Biogranulation Process for Simultaneously Quick Harvesting of Oil-producing Microalgae and Digestion Liquor Treatment

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Abstract

Resource depletion and environmental pollution issues have been focused recently along with the rapid increase of global population. Thus, the exploration of environmentally friendly technologies that facilitate waste treatment and resource recovery is an essential part for sustainable development. Recently, microalgae-based technologies have attracted lots of interests owing to its high efficiency on wastewater remediation and simultaneous nutrients recovery, and production of alternative biofuel or other high-value products. However, microalgae biomass harvesting is still the bottleneck for its large-scale application, which accounts for 20-30% of the operation cost for microalgae cultivation. Though several methods such as centrifugation, filtration, coagulation and flocculation, etc. have already been applied for microalgae harvesting, till today none of them has been proven economical and efficient at large scale. Further investigation and development of large-scale applicable harvesting methods is necessary for microalgae-based wastewater treatment or bioproducts production.

Previous studies revealed that microalgae and bacteria can form granular symbiosis consortia through biogranulation, i.e., algal-bacterial aerobic granular sludge (AGS). In this study, the feasibility of granule formation using unicellular oil-producing microalgae *Ankistrodesmus falcatus* var. *acicularis* for simultaneously quick harvesting of biomass and digestion liquor treatment was investigated in lab-scale sequencing batch reactors (SBRs). Additionally, the mechanisms involved in microalgae biogranulation, and its cost-effectiveness analysis were carried out. The main results can be summarized as follows.

(1) Easily settled algae granules with compact structure appeared around day 90 and mature granules were obtained after 150 days' operation. The microalgae settleability was remarkably improved, signaling by the substantial decrease of sludge volume index (SVI_{30}) from initially >3000 to 53.44 ± 3.31 mL/g, with settling velocity correspondingly increased from nearly 0 to 18.47 ± 0.23 m/h. Although the percentage of the target microalgae (*A. falcatus* var. *acicularis*) decreased along with the granulation process, the biomass concentration (2-4 g/L) and biomass productivity (130-270 mg/L/d) using biogranulation were 10-20 times and 16-34 times than that by the traditional suspension method. Compared to the seed microalgae cells, more extracellular polymeric substances (EPS) (162.54 ± 3.60 mg/g-volatile suspended solids (VSS)) with a higher proteins/polysaccharides ratio (7.62) were excreted from the mature algae granules. Moreover, the mature microalgae granules showed comparable nutrients removal, averagely 96% and 86% of dissolved organic carbon (DOC) and ammonia nitrogen

(NH_4^+-N) from the digestion liquor, reflecting its great potential for simultaneous microalgae cultivation, harvesting and wastewater treatment.

(2) Aggregates ranging from 0.5 to 2 mm with excellent settling capability were obtained in the group with liquid extract as additive, achieving harvesting efficiency in 30 min about 90.75 ± 1.23%. The continuous increase in polysaccharides in soluble EPS might be responsible for cells capture and adhesion at the early stage of aggregation, while the increase in concentration of bound EPS from 41.86 ± 5.08 mg/g-VSS to 264.05 ± 6.41 mg/g-VSS favored the stabilization of aggregates integrity, in which 93.8% was attributed by tightly bound EPS. The aromatic proteins in loosely bound EPS as well as tightly bound EPS were dramatically enhanced in response to the liquid extract, and N-acyl-homo-serine lactone (AHLs) mediated quorum sensing might be involved in this granulation process.

(3) Comparison in cost effectiveness between this novel biogranulation system and the conventional suspended cultivation for oil-producing microalgae (*A. falcatus* var. *acicularis*) cultivation and harvesting was conducted in regard of biomass accumulation, energy consumption and nutrients removal efficiency towards practical application. Results revealed that the biogranulation system achieved enhanced volumetric biomass productivity (223.17 ± 11.82 g/m^3/day) by 4.4 times when compared to the suspended system (41.57 ± 2.08 g/m^3/day) under the same environmental conditions. The biomass mass-based biofuel production was comparable between the two systems. More importantly, use of biogranulation improved the volumetric energy production by 3.3 times (3.44 ± 0.29 MJ/m^3/day versus 0.81 ± 0.06 MJ/m^3/day in the suspended system). Additionally, the biogranulation system can reduce biomass-mass based electricity consumption by 58% and footprint demand by 76%, respectively, demonstrating its great superiority in microalgae biomass cultivation and harvesting for large scale application.

The findings from this work are expected to provide the guideline for a cost-effective and sustainable cultivation and harvesting approach for scalable microalgae-based wastewater treatment and biofuel production. This work also sheds light on the mechanisms involved in microalgae granulation and the major contributors to this novel cultivation and harvesting technology for microalgae.

Keywords: Microalgae harvesting; Wastewater treatment; Biofuel production; *Ankistrodesmus falcatus* var. *acicularis*; Extracellular polymeric substances

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Chapter 1 Introduction

The global population is estimated to reach an astonishing 9.9 billion by 2050, increasing by > 25% when compared to 7.8 billion in 2020 (IISD, 2020), which will trigger more challenges including resources depletion and environmental pollution. Therefore, the development of environmentally friendly technologies that facilitate waste/wastewater treatment and resources recovery is essential for the sustainable development of the society. Recently, more attention has been paid to microalgal-based technologies due to their high efficiencies of wastewater remediation with simultaneous recovery of nutrients and production of alternative biofuel or other high value-added materials (Xiao and Zheng, 2016). Microalgae, a kind of photosynthetic microorganisms, can utilize solar energy for chemical energy production in the presence of carbon dioxide and nutrients, providing possibility for simultaneously wastewater purification, biomass accumulation and CO_2 sequestration. Thus, microalgae-based technology is a promising method for solving environmental problems in a sustainable, cost-effective and environmentally friendly way.

1.1 Microalgae-based wastewater treatment

1.1.1 Biofuel production

In order to overcome the depletion of natural resources especially dramatic reduction of fossil fuels, attention has been paid to renewable energy resources including geothermal, wind, solar, hydro-energy, and bioenergy (Demirbas, 2011). Bioenergy is regarded as one of the most potential energy alternatives, and biofuels have been promoted as energy substitute in many countries. As known, various kinds of lipid-rich materials can be used for biofuel production, including naturally derived crops, refined/used/waste vegetable oils, animal fats, algae lipids and sewage sludge (Tasić et al., 2016). However, recently microalgae have been focused by the researchers worldwide over other land-based crops for biofuel production due to it possesses several irreplaceable advantages. Firstly, relying on its unicellular structure and high photosynthetic efficiency, the productivity of oil for biofuel utilization is higher than other excellent oil seed crops, around 7-31 times greater compared to soy oil and palm oil per unit (Demirbas, 2011; J. et al., 2011). Also, microalgae can be cultivated without competing with food consumption and disruption of the environment. Independent on herbicide or pesticide and consumption of CO_2 during photosynthesis, making it deserved to be called an environmentally friendly choice for biofuel production. Additionally, some high-valued coproducts will be produced besides oil production, such as vitamins, pigments, omega-3 fatty acid and protein (Chen et al., 2018). However, the large-scale application of microalgae-based technologies are

hampered by the costs including cultivation and harvesting. Recently, microalgae cultivation in wastewater has been proposed, which can realize nutrients removal and microalgae-based biofuels production simultaneously.

1.1.2 Wastewater treatment

The feasibility of microalgae utilization for wastewater treatment has been proven by many researchers for it can efficiently reuse and recover nitrogen or phosphorus from wastewater without extra addition of carbon and nitrogen resources, and if been harvested, can be utilized as feedstock for biofuel production (Cai et al., 2013; de-Bashan and Bashan, 2010; Wang et al., 2010). Also, compared to conventional nutrients removal methods, such as activated sludge-based process applied over 100 years for municipal wastewater treatment (van Loosdrecht and Brdjanovic, 2014), microalgae can assimilate carbon dioxide from heterotrophs respiration to generate oxygen through photosynthesis, reducing greenhouse gases (GHGs) emission and cost for external oxygen supply to treatment plants. Otherwise, this approach can lower overall production costs and freshwater requirement for obtaining valued compounds or biofuels from microalgae biomass (Cai et al., 2013).

1.1.3 Feasibility of digestion liquor treatment

International concerns over energy and resources depletion are the main drivers for resource recovery from waste. Anaerobic digestion (AD), is one of the most commonly and effective used stabilization method for solid waste (Cai et al., 2016; Wang et al., 2009), leaving abundant nutrients in the digestate. The huge amount of digestion liquor produced from AD is rich in ammonia and phosphate triggering eutrophication and negative environmental impacts if not properly treated. For traditional treatment methods such as spreading as liquid fertilizer, land availability and long-distance transportation make them not cost-effective (Möller and Müller, 2012). It is essential to propose cost-effective methods for nutrients removal and resource recovery from digestion liquor.

As reported, a consortium of 15 native isolated microalgae showed over 96 % nutrient removal treating carpet mill effluent and 6.82 % oil lipid content was obtained (Chinnasamy et al., 2010). Metal ions, including Al, Ca, Fe, Mg and Mn were efficiently removed from municipal wastewater ranging from 56.5% to 100% by *Chlorella* sp. (Wang et al., 2010). Parsy et al. (2020) confirmed the feasibility of growing green microalgae, *Nannochloropsis oculata* in a mixture of seawater and liquid digestate from anaerobic digestion and approximately 100% ammonia and 40% carbon were efficiently removed. Thus, the above studies clearly show that

the microalgae are efficient to remove nutrients even metals from high pollutant loading wastewater, providing evidence for utilization of microalgae in digestion liquor treatment.

1.1.4 Bottleneck for large-scale application

Owing to its substantial costs and energy input, microalgae harvesting is another obstacle to develop microalgae biotechnologies into a commercial scale (Molina Grima et al., 2003; Pragya et al., 2013). While harvesting microalgae biomass, one or more solid-liquid separation steps will be involved, requiring high energy input as large amount of water need to be removed for obtaining concentrated biomass, especially large-scale microalgae biodiesel production and microalgal-based wastewater treatment (Kadir et al., 2018; Molina Grima et al., 2003). Small size (3-30 μm), much diluted culture (normally ranging around 0.3-5 g/L) in the cultivation medium, low settleability (settling velocity < 0.0036 m/h) and negative charged surfaces in neutral pH, are the main reasons for inefficiency in biomass harvesting (Barros et al., 2015; Hadjoudja et al., 2010; Molina Grima et al., 2003). It is claimed that the current microalgae harvesting step accounts for 20-30% of the operation cost for microalgae cultivation, mainly through coagulation/flocculation, centrifugation, flotation, etc.(Chen et al., 2011). Among these technologies, centrifugation is regarded as the most time-efficient method for microalgae harvesting, which has been widely applied for the separation of microalgae biomass (Barros et al., 2015; Molina Grima et al., 2003). However, the main drawback of centrifugation is energy intensive, greater than 3000 kWh/t as reported (Schenk et al., 2008). Hence, the microalgae type, value, and properties in addition to its sedimentation velocity, cell viability, recyclability of culture medium, cell density and size should be considered together when making decision on the suitable harvesting method for a large- scale application (Barros et al., 2015). Based on the hitherto reports, till today none of the commonly used harvesting technologies have been proven economical and efficient at large scale (Mathimani and Mallick, 2018; Shi et al., 2019). Therefore, seeking a proper harvesting technology is still crucial for a sustainable and cost-effective biofuel production.

1.2 Non-suspended cultivation and harvesting technologies of microalgae

1.2.1 State-of-the-art

In order to accelerate the application of microalgae biomass production coupled with wastewater purification technology, and minimize energy input in harvesting process, non-suspended microalgae cultivation was proposed (Ruiz-Güereca and Sánchez-Saavedra, 2016; Zhuang et al., 2020). The non-suspended microalgal cultivation, as described by Zhuang et al. (2020), is a new microalgae cultivation method including immobilizing microalgae in gels (e.g.

chitosan or alginate) or attaching microalgae on the surface of solids for forming biofilms. Compared to suspended cultivation methods, harvesting of biomass in non-suspended microalgae cultivation became easier and cheaper as the biomass is more compact (Roostaei et al., 2018; Wu et al., 2020). Some reports also noted that biomass accumulated in immobilized ways are higher than cultivated in suspended method. Wu et al. (2020) pointed out that *Chlorella* sp. can be successfully immobilized in alginate and the immobilized granules exhibited marginally higher growth rate than the suspended method when treating textile wastewater. 2.8 times higher biomass productivity was obtained in tubular chained systems, when cyanobacteria was immobilized by agar-alginate during purification of swine farm wastewater (Lee et al., 2013). Moreover, the immobilized or attached microalgae can tolerate higher pollutant concentration even toxic pollutant compared to the original microalgal suspension, as they are protected by the exotic polymeric substances (alginate) or self-secretion polymeric substances (Roostaei et al., 2018; Yang et al., 2018). However, some reports reveal that polymeric substances affect mass transfer as well as light transmission, which in turn influence the lipid contents especially in the immobilized form (Ruiz-Güereca and Sánchez-Saavedra, 2016; Zhang et al., 2008). Still, the issues relating to high operation cost (mainly from materials addition), residual polymeric materials added to biomass and worsen stability after long-time operation limit their large-scale applications.

1.2.2 Biogranulation, a novel alternative for non-suspended cultivation methods

The concept of algal-bacterial aerobic sludge system was proposed by Huang et al.(2015) when aerobic granular sludge (AGS) in lab-scale batch reactors (SBRs) was exposed to natural sunlight, achieving excellent biomass settleability (sludge volume index in 30 min or SVI_{30}=37-40 mL/g after maturation), and relatively dense and compact granule structure. It is implies algae and bacteria can form immobilized granular consortia without physical-chemical materials addition (Huang et al., 2015b). Liu et al. (2017) successfully formed mature algal-bacterial AGS with target strains (*Chlorella* and *Scenedesmus*) predominated when AGS served as the immobilized material, achieving quick and low cost harvesting of biomass only through gravity settling. Cai et al. (2019) and Tiron et al. (2017) achieved algal granules formation from activated algae or natural microalgae/bacteria consortia from previous algal-bacterial AGS systems as inoculation, demonstrating that algal granules can form without mature AGS as a carrier. Compared to the suspended microalgae cultivation systems, the microalgae biomass in the biogranulation system can be naturally concentrated and more easily harvested, leading to less expensive removal from the treated wastewater with resultant to microalgae feedstock

suitable for further processing, production of biofuel for instance. However, it is not clear whether this biogranulation can be efficient for pure single cell microalgae strains with no inoculation of the original aerobic granules.

(1) Bottleneck for biogranulation systems

Compared to conventional activated sludge (CAS) widely applied in wastewater treatment plants (WWTPs), AGS was proposed around 30 years ago (Mishima and Nakamura, 1991) and gained widespread attention from then, it possesses denser and compact structure, excellent settleability as well as awesome nutrients removal performance (Huang et al., 2015a). Algal-bacterial AGS was originated from bacterial AGS under sunlight exposure, it is a kind of microalgal-bacterial symbiosis obtained from lab-scale sequencing batch reactors (Huang et al., 2015b). The carbon, nitrogen phosphorus removal efficiency can be enhanced due to microalgae co-existence and the requirement of oxygen in this system can be reduced as microalgae can utilize CO_2 released by heterotrophs respiration and produce O_2 (Ahmad et al., 2019; Quijano et al., 2017). However, it is still the major challenge for the wide application of bacterial AGS-based or algal-bacterial AGS-based technologies, long start-up time for mature granule systems. Further investigation of granular formation mechanisms is crucial for accelerating granule formation, or extending the self-immobilization for microalgae harvesting.

(2) Possible mechanisms involved in biogranulation systems

It is claimed that shear force obtained by dividing the aeration rate over the cross sectional aera of SBR, is one of the triggering factors for granule formation. Beun et al. (1999) firstly described that higher superficial gas velocity of 0.041 m/s, benefited the detachment of filamentous outgrowth on the surface of granules preventing loosely structure formed. No AGS formed with the upflowing rate of 0.3 cm/s, while compact and regular granules were obtained when the upflowing rate increased was increased to 1.2 cm/s (Tay et al., 2001a). Higher shear force represented by gas velocity favored the formation of compact and stable structure by detaching loosely organisms on the surface, enhancing extracellular polymeric substances (EPS) secretion as well as improving cell surface hydrophobicity as described by above researchers.

Settling time or volume exchange ratio is another important parameter as it can control washing out rate of aggregates or granules in the reactors and the aggregates with good settling capability are selected for achieving AGS dominated under short settling time and higher exchange ratio (Adav et al., 2008; Nancharaiah and Kiran Kumar Reddy, 2018; Wang et al., 2006a).

Extracellular polymeric substances (EPS), as described (Aqeel et al., 2019; Felz et al., 2016; Zhang et al., 2017), are polymers secreted by microorganisms involved in granulation process and are important for aggregation, granulation as well as maintenance of granular stability. Proteins (PN) and polysaccharides (PS) are the major components in EPS, and PN contents are higher than PS in stable AGS (Adav et al., 2008). PS play a leading role in the formation and stability of AGS (Lin et al., 2020), however, there are some contrary viewpoints that PN play significant role over PS in aerobic granulation and in maintaining the stability of AGS (Zhang et al., 2007; Zhu et al., 2012), through increasing hydrophobicity and surface charge of sludge. Adav et al. (2008) noticed that α-polysaccharides, proteins, and lipid had minimal effect on structure integrity of granules while β-polysaccharides served as backbone for a network-like outer layer combined with protein, lipid, α-polysaccharides and microbial cells in the stable granules. No consistent conclusion has been drawn regarding the effect of EPS on granulation because various components of EPS, multiple set-up strategies and colorful microorganisms are involved in aerobic granulation. Hence, understanding the link between EPS components and microorganisms as well as operating strategies for the reactor are crucial for ascertaining mechanisms involved in granule formation.

Famine-feast regimes controlled by primary nutrients concentration in influent, biomass concentration and cycle set-up, can affect EPS concentration and bacterial constitutes in granules resulting in achieving changes of structure stability (Aqeel et al., 2019). Complex polymers can be produced in feast conditions and utilized as carbon resources when easily biodegradable organic substances are not available during famine conditions (van Loosdrecht et al., 1997). Moreover, feast-famine strategy may ensure the optimum growth of heterotrophs in the oxic zone, as there are competition between non-denitrifying heterotrophs and denitrifying heterotrophs under limited organic loading and aeration (Mozumder et al., 2014).

As noticed, K^+, Ca^{2+} and Mg^{2+} were closely related to microbial aggregates and stability of aerobic granulation (Wang et al., 2020a), while it was confirmed that feeding with metals in influent wastewater can reduce granulation time of AGS (Sarma et al., 2017). Metals can disrupt electrostatic repulsion between microorganisms and then increase surface charge and hydrophobicity. Also, those cationic ions can be involved into bridging between EPS and cells, reducing negative charge on cell surface that may induce aggregates formation (Sheng et al., 2010).

Besides, some creatures are reported to get engaged into granulation, such as filamentous fungi, protozoa, and aggregating strains (Beun et al., 1999; Ivanov et al., 2006).

(3) Preliminary strategies for accelerating start-up in biogranulation systems

Beun et al. (1999) reported that quicker and stable granule formation occurred under a short settling time of 4 min, while the same result was obtained when settling time was adjusted into gradual reduction (Nancharaiah and Kiran Kumar Reddy, 2018).

Considering cationic effect involved in granule formation, 10 mg/L Mg^{2+} was added into synthetic wastewater and the time for establishment of mature granular system was shortened around 14 days compared to the groups without Mg^{2+} addition (Li et al., 2009). Novel operation mode was proposed with air recycling, and Ca^{2+} accumulation inside aerobic granules resulted in a quicker formation and more compact structure (Zhang et al., 2019a). Aerobic granulation was accelerated and enhanced under a higher volume exchange ratio, in which more EPS was excreted and calcium was accumulated (Wang et al., 2006a). Mg^{2+}/Ca^{2+} ratio was the highest in the higher stability of phosphate granules with enhanced magnetic concentration (Schönborn et al., 2001). Cai et al.(2018) suggested that aerobic granules formed faster with pulse dosing of 30 mg/L Fe^{2+} than constant dosing with 5 mg/L Fe^{2+} in the influent. Some coagulants also were used for promoting granule formation and those coagulants can serve as a nuclei of granule for rapid formation, when polyaluminum chloride (PAC) was used (Liu et al., 2014), the granulation time was reduced to 7 days with 500 mg/L PAC. Moreover, it is confirmed that activated carbon (GAC) addition could greatly reduce granulation time from 6 weeks to 3 weeks due to its specific aera and porous structure for bacteria attachment (Li et al., 2013).

Considering exotic addition as described above can lead to increased costs or possible contamination of biomass, some studies applied biomass carriers in AGS systems for their quicker establishment. Mycelial pellets (MPs) were inoculated and the granules appeared earlier higher biomass (Geng et al., 2020). Novel strategy has been applied with addition of crushed granules into flocculent sludge, reducing start-up time and minimizing biomass loss during granule formation period when treating nutrient-rich abattoir wastewater or domestic wastewater (Coma et al., 2012; Pijuan et al., 2011). Fluorescent microbead particle were utilized for explaining mechanisms attributing to fast establishment of AGS systems through crushed granules (Verawaty et al., 2012), revealing that granules acted as nuclei for floccular particle attachment and then accelerating granule formation.

As for algal-bacterial AGS, there was not so much research on how to accelerate granule formation because most studies started from mature AGS, or it means nuclei already existed. However, in order to obtain algal granules with higher target strains dominated which can benefit for the subsequent biofuel or other high-valued substances extraction, it is crucial to

investigate the mechanisms involved in granule formation, which can help find methods for accelerating granular formation.

1.3 Research objectives and thesis structure

1.3.1 Research objectives

Therefore, the objectives of this research are as follows.

(1) To investigate the feasibility of granular formation using unicellular oil-producing microalgae *Ankistrodesmus falcatus* var. *acicularis* for simultaneously quick harvesting of biomass and digestion liquor treatment.

(2) To explore methods for rapid start-up of algal granular systems.

(3) To achieve further understanding of crucial parameters and mechanisms involved for biogranulation.

(4) To compare energy consumption between conventional suspended cultivation and biogranulation methods along cultivation and harvesting process.

All in all, results from this work are expected to provide scientific data for functional microalgae cultivation and fast harvesting through biogranulation with low energy and time consumption, and finally to promote microalgae-based technologies into large-scale application.

1.3.2 Thesis structure

In the first research (Chapter 2), attention was paid to the microalgal granule formation process of oil-producing microalgae *Ankistrodesmus falcatus* var. *acicularis* together with the quantification of nutrients removal from digestion liquid and identification of biological community changes.

In the second research (Chapter 3), quicker establishment and mechanisms of algal granular systems was concerned by using intact and crushed mature microalgae granules as additives and mixed with the suspended-growth microalgae to investigate the contribution of each fraction from crushed microalgae granules to the rapid harvesting process.

In the third research (Chapter 4), emphasis has been put on cost-effectiveness comparison between conventional suspended cultivation and novel biogranulation systems with footprint demand, electricity consumption, nutrients removal and energy efficiency being considered. This work also examined the feasibility of biogranulation system for simultaneous wastewater treatment and microalgae biofuel production.

The whole structure of this thesis is illustrated in Figure 1-1.

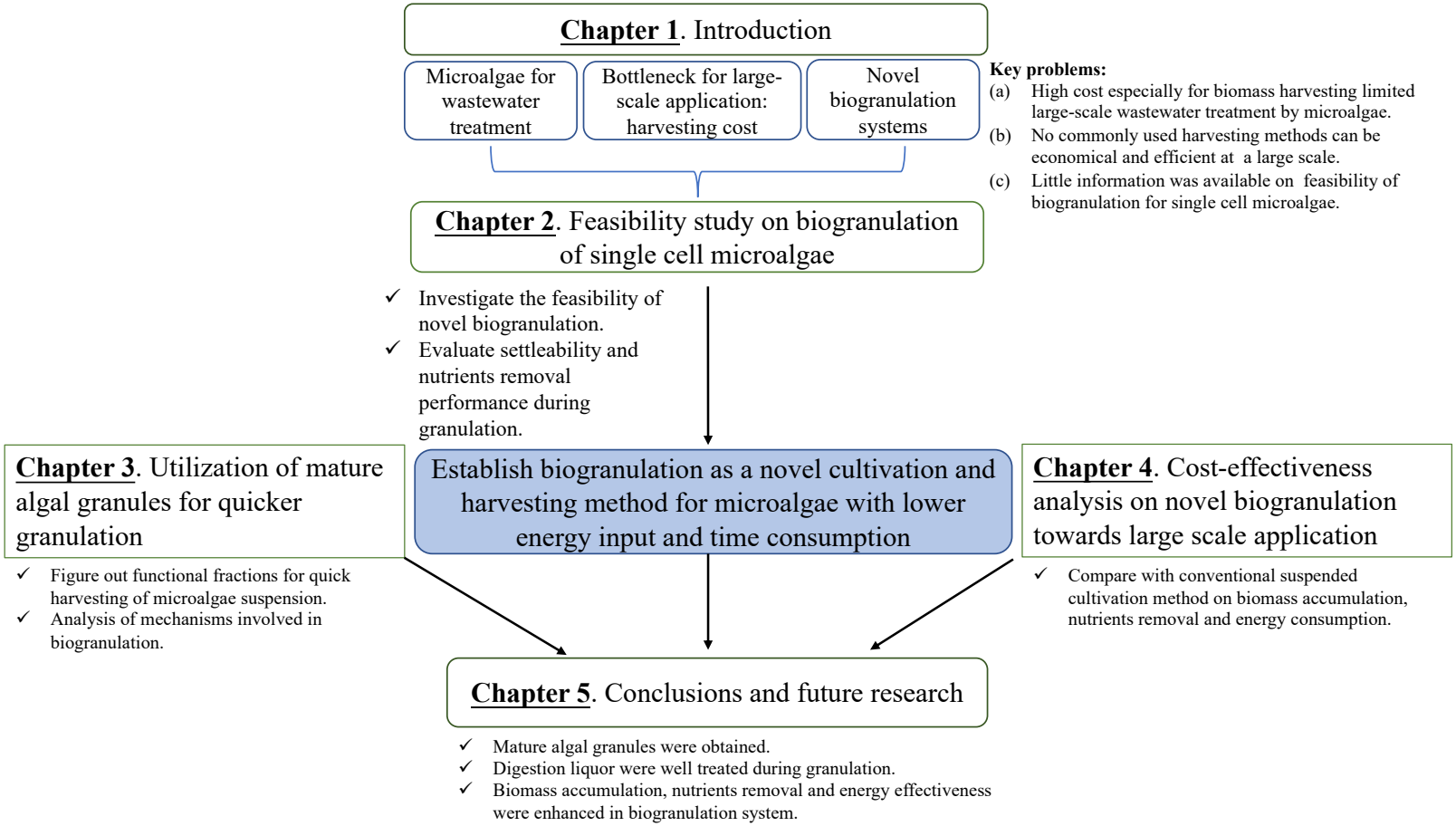


Figure 1-1 Framework of this thesis.

Chapter 2 Fast cultivation and harvesting of oil-producing microalgae *Ankistrodesmus falcatus* var. *acicularis* fed with anaerobic digestion liquor via biogranulation in addition to nutrients removal

2.1 Background

Anaerobic digestion (AD) process has been widely applied in the world as an effective energy and resources recovery approach from organic wastes including waste activated sludge (WAS), livestock manure or other organic solid wastes, and high-strength organic wastewaters. However, AD process can also release secondary pollutants to the environment if its liquid effluent (digestate, or digestion liquor) being discharged without proper treatment. Various components including mineral materials, non- or slowly biodegradable organics, and some intermediate products like volatile fatty acids (VFAs) are still remained in the digestion liquor after AD process (Barker et al., 1999; Cai et al., 2016; Ji and Chen, 2010). If microalgae can be cultivated with wastewaters like digestion liquor, the microalgal-based technology can be more beneficial for the establishment of a sustainable society due to its low cultivation cost and great potential for pollution amelioration.

Biofuels from microalgae haven't been commercialized on a large scale yet, mainly due to the high cost and energy input for the production of algae biomass (Borowitzka, 2013). Microalgae strains and their cultivation medium selection are identified as the important contributors to the reduction of overall production costs (Kadir et al., 2018). Among the various microalgae, *Ankistrodesmus falcatus* var. *acicularis* used in this research is more promising due to its high contents of lipids, carbohydrates and proteins in the cells (George et al., 2014). However, the harvesting of *Ankistrodesmus* sp. has also been challenging given its small size, low density and low solid concentrations.

2.2 Materials and methods

2.2.1 Synthetic digestion liquor and seed microalgae

The synthetic digestion liquor was prepared according to a previous study (Cai et al., 2016), which was mainly composed of 500 mg chemical oxygen demand (COD)/L, 50 mg PO_4^{3-}-P/L, and 100 mg NH_4^+-N/L to mimic the fermentation liquor after ammonia recovery by stripping. The trace elements solution contained (in mg/L) H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2$ (30), $MnSO_4 \cdot H_2O$ (50), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3$ (50), $CoCl_2 \cdot 6H_2O$ (50), and $NiCl_2$ (50) (Adav et al., 2008). The influent pH to the reactors was adjusted with sodium bicarbonate to be within 7.0-8.3.

The microalgae strain, *Ankistrodesmus falcatus* var. *acicularis* (NIES-2195) was supplied by the Microbial Culture Collection at the National Institute for Environmental Studies (NIES), Japan. All the stock and experimental algae were maintained at room temperature (25 ± 2ºC) in the artificial CT medium containing the following ingredients (per liter): $Ca(NO_3)_2 \cdot 4H_2O$ (150 mg), KNO_3 (10 mg), β–Na_2glycerophosphate$\cdot 5H_2O$ (50 mg), $MgSO_4 \cdot 7H_2O$ (40 mg), Vitamin B_{12} (0.1 μg), Biotin (0.1μg), Thiamine HCl (10 μg), $Na_2EDTA \cdot 2H_2O$ (3 mg), $FeCl_3 \cdot 6H_2O$ (0.59 mg), $MnCl_2 \cdot 4H_2O$ (108 μg), $ZnSO_4 \cdot 7H_2O$ (66 μg), $CoCl_2 \cdot 6H_2O$ (12 μg) and $Na_2MoO_4 \cdot 2H_2O$ (7.5 μg) (Waterbury and Stanier, 1981).

2.2.2 SBRs set-up and their operation strategies

Two identical laboratory-scale cylindrical column SBRs with a working volume of 0.9 L each (5.7 cm in diameter and 36 cm in effective height) were operated in parallel with their average results being reported. The SBRs were operated at room temperature (25 ± 2 ºC) and illuminance of 146-157 μmol/m^2·s for 24 hours at the top and 88-97 μmol/m^2·s at the bottom by two artificial solar lights fixed above the SBRs. The light illuminance was measured by a pocket digital lux meter (ANA-F11, Tokyo Photo-electric Co., Ltd., Japan). Before being inoculated to the SBRs for biomass accumulation, microalgae were centrifuged at 3000 rpm for 5 min after being washed with 0.9% sodium chloride to exclude the influence of CT medium.

Before the SBRs being operated in a 6 h cycle, pre-cultivation for biomass accumulation and settleability enhancement was performed for 10 days in both SBRs. During this pre-cultivation period, the influent was manually filled and discharged (after centrifugation). And non-aeration and aeration periods were alternatively operated every 3 hours. An air pump (AK-30, KOSHIN, JAPAN) was used for air supply at an air uplift velocity of 0.98 cm/s. After SBRs being switched to automatic operation, the cycle duration was set as 6 h, consisting of 2 min filling, 90 min non-aeration, 146 min aeration, 120 min settling and 2 min discharging at the very beginning. During the operation, the settling time was stepwise reduced from initial 120 min to 10 min, 5 min and 2 min, respectively. Meanwhile, the aeration time was increased accordingly with the reduction of settling time. The volumetric exchange ratio was kept at 50%, equivalent to a hydraulic retention time (HRT) of 12 h. After the initial granules appeared in the reactors on day 90, the algal biomass in the reactors was discharged daily to control the sludge retention time (SRT) at around 15 days.

2.2.3 Analytical methods

Mixed liquor (volatile) suspended solids (ML(V)SS) were measured to represent the biomass of microalgae or algal granules according to standard methods (APHA, 2012).

Chlorophyll *a* (Chl-*a*) content was used to estimate the algal biomass according to the modified method proposed by Ngearnpat et al. (2018). Briefly, 10 mL sample of microalgae or algal granules after crushed completely with a glass rob was centrifuged at 3000 rpm for 5 min. Then 10 mL of 90% methanol solution containing 0.2% $MgCl_2$ was used to suspend the settled fraction after centrifugation, and the mixture was heated at 70°C for 30 min in the dark for extraction. The extract was centrifuged at 3000 rpm for 10 min after cooling down. The Chl-*a* concentration was determined at the wavelengths of 750, 665, 645 and 630 nm by a spectrophotometer (UV 1800, Shimadzu, Japan), and then calculated using Eq.2-1:

$$\text{Chl-}a\ (mg/L) = (11.6\times(A_{665}-A_{750}) - 1.31\times(A_{645}-A_{750}) - 0.14\times(A_{630}-A_{750}))\times\frac{Vm}{Vf} \quad (2\text{-}1)$$

where *Vm* (mL) and *Vf* (mL) are the volume of the extraction methanol and sample, respectively.

Sludge volume index (SVI) (APHA, 2012) and settling velocity (Ghangrekar et al., 2005) were used to indicate the settleability of microalgae or algae granules. Integrity coefficient (%) is defined as the ratio of solids in the supernatant after being shaken on a platform shaker at 200 rpm for 5 min to the total solid weight of the determined sample (Ghangrekar et al., 2005), which was used to estimate the strength of algal granules in this study. Dissolved oxygen (DO) concentration in the reactors was measured with a DO meter (HQ40d, Hach, USA), and pH was monitored using a compact pH-meter (Horiba, Japan).

The size of granules was measured using the wet sieve separation method as previously described (Cai et al., 2018), and the morphology of microalgae or algal granules was observed by Leica M205 C Microscope (Leica Micro-systems, Switzerland).

As for the evaluation of nutrients removal, the suspension samples were filtrated through 0.45 μm filter and the filtrates were used for determination. Ammonia nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N) and total phosphorus (TP) were analyzed according to standard methods (APHA, 2012). Dissolved organic carbon (DOC) was quantified by a TOC analyzer (TOC-V_{CSN}, Shimadzu, Japan) equipped with an auto-sampler (ASI-V, Shimadzu, Japan).

The extraction of loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) was performed using the heating method (Morgan et al., 1990). Polysaccharides (PS) and proteins (PN) contents were determined by the phenol-sulfuric acid method (DuBois et al., 1956) and Lowry-Folin method (Lowry et al., 1951) with glucose and bovine serum albumin (BSA) as the standards.

As for heat value, the samples were firstly dried at 105°C for 24 h to constant weight, and then determined using Auto-Calculating Bomb Calorimeter (CA-4AJ, Shimadzu, Japan)

Samples for microbial community analysis were harvested on day 0, day 25, day 105 and day 155, respectively, and the methods for DNA extraction and PCR were described elsewhere (Zhang et al., 2020). The specific primers for bacteria and algae to amplify the hypervariable region of 16S rRNA and 18S rRNA were also the same as Zhang et al. (2020). After quantification using QuantiFluorTM-ST (Promega, USA), the PCR products of all samples were taken for high-throughput sequencing on Illumina platform (Illumina PE250, USA). MOTHUR (version: 1.31.2) was used for analyzing the biodiversity of bacteria and eukaryote in the samples.

2.2.4 Calculations

DOC, NH_4^+-N, and TP removal efficiencies were calculated according to Eq. 2-2

$$\text{Removal rate (\%)} = 100\times \ (\rho_{inf} - \rho_{eff}) / \rho_{inf} \quad (2\text{-}2)$$

in which ρ_{inf} (mg/L) and ρ_{eff} (mg/L) are the influent and effluent DOC, NH_4^+-N or TP concentrations, respectively.

TP removal capacity was calculated according to Eq. 2-3 modified from Cai et al. (2016), which was used to describe P removal on the basis of MLSS.

$$\text{TP removal capacity (mg/g-MLSS·d)} = 4\times 0.9 \times(TP_{inf} - TP_{eff})\times 0.5/(MLSS\times 0.9) \quad (2\text{-}3)$$

where TP_{inf} (mg P/L) and TP_{eff} (mg P/L) are the average influent and effluent TP concentrations during the operation period, respectively; MLSS (g/L) is the average MLSS concentration in SBR during this period, and 4, 0.5 and 0.9 are the number of cycles per day, exchange ratio and working volume (L) of SBR, respectively in this study.

2.3 Results and discussion

2.3.1 Morphological changes and formation of microalgae granules

A. falcatus var. *acicularis* is a kind of unicellular green microalgae and its cells are acicular to narrowly fusiform with the ends of taping to acute apices, 2-4 μm wide and 25-45 μm long. It represents one of the smallest taxa from microalgae category. The seed algae are independently single cells smaller than 50 μm under microscopic observation (Figure 2-1a). After 40 days' operation, the microalgae were aggregated with white organisms to form activated algae flocs, exhibiting irregular and mainly opened flocs, and filamentous microorganisms and the inoculated algae were frequently observed (Figure 2-1b and Figure 2-1f). Along with the operation, an obvious decrease in the number of free microalgae was noticed. The mean diameter of activated algae flocs was about 480 ± 11.2 μm after 90 days' operation

and irregular algae granules appeared with loose structure (Figure 2-1c and Figure 2-1g). Some vorticella and rotifers anchored into the granules or adhered to the surface of granules under microscope observation, which is in agreement with Cai et al. (2018). After 150 days' operation, regular algae granules with a compact structure and average size of 2.25 mm predominated the reactors (Figure 2-1d and Figure 2-1h).

According to the experimental records (Figure 2-2), only a small percentage (6.10 ± 0.09%) of total biomass volume were granules < 1 mm, and the largest proportion (50.00 ± 1.12%) were granules > 2 mm. The remaining biomass (43.91%) were composed of larger granules > 3 mm (32.93 ± 1.12%), and medium-sized granules ranged between 1 mm and 2 mm (10.98 ± 0.90%). The above size change revealed an evolution of microalgae from single cells to microalgae granules with round shape and compact structure (Figure 2-1). Compared to the light green color of the single cells of *A. falcatus var. acicularis*, the mature microalgae granules reflected a dark-green color (Figure 2-1h).

2.3.2 Changes in biomass concentration during granulation in the reactors

The initial MLSS of seed algae were around 0.32 g/L in each SBR (after pre-cultivation), which remarkably increased to 2.19 g/L within 10 days after being inoculated to the SBRs fed with the synthetic digestion liquor containing high nutrients concentration (Figure 2-3a). A slight decrease (to 1.59 g/L) was detected on day 14 due to a shorter settling time applied (from 2 h to 10 min). After that, the biomass concentration steadily increased till day 95 (5.45 g/L), and then decreased with some fluctuations due to the start of SRT control at 15 days. After day 112, the biomass concentration increased again and peaked at 4.0 g/L on day 129 when 5 min settling time was applied. From day 130 on, when the settling time was decreased to 2 min, the biomass concentration decreased to around 2.0 g/L and kept stable. According to the experimental results, the biomass concentration (2-4 g/L) and productivity (130-270 mg/L/d) of *A. falcatus var. acicularis* in this study are respectively around 10-20 times and 16-34 times that cultivated with traditional suspension method under the optimum conditions (0.21g/L and 7.9 mg/L/d)(George et al., 2014). This observation implies that biogranulation process for microalgae cultivation can achieve much higher biomass concentration and biomass productivity in the reactors, reflecting great potential for large reductions in reactor volume and land occupation, and energy saving.

The ratio of MLVSS to MLSS (MLVSS/MLSS) denotes the organic proportion of the total biomass. The MLVSS/MLSS ratio in this study was observed to decrease sharply from around 0.8 to lower than 0.6 during the first 20 days, then increase continuously during the granulation

period till the appearance of mature granules (Figure 2-3a). This ratio kept greater than 0.7 from day 60 to day 100; and a relatively stable MLVSS/MLSS ratio ranging between 0.58 to 0.67 was noticed after day 130, possibly attributable to the balance between algal-bacterial symbiosis growth and minerals accumulation. This observation agrees with Cai et al. (2018) who cultivated algae granules by using natural microalgae. As noticed, Chl-*a* content showed almost the same trend as the biomass concentration in the reactors. In addition, a higher Chl-*a* content was also detected during the granulation period. As Hu et al. (2017) pointed out that, the decrease of Chl-*a* content is associated with the combination effect of community change, nutrients deficiency, aging and the accumulation of EPS and chemical precipitates in the biomass. Thus, further characterization of the biomass or microalgae granules should be conducted.

2.3.3 Changes of microalgae settleability during the granulation

The pure *A. falcatus* var. *acicularis* cells are generally suspended in the culture medium, which are difficult to be separated. As seen, along with the formation of granules, more biomass can be easily separated from the liquor only after 5 min sedimentation. Also, the SVI_{30} value showed a decline trend during the granulation, from > 3000 mL/g of the seed algae cells to 205.48 ml/g on day 10, and finally to 53.44 ± 3.31 mL/g for the mature granules (Figure 2-3b), revealing the increase of settling efficiency during operation. The SVI_{30} data of mature granules are slightly greater than those conventional bacterial AGS (~ 25 mL/g) and algal-bacterial AGS (~38 mL/g)(Huang et al., 2015b), reflecting their different structure compactness. A higher proportion of algae in the granules obtained in this study might be the major reason for this settleability difference. While this observation to some extent is in agreement with the findings by Choi et al. (2010) and Su et al. (2012). However, compared to the seed microalgae cells in this study, the settleability of microalgae granules was remarkably improved, and almost all the biomass can be harvested within 2 min after maturation, which can also be reflected by the changes of settling velocity (Figure 2-3b). Tiron et al. (2017) stated that the settling velocity of microalgae cells (*Chlorella* sp.) was lower than 0.54×10^{-2} m/h. In another previous study, microalgae settleability was claimed to efficiently achieve with a settling velocity of 0.36 m/h (Granados et al., 2012). The biomass settling velocity in this study reached averagely 18.47 ± 0.23 m/h after the algae cells being transformed to activated algae granules, which is also faster than the algae granules achieved from natural microalgae flocs (Cai et al., 2019).

2.3.4 Overall performance on nutrients removal

Microalgae can utilize carbon, nitrogen, phosphorus and other nutrients for biomass accumulation simultaneously from wastewater. Nutrient removal efficiencies during the operation are illustrated in Figure 2-4. The granules exhibited excellent organic removal efficiency: The effluent DOC was always less than 5 mg/L after day 60, achieving 95.70 ± 1.22% of DOC removal till the end of experiments (Figure 2-4a).

P removal capability was 7.34 ± 0.53 mg-P/g-MLSS·d, which is higher than the bacterial AGS (6.15 mg-P/g-MLSS·d) treating the same synthetic digestion liquor (Cai et al., 2016). During day 66 to day 86, the SBRs showed better phosphorus utilization capacity and produced effluent with a lower TP concentration. This observation is closely related to the relatively high algae biomass concentration during this period (Figure 2-4a) as no SRT control was applied, suggesting this microalgae strain has high potential for phosphorus uptake from wastewater. The TP content in biomass was also monitored during the operation. After mature granules formed, the granular TP content kept relatively stable, around 53.33 ± 3.48 mg/g-MLSS in the granules (Table 2-1).

As for N removal, after 30 days' operation, the effluent NH_4^+-N concentration was <14.41± 2.71 mg/L, averagely achieving 85.59 ± 2.71% of NH_4^+-N removal till the end of experiments. This observation indicates that a stable nitrification could be quickly established in this reactor system. However, TN removal was about 54.66 ± 0.86 % after reaching a stable state, due to nitrite accumulation occurred under the test conditions. In this study, nitrite concentration (26.06 ± 1.49 mg/L) higher than nitrate concentration (4.50 ± 1.93 mg/L) was always detected in the effluent. This phenomenon is to some extent in agreement with the finding by Liu et al. (2017) who inoculated two microalgae strains (*Chlorella* and *Scenedemus*) into aerobic granules.

In order to better understand carbon, phosphorus and nitrogen uptake by the mature algae granules, typical cycle tests were also conducted on day 150 (Figure 2-5). The DOC concentration decreased sharply from 43.83 ± 1.17 mg/L to 11.59 ± 0.12 mg/L within 120 min, revealing that organic carbon can be utilized efficiently during the non-aeration period, possibly attributable to the co-existing bacteria. DO level was detected to decrease from 3.32 mg/L to 0.32 mg/L during the non-aeration phase, suggesting the possible existence of some heterotrophs in the reactors, and then increased to 6.36 ± 0.10 mg/L during the aeration period. A higher P uptake was detected in the aeration phase than in the non-aeration phase, implying that P was consumed by algae as well as by phosphorus accumulating organisms (PAOs). With

the decrease of NH_4^+-N concentration, NO_2^--N accumulated gradually with no obvious change of NO_3^--N during the aeration phase. That is, partial nitrification may occur in the reactors.

2.3.5 Variation of EPS extracted from algae biomass

EPS are metabolic products accumulated on the surface of algal and bacterial cells, which contain a variety of organic substances such as exopolysaccharides (PS) and exoproteins (PN) (Wang et al., 2006b). EPS are considered to play an important role in the granulation and flocculation of activated sludge. EPS can be mainly divided into two fractions, loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS), which are identified by their different distribution in the layered structure of AGS (Adav et al., 2008; Li and Yang, 2007). LB-EPS and TB-EPS contents and their major components (PN and PS) were also quantified during the granulation in this study (Figure 2-6). It can be clearly seen that the amount of total EPS increased remarkably from 63.06 ± 3.42 mg/g-MLVSS of seed algae (day 0) to 162.54 ± 3.60 mg/g-MLVSS of mature algae granules (day 150). During the granulation period, however, a slight decrease of total EPS on day 90 (123.78 ± 5.30 mg/g-VSS) was detected when compared to day 40 (131.93 ± 1.37 mg/g-VSS), mainly contributed by the decrease in PS content (LB-PS and TB-PS, from 29.12 ± 0.39 mg/g-VSS to 13.21 ± 0.13mg/g-VSS) in the aggregates (Figure 2-5). Wang et al. (2006b) claimed that EPS can serve as nutrients and energy source in the starvation phase, in which PS are easier to be utilized than PN. In this study, the biomass concentration was maintained at a relatively high level around day 90 (Figure 2-3a) due to no SRT control was applied, providing more possibility for microorganisms' starvation and EPS consumption. The good correlation between EPS and MLSS and/or SVI indicates that EPS could accelerate the accumulation of biomass, thus promoting algae granulation. Both LB-EPS and TB-EPS showed a similar increase trend with the total EPS content, while TB-EPS were observed to be more sensitive to algae granulation in this study. In addition, a continuous increase in PN/PS ratio was noticed during the granulation process, from 0.81 to 6.05 as per LB-EPS, from 2.19 to 8.54 for TB-EPS, and from 1.40 to 7.42 for the total EPS, reflecting that a higher PN/PS ratio is beneficial for algae granulation. The above results imply that a higher content of EPS is advantageous for algae granulation, and TB-EPS may play an important role in the maintenance of stable algae granules. Further research is still necessary on the functions of the various kinds of PN and PS in EPS.

2.3.6 Changes in biological community during granulation process

The changes in biological community structure from seed algae to algal granules were explored through high-throughput sequencing technology. The dominant eukaryote at genus

level and bacteria at phylum level are shown in Figure 2-7. As expected, the seed algae *Ankistrodesmus* genus kept predominant in the eukaryote community of granular consortia. No obvious accumulation of other harmful algae that would greatly affect the harvesting and algal composition was observed. The proportion of *Ankistrodesmus* genus was found to gradually decline from 99.99% (day 0) to 96.61% (day 25), 58.92% (day 105), and 17.77% (day 155), while the eukaryote species quantity and diversity increased as seen from the increased operational taxonomic units (OTUs) and the decreased Simpson index (Table 2-2). Unclassified eukaryote and chlorella emerged after day 25, occupying about 2.20% and 1.19%, respectively. It was noticed that the percentage of unclassified eukaryote increased to 40.99% on day 105 after the formation of algae granules, most probably attributable to some planktons such as vorticella and rotifer as they were frequently observed under microscope. Li et al. (2013) also reported that vorticella and rotifer appeared during aerobic granulation, which showed some positive effect on reduction in effluent suspended solids. Beun et al. (1999) claimed that protozoa enhanced the granulation process at the beginning of AGS system. Further research should also be concentrated on the identification of unclassified eukaryote and their effects on granule formation. The genus *Chlorella* from family *Chlorellaceae* occupied about 15.85% in the mature granules. As Nuramkhaan et al. (2019) pointed out, *Chlorella* sp. has excellent auto-aggregation capability with a higher EPS excretion, which may accelerate the formation and stable operation of algal-bacterial AGS. Results from this study indicate that *Chlorella* may also play an important role in the formation of algae granules from single cell suspension of *Ankistrodesmus*.

The predominant bacteria cover Proteobacteria, Firmicutes, Cyanobacteria and Bacteroidetes, accounting for 92.80%, 99.25%, 97.45% and 97.76% on day 0, day 25, day 105 and day 155, respectively. No significant difference was found in the proportion of dominant bacteria between the seed algae and the mature granules except the phylum Actinobacteria which almost disappeared after granules formed. Nitrite oxidizing bacteria (NOB) belonging to phylum Nitrospirae shortly appeared in the reactors, accounting for 0.41% in the initial granules, while vanished in the mature granules. This observation suggests that this kind of bacteria were inhibited under the symbiotic growth of algae and bacteria, in agreement with the finding from Huang et al. (2015). The disappearance of NOB can also be reflected by the accumulation of NO_2^--N in the effluent (Figure 2-4 and Figure 2-5). In the mature granules, the proportion of phylum Proteobacteria was about 48.74%, the largest one in bacterial community, and the percentage of order Betaproteobacteriales from this phylum was found to largely increase from

6.20% in the seed algae suspension to 19.49 %. Two genera, *Thauera* and *Zoogloea* from the family Rhodocyclaceae belonging to the order Betaproteobacteriales appeared after granulation, about 2.28% and 4.84%. These two genera are reported to closely associate with EPS excretion (Allen et al., 2004; Shao et al., 2009). The secondly higher percentage phylum was Bacteriodetes, about 34.46%, were reported to have capability of complex biopolymers degradation (Weon et al., 2005), providing the possibility for the treatment of wastewater containing higher organic compounds by using this algae-based technology. Moreover, the phylum Cyanobacteria increased from 3.04 % to 13.34%, in which class Oxyphotobacteria occupied 13.33%. According to the OTUs and Simpson index results, the bacterial community diversity was greatly improved during the granulation process (Table 2-2). Restated, further works are still necessary on the optimization of algae cultivation conditions to enhance the target microalgae proportion in the formed granules and the functional identification of algal and bacterial species involved in the formation of granules.

2.3.7 Preliminary analysis on energy consumption for microalgae harvesting in this study

The preliminary analysis on energy consumption for microalgae harvesting was conducted among this novel biogranulation and conventional harvesting methods according to the energy and materials consumption data in this experiment and previous studies (Brentner et al., 2011; Shi et al., 2019) with the algae harvesting step being only considered (Table 2-3). The electricity requirement was calculated based on per kg of algae output, and the energy consumption in this study was assumed to be mainly from artificial light, air pump, influent and effluent pumps. Daily energy consumption of each equipment was calculated as the total operation time per day multiplied by energy consumption rate mentioned in the device specification sheet.

As shown in Table 2-3, centrifugation process consumes 10.8 MJ/kg-algae output, which is much higher than other technologies. Probably this is the major reason why centrifugation is generally applied for harvesting algae cells rich in more valuable compounds other than those for biofuels (Brentner et al., 2011). Electrocoagulation requires pretty lower electricity, but its downstream process is usually affected by the metal (like Fe) retained in the algae biomass (Richardson et al., 2014; Richardson and Johnson, 2015). As for the chitosan flocculation method, the algae harvesting efficiency is proportional to the initial biomass concentration and the highest 95% can be achieved under optimum chitosan addition at a relatively low energy consumption (1.98×10^{-1} MJ/kg-algae output). The main barriers for its large-scale application are the cost for chemicals addition and the residual co-product added into the culture medium (Beach et al., 2012). Ultrasonic technology can concentrate algae cells and continuously

separate lipids and proteins by applying a standing acoustic wave, and no chemicals and materials are consumed at a relatively low energy consumption. However, the average harvesting efficiency (64%) by this technology is relatively low compared to other technologies. As for biogranulation, microalgae granules can be harvested by gravity sedimentation easily within 2 min without energy consumption, and only wastewater (digestion liquor in this study) is fed with no other materials addition. In addition, this biogranulation technology can maintain much higher biomass concentration at 2-4 g/L with a much higher biomass productivity (130-270 mg/L/d) in the reactors, which can definitely compete against all the other algae harvesting technologies in large scale applications.

On the other hand, the heating value of microalgae was detected to decrease from the initial 22.79 MJ/kg (day 0) to 13.33 MJ/kg-MLSS on day 150 (Table 2-1), which is still much higher than 9.45 MJ/kg-cultivated or harvested algae by other technologies (Shi et al., 2019). When taking its much higher biomass productivity into consideration, biogranulation process using digestion liquor can realize quick cultivation and harvesting, which is more promising than other microalgae cultivation and harvesting methods. Still, it should be further addressed whether the biogranulation process influence the lipid production from *A. falcatus* var. *acicularis* and more in-depth research works should be conducted on the optimization of granulation conditions (light-on/light-off cycle, aeration time, etc.), targeting higher oil production capacity.

2.4 Summary

Ankistrodesmus falcatus var. acicularis can be harvested within 2 minutes by simple gravity sedimentation after microalgae granule formation, which was stably achieved around 150 days' operation of SBRs. The granulation system can maintain much higher algae biomass concentration (2-4 g/L) with much higher biomass productivity (130-270 mg/L/d) compared to the traditional suspension cultivation method under optimum conditions. The microalgae granules showed great potentials for nutrients removal from digestion liquor with excellent and stable DOC (95.7± 1.22%) and NH_4^+-N (85.59 ± 2.71%) removals. Future research is directed to the optimization of granulation process for a stable lipid content in microalgae granular biomass and function identification of algal and bacterial species in the granulation process.

Table 2-1 Main characteristics of seed algae and mature algae granules in the reactors before and after granulation.

Parameters	Day 0	Day 150
Mean size	25-45 μm	2.25 mm
SVI_{30} （mL/g）	3125	53.44 ± 3.31
Settling velocity (m/h)	~ 0	18.47 ± 0.23
Integrity coefficient (%)	--	6.18 ± 0.01
Total phosphorus content (mg/g-TSS)	3.32 ± 0.18	53.33 ± 3.48
Total nitrogen content (mg/g-TSS)	11.19 ± 0.98	24.99 ± 1.65
Heating value	22.79 MJ/kg	13.33 MJ/kg

All data are the average values of triplicate tests.

Table 2-2 Changes in OTUs and biological community diversity of eukaryote and bacteria

		Day 0	**Day 25**	**Day 105**	**Day 155**
Eukaryote	OTU[a]	9009	9334	21344	34100
	Simpson diversity index[b]	2.098	2.257	0.979	1.793
Bacteria	OTU[a]	43411	41177	36343	67874
	Simpson diversity index[b]	0.164	0.124	0.238	0.057

Note: [a] OTU, operational taxonomic units

[b] Simpson diversity index is a measure of diversity. The greater the value, the lower the sample diversity

Table 2-3 Comparative analysis of performance, chemicals consumption and energy inputs among algae harvesting technologies

Harvesting technologies	Centrifugation[a]	Membrane filtration[b]	Electrocoagulation[b]	Chitosan flocculation[b]	Ultrasonic[b]	Biogranulation (this study)
Electricity required (MJ/kg-algae output)	10.8	7.45×10^{-1}	9.55×10^{-2}	1.98×10^{-1}	1.29×10^{-1}	0 (only gravity sedimentation used for harvesting step)
Chemicals/ materials consumption	No	Porous Ni membrane	Fe	Chitosan	No	No
Harvesting efficiency	~95%	~100%	~98%	~95%	~64%	~100%
Time consumption (min/ton-algae)	10 ~ 30 (based on centrifuge capacity)	21.4	146.77	20 ~ 30	2.18	$\leq$ 2

[a] Brentner et al. (2011); [b] Shi et al. (2019)

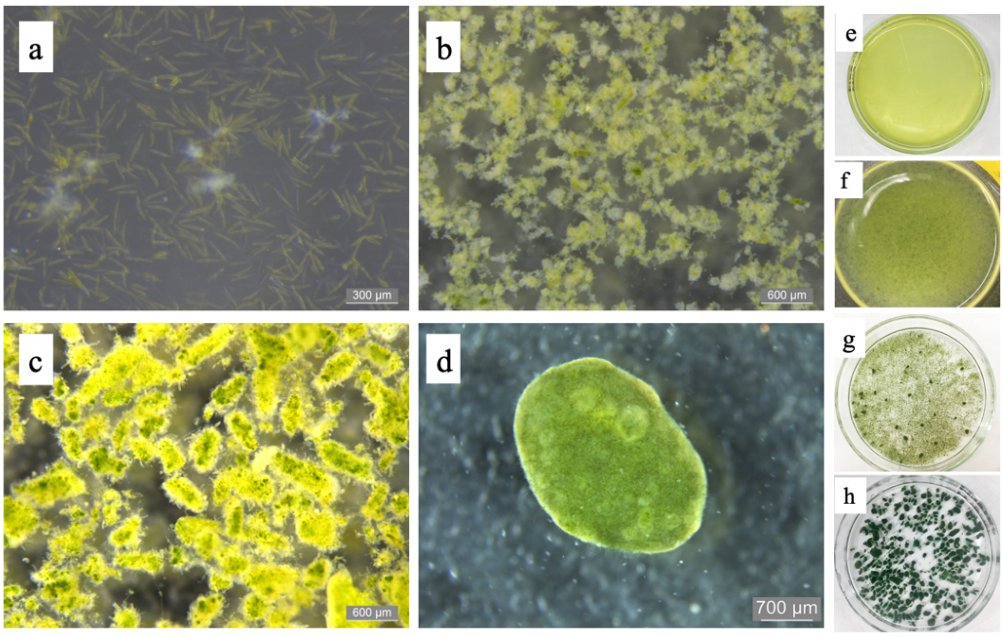


Figure 2-1 Transition from single cells of microalgae to granular structure. Light microscopy images of algae and algae aggregates during granulation: (a, e) seed algae, (b, f) activated algae flocs on day 44, (c, g) developed activated algae aggregates on day 90, and (d, h) mature activated algae granules on day 150.

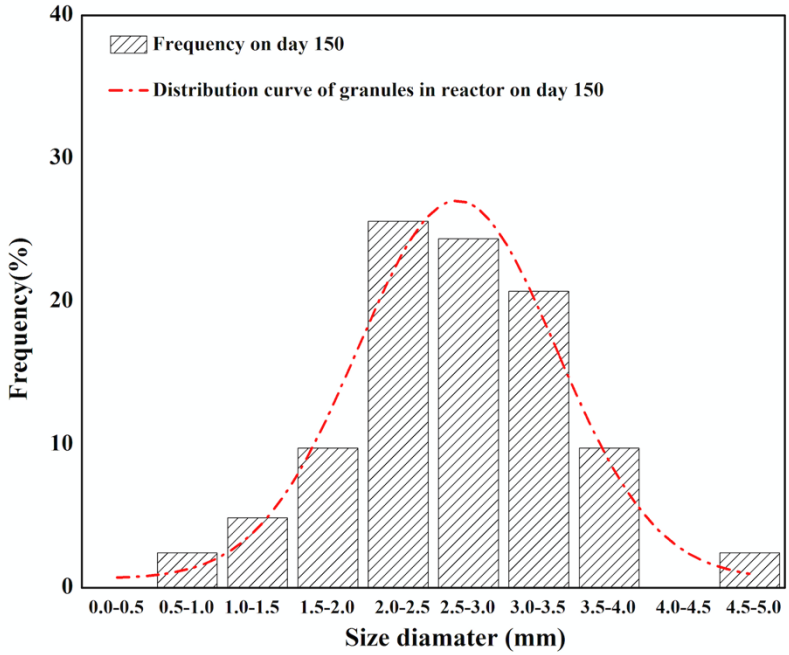


Figure 2-2 Size distribution of algae granules on day 150.

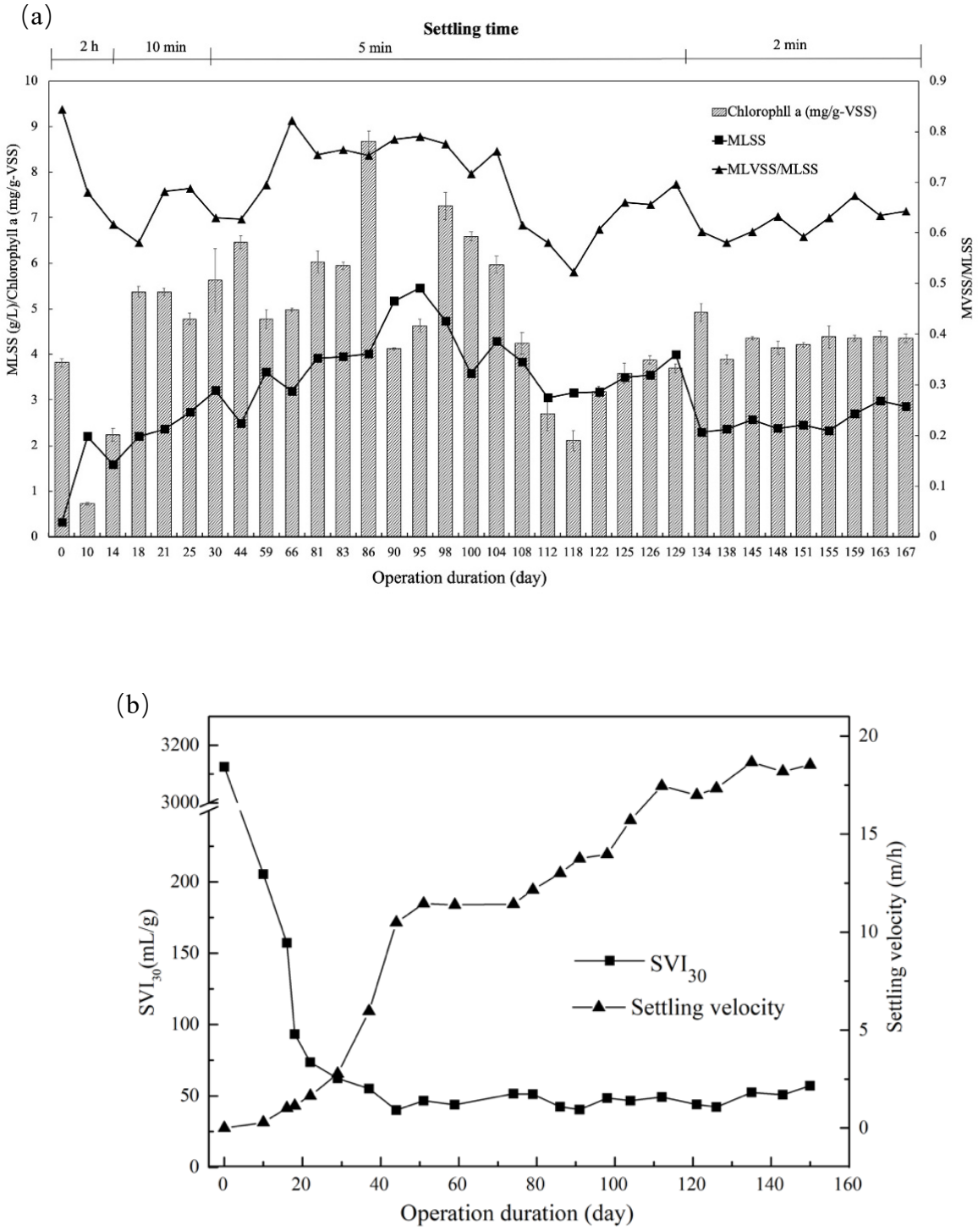


Figure 2-3 Variations of MLSS, MLVSS/MLSS, chlorophyll a (a), and SVI_{30} and settling velocity during algae granule formation.

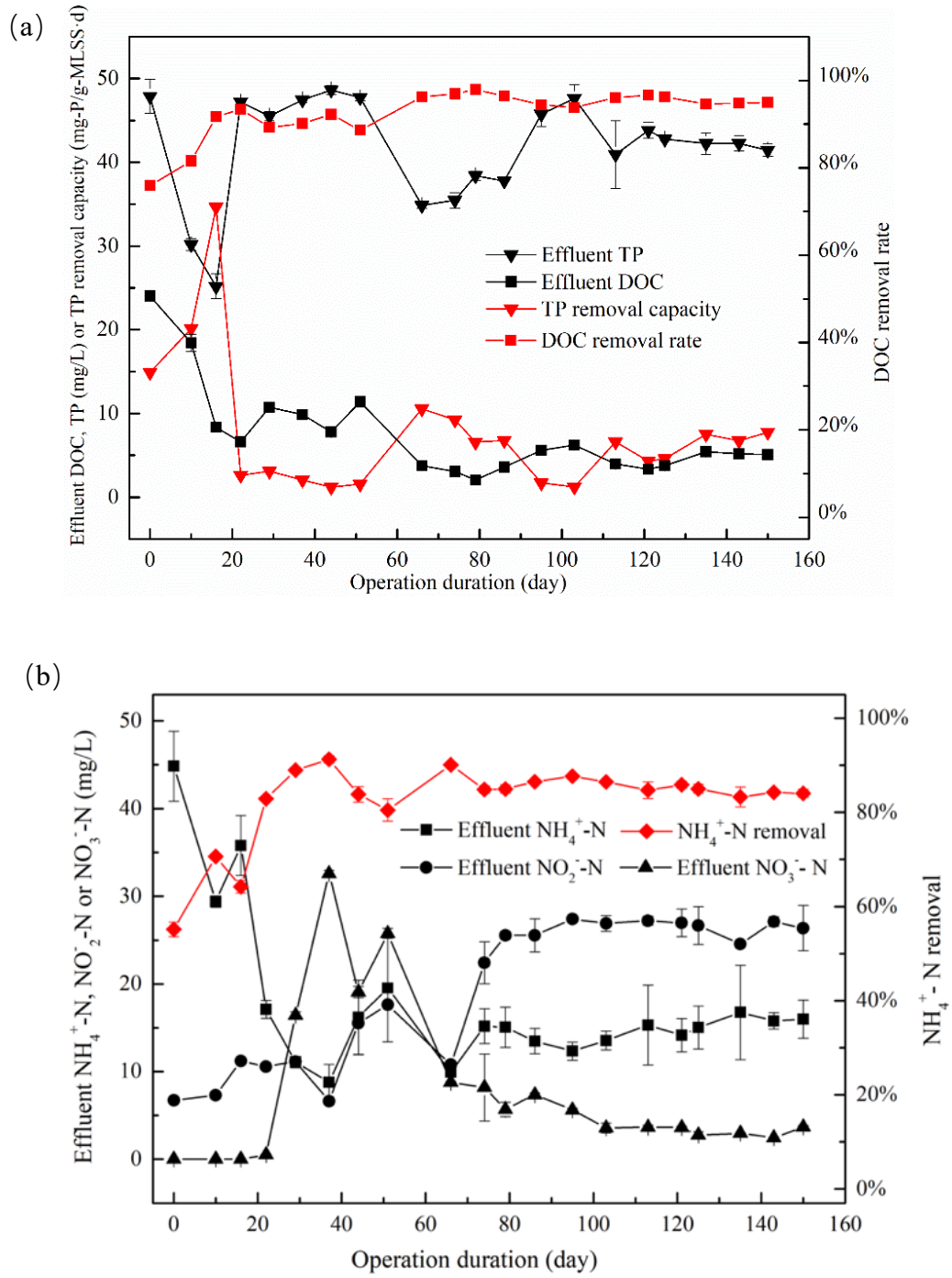


Figure 2-4 Nutrient variation and removal during operation. (a) Effluent TP and DOC concentrations, TP removal capacity and DOC removal rate; (b) Effluent ammonia nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N) and nitrite nitrogen (NO_2^--N) concentrations and NH_4-N removal rate.

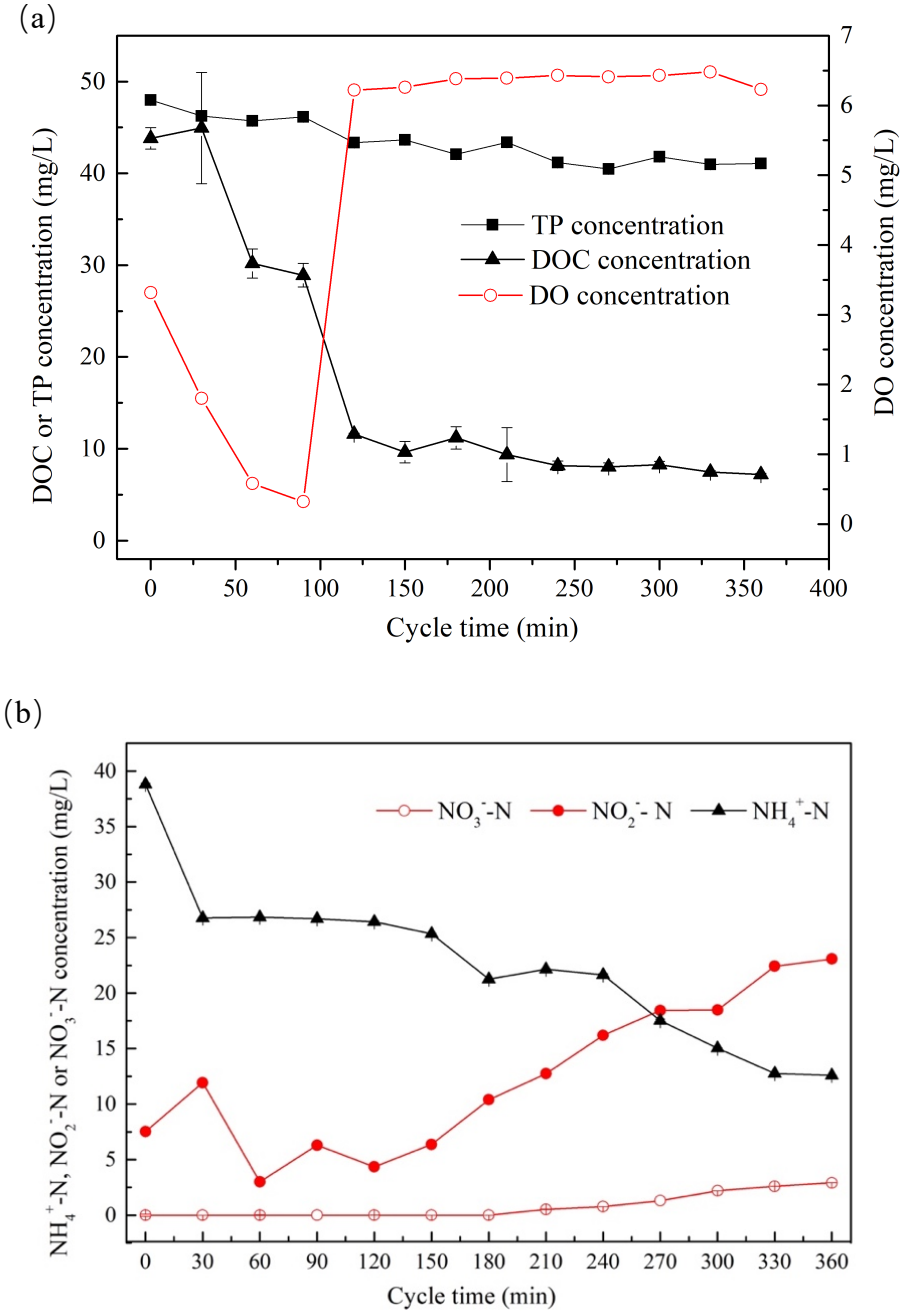


Figure 2-5 Variations of DOC, DO, and TP (a), and N species (b) in one typical cycle on day 150.

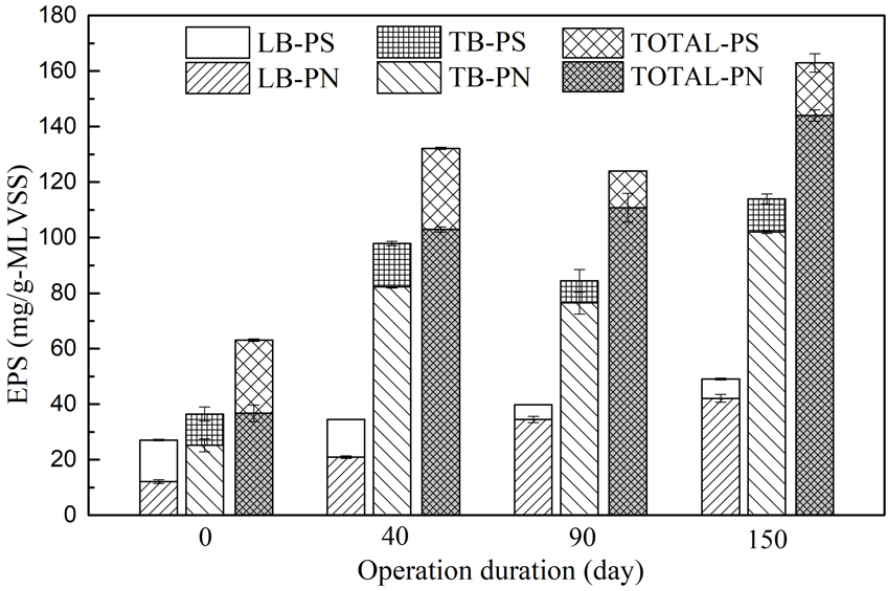
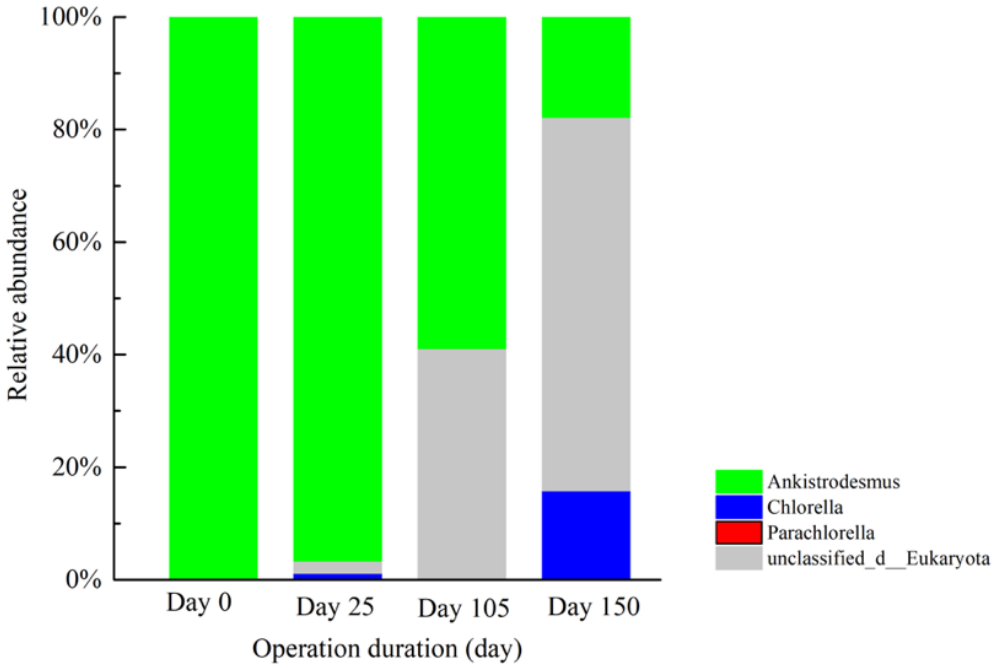


Figure 2-6 Changes of loosely bound EPS (LB- EPS), tightly bound EPS (TB-EPS), and total EPS contents, and their major components (proteins (PN) and polysaccharides (PS)) in the algae biomass or granules.

(a)



(b)

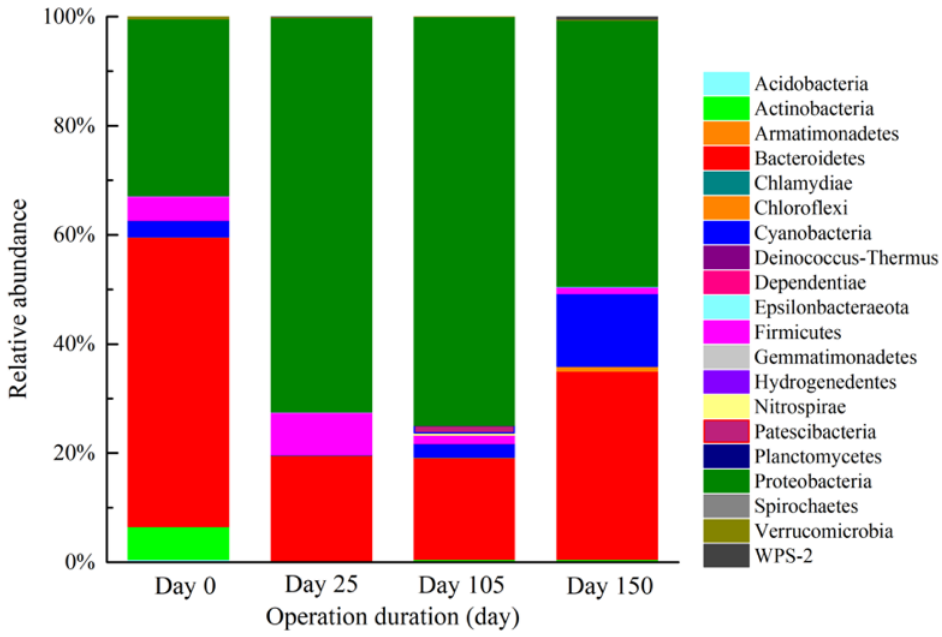


Figure 2-7 Abundance of dominant eukaryote at genus level (a) and bacteria at phylum level (b) in the algae biomass or granules.

Chapter 3 Utilization of mature algal granules for microalgae suspension harvesting: focused on mechanisms involved for quicker granulation

3.1 Background

Compared to conventional suspended cultivation systems, microalgae biomass in biogranulation system can be naturally concentrated and more easily harvested, leading to less expensive removal from treated wastewater and improving an microalgae feedstock suitable for further processing, production of biofuel for instance. However, limited understanding of fundamental mechanisms and long start-up (about 150 days) for algal granular systems remain main challenges in practical applications.

In this study, intact and crushed mature microalgae granules were used as additives and mixed with the suspended-growth microalgae to investigate the contribution of each fraction from crushed microalgae granules to the rapid harvesting process. More specifically, the microalgae suspensions were inoculated with intact, crushed mature microalgae granules and the separated fractions (solid and liquid) from the crushed granules with their effects on microalgae sedimentation performance being recorded. Extracellular polymeric substances (EPS) content and composition, zeta potential as well as nutrients removal and cationic uptake were monitored along with the 24-h batch aggregation test. In this study, three-dimensional excitation emission matrix (3D-EEM) technology was also applied for the analysis of EPS components stratified in the different layers including soluble EPS (S-EPS), loosely bound (LB-) and tightly bound (TB-) EPS during the formation of aggregates. Restated, up to now, little information could be found on EPS subfraction characteristics during the start-up period of microalgae granulation. This work is expected to shed light on the mechanisms of rapid microalgae granulation, paving the way for application of biogranulation in microalgae harvesting in practice.

3.2 Materials and methods

3.2.1 Target microalgae and synthetic digestion liquor

The seed microalgae, *A. falcatus* var. *acicularis* was obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES), Japan. The *A. falcatus* var. *acicularis* were preserved on agar jelly. In order to activating axenic strains, it was inoculated and batch cultivated in the CT medium under photoautotrophic conditions for 15 days, while the following ingredients were used for medium (per liter): $Ca(NO_3)_2 \cdot 4H_2O$ (150

mg), KNO_3 (10 mg), β–Na_2glycerophosphate·$5H_2O$ (50 mg), $MgSO_4$·$7H_2O$ (40 mg), Vitamin B_{12} (0.1 μg), Biotin (0.1μg), Thiamine HCl (10 μg), Na_2EDTA·$2H_2O$ (3 mg), $FeCl_3$·$6H_2O$ (0.59 mg), $MnCl_2$·$4H_2O$ (108 μg), $ZnSO_4$·$7H_2O$ (66 μg), $CoCl_2$·$6H_2O$ (12 μg) and Na_2MoO_4·$2H_2O$ (7.5 μg) (Waterbury and Stanier, 1981). To adapt experimental environment, activated seed strain was pre-cultivated under mixotrophic conditions with synthetic digestion liquor supplied for two generations (around 15 days) before aggregation test. Both cultures were performed in a 500 ml flask with a working volume of 350 ml at 25 ± 2 °C with shaking artificially twice one day to prevent adhesion on the wall of microalgal cells. Light was provided by LED lamps (NLSS20C, NIKKI, Japan) with 24 h light on and the intensities were measured around 146-157 μmol/m^2·s at the top by pocket digital lux meter (ANA-F11, Tokyo Photo-electric Co., Ltd., Japan).

The synthetic digestion liquor mainly consisted of 500 mg chemical oxygen demand (COD)/L, 50 mg PO_4^{3-}-P/L, and 100 mg NH_4^+-N/L to mimic digestion liquor after ammonia recovery by air stripping according to a previous study (Cai et al., 2016) and detailed components were described in Table 3-1.

3.2.2 Seed mature algal granules

Prior to the experiments, algal granules were cultivated in lab-scale sequencing batch reactors which has been stably operated for over 3 months with a sludge retention time (SRT) of 15 days. The target microalgal strain, *A. falcatus* var. *acicularis*, dominated the obtained microalgae granules. The mean diameter of granules was 2.25 ± 0.12 mm with a sludge volume index at 5 min (SVI_5) around 45.20 ± 0.81 mL/g, while chlorophyll-a (Chl-*a*) concentration was determined about 1.69 ± 0.02 mg/g-VSS.

3.2.3 Functional fractions of crushed microalgae granules and batch aggregation tests

Figure 3-1 and Table 3-2 summarize the details of the experimental framework and the characteristics of the target microalgae and mature microalgae granules used in this study. A glass homogenizer (SANSYO, JAPAN) was used for crushing the intact granules into homogenized suspension.

Batch aggregation tests were carried out to identify the functions of the separated fractions from the crushed mature microalgae granules during the rapid harvesting of suspended microalgae. All the batch tests were conducted under the same light and temperature conditions as those during the pre-cultivation of the target microalgae except the shaker (EYELA multi shaker MMS 210) which was used for agitation at 140 rpm during the aggregation test. As noted, 15 identical flasks (500 mL) were used in this study for the 5 group tests (in triplicate).

For each group test, 30 mL of microalgae suspension was taken out from the pre-culture and then centrifuged at 3500 rpm for 15 min (KUBOTA centrifuge 6200, Japan) after being washed with deionized water to exclude the effect of the previous medium, and then inoculated into a 500 mL Erlenmeyer flask containing 300 mL synthetic digestion liquor, resulting in a mixed liquor suspended solids (MLSS) or suspended microalgae concentration of 0.17 ± 0.02 g/L. Some other information about the batch tests was as follows: (1) No additive was added in the control group except 10 mL of deionized water to reach an equal working volume for all the different test flasks; (2) 8 grams of wet intact microalgae granules and well-crushed particles were added into the flasks, denoted as Add_{IG} and Add_{CG}, respectively; and (3) another 8 grams of wet pellets were crushed evenly with 10 mL deionized water and then the homogenous mixture was centrifuged at 3000 rpm for 15 min to separate the solid fraction from the liquid fraction, and both of them were used as additives, denoted as Add_S and Add_L, respectively. The volume of each additive containing different functional fraction was fixed at 10 mL with deionized water addition before being dosed into the test flask containing microalgae suspension.

3.2.4 Analytical methods

Twenty-five mL of culture samples were taken out at 0 h, 2 h, 4 h, 6 h, 12 h, and 24 h from the triplicate test flasks and used for evaluation of the harvesting efficiency, and determinations of S-EPS, zeta potential, pH, dissolved oxygen (DO), ionic variation and nutrients consumption. Before and after the tests, the homogenous mixture was sampled for the quantifications of mixed liquor (volatile) suspended solids (ML(V)SS), chlorophyll *a* (Chl-*a*), and bound EPS (LB-EPS and TB-EPS).

The morphologic change of the microalgal cells or aggregates, and the final microalgae granules was observed using Leica M205 C Microscope (Leica Micro-systems, Switzerland).

MLSS, MLVSS as well as Chl-*a* content can be used to reflect biomass concentration. MLSS and MLVSS were determined according to the Standard Methods (APHA, 2012). The determination of Chl-*a* was described detailed in Section 2.2. In brief, 5 mL of evenly crushed mixture was centrifuged at 3500 rpm for 15 min and the residue was resuspended in 10 mL of 90% methanol containing $MgCl_2$ solution. Then the mixture was heated at 70°C for 30 min in the dark for extraction. Later, centrifugation was performed at 3500 rpm for 10 min to separate the supernatant after being cooled down to room temperature, with the absorbance being recorded at the wavelengths of 750, 665, 645, and 630 nm, respectively using the UV-Visible

spectrophotometer (UV 1800, Shimadzu, Japan). Chl-*a* concentration in the separated supernatant can be calculated according to Eq. 3-1.

$$\text{Chl-}a\ (mg/L) = [11.6\times(A_{665}-A_{750}) - 1.31\times(A_{645}-A_{750}) - 0.14\times(A_{630}-A_{750})]\times \frac{Vm}{Vf} \quad (3\text{-}1)$$

where *Vm* (ml) and *Vf* (ml) are the volume of the extract methanol and the sample, respectively. Chl-*a* content (mg/g-VSS) in microalgae was obtained through dividing Chl-*a* concentration (mg/L) by MLVSS (g/L) of the corresponding test sample.

The harvesting efficiency of the suspended microalgae was also evaluated along with the batch aggregation test, which was measured according to the method described by Nuramkhaan et al. (2019) and Salim et al. (2011) with some modifications. Briefly, 3 mL aliquot of homogenous mixture was transferred into a cuvette for optical density (OD) determination at 682 nm (with the strongest peak at this wavelength) by the above-mentioned spectrophotometer. The harvesting efficiency was calculated according to Eq.3-2 by comparing the initial OD value (OD_0) and the OD value after 30 min or 90 min settlement (OD_{30} or OD_{90}). For the test groups Add_{IG}, Add_{CG}, and Add_S, the effect of the solid part of the microalgae granules on the absorbance determination was deducted to ensure the results that could represent the changes in the harvesting of suspended microalgae.

$$\text{Harvesting efficiency}_{30\text{ or }90}\ (\%) = 100\times(OD_0 - OD_{30\text{ or }90})/OD_0 \quad (3\text{-}2)$$

As for nutrients removal capability and ions variation during the batch tests, water samples were sampled and filtrated through 0.22 μm membrane filters and the filtrates were used for PO_4^{3-} - P, NH_4^+- N, NO_2^- - N, NO_3^- - N, K^+, Ca^{2+}, and Mg^{2+} determination by Ion Chromatography (IC, Shimadzu, Japan). Dissolved total organic carbon (DOC) and dissolved inorganic carbon (DIC) were measured by a TOC analyzer (TOC-VCSN, Shimadzu, Japan) coupled with an auto-sampler (ASI-V, Shimadzu, Japan). In addition, pH and dissolved oxygen (DO) in the mixture were monitored with pH meter (Hiroba, Japan) and DO meter (HQ40d, HACH, USA), respectively.

S-EPS were directly quantified by using the supernatants from centrifugation of the samples at 3000 rpm for 15 min followed by filtration through 0.22 μm membrane filter. The remaining residue was re-suspended with 0.9% NaCl solution and evenly homogenized by a glass rod, which was used for subsequent extraction of bound EPS according to the method described by Forster (1990). Briefly, the mixture was centrifuged at 7500 rpm for 20 min and the supernatant was filtrated through 0.22 μm membrane filter for LB-EPS determination. Again, the residue left in the centrifuge tube was washed and re-suspended with 0.9% NaCl solution, and then heated on the oven at 80°C for 30 min followed by centrifugation at 8500

rpm for 20 min with the supernatant being collected and filtrated through 0.22 μm membrane filter for TB-EPS determination. Phenol-sulfuric acid method (Dubois et al., 1956) and Lowry-Folin method (Lowry et al., 1951) were used for the quantification of polysaccharides (PS) and proteins(PN) in the S-EPS, LB-ESP and TB-EPS samples, with glucose and bovine serum albumin (BSA) being as the standard, respectively.

The 3D-EEM spectra of EPS samples were obtained using Hitachi F-4500 (Hitachi, Japan), which can provide detailed information of presence and concentration of fluorescent organics (Zhang et al., 2017). The emission wavelength (Em) was set to increase from 200 nm to 500 nm with 5 nm as an increment, and the excitation wavelength (Ex) was increased from 200 nm to 400 nm with the same increment unit at a fixed scan speed of 1200 nm/min. In addition, the fluorescence regional integration (FRI) was utilized to analyze the EEM spectra and calculate the fluorescent intensity of each region.

Zeta potential was determined by Zetasizer (Malvern, UK) according to the method described previously (Zhang et al., 2019) with some modifications on sample preparation. The sample used for determination was washed three times with deionized water and then centrifuged at 3000 rpm for 15 min to remove the supernatant. The residue was re-suspended in deionized water and homogenized by a glass homogenizer, followed by filtration through a 200 μm mesh screen to remove bulky particles.

3.2.5 Statistical analysis

There were at least three biological replicates for all of the cultivation and experiments, and results were all achieved from batch cultivation or reaction. Results were expressed as mean ± standard deviation. Analysis of Variance (ANOVA) was carried out to examine the differences between groups with or without addition of functional fractions from mature algal granules using IBM SPSS 27.0. Spearman correlation analysis was conducted in SPSS statistical software (version 24) in order to assess the correlation of harvesting efficiency and EPS variation. A P value less than 0.05 was considered statistically significant.

3.3 Results and discussion

3.3.1 Enhanced harvesting of suspended microalgae by addition of mature microalgae granules

Generally, the poor settleability of pure microalgae suspension is mainly caused by their small size and low biomass concentration (e.g. less than 1 g/L) (Grima et al., 2003; Salim et al., 2011). At the beginning of the batch tests, the harvesting efficiencies after 30 min (i.e. harvesting efficiency$_{30}$) and 90 min (harvesting efficiency$_{90}$) settling of the suspension with

original target microalgal strain were determined as 2.66 ± 0.31% and 7.70 ± 0.10%, suggesting the poor biomass separation potential through gravity settling. Figure 3-2 illustrates the variations of settling capability in terms of the harvesting efficiency$_{30}$ or harvesting efficiency$_{90}$ of the target microalgae, *A. falcatus* var. *acicularis* after being inoculated with the 5 different additives as defined in section 3.2.3 and Figure 3-1. It was obvious that the settleability of microalgae was improved significantly along with the batch tests for all the test groups including the control (without additive addition, increased from the initial harvesting efficiency$_{30}$ of 2.45 ± 0.12% to 17.74 ± 0.29% at 24 h), especially during the first 6 hours (Figure 3-2b). This observation was associated with the slightly elevated pH values with CO_2 depletion (Figure 3-3) and EPS excretion by bacteria under the continuous agitation condition. As reported, auto-flocculation or enhanced sedimentation of microalgae can occur naturally in the absence of organic or inorganic coagulants/flocculants, due to the consumption of CO_2 by photosynthesis of microalgae resulting in alkaline condition that favors precipitation (Sukenik et al., 1985; Sukenik and Shelef, 1984). On the other hand, some bacteria present in the medium (as this experiment was performed under non-sterile condition) could excrete EPS (in response to the stimulation by continuously shaking) to bind or flocculate with the microalgal cells in suspension. This kind of EPS secretion might mainly occur for self-protection in a stressful environment (Trunk et al., 2018). However, the microscopic observations (Figure 3-4) showed that most of the microalgal cells were still remained in suspension though some of them intertwined with bacteria into small flocs. In addition, the unstable harvesting efficiency further implies the non-persistence of this kind of auto-aggregation under the test condition (Figure 3-2b and Figure 3-2c). In contrast, as observed in the settling test (Figure 3-5), almost no biomass settled down within 15 min in the control, while complete settling of microalgae was achieved after addition of Add$_L$ (i.e. the liquid fraction of the crushed mature microalgae granules), resulting in a clear interphase between the settled biomass and culture medium. Among the test groups, Add$_L$ addition showed the best improvement efficiency on the settleability of the target microalgae, with the harvesting efficiency$_{30}$ and harvesting efficiency$_{90}$ increased from the initial 1.69 ± 0.10% (0 h, i.e., the moment right after homogeneously mixing target microalgae suspension with Add$_L$ on the shaker) to 90.75 ± 1.23% (24 h) and from initial 11.37 ± 2.82% (0 h) to 92.97 ± 0.59% (24 h), respectively. Besides, the images under microscope indicated the typical formation of aggregates with size varying from 0.5 mm to 2 mm in the flask (Figure 3-2a and Figure 3-4). It was found that the harvesting efficiencies between the control group and Add$_L$ addition groups were significantly different ($p < 0.01$), while there was no significant

difference in harvesting efficiency among the Add_{IG}, Add_{CG}, and Add_S groups ($p > 0.05$) whereas all of them were the original algal-bacterial symbiosis additives. For Add_{IG} (intact microalgae granules) addition, the harvesting efficiency$_{30}$ increased from the initial 7.27 ± 0.32% to 35.98 ± 0.60 % at 4 h and then decreased to 31.69 ± 1.84 % at 24 h, with the similar trend being noticed in the Add_{CG} and Add_S groups. This observation may be attributed to the presence of filamentous microorganisms that can serve as backbone and interact with the excreted EPS for dispersed cells attachment and adhesion (Aqeel et al., 2019; Beun et al., 2002), resulting in settleability improvement of microalgae. However, EPS matrix can also function as carbon or energy source for host cells under nutrients-deprived condition (Flemming and Wingender, 2010; Xiao and Zheng, 2016), which may explain the decline of harvesting efficiency in the Add_{IG}, Add_{CG} and Add_S added flasks after nutrients being exhausted (Figure 3-2 and Figure 3-10). As such, in comparison to the other three additives addition, the significant increase ($p < 0.01$) in harvesting efficiency by Add_L addition to the microalgae suspension demonstrates that the liquid fraction (Add_L) from the crushed microalgae granules greatly favors the rapid aggregation of the single-cell microalgae suspension.

3.3.2 Response of soluble EPS (S-EPS) and bound EPS (LB-EPS or TB-EPS) to addition of crushed microalgae granules

EPS play an important role in the formation and settleability of microbial aggregates, and can be classified into two major fractions: soluble EPS (including soluble macromolecules, slimes and colloids) and bound EPS (including loosely bound and tightly bound polymers)(Comte et al., 2006; Shi et al., 2017; Yu et al., 2008). In order to explore the function of each fraction of EPS in the mature microalgae granules, the EPS composition and concentration were determined during the batch tests.

As shown in Figure 3-6, the S-EPS concentration in the control group increased from the initial 11.73 ± 3.95 to 103.41 ± 7.03 mg/g-VSS at 24 h along with the harvesting efficiency improvement, to which PN contributed more, about 82%. As for the Add_L group, the S-EPS reached the highest level at 218.28 ± 8.86 mg/g-VSS right after the addition, and then decreased sharply to 61.54 ± 3.76 mg/g-VSS with substantial PN consumption after test for 2 h. However, it increased to 188.80 ± 7.12 mg/g-VSS with a higher PS content of 155.57 ± 1.75 mg/g-VSS in the harvested microalgae biomass, achieving a harvesting efficiency$_{30}$ of 90.75 ± 1.22%. It can be clearly seen that the PS content kept increasing after the test for 6 h in the Add_L group, which might be associated with the formation of granules according to Tay et al. (2001) who

pointed out that the continuous increase of PS could induce the initial formation of microbial aggregates.

The initial LB-EPS of the inoculated microalgae suspension was 2.83 ± 1.08 mg/g-VSS consisting of PS about 1.23 ± 0.71 mg/g-VSS and PN about 1.60 ± 0.37 mg/g-VSS. Seen from Figure 3-7a, the LB-EPS contents increased to 11.46 ± 0.42 mg/g-VSS and 16.58 ± 0.58 mg/g-VSS in microalgae biomass in the control and Add_L groups at the end of the test (24 h), in which PS contributed more to the enhancement, accounting for 74.80% and 84.19%, respectively. A declined trend of LB-EPS content was noticed in other test groups including $Add_{IG,}$ Add_{CG} and Add_S, in which a much higher LB-EPS content (averagely 42.75 ± 3.19 mg/g-VSS) contained in the seed original microalgae granules or crushed granules and decreased to 36.76 ± 0.70, 7.01 ± 0.59, and 3.10 ± 0.73 mg/g-VSS after the test for 24 h. This decrease was likely due to the followings: (1) lack of shear force generally provided by bubble aeration which can stimulate more EPS excretion to maintain the compact granular structure (Feng et al., 2021; Tsuneda et al., 2003), and (2) the easy utilization of released PN or PS by heterotrophs to support their endogenous respiration after microalgae granules being evenly crushed (Add_{CG} and Add_S groups). Moreover, the PN contents drastically reduced in Add_{CG} and Add_S groups to 5.77 ± 0.45 mg/g-VSS and 2.59 ± 0.37 mg/g-VSS, respectively, while almost no change was detected in PN content in Add_{IG} group with granular structure maintained, from the initial 33.79 ± 0.73 mg/g-VSS to 33.68 ± 0.65 mg/g-VSS at 24 h, suggesting that PN content in LB-EPS may be used as an indicator for granular disintegration and structural stability.

On the other hand, the TB-EPS content in Add_L group was found to remarkably increase from 39.04 ± 4.00 mg/g-VSS to 247.47± 5.83 mg/g-VSS with the PN/PS ratio increased from 0.76 to 4.66 (Figure 3-7b). Despite the increase in TB-EPS content from 39.04 ± 4.00 to 82.98 ± 2.31 mg/g-VSS) in the control, the PN/PS ratio kept relatively stable from 0.76 to 1.22 with some increase in both PN and PS contents. As for Add_{IG} group, the TB-EPS content showed some increase from 193.45 ± 7.62 to 238.79 ± 5.25 mg/g-VSS, in which 71% increment was contributed by PN, in agreement with a previous study (Zhao et al., 2019) when algal-bacterial granules were loaded into flasks under the same shaking condition. This observation implies that PN may function as protective agents to maintain the stable operation of algal-bacterial granules under the test condition. As for Add_{CG} and Add_S groups, the contents of TB-EPS as well as the PN/PS ratio decreased at the end of test. In addition, a strongly positive correlation ($r = 0.90$, $p < 0.05$) was found between the PN content in TB-EPS and the harvesting $efficiency_{30}$, suggesting that the increased PN content in TB-EPS favored the microalgae

granule formation and its granular stability, which is beneficial for the subsequent microalgae biomass separation from the culture medium.

More importantly, Add_L group achieved the highest harvesting efficiencies in 30 min and 90 min sedimentation. The microalgae biomass showed the dramatic and largest increase in bound EPS content, about 94% contributed by TB-EPS (Figure 3-7c). It is speculated that TB-EPS are more important than LB-EPS to the stable granule formation, which may have a closer correlation with the characteristics of aggregates/granules in the flocculation, sedimentation and dewatering units (Wang et al., 2020b; Yu et al., 2009). As TB-EPS are the tightly bound substances surrounding the cells (Poxon and Darby, 1997) and LB-EPS are those loosely bound to the cells, a higher TB-EPS content was likely favorable for the formation of a thicker EPS layer which was essential for maintaining a more stable granular structure.

3.3.3 3D-EEM assays

Figure 3-8 show the 3D-EEM spectra of LB-EPS and TB-EPS in the initial microalgae suspension, the control and Add_L test groups at 24 h with the related fluorescence spectra parameters including peak location, fluorescence intensity, and probable compositions being summarized in Table 3-3. According to the wavelength range defined, the spectrum was divided into five valuable regions, namely, I (Ex/Em wavelengths, 200-250/200-330 nm), II (200-250/330-380 nm), III (200-250/380-500 nm), IV (250-280/200-380), and V (250-400/380-500) where substances with fluorescence spectra are identified as aromatic proteins (I and II), fulvic acid-like organics (III), soluble microbial by-product (IV), and humic acid-like organics (V), respectively (Chen et al., 2003). As shown in Figure 3-8, peaks A and C representing microbial by-products and humic acid-like proteins, respectively appeared in the LB-EPS of the initial microalgae. At the end of test, the microalgae cells in the control kept almost suspended with very few small aggregates being observed under microscope; while peak A disappeared, instead, other similar peaks (C, D and E) representing humic acid-like proteins were observed. As noticed, there was no significant enhancement in fluorescent intensity from 11.19 AU of peak A in the initial microalgae to the average value of the three peaks (6.384 AU, 11.81AU and 17.55AU at peaks C, D and E, respectively) for the control after 24-h batch test. In contrast, as for the Add_L group, after 24-h test, the single-cell microalgae were clearly observed to fully form large aggregates with good settleability; the fluorescence intensity of peaks A and C were remarkably enhanced from the initial values of 11.19 AU and 3.317AU to 102.2AU and 44.3 AU, respectively. At the same time, a new peak B representing aromatic proteins appeared in the Add_L group with an intensity of 50.85 AU at 24 h. These macromolecular substances have

been reported to function as adsorption bridge and/or mesh sweeping that are beneficial for electrostatic force decrease and hydrophobicity enhancement on cells surface in the process of flocculation (Wang et al., 2020b; Yu et al., 2009). It is worth mentioning that all the TB-EPS samples have the same peaks A and B with only some changes in fluorescence intensity being observed among the initial microalgae, the control, and Add_L groups at 24 h. The aromatic proteins (peak B) were greatly enriched in the TB-EPS of Add_L group (added with the liquid fraction from the crushed mature microalgae granules), about 14 times that the initial microalgae value (from 46.38 AU to 666.7 AU). In the case of microbial by-products (peak A), the fluorescence intensity of TB-EPS in the Add_L sample (146.8 AU) increased to about 9 times that of the initial microalgae (16.87 AU). In contrast, the TB-EPS from the control group at the end of test exhibited relatively low enhancement in fluorescence intensity at peaks A and B when compared to the Add_L group.

Additionally, Figure 3-9 shows the dynamic changes of fluorescent substances in S-EPS in the control and Add_L groups throughout the test. In the control group, no valuable peaks were observed till peak A (microbial by-product substances) appeared at 12 h with an intensity of 4.477 AU, which was sharply increased to 111.0 AU at 24 h accompanied by peak B (aromatic proteins) appearance at a higher intensity of 305.9 AU. In the case of Add_L group, on the other hand, aromatic proteins (peak A) and microbial by-products (peak B) kept predominance along with the whole test, although a decline trend was notice in fluorescence intensity, from 149.0 AU to 15.38 AU and from 74.39 AU to 12.47 AU, respectively. Both peaks C and D representing humic acid-like substances started to appear after starting the test for 4 h with their intensity kept almost stable around 9-11 AU.

In summary, as aromatic proteins were detected to remarkably enrich in both LB-EPS and TB-EPS in the Add_L group that achieved the highest harvesting efficiency, it is inferred that aromatic proteins play a crucial role in the initialization of microalgal aggregate formation and the maintenance of granular stability. This observation to some extent agrees with the phenomenon noticed in the activated sludge systems by Görner et al. (2003). Zhu et al. (2012) also claimed that aromatic protein-like substances especially tyrosine are important in the maintenance of AGS structure. This study for the first time found that during the granulation process of microalgae, excessive secretion of aromatic proteins in S-EPS is not beneficial for cells aggregation as the highest aromatic proteins concentration were detected in S-EPS of the control group with very low harvesting efficiency. This observation was in accordance with the

quantitative results of EPS in Section 3.3.2, i.e., the extracellular proteins in bound EPS could contribute more to aggregate formation and granular structure stability.

3.3.4 Changes in nutrients removal and related cations

Figure 3-10 illustrates the concentration profiles of DOC, ammonium-N and phosphate-P. In general, after being quickly mixed with the additives, all the flasks showed a decreasing trend of DOC and total N (NH_4^+-N + NO_2^--N + NO_3^--N) concentrations. Over 90% DOC were removed in 2 h of test by the Add_{IG}, Add_{CG}, and Add_S groups, resulting in carbon starvation phase in the subsequent test period, which might be one major reason for the decrease of bound EPS as discussed in section 3.2.2. Xiao and Zheng (2016) claimed that EPS can be used as carbon or energy source. Aqeel et al. (2019) also pointed out that these polymeric substances can be produced and stored under feast conditions, while utilized when easily degradable organics are not available under famine conditions. After test for 24 h, the lowest TC (DOC+DIC) level, about 10 mg/L was detected in the Add_L group, much lower than those in other test groups, showing its rapid inorganic and organic carbon consumption probably due to the quick formation of microalgae granules and high bioactivities. Obviously, P release occurred in the Add_{IG}, Add_{CG}, and Add_S groups at the beginning and during the initial 6 h test due to relatively low DO levels inside the solid portion although the bulk DO varied from 1.28 to 6.39 mg/L (Figure 3-3), or lack of carbon source due to the presence of phosphorus accumulating organisms (PAOs) (Huang et al., 2015a).

On the other hand, partial accumulation of NO_3^--N and NO_2^--N was noticed along with the test in the test groups when compared to the control, signaling the existence of nitrifying bacteria in the seed mature microalgae granules and in the liquid portion of the crushed granules, among which the Add_L group reflected the lowest (NO_3^--N + NO_2^--N) concentration and the highest total N removal (70-80%) at the end of test.

In order to link Add_L to the quick aggregation of microalgae, additional experiment was performed to compare the effect of liquid fraction from the crushed microalgae granules before (Add_L) and after removing bacteria via 0.22 μm membrane filtration (denoted as Add_{LF} with the absence of bacteria including nitrifying bacteria). Results showed that addition of Add_{LF} could also achieve microalgae harvesting efficiency > 92 %. In addition, there was no significant difference in EPS concentration and composition between Add_L and Add_{LF}, although some slight increase of aromatic proteins in LB-EPS and decrease of aromatic proteins in TB-EPS were discerned (Figure 3-11 and Table 3-4).

It had been reported that K^+, Ca^{2+} and Mg^{2+} were closely associated with microbial aggregates and granular stability (Wang et al., 2021), which were also monitored in this study (Figure 3-12). Not much difference was detected in K^+ concentration in the bulk among all the test groups, except the Add_{IG} group in which K slightly increased during the first 2 h and then decreased as in other groups. As for Ca^{2+} and Mg^{2+} concentrations, in comparison to a continuous decline in the control group, the Add_{IG}, Add_{CG} and Add_S groups reflected a similar variation trend, i.e., firstly decreased till the test for 2-6 h and then increased, reaching relatively higher levels in the bulk at 24 h. As for Add_L addition, the Ca^{2+} and Mg^{2+} concentrations kept relatively stable throughout the batch test. More specifically, Ca^{2+} and Mg^{2+} concentrations in the Add_L group showed almost the same variation trend that kept increasing till 4 h, then slightly decreased at 6 h, followed by a large reduction at 12 h, finally increased and returned to the same level as its initial value.

The above variations of the three cations may have strongly close relationship with the change of PO_4^{3-}, especially K^+ and Mg^{2+}, due to their similar curve shape (Figure 3-10c and Figure 3-12) and very high coefficients ($r = 0.82$ and 0.92, respectively, $p < 0.05$). This observation is in agreement with the findings in anaerobic or aerobic granular systems (Schönborn et al., 2001; Wang et al., 2021) where K^+ and Mg^{2+} are regarded as counter-ions in the polyP chain during its hydrolysis and synthesis.

On the other hand, cations such as Ca^{2+} and Mg^{2+} can also be uptaken and utilized by microorganisms during the operation of biological processes, which were found to strongly correlate with each other in this study ($r = 0.95$, $p < 0.05$). As summarized by Sheng et al. (2010), the binding between EPS and divalent cations is one of the most important intermolecular interactions beneficial for the maintenance of microbial aggregate structure. This kind of binding may reduce electrostatic repulsion and then induce aggregation of suspended cells. This was evidenced by the larger reduction in absolute value of zeta potential of microalgae sample of the Add_L group from -23.23 ± 1.40 mV to -10.26 ± 1.88 mV, in comparison to the control group (from -23.50 ± 1.42 mV to -16.00 ± 1.41 mV) (Table 3-5). This phenomenon also implied that the microalgae biomass in the Add_L group was more easily to destabilize, resulting in the enhanced aggregation and better settleability of the microalgae.

3.3.5 Preliminary analysis involved in this study

The current study investigated the effect of intact mature microalgae granules, crushed microalgae granules, and its solid or liquid fraction on microalgae settleability, targeting the rapid formation of aggregates/granules and harvesting of microalgae biomass. As shown in

Figure 3-2, the harvesting efficiency$_{30}$ can be substantially improved from 1.69 ± 0.10 % (initial original microalgae) to 90.75 ± 1.23% (Add_L group) during the 24 h batch test, and a complete (100%) harvesting of microalgae biomass can be realized using Add_L within 15 min through gravity settling (Figure 3-5). This observation implies the liquid fraction from the crushed granules is the most important fraction for microalgal cells aggregation over the intact granules or solid fraction of the crushed granules. PS in S-EPS are responsible for cohesion and adhesion of suspended cells in broth and thus crucial at the beginning of granulation as the PS content in S-EPS kept increasing along with the self-aggregation improvement in the Add_L group (Figure 3-6). Although the network structure and large specific surface area of the intact granules or crushed granules may contribute to flocs aggregation, their relatively incompact structure under low shear force would make the aggregation strength relatively weak. Additionally, EPS can be consumed as nutrients source during the long-term famine in the Add_{IG}, Add_{CG} and Add_S groups, making the beneficial effects of intact granules or crushed granules on cells attachment reversible. Bound EPS are mostly related to the formation and stability of aggregation/granules, which were detected to increase obviously in the Add_L group from 41.86 ± 5.08 mg/g-VSS to 264.05 ± 6.41 mg/g-VSS, with 93.8% increment contributed by TB-EPS (Figure 3-7), resulting in the rapid formation of microalgal aggregates (Figure 3-2 and Figure 3-4). This observation may be attributed to the spatial distribution of LB-EPS and TB-EPS, and LB-EPS are loosely bound and rather slippery to cells and possess higher bound water content (Li and Yang, 2007). Thus LB-EPS can to some extent influence the initial formation of aggregates, but TB-EPS content may contribute more to the stabilization of formed aggregates or granules. PN content as well as PN/PS ratio in TB-EPS may attribute to the integrity of aggregates/granular structure. In this study, a strong correlation was found between the PN content and microalgae harvesting efficiency ($r = 0.90$, $p < 0.05$). As reported by Zhang et al.(2007), a higher relative hydrophobicity is associated with a higher PN/PS ratio ($r = 0.969$), and the former is considered as a triggering force for granulation. The obvious decline in absolute value of zeta potential from -23.23 ± 1.40 mV to -10.26 ± 1.88 mV in addition to the binding potential between EPS and the cations (K^+, Ca^{2+} and Mg^{2+}) may accelerate this aggregation process. Due to the high content of negatively charged amino acids, PN are more involved in electrostatic bonds with multivalent cations than PS, resulting in decreased negative surface charge density surrounding the cell surface, easy aggregation and subsequent sedimentation (Sheng et al., 2010; Zhang et al., 2007; Zhang et al., 2019).

As revealed by 3D-EEM spectra (Figure 3-8 and Figure 3-9), after the formation of large aggregates, the peaks representing aromatic proteins firstly appeared in LB-EPS and were strengthened in TB-EPS in the Add_L group, indicating aromatic proteins located in regions I and II (Ex/Em: 200-250/200-380) in bound EPS are favorable for stable microalgae aggregation. It is hypothesized that there are some biological processes that stimulated aromatic proteins secretion as the fluorescence intensity of aromatic proteins in the Add_L group was strengthened when compared to the original sample of the liquid fraction from the crushed granules, accompanied by the appearance of some new peaks. In the additional experiment on Add_{LF}, similar phenomenon also occurred when the liquid fraction was sterilized via 0.22 μm membrane filtration, suggesting the stimulation process was not contributed by the bacteria in the liquid. As previously reported, aromatic protein-like substances can preferentially induce and maintain structural stability by quorum sensing (QS) when N-acyl-homo-serine lactone (AHL) supernatant was added into bacterial AGS systems (Zhang et al., 2020). Many studies have found that AHL-mediated QS is closely associated with granulation (Dandekar et al., 2012; Fuqua and Greenberg, 2002; Tan et al., 2014), and AHLs can regulate the microorganisms through EPS, especially tryptophan and protein-like substances secretion, contributing to microbial aggregation in aerobic granular systems. Up to the present, few research has been conducted on microalgae aggregation/granulation regulated by AHLs, except Zhou et al. (2017) who mentioned that AHLs extracted from activated sludge may favor the aggregation of microalga. Therefore, further in-depth research is necessary on the regulation of AHLs during the rapid granulation of microalgae, targeting the large-scale application of microalgae harvesting via biogranulation.

3.4 Summary

As Figure 3-13 illustrated, microalgae biomass was successfully harvested from its suspension culture through the addition of liquid fraction (Add_L) of the crushed mature microalgae granules, achieving > 90% harvesting efficiency in 30 min. The formed aggregates with stable structure enhanced bound EPS production from the initial 41.86 ± 5.08 mg/g-VSS in the original microalgae to 264.05 ± 6.41 mg/g-VSS in the Add_L group at the end of 24-h batch test. PN content, especially PN/PS ratio in TB-EPS can significantly influence the formation of microalgae aggregate and its settleability, and the latter increased from 0.76 in the original microalgae suspension to 4.67 in microalgae granules in the Add_L group. More attention should be paid to aromatic proteins in LB-EPS and TB-EPS, due to the fact that their fluorescence intensities were dramatically strengthened after aggregates formation. That is,

aromatic proteins could play an important role in microalgae aggregation and granulation. Consequently, a novel startup strategy can be proposed for the rapid microalgae aggregates formation and biomass harvesting, which can be realized by adding the liquid fraction from the crushed mature microalgae granules. This novel strategy can greatly shorten the start-up time for settleability enhancement and then microalgae granulation from several months to 1-2 days, definitely and effectively avoiding microalgae biomass loss with 100% of microalgae harvested in 15 min after granulation achieved.

Table 3-1 Components of synthetic digestion liquor after air stripping for microalgae cultivation as well as aggregation test.

Parameters	Concentration in the liquor (mg/L)	Chemicals (concentration in the liquor)
COD	500	CH_3COONa (640 mg/L)
NH_4^+-N	80	NH_4Cl (303 mg/L)
PO_4^{3+}-P	50	KH_2PO_4 (219 mg/L)
Ca^{2+}	10	$CaCl_2 \cdot 2H_2O$ (41 mg/L)
Mg^{2+}	5	$MgSO_4 \cdot 7H_2O$ (51 mg/L)
Fe^{2+}	5	$FeSO_4 \cdot 7H_2O$ (25 mg/L)
Trace metals	1ml/L	H_3BO_3 (50mg/L), $ZnCl_2$ (50mg/L), $CuCl_2$ (30mg/L), $MnSO_4 \cdot H_2O$ (50mg/L), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50mg/L), $AlCl_3$ (50mg/L), $CoCl_2 \cdot 6H_2O$ (50mg/L), and $NiCl_2$ (50mg/L)

Table 3-2 Characteristics of initial microalgae suspension and functional fractions of mature algal granules in different groups.

Parameters	Suspended microalgae	Mature algal granules
Particle size	25~ 45 μm	2.25 ± 0.98 mm
Settling velocity (m/h)	~ 0	18.47 ± 0.23
Mixed liquor suspended solids (MLSS, g/L)	0.17 ± 0.02	1.10 ± 0.22
Mixed liquor volatile suspended solids (MLVSS, g/L)	0.14 ± 0.01	0.83 ± 0.16
MLVSS/MLSS ratio	82.81 ± 3.86%	75.6 ± 0.57%
Chlorophyll *a* (Chl-*a*, mg/g-VSS)	23.73 ± 0.64	1.69 ± 0.02
Loosely bound EPS (LB-EPS, mg/g-VSS)	2.83 ± 1.08	42.75 ± 3.19
Tightly bound EPS (TB-EPS, mg/g-VSS)	39.03 ± 3.99	193.46 ± 7.62

Table 3-3 Fluorescence spectra parameters of bound EPS (LB-EPS or TB-EPS) in response to liquid extraction from mature algal granules.

Groups		Peak	Ex/Em(nm)	Intensity	Probable composition
LB-EPS	Original	A	275/325	11.19	Microbial by-products
		C	360/455	3.317	Humic acid-like proteins
	Control	C	355/445	6.384	Humic acid-like proteins
		D	275/445	11.81	Humic acid-like proteins
		E	375/460	17.55	Humic acid-like proteins
	Add_L	A	280/335	102.2	Microbial by-products
		B	235/320	50.85	Aromatic proteins
		C	355/440	44.3	Humic acid-like proteins
		D	265/455	62.2	Humic acid-like proteins
TB-EPS	Original	B	225/300	46.38	Aromatic proteins
		A	280/355	16.87	Microbial by-products
	Control	B	225/300	134.1	Aromatic proteins
		A	280/340	32.48	Microbial by-products
	Add_L	B	225/300	666.7	Aromatic proteins
		A	280/345	146.8	Microbial by-products

Table 3-4 Fluorescence spectra parameters of bound EPS in Add_{LF}.

		Peaks	Ex/Em(nm)	Intensity	Probably composition
Add_{LF}	LB-EPS	A	280/335	84.92	Microbial by-products
		B	235/320	71.2	Aromatic proteins
		C	355/440	44.3	Humic acid-like proteins
		D	265/455	68.07	Humic acid-like proteins
	TB-EPS	A	280/345	75.1	Microbial by-products
		B	225/300	502.3	Aromatic proteins

Table 3-5 Zeta potential of the sampled microalgae from different groups before and after the 24-h test (unit: mV).

	Control	Add_{IG}	Add_{CG}	Add_{S}	Add_{L}
Initial	-23.5 ± 1.42	-26.47 ± 3.66	-25.2 ± 1.51	-22.03 ± 1.31	-23.23 ± 1.40
Final	-16.0 ± 1.41	-21.8 ± 0.78	-19.63 ± 2.15	-19.67 ± 0.89	-10.26 ± 1.88

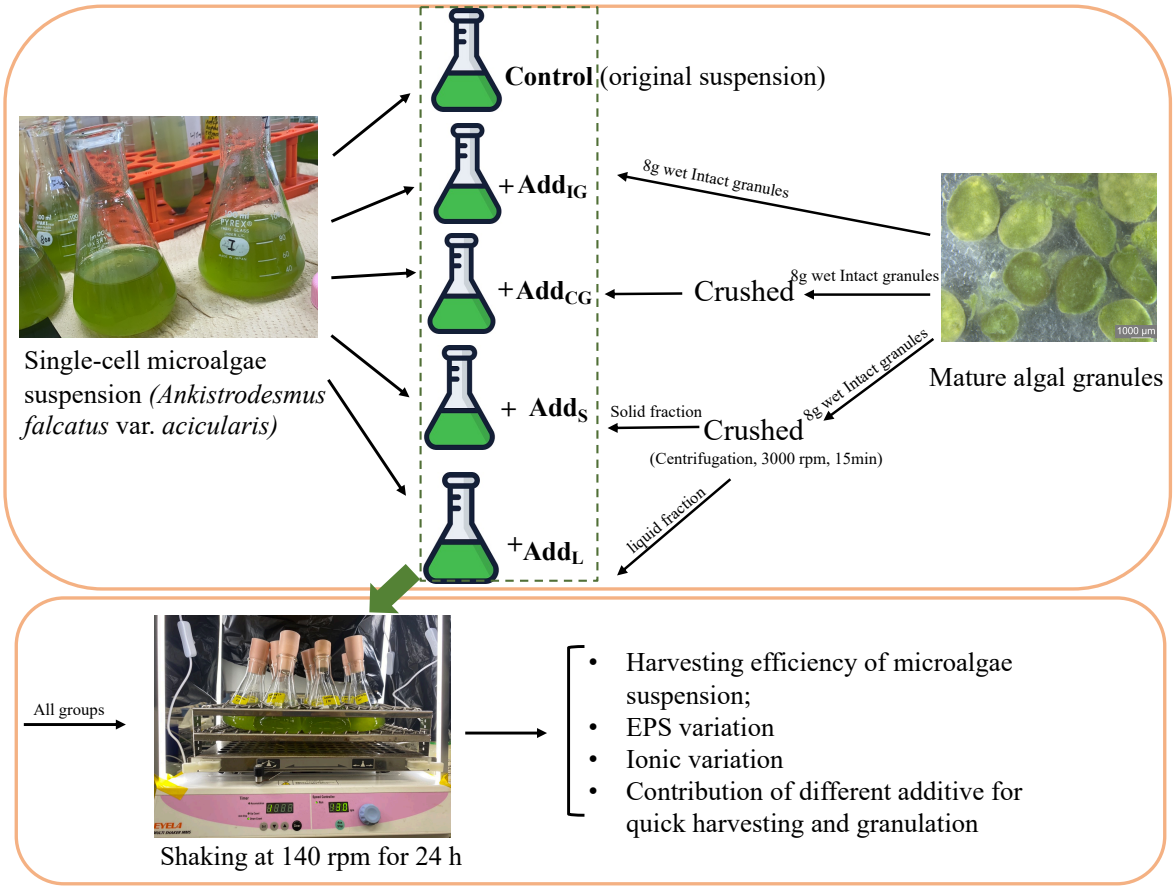


Figure 3-1 Experimental framework. EPS, extracellular polymeric substances. The lower letters right after Add are as follows: IG, intact granules; CG, crushed granules; S, solid fraction of the crushed granules; L, liquid fraction of the crushed granules.

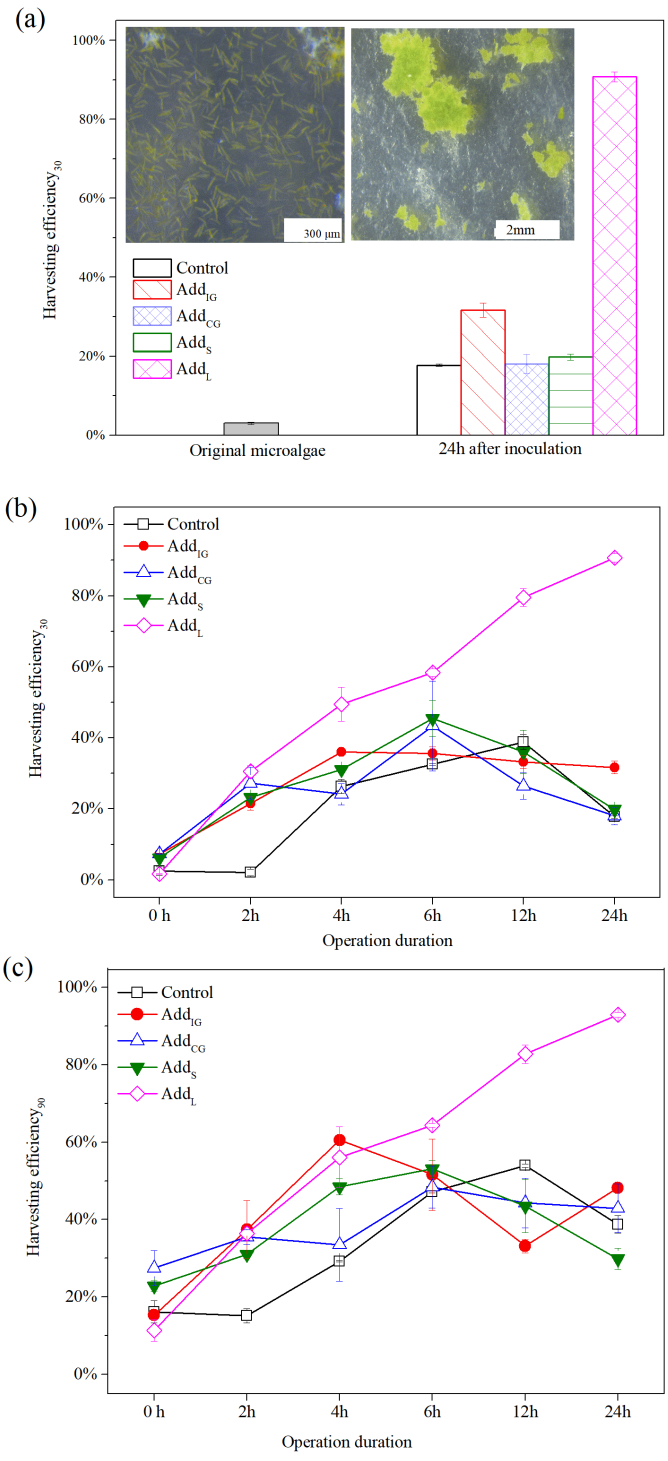


Figure 3-2 Changes in harvesting efficiency of *Ankistrodesmus falcatus* var. *acicularis* (NIES-2195) after the inoculation of different fractions from mature microalgae granules. (a) Harvesting efficiency in 30 min (main plot, in %) and microscopic images (inset plot) before and after inoculation, (b) variation of settleability in 30 min (harvesting efficiency$_{30}$), and (c) variation of settleability in 90 min (harvesting efficiency$_{90}$).

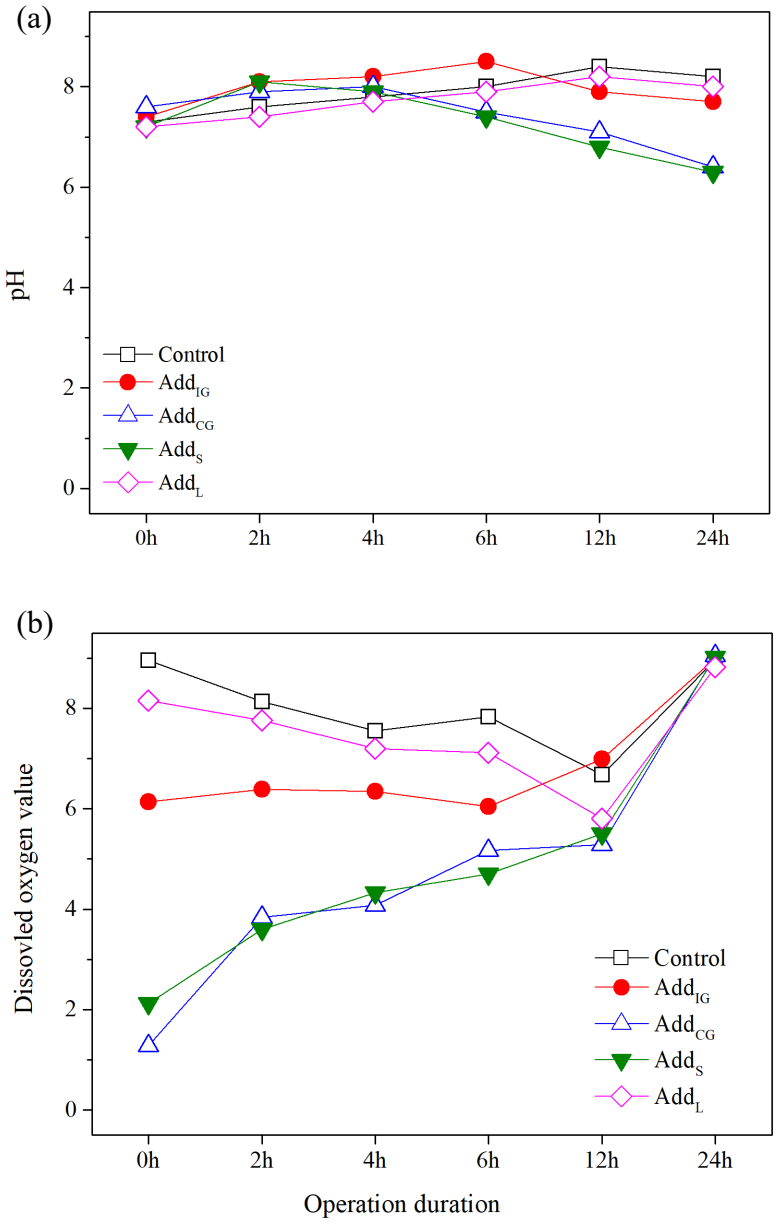


Figure 3-3 Variation of pH and dissolved oxygen (DO) variation along with the batch test.

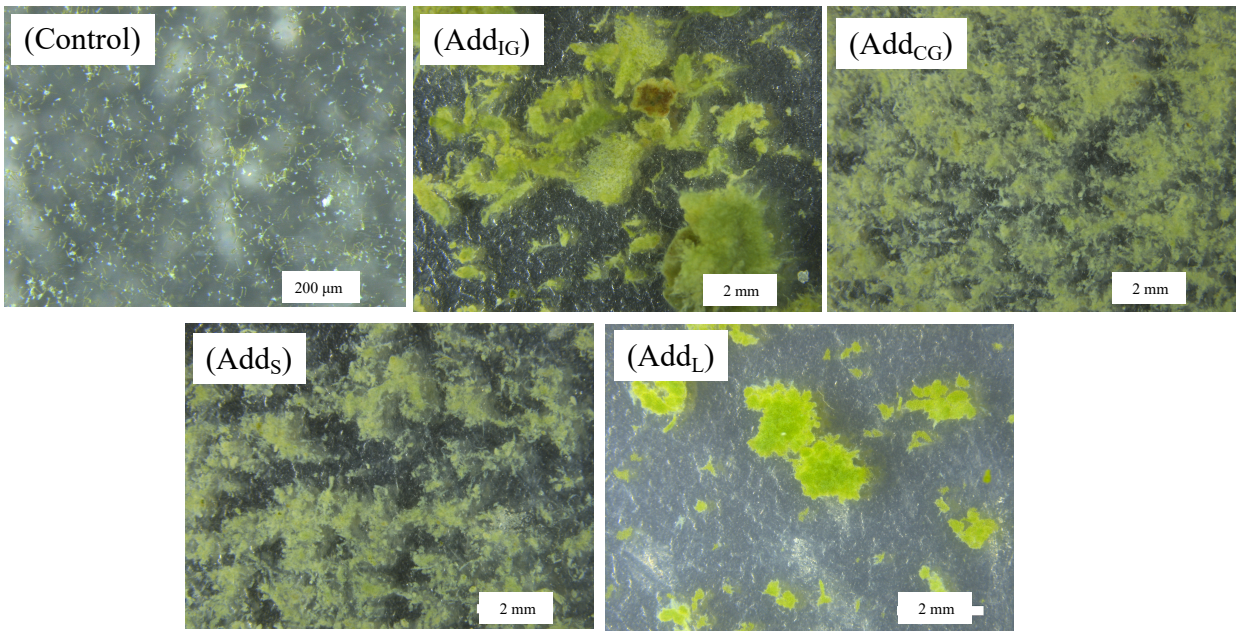


Figure 3-4 Microscopic observations of *A. falcatus* var. *acicularis* suspension after addition of the different additives for 24 h.

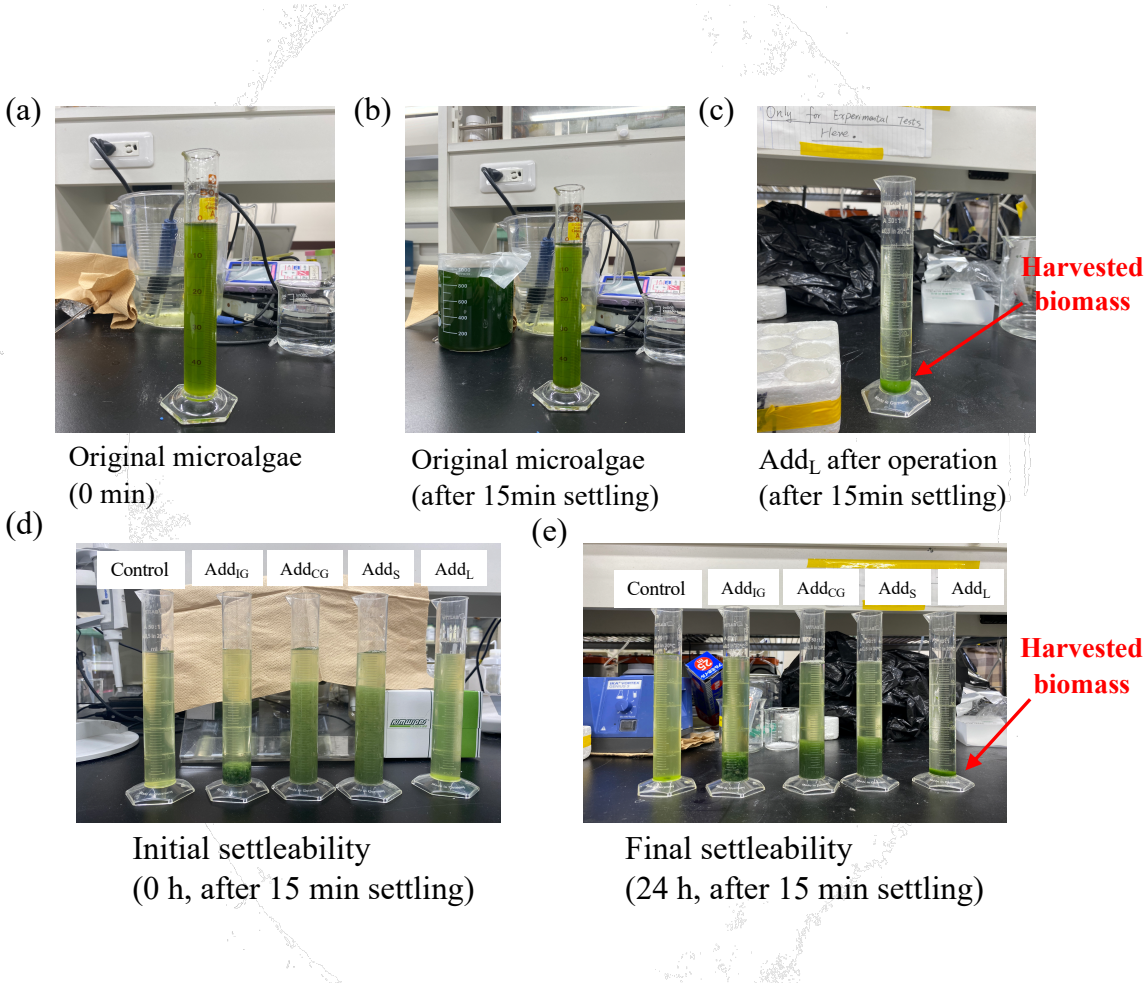


Figure 3-5 Settling tests in measuring cylinders.

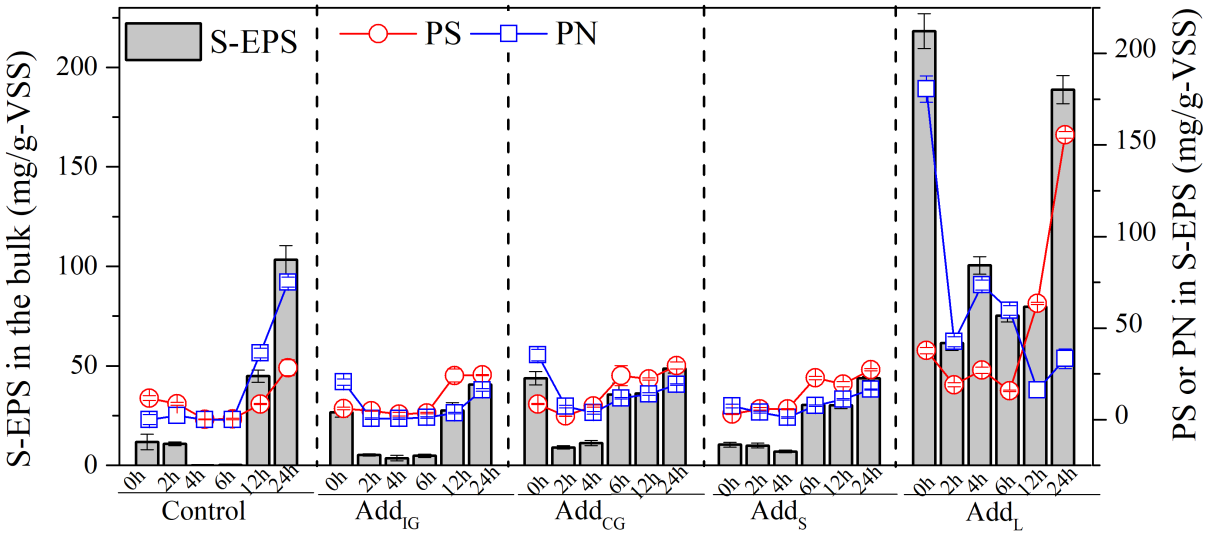


Figure 3-6 Variation of content and composition in soluble extracellular polymeric substances (S-EPS) during the 24-h batch test.

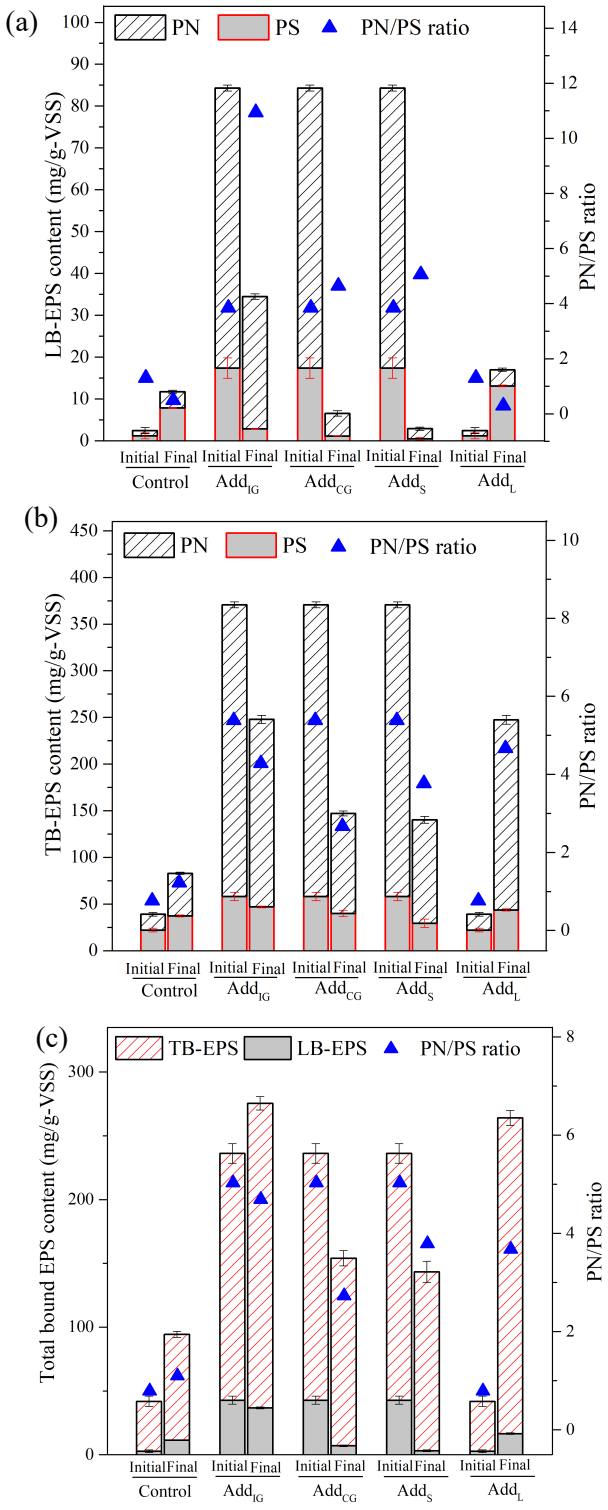


Figure 3-7 Bound EPS contents and its major components, proteins (PN) and polysaccharides (PS) contents, and PN/PS ratio of loosely bound EPS (LB-EPS) (a) and tightly bound EPS (TB-EPS) (b) and the total bound EPS (c) before and after batch test.

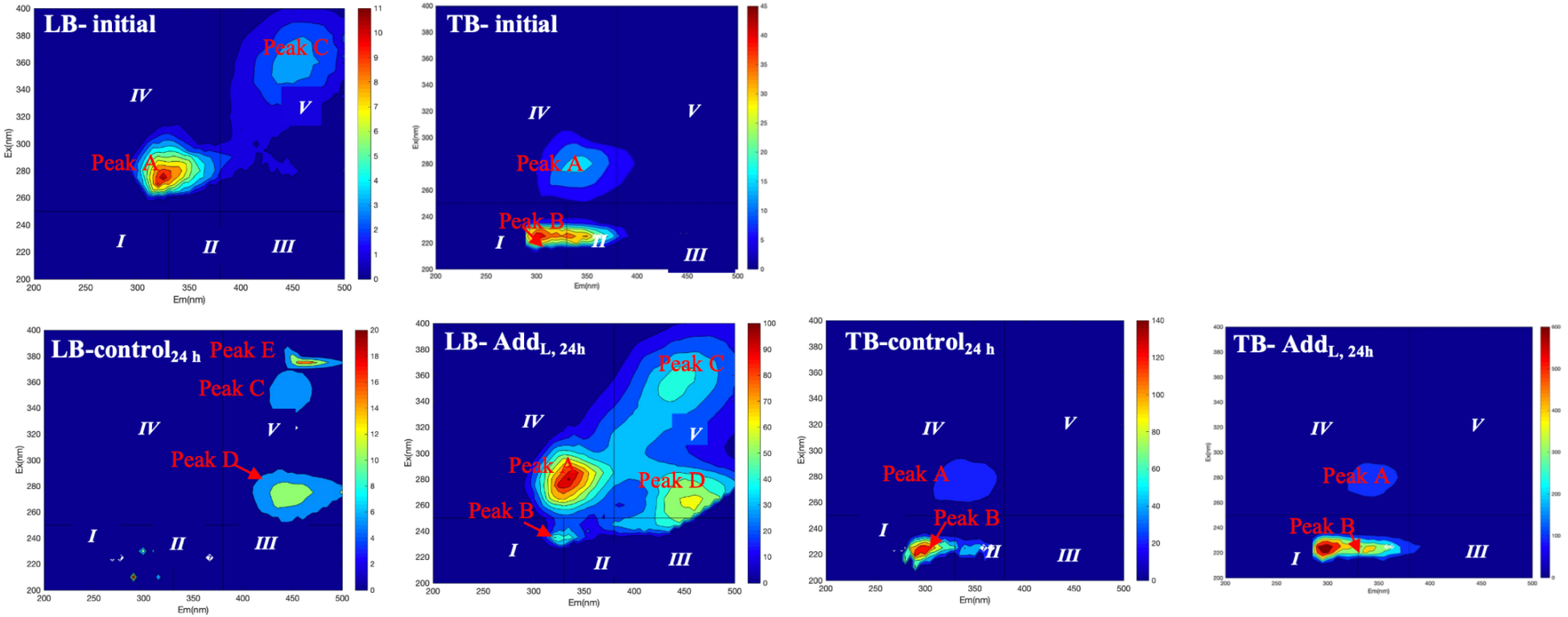


Figure 3-8 Variation of 3D-EEM fluorescence spectra in bound EPS in the control and Add_L groups (Add_L, the liquid fraction of the crushed mature microalgae granules; Initial, initial microalgal suspension).

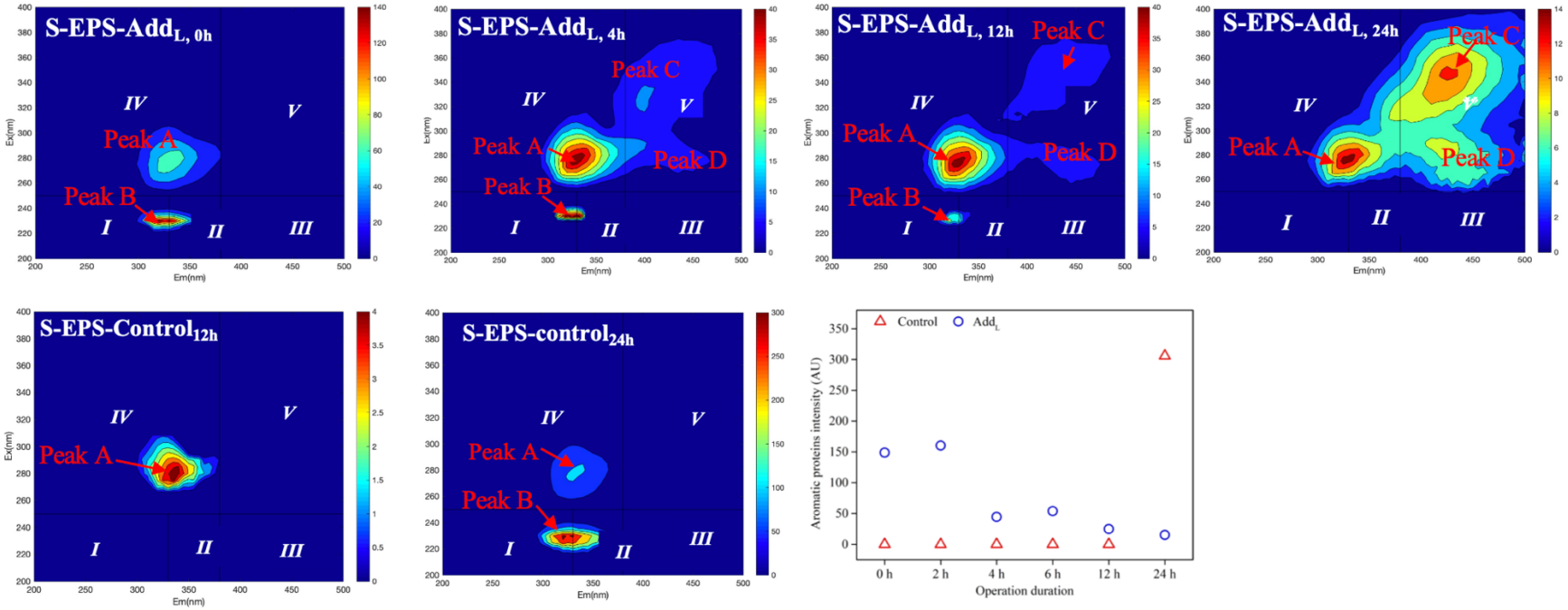


Figure 3-9 Variation of 3D-EEM fluorescence spectra and fluorescence intensity of aromatic proteins (Peak B) in soluble EPS (S-EPS) in the control and Add_L groups.

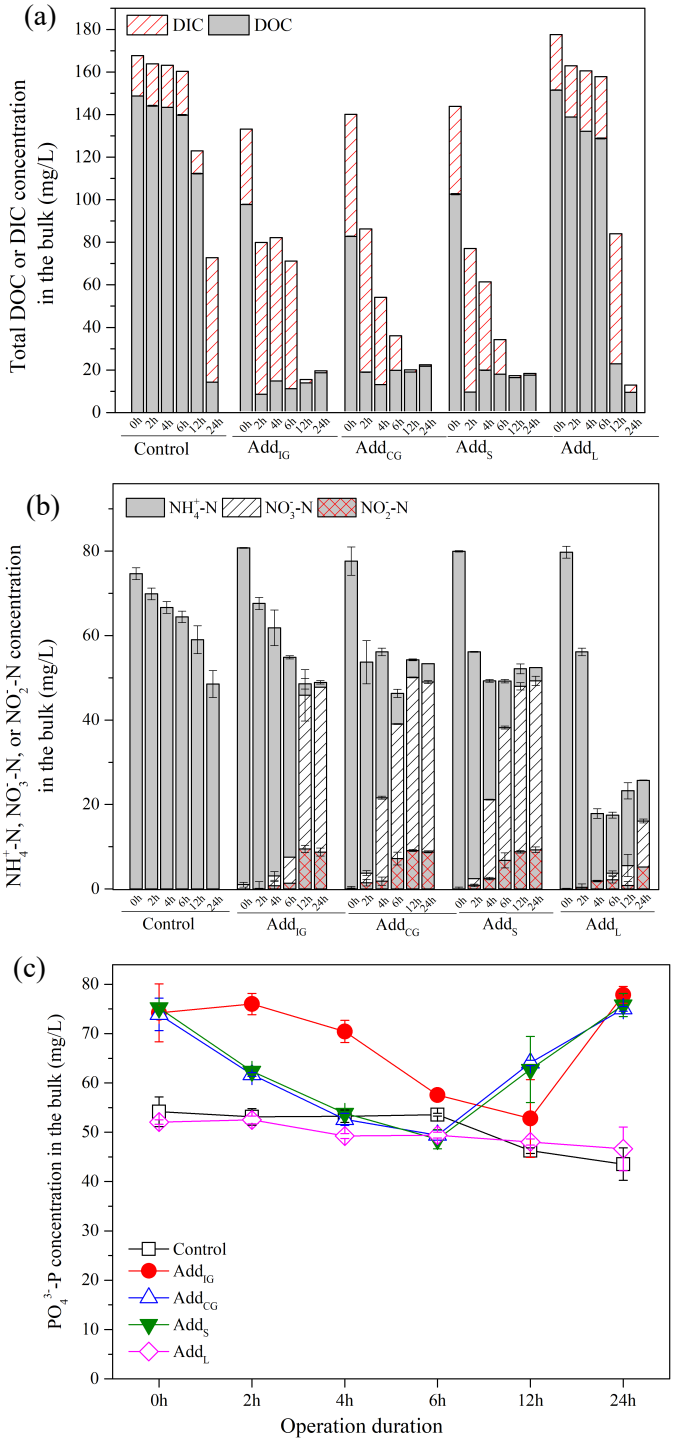


Figure 3-10 Profiles of nutrients concentration during the 24-h batch test. (a) Total dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC); (b) Ammonia nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N) and nitrite nitrogen (NO_2^--N); and (c) phosphate phosphorus (PO_4^{3-}-P).

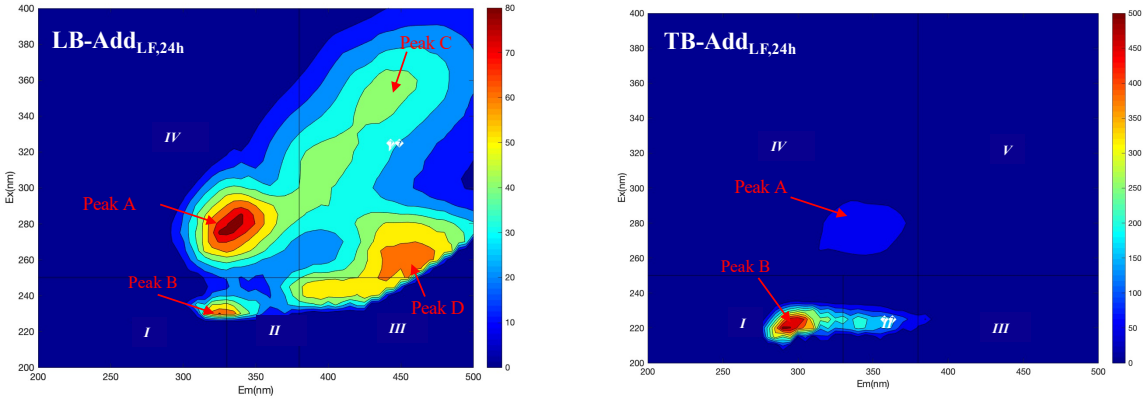


Figure 3-11 3D-EEM fluorescence spectra in bound EPS in the Add_{LF} group (sterilized liquid fraction) after the test.

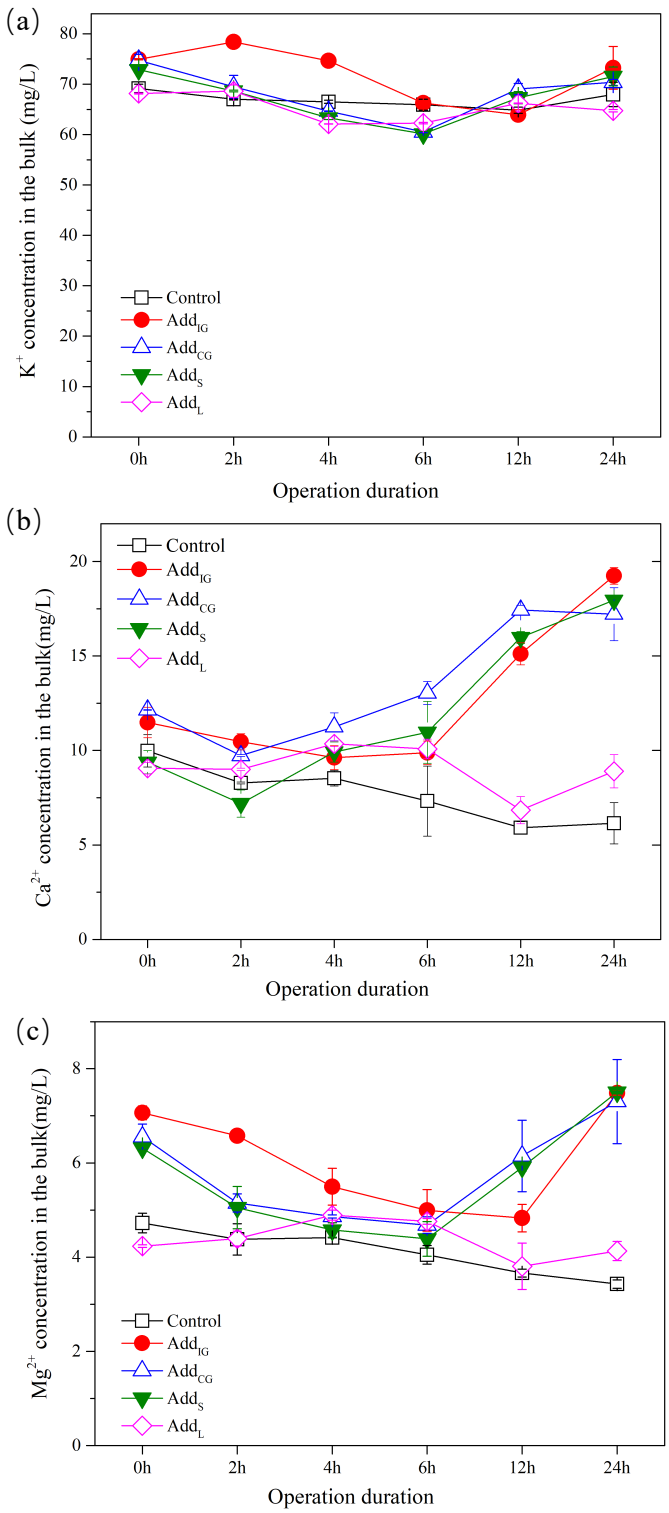


Figure 3-12 Variations of cations, i.e., K^+ (a), Ca^{2+}(b), and Mg^{2+} (c) concentrations in the bulk during the 24-h batch test.

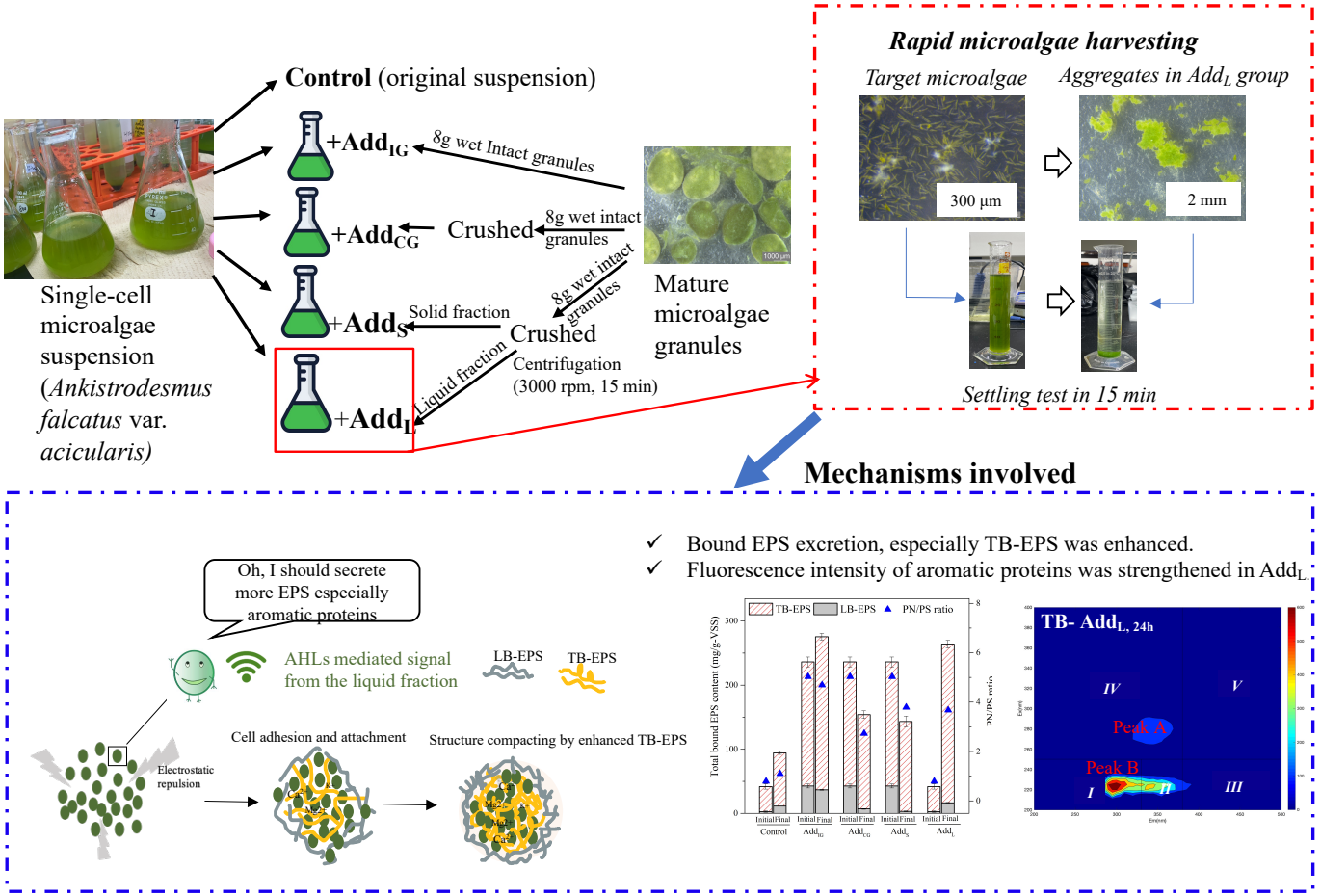


Figure 3-13 Graphical abstract of this part and mechanisms involved.

Chapter 4 Comparison between biogranulation and suspended systems towards practical application

4.1 Background

As discussed by Zhuang et al. (2020), non-suspended microalgae cultivation such as immobilization cultivation through addition of alginate or chitosan, or attached cultivation on membrane can accelerate the application of microalgae biomass production from wastewater treatment, which can achieve higher biomass productivity and footprint reduction with simplified biomass separation from the treated wastewater. Still, the issues relating to high operation cost (mainly from materials addition), residual polymeric materials added to biomass and worsen stability after long-time operation limit their large-scale applications. A previous work in Chapter 2 shows that biogranulation may potentially be utilized for simultaneous microalgae cultivation and harvesting in addition to wastewater treatment. However, up to now, little information is available on the comparison of biomass accumulation, nutrients removal and energy efficiency between the novel biogranulation and conventional suspended cultivation systems under the same experimental conditions. Thus, this research aimed for above parameters analysis and the results of this chapter could provide information of biogranulation in practical application.

4.2 Materials and methods

4.2.1 Synthetic digestion liquor and seed microalgae

It was same as described in Section 2.2.1.

4.2.2 Biogranulation system

Mature algal granules were obtained from the previous study in Chapter 2 and operation strategies can refer to section 2.2.2. Energy consumption was estimated and compared with the suspended cultivation. During the stable operation, sludge retention time (SRT) was controlled around 15 days through regular discharge of granules.

4.2.3 Suspended cultivation system

As for the batch-mode suspended systems, the seed microalgae after pre-cultivation were inoculated to 300 mL Erlenmeyer flasks containing 200 mL synthetic digestion liquor on a shaker (EYELA multi shaker MMS 210) at 180 rpm. Light illuminance and room temperature were the same as the two SBRs in section 2.2, 146-157 μmol/m^2·s for 24 hours at the top and 25 ± 2 °C, respectively. The initial mixed liquor suspended solids (MLSS) of microalgae was 0.33 ± 0.03 g/L, at which the microalgae exhibited the highest growth rate according to the

preliminary tests. The whole test with twenty flasks operated in parallel lasted about 16 days till the stationery phase under same conditions without exchange of synthetic wastewater.

4.2.4 Analytical methods and calculations

Every two days all 200 mL culture samples from two parallel flasks were used for quantification of biomass (dry weight (DW), chlorophyll *a* (Chl-*a*), optical density (OD) and heat value) and determination of water quality. The same sampling interval was done to the SBRs. Before heat value determination, homogenous liquors were sampled from both flasks and SBRs at the end of operation and dried to constant weight; and the residues were used for heat analysis by Auto-Calculating Bomb Calorimeter (CA-4AJ, Shimadzu, Japan).

As for the determinations of other parameters including mixed liquor (volatile) suspended solids (ML(V)SS), Chl-*a*, granule size and distribution, and water quality such as carbon (dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), dissolved total carbon (DTC)), nitrogen (NH_4^+-N, NO_2^--N, NO_3^--N, total nitrogen (TN)), and phosphorus (PO_4^{3-}-P, total phosphorus (TP)) have been detailed described in section 2.2. And nutrient removal capacity was calculated according to Eq.4-1 modified from Nguyen et al. (2020).

$$\text{Nutrient removal capacity (mg/g-biomass/day)} = \frac{(C-C0)}{(X-X0)\Delta t} \quad (4\text{-}1)$$

where X_0 and X are the initial and final biomass concentration (g/L, VSS basis), respectively; C_0 and C are initial and final concentration (mg/L) of specific nutrient, respectively; Δt is the operation duration (day), i.e. from the initial time to the date when the total biomass reaches the steady phase in the suspended cultivation. Volumetric biomass productivity ($g/m^3/d$) was calculated according to Eq. 4-2.

$$\text{Volumetric biomass productivity (g/m}^3\text{/d)} = 1000\times (DW_2\text{-}DW_1)/(t_2\text{-}t_1) \quad (4\text{-}2)$$

where DW_1 and DW_2 are the dry biomass concentration (g/L, constant at 105 ± 2°C) at two sequential sampling time t_1 and t_2, respectively; and t_2-t_1 (d) is the interval time between the two samplings.

Energy consumption was estimated from the electricity input for per kilogram algae output or per gram nutrient removed, mainly from artificial light, shaker and centrifuge for the suspended system, and artificial light, air pump, influent and effluent pumps for the biogranulation system, respectively. The energy consumption by each device was calculated as the total operation time multiplied by energy consumption rate mentioned in the device specification sheet.

4.3 Results and discussion

4.3.1 Biomass accumulation

In the suspended system, two peak biomass concentrations appeared in the suspended system, 0.75 ± 0.04 g/L and 0.82 ± 0.18 g/L of DW on days 6 and 12, respectively (Figure 3-1a), in agreement with previous findings (Lananan et al., 2016; Salim et al., 2011). This observation might be attributable to the fact that the nutrients released from dead cells were re-utilized by alive cells of microalgae for growth when nutrients were exhausted, which can be partially evidenced by the increase in Chl-*a* concentration in the system after day 8 (Figure 3-1b). In this study, some heterotrophic bacteria co-existing in the system after pre-cultivation might compete with the target microalgae for nutrients capture. At the early phase, bacteria can utilize abundant organic carbon in the bulk for quicker biomass accumulation. To some extent, the fluctuation of Chl-*a* content can reflect the variations of microalgae to bacteria ratio, and microalgae have better performance for reproduction under organic carbon source deficient condition. Results show that, the maximum specific growth rate (μ_{max}, d^{-1}) in this study, 0.32 d^{-1}(according to the changes in dry biomass weight during cultivation), is lower than 0.43 ± 0.04 d^{-1} obtained under mixotrophic cultivation condition with 1.0 g/L glucose as carbon source ((Mansa et al., 2018), likely due to the different nutrients composition and cultivation strategy adopted.

As for the biogranulation system, starting from the same initial inoculated biomass of 0.33 g/L, the biomass concentration was maintained at 3.37 ± 0.18 g/L by discharging granules regularly after mature granules being formed. During the followed-up stable operation, the highest specific growth rate was 0.12 ± 0.04 d^{-1}, lower than the cell-free suspended cultivation method. This result is similar to *Scenedesmus obliquus* (0.157 d^{-1}) using alginate immobilization (Ruiz-Marin et al., 2010), likely due to microalgal self-shading effect for light and nutrients uptake because of the high biomass concentration in the system. On the other hand, a remarkably higher and stable Chl-*a* concentration (17.39 ± 0.31 mg/L) was detected in this system compared to the suspended conditions (12.08 ± 0.14 mg/L) (Figure 4-1b), reflecting more and stable growth of microalgae in the tested granule system. However, due to the more accumulated granular biomass, the relatively stable Chl-*a* content (7.73 ± 0.53mg/g-VSS) was lower than that in the suspended system (17.85 ± 2.32 mg/g-VSS). Probably bacterial growth was favored, and more heterotrophic bacteria were aggregated with the target microalgae in the open biogranulation system under the test condition process. Also, the decline of light penetration caused by self-shading due to cells self-aggregation will be another important

reason for lower Chl-*a* content in granular system. Interestingly, unlike the biomass in the suspended system, mature algal granules contained relatively stable Chl-*a* content (Figure 4-1b), suggesting the potential for stable nutrients removal and easier management if being applied for wastewater treatment in practice.

4.3.2 Nutrients removal

Both cultivation systems were fed with the same synthetic digestion liquor and operated under the same environmental conditions. Results were showed on Figure 4-2. The biogranulation system kept at stable nutrients removal, with effluent DOC, PO_4^{3-}-P, NH_4^+- N concentrations averagely being 4.86 ± 0.95 mg/L, 43.45 ± 0.47 mg/L and 14.85 ± 1.35mg/L at the end of 6-h cycle, respectively. As for the suspended system, the DOC, PO_4^{3-}-P and NH_4^+-N concentrations in the bulk were 34.93 ± 1.98 mg/L, 28.76 ± 0.74 mg/L and 0.96 ± 0.02 mg/L, respectively after reaching the highest biomass concentration. In order to minimize the influence of different biomass concentrations in the two systems on nutrients removal performance evaluation, removal capacity was calculated and compared between the two systems (Table 4-1). Results show that the consortia in the biogranulation system exhibited much better performance for nutrients removal in terms of removal capacity, especially on organics and nitrogen. While not so much difference in P removal capacity was noticed in the two systems, about 4.04 ± 0.50 mg/g-VSS/day in the biogranulation and 5.73 ± 0.13 mg/g-VSS/day in the suspended systems, attributable to the operation strategies and biomass retention time adopted.

4.3.3 Energy production and consumption

In this study, heat value was measured to represent energy production capability of the obtained biomass from the two cultivation systems (Table 4-1). As seen, the heat values of biomass from the two systems were comparable, 19.29 ± 0.34 MJ/kg in the suspended system and 15.37 ± 0.52 MJ/kg in the biogranulation system, suggesting the microalgae cells in granules are basically suitable for biofuel production even though some decrease in microalgae content (indicated by lower Chl-*a* content) in the biomass occurs due to self-shading effect in the granules. When taking the biomass productivity into consideration, the volumetric energy production in the biogranulation system (3.44 ± 0.29 MJ/m^3/day) was increased by 330% compared to the suspended one (0.81 ± 0.06 MJ/m^3/day), mainly due to the former had 440% higher biomass productivity (223.17 ± 11.82 g/m^3/day) than the latter (41.57 ± 2.08 g/m^3/day) during the treatment of the same digestion liquor.

As lipid extraction and transesterification are already mature technologies with relatively high efficiencies of 90-95% (Harun et al., 2011; Shi et al., 2019), in this study only cultivation and harvesting were considered for energy consumption analysis. Centrifugation was chosen for biomass separation from broth in the suspended system as it is widely accepted as the fastest and efficient method for microalgae harvesting (Grima et al., 2003). The energy consumption by the biogranulation system, about 2.19×10^3 kWh/kg-algae output, is 58% lower than the suspended system (5.18×10^3 kWh/kg-algae output), due to the former can realize complete harvesting within 2 min through gravity sedimentation with no energy or materials input. When the energy consumption was calculated based on unit nutrient removed, much lower energy consumptions were also obtained by the biogranulation system, about 18.36, 1.43, 2.22 and 0.67 kWh for removing per gram TP, NH_4^+-N, TN and DOC, in comparison to 199.98, 42.89, 64.12 and 27.84 kWh by the suspended system, respectively.

4.3.4 Implication of this study

Conventional microalgae-based wastewater treatment requires a large footprint due to the lower biomass density and poor settling property, thus additional land is demanded for thickening and dewatering in applicated scale (Barros et al., 2015; Yin et al., 2020). Owing to the denser biological structure and outstanding settling property of microalgae granules, use of biogranulation can remarkably reduce the footprint for cultivation ponds when compared to the suspended system. Seen from Table 3-1, the highest biomass concentration in the biogranulation system can be increased by 300%, about 3.35 ± 0.18 kg/m^3 versus 0.82 ± 0.02 kg/m^3 in the suspended one. This means, 76% reduction in land area requirement can be achieved for producing the same amount of biomass that could be separated through only 2 min gravity sedimentation. In addition, the biogranulation process can be operated in open systems dominated by the inoculated strain (~97%) if SRT being controlled within 25 days. Still, further works are necessary for practical application, including acceleration of granule formation and optimization of operational/environmental parameters like HRT, SRT and light illumination to realize its highly efficient and stable operation.

4.4 Summary

Final results were concluded in Figure 4-3. This study compared biogranulation and conventional suspended cultivation systems on microalgal biomass accumulation, nutrients removal capability and energy production/consumption. Use of biogranulation can achieve 440% increase in volumetric biomass productivity and 330% increase in volumetric biomass energy production while with 58% less energy consumption for per unit biomass production and 76%

less footprint when compared to the suspended system in addition to stable organics and nutrients removal. This novel biogranulation process may be further developed as a practical and sustainable approach for biofuel production from wastewater treatment.

Table 4-1 Comparison of biomass production, nutrients removal performance and energy efficiency between the suspended cultivation and biogranulation systems.

System	Biomass accumulation			Energy production		Nutrients removal capacity				Energy consumption				
	Highest biomass conc. (kg/m^3)	Volumetric biomass productivity ($g/m^3/day$)	Chl-*a* content (mg/g-VSS)	Heat value (MJ/kg-biomass)	Volumetric energy production ($MJ/m^{3}/day$)	NH_4-N	TP	TN	TOC	Biomass based (KWh/kg-algae output)	Nutrients removal based (KWh/g-$nutrient_{removed}$)			
						(g-$nutrient_{removed}$/kg-VSS/day)					TP	NH_4-N	TN	DOC
Suspended	0.82 ± 0.02	41.57 ± 2.08	17.85 ± 2.32	19.29 ± 0.34	0.81 ± 0.06	18.84	4.04	12.60	29.02	5.18×10^3	199.98	42.89	64.12	27.84
Biogranulation	3.35 ± 0.18	223.17 ± 11.82	7.73 ± 0.53	15.37 ± 0.52	3.44 ± 0.29	73.40	5.73	47.38	157.45	2.19×10^3	18.36	1.43	2.22	0.67

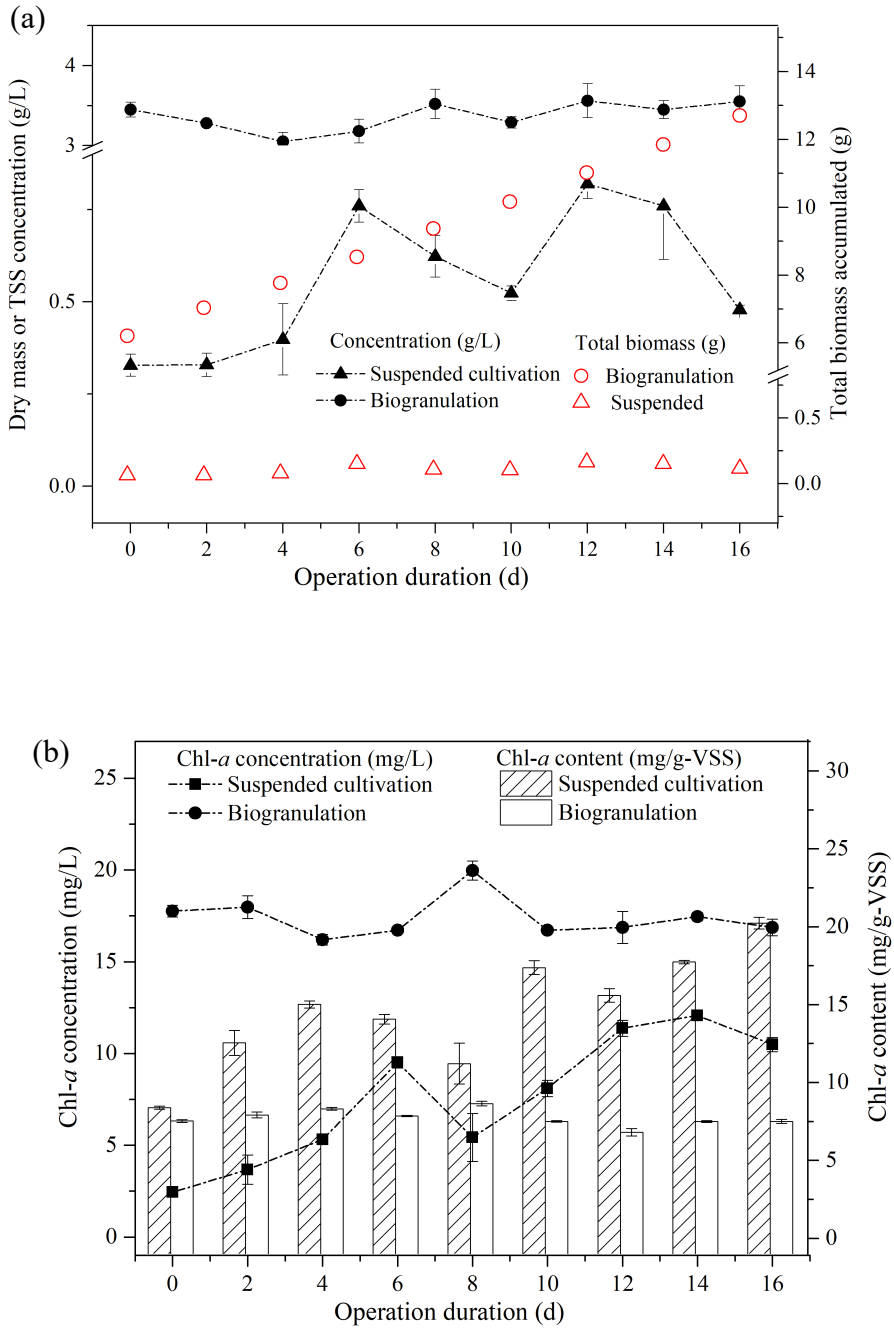


Figure 4-1 Variations of dry mass or total suspended solids (TSS, (a)) and Chl-*a* content or concentration (b) in the suspended cultivation and biogranulation systems.

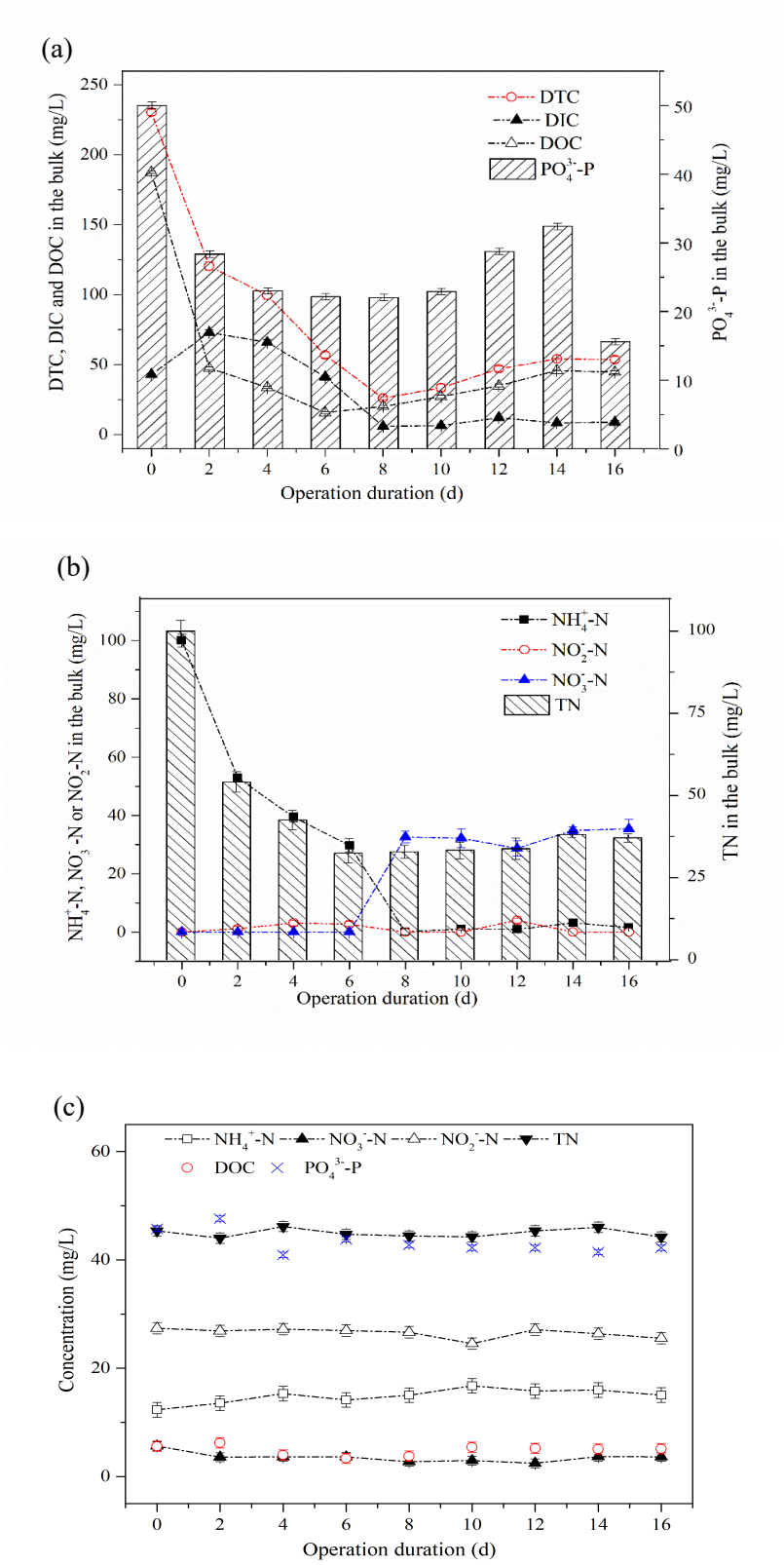


Figure 4-2 Nutrients concentration in the bulk during the suspended cultivation (a,b) or effluent nutrients concentration from the biogranulation system during operation (c). DOC, dissolved organic carbon; DIC, dissolved inorganic carbon; DTC, dissolved total carbon; NH_4^+-N, ammonia nitrogen; NO_2^- -N, nitrite nitrogen; NO_3^- -N, nitrate nitrogen; TN, total nitrogen; PO_4^{3-}-P, phosphate phosphorus.

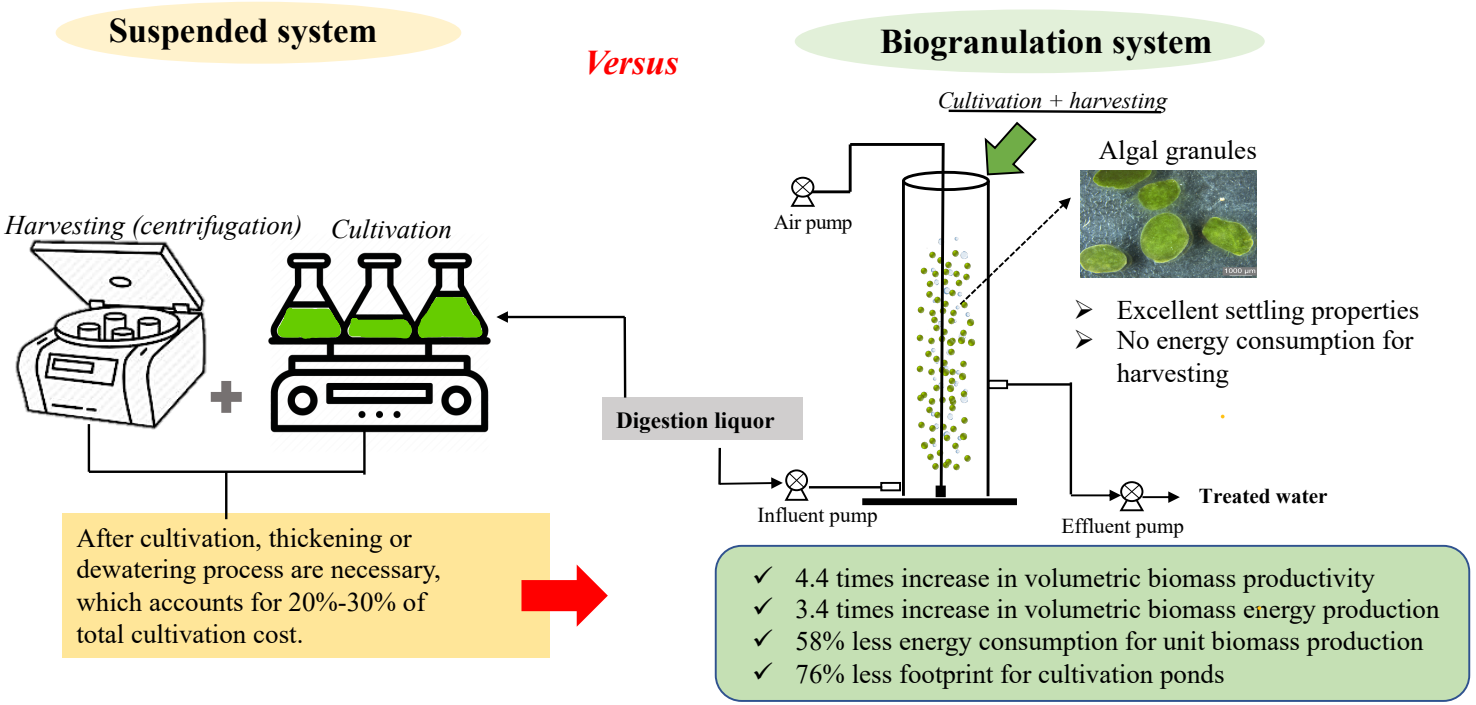


Figure 4-3 Graphical abstract and main results obtained.

Chapter 5 Conclusions and future works

5.1 Conclusions

5.1.1 Successful biogranulation of single cell microalgae for quicker harvesting

Mature algal granules were obtained after 150 days' operation with single cell oil-producing microalgae, *Ankistrodesmus falcatus* var. *acicularis* as the only inoculum, in lab-scale SBRs. The settling capability remarkably improved indicated by substantial decrease of SVI_{30} and increase of settling velocity. Simultaneously, excellent performance was observed along biogranulation process, achieving averagely 96% and 86% removal rate of DOC and NH_4^+-N from the digestion liquor. Higher biomass accumulated in reactors around 2-4 g/L in stable phase with SRT controlled in 15 days. These results provided evidence for feasibility of algal biogranulation for single cell microalgae with dual targets of biomass harvesting and wastewater remediation.

5.1.2 Novel biogranulation with lower energy and materials consumption in biomass harvesting

As summarized in Table 2-3, compared to conventional harvesting methods (centrifugation, membrane filtration, electrocoagulation, chitosan flocculation and ultrasonic), this novel biogranulation system showed irreplaceable advantages with lower energy and materials consumption as microalgae granules can be harvested by gravity sedimentation easily within 2 min without energy consumption, and only wastewater (digestion liquor in this study) is fed with no other chemicals or materials addition. Moreover, the harvesting efficiency approximately 100% is comparable to centrifugation which is considered as the most efficient method for microalgae biomass harvesting with high electricity consumption about 10.8 MJ/kg-algae output. Thus, the novel biogranulation can definitely compete against all the other algae harvesting technologies in large scale applications.

5.1.3 Novel biogranulation system with higher biomass accumulation, energy production and nutrients removal capability in microalgae cultivation process

The highest biomass concentration in the biogranulation system can be increased by 300%, about 3.35 ± 0.18 kg/m^3 versus 0.82 ± 0.02 kg/m^3 in the suspended one. This means, 76% reduction in land area requirement can be achieved for producing the same amount of biomass that could be separated through only 2 min gravity sedimentation. Moreover, the volumetric energy production in the biogranulation system (3.44 ± 0.29 MJ/m^3/day) was increased by 330% compared to the suspended one (0.81 ± 0.06 MJ/m^3/day). While considering nutrients removal

capability from wastewater, the consortia in the biogranulation system exhibited much better performance on nutrients especially on organics and nitrogen removal, which were 157.45 and 47.38 g-nutrient$_{removed}$/kg-VSS/day over 29.02 and 12.60 in the suspended systems. These results suggest that this novel biogranulation process may be further developed as a practical and sustainable approach for biofuel production from wastewater treatment.

5.1.4 Quicker microalgae aggregation/granulation via addition of liquid fraction from mature algal granules

Under stimulation of liquid extract, more EPS especially LB-EPS and TB-EPS were secreted by microalgae which might be regulated by AHL-mediated QS. Moreover, aromatic proteins enhanced in bound-EPS and benefited cells adhesion or cell to cell bridging, resulting to hydrophobicity improvement and then aggregates formation. This work helped to understand algal granule formation and provide solutions for long start-up of biogranulation system.

Above all, this research is expected to promote the novel cultivation and harvesting method through biogranulation, which may help solve bottlenecks existed in microalgal-based technologies.

5.2 Future works

Future works will be focused on the following aspects:

(1) Wider application of this novel biogranulation method for other microalgae species will be conducted to explore its potential for general utilization.
(2) Experiments will be designed for further investigation on AHLs production and regulation in algal granule systems.
(3) The spatial distribution of PS, PN and microorganisms in algal granules should be explored through confocal laser scanning microscopy, to provide information for mechanisms involved.
(4) The attribution of humic acid-like proteins or microbial by-product substances will be further investigated during algal granulation.

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