BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, March 2024. Vol. **21**(1), p. 99-107

A Bioflocculant Made from Chitosan and Modified Shrimp Waste Might Collect Fresh and Saltwater Microalgae

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https://dx.doi.org/10.13005/bbra/3206

(Received: 18 November 2023; accepted: 26 January 2024)

The present study aimed to investigate the flocculation efficiency in the harvesting of microalgae cultures, specifically Scenedesmus sp, Chlorella sp, Chlorococcum sp, and Teraselimus sp, cultivated in both freshwater and marine water. This investigation involved the addition of varying dosages (ranging from 0.1 to 100 mg/L) of the bioflocculant chitosan. The specific experimental conditions included a 30-minute treatment with 10 mg/L chitosan for Scenedesmus sp, a 60-minute treatment with 10 mg/L chitosan for Chlorella sp and Chlorococcum sp, and a 60-minute treatment with 100 mg/L chitosan for Teraselimus sp. The achievement of sedimentation efficiency was observed for 60 minutes while using a dose of 8.0 mg/L chitosan at a pH level of 8.0, as a consequence of the flocculation of all four algal biomass. The utilization of chitosan as a bioflocculant under alkaline circumstances resulted in the most significant documented recovery of microalgae. Moreover, the bioimaging assay conducted to assess cell viability provides evidence that the utilization of chitosan does not result in any detrimental effects on the four microalgae cultures, even when administered at elevated concentrations. Therefore, this method is regarded as an energy-efficient and cost-effective approach to biomass harvesting, offering an alternative to traditional approaches that include the use of chemical flocculants.

Keywords: Bioflocculation; Chitosan; Harvesting; Microalgae; Modified shrimp waste.

Microalgae, both marine and freshwater, with their nutritional value can produce valuable chemical compounds [1], polysaccharides [2], pigments [3,4] and polyunsaturated fatty acids [5]. Due to their high biomass productivity, these substances have lately gained widespread recognition as feedstocks for the generation of biodiesel [6]. Harvesting the algae, which is more expensive, is a significant bottleneck in the microalgae industry. Cells range in size from 1 to 30 µm in diameter, and the concentration of biomass in the culture broth is deficient 0.5 to 2.0 g/L, depending on the cultivation techniques employed [7]. Different microalgae's flocculation capacity is influenced by a variety of elements, including age, physiological conditions, the composition of their cell walls, the amount of excretions they make, and other characteristics [8]. Since microalgae have certain features, it is essential to choose the best flocculation technique for harvesting them. Several methods are adopted for the efficient harvesting of microalgae [7], centrifugation [9], foam fractionation [10], filtration [11],

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flocculation [12] and gravity sedimentation [13]. Even more energy-intensive and costly than modern cell harvesting techniques like centrifugation and filtering [14].

Furthermore, centrifugation is the method used by current commercial systems to harvest microalgae despite the fact that it uses a lot of electricity and can break cells, releasing their contents into the medium [15]. In order to address the issues above associated with the harvesting process, it is imperative to devise a downstream procedure that is both cost-effective and highly efficient. This procedure should focus on effectively extracting microalgae cells from the growth medium while ensuring their viability and bioactivity are maintained prior to use [16]. The flocculation harvesting process is one such efficient process to minimize or overcome the barrier in harvesting the algae. Flocculation has the potential to be an efficient and practical approach for the harvesting of microalgae from vast volumes of microalgae cultures, provided that it is both economically and technologically feasible [17]. Flocculation is the formation of more giant, loosely bound conglomerates from smaller, suspended microalgal cells. Numerous types of flocculants employed, including organic polymers, bio-based flocculation, and inorganic multivalent metal salts [18].

Because of its high molecular weight and charge density, chitosan is one of the most often used bio-flocculents. It has amino groups that are positively charged (NH^{3+} and NH^{2+}), which tend to bind to negatively charged microorganisms like microalgae [19]. Chitin, the structural component of crustaceans' exoskeletons (such as crab, shrimp, and others), can be converted into chitosan via deacetylation [20]. Comparatively to inorganic flocculants such as ferric chloride, aluminum chlorides, and aluminum sulfates, waste products from the seafood industry and shellfish wastes are inexpensive sources for the commercial manufacture of chitosan. Because chitosan doesn't contaminate recovered biomass during synthesis, the products can be employed right away in the food and fuel production industries [21].

In this study, chitosan was utilized as a flocculant for harvesting freshwater microalga, *Scenedesmus sp, Chlorella sp, Chlorococcum sp,* and marine microalgae *Tetraselmis sp*. The effect of different dosages of the flocculants during a period was investigated for biomass recovery. The re-usability of the culture medium after harvesting the algae provides a promising technology for economical and low-cost harvesting of microalgae.

EXPERIMENTAL METHODS

Microalgae and culture conditions

In the current investigation, marine microalgae *Tetraselmis sp*. and freshwater microalgae *Scenedesmus sp., Chlorella sp.,* and *Chlorococcum sp.* were both used. In 5 L conical flasks containing 3 L of sterile culture media, each species' culture was cultured in triplicate. According to [23], freshwater algae were grown in Bold Basal media (BBM) and marine algae in f/2 media. Algal cultures were created by adding 100 mL of a mother culture (2×10^6 cells mL-1) that was in the exponential phase of growth. These were grown in a unialgal condition in the lab and were kept at a pH of 8.2±1, with enough light (2000 lux), temperature (26°C), and conditions [8].

Morphological observation by microscopy

Algal cultures were monitored daily under a light microscope (CH2 Oi, Olympus microscope) to observe morphological features and microbial contamination changes. Images of algal cells were photographed using a celestron digital microscope imager (#44421) USA. A hemocytometer (Neubauer Chamber) was used to directly count the cells in order to track the microalgae's growth. For each sample, the mean value of four counts was calculated.

Synthesis of modified shrimp waste chitosan solution

The shrimp waste material was modified to chitosan (powder). Chitosan in low molecular weight 50,000-190,000 Da (based on viscosity). In order to make a stock solution of chitosan, 1.0 mg/mL of chitosan flakes were dissolve in 1% (v/v) acetic acid and then thoroughly stirred with magnetic beads until the flakes were dissolved. The pH of microalgae culture was maintained at a standard pH of 8.0 and if dropped, the culture was adjusted using NaOH to reach the average pH level.

Effect of different concentrations of chitosan flocculants

All of the algal biomass was harvested using chitosan flocculants made from shrimp

waste. Different chitosan concentrations (0.1, 1.0, 10 and 100 mg/L) of other common compounds were examined. Effects of chitosan flocculation on *Scenedesmus sp., Chlorella sp., Chlorococcum sp.,* and *Tetraselmis sp.,* freshwater microalgae. The shrimp waste materials chitosan flocculants, were used to harvest all the algal biomass.

Determination of flocculation efficiency

For each microalga*, Scenedesmus sp, Chlorella sp, Chlorococcum sp,* and *Tetraselmis sp,* tiny amounts of media (50 mL) were placed in cylindrical glass tubes for flocculation studies. Chitosan flocculants were gradually introduced to accomplish flocculation for all five microalgae. After applying the flocculants, the glass tubes were thoroughly vortexed for 30 seconds and then allowed to stand at room temperature for 10 minutes. The flocculation efficiency was measured by comparing the density of surviving microalgal cells in the transparent area with the concentration before treatment. The following equation was used to obtain the flocculation efficiency (%).

Flocculation efficiency (%) = Initial AB - Final AB / Initial AB × 100%

Where A is the optical density of the initial culture medium at 665 nm and B is the optical density of the sample measured at 665 nm using a Shimadzu UV-1601 UV-Visible spectrophotometer.

Cells viability assay

Trypan blue dye, which is rejected by live cells, was used to assess the vitality of cells [24]. After each flocculated media had been flocculated by lowering the growth medium's pH to the necessary level after two hours, before discarding the supernatant, 1 mL samples of each flocculated media were centrifuged. The cells were then incubated for an additional 30 minutes at room temperature while 100 L of a 1% Trypan blue solution was added. The excess and detached pigment was then removed from the cells by washing them twice with deionized water. Finally, an optical microscope was used to check the viability of fresh preparations of the centrifuged samples. Cells stained blue by trypan blue solution that had diffused into the protoplasm of the cells seemed to have shattered cell walls.

RESULTS AND DISCUSSION

Fresh and Marine water microalgae

The most significant primary biomass is made up of marine microalgae, which are also drawing attention as a source of new metabolites and essential biotechnological genes. Divers who study marine environmental habits have discovered a wide range of microalgae. To the aquatic environment, microalgae might be the next big thing for competitive global warming and biomass remaining after the microalgal lipids have been removed to make the fuels could be converted to the nutritional feeds for domestic animals such as chickens and pigs and others, aquaculture animals such as salmon and shrimp. The most promising methods for capturing the algae produced by chemical flocculation still rely heavily on marine microalgae as a food source [22]. In comparison to freshwater microalgae, there is more economic and environmental potential when biodiesel is produced from marine microalgal species that develop quickly and contain lipids. However, just like with freshwater microalgae, the process is constrained by a variety of factors,

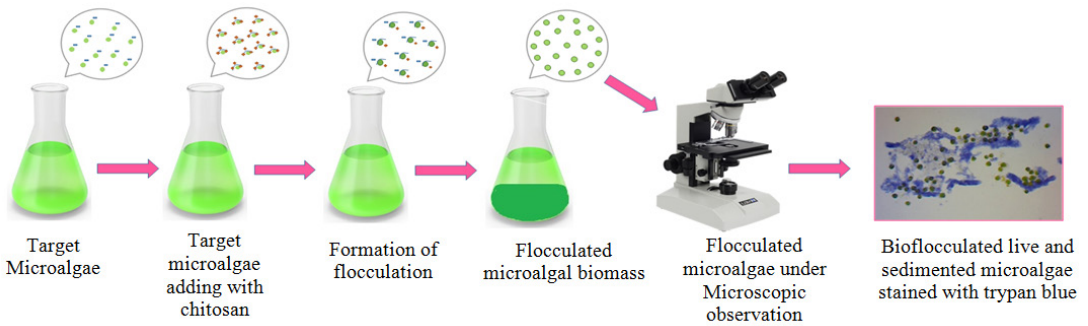

Fig. 1. Schematic diagrams illustrating the application of chitosan biofloculant in the method of harvesting algal biomass

including the small size of microalgal cells and the generally diluted nature of microalgal cultures [25]. In this investigation, the impact of chitosan dose on the flocculation effectiveness of freshwater microalgae *Scenedesmus sp., Chlorella sp., Chlorococcum sp.,* and marine microalgae *Tetraselmis sp.* was looked into (Fig. 1). Chitosan concentrations of 0.1, 1.0, 10 and 100 mg/L were investigated. An additional control experiment (one without chitosan) was conducted to serve as a benchmark for Auto-flocculation. After 30 minutes at 10 mg/L for *Scenedesmus sp.,* 60 minutes at 10 mg/L for *Chlorella sp.,* and *Chlorococcum sp.,* and 60 minutes at 100 mg/L for *Tetraselmis sp.,* the harvesting was nearly finished. (Fig: 2 & 3). The results of the experiment represent the efficiency of the chitosan. It was also evident from the experiment that increasing the chitosan concentration increases the harvesting efficiency of the algae. As the concentration of chitosan dose increases, the harvesting efficiency also increases and it takes a longer duration for very low concentration of chitosan.

Based on the previous experiment, various concentrations (1.0-10 mg/L) of chitosan were tested for 0-180 min for the freshwater microalga, *Scenedesmus sp., Chlorella sp*. and *Chlorococcum sp.* The optical density decreased with an increase in chitosan and the duration decreased. The harvesting efficiency of *Scenedesmus sp.*, was (99%) at 3.0 mg/L at 120 min, whereas it requires 8.0 mg/L at 30 min.The harvesting efficiency of *Chlorella sp*., was (99%) at 8.0 mg/L at 120 min, whereas it requires 10 mg/L at 60 min. The harvesting efficiency of *Chlorococcum sp.*, was (99%) at 7.0 mg/L at 120 min, whereas it requires 5.0 mg/L at 180 min. The harvesting efficiency of *Tetraselmis sp*., was (98%) at 50 mg/L at 90 min, whereas it requires 100 mg/L at 30 min.

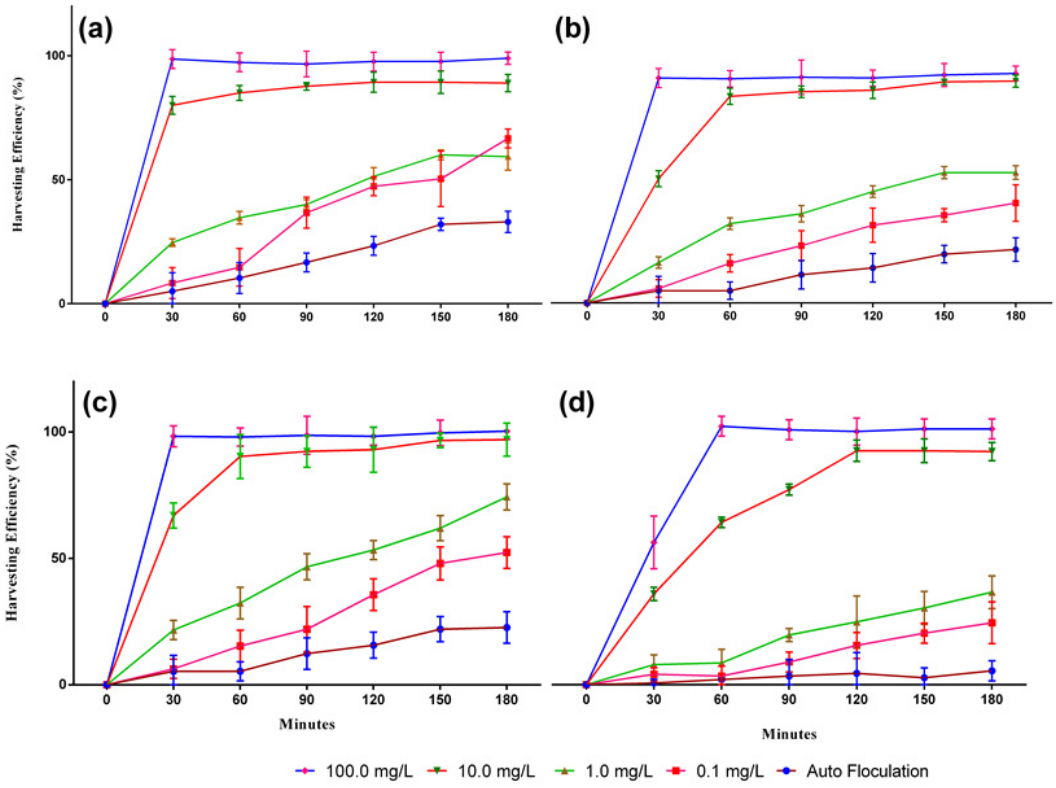

Fig. 2. Assessing the impact of different amounts of chitosan (ranging from 0.1 to 100.0 mg/L) dissolved in acetic acid on the flocculation efficiency of *Scenedesmus sp*, *Chlorella sp*, *Chlorococcum sp* in freshwater, and *Tetraselmis sp* in marine water at a pH of 8.0 during their stationary growth phase

The study findings confirm that the rapid harvest of algae can be achieved in less time if the concentration of the flocculants is increased. The experiment also noted that even at a low dose, the harvesting efficiency could be achieved by increasing the duration of time for settling. The stationary phase yielded 92% *Chlorella vulgaris* at 30 mg/L of chitosan, 300 rpm of mixing speed, and 10 minutes of paying time [26]. Within three minutes, the *Chlorella vulgaris* 120 mg/L of chitosan exhibited the best effectiveness (92%) [26]. In the flocculation of using chitosan as a flocculant, more than *Rhodomonas baltica* 75% flocculation efficiency was obtained at 80 mg/L of chitosan [27,28] found that adding 200 mg/L of chitosan to the mixture and adjusting the pH to 7.5 improved the flocculation efficiency (96–98%) of *Euglena gracilis*. However, several Tetraselmis species required a higher dosage of chitosan (150 mg/L), *Chaetoceros muelleri* required a lower dosage (40 mg/L) [29,30] found that employing a high dose of chemical coagulants, such as 1000 mg/L of $Al_2(SO4)3$ and a 6-hour incubation period, only 60% flocculation effectiveness was obtained with C. vulgaris. By lowering the viscosity and mean surface charge of algal cells, [22] proposed that a pH drop below 7.0 enhanced chitosan activity and flocculation efficiency.

The effectiveness of the process depends on the features of the algal species and the culture conditions used in each study; thus, even with extensive research, the mechanism of chitosan flocculation of algal cells is still not well known [31,32]. Chitosan has reportedly proven to be highly effective for the flocculation of freshwater algae [33,34] but not for marine microalgae [35]. Similar

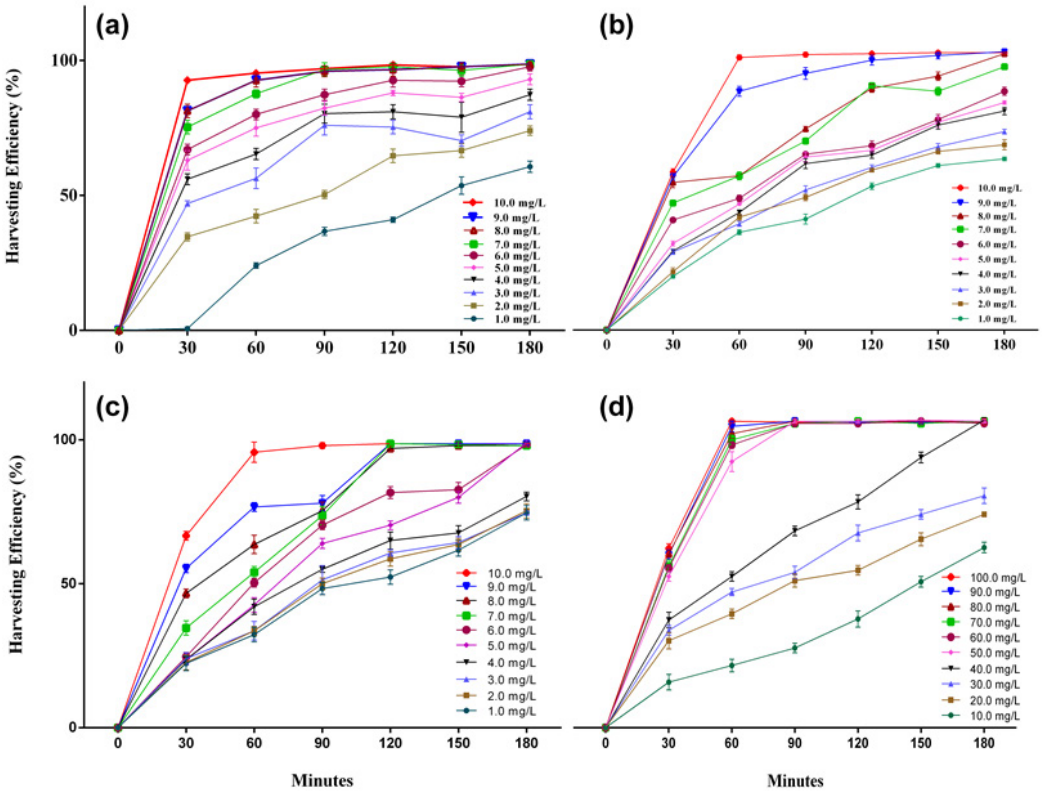

Fig. 3. The impact of different amounts of chitosan on the ability to induce flocculation in freshwater microalgae such as *Scenedesmus sp, Chlorella sp*, and *Chlorococcum sp*, as well as in marine microalgae *Tetraselmis sp* under pH 8.0 conditions, was examined. Chitosan was utilized as a flocculant within the range of 1.0 to 10 mg/L for groups a, b, and c, while a concentration of 10 to 100 mg/L was applied for group d. This study was conducted during the stationary growth stage and employed acetic acid as the solvent.

results were seen when cationic starch was used to flocculate microalgae [36,37]. Due to salinity's effect on seawater's high ionic strength. As previously indicated, some biopolymers undergo partial collapse at high ionic strength, which lessens the likelihood that they would interact with algal cells [28]. Because seawater is more salinity-sensitive than freshwater algae media, greater flocculent dosages are needed [38,35,36]. It's also critical to note that studies show that marine microalgae flocculated with chitosan had a 100% flocculation efficiency [22].

Evans blue staining was used to examine the flocculated microalgae's structural integrity. In microalgal cells, there was no evidence of cell lysis. 100 L of 1% tryphan blue dye was added to determine the vitality of the cells, and they were then incubated at room temperature for 1 hour. To get rid of the surplus unbound dye, deionized water was used to wash the incubated microalgal cells twice. The influence of different chitosan dosages on the flocculation effectiveness in binding trypan blue stain to various microalgae species was shown in Figure 4. After chitosan was added, the cells clumped together, leaving no single, distinct cell behind. The microscopic examination revealed that the main mechanism of flocculation used in the chitosan-based microalgae harvesting was bridging.

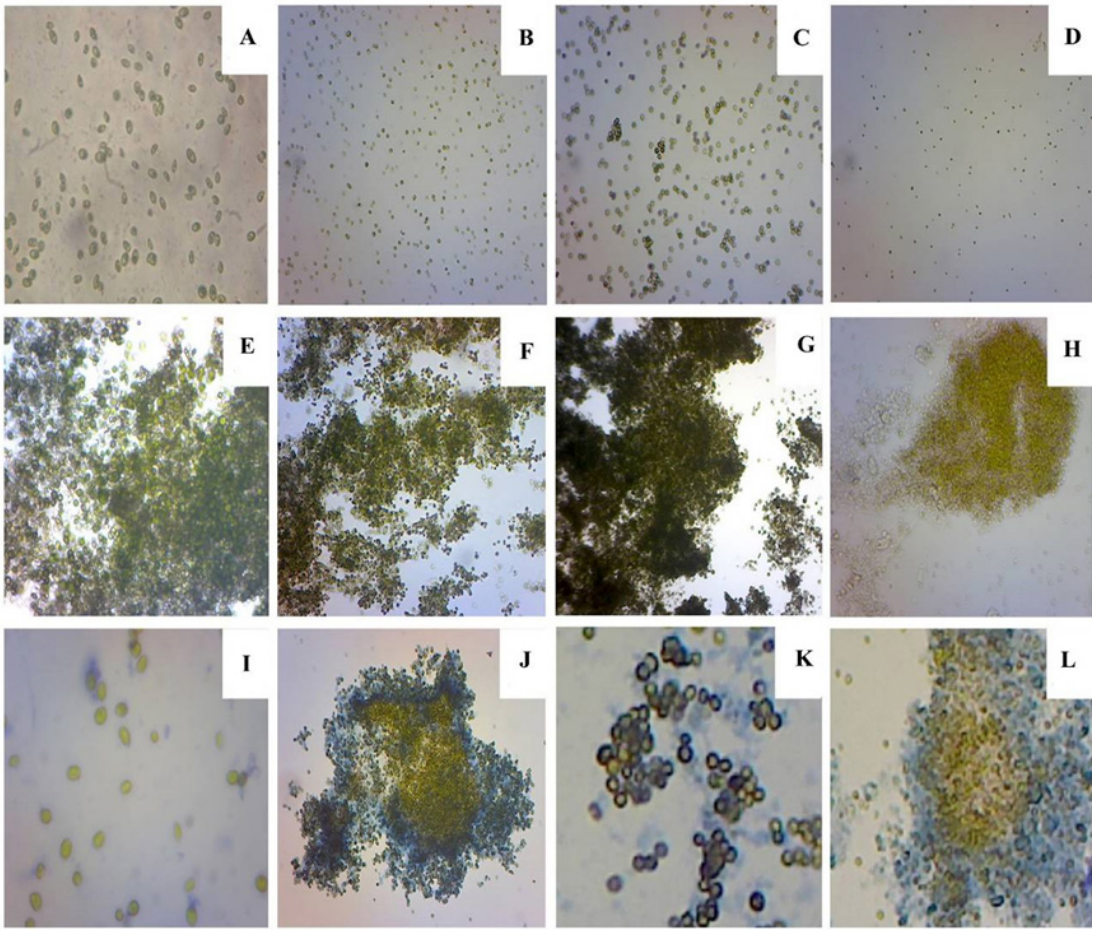

Fig. 4. The influence of different chitosan dosages on the flocculation effectiveness in binding trypan blue stain to various microalgae species was investigated. The study involved control groups with *Scenedesmus sp* (A), Chlorella sp (B), *Chlorococcum sp* (C), and *Tetraselmis sp* (D) for biomass flocculation. Additionally, trypan blue staining was performed on *Scenedesmus sp* (I), *Chlorella sp* (J), *Chlorococcum sp* (K), and *Tetraselmis sp* (L) to assess staining efficiency

The positively assigned group of chitosan enables it to adhere to the negatively charged algal surface. When the chain was long enough to attach more than one cell, algal cells were able to construct bridges between one another. As a result, there is less flocculation when the environment is acidic. An alkaline pH neutralized the positive charge, and pH 8.0 was roughly the highest neutralizing point. At this pH, the most negatively charged cells are microalgal. This led to an improvement in flocculation efficiency. The neutralization point at pH 7.9 marks the progressive disappearance of the positive control, and chitosan tends to coil and leave a residue [39,40]. Compared to algal cells, chitosan polymers are much smaller, which results in partial charge neutralization and static patch effects rather than bridging and netting as described in numerous previous articles and are more likely to be at play in the mechanism governing the flocculation of chitosan-algal cells [18,41]. Deacetylated units that are positively charged are dispersed over the entire backbone of chitosan chains in a mild acid environment, creating repelling forces between these units. The polymer is kept in an extended linear configuration by these repelling forces as opposed to a more coiled structure [42,43]. All of the aforementioned characteristics make it abundantly evident that chitosan is the best option for the algae harvesting process because it is cost-effective and ecologically benign. Chitosan requires less of dosage than inorganic metal salts because it has a more significant number of functional groups, such as free amino groups [44,45].

CONCLUSION

Chitosan exhibits considerable promise in facilitating the efficient retrieval of substantial biomass from microalgae cultivation. Enhanced flocculation efficiency can be attained through extended settling durations, even when dealing with low concentrations. Consequently, a plethora of studies have been initiated to investigate the diverse techniques employed for the flocculation of microalgae. The three primary ways of low-cost flocculation include chemical flocculation, alkaline flocculation, and polymer-based flocculation. The current work utilized chitosan, a natural bio-polymer known for its low toxicity, as a flocculant. The examination was conducted using a small quantity of chitosan. The efficacy of chitosan as a bio-flocculant has been demonstrated through its ability to reduce contamination, resulting in reduced flocculation expenses. This has subsequently led to the widespread use of chitosan as a commercially viable and efficient bio-flocculant.

ACKNOWLEDGMENTS

The authors gratefully acknowledge to University of Madras, for instrumental analysis.

Conflict of interest

We, at this moment, declare we have no conflict of interest.

Author contributions

The manuscript was revised and agreed upon by all authors.

Funding Sources

There are no funding source

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