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# Harvesting of *Scenedesmus obliquus* by Bioflocculation: Appropriate Chitosan Concentrations with Various pH Values at Different Growth Stages

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**Abstract**

The current study evaluates harvesting microalga *Scenedesmus obliquus* at different growth phases using chitosan, an anionic bioflocculating polymer. Different concentrations of chitosan were applied at different cultural pH levels and at different growth stages. The flocculation efficiency was evaluated in *S. obliquus* cultures with various cell densities. The optimum chitosan level for flocculating *S. obliquus* varied according to the cultural growth phase, cultural pH, and cells density. The highest flocculation efficacies (89.07 % and 88.48 %) at the late log phase were obtained when 80 ppm and 10 ppm of chitosan were applied to cultures with pH 10 ($OD_{680nm} \approx 1$) and pH 7 ($OD_{680nm} \approx 3$), respectively. Moreover, during the early stationary phase, the maximum coagulation value was achieved when 40 ppm of chitosan was used for cultures with pH 9 and $OD_{680nm} \approx 3$. At the late stationary growth phase, the highest flocculation efficacy (81.68% and 81.19 %) was achieved in cultures adjusted to pH 6 and treated with 20 ppm of chitosan and in the culture with pH 9 treated with 60 ppm of chitosan, correspondingly. Flocculation efficiency of *S. obliquus* could be improved by selecting a proper chitosan concentration according to the culture conditions (growth phase, pH, and cells density).

**Keywords:** *S. obliquus,* Bioflocculation, Chitosan, Growth stages, culture densities

## 1. Introduction

Microalgal biomass is considered as a sustainable feedstock for biofuel production, feed staff, nutraceutical and pharmaceutical products (El-Baz *et al.,* 2016; Hoh *et al.,* 2016; Lal and Das, 2016; Eida *et al.,* 2018). However, the energy richness of algal biomass, its diluted growth being around 0.02–0.05 % dry solids (Zamalloa *et al.,* 2011) and the small cell size being mostly below 30 µm (Grima *et al.,* 2003) represent the biggest challenges for microalgae harvesting, its commercial production, utilization and application.

The negative charge of the algal cells surface, that makes it well dispersed in suspension growth, in addition to the low density of algal cells in growth medium are considered among the imperative barriers standing in the way of expanding and scaling up microalgae production (Reynolds, 1984; Edzwald, 1993; Milledge and Heaven, 2013; Xia *et al.,* 2017). Such harvesting difficulties make the discovery and adoption of a harvesting technique that is efficient, cost-effective and environment-friendly extremely problematic for commercializing the microalgae production especially in biofuel production which requires a mass production of algal cells (Ghernaout and Ghernaout, 2012; Milledge and Heaven, 2013; Alam *et al.,* 2016).

Several methods can be implemented for harvesting microalgae from their cultures. These techniques include centrifugation, filtration, flocculation, flotation and sedimentation or a combination of two or more of these methods; although there is no common scheme for harvesting all microalgal species yet (Mata *et al.,* 2010; Milledge and Heaven, 2013; Shen *et al.,* 2009).

Flocculation is one of the most commonly applied techniques. It takes advantage of flocculant positive charge to form flocs with the negatively charged cells and improve its settling rate (Xu *et al.,* 2013; Matter *et al.,* 2018). Different inorganic chemical materials (including aluminum and ferric based salts) have been recognized for the coagulation of microalgae. In spite of the prevalence and the high efficiency of these methods, they have several possible environmental disadvantages (Fast and Gude, 2015). Thus, flocculation using natural polymers such as chitosan, cationic starch, and cellulose has been reported, emphasizing these polymers' universal availability, nontoxicity, biodegradability and low price (Yang *et al.,* 2016).

The cationic polyelectrolyte biopolymer, chitosan, has been efficiently utilized in the coagulation of organic and biological contaminants in wastewater treatment in addition to the flocculation of algae, bacteria and other applications (Divakaran and Pillai, 2002; Chen *et al.,* 2014; Wan *et al.,* 2015; Darwesh *et al.,* 2018). Several studies have reviewed the utilization of chitosan for the

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harvesting of microalgae (Chen *et al.,* 2014; Matter *et al.,* 2016; Yang *et al.,* 2016; Shuba and Kifle, 2018).

The microalgal cell morphology, its cell wall composition or structure, and its extracellular polymeric substances (EPS) are varied during different growth phases. These changes may affect the flocculation efficiency of the growing cells (Danquah *et al.,* 2009). Thus, the growth phase is one of the parameters that affect the harvesting of microalgae. Choi *et al.,* (2006) revealed that the precipitation rate of algae is significantly elevated in the stationary growth phase. Furthermore, the settling of *Scenedesmus* sp. was very little over a two-hour period in the exponential growth phase (day 6), while the highest sedimentation efficiency was recorded after fifteen days in the stationary phase (Manheim and Nelson, 2013). Emphasizing the previous reports, Danquah *et al.,* (2009) found that microalgae was settled down during the earlier growth phase (4-10 days) which is slower than in the stationary phase (10-12 days).

In a previous study, the utilization of different chitosan concentrations for harvesting *Scenedesmus* sp. at different pH values have been examined (Matter *et al.,* 2016). Whereas harvesting of microalgae could be required at different growth stages according to their application, the main objective of the current study focused on the determination of the appropriate chitosan concentration(s) for efficient flocculation of *S. obliquus* at different growth phases. The influence of different pH values and cell densities on microalgal flocculation using chitosan at different growth phases were also investigated in this study as well.

## 2. Materials and Methods

### *2.1. Microalgal Strain and Cultivation Conditions*

*Scenedesmus obliquus* NRCIbr1 (KY621475) was previously isolated and identified (Eida *et al*., 2018). The microalga was maintained on Bold Basal Medium (BBM) (Barsanti and Gualtieri, 2014), while it was cultivated on modified Bold Basal Medium where urea was used as a sole nitrogen source instead of sodium nitrate (Amin *et al.,* 2013). An air bubble photobioreactor with continuous illumination (white fluorescent light at the intensity of 2000 Lux) was used for the cultivation of microalgae. The cultures were collected at different growth phases for the harvesting processes. The three stages of harvesting are: the late log phase (after ten days), the early stationary phase (after fifteen days) and late stationary phase (after twenty days). These growth stages for *S. Obliquus* were designated according to the previous reports of Oukarroum (2016).

### *2.2. Preparation of the Chitosan Solution*

High molecular weight chitosan was purchased from Sigma–Aldrich (Steinheim, Germany) and was used to prepare 1 % (w/v) stock solution in 1 % acetic acid.

### *2.3. Harvesting Experiment*

All harvesting experiments were performed in 20 mL test tubes containing 10 mL of microalgal cultures. Before dispensing the culture in tubes, the optical densities ($OD_{680nm}$) of the cultures, at different growth phases, were adjusted to be around 1, 2 and 3 by centrifugation or dilution with water. The pH was also adjusted to 6, 7, 8, 9 and 10 using 5 N sulfuric acid or 2 N sodium hydroxide solutions. Specific amounts of chitosan solution were added to deliver the targeted concentrations (10, 20, 40, 60 and 80 ppm) to the culture tubes. Then, the mixtures were subjected to five seconds of vortexing. After mixing, the microalgal cells were allowed to settle down for one hour before sampling.

### *2.4. Flocculation Efficiency*

The Flocculation efficiency was measured by taking 2 ml of the samples from the middle of each sample tube at the same level to measure the optical densities at 680 nm ($OD_{680nm}$) using the spectrophotometer (SHIMADZU UV-2401PC, Japan). Flocculation efficiency (FE) was calculated by comparing the initial turbidity ($OD_{680nm}$) of the cultures with that measured after sedimentation (Farid *et al.,* 2013).

### *2.5. Statistical Analysis*

The results were expressed as mean of three measurements ± standard division. The one-way ANOVA test and post hoc test Duncan's test (for multiple comparisons) were performed using SPSS v.20 software (IBM-SPSS, Chicago, IL, USA).

## 3. Results

The flocculation efficiency (FE) was studied to indicate the harvesting of *S. obliquus* induced by chitosan as a natural flocculating agent at different growth stages, culture densities and pH values.

### *3.1. Flocculation Efficiency of S. obliquus at Late Log Growth Phase*

Figure 1 demonstrates the flocculation efficiency using different chitosan levels for *S. obliquus* cultures at late log growth phase under different pH values and culture $OD_{680nm}$ adjusted to 1, 2, and 3. At culture $OD_{680nm}$ adjusted to 1, the application of different chitosan concentrations (0-80 ppm) showed low flocculation efficiency (FE) percentage (lower than 38.00 %) at pH values below 10 (Figure 1a). At pH 10, a significant difference in FE was detected among all chitosan treatments, and the maximum FE value (76.60 %) was achieved when 80 ppm chitosan was applied. Through this growth stage, FE % generally trended to decline with increasing the chitosan concentrations. These results were confirmed at different studied pH values except pH 10 which displayed a significant increase in FE % when rising the chitosan concentration.

The harvesting efficacy at the late log growth phase for cultures with cells density of $OD_{680nm} \approx 2$ was evaluated at different pH and chitosan concentrations (Figure 1b). The obtained data exhibited general increase in FE % with doubling the culture density. Similar to the tendency noticed in $OD_{680nm} \approx 1$, the 80 ppm chitosan treatment at pH 10 revealed an FE % reaching 89.07 %. On the other hand, the highest harvesting efficiency at the neutral and slightly acidic conditions (pH 6 and 7) was obtained through applying 10 and 20 ppm chitosan followed by a decline in FE % when increasing the chitosan rates. However, the maximum flocculation efficiency at pH 8 reached 70.63 and 67.63 % at 20 and 40 ppm chitosan, respectively without significant difference. Nevertheless,

the 69.67 % efficiency was reached when 40 ppm chitosan was applied at pH 9.

Moreover, increasing the culture density of *S. obliquus* to $OD_{680nm}$ ≈3 was accompanied by a further increase in the flocculation rate at different chitosan concentrations within all manipulated pH values except pH 10 which showed lower FE % (Figure 1c). Increasing the pH of the culture required a higher chitosan concentration. Twenty ppm of chitosan accomplished 83.20 % and 88.50 % flocculation rate at pH 6 and 7, respectively, while pH 8 and 9 required 40 ppm of chitosan to achieve 78.20 % and 83.2 % flocculation efficacy, respectively. Furthermore, up to 80 ppm chitosan were needed to harvest 79.00 % of *S. obliquus* cells from the culture with $OD_{680nm}$ ≈3 and pH 10 during the late log phase. The highest flocculation rate (88.48 %) at the late log phase and $OD_{680nm}$ ≈3 was recorded for a 20 ppm of chitosan and pH 7.

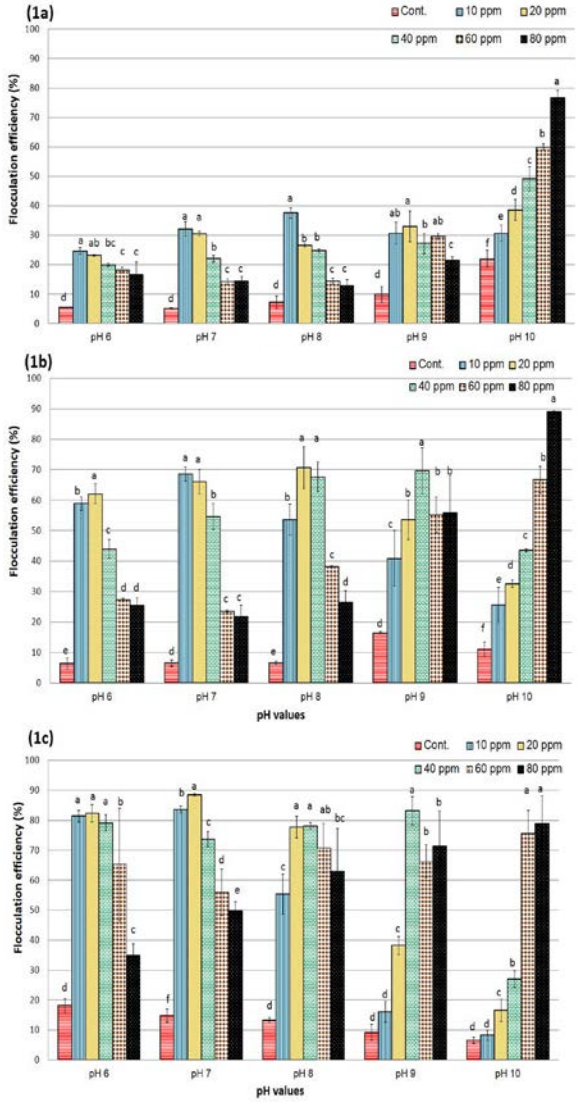


**Figure1**. *Scenedesmus obliquus* flocculation efficiency by various chitosan concentrations at different pH values and culture cells densities ($OD_{680nm}$ ≈1, "1a"; $OD_{680nm}$ ≈2, "1b" and $OD_{680nm}$ ≈3, "1c") at late log phase. Bars are average ± standard deviation of three experiments. One-way ANOVA and post hoc test Duncan's test were done. Different letters represent significant differences ($P$< 0.05).

### *3.2. Flocculation Efficiency of S. obliquus at Early Stationary Growth Phase*

The flocculation efficiency of *S. obliquus* at the early stationary growth phase was studied using different concentrations of chitosan at different growth condition including culture pH and density. The flocculation efficiency of chitosan concentrations at this growth stage differed according to cell densities and pH values as presented in Figure 2. The obtained data revealed that chitosan concentrations significantly increased the FE % at different pH values and culture densities.

At the lowest studied cells density ($OD_{680nm}$ ≈ 1), the flocculation efficiency of algal cells using chitosan improved with the increase in cultural pH value up to 9 (Figure 2a). The highest flocculation efficiency (69.93 %) was recorded for 40 ppm of chitosan treatment at pH 9 then declined with raising the chitosan level. When the cultural pH was neutral or marginally acidic, 20 ppm of chitosan was statistically a superlative treatment although the FE % did not exceed 30 %. Additionally, once the cultural pH was adjusted to 10, the FE % increased significantly with increasing the chitosan concentration to reach its maximum value (65.21 %) after the addition of 80 ppm of chitosan. During this growth stage, the auto-flocculation (0 chitosan) showed the lowest sedimentation values (didn't exceed 18.84 %).

Increasing the cell density of *S. obliquus* culture up to $OD_{680nm}$ ≈2 resulted in a slide increase in flocculation efficacy although the FE was trended similar to $OD_{680nm}$ ≈1 (Figure 2b). When the pH value of the culture was lower than 9, the concentration of 20 ppm of chitosan displayed significantly greater FE % followed by a gradual decline when increasing the chitosan concentration. At this chitosan concentration, the recorded FE reached 45.47, 55.25 % and 51.50 % at pH 6, 7, and 8, respectively. Like at $OD_{680nm}$ ≈1, 40 ppm chitosan at pH 9 exhibited the highest flocculation efficacy (70.00 %). At high alkaline condition (pH 10), the FE % was inferior to its corresponding chitosan rates at lower pH values and did not surpass 42.42 %. There was no significant difference in FE % at 0, 10, and 20 ppm chitosan in addition to the insignificancy found among 40, 60, and 80 ppm chitosan at pH 10.

The effect of chitosan concentrations on the flocculation efficiency of *S. obliquus* with cells density adjusted to $OD_{680nm}$ ≈3 (during the early stationary growth phase) at different pH values is presented in Figure 2c. The illustrated data revealed that the FE % in cultures with OD680nm ≈3 at designated pH was higher than its value at corresponding pH level in cultures with OD680nm ≈1 and OD680nm ≈2 except at pH 10. At this culture density, the application of 20 ppm chitosan showed the maximum flocculation efficiency at pH 6 (68.90 %), although the 40 ppm chitosan at pH 7, 8 and 9 retained superior to other treatments achieving 64.90 %, 72.80 % and 77.65 % harvesting effectiveness, respectively. Increasing chitosan concentration above 40 ppm significantly decreased the FE % except at pH 10 where the opposite trend occurred. Overall, the culture with pH 10 showed the bottommost flocculation efficiency (not more than 39.22 % at 80 ppm chitosan). It was notable that increasing the culture density from $OD_{680nm}$ ≈1 to $OD_{680nm}$ ≈3 at pH 10 reduced the effectiveness of chitosan in the harvesting process.

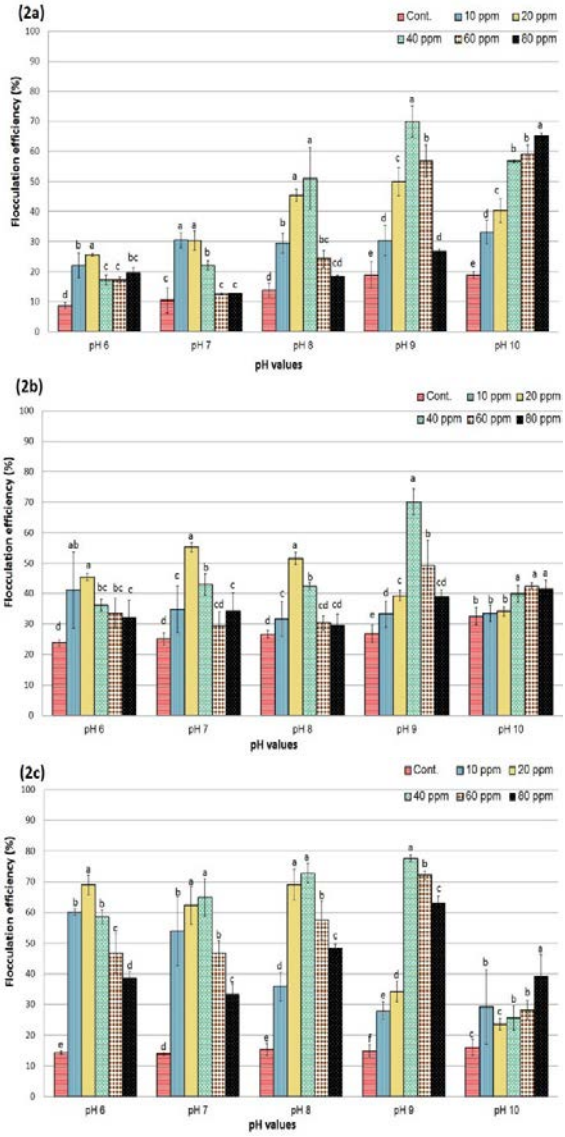


**Figure 2**. *Scenedesmus obliquus* flocculation efficiency by various chitosan concentrations at different pH values and culture cells densities ($OD_{680nm}$ ≈1, "2a"; $OD_{680nm}$ ≈2, "2b" and $OD_{680nm}$ ≈3, "2c") at late early stationary phase. Bars are average ± standard deviation of three experiments. One-way ANOVA and post hoc test Duncan's test were done. Different letters represent significant differences ($P< 0.05$).

### *3.3. Flocculation Efficiency of S. obliquus at Late Stationary Growth Phase*

The data acquired from the utilization of chitosan for harvesting the microalgal isolate *S. obliquus* during the late stationary phase revealed a further increase in the flocculation efficacy at the neutral, moderate acidic and moderate alkaline pH values (figure 3).

The ability of chitosan to coagulate *S. obliquus* declined generally when cultures tended to alkalinity. It was observed that during this growth stage (at different cultural ODs), increasing the pH value led to the increasing of the auto-flocculation although it retained the lowest flocculation efficacy using different chitosan concentrations.

Concerning the FE at culture $OD_{680nm}$≈1, the application of 10 ppm chitosan to the microalgal culture at pH 6 was the leading treatment (FE= 62.61 %) amongst all chitosan rates and pH values (Figure 3a), whereas, increasing the chitosan concentration was combined by a drop in the flocculation rate at the same conditions. Converting the culture reaction toward alkalinity required higher concentration of chitosan to achieve a reasonable FE. Regarding the culture with pH 7, the highest FE (51.78 %) was achieved using 20 ppm of chitosan while it was slightly higher (52.00 %) when 60 ppm chitosan was applied to the culture with pH 8. At pH 9, the harvesting rate of 40 ppm chitosan reached 61.68 % and was significantly superior to other chitosan concentrations. Similar to the results of FE at the early stationary phase especially at $OD_{680nm}$ ≈2 and $OD_{680nm}$ ≈3, the lowest harvesting efficiency with chitosan in the late stationary growth phase was found at pH 10, which mostly peaked (35.90 %) at 80 ppm of chitosan.

Furthermore, increasing the culture's OD to reach the value of around 2, the addition of 20 ppm of chitosan displayed a harvesting efficiency significantly exceeding other concentrations at pH 6 and 7 which was followed by a decline in FE when increasing the chitosan rate. The highest flocculation efficiencies (81.68 % and 81.19 %) were achieved in cultures adjusted at pH 6 treated with 20 ppm of chitosan and the culture with pH 9 treated by 60 ppm of chitosan. Correspondingly, the least flocculation efficiency (33.94 %) was observed at pH 10 utilizing 60 ppm of chitosan (Figure 3b).

However, as a result of condensing microalgal cells to $OD_{680nm}$ ≈3, the optimum harvesting efficacy was recorded at pH 6 (81.08 %) followed by pH 7 (77.88 %) when 20 ppm and 40 ppm of chitosan were added, respectively. In the above conditions, the FE % decreased significantly with stepping up the chitosan levels at pH 6 and insignificantly at pH 7. On the other hand, the increment of cultural pH demanded higher chitosan concentrations to perform a reasonable FE (70.61 % and 74.90 % at 80 ppm of chitosan and pH 8 and 9, respectively). Similar to the above-mentioned culture density, the lowest flocculation efficiency was observed at pH 10; even the highest chitosan concentration (80 ppm) did not harvest more than 22.83 % of *S. obliquus* cells from the culture medium (Figure 3 c).

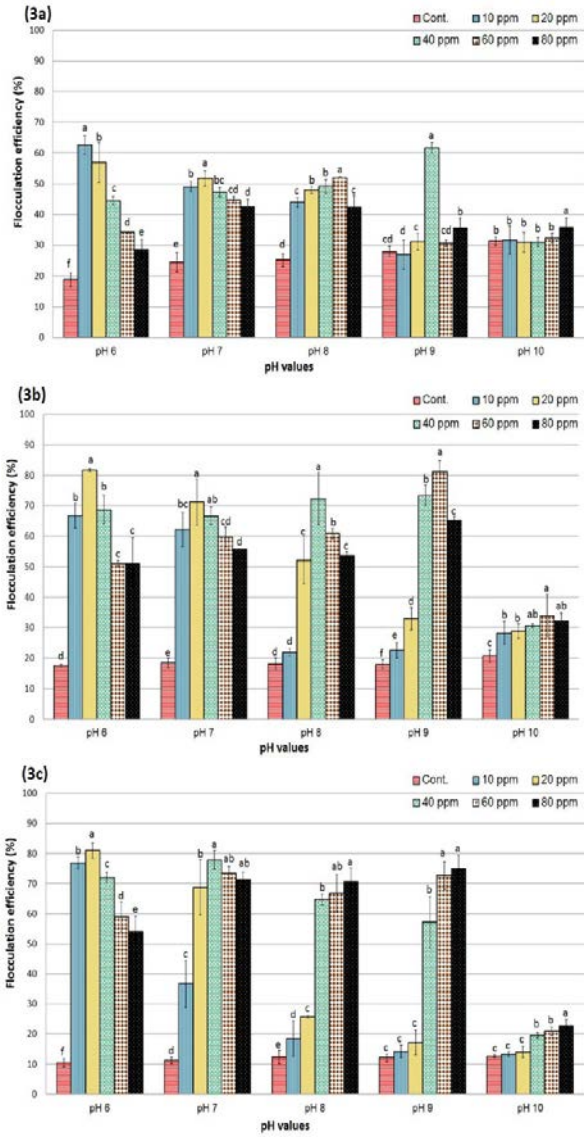


**Figure 3**. *Scenedesmus obliquus* flocculation efficiency by various chitosan concentrations at different pH values and culture cells densities ($OD_{680nm}$ ≈1, "3a"; $OD_{680nm}$ ≈2, "3b" and $OD_{680nm}$ ≈3, "3c") at late stationary phase. Bars are average ± standard deviation of three experiments. One-way ANOVA and post hoc test Duncan's test were done. Different letters represent significant differences ($P$< 0.05).

## 4. Discussion

The evaluation process of utilizing various chitosan concentrations to flocculate microalgae at the late log growth phase, and early and late stationary phases is performed in this study. The performance of chitosan concentrations was determined in *S. obliquus* cultures adjusted to different pH values (6-10) and cell densities ($OD_{680nm}$ ≈1-3) at each growth stage. In a previous study of the bio-flocculation of *S. obliquus* grown at the early logarithmic growth phase, chitosan was proven to be an effective bio-flocculating agent and the pH was found to be a major influencing factor to determine its efficacy (Matter *et al.*, 2016). Microalgal harvesting stages generally differ according to the purpose of biomass uses (Chang and Lee, 2012; Khanra *et al.*, 2018).

The obtained results revealed low sedimentation efficacy (5.5 % - 32.49 %) when chitosan was not applied (control) at all pH values and at all growth stages. In this case, the highest precipitation percentages of the studied microalgae were recorded at the highest pH values. In spite of the limited available information concerning the autoflocculation, Ummalyma *et al.* (2016) suggested the self-production of flocculating agents (e.g., poly-saccharides and glycoprotein) or a bridge-forming ability between algal cells through charge neutralization with modifying culture pH. Similar results were stated by Gerchman and the co-authors (Gerchman *et al.*, 2017).

Chitosan is a promising flocculating agent for coagulating microalgae harvesting, but its optimized application conditions are not steeled so far (Sajjad *et al.*, 2017; Matter *et al*., 2018). Generally, the application of various rates of chitosan improved the flocculation efficiency at different growth phases, culture densities, and pH values. The most consistent mechanism for harvesting microalgae using chitosan is the electrostatic interaction between its amino groups with the negatively charged groups (e.g. amide and carboxylic groups) on the algal cell surface (Pranowo *et al*., 2013) leading to adsorption and charge neutralization (Tran *et al.*, 2013). This fact was supported by the results of Lu *et al.* (2017) who studied the changes in Zeta potential during chitosan flocculation and their results indicated that the adsorption-bridging and charge neutralization were the essential mechanisms for algal flocculation.

The obtained data obviously showed that the FE increased with increasing the chitosan concentration at all growth phases and different pH levels to reach a maximum then a decline with any further increase in the chitosan dose. These results may refer to the charge neutralization and bridging phenomena and the decline in harvesting ability with chitosan over application could rely on the role of excess amino groups in the restabilization of the cells resulting in a decrease in the coagulation percentage (Yang *et al.*, 2016; Yunos *et al.*, 2017). Similar results were stated by Rashid *et al.* (2013) who reported that the application of chitosan overdose dramatically reduced the harvesting efficiency. It was reported that the coagulation efficacy of microalgae utilizing chitosan as a flocculating agent is very sensitive to pH (Rashid *et al.*, 2013; Xu *et al.*, 2013; Matter *et al*., 2018)

Results of the current study exhibited that while 10 to 20 ppm of chitosan achieved the greatest flocculation efficiency at neutral and mild acidic pH (6-7), cultures with higher pH (8-9) required superior chitosan concentrations (20 to 40; even 60 ppm) to flocculate reasonable amounts of *S. obliquus.* The advanced capability of chitosan in the bioflocculation process at a low pH could be explained by its enhanced charge neutralization and bridging effects at this condition (Yang *et al.*, 2016). In this respect, Xu *et al.* (2013) indicated that reducing the cultural pH can decrease the required chitosan concentrations to motivate an effective flocculation.

In accordance with our data Wu *et al.* (2012) reported that at the same pH value, the harvesting efficiency increased with increasing the biomass concentration which increased with the culture aging. In another study which agreed to these results, it was observed that at high pH

value (10), a general decrease in the flocculation efficiency was noticed with increasing the culture density. The increase in the harvesting percentage could be explicated by the fact that, high opaque algal cultures have higher negative charge that can strongly attach to positively charged biopolymers such as chitosan and neutralize its positive surface charge (Farid *et al*., 2013).

## 5. Conclusion

The current study concludes that different chitosan concentrations could be required for achieving a better and economical harvesting of *S. Obliquus* by bioflocculation. Harvesting *S. Obliquus* using chitosan for producing bioactive products as well as biofuels is depending on growth phases, cultural pH, and cell densities. The efficacy of chitosan in harvesting *S. obliquus* could be improved by selecting the most suitable concentration according to the harvesting conditions (pH, cells density and growth phase). The recommended chitosan concentrations (ppm) for the best harvesting of *S. obliquus* under the study conditions are summarized in table (1).

**Table 1**. Recommended chitosan concentrations for the best bioflocculation of *S. obliquus* at different growth conditions.

| | Growth Phase | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Late log phase | | | Early stationary phase | | | Late stationary phase | | |
| pH | OD 1 | OD 2 | OD 3 | OD 1 | OD 2 | OD 3 | OD 1 | OD 2 | OD 3 |
| 6 | 10 | 20 | 10 | 20 | 10 | 20 | 10 | 20 | 20 |
| 7 | 10 | 10 | 20 | 10 | 20 | 20 | 20 | 20 | 40 |
| 8 | 10 | 20 | 20 | 20 | 20 | 20 | 60 | 40 | 60 |
| 9 | 10 | 40 | 40 | 40 | 40 | 20 | 40 | 60 | 60 |
| 10 | 80 | 80 | 60 | 80 | 40 | 80 | 80 | 60 | 80 |

In the table, when there is no significant difference in flocculation efficiency between two chitosan concentrations, the recommended chitosan level was the lower concentration.

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