

## MARINE MICROALGAE FLOCCULATION USING PLANT: THE CASE OF *NANNOCHLOROPSIS OCULATA* AND *MORINGA OLEIFERA*

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### Abstract

Marine microalgae have been commercially used as live feed for aquaculture and nutritional supplements. However, harvesting of marine microalgae is a major obstacle for industrial scale and one of the promising harvesting techniques is bio-flocculation. *Nannochloropsis oculata* from the culture broth was investigated. The potential of *Moringa oleifera* as a flocculant has been evaluated using jar test experiments. *Moringa oleifera* after oil extraction (MOAE) and with non-extracted *Moringa oleifera* (MOWE) have been studied and compared to chemical flocculant, aluminium sulphate. Three parameters involved: pH, settling time and flocculant dosage. When MOAE and MOWE were used as flocculants, the highest flocculation efficiency of *Nannochloropsis oculata* was observed at 93.77% (pH 7, 150 minutes, 5000 mg/L) and 70.56% (pH 7, 90 minutes, 4000 mg/L) respectively. Harvesting efficiency of 99.98% with short settling time, 30 minutes and 2000 mg/L of flocculant dosage at pH 6 was achieved using aluminium sulphate. The concentrate of *Nannochloropsis oculata* was then fed to the *Brachionus plicatilis* (rotifers) to observe the growth characteristics in 12 days period. Concentrates of MOWE gave better growth of *Brachionus plicatilis* than growth in concentrates of MOAE and live *Nannochloropsis oculata*. In contrast, growth of *Brachionus plicatilis* in aluminium sulphate was tremendously decline. In conclusion, bio-flocculation using *Moringa oleifera* was rapid, inexpensive and eco-friendly technology as no addition of chemical flocculants was required.

**Key words:** *Brachionus plicatilis*, Flocculation, *Moringa oleifera*, *Nannochloropsis oculata*.

### Introduction

Currently, microalgae have been largely commercialized and cultured for their numerous applications including live feed for a wide variety of animals in aquaculture. Microalgae are also used in cosmetics, health and food products (Spolaore *et al.*, 2006). Microalgae are mainly used as a precious source of energy due to quick growth process, high oil production, low carbon dioxide (CO<sub>2</sub>) emissions and high protein content as compared to the traditional crops (Demirbas, 2006; Chisti, 2007). Harvesting of microalgae is a major obstacle for industrial scale processing. Recently, research has focused on the harvesting and biomass recovery of microalgae (Granados *et al.*, 2012; Zheng *et al.*, 2012; Lee *et al.*, 2013; Xu *et al.*, 2013), due to the characteristics of microalgae such as small cells size (2–20 µm in diameter), similar cells density to water, and large volumes of water need to be removed during microalgae biomass recovery. Hence, harvesting technique requires one or more solid-liquid separation steps, including the concentration and drying processes (Zhang *et al.*, 2010). There are various concentration technologies applied which is mostly relied on complex and sophisticated approaches, for instance coagulation, flocculation, centrifugation, gravity sedimentation, filtration, Alfa Laval decanter technology and high pressure membrane molecular sieving (Amin, 2009; Christenson & Sims, 2011; Suali & Sarbatly, 2012). However, these technologies have contributed to very high maintenance and operational cost. The total of 20–30% biomass production cost applied due to the energy requirement for the machinery especially in large scale

(Grima *et al.*, 2003; Bahadar & Khan, 2013; Razzak *et al.*, 2013). Additionally, harvesting of microalgae is difficult and energy intensive processes (Pimentel *et al.*, 2004). Therefore, an integrated approach is required in order to minimize the energy consumption in the harvesting process. Efficient system for harvesting is extremely essential for commercial production of microalgae. Flocculation is one of the most economical and energy efficient methods for harvesting microalgae (Schenk *et al.*, 2008). Harvesting microalgae by flocculation has been successfully applied in aquaculture (Knuckey *et al.*, 2006), biofuel production (Danquah *et al.*, 2009), wastewater treatment (Godos *et al.*, 2011) and river silt treatment (Divakaran *et al.*, 2002).

There are two main classifications of flocculants that are organic or natural flocculants and inorganic or polyelectrolyte flocculants (Chen *et al.*, 2011). From the study of Thapa *et al.* (2009), a better flocculation performance is dependent on the molecular weight of the polymer flocculants. In addition, charge density, dosage, pH, salinity and characteristics of the microalgae are also the factors that may influence the flocculation performance. Inorganic flocculants such as aluminium sulphate and ferric chloride are frequently used due to their proven capabilities, effectiveness and lower costs especially in wastewater treatment (Ahmad *et al.*, 2011). Nevertheless, Schenk *et al.* (2008) proved that, this is an easy and effective method but unlikely, not suitable for economical and sustainable harvesting of microalgae in large scale production plants because the flocculants are required in high doses and it will result in contamination of the microalgae biomass and that will lead to extra operational costs.

Basically, scientific improvements for flocculating microalgae often seek ways in producing flocculants that poses less harm to the organisms and environment as well as economical. As reported by Salim *et al.* (2011), organic flocculants are the method that spontaneously flocculates without chemical addition which is applicable at industrial scale. Organic flocculants are also sustainable and cost effective as no costs are involved in the pre-treatment process. The technology shift from biological and chemical treatment towards natural coagulants is believed to have bright potential aspect in reducing the cost of harvesting for oil extraction and no apparent toxic effect on aquaculture feeds (Knuckey *et al.*, 2006). According to Pritchard *et al.* (2010), natural plants extract such as *Moringa oleifera*, *Jatropha curcas*, *Strychnos potatorum*, *Hibiscus sabdariffa* and *Clidemia angustifolia* have been used for water purification. Moreover, chitosan and starch also have been explored in harvesting microalgae and shown to be effective in biomass recovery (Ahmad *et al.*, 2011). Nevertheless, both of the natural coagulants are expensive and utilize potential food supply (Hamid *et al.*, 2014). Among different crops, *M. oleifera* is the most studied plant for waste water treatment (Pritchard *et al.*, 2009), the plant is a small, fast growing drought deciduous tree that commonly used for water treatment. *M. oleifera* considered a natural flocculant that promote the floc formation due to its effective flocculant properties which act as clarifying agent (Madrona *et al.*, 2010). *M. oleifera* showed better results for dye removal than flocculation treatment with FeCl<sub>3</sub> and polyelectrolyte (Vilaseca *et al.*, 2014). According to Okuda *et al.*, (1999), the oil extraction of *M. oleifera* will generates a waste (65%–75% of seeds weight) that good for use as fertilizer in agriculture or as animal feed (Vilaseca *et al.*, 2014). Furthermore, *M. oleifera* can be used as a flocculant after extraction of edible vegetable oil (Ndabigengesere *et al.*, 1995). In Malaysia, *M. oleifera* have the potential to be used as bio-flocculant for harvesting microalgae due to its outstanding properties (Hamid *et al.*, 2014).

Applicable flocculants are needed to harvest microalgae at reasonable cost to make commercial production feasible. The selection of flocculants is extremely important as it will adversely affect the downstream process. The main objective of this study was to compare between the uses of *M. oleifera* as bio-flocculants and inorganic flocculant. *M. oleifera* after oil extraction (MOAE) and with non-extracted *M. oleifera* (MOWE) have been investigated. There were three parameters involved in this study; pH, settling time and flocculant dosage. Analysis was based on the percentage of microalgae cells removal by *M. oleifera*. The concentrated microalgae after flocculation were then fed to the zooplankton to observe the growth characteristics.

## Material and Methods

**Cultivation of marine microalgae, *Nannochloropsis oculata*:** The *N. oculata* was used as a model microalga for its ability to be concentrated by flocculation. This marine microalga was obtained from Agro-Biotechnology Institute, (ABI) Malaysia culture collection in Serdang, Selangor. The predominant species of marine microalgae, *N. oculata* was cultivated in 10 L glass tank filled with 29 ppt seawater containing f/2 medium for 9 days (Tompkins *et al.*, 1995).

The cultured was stored at room temperature (20–24°C) with continuous illumination in laboratory. The aeration was supplied by bubbling air at constant pressure. The average size of the particles in suspension was closed to 2 – 4 µm and the inoculums size was approximately  $22 \times 10^6$  cells/mL. The number of microalgae cells was determined using a Neubauer haemocytometer (Assistant, Germany) and cell concentration will be measured using a UV spectrophotometer (Lambda 25, Perkin Elmer, USA) from the optical density at 500 nm. All experiments were performed with cells from a single harvest in order to avoid any variations.

**Collection of *Moringa oleifera*:** The good quality of *M. oleifera* pods were collected in three months period from Taman Pertanian University (UPM), Serdang, Selangor. The hulls and wings were removed from the kernels to get the dry seeds of *M. oleifera*. The seeds were dried in the oven (Memmert, Germany) overnight at 50°C. Two types of flocculants were prepared; *M. oleifera* seeds after extraction (MOAE) and *M. oleifera* seeds without extraction (MOWE).

**Preparation of *Moringa oleifera* after extraction (MOAE):** The method of extraction was adopted from Bhatia *et al.*, (2007) with some modifications. The good quality seeds were grounded to a fine powder using ordinary electric blender. Parts of the powder grounded earlier were used for oil extraction with *n*-hexane (96% purity) as a solvent using Soxhlet apparatus. The extraction process was carried out for 8 hours. Stock solution of the *M. oleifera* cake after oil extraction (MOAE) was prepared by dissolving 5 g of the dried cake in 100 mL seawater. Then, the mixture was blended for 2 minutes at high speed to extract the *M. oleifera* active ingredient. Finally, the suspended *M. oleifera* was filtered through a muslin cloth and the residue will be discarded.

**Preparation of *Moringa oleifera* without extraction (MOWE):** The method was adopted from Katayon *et al.*, (2006) with some modifications. To prepare the stock solutions for MOWE, 5 g powder of *M. oleifera* was dissolved in 100 mL seawater in a beaker. Then, the mixture was blended using ordinary electric blender for 2 minutes at high speed in order to extract the active ingredient of *M. oleifera*. The suspension was filtered through muslin cloth and the filtrate made up to 100 mL using seawater to give stock solution of *M. oleifera*.

**Flocculation process:** Process of flocculation was modified from the article by Bhatia *et al.* (2007). The flocculation process was performed using a conventional Jar test (Velp Scientifica, Italy), equipped with 1000 mL beakers filled with 500 mL of *N. oculata* for each run. The stock solution of MOAE and MOWE were used to run the flocculation tests for 5 sets each with different dosage; 1000 mg/L, 2000 mg/L, 3000 mg/L, 4000 mg/L and 5000 mg/L. The pH value (Mettler Toledo 320 pH meter) of each Jar test was adjusted by using either hydrochloric acid (HCl, 3M) or sodium hydroxide (NaOH, 5M) within the range of 4 - 9. *N. oculata* sample

was agitated at 150 rpm for 5 minutes (rapid mixing). Then the speed was reduced to 30 rpm for 30 minutes (slow mixing). The contents of each beaker were allowed to sediment with different settling time; 30 minutes to 150 minutes. Next, the Jar test was repeated using MOWE followed by using inorganic flocculant. For comparative study, the stock solution of aluminium sulphate powder was prepared by dissolving 5g of  $Al_2(SO_4)_3 \cdot 18H_2O$  in 100 mL distilled water with similar dosage range of 1000 mg/L to 5000 mg/L.

### Statistical analysis

In this study, Design-Expert software (version 9.0, Stat-Ease, Inc., Minneapolis, Minnesota, United States) was used to observe the effects of pH, settling time and dosage of *M. oleifera* as the process variables on the harvesting microalgae. The ranges of parameters were analyzed using Response Surface Methodology (RSM) and applied one factorial design which capable in generating number of experiments with different formulation in order to screen the effectiveness of the flocculant parameters. The experiment was based on three factors and five levels, which are listed in Table 1. Results of this statistically analysis were evaluated using one-way analysis of variance (ANOVA).

**Flocculation efficiency:** The supernatant of microalgae concentration can be determined by using UV spectrophotometer at 500 nm of optical density and compared to the initial value. The pH value of supernatant was measured using a pH meter. The microalgae floc after sedimentation was dewatered in the filter and dried in the oven for 24 hours at 100°C. The sample was cooled in desiccator for about 30 minutes and weighted to determine microalgae mass. The data was collected in triplicate. In the present study, flocculation efficiency is defined as follows:

$$\text{Cell removal efficiency (\%)} = \frac{I_{\text{control}} - I_{\text{sample}}}{I_{\text{control}}} \times 100\%$$

where  $I_{\text{control}}$  is the absorbance intensity of the reference culture with no flocculant added and  $I_{\text{sample}}$  is the absorbance intensity of the sample after the flocculation process.

### *Nannochloropsis oculata* concentrates as rotifer feeds:

Finally, the concentrated *N. oculata* as in Fig. 1A was further analyzed by feeding on rotifers and compared with live *N. oculata*. The concentrated *N. oculata* after flocculation was filtered using vacuum pump (Rocker 600, Taiwan) and kept in the chiller (Sanyo, Japan) with temperature of -80°C for 24 hours. It was then freeze dried for 3 days using Free Zone Bench Top Freeze Dry System (Labconco, USA). Live *N. oculata* and the powdered form of aluminium sulphate, MOAE and MOWE as in Fig. 1B fed to the rotifers with density of 20 individuals/mL. The experiments were carried out in triplicate for 12 days.

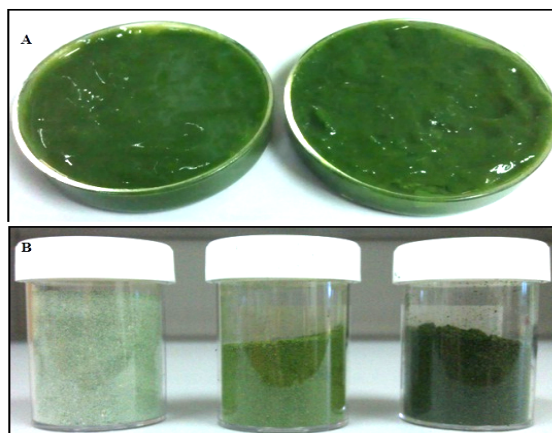


Fig. 1. A concentrated *N. oculata* after flocculation: Fig. 1.B powdered form of Alum, MOAE, MOWE (from left).

**Table 1. Process variables (settling time, pH and flocculant dosage) for design of experiment.**

Std.	Factor 1	Factor 2	Factor 3
	A: settling time min	A: pH	A: dosage mg/L
1	30	5	1000
2	30	5	1000
3	60	6	2000
4	90	7	3000
5	90	7	3000
6	120	8	4000
7	150	9	5000
8	150	9	5000

### Results and Discussion

**Effect of process variables on MOAE:** The quadratic graph in Fig. 2A shows the increasing percentage of cell removal with the increase in settling time 30 minutes to 150 minutes with fixed MOAE dosage 5000 mg/L at pH 9. Dosage of *M. oleifera* and pH were selected based on the study done by Bhatia *et al.*, (2007). In the Fig. 2A shows a curvature at the end of the graph. At 30 minutes settling time, it shows 83.89% of cell removal and increasing up to 90.65% of cell removal at 150 minutes. This indicates that interaction effect between settling time and MOAE dosage on cell removal was greatly visible. Thus, to proceed in determining the best value for pH, dosage of 5000 mg/L was used with settling time of 150 minutes.

Fig. 2B shows the effect of pH on percentage of cell removal in one factorial plot. The flocculation efficiency was observed for all these pH values on the basis of optical density fluctuations of the supernatant. The graph shows a curvature with maximum value of parabola. An increase in pH with fixed MOAE dosage of 5000 mg/L and settling time at 150 minutes will decrease the percentage of cell removal. In the range of pH 5 to 7, the percentage of cell removal increased. At pH 7, the maximum percentage of cell removal was 90.57%. However, the percentage of cell removal decreased tremendously above pH 8. This is parallel to the study done by Knuckey *et al.* (2006) which proved that less than 30% of *N. oculata* cells removal was achieved with increasing pH 8-11. The response surface plot reveals that the flocculation efficiency was greatly influenced by the pH.

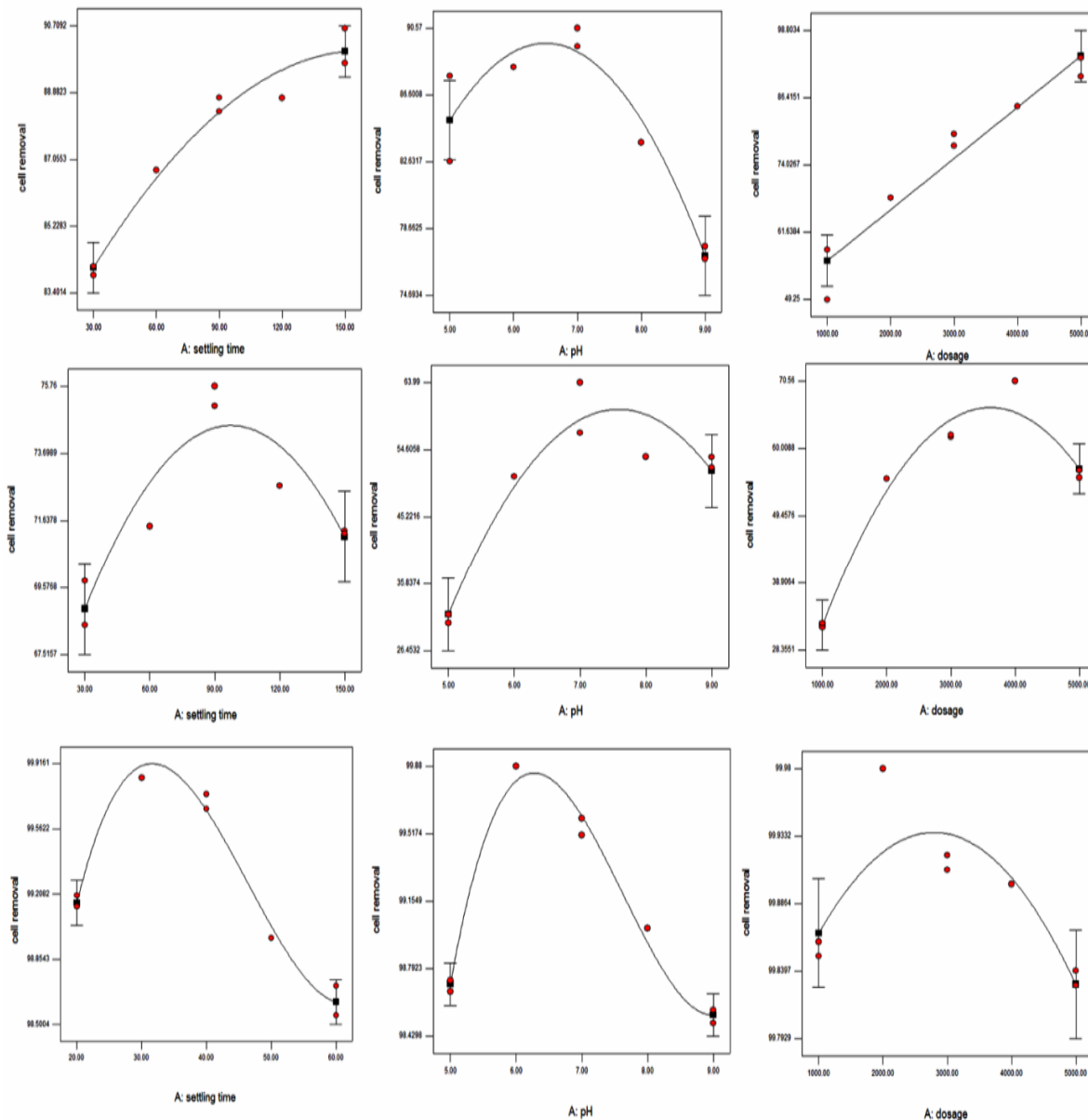


Fig. 2. Effect of settling time, pH and dosage on removal efficiency (from left to write) of MOAE (A-C), MOWE (D-F) and aluminium sulphate (G-I).

In follow up experiments, the best value of pH at 7 and 150 minutes settling time were carried from the previous experiment. Fig. 2C shows a linear interaction effect between MOAE dosage and percentage of cell removal. It can be seen that the percentage of cell removal increased with an increase in flocculant dosage. From the graph, 49.25% of cell removal achieved at 1000 mg/L MOAE dosage while 5000 mg/L dosage of MOAE was needed to achieve 93.77% of cell removal. It can be concluded that, the marine microalgae were fully flocculated as the flocculant dosage increased. Although high dosage required from *M. oleifera* for flocculation process, the removal of *N. oculata* cells was improved. The study was also confirmed by Teixeira *et al.* (2012) which mentioned that *M. oleifera* was able to harvest marine microalgae more than 90% of removal efficiency.

**Effect of process variables on MOWE:** Fig. 2D demonstrates the correlation between settling time and percentage of cell removal on MOWE with fixed 5000 mg/L MOWE dosage at pH 9. The graph shows a curvature in which the maximum point is at the peak. The highest percentage of cell removal was 75.16% at 90 minutes while the lowest was 69.78% at 30 minutes. The percentage of cell removal increased from 30 minutes to 90 minutes of settling time and dramatically decreased when above 95 minutes of settling time. According to the observation, before 90 minutes of settling time, larger flocs was formed while after 100 minutes, the broth appeared hazy and the small floc of microalgae floated at the broth surface.

Fig. 2E illustrated the interrelation between pH and settling time. From the previous experiment, the best settling time was at 90 minutes and flocculant dosage was fixed at 5000 mg/L. In this study, the percentage of harvested microalgae decreasing as the value of pH increased. The peak value was at pH 7 with 63.99% of cell removal. However, at pH 8 the percentage of cell removal decreased to 53.65%. *M. oleifera* and *N. oculata* interact with each other through electrostatic interaction. *M. oleifera* attached to the negatively charged microalgae surface via its positive charged group. Bridges were formed between microalgae cells when the chain had sufficient length to bind more than one cell. Therefore, during acidic condition the degree of flocculation is very weak. As pH increase in alkaline, the positive charge was neutralized and the highest neutralizing point was approximately achieved at pH 7. At this pH, *N. oculata* cells have the highest negative charge, thus the flocculation efficiency was enhanced (Harith *et al.*, 2009).

Fig. 2F shows the effect of MOWE dosage with percentage of cell removal with fixed pH of 7 and settling time of 90 minutes. From the quadratic graph it demonstrates that the best flocculant dosage for MOWE was 4000 mg/L which gave 70.56% of cell removal. However, flocculation efficiency decreased as flocculant dosage increased. MOWE cannot perform very well in lower dosage of flocculant since only 31.88% of cell removal at 1000 mg/L. The color of marine microalgae was cloudy and turbid after flocculated with 1000 mg/L MOWE dosage at 90 minutes of settling time and was highly turbid when it leaves to floc until 150 minutes. While, the flocculation process ended after 90 minutes settling time with dosage of 4000 mg/L. This result is in agreement with study done by Ndabigengesere & Narasiah (1998), whereby excess coagulant residue will form after continuous addition of coagulants. This happens because of the microalgae particles form larger colloids when coagulant exceed the optimum dosage.

**Effect of process variables on aluminium sulphate:** In the study, the effect of settling time was monitored in the range of 20 to 60 minutes at constant pH of 6 and fixed coagulation dosage of 5000 mg/L respectively. Fig. 2G shows the cubic graph of percentage removal on settling time using one factor design. For overall observation, the solutions appeared clear but showed the presence of very small colloidal particles. It can be seen from the Fig. that the reduction of suspension cells as 98% to 99% and were very consistent. The highest cell removal percentage as 99.84% at settling time of 30 minutes and it was carried out to the study on effect of pH. As the settling time increased, the *N. oculata* broth turned into a darker color. It indicates that the removal of *N. oculata* cells became poorer.

The effect of pH using constant dosage of 5000 mg/L and settling time of 30 minutes was studied on flocculation of aluminium sulphate. Fig. 2H shows the effect of pH on removal of harvested microalgae. It can be observed from the Fig. that an increase pH of 5 to 6, increase the efficiency of flocculation process for the removal of *N. oculata* cells. Then, the flocculation strength became weaker with the increase of pH 7 to 9. It can be said that

adding extra bases to the culture medium did not improve the flocculation efficiency but it increased in the formation of precipitation and loose flocs. Hence, the removal of *N. oculata* cells was as efficient as 99.88% at pH 6, with the combination of 5000 mg/L aluminium sulphate dosage and settling time of 30 minutes.

Fig. 2I illustrates the effect of different dosage of aluminium sulphate flocculant over percentage of cell removal at constant pH of 6 and settling time of 30 minutes. In the study, the best aluminium sulphate dosage was determined when there was insignificant increase in the removal efficiency with further addition of flocculant. The best dosage for aluminium sulphate was observed at 3000 mg/L with 99.92% of cell removal and further addition, there was no significant increase of cell removal. Nevertheless, the lowest cell removal was obtained at 5000 mg/L of aluminium sulphate dosage with 99.83% of flocculation efficiency. The culture broth in six different beakers appeared to be crystal clear with several colloidal particles.

**Optimization analysis:** In the present study, Response Surface Methodology (RSM) with one factorial design was applied in order to obtain the relationship between the variables and responses. The range and levels of the variables were investigated. Eight experiments were carried out to represent every process variable as discussed previously. Generally, these parameters range were generated based on the previous study of Bhatia *et al.* (2007). From the previous study, it is proven that the flocculation efficiency increased when adding of polymer aid (NALCO 7751). However, for this study, polymer aid was not used because it decreased the flocculation efficiency and brought difficulties in filtering the concentrated *N. oculata* after the process.

In order to verify of the model developed, 15 more experiments need to be performed for harvesting microalgae by using the numerical optimization of the Design-Expert software based on the Central Composite Design (CCD) as in Table 2. A set of solution will be generated by the Design-Expert 6.0.6 software to determine the optimum conditions of flocculation process using MOAE. The experiments will be conducted at these conditions and comparison between the experimental results with the predicted results from the model will be analyzed.

**Comparison on performance of MOAE and MOWE:**

The efficiency of the flocculation process was studied using MOAE, as well as in MOWE in order to improve the removal efficiency of *N. oculata* cells. As according to Krentz *et al.*, (2006) bio-flocculant does not contaminate the microalgae biomass as it is approved for food contact and for use in treatment of drinking water. Bio-flocculants were added to *N. oculata* broth to neutralize the negative charges on dispersed non-settleable cells. When the charges are neutralized, the small suspended particles are capable to interact together through rapid mixing. When the floc reached its optimum size and strength, the broth is subjected to the sedimentation process.

**Table 2. Experimental factors for optimization analysis using RSM in harvesting microalgae.**

Std	Settling time min	pH	Flocculant dosage mg/L
1	100	5.5	5000
2	200	5.5	5000
3	100	7.5	5000
4	200	7.5	5000
5	100	5.5	10000
6	200	5.5	10000
7	100	7.5	10000
8	200	7.5	10000
9	50	6.5	7500
10	250	6.5	7500
11	150	4.5	7500
12	150	8.5	7500
13	150	6.5	2500
14	150	6.5	12500
15	150	6.5	7500

From observation, the *N. oculata* broth that was added with MOAE as bio-flocculant, appeared clearer as compared to MOWE. The extraction of the active flocculant component from *M. oleifera* seeds has attracted more *N. oculata* interact. It is known that the *M. oleifera* seeds protein is the most important compound in the process of clarifying the water (Madrona *et al.*, 2010). Hamid *et al.* (2014) also stated that MOAE consists of pure coagulant polymer which allows the suspended particles to bind completely. MOAE was utilized as an alternative to MOWE.

Comparison between MOAE and MOWE revealed that extraction of *M. oleifera* had more positive effect on the removal efficiency of *N. oculata*. The flocculation efficiency using MOAE as high as 93.77% at pH 7 with long settling time as 150 minutes in 5000 mg/L dosage. In contrast, cell removal efficiency using MOWE as 70.56% with same pH and shorter period for settling time, 90 minutes in 4000 mg/L dosage. In the flocculation process using MOWE; it is observed that as settling time and flocculant dosage increased, it resulted in cloudy broth solution of *N. oculata* which no flocculation occurred. In conclusion, MOAE was found useful to achieve high flocculation efficiency as bio-flocculant.

**Influence of settling time on flocculation efficiency:** In the present study, the effect of settling time was demonstrated in the range of 30 to 150 minutes using MOAE and MOWE. The experiments were performed with a fixed flocculant dosage of 5000 mg/L. Fig. 3A shows the dependence of cell removal by MOAE and MOWE on the settling time. It can be seen that MOAE and MOWE gave the removal of cells as 93.77% at settling time of 150 minutes and 75.76% with settling time of 90 minutes, respectively. It was observed that MOWE produced larger flocs as compared to MOAE. An increased floc size was observed to increase the free settling velocity compared to the individual particles that do not form flocs.

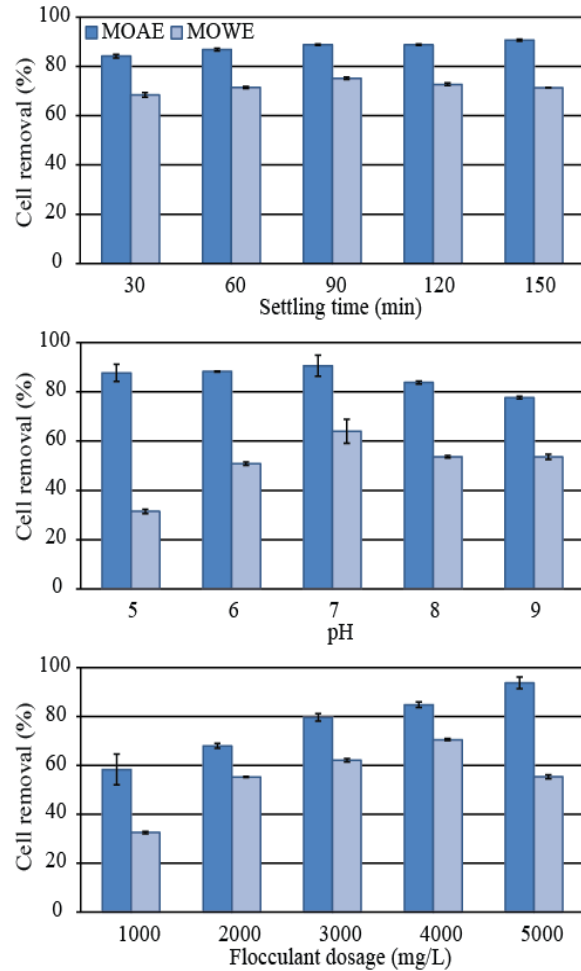


Fig. 3. Comparison on performance efficiency of MOAE and MOWE with respect to settling time (A), pH (B), and dosage (C).

This is similar with the study of Ahmad *et al.* (2006), which agreed the settling time depends on the floc size. The study concluded that Chitosan encouraged faster aggregation of cells through the formation of bridges between the dispersed cells. It allowed the formation of particles of a sufficient size that can settle faster or sedimented. This is due to the bridging mechanism that occurs in the removal process. The sedimentation time decreased as the bridged particles and flocs settled to the bottom of the beaker and this effect is mainly driven by the gravitational force.

**Influence of pH on flocculation efficiency:** The pH is one of the significant factors affecting the performance of the flocculation process. It affects the effectiveness of the charge adsorption - neutralization in mechanism. Higher pH will sweep the influential floc coagulation mechanism. The influence of pH on the sedimentation was studied in Fig. 3B. As the pH increased from 5 to 7, percentage of cell removal was increased, probably due to destabilization or neutralization of the particle's charges. Once the pH 7 was increased, it is noticed there was slight decrease in flocculation efficiency. This is compatible to flocculation of microalgae using chitosan, which is only efficient at a pH below 8 (Divakaran & Pillai, 2002; Liu *et al.*, 2009).



During the initial stage of flocculation process, when the pH of medium was increased, the small particles aggregated and slowly settled due to gravitational force. The cells formed large loose and dense packed aggregates that settled under gravitational force. Once the fine capture achieved equilibrium, further addition of flocculant might lead to the formation of larger aggregates.

**Influence of flocculant dosage on flocculation efficiency:** The influence of the flocculant dosage of MOAE and MOWE were investigated as in Fig. 3C, varying the dosage from 1000 mg/L to 5000 mg/L keeping all other variables maintained constant. In the flocculation of *N. oculata* using *M. oleifera* as flocculant, more than 93.77% flocculation efficiency was obtained at 5000 mg/L of MOAE. On the other hand, lower dosage of MOWE (4000 mg/L) was required to obtain 70.56% of cell removal. The result is same to the study of Bhatia *et al.* (2007) that required high dosage of MOAE (6000 mg/L) to remove 95% of cell removal.

For present study, higher dosage of *M. oleifera* was required to induce flocculation due to small size of *N. oculata*. This is supported by Bratby (2006) that smaller size of microalgae species requires higher flocculant dosage for flocculation than larger size microalgae.

**Comparison on performance of MOAE and aluminium sulphate:** Various chemical flocculants are widely used in harvesting microalgae. These flocculants are classified into inorganic and synthetic organic polymer. The most commonly used primary flocculant is aluminium sulphate (Okuda *et al.*, 1999). Therefore, the comparison between the performances of MOAE with aluminium sulphate as flocculant can be made.

From the experiments, the percentage of harvested microalgae was achieved at 93.77% (pH 7, 150 minutes settling time, 5000 mg/L dosage) when *M. oleifera* was used as flocculant. *M. oleifera* contain cellulose, hemicellulose, lignin, and crude fiber. Its matrix network contains carboxylic, fiber carbonaceous, and amino functional groups. These functional groups may influence the adsorption of components onto *M. oleifera* (Akhtar *et al.*, 2007). The observation is quite better in the experiment using aluminium sulphate where 99.98% of cell removal efficiency was achieved at pH 6 with short period of settling time (30 minutes) and in small dosage (2000 mg/L). Although aluminium sulphate is efficient flocculant but it results in contamination of the microalgae biomass due to high toxic content (Becker, 1994).

For *M. oleifera*, larger dosage resulted in highest efficiency of cell removal and the percentage of removal varied with different pH and settling time. When overdose of *M. oleifera* was used, the percentage of cell removal increased. Furthermore, *M. oleifera* is reported to have a cationic dimeric protein of high molecular weight, which destabilizes the particles in the water through a process of neutralization and adsorption, flocculate colloids followed by sedimentation (Vieira *et*

*al.*, 2010). Whilst for aluminium sulphate the efficiency of removal decreased with increasing dosage applied. Observation during the experiments showed that when increase dosage of aluminium sulphate was applied, the broth appeared to be cloudy and the floc of microalgae floated at the broth surface.

As a final point, the results showed that *M. oleifera* and aluminium sulphate had similar conditioning performance for flocculation of *N. oculata* and could be effectively used industrially. However, due to high toxic content of aluminium sulphate, *M. oleifera* has the potential to become new source of environmental friendly and natural flocculant.

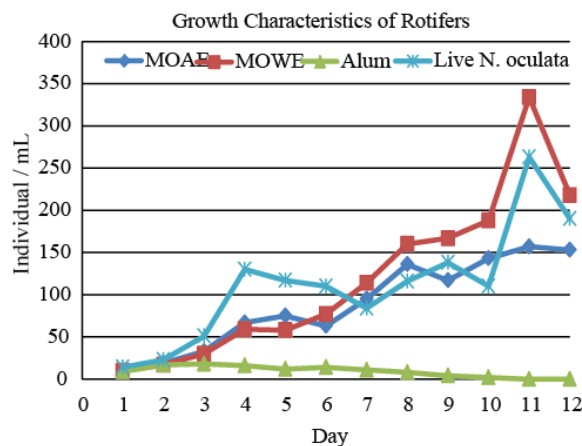


Fig. 4. Daily growth rate for *Brachionus plicatilis*, fed diets with MOAE, MOWE, Alum and Live *N. oculata*.

**Growth analysis as a comparative study:** Fig 4. Shows different dietary treatments feeding to the zooplankton. 100 mg/mL per day of live *N. oculata* diet and microalgae concentrate (MOAE, MOWE, and aluminium sulphate) diets were fed into 20 individuals/mL euryhaline rotifer, *Brachionus plicatilis* for 12 consecutive days respectively. When fed to the rotifers, the MOWE diet enhanced the highest growth, 334 individuals /mL and survived in longer time up to 3 weeks as compared to other diets. Similar trends were observed in live *N. oculata* diet with 263 individuals /mL. MOAE diet came at third with the highest growth as 157 individuals/mL. Highest growth of rotifers with Live *N. oculata*, MOAE and MOWE were observed at day 11. Finally, concentrated microalgae with aluminium sulphate diet showed the poorest growth of rotifers as the best growth can be achieved was only 18 individuals /mL at day 3 and the growth of rotifers started to decrease after prolonged experiments carried out.

As observed in this study, MOWE diet produced higher growth of rotifers than live *N. oculata*. The reason being, *M. oleifera* contains dietary polyunsaturated fatty acid (PUFA) which is essential for rotifers' growth (Moyo *et al.*, 2011). This statement is similar to the study of Foidl *et al.* (2001) that proved *M. oleifera* has high quality protein which is easily digested. Abdulkarim *et al.* (2005) reported *M. oleifera* contains 38.3% protein, 16.5% carbohydrate and 30.8% lipid.

According to Reboloso-Fuentes *et al.* (2001), in *N. oculata*, composition of protein, lipid and carbohydrate are 28.8%, 18.4% and 37.6% respectively. This indicates that MOWE diet can show qualitatively equal production as live *N. oculata*. Hence, for this study, comparison of MOWE diet and the composition of *N. oculata* can be made. However, since large quantities of microalgae are needed for marine hatcheries, Live *N. oculata* cannot live longer for continuous feeding. Thus, another alternative is by feeding with MOWE diet which had a good nutritional value for rotifers' growth and can be stored in long time.

## Discussion

The study was performed attempting to characterize the effectiveness of *M. oleifera* as bio-flocculant in the process of harvesting marine microalgae. *M. oleifera* causes satisfactory removal of the marine microalgae cells due to its high protein content. Destabilization of the microalgae cells was achieved successfully by the application of *M. oleifera*, which caused a synergistic enhancement of the agglomeration, flocculation and adsorption (Ahmad *et al.*, 2011). The use of *M. oleifera* can be considered an advantageous and an alternative option when microalgae are grown at commercial scale (Vandamme *et al.*, 2010). It can be considered as promising step towards improving the processes of flocculation. *M. oleifera* are non-toxic, indigenous, and easily available in large quantities raw material that may be used for different industrial applications. The flocculation efficiency of marine microalgae for separation of cells from the culture broth was greatly influenced by the settling time, pH adjustment and flocculant dosage (Harith *et al.*, 2009). Enhanced sedimentation rate and adjustment of pH were the obvious improvement of flocculation process with modification in flocculant dosage, which significantly increased the percentage of harvested microalgae. Efficient flocculation process that can maintain high cell viability could be a method of choice due to rapid, inexpensive and simple method for harvesting large quantity of marine microalgae cells such as *N. oculata* from the culture broth prior to commercial formulation.

Comparable flocculation efficiency (more than 90%) was obtained in flocculation with MOAE and aluminium sulphate. In this study, flocculation in MOAE can achieved as 93.77% of cell removal and this showed similarity with flocculation using chemical flocculant, aluminium sulphate that can achieved as 99.98% of cell removal. Although aluminium sulphate is more efficient as flocculant compared to MOAE, it may not be economical for microalgae separation from culture broth due to higher price of aluminium sulphate and high toxicity content (Zhang *et al.*, 2014). The process appeared to be less damaging to cells than chemical flocculant, aluminium sulphate. This was observed when microalgae concentrate with *M. oleifera* and aluminium sulphate was fed into rotifers, *Brachionus plicatilis*.

MOWE and MOAE diets enhanced better growth of rotifers as compared to aluminium sulphate diet. The data derived from the study are clear indications that *M. oleifera* is rich in nutrients and has potential to be used as a nutritional quality of feed additive with multiple purposes (Moyo *et al.*, 2011). These include serving as a protein, fatty acid, mineral and vitamin resource for animal feed formulations. The study is extendable and has a lot of aspects for improvement. There are several other parameters that can be optimized in order to achieve high cell removal, for example effect of mixing rate, mixing time and temperature. Extent study should be carried out in larger scale in order to prove that *M. oleifera* can be applied as bio-flocculant for harvesting marine microalgae industrially and commercially. This study has proved that *M. oleifera* not only can be used as treatment in wastewater but also can be used as flocculant in high salinity of water. In conclusion, a novel process for flocculation of marine microalgae using *M. oleifera* was developed as a cost-effective alternative for preparing microalgae concentrate as well as non-toxic for the aquaculture industry and is suited for on-site production by hatcheries.

## Acknowledgments

This research was funded by the Malaysian Ministry of science and innovation (MOSTI) through the project no. 08-05-ABI. The authors would like to thanks the management of Agro-biotechnology Institute Malaysia (ABI) and the National Institiuet of Biotechnology Malaysia (NIBM) for facilitating the work.

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(Received for publication 8 March 2015)