

Harvesting effectiveness of *Chlorella* sp. biomass using different flocculation treatments of *Moringa oleifera* extract and pH conditions

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Abstract. *Chlorella* sp. is microalgae species that is commonly found in seawater. *Chlorella* biomass have been produced worldwide and considered as sources for commercial high value bioproducts and biofuels for the last few decades. However, its microscopic size and negatively charged cell surface causes the difficulties of harvesting process or separation of its biomass from the culture medium. The common techniques for harvesting microalgae biomass are centrifugation, filtration, and flocculation. The determination of efficient harvesting methods was the aim of this study to lower the cost of harvesting. In this study, the flocculation harvesting method was used by applying several flocculant doses of moringa seeds extract (*Moringa oleifera*) and adjusted pH. Moringa seed was used as a bioflocculant to reduce the use of synthetic flocculant materials, especially metals, in order to achieve an environmentally friendly, more efficient, and safe biomass harvesting method for uses as aquaculture feed. The results showed that the moringa seeds are considered efficient for use as bioflocculants of *Chlorella* biomass. This bioflocculant achieved the optimum performance in the treatment of pH 11 with a dose of 0.15 grams moringa seeds bioflocculant in 1 Liter of *Chlorella* sp. culture.

1. Introduction

Chlorella sp. is a microalga species that is commonly found in seawater [1]. *Chlorella* has been widely known and cultivated for many applications including food sources for hatcheries [2], animal food additives, human dietary supplements [3, 4], and biodiesel feedstock [5, 6]. The green color of chlorophyll in *Chlorella* sp. is called green blood has a hemoglobin-forming iron substance that can be used for medical treatments [7]. As development of microalgae biomass production and processing technology, microalgae harvesting methods are also developing. Various methods have been used, from methods that work mechanically or physically to chemical methods. Several methods that have been commonly performed are filtration, centrifugation, and flocculation [8].

Techniques that generate the highest harvesting efficiency for microalgae biomass are centrifugation, filtration and flocculation [8]. The identification of efficient methods with low



operating cost was the aim of this study to lower the harvesting cost [9]. In the harvesting process, there are several aspects that must be considered. Firstly, the harvesting process should be easily operated, the biochemical compounds in microalgae biomass should not be changed or damaged after applying the harvesting treatment, and the process should generate a large quantity of biomass yields [10].

One of the most effective methods that can reduce the cost of microalgae biomass harvesting is coagulation-flocculation [9-11]. Flocculation is a harvesting method by forming microalgae cells in larger clusters making it easy to collect the biomass [9]. To form clusters, microalgae are given flocculants. Based on previous research the commonly used flocculants are synthetic materials such as Al_2SO_4 and nanomagnetic chitosan. Disadvantages of these flocculant materials are that the biomass should not be used as food, animal feed, organic fertilizer, and in aquaculture [11]. This study used flocculant that is environmentally friendly, easy to obtain and has more energy efficient. The flocculant made from natural materials is commonly referred to as bioflocculants. The bioflocculant used in this study is *Moringa oleifera* seed powder which was derived from dried moringa seed which was pounded until smooth (6 μm).

The use of natural materials 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 *Moringa oleifera* matured and dried in the microalgae *Chlorella* flocculation process. This research was conducted to investigate the effectiveness of harvesting *Chlorella* sp. biomass using moringa seed bioflocculant with varied dose and predetermined pH levels. The experimental parameters tested of *Chlorella* cultures included treatments of pH cultures and flocculant dose.

2. Materials and Methods

2.1. Equipment and Materials

The equipment used were porcelain mortar and pestle, small jar, aerator, aerator tubes, autoclave, Haemocytometer, refractometer, pH-meter, glass cuvette, drop pipettes, filter paper Whatman No. 41, light microscope, 20 liter capacity cultivation container, 100 mL beaker glass, and 2000 mL Erlenmeyer flasks. The materials used were *Chlorella* sp. originated from Surfactant and Bioenergy Research Center - Bogor Agricultural University, fertilizer of TSP, ZA and urea, seawater at salinity 25 ppt, aquadest, alcohol 70%, and NaOH 3M.

2.2. Preparation of Moringa Seed Powder

Brown color dried moringa seeds were obtained from Cirebon, Indonesia. The seed shell was peeled, then its yellowish white contents were dried for 24 hours. After drying, the moringa seeds were put into a porcelain mortar then pounded until smooth with a pestle. The fine powder was then sieved with a 60 mesh-sieve. The moringa seeds powder produced was in the form of fine yellowish grains. The moringa seeds powder was immediately weighed and stored in a closed container.

2.3. Proximate Analysis of Moringa Seed Powder

Proximate analysis was used to determine the characteristics of moringa seed samples used in this study. The tests included analyses of water content and ash content.

1. Determination of Water Content

Water content was determined by gravimetric method [12]. Porcelain cup was weighed and coded, then the cup was dried at 105°C in the oven for 30 minutes and cooled in a desiccator for 20 minutes. One gram biomass was inserted into a porcelain cup and weighed, then dried at 105°C for 24 hours and then cooled in a desiccator. Determination of moisture content was performed with the calculation using the formula as follows [12]:

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

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 by dry ashing method [12]. A porcelain cup that has been washed, dried in the oven about 1 hour at temperature 105°C then cooled in a desiccator about 10-30 minutes and carefully weighed (A gram). Two grams of moringa seeds was weighed, inserted into porcelain cup (B gram). Then it was put into the furnace, and burned at 600°C for 5 hours until all the moringa seeds was burned and the color turned white. After cold, the samples were transferred into a desiccator. The weight of obtained ash was weighed (C gram). Ash content was calculated using the formula as follows [12]:

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

2.4. Cultivation of *Chlorella* sp.

Seawater with salinity of 25 ppt was taken from the seawater storage tanks at Surfactant and Bioenergy Research Center (SBRC), Bogor Agricultural University, as much as 15 L seawater was sterilized using Autoclave for 120 minutes with a temperature of 121°C (Autoclave capacity was 5 L per 120 minutes). 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 and scaled up in a sterile algae culture room and aerated for 24 hours for 5 days until reached 18 L *Chlorella* sp. After culturing, 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. Fertilizer/Culture Medium

The culture medium was comprised 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 stages of 900 mL *Chlorella* sp. starter culture.

2. Growth Curve of *Chlorella* sp.

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

n : Number of observation boxes observed

DF: Dilution factor

25×10^4 = Haemocytometer constant

3. pH and Salinity Measurements

During *Chlorella* sp. cultivation period, pH and salinity were frequently measured. Measurement of pH was performed using pH-meter pHep® by HANNA instruments, whereas for salinity Refractometer MASTER REFRACTOMETER ATAGO® was used.

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

1500 mL of *Chlorella* sp. culture was taken and transferred into three 500 mL beaker glasses. These culture samples with predetermined pH were divided into 5 of 100 mL beaker glasses each. Afterwards, the bioflocculant of moringa seed powder with predetermined dose was added. The culture suspensions were stirred and allowed to settle at room temperature 25°C for 1 hour. The culture suspensions were further separated, and each of the supernatant was taken. The flocculation efficiency was calculated from changes in cell density between before and after flocculation process. The change in cell density was measured by 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 efficiency of flocculation (Ef) or percentage of microalgae biomass removed from the suspension was calculated using the harvesting efficiency formula as follows:

$$Ef = \frac{A0 - Af}{A0} \times 100\%$$

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

Lipid extraction was performed according to the Bligh and Dyer method (1959) [13] by modifying the solvent. The determination of % total lipid weight was performed using the formula:

$$\text{Total lipid (5)} = \frac{Lw}{Bw} \times 100\%$$

Where Lw = weight of lipid sample (gram); Bw = weight of biomass sample (gram)

3. Results and Discussions

3.1. Proximate Analysis of Moringa Seeds Bioflocculant

In this study, the proximate analysis includes water content and ash content shown in Table 1 and 2.

Table 1. Water content of moringa seeds bioflocculant

No.	A (g)	B (g)	C (g)	Water Content (%)
1	43.8	45.6	45.5	0.21
2	66.3	68.3	83.2	0.15
3	58.5	60.5	60.4	0.17
Mean of water content (%)				0.177

Table 1 showed that water content in 2 grams of moringa seed bioflocculant powder was 0.177%. Based on the literature review, generally 1 gram of fresh moringa seeds contain 7.9% water.

Table 2. Ash content of moringa seeds bioflocculant

No.	A (g)	B (g)	C (g)	Ash Content (%)
1	34.52	36.52	34.88	4.49
2	37.17	39.17	37.83	3.42
3	61.26	63.26	61.94	2.08
Mean of ash content (%)				3.33

Table 2 showed that ash content in 2 grams of moringa seed bioflocculant powder was 3.33%.

3.2. Cell Density and Growth of *Chlorella* sp.

The growth curve of *Chlorella* sp. in this study is presented in Figure 1. The Observations on days 0 to 14 days of culture showed the growth of *Chlorella* sp. that was very high at the beginning of the cultivation period, but it was decreased daily with total cell density reaching $2\text{--}2.5 \times 10^4$ cells mL^{-1} in the first three days. This decrease in cell density may be due to the reduced nutrient present in the media because the growth *Chlorella* sp. The increase of density generate a competition between cells to obtain nutrients. This was occurred because cells that have larger sizes have a smaller growth rate. Larger cell sizes required more nutrients to grow and reproduce.

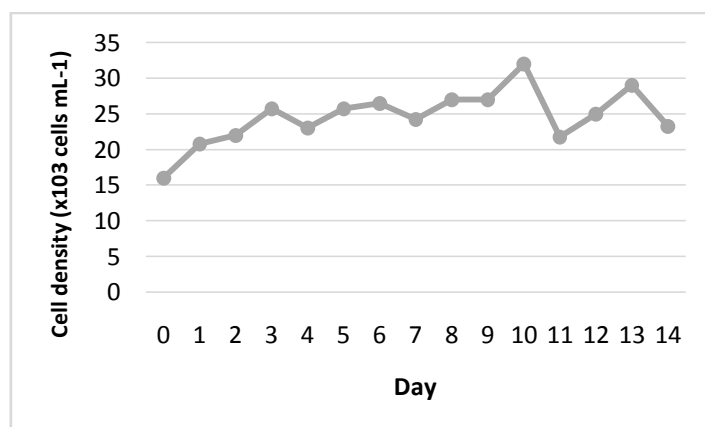


Figure 1. Growth curve of *Chlorella* sp. culture during the cultivation in this study.

Salinity and pH of the experimental culture were shown in Figure 2 and 3. Salinity was one of important factors that affected the growth of *Chlorella* sp. The level of salinity varies, depending on evaporation and precipitation. Microalgae living in waters have different tolerance and sensitivity to salinity, depending on the species and the stage of life cycle. The marine microalgae *Chlorella* sp. is tolerant in the environment with salinity of 0-70 ppt. During the cultivation period, the salinity of culture recorded during observations ranged from 24-26 ppt. The increase of culture salinity was accompanied by an increase in pH culture. The pH recorded during cultivation period ranged from 7.6 to 9.3.

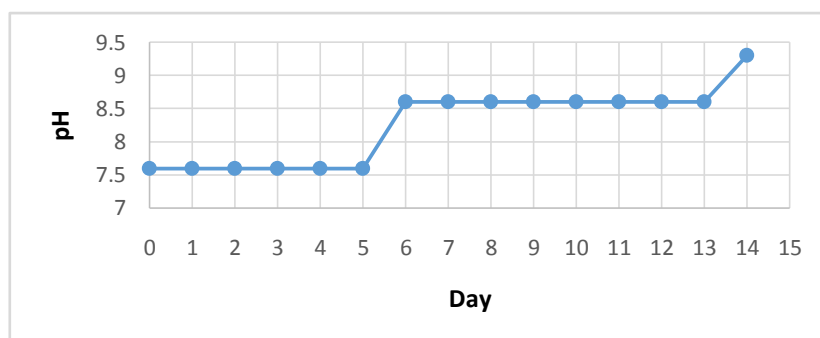


Figure 2. pH condition of *Chlorella* sp. culture during the cultivation in this study.

The increase in pH increased with the growth of *Chlorella* sp biomass. pH began to rise on day 6 and continue to be constant and peaked on day 14.

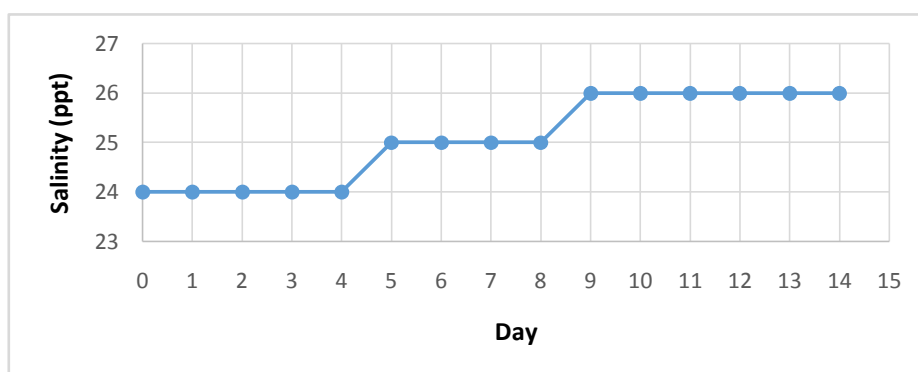


Figure 3. Salinity of *Chlorella* sp. culture during the cultivation in this study.

Figure 3 indicated that the salinity was increased after 5 days of culture from 24 ppt to 25-26 ppt showing the most significant period indicated in the range from day 5 to day 9 from 25 ppt to 26 ppt. When salinity exceeded the growth range, it was apparently that *Chlorella* sp. cannot tolerate, reduce the growth and achieve the death phase. The measured salinity in this study was within the optimum salinity for *Chlorella* sp. culture, i.e. 25-28 ppt [14], as well as within the range of salinity tolerance for a good growth at 15-35 ppt [14].

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

The harvesting of *Chlorella* sp. biomass using moringa seeds bioflocculant 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 this study, the pH value of the culture was adjusted to the pH range of bases by adding NaOH into the culture suspension in such a way that it reached the expected pH treatments concentration, i.e. pH 9, 10 and 11. The process of flocculation of microalgae cells was using sodium ions. It aimed to initiate the precipitation of magnesium hydroxide and to stimulate the occurrence of cell flocculation by magnesium ions [15]. In the flocculation process, the amount of bioflocculant applied to the *Chlorella* sp. biomass must be determined, so that it can achieve the optimum dose. Flocculation efficiency is a measure of how many microalgae cells are successfully removed from culture and expressed in percentages [15].

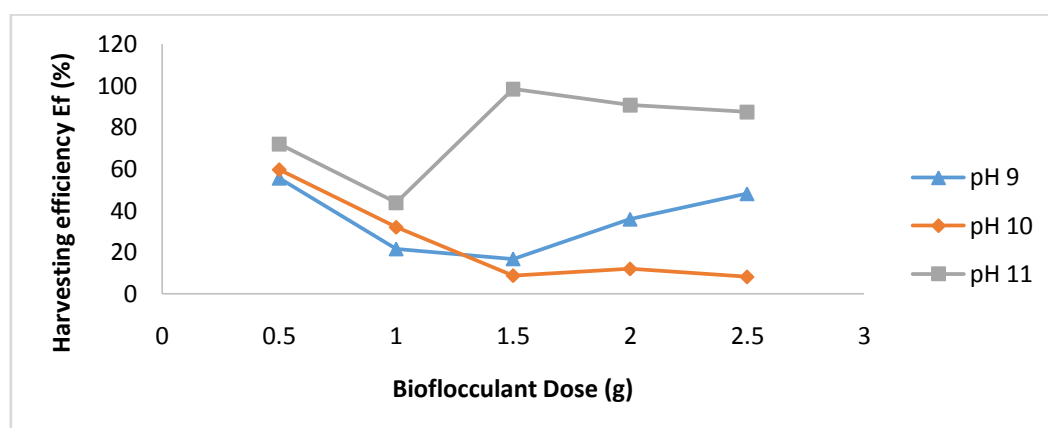


Figure 4. Harvesting efficiency of *Chlorella* sp. biomass using biofloculant moringa seeds.

Figure 4 showed that the higher the flocculation efficiency, the more microalgae cells were flocculated in the culture medium. The optimum pH for harvesting *Chlorella* sp. using moringa seeds biofloculant was at pH 11 with the biofloculant concentration of 0.15 gL^{-1} . The flocculation efficiency value calculated after harvesting *Chlorella* sp. at pH 11 with a dose of 0.15 gL^{-1} was higher than that of pH 9 and pH 10 with the same dose (0.15 gL^{-1}). At pH 11, the amino acid was ionized to produce carboxylic and proton ions, the proton charge attracts electrons (colloids) to form neutral groups and then produce flocs.

The absorbance value was influenced by the culture concentration in the flocculation process. The number of cells in contact with the flocculant particles was limited to the ability of the flocculant. The unaffected cells of the flocculant mostly did not form flocs and are unsedimented. The more biomass that was flocculated, the lower the absorbance of the suspension. The effect of dose of moringa seeds powder biofloculant showed decreasing of biomass concentration even though the decrease was gradually. One of the mechanisms of flocculation is the charge neutralization (Figure 5). 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 until finally be flocculated [10].

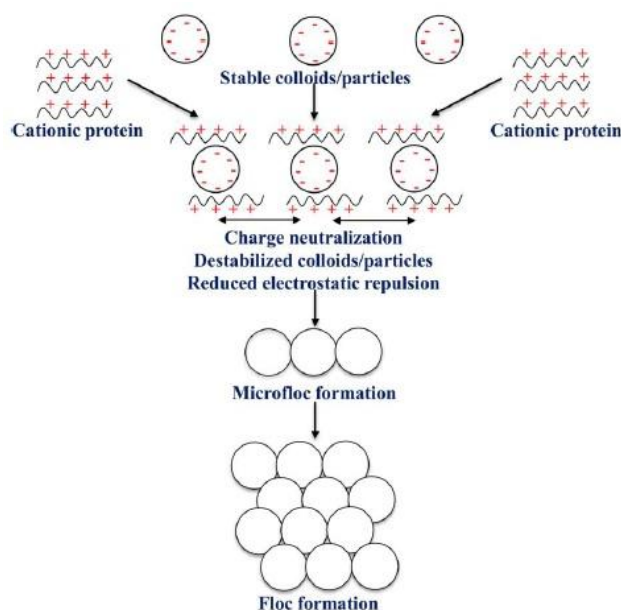


Figure 5. The flocculation mechanism using the cationic protein of *Moringa oleifera*.

The optimum pH condition of harvesting efficiency at pH 11 may be attributed to the low dose of the bioflocculant ($0.05\text{--}0.15\text{ gL}^{-1}$), the pH required to induce the higher flocculation, thus at pH 9 and 10, the flocculation efficiency was low, i.e. only 30-50% with its optimum dose at 0.05 gL^{-1} . However, at pH 11 with the lowest dose of 0.05 gL^{-1} , harvesting efficiency achieved up to 71% and can be used to induce bioflocculation to reach the optimum with a dose of 0.15 gL^{-1} with harvesting efficiency value higher than 98%. The lipid yields of *Chlorella* sp. in pre-flocculation and post-flocculation were 60% and 22%, respectively.

4. Conclusions

In conclusion, this study showed the potential of moringa seeds powder as a natural bioflocculant to harvest microalgae biomass. The efficiency of harvesting of *Chlorella* sp. was optimum at 98.44% with pH 11 and the dose moringa seeds bioflocculant was 1.5 g. The biomass yield obtained at the optimum flocculation efficiency condition was 0.069 gL^{-1} . The lipid yields of *Chlorella* sp. in pre flocculation and post-flocculation were 60% and 22%, respectively.

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