Journal of Cleaner Production 234 (2019) 150–156

Contents lists available at ScienceDirect

Journal of Cleaner Production

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

Efficient microalgae harvesting mediated by polysaccharides interaction with residual calcium and phosphate in the growth medium

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ARTICLE INFO

Article history:
Received 11 February 2019
Received in revised form
13 June 2019
Accepted 14 June 2019
Available online 15 June 2019

Handling Editor: Prof. Jiri Jaromir Klemeš

Keywords:
Microalgae
Harvesting
Auto-flocculation
PSAs
Calcium
Phosphate

ABSTRACT

This study sets out to harvest microalgae through auto-flocculation by manipulating the interactive effect of calcium, phosphate, and polysaccharides (PSAs). The harvesting efficiency (H.E) of 91 ± 2.7% was achieved for *Ettlia* sp. through auto-flocculation. The H.E was attributed to the chelation of calcium and PSAs present in microalgae medium. In the absence of PSAs, the H.E reduced to 64 ± 05% only. The addition of phosphate (34 ± 0.13 mg L^{-1}) increased the H.E to 73 ± 1.5%. Zeta-potential measurements showed that the harvesting was induced by charge neutralization and inter-particle bridging. The PSAs-based auto-flocculation was tested for *Chlorella* sp. too but it turned out the H.E of 51 ± 1.3% only. The flocculation did not take place when the co-harvesting of *Ettlia* sp. and *Chlorella* sp. was carried out. It is concluded that each microalgae specie shows different auto-flocculation mechanism due to variation in their PSAs characteristics. It triggers up the need for setting up a distinct protocol for different microalgae species to assess their auto-flocculation potential.

1. Introduction

Harvesting or dewatering is a critical step of microalgae bio-processing (Gejji and Fernando, 2018; Pradhan et al., 2019). Harvesting accounts for 20–30% of the total cost of microalgae bio-processing (Sahoo et al., 2017a; Yoo et al., 2013). So far, various methods for microalgae harvesting including centrifugation, dissolved air flotation, and filtration have been developed; however, none of them is cost-effective yet (Sahoo et al., 2017b). To address this challenge, auto-flocculation has been recognized as an alternative method for microalgae harvest (Raheem et al., 2018). Auto-flocculation is a cheap and efficient method (Lai et al., 2016; Mathimani et al., 2018; Wang et al., 2018). It demands less resource input than any other method of microalgae harvest (Ding et al., 2017; Jiménez et al., 2017). Auto-flocculation is based on the principle of the well-known chemical flocculation process; however, it does not involve the addition of expensive chemicals. Auto-flocculation is mainly driven by the interaction of polysaccharides (PSAs), calcium, and phosphate (Tran et al., 2017; Ummalyma et al., 2017; Vandamme et al., 2012). PSAs are the form of polymeric carbohydrates, which are produced by microalgae under specific cultivation conditions. PSAs are composed of glucose, arabinose, galactose, xylose, mannose, and fructose. Each sugar group has a different role in cell metabolism. For example, arabinose controls cell aggregation properties, and galactose is responsible for providing energy. It is widely known that microalgae produce PSAs under light and nutrients stress condition. The yield and the

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https://doi.org/10.1016/j.jclepro.2019.06.154
0959-6526/

characteristics of PSAs depend on the microalgal species (Lee et al., 2016; Vandamme et al., 2012).

Microalgae release PSAs, so they are widely available in the microalgae culture; the un-used calcium and phosphate also remain present in the culture in trace amount which can facilitate auto-flocculation. Since auto-flocculation relies on the resources which are already available in the microalgae culture, thus it can be considered more economical than the chemical flocculation.

Auto-flocculation is prompted by several mechanisms namely charge neutralization, inter-particle bridging, and sweep flocculation (Brady et al., 2014; Liu et al., 2018). These mechanisms are influenced by a number of factors such as particle size, charge density, water chemistry, ionic strength, and most of all the surface properties of microalgae cells (Brady et al., 2014). Thus, it is hard to identify the exact underlying mechanism of auto-flocculation (Alimoradi et al., 2017; Ma et al., 2017; Rezvani et al., 2017). Setting aside the intrinsic properties of microalgae cells, auto-flocculation is mainly controlled by the chelation of calcium with phosphate, to form positively charged $Ca_3(PO_4)_2$ crystal. It serves as a nucleation site to interact with microalgae cells to flocculate them (Ummalyma et al., 2017; Vandamme et al., 2012, 2018).

Previous studies have demonstrated the use of $Ca_3(PO_4)_2$ to induce auto-flocculation (Tran et al., 2017; Vandamme et al., 2012). It was found that the presence of PSAs in microalgae culture inhibit flocculation, arguing that PSAs might compete with phosphate and interfere with crystal growth of $Ca_3(PO_4)_2$. Moreover, PSAs compete with microalgae cells and increase the required dose of the flocculant to establish electroneutrality (Vadlamani et al., 2017; Vandamme et al., 2018). Thus, the presence of PSAs in microalgae culture limits the application of auto-flocculation. On the other hand, the removal of PSAs from the microalgae culture is difficult. It triggers up the need for developing an efficient method of microalgae harvest, which is not affected by the presence of PSAs.

To address these challenges, we targeted to induce flocculation in the presence of PSAs. To this end, it was postulated that calcium ions should show an affinity to PSAs to form Ca–PSAs crystals. These crystals acquire a positive charge and become readily available to interact with microalgae cells to flocculate them. To prove this concept, experiments were carried out under various combinations of calcium, phosphate, and PSAs concentration. The proposed concept was tested for *Ettlia* sp. and *Chlorella* sp. These species are widely studied due to their applications in bio-refinery (Lee et al., 2016; Yoo et al., 2013). They are freshwater microalgae and reportedly produce polysaccharides during growth, and thus, expected to offer auto-flocculation potential. The auto-flocculation experiment was carried out for pure cultures of these species and the mixed culture too.

2. Material and methods

2.1. Microalgae growth and culture conditions

Microalgal strains *Ettlia* sp. and *Chlorella* sp. used in this study were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB). The experiments were mainly performed with *Ettlia* sp. *Chlorella* sp. was used only to ascertain the extensibility of the PSAs-based auto-flocculation concept (see section 5). *Ettlia* sp. cells were cultivated autotrophically in a 5 L glass bottle in BG-11 medium containing (g L^{-1}) 1.5 $NaNO_3$, 0.12 K_2HPO_4, 0.075 $MgSO_4{\cdot}7H_2O$, 0.036 $CaCl_2{\cdot}2H_2O$, 0.02 Na_2CO_3, 0.0006 Ferric ammonium citrate, 0.001 EDTA (disodium salt), 0.061 H_3BO_3, 0.169 $MnSO_2{\cdot}2H_2O$, 0.287 $ZnSO_4{\cdot}7H_2O$, 0.012 $NaMoO_4{\cdot}2H_2O$, 0.0025 $CuSO_4{\cdot}5H_2O$, 0.001 $MnCl_2.4H_2O$, and 0.049 $Co(NO_3)2.6H_2O$ (Yoo et al., 2013). CO_2-mixed air (2%) with a flow rate of 70 mL min^{-1} was supplied continuously under the illumination of 200 μmol photons $m^{-2}s^{-1}$ (Lee et al., 2016; Nayak et al., 2018). The cell growth was monitored by measuring the optical density at 680 nm (OD_{680}) using UV spectrophotometer (HACH, Japan). The cells growth was terminated in early stationary phase ($OD_{680} = 1.60$) Figure-1. At this stage, no nutrients were available to promote growth, and thus, the minimum changes in cell properties and the medium composition were expected.

2.2. Analytical methods

Calcium and phosphate removal was assessed by determining their concentrations before and after flocculation. The microalgae samples were centrifuged (Hanil Combi 514R Centrifuge) at 4500 rpm for 15 min at room temperature (25 °C ± 2 °C). The supernatant was filtered through a syringe filter (0.2 μm). The filtrate was collected in a washed and dried glass bottle.

The filtrate was analyzed on an inductively coupled plasma optical emission spectroscopy (Specto Blue-EOP-TI, Germany) to measure the concentrations of calcium and phosphate (Tran et al., 2017). Analytical triplicates were performed for each sample. Average values of analytical triplicates with standard deviation are reported. For zeta-potential (ZP) measurement, the supernatants of the treated samples (after 60 min settling time) were withdrawn from the aqueous phase. The ZP was measured by using a Zeta-sizer (Photal, ELS-Z). The ZP value was calculated based on Smoluchowski equation (Rashid et al., 2013). Measurements were taken in duplicate and the values are reported with standard deviation. PSAs were measured by following the method as described in the literature (Beuckels, 2013; Lee et al., 2016). Briefly, microalgae suspension was centrifuged at 3500 rpm for 10 min. The supernatant was re-centrifuged at 10,000 rpm for 2 min to remove cell debris. The supernatant was used to measure total PSAs by phenol-sulfuric acid assay, using glucose as a standard.

Different pH values of the culture were obtained by using an appropriate volume of 4 M NaOH solution. The pH was measured by a pH meter. The pH meter was calibrated before use.

2.3. Harvesting assay

The harvesting test was performed in 15 mL falcon tubes. After pH adjustment (as per experimental design), the microalgae culture was immediately transferred to the falcon tubes. The cells were allowed to settle for 60 min. A 60 min cut-off time was selected, as the minimal changes were observed beyond this time point (data not shown). The samples were carefully drawn from the mid-height

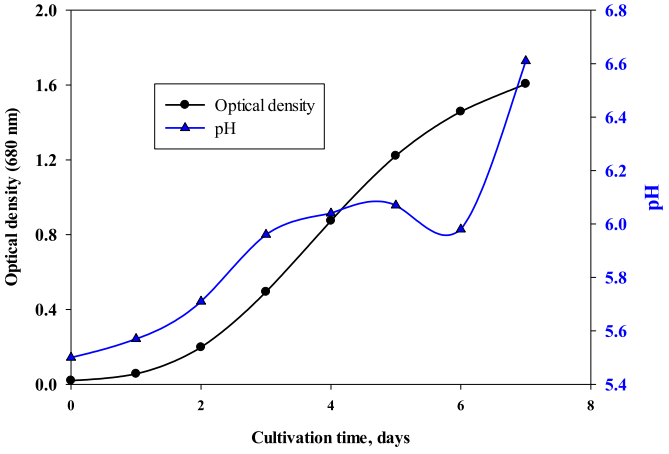

Fig. 1. Growth of *Ettlia* sp. represented in terms of daily optical density and pH change.

of the culture volume (Lai et al., 2016). All harvesting experiments were performed in triplicate. The harvesting efficiency was calculated as follows:

$$Harvesting\ efficiency\ (H.E)\ \% = \frac{OD_{to} - OD_{60}}{OD_{to}} \times 100 \quad (1)$$

where OD_{to} is the optical density at the initial time point and OD_{60} is the optical density of the sample after 60 min.

2.4. Experimental design

To know the effect of calcium, phosphate, and PSAs on the H.E. The following medium were prepared and the harvesting assay was performed for each treatment:

- Medium-1 (M1): Natural growth medium
- Medium −2 (M2): Medium without PSAs and phosphate
- Medium −3 (M3): Medium with calcium (80 mg L^{-1})
- Medium −4 (M4): Medium with phosphate (34 mg L^{-1}) and without PSAs

M1 was set as a control to compare the H.E of natural growth medium with other treatments. Since M1 was the natural growth medium, it contained PSAs which were naturally produced by the microalgae. To underline the impact of PSAs on the H.E, M2 treatment was employed in which PSAs were removed. Thus, M1 treatment served as a positive and M2 as a negative control of PSAs. M2 was prepared by centrifuging the microalgae suspension at 3000 rpm and discarding the supernatant. The pellets were further washed three times with distilled water to remove cell-bound PSAs. After washing, the pellets were re-suspended in an equivalent volume of fresh BG-11 medium except for phosphate.

To enhance the interaction between calcium and PSAs, and its subsequent impact on the H.E, M3 was prepared. M3 was simply prepared by adding calcium to the natural growth medium (Beuckels, 2013) to make a final concentration of 80 mg L^{-1}. To ascertain the effect of phosphate and calcium interaction on the H.E, M4 was prepared. For M4, the same procedure was repeated as for M2; however, a high concentration of phosphate (34 ± 0.13 mg L^{-1}) was added (Lee et al., 2016). The concentration of calcium and phosphate was selected based upon the literature review and stoichiometric calculation of phosphate and calcium bio-chemical reaction (Beuckels et al., 2013; Lee et al., 2016). Table-1 provides the detail of various treatments employed in this study. The concentration of calcium and phosphate was measured before and after harvesting test. For all treatments, the harvesting tests were carried out at different pHs (7.0–11.0). The culture without pH adjustment was used as a control.

3. Results

3.1. Auto-flocculation experiment

Auto-flocculation of microalgae is influenced by a number of parameters. However, in this study, we restricted our focus only onto the most influencing factors including calcium, phosphate, PSAs and the pH (Gani et al., 2016; Ma et al., 2017; Ummalyma et al., 2017). The interactive effect of these parameters was demonstrated by performing flocculation in different sets of treatments at different pHs. As mentioned earlier, four different solutions (M1, M2, M3, M4) were prepared with various combinations of phosphate, calcium, and PSAs. The harvesting assay was performed for each solution to measure the H.E.

The results show that the M1 containing 13.8 ± 1.3 mg L^{-1} of calcium (after growth termination) 12.4 ± 0.6 mg L^{-1} of phosphate, and 76.5 ± 7.2 mg L^{-1} of PSAs showed the highest H.E of 91 ± 2.7% at pH 11.0. After harvesting, the dissolved calcium concentration reduced to 4.7 ± 1.7 mg L^{-1}, phosphate 9.8 ± 0.44 mg L^{-1}, and PSAs 64.5 ± 14.2 mg L^{-1} (Figure-2). The cells aggregation started at pH 10.0 (H.E = 19.1 ± 2.7%) and reached the maximum at pH 11.0. Below pH 10.0, the cell suspension was stable and no flocs were observed apparently, also confirmed by H.E measurements. Thus, pH 11.0 turned out to be the threshold point to induce flocculation for this treatment.

M2 showed the maximum H.E of 64 ± 05% only at pH 11.0. It demonstrates that in the absence of PSAs the H.E decreases. Low H.E in M2 can be attributed to the non-availability of PSAs to interact with calcium and to make Ca–PSAs ligand, which could serve as a flocculating agent. Lesser removal of calcium (3.2 mg L^{-1}) in M2 than M1(9.1 mg L^{-1}) also evidenced that the calcium did not interact with other compounds and remain freely available in the solution. The availability of calcium in the solution might have caused an increase in the repulsion forces, which inhibit cell aggregation and decrease H.E.

These two treatments (M1, M2) results established that the H.E was attributed to the formation of Ca–PSAs ligand. Thus, we posited that the provision of abundant calcium would enhance its

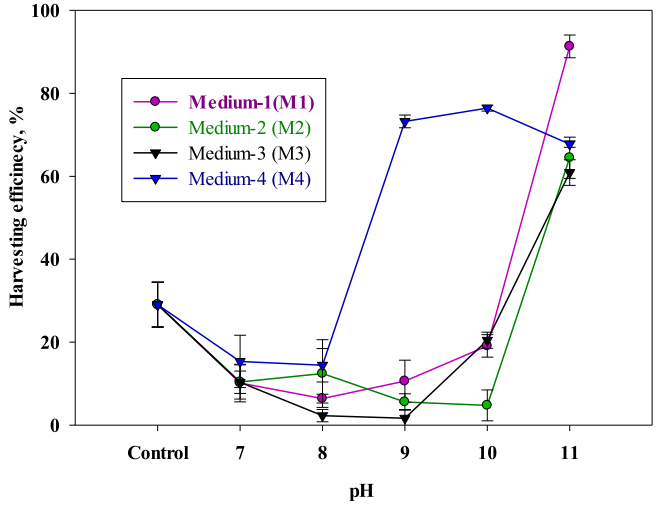

Fig. 2. Harvesting efficiency of *Ettlia* sp. under different set of treatments. M1-natural culture; M2-culture without PSAs; M3-culture with excess calcium; M4-culture with excess phosphate. Error bars show the standard deviations (n = 3).

Table 1
Different treatments employed for the evaluation of microalgae harvesting efficiency.

Treatment	Calcium, mg L^{-1}	PSAs, mg L^{-1}	Phosphate, mg L^{-1}	Harvesting efficiency, %	Optimized pH
M1	13.8 ± 1.3	76.5 ± 7.2	12.4 ± 0.6	91 ± 2.7	11.0
M2	13.8 ± 1.3	0	0	64 ± 5.0	11.0
M3	80 ± 0	76.5 ± 7.2	12.4 ± 0.6	61 ± 3.0	11.0
M4	13.8 ± 1.3	0	34 ± 0.13	73 ± 1.5	9.0

interaction with PSAs to form Ca–PSAs; the abundance of Ca–PSAs ligand would impart a positive impact on the H.E too. With this in mind, in M3 the concentration of calcium was kept high (80 mg L^{-1}). The results show that the H.E in M3 was only 61 ± 3.0% which was against our set hypothesis. The removal of calcium and phosphate in this treatment were 76 mg L^{-1} and 11.01 mg L^{-1} respectively, which are much higher than in M1 and M2. This observation led us to conclude that when the calcium is provided in excess, it forms Ca–PSAs ligand as well as Ca-phosphate ligands. Both ligands, being positively charged, repel each other resulting in an increase in repulsion forces, which pose a negative impact on the H.E.

In M4, the H.Es were 73.2 ± 1.5%, 76.4 ± 0.72%, and 67.7 ± 0.8% at pH 9.0, 10.0 and 11.0, respectively. Only 1.3 mg L^{-1} of calcium and 6.5 mg $L^{-1}$of phosphate were removed in this treatment. Apparently, pH 10.0 showed the highest H.E. However, the H.E at pH 9.0 and pH 10.0 were statistically insignificant ($P > 0.05$). We considered pH 9.0 as the optimized pH value; because from an economic viewpoint the amount of chemical (NaOH) required to raise the pH of the culture up to this pH (9.0) will be lesser than what required for pH 10.0 (Table-1).

3.2. Screening calcium dose for auto-flocculation

Aforementioned experiments revealed that the concentration of calcium plays an influential role in auto-flocculation process. The concentration of calcium dictates the possible mechanism of harvesting including inter-particle bridging, charge neutralization, and sweep flocculation. Thus, it was important to optimize calcium concentration to achieve efficient harvesting. A dose-response relationship was developed between calcium concentration and H.E.

All dosage tests were performed using natural growth medium (M1). An appropriate amount of calcium was added to M1 to obtain required concentrations ranging from 10 to 80 mg L^{-1}. The harvesting test was performed at the optimized pH (11.0) only. The results showed the H.Es of 28 ± 3%, 91 ± 3%, 37 ± 6%, 29 ± 1%, 32 ± 1%, and 61 ± 4% with 0, 9, 18, 35, 50 and 80 mg L^{-1} of calcium, respectively (Figure-3).

3.3. Zeta-potential

Zeta-potential is an important parameter to identify the mechanism under which the flocculation takes place. Figure-4 shows the ZP measurements at the optimized pHs in different treatments. Microalgae culture without any pH adjustment was used as a control. The control showed the ZP of −12 ± 0.7 mv. The ZP value of microalgae culture M1 was −16.2 ± 0.2 mv. M2 showed the ZP value of −15.6 ± 0.1 mv. ZP in M3 and M4 were −22.4 ± 0.2 mv and −22.5 ± 0.8 mv, respectively. Increase in ZP in M3 and M4 can be attributed to the repulsion forces generated due to the addition of excessive counter-ions (calcium/phosphate).

4. Discussion

Figure-2 shows that the highest H.E was achieved in M1 at pH 11.0. Below this pH, no cell aggregation was observed. In this treatment, the flocculation was induced by the chelation of PSAs with calcium. Calcium encapsulates PSAs through charge neutralization and inter-particle bridging creating a net positive charge on Ca–PSAs nuclei (Vandamme et al., 2012; Xia et al., 2018; Yoo et al., 2013). Fig. S1 illustrates this mechanism. Ca–PSAs nuclei interact with negatively charged microalgae cells and destabilize them to precipitate. Phosphate ions present in the medium might have competition with PSAs to interact with calcium, forming $Ca_3(PO_4)_2$ crystal. However, it appeared that only 2.6 mg L^{-1} of phosphate was removed in this treatment. This small amount of phosphate can't create enough $Ca_3(PO_4)_2$ nuclei which could interact with microalgae cells and force them to precipitate. Thus, the flocculation was thought to be predominately induced by Ca–PSAs interaction with microalgae cells. M2 results confirmed this mechanism. M2 showed that in the absence of PSAs, the H.E reduced to 64 ± 05% only, at pH 11.0. Low H.E in the absence of PSAs supported our concept that PSAs are a complementary part of Ca–PSAs based auto-flocculation process. It found that calcium removal in M2 was lower than M1. Thus, calcium removal was attributed to the presence of PSAs. It implies that at high pH, calcium and PSAs had an affinity for each other to form Ca–PSAs nuclei. Since M2 was carried out in the absence of PSAs, and hence, there were no chances of Ca–PSAs formation. Ultimately, the H.E reduced. Lee et al. (2016) also showed that *Ettlia* sp. display higher H.E in the presence of PSAs (Brady et al., 2014; Choi et al., 2016a,b; Yoo et al., 2013). The distinct feature of this study is that microalgae removal is achieved

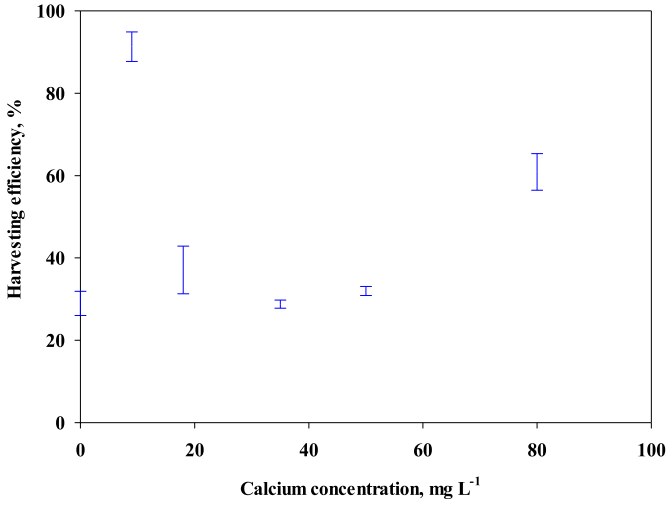

Fig. 3. The effect of calcium concentration on the harvesting efficiency of *Ettlia* sp. Error bars show the standard deviations (n = 3).

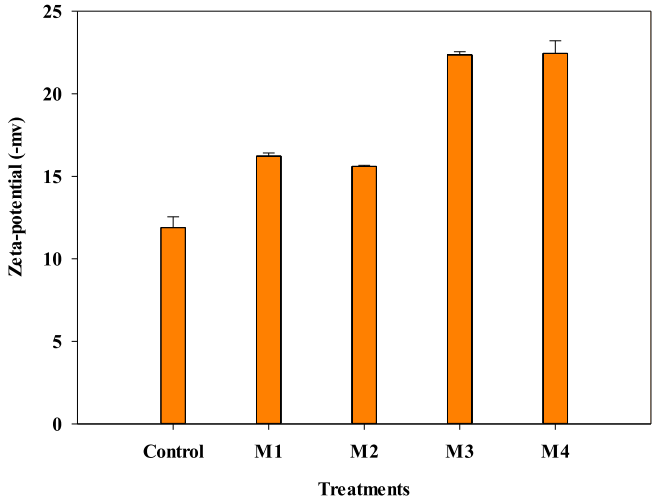

Fig. 4. Zeta-potential measurements in different treatments. Control-natural culture without pH adjustment; M1-natural culture; M2-culture without PSAs; M3-culture with excess calcium; M4-culture with excess phosphate and without PSAs. Zeta-potential was measured at the optimized pHs. Y-axis shows the negative value of zeta-potential (-mv). Error bars show the standard deviations (n = 2).

without providing additional flocculant (refer to M1 & M2 treatments). Previous studies reported that the microalgae harvest was achieved by adding calcium and phosphate as flocculants and maintaining their ratios; however, these methods showed less efficacy when PSAs were present in the medium (Beuckels, 2013; Choi et al., 2016a,b). PSAs are produced during microalgae growth and are an integral part of microalgae culture. Thus, it was important to develop a technique which could efficiently remove microalgae in the presence of PSAs. This work suggests that PSAs show a positive impact on microalgae harvest if the residual calcium is utilized to form Ca–PSAs. Since low concentration of calcium present in microalgae suspension (the residual calcium) was enough to induce flocculation; therefore, charge neutralization and inter-particle bridging can be considered as the dominant harvesting mechanisms in Ca–PSAs auto-flocculation process (Beuckels, 2013; Shin et al., 2016).

In this experiment, microalgae removal was achieved without any flocculation aid; however, flocculation took place at high pH only. From an economic and sustainability viewpoint, a rise in pH to induce flocculation is not affordable at large-scale due to the cost vested on chemical (NaOH) supply. Thus, it was targeted to achieve flocculation at low pH. In this regard, we thought to achieve it by providing an additional dose of calcium to the microalgae suspension. It would increase the availability of positive ions to interact with PSAs (M3 treatment). It turned out that provision of calcium ions rather hindered flocculation, resulting in the H.E of $61 \pm 3.0\%$ only even at pH 11.0. Flocculation did not take place at lower pHs (<10.0). The removal of calcium ($76\ mg\ L^{-1}$) and phosphate ($11\ mg\ L^{-1}$) in M3 was much higher than that observed earlier in M1 and M2, still, the H.E was low. High removal of calcium and phosphate provided evidence of $Ca_3(PO_4)_2$ formation. Calcium would have interacted with PSAs also to form Ca–PSAs. The presence of $Ca_3(PO_4)_2$ and Ca–PSAs caused excess positive nuclei, resulting in electrostatic repulsion, charge reversal, and weak interaction with microalgae cells, which reduce the H.E.

M3 results led us to investigate the dose-response relationship of calcium and the H.E. Figure-3 shows that low calcium concentration ($13.8 \pm 1.3\ mg\ L^{-1}$) resulted in the highest H.E. Inter-particle bridging seems to be the underlying flocculation mechanism. High calcium concentration caused overdosing, charge reversal, and re-stabilization of the particles. Also, at high concentration, the interaction between PSAs and calcium would be weak because of excessive positive ions, which result in repulsion forces. A little increase in the H.E could be observed at $80\ mg\ L^{-1}$ of calcium, which is probably because of the increase in ionic strength (Choi et al., 2016a,b). High ZP (-22.4 ± 0.2 mv) also showed that excess provision of calcium had caused repulsion between indifferent ions leading to charge reversal. The charge reversal corresponded to low H.E. M4 shows that H.E of $73 \pm 1.5\%$ could be achieved at pH 9.0 with the provision of high phosphate ($34 \pm 0.13\ mg\ L^{-1}$) concentration but in the absence of PSAs. Only $1\ mg\ L^{-1}$of calcium was removed in M4 treatment. It supported our pre-set hypothesis that calcium removal corresponds to the presence of PSAs. In this treatment, charge neutralization and inter-particle bridging mechanism can be ruled out. Flocculation was likely to be governed by sweep flocculation (Beuckels, 2013; Ma et al., 2017; Yang et al., 2018). ZP measurements well-explain this mechanism. ZP value (-22.5 ± 0.8 mv) in this treatment (M4) was significantly ($P < 0.05$) higher than in M1; however, H.E was low. It provided an understanding that phosphate ions were in excess, which repels each other and increases net repulsion forces. Ultimately, zeta-potential also increases. As stated above, the residual phosphate concentration dictates that only a small concentration of phosphate was removed in this treatment, thus, the ions remain un-attached into the suspension causing repulsion and an increase in ZP. Beuckels et al. (2013) also showed flocculation mediated by a high concentration of phosphate (Beuckels, 2013), but the presence of PSAs inhibited auto-flocculation. However, this approach has less practical significance because PSAs are released during microalgae growth, and are difficult to remove (Xia et al., 2018; Yoo et al., 2013). The efficient microalgae harvesting at low pH with the excess provision of phosphate (M4 treatment) ions provides a clue that the use of wastewater containing a high concentration of phosphate can be a promising approach. Moreover, the cultivation of microalgae in high phosphate-containing wastewaters would provide an additional advantage of auto-flocculation.

5. Extensibility of PSAs-based auto-flocculation concept

The above-mentioned results show that the PSAs help *Ettlia* sp. to flocculate. In this experiment, we were interested to investigate the extensibility of PSAs-based auto-flocculation concept to other microalgae species. The concept was applied to *Chlorella* sp. having same culture age (7 days) as that of *Ettlia* sp. To ascertain the effect of PSAs, the experiments were carried out to harvest *Chlorella* sp. with PSAs (PSAs+), and without PSAs (PSAs-). For PSAs+, the natural culture was used, whilst PSAs- culture was achieved by removing PSAs through centrifugation and re-suspending the pellets into fresh medium. Flocculation was carried out at the optimized pH only (11.0). The results showed that the H.Es were $91.3 \pm 0.4\%$, and $51 \pm 1.3\%$ in PSAs-, and PSAs + medium, respectively (Figure-5). It reflects the negative impact of PSAs on microalgae *Chlorella* sp. harvest. This role of PSAs is opposite to what was observed for *Ettlia* sp. in the aforementioned experiment. It provided an understanding that the role of PSAs in auto-flocculation can't be generalized. Auto-flocculation depends on the intrinsic properties of PSAs and the species themselves. Microalgae species produce different types of PSAs having varied composition, and thus, show different behavior towards flocculation (Pivokonsky et al., 2015; Sahoo et al., 2017a; Ummalyma et al., 2017). Further studies should be carried out demonstrating the role of PSAs composition and the morphology of microalgae species to unveil the mechanisms of auto-flocculation.

5.1. Investigating co-harvesting of two microalgae

Aforementioned experiments showed the negative impact of

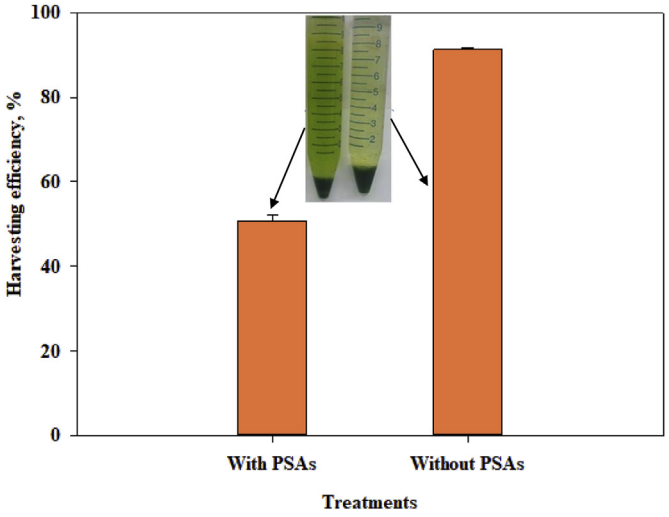

Fig. 5. Harvesting efficiency of *Chlorella* sp. with polysaccharides (PSAs+) and without polysaccharides ($PSAs^-$) in the medium. Error bars show the standard deviations (n = 3).

PSAs on *Chlorella* sp. harvesting and the positive impact on *Ettlia* sp. harvest. In this part of the experiment, it was targeted to co-harvest these two species. The basic idea was that the PSAs of *Ettlia* sp. might be able to flocculate *Chlorella* sp. cells also when mixed together (since *Ettlia* sp. PSAs facilitate flocculation). This way co-harvesting would be more advantageous than harvesting the species separately. To test co-harvesting, *Ettlia* sp. and *Chlorella* sp. were mixed together with 1:1(v/v) ratio and the harvesting assay was performed. The pure culture of *Ettlia* sp. and *Chlorella* sp. were set as controls. This time the harvesting was carried out without pH change since the objective was only to know if two species offer high H.E potential instead of recognizing the effect of pH change. The results show that the H.Es of *Ettlia* sp., *Chlorella* sp. and the mixed culture were $22 \pm 4\%$, 0.3 ± 0, and 10.4 ± 1.4, respectively (Figure-6). The low efficiency in the mixed culture indicates that the addition of *Chlorella* sp. to *Ettlia* sp. rather reduced its harvesting. It was against our hypothesis. The possible reason for this phenomenon can be that the PSAs of *Chlorella* sp. overplay *Ettlia* sp.'s PSAs. Since PSAs of *Chlorella* show a negative impact on the H.E; therefore, in the mixed culture, they inhibited the flocculation of *Ettlia* sp. too. Ultimately the H.E of the mixed culture decreased.

Thus, it can be concluded that the co-harvesting of two microalgae species was not an effective method to improve the H.E since both species had different intrinsic properties as well as variation in PSAs behavior.

6. Economic and environmental sustainability

Auto-flocculation offers great promise for microalgae harvest. Unlike typical flocculation techniques, it has the potential to displace the need for flocculant aid, and thus, making it cost-effective technology. Our results show that microalgae can be successfully removed from water by manipulating the interactive effect of calcium, phosphate, and PSAs. The residual calcium and PSAs are naturally produced by microalgae during their growth; the PSAs effectively interact, form a positively charged crystal and co-precipitate with microalgae cells. This process does not require additional flocculant; however, it requires elevated pH. In this experiment, the natural pH of *Ettlia* sp. was 6.61 ± 0.10 and it needs to be raised up to 11.0 to induce flocculation. Raising pH requires chemical addition which is not economically supported. An increase in pH of microalgae culture can be possible by allowing them to do photosynthesis for an extended time (Tran et al., 2017), but it also poses cost due to prolonged retention time and the system loading. This work provides a strategy to achieve microalgae harvest at relatively low pH. We found that phosphate addition to the microalgae culture helps to attain flocculation at relatively low pH (9.0). Unfortunately; however, phosphate-based flocculation takes place in the absence of PSAs only. In practical, it is not possible to attain PSAs-free microalgae culture, since PSAs are ultimately produced during their growth. Also, the removal of PSAs is not cost-effective. Yet, phosphate-based harvesting would be applicable to the dilute microalgae cultures having a low concentration of PSAs. In the future, several other techniques can also be applied to achieve auto-flocculation at low pH. For instance, selecting such species of microalgae which grow at pH closer to threshold pH of flocculation. Tran et al. (2017) showed that *Nannochloropsis oculata* can naturally attain pH 10.4 during their growth (Tran et al., 2017). They found that the pH of microalgae culture can be increased by controlling carbon and air supply and by re-modeling photosynthesis parameters. *Chlorella sorokiniana* is also reported to grow at $pH > 10$ (Liu et al., 2018) and their threshold flocculation pH is 11.0. Modulation in the medium composition can also help in attaining flocculation at natural pH due to the distinct nature of the electrolytes present in the growth medium. Generally, microalgae culture contains low colloid concentration and low alkalinity, which is considered the most challenging medium in flocculation study (Brady et al., 2014). Probably, the addition of alkalinity would help to attain flocculation at low pH. However, the economics of this process would dictate whether an increase in alkalinity by chemical aid or raise in pH is a favorable choice.

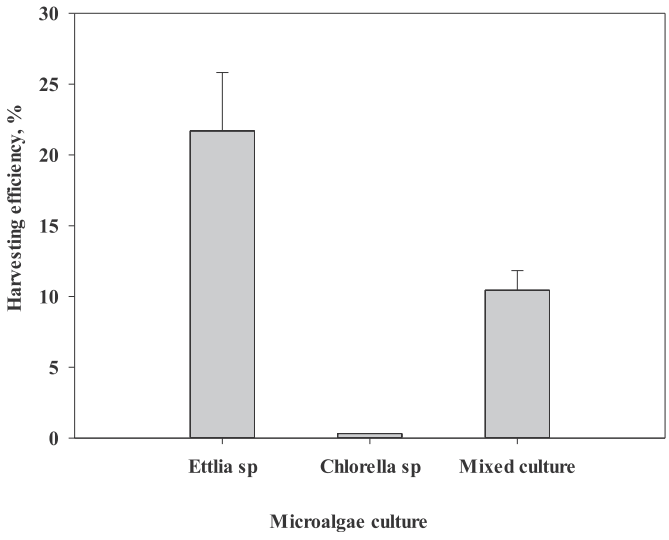

Fig. 6. Harvesting efficiency of *Ettlia* sp, *Chlorella* sp, and the mixed culture (without pH adjustment). Error bars show the standard deviations (n = 3).

7. Conclusions

This study highlights that the auto-flocculation of microalgae is driven by PSAs. PSAs showed a positive impact on harvest *Ettlia* sp. but the negative impact on *Chlorella* sp. harvest. The positive role of PSAs is attributed to their interaction with calcium to form Ca–PSAs ligand, which flocculates microalgae. Efficient Ca–PSAs interaction required an optimized concentration of calcium. Ca–PSAs based auto-flocculation was not suitable for *Chlorella* sp. due to non-flocculating characteristics of *Chlorella* cells. Co-harvesting of flocculating (*Ettlia* sp.) and non-flocculating microalgae (*Chlorella* sp.) species did not improve the harvesting efficiency. This work provided an understanding that auto-flocculation is a species-dependent process, and thus, each species would need a distinct harvest protocol.

Declaration of interest

The authors declare no declaration of interest and the authors don't have any copyright issues with the publication of this article.

Acknowledgments

This work was supported by the Advanced Biomass R&D Center (ABC) of the Global Frontier Project, funded by the Ministry of Science, ICT and Future Planning (ABC-2010-0029728).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2019.06.154.

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