Research Article

Effects of pH-induced technique on flocculation efficiency and fatty acids profile of marine microalgae, *Thalassiosira sp*

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Abstract

Microalgae contain many valuable biological compounds, so their commercial production is of great importance. One of the important challenges in microalga production is their high cost associated with their harvesting processes. Therefore, finding efficient and cost-effective technologies for algae biomass separation is essential for achieving an operational process. The study was conducted to find the influence of pH variation from 4 to 11 on harvesting efficiency and fatty acids component of marine microalgae *Thalassiosira* sp under laboratory condition. The NaOH and HCl were added to adjust pH value in each treatment. Data was presented as mean ± SD. Comparison between treatments was performed using one-way analysis of variance ANOVA. The results showed that with increasing the pH up to 10, flocculation processes (92%) and concentration factor (20) increased significantly. While, lowering the pH from 8.2 to 4 was not effective in isolating algal biomass. In the next stage, the biomass fatty acids content was measured by both alkaline pH induction method and the centrifuge method. Analyzes showed that the percentage of lipid and saturated and unsaturated fatty acids in biomass collected by alkaline pH induction technique was lower than the centrifugation method. In general, the results of the present study showed that clotting in alkaline pH up to 10 can be suggested as a suitable method for harvesting of *Thalassiosira* sp microalgae, and if the production of polyunsaturated acids is considered, of consuming high amounts of sodium hydroxide should be avoided.

Keywords: Microalgae, *Thalassiosira* sp, pH-induced, Harvesting efficiency, Fatty acids

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Introduction

Microalgae can produce many high-value

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biological compounds. In order to produce metabolites derived from microalgae, economic microalgae cultivation technologies should be used (Borowitzka and Borowitzka, 1988). Commercial production of microalgae metabolites requires the following process: (1) large-scale single-species production. (2) biomass recovery from diluted culture medium. (3) extraction of metabolites from biomass and (4) purification of crude and fraction extracts (Maji *et al*., 2018). In the production of beneficial biological metabolites, downstream processes of microalgae recovery can be much more expensive than algae cultivation. The biomass retrieval involves of a primary concentration step, after that a separation step for isolation the microalgal biomass from its aquatic environment (Milledge and Heaven, 2013). This step represents a critical bottleneck for large-scale algal bio-refinery process and accounts about 20 to 30 % of the total biomass production custom depending on the algal species and culture process used (Uduman *et al*., 2010).

In general, harvesting methods are founded on biological, chemical, or physical processes that are included gravity sedimentation, screening, filtration, and air flotation techniques. However, there is no single universal technique for all algae species or application, and the appropriate technique should be used depend on the metabolites derived from microalgae (Show *et al*., 2013).

Flocculation technique refers to the accumulation of unstable and small particles by neutralizing surface charge, electrostatic patching or bridging after the addition of flocculants. Since, flocculants play an important role in the harvesting process, finding an efficient and low-cost flocculent is a serious challenge (Barros *et al*., 2015). Ideally, in addition to excellent harvest efficiency and promising scalability, flocculants should have low toxicity, good recyclability and satisfy the demands for commercial production. Many chemical products such as organic polymers / organic polyelectrolytes and multivalent metal salts have been evaluated as coagulants. Several studies have shown that pH changes are very effective in the clotting of microalgal biomass (Wu *et al*., 2012). It should be noted that flocculants resulting from pH manipulation is mainly dependent on the algae species (Wan *et al*., 2015).

Microalgae *Thalassiosira* sp is a unicellular microalgae distributed globally and are a key group of the phytoplankton in the ocean which primarily utilized as shrimp and shellfish larval feed (Lang *et al*., 2013). *Thalassiosira* species has the ability to secrete diverse secondary metabolites according to the media condition. Also this species can be treated different bacterial infections and different abnormality such as cancer, aging and neurological abnormality (García *et al*., 2012). Therefore, in order to use of the beneficial metabolites of this microalgae, it is necessary to mass-produce it with the appropriate harvesting technique. So, in this study, an attempt was made to examine the effect of flocculation technique through pH changes on harvesting efficiency and the fatty acids content of microalgae *Thalassiosira sp.*

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Materials and methods

Microalgal strains and culture conditions

The algae *Thalassiosira* sp was collected from private shrimp hatchery near to Chabahar Bay, Iran, Oman Sea. The diatom was acclimatized and grown in laboratory with f/2 medium prepared with seawater (Guillard, 1975). The cultures have been incubated in 500-mL Erlenmeyer flasks with 300 mL of culture media at 27 ± 2 °C, and illuminated by fluorescent lamps for 12-h light:12-h dark cycle at 50 μmol photons m^{-2} s^{-1} light intensity. The culture has been continuously aerated and blended by the gentle bubbling filtered air. Cell concentration has been measured turbidometrically at 680 nm (Evolution™ 300 UV-Vis Spectrophotometer). One unit of OD 680, corresponds to 0.2 g-dry cell wt/l in this culture.

Flocculation experiments

Five hundreds ml of the broth were poured into 1000 ml beakers then, added 1.0 M HCl or 1.0 M NaOH solution to regulate pH. The samples were blended for 3 min. The mixed suspensions have been transferred into 100-ml measuring cylinders and retained for seeing. After 1, 3, 5 and 7h of algal cell flocculation, an aliquot 5 mL of the culture has been pipetted from a height of two-thirds to measure the flocculation efficiency, at a wavelength of 680 nm (Evolution™ 300 UV-Vis Spectrophotometer). The flocculation efficiency and concentration factor were calculated according the following equations. A control without added coagulant has been used as a reference (Wu *et al*., 2012):

Flocculating efficiency (%) = (1-B/A) * 100

Concentration factor (CF) = V0/Vf

A= OD680 of the control group

B= OD680 of the pH-regulated group

V0= the initial volume of algae solution

Vf= the final volume of condensed algae solution

Microscopic findings

The reaction between algal cells and clotting agents was examined by light microscope .At the end of coagulation, samples were gathered from the bottom and seen under a light microscope (ECLIPSE E200, Nikon).

Lipid extraction and fatty acid composition

To investigate the flocculation techniques on lipid content and profile of biomass fatty acids, effects of harvesting method on the lipid and fatty acid content of experimented algae, the algae cells samples harvested from flocculation and centrifugation method were gathered. In the centrifugation method, the algae cells were centrifuged at 4,000×g for 6 min (Santriphoge, Labofge 200). In the treatments with flocculant, the basic pH-induce sample (9, 9.3, 9.6, 9.8,10, 10.5 and 11) were chosen for lipid and fatty acid analysis.

Two techniques of pH induction and centrifugation were used to harvest algae cells. The cultures were settled then the supernatant was taken away and algal cells were gathered. To reduce salinity, the collected algal cells were washed with 0.5 M (NH4) HCO3 and then lyophilized (Jalteb, Iran). Fatty acid methyl esters (FAME)

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preparation was carried out using transmethylation protocol published by (ISO, 2011) . The quantitative detection of fatty acids has been performed using Shimadzu gas chromato-graph (GC-2010, Shimadzu; Tokyo: Japan) that has been equipped with the auto-injector (AOC-20i; Shimadzu). The temperature of the flame ionization detector and injector equaled 260 °C. Therefore, temperature of silica capillary column (Supelco SP-2560; 100 m9 0.25 mm with a film thickness of 0.20 lm) has been programmed from 140 - 240°C at 4°C/min. In addition, nitrogen has been utilized as a carrier gas. Moreover, relative retention time with a reference standard (Supelco; 37 components FAME Mix, Supelco Inc., Bellefonte: PA: USA) has been compared by the fatty acids. Furthermore, area and internal standard have been compared to quantify the fatty acid contents and has been written as the dry weight percent.

Statistical analysis

Data was presented as mean ± SD. Effects of treatments were evaluated using one-way analysis of variance ANOVA. Duncan's multiple range test was use to compare differences among treatments and results were determined as significant at $p<0.05$. The data were investigated by the SPSS software version 22 (Armonk, NY, USA).

Results

Comparison of flocculation efficiency at different pH

The pH changes using 1M solution of sodium hydroxide and hydrogen chloride on the flocculation rate of *Thalassiosira* Sp microalgae culture medium was shown in Fig. 1. Calculation of flocculation efficiency in alkaline medium showed an increasing trend of 50% at pH 8 (in the natural environment) continued up to about 90% at an alkaline pH of 10. From pH 10 up to pH 11, the process of mass formation remained constant and no significant increase in flocculation was observed ($p>0.05$). Meanwhile, the culture medium from pH 10 to 11 became gel-like and milky. So, there was no significant difference in the method of cell coagulation ($p>0.05$). The results of the pH change test in the acidic range, which was performed by adding hydrogen chloride up to pH 4, showed no coagulation or clotting in the samples. The cells remained floating in the culture medium.

Comparison of concentration factor (CF) at different pHs

Concentration factor at different pHs was presented in Fig. 2. The results showed that with increasing the pH, the concentration coefficient increased and the highest CF was obtained at alkaline pH 10 ($p<0.05$).

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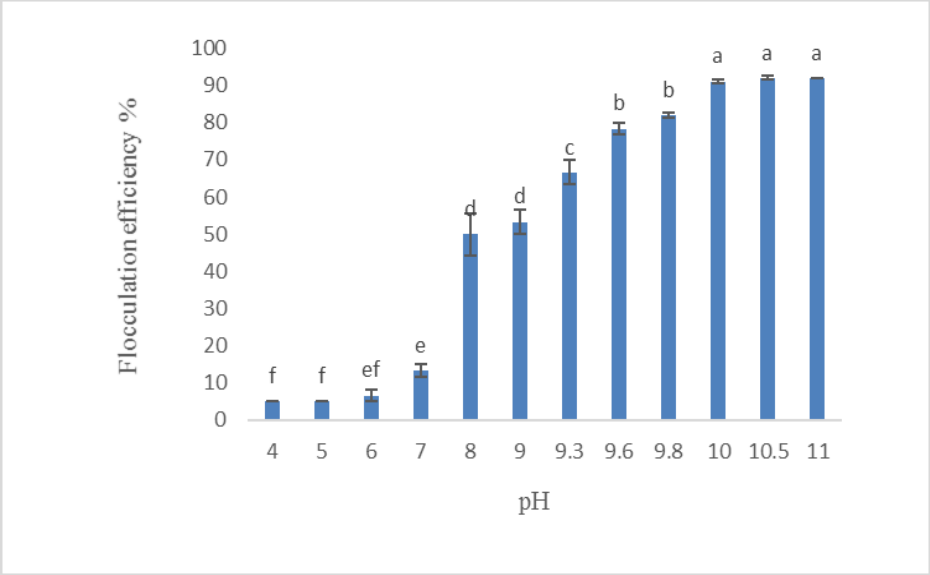

Figure 1. Flocculation efficiency of algae at different pHs. Different lowercase letters at different pHs show significant ($p<0.05$) differences.

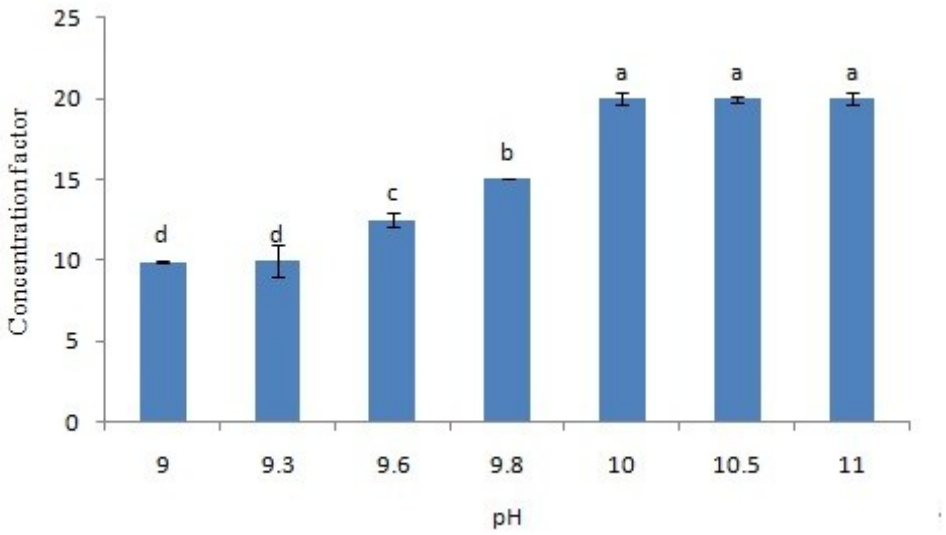

Figure 2. Concentration factor of algae at different pH. Different lowercase letters at different pHs show significant ($p<0.05$) differences.

Flocculation efficiency at different settling times

Flocculation efficiency at different settling times for different pHs were displayed in Fig 3. It was observed that there was no significant difference at different time intervals in treated groups and biomass settlement took place under 1, 3, 5 and 7 h at high pH values (> 9) was similar.

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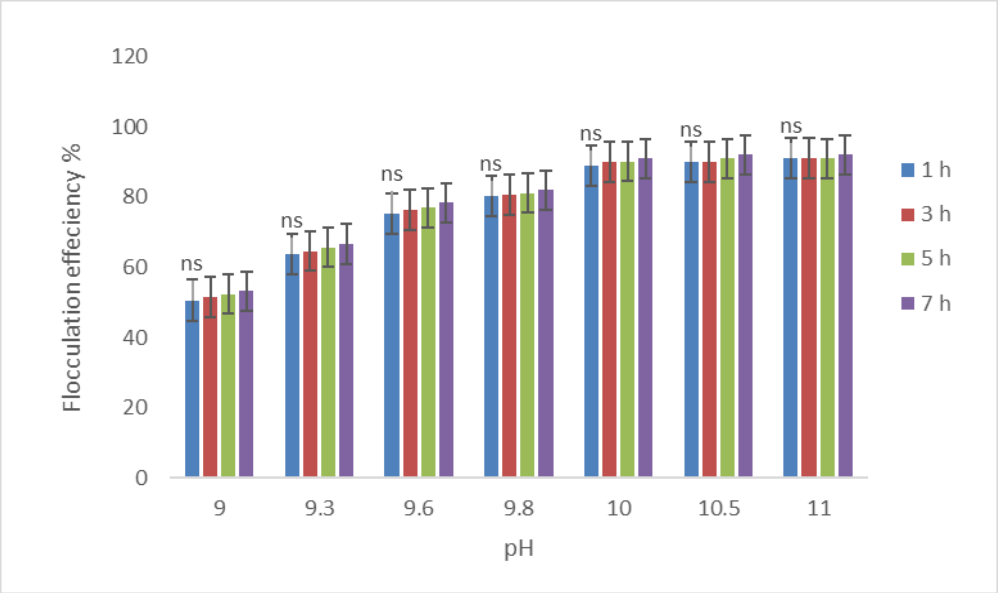

Figure 3. Flocculation efficiency at different settlement time.

The effect of flocculation techniques on lipid content and profile of biomass fatty acids

The percentage of lipid content and profile of biomass fatty acids collected of *Thalassiosira* sp. differed among centrifuge and pH-induced technique shown in Table 1and 2. The results showed that the lowest lipid content (0.08% dry weight) at pH value 11 and the highest lipid content (1.09% dry weight) for centrifuge technique were seen (Table 1). C18:1, C18:0 and C16:0 fatty acids collected by centrifuge technique were higher percentages than those of pH-induced technique (Table 2). A greater difference was seen for highly unsatured fatty acids such as C20:4 and C20: 5 after the addition much more flocculants.

Table 1. Lipid yield, dry weight, total SFA, MUFA and PUFA (% dry weight) of *Thalassiosira* sp. (mass fraction in %) for different treatments

			Biomass (%)	Lipid (%)	SFA (%)	MUFA (%)	PUFA (%)
Harvesting methods	**Centrifugation**		1.82±0.15^{a}	1.09±0.09^{a}	42.85±10.06^{a}	51.23±9.47^{a}	4.98±0.20^{ab}
	pH-Flocculation	9	1.03±0.14^{b}	0.60±0.08^{b}	42.65±8.49^{a}	35.61±5.85^{b}	5.33±0.68^{a}
		9.3	0.88±0.04^{bc}	0.49±0.01^{c}	39.45±6.14^{b}	42.59±8.38^{b}	4.41±0.18^{b}
		9.6	0.73±0.07^{cd}	0.37±0.05^{d}	38.17±7.40^{c}	31.69±3.34^{e}	3.58±0.12^{c}
		9.8	0.59±0.07^{de}	0.23±0.02^{e}	37.99±3.18^{c}	35.92±8.21^{d}	2.47±0.22^{d}
		10.0	0.44±0.04^{ef}	0.15±0.01^{ef}	39.60±3.08^{b}	37.28±7.34^{c}	1.66±0.39^{e}
		11	0.29±0.02^{f}	0.08±0^{f}	40.47±1.52^{b}	25.86±2.88^{f}	1.07±0.04^{f}

Data are shown as means ± S.D (standard deviation). Different letters in the same columns indicate significant differences between treatments ($p<0.05$).

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Table 2. Fatty acid content (%) of *Thalassiosira* sp in various treatments

Fatty acid	Centrifugation	pH- Flocculation					
		9	9.3	9.6	9.8	10	11
C14:0	20.63±2.05^{a}	18.32±1.08^{b}	14.16±0.84^{c}	13.10±2.11^{d}	11.22±1.04^{e}	10.93±0.74^{f}	9±0.54^{g}
C16:0	20.93±1.54^{a}	18.92±0.13^{b}	17.66±0.30^{c}	15.40±1.09^{d}	14.01±0.94^{e}	13.33±0.15^{f}	10.93±0.66^{g}
C17:0	3.04±0.19^{a}	2.45±0.26^{b}	2.07±0.18^{c}	1.74±0.54^{d}	1.41±0.12^{e}	1.11±0.53^{f}	0.94±0.04^{g}
C18:0	10.60±0.73^{a}	8.74±0.34^{b}	7.33±0.64^{c}	5.39±0.84^{d}	5.15±0.51^{e}	4.84±0.14^{f}	3.86±0.3^{g}
C15:1	2.70±0.10^{a}	2.02±0.16^{b}	1.83±0.06^{c}	0.54±0.02^{d}	0.29±0.03^{e}	0.32±0.06^{e}	nd
C16:1	9.94±0.60^{a}	9.70±0.36^{a}	9.89±0.11^{a}	7.46±0.25^{b}	4.43±0.56^{c}	4.05±0.15^{d}	2.90±0.09^{e}
C17:1	1.76±0.04^{a}	1.64±0.03^{a}	1.73±0.06^{a}	1.68±0.08^{a}	0.98±0.04^{b}	0.74±0.04^{b}	0.70±0.05^{b}
C18:1n9	29.60±3.36^{a}	28.89±5.17^{b}	24.73±1.18^{c}	22.90±8.09^{d}	22.49±4.45^{d}	21.23±5.06^{e}	7.87±1.14^{f}
C18:2	4.41±0.18^{a}	3.58±0.12^{b}	3.32±0.06^{bc}	2.88±0.58^{c}	1.66±0.39^{d}	1.72±0.08^{d}	1.07±0.34^{e}
C20:4	0.31±0.03^{a}	0.09±0.03^{b}	nd	nd	nd	nd	nd
C20:5	0.74±0.08^{a}	0.03±0.07^{b}	nd	nd	nd	nd	nd

Data are shown as means ± S.D (standard deviation). nd: Not defined. Different letters in the same rows indicate significant differences between treatments ($p<0.05$).

Discussion

Microalgae are microscopic plants that grow in water and produce high lipid up to 20 to 50 % of their dry weight. Therefore lipid synthesis is much greater in microalgae than in terrestrial plants (Becker, 2004). Among microalgae, diatoms are a very promising source of oil, since they produce various types of natural oils (triacylglycerols and either saturated, unsaturated, or polyunsaturated fatty acids) (Borowitzka and Borowitzka, 1988). In the current study, the flocculation efficiency and changes in fatty acids of marine diatom *Thalassiosira* sp. during algae harvesting were investigated. The observation displayed that the measured size of the *Thalassiosira* sp. cells was about 10 µm in diameter during culture period, so separation of microalgae from the growth medium could be a difficult task. Scientists believe that in addition to their small size, negative surface charge and similar densities to their medium water are also a limiting factor in harvesting process (Horiuchi *et al*., 2003).

In current study, no significant fluctuation was observed in acidic and neutral pH level from 4 to 8.2. This means that under acidic conditions, clot formation in algae medium was not observed. However, by pH increasing from 9 to 10 floe formation of microalgal cells and fast flocculation happened and harvesting efficiency reached to maximum 90 % and after that remained constant when the pH was arrived to 11. These results suggest that the alkaline pH is a relatively good method for the formation of clots in microalgae, *Thalassiosira* sp. cells. At pH 11, flocculation happened, but, the settling rate was more gradual than that at pH 9 to 10, which proposed that higher pHs can inhibit favorable flocculation. While the pH: 10 showed the concentration 20 times

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higher than the initial concentration of the cell. It is apparent that favorable flocculation caused high recovery with a fast settling rate of the cell. Also in this study, a significant color change was observed in the pH between 10 and 11. The reason for the change of color could be due to the saponification of chlorophyll in the presence of sodium hydroxide. The pH based (acidic and alkaline) flocculation method was performed for various species of microalgae such as *Dunaliella tertiolecta* (Horiuchi *et al*., 2003); *Phaeodactylum tricornutum* (Spilling *et al*., 2011); (*Chlorella vulgaris*, *Scenedesmus* sp., *Chlorococcum* sp.) and marine microalgae (*Nannochloropsis oculata*, *Phaeodactylum tricornutum*) (Wu *et al*., 2012); *Chlorococcum ellipsoideum (Liu et al., 2013)*; *Dunaliella salina* (Besson and Guiraud, 2013); *Dunaliella viridis* (Mixson *et al*., 2014); *Nannochloropsis oculata* (Sales and Abreu, 2015); *Chlorella* sp. (Yang *et al.*, 2016); *Skeletonema costatum and Chaetoceros gracilis* (Pérez *et al*., 2017). The comparison of different studies show that the effect of pH on microalgae harvesting varies between species and the suitable pH for one species of microalgae may not necessarily be effective for other species of microalgae. For example, the optimum pH for *Phaeodactylum tricornutum*, was 10.5, while pH for *N. oculata* was around 10.4 to 10.7. This could be due to changes in surface functional groups in different strains of microalgae cells. Differences in the results of several studies can be related to the variation in age of the cell culture at sampling time (Kwon *et al*., 2014).

In the study of Besson and Guiraud (2013), also the pH buffer zone has been observed in pH value 10.5 >. In the buffer zone, flocs formation also changes and cell mass separation becomes more difficult than lower pH value.

Some of these reports indicated that the concentrations of Fe^{3+}, Ca^{2+} and Mg^{2+} as the polyvalent cations in the growth medium played a main role in the coagulation action and the alkaline pH range is caused the precipitation reaction of magnesium and formed magnesium hydroxide. Finally, the negative charges on the surface of microalgal cell are neutralized by the positive charges of the metal hydroxides, leading to clotting. Generally these researches have proven that coagulation is pertained to cell surface properties (Wu *et al*., 2012, Besson and Guiraud, *2013*, Krishnakumar *et al.*, 2013).

Some researchers believe that in the harvesting technique, by inducing pH, since the contaminant chemical is not added to the culture medium, the culture medium can be reused. In this respect, the possibility of recycling the flocculated medium was examined. However, in the present study only the clear water area of the upper third part could be reused in alkaline pH <10. In addition, microscopic observations also showed that microalgae cells have low viability from pH value more than 9 and they are not suitable for re-inoculation exhibited that increasing pH leads to a cell lysis during the clotting, the cells seemed to be very sensitive to relatively high pH values and the

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molecular function and structure of cells were affected.

The method of harvesting microalgae cultures plays a crucial part in the cost and quality of the final products. Therefore, this study looked at whether the pH-induce method can affect lipid and fatty acids content of microalga, *Thalassiosira* sp. Our result showed that, A minimum mass fraction for pH-induced treatment at pH value 11 and maximum mass fraction for centrifuge technique were seen. The fatty acid profiles of *Thalassiosira* sp. harvested by centrifuge showed higher percentages of C18:1, C18:0 and C16:0 fatty acids. A greater difference was seen for highly unsatured fatty acids such as C20:4 and C20: 5 after the addition much more flocculants. Our results were in line to the cationic coagulants in microalgae *N. oculata.* Scientists believe that environmental factors including light, temperature and pH are strongly effective on the fatty acids in microalgae (Maji et al., 2018, Pérez et al., 2017). But this change is much greater in unsaturated fatty acids since; polyunsaturated fatty acids (PUFAs) as main ingredients of the microalgae membranes, react quickly to environmental changes such as pH variation. The alkaline pH may lead a decrease in membrane lipids. Therefore, if the purpose of microalgae cultivation is to produce specific commercial fatty acids such as oleic acid (C18: 1n9c) or EPA (C20: 5), the use of coagulants must be limited because coagulants are significantly reduces these fatty acids. If microalgae are produced for use in biodiesel, low levels of unsaturated fatty acids are not restrictive and pH flocculation is recommended.

In conclusion, this research found that with increasing the pH up to 10, flocculation processes (92%) and concentration factor (20) increased significantly. While, lowering the pH from 8.2 to 4 was not effective in isolating algal biomass. In the next stage, Analyzes explained that the percentage of lipid and saturated and unsaturated fatty acids in biomass collected by alkaline pH induction technique was lower than the centrifugation method. In general, the results of the present research showed that clotting in alkaline pH up to 10 can be suggested as a suitable method for harvesting of *Thalassiosira* sp microalgae, and if the production of polyunsaturated acids is considered, of consuming high amounts of sodium hydroxide should be avoided.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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