Bioresource Technology 213 (2016) 216–221

Contents lists available at ScienceDirect

Bioresource Technology

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

Harvesting of microalgal biomass: Efficient method for flocculation through pH modulation

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HIGHLIGHTS

- Demonstration of the auto-flocculation capability of *Chlorococcum* sp. R-AP13.
- Demonstration of the use of chitosan for flocculating *Chlorococcum* sp.
- 94% efficiency in cell harvesting achieved through flocculation by modulation of me l.
- Medium after flocculation re-used for cultivation without significant reduction in densities.
- No significant change in fatty acid profiles for cells flocculated by pH change, ch an or by auto-flocculation.

ARTICLE INFO

Article history:
Received 9 January 2016
Received in revised form 17 March 2016
Accepted 21 March 2016
Available online 23 March 2016

Keywords:
Microalga
Chlorococcum
Flocculation
Biofuel
Algal cultivation

ABSTRACT

Harvesting of the mic g *coccum* sp. R-AP13 through autoflocculation, chemical flocculants or by change in medium p was e Surface charge of algal cells changed in response to the method used and affected floccu on ciency. While aluminum sulfate and $FeCl_3$ supported 87% and 92% efficiency, au culation recover 75% of biomass in 10 min. Maximum efficiency (94%) was obtaine ge in m um pH from 8.5 to 12.0 achieved through addition of 40 mg l^{-1} of NaOH. Sinc h conc rations o Cl_3 and $AlSO_4$ were toxic to the cells, flocculation induced by pH change m consi most effective strategy. Residual medium after flocculation could be reused effi- ient , minimizing the demand for fresh water.

1. Introduction

Microalgal h roduc ms generally involve cultivating them in env ment t mulates the accumulation of target me lites a the recovery of the biomass for the downstream proc 2011). However major bottleneck in the algal bio based product development is the recovery of biomass from the duction medium, mainly due to the smaller size (5–50 μm), pres e of negative surface charge, low biomass concentrations, and similarity of the density of algal cells to the growth medium (Garzon-Sanabria et al., 2012; Milledge and Heaven, 2013). Key factor limiting the commercial use of microalgal biomass is cost effective harvesting, which is considered to be the most challenging area in algal based biofuels (Georgiana and Mayfield, 2012). It has been suggested that 20–30% of the cost of algal biomass is the cost of harvesting (Mata et al., 2010). Harvesting technology is an important factor in the production of algal based biofuels, and an effective, convenient and economical method of microalgal harvesting is yet to evolve. The high costs involved in harvesting are acceptable only in cases where the target microalgal products are of high value. For low-value bulk products, both the investment as well as the operational costs should be drastically reduced to make commercial production feasible (Wijffels and Barbosa, 2010). So it is necessary to develop cost effective techniques that can permit efficient harvesting of microalgal biomass from culture systems.

Several methods have been tested for the harvesting of algal biomass, which includes centrifugation, filtration, flotation and flocculation (Uduman et al., 2010; Milledge and Heaven, 2013). Flocculation is a chemical based separation process that needs less energy than centrifugation and filtration, and thus it is regarded as one of the most promising means of dewatering algal biomass

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http://dx.doi.org/10.1016/j.biortech.2016.03.114
0960-8524/

(Wan et al., 2015). A large number of chemical products have been tested as flocculants including various inorganic multivalent metal salts (Duan and Gregory, 2003) and organic polymers/polyelectrolytes (Vandamme et al., 2010). A variety of flocculation strategies, such as physical, chemical and biological methods have been developed for microalgal harvesting as summarized in recent reviews (Vandamme et al., 2013; Wan et al., 2015).

The mechanism of flocculation depends on the interaction of cell surface charge and flocculent charges. Metal salts such as aluminum sulfate, ferric chloride, ferric sulfate, etc. are generally employed in flocculation processes, since they lead to improved harvesting efficiencies. One of the disadvantages of these inorganic flocculants is that they are required in high doses and results in contamination of the biomass with aluminum or iron (Wyatt et al., 2012). Chitosan has recently emerged as a favorable organic flocculating agent for harvesting of microalgae. Compared with other flocculants, it presents various advantages, including formation of larger flocs, resulting in faster sedimentation of biomass and providing a clearer residual solution. Chitosan is also non toxic and biodegradable which makes it possible to reuse the flocculated medium for algal cultivation (Chen et al., 2014). Another strategy being actively investigated is the use of auto-flocculation for harvesting of algal cells. Auto-flocculation can occur naturally in some microalgae and they flocculate in response to certain environmental stresses such as change in nitrogen concentrations, pH, dissolved oxygen and the amount of some metal ions in the medium (Uduman et al., 2010).

It is well known that flocculation of algal biomass is sensitive to pH of the culture medium and enhancement in flocculation efficiency with increase in pH increase has been reported (Wu 2012). Recently, several studies have revealed that micr can be successfully flocculated by adjusting the pH. The pH th old for flocculation may vary with several parameters, such properties of cell surface, biomass concentration sition, and flocculation time (Yang et al., 2). W the p increased from 8.5 to 10.5, the flocculati fficien of *Phaeodactylum tricornutum* was higher than 90% (this context, flocculation by simple i ease o medium pH could be an attractive alternative b e it is low low energy and non toxic to microalgal cells use of floc ts can be avoided. Another advantage o his st y is that the growth medium can be recycled r flocculatio nce no flocculants are used and medium ot contaminated toxic chemicals. However, this metho s test nly in a few number of microalgal strains (Castrillo ., 2 ; Yang et al., 2015). The method was also successfully nstrate *Chlorococcum* sp. (Wu et al., 2012; 201

Present estig n high the potential of physical conditions/fe es like change of medium in flocculating the algal cells. Di t culants including aluminum sulfate, ferric chlor itosan and auto-flocculation and pH change of medium was pared for harvesting of *Chlorococcum* sp. R-AP13 biomass. F dation efficiency, dose, and zeta potential of algal biomass during flocculation were studied and fatty acids profiling was conducted in the optimized flocculated biomass. Recycling of the residual medium after flocculation for the cultivation of alga was also investigated. For the first time, we demonstrate that the residual medium after flocculation using pH change, autoflocculation or using chitosan as flocculent supports similar algal growth as fresh medium and there are no significant differences in the fatty acid profiles of algal cells grown in fresh or re-used medium. On the other hand, the fatty acid profiles of algal cells grown in residual medium from chemically flocculated cultures were considerably different from those of their counter parts grown in fresh medium.

2. Materials and methods

2.1. Microalga and culture conditions

Chlorococcum sp. RAP-13 (Ummalyma and Sukumaran, 2014) was used for flocculation studies. The alga was maintained in MA medium, with a composition in mg l^{-1}: Ca $(NO_3)_2{\cdot}4H_2O$-50, KNO_3-100, $NaNO_3$-50, Na_2SO_4-50, $MgCl_2{\cdot}6H_2O$-50, Na-β-glycerophosphate$\cdot 5H_2O$-100, $Na_2EDTA{\cdot}2H_2O$-5, $MnCl_2$-5, $FeCl_2{\cdot}6H_2O$-5, $Na_2MoO_4{\cdot}2H_2O$-0.8, H_3Bo_3-20. pH of the medium was adjusted to 6.8. Cells for the study were grown in 5 L flasks containing 3 L medium and incubated in a climate contr ber at 30 °C with diurnal cycle of 14/10 h. Flocculatio udies w erformed after the cells reached stationary phase e growth. F ulants tested were procured either from M ck, In r Sigma– rich, India.

2.2. Flocculation experime

The effect of flo ule nd conc tration on the flocculation efficiency w determi using r test (Vandamme et al., 2010; Gerde , 2014). B algal suspension (100 ml) was stirred pm in a 10 beaker, while the flocculent was added slowly fter this, the stirring was continued for 2 min then stopped a llowed to settle for 10 min. Then an aliq e supernatant s taken at a depth of ~2.0 cm from the s ace of the liquid and its absorbance was measured at 680 nm in visible spe photometer. Absorbance of the original suspens was also en before addition of the flocculent. The absorba values ere extrapolated to cell numbers based on a standa e constructed with algal cell suspensions having different cell densities. Flocculation efficiency of *Chlorococcum* sp. was d as below (Eq. (1))

$$\frac{(\text{Initial Cell Conc.} - \text{Cell Conc. in Supernatant})}{\text{Initial Cell Conc.}} \times 100 \quad (1)$$

2.3. Zeta potential measurement

Zeta potential of the *Chlorococcum* sp. R-AP13 was measured before and after the addition of various flocculants into the medium using Malvern Zetasizer 90 (Malvern Instruments Ltd., USA). Zeta potential was analyzed in triplicates at room temperature and the mean values were taken.

2.4. Cell viability

The viability of flocculated cells was tested by dye exclusion method using 1.0% Trypan blue, which is excluded by viable cells. One milliliter samples of each experiment were centrifuged at 6000 rpm for 5 min and the supernatant was discarded. Then 100 µl of the 1.0% Trypan blue solution was added, and the cells were incubated for 3 h at room temperature. Next, the cells were washed twice using deionized water to remove excess of unbound dye. Finally, the fresh preparations of cells were examined for dye exclusion under a Phase contrast Microscope (Leica DMLS2000, Germany). Cells with intact cell wall (live cells) exclude Trypan Blue, while the dead cells take up the dye, differentiating viable and non-viable cells.

2.5. Recycling of flocculated medium

Flocculated biomass and medium were separated by aspirating the medium. pH of the residual medium was adjusted to 6.8–7.0 using 1N NaOH or HCl. After that, components of MA medium were added and used for cultivation of the next batch of cells. Fresh MA

medium was used as control. The control and recycled media were inoculated with 10% v/v of an inoculum containing 3×10^6 cells ml^{-1}. Biomass production was monitored as cell density at two days interval.

2.6. Fatty acids profiling

Fatty acid profile of oil from different flocculated biomass were done by acid mediated trans-esterification for FAME generation followed by gas chromatography methods as described in Ummalyma and Sukumaran (2014). FAME was identified by comparing their fragmentation pattern with internal standards (Sigma Aldrich, India).

3. Results and discussion

3.1. Evaluation of inorganic flocculants for harvesting microalgal cells

Among the inorganic flocculants-aluminum sulfate and ferric chloride tested for flocculation of *Chlorococcum* sp. R-AP13 cells, $FeCl_3$ was found to be more effective than aluminum sulfate. $FeCl_3$ supported a flocculation efficiency of 92% at concentrations of 70–80 mM while aluminum sulfate had an efficiency of 87% at 120 mM concentration (Fig. 1A and B). Initial zeta potential of the algal cells was found to be −20 mV. The surface charge of the cell changed after the addition of flocculants. Flocculation efficiency was increased near to the neutralization point. Higher concentrations of aluminum sulfate and ferric chloride increased the positive charges in the medium which affected the flocculation efficiency

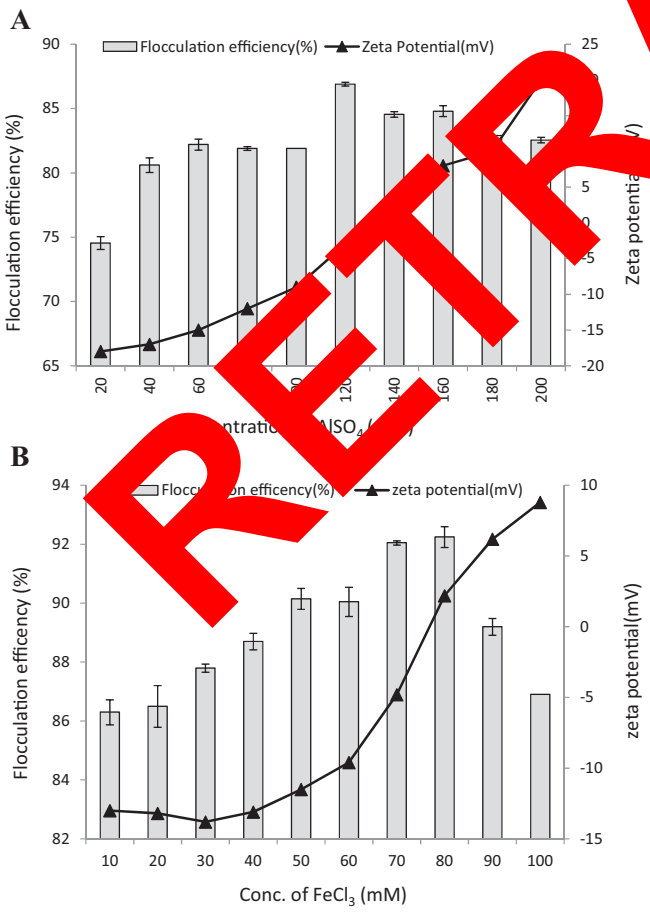

Fig. 1. Flocculation of *Chlorococcum* sp. RAP-13 cells using inorganic flocculants. A: Aluminum sulfate, B: Ferric chloride.

of cells (Fig. 1A and B). Possible explanation for this could be that the amount of flocculent that exceeded the optimum concentration could contribute to excess of positive charges, thus stabilizing the cell particles in suspension by charge repelling, as well as by stearic hindrance (Vandamme et al., 2010).

The flocculation mechanism depends on the nature of the algal cells and the charge of the flocculent. Numerous chemical coagulants or flocculants have been tested for microalgal flocculation (Rakesh et al., 2014). Metal salts (aluminum sulfate, ferric chloride, etc.) are generally preferred because they lead to improved harvesting efficiency. The results of $FeCl_3$ as flocculent showed an almost comparable efficiency with the [illegible] efficiencies for flocculation of *Chlorella zofingiensis* ([illegible] et al., [illegible]12). For any given algae species, effective floc[illegible]on with Fe[illegible] might be obtained if the conditions of neg[illegible]e charge [illegible] sufficient flocculent concentrations are a[illegible]able in t[illegible]edium. Since different algal species vary in th[illegible]concentration[illegible]tional groups on the cell surface, the [illegible]imum [illegible]ount of $FeCl_3$ required for effective flocculation may [illegible]yatt et [illegible], 2012). When compared with alumin[illegible]sulfate[illegible]ic chlo[illegible]e is generally required in minimum co[illegible]rations to [illegible]coagulation of algal cells. In solution, fe[illegible]ide forms po[illegible]ely charged hydroxide precipitate (at p[illegible] < 8) w[illegible] associates with the negative algal cell surface. The ferric hydr[illegible]e precipitates form bridges between algal [illegible]hich bind the[illegible]ogether into flocs. At low algal concen[illegible]tions, the amount of $FeCl_3$ required to achieve coagulation inc[illegible]ses linearly [illegible]ith algal concentration. However, at higher con[illegible]trations, th[illegible]minimum amount of $FeCl_3$ required for flocculation [illegible]omes [illegible]ependent of algal concentration, as the dominant me[illegible] changes from electrostatic bridging to sweep [illegible]ulation by large coagulated algal flocs (Wyatt et al., 2012). M[illegible]dvantage of inorganic flocculants such as alum and iron [illegible]oride is that it may lead to contamination of growth medium with aluminum or iron (Oh et al., 2001). Nevertheless, they may be useful in treatment of wastewaters, wherein the spent water after mass multiplication of microalgae can be passed through columns to remove the Fe ions and then reused for algal cultivation. In this present study, ferric chloride was found to be a more effective flocculent for harvesting of microalgae compared to alum.

3.2. Evaluation of chitosan for flocculation

Chitosan is a cationic polysaccharide, which has emerged as a favorable flocculating agent in the harvesting of microalgae (Xu et al., 2013). Compared with other commercial flocculants, it has various advantages, including production of larger flocs (Zeng et al., 2008) resulting in faster sedimentation rates and providing a clearer residual solution after harvesting, and being nontoxic and biodegradable (Knuckey et al., 2006). Use of chitosan as flocculent makes it possible to reuse the residual solution to grow microalgae. Chitosan mediated flocculation of *Chlorococcum* sp. R-AP13 was tested at concentrations of 20–120 mg l^{-1}. Flocculation efficiency of 84% was obtained at a concentration of 40 mg l^{-1} and the zeta potential of algal cell was changed from −20 mV to +5 mV (Fig. 2). Further increase in the concentration of chitosan increased the positive charges on the cells which affected further flocculation. This drastic decrease in performance could have resulted when the chitosan overdose caused an overload of positive charges, which were retained on the surface of the cell causing repulsion between positively charged microalgal cells resulting in re-stabilization.

3.3. Flocculation of algal cells by changing pH of the medium

Recently, flocculation induced by increase in pH has gained more attention for algal flocculation (Wu et al., 2012; Rakesh

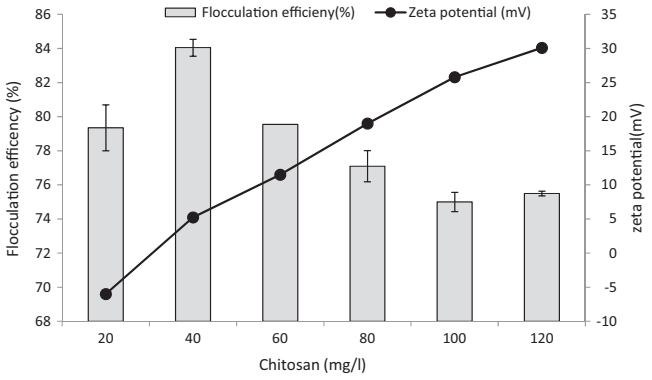

Fig. 2. Flocculation of *Chlorococcum* sp. RAP-13 cells using chitosan.

et al., 2014). In this present study, increase in medium pH as a flocculation agent was evaluated for *Chlorococcum* sp. R-AP13 cells. Flocculation efficiency increased as the medium pH was increased to the alkaline range of 11–12. Maximum efficiency of 94% was obtained with the pH increased to 12. Zeta potential of the algal cell varied with different pH, but the surface charge of the algal cells was negative in the alkaline pH (Fig. 3).

The zeta potentials were pH dependant and negative at different pH values. For freshwater microalgal systems, the zeta potential was shown to initially decrease with increase in pH, but increasing on further increase of pH. The decrease in zeta potential with pH increase indicated the decrease of the cell surface charges, possibly due to charge neutralization in this range. Possible mechanisms of pH mediated flocculation is the formation of M precipitate from Mg^{2+} in the growth medium as the pH incre d. The $Mg(OH)_2$ precipitate has a large adsorptive surface area a positive superficial charge (Parks, 1967). This pr te attra the negatively charged microalgal cells, thus re he cor pression of the electrical double-layer and ca g them become destabilized and hence to flocculate. For wate zeta potential increased after the initial cline n was attributed to the dissociation of carboxyli d groups he surface of microalgal cells (Henderson et al. However, t culation efficiency was significantly hig indi g that swe p flocculation was active in this pH ra e (Wu et a 12). $Mg(OH)_2$ precipitates tend to have a rat open structure, that even a small mass could give a larg fective olume concentration and hence it has a high probab of c ring microalgal cells (Duan and Gregory, 2003). The flo n efficie was therefore considerably improved n wh particl were destabilized just by charge neu izat Prese ts agreed with the previous reports h u et a 2012) and andamme et al. (2011).

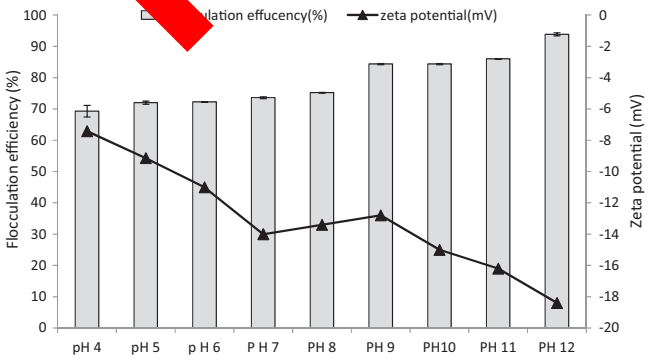

Fig. 3. Flocculation of *Chlorococcum* sp. RAP-13 cells by increase in medium pH.

Flocculation induced by high pH is considered as a potentially useful method to pre-concentrate fresh water microalgal biomass during harvesting (Vandamme et al., 2011). However, as microalgae usually carry a negative surface charge, an increase in pH will cause an increase in surface charge rather than a decrease, which might be the possible cause for flocculation induced by high pH. The use of flocculation induced by high pH for harvesting microalgae may have an additional advantage that the high pH may effectively sterilize the microalgal biomass as well as the process water. This may be advantageous when microalgae are used in wastewater treatment, as the high pH may kill pathogenic microorganisms (Semerjian and Ayoub, 2003). It has be d that an increase in pH within the range of 8.5–11.0 a s the r ery of microalgae (Horiuchi et al., 2003; Sirin l., 2012), a has biomass recovery efficiencies higher th n 90

3.4. Autoflocculation

Autoflocculation of *um* sp. AP13 was evaluated by culturing the c up to week incubation under photoautotrophic con n and floc ic fficiency was tested every week. Floc fficiencies eased as the incubation time increased from in the initial week to maximum efficiency of 75% in the 3rd wee ncubation. Zeta potential of cells became m gative with in ase in incubation time. Potential of roalgae to autoflocculate depends on their physiological conditions. Autofloc ation may be induced by end of the exponential p e and coul e resultant of the pH changes in the culture broth (W et al. 2). Algal surface charge and suspension stability is clearly ated to functional groups on the cell wall and zeta-potential is often used as an indicator of cell stability. The decline potential from the exponential to stationary phase has been correlated to surface functional groups in *C. zofingiensis* (Zhang et al., 2012). Therefore, micro algal cell instability is presumed to increase in the later growth phase.

Cell flocculation widely occurs in microorganisms and several self-flocculating microalgae have also been discovered, such as *Chlorella vulgaris* JSC-7 (Alam et al., 2014), *Scenedesmus obliquus* AS-6-1 (Guo et al., 2013). Limited reports are available in the literature regarding the auto flocculation of cells and actual mechanism of auto flocculation is still obscure. Guo et al. (2013) and Alam et al. (2014) had studied the biochemical basis of auto flocculation in the micro algae *S. obliquus* AS-6-1 and *C. vulgaris* JSC-7 respectively. They found that the polysaccharides biosynthesized by these two strains were responsible for self-flocculation. Another recent report proposed that glycoproteins are involved in cell flocculation of the green microalga *Ettlia texensis* SAG79.80 (Salim et al., 2014). Therefore, microalgal self-flocculation may occur when the flocculating agents (e.g., polysaccharides and glycoprotein) produced by microalgal cells themselves patch adjacent cells, or it may be due to formation of bridges between the cells via charge neutralization with changes in medium pH, promoting self-flocculation. More research is needed in this area to understand the exact mechanism of self flocculation of microalgal cells. Microalgal self-flocculation, differing from the flocculation induced by pH adjustment, can occur naturally via interaction of adjacent cells without acid, alkaline, or metal ion addition. Moreover, harvesting microalgae using self-flocculation, which requires no extra expenditure in cultivation of microalga or purification of bio-flocculent, is a promising method for low-cost harvesting.

3.5. Viability of flocculated biomass

Viability assay of flocculated biomass was carried out by Trypan blue staining of the cells. Auto flocculated cells, cells flocculated by chitosan and through change in medium pH were found to be

viable. However, at least some cells flocculated through aluminum sulfate and ferric chloride showed dye uptake indicating the presence of dead cells and the percentage of dead cells were proportionate to the concentration of the flocculent. Inorganic flocculants, including alum and iron chloride, may also lead to contamination of the growth medium with aluminum or iron (Oh et al., 2001). Flocculation by alum or ferric chloride therefore cannot be considered as a preferred method for algal biomass recovery in this case, since it was found to be toxic to the cells besides contaminating the residual medium. Flocculation mediated by chitosan was very effective for harvesting the biomass, with the added advantages of non toxicity and complete clarification of medium after flocculation. However, the cost of chitosan is high making it not a feasible option for large scale usage. Flocculation mediated by auto flocculation or induced by pH increase may be considered as effective strategies for harvesting the microalgal biomass since these are low cost processes and no extra flocculants are required for harvesting of the biomass.

3.6. Recycling of flocculated medium for algal cultivation

Medium recovered from flocculation could preferably be recycled for next round of cultivation. In the flocculation studies performed, the medium was recovered after flocculation and then were supplemented with nutrients (Components of MA medium). The medium pH was adjusted to 6.8–7.0, and was used for algal cultivation so as to evaluate the possibility for medium recycling. *Chlorococcum* sp. R-AP13 cells were cultivated in the recycled medium (Fig. 4). It was observed that the cell densities of *Chlorococcum* sp. R-AP13 cultivated in the recycled growth medium were clos that cultivated in fresh MA medium, indicating that the resid medium after flocculation and separation of cells could be succes fully recycled for cultivation of the alga. Previous studies con ducted with *P. tricornutum*, *Nannochloropsis* and *Chlorococcum* sp. have also concluded that the mass overy from fresh or recycled media were similar (et al., 2; Liu et al., 2013).

3.7. Fatty acids profiling of flocculated mass

Fatty acids profiling of floccula ed biom was carried out to check any changes in the lipid ofile of biom fter the addition of flocculants in the medi Results showed th atty acids profile of auto flocculated b ass, p duced and chitosan mediated flocculated biomass wer a ed, while the biomass from aluminum sulfate and ferric c e floc d cultures showed differences in the ids p (T e 1). Fatty acids profile of biomass fro esh m um and dual medium from pH treatment showe imilar profi whereas biomass grown in residual medium fro itosan treatment showed longer chain fatty ac th oleic ac the major fatty acid. Lipids profile of alga s grown in residua edia from aluminum sulfate and ferric oride treatments also showed elevated oleic acid content wh was signifi tly higher that the levels of this fatty acid from cell wn in fre media. Oleic acid production might have a protectiv to e up with the toxic chemicals in the medium. Increase in unsaturated fatty acid content of algae grown in media could also be a sign of stress and may be recognized anism of adaptation to the environmental conditions. It as been suggested that algal TAG serves as a depot of PUFA, which can be mobilized for the construction of chloroplast membranes under certain environmental conditions (Khozin-Goldberg et al., 2005).

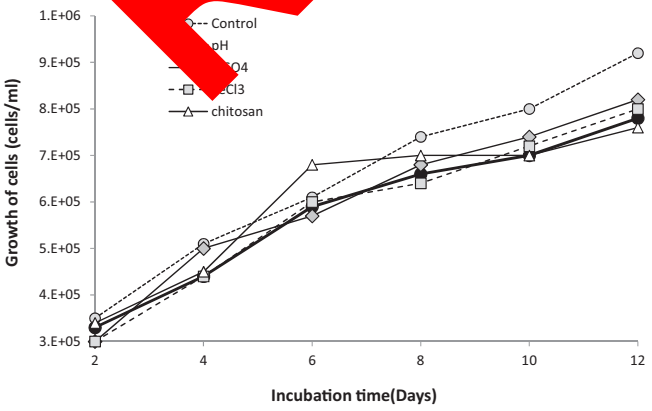

Fig. 4. Growth of *Chlorococcum* sp. R-AP13 in recycled medium.

Table 1
Fatty acid profile of algal biomass cultivated in fresh medium and residual medium from different flocculation treatments.

Fatty acid type	Fatty acid content in the oil (%)									
	Auto flocculation		Chitosan		pH 12		$FeCl_3$		$AlSO_4$	
	F	R	F	R	F	R	F	R	F	R
C12	3.7	2.8	2.8	1.8	4	3.6	3.0	7.2	1.6	5.6
C14	2.3	3.4	3.2	1.6	3	2.8	–	2.6	–	0.8
C15	1.7	1.8	1.8	–	3.2	3.2	1.8	–	–	–
C16	39.7	39.8	42	22.6	41.2	44.2	44	10.2	38	6
C16:1	3.6	5.2	2.8	2.8	3.7	3.8		2.2	–	–
C17	2.1	1.2	3.7	2.6	3.1				–	–
C18:0	8.1	9.2	6.2	8	3.8		20		18	8.2
C18:1	22.0	16.2.	20	34.2	16.	8	24	47.	26	57.3
C18:2	3.2	4.6	7.8	6.2	7.1		8	4.4	12	2.8
C18:3	7.5	6	3.1	5.6	2	2.	12	9.9	8	12.7
C22	–	–	–	–	–	–			–	2.3
C22:1	5.9	4.6	5.8		5	2.8			–	2.6
C24	–	–	4		–	–	–	–	–	–

F – Fresh medium; R – R ed m residual um after flocculation).

4. Conclusion

Development of economically feasible flocculation technology can significantly reduce the cost of microalgal biomass production. pH modulation as a flocculation method seems to be a feasible strategy since it attained a flocculation efficiency of 94%, and allowed re-use of the medium. pH-induced flocculation and auto-flocculation therefore can be considered as best possible options for cost effective and efficient harvesting of *Chlorococcum* cells. These methods also allow the re-use of media for further cycles of cultivation thereby minimizing fresh water requirement. The self flocculating micro alga – *Chlorococcum* sp. R-AP13 can be used for various applications such as biofuels and nutraceuticals.

Acknowledgements

SBU would like to acknowledge financial support from University Grants Commission (UGC), Govt. of India in the form of Research Fellowship for her Ph. D work. We thank the Department of Forests and Wildlife Kerala for permission and assistance in collection of the sample from deep forest locations from which *Chlorococcum* sp. R-AP13 was isolated.

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