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Evaluating Flocculation Efficiency for Harvesting *Chlorella vulgaris* Using Different Flocculants: Inorganic Salts, Natural Polymer, Carbon Sources and Probiotic Bacteria

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Abstract *Chlorella vulgaris* has received considerable attention for various applications, including biofuel production, animal feed, and wastewater treatment. Flocculation is a preferable method for efficient harvesting of Chlorella biomass. In this study, metal coagulants such as $AlCl_3$, $Al_2(SO_4)_3$, $FeCl_3$, $FeSO_4$, chitosan as a natural polymer, mollasses, wheat flour and *Bacillus Subtilis* were employed to enhance flocculation efficiency of microalgae. A series of concentrations prepared for each flocculant at different pH values was conducted to determine the optimal conditions for flocculating activities. The results showed that five flocculants - $AlCl_3$, $Al_2(SO_4)_3$, chitosan, $FeCl_3$, and $FeSO_4$, were found as efficient agents for the flocculation of *Chlorella vulgaris*, with an efficiency exceeding 98% at the optimal concentrations of 70, 150, 15, 200, and 150 mg/L, respectively. The pH 10 was found as the best condition for flocculation efficiency of Chlorella cells in the culture medium. Among the tested flocculants, chitosan is an effective agent for aggregation of microalgae *Chlorella vulgaris* due to its nontoxic nature, bio polymer composition and the requirement for a low dosage. Additionally, *Bacillus Subtilis*, a probiotic bacterium, was found to slightly enhance the flocculation efficiency after 24 hours of incubation with an inoculation percentage of 3% Bacillus culture. Further study to optimize the addition rate and incubation time of *Bacillus* bacteria is necessary to enhance the flocculation efficiency of microalgae *Chlorella vulgaris*.

Keywords Flocculation, Chitosan, Bacillus, Microalgae, Chlorella

1. Introduction

Microalgae, unicellular photosynthetic organisms, have emerged as pivotal contributors to ecological balance, industrial applications, and human well-being [1]. Among these microalgae, the genus Chlorella has received increasing scientific interest due to its unique biochemical composition, rapid growth rates and adaptability to diverse environmental conditions [2].

The biomass of the microalga *Chlorella vulgaris* primarily consists of a varied composition of essential primary and secondary metabolites, dependent on the cultivation conditions such as light intensity, salinity, medium nutrients, temperature, and pH [3]. On average,

they include protein (40–70% dry weight), lipid (5-58% dry weight), carbohydrates (12-55% dry weight), pigments (1-2% dry weight, encompassing chlorophyll, astaxanthin, lutein, β-carotene, lycopene and cantaxanthin), minerals (e.g., calcium, potassium, magnesium and zinc), and vitamins (e.g., vitamin A, B, C and E) [4-6]. The nutrient-rich composition of microalgal biomass has enabled diverse applications in health supplements, food additive, and animal feeds [7,8]. Furthermore, Chlorella species have demonstrated their capacity to effectively remove both inorganic and organic contaminants from various types of wastewater, including municipal waste water, agriculture wastewater, and food industry wastewater, as well as greenhouse gases from the industrial flue gas emissions [9-11]. By utilizing organic and nutrients source in wastewater for the growth, the production of this microalgal biomass is considered as a cost-effective and environmentally friendly approach to provide resources for the biofuel industry[7,11].

However, a major challenge in large-scale production of microalgal biomass is minimizing the cost of harvesting microalgal cell due to low cell concentrations in culture medium, and small cell size [12,13]. Currently, numerous harvesting techniques have been studied, encompassing physical methods (e.g., centrifugation, gravity sedimentation, filtration, and electroflocculation), chemical approaches (e.g., metal flocculants, chitosan, cationic starch), biological methods (e.g., bacteria, fungi) [14-16]. Among these techniques, flocculation is considered as a promising method for efficiently harvesting a large volume of microalgae culture due to its simple operation and less time- and energy- consuming approach [17,18].

The iron and aluminum salts are the most common used inorganic flocculants for flocculation of microalgae cells due to their cost effectiveness. However, they require a higher dose, and raise significant concerns regarding metal contamination and discolouration of microalgal biomass, limiting its applications for biofuel and pigment extraction [13,19]. To address concerns about quality of harvested biomass, some organic flocculants like chitosan and cationic starch have been investigated for microalgae cell flocculation, given their non-toxic, and biodegradable nature [20]. Chitosan, derived from chitin, the second most abundant natural polymer on Earth, contains numerous amino groups on its surface, and can stimulate microalgal floc formation through various mechanisms such as charge neutralization, bridging, and adsorption [21,22]. Despite being considered an effective and non-toxic flocculant, chitosan has some disadvantages such as pH dependent, and poor solubility in water, which may hinder its industrial-scale applications [23]. In addition to inorganic flocculants, and chitosan, certain microorganisms have been reported to stimulate the aggregation of microalgae cells. A 2h inoculation of activated sludges with several microalgae species, such as *Chlorella vulgaris*, Chlorella sorokiniana, Scenedesmus dimorphus, and Neochloris oleoabundans could initiate formations of microalgae-bacterial flocs. Among these microalgae, *Chlorella vulgaris* was found to incorporate into activated sludge to form microalgae-bacteria flocs with the highest efficiency, while C. sorokiniana the least efficiency [24] Citrobacteria, isolated from microalgae sewage culture system, was observed to strongly interact with Chlorella pyrenoidosa, resulting in the formation of microalgae-bacteria flocs. This interaction was facilitated through some key components, including protein, polysaccharides and carboxylic acid [25]. A recent study reported an impressive flocculation efficiency of 97.45% for Chlorella pyrenoidosa, achieved by employing both bacteria (Citrobacter) and a filamentous fungi for flocculating and harvesting this microalgae species [26].

This study aims to examine capacity of certain metal flocculants, chitosan, and *Bacillus Subtilis* in flocullation of microalgae *Chlorella vulgaris*. Four metal flocculants including $AlCl_3$, $Al_2(SO_4)_3$, $FeCl_3$, and $FeSO_4$, along with chitosan, were prepared at various concentrations and pH values to determine the optimal conditions for flocculation efficiency. Additionally, *Bacillus Subtilis* and two carbohydrate sources, molasses and wheat flour were investigated to stimulated biological flocculation of *Chlorella vulgaris*.

2. Material and Methods

2.1. Microorganism Strain and Culture Conditions

The strain of *Chlorella vulgaris* was obtained from the Institute of Applied Technology and Sustainable Development at the Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam. This microalgae strain was preserved and cultured in the Blue-Green 11 (BG11) medium, which was prepared with the composition: $NaNO_3$ (1.5 g/L), K_2HPO_4 (0.04 g/L), $MgSO_4.7H_2O$ (0.075 g/L), $CaCl_2.2H_2O$ (0.036 g/L), Citric acid (0.006 g/L), Ammonium ferric citrate green (0.006 g/L), EDTANa2 (0.001 g/L), Na_2CO_3 (0.02 g/L), H_3BO_3 (2.86 mg/L), $MnCl_2.4H_2O$ (1.81 mg/L), $ZnSO_4.7H_2O$ (0.222 mg/L), $Na_2MoO_4.2H_2O$ (0.39 mg/L), $CuSO_4.5H_2O$ (0.08 mg/L), and $CoCl_2.6H_2O$ (0.05 mg/L). Each chemical was prepared in a separated stock solution, except for the stock solution of trace metal mix consisted of H_3BO_3, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, and $CoCl_2.6H_2O$. The prepared medium was autoclaved at 121°C, for 15 min for sterilization. *Chlorella vulgaris* was cultured at the temperature of 25 ± 2 ℃, under continuous air aeration, and white fluorescence with the light intensity of 2500 lux. The density and possible contamination of Chlorella culture were monitored daily by observation under an optical microscope (Fig. 1).

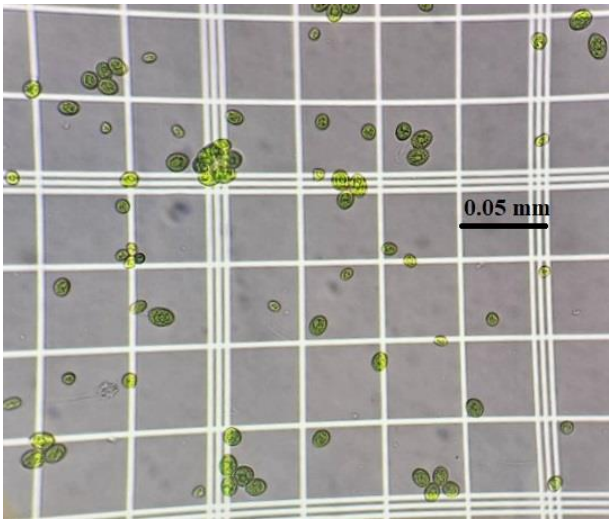

Figure 1. Microscopic picture of *Chlorella vulgaris* cells cultured in the medium of BG11

The strain of *Bacillus Subtilis* was provided from the Center for Science and Biotechnology, at the University of Sciences, Vietnam National University Ho Chi Minh City, Vietnam. Bacteria were regularly preserved and cultured in the LB medium (Himedia, India) broth containing tryptone (10g/L), yeast extract (5 g/L), and NaCl (10g/L), or LB solidified with 1.5% agar (Himedia, India). For sterilization, the LB medium was autoclaved at 121°C, for 15 min. *Bacillus Subtilis* was cultured at the temperature of 37 ±1 ℃, and for 24h.

2.2. Effects of Inorganic Salt, and Chitosan on Flocculation of *Chlorella vulgaris*

The flocculation of microalgae *Chlorella vulgaris* were examined using some inorganic salts such as $FeCl_3$ (Chemsupply, Australia), $FeSO_4$ (Chemsupply, Australia), $Al_2(SO_4)_3$ (Sigma-Aldrich, Australia), $AlCl_3$ (Sigma-Aldrich, Australia), and chitosan (Sigma-Aldrich, Australia). A series of working concentrations for $FeCl_3$, and $FeSO_4$ were investigated as 0, 50, 100, 150, 200, and 250 mg/L; while those for $AlCl_3$, and $Al_2(SO_4)_3$ were 0, 10, 30, 50, 70, and 100 mg/L. Each of these inorganic chemicals was prepared in the 100-fold stock solutions using deionized water from a Milli-Q IX 7015 Pure Water Purification System (Merck, Germany). For chitosan, several working concentrations were examined such as 0, 5, 10, 15, 20, and 30 mg/L, and its 100-fold stock solution was prepared in 0.1% HCl. All the stock solutions were stored at room temperature and used for further experiments within 1 week. The natural polymer flocculation used in experiments was chitosan (95% purity, Chitosan Kien Giang company, Vietnam).

To examine the effects of these chemicals on the flocculation of *Chlorella vulgaris*, the microalgae strain was initially inoculated at the rate 5% into 1.5L fresh BG11 medium contained in a 2L glass. Chlorella microalgae was grown in the culture conditions described above for 7-10 days until the value of optical density (OD) at the wavelengths of 680nm reached 0.8, which the cell density was approximately 106 cells/ml. Next, the 100-fold stock solutions for each investigated flocculants were gently added with the volume of 15 ml into the cultured microalgae bottle. The air aeration was kept running for 10 min to allow a complete mixing. After that, the mixture was transferred into a 100ml cylinder kept still on the flat surface for 30 min to allow the flocculation of microalgae occur. The OD values at 680nm were measured for the samples before an addition of the flocculants and for the samples after flocculation time in order to calculate the flocculation efficiency according to the following equations:

$$Flocculation\ \ efficiency\ \ (\%) = \frac{(OD_{before} - OD_{after}) \times 100}{OD_{before}}$$

To examine the effect of pH on the flocculating activities of $AlCl_3$, $Al_2(SO_4)_3$, chitosan, $FeCl_3$, and $FeSO_4$, the optimal concentrations of these flocculants determined in the previous experiments were prepared. Subsequently, the pH values were adjusted using 2M HCl (Sigma-Aldrich, Australia) and 2M NaOH (Sigma-Aldrich, Australia) solutions. Four pH values, namely pH 8, 9, 10, and 11, were tested for each flocculant.

2.3. Effects of *Bacillus Subtilis*, Wheat Flour and Molasses on the Flocculation of *Chlorella vulgaris*

Chlorella vulgaris was grown in a 2L glass bottle containing 1.5 L BG11 medium at the culture condition described above for 7-10 days until the OD value at the wavelengths of 680 nm reached 0.8, corresponding to a cell density of approximately 10^6 cells/ml. *Bacillus Subtilis* was grown at 37°C in the LB medium broth for 24h until the OD value at the wavelength of 600 nm reached 1.0, indicating a cell concentration of approximately 10^8 cells/ml. Subsequently, Bacillus culture both was added into the 1.5L culture volume of microalgae with the rate of 0, 0.5, 1.5, and 3% of volume. Besides, wheat flour and molasses, two carbon sources regularly used for biofloc formation, were employed to add into microalgae culture with the rate of 0, 0.5, 1.5, and 3% microalgae volume. After supplemented with *Bacillus Subtilis*, wheat flour (Meizan, Vietnam), or molasses (Thien Thao Han company, Vietnam), the *Chlorella vulgaris* were continuously maintained at the microalgae culture conditions for 24h before flocculation analyses. The rate of flocculation was determined in the same method described in the section of 2.2.

2.4. Data Analysis

For each tested flocculants, at least 4 concentrations and

control (no additions) were used in an experiment investigating their effects on the flocculation of *Chlorella vulgaris*. Each flocculant concentration was performed in triplicate. The measurement of OD value at 680nm were performed in duplicate. The average data was expressed in the tables using Microsoft Excel 2016 and figures using the software of Veusz (version 3.6.2, 2023).

3. Results and Discussion

3.1. Effect of Metal Coagulants and Chitosan on the Flocculation of *Chlorella vulgaris*

The flocculating efficiency of *Chlorella vulgaris*, which is useful for the harvest process of this microalgae, was evaluated in an experiment supplemented with several flocculants including $AlCl_3$, $Al_2(SO_4)_3$, $FeCl_3$, $FeSO_4$, and chitosan, as shown in the Fig. 2 and Table 1. Generally, all these chemicals were observed to significantly induced flocculation of microalgae with the efficiency of more than 95%. Among the tested flocculants, chitosan was found as the best one with the flocculating efficiency of 98% at the concentration of 15 mg/L, which was much lower in comparison with all metal coagulants. The flocculation efficiency of chitosan in this study was a little lower than that in a study in Malaysia, which reported a flocculating percentage of 99.3% for Chlorella microalgae at the chitosan concentration of 10 ppm [27]. It is believed that an interaction between positively charged function group on chitosan molecule and negatively charged microalgae cell leads to charge neutralization and formation of flocs [28-30]. The concentration of chitosan required for efficient flocculation of microalgae in sea water was found to be higher than that in the freshwater [31]. Perhaps, the presence of different abundance and types of salts in the microalgae culture medium affected on the concentration of chitosan required for flocculation. Following chitosan, the $AlCl_3$ showed a good flocculation with the efficiency of 94.7% at the concentration of 70 mg/L, while the $Al_2(SO_4)_3$, $FeCl_3$, and $FeSO_4$ shared a similar removal efficiency from 93 to 95% at the concentration from 150 to 250 mg/L (Fig. 2, Table 1). This result suggested that among the tested metal coagulants, the $AlCl_3$ was the best flocculants for harvesting the microalgae *Chlorella vulgaris*.

Table 1. The optimal concentrations of chemicals for the flocculation

Chemicals	Concentration (mg/L)	Flocculation efficiency (%)
$AlCl_3$	70	94.7
$Al_2(SO_4)_3$	150	91.0
Chitosan	15	98.0
$FeCl_3$	200	95.0
$FeSO_4$	150	93.0

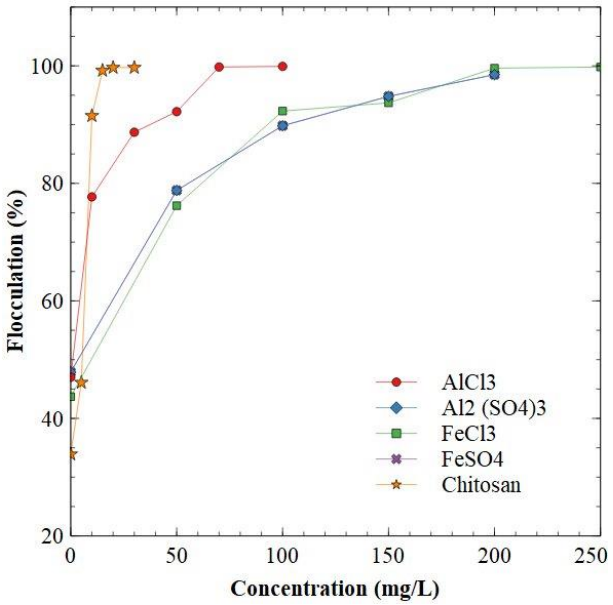

Figure 2. Effect of inorganic and chitosan flocculants on the flocculation of *Chlorella vulgaris*

3.2. Effect of pH on the Flocculation of *Chlorella vulgaris by $AlCl_3$, $Al_2(SO_4)_3$, Chitosan, $FeCl_3$, and $FeSO_4$*

The flocculation of *Chlorella vulgaris* can be influenced by various factors, with pH playing an important role in modulating this phenomenon [32]. In the context of employing different flocculants such as $AlCl_3$, $Al_2(SO_4)_3$, Chitosan, $FeCl_3$, and $FeSO_4$, understanding the effect of pH becomes useful to explain the mechanism and determine the optimal conditions for flocculation of *Chlorella vulgaris*. Table 2 showed the flocculation efficiency of *Chlorella vulgaris* under varying pH conditions using different flocculants such as $AlCl_3$, $Al_2(SO_4)_3$, Chitosan, $FeCl_3$, and $FeSO_4$, which were supplemented with their optimal concentrations determined in the above experiment, including 70, 150, 15, 200, and 150 mg/L, respectively. At pH 8, $AlCl_3$ exhibited a significant efficiency of 63.98%, while $Al_2(SO_4)_3$, Chitosan, $FeCl_3$, and $FeSO_4$ showed lower efficiencies ranging from 22.14% to 43.16%. As the pH increases to 9 and 10, the flocculation efficiency improves dramatically for all flocculants, reaching peak values at pH 10. Notably, flocculation efficiency of $AlCl_3$, $Al_2(SO_4)_3$, chitosan, $FeCl_3$, and $FeSO_4$ were higher than 90% at pH 10. However, at pH 11, decline in efficiency was observed for $AlCl_3$, $FeCl_3$, and $FeSO_4$, while the flocculating activity were maintained higher than 90% for $Al_2(SO_4)_3$, and chitosan. One of the main mechanisms causing flocculation of microalgae is charge neutralization of the cell surface, which diminishes the repulsive force between microalgae cells, thus stimulates attractive forces between neutralized microalgae cell and the cationic flocculants [32,33]. The difference in pH can alter the charge on both microalgae cells and flocculants. At certain pH values, the microalgae cells may carry a net positive or negative charge, affecting

their repulsion or attraction to the charged flocculants. A recent study reported that the zeta potential for *Chlorella vulgaris* cell become more negative when pH in the medium increased [17]. It reached a highest negative value at pH 10 and gradually reduce its negative charge when the pH value increased to 11. It indicated that the highest quantity of functional groups on the *Chlorella vulgaris* cell surface will be negatively charged at the pH 10, and thus be neutralized by the cationic flocculants in the solution, which resulted in flocculation of the microalgae cells. Besides, it was reported that in the pH range of 6-10, cationic ions of the used flocculants, for examples aluminium ions, are more soluble and positively charged at the lower pH conditions, and tend to change to less soluble and positively charged at a higher pH [17]. In our study, all coagulants including $AlCl_3$, $Al_2(SO_4)_3$, Chitosan, $FeCl_3$, and $FeSO_4$ were used at their optimal dosages and showed the highest efficiency at the pH 10. These findings indicated the importance of pH optimization for enhancing the flocculation of *Chlorella vulgaris* using specific flocculants.

3.3. Microscopic Observation of Flocculation for *Chlorella vulgaris* Using Inorganic Salts and Chitosan

The flocculation phenomenon of the microalgae *Chlorella vulgaris*, stimulated by various flocculants under pH 10 condition, was observed through an optical microscope, as illustrated in Figure 3. In general, Chlorella cells significantly formed aggregates and clumps in the culture medium supplemented with tested flocculants, such as $AlCl_3$, $Al_2(SO_4)_3$, Chitosan, $FeCl_3$, and $FeSO_4$, at their optimal dosages and a pH of 10, in comparison to the control without any added chemicals. Among the flocculants used, chitosan appeared to be the most effective in supporting the flocculation of Chlorella cells, as evidenced by the formation of the largest and thickest clumps in both the culture medium and under the microscope (Fig. 3 D1 and D2). Following chitosan, aluminum sulfate $Al_2(SO_4)_3$ stimulated a substantial cloud of microalgae cells in the culture medium, although the cloud was not as dense as the one formed by chitosan (Fig. 3 C1 and C2). The flocculants $AlCl_3$, $FeCl_3$, and $FeSO_4$ were effective in forming aggregates of chlorella cells in the solution; however, these clumps were smaller than those formed by chitosan and aluminum sulfate (Fig. 3 B1, E1, F1, B2, E2, F2). Chitosan, a natural polysaccharide derived from chitin, has been reported for its ability to stimulate the flocculation of microalgae cells. Some main mechanisms by which chitosan induces flocculation were widely studied such as charge neutralization, and polymer bridging [34]. Our results indicated that the chitosan is an effective agent for flocculation of microalgae *Chlorella vulgaris* due to it is a nontoxic nature, being bio polymer and required low dosage.

Table 2. Effect of pH on the flocculating activities of $AlCl_3$, $Al_2(SO_4)_3$, Chitosan, $FeCl_3$, and $FeSO_4$

	Average flocculation efficiency (%)				
pH	**$AlCl_3$ (70 mg/L)**	**$Al_2(SO_4)_3$ (150mg/L)**	**Chitosan (15mg/L)**	**$FeCl_3$ (200 mg/L)**	**$FeSO_4$ (150 mg/L)**
8	63.98	29.69	43.16	33.16	22.14
9	90.71	44.29	76.12	80.00	60.31
10	99.59	98.78	98.16	95.00	93.06
11	53.06	95.51	95.41	55.82	54.08

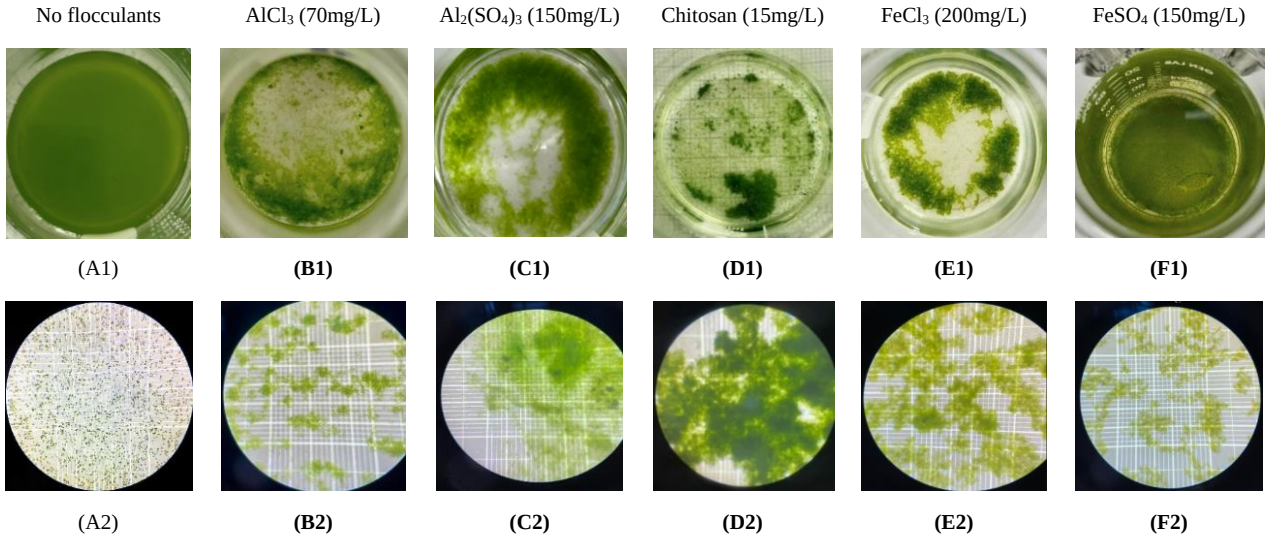

Figure 3. Flocculation of *Chlorella vulgaris* enhanced by different flocculants observed by naked eyes (A1, B2, C1, D1, E1, F1) and under a microscope (A2, B2, C2, D2, E2, F2). No flocculants (A1, A2); 70mg/L $AlCl_3$ (B1, B2); 150 mg/L $Al_2(SO_4)_3$ (C1, C2); 15 mg/L Chitosan (D1, D2); 200 mg/L $FeCl_3$ (E1, E2); 150 mg/L $FeSO_4$ (F1, F2)

3.4. Effect of *Bacillus Subtilis*, Wheat Flour, and Molasses on the Flocculation of *Chlorella vulgaris*

Bacteria have been reported to engage in biological interactions with microalgae cells that significantly influence the flocculation of microalgae, thereby impacting the harvesting of *Chlorella vulgaris* [26,35]. In this study, the effects of *Bacillus Subtilis* were explored alongside two commonly used carbon sources that support bacterial growth, namely wheat flour and molasses. The results, as shown in Fig. 4, reveal a noteworthy trend in the flocculation efficiency of *Chlorella vulgaris* in response to varying concentrations of *Bacillus Subtilis*. Specifically, the flocculation efficiency increased from 18% to 30% as the addition percentage of *Bacillus Subtilis* rose from 0% to 3% of volume. This observed trend suggests a potential correlation between bacterial concentration and enhanced microalgae flocculation. In the case of wheat flour and molasses, both of these carbon sources exhibited a similar pattern in the flocculation of microalgae. Flocculation efficiencies were induced at the addition percentage of 0.5% for both carbon sources but experienced a notable reduction at higher addition (1.5% and 3%), as illustrated in Fig. 4. At the percentage of 0.5%, molasses emerged as the better carbon source, inducing a higher flocculation percentage (45%) compared to wheat flour (32%) in promoting the formation of Chlorella flocs. Wheat flour and molasses, the two carbon sources employed for growing microbial community in the aquatic environments, are widely applied in biofloc technology to enhance water quality of the aquaculture cultivation pond and to reduce feed cost [36,37]. The primary mechanism behind biofloc formation involves the extracellular polymeric capsule of the microbial cell, which facilitates the binding of biofloc components in the aquaculture ponds, such as microbial communities, and metabolites [38–40]. A high density of microorganisms can increase the likelihood of bacterial aggregation, thereby stimulating the formation of bioflocs that include microbial cells, organic solid suspense, and zoo/phyto-planktons in the aquatic environment. Our results indicated that while both wheat flour and molasses can stimulate the biofloc formation, addition of *Bacillus Subtilis* represents a promising method for enhance biofloc formation, with the flocculation efficiency increasing from 18% to 30% as the bacteria addition rose from 0 to 3%. These findings provide valuable insights into the optimization of bacterial and carbon source parameters for efficient bioflocculation processes in microalgae harvesting systems.

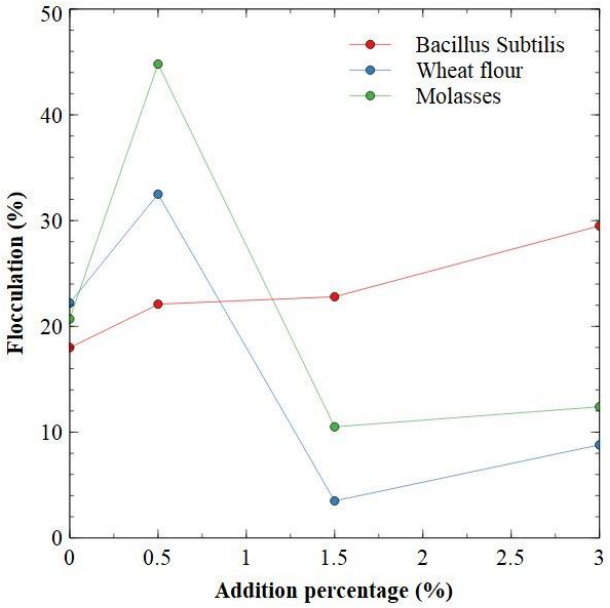

Figure 4. Effect of *Bacillus Subtilis*, wheat flour, and molasses on the flocculation of *Chlorella vulgaris* after 24h incubation

4. Conclusions

As microalgae have been widely applied in various fields, ranging from renewable energy to environmental remediation and human health, efficient flocculation of microalgae represents a cost-effective and environmentally sustainable method for harvesting and processing microalgae in a range of industrial applications. In this study, five flocculants, including $AlCl_3$, $Al_2(SO_4)_3$, chitosan, $FeCl_3$, and $FeSO_4$ were proved to be efficient agents for the flocculation of microalgae *Chlorella vulgaris*, with optimal concentrations of 70, 150, 15, 200, and 150 mg/L, respectively. In the pH range from 8 to 11, the optimal flocculation efficiency for Chlorella occurred at a pH value of 10, corresponding to the literature-reported most negative charge on the Chlorella cell surface in the solution. In addition to these flocculants, the *Bacillus Subtilis* contributed to a slight improvement in Chlorella cell flocculation, achieving an efficiency of up to 30% after 24 h incubation with a 3% addition of Bacillus. Given that *Bacillus Subtilis* is a probiotic bacterium with numerous benefits for aquaculture animals, such as improving growth performances, disease resistance, and water quality, the flocculation of microalgae by Bacillus presents a promising method for harvesting microalgae cells applicable to the aquaculture industry.

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