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CHLORELLA VULGARIS AUTO-FLOCCULATION IN WASTEWATER TREATMENT. PREFACE TO GRANULATION

Microalgae wastewater treatment technology has not only the function of wastewater treatment but also biomass production, resource recovery, and biological carbon fixation with significant economic and environmental benefits. Good sedimentation of microalgae cells is the key to realize wastewater treatment and microalgae cell proliferation. In this study, short settling time in sequence batch reactors (SBRs) was utilizable as an environmental selection pressure to promote the auto-flocculation of *Chlorella vulgaris* treating synthetic domestic wastewater. After 60 days of operation, bacteria-microalgae consortia formed in the reactors, improving the settling efficiencies. Microalgae cultivation reactor with 30 min settling time had the largest flocs size and highest settling efficiency. Bacteria-microalgae granular sludge had a relatively high content of P, Fe, Mg, and Ca elements that both bacteria and microalgae coexisted and adhered to each other. The dominant bacteria distribution of bacteria-microalgae granular sludge was like that of aerobic granular sludge, which implied bacteria played a vital role in *Chlorella vulgaris* auto-flocculation. Lastly, the mechanism of *Chlorella vulgaris* auto-flocculation in wastewater treatment was interpreted.

1. INTRODUCTION

Wastewater treatment by microalgae uses algal cells as the main body to metabolize and utilize C, N, P, and other substances in wastewater [1]. The microalgae cells can be used for biomass energy production, industrial raw materials to extract algal protein, microbial flocculant, etc. The absorption of CO_2 by the microalgae photosynthesis process is also considered a possible way to achieve carbon fixation in the atmosphere [2]. Many studies have successfully shown the efficiency of microalgae for treatment and resource recovery via biomass production and valorization. Using microalgae for waste-

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water treatment can be carried out at a large scale in closed systems (photobioreactors) or open systems (raceway ponds) and be suitable for various sewage [1, 2].

The main bottlenecks are difficulties in separation and harvest, low density of suspended growth cells and limited resource recovery value [3, 4]. Microalgae granulation is one of the possible ways to solve this problem [5]. As to the activated sludge process, aerobic granulation has become a mature technology for municipal wastewater treatment [6]. Microalgae granulation could increase the cell density and effectively improve the rapid settling velocity to overcome the difficulty in separation and harvest. However, the reports of microalgae granulation at present are numbered. Tiron et al. [7, 8] first showed that microalgae filaments developed a dense, stable, and granular biological matrix for the pressure of stirring force. A high density of the biomass within the granules' structure (with 80–300 μg dry weight/granular) and large granules' sizes (500 –3000 μm) ensured a high settling velocity of the granules (18–29 m/h). Regardless, the granulation mechanism still needs future research for prominent clarification. In this study, settling time was used to promote the microalgae auto-flocculation as an environmental selection pressure. Although, the settling efficiency, particle size distribution, inorganic elements content and microbial community structure were interrogated for the determination of the microalgae auto-flocculation mechanism.

2. MATERIALS AND METHODS

Microalgae strain, medium, and maintenance. Chlorella vulgaris (FACHB-8) was used in this study and bought from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-collection). The strains grew on the agar medium in test tubes and were kept at 10 °C in an illuminating incubator for standby.

Wastewater source. CH_3COONa, NH_4Cl, and K_2HPO_4 were used to make up the synthetic domestic wastewater, with chemical oxygen demand (COD) of 250 mg/dm^3, ammonia (NH_4^+-N) of 60 mg/dm^3, and total phosphorus (TP) of 9 mg/dm^3. Other trace chemicals such as 30 mg/dm^3 of $CaCl_2 \cdot 2H_2O$, 25 mg/dm^3 of $MgSO_4 \cdot 7H_2O$, and 20 mg/dm^3 of $FeSO_4 \cdot 7H_2O$ were contained in wastewater.

Experimental design. 50 cm^3 of standby microalgae strains was inoculated in four 500 cm^3 reactors separately (open system, not sealed) filling synthetic domestic wastewater (no bacteria strain was inoculated). The reactors were set with 5-, 15-, 30-, and 45-min settling times, respectively, labeled R1, R2, R3, R4. The cultivation process was carried out in MGC-350B illumination incubator (Hucheng, Shanghai, China) under the following cultivating conditions: light intensity 50–60 μmol/(m^2·s), light/dark ratio 13:11, shaking frequency 150 1/min, and temperature 20 °C.

The reactor operation cycle was 48 hours (only stirring, including settling time). The differences between the settling times of the reactors were compensated by the idle time. The drainage ratio was 60%.

Analytical methods. Optical density at a wavelength of 600 nm was used to characterize microalgae density. Before chemical composition analysis, the mixed liquor was filtrated through a 0.45 μm membrane (Millipore Co., USA). Standard methods [9] were applied to determine COD, NH_4^+-N, TP and MLSS (mixed liquid volatile suspended solids). The morphology of bacteria-microalgae consortia was observed under an Olympus BX51 microscope (Olympus, Japan), and the size distribution was measured by Malvin Mastersizer 2000 (Malvin, UK).

The removal efficiency (*RE*) was calculated from

$$RE = \left(1 - \frac{MLSS_e}{MLSS_r}\right) \times 100\%$$

where $MLSS_e$ is discharged after sedimentation, and $MLSS_r$ – at the end of the operation cycle, both in mg/dm^3.

Scanning electron microscopy (SEM) observations were performed after the dehydration of bacteria-microalgae consortia using the Hitachi S-4300 apparatus (Hitachi, Japan) equipped with an energy-dispersive X-ray analyzer (EDX) to determine the elemental composition of the sludge.

Dry microalgae flocs were analyzed by X-ray diffraction (XRD) (XRD-6000, Shimadzu, Japan). Jade 6.0 software was used to analyze the data and find the chemical structure of the precipitate.

Bacteria-microalgae consortia after 60 days of operation in reactor R3 were centrifuged first and then sent to Shanghai Majorbio Biomedical Technology Co., Ltd. for high-through--put sequencing analysis. The main processes included sludge DNA extraction, design of synthetic primer connectors, PCR (polymerase chain reaction) amplification and product purification, PCR product quantification and homogenization, construction of PE (paired end) library, and Illumina sequencing. The primers for 16S rRNA sequencing were 515F (5′-TGCCAGCGCCGG-3′) and 907R (5′-CGTCAATTCMTTTRAGTTT-3′) in the V4–V5 region.

3. RESULTS

The developed bacteria-microalgae consortia showed different aggregation states depending on the settling time (Fig. 1). According to the micrograph, *Chlorella vulgaris* in reactor R3 had the largest flocs in size, followed by the floc size in the reactor R4, then R2 and R1 with the least. The granular sludge in reactor R3 could be visible to the

naked eye with a size greater than 100 μm. The granular sludge in reactor R4 was composed of smaller particles around 50 μm. In reactors R2 and R1, *Chlorella vulgaris* showed a scattered flocs state but more aggregated than incubated *Chlorella vulgaris*.
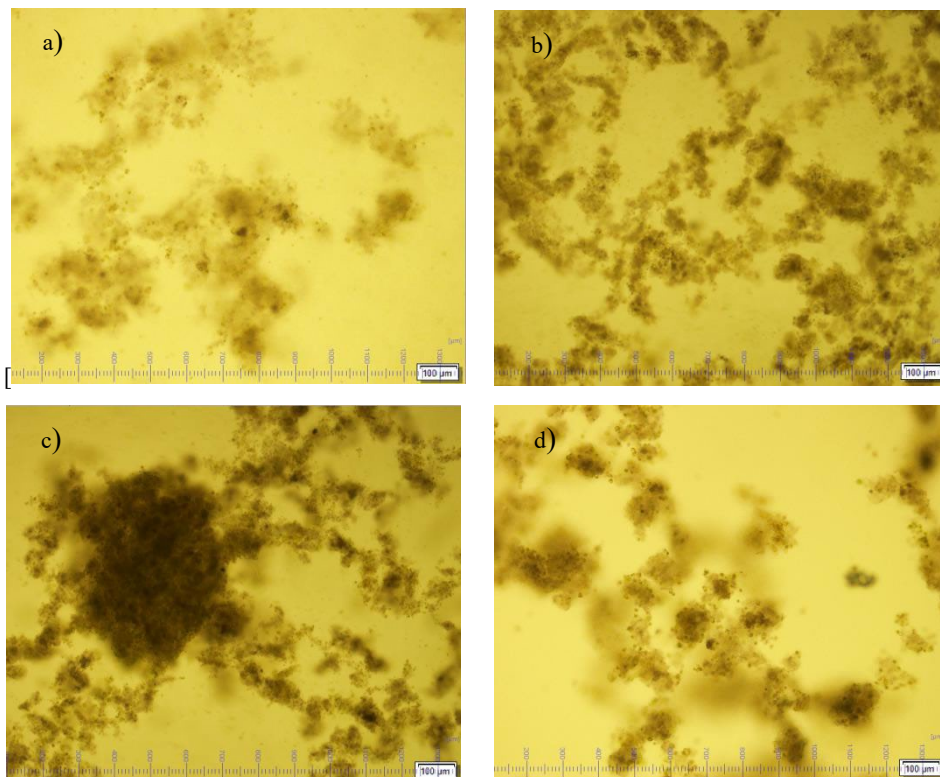

Fig. 1. Morphologies of bacteria-microalgae consortia varying in the settling time;
a) reactor R1, 5 min, b) reactor R2 – 15 min, c) reactor R3 – 30 min, d) reactor R4 – 45 min

Technically speaking, the activated sludge in aerobic granulation 33 environmental selection pressures (short settling time in SBRs) could promote the aggregation of activated sludge microorganisms and accelerate the aerobic granulation process. This study was confirmed to be an alternative driving pressure hypothesized in the aerobic granulation mechanism [10, 11]. Setting a short settling time in SBR was a simple method that could form the environmental selection pressure [12, 13]. Therefore, to conclude this study, the settling time evidently influenced *Chlorella vulgaris* auto-flocculation.

Figure 2 shows the time dependences of the settling efficiency (*RE*) of bacteria-microalgae consortia formed in reactors R1, R2, R3 and R4. A slight increase in *RE* was observed in all reactors during the experiment. The adaptation process occurred within 15 days, and the average settling efficiency was 33.5%, without any visible changes. On

day 15th, it began to increase in a significant manner until day 40th. Reactor R3 has the highest settling efficiency out of the four, exceeding 70%.
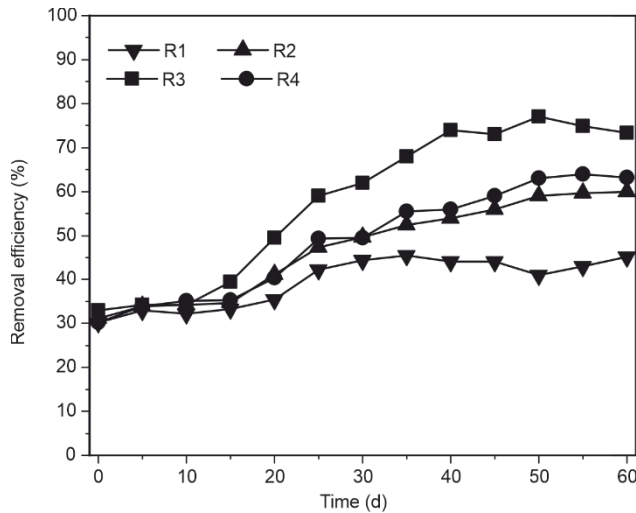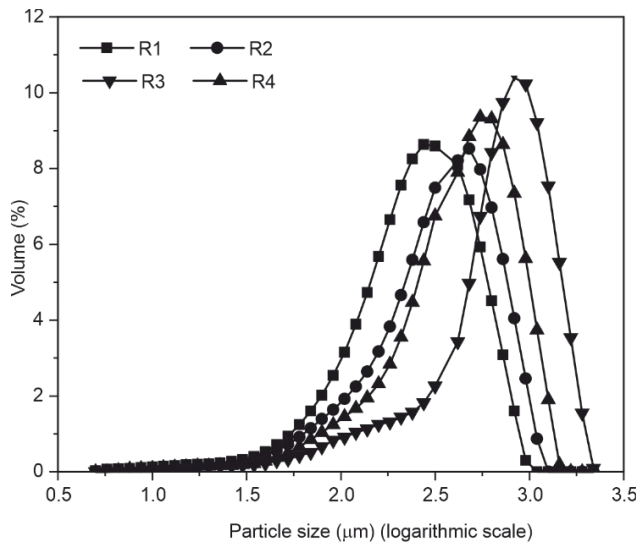

Fig. 2. Settling efficiencies versus time

Fig. 3. Particle size distribution of bacteria-microalgae consortia
depending on the settling time

Whereas high settling efficiency for both microalgae separation and harvesting in wastewater treatment by the flocculation method was evident, its disadvantage was higher project costs [14, 15]. Anyway, microalgae application in advanced wastewater

treatment and renewable energy production is a promising technology [2]. However, its industrial application had been vastly hindered due to the difficulty in biomass separation and harvesting [1, 16]. Thus, the auto-flocculation of microalgae can improve the settling of algal cells, providing a solution to biomass separation and harvesting [5].

The flocs size distributions are shown in Fig. 3. The flocs in reactor R3 had the biggest particle size, 736.56 μm on average. The average particle sizes in reactors R1, R2, and R4 were 206.99 μm, 325.74 μm and 480.73 μm, respectively. The settling property of flocs determined the biomass separation efficiency. The structure and particle size seem to have an effect showing that the larger the size of the flocs, the faster the settling velocity is. The higher settling rate increased the residence time of organisms, maintained more biomass in the reactor, and improved pollutant removal efficiencies.
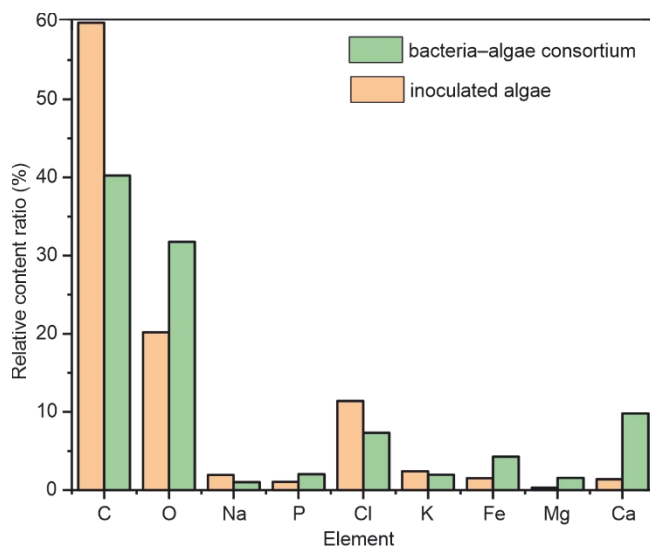

Fig. 4. Eelement's contents in bacteria-microalgae consortia and inoculated algae

Eelement's contents in bacteria-microalgae consortia and inoculated algae in reactor R3 are shown in Fig. 4. The inoculated consortia had relatively high quantity of Cl and K elements, on the same pattern of the bacteria-algae consortia with P, Fe, Mg and Ca elements as above. Therefore, P, Fe, Mg and Ca were the main elements reported in the aerobic granular sludge [17, 18], which indicated that the increased availability of these elements could improve the sedimentation of microalgae flocs. As shown in Fig. 5, compared with the standard documents in Jade 6.0 software, the peaks in the XRD pattern were consistent with tetra-calcium phosphate(($Ca_4O(PO_4)_2$). In addition to the energy spectrum, we could conclude that Ca and P played a vital role in microalgae auto-aggregation.
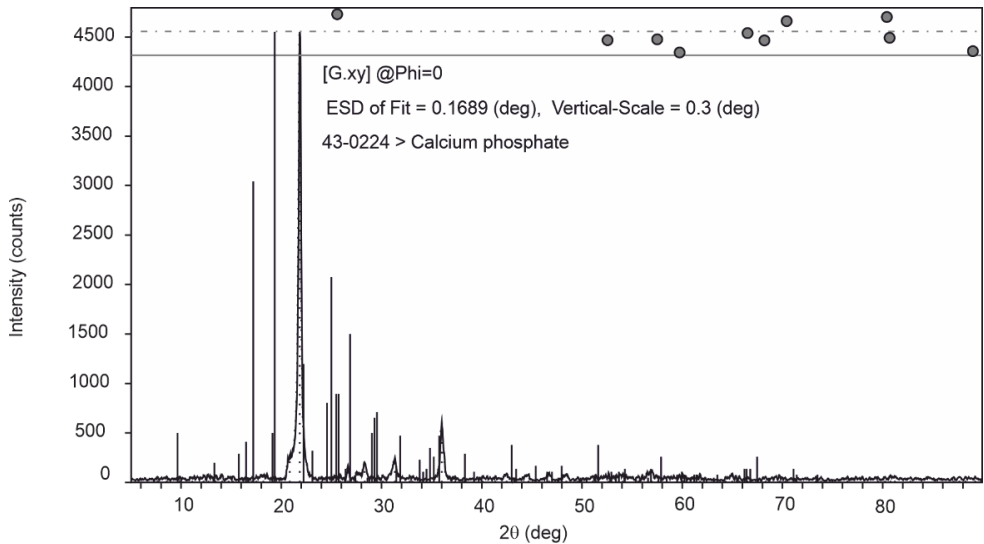
Fig. 5. X-ray diffraction patterns of bacteria-microalgae consortia

In Figure 6, scanning electron microscopy images of the flocs and internal micro-structure of the *Chlorella vulgaris* flocs are shown. The cell surface of inoculated *Chlorella vulgaris* is relatively smooth in the absence of other microalgae and bacteria while the flocs of *Chlorella vulgaris* show that algae's cell surface was rough and adhered towards some bacteria and colloidal substances (extracellular polymeric substances, EPS). Microalgae cells cohere to flocs, and the flocs have many pores. It is clear that bacteria play a vital role in the *Chlorella vulgaris* auto-flocculation.
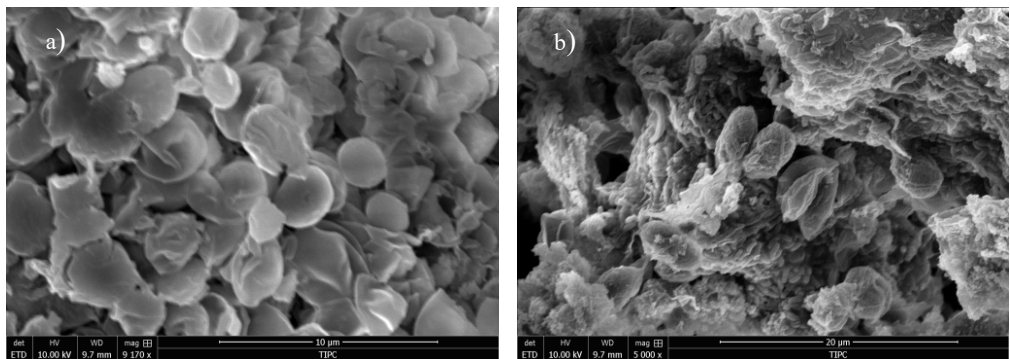

Fig. 6. Scanning electron microscopy images: a) inoculated algae, b) bacteria-microalgae consortia

The microbial community distributions of flocs *Chlorella vulgaris* in rector R3 are shown in Fig. 7. *β-Proteobacteria* and *γ-Proteobacteria* were the dominant bacteria, and

their relative contents were 46.15 and 23.08% respectively. *α-Proteobacteria*, *γ-Proteobacteria*, *Clostridia* and *Acidobacteria* the contents from 7.2% to 8.3%. The dominant bacteria distribution of flocs in reactor R3 is similar to that in aerobic granular sludge [19, 20], indicating that bacteria played a vital role in the formation of bacteria-algae consortia.
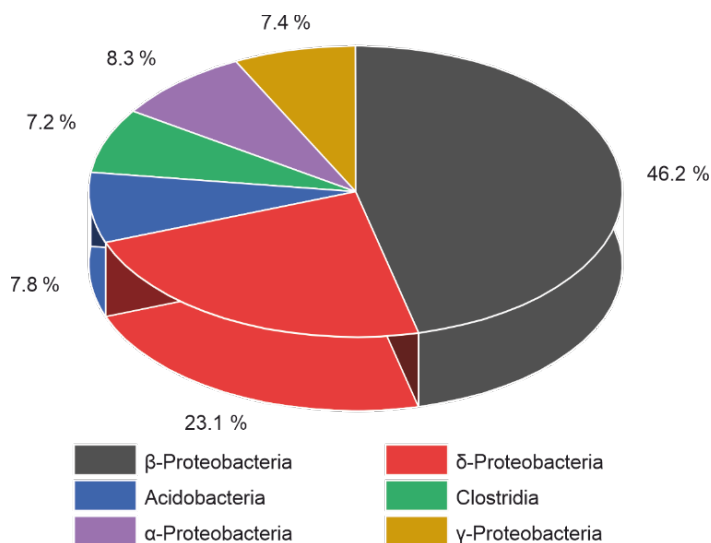

Fig. 7. Microbial community structure of bacteria-microalgae consortia

4. DISCUSSION

Microalgae auto-flocculation refers to the flocculation of microalgae without adding any external flocculant [21]. Since Golueke and Oswald first reported the auto-flocculation of microalgae in 1965, many studies confirmed the existence of this phenomenon [15, 16, 21]. At present, the two major external factors believed to be involved in the auto-flocculation of microalgae are high pH-induced flocculation and EPS-initiated flocculation [22, 23].

In 1984, Sukenik and Shelef [21] first quantitatively and systematically studied the self-flocculation of microalgae under high pH expressing an opinion that calcium phosphate was crucial in sediment to induce self-flocculation. Vandamme et al. [22] found that pH significantly affected the flocculation effect when they studied the auto-flocculation of *Chlorella*. Sirin et al. [14] found that the sediment was mainly magnesium sediment under pH 10.5–11.0 when they examined the auto-flocculation of *Phaeodactylum deltoids*. In the above study, the auto-flocculation of microalgae was regarded a pure chemical reaction process, ignoring the changes of pH in the internal microenvironment of the flocs caused by the microorganism biochemical processes. In this study,

the contents of P, Fe, Mg, and Ca elements in the consortia of the R3 flask were relatively high. Previous studies [17, 18] have shown that elements P, Fe, Mg, and Ca play a vital role in activated sludge flocculation and aerobic granulation. Based on the microalgae auto-flocculation mechanism, P, Fe, Mg and Ca played a vital role in the formation of microalgae flocs in this study, even though the method might not be limited to the chemical precipitation caused by the change of pH conditions.

EPS is well known as a layer of sticky matrix outside the microbial cells, which can affect the characteristics of the cell surface [24, 25]. It was closely relevant to the morphology, structure, function, and ecology of microbial aggregates in the wastewater treatment reactor. It also played a vital role in wastewater biological treatment [26, 27]. Zhang et al. [28] found that phosphorus concentration could increase the growth of microalgae cells and EPS secretion. Boonchai et al. [29] found that hunger treatment could increase the production of microalgae EPS, and the microalgae EPS under nitrogen starvation conditions had relatively high protein content. Salim et al. [23] found that EPS played a crucial role in the self-aggregation of *E. textensis* cells, and the main substance adhered to the cell surface was glycoprotein. Ge et al. [30] found that the higher ratio of carbohydrate/protein in EPS could receive a much more advanced performance of the settling in microalgae progress rather than the usual amount of ESP. These studies showed that EPS has a significant effect strategy on both surface properties and flocculation of microalgae cells.

These two auto-flocculation mechanisms mentioned above could explain some microalgae auto-flocculation phenomena under the pure culture in laboratory conditions. However, in recent years, more and more evidence proves that bacteria play a vital role in the process of microalgae auto-flocculation in wastewater treatment. The flocculation of the symbiotic system of bacteria and microalgae gained the attention of researchers for a long time. Lee et al. [31] found that *Flavobacterium*, *Terrimonas,* and *Sphingobacterium* had a similar effect on the flocculation activity of *Chlorella vulgaris* culture. Some filamentous fungi have the ability to combine with algal cells, such as *Rhizopus oryzae*, *Penicillium expansum,* and *Mucor circunelloides* and when cocultured with microalgae, they could form large particles (2–5 mm in diameter) under laboratory-optimized conditions [32]. The actual microalgae wastewater treatment system was an open system, inducing the natural form of a bacteria and algae symbiotic system. As shown in Figs. 6 and 7, bacteria have vital roles in microalgae auto-flocculation.

The phenomenon of microalgae auto-flocculation could not be explained by any of the above single mechanisms, especially for the open microalgae sewage treatment system. The process included multiple functions between bacteria–bacteria, bacteria–algae and algae–algae. Based on this current study, the mechanism of *Chlorella vulgaris* auto--flocculation in wastewater treatment was: the environmental selection pressure (settling time in this study) promoted microalgae cells to alter the physiological process. Microalgae cells secreted more flocculating EPS resulting in a strengthened adhesion

and biochemical processes enriched in P, Fe, Ca, and Mg elements, and the multiplication of flocculating bacteria promoted the co-flocculation of bacteria and microalgae.

5. CONCLUSION

Settling efficiencies of microalgae–bacteria consortia were improved by setting a short settling time in SBRs with *Chlorella vulgaris* treating synthetic domestic wastewater. Although improvement was clear in this experiment, the best feedback did not favour the minimum duration. The reactor with 30 min settling time had the largest flocs size and highest settling efficiency. Bacteria–microalgae granular sludge in this reactor had a relatively high content of P, Fe, Mg and Ca elements, making bacteria and microalgae coexist and cling to each other. The dominant bacteria distribution of bacteria–microalgae granular sludge in similar to that of aerobic granular sludge implied bacteria played a vital role in *Chlorella vulgaris* auto-flocculation.

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