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A rapid inoculation method for microalgae biofilm cultivation based on microalgae-microalgae co-flocculation and zeta-potential adjustment

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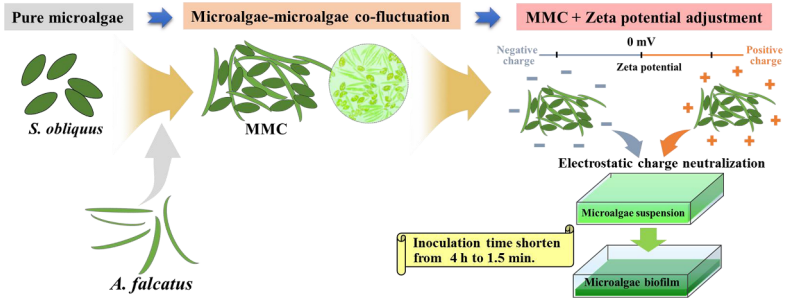
GRAPHICAL ABSTRACT



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ABSTRACT

Due to the small size, similar density to water, cells inoculating onto the solid carrier is a major challenge for microalgae biofilm cultivation. To reduce biofilm inoculation time, *A. falcatus* with long stripe were chosen as the bond linking with the main microalgae cells forming microalgae-microalgae co-flocculation by bridging and twining. The optimal matching species were *S. obliquus* and *A. falcatus* with the volume ratio of 4–1. By changing the zeta-potential of the microalgae-microalgae co-flocculation to positive and negative through pH regulating, the inoculation time was significantly shorted from 4 h to 1.5 min due to the charge neutralization. Fortunately, the added *A. falcatus* and pH regulation has no negative effects on biofilm growth. Inversely, the porous microstructure of microalgae-microalgae co-flocculation improve the transfer efficiency of nutrients, resulting a 90.15% increase on biomass productivity (229.15 g m^{-2}) comparing to pure microalgae species.

1. Introduction

Microalgae has been increasingly considered as a promising alternative to deal with the energy crisis and global climate change (Sun et al., 2018). Microalgae biofilm cultivation has become a cost-effective approach for bioenergy production and waste treatment due to its advantages of high biomass productivity, and low harvesting consumption (Miranda et al., 2017).

As the first step of microalgae biofilm cultivation, the inoculation step was the critical process for microalgae biofilm formation and growth. Thus, the inoculation method had significant effects on biofilm attachment strength and biofilm microstructure (Wang et al., 2017; Zhang et al., 2011), which were the basic factors for biofilm development. However, at present, due to the lack of relative research, the inoculation methods applied for microalgae biofilm cultivation almost drew on the experience of suspended biomass harvesting. For example,

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the high concentrated microalgae paste was sprayed on the substrate surface by a sponge roller (Naumann et al., 2013; Shi et al., 2014). Although the biomass cells could be simply and fast inoculated on the large-scale surfaces, the process of spraying inoculation not only resulted in uneven distribution of microalgae biomass, but also made it unquantifiable for quantifying the microalgae biomass. Furthermore, to obtain the high concentrated microalgae paste for this inoculation process, some microalgae cells enrichment methods, such as centrifugation, was used before inoculation, which increased the operating difficulty and cultivation cost. For these reasons, the filtration method was developed for quantitative inoculation (Cheng et al., 2014; Schultze et al., 2015). Certain concentration of microalgae was fast inoculated on the membrane via a filter unit with the consumption of pumping power, so that the dense biofilm formed on the membrane uniformly. The dense biofilm had stronger attachment strength on the substrate, but the tight microstructures enhanced the resistance of nutrients transfer, which against microalgae biofilm growth (Tian et al., 2010; Zheng et al., 2017). On the other hand, the energy consumption for pumping power increased the microalgae cultivation cost. And the filter unit strictly limited the size, shape and materials of the substrate membranes. Thus, this method was just used in laboratory studies and couldn't be extended application cosmically.

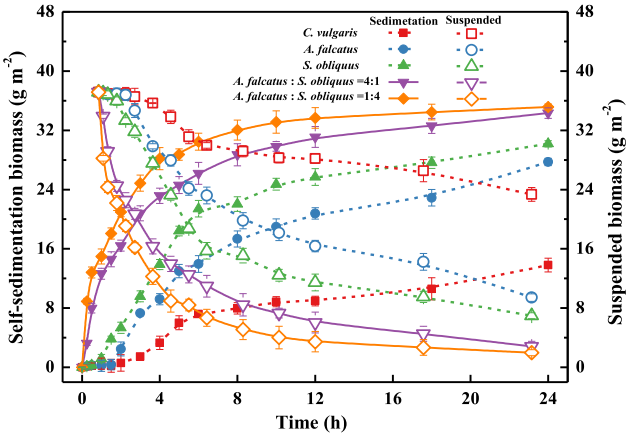
Therefore, to liberate inoculation process from the substrate constraints and lower the energy cost, microalgae suspension was applied to directly flowed through substrate surfaces, so that microalgae cells could attract onto the substrate surface under the action of electrostatic force, van der waals force and so on (Ozkan and Berberoglu, 2013). However, comparing with the flow shear force, these micro forces were very small. It was difficult for microalgae cells in the suspension to attachment on the substrate surfaces. Thus, the inoculation process always took a long time with this inoculation method (Choudhary et al., 2017). On the other hand, for this reason, the substrate surfaces used in this inoculation method should be strictly modified, for example increasing the roughness and designing the surface morphology, to enhance the microalgae attachment (Gross et al., 2015). Obviously, this method still couldn't be a universal choose for biofilm cultivation.

Thus, to reduce the energy consumption, Rincon et al., (2017) settled microalgae cells onto substrate surfaces by self-sedimentation. The sedimentation rate is calculated by Stokes formula, $u_0 = d_e^2(\rho_s - \rho)g/18\mu$ (u_0 is the sedimentation rate, d_e is the particle equivalent diameter, ρ_s is the particle density, ρ is the liquid density, g is the gravitational acceleration and μ is the liquid viscosity) (Stokes, 1851). Due to the microalgae characteristics of small size (2–30 μm) and similar density to water (González-Fernández and Ballesteros, 2013), the sedimentation rate of microalgae cells was quite low. The inoculation time usually took 6–24 h or even longer for with the natural sedimentation before microalgae biofilm cultivation. Thus, according to the guiding of Stokes formula, increasing particle diameter is an effective way to improve the sedimentation rate and shorten the biofilm inoculation time. For instance, Heng et al., (2009), Kim et al., (2012), and Tran et al., (2013) adopted extra ultrasound, electric field, and some chemicals to assist microalgae cells flocculating to big particle, and then improve the sedimentation rate. Nonetheless, these extra driving fields and chemicals not only increased the cultivation cost, but also damage the microalgae cells in a certain degree. As a low-cost and nontoxic method, bio-flocculation was obviously chosen to use for enhancing sedimentation rate during the process of biofilm sedimentation inoculation. High fluctuation efficiency of microalgae assisted with bacteria was obtained when mixture of bacteria and microalgae due to the mechanism action of trap and bridging via muciliage including of mucooplysaccharide, glycoprotein etc. on the cell surfaces (Wang et al., 2012). On the other hand, for some bacteria or fungal, the surface charges were active. The electrostatic neutralization would break the stability of the suspension and lead to flocculation and sedimentation. However, the assisted bacteria or fungal would degrade the quality of microalgae biomass, which was negative for microalgae growth and the subsequent bioproducts production, especially for high-value bioproducts (Wan et al., 2015). Fortunately, muciliage was found on the cell surfaces of some microalgae species, such as *Scenedesmus obliquus* (Gou et al. 2013). And its surface charge also could be adjustment by pH. Therefore, it seemed that choosing another microalgae as the addictive bio-flocculant was an optimal way to shorten the inoculation time and promote biomass accumulation of microalgae biofilm cultivation at the same time.

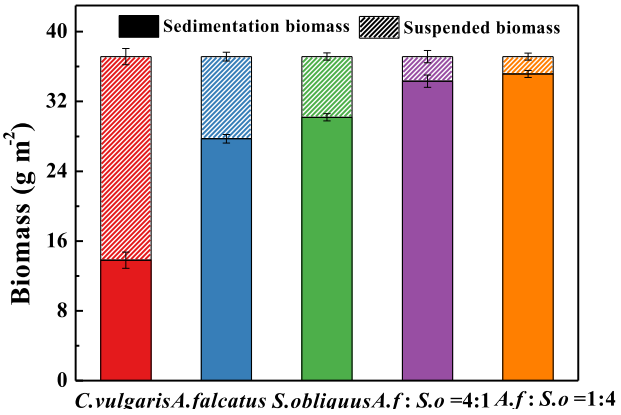
Therefore, in this study, a rapid inoculation method for microalgae biofilm cultivation was proposed by choosing another microalgae species to form the bigger floccule, the microalgae-microalgae co-

Table 1
The effects of microalgae mixing ratio on the self-sedimentations of microalgae-microalgae co-flocculation.

Microalgae Species	Self-sedimentation efficiency (%)					
	1 : 0	1 : 1	1 : 2	1 : 3	1 : 4	1 : 5
C. vulgaris : *S. obliquus*	37.17%	54.08%	64.38%	72.90%	80.40%	81.64%
S. obliquus : *C. vulgaris*	87.25%	54.08%	53.30%	50.12%	44.08%	40.54%
C. vulgaris : *A. falcatus*	37.17%	62.65%	70.15%	73.65%	77.71%	78.15%
A. falcatus : *C. vulgaris*	74.65%	62.65%	51.26%	47.30%	45.29%	42.45%
A. falcatus : *S. obliquus*	74.65%	89.47%	90.69%	90.76%	94.66%	92.89%
S. obliquus : *A. falcatus*	87.25%	89.47%	91.02%	90.85%	92.41%	90.88%



(a) The changes of self-sedimentation and suspended biomass

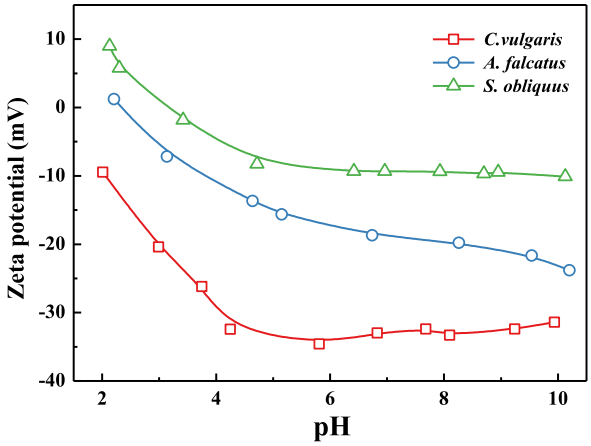


(b) The comparison of sedimentation and suspended biomass

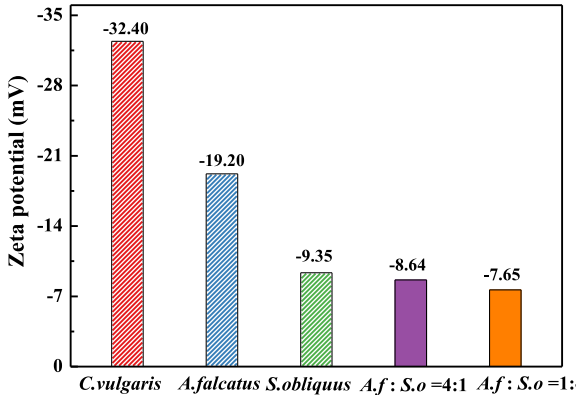
Fig. 1. Comparison of self-sedimentation characteristics between pure microalgae species and microalgae-microalgae co-flocculation. (a) The changes of self-sedimentation and suspended biomass. (b) The comparison of self-sedimentation and suspended biomass.

Table 2
Comparison of growth characteristics of three microalgae species cultivated in suspension for 7 days.

	C. vulgaris	*S. obliquus*	*A. falcatus*	*A. falcatus: S. obliquus* = 1:4
Floc porosity (%)	–	13.38	17.77	26.61
Size (μm)	5–7 (6.23)	9–12 (10.36)	28–50 (37.27)	–
Dry weight ($g\,L^{-1}$)	3.14	3.08	3.26	3.61



(a) Zeta potential of pure microalgae cells changes with pH



(b) Comparison of zeta potential under pH=7.0

Fig. 2. Effect of pH (a) and microalgae-microalgae co-flocculation (b) on Zeta potential under pH = 7.0.

flocculation (MMC). Subsequently, the zeta potential of the MMC was adjusted to negative charge and positive charge via pH adjustment respectively. And the effects of species ratios and pH on sedimentation rates were also investigated. Thereafter, a long-term microalgae biofilm cultivation was conducted using this inoculation method.

2. Materials and methods

2.1. Microorganisms and culture conditions

Three microalgae species, *Chlorella vulgaris* FACHB-31 (*C. vulgaris*), *Scenedesmus obliquus* FACHB-417 (*S. obliquus*) and *Ankistrodesmus falcatus* FACHB-20 (*A. falcatus*), were chosen for this study. They were purchased from the institute of hydrobiology, Chinese Academy of Sciences (Wuhan, China). All the microalgaes were maintained in Blue-Green medium (BG11 medium) under a light intensity of 90 μmol $m^{-2}s^{-1}$ in the artificial greenhouse (25 ± 0.5 °C) (Zheng et al., 2016). The initial pH of the medium was adjusted to 7.0 with 0.1 M HCl, and the medium was autoclaved before use.

2.2. Experiments setup

The experiments for self-sedimentation characteristics of microalgae were investigated in the cylindrical serum bottles (length of 180 mm, width of 60 mm, and height of 310 mm). 500 mL of microalgae suspension was put into the serum bottle for self-sedimentation testing and the serum bottle was sealed and covered by silver paper to block out light and reduce the effects of microalgae proliferation on the obtained results. The suspensions of 1.5 mL were gently sampled using a pipettor every several minutes using for the measurement of self-sedimentation characteristics and flocs analysis. The experiment of self-sedimentation was lasted 24 h. In this study, self-sedimentations of the three pure microalgae species were tasted in the above experiments system, firstly. And then, the flocculation and sedimentation of two mixing microalgae species with different mixing ratio (1:1, 1:2, 1:3, 1:4 and 1:5) were also tested Thirdly, to further control the flocculation and sedimentation process of mixing microalgae species, the initial pH of the mixing microalgae suspension was regulated in the range of 2–9. In order to clarify the effects of initial pH on microalgae activity, the growth experiment was arranged after the sedimentation process control by pH adjustment. The growth of microalgae with overly basic or acidic inoculation conditions were compared with that in neutral inoculation conditions. Finally, the biofilm growth characteristics of MMC were investigated in the biofilm cultivation system, which was represented in detail in the authors' previous paper (Zheng et al., 2016). The precultured *A. falcatus* and *S. obliquus* suspensions were mixed together and inoculated into the bioreactor with substrate and then the mixed microalgae suspension were settled on the substrate, so that the concentrated mixed microalgae paste were formed on the substrate. Furthermore, these microalgae paste also acted as the inoculation species for the following biofilm cultivation.

2.3. Analysis methods

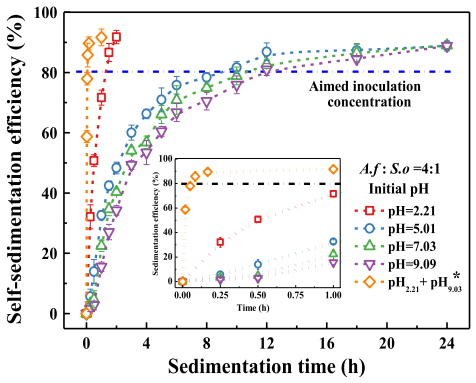
2.3.1. Analysis of self-sedimentation characteristics of microalgae suspension

Self-sedimentation efficiency (SE) was calculated based on changes of the optical density of microalgae suspension at an absorbance of 685 nm (OD_{685}) using a UV-Visible spectrophotometer (Persee TU-1901, China) during the experiments according to the following formula (Miranda et al., 2017):

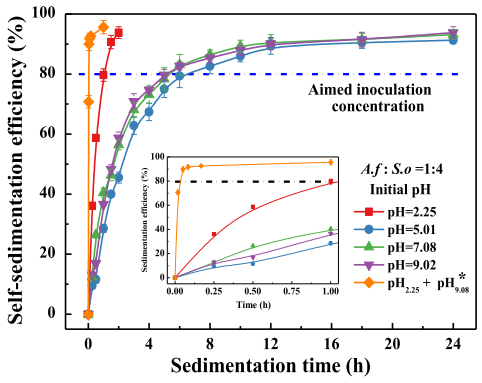
$$SE = (1 - OD_A/OD_0) \times 100\% \quad (1)$$

where OD_A is the average OD_{685} of microalgae suspension sampled from the upper layered, the middle layer and the bottom layer in the self-sedimentation bottles, OD_0 is the OD_{685} at the beginning of the experiment.

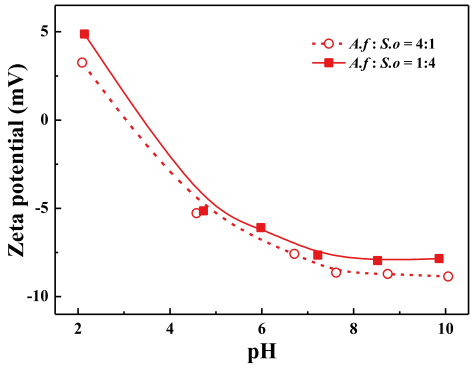
Zeta-potential is an important indicator of the degree of repulsion among the charged particles in the suspension. It is an important parameter to characterize the stability of liquid dispersion. In this study, in order to measure the self-sedimentation characteristics of microalgae suspensions, the zeta-potential was measured with a zeta-potential-analyzer (Mastersizer 2000, UK) at different pH ranges. The zeta-potentials were evaluated at a room temperature of 20 ± 2 °C. For each species, triplicate cultures were taken for measurements, and each dataset, 10–20 readings were taken for each sample (Wrede et al., 2014).



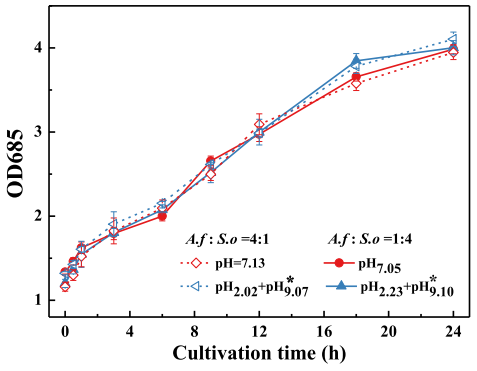
(a) *A. falcatus* mixed with *S. obliquus* (4:1)



(b) *A. falcatus* mixed with *S. obliquus* (1:4)



(c) Zeta potential of co-flocs



(d) Effect of inoculation pH on growth

Fig. 3. Effects of initial pH on the self-sedimentation of microalgae-microalgae co-fluctuation. (a) The self-sedimentation characteristics of *A. falcatus* mixed with *S. obliquus* (4:1). (b) The self-sedimentation characteristics of *A. falcatus* mixed with *S. obliquus* (1:4). (c) Zeta potential of microalgae-microalgae co-flocculation. (d) Effect of inoculation pH on microalgae-microalgae co-flocculation growth.

2.3.2. Floc morphology analysis

The flocs were gently picked up from the microalgae suspension with an inoculating loop. Then, floc samples were fixed, stained and dehydrated in the proper sequence as described by Surman et al. (1996). The samples remained desiccated and were gold sputtered using a sputter coater and viewed in a scanning electron microscopy (SEM) (TESCAN, VEGA3). From the SEM images of floc sections, the porosity of flocs was calculated with the digital image processing program Adobe Photoshop (Zheng et al., 2017).

2.3.3. Microalgae biofilm growth analysis

During microalgae biofilm cultivation, two parallel samples were sampled every two days as follows: first, removed the supernatant in each tested PRB, then washed down the attached biofilm on the substrates with sterile distilling water, and dilute the resuspension to 25 mL in a volumetric flask. This microalgae resuspension were used for subsequent measurements and analysis.

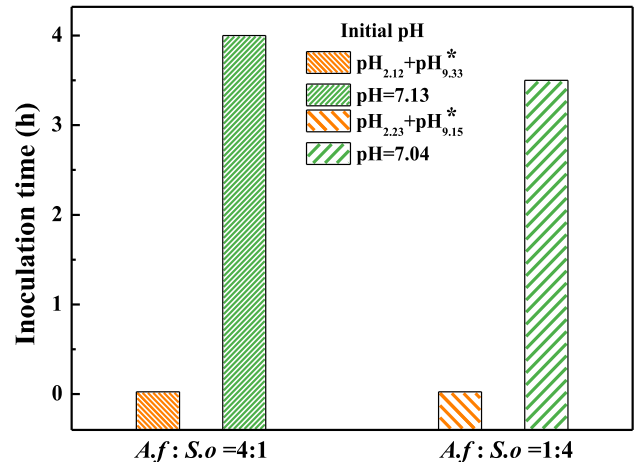
Firstly, the microalgae resuspension was sampled to measure the cell ratio of the mixed two microalgae by cell counts with microscope (Olympus IX81, Japan). The microalgae cells were counted in the microscope images. Then, the biomass concentration of microalgae biofilm was determined by weight method. The resuspension samples were dewatered by centrifugation and dried at 85 °C for 24 h to constant weight. And the obtained dry biomass was weighted by the electronic balance. Biomass concentration ($g\,L^{-1}$) was calculated from the microalgae dry weight produced per liter. And for the biofilm cultivation, the biomass concentration was then converted to areal density ($g\,m^{-2}$) based on the substrate area and resuspension volume. Finally, the resuspension was dried and ground into powder for lipid extraction, which was performed through direct transesterification (Cheng et al., 2016).

As for the parameter of Fv/Fm, Fv and Fm represent fluorescence parameter and maximum fluorescence, respectively. The ratio of Fv/Fm that represents the maximum quantum yield of photosystem II (PS II) was also calculated to determine the microalgae photosynthetic activity. The microalgae suspension first underwent dark adaptation for 15 min. The fluorescence parameters were subsequently measured with an algal efficiency analyzer (AquaPen AP-C100).

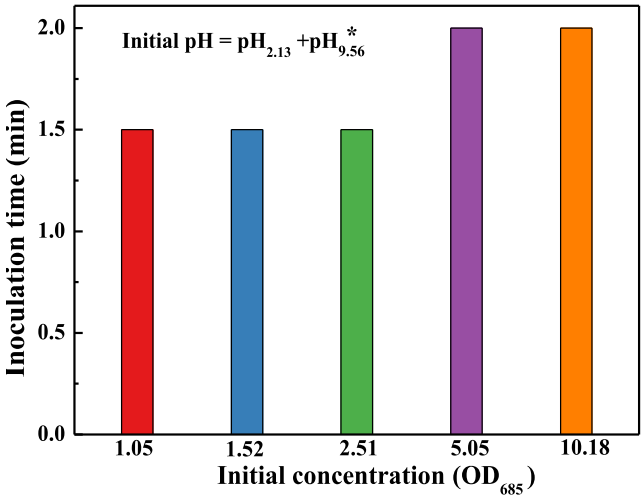
3. Results and discussion

3.1. Self-sedimentation characteristics of microalgae-microalgae co-flocculation with different mixing ratio

It can be consulted on Table 1 that self-sedimentation efficiency of *C. vulgaris* was only 37.17% within 24 h. Conversely, the self-sedimentation efficiencies of *A. falcatus* and *S. obliquus* reached to 74.65% and 87.25%, respectively. As for the self-sedimentation biomass concentration (Fig. 1a), the curves of self-sedimentation biomass showed that self-sedimentation rates were faster at the earlier inoculation period. While, the curves of suspended biomass were decreased over time. It could be attributed that this period was the accelerating sedimentation with the action of gravity, buoyancy and drag force (Ghosh and Stockie, 2015). Subsequently, with the increasing of self-sedimentation rate, the drag force increased and gradually balanced with gravity and buoyancy, and the self-sedimentation process stepped into the constant self-sedimentation (Xu et al., 2017). At the end of the self-sedimentation period, more *A. falcatus* and *S. obliquus* biomass settled down to the bottom, while more *C. vulgaris* reminded in the suspension (Fig. 1b). The sedimentation biomass of these three pure microalgae species achieved 13.81 $g\,m^{-2}$ (*C. vulgaris*), 27.73 $g\,m^{-2}$ (*A. falcatus*), 30.18 $g\,m^{-2}$ (*S. obliquus*). It was affected by the microalgae characteristics including shape, size, density, zeta-potential, and so on. On the one hand, according to the Stokes formula, if the particles obtained small size diameter, similar density to water, the self-sedimentation rate would be slow. From the results in Table 2, the size of *C. vulgaris* was



(a) Inoculation time with different inoculation methods



(b) Inoculation time with different initial concentration

Fig. 4. Effects of pH regulation method (a) and initial microalgae-microalgae co-flocculation concentration (b) on the inoculation time in biofilm cultivation PBRs.

smaller than those of *A. falcatus* and *S. obliquus*. In addition, *C. vulgaris* were individual, while *A. falcatus* and *S. obliquus* can form larger flocs. On the other hand, absolute value of zeta-potential can be used as an indicator of suspension stability. With the increasing of the absolute value of zeta-potential, electrical repulsion among particles became stronger and stronger, which the suspension trended to be stable. When the zeta potential is close to zero, particles can approach each other to a point where they will be attracted by Van der Waals forces (Henderson et al., 2008). The zeta-potential of *C. vulgaris* was larger than those of *A. falcatus* and *S. obliquus* (Fig. 2a), which indicated that *C. vulgaris* suspension were more stable than other two species. Thus, the self-sedimentation efficiency and biomass concentration of *C. vulgaris* were lower than others. However, although the size of *A. falcatus* was bigger than *S. obliquus*, the self-sedimentation efficiency and concentration of *A. falcatus* were lower than that of *S. obliquus* owning to the higher zeta-potential.

Based on the self-sedimentation characteristics of pure microalgae species, any two of them were mixed with different ratios. The large flocs formed by flocculating microalgae (*A. falcatus* and *S. obliquus*) could trap the non-floc microalgae (*C. vulgaris*) and settled faster than pure *C. vulgaris* microalgae cells. Furthermore, an increase in the ratio of the flocculating microalgae leaded to higher self-sedimentation rates (Table 1). Especially, *A. falcatus* in shape of long strip acted as the bond and linked the fusiform *S. obliquus* or spherical *C. vulgaris*, so that they could twine together and form the microalgae-microalgae co-flocculation (MMC). However, the self-sedimentation efficiencies of MMC were affected by the species ratio. The results in Fig. 1 showed that the self-sedimentation concentration reached to 34.33 g m^{-2} (*A.f* : *S.o* = 4:1) and 35.16 g m^{-2} (*A.f* : *S.o* = 1:4). The self-sedimentation efficiencies (Table 1) were relatively higher when the microalgae mixing ratio of *A. falcatus* and *S. obliquus* were 1:4 (94.66%) and 4:1 (92.41%). Furthermore, the absolute values of MMC zeta-potential were lower than pure microalgae, which indicated that the MMC suspensions were instability and were more accessible to flocculation. Particularly, it can be seen in the Table 2 that the average sizes of *S. obliquus* and *A. falcatus* were 10.36 μm and 37.27 μm. Obviously, the size ratio of these two species was about 1:4. This suggested that the optimal mixing ratio and microalgae sizes had close relations. On the other hand, the dry weights of these microalgae species were approximately the same (Table 2). As for *S. obliquus* and *A. falcatus*, the dry weights and lipid contents were even higher. Therefore, from the experimental results, *S. obliquus* and *A. falcatus* were the optimal microalgae species for the application in the following experiments with the mixing ratio of 1:4 and 4:1.

3.2. Self-sedimentation of microalgae-microalgae co-flocculation with different inoculation pH

According to the results of Section 3.1, zeta-potential was an important factor affecting the microalgae self-sedimentation efficiency. It was known that zeta potential could be adjusted by changing pH (Roselet et al., 2017). In this section, the self-sedimentation efficiency of MMC was investigated with different inoculation pH. The MMC were mixed by *S. obliquus* and *A. falcatus* with the ratios of 1:4 and 4:1, respectively. From the results in Fig. 3a and b, self-sedimentation efficiencies and rates were both decreased with the increasing of inoculation pH. The self-sedimentation efficiencies could all achieve above 90% within 18 h. And when *S. obliquus* and *A. falcatus* mixed with the ratio of 1:4, the microalgae cells settled on the surfaces of the bottom of the reactor and accumulated to inoculation concentration with 8 h (Fig. 3a). While, for the mixing ratio of 4:1, the inoculation concentration reached within 12 h (Fig. 3b). In particular, the self-sedimentation efficiencies of MMC could exceeded 90% in 2 h with the inoculation pH of about 2. However, as it was known microalgae biofilm were not able to grow well in the overly acidic medium. Fortunately, the overly acidic medium could be neutralized with alkaline medium. Thus, the pH of MMC suspensions were firstly adjusted around 2 and 9 respectively, then mixed the medium together. In this case, the self-sedimentation efficiencies rapidly reached more than 90% in several minutes (orange diamond symbol in Fig. 3a and b), which greatly reduced the self-sedimentation time. This phenomenon may be attributed to two main reasons by the means of the results in Fig. 3c. On one hand, the MMC mixed with *S. obliquus* and *A. falcatus* shown as the porous and multilayer flocs, this construct was benefit for self-sedimentation. On the other hand, the MMC possessed negative charge in the pH range from 4 to 10, while turned to be positive charge below the pH of 3. Microalgae possessed a negative charge on their cell surface due to the presence of carboxylic (−COOH), amine ($-NH_2$) and phosphate ($-PO_4$) groups. While, the surface groups would be protonated which imparted a net positive charge in the overly acidic medium (Bhattacharya et al., 2017; Tian et al., 2009). Thus, in accordance with the principle of adsorption coagulation with charge neutralization (Bernhardt and Clasen, 1994), the stability of MMC suspension was broken when the MMC of opposite charges were mixed together. In addition, a neutralization process of acid and alkali occurred during the mixing process, which avoid the inhibition of overly acidic and alkali medium on microalgae activity and made the mediums were more appropriate for microalgae biofilm growth. To test the microalgae activity after this mixing process, a growth recovery experiment was

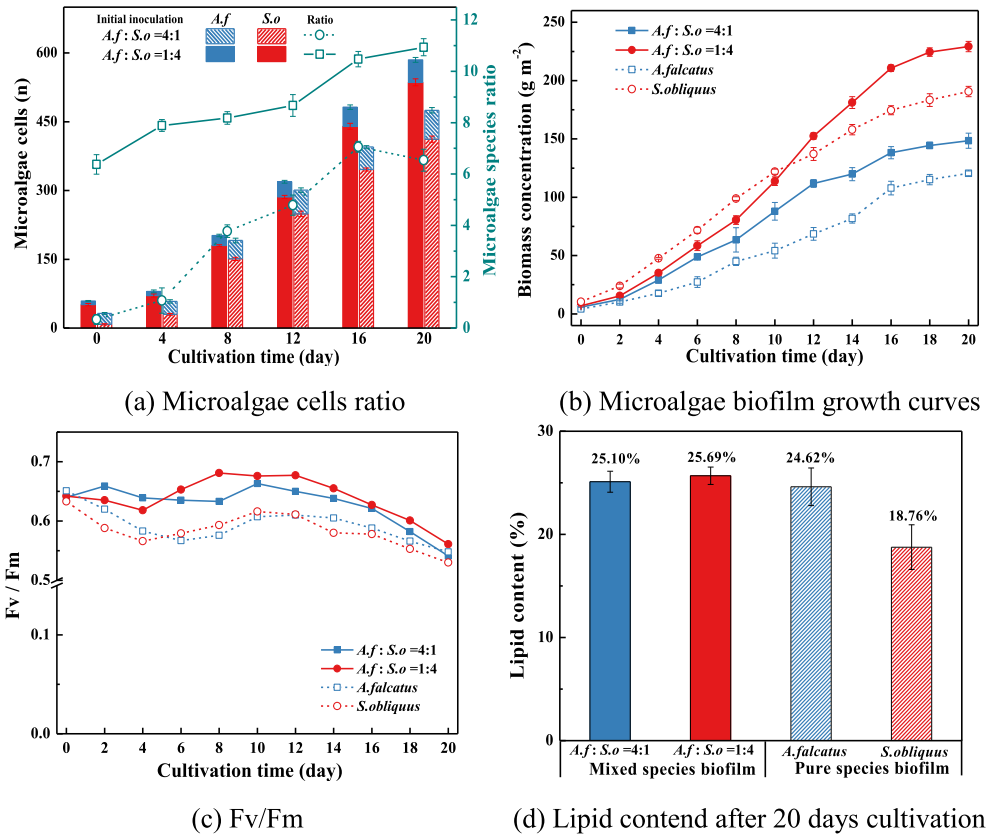


Fig. 5. Comparison of microalgae ratio (a), biomass concentration (b), Fv/Fm (c) and lipid contend (d) among pure microalgae species and different microalgae-microalgae co-flocculation in biofilm cultivation PBR.

conducted in the microalgae cultivation system. In the Fig. 3d, the growth curves of MMC suspension with inoculation of acid-alkali mixture were compared with the neutral inoculation. Obviously, the growth curves almost showed the same trend between experimental groups and control group. Therefore, this inoculation method of zeta-potential adjustment could significantly enhance the self-sedimentation rate, so that the inoculation time and film-form time of microalgae biofilm would be shortened. Furthermore, this method didn't affect the microalgae growth.

3.3. The growth of microalgae-microalgae co-flocculation biofilm

In this section, the MMC were inoculated in the cycling biofilm cultivation system based on the optimal methods concluding from the experiments of Sections 3.1 and 3.2. Fig. 4 showed the effects of inoculation method (Fig. 4a) and biomass concentration (Fig. 4b) on inoculation time. Comparing with the inoculation method of neutral self-sedimentation, the inoculation time significantly was shortened from 4 h to 1.5 min with the inoculation method of zeta-potential adjustment. It could be obtained from Fig. 4b, although inoculation time increased with the increasing of biomass concentration, the inoculation processes only lasted less than 2 min for every testing inoculation concentration due to the rapid self-sedimentation process with the zeta-potential adjustment method. Thus, the inoculation concentration had no significant influence on inoculation time, and the inoculation method of zeta-potential adhesion could equally apply to high concentration microalgae.

After inoculation, the long-term cultivation of microalgae biofilm were continually proceeded in the cycling biofilm cultivation system. As Fig. 5a showed, the ratios of *S. obliquus* to *A. falcatus* kept on changing during the cultivation period. No matter the inoculation ratio was, *S. obliquus* grow faster than *A. falcatus*. Thus, the microalgae species ratio increased along the cultivation time. When the inoculation ratio of *S. obliquus* to *A. falcatus* was 1:4 (dotted line in Fig. 5a), *A. falcatus* were the dominant species in the first 4 days. Then, *S. obliquus* grew to become the dominant species. This phenomenon indicated that *S. obliquus* were more suitable for biofilm cultivation, and *A. falcatus* only played a critical role in the optimization of MMC. Combined with the results in Fig. 5b–d, the biomass concentration, Fv/Fm and lipid content of MMC were all higher than those of their main species (a major part of microalgae species in MMC). When the inoculation ratio of *A. falcatus* and *S. obliquus* was 1:4, the yield biomass concentration and lipid content achieved to 229.15 g m^{-2} and 25.69%, which increased by 26.88% and 36.94% than pure *S. obliquus*, respectively. Comparing with pure *A. falcatus*, the biomass concentration even increased by 90.15%. As for Fv/Fm (Fig. 5c), the decreaing of Fv/Fm after inoculation, it might be a result of the changing cultivation environment from maintained environment to biofilm cultivation system, microalgae required some time to adapt to the new growth environment (Lan et al., 2017). During the cultivation period, Fv/Fm increased gradually, which indicated that the photosynthetic activity of microalgae gradually recovered. Later the Fv/Fm stared to decrease due to suppression of photosynthesis occurring when nutrition and living space were consumed by the microalgae (Guo and Tong, 2014). On the other hand, Fv/Fm of MMC were higher than that of pure species, which implied MMC possessed higher activity. The inserting SEM images showed that the microstructure of MMC was porous and multilayer. This structure could minimize transfer resistance and improve the mass transfer efficiency between biofilm and the surrounding environment (Wu et al., 2016). As a result, MMC could absorb nutrients and output metabolism wasted well. However, because of the unsuitability of *A. falcatus* for biofilm cultivation, Fv/Fm of MMC with the 1:4 ratio of *S. obliquus* and *A. falcatus* were lower than that with the 1:4 ratio of *A. falcatus* and *S. obliquus*.

Finally, the results of microalgae biofilm biomass productivity in this study were compared with that using other optimal methods in the previous researches. Rajendran and Hu (2016) mixed microalgae and fungal (microalgae-bacterial symbiosis), and the yield biomass concentration and biomass production was 25.8 g m^{-2} and 3.69 g m^{-2} day^{-1}. Rincon et al. (2017) cultivated microalgae biofilm under mixotrophic growth conditions, the biomass concentration reached to 101.12 g m^{-2}. (Rincon et al., 2017) Notably, through the above analysis, it can be concluded that the method combined with MMC and zeta potential adjustment could not only significantly save biofilm inoculation time, but also lead to a porous and multilayer biofilm microstructure. These key factors were all benefit for microalgae biofilm growth.

4. Conclusions

The microalgae biofilm inoculation time could be significantly shortened from 4 h to 1.5 min using the microalgae-microalgae co-flocculation by zeta-potential adjustment. The optimal mixture ratio formed microalgae-microalgae co-flocculation was 1 volume *A. falcatus* to 4 volume *S. obliquus*. Furthermore, the porous and multilayer microalgae-microalgae co-flocculation could minimize transfer resistance of nutrients and improve the their transfer efficiency, which benefitted microalgae biofilm growth. The biomass concentration and lipid content achieved to 229.15 g m^{-2} and 25.69%, increased by 90.15% and 36.94% than pure microalgae species, respectively.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.01.083.

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