Effect of Process Parameters on Chitosan-mediated Microalgae Flocculation

S. Akış, B. İnan, and D. Balkanlı Özçimen*
Bioengineering Department, Faculty of Chemistry and Metallurgy, Yildiz Technical University, Esenler, 34220, Istanbul, Turkey

doi: https://doi.org/10.15255/CABEQ.2022.2152

Original scientific paper
Received: October 18, 2022
Accepted: May 2, 2023

Today, microalgae have received considerable interest as an alternative feedstock for biofuel, animal feed, human food, and pharmaceuticals because they possess valuable oils and biomolecules. The main problem of the cost of microalgae production is generally associated with the harvesting process. Flocculation is an effective method to harvest microalgal biomass and minimize the operating cost. In this study, the effect of the chitosan solution, different pH conditions, and flocculation time on flocculation process of *Chlorella minutissima* and *Nannochloropsis oculata* were investigated, and the obtained data were evaluated statistically. Flocculation efficiency of *C. minutissima* and *N. oculata* were the highest under the conditions of 10 pH, 100 mg L^{-1} chitosan concentration, and flocculation time of 60 min, and found as 97 % and 85 %. It was also found that chitosan flocculation could be improved with pH increase. This study showed that chitosan is a favorable flocculant because of its high efficiency, being non-toxic, and enabling the reusability of the growth medium after flocculation.

Keywords
N. oculata, C. minutissima, flocculation, chitosan, microalgae

Introduction

Separating microalgae from the growth medium emerges as the biggest problem in the microalgae process. High biomass concentration causes the microalgae cells to shade each other, and use only the growth media to grow algae at low concentrations. Microalgae are used for high-value products, which exhibit antibacterial, antifungal, anticoagulant, antiviral, antioxidant, anticancer, and anti-inflammatory properties.[1] In order to obtain high-value products, harvesting of microalgae is generally carried out by centrifugation. However, if the biomass is processed for lower valued products, such as biofuels, centrifugation for harvesting is quite expensive and requires a lot of energy. It is important to find an alternative technology that requires minimal cost to harvest microalgae from large-scale cultivation, to reduce process cost, and increase microalgae biomass production.[2–5] Flocculation has come into prominence for microalgal production to reduce the operating costs, which mainly consist of dewatering process due to its effectiveness, maturity, and energy-efficiency.[6] Flocculation process can be performed via pH adjustment, using flocculants such as aluminum and ferric salts, natural polymers such as chitosan or taking advantages of fungus or bacteria which induce flocculation biologically.[7–9] Flocs can be formed by flocculants such as aluminum and ferric salts with microorganisms such as microalgae.

Chitosan is a natural biopolymer, one of the most abundant in the world, and is a linear copolymer synthesized by the alkaline deacetylation of chitin. It is a cationic polyelectrolyte due to free amino groups, which are protonated in acidic medium. Being a cationic biopolymer, it enhances flocculation, including efficient charge neutralization and bridging effects.[10] Since microalgae cells are charged negatively, charge neutralization occurs during the interaction within chitosan and microalgae cells, which causes flocculation of microalgae cells. In the literature, studies investigate flocculation of microalgae cells using chitosan.[9,11–13] These studies show efficient flocculation performance of chitosan for some species of microalgae. However, without a statistical evaluation, it is hard to assess the effect of chitosan under different conditions, examine the relation between process parameters, and see the interactive effects of chitosan and other parameters such as pH, especially. In order to interpret the results comprehensively, a statistical evaluation of the flocculation process should be carried out.

Although there are a few studies on flocculation of *Chlorella* sp. and *Nannochloropsis oculata,* with chitosan, no study evaluates the effect of experimental parameters (chitosan concentration, pH, and flocculation time) and their interactive effects

*Corresponding author: ozcimen@yildiz.edu.tr, didem.ozcimen@gmail.com; Phone : +90 212 383 4635

on flocculation efficiency with analysis of variance. In this study, *Chlorella minutissima* and *N. oculata* were flocculated via different concentrations of chitosan solution under different pHs and flocculation times. These microalgae species were selected to examine the effects of parameters on flocculation of both freshwater and marine microalgae. In addition, utilizability of the microalgal cells and culture medium that remain after this dewatering process were investigated. Box-Behnken experimental design was used to examine the results statistically. In the literature, it was observed that the remaining chitosan residue in the medium after collecting microalgae is not investigated in most of the studies. However, whether chitosan remains in the solution or becomes floc with the microalgae may change the results of further applications. Therefore, in this study, the presence of chitosan in the remaining medium was also tested after flocculation of microalgae.

Materials and methods

Microalgae cultivation

C. minutissima and *N. oculata* were selected as fresh and marine microalgae for the flocculation experiments. Cultures were obtained from Algal Biotechnology Laboratory of Yildiz Technical University, TURKEY. *N. oculata* and *C. minutissima* were grown in f/2 medium and BG-11, respectively. Microalgae were cultivated in a 250-mL glass flask at 25±3 °C under continuous white illumination (8000 lx) for 15 days. Initial number of living microalgae cells was $7.02–8.01 \cdot 10^5$ mL^{-1}. Microalgal growth was measured by UV–visible spectrophotometer (PG Instruments T60, UK) at the wavelength of 680 nm. When the cells entered the stationary phase on day 15, flocculation with chitosan was carried out in triplicate.

Flocculation experiments

Box-Behnken experimental design was carried out to investigate the effect of the process parameters on concentration of chitosan solution, pH, and flocculation time. To evaluate the effects of these parameters on flocculation, different concentrations of chitosan solutions were added to the microalgal cultures in beakers. The pH of the cultures were adjusted within the range of 8–10 using 0.1 M NaOH solution. The samples were then mixed at 100 rpm, and different flocculation times were measured at 750 nm at room temperature. Efficiency of flocculation was determined with the following equation:

$$\text{Flocculation efficiency}(\%) = (1 - A/B) \cdot 100 \quad (1)$$

A: Absorbance of the culture suspension at different times during the flocculation experiment

B: Absorbance of original culture suspension at the beginning.

The factors were studied at three levels and three replicates at the center points. The three factors and their levels are listed in Table 1.

Table 1 – *Parameters and their levels*

Parameter	Unit	–1	0	+1
Chitosan concentration (X_1)	mg L^{-1}	50	100	150
pH (X_2)		8	9	10
Flocculation time (X_3)	min	30	45	60

The following model was used:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (2)$$

where Y is a response; β_0, β_1, β_2, β_3, β_{12}, β_{13}, β_{23}, β_{11}, β_{22}, and β_{33} are constant factors. X_1, X_2, X_3, which are defined as the independent factors, are the concentration of chitosan, pH, and flocculation time, respectively.

The best-fitting models were established via quadratic regression, and significant model parameters were chosen only by removing the insignificant model parameters from the models. The computational work, including the designation of experimental points, randomization, analysis of variance fitting of the quadratic models, and graphical representations, as well as optimization, was performed using a statistical package Design-Expert version 7.0 (Stat-Ease In., Minneapolis, USA).

Determination of zeta potential

Malvern Zetasizer Nano ZS instrument was used to determine the zeta potentials of the microalgae culture. Zeta potential measurements were performed at ambient conditions, and three measurements were conducted.

Viability of harvested microalgae cells

Flocculated microalgal biomass was grown again under the conditions mentioned in the microalgae cultivation section to observe their viability and physiological activity. In order to observe the microalgae growth, the optical density of the cultures was determined at 680 nm. Moreover, biochemical content of the cultures was evaluated. Total carbohydrate content in microalgae was measured using the phenol-sulfuric acid method.[14] β-carotene and chlorophyll-α contents were also determined, as given in the study of Zou and Richmond.[15]

Utilizability of the culture medium

The remaining medium after harvesting of microalgal cells was investigated to determine whether the chitosan solution had changed the medium or it was adequate for the next growth process to decrease the microalgae cultivation cost. Approximately 90 % of the supernatant was recovered with flocculation, and whether the chitosan remained in the medium was investigated. The pH of the recovered solution, which was used as growth medium, was set to pH level which was optimal for microalgae cultivation, with 0.1 M HCl solution. Compounds used for the formulation of BG-11 and f/2 medium were added to the recycled medium, and a new medium for cultivation was prepared as a control. Cultures were grown under the same conditions mentioned in the microalgae cultivation section. The growth of microalgae was monitored using spectrophotometer at the wavelength of 680 nm.

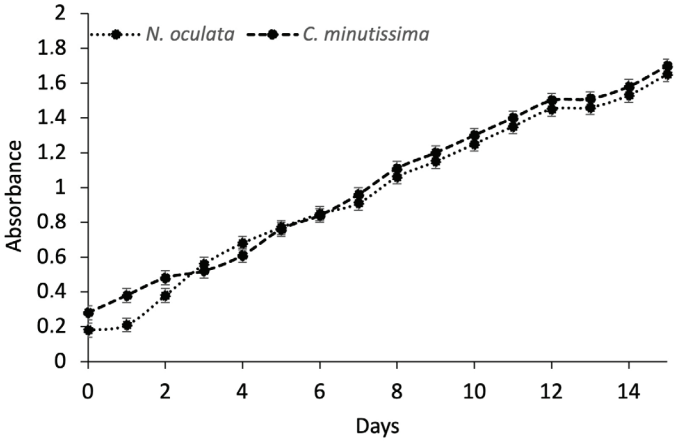

Fig. 1 – *Growth curves of C. minutissima and N. oculata*

Results and discussion

Effect of process parameters on flocculation efficiency

In this study, microalgae species were firstly grown until they reached stationary phase. The growth curves of the microalgal species are given in the Fig. 1. Statistical evaluation of the effects of the process parameters (chitosan concentrations, pH level, and flocculation time) on the flocculation efficiency for *C. minutissima* and *N. oculata* are given in Table 2. The highest flocculation efficiencies were determined as 85 % and 97 % for *C. minutissima* and *N. oculata,* respectively. The relationship between flocculation efficiency and the selected parameters was fitted to the quadratic regression model equation. Based on the coded levels of these parameters, regression model equations were represented as Eq. 3 and Eq. 4 for *C. minutissima* (CM) and *N. oculata* (NO), respectively.

$$Y_{\mathrm{FE,\,CM}} = 50.00 + 13.63X_1 + 6.62X_2 + 17.75X_3 - 4.00X_1X_2 - 10.25X_1X_3 + 16.75X_2X_3 + 6.50X_1^2 - 5.50X_2^2 + 0.75X_3^2 \quad (3)$$

$$Y_{\mathrm{FE,\,NO}} = 76.00 - 2.00X_1 + 31.75X_2 + 6.75X_3 - 2.50X_1X_2 + 11.00X_1X_3 - 11.25X_1^2 - 8.25X_2^2 - 6.25X_3^2 \quad (4)$$

Table 2 – *Flocculation efficiency of C. minutissima and N. oculata by chitosan under different flocculation times and pH conditions*

Batch number	Process conditions			Coded factors			Flocculation efficiency (%)	
	Chitosan concentration (mg L^{-1})	pH	Flocculation time (min)	X_1	X_2	X_3	*C. minutissima*	*N. oculata*
1	100	8	60	0	–1	1	38	33
2	50	8	45	–1	–1	0	24	20
3	150	9	30	1	0	–1	59	31
4	150	10	45	1	1	0	70	88
5	100	8	30	0	–1	–1	39	26
6	100	10	60	0	1	1	85	97
7	50	9	30	–1	0	–1	17	66
8	150	8	45	1	–1	0	65	30
9	100	10	30	0	1	–1	19	90
10	100	9	45	0	0	0	50	76
11	150	9	60	1	0	1	77	73
12	50	9	60	–1	0	1	76	64
13	50	10	45	–1	1	0	45	88

Table 3 – *ANOVA results of statistical evaluation for the flocculation process of N. oculata and C. minutissima*

	Sources of variations	Degree of freedom	Sum of squares	Mean square	F-value	Probability
C. minutissima	Regression model	9	6252.67	694.74	24.74	0.0116
	Error	3	84.25	28.08		
	Corrected total	12	6336.92			
N. oculata	Regression model	9	9292.69	1032.52	12.54	0.0306
	Error	3	247	82.33		
	Corrected total	12	9539.69			

Analysis of variance (ANOVA) method was used to study the design matrix and the main effects quantitatively. The ANOVA results of the statistical evaluation are shown in Table 3. Table 3 shows that the R^2 values for flocculation of *C. minutissima* and *N. oculata* were found to be very close to 1. Furthermore, since the gap between Pred-R^2 and Adj-R^2 was less than 0.2, the values of Pred-R^2 were most likely in agreement with the values of Adj-R^2. Thus, the regression models were found to be important since the p values were less than 0.01. The coefficients of determination for the equations were calculated as 0.98 and 0.97, for *C. minutissima* and *N. oculata,* respectively. The response surface plots of *N. oculata* and *C. minutissima* flocculation using different concentrations of chitosan under different flocculation times and pH conditions are presented in Figs. 2–3.

As seen from equation (3), the coefficient of flocculation time was the highest, thus, its effect on flocculation was the strongest. It was found that, the chitosan concentration, pH, and flocculation time had positive influence on the flocculation efficiency. Interactive effects of X_1X_2 (chitosan concentration and pH), X_2X_3 (pH and flocculation time) and X_1X_3 (chitosan concentration and flocculation time) had considerable coefficient values. The interactive effects of chitosan concentration and pH, and chitosan concentration and flocculation time, affected flocculation negatively. Moreover, the square coefficient of the pH, which was also negative, was high compared to the coefficient of the individual and interactive effect of pH. Despite the fact that the pH affects flocculation of *C. minutissima* positively, it was found that increasing the pH level above 10, would affect the flocculation efficiency negatively, as can be seen with the effect of pH squared given in the equations. Xu *et al.* studied harvesting of green microalga *Chlorella sorokiniana* via flocculation using chitosan as flocculant. The author reported that almost 99 % efficiency was achieved under acidic conditions.[12] Morales *et al.* investigated harvesting of various microalgae species using chitosan. They found that by using high chitosan concentrations, flocculation efficiency of up to 100 % was achieved without changing the pH. When the pH was around 7.8–8.0, a full harvesting process was achieved with 40 mg L^{-1} or more chitosan concentrations. Nevertheless, it was stated that, in acidic conditions, 95–100 % flocculation efficiency was achieved using less concentrations of chitosan solution in comparison to the neutral conditions.[16]

On the other hand, as for the regression equation of the flocculation process of *N. oculata,* the coefficient of pH is the highest and its effect on the flocculation is the strongest. It was observed that, increasing the pH caused an increase in the flocculation efficiency. At pH 8, flocculation efficiency was very low, and it can be said that the chitosan concentration had little or no effect on the flocculation process. Similar to *C. minutissima,* flocculation time had positive effect on flocculation process. However, unlike *C. minutissima,* chitosan concentration had negative effect on the flocculation of *N. oculata.* It was seen that, up to a certain chitosan concentration (100 mg L^{-1}), flocculation efficiency increased with increasing pH and time. Above this concentration, the flocculation efficiency was still high in alkaline conditions, but it was lower than the flocculation efficiency of the experiment using 100 mg L^{-1} chitosan. This result showed that, at higher pH levels, flocculation process occurred with a mechanism independent of chitosan concentration, which was in agreement with the study of Blockx *et al.*[17] Blockx *et al.*[17] investigated the most suitable parameters for harvesting of *N. oculata* using chitosan. It was stated that, in order to hydronate the amine groups and induce the activity of flocculation toward charge-neutralization and bridging, acidic conditions are necessary in freshwater. In contrast, this process can be only performed effectively in alkaline conditions. According to Blockx *et al.*, in order to harvest *Nannochloropsis* using chitosan, higher concentrations of chitosan solutions were required in comparison to the freshwater species.[17] In another study, Farid *et al.* investigated the effect of chitosan and nano-chitosan on flocculation of *Nannochloropsis* sp. The authors re-

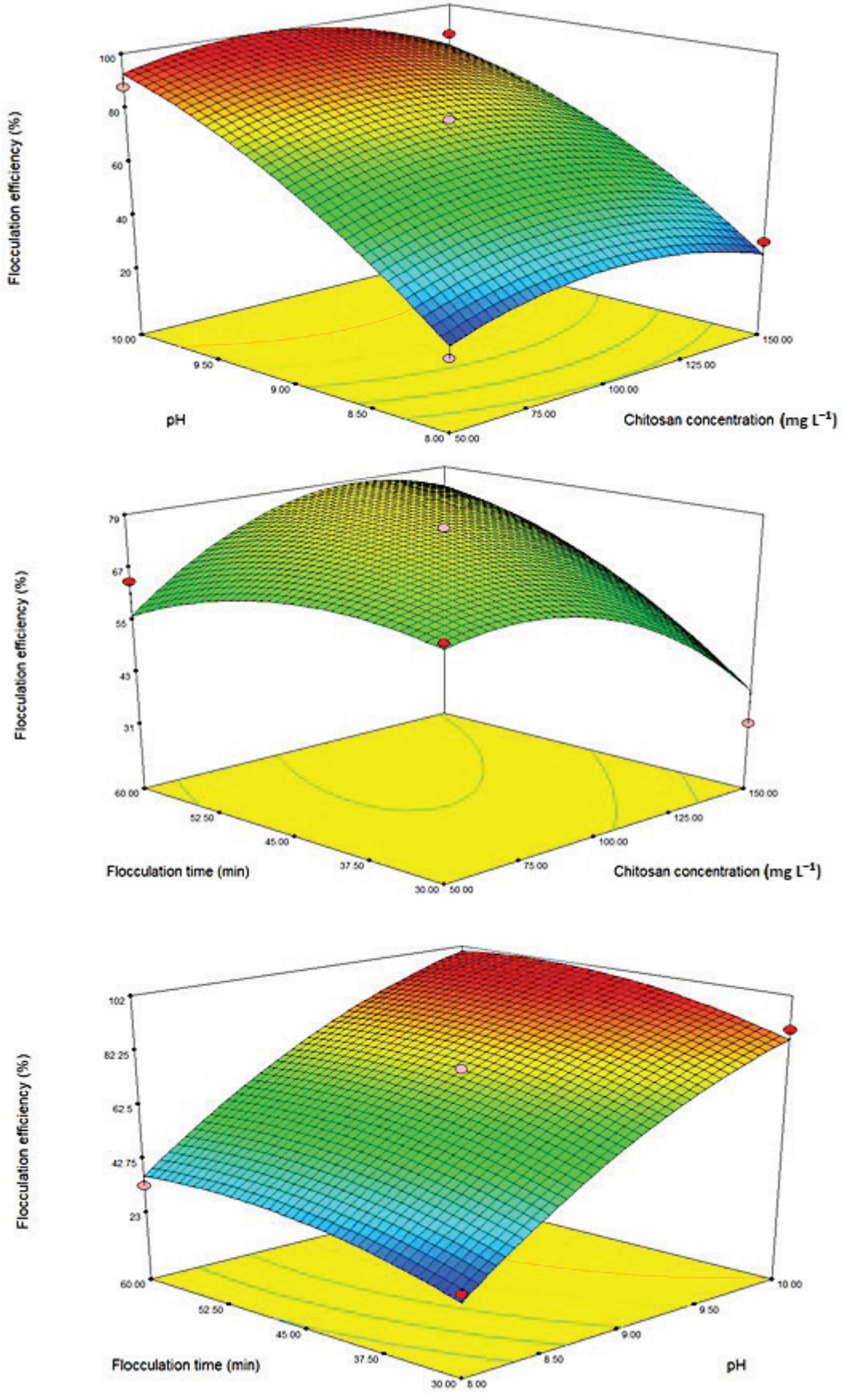

Fig. 2 – *Response surface plots showing flocculation of N. oculata at different concentrations of chitosan under different flocculation times and pH conditions*

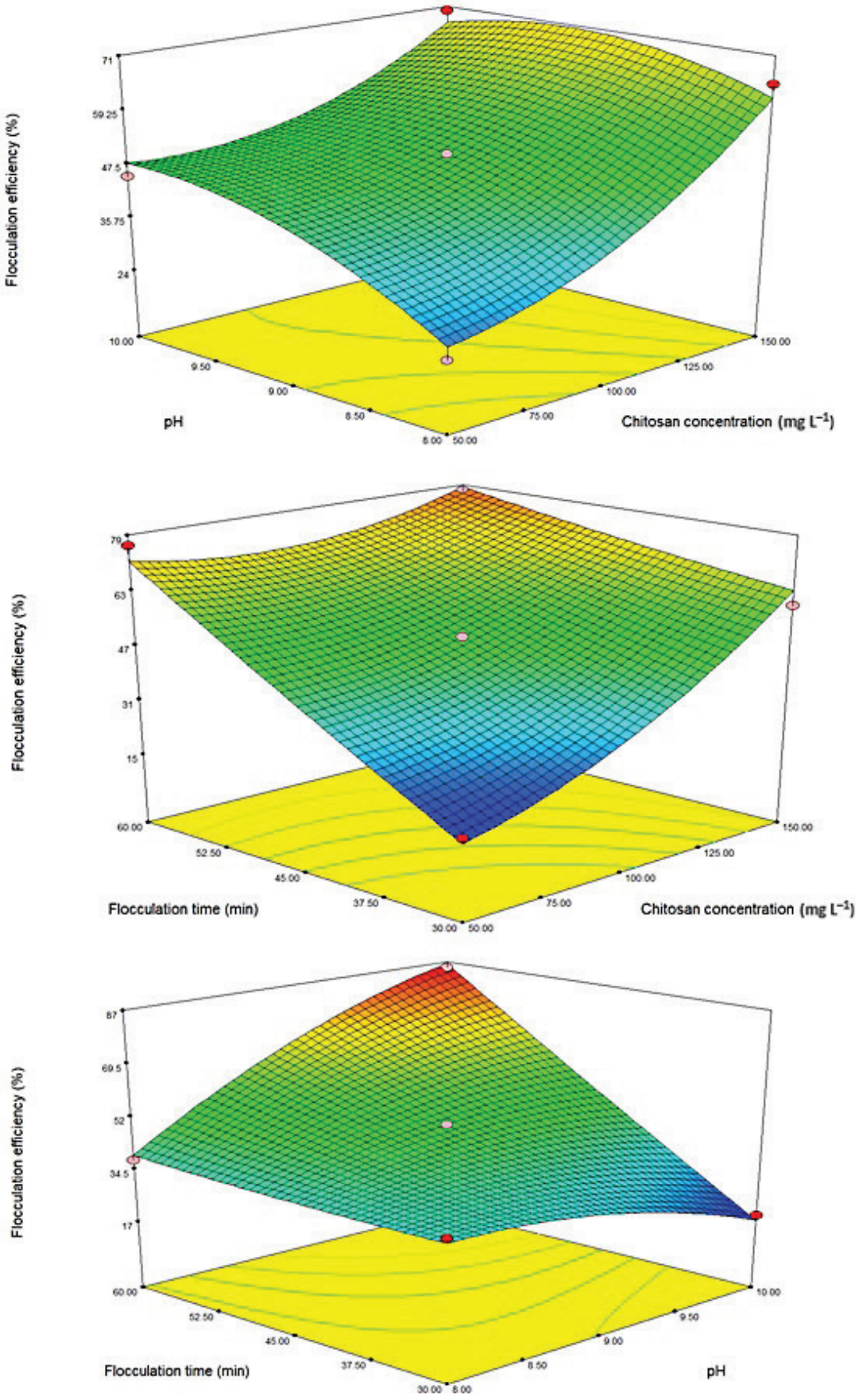

Fig. 3 – *Response surface plots showing flocculation of C. minutissima at different concentrations of chitosan under different flocculation times and pH conditions*

ported that flocculation efficiency carried out under the conditions of pH 9.0 and chitosan concentration of 100 mg L^{-1} was the highest. Similar to this study, a slight decrease was observed for flocculation efficiency when the chitosan concentration was 120 mg L^{-1}.[18]

As may be seen with the regression equations, harvesting of *C. minutissima* and *N. oculata* via chitosan flocculation were more affected by ionic strength or interactive effects of ionic strength and flocculation time. Therefore, different results can be obtained for different microalgae species, depending on their response to the chitosan concentrations. According to Sirin *et al.*, many parameters affect the efficiency of flocculation, such as pH and ionic strength of the environment, and type of microorganism to be harvested. The authors stated that, in alkaline pH, remarkable increase in flocculation with chitosan was observed. Because positive charge started to decrease (neutralization point at pH 7.9), the chitosan started to coil and then precipitate. The variation of the flocculation efficiency in chitosan experiments can also be explained with the results of using the remaining chitosan. To stabilize it, repulsion was performed between positively charged microalgae cells.[13]

After providing Eq. 3 and Eq. 4, the optimal conditions were predicted. As for *C. minutissima,* in order to achieve maximum flocculation efficiency, concentration of chitosan solution should be 55 mg L^{-1}, pH level, and flocculation time should be 10 and 59 min, respectively. As for the *N. oculata,* these conditions were predicted as concentration of chitosan solution of 80 mg L^{-1}, pH level of 10, and flocculation time of 46 min, respectively. In these process conditions, flocculation efficiency was predicted at 90 % and 97 % for *C. minutissima* and *N. oculata,* respectively.

Zeta potential measurement

Increase in the chitosan concentration increased the zeta potential of the microalgal culture. However, according to the results of the zeta potential determination, a negative correlation with the chitosan concentration was observed, as shown in Fig. 4. It can be stated that the separation of carboxylic acid groups caused this decrease, which induced the release of negative ions into the solution. In the literature, some studies report zeta potential decreases with increases in flocculant concentration, which is in agreement with this study.[9,19] According to Rashid *et al.*, flocculant dose affected zeta-potential negatively. They reported that a decrease in the zeta-potential was observed at high concentrations of chitosan solution. Such a result indicated that the positive charge of chitosan neutralized the negative charge on *C. vulgaris* cell.[9] Wu *et al.* also indicated

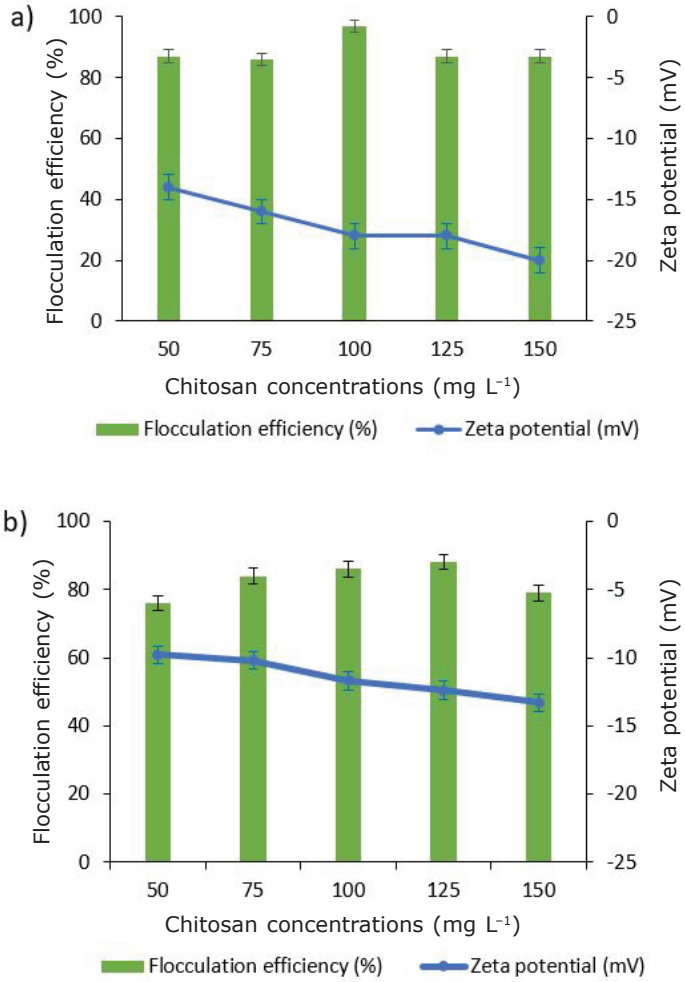

Fig. 4 – *Zeta potential of a) N. oculata, and b) C. minutissima after flocculation with chitosan at pH 10 for 60 min*

that the zeta potentials changed according to pH of the medium, tending to first decrease, and then increase.[19]

Viability of harvested microalgae cells

At this stage of the study, cells that were harvested with chitosan solution were grown again to observe their viability and changes in their biochemical content. As may be seen from Fig. 5, the microalgal cells began to proliferate, and no changes were observed in the molecular structure or function of the cells. These results are comparable to those reported in the literature.[20,21]

Besides the growth curves, biochemical content of the harvested and naturally sedimented microalgae were compared. Content of carbohydrate, chlorophyll-α, and β-carotene of *N. oculata* sedimented naturally were found as 27 %, 3.3 %, and 1.1 %, respectively. The contents of carbohydrate, chlorophyll-α, and β-carotene of *N. oculata* flocculated with chitosan were determined as 28 %, 3.2 %, and 1 %, respectively. As for the *C. minutissima,* the

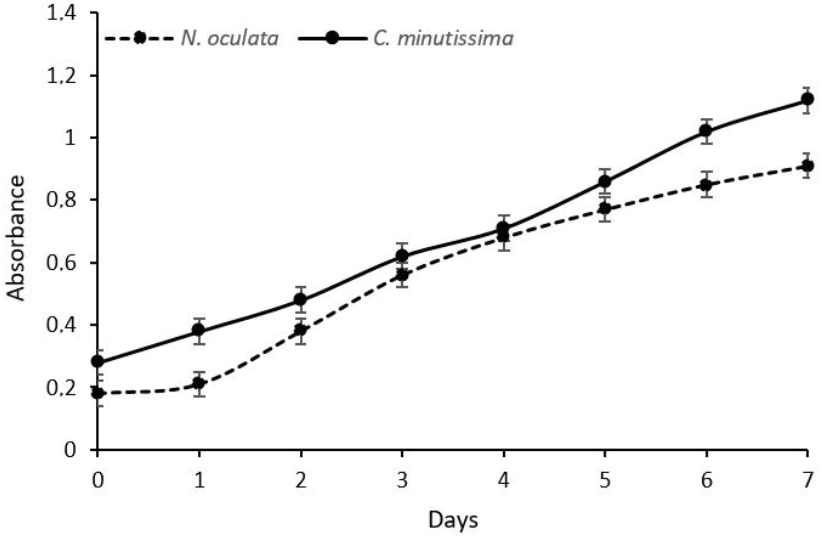

Fig. 5 – *Growth curves of N. oculata and C. minutissima obtained after flocculation process*

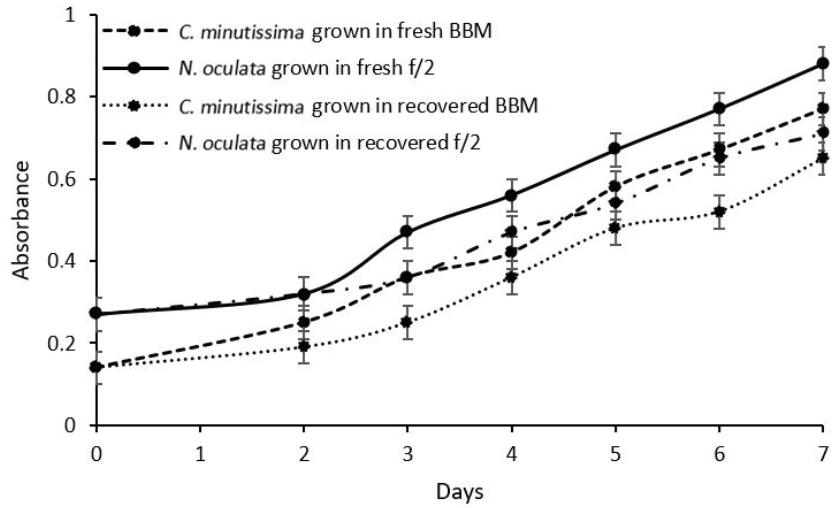

Fig. 6 – *Growth curves of N. oculata and C. minutissima cultivated in fresh medium and the recovered medium obtained after flocculation*

chlorophyll-α, β-carotene, and carbohydrate contents of naturally sedimented and flocculated cells were determined as 4.4 %, 1.7 %, and 14 %, and 4.5 %, 1.6 %, and 13 %, respectively. In the flocculation process carried out with chitosan, chitosan cannot be removed from the flocculated microalgae at the end of the process. Therefore, some amount of chitosan remains with the microalgal biomass.[17] However, no significant difference between the carbohydrate, clorophyll-α, and β-carotene contents of naturally sedimented and flocculated *N. oculata* and *C. minutissima* were observed.

Utilizability of the culture medium

The culture medium recovered after harvesting should be utilizable for the next cultivation in order to achieve a cost-effective microalgae cultivation. Apart from investigating the biochemical content and viability of the cells, it is required to evaluate whether the recovered medium was adversely affected by the chitosan solution. According to the method carried out by Blockx *et al.* in their study, at high pH, chitosan precipitation should be seen clearly in pure chitosan solutions in deionized water.[17] In this study, the recovered medium was evaluated for chitosan precipitation, and no precipitation was observed. The recovered medium was then evaluated for subsequent cultivations. Based on the results presented in Fig. 6, the microalgae growth carried out in the recovered solution and the freshly prepared solution was similar. Therefore, both microalgae species can be grown in recovered medium. These findings are in agreement with the existing literature.[19,21,22]

Conclusion

This study used chitosan for harvesting *C. minutissima* and *N. oculata* by flocculation. Based on the flocculation efficiency and reusability results, chitosan can be considered as a potential candidate for harvesting microalgae cultures. The results also showed that flocculation with chitosan was more complete at alkaline pH for the microalgae species used in this study. Statistical analysis indicated the difference in flocculation between marine and freshwater microalgae. Chitosan, being a natural biopolymer, is non-toxic and has no negative effects on the growth medium and microalgae cells. However, the use of chitosan in microalgae flocculation is unattractive due to its high costs. Therefore, microalgae flocculation using chitosan can be suggested only for obtaining high-value algal products.

References

1. *Vehapi, M., Koçer, A. T., Yılmaz, A., Özçimen, D.,* Investigation of the antifungal effects of algal extracts on apple-infecting fungi, Arch Microbiol **202** (2020) 455. doi: https://doi.org/10.1007/s00203-019-01760-7
2. *Schlesinger, A., Eisenstadt, D., Bar-Gil, A., Carmely, H., Einbinder, S., Gressel, J.,* Inexpensive non-toxic flocculation of microalgae contradicts theories; overcoming a major hurdle to bulk algal production, Biotechnol. Adv. **30** (2012) 1023. doi: https://doi.org/10.1016/j.biotechadv.2012.01.011
3. *Uduman, N., Qi, Y., Danquah, M. K., Forde, G. M., Hoadley, A.,* Dewatering of microalgal cultures: A major bottleneck to algae-based fuels, J. Renew. Sustain. Energy **2** (2010) 1. doi: https://doi.org/10.1063/1.3294480
4. *Grima, E. M., Belarbi, E.-H., Fernández, F. G. A., Medina, A. R., Chisti, Y.,* Recovery of microalgal biomass and metabolites: Process options and economics, Biotechnol. Adv. **20** (2003) 491. doi: https://doi.org/10.1016/S0734-9750(02)00050-2
5. *Hesse, M. C. S., Santos, B., Selesu, N. F. H., Corrêa, D. O., Mariano, A. B., Vargas, J. V. C., Vieira, R. B.,* Optimization of flocculation with tannin-based flocculant in the water reuse and lipidic production for the cultivation of *Acutodesmus obliquus*, Sep. Sci. Technol. **52** (2017) 936. doi: https://doi.org/10.1080/01496395.2016.1269130
6. *Shin, H., Kim, K., Jung, J. Y., Bai, S. C., Chang, Y. K., Han, J. I.,* Harvesting of *Scenedesmus obliquus* cultivated in seawater using electro-flotation, Korean J. Chem. Eng. **34** (2017) 62. doi: https://doi.org/10.1007/s11814-016-0251-y
7. *Vandamme, D., Foubert, I., Muylaert, K.,* Flocculation as a low-cost method for harvesting microalgae for bulk biomass production, Trends Biotechnol. **31** (2013) 233. doi: https://doi.org/10.1016/j.tibtech.2012.12.005
8. *Cheng, Y. S., Zheng, Y., Labavitch, J. M., Vandergheynst, J. S.,* The impact of cell wall carbohydrate composition on the chitosan flocculation of *Chlorella*, Process Biochem. **46** (2011) 1927. doi: https://doi.org/10.1016/j.procbio.2011.06.021
9. *Rashid, N., Rehman, S. U., Han, J. I.,* Rapid harvesting of freshwater microalgae using chitosan, Process Biochem. **48** (2013) 1107. doi: https://doi.org/10.1016/j.procbio.2013.04.018
10. *Yang, R., Li, H., Huang, M., Yang, H., Li, A.,* A review on chitosan-based flocculants and their applications in water treatment, Water Res. **95** (2016) 59. doi: https://doi.org/10.1016/j.watres.2016.02.068
11. *Morales, J., de la Noüe, J., Picard, G.,* Harvesting marine microalgae species by chitosan flocculation, Aquac. Eng. **4** (1985) 257. doi: https://doi.org/10.1016/0144-8609(85)90018-4
12. *Xu, Y., Purton, S., Baganz, F.,* Chitosan flocculation to aid the harvesting of the microalga *Chlorella sorokiniana*, Bioresour. Technol. **129** (2013) 296. doi: https://doi.org/10.1016/j.biortech.2012.11.068
13. *Şirin, S., Trobajo, R., Ibanez, C., Salvadó, J.,* Harvesting the microalgae *Phaeodactylum tricornutum* with polyaluminum chloride, aluminium sulphate, chitosan and alkalinity-induced flocculation, J. Appl. Phycol. **24** (2012) 1067. doi: https://doi.org/10.1007/s10811-011-9736-6
14. *Dubois, M., Gilles, K., Hamilton, J. K., Rerbers, P., Smith, F.,* Colorimetric method for determination of sugars and related substances, Anal. Chem. **28** (1956) 350. doi: https://doi.org/10.1021/ac60111a017
15. *Zou, N., Richmond, A.,* Light-path length and population density in photoacclimation of *Nannochloropsis* sp. (*Eustigmatophyceae*), J. Appl. Phycol. **12** (2000) 349. doi: https://doi.org/10.1023/A:1008151004317
16. *Morales, J., Noüe, J. de la., Picard, G.,* Harvesting marine microalgae species by chitosan flocculation, Aquac. Eng. **4** (1985) 257. doi: https://doi.org/10.1016/0144-8609(85)90018-4
17. *Blockx, J., Verfaillie, A., Thielemans, W., Muylaert, K.,* Unravelling the mechanism of chitosan-driven flocculation of microalgae in seawater as a function of pH, ACS Sustain. Chem. Eng. **6** (2018) 11273. doi: https://doi.org/10.1021/acssuschemeng.7b04802
18. *Farid, M. S., Shariati, A., Badakhshan, A., Anvaripour, B.,* Using nano-chitosan for harvesting microalga *Nannochloropsis* sp., Bioresour. Technol. **131** (2013) 555. doi: https://doi.org/10.1016/j.biortech.2013.01.058
19. *Wu, Z., Zhu, Y., Huang, W., Zhang, C., Li, T., Zhang, Y., Li, A.,* Evaluation of flocculation induced by pH increase for harvesting microalgae and reuse of flocculated medium, Bioresour. Technol. **110** (2012) 496. doi: https://doi.org/10.1016/j.biortech.2012.01.101
20. *Ummalyma, S. B., Mathew, A. K., Pandey, A., Sukumaran, R. K.,* Harvesting of microalgal biomass: Efficient method for flocculation through pH modulation, Bioresour. Technol. **213** (2016) 216. doi: https://doi.org/10.1016/j.biortech.2016.03.114
21. *Wu, J., Liu, J., Lin, L., Zhang, C., Li, A., Zhu, Y., Zhang, Y.,* Evaluation of several flocculants for flocculating microalgae, Bioresour. Technol. **197** (2015) 495. doi: https://doi.org/10.1016/j.biortech.2015.08.094
22. *Liu, J., Zhu, Y., Tao, Y., Zhang, Y., Li, A., Li, T., Sang, M., Zhang, C.,* Freshwater microalgae harvested via flocculation induced by pH decrease, Biotechnol. Biofuels **6** (2013) 1. doi: https://doi.org/10.1186/1754-6834-6-98