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Original Article

Assessment of flocculation induced by pH increase for harvesting microalgae *Cyanothece* sp.

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Abstract: One of the most important challenges lies in the microalgae mass production is the high cost of harvesting process which is the separation of a low amount of biomass consisting of small individual cells from a large volume of culture medium. Therefore, finding an efficient and cost-effective technique for harvesting microalgae is important issue. In the current study, pH-induced flocculation method was tested for microalgae *Cyanothece* sp. harvesting. The halophilic microalgae were cultured and grown in laboratory with hypersaline water in F/2 medium. After reaching the stationary phase, the impact of pH induction (from natural medium culture pH:8.2 to pH:11) on flocculation efficiency, chlorophyll a, chlorophyll b, total carotenoid, β-Carotene and phycocyanin component and the possibility of reuse flocculated medium of microalgae *Cyanothece* sp. were evaluated. The results indicated that the increasing the medium pH value by adding NaOH from pH natural at 8.2 to 9.4 increased flocculation efficiency significantly from 10 up to 90% ($P<0.05$), but after that remained stable up to pH: 11. Regarding the pigment content, the increase in pH value from natural pH: 8.2 to pH: 9.1 had a relatively a medium effect on pigment components, including chlorophyll a, chlorophyll b, total carotenoid, β-Carotene and phycocyanin amount of the harvested biomass of *Cyanothece* sp, but after that from pH: 9.4 to 11 the reduction was severe. The medium culture from pH: 8.5 to pH: 11 was reusable for new culture of microalgae. Thus, the flocculation induced by pH increase up to pH: 9.1 is a suitable method for harvesting microalgae *Cyanothece* sp. with no serious adverse effect on pigment component.

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Introduction

The commercial culture of microalgae is now over 60 years old with the main culture in large shallow open-air ponds. In recent years too much attention has been paid in research and development for increasing the yield of microalga biomass production through open pond raceway and photo bioreactor design (Helical tubular and flat panels systems), strain screening and selection, water quality, nutrients composition and climate condition (Ben-Amotz, 1999; Borowitzka and Borowitzka, 1988; Borowitzka, 2013b; Moheimani, 2013). But, much less work has been made on research and innovation in downstream processing such as harvesting and drying processes which is essential to reduce the cost of the production process. In order to moving microalgae production from laboratory to pilot scale and commercial-scale, we need to find cost- and energy efficient downstream processing technologies.

The separation and recovery of small microalgae biomass (about 0.5 g/l in open pond and 5 g/l in photobioreactors) from their large volume of culture medium is a challenging area and the costs related to harvesting, thickening and dewatering of microalgae biomass are about 20-30% of the total production cost (Horiuchi et al., 2003; Kwon et al., 2014). Several separation processes are currently used, including sedimentation, centrifugation, filtration and flocculation of microalgae in their water culture medium. Centrifugation and filtration methods are usually used for high-value products which is too expensive and energy-intensive. The flocculation is

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seen a low cost and convenient harvesting method. Flocculation refers to the aggregation of unstable and small cells through surface charge neutralization, electrostatic patching and/or bridging after addition of flocculants to spate cells from their medium culture (Spolaore et al., 2006; Wu et al., 2012; Ahmed et al., 2017). Usually flocculation is proposed for the first step to concentrate a dilute suspension of 0.5 g/l dry matter 20-100 times to a slurry of 10-50 g/l (Branyikova et al., 2018).

The current study was designed to study flocculation method by pH inducing for unicellular cyanobacterium *Cyanothece* sp. harvesting. *Cyanothece* sp. is an aerobic, diazotrophic and halophyte cyanobacterium species that can grow in a wide range of salinities, light intensities, temperatures and nutrient concentrations. Recent decade studies on different genera of Cyanothece have shown that this genus has very important role in nitrogen cycle in aquatic and terrestrial environments. (Bandyopadhyay et al., 2011). Based on biotechnology research, there are several studies showed that Cyanothece strains isolated from saline environments can produce high amount of extracellular polymeric substances which has characteristics suitable for food and pharmaceutical applications (Chi et al., 2007; Ohki et al., 2014). However, despite the great importance of this algae in the production of beneficial compounds, studies on suitable harvesting methods are still limited. Therefore, this work was designed for evaluating the effect of pH-induced on harvesting efficiency, total carotenoid and β-Carotene content of hypersaline microalgae, *Cyanothece* sp.

Materials and Methods

Microalga strain and culture conditions: The strain of *Cyanothece* sp. was isolated from Lipar lagoon in Chabhar, Iran. The halophilic microalgae were cultured and grown in laboratory with medium prepared with hypersaline water in F/2 medium (Guillard and Ryther, 1962) .The cultures were incubated in 500 mL Erlenmeyer flasks with 300 mL of culture media at 29±1°C, and illuminated by fluorescent lamps for 24 h in 4300 lux (Chi et al., 2007). The cultures were scaled up in 10 L and continuously aerated and mixed by the gentle bubbling filtered air. The seawater pH is typically limited to a range between 7.9 and 8.2. The growth was monitored by measuring the absorbance at 680 nm (OD680) by a spectrophotometer (Evolution™ 300 UV-Vis Spectrophotometer). Dry weight biomass was determined by filtering a fixed volume of the algae suspension through a pre-weighed filter with a 0.45 μm porous membrane. Then the filter and algal cells were dried at 105°C for 48 h (Zhu and Lee, 1997).

Determination of flocculation efficiency: Flocculation tests were performed after reaching the stationary phase. Flocculation experiments were carried out in cylindrical laboratory glass beakers of 1 L volume. 0.1-1 M sodium hydroxide (NaOH) and Hydrochloric acid (HCl) were used to adjust the pH value (from natural medium culture pH:8.2 to pH:11). The microalgae suspensions were mixed quickly just after pH adjustment and then allowed to settle at room temperature observe the behavior of flocs. An aliquot of the suspension at a height of two-thirds of the glass beakers was collected and flocculation efficiency percentage was calculated from the absorbance measured at 680 nm by a spectrophotometer (Evolution™ 300 UV-Vis Spectrophotometer). A control biomass was collected by centrifuging as a reference (Spilling et al. 2011).

$$\text{Flocculating efficiency (\%)} = (1-B/A) * 100$$

Where A represents the OD680 of the control treatment and B is the OD680 of the pH-adjusted treatment. The efficiency, in terms of biomass recovery percentage was determined for different samples at different time intervals 1, 3, 5 and 7 hours.

Floc Visualization: After the sedimentation, flocs of the different treatments were observed under the optical Microscope (ECLIPSE E200, Nikon).

Reuse of flocculated medium: After flocculation, the flocs and the growth medium were separated. The growth medium was return to refresh new alga culture and adjusted to the original pH value by adding the necessary amount of HCl and nutrients.

Pigment extraction and analysis: After flocculation, the microalgae biomass was collected and pigments

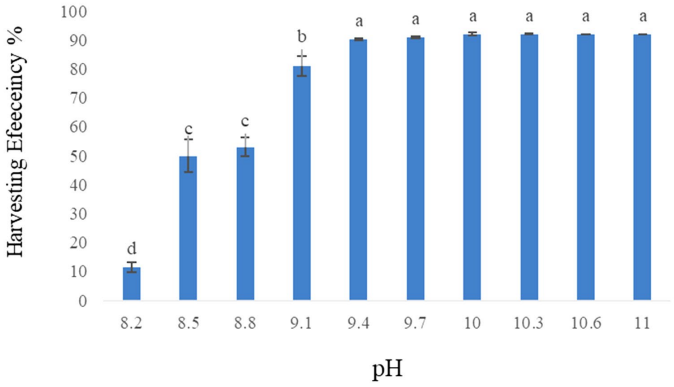

Figure 1. Harvesting efficiency of pH-induce flocculation in microalga *Cyanothece* sp.

content including, chlorophyll a, chlorophyll b, carotenoid, β-carotene and phycocyanin concentrations were measured. The algal biomass harvested was washed with 0.5M ammonium bicarbonate until total reduction of the salinity. After washing, the biomass was lyophilized (Jalteb, Iran). A sample of 0.1 g of lyophilized microalgae was suspended in 5 mL of the acetone 100%. The suspension was sonicated for 3 minutes in an ultrasound apparatus (Elma Ultrasonic) and stored for 24 h at 4°C. The extract was separated from the pellet and recovered by centrifugation, immediately filtered through a 0.22 µm filter (Cardoso et al., 2012). The absorbance of supernatant was noted at 660, 645, 470, 460, 431 and 412 nm using UV-Visible spectrophotometer (Evolution™ 300 UV-Vis Spectrophotometer). The amount of total carotenoids was determined using following equations (Lichtenthaler, 1987; Kumar et al., 2013):

Chlorophyll a (µg mL^{-1}) = (12.25 A 663) – (2.79 A 646)

Chlorophyll b (µg mL^{-1}) = (20.50 A 646) – (5.10 A 663)

Total carotenoids (µg mL^{-1}) = (1000 A 470 – 1.82 Chl a – 85.02 Chl b) / 198

β-carotene (µg/ mL) = -0.430 (A412) + 0.251(A431) - 4.376A (460) + 13.216A (480)

Phycocyanin = (mg/ mL) = (A615 A 720) -0.474× (A652- A720))/5.34

Where A663, A646, A470 and A412 represent absorbance at 663, 646 nm; 470 nm; 412 nm, respectively. The obtained extract was processed on the High-Performance Liquid Chromatography (HPLC) system for the separation and identification of β-carotene by comparison of their retention times with those of the commercial standards.

Statistical analysis: One-way analysis of variance was applied to analyze the experimental data using SPSS 16.0 software (SPSS, USA). The differences were considered significant at $P<0.05$. All the treatments were repeated three times, and data are reported as the mean±SD values.

Results

Effect of pH inducing on flocculation efficiency of microalgae *Cyanothece* sp.: The effect of pH on the flocculation efficiency of marine microalgae *Cyanothece* sp. was studied using 0.1-1 M NaOH and HCl when the culture was in stationary phase (day 10). The results indicated that no significant automatic precipitation occurs in the culture medium without use of flocculating agent over time. The microalgae cells began to agglomerate when NaOH was added and induced destabilization that led to the progressive formation of algal flocs. As, pH increased from pH: 8.2 in natural medium culture to 9.4, the flocculation efficiency was greatly raised to around 90% and reached plateaus. After that, with adding larger quantities of NaOH, the solution behaved like a gel and no more sedimentation was observed from pH 10 to 11 (Fig. 1). The method of adding NaOH, either at low flow rate or at once, has no significant effect on recovery efficiency.

Reuse of growth medium for cultivation: It has been observed that the biomass of microalgae grown in a re-used medium from pH: 8.5 to pH: 11 is close to that of fresh medium culture.

Effect of flocculants on pigment component including chlorophyll a, chlorophyll b, total carotenoid, β-Carotene and phycocyanin of harvested biomass of *Cyanothece* sp.: Chlorophyll a, chlorophyll b, total carotenoid, β-Carotene and phycocyanin of microalgae *Cyanothece* sp. biomass which was collected from different pH induced treatment (Figs. 2-5). Chlorophyll a, chlorophyll b,

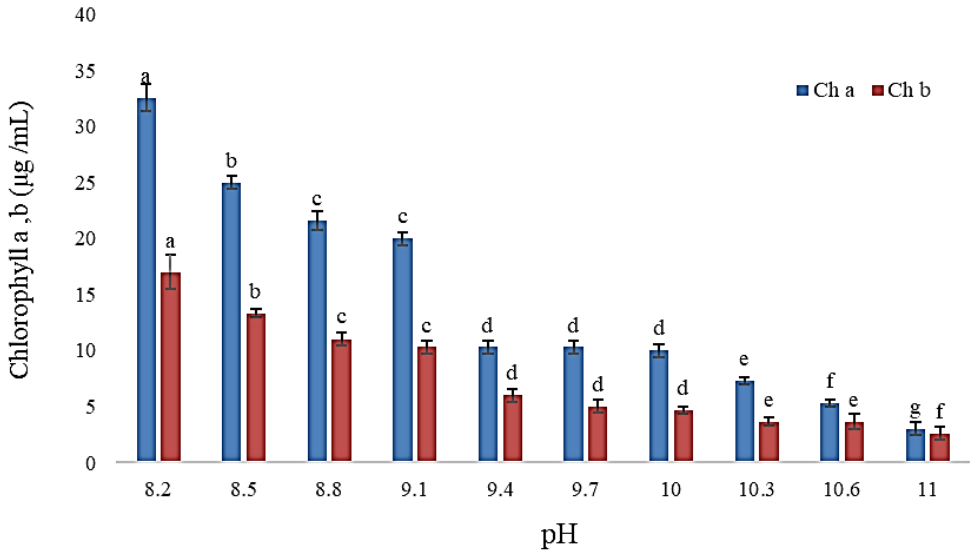

Figure 1. Chlorophyll a, b of *Cyanothece* sp. harvested with pH-induce flocculation

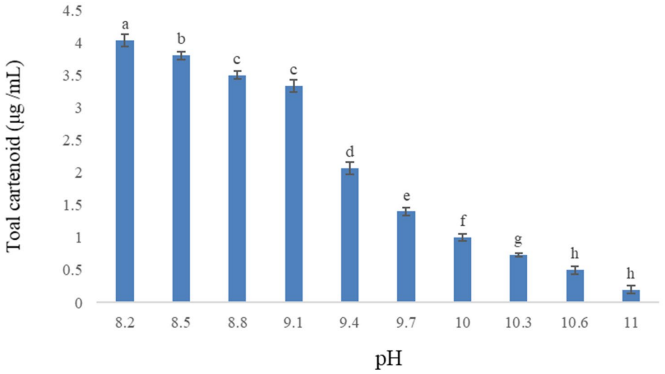

Figure 3. Total carotenoid of *Cyanothece* sp. harvested with pH-induce flocculation.

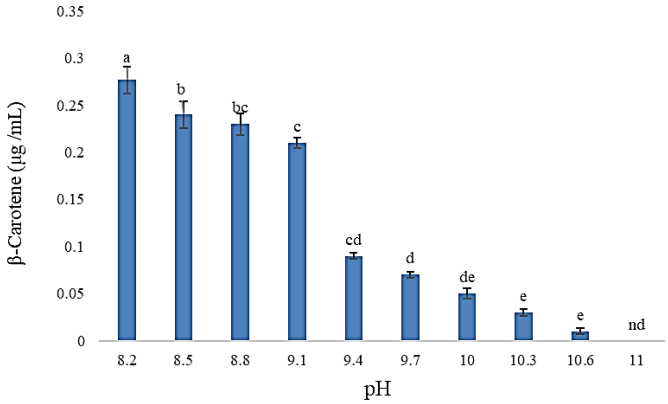

Figure 4. β-carotene of *Cyanothece* sp. harvested with pH-induce flocculation.

total carotenoid, β-Carotene and phycocyanin content of the different pH induced treatment were significantly different (P<0.05). The chlorophyll a content experienced a relatively small decrease from 32.6 in pH: 8.2 to 20 µg/mL at pH: 9.1 after that sever reduction was observed up to pH: 14 and reached 3 µg/mL. The chlorophyll b also decreased very strongly from 10.3 to 2.6 µg/mL. The total carotenoid content was decreased from pH: 8.2 to 8.8. and there was no significant difference between pH; 8.8 and 9.1 However, a fast downward trend from pH 9.1 to 11 was observed from 3.3 to 0.2 µg/mL. β-carotene content of microalgae *Cyanothece* sp. was very sensitive to adding NaoH and pH increase specially in high pH value and it was reached to 0.02 µg/mL at pH: 10.6 and non-detected at pH: 11. Changes in phycocyanin amount of microalgae *Cyanothece* sp. biomass were also very evident with increasing pH of the culture medium and decreased from 13 to 1 µg/mL from pH: 8.2 to pH:11

Discussions

Microalgae have been considered as the third generation sources of biomass production for biofuels. The algal biomass and extracted pigment have wide range of industrial applications, including animal feed and aquaculture, food supplements, nutrients and medicine. However, in the present, several important challenges related to algae cultivation, such as small size and negative surface of algae cells, as well as the dilution of the culture medium have led to their high-cost commercial production. Therefore, the method of

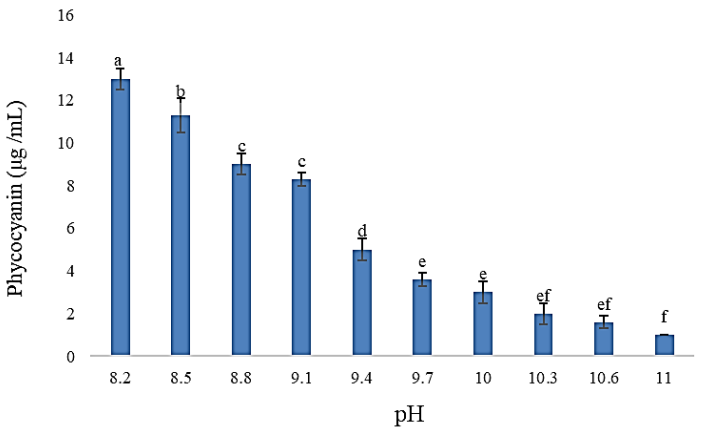

Figure 5. Phycocyanin of *Cyanothece* sp. harvested with pH-induce flocculation.

harvesting of microalgae is an important bottleneck for the large-scale industrial production process (Wu et al., 2012). The results of this study showed that increasing the pH from 8.2 to 9.4 cause cells to coagulate and precipitate to the bottom in the culture medium and increases the harvest efficiency from 18 to 92%; however, an increase in pH to 11 did not have a significant effect on increasing harvesting efficiency. Similarly, Wu et al. (2012) showed that increasing the pH to 8-9 in saline aquatic species e.g. *Nannochloropsis oculataa* and *Phaeodactylum tricornutum* increased flocculation efficiency up to 90%. Also, pH-induced flocculation method was successfully applied to harvest marine *Chlorella* sp. and the flocculation efficiencies were up to 90% within 10 min (Yang et al., 2016). Additionally, based on Perez et al. (2017), acid pH values (2 to 6) and basic pH values (8 to12) have been tasted for *Skeletonema costatum* and *Chaetoceros gracilis* microalgae and greater flocculation efficiency was achieved at high pH values whereas the lowest pH values reached a maximum algal biomass separation around 60% (Pérez et al., 2017). The flocculation mechanism is related to the presence of metal cations in the culture medium, which are hydrolyzed by increasing pH and produce the hydroxide of metal cations. It has been proposed that this enhancement may be attributed mainly to the co-precipitation of Ca^{2+} and Mg^{2+} ions dissolved in the medium with algal cells. Also, the algae cells with a negative charge become unstable with the formation of hydroxide of metal ions with a large adsorptive surface area and a positive superficial charge in basic pH (Liu et al., 2013). In our experiment, adding sodium hydroxide to the culture medium naturally increased the pH from 8.2 to 9.4, but after that high amounts of sodium hydroxide were required to achieve pH 10 to 11, indicating a buffer state in this area. By increasing the larger quantities of NaOH, the algae culture medium behaved like a gel and slow separation became due to the gelification. Therefore, no significant difference was observed in the flocculation efficiency from pH 9.4 to 11.

The reuse of water and viable algae cells fraction remaining after flocculation is an important issue for researchers and operators since, the recycle of flocculated medium could minimize the cost of nutrients and the demand for water (Maji et al., 2018). In the present study, in pH-induced treatments, the culture medium was divided in to two parts, the upper part of which was clear and completely separate water, and the lower part of which was a sedimentary cell. The upper phase cultivated in the reused growth medium was close to that cultivated in the fresh solution, indicating the culture solution could be recycled, but the settling phase could not be reused due to the presence of non-living and cells lysis during the flocculating by pH increase. In term of inoculating cell culture to the new cell culture medium, this capability was eliminated in high pH value more than 9.4. The flocculated medium, after neutralizing the pH, could be reused for cultivating the microalgae.

Aluminum and ferric salts are of the most popular inorganic flocculants which have been used for long time in wastewater clarification. Recently these multivalent inorganic chemicals used for algal biomass recovery of several species such as, *Tetraselmis* sp., *Chlorella* sp. and *Scenedesmus* sp. with respectively 86, 100 and 90% flocculation efficiency (Sanyano et al., 2013; Gerde et al., 2014; Kwon et al., 2014). The harvesting efficiency of inorganic flocculants depends largely on their physicochemical properties such as solubility and electronegativity. However economically, the cost of using aluminum salt is relatively low, which could be

a potential choice for biomass recovery, but the main problem for using them is due to contamination and discoloration of the culture medium the release of such chemicals into the environment is a critical environmental and health concern (Vandamme et al., 2015).

Effect of pH inducing flocculation method on pigment component of *Cyanothece* sp.: Due to the fact that the most important commercial and desired composition in microalgae *Cyanothece* sp. algae are its pigments, especially carotenoid, β-carotene and phycocyanin. Determining the effect of harvesting technique on this pigment is important for final use. The results of microalga biomass analysis after harvesting indicated that total carotenoid, β-carotene and phycocyanin content of *Cyanothece* sp. were affected by changes in pH medium culture, but the rate of change was different and analyzable at different treatments. The increase in pH value from 8.2 to 9.1 had 80% harvesting efficiency with a reasonable decrease in total carotenoid, β-carotene and phycocyanin, however, the more increases in pH up to 9.4 improved harvesting efficiency till 90% with serious negative effect on total carotenoid, β-carotene and phycocyanin. Adding high amounts of sodium hydroxide in pH 10 to 11 appear to damage pigment microalgae cells and it can have serious negative impacts on the down-stream processing and quality of products. There are some cases has been studied lipid component of microalgae after pH inducing harvesting. For instant, pH induced flocculation was used for harvesting *Chlorella* sp. 725 and a high lipid extraction yield was obtained for the NaOH flocculated cells, which was similar to the yield of the cells harvested by centrifugation, indicating that the method was suited for harvesting marine *Chlorella* sp., for biofuel production (Yang et al., 2016). Therefore, it can be concluded that depending on the purpose of collecting algae, the harvesting method should be chosen.

As conclusion, the results of this study showed that flocculation method induced by pH increase up to 9.1 is effective for harvesting microalgae *Cyanothece* sp. with relatively low effect on its chlorophyll, total carotenoid, β- carotene and phycocyanin pigments content.

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