



The Application of Chitosan as a Natural Flocculant Material to *Chlorella* sp. Abundance

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ABSTRACT

Natural feed must be available in order to give high-quality seeds to support aquaculture activities. *Chlorella* sp. is a natural food source for fish larvae and zooplankton. *Chlorella* sp. is more effective and efficient when stored in the form of floc. The purpose of this research was to obtain an effective dose of chitosan for flocculation of *Chlorella* sp. The study used a completely randomized design (CRD) with 5 treatments and 3 replications, i.e. A. 150 mg L⁻¹, B. 200 mg L⁻¹, C. 250 mg L⁻¹, D. 300 mg L⁻¹, E. 350 mg L⁻¹. The abundance of *Chlorella* sp. before and after flocculation measured as the main parameter. Water quality parameter such as temperature, pH, dissolved oxygen, total ammonia nitrogen, and nitrite function as supporting parameters. The results showed that the abundance of *Chlorella* sp. before flocculation increased from the beginning of stocking until the 6th day. The best results of the abundance of *Chlorella* sp. found in the C treatment (54.4-74.5%), then followed with B, A, D, and E treatments (31.2-55.1%, 22.8-51.2%, 19.0-45.6 %, and 13.3-44.6%) respectively. The most effective dose of chitosan in the flocculation process of fresh water *Chlorella* sp. was 250 mg L⁻¹ with an abundance of 39.1x10⁵ cells mL⁻¹. Water quality in culture media of the *Chlorella* sp. showed that the range of measured values were still within the optimum range.

Keywords: Abundance, Chitosan, *Chlorella* sp., Flocculation

ABSTRAK

Pakan alami harus tersedia agar dapat menghasilkan benih yang berkualitas untuk menunjang kegiatan budidaya. *Chlorella* sp. merupakan sumber makanan alami bagi larva ikan dan zooplankton. *Chlorella* sp. lebih efektif dan efisien bila disimpan dalam bentuk floc. Tujuan dari penelitian ini adalah untuk mendapatkan dosis kitosan yang efektif untuk flokulasi *Chlorella* sp. Penelitian menggunakan Rancangan Acak Lengkap (RAL) dengan 5 perlakuan dan 3 ulangan, yaitu A. 150 mg L⁻¹, B. 200 mg L⁻¹, C. 250 mg L⁻¹, D. 300 mg L⁻¹, E. 350 mg L⁻¹. Kelimpahan *Chlorella* sp. sebelum dan sesudah flokulasi diamati sebagai parameter utama. Parameter kualitas air seperti suhu, pH, oksigen terlarut, total amonia nitrogen, dan nitrit berfungsi sebagai parameter pendukung. Hasil penelitian menunjukkan bahwa kelimpahan *Chlorella* sp. sebelum flokulasi meningkat dari awal penebaran sampai hari ke-6. Hasil terbaik dari kelimpahan *Chlorella* sp. ditemukan pada perlakuan C (54.4-74.5%), kemudian disusul dengan perlakuan B, A, D, dan E (31.2-55.1%, 22.8-51.2%, 19.0-45.6%, dan 13.3-44.6%). Dosis kitosan yang paling efektif dalam proses flokulasi air tawar *Chlorella* sp. adalah 250 mg L⁻¹ dengan kelimpahan 39.1x10⁵ sel mL⁻¹. Kualitas air di media kultur *Chlorella* sp. menunjukkan bahwa nilai terukur masih dalam kisaran yang optimum.

Kata kunci: Kelimpahan, Kitosan, *Chlorella* sp., Flokulasi

1. Introduction

Nowadays freshwater aquaculture activities are currently being carried out intensively. The availability of natural feed is needed to produce quality seeds in order to support aquaculture activities. Until now, natural food cannot be replaced by artificial feed because it contains digestive enzymes that make it easier for larvae to digest their food

(Abdel-Ghany and Salem., 2019). The advantages of natural feed compared to artificial feed are that it is generally small in size so that it can be adapted to the larva's mouth opening, has a color that attracts the attention of the larvae, has a slow movement so that it attracts attention and makes the fish easier to catch it, naturally a feed that is usually eaten by larvae, its nutritional quality can be improved through enrichment, and can be cultivated intensively.

One of the natural food that feeds fish larvae and zooplankton was microalgae. Microalgae are unicellular or multicellular organisms that have the capacity to grow with little water, nutrients, or carbon dioxide, can absorb solar energy, and have the capacity to use photosynthesis as a mechanism to acquire energy (Ahmad et al., 2011). Microalgae can be found mostly in fresh or salty water, but also on surfaces of different types of soil (Katarzyna et al., 2015). They are of great ecological importance for their ability to adapt to conditions with adverse temperature variations, light, pH, salinity, and humidity, as well as their reduced need for nutrients and their ability to grow in inhospitable environments such as deserts (Guedes et al., 2014; Katiyar et al., 2017).

One of the potential and easy to develop microalgae is of the genus *Chlorella*, which is unicellular green algae, spherical, showing asexual reproduction (Silva et al., 2019). The demand for this type of microalgae has been continuously increasing due to its high nutritional value, leading to its addition to diathetic substitutes, cosmetic products, anti inflammatory products, among others (Katiyar et al., 2017). The interest in these microalgae is due to their rapid growth and simple life cycle, high protein and carotenoids, vitamin, and mineral content (Silva et al., 2019).

Algae can be harvested or yield by using various technique, such as air floatation, filtration, adsorption, chemical peroxidation, and coagulation-flocculation (Sun et al., 2017). Among these technique, flocculation methods are generally considered to be reliable and high-yielding (Beach et al., 2012), the safest, most effective, and widely used because it does not destroy algal cells (Sun et al., 2017). Some reference mention that flocculation of microalgae had been doing using NaOH (Puspaningsih and Saputra., 2014); FeCl_3 (Wyatt et al., 2012); Magnesium (Smith et al., 2012.); NaOH and $\text{Al}_2(\text{SO}_4)_3$ (Ferriols and Aguilar., 2012). Applying chemistry thus given another problems regarding with negative impacts and minimum chemical required (Wyatt et al., 2012). Chitosan is a naturally large molecule with many functional groups in its molecular chain, and thus molecule has been extensively investigated as potential flocculants (Lu et al., 2017). Chitosan has a variety of applications in different fields such as water treatment, agriculture, fabric and textiles, cosmetics, nutritional enhancement and food processing (Abdel-Ghany and Salem., 2019). Flocculation by chitosan was found to have superior technical and environmental performance (Beach et al., 2012). Therefore, the

experiment focused in the flocculation process of *Chlorella* sp is a need in order to get the effective dose. The purpose of the present experiment is to obtain an effective dose of chitosan for flocculation of *Chlorella* sp.

2. Materials and Methods

2.1 Experimental time and location

The experiment conducted from June to August 2020 at Research Station for Environmental Technology And Toxicology Freshwater Aquaculture, Research Institute for Freshwater Aquaculture and Fisheries Extension, Bogor, West Java, Indonesia.

2.2 Materials

The equipment used is 15 glass jars with a volume of 3 liters, aeration equipment, measuring cups, PVC pipe 1", blower cap. 50 L min^{-1} , Zeiss binocular microscope, cover glass, Sedgwick Rafter (SR), 6 pieces of 40 watt TL lamps, magnetic stirrer, hand counter, 600 mL plastic bottle and refrigerator.

2.3 Algal culture

The materials used were *Chlorella* sp inoculants obtained from laboratorium of Research Station for Environmental Technology and Toxicology Freshwater Aquaculture at Cibalagung, Bogor, West Java, Indonesia. Chlorine, sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$), chitosan, distilled water, 1% acetic acid and PHM media (Provasoli Haematococcus Media) were used as fertilizer when *Chlorella* sp. culture. The flocculation method was carried out according to Beach et al. (2012).

2.4 Experimental design

The research used a Completely Randomized Design (CRD) consisted of three treatments with three replication. The treatment of different dose of chitosan as followed:

- A). 150 mg L^{-1}
- B). 200 mg L^{-1}
- C). 250 mg L^{-1}
- D). 300 mg L^{-1}
- E). 350 mg L^{-1}

2.5 Research procedure

2.5.1 Equipment sterilization

Sterilization was conducted in several steps, including: a). Prepared the necessary equipment (aeration hose, aeration stone, PVC pipe, and glass jars with volume of 3 L), b. Soaked all equipment for 24 hours in 3.75 mL/L chlorine water, then rinsed with clean water, c). Soaked for 24 hours in 40 mg/L sodium thiosulfate to neutralized chlorine and then rinsed with clean water, d). Soaked for 24 hours

Table 1. Average daily abundance of *Chlorella* sp. after flocculation and storage

Treatment	Abundance \pm SD ($\times 10^5$ cell mL ⁻¹)					
	1	2	3	4	5	6
A	24.7 \pm 6.36 ^a	14.9 \pm 5.34 ^a	11 \pm 4.01 ^a	12.6 \pm 4.21 ^{ab}	16.8 \pm 4.35 ^{ab}	21.7 \pm 3.72 ^{abc}
B	29.5 \pm 9.44 ^a	25.2 \pm 10.7 ^a	16.7 \pm 6.89 ^{ab}	23.1 \pm 7.70 ^{bc}	23.7 \pm 6.26 ^{bc}	27.7 \pm 8.22 ^{bc}
C	40.1 \pm 19.5 ^a	34.8 \pm 19.2 ^a	29.3 \pm 16.4 ^b	33.8 \pm 18.2 ^c	37.3 \pm 18.5 ^c	39.1 \pm 19.4 ^c
D	16.3 \pm 5.21 ^a	10.5 \pm 2.68 ^a	6.79 \pm 3.49 ^a	7.40 \pm 1.82 ^{ab}	8.06 \pm 1.52 ^{ab}	10.8 \pm 2.54 ^{ab}
E	14.6 \pm 7.68 ^a	9.12 \pm 5.95 ^a	4.35 \pm 2.95 ^a	4.67 \pm 2.96 ^a	5.21 \pm 3.16 ^a	7.3 \pm 2.35 ^a

Remarks: The value followed with the same letter are not significantly different ($p > 0.05$)

in clean water, e). After rinsed with clean water, all equipment was ready to used.

2.5.2 Culture of *Chlorella* sp.

Culture of *Chlorella* sp. was conducted in a view steps: a). Prepared all the instruments, which included glass jars with a volume of 3 L as a culture container, aeration equipment as an oxygen supply, and 40 watt TL lamp as a substitute for sunlight for photosynthesis. b). The culture container was filled with 2 L of distilled water, modified PHM fertilizer was used as a medium, and it was aerated for 24 hours. c). Inoculated the culture container with 200 ml of *Chlorella* sp. inoculant, d). The quantity of *Chlorella* sp. was measured daily with a microscope on a Sedgewick rafter.

2.5.3 Chitosan solution

1 gram of chitosan was dissolved in 100 mL of 1% acetic acid and stirred for 6 hours with a magnetic stirrer to ensure total dissolution.

2.5.4 Flocculation and storage

Chlorella sp. flocculation was conducted out with varied doses of chitosan treatment after reaching the maximal abundance on day 6. After mixing *Chlorella* sp. with chitosan with a magnetic stirrer, the flocculation procedure was carried out. The flocculation approach used by Beach et al. was followed (2012). After *Chlorella* sp. solidified and sedimented, it was removed and stored in a 400 ml bottle. The abundance of

Chlorella sp. flocculation was calculated using a Sedgewick rafter and a microscope. Furthermore, floc *Chlorella* sp. was kept in a refrigerator at 4 °C for one week. After storage, *Chlorella* sp. was re-cultured in PHM media and its abundance was monitored every day for 6 days. The main parameter observed of this research is the abundance of *Chlorella* sp. The water quality parameters measured were temperature and dissolved oxygen (DO) (DO meter digital, Lovibond), pH (pH-meter digital, Lovibond), total ammonia nitrogen (TAN) and nitrite were measured using spectrophotometer PG Instrument.

2.6 Statistical analysis

The data for daily abundance of *Chlorella* sp. were analyzed by one-way ANOVA followed by Duncan's Multiple Range Test at a confidence level of 95%. Data on the water quality parameters were analyzed descriptively.

3 Result and Discussion

3. 1 Daily abundance of *Chlorella* sp. before flocculation

Based on the graph of *Chlorella* sp. before flocculation, increased abundance of *Chlorella* sp. from the beginning of culture until the 6th day can be seen in Figure 1. This graph is shown until the 6th day, because the flocculation process was carried out on the 6th day. The flocculation day was held on the 6th day or at the exponential phase, because after that the abundance of cells will decreased, indicating that the culture had entered a stationary phase. The stationary phase occurs because the nutrients in the media have been greatly reduced so that they are not sufficient for cell growth and division. *Chlorella* sp. was re-cultivated and until its growth was at stationary phase before the microalgae was harvested via coagulation-flocculation, is the best time which show high flocculation efficiency (Yunos et al., 2017). The result showed that on day 1 to 3 *Chlorella* sp. undergoing an adaptation phase, it is assumed that there are enough nutrients available so that the growth of *Chlorella* sp. grew fast. The availability of nutrient elements affects the

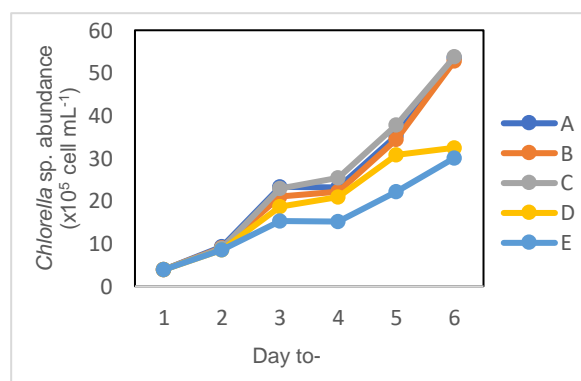


Figure 1. Daily abundance of *Chlorella* sp. before flocculation

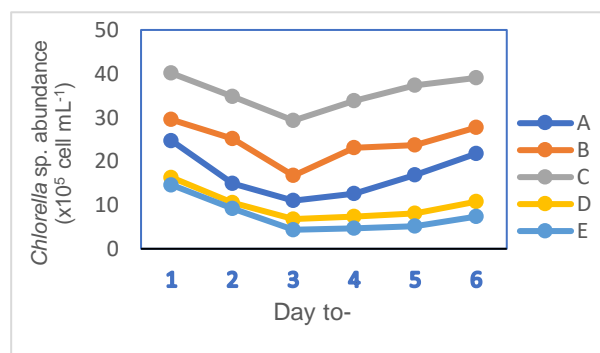


Figure 2. Daily abundance of *Chlorella* sp. after flocculation and storage

growth of *Chlorella* sp. (Silva et al., 2019). In this phase the cell size increases, phytoplankton becomes active and protein synthesis occurs. Organisms undergo metabolism but have not undergone division.

3.2 Daily abundance of *Chlorella* sp. after flocculation and storage

Daily abundance of *Chlorella* sp. after flocculation and storage for one week can be seen on Figure 2. *Chlorella* sp. which has been flocculated and stored for one week in the refrigerator, can still be cultured again until the 6th day and showed different daily abundances of cells in each treatment (Table 1). The average value of the increase or decrease percentage in daily abundance of *Chlorella* sp. in all treatments, when culture after flocculation and storage decreased in the range of 1%-20%. Storage in low temperature and administration of chitosan can inhibit the process of cell metabolism and bacterial growth, so it is thought

to affect the daily abundance of *Chlorella* sp. during culture after flocculation and storage.

The adaptation phase at the time of recultivated was different, this was thought to be the effect of different doses of chitosan. On the 4th day of recultivated, all treatments showed an increase in abundance, this was happen because *Chlorella* sp. has passed the adaptation phase and entered the exponential phase. The highest abundance was seen in treatment C, which was 33.8×10^5 cell mL⁻¹ using a dose of chitosan 250 mg L⁻¹, then treatment B (200 mg L⁻¹), A (150 mg L⁻¹), D (300 mg L⁻¹), and E (350 mg L⁻¹), namely 23.1×10^5 , 12.6×10^5 , 7.40×10^5 , and 4.67×10^5 cell mL⁻¹. In treatment C, with a dose of chitosan 250 mg L⁻¹, it resulted a high increase compared to other treatments. This was thought because when the addition of chitosan in the flocculation process, the abundance did not decrease much. As more flocculants are added to the solution, the number of chemicals that are able to reduce the electrical charge on the surface of the microalgae particles also increases so that the repulsive forces between microalgae particles will weaken and the particles will be close together and will then combine to form flocs (Aji et al., 2012).

The same thing happened on 5th day, the highest abundance was obtained in treatment C, namely 37.3×10^5 cell mL⁻¹ which was significantly different ($P < 0.05$) with treatment A, D and E and not significantly different ($P > 0.05$) with treatment B. Other result showed that optimal dose of chitosan varied from 40 mg L⁻¹ up to >200 mg L⁻¹ (Beach et al., 2012). This difference is thought to be the effect of different initial abundances of inoculants during

Table 2. Water quality parameters before flocculation and after flocculation and storage

Treatment		Parameters				
		Temperature °C	DO (mg L ⁻¹)	pH	TAN (mg L ⁻¹)	Nitrite (mg L ⁻¹)
Before flocculation	A	20.0-23.6	3.55-6.19	6.28-7.85	0.149-.385	0.47-1.25
	B	20.7-24.1	4.07-6.31	6.38-7.73	0.750-.268	0.88-1.38
	C	19.8-23.4	4.68-6.07	6.29-8.42	0.104-.463	0.45-1.20
	D	19.3-23.1	4.77-6.17	6.35-8.75	0.126-.465	0.16-0.98
	E	19.4 -23.5	4.27-6.85	6.22-8.79	0.810-.316	0.48-1.25
After flocculation and storage	A	19.8-23.8	4.47-7.02	6.70-8.93	0.012-0.89	0.06–0.19
	B	19.8-24.2	4.53-7.74	7.81-9.01	0.840-6.30	0.05–0.16
	C	19.4-24.6	4.17-7.00	7.95-9.41	0.212-5.50	0.05–0.14
	D	19.9-24.1	4.26-7.86	6.41-9.76	0.120-2.40	0.22–0.35
	E	19.3-23.5	4.51-7.86	6.76-9.63	0.140-2.11	0.12–0.18

re-cultivated. The difference in the density of the microalga culture was caused by the emergence of new individuals and the occurrence of cell death. The specific growth rate describes the number of new individuals that appear per unit of time. The specific growth rate of microalgae culture will generally increase until it reaches the maximum growth rate, then decrease due to a decrease in the quality and quantity of nutrients, as well as various other abiotic factors. In addition, the specific growth rate of microalgae culture is influenced by the initial density of the inoculum. This is related to the carrying capacity of the medium which is a limiting factor for the growth of microalgae.

The abundance of 4th day to 6th day in each treatment increased. Treatment C gave the best results with the highest abundance compared to treatments A, B, D and E. Treatment E got the lowest abundance value, which was 7.3×10^5 cell mL⁻¹. This is thought to be the effect of high doses of chitosan. The addition of high flocculants will not cause sediment but will further break down the precipitate and cause turbidity in the solution because in these conditions an excessive number of flocculants can cause deflocculation or unstabilization particle due to the repulsion between the positive charges of the chitosan particles (Aji et al., 2012). So that at the time of flocculation, treatment E gave the lowest abundance which resulted at the time of re-activated which resulted in the lowest abundance as well. Chitosan knew has various favourable biological properties including biosafety, biodegradability and biocompatibility, also has many physiological functions in fish including growth promoting, antioxidation, antimicrobial effect and immunostimulation (Abdel-Ghany and Salem., 2019). For the harvesting process, chitosan was proven to be effective in harvesting *Chlorella* sp. (Yunos et al., 2017). For terrestrial animals, chitosan has been widely used as a feed additive because enhancing the growth performance, improving immune functions, inhibition intestinal microbial pathogens and lowering cholesterol (Abdel-Ghany and Salem., 2019.). Recently chitosan also used in fish diets, aquaculture water management and other aquaculture activities.

3.3 Water quality parameters

Water quality parameters measured during the experiment can be seen on Table 2. Water quality parameters measured during the experiment showed that temperature range between 19.3-24.1 °C (before flocculation), 19.3-24.6 °C (after flocculation and storage); DO

range between 3.55-6.85 mg L⁻¹ (before flocculation), 4.17-7.86 mg L⁻¹ (after flocculation and storage); pH range between 6.22-8.79 (before flocculation), 6.41-9.76 (after flocculation and storage). The pH range in this experiment showed higher than that of (Yunos et al., 2017), mentioned that final pH in harvesting *Chlorella* sp. using chitosan between 7-8.

However this is supposed to happen because it used HCl as a solvent in the research. In this experiment, measurement of pH for all treatment showed changes after flocculation. This phenomena also happen according to (Ferriols and Aguilar., 2012), which stated that measurement of pH for all treatments showed significant changes in the pH of the culture media after the addition of flocculants. Result in this experiment was also the same with Cheng et al. (2011) which stated that higher pH at 8.5 was optimal for *C. sorokiniana*, and these differences might be due to the difference in culture media, growth conditions and unique strain properties, such as cell morphology, extracellular organic matter and cell surface.

4. Conclusion

The results showed that the inoculants of fresh water *Chlorella* sp. that has been flocculated and stored for a week can be cultured up to 6th day. The most effective dose of chitosan in the flocculation process of fresh water *Chlorella* sp. to the highest abundance is 250 mg L⁻¹ with an abundance of 39.1×10^5 cells mL⁻¹.

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