

EFFECT OF FLOCCULATION ON LIPID EXTRACTION FROM *CHLORELLA VULGARIS* UTEX 1803 USING RESPONSE SURFACE METHODOLOGY

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Abstract

Microalgae are an attractive source of metabolites, such as lipids, proteins, pigments and carbohydrates, of potential interest to the pharmaceutical, food and energy sectors. The aim of this study was to evaluate the effect of flocculation on lipid extraction from *Chlorella Vulgaris*. A 3³ experimental design was performed with STATISTICA 7.0 software in order to determine the effects of flocculant (AlCl₃) addition, pH and time. The best strategy of pH adjustment-flocculant addition was evaluated, followed by lipid extraction under optimal conditions. pH adjustment after the addition of flocculant provided higher flocculation efficiencies (87.2-98.9%) compared to adjustments made before adding the flocculant (67.8-85.9%). Experiments performed according to the experimental design led to a flocculation efficiency of 99.7% when 100 mg/L of AlCl₃, pH 7 and a cultivation time of 18.3 days were used. The percentage of a lipid extract from the flocculated biomass was 2.7% and the flocculant did not affect the production of fatty acid methyl esters.

Keywords: *Chlorella vulgaris*, Flocculation, Lipid extraction, Microalgae, Sedimentation.

1. Introduction

Energy is essential for any household, industrial and agricultural applications [1]. Energy consumption is constantly increasing all over the world due to population growth, hence, many researches have focused on sustainable alternatives for replacing fossil fuels [2-5]. Joshi et al. [6] reported that among alternative energy sources, biofuels are gaining increasing attention because of their sustainable and environmentally friendly features. In addition, they can be easily obtained from different types of biomass [7], including by-products and wastes [8].

Microalgae are considered as one of the most promising lipid feedstocks due to their high photosynthetic efficiency, biomass productivity and oil content [9]. From these photosynthetic microorganisms, several types of renewable biofuels, such as methane, biodiesel and hydrogen, can be obtained [10]. Compared to common oil plants, microalgae can produce much more lipids and can potentially satisfy the world demand for biodiesel [11]. In addition, novel green technologies have recently been developed, i.e., which provide a significant enhancement in lipid recovery [12, 13]. However, in order to carry out the production of biodiesel, the biomass must be separated from the culture medium through appropriate methods, such as centrifugation [14], filtration [15], flotation [16] and flocculation [17].

According to Borges et al. [18], from a technical and economical point of view, flocculation is often the preferred option, since it allows the collection of biomass in large quantities and at low cost. Furthermore, it is easily scalable and can be used for a wide variety of microalgae species. Flocculation acts on the aggregation of suspended cells to form larger particles. The flocculant molecules interact with the surface charges of microalgae causing their neutralization and facilitating cell settling [19]. Flocculation by metal salts can be carried out under acidic or alkaline conditions [20]. Wu et al. [21] showed that, in a study performed on three species of freshwater microalgae and two marine species, in which, flocculation efficiencies of up to 90% can be achieved by increasing the pH of the medium at low to medium concentrations of biomass. This result can be explained by the fact that, under alkaline conditions, the Mg^{2+} ions present in the growth medium can form magnesium hydroxide precipitates trapping the cells and favouring sedimentation. Factors like pH, biomass concentration, type and amount of flocculant can influence the flocculation efficiency of microalgae and lipid recovery [20-22].

The objective of this paper was to evaluate the effect of flocculation with $AlCl_3$ on the production of lipids for freshwater species, *Chlorella Vulgaris* UTEX 1803. Scaling effects for biomass flocculation were also investigated.

2. Experimental

2.1. Culture methods

Chlorella Vulgaris UTEX 1803 was obtained from the strain collection of the University of Texas (USA) and cultivated in Bold's Basal Medium (BBM). The main components of BBM (in mg/L) were: $NaNO_3$ (2.94), $MgSO_4 \cdot 7H_2O$ (3.04×10^{-1}), $NaCl$ (4.28×10^{-1}), K_2HPO_4 (4.31×10^{-1}), KH_2PO_4 (1.29), $CaCl_2 \cdot 2H_2O$ (1.70×10^{-1}), H_3BO_3 (1.85×10^{-1}), EDTA (1.71×10^{-1}), KOH (5.53×10^{-1}) and $FeSO_4 \cdot 7H_2O$ (1.79×10^{-2}). The culture was kept in a 12 L glass reactor coupled to a bubble aeration system for air injection ($0.6 L_{air}/L_{media}$) with a light/dark cycle of 12:12 h.

2.2. Experimental design

Borges et al. [18] described that the experimental design used was based on the procedure and carried out with STATISTICA 7.0 software. The effects of the following variables were evaluated: cultivation time (5, 10 and 15 days), pH (4, 7 and 10) and flocculant concentration (50, 100 and 150 mg/L).

2.3. Determination of optimal conditions for addition of flocculant and pH adjustment

Since pH has a significant effect on flocculation [23], preliminary experiments were carried out to determine if the optimal flocculation conditions were achieved by stabilizing the pH before or after the addition of the flocculant. A solution of 40 g/L of aluminium chloride (AlCl_3) was used as a flocculating agent. Aliquots of 50, 100 and 150 mg/L of flocculant were added to 100 mL of samples, with three replicates for each treatment. The pH was adjusted to 4, 7 and 10 with 1 M HCl or 1 M NaOH. The experiments were performed by adding the flocculant before or after pH adjustment. The samples were magnetically stirred for 2 min at 500 rpm followed by slower mixing at 60 rpm for 15 min to ensure hydrolysis of flocculant and aggregate formation [20, 24]. Then, the pH was measured and aliquots of 10 mL were taken to monitor the optical density of the solution at 500 nm. Data points were taken over 5 h at 1-h intervals.

2.4. Flocculation tests for the experimental design

Chlorella Vulgaris was cultivated with an inoculum of 1 g/L wet biomass. Flocculation tests were carried out under the experimental design conditions. Addition of flocculant, pH adjustment and agitation of samples were performed according to the optimal conditions found in the experiments described in the previous section. Dry weight biomass concentration was determined from absorbance measurements [25].

Flocculation efficiency was calculated using the following equation:

$$\text{Efficiency (\%)} = \left(1 - \frac{\text{Final absorbance}}{\text{Initial absorbance}}\right) \times 100 \quad (1)$$

while the concentration of the recovered biomass was obtained from the following mass balance:

$$\text{Biomass recovered (g/L)} = \text{Initial biomass} - \text{Final biomass} \quad (2)$$

2.5. Experimentation for evaluation of scaling effects

After determining the optimal flocculation conditions through the development of the experimental design, the process scaling was investigated in culture volumes of 100, 250, 500 and 1000 mL. For the volume of 1000 mL, the flocculated biomass was separated from the supernatant by decantation prior to performing lipid extraction.

2.6. Influence of the amount of metals and other chemicals used for flocculation

Ash quantification [26] was performed to determine the influence of hydroxides, metal salts and other chemicals used for flocculation and pH adjustment. Samples of supernatant and flocculated biomass were taken and weighed together with

control samples of biomass and supernatant without flocculant. The samples were put in an oven at 105 °C for 24 h. A muffle furnace was then used to obtain ashes. In particular, biomass samples were heated to 105 °C for 12 min, then at 250 °C for 30 min and finally at 575 °C for 180 min, according to the analytical procedure described elsewhere [27]. Ash content was calculated as:

$$\text{Ash (\%)} = \frac{\text{Ash weight}}{\text{Initial sample weight}} \times 100 \quad (3)$$

2.7. Lipid extraction

Flocculated biomass was dried in an oven at 60 °C for 12 h. The dried biomass was weighed, homogenized, wrapped in a filter paper and introduced into a Soxhlet tube. The flask was weighed and filled with 250 mL of n-hexane. Soxhlet extraction was performed at 69 °C for 16 h and the extracted lipids were weighed and stored in sealed vials for further characterization. The percentage of extracted lipids was calculated from the final and initial mass in the flask as:

$$\text{Lipid (\%)} = \frac{\text{Final mass} - \text{Initial mass}}{\text{Initial mass}} \times 100 \quad (4)$$

This procedure was also performed on the biomass concentrated by centrifugation at 3400 rpm for 15 min.

2.8. Analysis of extracted lipids

Analysis of fatty acids was carried out by gas chromatography on their methyl esters. Analytical determinations were performed according to NTC 4967 and NTC 5013 standards on two samples of lipid extracts obtained from the biomass flocculated with AlCl₃. A gas chromatograph (GC) AT 7890N (Agilent Technologies, Palo Alto, California, USA) with a flame ionization detector (FID) was used. The GC contained an HP 88 column (J & W Scientific, Folsom, CA, USA) (88%-cyanopropyl-aryl-polysiloxane, 60 m × 0.25 mm × 0.20 μm). The injection port was operated in split mode with a split ratio of 10:1 and a sample volume of 2 μL.

3. Results and Discussion

3.1. Addition of AlCl₃-pH adjustment

The procedure for pH adjustment showed an initial acidification of samples when the flocculant was added. This can be explained by considering that the hydrolysis reaction of metal salts leads to the formation of metal hydroxides, in this case aluminum hydroxides. From the hydrolysis reaction, cationic products are formed that are strongly adsorbed on negative particles, causing their destabilization [25]. Hydrolysis is particularly prominent in acidic media. Thus, additions of AlCl₃ reduce to a lower extent the pH of samples characterized by higher pH values. As the pH increases, less positively charged hydroxides are produced, hence, AlCl₃ reduces the pH to a lower extent in samples at more alkaline pH values. As is shown in Fig. 1(a), no significant pH variations were observed after 1 h, when flocculation started. When the flocculant was initially added in Fig. 1(b), the pH decreased in equal proportions in all samples, because their initial pH values were the same. Then, the pH was adjusted according to the conditions established to start the flocculation, and no significant changes were observed throughout the process. In both cases, the pH was stable during flocculation, regardless of the pH value. Therefore, the optimal method was selected by considering the highest flocculation efficiency.

Figure 2 shows that the efficiency of biomass recovery was always higher when the pH was adjusted after the addition of flocculant. At present, no reports are available in the literature on this strategy, being the pH usually adjusted before adding the flocculant. As can be seen, a pH adjustment after addition of $AlCl_3$ resulted in efficiencies ranging from 87.2% to 98.9%, while in the second case the efficiencies varied between 67.8% and 85.9%. For this reason, the design of experiments was developed using the former strategy.

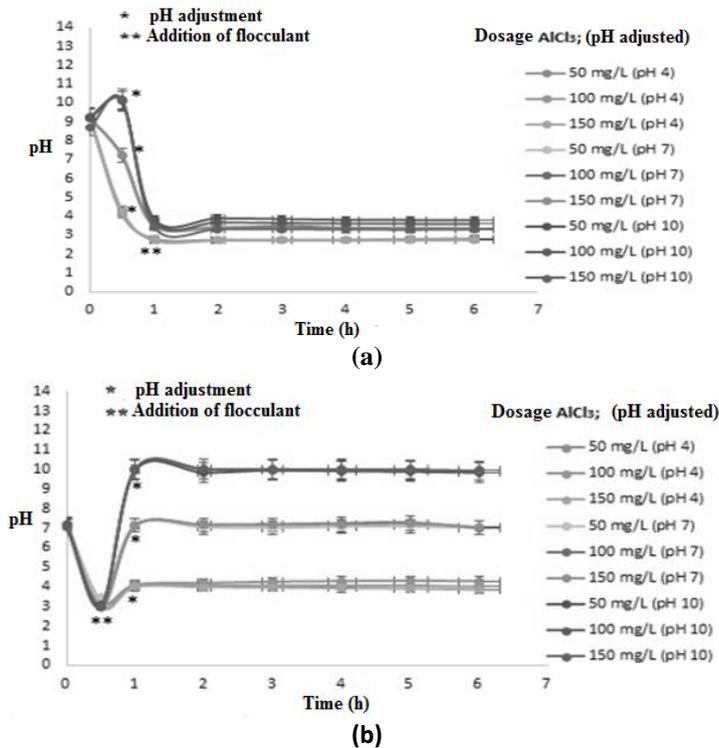


Fig. 1. Variation of pH with time: (a) pH adjustment before addition of $AlCl_3$ and (b) pH adjustment after addition of $AlCl_3$.

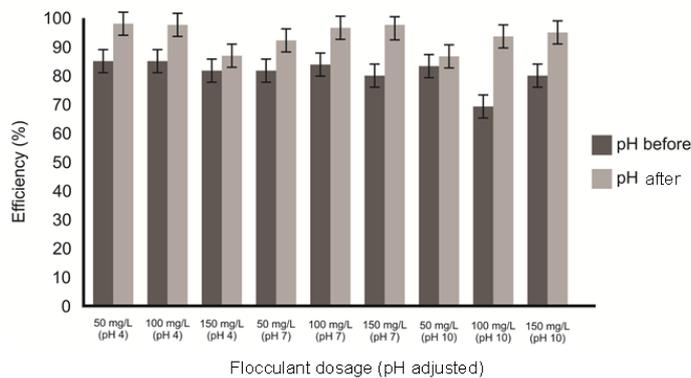


Fig. 2. Flocculation efficiencies for two methods of flocculant addition-pH adjustment.

3.2. Flocculation development according to experimental design

As shown in Table 1, several tests of the experimental design gave flocculation efficiencies greater than 95%. The highest values were obtained in test #8 (99.5%) and test #17 (99.7%). Since the flocculant concentration and the pH at the start of the process were the same for both tests, the conditions of test #17 were considered as the optimal ones. In all tests, the pH value at the end of the process was very close to that at the beginning of flocculation.

Overall, neutral and basic pH resulted in higher efficiency. The high efficiency values at pH 7 in Figs. 3(a), (c) and (d) are likely due to effective charge neutralization and precipitation of large amounts of aluminum hydroxides [28]. At alkaline pH as in Figs. 3(b), (c) and (d), high cell removal occurs because the use of a base for the adjustment of pH leads to the formation of polynuclear aluminum hydroxide compounds.

These compounds can neutralize the charges of colloidal hydroxide particles, contributing to the formation of precipitated hydroxides. Ohman et al. [29] consistently with this, carried out the experimentation for the neutralization of aluminum by addition of a base, which showed that settleable solids were formed at pH 7 and higher.

Based on studies by Henderson et al. [28], it was shown that charge neutralization of the biomass is not necessary to obtain efficiencies above 90%, as the sedimentation can occur through sweep flocculation mechanisms. As apparent from Figs. 3(b), (c) and (d), the efficiency decreased at acidic pH values, since under these conditions the cells are not sufficiently destabilized to result in massive flocculation and sedimentation.

Table 1. Flocculation efficiencies and pH at end of process.

Test	Cultivation time (days)	AlCl ₃ (mg/L)	pH	Efficiency (%)
1	1.6	100.0	7.5	98.6
2	5.0	50.0	4.1	66.2
3	5.0	50.0	4.0	63.3
4	5.0	150.0	9.7	93.8
5	5.0	150.0	9.9	95.2
6	10.0	16.3	7.4	98.9
7	10.0	100.0	2.0	78.6
8	10.0	100.0	6.6	99.5
9	10.0	100.0	6.6	99.5
10	10.0	100.0	6.6	99.5
11	10.0	100.0	11.8	97.7
12	10.0	183.6	7.2	99.1
13	15.0	50.0	4.3	67.2
14	15.0	50.0	4.2	61.4
15	15.0	100.0	9.5	88.8
16	15.0	100.0	9.8	94.7
17	18.3	100.0	7.5	99.7

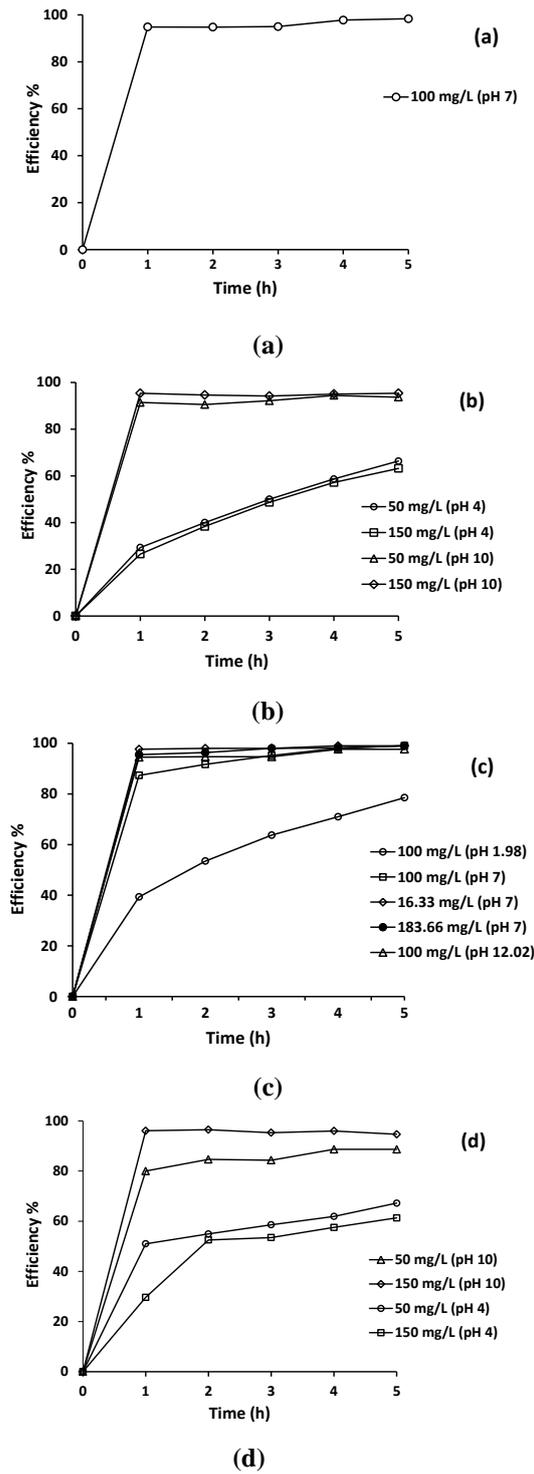


Fig. 3. Flocculation efficiencies for the two methods of flocculant addition-pH adjustment.

3.3. Statistical analysis

In order to analyse the influence of the variables considered in the experimental design on the amount of recovered biomass, a Pareto diagram was constructed as shown in Fig. 4. It can be seen that cultivation time was the most significant factor, with a large positive effect on biomass recovery, suggesting that this latter is primarily affected by the initial biomass concentration.

The response surface in Fig. 5 shows that the highest biomass recovery was achieved at long cultivation times and high pH values, approximately between 7 and 14.

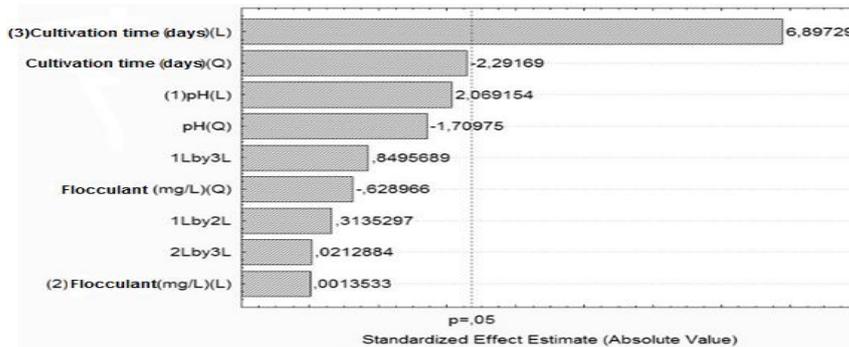


Fig. 4. Pareto diagram for recovered biomass.

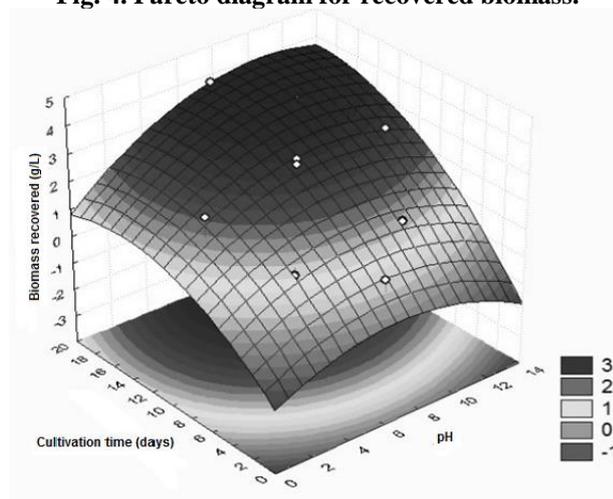


Fig. 5. Response surface for recovered biomass: Dependence of amount of recovered biomass on pH and cultivation time.

3.4. Evaluation of flocculation under optimal conditions for different sample volumes

Experiments performed under optimal conditions by varying the culture volume showed that the process can be easily scaled-up to volumes greater than 100 mL without significant changes in flocculation efficiency in Table 2. In particular, the efficiency remained almost constant, at or above 99.5%, when the volume was increased up to 1000 mL.

Table 2. Flocculation efficiency and amount of biomass recovered at different culture volumes.

Volume (mL)	Efficiency (%)	Biomass recovered (g/L)
100	99.7	4.2
250	99.5	4.2
500	99.5	4.2
1000	99.6	4.2

3.5. Ash content in flocculated samples

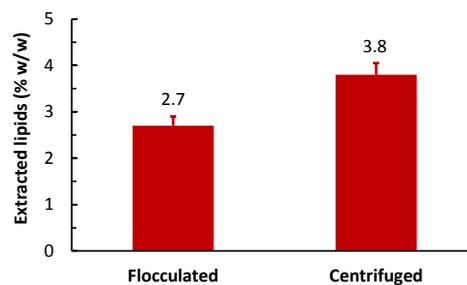
In Table 3 it shows that the ash content of flocculated samples was only slightly affected by the culture volume. The higher values observed in flocculated samples, compared to control samples, can be explained by the presence of the flocculant and the use of sodium hydroxide for adjusting the pH. The amount of ash in the biomass of flocculated samples was higher than that in the supernatant, which can be ascribed to the precipitation of hydroxides, causing cell sedimentation.

Table 3. Ash content of flocculated and control samples at different culture volumes.

Volume (mL)	Sample	Flocculated sample ash (%)	Control sample ash (%)
100	Supernatant	0.18	0.02
	biomass	0.40	0.08
250	Supernatant	0.15	0.02
	biomass	0.32	0.08
500	Supernatant	0.16	0.02
	biomass	0.35	0.08
1000	Supernatant	0.16	0.02
	biomass	0.30	0.08

3.6. Lipid extraction

From Fig. 6, it can be seen that the centrifuged biomass resulted in a higher percentage of extracted lipids (3.8%) compared to the flocculated sample (2.7%). Lee et al. [30] found that these results are similar to those who extracted about 4% (by weight) of lipids from *Chlorella Vulgaris* cells harvested by centrifugation. Despite the amount of lipids extracted from flocculated samples is lower than that from centrifuged samples, the advantages on biomass recovery offered by flocculation should be taken into account when considering the industrial process development.

**Fig. 6. Comparison of the percentage of extracted lipids in flocculated and centrifuged biomass samples.**

3.7. Characterization of extracted lipids

Quantification of fatty acid methyl esters gave the results presented in Table 4. The total amount of methyl esters was 52.8% in the original sample and 57.1% in the replicated one, which indicates that the flocculant does not affect the ability of *Chlorella Vulgaris* to produce lipids.

Nervonic, docosadienoic, arachidonic and heneicosanoic acids were the most abundant (>5%) fatty acids, followed by the others listed in the table.

Table 4. Quantification of fatty acid methyl esters in lipid extracts.

	Relative amount	
	Original %	Replica%
Undecanoic (C11:0)	0.1	-
Lauric (C12:0)	0.3	-
Tridecanoic (C13:0)	0.5	0.9
Myristic (C14:0)	1.2	1.3
Pentadecanoic (C15:0)	1.6	2.0
Palmitic (C16:0)	2.1	2.3
Palmitoleic (C16:1)	-	2.4
Heptadecanoic (C17:0)	2.8	2.3
Stearic (C18:0)	3.1	2.6
Linoleic (C12:2n6c)	3.3	3.5
Arachidic (C20:0)	3.9	3.8
Heneicosanoic (C21:0)	5.0	5.5
Arachidonic (C20:4)	7.5	8.7
Docosadienoic (C22:2n6)	10.0	11.5
Nervonic (C24:1)	11.4	10.3
Total	52.8	57.1

4. Conclusions

In this research, the effects of flocculation on lipid extraction from *Chlorella Vulgaris* microalgae were investigated. The flocculant used ($AlCl_3$) did not affect the production of lipids by the microalgae, suggesting that it can be effectively utilized to preconcentrate the biomass prior to lipid extraction. Adjusting the pH after the addition of flocculant was found to be the best strategy to achieve high flocculation efficiencies. Under optimal process conditions, with a sufficiently high biomass concentration, up to 99.7% of the biomass was recovered.

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