Algal Research 57 (2021) 102358

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Harvesting freshwater microalgae with natural polymer flocculants

Zijun Yang, Jun Hou *, Lingzhan Miao

Key Laboratory of Integrated Regulation and Resources Development on Shallow Lakes of Ministry of Education, College of Environment, Hohai University, Nanjing 210098, China

ARTICLE INFO

Keywords:
Natural polymers
Tanfloc
Flocculation performance
Flocculation mechanism
Cell viability
Media recycling

ABSTRACT

This study revealed the differences in harvesting performance and potential mechanisms of four natural flocculants - Chitosan, Tanfloc, Cationic starch, *Moringa oleifera* - on microalgae cells and explored the potential of four flocculants for harvesting microalgae. Influence of dosage, culture pH, cell density and algal organic matter (AOM) on harvesting using four flocculants were compared. The effects of the flocculants on cell viability and the reusability of separated medium were also investigated. The results demonstrated that the optimal dosages of Chitosan/Tanfloc/Cationic starch for harvesting *Chlorella vulgaris* and *Scenedesmus obliquus* were much lower than *Moringa oleifera*. Chitosan/Tanfloc/Cationic starch and *Moringa oleifera* improved microalgae harvesting through electrostatic binding and bridging, respectively. The harvesting of other flocculants were affected by culture pH except for *Moringa oleifera*. Nevertheless, as biomass concentration increased from ~0.3 g L^{-1} to ~1.5 g L^{-1}, the optimal dosage of *Moringa oleifera* increased significantly, which was about 20–100 times than other flocculants. AOM could interfere all harvesting processes to varying degrees. Moreover, the flocs formed from Chitosan/Tanfloc/Cationic starch were smaller but more compacted compared to *Moringa oleifera*, thus possessing a better dewatering functionality. The harvesting process of four flocculants did not affect cell viability or lead to the loss of cell extractions including lipid, carbohydrates and protein. In addition to Chitosan, the medium separated from the other flocculants after harvesting could be recycled for next cultivation, reducing costs of microalgae cultivation. Amongst four natural flocculants, Tanfloc displayed >98% high harvesting efficiency with low dosages (30 mg L^{-1} for *Chlorella vulgaris* and 20 mg L^{-1} for *Scenedesmus obliquus*). It could effectively harvest microalgae in a wide pH range (pH 4.0–9.0) and showed a good dewatering potential. More importantly, the lowest harvesting cost of the four flocculants facilitates its application for large-scale harvesting. We therefore recommend Tanfloc for microalgae harvesting of the four flocculants.

1. Introduction

Microalgae is considered as a great fortune with its usage in food, feed, fuel and wastewater treatment [1,2]. Therefore, more and more attention has been paid to the cultivation of microalgae for these purposes in recent years [3,4]. However, as a crucial step during microalgae biomass production, microalgae biomass harvesting from growth medium is still a challenge, owing their negatively charges surface (−7.5 to −40.0 mV), small size (3–50 μm) and low biomass concentrations (0.1–5.0 g L^{-1}) [1,5]. Methods to reduce these costs are highly desirable in the biomass industry.

Several methods including centrifugation [6], filtration [7], gravity sedimentation, autoflocculation and induced flocculation [7] have been employed in microalgae harvesting. Amongst these methods, there are membrane fouling and large power input in the filtration and centrifugation process [8]. Although autoflocculation is a simple, inexpensive and chemical-free nontoxic process, it is not suitable for industrial-scale harvesting because it is time consuming, unreliable and fits only a few microalgae species. In contrast, induce flocculation can be an convenient and effective method to harvest microalgae from large quantities of microalgae cultures. Inorganic chemical flocculants, such as ferric chloride ($FeCl_3$), aluminum sulfate ($Al_2(SO_4)_3$) and polymeric aluminum (PAC), have been successfully applied for harvesting microalgae [9,10]. However, these compounds have the disadvantage of requiring a relatively high dosage and that the biomass is contaminated with high concentrations of metals, limiting the application of the biomass due to metal toxicity [11]. Organic polymers, such as polyacrylamide-based flocculants are generally preferred over inorganic flocculants due to their lower dosage [12]. However, they may contain acrylamide residues that are presumably carcinogenic or display a high

* Corresponding author at: College of Environment, Hohai University, 1 Xikang Road, Nanjing 210098, China.
E-mail address: hjy_hj@hhu.edu.cn (J. Hou).

https://doi.org/10.1016/j.algal.2021.102358
Received 16 August 2020; Received in revised form 5 May 2021; Accepted 12 May 2021
Available online 28 May 2021

toxicity towards aquatic organisms [13]. In recent years, due to their biodegradability and eco-friendly nature, natural polymer based flocculants are getting more and more attention [1]. In addition, their powerful potential for flocculation make them possible candidates for large-scale microalgae harvesting [14].

Chitosan, produced from the deacetylation of chitin, has high charge and molecular weight (MW), making it a potential candidate for microalgae harvesting [15,16]. Yunos [17] demonstrated that Chitosan at 30 mg L^{-1} achieves a harvesting efficiency (HE) of 98% for *Chlorella* sp. and 80% of biomass recovery. Cationic starch, which is obtained from the introduction of quaternary ammonium groups, has a charge that is independent of pH, and can be used to efficiently harvest microalgae at a broad pH range [18,19]. An additional natural flocculent commonly used to harvest microalgae are the seeds of *Moringa oleifera* [20]. *Moringa oleifera* are tropical multipurpose plants the seeds of which contain an active bioflocculant. The low cost of the plants make them an ideal natural flocculant for microalgae harvesting [21,22]. Tanfloc, a tannin modified product, is manufactured from tannin through the addition of amino groups [23]. Tanfloc has received intense research attention due to its efficiency and low costs [24,25]. Barrado-Moreno M M [26] observed a HE greater than 94% on *C. vulgaris* cells using 10 mg L^{-1} Tanfloc. Moreover, Tanfloc could efficiently harvest microalgae at a wide pH range (5.0–9.0). Furthermore, algae organic matter (AOM) secreted from the growth of microalgae tend to hinder the harvesting process requiring a higher dosage of the flocculent. However, there is a lack of research to compare the performance and mechanism (s) of four natural flocculants on the freshwater microalgae harvesting.

Microalgae cells must be harvested intact. Intracellular compounds can be released into the environment following cell rupture. Chemical flocculants produces chemical stress to cell membranes leading to cell damage. The low concentration of calcium hydroxide ($Ca(OH)_2$) and direct effects of copper sulfate ($CuSO_4$) causes physical damage to cells [27,28]. Additionally, to reduce the costs of microalgae cultivation, the growth media after harvesting can be recycled for subsequent cultivation [11]. However, as far as we know, there are no studies comparing the effects of natural flocculants on microalgae cell integrity and cell extractions such as lipid and protein until now. In addition, few studies have focused on the recyclability of the medium harvested by natural flocculants.

Chlorella vulgaris (*C. vulgaris*) and *Scenedesmus obliquus* (*S. obliquus*) are microalgae with wide range of applications as live feed, food, cosmeceuticals, nutraceuticals, etc. This study aimed to reveal the differences in harvesting performance and potential mechanisms of four natural flocculants on microalgae cells and explored the potential of four flocculants for harvesting microalgae. The specific objectives were to: (1) reveal the effects of dosage, culture pH, cell density, and AOM on the harvesting performances of four flocculants and illustrate their harvesting mechanisms on *C. vulgaris and S. obliquus*; (2) compare the dewatering performance of their flocs; (3) To investigate the influence of four flocculants on cell viability and cell extractions; and (4) explore the reusability of the separated medium for each flocculant. These studies deepen our understanding of the harvesting process with natural flocculants and provides a theoretical basis for their selection.

2. Materials and methods

2.1. Resources

2.1.1. Microalgae culturing

C. vulgaris (FACHB-8) and *S. obliquus* (FACHB-12) were obtained from the Institute of Hydrobiology, Wuhan. The microalgae cultures were grown in a standard BG-11 medium and cultured at 25 ± 1 °C under 2000 lx illumination for 12 h and in the dark for 12 h [17,18]. The microalgae suspension at the late exponential growth phase (approximately 21 days) was collected by centrifugation at 1006.2*g* for subsequent experiment. Characteristics of the microalgae cultures are shown in the Supplementary material.

2.1.2. Flocculant preparation

Tanfloc was obtained from TANAC S.A. (Montenegro, Brazil). 0.5 g of Tanfloc power was dissolved in 500 mL water at 300 rpm for 1 h to obtain Tanfloc stock solutions of 1 g L^{-1}. Chitosan was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and 0.5 g of Chitosan power was dissolved in 500 mL water to produce 1 g L^{-1} Chitosan stock solution.

Cationic starch: 2 g 2,3-epoxypropyl trimethyl ammonium chloride (GTA) was added to 100 mL 5.0 g L^{-1} NaOH solution and was stirred with 10 g of corn starch for 30 min at 70 °C. Samples were cooled at room temperature and microwaved under a set output power of 300 W for 1 min on five occasions. Afterwards, samples were washed with 95% ethanol three times and the precipitate were vacuum dried for 6 h, crushed and passed through a 100-mesh sieve [29].

Moringa oleifera seeds: 1 g of *Moringa oleifera* seed powder (Moringa Smart Technology Co., Ltd., Beijing, China) was added to 95% ethanol with stirring for 30 min and then 200 mL of 0.5 mol L^{-1} NaCl solution was added to the evaporated residue. After stirring for 30 min, the mixture was passed through a 0.45-μm Whatman OE67 cellulose acetate membrane. The resultant filtrate solution diluted to 5 g L^{-1} was then used as a *Moringa oleifera* stock solution [21].

2.2. Flocculation experiments

A programmed six-in-one electromotive stirrer (ZR4-6, Zhongrun Water Technology Development Co., China) was used for all flocculation assessments, the photo of the experimental set up was provided in the Supplementary material (Fig. S1). Assessments were performed with 600 mL of microalgae culture. Following the addition of Tanfloc solution, cultures were mixed at 300 rpm for 2 min for uniform dispersion of the flocculant. Cultures were then gently mixed at 50 rpm for 20 min to permit floc formation. Subsequently, samples were left for 30 min to settle. The optical density (OD) at 690 nm of the supernatant was determined and used to calculate the HE based on Eq. (1) [24]. 1 mL of supernatants was collected in the middle of the treated water and used to measure Zeta potentials. After removing the supernatant, the microalgae-containing floc samples at the bottom of the beaker were collected to analyse cell viability through cell staining and photochemical activity determination. The measurement details was shown in the Section 2.3.3. Floc size was analyzed by Mastersizer 2000 (Malvern, UK) using the results of median average equivalent diameter (d_{50}) of the flocs. Image was used to measure the porosity of flocs.

$$\mathrm{HE}\ (\%) = \left[\left(\mathrm{OD_i} - \mathrm{OD_f}\right)/\mathrm{OD_i}\right] \times 100 \quad (1)$$

where OD_i is initial OD of the microalgae medium; OD_f is OD of the supernatants at 690 nm.

The settleable solid volume fraction (SSVF) is the ratio of the volume of the microalgae slurry to the initial microalgae culture [30]:

$$\text{Settleable solid volume fraction} = \mathrm{h_f}/\mathrm{h_o} \quad (2)$$

where h_o is initial height of the microalgae solution; h_f is final height of the concentrated microalgae culture at the end of harvesting; concentration factor (CF) is the ratio of the final product to the initial concentration, calculated as [31]:

$$\text{Concentration factor (CF)} = \mathrm{HE/SSVF} \quad (3)$$

The harvesting experiments were first tested with different flocculant dosages in the range of 1–100 mg L^{-1}. To explore the effects of pH on harvesting, the medium pH was adjusted ranging from 4.0 to 10.0 using 0.1 M HCl and 0.1 M NaOH. To study the effects of cell density, the *C. vulgaris* and *S. obliquus* medium were diluted to obtain ~0.3 g L^{-1}, ~0.6 g L^{-1} and ~ 1.5 g dry cell weight/L with different volume of 0.5% NaCl solution. To the effect of AOM, the microalgae medium was

centrifuged (4500 ×*g*, 10 min) and then resuspended in 0.5% NaCl (m/v) solution to the original volume to maintain the osmotic equilibrium and prevent cell lysis [32].

2.3. Analyses

2.3.1. Fourier transform infrared spectroscopy

Freeze-dried flocculent samples were ground with a KBr (mass: ratio = 1:100) and assessed via Fourier transform infrared spectroscopy (FTIR) measurements to analyse the functional groups of four flocculants. The parameters were set as follows: Infrared spectra were recorded at 4000 to 400 cm^{-1}; resolution: 4 cm^{-1} from a total of 32 scans.

2.3.2. Determination of dry weight

Microalgae dry weights were used to represent the microalgae biomass. The samples were centrifuged at 1341.6*g* for 15 min and dried to a constant weight at 60 °C for 24 h. The dried weight of the cell biomass was determined gravimetrically to represent the microalgae dry weight.

2.3.3. Cell viability assessments

2.3.3.1. Cell staining. The cell vitality was tested using 1% Evans Blue dye, which is excluded by viable cells [33]. Samples (1.0 mL) were pelleted and stained with 1% Evans blue solution for 10 min at 25 °C. Cells were washed in water, pelleted and stained cells were examined on an optical microscope (Zeiss Axioskop 40, Shanghai).

2.3.3.2. Pulse amplitude modulation (PAM). The photochemical activity of photosystem II (PSII) of the microalgae cells were determined using Phyto-PAM-II (Hein Walz GmbH, Germany). Samples (2 mL) were added to glass cuvettes after 15 min of dark adaption and the initial (F_0) and maximum (F_m) fluorescence levels were determined. The F_v/F_m values (with $F_v = F_m - F_0$) were calculated to quantify the effective quantum yield of PSII.

2.3.4. Determination of total lipids

Lipid content was measured by solvent extraction and gravimetric methods [34]. Approximately 0.1 g of dry microalgae powder was mixed with 10 mL of chloroform methanol solution (< 2:1, *v*/v) in a centrifuge tube. After untrasonication for 10 min, the mixture was centrifuged at 1341.6*g* for 10 min at 4 °C and supernatant was transferred to fresh 60 mL separation funnels. The extraction process was repeated three times. Sodium chloride solution (0.9%, volume ratio of supernatant to sodium chloride: 5:1 v/v) was added to the separating funnel following extraction. The mixture was shaken for 1 min and subsequently left for 15 min to stratify. Most of the lipids in the microalgae cells were dissolved in the underlying solvent phase and the volume of the underlying solvent phase solutions were determined. The 5 mL low-phase solution was transferred to pre-weighed 15-mL glass tubes. The solvent was evaporated with a nitrogen stream, and glass tubes were dried at 60 °C in the oven until the weight was stable. The liquid content of the microalgae cells (LC, % DW) were calculated based on the following equation (Eq. (4)) [35]:

$$LC = (m_2 - m_1) \times v/(5 \times m_0) \quad (4)$$

where m_0, m_1, m_2 and v represent the biomass dry weight, the weight of the clean glass tube, the weight of the lipid containing tube, and the volume of the low-phase solution, respectively.

2.3.5. Determination of total carbohydrate and protein levels

The carbohydrates of microalgae cells were extracted by the hydrolysis of hydrochloric acid in a boiling water bath for 20 min. Total carbohydrates and total protein were determined using the modified anthrone method and bicinchoninic acid (BCA) method, respectively [36]. The range of protein determination using BCA reagent was 20–2000 μg / mL.

2.4. Separated medium reuse experiments

Separated supernatant was collected after microalgae harvesting. The pH of the supernatant was readjusted to ~7.0 and filtered to remove some algal metabolites. In the experimental group - 600 mL erlenmeyer flasks were filled with 350 mL separated medium. Fresh media was added to the control group. All media types were shaken every day to simulate actual cultivation process. The initial optical density of the microalgae cells was approximately OD_{690nm} = 0.2 in all flasks cultivated under the described conditions. Data are the means ± standard deviation (SD) (n = 3 for all experiments) and were performed using Origin v. 9.0. The parameters were compared across treatments with one-way ANOVA using SPSS v.22.0, and the statistical significance levels were set to $P < 0.05$.

3. Results and discussion

3.1. Characteristics of four flocculants

The variation of Zeta potentials of the four flocculants are shown in Fig. 1. The surface of three flocculants including Tanfloc, Chitosan and Cationic starch exhibited positive charges below the isoelectric points (pH_{pzc}), whilst the pH_{pzc} of Tanfloc (pH 8.0) is higher than that of Chitosan (pH 7.6) and Cationic starch (pH 7.58). Moreover, the negative charge reversed after exceeding the pH_{pzc}, consistent with previous studies [37]. Noted that the Zeta potential of Chitosan was close to zero after exceeding its zero potential point, mainly due to the insolubility of Chitosan under alkaline conditions [38]. In contrast to the three flocculants discussed above, *Moringa oleifera* was negative across the range of pH values, whilst its Zeta potential was only −2.29 mV even at low pH values of 2.11. The FTIR spectra of four flocculants was presented in the Fig. S2. The peak at 1476 cm^{-1} and 1325 cm^{-1} in Tanfloc represented the bending vibration C-H/N-C on the amino group [23]. The absorption peaks at 1466 cm^{-1} and 1305 cm^{-1} in Chitosan and the peak at 1468 cm^{-1} in Cationic starch were attributed to the N—H bending vibration, respectively [39].

3.2. Harvesting performance

3.2.1. The effects of dosage

Flocculant dosages are an important indicator to evaluate flocculation performance. The changes in HE of the four flocculants on

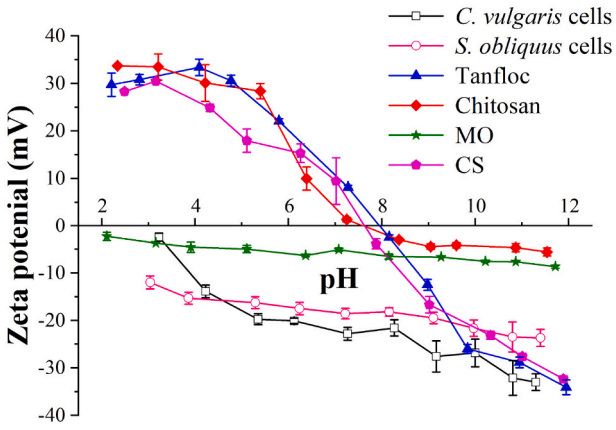

Fig. 1. The Zeta potential of two microalgae (*Chlorella vulgaris* (*C. vulgaris*) and *Scenedesmus obliquus* (*S. obliquus*)) and four natural flocculant (Chitosan, Tanfloc, Cationic starch (CS) and *Moringa oleifera* (MO)). Data are mean ± standard deviation (n = 3).

C. vulgaris and *S. obliquus* at specific flocculant dosages are shown in Fig. 2. It can be seen that all four flocculants effectively harvested the microalgae. At specific dosages, all four flocculants achieved HEs of more than 90%. The optimal dosage of the flocculants assessed by the minimum dosage required to reach a stable HE, is often used to evaluate flocculation capacity. The lower the optimal dosage, the higher the flocculation capacity. The optimal doses of the four flocculants were as follows: Chitosan (5 mg L^{-1} and 3 mg L^{-1} for *C. vulgaris* and *S. obliquus*) < Tanfloc (30 mg L^{-1} and 20 mg L^{-1}) < Cationic starch (60 mg L^{-1} and 40 mg L^{-1}) < *Moringa oleifera* (600 mg L^{-1} and 400 mg L^{-1}). Statistical analysis indicated that there were significant differences ($P < 0.05$) in the optimal doses of four flocculants and the order of harvest capacity was as follows: *Moringa oleifera* < Cationic starch < Tanfloc < Chitosan. This was similar to previous studies. Xu [40], Roselet [24], Letelier-Gordo [19] and Teixeira and Teixeira [20] that used Chitosan, Tanfloc, Cationic starch and *Moringa oleifera* to harvest *C. vulgaris* showed optimal doses of 5 mg L^{-1}, 30 mg L^{-1}, 40 mg L^{-1} and 600 mg L^{-1} in neutral conditions, respectively. Under optimal dosages, HE values of more than 80% were obtained by all four flocculants. The optimal dose of MO was higher than that of Chitosan, Tanfloc and Cationic starch, with high *Moringa oleifera* doses resulting in high levels of microalgae-containing sludge, increasing the costs of the dewatering process. Furthermore, the HE of the four flocculants on *S. obliquus* was significantly ($P < 0.05$) higher than that of *C. vulgaris.* The optimal dosage of the four flocculants on *S. obliquus* was lower than that on *C. vulgaris,* indicating that *S. obliquus* is more easily harvested than *C. vulgaris*.

To investigate the flocculation mechanism, variations in the Zeta potential of the four flocculants and their dosages are shown in the Fig. 2. For Chitosan, Tanfloc and Cationic starch, the Zeta potentials were ≤ 0 at optimal doses, with opposite findings observed for high doses of the flocculants (Fig. 2a-c, e-g). This indicated that electrostatic patching may be the main flocculation mechanism for Chitosan, Tanfloc and Cationic starch [38]. The negative microalgae cell attached itself to the positive flocculant molecular chain, which brought about the development of negatively charged regions on the surface of flocculant. A simultaneous attachment of oppositely charged regions happened when the charged flocculant molecular chains closed to each other. For *Moringa oleifera* (Fig. 2d & h), the Zeta potential did not respond to increased doses and did not approach 0 as positive charges were lacking. However, it maintained as high harvesting capacity on microalgae cells, attributed to the bridging mechanism [38]. Due to Van der Waals forces with hydraulic agitation, numerous negatively charged microalgae cells were simultaneously adsorbed onto the stretched *Moringa oleifera* chains. Thus, numerous microalgae cells bound to the extended *Moringa oleifera* chains, increasing the size of the flocs. As the surface charges of microalgae were shielded by these interactions, electrostatic repulsion had little effect and the Zeta potentials remained ≤0.

As shown in Fig. 2, particularly for Chitosan, the HE on *C. vulgaris* increased at low dosages (0–5 mg L^{-1}), but decreased at higher dosages (5–10 mg L^{-1}). A similar phenomenon occurred during Tanfloc and Cationic starch flocculation. This may be explained by the flocculation mechanism(s) of Chitosan, Tanfloc and Cationic starch. These flocculants display characteristically high cationic charge densities. At increasing dosages, the positive charges accumulate, leading to a higher likelihood of flocculant-microalgae collision. This is conductive to improvements in HE. As the flocculant doses further rise, excessive cationic charges may enhance stability and reduce the HE [41]. However, for *Moringa oleifera*, the HE of microalgae cells did not obviously decrease at high doses. The reasons may be that the bridging effect represents the main mechanism for microalgae harvesting, and that electrostatic repulsion caused by the excessive charge accumulation when adding high doses of flocculant do not offset the adsorption force of the bridging effect, meaning the flocculation performance does not decrease.

The fact that *S. obliquus* was more easily harvested than *C. vulgaris* could be explained by the flocculation mechanism. Changes in the Zeta potential of *C. vulgaris* and *S. obliquus* with pH were investigated to explain these differences (Fig. 1). The Zeta potential of *C. vulgaris* was lower than that of *S. obliquus* at a pH range of pH 4–11. For CTS, Tanfloc and Cationic starch, electrostatic interactions were the main factor affecting the harvesting process. The higher levels of negative charge on *C. vulgaris* was needed more positive charge to neutralize, thus leading to more dosage adding compared to *S. obliquus*. For *Moringa oleifera*, the bridging effect was the main flocculation mechanism. The diameter of *S. obliquus* used in the study (8–10 μm) were larger than those of *C. vulgaris* (3–5 μm), with the larger diameter meaning that single microalgae cells had a higher surface area and more active binding sites. This was conducive to the combination of microalgae cells and flocculants, and improved the HE.

3.2.2. The effects of culture pH

Algal harvesting is strongly affected by the pH of the culture media, because pH not only influences the charge of polymers but also the surface charge of microalgae cells [42]. As shown in Fig. 3, the HE obtained by four flocculants at pH 4.0–5.0 were similar, all of which were about 80% for both *C. vulgaris* and *S. obliquus*. When the culture pH increased to 6.0–7.0, the HE of four flocculants for two microalgae

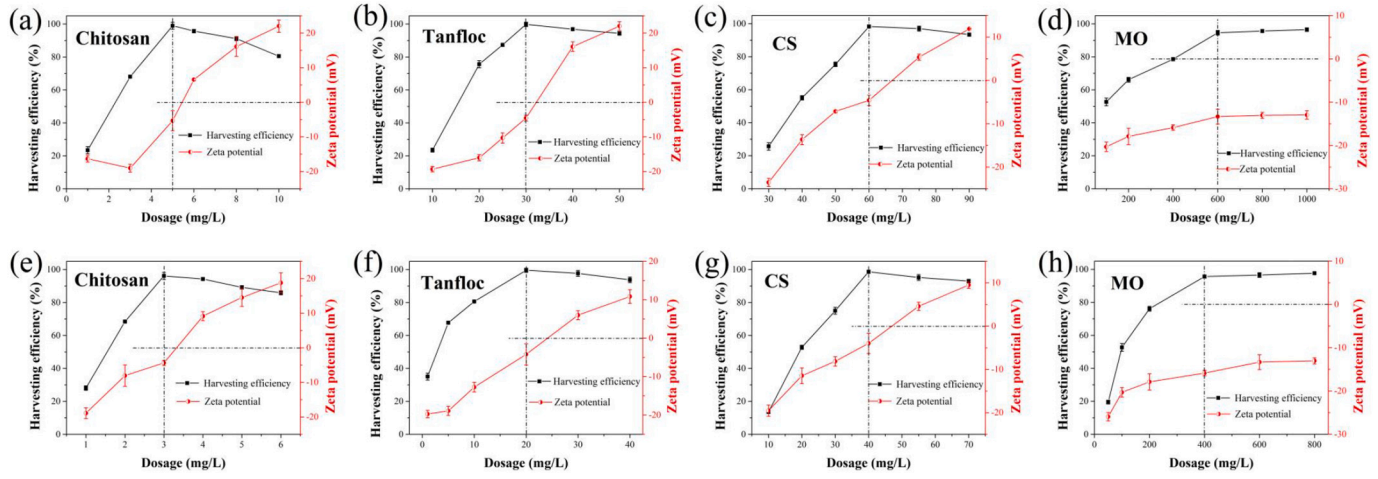

Fig. 2. The harvesting efficiency and Zeta potential of the supernatant as a function of dosage of four flocculant: (a) Chitosan, (b) Tanfloc, (c) Cationic starch (CS) and (d) *Moringa oleifera* (MO) for harvesting *Chlorella vulgaris*, and (e) Chitosan, (f) Tanfloc, (g) CS and (h) MO for harvesting *Scenedesmus obliquus*. (biomass concentrations: ~0.6 g dry cell weigh/L; culture pH: ~7.0; the microalgae cells are not treated to remove AOM (algal organic matter)). Data are mean ± standard deviation (n = 3).

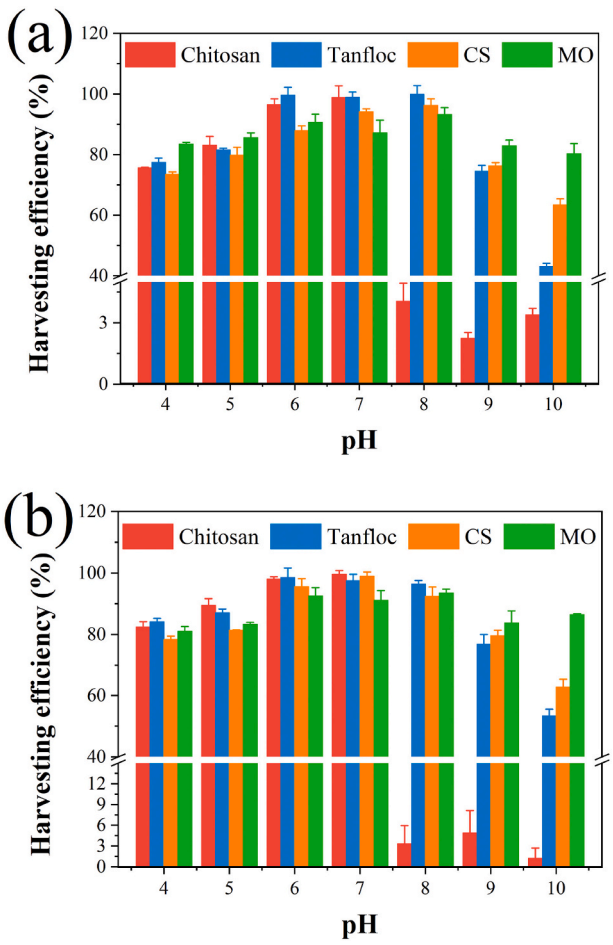

Fig. 3. The harvesting efficiency of (a) *Chlorella vulgaris* and (b) *Scenedesmus obliquus* from culture broth (~0.6 g dry cell weight/L) at different pH values. (biomass concentrations: ~0.6 g dry cell weigh/L; the microalgae cells are not treated to remove AOM (algal organic matter)). Data are mean ± standard deviation (n = 3). CS and MO represent Cationic starch and *Moringa oleifera*, respectively.

reached the highest, and all of them were more than 90%; however, as the culture pH further increased (pH > 7.0), the HE of four flocculants appeared differences: Chitosan was insoluble in water under alkaline condition, so its harvest performance on microalgae could be negligible when the culture pH > 7.0; Tanfloc and Cationic starch could efficiently harvest two microalgae with >90% of HE at pH 8.0. However, when the culture pH increased to 10.0, the HE obtained by Tanfloc and Cationic starch significantly reduced and were only 43.07% and 63.34% for *C. vulgaris,* and 53.32% and 63.72% for *S. obliquus* respectively. Similar findings have been found in the results of [26], that the HE of Tanfloc on *C. vulgaris, Microcystis aeruginosa* (*M. aeruginosa*), *Oocystis solitaria, Scenedesmus smithii* at culture pH < 8.0 are higher than 90%, but it decreases to <60% at culture pH > 9.0. In contrast, MO still had a HE of 80.34% for *C. vulgaris* and 86.42% *S. obliquus* even at pH 10.0, respectively. This is mainly due to the differences in the harvesting mechanisms. The microalgae harvest by Tanfloc and Cationic starch mainly depended on electrostatic patching. The pH_{pzc} of both Tanfloc and Cationic starch were between 7.6 and 8.0 according to the Fig. 1, therefore the OH- in the solution prevailed compared to the H^+ when the pH ≥ 8.0. The - NH_2 group on the surface of Tanfloc and Cationic starch could not undergo the protonation to form positively charged - NH_4^+ (Fig. S1), thus neither Tanfloc or Cationic starch could bind to microalgae cells by electrostatic interaction. *Moringa oleifera* mainly captured microalgae cells through bridging effect. There was no obvious differences in hydraulic radius of *Moringa oleifera* under different culture pH (Fig. S3), indicating that its molecular chain was less affected by the culture pH. Therefore, no obvious HE decrease of *Moringa oleifera* on microalgae cells was observed in high pH value. In short, Chitosan, Tanfloc and Cationic starch could flocculate microalgae cells efficiently under acidic and neutral conditions, but their harvest performances were restricted by alkaline environment to varying degrees. In contrast, *Moringa oleifera* is almost not affected by culture pH although its HE for two microalgae cells was lower.

3.2.3. The effects of biomass concentration

In order to explore the effects of biomass concentration during the harvesting process, the flocculation harvesting were studied over a density range from ~0.3 g L^{-1} to ~1.5 g L^{-1} (Fig. 4). When the biomass concentration was ~0.3 g L^{-1}, the optimal dosage of four flocculants for *C. vulgaris* and *S. obliquus* were 3 and 1 mg L^{-1} (Chitosan), 5 and 3 mg L^{-1} (Tanfloc), 10 and 5 mg L^{-1} (Cationic starch) and 300 and 200 mg L^{-1} (*Moringa oleifera*), respectively, As the cell density increased to ~0.6 g L^{-1}, the optimal dosage of all four flocculants increased to varying degrees, but the order of harvest performance was still Chitosan < Tanfloc < Cationic starch < *Moringa oleifera*, which was confirmed by statistical analysis that there were significant differences ($P < 0.05$) in the optimal doses of four flocculants. This indicated that the increase in biomass concentration will result in the increase of optimal dosage, but does not affect its relative orders. When biomass concentration further increased to ~1.5 g L^{-1}, the optimal dosage of Chitosan, Tanfloc, Cationic starch and *Moringa oleifera* reached 10, 40, 70 and 1000 mg L^{-1} for *C. vulgaris*, 7, 25, 50 and 800 mg L^{-1} for *S. obliquus*, respectively.

With increased biomass concentration, the dosage of polymers usually increases. For example, 4-fold synthetic polyacrylamide polymers dosage was required as cell density increased from 0.315 g L^{-1} to 1.46 g L^{-1} [24]. In our research, as cell density increased from ~0.3 g L^{-1} to ~1.5 g L^{-1}, the optimal dosages of microalgae harvesting by Chitosan were less than 10 mg L^{-1}, indicating that even a small amount of Chitosan can efficiently collect microalgae cells within a wide range of biomass concentration; for Tanfloc and Cationic starch, their optimal dosages significantly increased when the biomass concentration increased from ~0.3 g L^{-1} to ~1.5 g L^{-1}, which was increased by 8 and 7 times (*C. vulgaris*) and 8 and 10 times (*S. obliquus*), respectively. Noted that at low biomass concentration (~0.3 g L^{-1}), the optimal dosages of both Tanfloc and CS were closed to Chitosan, which was 1.7 and 3 times (*C. vulgaris*), 3.3 and 5 times (*S. obliquus*) as much as Chitosan, respectively; while at high cell density (~1.5 g L^{-1}), their optimal dosages were significantly higher than Chitosan, which were 4 and 7 times (*C. vulgaris*), 3.6 and 7.1 times (*S. obliquus*) that of Chitosan. This meant that there was no significant ($P < 0.05$) dosage differences for Chitosan, Tanfloc and Cationic starch when collecting low-concentration microalgae cells, while Chitosan showed a greater advantage in harvesting high concentration microalgae cells. In contrast, when the concentration increased from ~0.3 g L^{-1} to ~1.5 g L^{-1}, the optimal dosage of *Moringa oleifera* increased significantly, which was more than 100 times that of Chitosan, 24 times and 16 times that of Tanfloc and Cationic starch, respectively. It suggested that no matter low or high biomass concentration, the dosage required for *Moringa oleifera* to obtain high HE was much higher than that of the other natural flocculants.

3.2.4. The effects of algal organic matter

To study the effects of AOM, the composites were applied to algal samples with or without AOM. Since 3DEEM could provide specific information on the organic characteristics of protein-like substances, the relative intensity of the characteristic peaks was used to reflect the relative content of AOM [36]. It could be seen that the peak intensity of protein-like substances (Peak 1) in the microalgae medium was about 1.6 times than that without AOM (Fig. S4), indicating that most of the AOM were removed after centrifugation.

As shown in Fig. 5, the optimal dosage of all four flocculants

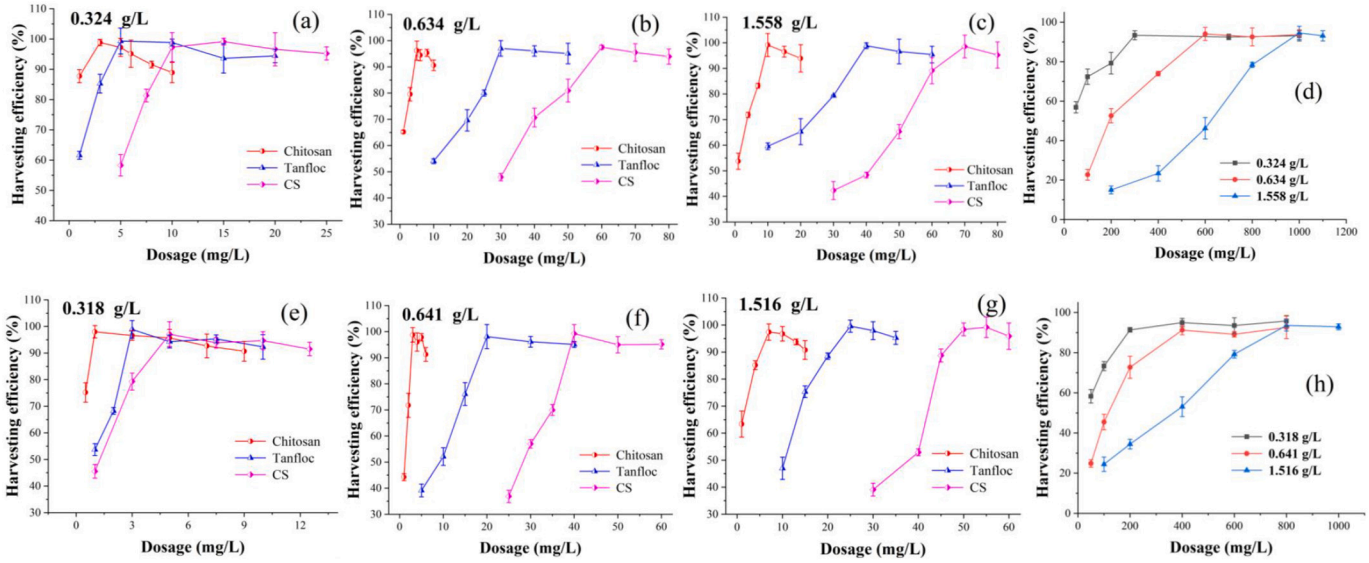

Fig. 4. Effect of different biomass concentrations on harvesting of *Chlorella vulgaris* using Chitosan, Tanfloc and Cationic starch (CS): (a) 0.324 g dry cell weight/L; (b) 0.634 g dry cell weight/L; (c) 1.558 g dry cell weight/L; *Scenedesmus obliquus*: (e) 0.318 g dry cell weight/L; (f) 0.641 g dry cell weight/L; (g) 1.516 g dry cell weight/L. Effect of different biomass concentrations on harvesting of *Chlorella vulgaris* (e) and *Scenedesmus obliquus* (h) using *Moringa oleifera* (MO). (Culture pH: ~7.0; the microalgae cells are not treated to remove AOM (algal organic matter)). Data are mean ± standard deviation (n = 3).

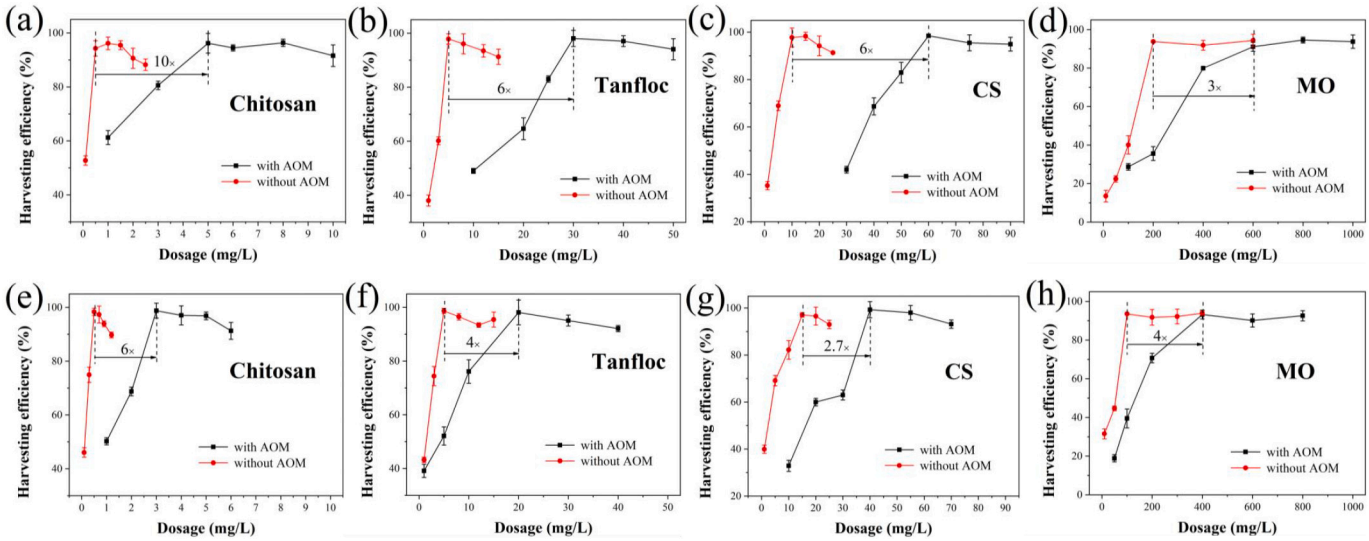

Fig. 5. Effects of algal organic matter (AOM) on harvesting of *Chlorella vulgaris* by (a) Chitosan, (b) Tanfloc, (c) Cationic starch (CS) and (d) *Moringa oleifera* (MO); and *Scenedesmus obliquus* by (e) Chitosan, (f) Tanfloc, (g) CS and (h) MO. (biomass concentrations: ~0.6 g dry cell weigh/L; culture pH: ~7.0). Data are mean ± standard deviation (n = 3).

decreased after removing the AOM. For instance, the optimal dosages of *C. vulgaris* harvested by Chitosan, Tanfloc, Cationic starch and *Moringa oleifera* were only 1/10, 1/6, 1/6 and 1/3 than that without AOM, respectively (Fig. 5a-d). It indicated that AOM could interfere with harvesting process by natural flocculant, and the interference was affected by the flocculant and microalgae species. It is consisted with previous research. Vandamme [10] demonstrated AOM could influence the harvesting process of five different modes including aluminum sulfate, Chitosan, Cationic starch, pH-induced flocculation and electrocoagulation-flocculation (ECF) on *C. vulgaris*, and they deemed the degree of inhibition of flocculation by AOM is most likely related to the quantity and the composition of the AOM present in the medium. Further, Wang [37] studied the main components of AOM interfering with quaternary ammonium-salt-modified tannin (Q-TN) harvesting *M. aeruginosa*, and found simple aromatic proteins and protein-like substances are the main reason for the increase in dosage due to consumption of Q-TN. However, after studying the components of *Nannochloropsis salina*, Garzon-Sanabria [43] believed that the high concentration of carbohydrates is the main reason for increased dosage requirements in harvesting using synthetic cationic polymers. In short, the differences in microalgae types will lead to the differences in the composition and quantity of AOM produced by them, and the binding ability of different flocculants with AOM components also differed. These reasons ultimately determined the degree of inhibition of harvest process by AOM. In our study, according to the optimal dosage of four flocculants before and after AOM removal, AOM had the greatest interference on the harvesting process by Chitosan, while the harvesting process by *Moringa oleifera* was least affected by AOM. Nevertheless, all four flocculants could overcome this interference by increasing the dosage.

3.3. Flocs properties

During flocculation, flocs with compact structures can easily sediment, resulting in a high removal efficiency. Flocs formed by flocculants with poor dehydration ability tend to have loose structure, causing difficulties in collection and requiring large investment for the subsequent dehydration treatment. Besides, microalgae dewatering is a primary challenge in large-scale harvesting and processing of microalgae from water environment. Therefore, the structures and dewatering performance of the flocs formed by four flocculants were compared.

As can be seen from Table S2, there was no obvious differences between the size of flocs formed from Chitosan, Tanfloc and Cationic starch, while the size formed from *Moringa oleifera* was obviously larger than other flocculants. For example, the size range of *C. vulgaris* flocs harvested by Tanfloc under shear rates was 618–805 μm, while the size range of *C. vulgaris* flocs harvested by *Moringa oleifera* increased to 998–1217 μm. SSVFs and CFs of the flocs are often used to evaluate the flocs compactness [14]. Higher SSVF values indicate higher volumes of microalgae slurry and lower flocculation performance. Low CF values indicate low HE and high SSVF values [16]. As shown in the Fig. 6(a) & (b), SSVF and CF values were as follows: (SSVF) Chitosan < Tanfloc < Cationic starch < *Moringa oleifera* and (CF) *Moringa oleifera* < Cationic starch < Tanfloc < Chitosan. Therefore, the concentrating ability of the flocs were as follows: *Moringa oleifera* < Cationic starch < Tanfloc < Chitosan. A CF <10 results in impractical levels of sludge relative to the culture volume [16]. The CF values harvested by Chitosan were as high as 16.17 and 18.07 respectively, indicating the highest sedimentation and concentrating ability. The flocs harvested by Tanfloc and Cationic starch also showed good concentrating abilities, with CF values higher than 10. However, the CF values of *C. vulgaris* and *S. obliquus* flocs harvested by *Moringa oleifera* were only 9.14 and 8.80 (⟨10), indicating that the flocs were loose and that the cells may escape from the harvested flocs.

Additionally, the moisture and porosity of the flocs can reveal the dewatering functionality of the flocculants. As shown in Fig. 6(c) & (d), there were significant differences ($P < 0.05$) in the moisture and porosity of the flocs formed by four flocculants and their orders were as follows: Chitosan < Tanfloc ≈ Cationic starch < *Moringa oleifera*. It indicated that the flocs formed by Chitosan were more stable and compact than other flocculants, while the more porous and more moisture flocs was obtained by *Moringa oleifera* flocculated. The differences in the concentrating ability, moisture and porosity of four flocculants could be explained by their diverse flocs formation mechanism. The electrostatic patching was the main harvesting mechanism of Chitosan, Tanfloc and Cationic starch [44,45]. Positively charged flocculant could integrate with the negatively charged microalgae cells forming homogeneous and tight microflocs through charge neutralization. Numerous microflocs collide to form compacted macroflocs. Furthermore, the main flocculation mechanism for *Moringa oleifera* is the bridging effect [44,45]. Compared to electrostatic interactions, although the long molecular chain of *Moringa oleifera* allowed it simultaneously capture the multiple cells to form larger flocs, the interaction between different molecular chains is relatively weak, which results in the lack of tight bonding between different molecular chains and thus forms a relatively loose floc. On the whole, the microalgae-dewatering capability of Chitosan, Tanfloc and Cationic starch enables high-performance flocculation, which can dramatically reduce the cost of further industrial processing of dehydration.

3.4. Effects on the cell viability of flocs

In addition to harvesting performance, the effects of harvesting on cell viability and physiological activity was undertaken to evaluate the performance of the flocculants. Evans blue assays are often used to

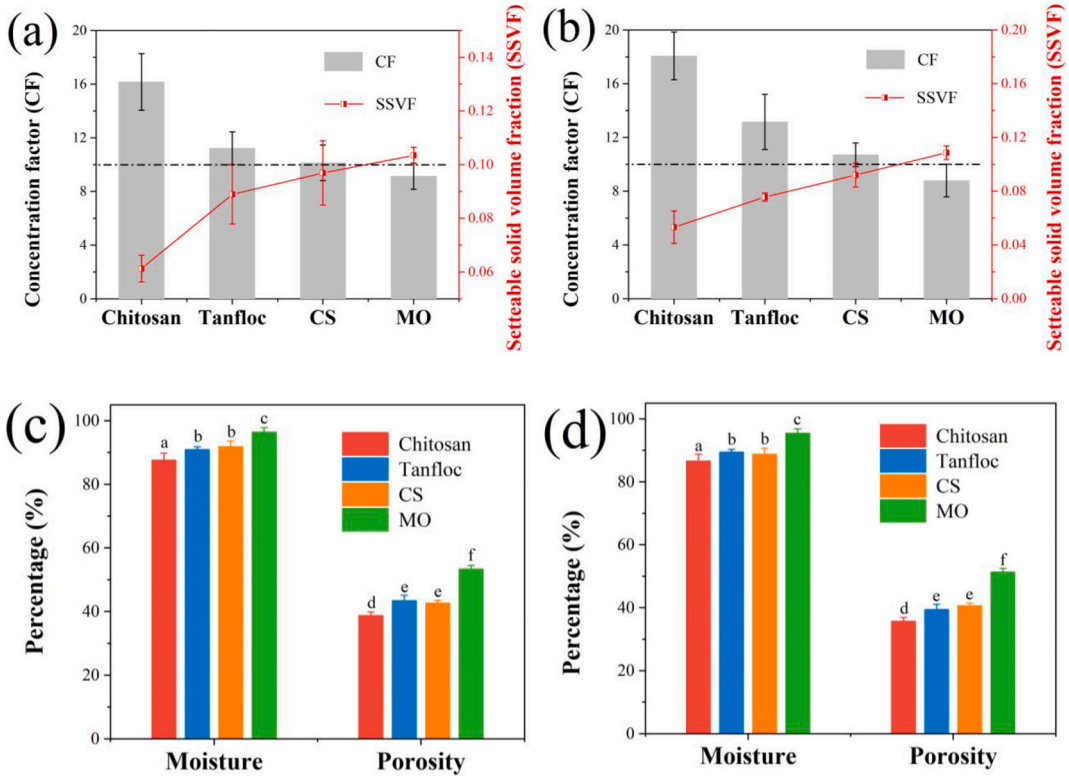

Fig. 6. Settleable solid volume fractions (SSVF) and concentration factor (CF): (a) *Chlorella vulgaris*; and (b) *Scenedesmus obliquus*. Moisture and Porosity of (c) *Chlorella vulgaris* flocs; and (d) *Scenedesmus obliquus* flocs formed by four flocculants. Data are mean ± standard deviation (n = 3). The parameters were compared across treatments with one-way ANOVA using SPSS v.22.0, and the same letters represents no significant differences ($P > 0.05$) between the treatment means. CS and MO represent Cationic starch and *Moringa oleifera*, respectively.

assess cell integrity and viability. Viable cells efflux the Evans blue dye and remain unstained [15]. Images of stained microalgae cells are shown in Fig. S5–6. Using all four flocculants, low numbers of *C. vulgaris* and *S. obliquus* cells was dyed, indicating no obvious cell lysis and intact cell membranes. The harvesting process of all four flocculants therefore caused no damage to *C. vulgaris* and *S. obliquus* cells. In contrast, Daniel [28] found that inorganic chemical flocculants, such as $CuSO_4$, may distress cell membranes of and cause lysis.

From fluorescence quenching analysis, F_v/F_m reflects the potential of microalgae photochemistry and it is often used to reflect the cell stress induced by the harvesting procedure [46]. If microalgae cells are subject to environmental stress induced by harvesting, the F_v/F_m decreases (Table 1). Previous studies found that the F_v/F_m (effective quantum yield of PSII) is stable at ~0.5–0.7 if microalgae cells are in a good physiological state [47,48]. Both *C. vulgaris* and *S. obliquus* maintained a good physiological state prior to flocculation, and the range of F_v/F_m was 0.48–0.67. Following flocculation, excluding the F_v/F_m of microalgae cells harvested by *Moringa oleifera* which slightly decreased, the other three flocculants showed no significantly ($P > 0.05$) changes and their range of F_v/F_m was 0.51–0.65. This indicated that the harvesting process of Chitosan, Tanfloc and Cationic starch caused no cell stress. Although *Moringa oleifera* leads to cell pressure, the F_v/F_m values of *C. vulgaris* (0.63 ± 0.01) and *S. obliquus* (0.49 ± 0.03) after flocculation ranged from 0.5–0.7, indicating low cell pressure. This further demonstrated that the harvesting process of four flocculants had no obvious effects on the viability of *C. vulgaris* and *S. obliquus*.

3.5. Effects on the cell extractions

The effects of the harvesting process on total lipid, carbohydrates and protein are also important indicators when evaluating the performance of a flocculant. Lipid, carbohydrates and protein are the raw materials in food, feed, cosmetics and other fields [49–51].

The extractions of cells harvested by four flocculants was compared. As is shown in the Fig. 7. there was no significant differences ($P > 0.05$) in the total lipid, total carbohydrates and total protein content after all four flocculants were collected compared with the control group - natural sedimentation, which indicated that all four natural flocculants would not cause the loss of cell extractions. This may be due to the fact that none of four flocculants would cause cell lysis or affect cell viability in harvesting process, so intracellular substances can be completely recovered. It is consistent with the studies of [15], that both *Scenedesmus* sp. cells and their intracellular extractions could be intactly harvested by Chitosan. Hou [23] also found the intracellular organic matter including microcystin and proteins from *M. aeruginosa* would not be released into water after Tanfloc flocculation. Moreover, other natural flocculants had similar results. U. Suparmaniam [52] used chicken's eggshell- and clam shell-derived bio-flocculants to harvest *C. vulgaris*. They found the percentage of lipid yield obtained in the study was aligned with for biomass, and the harvesting process did not significantly alter the fatty acid composition of microalgae lipid. In brief, natural flocculant harvesting may cause no loss of lipids, carbohydrates or proteins, which was beneficial to maximization of microalgae resources in succeeding biodiesel production.

Table 1
The F_v/F_m value of microalgae cells before and after flocculation.

		Chitosan	Tanfloc	Cationic starch	*Moringa oleifera*
Chlorella vulgaris	Before flocculation	0.54 ± 0.02^a	0.64 ± 0.05^a	0.65 ± 0.04^a	0.67 ± 0.01^a
	After flocculation	0.53 ± 0.03^a	0.63 ± 0.03a	0.65 ± 0.02^a	0.63 ± 0.01^a
Scenedesmus obliquus	Before flocculation	0.49 ± 0.04^b	0.48 ± 0.04^b	0.52 ± 0.06^b	0.61 ± 0.01^b
	After flocculation	0.59 ± 0.03^b	0.54 ± 0.03^b	0.51 ± 0.04^b	0.49 ± 0.03^c

Notes: Significant differences ($P < 0.05$) between the treatment means were represented by different letters.

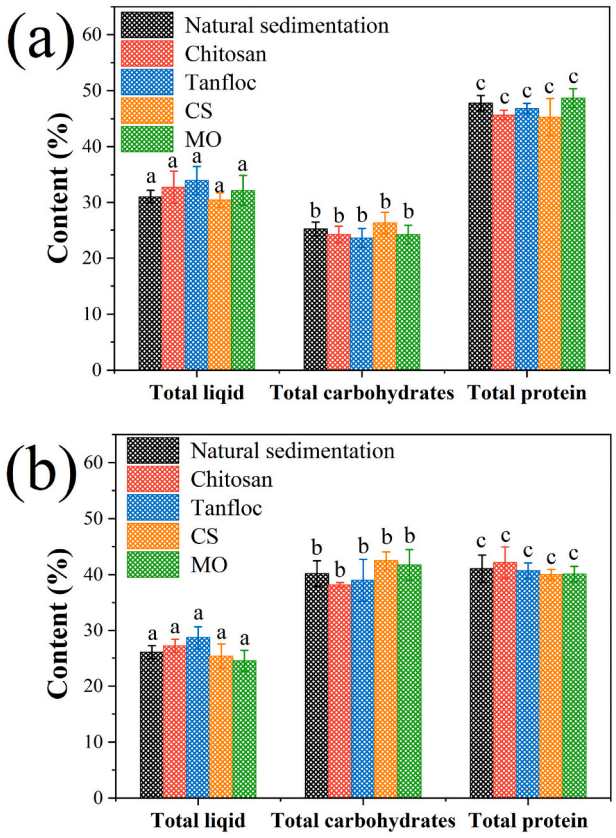

Fig. 7. Extractions (total lipid, carbohydrates and protein) of microalgae cells harvested by four flocculants and natural sedimentation: (a) *Chlorella vulgaris*; (b) *Scenedesmus obliquus*. Data are mean ± standard deviation (n = 3). The parameters were compared across treatments with one-way ANOVA using SPSS v.22.0, and the same letters represents no significant differences ($P > 0.05$) between the treatment means. CS and MO represent Cationic starch and *Moringa oleifera*, respectively.

3.6. Reuse of the separated medium

Recycling of the harvesting water from microalgae production systems can economize water resources and reduce the costs of microalgae cultivation [2]. It is therefore valuable to investigate the reusability of separated medium following flocculation. The media were recovered after flocculation and nutrient integration from the fresh BG-11 medium was added for supplementation. Separated and fresh media were used to recultivate *C. vulgaris* and *S. obliquus,* respectively. The growing states of microalgae cells in the separated and fresh media were investigated (Fig. 8). The OD_{690nm} values were used to reflect cell growth and the slope of the growth curves represented the growth rates.

As shown in Fig. 8, microalgae cells in the separated medium from both Tanfloc and Cationic starch flocculation showed robust growth that was modestly lower than fresh medium. In contrast, microalgae in the media separated by *Moringa oleifera* flocculation had the highest growth rates. The OD of microalgae growing in the separated medium was comparable to that of fresh medium, indicating that the separated medium from Tanfloc, *Moringa oleifera* and Cationic starch flocculation could be recycled for further cultivation. Noted that the growth of microalgae cells in the separated medium from Chitosan flocculation stagnated, with no increase in OD over the 15-day culture period, indicating that the separated medium of Chitosan may be unsuitable for

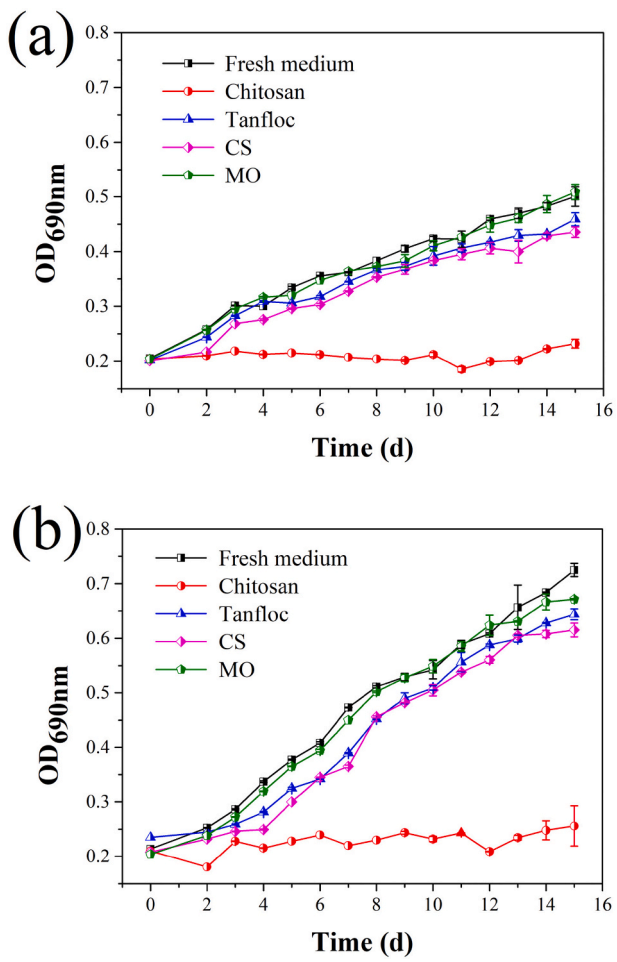

Fig. 8. Growth curves of (a) *Chlorella vulgaris* and (b) *Scenedesmus obliquus* harvested by four flocculants and natural sedimentation during culturing process in fresh medium. Data are mean ± standard deviation (n = 3). CS and MO represent Cationic starch and *Moringa oleifera*, respectively.

the re-culture of microalgae cells.

3.7. Bioeconomic comparison of the four natural flocculants

Although the four natural flocculants possess differing flocculation mechanisms, they showed high harvesting performance and concentrating abilities, allowing the flocculants to prevent unstable aggregation and improve the separation performance of flocs from the medium. Furthermore, the harvesting process caused no cell lysis and did not affect the physiological status of microalgae cells. The medium separated from Tanfloc, *Moringa oleifera* and Cationic starch after harvesting could be used for the re-culture of microalgae, reducing the costs of the culture process.

The harvesting performance and cost analysis of the four natural flocculants were next compared (Table 2). As a common natural flocculant, Chitosan efficiently harvested microalgae cells. A high HE of 98.9% for *C. vulgaris* and 96.1% for *S. obliquus* was obtained at low Chitosan dosages (5 mg L^{-1} and 3 mg L^{-1}). Meanwhile, good dewatering functionality was observed. However, the cost per ton of biomass harvested ($176.81 US and $106.95 US) was high and almost double that of Tanfloc. A further disadvantage was that Chitosan could hardly harvest microalgae under alkaline conditions. Additionally, Chitosan medium could not be recycled for the re-growth of microalgae cells. Although the *Moringa oleifera* unit price was low (1259 US$/ton), the cost per ton of biomass harvested ($1332.28 US and $877.96 US) was higher than other flocculants, and about 20-fold higher than Tanfloc due to the poor harvesting performance. In addition, higher doses were required (600 mg L^{-1} for *C. vulgaris* and 400 mg L^{-1} for *S. obliquus*) to achieve high HE values. Moreover, the large doses result in a mass of microalgae-containing sludge and low flocs compactness lead to the loose flocs, both of which increases the cost of the dewatering process. Cationic starch had the lowest unit price amongst the four flocculants, with a cost per ton of biomass harvested second only to Tanfloc. Its harvesting performance was superior to *Moringa oleifera*, however, compared with Chitosan and Tanfloc, higher dosages and a larger production of microalgae-containing flocs occurred.

In contrast, Tanfloc achieves good harvesting performances at lower dosages (30 mg L^{-1} for *C. vulgaris* and 20 mg L^{-1} for *S. obliquus*). Although it consumes higher dosages than Chitosan to obtain the same

Table 2
Harvesting performance and cost analysis of four natural flocculant.

Flocculant	Microalgae	Optimal dosage (mg L^{-1})[1]	HE[2] (%)	Optimal pH range[3]	Flocs concentrating abilities[4]	Cell viability	Cell extraction	Reusability	Flocculant cost (US$/ton)	Biomass harvested (g L^{-1})	Flocculant cost per ton of biomass harvested (US$)
Chitosan	*Chlorella vulgaris*	5	98.9	4.0–7.0	Compact	√[5]	√[5]	×[5]	20,984	0.59	176.81
	Scenedesmus obliquus	3	98.1	4.0–7.0	Compact	√	√	×		0.59	106.95
Tanfloc	*Chlorella vulgaris*	30	99.8	4.0–9.0	Compact	√	√	√	1500	0.60	75.15
	Scenedesmus obliquus	20	99.5	4.0–9.0	Compact	√	√	√		0.59	50.25
Cationic starch	*Chlorella vulgaris*	60	98.3	4.0–9.0	Compact	√	√	√	839	0.59	85.35
	Scenedesmus obliquus	40	98.6	4.0–9.0	Compact	√	√	√		0.59	56.73
Moringa oleifera	*Chlorella vulgaris*	600	94.5	4.0–10.0	Loose	√	√	√	1259	0.57	1332.28
	Scenedesmus obliquus	400	95.6	4.0–10.0	Loose	√	√	√		0.57	877.96

Notes: 1. The optimal dosage were obtained in the harvesting conditions (Cell density: ~0.6 g dry cell weigh/L; culture pH: ~7.0; the microalgae cells are not treated to remove AOM (algal organic matter)). 2. The harvesting efficiency (HE) was obtained at the optimal dosage. 3. Optimal pH range is the pH range corresponding to HE greater than ~75%. 4. the compact (or loose) flocs represents its concentration factor (CF) >10 (or < 10), respectively. 5. √ represents one of three cases: (a) the harvesting process could not affect cell viability; (b) the harvesting process could not cause the loss of cell extractions; or (c) the separated medium could be reused for next cultivation. × represents one of three cases: (a) the harvesting process could affect cell viability; (b) the harvesting process could cause the loss of cell extractions; or (c) the separated medium could not be reused for next cultivation.

HE, the dosages were lower than the other flocculants, particularly *Moringa oleifera*. Tanfloc could effectively harvest microalgae in a wide pH range (pH 4.0–9.0) and showed a good dewatering potential. The harvesting costs of Tanfloc were the lowest amongst the four flocculants, with a cost per unit weight of biomass of only $75.15 US for *C. vulgaris* and $50.25 US for *S. obliquus*. Additionally, Tanfloc flocculation did not damage cell integrity, cause the loss of cell extractions and the separated medium could be recycled. In summary, Tanfloc is the best choice for microalgae harvesting amongst the four natural flocculants.

4. Conclusions

Although part of microalgae including some of *Scenedesmus* sp. can be harvested through settling, it is a slow process on its own and generally needs to be induced chemically or by introducing stress to the culture. Here, we investigated the potential of four natural flocculants for harvesting microalgae. The main conclusions were as follows:

The optimal dosages of Chitosan/Tanfloc/Cationic starch for harvesting *C. vulgaris* and *S. obliquus* were much lower than *Moringa oleifera*. Electrostatic patching and bridging effects were the main harvesting mechanisms for Chitosan/Tanfloc/Cationic starch and *Moringa oleifera*, respectively. The culture pH could interfere the harvesting processes of the flocculants except for *Moringa oleifera*. Nevertheless, its optimal dosages at high biomass concentration (~1.5 g L^{-1}) were as high as 1000 mg L^{-1} for *C. vulgaris* and 800 mg L^{-1} for *S. obliquus*, which was much more than other flocculants. AOM could hinder all harvesting processes, but its disturbance can be overcome by increasing dosage.

With the help of electrostatic attraction, Chitosan/Tanfloc/Cationic starch obtained smaller but denser flocs, which endows them better dewatering functionality. Harvesting using all four flocculants did not affect cell viability or cause the loss of cell extractions. In addition to Chitosan, the medium separated from the other flocculants could be recycled for subsequent cultivations, reducing the cost of microalgae cultivation greatly.

Amongst four natural flocculants, high HE (> 98%) could be obtained by Tanfloc with low dosages (30 mg L^{-1} for *C. vulgaris* and 20 mg L^{-1} for *S. obliquus*). Tanfloc could also effectively harvest microalgae in a wide pH range (pH 4.0–9.0) and was less disturbed by AOM. More importantly, the harvesting costs of Tanfloc were the lowest amongst the four flocculants, with a cost per unit weight of biomass of only $75.15 US for *C. vulgaris* and $50.25 US for *S. obliquus*. Therefore, Tanfloc is the best choice for microalgae harvesting of the four flocculants.

CRediT authorship contribution statement

Zijun Yang, and Jun Hou performed analysis and data interpretation, and Zijun Yang drafted the manuscript. Lingzhan Miao performed statistical analysis. All coauthors contributed to data interpretation and made critical revisions in the manuscript. Jun Hou, Lingzhan Miao provided funding support.

Declaration of competing interest

No conflicts, informed consent, human, or animal rights are applicable.

Acknowledgements

We are grateful for the grants for Project supported by the National Key Research and Development Program of China (2016YFC0401709), the National Science Funds for Creative Research Groups of China (No. 51421006), the National Natural Science Foundation of China (No.52039003), the National Natural Science Foundation of China (No.51979075) and PAPD.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102358.

References

[1] M.N. Mathimani T, A comprehensive review on harvesting of microalgae for biodiesel–key challenges and future directions, Renew. Sust. Energ. Rev. 91 (2018) 1103–1120.
[2] S.Y. Chiu, C.Y. Kao, T.Y. Chen, Y.B. Chang, C.M. Kuo, C.S. Lin, Cultivation of microalgal Chlorella for biomass and lipid production using wastewater as nutrient resource, Bioresour. Technol. 184 (2015) 179–189.
[3] L.B. Yang, X.B. Tan, D.Y. Li, H.Q. Chu, X.F. Zhou, Y.L. Zhang, H. Yu, Nutrients removal and lipids production by Chlorella pyrenoidosa cultivation using anaerobic digested starch wastewater and alcohol wastewater, Bioresour. Technol. 138 (2013) 95–100.
[4] N. Abdel-Raouf, A.A. Al-Homaidan, I.B. Ibraheem, Microalgae and wastewater treatment, Saudi J. Biol. Sci. 19 (2012) 257–275.
[5] J.S. García-Pérez, A. Beuckels, D. Vandamme, O. Depraetere, I. Foubert, R. Parra, K. Muylaert, Influence of magnesium concentration, biomass concentration and pH on flocculation of Chlorella vulgaris, Algal Res. 3 (2014) 24–29.
[6] A.J. Dassey, C.S. Theegala, Harvesting economics and strategies using centrifugation for cost effective separation of microalgae cells for biodiesel applications, Bioresour. Technol. 128 (2013) 241–245.
[7] S.V.D. Hende, V. Han, S. Desmet, N. Boon, Bioflocculation of microalgae and bacteria combined with flue gas to improve sewage treatment, New Biotechnol. 29 (2012) 23–31.
[8] W. Lei, D.S. Dandy, A microfluidic concentrator for cyanobacteria harvesting, Algal Res., (2017) S2211926416304362.
[9] A. Sukenik, D. Bilanovic, G. Shelef, Flocculation of microalgae in brackish and sea waters, Biomass 15 (1988) 187–199.
[10] D. Vandamme, I. Foubert, I. Fraeye, K. Muylaert, Influence of organic matter generated by Chlorella vulgaris on five different modes of flocculation, Bioresour. Technol. 124 (2012) 508–511.
[11] W. Farooq, M. Moon, B.G. Ryu, W.I. Suh, A. Shrivastav, S.P. Min, S.K. Mishra, J. W. Yang, Effect of harvesting methods on the reusability of water for cultivation of Chlorella vulgaris, its lipid productivity and biodiesel quality, Algal Res. 8 (2015) 1–7.
[12] G.P.T. Lam, M.H. Vermuë, G. Olivieri, L.A.M.V.D. Broek, M.J. Barbosa, M.H. M. Eppink, R.H. Wijffels, D.M.M. Kleinegris, Cationic polymers for successful flocculation of marine microalgae, Bioresour. Technol. 169 (2014) 804–807.
[13] J.G.B. Bolto, Organic polyelectrolytes in water treatment, Water Res. 41 (2007) 2301–2324.
[14] V. Dries, F. Imogen, M. Koenraad, Flocculation as a low-cost method for harvesting microalgae for bulk biomass production, Trends Biotechnol. 31 (2013) 233–239.
[15] J. Wu, J. Liu, L. Lin, C. Zhang, A. Li, Z. Yi, Y. Zhang, Evaluation of several flocculants for flocculating microalgae, Bioresour. Technol. 197 (2015) 495–501.
[16] S. Lama, K. Muylaert, T.B. Karki, Flocculation properties of several microalgae and a cyanobacterium species during ferric chloride, chitosan and alkaline flocculation, Bioresour. Technol. 220 (2016) 464–470.
[17] F.H.M. Yunos, N.M. Nasir, H.H.W. Jusoh, H. Khatoon, S.S. Lam, A. Jusoh, Harvesting of microalgae (Chlorella sp.) from aquaculture bioflocs using an environmental-friendly chitosan-based bio-coagulant, Int. Biodeterior. Biodegradation 124 (2017) 243–249.
[18] D. Vandamme, I. Foubert, B. Meesschaert, K. Muylaert, Flocculation of microalgae using cationic starch, J. Appl. Phycol. 22 (2010) 525–530.
[19] C.O. Letelier-Gordo, S.L. Holdt, D.D. Francisci, D.B. Karakashev, I. Angelidaki, Effective harvesting of the microalgae Chlorella protothecoides via bioflocculation with cationic starch, Bioresour. Technol. 167 (2014) 214–218.
[20] C.M.L.L. Teixeira, P.C.N. Teixeira, Evaluation of Moringa oleifera seed flour as a flocculating agent for potential biodiesel producer microalgae, J. Appl. Phycol. 24 (2012) 557–563.
[21] M.M. Barrado-Moreno, Beltran-Heredia, Martín-Gallardo, microalgae removal with Moringa oleifera, Toxicon 110 (2016) 68–73.
[22] S.H.A. Hamid, F. Lananan, H. Khatoon, A. Jusoh, A. Endut, A study of coagulating protein of Moringa oleifera in microalgae bio-flocculation, Int. Biodeterior. Biodegradation 113 (2016) 310–317.
[23] J. Hou, Z. Yang, P. Wang, C. Wang, Y. Yang, X. Wang, Changes in Microcystis aeruginosa cell integrity and variation in microcystin-LR and proteins during Tanfloc flocculation and floc storage, Sci. Total Environ. 626 (2018) 264.
[24] F. Roselet, D. Vandamme, M. Roselet, K. Muylaert, P.C. Abreu, Effects of pH, salinity, biomass concentration, and algal organic matter on flocculant efficiency of synthetic versus natural polymers for harvesting microalgae biomass, Bioenergy Res. 10 (2017) 1–11.
[25] Á. Cancela, Á. Sánchez, X. Álvarez, A. Jiménez, L. Ortiz, E. Valero, P. Varela, Pellets valorization of waste biomass harvested by coagulation of freshwater algae, Bioresour. Technol. 204 (2016) 152–156.
[26] M.M. Barrado-Moreno, J. Beltrán-Heredia, J. Martín-Gallardo, Microalgal removal with natural coagulants, Phycologia 55 (2016) 688–695.
[27] H.G. Peterson, S.E. Hrudey, I.A. Cantin, T.R. Perley, S.L. Kenefick, Physiological toxicity, cell membrane damage and the release of dissolved organic carbon and geosmin by Aphanizomenon flos-aquae after exposure to water treatment chemicals, Water Res. 29 (1995) 1515–1523.

[28] D. Jančula, B. Maršálek, Critical review of actually available chemical compounds for prevention and management of cyanobacterial blooms, Chemosphere 85 (2011) 1415–1422.
[29] S. Feng, W.U. Xingqiang, C. Wang, Y. Peng, Q. Hui, Y. Liu, B. Xiao, Optimization of preparation conditions of cationic starch and its flocculation efficiency on field cyanobacteria, Environ. Sci. Technol, 2018.
[30] S. Şirin, R. Trobajo, C. Ibanez, J. Salvadó, Harvesting the microalgae Phaeodactylum tricornutum with polyaluminum chloride, aluminium sulphate, chitosan and alkalinity-induced flocculation, J. Appl. Phycol. 24 (2012) 1067–1080.
[31] A.K. Lee, D.M. Lewis, P.J. Ashman, Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel, J. Appl. Phycol. 21 (2009) 559–567.
[32] X. Wang, Y. Zhao, X. Jiang, L. Liu, X. Li, H. Li, W. Liang, In-situ self-assembly of plant polyphenol-coated Fe3O4 particles for oleaginous microalgae harvesting, J. Environ. Manag. 214 (2018) 335–345.
[33] A. Papazi, P. Makridis, P. Divanach, Harvesting Chlorella minutissima using cell coagulants, J. Appl. Phycol. 22 (2010) 349–355.
[34] J. Folch, M. Lees, G.S. Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[35] W. Zhou, Y. Li, M. Min, B. Hu, P. Chen, R. Ruan, Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production, Bioresour. Technol. 102 (2011) 6909–6919.
[36] Z. Yang, J. Hou, L. Miao, Y. Yang, G. You, D. Jia, M. Gao, Removing specific extracellular organic matter from algal bloom water by Tanfloc flocculation: performance and mechanisms, Sep. Purif. Technol. 212 (2019) 65–74.
[37] W. Li, L. Wenyan, Y. Jian, L. Zhixia, R. Lingling, Z. Yuanchun, Flocculation of Microcystis aeruginosa using modified larch tannin, Environ. Sci. Technol. 47 (2013) 5771–5777.
[38] C. Dong, W. Chen, C. Liu, Flocculation of algal cells by amphoteric chitosan-based flocculant, Bioresour. Technol. 170 (2014) 239–247.
[39] S. Feng, W.U. Xingqiang, C. Wang, Y. Peng, Q. Hui, Y. Liu, B. Xiao, Optimization of preparation conditions of cationic starch and its flocculation efficiency on field cyanobacteria, Environ. Sci. Technol. 41 (2018) 37–42.
[40] Y. Xu, S. Purton, F. Baganz, Chitosan flocculation to aid the harvesting of the microalga Chlorella sorokiniana, Bioresour. Technol. 129 (2013) 296–301.
[41] F. Roselet, J. Burkert, P.C. Abreu, Flocculation of Nannochloropsis oculata using a tannin-based polymer: bench scale optimization and pilot scale reproducibility, Biomass Bioenergy 87 (2016) 55–60.
[42] L. Van Haver, S. Nayar, Polyelectrolyte flocculants in harvesting microalgal biomass for food and feed applications, Algal Res. 24 (2017) 167–180.
[43] A.J. Garzon-Sanabria, S.S. Ramirez-Caballero, F.E. Moss, Z.L. Nikolov, Effect of algogenic organic matter (AOM) and sodium chloride on Nannochloropsis Salina flocculation efficiency, Bioresour. Technol. 143 (2013) 231–237.
[44] C. Dong, W. Chen, C. Liu, Flocculation of algal cells by amphoteric chitosan-based flocculant[J], Bioresour. Technol. 170 (2014) 239–247.
[45] Z. Yang, H. Wu, B. Yuan, et al., Synthesis of amphoteric starch-based grafting flocculants for flocculation of both positively and negatively charged colloidal contaminants from water[J], Chem. Eng. J. 244 (2014) 209–217.
[46] K. Pirwitz, L. Rihko-Struckmann, S. Kai, Comparison of flocculation methods for harvesting Dunaliella, Bioresour. Technol. 196 (2015) 145–152.
[47] O. Bjorkman, B. Demmig, Photon yield of O2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins, Planta 170 (1987) 489–504.
[48] Z. Kolber, J. Zehr, P. Falkowski, Effects of growth irradiance and nitrogen limitation on photosynthetic energy conversion in photosystem II, Plant Physiol. 88 (1988) 923–929.
[49] M.B. Johnson, Z. Wen, Development of an attached microalgal growth system for biofuel production, Appl. Microbiol. Biotechnol. 85 (2010) 525–534.
[50] J. Li, X. Wang, J. Wang, et al., Simultaneous recovery of microalgae, ammonium and phosphate from simulated wastewater by MgO modified diatomite[J], Chem. Eng. J. 362 (2019) 802–811.
[51] I.-C. Sheih, T.-K. Wu, T.J. Fang, Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems, Bioresour. Technol. 100 (2009) 3419–3425.
[52] U. Suparmaniam, M.K. Lam, Y. Uemura, S.H. Shuit, J.W. Lim, P.L. Show, K.T. Lee, Y. Matsumura, P.T.K. Le, Flocculation of Chlorella vulgaris by shell waste-derived bioflocculants for biodiesel production: process optimization, characterization and kinetic studies, Sci. Total Environ. 702 (2020) 134995.