JIPK. Volume 17 No 2 June 2025
Sinta 1 (Decree No: 158/E/KPT/2021)
e-ISSN:2528-0759; p-ISSN:2085-5842
Available online at https://e-journal.unair.ac.id/JIPK

JIPK
(JURNAL ILMIAH PERIKANAN DAN KELAUTAN)
Scientific Journal of Fisheries and Marine

Research Article

Harvesting Optimization, Biomass, and Lipid Content Analysis of *Euglena* sp. Culture with *Ettlia texensis* Bioflocculant and Commercial Chitosan

Eko Agus Suyono[1,5]*, Siti Mudrikah[1], Khalid Erlangga Karilanata[1], Dedy Kurnianto[2], Arief Budiman[3,4,5], Nugroho Dewayanto[3,5], Renata Adaranyssa Egistha Putri[6], Brilian Ryan Sadewo[3,5], and Tia Erfianti[1]

[1]Faculty of Biology, Universitas Gadjah Mada, Sleman, Yogyakarta. Indonesia
[2]Research Centre for Food Technology and Processing, National Research and Innovation Agency, Gunungkidul, Yogyakarta. Indonesia
[3]Department of Chemical Engineering, Faculty of Engineering, Universitas Gadjah Mada, Yogyakarta. Indonesia
[4]Master Program in System Engineering, Universitas Gadjah Mada, Yogyakarta. Indonesia
[5]Center of Excellent of Microalgae, Universitas Gadjah Mada, Sleman, Yogyakarta. Indonesia
[6]Study Program of Biotechnology, Graduate School of Universitas Gadjah Mada,Yogyakarta. Indonesia

OPEN ACCESS

ARTICLE INFO

Received: May 08, 2024
Accepted: Oct 02, 2024
Published: Jan 22, 2025
Available online: May 25, 2025

*) Corresponding author:
E-mail: eko.suyono@ugm.ac.id

Keywords:
Flocculant agents comparison
Growth kinetic model
Microscopic observation
Recovery efficiency

Abstract

Euglena sp. has a high potential to be developed as biofuel. However, the high cost and energy required for the harvesting process are hindering the production. Flocculation using natural substances, such as microorganisms and biopolymers, offers a promising solution to minimize energy and production costs, so it is applicable on a mass scale. *Ettlia texensis* is one of the autoflocculating microalgae that can excrete extracellular polymeric substances (EPS). Chitosan is a linear copolymer of D-glucosamine and N-acetyl-D-glucosamine produced by the deacetylation of chitin, which is usually exploited by marine crustaceans, shrimp, and crabs. Chitosan has a very high cation load, so it is often used for coagulation or flocculation. This study explores the potential of *E. texensis* and chitosan as flocculant agents to harvest the mass culture of *Euglena* sp. by giving different doses *E. texensis* with 1:0.25 (E3), 1:0.5 (E4), 1:1 (E5), and 1:2 (E6), and chitosan with 1.25 mg (C1), 2.5 mg (C2), 3.75 mg (C3), and 5 mg (C4). This research began with the cultivation of *Euglena* sp. and *E. texensis* on a 50 L scale for 12 days. The effectiveness of flocculation was measured by the spectrophotometric method. Based on this research, the best treatment for harvesting *Euglena* sp. culture by bioflocculation was shown by the addition of chitosan (5 mg) with the recovery of 84.83%, 0.2213 mg/mL biomass, and 0.2117 mg/mL lipid content. Meanwhile, with *E. texensis,* the best was shown by the ratio of 1:2 with recovery 84.71%, 0.2053 mg/mL biomass, and 0.1753 mg/mL

Cite this as: Suyono, E. A., Mudrikah, S., Karilanata, K. E., Kurnianto, D., Budiman, A., Dewayanto, N., Putri, R. A. E., Sadewo, B. R., & Erfianti, T. (2025). Harvesting Optimization, Biomass, and Lipid Content Analysis of *Euglena* sp. Culture with *Ettlia texensis* Bioflocculant and Commercial Chitosan. *Jurnal Ilmiah Perikanan dan Kelautan,* 17(2):260–275. https://doi.org/10.20473/jipk.v17i2.57417

1. Introduction

Microalgae is one of the organisms that can be used as a biofuel feedstock because it is supported by its ability to produce high amounts of lipids. One of the microalgae showing high potential for lipid production is *Euglena* sp., as demonstrated in various studies (Gris *et al*., 2014). One of the microalgae with this potential is *Euglena* sp.. *Euglena* sp. has significant industrial potential due to its ability to produce various metabolites, including proteins, lipids, fatty acids, carbohydrates, and different types of pigments (Inwongwan *et al*., 2019; Asiandu *et al*., 2023). A recent study by Erfianti *et al.* (2024) found that *Euglena* sp. contains metabolite concentrations, including 0.387 g/L of lipids, 0.366 g/L of carbohydrates, and 0.542 g/L of proteins, highlighting its potential for biofuel production.

Harvesting microalgae requires significant energy, which increases production costs. This challenge affects both cultivation and product development. Studies have shown that microalgae harvesting can account for up to 20-30% of the total open pond production cost, and in some cases, it can reach as high as 50%. One of the main challenges in large-scale microalgae cultivation is the difficulty in harvesting due to the small size, negative charge, and low density of microalgae cells, making them hard to separate from the culture medium (Li *et al.,* 2021). To address this, several harvesting methods have been developed, including centrifugation, ultrafiltration, and flocculation (Babakhani *et al.,* 2022). Among these, bioflocculation, which uses biobased agents to facilitate the aggregation of microalgae cells, is emerging as a promising technique. In the current development of microalgae products, more efficient harvesting techniques are needed regarding cost and time, including the bioflocculation technique. Bioflocculation is a flocculation technique for harvesting microalgae using biobased agents. Harvesting effectiveness can be known from the percentage of microalgae cell precipitation, which is how fast microalgae cells separate themselves from the culture medium (Van Anh *et al*., 2022).

Previous studies have shown that the effectiveness of harvesting *Euglena* sp. by bioflocculation method is very high. Bioflocculant agents can be microorganisms or other biological products. Autoflocculating microalgae can be used as a bioflocculant agent in harvesting *Euglena* sp. Bioflocculation of *Euglena* sp. with bioflocculant microalgae *Skeletonema* sp. showed the highest flocculation power achieved at a ratio of 1: 1 with a value of 94.31%. The lowest flocculation power was demonstrated by the 1:0.25 flocculant ratio treatment with a value of 80.89%. The study proved that the ability of floc formation and sedimentation will increase in line with the ratio of microalgae flocculants during mixing (Indahsari *et al*., 2022). One of the autoflocculating microalgae that can be used as a bioflocculant in *Euglena* sp. harvesting is the microalgae *Ettlia texensis*. *E. texensis* is a microalgae that can combine excellent autoflocculation and sedimentation potential with high lipid content so that it is suitable for supporting *Euglena* sp. biomass production (Salim *et al*., 2014). Through the mechanism of automatic floc formation, the recovery of the biomass removed from the microalgal suspension with *E. texensis* was higher than that of three other microalgal species, namely *Ankistrodesmus falcatus*, *Scenedesmus obliquus*, and *Tetraselmis suecica*. Biomass recovery of *C. vulgaris* culture with the addition of *E. texensis* reached almost 60% based on previous studies. Harvesting with the formation of flocs can occur because non-flocculated microalgae cells are trapped by flocs produced by flocculated microalgae, namely *E. texensis* (Salim *et al*., 2012).

Bioflocculation techniques in microalgae harvesting can also be pursued using natural biopolymer materials. One natural biopolymer that has the potential to increase the effectiveness of microalgae harvesting is chitosan. Another study mentioned a natural biopolymer that offers a greater favorable impact on the end product of algae and on altering the waste produced by industry so it can degrade naturally on the environment. Chitosan is a linear copolymer of D-glucosamine and N-acetyl-D-glucosamine soluble in acidic media. Chitosan is not found frequently in nature but is mainly produced from the chemical deacetylation of chitin from marine crustaceans, shrimp, and crabs (Aranaz *et al*., 2021). Chitosan has very high amino groups on its surface, which bind the microalgae cells, so it is often used for coagulation/flocculation processes with its most important properties, no toxicity and biodegradability (Yin *et al*., 2021). Numerous studies stated that the effectiveness of the chitosan harvesting method of algae culture, mostly using *Chlorella* sp., is greater than 95% (Xu *et al*., 2021; Hadiyanto *et al*., 2022; Liang *et al*., 2022;Wang *et al*., 2022), but none of the studies is using *Euglena* sp. with the same method yet. Chitosan is often used in the food industry as a natural preservative to prevent the loss of vitamins and increase the fiber content which also increases the nutritional value of the food (Zhou *et al*., 2021). In a prior study conducted by Rashid *et al.* (2013), the efficacy of using chitosan as a bioflocculant for harvesting microalgae biomass was investigated. The research explored varying chi-

tosan concentrations of 30 mg/L, 60 mg/L, 90 mg/L, and 120 mg/L in the context of the microalgae species *Chlorella vulgaris*. The findings demonstrated that the optimal biomass recovery efficiency reached 99 ± 0.5% at a chitosan concentration of 120 mg/L. Thus, this research aims to investigate chitosan doses in smaller quantities than previous studies.

Research on the use of autoflocculating microalgae *E. texensis* and commercial chitosan as flocculant agents in the harvesting of *Euglena* sp. semi-mass culture has never been conducted. Both materials have a high potential to increase the effectiveness of microalgae culture harvesting. This study was conducted to explore the potential of *E. texensis* and chitosan as flocculant agents to optimally harvest semi-mass cultures of *Euglena* sp. by providing different doses of both flocculant agents. Through this research, the flocculation efficiency with two different flocculant agents can be known. In addition, this study also aims to compare the two flocculant agents in their effect on biomass and lipid content produced by *Euglena* sp. culture.

2. Materials and Methods

2.1 Materials

2.1.1 The equipments

Some of the equipments used in this study consist of analytical scales (AND), autoclave (Tomy), laminar airflow (Gelman Sciences), multiwell plate (Biologix), inverted microscope (Falcon), micropipette (Eppendorf), centrifuge (Hettich University), buchner funnel, UV-Vis spectrophotometer (Genesys), and hemocytometer (Neubauer).

2.1.2 The materials

Microalgae *Euglena* sp. IDN 22 was obtained from the Biotechnology Laboratory, Faculty of Biology, Gadjah Mada University, Indonesia, and microalgae *E. texensis* was obtained from the Indonesian Culture Collection (InaCC). The composition of the Cramer Myers medium includes ammonium sulfate fertilizer (Petrokimia Gresik), mono potassium phosphate fertilizer (Pak Tani), $MgSO_4$, KCl, $Fe_2(SO)_4 \cdot 7H_2O$, $MnCl_2$, $CoSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$, Vitamin B1, and Vitamin B12 (Merck). The composition of the AF-6 medium (artificial fluid medium for freshwater algae) includes MES Monohydrate (Hymedia), $NaNO_3$, NH_4NO_3, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, KH_2PO_4, $CaCl_2 \cdot 2H_2O$, Fe-citrate, citric acid, $Na_2EDTA \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$, Vitamin B1, Vitamin H, Vitamin B12, and Vitamin B6 (Merck). For bioflocculation test and metabolite analysis using commercial chitosan (Phy Edumedia), chloroform, and methanol (Merck).

2.1.3 Ethical approval

This study does not require ethical approval because it does not use experimental animals.

2.2 Methods

2.2.1 Microalgae cultivation

Microalgae *Euglena* sp. IDN 22 was obtained from the Biotechnology Laboratory, Faculty of Biology, Gadjah Mada University, Indonesia, and cultivated on a semimass scale with a volume of 50 L using Cramer and Myers (CM) medium modification. Microalgae *E. texensis* was obtained from the Indonesian Culture Collection (InaCC) and cultivated on a 50 L semi-mass scale using AF-6 medium based on Watanabe *et al.* (2000). Cultivation was carried out for 12 days in a greenhouse, and growth data were collected daily.

2.2.2 Measurement of the growth

This study involved two species of microalgae, *Euglena* sp. as non-flocculating microalgae and *E. texensis* as autoflocculating microalgae (one of the flocculant agents used). The growth curves of both microalgae species were obtained from cell counts using a Neubauer hemocytometer counting chamber. A total of 1 mL of sample was put into the counting chamber, covered with a cover glass, and observed with a Falcon light microscope. Cells were counted in four quadrants. Doubling time and specific growth rate were calculated with the following formula (Krzemińska *et al.*, 2014).

$$\mu = \ln (N_2/N_1) / (t_2-t_1) \quad \text{.....(i)}$$

Where :

μ = the specific growth rate

N_2 = the number of cells acquired during the final phase of the logarithmic phase

N_1 = the number of cells acquired during the beginning of the logarithmic phase

t_2 = time 1

t_2 = time 2

2.2.3 Growth kinetic modeling

Semi mass or mass scale cultivation requires

a suitable kinetics model to understand the growth dynamics of microalgae cultures so that microalgae growth can be predicted, and culture conditions can be optimized. Several nonlinear models, such as the logistic and Gompertz models have been widely used because they are simple growth models for microorganisms, such as microalgae, that are not limited to the type of substrate. The logistic model was chosen to see the stable growth kinetics of *Euglena* sp. and *E. texensis* concerning the daily maximum growth rate of the culture. The logistic model is obtained by applying the following formula (Erfianti *et al*., 2023; Nurafifah *et al*., 2023).

$dx/dt = \mu max\ (1-x/\mu max\ )x$.....(ii)

$x = ((X_0.\exp(\mu_{max}.t))/(1-[(X_0/X_{max}\ )(1-\exp(\mu max-.t)])$.....(iii)

Where :

X = cell density

X_0 = initial cell density

X_{max} = maximum cell density

μ_{max} = the maximum specific growth rate of microalgae

In line with this model, the Gompertz model is also used for cell population calculations during the logarithmic phase. In this model, some more complex parameters are used, including maximum cell production and lag time. Based on Eq v, it is known that *SSR* is the sum square residual, while *SST* is the sum square total. These formulas are used to determine the value of the R^2 coefficient which indicates the level of fit of the model to the growth patterns of *Euglena* sp. and *E. texensis* (Hanief *et al*., 2020).

$X = X_0+[X_{max}.\exp[-\exp(((r_m.\exp(1))/X_{max})(t_L-t)+1)]]$.....(iv)

$R^2 = (1-SSR/SST)$.....(v)

2.2.4 Biomass measurement

The biomass of *Euglena* sp. and *E. texensis* was determined using filter paper that had previously been weighed with analytical scales. A total of 50 mL of sample was poured into a Buchner funnel that had been given filter paper. The tool was turned on and waited until the green biomass was filtered. The filter paper was reheated at 100°C for 1 hour; then the paper was weighed again. The following formula was used to calculate the biomass produced (Asiandu *et al*., 2023).

Biomass (mg⁄mL) = (Filter paper final weight-Filter paper initial weight) / (Sample Volume).....(vi)

Biomass Productivity ((mg/mL)/day)=(Maximum biomass-Initial biomass)/(Day max-Day 0).....(vii)

2.2.5 Measurement of lipid content

The lipid content test was conducted using the method of Bligh and Dyer (1959). Empty petri dishes were weighed with an analytical balance. Each 40 mL sample was centrifuged at 4000 rpm for 10 minutes (28°C). The supernatant was discarded, and the pellet was added with 2 mL of 100% methanol and 1 mL of 100% chloroform and mixed by vortex. A total of 1 mL chloroform and 1 mL sterile distilled water were added and homogenized again. After that, the mixture was centrifuged at 4000 rpm for 10 minutes (28°C). The results of centrifugation into 3 layers, the yellow lipid at the bottom was taken and placed on a petri dish, the lipid was incubated. The petri dish was weighed, calculated the lipid content by dividing the difference between the final and initial petri dish weight by the sample volume (Erfianti *et al*., 2024).

2.2.6 Preparation of chitosan solution

The chitosan that will be used is obtained from the marketplace and is of pharmaceutical grade. This process was carried out following research by Rashid *et al*. (2013). A total of 1000 mg dry weight of chitosan was mixed with 100 mL 0.1% HCl solution in a 1000 mL beaker which was homogenized with a heated magnetic stirrer for 30 minutes continuously. After that, the mixture was diluted with 1000 mL of deionized water to make a chitosan solution with a concentration of 1000 mg/L.

2.2.7 Microalgae flocculation process

After the *Euglena* sp. culture reached the stationary phase on the 12th day of cultivation, harvesting was carried out by flocculation technique using several variations of flocculant, namely other microalgae species *E. texensis* and the use of commercial chitosan. The treatment variations followed the combinations in Table 1.

Samples were put into a conical tube with a total volume of 50 mL according to the treatment groups in Table 1. The flocculation process was observed at hours 0, 1, 2, 3, 4, 5, and 6 by measuring optical density at a wavelength of 680 nm. The percentage of precipitation in the flocculation was calculated by the following formula (Salim *et al*., 2012).

Recovery = $(OD_{680}(t_0)-OD_{680}(t))/(OD_{680}(t_0)\ )\times100\%$....

.....(viii)

Where :

$OD_{680}(t_0)$ = the initial optical density of the sample taken at 0 hours

$OD_{680}(t)$ = the optical density of the sample taken at *t* hour

Table 1. Bioflocculation test treatment groups in the form of the ratio of *Euglena* sp. culture and bioflocculant agent used.

Treatments	Ratio of *Euglena* sp. and *Ettlia texensis* (v/v)	Treatments	Weight of the Commercial Chitosan (mg)
E1 (Control)	1:0	Control	0
E2	0:1	C1	1.25
E3	1 : 0.25	C2	2.5
E4	1 : 0.5	C3	3.75
E5	1:1	C4	5
E6	1:2		

2.3 Analysis Data

All treatments were analyzed by One-Way Analysis of Variance (ANOVA) ($P < 0.05$), Tukey post-hoc multiple comparison test, and Duncan Multiple Range Test (DMRT) with IBM SPSS Statistics 25. A p-value smaller than 0.05 was considered for the treatment to be statistically significant.

3. Results and Discussion

3.1 Results

3.1.1 Growth rate Euglena sp. *and E. texensis*

Euglena sp. belongs to non-flocculating microalgae and in this study, flocculating agents were used for its harvesting, namely autoflocculating microalgae *E. texensis* and commercial chitosan. Both microalgae species, *Euglena* sp. and *E. texensis*, were cultivated for 12 days simultaneously. *Euglena* sp. and *E. texensis* reached the logarithmic phase on the 1st day of cultivation. On the 12nd day of cultivation, the decreasing cell number was declared as the beginning of the stationary phase. The highest cell number of *Euglena* sp. was 494,583 ± 20,552 cells/mL, while *E. texensis* was 977,708 ± 28,982 cells/mL (Figure 1). In the stationary phase, the rate of cell division is balanced with the rate of cell death. This stationary phase was reached on the 12th day of cultivation, and this time was set as the harvesting period for *Euglena* sp. and *E. texensis* cultures. This follows the harvesting technique used, namely by bioflocculation. Harvesting carried out in the stationary phase is more favorable because microalgae during this phase have lower metabolic activity and cell mobility to increase the ability of intercellular interactions due to low zeta potential (Barros *et al*., 2015). In addition, previous studies have also shown that lipid accumulation can be maximized in the exponential phase and continue until the stationary phase (Teh *et al*., 2021).

The growth of *Euglena* sp. and *E. texensis* can be projected with growth kinetics models, namely using the Logistic model and the Gompertz model. Based on logistic modeling, the culture-specific growth rate (μ_{max}) of *Euglena* sp. is 0.379/day (Figure 2a) with the R^2 errors was 0.988. Meanwhile, based on the Gompertz model with an R^2 value of 0.946, the specific growth rate (μ_{max}) is 3.389/day (Figure 2b). The R^2 value indicates the suitability of the kinetic growth model, i.e., the higher the R^2 value, the better the suitability of the model (Erfianti *et al*., 2024). Similar to the *Euglena* sp. culture, the growth pattern of *E. texensis* also shows that the logistic model is the most suitable growth kinetics model for *E. texensis*. This is indicated by the R^2 coefficient value (0.851), which is higher than the R^2 value of the Gompertz model (0.828) (Figure 2c and 2d). Based on the logistic model, *E. texensis* has a μ_{max} of 0.346/day while based on the Gompertz model it has a μ_{max} of 8.231/day.

3.1.2 Flocculation of Euglena sp. *using autofloccula*

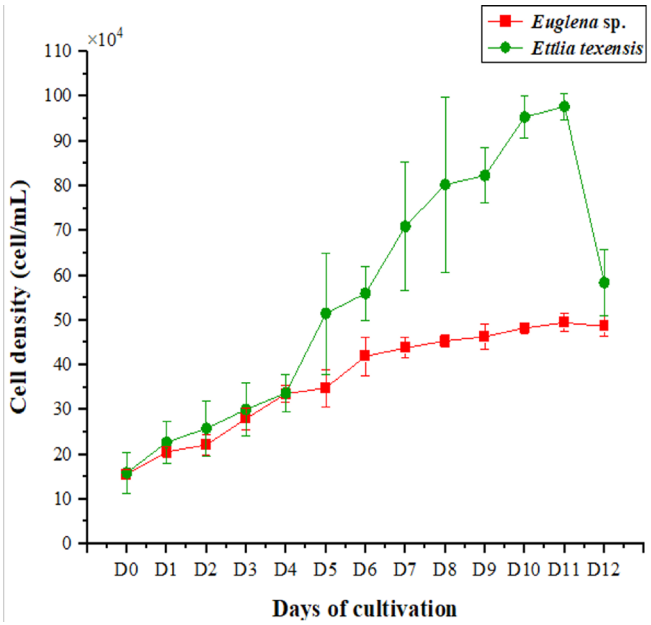


Figure 1. Cell density of *Euglena* sp. and *E. texensis.*

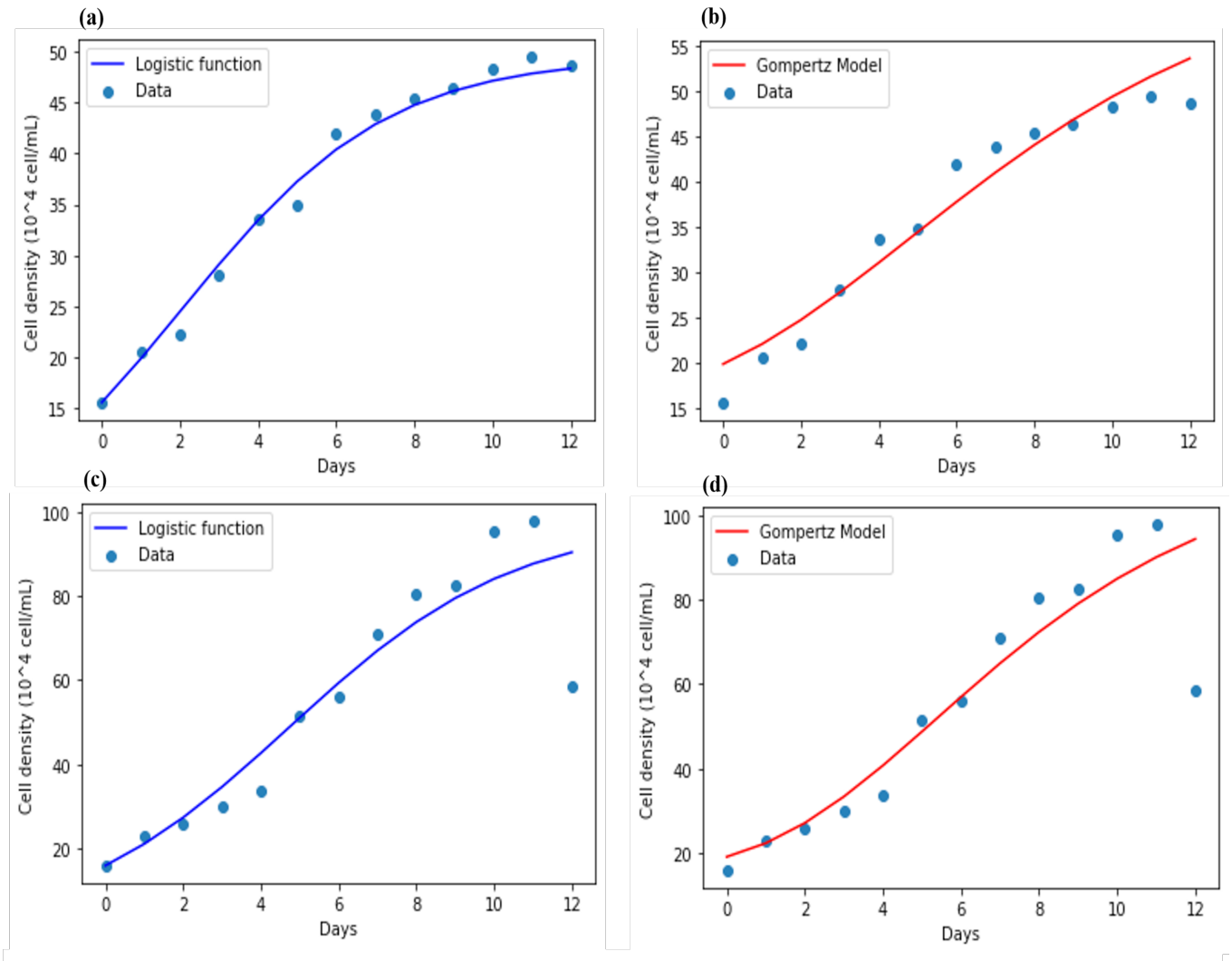


Figure 2. The growth kinetic model of *Euglena* sp. and *E. texensis* (a) *Euglena* sp. Logistic Model; (b) *Euglena* sp. Gompertz Model; (c) *E. texensis* Logistic Model; (d) *E. texensis* Gompertz Model.

tion microalgae E. texensis

The effectiveness of flocculation of *Euglena* sp by using *E. texensis* varied in each dose. Figure 3 showed that both *Euglena* sp. and *E. texensis* control treatment (E1 and E2, respectively) had an initial optical density of 0.472 and 0.247, which decreased further until the 6th hour to 0.123 and 0.0407 respectively. A decrease in optical density with time was also seen in the culture mixture treatments E3 (from 0.412 to 0.147), E4 (from 0.350 to 0.085), E5 (from 0.315 to 0.085), and E6 (from 0.298 to 0.074). However, there was a slight difference in the *Euglena* sp. control treatment (E1) and the 1:0.25 ratio treatment (E3), which showed that in both treatments, the optical density showed an increase in the 6th hour when compared to the 5th hour. The increase in optical density in E1 and E3 was 0.023 and 0.032, respectively. This is likely due to *Euglena* sp. which reproduces after six hours of standing. *Euglena* sp. can divide cells relatively quickly so that an increase in optical density in the culture can occur at that hour (Xin *et al*., 2024).

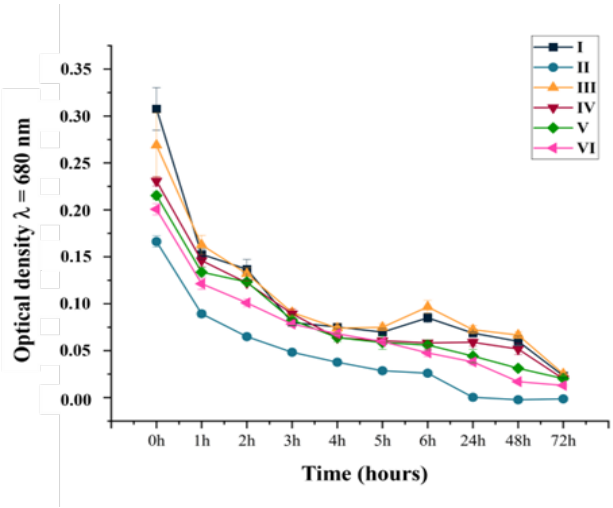


Figure 3. Optical density at 680 nm of *Euglena* sp. with *E. texensis* flocculant agent.

In contrast to the optical density value, the flocculation efficiency showed that the longer the flocculation time, the higher the recovery (Figure 4). The highest flocculation efficiency based on percentage recovery was achieved in treatment E6, from 60.79% at hour 1 increase to 84.22% at hour 6. The results show that the higher the volume of *E. texensis* added, the more *Euglena* sp. precipitation will also increase. The higher percentage of recovery indicates that the effectiveness of *E. texensis* bioflocculant in harvesting non-flocculant microalgae *Euglena* sp. is improving. Through the percentage value of recovery, it can be seen to what extent the microalgae cells of *Euglena* sp. and *E. texensis* have aggregated to form flocs or larger particles so that they can more easily settle, which has an impact on the harvesting process to be more efficient (Molitor *et al.*, 2021).

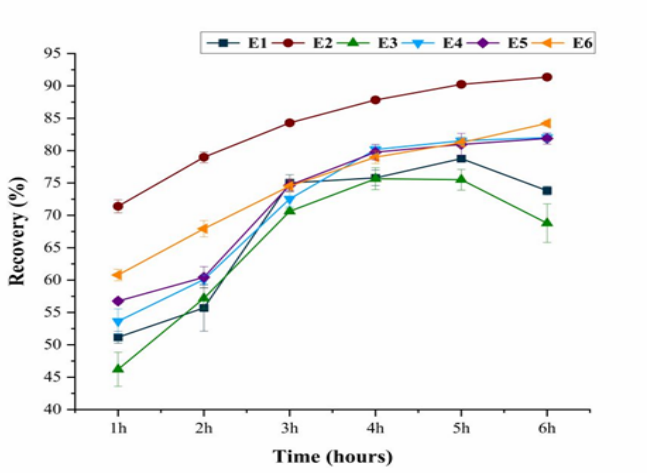


Figure 4. Recovery of *Euglena* sp. with *E. texensis* flocculant agent.

3.1.3 Flocculation of Euglena sp. *using commercial chitosan*

Microalgae flocculation using polymer has a different mechanism compared to living flocculants. *Euglena* sp. with Control and C4 treatments had optical densities of 0.3177 and 0.3210, which decreased over 5 hours and then became 0.0530 and 0.0487 (Figure 6). The same thing can also be observed in other treatments such as C1, C2, and C3, which each have an initial optical density of 0.3193, 0.3220, and 0.3207, which then decreased at the end of the flocculation period to 0.0577, 0.0537, and 0.0490. Figure 7 shows the same thing as Figure 6 with Control, C1, C2, C3, and C4 experiencing an increase from the first hour, in percentage, namely 48.27, 43.84, 48.24, 45.95, and 51.61, respectively, to 83.32, 81.94; 83.33; 84.72; and 84.84%. The highest yield was obtained in C4 at 5 hours after flocculation began (84.84%), but the most efficient time yield compared to other treatments was in C4 at 2 hours after flocculation began (78.19%).

3.1.4 Biomass and lipid content produced by Euglena sp. *with E. texensis flocculant agent*

The biomass and lipid content of *Euglena* sp. control treatment E2 (1:0) were higher than *E. texensis* control treatment E2 (0:1) (Figure 5). Meanwhile, in terms of the combination of the two cultures, the results showed that the highest biomass and lipid con tent by *Euglena* sp. with *E. texensis* flocculant were achieved in E3 (1:0.25) at 0.2980 and 0.2747 g/mL, respectively. Then, the combination treatment E6 (1:2) showed the lowest biomass and lipid content, 0.2053

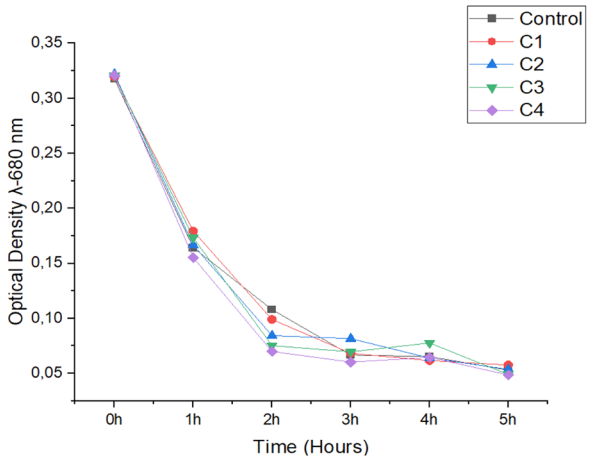


Figure 6. Optical density at 680 nm of *Euglena* sp. with chitosan flocculating agent.

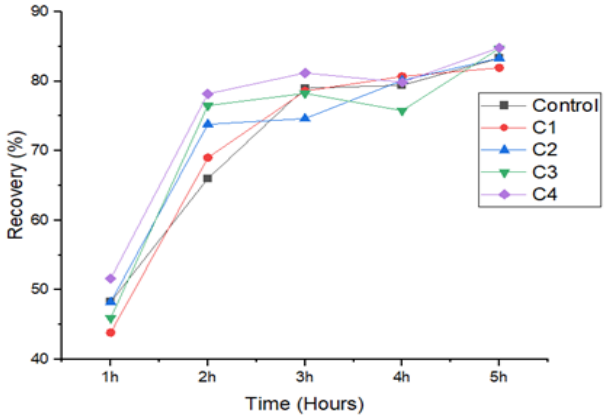


Figure 7. Recovery of *Euglena* sp. with chitosan flocculating agent.

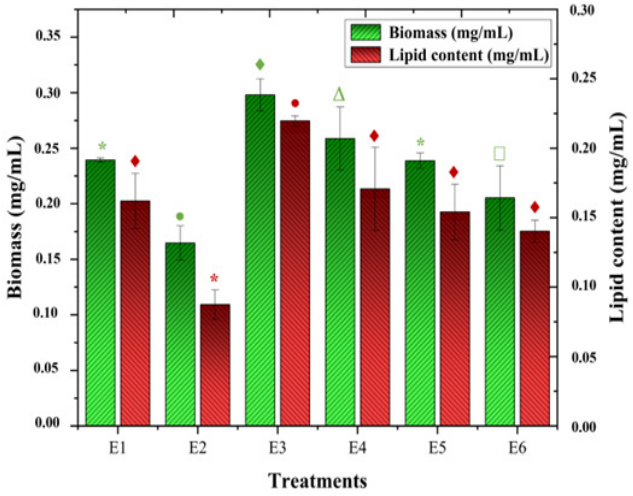


Figure 5. Biomass and lipid content of *Euglena* sp. with *E. texensis* flocculant agent. The results of statistical analysis with one-way ANOVA test are shown with some symbols of each parameter. Different symbols indicate the significance of $P < 0.05$.

and 0.1753 g/mL, respectively. The increase in biomass and lipid content is directly proportional to the increase in lipid content produced. Statistical analysis with one-way ANOVA showed that all treatments of the ratio combination were significantly different on the biomass and lipid content produced by *Euglena* sp. The significant difference was indicated by a significance value of $P < 0.05$ at the level of confidence $\alpha = 0.05$.

3.1.5 Biomass and lipid content produced by Euglena sp. with E. texensis flocculant agent

The graph (Figure 8) shows the results of biomass measurements (mg/mL) after treatment to several groups, namely control, C1, C2, C3, and C4. Biomass in the control group was recorded at around 0.201 mg/mL, the lowest value among all groups. In the treatment group, there was an increase in biomass compared to the control, with the highest increase in biomass achieved in group C4, with a value of around 0.221 mg/mL. The error bars in each group show relatively small standard variations, indicating consistent measurement results between replicates. Treatments C1 to C4 showed a trend of increasing biomass compared to the control, with the most significant effect seen in treatment C4. The graph also illustrates the lipid content (mg/mL). In the control group, lipid levels were recorded at approximately 0.172 mg/mL, representing the lowest concentration among all groups. The highest lipid content was recorded in treatment group C4, with a value of around 0.212 mg/mL. The relatively small error bars across all groups indicate consistency in the measurements obtained.

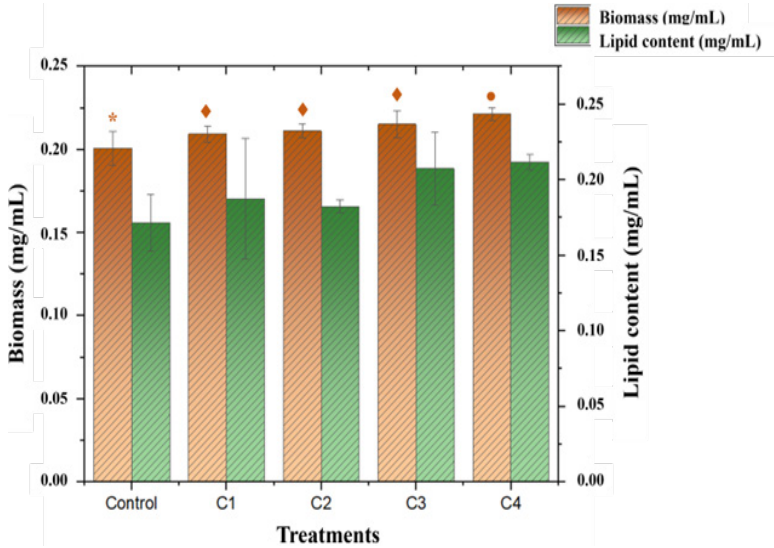


Figure 8. Biomass and lipid content of *Euglena* sp. with commercial chitosan. The results of statistical analysis with one-way ANOVA test are shown with some symbols of each parameter. Different symbols indicate the significance of $P < 0.05$.

3.2. Discussion

3.2.1 Effect of E. texensis as flocculant agent on the biomass and lipid content of Euglena sp.

This study showed that the biomass and lipid content produced by *Euglena* sp. with and without the addition of *E. texensis* flocculant agent showed the same pattern. The high biomass and lipid content produced was proportional to the high ratio of *Euglena* sp. to *E. texensis*. In this study, there were four variations of the ratio between *Euglena* sp. and *E. texensis*, with two control treatments consisting of *Euglena* sp. and *E. texensis* respectively. The total volume in the ratio was 50 mL, so the volume of *Euglena* sp. and *E. texensis* was determined based on the ratio.

Based on Figure 5, the biomass value of *Euglena* sp. (E1) was almost 1.5 times as high as the biomass of *E. texensis* (E2). This can be caused because there are differences in cell size when viewed from the morphology of *Euglena* sp. and *E. texensis* cells. *E. texensis* cells have a size range of 8 – 15 µm in diameter, while *Euglena* sp. has a diameter that can reach 20 µm with relatively long cells (35 – 50 µm) (Podwin *et al*., 2017; Purbani *et al*., 2019). The larger cell size of microalgae is positively correlated with the biomass it produces. The larger the cell size of microalgae, the more potential it has to produce higher biomass. Larger cell sizes lead to an increase in area for nutrient uptake and photosynthesis purposes of the cell. Previous research also supports this, that increasing cell concentration and cell size of microalgae *Chlorella* sp., *Nostoc* sp., and *Chlamydomonas* sp. over time showed an increase in biomass under the treatment of carbon dioxide availability in the culture environment (Lim *et al*., 2022). The high biomass of *Euglena* sp. significantly influenced the biomass yield in the mixed culture treatments of *Euglena* sp. and *E. texensis* with a significance of $P < 0.05$ (Figure 5). The mixed treatment of *Euglena* sp. and *E. texensis* with several ratio combinations showed that the higher the volume of *Euglena* sp. in the mixture, the higher the biomass produced. This study showed that the highest biomass in the mixed treatment was achieved by treatment E3 (0.298 mg/mL) almost one and a half times higher than treatment E6 with the lowest biomass (0.2053 mg/mL). Based on the results of this study, the ratio combination treatment showed higher biomass values compared to the control *Euglena* sp. (E1) seen in the E5 and E6 treatments. The biomass value in the combination treatments E3 and E4 were 1.2 times and 1.08 times higher than the control treatment *Euglena* sp. combination E1, respectively.

In line with these results, this study showed that flocculation treatment with the addition of *E. texensis* as a flocculant agent is positively correlated with the biomass produced in *Euglena* sp. The higher the volume of flocculant added up to a 1:0.5 ratio, an increase in the biomass produced compared to the control treatment E1. This study showed that the most optimal ratio combination treatment applied as a bioflocculation technique on *Euglena* sp. culture is a ratio of 1:0.25 in terms of biomass content produced. The biomass of *Euglena* sp. increased from the control treatment when added with the microalgae flocculant *E. texensis*. This can occur because microalgae *E. texensis* which an autoflocculating microalgae, can induce flocculation by the formation of extracellular polymeric substances (EPS). These polymers can bind to microorganism cells, including the microalgae cells themselves. Previous research also proved that the EPS produced by *E. texensis* can coat the entire surface of the microalgae cells. Not only that, but the EPS also produced can become a link between EPS-producing *E. texensis* flocculant microalgae and other non-flocculant microalgae. This causes the formation of aggregates to form a floc that is easier and faster to settle (Salim *et al*., 2014; Moreira *et al*., 2022).

Microalgae biomass contains various primary metabolites, one of which is lipid content. Based on the graph in Figure 5, lipid content in the control treatment *Euglena* sp. (E1) is 0.2025 mg/mL. These results are almost twice as high as the control treatment *E. texensis* (E2) which has a lipid content value of 0.1093 mg/mL. The results of this study indicate that the lipid content in *Euglena* sp. is quite high in line with the research conducted by Khanra *et al*. (2017), which can reach 24.6% of its dry weight. Meanwhile, the lipid content in *E. texensis* based on this study shows the opposite result, which is relatively low compared to lipids in *Euglena* sp. Another study mentioned that lipids in *Ettlia oleoabundans* only amounted to 1.5% of their dry weight (Yang and Weathers, 2015). Lipid content in photoautotrophic cultivation as in this study showed relatively low results. Research by Kim *et al*. (2019) shows that increasing lipid content can be done by heterotrophic cultivation.

In addition, the mixed culture treatment of *Euglena* sp. and *E. texensis* with the highest lipid content is the highest ratio treatment, namely the E3 treatment (1:0.25), followed by combination treatments E4 (0.2133 mg/mL), E5 treatment (0.1927 mg/mL), and finally the lowest was achieved by treatment E6 (0.1753 mg/mL). Statistical analysis with one-way ANOVA showed that the combined treatment of the ratio of non-flocculated microalgae *Euglena* sp. and

flocculated microalgae *E. texensis* had a significant effect on lipid content with a significance of $P < 0.05$ (Figure 5). Lipid content with values above the *Euglena* sp. control treatment (E1) was shown by treatments E3 and E4, each of which had a ratio of non-flocculant microalgae *Euglena* sp. and flocculant microalgae *E. texensis* of 1:0.25 and 1:0.5, respectively. The results of this lipid content study were positively correlated with the biomass produced by each treatment. This is following the biomass produced by each treatment. This is following research by Timotius *et al.* (2022), that higher biomass can produce higher lipid content because the metabolic pathway for cellular defense increases.

The flocculation treatment with *E. texensis* showed that the higher the ratio of non-flocculant microalgae *Euglena* sp. and flocculant microalgae *E.* texensis, the higher the lipid content produced. These results are not in line with research conducted by Indahsari *et al.* (2022) that bioflocculation of *Euglena* sp. with *Skeletonema* sp. with salinity treatment showed the highest and lowest lipid content obtained at ratios of 1:1 and 1:0.25, respectively. Cultures with the addition of other microalgae have more potential to produce more lipids. In mixed cultures nutrients become limited, causing nutritional stress conditions. The limited amount of nutrients can stimulate microalgae to accumulate lipids. This study did not show the same thing could be due to differences in autoflocculating microalgae species, different culture conditions (laboratory scale and semi-mass scale), and the presence or absence of additional treatments. In this study, *E. texensis* microalgae were used, both of which were cultured in open ponds without additional treatment as in previous research was given salinity treatment.

However, referring to previous studies, *E. texensis* lipids are produced in relatively small (Yang and Weathers, 2015) amounts and the EPS produced by these microalgae predominantly contains protein and carbohydrate groups (Salim *et al.*, 2014), not lipids. This may influence the lipid content of *Euglena* sp. harvested with the flocculant agent *E. texensis*. The more *E. texensis* is added to the culture during harvesting the maximum accumulation of metabolites not in lipids. Therefore, the ratio of 1:0.25 (E3 treatment) was the most optimal ratio of *Euglena* sp. microalgae and *E. texensis*. Although the addition of lipid content is not too high, the ratio can produce a higher lipid content than the control ratio of *Euglena* sp. (E1 treatment). This was also seen in the lipid content produced by treatment E4 (1:0.5).

3.2.2 Flocculation effectiveness of Euglena sp. *with commercial chitosan flocculant agent*

Chitosan is a biopolymer that contains a positively charged functional group, such as amine. When dissolved in an acidic solution, this amine group will be protonated, which gives a strong cationic characteristic to chitosan. This positive charge will bind to negatively charged functional groups in microalgae membranes, such as carboxylic and sulfate groups (Nicknig *et al.*, 2024). After the charge on the membrane becomes neutralized, the van der Waals force will occur between microalgae cells, which produce flocs. Moreover, chitosan as a polymer can also make floc formation efficiently. When the hydrogen of chitosan is damaged, the polymer chain becomes more flexible, so it forms a bridge between each chitosan molecule. This bridge will add molecular weight to microalgae cells, so gravity affects it more because of the increased surface area (Yang *et al.*, 2016). However, the result showed in Figure 6 that the floc forming of *Euglena* sp. on all treatments, including the control treatment, tends to settle to the bottom of the flask every hour regardless of the chitosan added. This settling happens because nutrient depletion can naturally induce flocculation. A nutrient-depleted microalgae causes them to be unable to move in the media, which leads to sedimentation at the bottom of the media, which is affected by gravity (Muir *et al.*, 2024)

3.2.3 Effect of commercial chitosan as flocculant agent on biomass and lipid content of Euglena sp.

In general, the increase in the effectiveness of biomass recovery had a positive trend (Figure 7) but occurred quite significantly in the first two hours in all treatments except the control treatment. Amine (NH_3^+) in the surface area of chitosan is the main cause of this floc forming. A lot of NH_3^+ ions cause this significant incline at the beginning of flocculation due to the addition of chitosan (Chang *et al.*, 2015). However, in the next hours, the effectiveness of floc formation slowed down, although the trend in all treatments increased, including the control treatment. This change in biomass recovery trend after the first 2 hours can be caused by the decreasing zeta potential value in the suspension, which results in a decrease in the effectiveness of floc formation, even though high doses of flocculant are given. The zeta potential of microalgal cultures increases positively as the flocculant dose increases. However, in our experiments, the decreasing trend in zeta potential at the last hours of flocculation was most likely caused by the dissociation of carboxylic acid groups on the microalgae cell surface, which generated negative ions. This phenomenon was

also found in the experiments of Wu *et al.* (2012) and Rashid *et al.* (2013), which have the same biomass recovery trend. This study showed that the biomass and lipid content produced by *Euglena* sp. which is affected with and without the addition of chitosan flocculating agent showed the same pattern. Biomass and lipid content was proportional to the increase of chitosan dose in each treatment. In this study, there were four doses of chitosan, with one control treatment. The total volume of *Euglena* sp. used in this flocculation was 50 mL.

Each treatment of chitosan shows an increasing biomass weight which was directly proportional to the increase in dose (Figure 8). The control treatment was lower than the chitosan treatment (Figure 8). Based on this table, biomass is influenced by the chitosan concentration given with a significance of $P < 0.05$. However, this was greatly influenced by the weight of the chitosan itself because the difference in weight between one treatment and another showed the same weight between the controls with increasing doses of chitosan. Giving chitosan does not have any effect on the growth of microalgae because chitosan will bind to the microalgae cell membrane, which can inhibit its growth. Meanwhile, overall lipid levels in the treatment with the addition of chitosan (0.1875 mg, 0.1825 mg, 0.2075 mg, and 0.2117 mg) increased compared to the control treatment (0.1717 mg).

In contrast to biomass, the difference between the weight of the control and the chitosan treatment was quite significant, so it can be confirmed that chitosan influences lipid extraction even though the significance is $P > 0.05$. This finding is in accordance with some literature that states microalgae with thin cell walls can be disrupted by chitosan (Martins *et al.*, 2018; Saliu *et al.*, 2021). *Euglena* sp. does not have a cell wall that can protect it from the external environment, so chitosan can easily enter the cell by disrupting the cell membrane, which releases the lipid content in the microalgae cells. Apart from that, the formation of the flocs also helps emulsify the fat that comes out of the cells (Saliu *et al.*, 2021). Chitosan can also act as a lipid binder, which can increase lipid extraction efficiency from the microalgae. In an acidic environment, chitosan is ionized into its oligomer, which contains an amine group. This amine group can be protonated by H^+ ions released by ionized acid in water so that the surface of the chitosan molecule has a positive charge. The ionized chitosan molecules attract negatively charged fat molecules, fatty acids (such as oleate, linoleate, palmitate, stearate, and linoleate), and bile acids (such as cholate, deoxycholate, and lithocholate), forming ionic complexes. Additionally, chitosan can disrupt the emulsification of neutral lipids (like cholesterol and other sterols) by binding to them through hydrophobic interactions, thereby forming hydrophobic complexes (Sapei *et al.*, 2022; Nie *et al.*, 2024).

3.2.4 Comparison of effectiveness of flocculant agents on Euglena sp. *harvesting*

From this study, the auto flocculation ability of *E. texensis* can indeed be used as a flocculant agent in the harvesting of *Euglena* sp. Autoflocculation of *E. texensis* can occur due to the process of production, adsorption, excretion, and bridging of polymers derived from these microalgae. The polymeric substances may be excreted by the microalgae in suspension, or the polymers may also adhere to the microalgae cells. *E. texensis* is known to produce polymers known as extracellular polymeric substances (EPS). These microalgae EPS can bind partially or completely to the microalgae cells. In addition, when the polymer is only partially bound, the remaining polymer can bind to other microalgae cells so that it can become a link between cells and form a network of polymers and microalgae cells. If the polymer fully binds to microalgae cells because it is too short to bind to others, the polymer will be fully attached to the microalgae surface (Salim *et al.*, 2014).

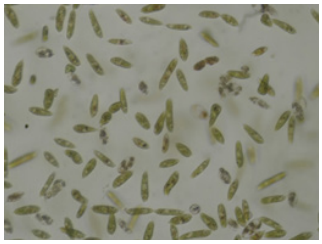
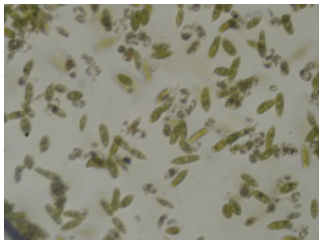
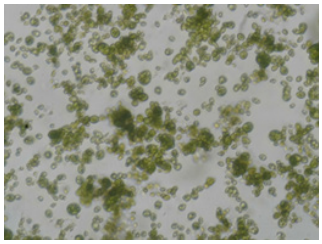
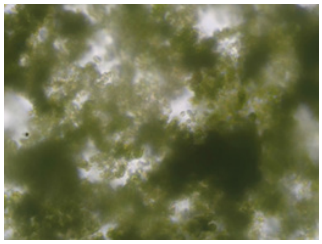
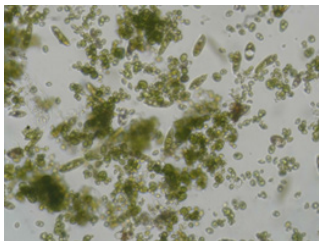
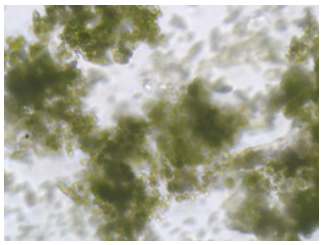
Previous research mentioned that the EPS matrix in *E. texensis* is not only on the surface of individual cells or between individual cells in the floc. The EPS matrix also forms an extra layer that envelops the entire floc. Observation using a scanning electron microscope (SEM) indicated that autoflocculation of *E. texensis* occurs due to polymers attached to the cell surface. Observations were also made on the suspension of *E. texensis* and *Chlorella vulgaris* showing images of large flocs of *E. texensis* with *C. vulgaris* cells trapped between the flocs. The SEM image shows that the EPS released by *E. texensis* makes two *C. vulgaris* cells stick together. The EPS attached to *E. texensis* was also attached to *C. vulgaris* cells by forming a fibrous structure (Salim *et al.*, 2012; Salim *et al.*, 2011).

Microscopic observation of the flocculation process in this study was also carried out using an inverted microscope. Table 2 shows the results of microscopic observations of *Euglena* sp. flocculation using *E. texensis* in the combined treatment of *Euglena* sp. control ratio (E1), *E. texensis* control (E2), and 1:2 ratio (E6). The addition of flocculant microalgae to non-flocculant microalgae can increase the settling rate considerably. This is because, in the culture mixture, there is an aggregation of *E. texensis* flocculant

microalgae cells, which can also cause *Euglena* sp. non-flocculant microalgae cells to become trapped by flocculant microalgae aggregates that form flocs. Thus, the formation of larger floc particles is easier to settle out (Djunaedi, 2016).

there is nothing to prevent the two colloidal particles from aggregating which causes the formation of larger particles or floc (Low and Lau, 2017). The flocculant dose influences the zeta potential value, so the larger the dose is given, the greater the value. However,

Table 2. Microscopic photos with a magnification of 10 x 20 on the flocculation process in the *Euglena* sp. control treatment (E1), *E. texensis* control treatment (E2), and the best ratio treatment of 1:2 (E6) at hour 0 and hour 6.

Treatments	0h	6h
E1 (*Euglena* sp.)		
E2 (*E. texensis*)		
E6 (1:2)		

On the other hand, it should be noted that floc formation is greatly influenced by the repulsive force between particles in a suspension, which is called zeta potential. *Euglena* sp. culture has a zeta potential value that is positively charged and almost neutral (Lewis and Guéguen, 2022). Also, the zeta potential of chitosan in a dissolved state at low pH has a positive charge. This positive zeta potential is due to the presence of amine (NH_3^+) ions on the surface of the chitosan molecule, which provides a repulsive force if there are positively charged molecules, in this case, *Euglena* sp. (Chang *et al*., 2015). To achieve optimal flocculation, the total zeta potential of the suspension from both flocculants and microalgae must neutralize each other or must approach zero due to the absence of repulsive forces between colloidal particles in the suspension so that the flocculation value using chitosan obtained in this experiment was not very significant. Some literature stated that chitosan flocculating capability is mostly influenced by pH range. Chitosan is dissolved at pH < 5.8 and precipitate at pH 6 (Sogias *et al*., 2010). Elcik *et al.* (2023) experiment found that at acidic pH chitosan as a floculating agent has a better biomass recov ery. This phenomenon is caused by increased chitosan surface area so that the amine group on the surface of chitosan can bind to negatively charged microalgae membrane. Based on the results obtained, chitosan offers a better cost and is more time-efficient than other flocculants.

The most optimal flocculant agent to be used in bioflocculation of *Euglena* sp. based on the recovery percentage is commercial chitosan with treatment

Table 3. Comparison of flocculant agent effectiveness for *Euglena* sp. harvesting.

Parameters	Control	The Best Treatments	
		E. texensis	Chitosan
Recovery (%)	78.57	E6 (84.71)	C4 (84.83)
Biomass (mg/mL)	0.22	E3 (0.2980)	C4 (0.2213)
Lipid content (mg/mL)	0.1871	E3 (0.2747)	C4 (0.2117)

C4 (Table 3), which is the addition of 5 mg of chitosan to the culture of *Euglena* sp. It can be seen in the table that the microalgae *E. texensis* also has a recovery percentage that is not much different from the chitosan treatment, which is only 0.12% difference. When viewed from the biomass and lipid content produced, *E. texensis* showed the best results for *Euglena* sp. flocculation because it produced higher biomass and lipids, namely in treatment E3 with a ratio of non-flocculant microalgae *Euglena* sp. and flocculant microalgae *E. texensis* 1: 0.25. The amount of biomass and lipid content produced by the E3 treatment was 1.3 times higher than that produced by the C4 treatment. This indicates the flocculant agent *E. texensis* more suitable than chitosan when *Euglena* sp. harvesting is targeted at increasing its biomass and lipids.

4. Conclusion

This study indicates that *E. texensis* and chitosan can be applied as flocculant agents in harvesting *Euglena* sp. microalgae by bioflocculation technique. The addition of both flocculant agents was equally able to increase the recovery percentage, biomass produced, and lipid content. The use of *E. texensis* as a flocculant agent resulted in significant percentage recovery, biomass, and lipid content with $P < 0.05$, while commercial chitosan as a flocculant agent was able to provide a high percentage recovery and significant biomass, but treatment with this flocculant had no significant effect on lipid content ($P > 0.05$). The best treatment for harvesting *Euglena* sp. culture by bioflocculation was shown by the addition of chitosan as much as 5 mg or it could also be done with the addition of *E. texensis* in a ratio of 1:0.25 (*Euglena* sp. to *E. texensis* v/v).

Acknowledgment

The authors would like to thank the Biotechnology Laboratory, Faculty of Biology, Gadjah Mada University for facilitating our research facilities and infrastructure.

Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author as follow, Eko; determined the mayor of topic, funding, provided advice, and proofread the manuscript. Mud and Khal; collected the data, analyzed the data, drafted the manuscript, and designed the figures. Ded and Tia; proofread the manuscript and provided advice. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Declaration of Artificial Intelligence (AI)

The author(s) affirm that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement of this manuscript. All content presented is the result of the independent intellectual efforts of the author(s), ensuring originality and integrity.

Funding Information

The authors would like to thank the Dana Padanan 2024 funded by the Ministry of Education, Cultural, and Higher Education Republic of Indonesia and PT Indoalgae Akuakultura.

References

Aranaz, I., Alcántara, A. R., Civera, M. C., Arias, C., Elorza, B., Heras Caballero, A. & Acosta, N. (2021). Chitosan: An overview of its properties and applications. *Polymers*, 13(19):1-22.

Asiandu, A. P., Nugroho, A. P., Naser, A. S., Sadewo, B. R., Koerniawan, M. D., Budiman, A., Siregar, U. J., Suwanti, L. T., & Suyono, E. A. (2023). The effect of tofu wastewater and pH on the growth kinetics and biomass composition of *Euglena* sp. *Current Applied Science and Technology*, 23(2):1-16.

Babakhani, P., Mahdavi, M. A., Gheshlaghi, R., & Karimian, A. (2022). The shift in carbon source

induces pH increase and autoflocculation in microalgal suspensions facilitating multi-approach biomass harvesting. *Biomass Conversion and Biorefinery,* 12(10):1-12.

Barros, A. I., Gonçalves, A. L., Simões, M., & Pires, J. C. M. (2015). Harvesting techniques applied to microalgae: A review. *Renewable and Sustainable Energy Reviews*, 41(1):1489-1500.

Bligh, E. G. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37(8):911-917.

Chang, S. H., Lin, H. T. V., Wu, G. J. & Tsai, G. J. (2015). pH Effects on solubility, zeta potential, and correlation between antibacterial activity and molecular weight of chitosan. *Carbohydrate polymers,* 134(20):74-81.

Djunaedi, A. (2016). Microalgae biomass production (*Tetraselmis chuii*) with different harvesting systems. *Jurnal Kelautan Tropis*, 18(2):107-111.

Elcik, H., Karadag, D., Kara, A.I. & Cakmakci, M. (2023). Microalgae biomass harvesting using chitosan flocculant: Optimization of operating parameters by response surface methodology. *Separations,* 10(9):1-14.

Erfianti, T., Daryono, B. S., Budiman, A., & Suyono, E. A. (2024). Growth and metabolite enhancement of acidophile *Euglena* sp. isolated from Indonesia under different photoperiod cycles. *Jurnal Ilmiah Perikanan dan Kelautan*, 16(1):15-30.

Gris, B., Morosinotto, T., Giacometti, G. M., Bertucco, A., & Sforza, E. (2014). Cultivation of *Scenedesmus obliquus* in photobioreactors: effects of light intensities and light–dark cycles on growth, productivity, and biochemical composition. *Applied Biochemistry and Biotechnology*, 172(5):2377-2389.

Hadiyanto, H., Widayat, W., Christwardana, M. & Pratiwi, M. E. (2022). The flocculation process of *Chlorella* sp. using chitosan as a bio-flocculant: Optimization of operating conditions by response surface methodology. *Current Research in Green and Sustainable Chemistry*, 5(1):1-11.

Hanief, S., Prasakti, L., Pradana, Y. S., & Cahyono, R. B. (2020). Growth kinetic of. *Botryococcus braunii* microalgae using logistic and gompertz models. *AIP Conference Proceedings*, 201(1):1-7.

Indahsari, H. S., Tassakka, A. C. M. A. R., Dewi, E. N., Yuwono, M., & Suyono, E. A. (2022). Effects of salinity and bioflocculation during *Euglena* sp. harvest on the production of lipid, chlorophyll, and carotenoid with *Skeletonema* sp. as a bioflocculant. *Journal of Pure and Applied Microbiology*, 16(4):2901-2911.

Inwongwan, S., Kruger, N. J., Ratcliffe, R. G., & O'neill, E. C. (2019). *Euglena* central metabolic pathways and their subcellular locations. *Metabolites*, 9(6):1-24.

Khanra, A., Vasistha, S., & Rai, M. P. (2017). Glycerol on lipid enhancement and FAME characterization in algae for raw material of biodiesel. *International Journal of Renewable Energy Research*, 7(4):1970-1978.

Kim, M., Lee, B., Kim, H. S., Nam, K., Moon, M., Oh, H. M., & Chang, Y. K. (2019). Increased biomass and lipid production of *Ettlia* sp. YC001 by optimized C and N sources in heterotrophic culture. *Scientific Reports*, 9(1):1-12.

Krzemińska, I., Pawlik-Skowrońska, B., Trzcińska, M., & Tys, J. (2014). Influence of photoperiods on the growth rate and biomass productivity of green microalgae. *Bioprocess and Biosystems Engineering*, 37(4):735-741.

Lewis, A. & Guéguen, C. (2022). Using chemometric models to predict the biosorption of low levels of dysprosium by *Euglena gracilis*. *Environmental Science and Pollution Research,* 29(39):58936-58949.

Li, T., Hu, J., & Zhu, L. (2021). Self-flocculation as an efficient method to harvest microalgae: A mini-review. *Water (Switzerland)*, 13(18):1-10.

Liang, C., Yang, Y., Xia, Y., Yuan, W., Chen, J., Zheng, Z. & Zheng, X. (2022). The optimization of *Chlorella vulgaris* flocculation harvesting by chitosan and calcium hydroxide. *Indian Journal of Microbiology*, 62(2):266-272.

Lim, Y. A., Khong, N. M. H., Priyawardana, S. D., Ooi, K. R., Ilankoon, I. M. S. K., Chong, M. N., & Foo, S. C. (2022). Distinctive correlations between cell concentration and cell size to microalgae biomass under increasing carbon dioxide. *Bioresource Technology*, 347(6):1-6.

Low, Y. J. & Lau, S. W. (2017). Effective flocculation of *Chlorella vulgaris* using chitosan with zeta potential measurement. *IOP Conference Series: Materials Science and Engineering*, 206(1):1-11. IOP Publishing.

Martins, D. B., Nasário, F. D., Silva-Gonçalves, L. C., de Oliveira Tiera, V. A., Arcisio-Miranda, M., Tiera, M. J. & dos Santos Cabrera, M. P. (2018). Chitosan derivatives targeting lipid bilayers:

Synthesis, biological activity and interaction with model membranes. *Carbohydrate polymers*, 181(3):1213-1223.

Molitor, H. R., Schaeffer, A. K., & Schnoor, J. L. (2021). Sustainably cultivating and harvesting microalgae through sedimentation and forward osmosis using wastes. *ACS Omega*, 6(27):17362-17371.

Moreira, J. B., Kuntzler, S. G., Bezerra, P. Q. M., Cassuriaga, A. P. A., Zaparoli, M., da Silva, J. L. V., Costa, J. A. V., & de Morais, M. G. (2022). Recent advances of microalgae exopolysaccharides for application as bioflocculants. *Polysaccharides*, 3(1):264-276.

Muir, E., Grossman, A.R., Chisti, Y., Fedrizzi, B., Guieysse, B. & Plouviez, M., (2024). Self-aggregation for sustainable harvesting of microalgae. *Algal Research*, p.103685.

Nicknig, M. A., Azevedo, A. C. D., de Oliveira, H. A. & Schneider, I. A. H. (2024). Cultivation of microalgae (*Scenedesmus* sp.) using coal mining wastewater and separation via coagulation/flocculation and dissolved air flotation (DAF). *Minerals,* 14(4):1-14.

Nie, C.Z., Li, Y., Huang, X.H., Wang, H.P., Wang, X.S., Dong, X.P., Zhu, B.W. & Qin, L., (2024). Multi-scenario application of chitosan emulsions as fat replacers: Based on the regulation of chitosan hydrophobicity and emulsion rheological properties. *Food Hydrocolloids*, 11(13):1-13.

Nurafifah, I., Hardianto, M. A., Erfianti, T., Amelia, R., Maghfiroh, K. Q., Kurnianto, D., Siswanti, D. U., Sadewo, B. R., Maggandari, R., & Suyono, E. A. (2023). The effect of acidic pH on growth kinetics, biomass productivity, and primary metabolite contents of *Euglena* sp. *Makara Journal of Science*, 27(2):97-105.

Podwin, A., Kubicki, W., & Dziuban, J. A. (2017). Study of the behavior of *Euglena viridis, Euglena gracilis* and *Lepadella patella* cultured in all-glass microaquarium. *Biomedical Microdevices*, 19(3):1-10.

Purbani, D. C., Ambarwati, W., Kusuma, A. B., & Herliany, N. E. (2019). Identification of marine microalgae from Tambrauw, West Papua. *Journal of Chemical Information and Modeling*, 11(3):777-790.

Rashid, N., Rehman, S. U. & Han, J. I. (2013). Rapid harvesting of freshwater microalgae using chitosan. *Process Biochemistry*, 48(7):1107-1110.

Salim, S, Bosma, R., Vermuë, M. H., & Wijffels, R. H. (2011). Harvesting of microalgae by bio-flocculation. *Journal of Applied Phycology*, 23(5):849-855.

Salim, S., Kosterink, N. R., Tchetkoua Wacka, N. D., Vermuë, M. H., & Wijffels, R. H. (2014). Mechanism behind autoflocculation of unicellular green microalgae *Ettlia texensis*. *Journal of Biotechnology*, 174(1):34-38.

Salim, S., Vermuë, M. H., & Wijffels, R. H. (2012). Ratio between autoflocculating and target microalgae affects the energy-efficient harvesting by bio-flocculation. *Bioresource Technology*, 118(16):49-55.

Saliu, F., Magoni, C., Torelli, A., Cozza, R., Lasagni, M. & Labra, M. (2021). Omega-3 rich oils from microalgae: A chitosan mediated in situ transesterification method. *Food Chemistry,* 337(4):1-8.

Sapei, L., Agustriyanto, R., Fitriani, E.W., Levy, Z. & Sumampouw, C. (2022). Enhancement of the stability of w/o/w double emulsion by chitosan modified rice husk silica. *International Journal of Technology*, 13(3):584-595.

Sogias, I. A., Khutoryanskiy, V. V. & Williams, A. C. (2010). Exploring the factors affecting the solubility of chitosan in water. *Macromolecular Chemistry and Physics*, 211(4):426-433.

Teh, K. Y., Loh, S. H., Aziz, A., Takahashi, K., Effendy, A. W. M., & Cha, T. S. (2021). Lipid accumulation patterns and role of different fatty acid types towards mitigating salinity fluctuations in *Chlorella vulgaris*. *Scientific Reports Nature*, 11(1):1-12.

Timotius, V., Suyono, E. A., Suwanti, L. T., Koerniawan, M. D., Budiman, A., & Siregar, U. J. (2022). The content of lipid, chlorophyll, and carotenoid of *Euglena* sp. under various salinities. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, 30(3):114-122.

Van Anh, L. T., Ngoc Thu, T. T., & Thi Dong Phuong, N. (2022). Investigation of microalgae culture by autoflocculation methodologies. *Vietnam Journal of Biotechnology*, 20(3):487-494.

Wang, Y., Wang, J., Feng, C., Li, J., Wang, N. & Cai, J. (2022). High-quality *Chlorella vulgaris* biomass harvesting through chitosan and polyacrylamide. *Environmental Science and Pollution Research*, 29(23):34651-34658.

Watanabe, M. M., Kawachi, M., Hiroki, M., & Kasai, F. (2000). NIES collection list of strains. 6th Ed. NIES, Japan, p. 159.

Wu, Z., Zhu, Y., Huang, W., Zhang, C., Li, T., & Zhang, Y. (2012). Evaluation of flocculation induced by pH increase for harvesting microalgae and reuse of flocculated medium. *Bioresource Technology*, 11(8):496-502.

Xin, K., Guo, R., Wang, X., Yu, Z., Mao, W., Cheng, C., Che, G., Qian, L., Cheng, J., Yang, W., & Gao, Z. (2024). Photoautotrophic growth and cell division of *Euglena gracilis* with mixed red and blue wavelengths. *Industrial and Engineering Chemistry Research*, 63(11):4746-4755.

Xu, K., Zou, X., Mouradov, A., Spangenberg, G., Chang, W. & Li, Y. (2021). Efficient biofloccu lation of *Chlorella vulgaris* with a chitosan and walnut protein extract. *Biology*, 10(5):1-13.

Yang, R., Li, H., Huang, M., Yang, H. & Li, A. (2016). A review on chitosan-based flocculants and their applications in water treatment. *Water research,* 95(8):59-89.

Yang, Y., & Weathers, P. (2015). Red light and car bon dioxide differentially affect growth, lipid production, and quality in the microalga, *Ettlia oleoabundans*. *Applied Microbiology and Biotechnology*, 99(1):489-499.

Yin, Z., Chu, R., Zhu, L., Li, S., Mo, F., Hu, D. & Liu, C. (2021). Application of chitosan-based flocculants to harvest microalgal biomass for biofuel production: A review. *Renewable and Sustainable Energy Reviews*, 145(11):1-15.

Zhou, D. Y., Wu, Z. X., Yin, F. W., Song, S., Li, A., Zhu, B. W. & Yu, L. L. (2021). Chitosan and derivatives: Bioactivities and application in foods. *Annual Review of Food Science and Technology*, 12(1):407-432.