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Algal-algal bioflocculation enhances the recovery efficiency of *Picochlorum* sp. QUCCCM130 with low auto-settling capacity

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ABSTRACT

Large-scale production of microalgal biomass is still considered non-viable due to the high cost and energy required for harvesting. A fast, cost-effective, and successful harvesting technique has become widely sought after in microalgal biotechnology applications. Algal-algal bioflocculation was adopted for the current study, pH and the ratio between species were selected as two parameters to be optimised. *Picochlorum* sp. QUCCCM130, *Nannochloris* sp. QUCCCM31 and *Tetraselmis* sp. QUCCCM50 presenting a cell size of ~2 μm, ~5 μm, and ~15 μm, respectively, were selected to be subjected to the harvesting optimisation experiments. Results showed that self-settlement capacity increased with cell size and can be indirectly related to a decreased zeta potential of larger cells which enhances the Van der Waals attractive forces. Furthermore, it was noted that pH enhanced the self-settlement capacity of small-sized cells as well that are unable to self-settle. Algal-algal bioflocculation efficiency is dependent on the ratio between species with different sizes, increasing with a higher proportion of larger size microalgal cells. Mixing three microalgae together at pH 10 led to the appearance of large flocs in which the larger cells surrounded the smaller cells. Microscopic observation confirmed that *Tetraselmis* sp. held the small cells inside the flocs using their flagella. Thus, we can conclude that mixing microalgal cells in a specific ratio and at a specific pH increases the recovery efficiency of small-sized microalgae that can be difficult to harvest, such as *Picochlorum* sp. QUCCCM130.

1. Introduction

Microalgal biomass has been very well exploited as feedstock for several applications, including food, animal feed, cosmetics, and pharmaceuticals [1]. This is due to its potential for producing high-value products with multiple health benefits in addition to the primary metabolites [2]. However, producing microalgae at a commercial scale for bioproducts is still considered unviable due to the high cost which can reach up to 5 $/70 kg [3]. One of the major challenges includes harvesting of microalgal biomass, limiting the sustainability of microalgal biomass production.

As microalgal cells have low density and size, high capital costs and energy expenditure is usually required to harvest cells from aqueous solutions to dry matter. According to Musa et al., [4], harvesting costs represent the largest component of the total cost required for producing algal biomass using an open raceway pond (ORP). Several studies have shown that harvesting costs can account for 20–30 % of total production costs, and in some cases, 50–60 % [5,6]. As a result, developing a cost-effective process for harvesting microalgal biomass has become crucial for sustainable biomass production.

Several technologies have been tested during the last decade such as cross-flow filtration [7], centrifugation, flotation, electrical based, and flocculation [8]. Interestingly, flocculation is considered the most advanced and cost-effective technology for harvesting microalgae [9]. There are many mediums used to induce flocculation of cells, such as direct current, high frequency ultrasound and smart and precise mixing strategy [10,11], metal and magnetic coagulants [12] and polymers such as chitosan and cationic polyacrylamide, which enhance cell agglomeration [4,6]. Overall, electrocoagulation or ultrasound are known to be efficient for harvesting certain microalgal species but are associated with contamination by metal electrodes and high cost [13,14]. Therefore, the development of a high-efficiency and cost-effective harvesting process becomes vital for sustainable biomass production.

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Bioflocculation is considered the safest and most cost-effective technique for harvesting microalgal biomass, since it can result from self-settling, and involves other microorganisms such as bacteria, fungus or algae [15,16] and/or bio-flocculants such as extracellular polymers [17,18]. However, it is also influenced mainly by factors such as: the small size of the microorganisms (5–50 μm), negative surface charge high pH requirements [8] and low biomass concentration (varying from 0.5 to 5 g/L) [19]. Moreover, the persistence of the coagulants in the final harvested biomass could limit its potential applications. Furthermore, increasing the pH of the medium is known to cause cells to flocculate and can be a cheaper method overall. This technique removes the need for introduction of foreign coagulating agents which may compromise the harvested biomass quality, and showcases similar harvesting efficiency as other methods [3]. For example, *Chlorella* sp. had a flocculation efficiency of 98.9 % in a medium containing 7 g L^{-1} $NaHCO_3$ [20]. Additional studies are required to improve the bioflocculation efficiency and scalability for successful industrial production of microalgal biomass.

Overall cell density, cell surface charge, concentration of nutrients and pH are determining factors for efficient bioflocculation Recent studies have proved that bioflocculation can be enhanced by altering the pH [21] or by increasing the cell density [22]. Furthermore, Salim et al., [23] demonstrated that the algae-algae flocculation is species and cell-ratio dependant. Kawaroe et al. [24] proved that mixing self-settling species (*Tetraselmis suecica*) with smaller celled species (*Chlorella* sp. and *Nanochloropsis* sp.) can enhance the recovery efficiency (16.2 % and 21.91 % in the first hour respectively) and reduce the energy required for cell harvesting while being an environmentally friendly alternative. Therefore, the implementation of algal-algal bioflocculation, with a specific ratio of flocculating and non-flocculating microalgae, could reduce energy consumption, and maintain the quality and safety of biomass. It is one of the most promising harvesting technologies for commercial algae production [25]. However, it involves the additional cultivation of self-flocculating microalgal species [23].

In the present study, for the first time both key factors influencing the algal-bioflocculation, the pH and cell ratio were combined. Three genera of various cell sizes were used in this investigation: *Tetraselmis* sp., *Nannochloris* sp., and *Picochlorum* sp. These unicellular chlorophytes presented a size of 15 μm, 5 μm, and 2 μm respectively. The selection of these species was based on three main factors: (i) their abundance on the Qatari coastline [26]; their thermo- and halotolerance capacities; and (iii) their growth performance and metabolite production [16]. Moreover, these strains generate a high concentration of lipids and proteins in addition to their ability to produce high-value products such as omega 3 and omega 9 [16]. In this experiment, a co-culture was also assembled. The recovery efficiency was determined during the incubation time and a morphological survey was also performed for the different conditions studied.

2. Materials and methods

2.1. Microalgal strains: identification and phylogeny

The strains used for the current study were locally isolated from the Qatari coastline and belong to the Qatar Culture Collection of Cyanobacteria and Microalgae (QUCCCM). *Tetraselmis* sp. (QUCCCM50), *Nannochloris* sp. (QUCCCM31), and *Picochlorum* sp. (QUCCCM130) (Table 1). The first two strains were isolated and identified by Saadaoui et al. [26]. QUCCCM130 was identified in the current study via 18S ribosomal DNA (rDNA) polymerase chain reaction (PCR) sequencing as described by Saadaoui et al. [26]. The genomic DNA of the strain was isolated then quantified. 10 μg of pure DNA was used to perform PCR amplification of the 18S rDNA gene using the following primers: "EAE3 (5′ TCGACAATCTGGTTGATCCTGCCAG 3′) and N1200R (5′ AACATCTAAGGGCATCACAGAC CTG 3′). The PCR cycles were performed as per the following program: an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at 54 °C and 1 min 30 s at 72 °C and a final extension step of 10 min at 72 °C. The PCR fragment was later purified from the agarose gel using Minielute Gel Extraction kit (Qiagen, USA) then quantified prior to being sequenced using Genetic Analyzer 3500 (Applied Biosystems, California, USA). The GenBank Accession number of the PCR fragment is MG149785. This sequence was used to perform a BLASTN. The closest sequences were selected to perform multiple alignment via Muscle (Edgard 2004). Phylogenetic and molecular evolutionary analyses were conducted using MEGA X [27,28].

Table 1
Description of the local microalgae isolates.

Strain name	Nature	Location of the isolation	Genbank accession #	Molecular classification	Reference
QUCCCM31	Marine	Qatar coastline	KM985399	*Nannochloris* sp.	[26]
QUCCCM50	Marine	Qatar coastline	KM985410	*Tetraselmis* sp.	[26]
QUCCCM130	Marine	Qatar coastline	MG149785	*Picochlorum* sp.	Current Study

2.2. Algae cultivation and growth assessment

The unialgal isolates have been maintained on solid f/2 growth media and incubated in an illuminated growth chamber (MRL-351/351H, Sanyo, Japan) under a temperature of 30 °C, a relative humidity (RH) of 50 % and an illumination of 100 μmol photon m^{-2} s^{-1} of light with a light-dark cycle of 12:12 h. A single colony of each strain was used to inoculate 10 ml of f/2 growth medium [29] and then incubated for 7 days in an illuminated shaker (Innova 44R, New Brunswick Scientific, USA) at 150 rpm and 30 °C with 100 μmol photon s^{-1} m^{-1} of light and a light-dark cycle of 12:12 h. The pH was fixed at 8 as it is known to be optimal for marine algal growth. Each culture was then scaled up to 100 ml then 1 l and incubated under the same previously described conditions for 12 days. The growth of the three microalgal isolates was monitored via optic density at 750 nm (OD_{750nm}) determination using a spectrophotometer (Jenway – #7200, UK). Growth rate was calculated with the following equations as it was described by Schoen and Guillard [30]

$$\text{Growth rate (u) is}: \mu = \ln X2 - \ln X1\ t2 - t1$$

where X1 and X2 are optic densities at times t1 and t2.

2.3. Morphological characterisation of the microalgal isolates

The morphology of the microalgal isolates was examined using light microscopy (Carl Zeiss with 100× magnification). The flocs of the mixed microalgae were viewed using the same microscopy and magnification.

2.4. Bioflocculation

The three selected microalgal isolates, QUCCCM50, QUCCCM31 and QUCCCM130, were cultivated separately using f/2 growth medium under the previously described conditions of temperature, light, and agitation. After a specific incubation time of 13 days or 15 days, an adequate volume of each algal culture was transferred to a new Pyrex glass beaker allowing an OD_{750nm} of 0.5, prior to being used for the flocculation assay. Then, the final volume in the Pyrex glass beaker was adjusted to 50 ml and the initial OD_{750nm} of the mixture was fixed at 0.5 as described by Salim et al. [23]. After a gentle and brief mixing, the 50 ml culture was incubated for 180 min at room temperature without agitation to study the spontaneous sedimentation capacity of each strain. One ml of supernatant was collected from the middle of the flask prior to being subjected to an assessment of the optical density OD_{750nm} at different time intervals of 30, 60, 90, 120, 150 and 180 min.

The recovery efficiency was determined using the following equation published by Salim et al. [23]:

$$\text{Recovery } (\%) = [OD_{750}(t_0) - OD_{750}(t)]/OD_{750}(t_0)*100$$

where OD_{750nm} t_0 corresponds to the initial OD_{750nm} of the culture before incubation (= 0.5), and OD_{750nm} (t) corresponds to the OD_{750nm} of the culture after the different incubation times (30, 60, 90, 120, 150 and 180 min).

To select the optimal pH for settlement, three different pH levels (8, 9, and 10) were tested for the smallest and largest cell-size strains, QUCCCM50 and QUCCCM130, respectively. The pH was adjusted by adding the required volume of NaOH (1 M) or HCl (3 N). The recovery efficiency was determined as described above. The optimal pH was then adopted for the bioflocculation experiment.

To enhance the recovery efficiency of the smaller celled species *Picochlorium* sp., we mixed it with QUCCCM50 possessing the largest cell size and highest capacity to self-settle. This mixing of monocultures was named algal-algal bioflocculation. For this purpose, an adequate volume was taken from both monocultures of 12 days allowing the following 'QUCCCM130/QUCCCM50' ratios of 20:80; 50:50 and 80:20. The final volume in the Pyrex glass beaker was also adjusted to 50 ml and the initial OD_{750nm} was fixed at 0.5 as it was described by Salim et al. [23]. After a gentle mixing of the cultures, the Pyrex glass was kept at room temperature. Next, an assessment of the OD_{750nm} of the supernatant was performed at the same previously described time intervals and the recovery efficiency was determined as previously described.

To further enhance the recovery efficiency of microalgal biomass, a third strain, *Nannochloris* sp. (QUCCCM31) was added to the mixture of the monocultures of QUCCCM130 and QUCCCM50. The optimal pH for harvesting was used for this algal-algal bioflocculation experiment, and pH 8 was used as control. Three replicates were performed for each single condition.

2.4.1. Lipid and protein characterisation

The total protein and lipid content were calculated for the three strains separately and in the various ratio of mixtures of the three microalgae.

2.4.1.1. Total lipid extraction. Lipid extraction method described by Folch et al. [31] with modifications by Saadaoui et al., [26] was used. The lipid content was calculated using the equation: Lipid content (%) = Total lipids (g) / Dry biomass (g).

2.4.1.2. Total protein extraction. 50 mg of dried algal biomass was hydrolyzed using 8 ml of sodium hydroxide (NaOH 0.1 M). The lysate was collected and centrifuged at 14,000 rpm for 10 min after overnight incubation at 60 °C. The supernatant was collected and used to determine the total protein content using the assay described by Lowry et al. [32].

2.5. Co-cultivation of the microalgal isolates

The co-culture of *Tetraselmis* sp. and *Picochlorium* sp. QUCCCM50 and QUCCCM130 was performed as follows. Inoculating loop was used to acquire algal biomass of equal amounts from both species separately. The biomass from both of these species was then added to a single flask containing 100 ml fresh growth medium. Equal cell density was used from both strains to inoculate a fresh growth medium allowing an initial OD_{750nm} of 0.1 in a final volume of 100 ml. This co-culture was incubated under the same previously cited condition for 13 and 15 days. Optical density was assessed during the growth. Then, the co-culture was stopped, and an adequate volume was transferred to Pyrex glass beaker allowing an initial OD_{750nm} of 0.5 in a final volume of 50 ml prior to being subjected to spontaneous sedimentation. 1 ml was used from the culture to measure the OD_{750nm} every 20 min. The duration of the experiment was also 180 min. Three replicates were performed for each single condition.

2.6. Statistical analysis

The means and standard deviation were determined using Microsoft Excel and the correlation Analysis was carried out using SPSS. All experiments were performed in triplicate. One-way ANOVA was used to determine a significant difference ($p = 0.05$) between the means of independent conditions. Analysis was conducted in R [33] and figures were produced using the package ggplot2 [34].

3. Results

3.1. Impact of cell morphology on the self-settlement capacity of the microalgal isolates

The morphological characterisation of QUCCCM50, QUCCCM31and QUCCCM130 using light microscopy showed decreasing cell sizes of ~15 μm, ~5 μm, and ~2 μm, respectively (Fig. 1). These strains were tested for their spontaneous settlement at pH 8, as this represents the optimal pH for cultivation. After 180 min of incubation QUCCCM50 presented the highest spontaneous recovery efficiency at 87 %, followed by QUCCCM31 (43 %), then QUCCCM130 (14 %).

3.2. Comparative analysis of the impact of co-culture and monoculture mixture bio-flocculation on the harvesting of local Picochlorum sp.

The monocultures of QUCCCM130 and QUCCCM50 presented a growth rate μ of 0.218 day^{-1} and 0.165 day^{-1} respectively. However, the co-culture presented lower growth rate of 0.147 day^{-1}. Furthermore, bioflocculation experiments showed interesting results. Recovery efficiency on day 13 for the 50:50 ratio co-culture samples (81 %, $p < 0.05$) was higher compared to 50:50 ratio mixture of separately cultured strains (76.5 %). (Fig. 2A). Contrarily, it was noted that on day 15, co-culture showed a decrease in the recovery efficiency (67.5 %), while mixture of separately cultured strains displayed a significantly higher recovery efficiency (78 %, $p < 0.05$) surpassing even that of QUCCCM50 (73 %) alone after 180 min (Fig. 2B). Furthermore, we noticed that QUCCCM50 cultivated for 15 days (73 %) presented a lower recovery efficiency ($p < 0.05$) compared to biomass obtained after 13 days (91.5 %). Accordingly, the ensuing algal-algal bioflocculation experiments were performed with biomass cultivated after 13 days of cultivation to better enhance the low recovery efficiency of QUCCCM130.

3.3. Assessment of the impact of pH on bioflocculation of Picochlorum sp. and Tetraselmis sp.

Two pH values were tested, pH 9 and pH 10, while pH 8 was shown as a standard control. Results showed that pH 9 did not affect the recovery efficiency of either of the microalgal strains (Fig. 3). However, cultivation in pH 10 led to an improvement in the recovery efficiency ($p < 0.05$) of both QUCCCM50 and QUCCCM130 by 10 % and 25 % increase, respectively (Fig. 3). This positive effect was observed starting at 60 min of the settlement experiment and increased gradually over the period of experiment. In fact, high recovery efficiency percentages of 84.1 % and 37.1 % were observed for the strains QUCCCM50 and QUCCCM130 at pH 10, respectively after 60 min compared to 65.7 % and 2.9 % at pH 8.

3.4. Study of the bioflocculation of Tetraselmis sp. and Picochlorum sp. at different pH levels

A mixture of QUCCCM130 to QUCCCM50 with a ratio of 20:80 led to enhancement of the recovery efficiency by approximately 75 % (pH 8)

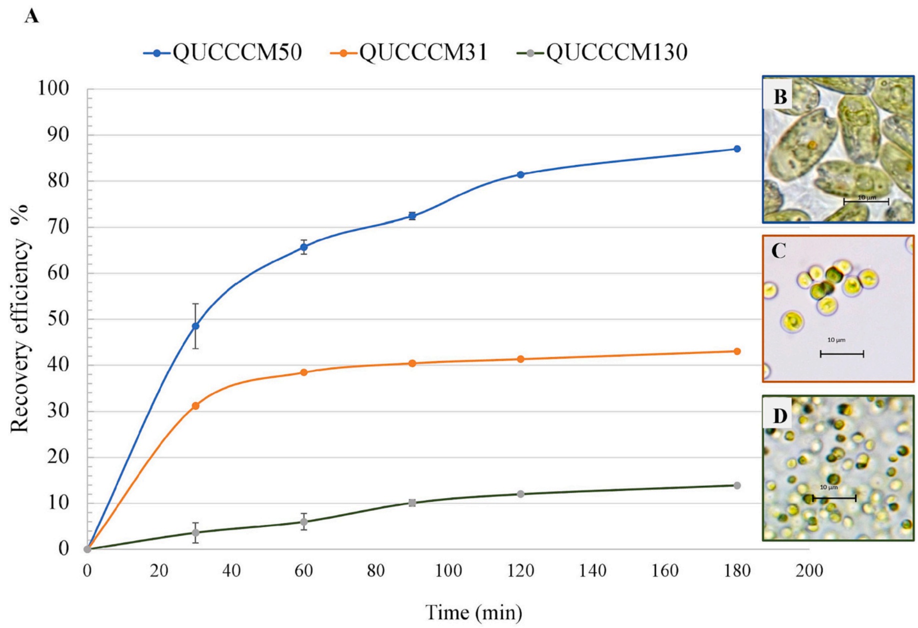

Fig. 1. Spontaneous self-settlement of microalgae at pH 8. A: recovery of the microalgal isolates during time. B, C and D: morphological characterisation using light microscopy with magnification of 100×. B: QUCCCM50; C: QUCCCM31, and D: QUCCCM130 (n = 3).

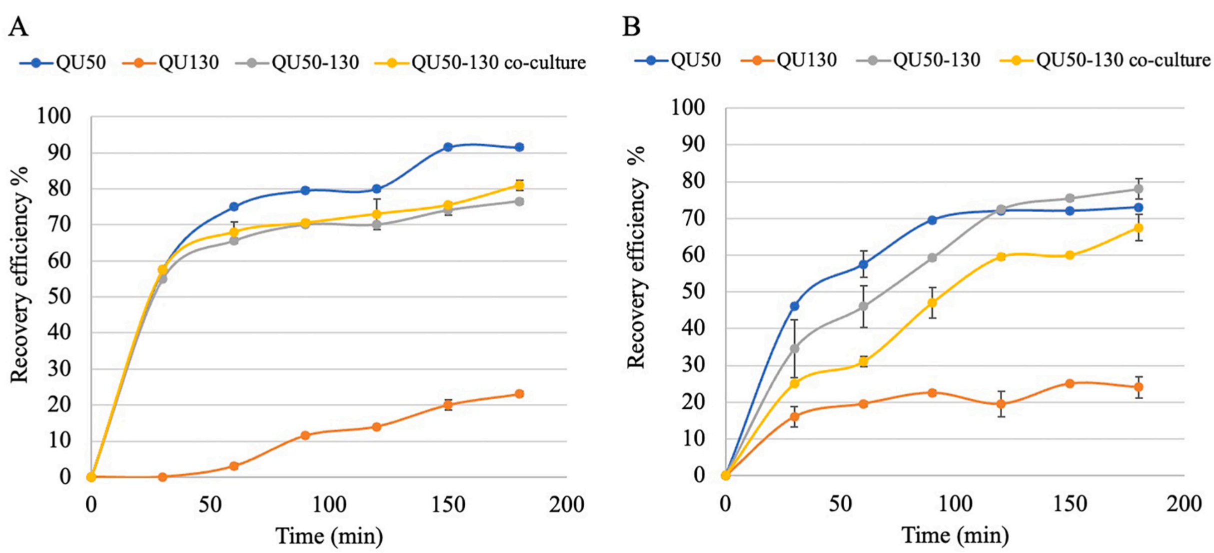

Fig. 2. Comparative analysis of the effect of bioflocculation and co-culture on the settlement efficiency at different cultivation time intervals: A – 13 days and B – 15 days. QU50: QUCCCM50; QU130: QUCCCM130. QU50–130: mixture of both strains cultured separately; QU50–130 co-culture where both strains were mixed with 50:50 ratio before experiment (n = 3).

and 87 % (pH 10) over the experimental period (Fig. 4A; B). Although bioflocculation recovery efficiency was already noted to be 60 % (pH 8) and 80 % (pH 10) with in the first 30 min incubation time. Furthermore, recovery efficiency of QUCCCM130 was 8 % (pH 8) and 55 % (pH 10), while introducing QUCCCM50 in a ratio of 80:20 (QUCCCM130: QUCCCM50) enhanced bioflocculation by increasing the recovery efficiency to 46 % (pH 8) and 65 % (pH 10). Therefore, adding a low percentage of QUCCCM50 to QUCCCM130 not only enhances its bioflocculation but also makes it faster. The effect of pH was also clearly visible in this experiment, as recovery efficiencies for all different experimental combinations were elevated at pH 10 (Fig. 4B) compared to at pH 8 (Fig. 4A). For example, efficiency at pH 8 for 50:50 ratio of QUCCCM130 to QUCCCM50 was 65 % while at pH 10 it was around 75 %.

3.5. Assessment of the bioflocculation of the three microalgal isolates with increasing size and self-settlement efficiency

The optimum mixing ratio for the 3 strains QUCCCM31: QUCCCM130:QUCCCM50 was 10:10:80, while the least bioflocculation efficient ratio was 40:40:20. With the decrease in ratio of QUCCCM50, the recovery efficiency decreased as well. Moreover in this experiment, replacing 10 % of QUCCCM130 with 10 % QUCCCM31 (presenting larger cell size) led to an increase in the recovery efficiency (Fig. 5), reaching ~96 %, compared to Fig. 4B, where 20:80 QUCCCM130: QUCCCM50 recovery was 87 %. The lipid content did not differ greatly for the three mixed cultures while the protein was noted to be highest in the 10:10:80 ratio which also had the highest recovery efficiency (Table 2).

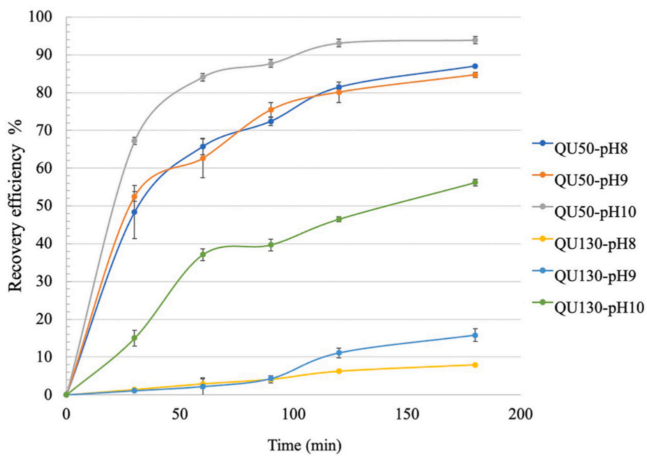

Fig. 3. Effect of pH on the self-settlement efficiency of QUCCCM130 and QUCCCM50 at different incubation time intervals. QU50 – QUCCCM50; QU130 – QUCCCM130; (n = 3).

The agglomeration of the cells was assessed using light microscopy (Fig. 6). This investigation indicated that the combination of QUCCCM50, QUCCCM31, and QUCCCM130 with ratio of 60:20:20, respectively, led to the appearance of large flocs of cells wherein QUCCCM50 having a larger cell size surrounded QUCCCM31 and QUCCCM130 which have a lower cell size. It was also noted that the flagella of QUCCCM50 cells were oriented toward the inner part of the flocs. Accordingly, we hypothesise that QUCCCM50 cells use their flagella to hold the non-flocculating microalgae; QUCCCM31 and QUCCCM130, inside the flocs (Fig. 6).

4. Discussion

Results showed for the first time that autoflocculation can also be positively correlated with cell size as the highest self-settlement efficiency was observed for QUCCCM50 (Fig. 2) compared to the other two smaller cell sized species. It has been suggested by Branyikova et al. [22] that due to smaller cell size of *Chlorella vulgaris*, sedimentation of species becomes neglected which over all reduces recovery efficiency of cells. The smaller size of cells can be a disadvantage also because the negative surface charge results in a stable dispersed algal solution which makes the harvesting process harder [21]. Although it has been mentioned before that size can be a factor contributing to the bioflocculation efficiency of cells, it has not been investigated thoroughly. On the other hand, zeta potential and Van der Waal forces have been showcased to affect the bioflocculation capacity of cells [35]. In a study by Deryabin et al. [36] it was demonstrated that the zeta potential of a particle decreases with an increase in particle size. Although not directly related it can be said that when a cell has a larger size, the zeta potential will be low, and this enhance the Van der Waal attractive forces leading to a successful aggregation of cells.

In our study, the best recovery efficiency results were noted for 13 day co-culture of QUCCCM50 and QUCCCM130 compared to 50:50 ratio mixture of monocultured microalge. Co-culturing cells enhanced the recovery efficiency of QUCCCM130 by 58 %. Similar results were noted in Zhao et al. [37] study, where co-cultures of *Desmodesmus* and *Monoraphidium* had high recovery efficiency of 85 % compared to monocultures of both strains. Higher bioflocculation activity of co-cultures can also be induced by production of extracellular polymeric substances (EPS) by microalgal cells as a stress response due to two species being cultured together [37]. EPS consist mainly of polysaccharides and proteins, which are secreted outside the cell, and are known to induce flocculation of cells [38]. Therefore, it can be said that the flocculation efficiency of species really depends on the properties of both species in co-culture and how they react to presence of the other strain in culture.

An incubation time of 13 and 15 days was chosen for this experiment, as these two days were previously studied to be the stationary phase (day 13) and late stationary phase (day 15) for the microalgal species. Previous studies have shown higher success rates for flocculation experiments at stationary phase and late stationary phase compared to exponential phase [6,23] This may be due to changes in cell size, cell zeta potential and production of lipids during the stationary phase [22]. According to Zhang et al. [39] study, the molecular weight of algal matter decreases due to biodegradation in the decline phase, which reduces flocculation efficiency. Furthermore, as the late stationary phase is reached, the carbon resources get depleted due to metabolic activities, and this causes a decrease in the production of EPS, which in turn decreases the bioflocculation of cells. In our study, the most suitable incubation time was 13 days (Fig. 2).

pH has an impact on the electrostatic properties of the cell surface, and might influence the interaction between cells, thereby affecting the flocculation capacity [25]. Increasing the pH from 8 to 9 did not significantly improve microalgal capacity for autoflocculation. However, at pH 10, there was a considerable increase (7 times) in the autoflocculation capacity of QUCCCM130 and a slight increase in the case of QUCCCM50. This result coincides with that of Shen et al. [40], confirming that a positive effect was observed only at pH 10 and that this effect is species dependent. Similarly, Tran et al. [8] proved that the autoflocculation of *Nannochloropsis oculata* reached a maximum of 90 %

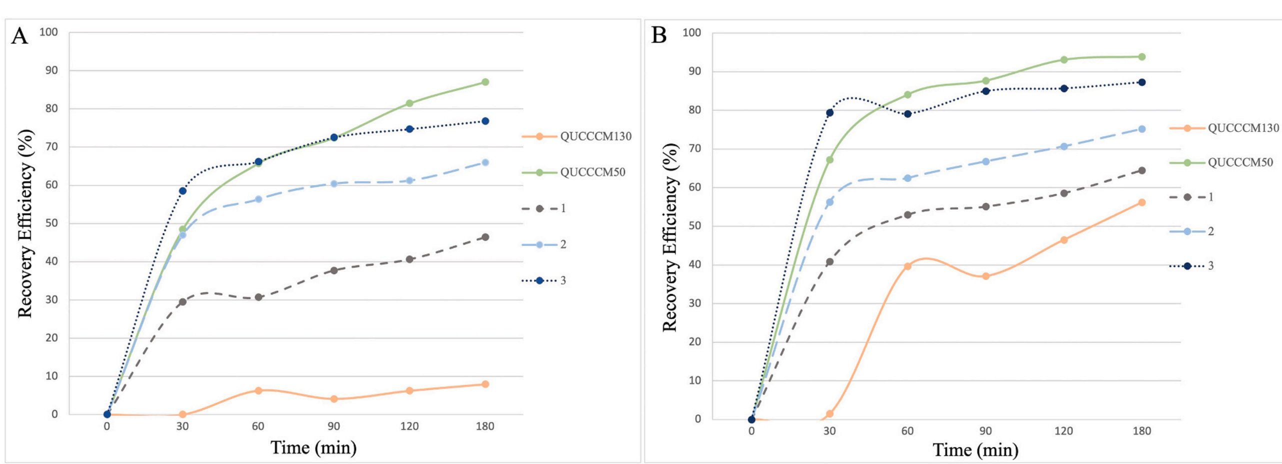

Fig. 4. Effect of pH and ratio of QUCCCM130 to QUCCCM50 on bioflocculation efficiency at different time intervals. The combinations 1, 2, and 3 correspond to the following proportions: **1**: 20:80; **2**: 50:50; **3**: 80:20. A: pH 8 and B: pH 10 (n = 3).

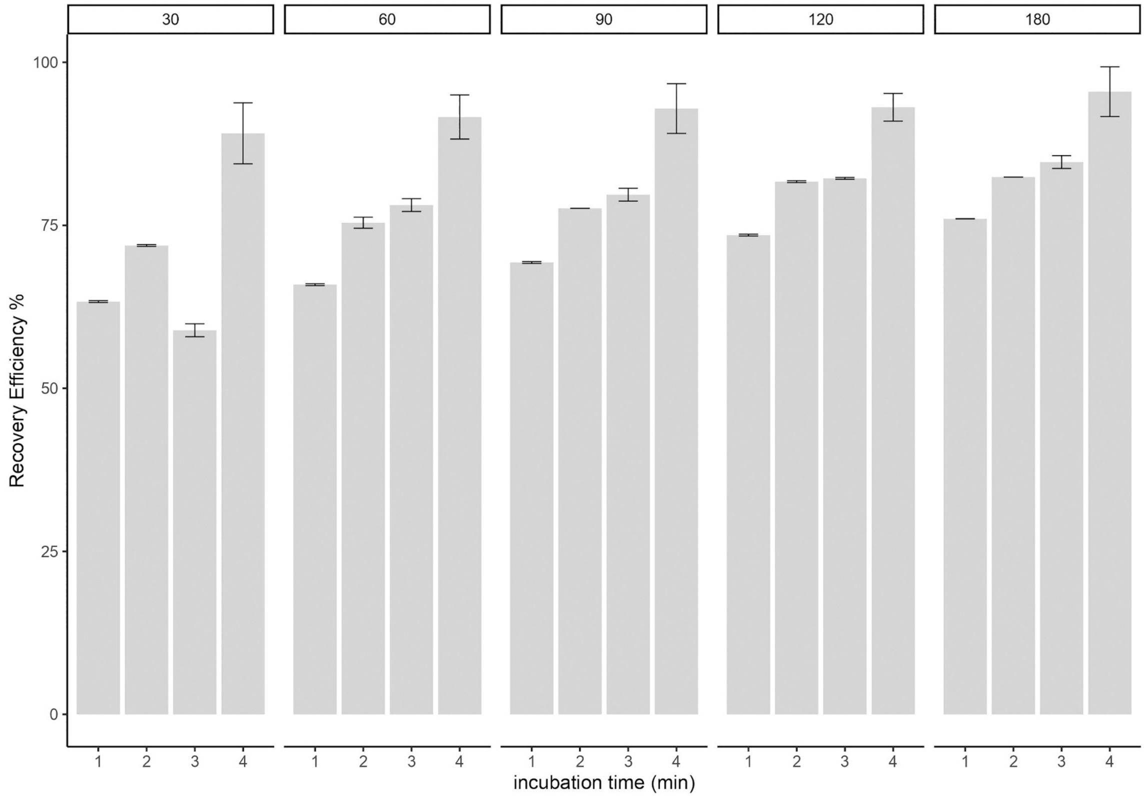

Fig. 5. Bio-flocculation of QUCCCM31, QUCCCM130 and QUCCCM50 at pH 10. The combinations 1, 2, 3 and 4 correspond to the combination of QUCCCM31: QUCCCM130: QUCCCM50 with the following proportions: **1**–40:40:20; **2**–30:30:40; **3**–20: 20:60; **4**–10:10:80.

Table 2
Total protein and lipid content of microalgal strains in different culture conditions measured in %/g wt.

Microalgae strain	Protein	Lipids
QUCCCM50	37 % ± 0.074	32 % ± 0.028
QUCCCM31	27 % ± 0.045	39.3 % ± 0.006
QUCCCM130	33 % ± 0.021	32.7 % ± 0.036
60 % QUCCCM50 + 20 % QUCCCM130 + 20 % QUCCCM31	32.97 % ± 1.027	34.20 % ± 0.6
40 % QUCCCM50 + 30 % QUCCCM130 + 30 % QUCCCM31	31.60 % ± 1.204	34.90 % ± 0.5
80 % QUCCCM50 + 10 % QUCCCM130 + 10 % QUCCCM31	37.51 % ± 1.908	34.09 % ± 1.315

at pH 10.4. This large increase in the agglomeration capacity might be explained by the increased negative charge of cell surface, by increasing the alkalinity of the media to pH 10, increasing cells self-aggregation. More recently, Branyikova et al. [22] proved that the negatively charged microalgal cells interact with negatively charged calcium phosphate particles during their pH-induced precipitation. Moreover, Tran et al. [8] stated that microalgal surface charge-neutralisation by calcium cations and sweep flocculation by calcium carbonate and calcium phosphate precipitates are the dominant flocculation mechanisms.

To test the bio-flocculation efficiency, one more strain was added to this test, *Nannochloris* sp. (QUCCCM30). The novelty of this study includes testing the possibility of mixing a multi-culture of the three microalgal isolates of increasing size. Accordingly, introducing a third microalgal strain enhances the flocculation capacity of the mixture and leads to an almost total recovery of the culture at pH 10 when the ratio is 10:10:80 of QUCCCM31:QUCCCM130:QUCCCM50. The high recovery efficiency can be explained by the increased protein content of this mixture which was noted to be the highest compared to the two other ratios. This technique can be considered of high potential to be used toward a fast, cost-effective technique for harvesting microalgal strains.

More importantly, a ratio of 40:40:20 of QUCCCM130:QUCCCM31: QUCCCM50 instead of using QUCCCM130 and QUCCCM50 with a ratio of 80:20 led to an improvement by 46 % in the settlement efficiency, reaching approximately 70 % at pH 8. This confirms that introducing a third microalgal strain can improve bioflocculation efficiency even at pH 8. Therefore, an alternative to increasing the pH can be mixing a multi-culture to avoid the negative impact on microalgal biomass associated with an alkaline pH [19].

Additionally, remarkable formation of large flocs wherein large cells of QUCCCM50 surround the small cells of QUCCCM31 and QUCCCM130 was observed (Fig. 6). The real mechanism of microalgal bioflocculation is not yet well understood, and different hypotheses have been suggested in the literature. From our investigations we have inferred the use of flagella by the *Tetraselmis* sp. in aiding the formation of flocs which held smaller sized microalgal cells, which are not self-flocculating naturally, inside the co-culture flocs that had formed. As this inference has not been viewed in previous studies, it should be tested by co-culturing other microalgae that are known for autoflocculation and possess flagella. Furthermore, the efficiency may differ based on the species used, hence it is important to conduct further research, with various microalgae, to confirm the exact mechanism contributing to efficient bioflocculation of microalgae.

5. Conclusions

Harvesting of microalgae is the most challenging and costly step in the large-scale production of microalgal biomass, especially with smaller celled species that are unable to self-settle. The novelty of this study lies in the consideration of combining two variables which are pH and cell ratio. The study was further enhanced by creating co cultures

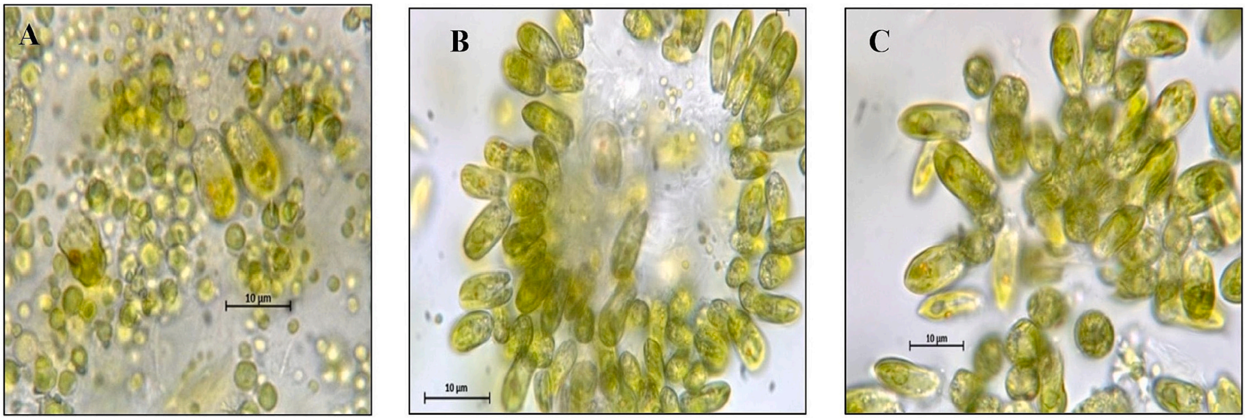

Fig. 6. Microscopic observation of bioflocculation of the three microalgal isolates using light microscopy with 100× magnification. Three combinations of QUCCCM130, QUCCCM31, and QUCCCM50 with the following frequencies: A – 30:30:40; B – 20:20:60; C – 10:10:80.

with three microalgal species whilst considering the pH of mixture as well. We have demonstrated in the current research that mixing small species such as *Picochlorum* sp. with a large celled species such as *Tetraselmis* sp. enhances its settlement through bioflocculation. Our results also suggest that high protein content is associated with increased bioflocculation efficiency. Furthermore, microscopic observation shows that both species interact together and form flocs wherein *Tetraselmis* sp. holds *Picochlorum* sp. cells inside the flocs with its flagella. Additionally, if a lower pH is desired, bioflocculation utilizing three microalgal strains can be considered as possible solution. This study provides a gateway for future large scale cultivation studies, where the different variables to consider can be deduced by the different experiments conducted in this research. Additionally larger scale experiments need to be conducted to further verify the bioflocculation usage, as other factors may need to be considered. Overall bioflocculation can be one of the most promising algal harvesting techniques and should be considered for commercialization.

CRediT authorship contribution statement

I.S.: Conceptualisation, Methodology, writing—original draft preparation, writing—review and editing and funding acquisition; M.C.: data acquisition, writing—review and editing, S.A.S.: writing—original draft preparation, writing—review and editing; H.M.J. and S.S.: writing—review and editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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