	19.33	7	2.76		
	Lack of fit	14.01	3	4.67		
	Pure error	5.33	4	1.33	3.51	0.1285
	Cor total	1653.09	12			
	R^2 = 0.9883, Adj. R^2 = 0.9800, Pred, R^2 = 0.9206, Adeq precision = 38.981					

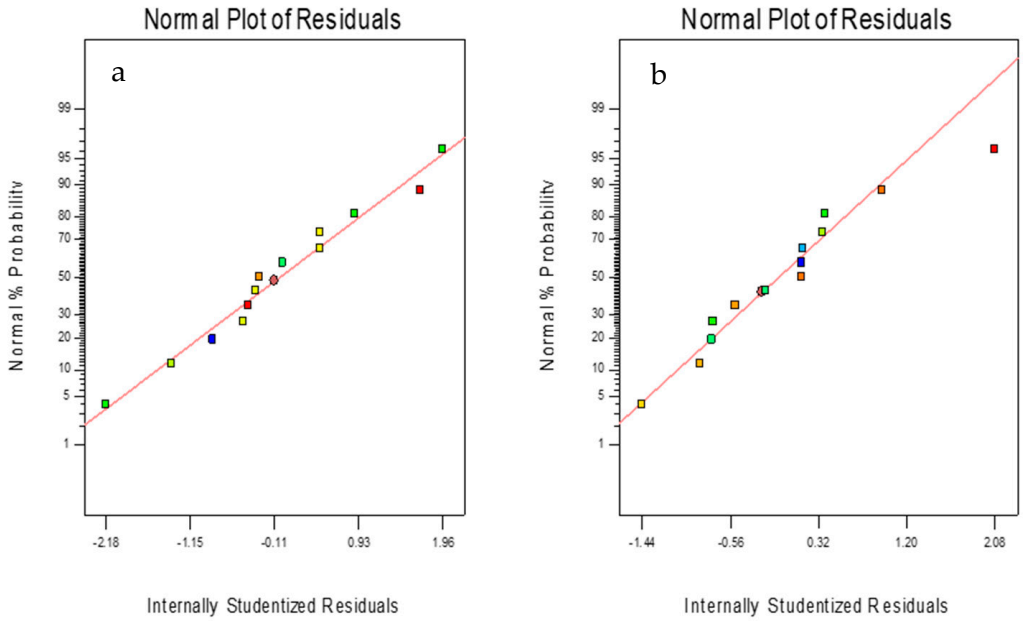


Figure 7. Design expert plot: normal probability plot of the internally standardized residual for harvesting efficiency using (**a**) CC and (**b**) CA coagulant.

Meanwhile, Figures 8 and 9 show the contour plot and three-dimensional graph that reflects the interaction of pH and CC dosage on the response for this study. The surface plot for the CC coagulant shows that as the pH and CC dosage increased, so did the harvesting efficiency.

Figure 10a shows the finest pH and CC capacity values were 11.72 and 178.75, respectively, while the highest efficiency was 89.29% with 1000 desirability. The final experiment was conducted by using the statistical model data to investigate the optimal condition of the process.

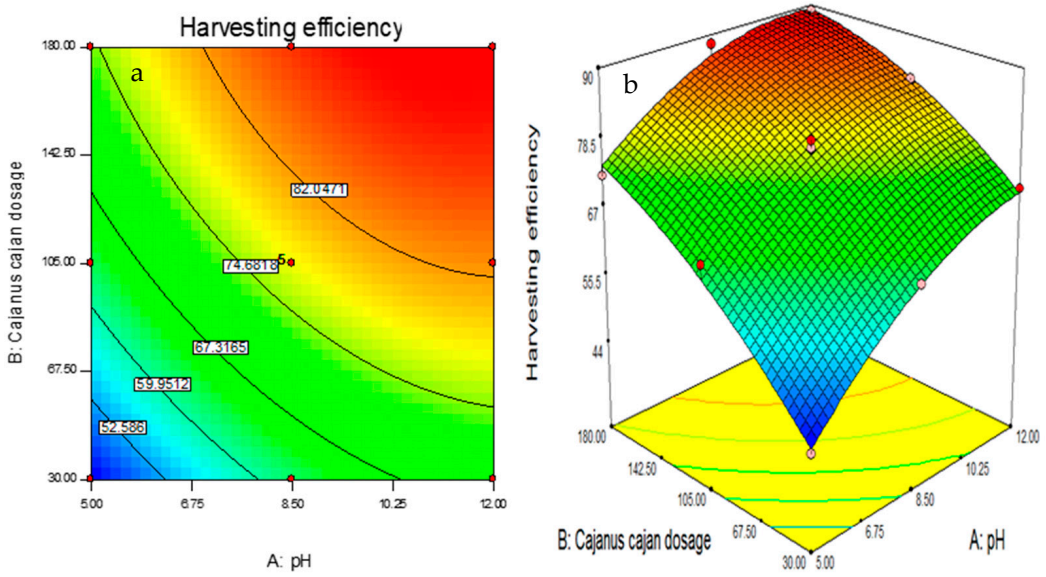


Figure 8. Design expert plot: (**a**) contour plot and (**b**) 3D response surface for maximum growth rate using CC coagulant.

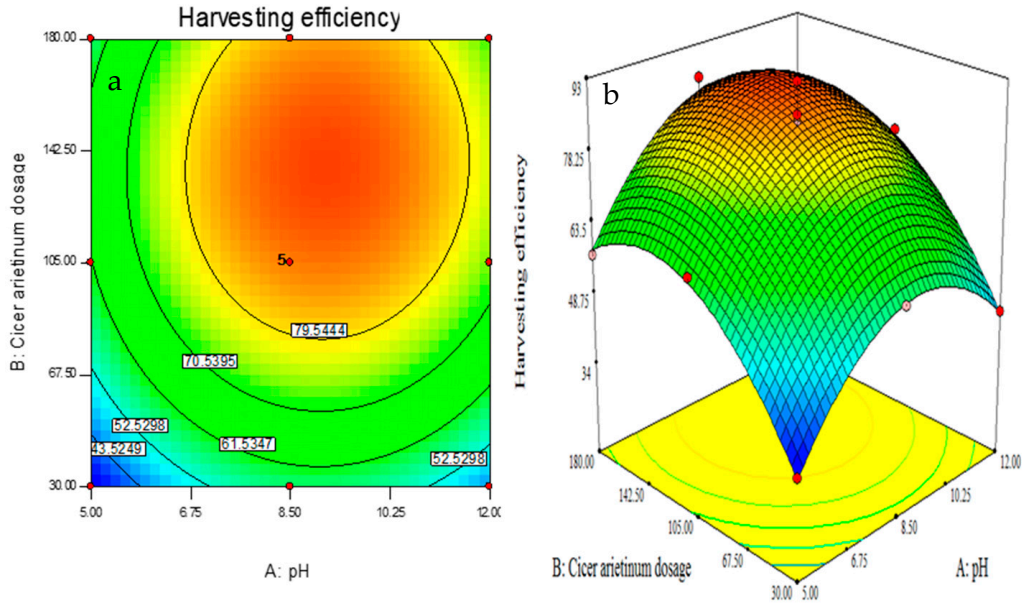


Figure 9. Design expert plot: (**a**) contour plot and (**b**) 3D response surface for maximum growth rate using CA coagulant.

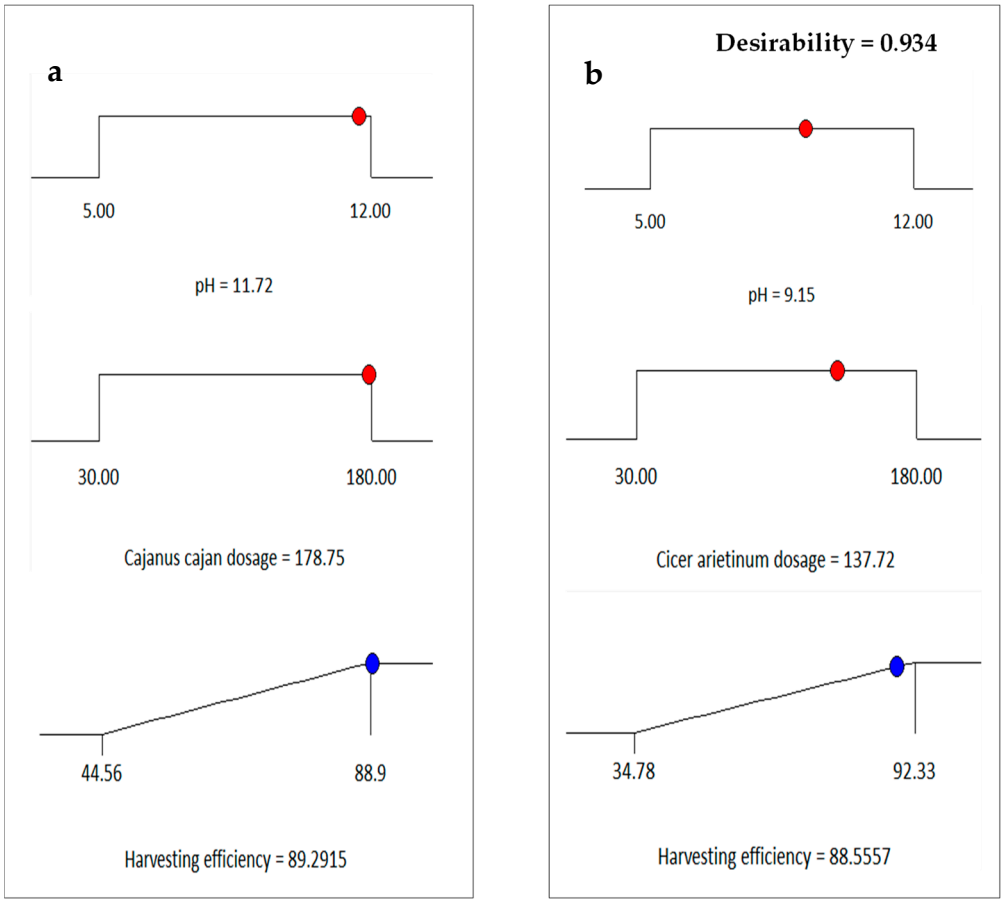


Figure 10. Harvesting optimization ramps for (**a**) *Cajanus cajan*, CC, and (**b**) *Cicer arietinum*, CA.

The actual average value in the optimization condition was 82.12%, which agreed with the predicted response obtained from the numerical optimization technique. The ANOVA regression for the quadratic model for CA coagulant to the response of harvesting efficiency is also stated in Table 5. When distinguishing actual values from anticipated values for harvesting efficiency with CA coagulant, the R^2 value was found to be 0.9731. The model was highly significant on a low probability and a high level of confidence in the results with a *p*-value of less than 0.0001 and an F-value of 50.60. Furthermore, the modified R^2 of 0.9538 was fairly close to the projected R^2 of 0.9353. As a result, the model's sufficient precision ratio was around 19,726, which was a good signal for the CA coagulant model. The required precision was larger than four, indicating that the anticipated model's desirability and confirmation may be used to explore the design space. This model implied that the 0.20 value for lack of fit was insignificant in comparison to the pure error for lack of fit. There was an 89.12 percent possibility of lack of fit due to numerical disagreement or noise, suggesting that the model was valid and fit. As a result, as shown in Table 5, by determining the reasonable agreement of the response values obtained through the experiment, the perfect combination of the variables of the two factors (pH and CA coagulant) was discovered. A quadratic equation was able to describe the greatest harvesting efficiency for CA coagulant since it contained all of the relevant factors in the equation.

Table 5. ANOVA for the response surface quadratic model of harvesting efficiency of *Scenedesmus* sp. in WMW for CA coagulant.

Type of Coagulant	Source	Sum of Squares	Df	Mean Square	F Value	*p*-Value
Cicer arietinum	Model	3615.16	5	723.03	50.6	<0.0001
	C—pH	225.22	1	225.22	15.76	0.0054
	D—Coagulant dosage	992.53	1	992.53	69.46	<0.0001
	CD	4.22	1	4.22	0.3	0.6036
	C^2	870.41	1	870.41	60.91	<0.0001
	D^2	617.08	1	617.08	43.18	0.0003
	Residual	100.03	7	14.29		
	Lack of fit	13.07	3	4.36		
	Pure error	86.96	4	21.74	0.2	0.8912
	Cor total	3715.19	12			
	R^2 = 0.9731, Adj. R^2 = 0.9538, Pred, R^2 = 0.9353, Ad. precision = 19.726					

Table 6 shows the actual value of the coded formulation of the optimization approach for harvesting efficiency. As a result, the harvesting efficiency may be predicted by altering the pH and the CA coagulant dosage (in milligrams per liter) in this quadratic equation. Meanwhile, the three-dimensional (3D) graphs and contour plot that reflects the interactions of harvesting efficiency in response to this model of CA are illustrated in Figure 9a,b. Lowering the CA coagulant from 180 to 30 (mg/L) resulted in a quadratic loss in harvesting efficiency, as seen in Figure 9a. However, pH levels as high as 8.5 were favorable and positive for harvesting efficiency. After that, there was a decrease in reactivity, which could be attributable to a low dose of CA coagulant. The best circumstances for harvesting efficiency of 88.56 percent were written as pH = 9.15 and CA dose = 137.77 with the desirability of 0.934, as represented in Figure 10b.

Table 6. The quadratic equation developed for harvesting efficiency of *Scenedesmus* sp. in WMW in terms of the coded and actual factor.

Type of Coagulant	Quadratic Equation (Coded Factors)	Quadratic Equation (Actual Factors)
Cajanus cajan	+77.52 + 9.89A + 12.11B − 2.27AB − 4.74A2 − 3.28B2	−5.56 + 10.31A + 0.36B − 8.65AB − 0.39A2 − 5.84B2
Cicer arietinum	+85.18 + 6.13C + 12.86D + 1.03CD − 17.75C2 − 14.95D2	−78.211 + 25.98C + 0.70D + 3.91CD − 1.45C2 − 2.66D2

It is important to note that as for harvesting efficiency, the optimum values of the factors were validated by the predicted optimal value as RSM optimized in a numerical setting to achieve the maximum harvesting efficiency [34]. To ensure that the anticipated ideal value was accurate, three separate tests were conducted. The extra test had a result of 80.5 percent, which was close to what was predicted (88.56%). Hence, it substantiated the usefulness of evidence of the model obtained, which also contributed to the significance of this model.

Comparing these two coagulants (CC and CA), the present study showed similar harvesting efficiency with 89.30% and 88.56% biomass recovery. Moreover, this study showed that these two significant factors significantly influenced the harvesting efficiencies of microalgae biomass. Gani et al. [22] previously employed alum to extract *Botryococcus* sp. from domestic wastewater at the ideal pH (8.24) and dosage (177.74 mg/L), with a flocculation efficiency of 99.3%. The study obtained 94.2% flocculation effectiveness using chitosan as a coagulant at the optimal pH (12) and dosage (169.95 mg/L). The biomass recovery in this study was slightly lower than Gani et al. [22], but the coagulants utilized (CC and CA) had a high harvesting potential. As a result, it is suggested that the coagulants be utilized as an alternative to alum due to their nonchemical and simple technique of biomass recovery.

3.3. Analyses of Microalgae Biomass Protein–Lipid Content

The increasing interest in soybean meal, cornmeal, fish feed formulation, and the potential of microalgae biomass as an alternative protein source have given commercialization a new boost. Biomass from *Scenedesmus* sp. harvested using natural coagulant flocculation (CC and CA) as well as centrifugation (CT) was compared and analyzed to fish feed standards in Malaysia by Yaakob et al. [23] to determine the nutritional proximate composition of biomass. Table 7 shows that the protein and lipid content of the microalgae *Scenedesmus* sp. found in this study was higher than that found by Yaakob et al. [23], who grew the algae using wastewater from chicken slaughterhouse and Malaysian quality fish feed standards. This could be as the protein content in CC and CA was increased, which led to changes in the protein and lipid content.

Table 7. Protein and lipid composition.

Composition	CT	CA	CC	Malaysian Quality Fish Feed Standards			Yaakob et al. [23]
				Grade A	Grade B	Grade C	
Protein (%)	36.5	45.8	43.6	65	60	55	29.9
Lipid (%)	16	15	13	12	13	13	25.75

However, harvested *Scenedesmus* sp. biomass by CT also has an excellent protein (36.5%) and lipid (16%) content. These results show that it might be due to higher nutrients in the WMW, higher nitrogen and phosphorus involvement achieved higher protein-lipid composition, or some other factor was involved during the phycoremediation process. Further experiments with microalgae *Scenedesmus* sp. as a fish feed replacement are thought to have met the Food and Agriculture of the United Nations (FAO) standard of protein and lipid percentage requirements for fish. Hence, the biomass of protein and lipid composition produced in WMW is sufficient for fish growth, especially fry and fingerlings, ranged between 35–50% for protein and 14–7% for lipids, respectively (FAO).

It can be inferred that the use of CA and CC is effectively suitable in the present study, followed by its natural material for microalgae biomass recovery. Thus, this study established that *Scenedesmus* sp. cultivated via WMW phycoremediation can be used as a partial or entire fish feed substitute. This might deliver more health advantages than current soy- or corn-based fish feed.

3.4. Analyses of Heavy Metal Content in Dry Microalgae Biomass

The heavy metals identified in WMW in this investigation are acceptable. Thus, *Scenedesmus* sp. could be used as fish feed and other commodities. However, before commercialization, the qualities of microalgae biomass must be investigated to ensure that they are safe, particularly for microalgae produced during wastewater treatment. Heavy metal contamination limits the end-use, including microalgae toxicity and cell characteristics of microalgae species. To preserve public health, maximum quantities of metals in foods that are safe for human and animal consumption must be limited [35]. Furthermore, the European Commission Regulation establishes specific contamination limits for microalgae biomass used as animal feed ingredients [36]. Thus, calculations of the heavy metal concentrations in the dry biomass of CA, CC, and CT were performed to confirm and validate that the metals contained in WMW did not affect the biomass quality and are shown in Table 8.

Table 8. Heavy Metals Content in Microalgae Biomass.

Heavy Metals	Microalgae Biomass Concentration (ug/kg as in ppb)			Remarks	Max. Content in ug/kg (ppb) (EU) 2015/186
	CA	CC	CT		
Ferum (Fe)	252 ± 1.3	325 ± 0.3	220 ± 2.41	Low	50,000
Chromium (Cr)	0.221 ± 0.66	0.114 ± 0.45	0.35 ± 0.75	Low	20
Cadmium (Cd)	0.513 ± 3.9	n.d.	0.152 ± 2.3	Low	10,000
Arsenic (As)	n.d.	n.d.	0.33 ± 3.1	Low	2000
Mercury (Hg)	1.41 ± 0.11	1.03 ± 0.25	1.22 ± 2.3	Low	25,000

According to Table 8, the heavy metal concentrations (Fe, Cr, Cd, As, and Hg) in CA, CC, and CT are mostly low; some are not detected and are below the European Commission Regulation limits. The phycoremediation experiment by *Scenedesmus* sp. showed that the cultivation of nontoxic algal biomass from WMW has a high potential for usage in future bioproducts such as fish feed.

3.5. Fatty Acid Analyses of Microalgae Biomass

GC–MS analysis of *Scenedesmus* sp. biomass cultivated in WMW revealed two primary fatty acid methyl esters (FAMEs) components, as opposed to three significant compounds in the CT and CC. The primary compounds discovered are listed in Table 9 along with their molecular formula, molecular weight, algal oil source, and potential applications. The fatty acid studies revealed numerous bioproduct potentials from microalgae biomass, which might be exploited for a variety of nutrition and medicinal goods [13]. The predominant fatty acid compounds present in CT were oleic acid (50%) and tridecanoic acid (24.5%), whereas the major compounds present in samples CA and CC were oleic acid (53–40%), linolenic acid (30%), trifuoroacetocetexy pentadecane (17%), and 6-Octadecenoic acid, (Z)-(15%). Table 8 shows that pure fatty acid (oleic acid) is commonly found in a variety of fats (animal and vegetable) with a lipid number of 18:1. As a result, it is classified as a monounsaturated omega-9 fatty acid [37].

Table 9. Major chemical compounds in microalgae biomass produced in WMW and BBM medium.

Compound	Molecular Formula	Molecular Weight	Algal Oil (%)	Compound Possible Applications (Dr. Duke's Phytochemical and Ethnobotanical Database, 1994)
		CT		
Oleic Acid	$C_{18}H_{34}O_2$	282.5	50	Antiandrogenic, preventive, antifungal, anti-inflammatory, cancer, polyunsaturated fatty acid, antiarthritic, antifungal
Tridecanoic acid	$C_{13}H_{26}O_2$	214.3	25	Enzyme inhibitors, antifungal agents, histamine antagonists
Compound	Molecular Formula	Molecular weight	Area (%)	Compound Possible applications (Dr. Duke's Phytochemical and Ethnobotanical Database, 1994)

Table 9. *Cont.*

Compound	Molecular Formula	Molecular Weight	Algal Oil (%)	Compound Possible Applications (Dr. Duke's Phytochemical and Ethnobotanical Database, 1994)
		CC		
Oleic acid	$C_{18}H_{34}O_2$	282.5	40	Antiandrogenic, preventive, antifungal, anti-inflammatory, cancer, polyunsaturated fatty acid, antiarthritic, antifungal
Linolenic Acid	$C_{18}H_{30}O_2$	278.4	30	Antioxidant, anti-inflammatory and antinociceptive activities
2-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	324.428	17	Antibacterial activity against Salmonella typhi, Pseudomonas eurogenosa, Bacillus subtilis, Streptococcus faecalis, and Staphylococcus aureus
		CA		
Oleic Acid	$C_{18}H_{34}O_2$	282.5	53	Antiandrogenic, preventive, antifungal, anti-inflammatory, cancer, polyunsaturated fatty acid, antiarthritic, antifungal
6-Octadecenoic acid, (Z)-	$C_{18}H_{34}O_2$	282.5	15	Antifungal, anti-inflammatory, anticancer

Oleic acid is usually the most dominant and major fatty acid compound in *Scenedesmus* sp. [38]. The primary sources of oleic acid can come from almond oil, olive oil, and other good sources of oil that are commonly employed as a food flavoring and emulsifying agents. It also can be found in the production of soap, detergents, and pharmaceutical products. One of the major significant findings that can be highlighted owing to the distinct fatty acid composition in *Scenedesmus* sp. is the expected biofuel produced from this microalga, which has strong potential due to the available fraction of fatty acid compounds such as oleic acid.

This result was higher than the *Scenedesmus* sp. fatty acid profiling cultivated in the BG-11 medium investigated by Gour et al. [39], which had only 16.12% oleic acid. Furthermore, Apandi et al. [21] reported that *Scenedesmus* sp. grown on BBM has four compounds of FAME compared to *Scenedesmus* sp. grown in wet market wastewater, which has 44 compounds. It was found that *Scenedesmus* sp. cultivated in WMW contained a sufficient amount of desirable fatty acids compared to the synthetic media such as BBM and BG-11 and was able to produce high-quality FAME. Prabakaran and Ravindran [40] discovered an 11.77 mg/g dry weight of oleic acid in *Scenedesmus* sp. from isolated water bodies at Gandhigram, Tamil Nadu, India. They found that the *Scenedesmus* sp. may be helpful for the production of by-products in addition to having a high oleic acid and lipid content. Following that, it is concluded that *Scenedesmus* sp. is a suitable candidate for fish feed utilization based on a total event of fourignificant fatty acids: the relative occurrence of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), and, most importantly, oleic acid, as literature reports. This compound has demonstrated that a single or a blend of this compound can easily substitute commercial fish feed. If the fish feed is replaced with an equal amount of alternative oil source, slight variations in the total dietary energy content may have varying digestibility within the content of FAMEs in CT, CC, and CA. As a result, fluctuations in feed intake may occur if the total energy of the meal is partially modified.

To determine whether these microalgae *Scenedesmus* sp. produced on WMW and harvested using CA and CC are safe to give to fish, more research on their application to the fish is required. *Scenedesmus* sp. cultivated on WMW performed better than those harvested with natural coagulants, according to the general basis data acquired on FAMEs. Furthermore, this research made several significant additions to recent findings from fatty acid molecules that might be employed as various chemicals in a variety of industries, including, cosmetology, agro feedstock, biodiesel, and pharmaceuticals.

3.6. FTIR Analyses

WMW nutrients and heavy metals can be absorbed and degraded by microalgae. WMW absorbs nutrients and metals, which causes active groups and chemical bonds to form on the microalgae's binding sites [41]. In this study, FTIR was used to identify the quantitative analysis of key functional groups in *Scenedesmus* sp. Figure 11a–c show the FTIR spectra of *Scenedesmus* sp. extracted by centrifugation (CT) and natural coagulants CA and CC after phycoremediation of heavy metals in WMW. The CT (Figure 11a) had a strong peak at 3294.24 cm^{-1}, indicating the presence of O-H stretching, and a rise indicated the presence of symmetrical and asymmetrical C-O at 1219.27 cm^{-1} and 1055.01 cm^{-1}, respectively. This wavelength was allocated based on the extending of vinyl ether and was most likely caused by alcohol and water in the biooil, as microalgae contain functional groups similar to biodiesel and hydrocarbons [42]. The lowest transmittance intensity was recorded on microalgae biomass based on the FITR spectrum as shown in Figure 11b,c with wavelengths of 3343.44 and 3344.40 cm^{-1}, respectively. The medium bonded amine groups (N-H) and hydroxyl groups (O-H) are found in the functional groups of both CA and CC within the band 3350–3310 cm^{-1}. The amount of nitrogen in wastewater absorbed by microalgae cells could explain the presence of this functional amine group. Sulaymon et al. [43] found that hydroxyl, sulfonate, and carboxyl functional groups are linked to cadmium, heavy metal, and other metal absorption by microalgae biomass. Apart from that, the hydroxyl group is found in the cell walls of microalgae.

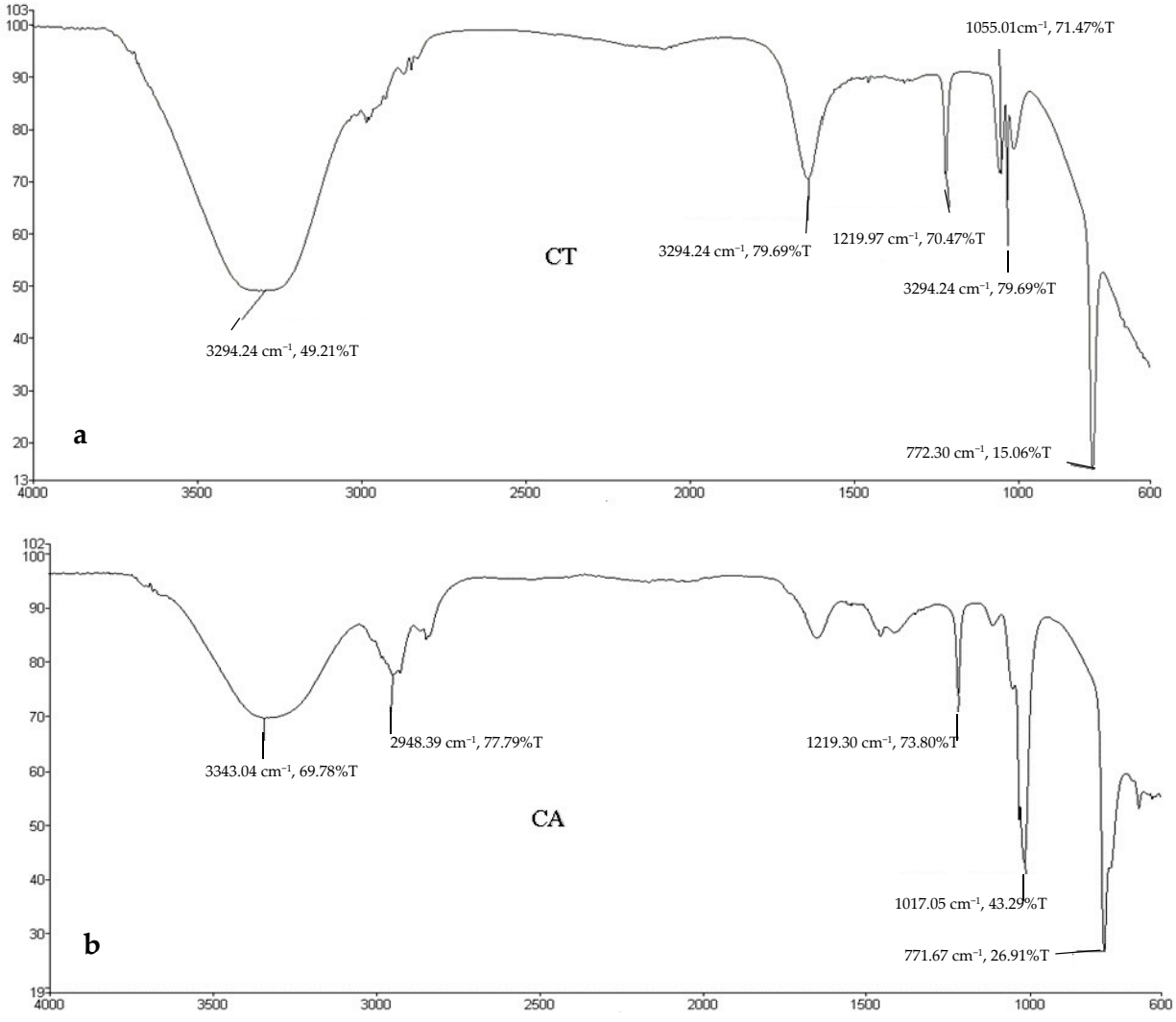


Figure 11. *Cont.*

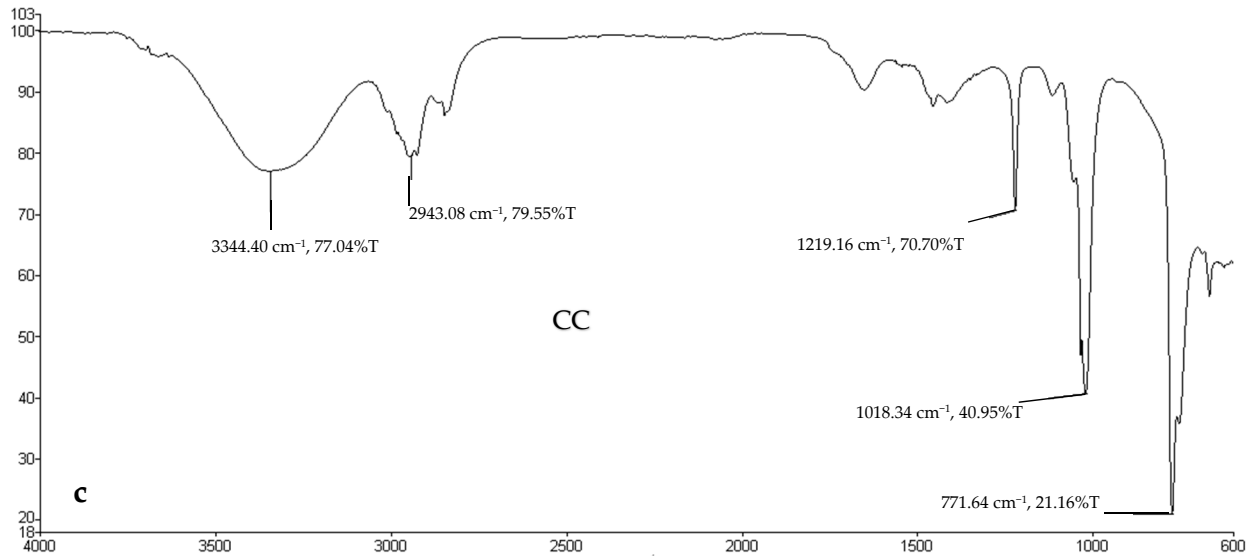


Figure 11. Comparison of the FTIR spectra of *Scenedesmus* sp. biomass harvested by (**a**) CT, (**b**) CA, and (**c**) CC.

The acyl chain stretches of C-H used to analyze lipids CA and CC were connected to wavelengths ranging from 3000 to 2840 cm^{-1} as shown in Figure 11. This study discovered a minor difference in the transmittance intensity of microalgae biomass generated in CA, CC, and CT. The 3350–3310 cm^{-1} spectral change in the stretching vibration of the amide group and the bending of NH functional groups increases the protein content of flocculants CA and CC. The FITR spectrum of microalgae biomass employed in this work could be used to assess protein concentration. According to Yaakob et al. [23], there is no wavenumber assigned to protein determination in commercial fish diets. However, the extraction procedure can be used to determine how much protein is in microalgae biomass and fish feeds, which can be supported by wavelength shifts caused by nitrogen-limited growing conditions [42].

Total carbohydrates can be estimated by measuring the absorption of the ether functional group (C-O-C) in the wavelength range of 1200–950 cm^{-1}. In this investigation, the transmittance intensity of microalgae biomass harvested by CA and CC was 771.67 cm^{-1} and 771.64 cm^{-1}, respectively, and that of CT was 772.30 cm^{-1}. When compared to the transmittance intensity of commercial fish feed (747.63 cm^{-1}) published by Yaakob et al. [23], the abundance of this functional group (C-H) in all samples had a greater transmittance intensity. The experimental results, which showed 15% (CA), 13% (CC), and 13–12% of lipids in microalgae biomass and commercial fish diets, respectively, support this assertion.

4. Conclusions

The experimental investigation concluded that *Scenedesmus* sp. has great potential for the next level of biotechnological applications. The present study also revealed more details on the heavy metal's removal efficiencies depending on the microalgae and WMW concentration applied. Both natural coagulants CA and CC were anticipated to successfully recover *Scenedesmus* sp. biomass that has the ability to provide high nutritional value for use in fish feeds. The application of the derived microalgae oil extracted by CA and CC has valuable nutritional demands for fish feed production, according to GC–MS, FTIR, protein and lipid profile, and heavy metals content in dry biomass analysis. The recovery of these valuable nutritional and other beneficial bio compounds is proven to be an alternative source. As a result, the consolidation method of using wastewater and natural coagulant is a more cost-effective strategy, and this eco-friendly approach might potentially replace the usage of fish feed partially or even entirely. These applications of microalgae must

be commercialized on a large scale, particularly as biological assessments and alternative biological treatment, as well as its biomass, can be used as a regulator of the environment. Furthermore, the implementation of this process could tackle any related industry without harming the global environment.

Author Contributions: All authors contributed to the study conception and design. Conceptualization, N.M.A. and N.M.S.; methodology, N.M.S., R.M.S.R.M. and A.A.; software, N.M.A.; validation, N.M.A.; formal analysis, N.M.A. and P.G.; investigation, N.MA. and R.N.; resources, N.M.A.; data curation, N.M.A.; writing—original draft preparation, N.M.A.; writing—review and editing, A.M.A., R.N., N.A.R.S., K.C., A.A. and R.A.R.; visualization, N.M.A.; supervision, R.M.S.R.M., N.M.S. and A.A.; project administration, N.M.A. and R.M.S.R.M.; funding acquisition, N.M.S., R.M.S.R.M. and A.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: We declare that this manuscript is original, has not been published before, and is not currently being considered for publication elsewhere. We have no conflict of interest to disclose with this publication. We have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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