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Efficiency of various flocculants in harvesting the green microalgae *Tetraselmis tetrahele* (Chlorodendrophyceae: Chlorodendraceae)

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Abstract. This study showed the efficiency of various compounds in flocculating cultures of the microalgae *Tetraselmis tetrahele* (G. S. West, 1916) for use as potential biodiesel feedstock. Of the five flocculants tested, NaOH and $Al_2(SO_4)_3$ at concentrations of 200 mgL^{-1} showed the highest ($p<0.05$) flocculating efficiencies at 96.15% and 98.65%, respectively. In terms settling time, $Al_2(SO_4)_3$ at concentrations of 150 and 200 mgL^{-1} showed the fastest reactions reaching stable algal flocs only 1 hour after the addition of flocculants. Microscopic examination of resulting flocs showed no signs of cell plasmolysis or structural damage to cell walls for all treatments. Although significant ($p<0.05$) changes in pH of the resulting supernatant were observed for all treatments, these still fell within the suitable range for algal culture indicating the possibility of reusing the waste water for further cultures.
Key Words: *Tetraselmis tetrahele*, flocculation, microalgae, harvesting.

Introduction. The green microalgae *Tetraselmis tetrahele* (G. S. West, 1916) is a widely used microalgal species in aquaculture. It is mainly used in hatcheries as natural food for several aquatic species such as shrimps, bivalves, abalone, and rotifers (Estacion et al 1986; De Pauw & Persoone 1988; Ronquillo et al 1997; Madrones-Ladja et al 2002). Aside from its role in aquaculture, it is also a potential biodiesel feedstock due to its high lipid content and fast growth rate (Ferriols & Aguilar 2008).

In order to use *T. tetrahele* or any other microalgae species as feedstock for biodiesel production, efficient methods for separating and harvesting the algal biomass from the culture media must be employed. Separation of algal biomass from the culture media can be achieved using several methods. Physical or mechanical means of separation include sedimentation, filtration, and centrifugation. The two latter methods entail high energy costs making the production of algal paste for drying expensive (Shelef et al 1984). Sedimentation, although relatively cheaper takes a long time due to the colloidal nature of algal suspensions. The electric repulsion interactions between algal cells and cell interactions with the surrounding water both contribute to the colloidal nature of microalgal cultures (Tenney et al 1969). In order to increase the rate of sedimentation, chemical means of separating the algal biomass from the culture media are employed. This involves the use of various compounds such as inorganic salts containing polyvalent metal ions and cationic polymers to induce cell flocculation and coagulation. Upon addition of flocculants, microalgal cells form clumps that easily settle to the bottom and can be further separated by simpler filtration methods due to increased particle size of the algal biomass or by decanting due to the settling of the algal biomass out of the culture medium (Shelef et al 1984). The harvesting of algal cells by flocculation is a more convenient process than conventional methods such as centrifugation and gravity filtration because it allows the treatment of large quantities of culture (Lee et al 1998). In using flocculants for harvesting algal cells, considerations

such as cost, efficiency, and overall effect on the algal biomass should also be noted (Grima et al 2003).

Inorganic salts such as aluminum sulfate ($Al_2(SO_4)_3$), ferrous sulfate ($FeSO_4$), and calcium chloride ($CaCl_2$) are examples of compounds containing polyvalent metal ions. These inorganic salts have been used as clarifying and flocculating agents in other processes such as wastewater treatment in order to remove particulate matter and other dissolved compounds such as phosphorous (Dao & Daniel 2002; Al Mubaddal et al 2008).

Flocculation of algal cells is also sensitive to adjustments in pH of the culture media. The addition of chemicals such as sodium hydroxide (NaOH) and calcium hydroxide ($Ca(OH)_2$) are sometimes used in wastewater treatment to raise pH levels to the point where magnesium hydroxide ($Mg(OH)_2$) is formed and acts as the primary flocculant (Friedman et al 1977). Aside from the simple adjustment of pH, striking a balanced addition of ions such as Mg^{2+} at optimum pH ranges allows for the formation of stable flocs with faster settling rates (Smith & Davis 2012). Although this method of treating water has been found to be effective, the resulting medium would have to be retreated to neutralize its pH in order to be reused.

Moringa oleifera, considered as a tree vegetable, originates from India and Pakistan but has already been widely introduced in the tropical and subtropical regions of Africa and Asia. Almost all parts of the plant are used for various purposes due to high levels of proteins, vitamins, and minerals (Bosch 2004). Of its many uses, *M. oleifera* seeds have also been employed as clarifying agents in the treatment of water for the removal of particulate matter including algal cells (Katayon et al 2004; Shehata et al 2008). *M. oleifera* seeds have been found to have high concentrations of dimeric cationic polymers which act as coagulating agents through the adsorption and neutralization of colloidal charges (Ndbigengesere et al 1995). The use of organic cationic polymers as primary coagulants such as those present in *M. oleifera* seeds have been considered as low cost water purification methods with potential large scale applications in developing countries.

Although many organic and inorganic flocculants have already been tested for their efficiency in treating wastewater for the removal of particulate matter (including algal cells), the flocculation of algal cells can vary due to several factors. The variable flocculation potential of various algal species depends on the composition and properties of the cell wall, the extent and type of excretions, physiological conditions, age, and other factors (Avnimelech et al 1982). Flocculation reactions of algal biomass are also particularly sensitive to pH, properties of the cellular surface, the concentration of flocculants and divalent cations, and the ionic strength of culture solution (Bilanovic et al 1988). In this study, the flocculating efficiencies of various compounds on *T. tetrahele* were studied in relation to their concentration, settling time, and effect on pH of the resulting media/supernatant. Microscopic examination of algal cells was also carried out to determine if the various flocculants caused algal cells to rupture.

Material and Method

Algal culture. This study was conducted at the Multispecies Hatchery of the Institute of Aquaculture, University of the Philippines Visayas, Miagao, Iloilo, Philippines during the month of May, 2011. *T. tetrahele* starters were obtained from the Phycology Laboratory of the same institute. Test organisms were grown under a batch culture method using TMRL (Tungkang Marine Research Laboratory) media under constant illumination and aeration at ambient temperatures. *T. tetrahele* starters were first cultured in three 1 L vessels for 3 days then inoculated and scaled-up into a single 15 L vessel and allowed to grow for another 3 days. This was done to ensure homogenous samples to be used for the flocculation experiments.

Flocculation methods. To determine their flocculating efficiency on *T. tetrahele* cultures, four inorganic compounds (aluminum sulfate, ferrous sulfate, calcium chloride, and sodium hydroxide) and one organic flocculant (aqueous extract of *M. oleifera*) were used in jar tests at various concentrations. These compounds were selected based on

previous studies on their use in either waste water treatment or harvesting other species of algae. Stock solutions for the four inorganic compounds were prepared at a concentration of 10,000 mgL^{-1} while the aqueous extract of *M. oleifera* was prepared following the methods of Katayon et al (2004).

Aliquots of algal culture (100 ml) were placed in 150 ml beakers. The test flocculants were then added accordingly to reach final concentrations of 100, 150, and 200 mgL^{-1}. Each concentration for every flocculant was carried out in triplicate. Upon addition of flocculants, beakers were stirred for 1 minute using a magnetic stirrer and left to settle over a 4 hour period. A control group with no flocculants added was also prepared to serve as a reference.

Determination of flocculation efficiency. Flocculation efficiency was determined by measuring the optical density of the cell cultures before flocculation and the residual optical density of the supernatant liquid every hour. Optical density (OD) was read using a spectrophotometer at 680 nm (Lee et al 1998). To determine the flocculating efficiency of the different compounds tested, the following formula was used:

Flocculating Efficiency = (Initial OD – Residual OD)/Initial OD x 100

Microscopic examination of algal cells and flocs. To determine whether the various flocculants used had any effect on the integrity of the cell walls of *T. tetrahele*, samples of settled material from each treatment after the 4 hour settling period were pipetted out and observed using a compound microscope under 100x magnification.

pH. The initial pH of the culture medium and the final supernatant were also monitored using a standard laboratory pH meter.

Statistical analysis. The data for flocculating efficiency (%) were arcsin transformed. The transformed data for flocculating efficiency and pH were analyzed by one-way ANOVA followed by Duncan's Multiple Range Test at a confidence level of 95%.

Results and Discussion

Flocculating efficiency. Figure 1 shows the different flocculating efficiencies of the test flocculants at different concentrations after a settling period of 4 hours. Highest flocculating efficiencies for all test flocculants were observed at concentrations of 200 mgL^{-1} except for that of $FeSO_4$ which had the highest flocculating efficiency at 100 mgL^{-1}. At concentrations of 200 mgL^{-1}, there was no significant difference ($p>0.05$) between flocculating efficiency of NaOH and $Al_2(SO_4)_3$ (98.7% and 96.2%, respectively) and both treatments were significantly higher ($p<0.01$) than the other flocculants tested.

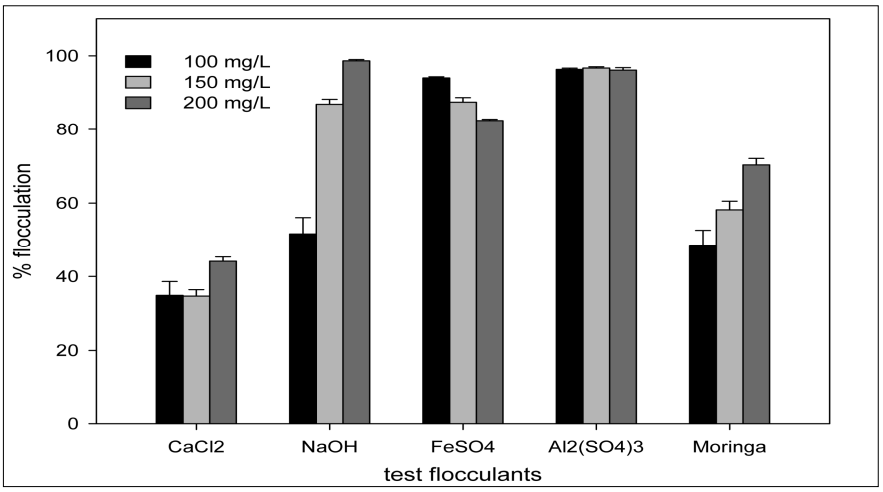

Figure 1. Flocculating efficiency (%) of various flocculants at different levels of concentration (mgL^{-1}) after the 4 hour settling period.

Comparing the flocculating efficiency of each flocculant at different concentrations, increasing efficiency was observed with increasing concentrations for NaOH, $CaCl_2$, and *M. oleifera* extracts. There was no significant difference ($p>0.05$) between the concentrations for $Al_2(SO_4)_3$. Treatments with $FeSO_4$ on the other hand showed lower flocculating efficiency as concentrations increased.

Settling time. Changes in the optical density of the algal culture under different flocculants throughout the 4 hour settling period are shown in Figure 2. Among the flocculants tested, treatments with $Al_2(SO_4)_3$ at 150 and 200 mgL^{-1} had the fastest settling times displaying stable optical densities 1 hour after the addition of flocculants. This was followed by $Al_2(SO_4)_3$ at 100 mgL^{-1}, $FeSO_4$ at 200 mgL^{-1}, and *M. oleifera* extracts at 100 and 150 mgL^{-1} which displayed stable optical densities after 2 hours. All the other treatments, including NaOH at all concentrations, reached stable flocculating efficiencies 3 hours after the addition of flocculants.

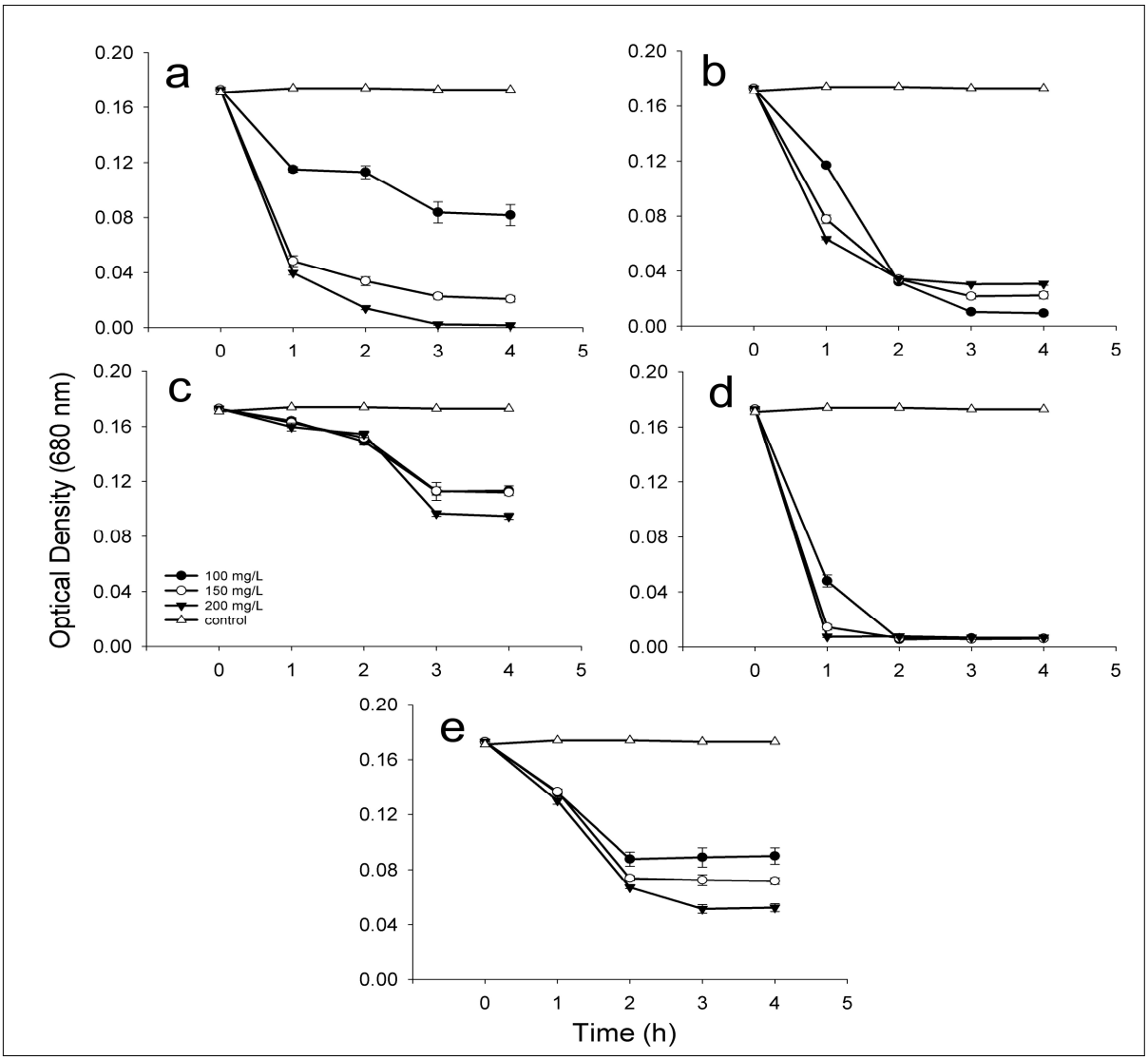

Figure 2. Changes in optical density of the algal culture with the addition of (a) NaOH, (b) $FeSO_4$, (c) $CaCl_2$, (d) $Al_2(SO_4)_3$, (e) *M. oleifera* extract at different concentrations over 4 hours.

Microscopic examination. Microscopic examination of cells treated with various flocculants showed that cells were in good physical condition. No signs of cell structural damage or plasmolysis were observed under all treatments (Figure 3). The formation of flocs were clearly observed for cells treated with NaOH, $FeSO_4$, and $Al_2(SO_4)_3$ (Figures 3a, 3b, 3c) while those treated with $CaCl_2$ and *M. oleifera* extracts, although indicating

reductions in optical densities, displayed no prominent aggregations of algal cells (Figures 3d, 3e). Compared to untreated cells (Figure 3f), flocculated cells exhibited no distinct changes in size and shape.

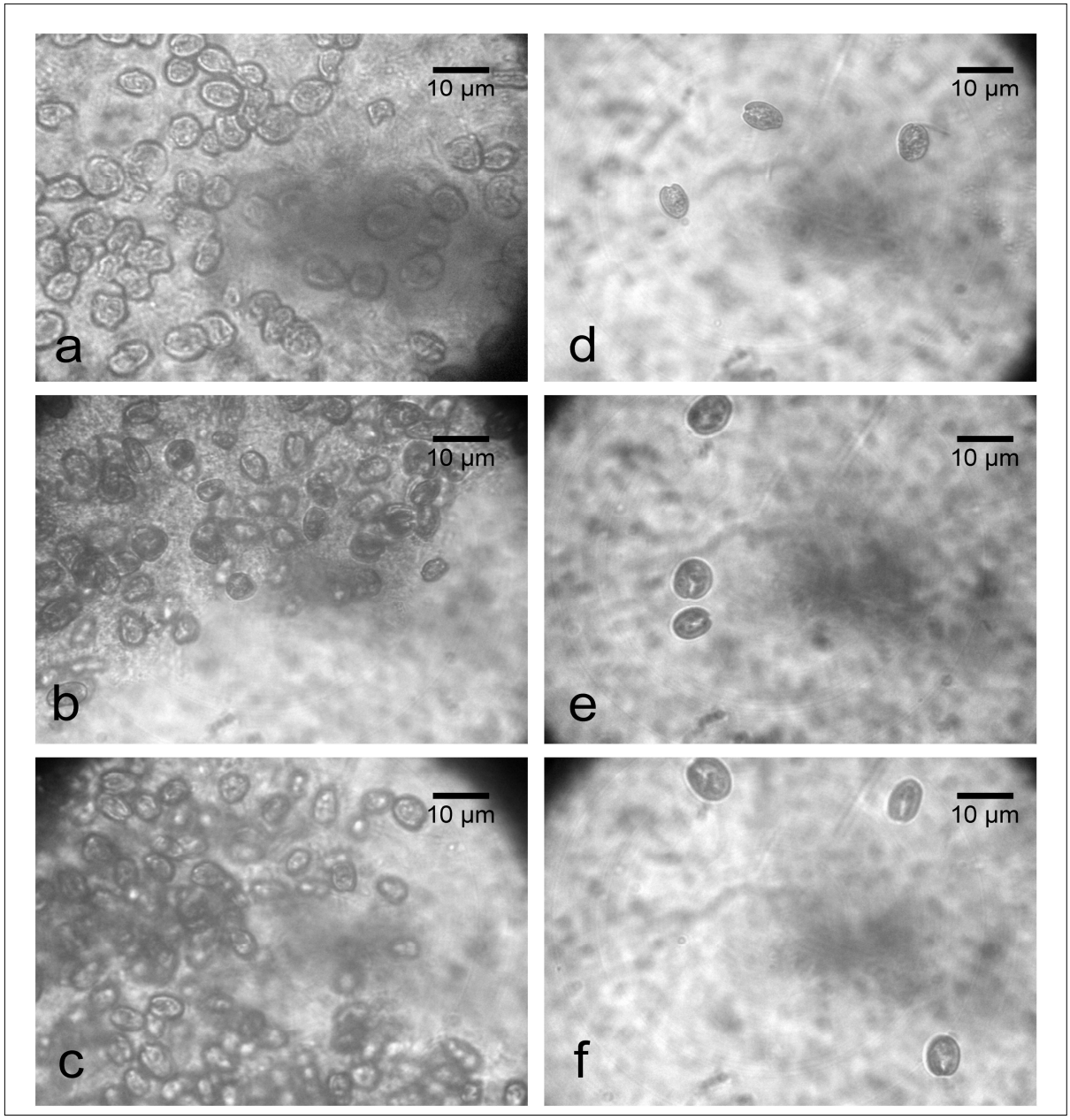

Figure 3. Microscopic examination of flocculated cells of *T. tetrahele* treated with (a) NaOH, (b) $FeSO_4$, (c) $Al_2(SO_4)_3$, (d) $CaCl_2$ and (e) *M. oleifera* showing no signs of cell plasmolysis. Comparison with untreated cells (f) shows similar cellular shape and size for all treatments.

pH. Mean pH levels of the resulting supernatants are shown in Table 1. Treatments with $FeSO_4$, $Al_2(SO_4)_3$, $CaCl_2$ and *M. oleifera* showed significantly lower ($p<0.01$) pH levels while treatments with NaOH showed significantly higher ($p<0.01$) pH levels when compared to the pH of the initial culture media. Although pH levels for the former flocculants were significantly lower, mean pH readings at all concentrations still showed that the resulting supernatants were neutral. Treatments using NaOH however resulted in supernatants that were slightly alkaline with mean pH levels ranging from 8.02 (for 100 mgL^{-1} NaOH) to 8.42 (for 200 mgL^{-1} NaOH). It was also observed that increasing concentrations of $FeSO_4$, $Al_2(SO_4)_3$, and *M. oleifera* had an inverse relationship with pH levels of the resulting supernatant while the opposite was observed for treatments with

NaOH. No significant difference was observed in the pH levels of samples treated with $CaCl_2$ at various concentrations.

Table 1

Mean pH of resulting supernatants after 4 hour settling period

Flocculant concentration (mgL^{-1})	*Flocculant*				
	$CaCl_2$	*NaOH*	*$FeSO_4$*	*$Al_2(SO_4)_3$*	*Moringa*
100	7.71±0.01	8.02±0.03	7.51±0.02	7.46±0.03	7.62±0.02
150	7.71±0.02	8.17±0.03	7.30±0.02	7.11±0.01	7.53±0.03
200	7.72±0.01	8.42±0.03	6.98±0.02	6.99±0.02	7.42±0.02

Note: mean pH of initial culture media is 7.79.

This study showed that the most effective flocculants for the separation of *T. tetrahele* cells from culture media were NaOH and $Al_2(SO_4)_3$. Knuckey et al (2006) reported that adjustment of pH using NaOH with subsequent addition of a non-ionic polymer was effectively applied to harvest cells of *Chaetoceros calcitrans, C. muelleri, Thalassiosira pseudonana, Attheya septentrionalis, Nitzschia closterium, Skeletonema sp., Tetraselmis suecica* and *Rhodomonas salina*, with efficiencies ≥80%. Similarly, a study by Lee et al (1998) also showed that pH adjustment using NaOH was more effective in harvesting *Botryococcus braunii* cells compared to aluminum sulfate and a cationic polyelectrolyte. It was also reported that aluminum sulfate was more superior in comparison with other inorganic salts in terms of optimal dose, pH, and the quality of the resulting water and algal slurry (Bare et al 1975; Moraine et al 1980). Lee et al (1998) also demonstrated that aluminum sulfate was more efficient in harvesting *Chlorella sp.* cells compared to pH adjustment using NaOH. Although the efficiency of one particular flocculant may vary from species to species depending on several factors (Avnimelech et al 1982; Bilanovic et al 1988), this study showed the efficiency of both aluminum sulfate and NaOH in separating *T. tetrahele* cells from the culture media.

This study also shows the direct relationship observed between concentration and flocculating efficiency for NaOH, $Al_2(SO_4)_3$, $CaCl_2$, and *M. oleifera*. Bilanovic et al (1988) and Wyatt et al (2012) reported flocculation reactions can be sensitive to a number of factors, one of which is flocculant concentration. As the trends in this study show, optimal doses for each flocculant could be determined in future studies. Although NaOH and $Al_2(SO_4)_3$ were shown to be more efficient flocculants, the use of aqueous extracts of *M. oleifera* could still be a cheaper alternative to chemical flocculants given higher concentrations. The use of aqueous extracts of *M. oleifera* as flocculants have been reported by Katayon et al (2004) with flocculating efficiencies of up to 94% at concentrations of 400 mgL^{-1}. Shehata et al (2008) also reported efficiencies of up to 97% in flocculating a mixture of algal cells using *M. oleifera* extracts. The decreasing flocculating efficiency of $Fe(SO_4)$ as concentrations increased was a notable observation since this digressed from the trend of all other tested flocculants. Similar observations by Aziz et al (2007) showed that using $Fe(SO_4)$ as a clarifying agent for landfill leachate at a controlled pH of 6 resulted in a notable reduction in the efficiency of $Fe(SO_4)$ as concentrations increased from 0 to 500 mgL^{-1}.

The maintenance of cell wall integrity during the harvesting process has been shown to improve the shelf life of harvested cells and the conservation of cell metabolites (Knuckey et al 2006; Knuckey 1998). In the process of extracting oil from algae, although the disruption of the cell wall is necessary to maximize lipid recovery, this is only done after cells have been sufficiently dewatered and dried. During the dewatering and drying stages of the algal biomass (and even during storage if extraction does not proceed directly), retention of cell wall integrity could help in minimizing the oxidation of lipids within the cells. This study showed that all flocculants used at varying concentrations did not exhibit any signs of cell wall disruption indicating the suitability of using these flocculants as a means of harvesting algal cells for oil extraction.

With regards to pH, this study showed that all flocculants used had a significant effect in changing the pH of the culture media after flocculation regardless of concentration. All

flocculants lowered the pH of the resulting supernatant except for NaOH which had the opposite effect. Monitoring the pH of the resulting supernatant was done in order to determine if the resulting water after flocculation was suitable for reculture. A study by Azov (1982) showed that lower pH ranges for culture media resulted in higher biomass concentrations. Although significant changes in pH were observed for all treatments, these levels were still within the tolerable range for algal culture of pH 7-9 (Coutteau 1996).

Conclusions. The present study showed the efficiency of various compounds in flocculating cultures of the microalgae *T. tetrahele*. Of the five flocculants tested, NaOH and $Al_2(SO_4)_3$ at concentrations of 200 mgL^{-1} showed the highest ($p<0.01$) flocculating efficiencies at 96.15% and 98.65%, respectively. In terms of settling time, $Al_2(SO_4)_3$ at concentrations of 150 and 200 mgL^{-1} showed the fastest reactions reaching stable algal flocs only 1 hour after the addition of flocculants.

All flocculants tested had no effect on the integrity of the cell walls of the test organism. Microscopic examination showed algal cells with no apparent structural damage or plasmolysis.

Measurement of pH for all treatments showed significant changes ($p<0.01$) in the pH of the culture media four hours after the addition of flocculants. Aqueous extract of *M. oleifera*, ferrous sulfate, aluminum sulfate, and calcium chloride all resulted in lower pH values for the resulting supernatant displaying an inverse relationship between flocculant concentration and pH. NaOH on the other hand had an opposite effect, raising pH levels of the resulting supernatant which was directly related with flocculant concentration. Despite significant changes in pH, all were still within the range suitable for algal culture indicating the possibility of reusing the supernatant for further cultures.

Further studies regarding optimum dosage of flocculants should still be carried out for *T. tetrahele*. This is of particular interest with regards to the use of *M. oleifera* since it has the potential to become a cheaper alternative to inorganic salts and other synthetic polymers. Although pH of the culture media plays a major role in the culture of algae, other parameters such as nutrient concentration (i.e. ammonia, nitrite, nitrate, and phosphorous) should also be included in future studies regarding the possibility of reusing waste water after flocculation.

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