

Article



Optimization of Microalgal Harvesting with Inorganic and Organic Flocculants Using Factorial Design of Experiments

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Abstract: Microalgae have a lot of potential as a source of several compounds of interest to various industries. However, developing a sustainable and efficient harvesting process on a large scale is still a major challenge. This is particularly a problem when the production of low-value products is intended. Chemical flocculation, followed by sedimentation, is seen as an alternative method to improve the energetic and economic balance of the harvesting step. In this study, inorganic (aluminum sulfate, ferric sulfate, ferric chloride) and organic (Zetag 8185, chitosan, Tanfloc SG) flocculants were tested to harvest *Chlorella vulgaris* in batch mode. Preliminary assays were conducted to determine the minimum dosages of each flocculant that generates primary flocs at different pH. Except for chitosan, the organic flocculants required small dosages to initiate floc formation. Additional studies were performed for the flocculants with a better performance in the preliminary assays. Zetag 8185 had the best results, reaching 98.8% and 97.9% efficiencies with dosages of 50 and 100 mg L⁻¹, respectively. Lastly, a 2⁴ full factorial design experiment was performed to determine the effects of the flocculant dosage, settling time, and mixing time on the Zetag 8185 harvesting efficiency. The harvesting efficiency of *C. vulgaris* was optimal at a dosage of 100 mg L⁻¹ and 3 min of rapid mixing.

Keywords: chemical flocculation; Chlorella vulgaris; flocculants; harvesting; microalgae

1. Introduction

Microalgae are photosynthetic microorganisms that have a wide variety of environmental applications: (i) the bioremediation of wastewater in terms of nutrient removal/recovery; (ii) carbon dioxide (CO_2) sequestration from biogas and industrial flue gases; and (iii) the bioenergy production of fuels such as biodiesel, bioethanol, biohydrogen, and others [1,2]. Besides their high content in polysaccharides, proteins, and lipids, microalgae also accumulate high-value products such as antioxidants, pigments, and vitamins that have already been commercially applied in supplementary food products, pharmaceuticals, and cosmetics [3]. Chlorella vulgaris is a green microalgal widely cultivated on a large scale and commercially produced because of its economic potential. It has a high photosynthetic rate and the ability to grow in different environmental conditions [4]. Maximum oxygen production rates of approximately 135 g O_2 kg cell⁻¹ h⁻¹ were estimated at different C. vulgaris cell concentrations [5]. Zheng et al. [6] reported carbon dioxide fixation rates of 454 mg CO₂ L⁻¹ d⁻¹ at a 5% CO₂ concentration. Furthermore, C. vulgaris is a carbohydrate-rich microalga with the potential to produce bioethanol [7]. Despite their numerous applications, microalgae are only commercially produced when they are intended for use in synthesizing high-value products due to the costly harvesting processes involved [8]. This step represents 20–30% of the total biomass production costs and may represent 90% of the equipment costs [9]. For low-value products, such as biofuels, the



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). production costs must be significantly reduced for the process to be economically viable and able to compete with actual fossil fuel prices.

Microalgae have a microscopic size (2-30 µm), and the cultures present low biomass concentrations with densities close to water, making sedimentation difficult [8,10]. Usually, the biomass concentration ranges from 0.2 to 6 g L^{-1} , being about 0.5 g L^{-1} in a typical 20 cm deep raceway pond [8,11]. Furthermore, microalgae present carboxylic and amine groups on the cell surface, having a negative surface charge at the common pH values (above 4–5) of the cultures since the groups tend to be deprotonated. Microalgal cells also carry algogenic organic matter (AOM), which includes extracellular organic matter (EOM), a product of algal metabolism released by diffusion, and intracellular organic matter, released during cell lysis [12]. During microalgal growth, AOM characteristics and quantity are considerably influenced by EOM [13]. Gonzalez-Camejo et al. [14] studied the impact of different stressing factors on EOM production in a *Chlorella*-dominated culture, concluding that nutrient limitation and sudden temperature changes should be avoided, as they seem to increase EOM production. AOM and the negative surface charge of the cultures do not allow microalgae to aggregate easily, remaining in stable suspensions [1,8]. These factors make the harvesting process challenging and consequently, expensive. Decreasing harvesting costs is thus seen as a critical factor in developing a long-term, full-scale production of microalgal biomass. At this moment, microalgal harvesting is typically performed by mechanical (gravity sedimentation, centrifugation, filtration, flotation), biological (bioflocculation), chemical (chemical flocculation), or physical methods (electroflocculation, magnetic separation). Although these methods can be employed individually, microalgal harvesting is usually a two-step concentration process: there is a thickening and a dewatering step. First, the diluted cell suspension is concentrated in a slurry of about 2–7% of the total suspended solids (TSS). Then, the slurry is concentrated by centrifugation or filtration, resulting in a cake with 15–25% TSS [1]. This allows for a more efficient and economical process, as the thickening step can be performed by a cost-effective process, such as flocculation, prior to the energy-consuming step of dewatering that requires expensive equipment [9].

Chemical flocculation, followed by sedimentation, is considered a low-cost, alternative method for improving the energetic and economic balance of microalgal harvesting [9,15]. Although some authors may differentiate flocculation and coagulation, most do not distinguish between these processes when reporting on microalgae harvesting. Thus, this differentiation was not made in this work either; we chose to use only the term flocculation. In this process, inorganic or organic compounds with flocculant activity allow floc formation by adsorption and charge neutralization, adsorption and interparticle bridging, and enmeshment in a precipitate (sweep flocculation) [16]. Flocculation usually includes a rapid mixing (RM) stage performed at 100–300 rpm for 1–5 min, where suspension destabilization occurs, and a slow mixing (SM) stage to allow the formation of larger agglomerates, performed at 20-50 rpm for 9-20 min [17-20]. Typically, inorganic flocculants based on iron or aluminum salts such as ferric chloride, ferric sulfate, and aluminum sulfate are used [18,21]. However, these flocculants are non-biodegradable; their use promotes secondary pollution and produces toxic sludge, which is expensive and complex to treat [22]. A study showed that the bioaccumulation of ferric ions is superior to that of ferrous ions in different microalgae, with *Chlorella* being the most resistant [23]. Other authors studied the adverse effects of ferric chloride and aluminum sulfate in microalgae, concluding that more than 95% of metals were transferred to the biomass [24]. Some studies also point to the harm associated with human exposure to aluminum, linking it to neurodegenerative diseases such as Alzheimer's disease [25,26]. On the other hand, organic flocculants are generally synthetic polymers that require lower dosages, reducing the production of contaminated sludge [27]. Nevertheless, they are more expensive than the inorganic ones. Zetag 8185 is a synthetic polyacrylamide-based polymer that proves to be efficient in the harvesting of the microalga Neochloris oleoabundans [28]. Organic flocculants can also be natural-based. There has been a growing interest in applying chitosan [20,29] and tannin-based flocculants [15] to harvest microalgae.

Although organic polymers have been reported in prior investigations, there is limited information about their use in harvesting *C. vulgaris*. Further, there have been almost no studies comparing the utilization of metallic salts with both natural and synthetic organic polymers, and none perform process optimization. For this purpose, this study aims to optimize chemical flocculation. Aluminum sulfate, ferric chloride, and ferric sulfate (inorganic flocculants), Zetag 8185 (a synthetic organic polymer), Tanfloc SG (a tannin-based flocculant derived from *Acacia mearnsii*), and medium molecular weight chitosan were tested for the harvesting of *C. vulgaris*. Preliminary assays were conducted to determine the minimum dosages of each flocculant that generated primary flocs at different pH levels. Additional studies were conducted for the flocculants that had a better performance in the preliminary assays. Lastly, a 2⁴ full factorial design experiment was performed to determine the effects of flocculant dosage, settling time, and RM and SM time on the harvesting efficiency of the best flocculant. In general, organic flocculants had a better performance, with Zetag 8185 being the most efficient flocculant.

2. Materials and Methods

2.1. Materials

2.1.1. Microalgal Culture

The microalgal *C. vulgaris* CCAP 211/11B used in this study was obtained from the Culture Collection of Algae and Protozoa (CCAP, Oban, UK). The microalgal was cultivated in 5 L bottles, stirred by air injection, and maintained under continuous artificial light with an intensity of 140 μ mol m⁻² s⁻¹. The biomass concentration was monitored by an optical density at 680 nm (OD₆₈₀). The relation between OD₆₈₀ and the biomass dry weight (DW) concentration was established through a calibration curve (Equation (1)). The cultures were collected at concentrations above 340 mg_{DW} L⁻¹.

 $OD_{680} = 3.7837 \times Biomass \text{ concentration } (g_{DW} L^{-1}) - 0.0016$ (R² = 0.9902, Limit of detection = 0.02 g_{DW} L⁻¹, Limit of quantification = 0.07 g_{DW} L⁻¹). (1)

2.1.2. Flocculants

The inorganic flocculants aluminum sulfate, ferric sulfate, and ferric chloride were supplied by VWR International, and solutions of 50, 12, and 20 g L⁻¹, respectively, were prepared in distilled water. Chitosan power with a deacetylation degree \geq 75% was obtained from Sigma–Aldrich (St. Louis, MO, US), and a solution of 2 g L⁻¹ was prepared according to the process described by Divakaran and Pillai [30]. A 20 g L⁻¹ solution of Tanfloc SG, obtained from TANAC (Montenegro, Brazil), and a 2 g L⁻¹ Zetag 8185 solution, supplied by BASF (Porto, Portugal), were also prepared by dissolving the required amount in distilled water.

2.2. Methods

2.2.1. Chemical Flocculation Assays

Flocculation assays were conducted in batch mode using Jar test equipment with six stirrers and a fluorescent lamp to observe floc formation. In all assays, 800 mL beakers containing 500 mL of microalgal culture were used. Preliminary assays were performed to determine the minimum dosages of each flocculant that generated primary flocs at pH levels of 5, 6, 7, 8, and 9. Increments of the coagulants were successively added to the 500 mL microalgal culture. After each addition, the solutions were stirred at 150 rpm for 3 min (RM) and at 20 rpm for 15 min (SM). Floc formation was then evaluated by visual observation. The assays were performed in duplicate.

Additional studies were conducted for the flocculants that had a better performance in the preliminary assays—Ferric chloride at pH 9, Zetag 8185 at pH 6, and Tanfloc SG at pH 9. Different flocculant dosages from 10 to 200 mg L⁻¹ were tested. The experiments also included the RM and SM stages, followed by 15 min of sedimentation, and were performed in duplicate. Flocculation efficiency (η) was calculated with Equation (2), knowing the absorbance at 680 nm and measured using a UV-vis spectrophotometer through wavelength scanning. The maximum absorbance was observed at this value. OD_i corresponds to the optical density of the microalgal culture prior to any flocculant addition, and OD_f is the optical density after the settling period. The samples to determine OD_f were taken from the supernatant, approximately 2 cm below the surface. The absorbance was measured in a UV-vis spectrophotometer (*UV-6300PC* double beam, *VWR*).

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

2.2.2. Zetag 8185 Optimization Assays

A 2^4 full factorial experimental design was performed to determine the effects of the flocculant dosage, settling time, RM time, and SM time on Zetag 8185 harvesting efficiency. Each factor was tested at low (-1) and high (+1) levels in duplicate. Table 1 presents the coded values with the respective real values in parentheses.

Table 1. Experimental design data for optimizing *C. vulgaris* harvesting with Zetag 8185 (coded levels and real values in parentheses).

Run	Dosage (mg L ⁻¹)	Settling Time (min)	RM Time (min)	SM Time (min)
1	-1 (10)	-1 (5)	-1 (0)	-1 (0)
2	+1 (100)	-1 (5)	-1(0)	-1 (0)
3	-1 (10)	+1 (15)	-1(0)	-1 (0)
4	+1 (100)	+1 (15)	-1(0)	-1 (0)
5	-1(10)	-1 (5)	+1 (3)	-1 (0)
6	+1 (100)	-1 (5)	+1 (3)	-1(0)
7	-1(10)	+1 (15)	+1 (3)	-1(0)
8	+1 (100)	+1 (15)	+1 (3)	-1 (0)
9	-1(10)	-1 (5)	-1(0)	+1 (15)
10	+1 (100)	-1 (5)	-1(0)	+1 (15)
11	-1(10)	+1 (15)	-1(0)	+1 (15)
12	+1 (100)	+1 (15)	-1(0)	+1 (15)
13	-1(10)	-1 (5)	+1 (3)	+1 (15)
14	+1 (100)	-1 (5)	+1 (3)	+1 (15)
15	-1(10)	+1 (15)	+1 (3)	+1 (15)
16	+1 (100)	+1 (15)	+1 (3)	+1 (15)

A four-way ANOVA (p < 0.05) was conducted, and an empirical first-order model characterizing flocculation was established. The model was then used to create a contour diagram to analyze the effects of the variables on Zetag flocculation efficiency. The statistical analysis and the model were constructed using Minitab[®] Statistical Software 21.1.0.0.

3. Results and Discussion

3.1. Minimum Dosage Determination

Preliminary studies aimed to determine the minimum flocculant dosage required to start the formation of primary flocs and the optimal pH for each flocculant. It was also our intent to select the flocculants with the most promising results to study the optimization of other parameters, such as dosage, in more detail. The results are presented in Table 2.

Ferric sulfate and ferric chloride showed better performance at a basic pH, with the optimum being pH 9. Despite the minimum dosages being very similar, ferric chloride allowed for the formation of larger flocs. Sanyano et al. [18] also obtained higher harvesting efficiency with ferric chloride at a basic pH (>8) for the harvest of *Chlorella* sp. The optimal pH for aluminum sulfate was 9 as well. Gani et al. [31] investigated the effect of pH and alum dosage in *Botrycoccus* sp. Harvesting, and the optimal pH was measured to be 9.2. Table 2 shows that aluminum sulfate needed a much higher dosage than the other inorganic flocculants to form flocs. Ferric salts were more effective than aluminum sulfate

since these flocculants are converted to ferric hydroxide at pH 9, causing microalgae to establish bridges together, forming flocs more easily [32]. In the study performed by Sanyano et al. [18], ferric chloride was also more efficient than aluminum sulfate, requiring an inferior dosage to generate denser flocs. Except for chitosan, organic flocculants required lower dosages than did inorganic flocculants to form flocs. Chitosan and Zetag allowed for the formation of larger flocs at an acidic pH, while Tanfloc worked best at a basic pH. In a study by Kirnev et al. [28] on the harvest of N. oleoabundans, the flocculation efficiency increased for Zetag 8185 and chitosan when the pH was below 6, while the maximum efficiency for Tanfloc was achieved at pH 10. Chitosan also showed better performance at pH < 7 on the harvest of *Chlorella sorokiniana* [33]. Chitosan, Zetag 8185, and Tanfloc SG are cationic polymers, so they are likely to bind to and between the negatively charged *C. vulgaris* cells. However, chitosan needed a higher dosage in order to neutralize cells. The polymer size and charge density may have influenced flocculation, since chitosan is less charged than the other organic flocculants studied [28]. On the other hand, being a flocculant that requires a lower dosage to initiate floc formation, Zetag 8185 has a high charge density.

Table 2. Optimal pH and minimum dosage for flocculation obtained for aluminum sulfate, ferric sulfate, ferric chloride, chitosan, Zetag 8185, and Tanfloc SG.

Flocculants	Optimal pH	Initial Biomass Concentration (mg $_{\rm DW}$ L $^{-1}$)	Minimum Dosage (mg L ⁻¹)	Minimum Dosage (mg g _{DW} ⁻¹)
Aluminum sulfate	9	342 ± 9	60.0 ± 0.0	175 ± 0
Ferric sulfate	9	342 ± 9	9.6 ± 0.0	28 ± 0
Ferric chloride	9	342 ± 9	9.2 ± 0.0	27 ± 0
Chitosan	5	349 ± 5	12.0 ± 0.0	34 ± 0
Zetag 8185	6	356 ± 9	4.0 ± 0.0	11 ± 0
Tanfloc SG	9	356 ± 0	8.0 ± 0.0	22 ± 0

3.2. Dosage Optimization

The flocculants with the most promising preliminary tests were selected for the dosage optimization tests. The organic flocculants Zetag 8185 and Tanfloc SG were selected, as they present with the lowest minimum dosages for the formation of flocs. Of the inorganic flocculants, ferric chloride was chosen. Despite the minimum dosage being very similar to that of ferric sulfate, ferric chloride allowed for the formation of larger flocs, which could enable a faster sedimentation process. The assays were performed at the optimal pH previously determined for each flocculant (Table 2), and the results related to the harvesting efficiency are shown in Table 3.

Table 3 shows that the dosage significantly affects the efficiency of the flocculation harvesting process. Once again, Zetag 8185 achieved the best results, reaching 98.8% and 97.9% efficiencies at 50 and 100 mg L⁻¹, respectively. The appearance of the culture after flocculation can be observed in Figure 1, which clearly shows the flocculated biomass and the transparency of the medium achieved with 50 and 100 mg Zetag L⁻¹. From 100 mg L⁻¹, there is a slight decrease in the efficiency, which indicates that the optimal dosage for this flocculant, under the conditions studied, is between 10 and 100 mg L⁻¹.

The harvesting efficiency achieved with the Tanfloc SG enhanced with the increasing dosage was 24.4% with a dosage of 200 mg L⁻¹. However, this value is still very low, especially when compared to the results obtained with Zetag. Even though some neutralization occurred, it was not enough to cause significant aggregation. Kirnev et al. [28] applied different organic flocculants to harvest *N. oleoabundans* and reported Zetag 8185 as the best flocculant at pH 6 and a concentration of 16 mg L⁻¹, reaching efficiencies above 95%. In the same study, Tanfloc SG only achieved a maximum efficiency of 13% at pH 6 and 14 mg L⁻¹. Tanfloc has a lower charge density compared to Zetag, requiring higher dosages to achieve cell neutralization [28]. In a study performed by Niemi and Gentili [34] to harvest *C. vulgaris*, grown in municipal wastewater for 2–6 days, a tannin-based flocculant

achieved efficiencies between 26% and 71% at 100 mg L^{-1} . Increasing the flocculant dosage to 300 mg L^{-1} allowed for reaching harvesting efficiencies from 81% to 99%. Therefore, a dosage superior to 200 mg L^{-1} Tanfloc is expected to achieve better efficiencies.

Table 3. Harvesting efficiency obtained in flocculation assays using concentrations of 10–200 mg L^{-1} of Zetag 8485 at pH 6 and Tanfloc SG and ferric chloride at pH 9.

Flocculant	pН	Biomass Concentration (mg $_{\rm DW}$ L $^{-1}$)	Dosage (mg L ⁻¹)	Final Biomass Concentration $(mg_{DW} L^{-1})$
Zetag 8185		344 ± 4	10	239 ± 27
			50	4 ± 1
	6		100	7 ± 1
			150	16 ± 2
			200	12 ± 1
Tanfloc SG		346 ± 2	10	313 ± 13
			50	321 ± 10
	9		100	314 ± 5
			150	291 ± 6
			200	262 ± 5
Ferric chloride	9	343 ± 7	10	338 ± 5
			50	332 ± 6
			100	311 ± 8
			150	316 ± 12
			200	353 ± 6



Figure 1. Flocculated *C. vulgaris* biomass after harvesting at: (a) 50 mg L⁻¹ Zetag 8185; (b) 100 mg L⁻¹ Zetag 8185.

Ferric chloride was the flocculant with the worst performance, only reaching a maximum efficiency of 9.4% with a concentration of 100 mg L^{-1} . For dosages above 100 mg L^{-1} , there was also a decrease in the efficiency. Due to the excessive positive charge around the negative charged cells, the higher flocculant concentration may have slowed down cell aggregation, destabilizing the microalgal cells because of electrostatic repulsion [31].

3.3. Zetag 8185 Optimization

Since Zetag 8185 was the flocculant that achieved better harvesting efficiencies, the impact of the other parameters was also studied. In addition to pH and dosage, the settling time and RM and SM time can also influence the harvesting efficiency. The results obtained regarding the efficiency of microalgae harvesting (response variable), which were obtained

Run	Dosage (mg L ⁻¹)	Settling Time (min)	RM Time (min)	SM Time (min)	Harvesting Efficiency (%)
1	10	5	0	0	0.0 ± 0.0
2	100	5	0	0	0.0 ± 0.0
3	10	15	0	0	0.0 ± 0.0
4	100	15	0	0	0.0 ± 0.0
5	10	5	3	0	26.4 ± 1.6
6	100	5	3	0	99.8 ± 0.2
7	10	15	3	0	27.0 ± 1.5
8	100	15	3	0	99.7 ± 0.0
9	10	5	0	15	28.7 ± 1.3
10	100	5	0	15	99.3 ± 0.2
11	10	15	0	15	27.5 ± 1.5
12	100	15	0	15	99.5 ± 0.1
13	10	5	3	15	19.9 ± 1.5
14	100	5	3	15	99.0 ± 0.5
15	10	15	3	15	19.9 ± 2.7
16	100	15	3	15	99.1 ± 0.1

for the 2⁴ full factorial experimental design, are in Table 4. Runs 17–32 are duplicates of runs 1–16 to perform statistical analysis.

Table 4. T	he harvesting efficiency	$_{\prime}$ obtained in the 2 4	full factorial	design experiment.	

The results were analyzed by ANOVA (p < 0.05) to assess the goodness of fit, and the main effects and interaction analysis between the factors are presented in Table 5. The effects indicate the significance of each variable. The signs + and – indicate that the variable directly or indirectly influences the harvesting efficiency, respectively. For instance, increasing the settling time from 5 to 15 negatively affects efficiency. According to the *p*-values obtained, the Zetag dosage and the RM and SM times are the most significant variables affecting the flocculation process.

Table 5. Effects and factor interaction analysis for C. vulgaris flocculation with Zetag 8185.

Factor	Effects	<i>p</i> -Value
Dosage (A)	57.006	0.000
Settling time (B)	-0.156	0.776
RM time (C)	28.844	0.000
SM time (D)	29.431	0.000
$A \times B$	0.331	0.548
$A \times C$	20.706	0.000
$A \times D$	19.744	0.000
$B \times C$	0.144	0.793
$B \times D$	-0.319	0.563
$C \times D$	-33.544	0.000
$A \times B \times C$	-0.094	0.864
$A \times B \times D$	0.494	0.374
$A \times C \times D$	-16.556	0.000
$B \times C \times D$	-0.019	0.973
$A \times B \times C \times D$	0.069	0.900

An empirical model (Equation (3)) describing the harvesting efficiency was obtained by eliminating the terms that are statistically insignificant (p > 0.05) that correspond to the terms that include the settling time. The R² coefficient obtained was 0.9987, indicating that the model appropriately fitted the data. Based on the experimental results, the harvesting efficiency of *C. vulgaris* was found to be optimum at a Zetag 8185 dosage of 100 mg L⁻¹, an RM time of 3 min, and without a SM phase. The settling time was not significantly relevant. The contour diagrams with the effects of the interactions between the statistically significant factors (Dosage vs. RM time; Dosage vs. SM time; and RM time vs. SM time) on the flocculation efficiency are in Figure 2. It is possible to observe that the diagrams Dosage vs. RM time and Dosage vs. SM time are very similar. In fact, from the diagram for RM time vs. SM time, it is clear that there is no need to include two mixing phases when Zetag 8185 is used as a flocculant, a simplification which would reduce time and energy costs at an industrial level, further facilitating the process of harvesting microalgae by flocculation.



Figure 2. Contour diagrams of *C. vulgaris* harvesting efficiency as a function of the statistically significant factors: (**a**) Dosage vs. RM time; (**b**) Dosage vs. SM time; (**c**) RM time vs. SM time.

4. Conclusions

Different organic and inorganic flocculants were studied for the purpose of harvesting *C. vulgaris* in batch mode. In general, organic flocculants needed small dosages to initiate floc formation. Zetag 8185 showed the best performance, only requiring a dosage of 11 mg g_{DW}^{-1} (4 mg L⁻¹). Ferric chloride was unable to harvest the strain in question, reaching only an efficiency of 9% for 100 mg L⁻¹. On the other hand, Zetag reached efficiencies close to 100% for 50 and 100 mg L⁻¹ dosages. Based on the empirical model obtained, the harvesting efficiency of *C. vulgaris* was optimal at a dosage of 100 mg L⁻¹ of Zetag 8185, 3 min of rapid mixing, and without a slow mixing step. The settling time was considered to be statistically insignificant. There is no universal harvesting process, hence, factors such as the microalgal species, cultivation system used, and the purpose of the biomass or product produced by the microalgae must be considered when choosing, for instance, a flocculant.

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