Effect of pH culture and dosage of chitosan nanoemulsion on the effectiveness of bioflocculation in harvesting *Chlorella* sp. biomass

Erdawati1, Mutia Kanza1, Ganjar Saefurahman2, Syarif Hidayatuloh2, and Mujizat Kawaroe2,3

1Department of Chemistry, Faculty of Mathematics and Sciences, Jakarta State University, Jl. Pemuda No.10, Rawamangun, Jakarta, 13220, Indonesia
2Surfactant and Bioenergy Research Center, IPB University, Baranangsiang Campus, Jl. Raya Pajajaran No. 1, Bogor, 16144, Indonesia
3Department of Marine Science and Technology, Faculty of Fisheries and Marine Sciences, IPB University, Dramaga Campus, Bogor, Indonesia

*Email: erda_wati_0912@yahoo.com*

**Abstract.** *Chlorella* sp. is a microalga with a size of less than 30 μm that requires a dewatering process to separate its biomass from the culture media, for instance using the flocculation method. In this study, chitosan nanoemulsion was used as the bioflocculant. The aim of this study was to investigate the effectiveness of harvesting *Chlorella* sp. biomass using chitosan nanoemulsion with varied doses and predetermined pH levels. The pH and bioflocculant dose affected the amount of biomass recovered during the harvesting process. The results showed that treatment at pH 9 and a dose of 2 g L⁻¹ bioflocculant achieved the highest harvesting efficiency of 98.7%. The characterization of bioflocculant was carried out in the form of proximate analyses on chitosan nanoemulsion which resulted in 95% ash and 90% water contents. The appearance tests showed characteristics of yellow in color, sour aroma, and gel-shaped texture. The physical tests were also performed resulting in a pH of chitosan nanoemulsion of 4.3, viscosity of 15.5 cps, and a density of 1.912 cm³g⁻¹. This study indicated that chitosan nanoemulsion is considered efficient for use as an alternative bioflocculant for harvesting *Chlorella* biomass.

1. **Introduction**

*Chlorella* sp. is an important microalgae species that usually lives in fresh and marine waters [1]. *Chlorella* sp. contains lipids, carbohydrates, proteins, pigments, vitamins, minerals, and amino acids [2]. *Chlorella* sp. biomass is useful for increasing immunity, increasing platelet levels, and binding toxins in the digestive tract [3]. Commercial *Chlorella* has been widely used as a supplement for health [2, 4]. *Chlorella* can also be used as food sources for hatcheries and as fish feed to increase the body’s resistance to the bacteria [5]. *Chlorella* also contains lipids, making *Chlorella* can be used as a feedstock for biodiesel [6]. The utilization of *Chlorella* as a biodiesel feedstock, for example, using 0.5 M HCl as a catalyst, biomass:methanol ratio of 1:5, reaction temperature of 65 °C and reaction time of 7 hours, can convert 95% lipids to biodiesel [7].

However, behind the excellence of *Chlorella* sp. as a biomass feedstock for a health supplement, aquaculture feed, and biodiesel, the drawback is its size smaller than 30 μm and its...
surface is negatively charged causing the process of harvesting or separation of biomass from culture media is difficult to be achieved [8]. The harvesting process is one of the most important stages in microalgae biomass production. Several methods that are commonly used are filtration, centrifugation, and flocculation [9].

Various methods have been developed to harvest *Chlorella* sp. biomass such as centrifugation, sedimentation, filtration, and flocculation [10, 11]. The electrical energy required for centrifugation is 8 kWh/m³, with filtration techniques requiring only 0.4 kWh/m³ of electricity, but the operational costs increase because screen filters need to be replaced periodically [12]. Poelman et al. [13] reported that the electrocoagulation method and without using coagulant required energy of 0.3 kWh/m³ with a yield of 95%. According to Salim et al. [14], the flocculation method can reduce electricity costs by 87% for centrifugation. Commonly used flocculants include cationic polymer flocculants, poly aluminum chloride, ferric chloride, aluminum chloride [15], α-poly-L-lysine biopolymers [16] and chitosan [17]. Aluminum sulfate and ferric sulfate salts are flocculants that are widely used to harvest microalgae because they are cheap. However, dissolved Fe²⁺ ions and Al³⁺ ions will contaminate the harvested biomass. Rzymski et al. [18] found that *Chlorella* and *Spirulina* powders had shown both biomass containing Al ions after flocculated using Aluminum sulfate.

One of the most effective methods that can reduce the cost of microalgae biomass harvesting is coagulation-flocculation [8, 10, 11, 19]. Flocculation is a harvesting method by forming microalgae cells in larger clusters making it easy to collect the biomass [10]. To form clusters, microalgae are given flocculants. Based on previous research, the commonly used flocculants are synthetic materials such as Al₃SO₄ or alum. The disadvantages of these flocculant materials are that the biomass should not be used as food, animal feed, organic fertilizer, and in aquaculture [19]. This study applied a bioflocculant that is considered environmentally friendly, easily available and more energy efficient. Flocculants made from natural ingredients are usually referred to as bioflocculants. The bioflocculant used in this research was chitosan nanoemulsion.

The use of natural flocculant aimed to reduce the use of synthetic materials, to obtain biomass that is environmentally friendly and safe to use as aquaculture feed, as well as lower production costs because it saves time and energy, and to determine the ability of chitosan nanoemulsion in the microalgae *Chlorella* flocculation process. This research was conducted to investigate the effectiveness of harvesting *Chlorella* sp. biomass using chitosan nanoemulsion with varied doses and predetermined pH levels.

### 2. Materials and Methods

**2.1. Equipment and Materials**

The equipment used in this research were 2000 mL beaker, 600 mL beaker, analytical balance, pipette, magnetic stirrer, hotplate stirrer, static ring and clamp, oven, aerator, small hose for aerator, funnel, spatula, 100 mL, 50 mL and 25 mL measuring cups, testing jar, spectrophotometer, LEICA ICC 50 HD microscope with camera, Haemocytometer, refractometer, centrifuge, centrifuge bottle, and Whatman filter paper No. 41.

The materials used in this study were STPP (sodium tripolyphosphate), aquadest, glacial acetic acid, urea, ZA, TSP (triple superphosphate, Merck) as fertilizer for Chlorella culture, and chitosan with 90% distillation degree and 35 µm size was obtained from the material laboratory, BATAN, Jakarta, Indonesia and microalgae *Chlorella* sp. which was originally cultivated at the Surfactant and Bioenergy Research Center, IPB University in Bogor, Indonesia.

**2.2. Preparation and characterization of Chitosan Nanoemulsion**

Six grams of chitosan was added to 1200 mL of 1% acetic acid solution then it was stirred until all the chitosan was dissolved, and left for 24 hours. In the condition of stirring with a magnetic stirrer, 20 ml of 0.5% STPP was gradually added right in the middle of the stirrer and it was stirred for 20 min. This step was performed until 400 mL of STPP was added. Afterward, chitosan nanoemulsion
was stored into a bottle and kept in the freezer to let it stand for 24 hours. After 24 hours, the chitosan nanoemulsion was taken out and then the formed water was decanted and centrifuged at a speed of 5000 rpm for 10 min. This step was performed until the chitosan emulsion was obtained. The characterization of chitosan nanoemulsion included the measurement of color, aroma, texture, viscosity, pH, and density. The proximate analysis was also performed to determine the water and ash contents of chitosan nanoemulsion.

2.3. Proximate Analysis of Chitosan Nanoemulsion

Proximate analysis was used to determine the characteristics of chitosan nanoemulsion samples in this study. The analyses performed were water content and ash content.

1. Determination of Water Content

Water content was determined using the gravimetric method [20]. Porcelain cup was weighed and labeled, then the cup was dried at 105 °C in the oven for 1 hour and cooled in a desiccator for 20 min. One gram biomass was put into a porcelain cup and weighed, then dried at 80 °C for 6 hours and then cooled in a desiccator for 30 min. The calculation of water content was performed using the formula as follows [20]:

\[
\text{\% of water content} = \frac{(A+B) - C}{B} \times 100\% 
\]

Note:
A = weight (porcelain cup + sample) before being dried (g)
B = weight (porcelain cup + sample) after being dried (g)
C = sample weight (g)

2. Determination of Ash Content

The ash content was determined using the dry ashing method [20]. A porcelain cup was washed, dried in the oven about 1 hour at a temperature of 105 °C then cooled in a desiccator about 10 min and carefully weighed (A gram). Two grams of chitosan nanoemulsion were weighed, inserted into a porcelain cup (B gram). Then it was put into the furnace, and burned at 400 °C for 2 hours until the sample was burned and the color turned white. After cooled, the samples were transferred into a desiccator. The weight of the obtained ash was weighed (C gram). Ash content was calculated using the formula as follows [20]:

\[
\text{\% of ash content} = \frac{(A+B) - C}{B} \times 100\% 
\]

2.4. Determination of Density and Viscosity of Chitosan Nanoemulsion

The empty pycnometer was dried in the oven and weighed. The pycnometer was filled with aquadest or nanoemulsion samples at 200 °C then heated in a water bath at 250 °C for 30 min. The pycnometer was then lifted, dried and weighed. Pycnometers containing samples or aquadest were then weighed. The viscosity of samples was measured using a rotary viscosimeter at room temperature (27 ± 0.20 °C).

2.5. Cultivation of Chlorella sp.

Seawater with a salinity of 25 ppt was taken from the seawater storage tanks at Surfactant and Bioenergy Research Center (SBRC), IPB University, as much as 15 L seawater was sterilized using an autoclave for 120 min with a temperature of 121 °C. The sterile seawater of 0.6 L with 25 ppt salinity was transferred into 2 L Erlenmeyer flasks containing 0.3 L of Chlorella sp. starter culture. Chlorella sp. was cultured in sterile algae growing room and aerated for 24 hours for 5 days until reached 18 L Chlorella sp. During the culture, the cell density and growth of Chlorella sp. was observed every day, and the culture was continuously aerated in the culture room until ready to be harvested.
1. Culture Medium

The culture medium consisted of ZA, Urea, TSP with a ratio of 2: 2: 1 in a culture of 300 mL of *Chlorella* sp. culture and 600 mL of seawater in the initial volume of 900 mL *Chlorella* sp. starter culture.

2. Growth of *Chlorella* sp.

The growth curve of *Chlorella* sp. culture was determined by measuring the cell density. Measurements of cell density were performed by taking 1 mL of samples daily. Daily cell density measurements were performed using the Haemocytometer and the samples were observed under the light microscope LEICA ICC 50 HD equipped with a camera. The cells in the microscope were manually counted by the number of cells, then the total number of cell density was calculated by the formula:

\[
D = \left( \frac{N_1 + N_2}{2} \times \frac{25 \times 10^4}{n} \right) \times DF
\]

Note:
- **D**: Cell density
- **N1**: Number of microalgae in the upper field of Haemocytometer
- **N2**: Number of microalgae in the lower field of Haemocytometer
- **n**: Number of observation boxes observed
- **25 \times 10^4**: Haemocytometer constant
- **DF**: Dilution factor

3. pH and Salinity Measurements

During the *Chlorella* sp. culture period, pH and salinity were measured daily. Measurement of pH was performed using pH-meter pHep® by HANNA instruments, whereas Refractometer MASTER REFRACOMETER ATAGO® was used to measure the salinity.

2.6. Harvesting and Lipid Extraction of *Chlorella* sp. Biomass

1500 mL of *Chlorella* sp. culture was transferred into three 500 mL beaker glasses. These culture samples with different pH were divided into 5 of 100 mL beaker glasses each. The bioflocculant of chitosan nanoemulsion with different dose was added. The culture suspensions were stirred and allowed to settle at room temperature of 25 °C for 1 hour.

The culture suspensions were separated, and each of the supernatants was taken. The harvesting efficiency was calculated from changes in cell density between before and after the flocculation process based on the measurement of spectrophotometry at a wavelength of 680 nm. Control of pH 9, 10, and 11 without the addition of flocculants was used as a reference. The harvesting efficiency (Ef) or percentage of microalgae biomass separated from the suspension was calculated using the formula as follows:

\[
Ef = \frac{A_0 - A_f}{A_0} \times 100\%
\]

Where **A0** is the cell density before flocculation while **Af** as cell density after flocculation.

3. Results and Discussions

3.1. Characteristics and Proximate Analysis of Chitosan Nanoemulsion

Chitosan nanoemulsion is a cross-link between sodium tripolyphosphate (STPP). The amino group from chitosan will be hydrated and produce NH3+ ions when dissolved in water with acidic pH. When STPP is dissolved in water, it produces hydroxyl and phosphate ions. Phosphate ions interact
with NH$_3$ from chitosan and cause a cross-link between chitosan and tripolyphosphate [21]. Characteristics and proximate analysis of chitosan nanoemulsion are shown in Table 1.

Table 1. Characteristics and proximate analysis of chitosan nanoemulsion

| No. | Characteristics | Result          |
|-----|-----------------|-----------------|
| 1   | Color           | Light yellow    |
| 2   | Aroma           | Sour            |
| 3   | Texture         | Gel             |
| 4   | pH              | 4.3             |
| 5   | Viscosity       | 15.5 cps        |
| 6   | Density         | 1.912 cm/g      |
| 7   | Water content   | 90%             |
| 8   | Ash content     | 95%             |

The results of nanoemulsion physical properties showed the color mixture of yellow chitosan and white STPP, and the color of nanoemulsion was found to be a light yellow. The aroma was sour originated from glacial acetic acid, and the texture was gel after the cross-linking between chitosan and tripolyphosphate. The water content is one of the important parameters to determine the quality of chitosan. In this research, the chitosan nanoemulsion had a high water content of 90%, the amount of water content in chitosan nanoemulsion is influenced by the process of using aquadest as the solvent of 0.5% STTP and 1% glacial acetic acid. The physical tests of chitosan nanoemulsion showed a pH of 4.3, a viscosity of 15.5 cps, and a density of 1.912 cm/g.

3.2. Cell Density and Growth of Chlorella sp.

The growth of Chlorella sp. in this study is shown in Figure 1. The growth curve showed that the density of Chlorella sp. that was already high at the beginning of the cultivation period. However, it continued to increase until the highest density of 3.2 $\times 10^4$ cells mL$^{-1}$ on day 10, and it was decreased daily with total cell density reaching 2.3 $\times 10^4$ cells mL$^{-1}$ on day 14. This decrease in cell density may be due to the reducing nutrient in the media because of the Chlorella sp. growth. The increase in density increased the use of available nutrients. The growth of Chlorella sp. in culture is influenced by several factors such as culture system, nutrients availability, light, temperature, and salinity [2, 22].

![Figure 1. Growth curve of Chlorella sp. culture during the cultivation in this study](image-url)
During the culture period, the salinity of culture ranged from 24-26 ppt. The increase in culture salinity was followed by an increase in pH culture. Salinity is one of the important factors that affected the growth of \textit{Chlorella} sp. Microalgae living in waters have different tolerance to salinity, depending on the species and the stage of the life cycle. Salinity can affect the growth and cell composition of microalgae [23, 24]. In general, an increase in salinity will reduce growth rates in almost all species [25]. The culture salinity in this study was within the optimum salinity for \textit{Chlorella} sp. culture, i.e. 25-28 ppt [26], and within the range of salinity tolerance for a good growth at 15-35 ppt [26]. The rise in pH increased with the growth of \textit{Chlorella} sp biomass. pH began to rise on day 6 and continue to be constant and reached the maximum on day 14. The pH during the cultivation period ranged from 7.6 to 9.3.

3.3. \textit{Harvesting and Lipid Extraction of Chlorella sp. Biomass}

The harvesting of \textit{Chlorella} sp. biomass using bioflocculant chitosan nanoemulsion was performed after the density of microalgae cells decreased constantly at the stationary phase closed to the death phase, i.e. on day 14. In the flocculation process, the amount of bioflocculant added to the \textit{Chlorella} sp. biomass was determined, so that it can achieve the optimum dose. Harvesting efficiency is a measure of how many microalgae cells are successfully removed from culture and expressed in percentages [27]. Harvesting efficiency of \textit{Chlorella} sp. biomass using chitosan nanoemulsion is shown in Figure 2.

![Figure 2. Harvesting efficiency of Chlorella sp. biomass using chitosan nanoemulsion](image)

Figure 2 showed that the higher the flocculation efficiency, the more microalgae cells were flocculated or recovered in the culture medium. Harvesting of \textit{Chlorella} sp. was carried out at pH 9, 10 and 11, wherein these pH showed different results of optimum harvesting efficiency. At pH 9, the optimum harvesting efficiency was achieved at a dose of 2 grams bioflocculant with a yield of 98.7%, while at pH 10 the optimum harvesting efficiency was at a dose of 1 gram with a yield of 96%, and at pH 11 the optimum harvesting efficiency was at a dose of 0.5 gram with a yield of 96%. It can be concluded that the greater the pH of \textit{Chlorella} sp. culture, the bioflocculant doses of chitosan nanoemulsion required were less. The most optimum pH condition was at pH 9 with the highest harvesting efficiency compared to pH 10 or 11, which was 98.7%. The optimum pH for \textit{Chlorella} sp. flocculation was at pH 9 because when the pH exceeded 10, the turbidity was increased and the culture density was gradually reduced.

Blockx et al. [28] indicated that flocculation induced by chitosan at varying pH was not related to precipitation of magnesium hydroxides or autoflocculation. Chitosan flocculation at high pH in microalgae culture was found to be caused by precipitation of chitosan due to partial deprotonation of the amine groups, resulting in a network formation that generates flocculation by a sweeping
mechanism. Visual observations and viscosity measurements also confirmed the occurrence of precipitation of chitosan at pH > 7.5 [28].

One of the mechanisms of polymeric flocculation is the charge neutralization [28, 29] (Figure 3). The surface of the cells will result in the reduction of electrostatic repulsion force between the cell particles so that the cells will be coagulated and finally be flocculated [29]. Stable microalgal cells which have negative charges are brought closer to the added acrylamide based cationic polyelectrolyte as bioflocculant to the culture. The surface charge of the negatively charged microalgal cells is neutralized by cationic bioflocculants via electrolytic repulsion which reduces the zeta-potential and enables the flocculation process [28]. The gentle mixing accelerates the rate of particle collision, while the mixing speed was increased to allow flocculant distribution evenly. Therefore, the positively charged flocculant is attracted to the negatively charged microalgal colloidal particles by means of some electrostatic forces of attraction via charge, dipole-dipole hydrogen bonding and van der Waals forces of interactions [29].

![Figure 3. The polymeric flocculation mechanism [29]](image)

4. Conclusions

This study showed the potential of chitosan nanoemulsion as a natural, non-toxic, energy-efficient, and environmentally friendly bioflocculant to harvest *Chlorella* sp. biomass. The greater pH of *Chlorella* sp. culture required fewer doses of chitosan nanoemulsion applied to achieve the optimum harvesting efficiency. The harvesting efficiency of *Chlorella* sp. was optimum at 98.7% with pH 9 and the dose of chitosan nanoemulsion bioflocculant was 2 gL⁻¹.
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