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Effect of nutrient starvation on nutrient uptake and extracellular polymeric substance for microalgae cultivation and separation

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ABSTRACT

The purpose of the present study is to investigate the effects of nutrient starvation of microalgae on its nitrogen (N) and phosphorus (P) uptake, characteristics of extracellular polymeric substances (EPS), and algal sedimentation. An experiment was carried out by starving the wastewater-tolerant microalgae *Chlorella* sp. ADE4. The algal cultivation was put under various conditions of nutrient starvation in order to enhance nutrient removal and algal cell separation. The experimental results showed that 40 h of nutrient starvation prior to the cultivation did affect nutrient removal of *Chlorella* sp. ADE4. When using synthetic wastewater, the N-starved algae was the most effective in removing 82% of N in 48 h and 92% of P in 24 h. However, the starvation conditions did not cause noticeable removal improvement when microalgae were tested with real wastewater effluent. N and P removal efficiencies of 57 and 100%, respectively, were achieved in 48 h in real wastewater effluent. The lower N removal efficiency was caused by P limitation in the real sewage effluent. EPS were analyzed to evaluate if they play a role in algal cell agglomeration and subsequent microalgal separation. Carbohydrates and protein were indicated as major components in soluble and bound EPS. It was found that starvation of microalgae for 40 h could induce higher EPS production. Interestingly, the N-starved microalgae contained a large protein fraction in their EPS and low N content in their biomass. However, a significant correlation between EPS content and sedimentation efficiency was not observed in this study.

Keywords: Extracellular polymeric substances (EPS); Microalgae; Nutrient uptake; Sedimentation; Nutrient starvation; Wastewater

1. Introduction

The demand for clean water is growing, along with an increase in the amount of wastewater that needs to be treated adequately to meet environmental discharge regulations. Treated wastewater effluents containing high concentrations of nitrogen (N) and phosphorus (P) can lead to eutrophication, and thus the depletion of oxygen in the water. To achieve high quality effluent, several advanced treatments have been studied for wastewater treatment. Microalgae can be used in tertiary treatment. Compared to biological nutrient removal or chemical precipitation, use of microalgae would provide advantages such as cost effectiveness, low energy requirements, reduction in sludge formations, greenhouse gas mitigation, and production of useful microalgal biomass [1–4]. However, the

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treatment process involving microalgae requires a long hydraulic retention time (HRT) of approximately 3–12 d [5–8], meaning this system requires a large area to accomplish target concentration, making microalgae impractical for large scale application. To minimize the HRT, microalgae technology needs to uptake N and P effectively and rapidly. Many strategies have previously been employed, such as a balanced N:P ratio in wastewater [6], microalgae/bacteria consortium [9], co-immobilization in alginate beads with microalgae growth-promoting bacterium *Azospirillum brasilense* [10], and P starvation [11]. Among these methods, P starvation is one of the simplest strategies. It was found that under a P-limited condition, microalgae can be triggered to uptake much more P than it needs for survival [11,12]. Thus, it is reasonable to expect that microalgal metabolism will be different under different growth and environmental conditions, including nutrient starvation. Changes in nutrient removal could result from altering microalgal metabolism.

Moreover, microalgae are like other micro-organisms in that it is recognized that they excrete a naturally adhesive organic material, extracellular polymeric substances (EPS). EPS have a profound impact on cell aggregation causing microalgae and other particulate organic carbon to settle [13,14]. Despite this important aspect of EPS, research has not been conducted on EPS production by microalgae in wastewater under the conditions of N and P starvation. Therefore, the present study attempts to investigate the effect of nutrient starvation on N and P uptake, EPS production, and microalgal sedimentation efficiency. The experiments were conducted by adding a nutrient starvation period to normal cell growth prior to cultivation in wastewater. It was hypothesized that the starved microalgae would result in high and rapid nutrient uptake, as well as in changes of the characteristics and quantity of EPS that may further affect algal biomass settling. Hence, cell growth rate, nutrient removal, EPS characteristics, and sedimentation efficiency in synthetic and real wastewater were all investigated in this study. If starvation of microalgae can improve nutrient uptake and algal sedimentation, reduction in time required for wastewater treatment and improvement of biomass separation will greatly improve the benefits of use of microalgae in wastewater treatment.

2. Materials and methods

2.1. Microalgae culture condition

Chlorella sp. was isolated from the effluent line of anaerobic digestion tanks at Su-young wastewater treatment plant in Busan, South Korea by the Department of Microbiology, Pusan National University, South Korea, and was named *Chlorella* sp. ADE4. Pure microalgae culture was maintained in BG11 medium [15]. The medium was adjusted to have a pH of 7.1 before autoclaving. The microalgae culture was kept at 25 ± 2°C and under a light intensity of 50 μmol m^{-2} s^{-1} for the entire experiment.

2.2. Wastewater characterization

In this study, the experiments were carried out using synthetic wastewater and real wastewater effluent. For the experiment using synthetic wastewater, modified BG11 medium was adjusted to contain N and P concentrations of 20 and 2 mg L^{-1}, respectively. These values are similar to the wastewater effluent standard quality in South Korea.

For the experiment using real wastewater, secondary wastewater effluent was collected from the Jinhae wastewater treatment plant of Changwon City, South Korea. The average concentrations of chemical oxygen demand (COD), suspended solids, total nitrogen (TN), and total phosphorus (TP) in the secondary effluent were 10.5, 2.3, 19.9, and 0.15 mg L^{-1}, respectively. As a result of the nature of real wastewater, there were a number of micro-organisms in the wastewater. The presence of indigenous bacteria and protozoa in the wastewater and the different chemical composition in real wastewater can interfere with microalgae growth, nutrient uptake, and EPS production. Therefore, in this study, the wastewater was filtered through a 0.22 μm membrane, through methods described by Cho et al. [2].

2.3. Experimental set-up

To investigate the effect of nutrient starvation on nutrient uptake and EPS production, four conditions of BG11 medium were used in this study, which included a control with sufficient concentrations of nitrogen and phosphorus (N+P+), nitrogen sufficient and phosphorus deficient concentrations (N+P−), nitrogen deficient and phosphorus sufficient concentrations (N−P+), and both nitrogen and phosphorus deficient concentrations (N−P−).

After a two-week cultivation period in the BG11 medium, microalgae were separated by centrifugation. The cells were then washed twice with distilled water. Starvation was conducted by inoculating the microalgae in four different conditions of media for 40 h. After the starvation period, the starved culture was again washed twice with distilled water and then

transferred either to new synthetic wastewater or real wastewater effluent in 1 L Pyrex bottles with a 700 mL working volume. Air was purged at the rate of 0.35 VVM to each flask. The mixing was provided at a speed of 150 rpm. The experiments were operated without controlling the pH. The experiments were carried out in duplicate.

2.4. Extraction method of EPS and analysis

Algal suspension was collected 120 h after the experimental set-up period. Samples from each of the conditions were separated into two portions. One portion of microalgal culture was centrifuged at 12,000 g for 15 min. The supernatant was filtered through a 0.22 μm membrane. The filtrate was collected as soluble EPS. Another portion was extracted to obtain bound EPS according to the instruction of Pan et al. [16]. This method was modified from that of Liu and Fang's method using formaldehyde (36.5%) for 1 h at 4℃, and then with 1 M NaOH for 3 h at 4℃ [17]. The extracted solution was then filtered through a 0.22 μm membrane. Both filtrates were used for carbohydrate and protein analysis of the EPS.

2.5. Analytical methods

The algal suspension was filtered using a 0.45 μm membrane. The filtrate was then collected for measurement of TN and TP. The dry weight content of algae was determined by filtering the microalgal culture through a glass microfiber filter (GF/C, Whatman®) and drying at 105℃ for 24 h. The specific growth rate, μ (d^{-1}), was expressed as

$$\mu\,(d^{-1}) = \frac{\ln(X2 - X1)}{t2 - t1} \quad (1)$$

where *X1* and *X2* represent dry weight or cell number at times *t1* and *t2*, respectively. To identify the functional groups of the algal cells, the freeze-dried cells were analyzed using Fourier transform infrared (FTIR) spectroscopy with a FT/IR 6300 spectrometer (Jasco, Japan). Freeze-dried cells were also collected for elements analysis using a CHNS/O element analyzer (2400 series II, PerkinElmer Inc., USA). The sedimentation efficiency test was conducted spectrophotometrically in a cuvette at 750 nm according to methods described by Salim et al. [18]. The sample was left untouched and measured every 5 min for 30 min. Carbohydrate concentration in the extracted EPS was measured using a phenol-sulfuric method [19] with glucose as the standard. Sample absorbance was measured at 490 nm. Meanwhile, protein concentration was measured by the Bradford method [20], using bovine serum albumin (Biorad, USA) as the standard. The color developed at the absorbance of 595 nm. All measurements were done in duplicate and data reported in this study represents the mean value of the duplicates.

3. Results and discussion

3.1. The effect of starvation on algal growth and nutrient uptake in synthetic wastewater

The biomass dry weights of *Chlorella* sp. ADE4 under the four conditions in synthetic wastewater are shown in Fig. 1. It was observed that *Chlorella* sp. ADE4 in the N−P+ condition presented the highest growth rate at 0.33 d^{-1} in synthetic wastewater. The growth rates of microalgae were correlated with N and P removal efficiency, as shown in Table 1. While the time required for removing N and P in other studies have been longer [7], the present study focused on rapid N and P uptakes and thus the period of treatment was relatively short, considering 48 h for N uptake and 24 h for P uptake. The nutrient removal rate of *Chlorella* sp. ADE4 under different starvation conditions did show significant differences, as well. Among all nutrient starvation conditions, the N−P+ condition of *Chlorella* sp. ADE4 was the most effective condition, demonstrating N and P removal efficiencies of 82.08% after 48 h and 92.09% after 24 h, respectively. The N and P uptakes were comparable to previous uptake measurements conducted by Cho et al. which reported more than 90% of TN and TP uptake within

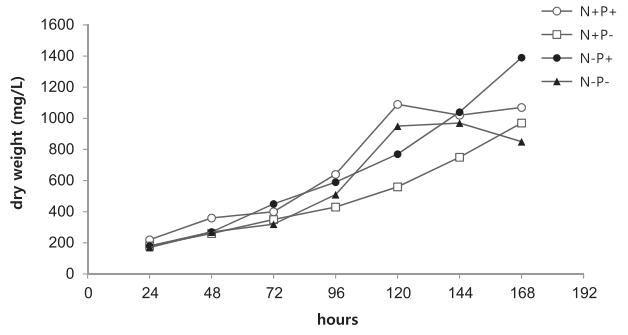

Fig. 1. Profiles of four conditions of starved microalgae *Chlorella* sp. ADE4 in synthetic wastewater, with initial concentrations of TN of 20 mg L^{-1} and TP of 2 mg L^{-1} (N+P+: both nitrogen and phosphorus sufficient (control); N+P−: nitrogen sufficient and phosphorus deficient; N−P+: nitrogen deficient and phosphorus sufficient; N−P−: both nitrogen and phosphorus deficient).

Table 1
Removal of TN, removal of TP, and specific growth rate of *Chlorella* sp. ADE4 under different nutrient starvation conditions in synthetic wastewater

Conditions	Specific growth rate μ (d^{-1})	TN removal (%)			TP removal (%)		
		24 h	48 h	168 h	24 h	48 h	168 h
N+P+	0.23	−0.61	60.81	95.42	89.08	89.56	97.51
N+P−	0.26	−4.82	38.78	89.48	73.97	90.57	98.48
N−P+	0.33	25.85	82.08	94.34	92.09	92.43	96.43
N−P−	0.25	9.01	63.13	93.34	75.79	91.41	97.63

40 h of cultivation using wastewater-isolated *Chlorella* sp. [21].

3.2. The effect of starvation on algal growth and nutrient uptake in real wastewater

The presence of indigenous bacteria and protozoa in real wastewater and the different chemical composition in real wastewater, compared to synthetic wastewater, can interfere with microalgae growth. Therefore, it would be meaningful to evaluate the microalgal growth and nutrient uptake in both synthetic and real wastewater.

Fig. 2 presents the cell growth characteristics of *Chlorella* sp. ADE4 in four different conditions cultivated in real wastewater. From the results, it was found that real wastewater could support the growth of *Chlorella* sp. ADE4. This result indicated that *Chlorella* sp. ADE4 was tolerant in wastewater, although a lag phase was observed in the first 48 h. It was then noticed that under the N−P+ and N−P− conditions, the algae reached its stationary phase after 96 h of cultivation. At the same time, the culture under the N+P+ and N+P− conditions were still in an exponential phase suggesting that the N-sufficient culture was able to support cell reproduction and would reach the stationary phase later than N-starved culture.

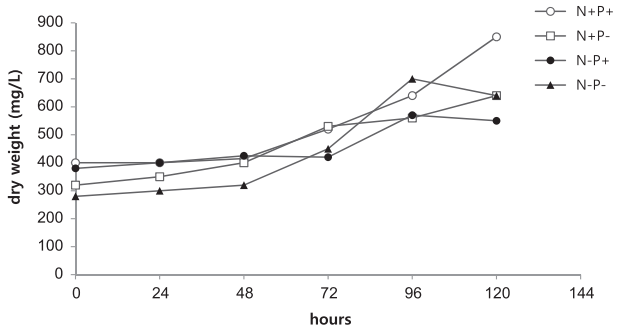

Fig. 2. Profiles of the four conditions of starved microalgae *Chlorella* sp. ADE4 using real wastewater.

N and P removal efficiencies can be seen in Table 2. Similar patterns of N and P removal trends occurred under all conditions except for the N+P− condition. The sample of secondary effluent used in this experiment presented initial concentrations of TN and TP of 19.9 and 0.15 mg L^{-1}, respectively. The average removal efficiency of TN was 57% after 48 h, while the average removal efficiency of TP was 70% after 24 h, with complete P uptake after 48 h of treatment. At the end of the experiment, the wastewater had an average N concentration of 6.61 mg L^{-1} for the N+P+, N−P+, and N−P− conditions, while the N+P− condition had an N concentration of 8.10 mg L^{-1}. As the secondary wastewater in this study contained a low concentration of P, the culture could completely remove P within 48 h, resulting in total depletion of the P source. The depletion of the P source was believed to be associated with the N assimilation observed. N concentration in the wastewater was rapidly removed in the first 48 h, and ceased thereafter (see Fig. 3). Previous studies have shown a longer time requirement for removal of N and P. In research conducted by Rasoul-Amini et al., *Chlorella* sp. exhibited an N removal efficiency of 51.41%, while *Chlamydomonas* sp. exhibited a P removal efficiency of 94.77% after 4 d [22].

3.3. Algal biomass element analysis

After observance of the N removal curves, as seen in Fig. 3, where *Chlorella* sp. ADE4 could not completely remove N after P in the wastewater was exhausted, the culture was analyzed for carbon (C), hydrogen (H), and nitrogen (N) contents using an element analyzer. The results of the element analyses can be seen in Table 3. The C content of the final biomasses ranged from 32.8 to 42.1% in the current experiment, which was lower than C content of biomasses in previous study [1]. Our results indicate that C content limited the microalgae due to a low concentration of organic C in the secondary wastewater (COD = 10.5 mg L^{-1}) with no additional CO_2 supply. The N and H contents of the microalgae were within

Table 2
Summary of TN removal efficiency, TP removal efficiency, and specific growth rate of *Chlorella* sp. ADE4 in real wastewater

Conditions	Specific growth rate μ (d^{-1})	TN removal (%)		TP removal (%)	
		24 h	48 h	24 h	48 h
N+P+	0.15	26.02	62.32	72.45	100
N+P−	0.10	19.16	52.17	67.78	100
N−P+	0.14	23.41	59.93	67.78	100
N−P−	0.17	27.00	61.13	73.61	100

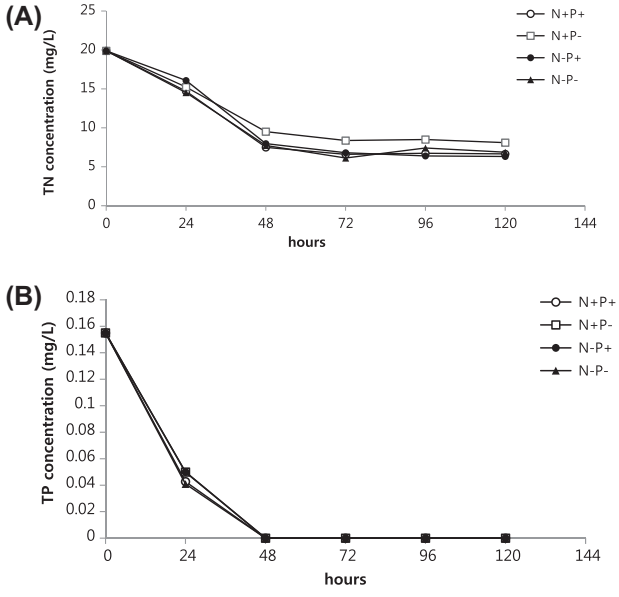

Fig. 3. TN removal (A) and TP removal (B) of the four conditions of starved microalgae *Chlorella* sp. ADE4 in real wastewater.

Table 3
Compositions of C, H, and N of microalgae *Chlorella* sp. ADE4 after 120 h in real wastewater

Conditions	C (wt%)	H (wt%)	N (wt%)
N+P+	34.2	6.1	4.1
N+P−	32.8	5.5	3.3
N−P+	41.6	7.4	3.0
N−P−	42.1	7.9	3.0

typical values, between 1–10% and 2.9–10%, respectively [23]. The N contents in the biomass obtained in this study agreed with previous results reported by Ruiz et al. of 3.1–5.9% in *Scenedesmus* sp. in secondary effluent [24]. For the N starved cultures (N−P+ and N−P− conditions), the algal biomass exhibited a lower N content and higher C content.

3.4. The effect of starvation on characteristics of EPS

The characteristics of the EPS response to environmental changes are critical. It has been reported that a higher yield of EPS of bacteria could be induced under starvation conditions because EPS helps in trapping and retaining the nutrients by the cells from surrounding environment [25].

Both EPS quality, type of component, and quantity, concentration, are important parameters of EPS. Generally, carbohydrates and proteins are considered the major components of EPS. However, to confirm the composition of the EPS of *Chlorella* sp. ADE4 under different nutrient starvation conditions, functional groups of EPS were analyzed. Fig. 4 shows the FTIR spectra of the EPS of *Chlorella* sp. ADE4 under different nutrient starvation conditions. It can be seen that all four conditions result in EPS with similar functional groups. All samples displayed strong absorption bands in the region of 3,400–3,425 cm^{-1} due to the OH hydroxyl group of polysaccharides. Weak absorptions in the range of 2,918–2,947 cm^{-1} were assigned to the aliphatic CH_2 group. Medium stretches observed in the range of 1,640 and 1,550 cm^{-1} were related to the peptide carbonyls (amide I) and the N–H (amide II) group, respectively, which are typical for proteins. Infrared (IR) peaks in the range of 1,015–1,150 cm^{-1} correspond to a polysaccharide-like substance. Based on these FTIR spectra, the presence of polysaccharides and proteins in the algal EPS was confirmed. Similar functional groups have also been reported in the EPS of microalgae in wastewater by previous works using *Chlorella* sp. [26,27] and *Dunaliella* sp. [28]. The form of the EPS was divided into bound EPS and soluble EPS. Bound EPS are closely attached with cells, while soluble EPS are weakly bounded with cells and dissolve in a solution [29]. In this study, bound EPS was extracted using chemicals, while soluble EPS was extracted using centrifugation. The results of the extraction processes can be seen in Table 4. Bound EPS from starved microalgae *Chlorella* sp. ADE4 from the N+P−, N−P+, and N − P− conditions were found to have higher amounts of carbohydrates as well as

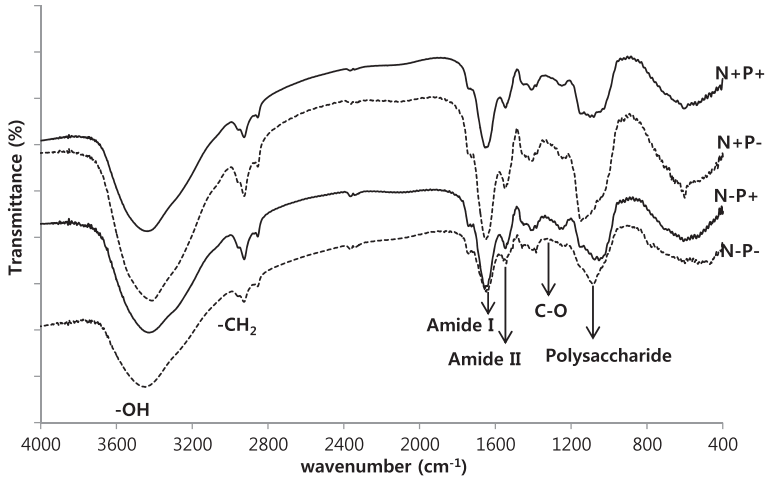

Fig. 4. Comparative FTIR spectra of EPS extracted from different nutrient starvation conditions of *Chlorella* sp. ADE4 culture grown in real wastewater after 120 h.

proteins than microalgae in the control condition, the N+P+ condition. Similar phenomenon was also observed in the soluble EPS where starved microalgae *Chlorella* sp. ADE4 from the N+P−, N−P+, and N−P− conditions showed higher amounts of proteins than those in the control group. The results agreed with a previous study by Wang et al., which showed that activated sludge under a stress condition produced larger amounts of EPS [30]. Both the carbohydrate and protein contents in the soluble EPS were highest in the N−P− culture among all of the conditions. In the previous study conducted by Wang et al., it was found that an unbalanced N/P ratio in wastewater tended to cause unfavorable conditions for algae, which led to the expression of higher protein fractions in EPS [13]. Through examination of the element analysis results and the amount of EPS, it was found that N-starved culture, which has a high protein fraction in both soluble and bound EPS, showed low N content. Since N is an essential element for protein, it can be implied that microalgae in an N-deficient condition would likely store N outside their cells in the form of EPS instead of inside the cells. This finding explains why the N-starved *Chlorella* sp. ADE4 had a large protein fraction in EPS and a low N content inside their cells.

3.5. The effect of nutrient starvation on microalgal sedimentation

Comparison of sedimentation ability of *Chlorella* sp. ADE4 under different starvation conditions in wastewater and in BG11 can be seen in Fig. 5. The sedimentation test was accomplished using the OD_{750} method [18]. Even though significant influence of starvation on the algal sedimentation was not observed, a difference in algal settling efficiency between the real wastewater and synthetic medium BG11 could be observed. A maximum sedimentation efficiency of 98% after 10 min of settling was achieved in all conditions in wastewater when the biomass reached approximately 600 mg L^{-1}. This value was higher than that in research conducted previously, which presented average algal settling efficiencies of 75.3 and 86%, achieved after 10 and 30 min of settling, respectively, when green algae *Pediastrum* sp. was dominant in a high rate algal pond [31]. These results suggest

Table 4
Contents of EPS of *Chlorella* sp. ADE4 after 120 h in real wastewater

	Bound EPS		Soluble EPS	
Conditions	Carbohydrate (wt%)	Protein (wt%)	Carbohydrate (wt%)	Protein (wt%)
N+P+	4.25	1.44	1.40	1.09
N+P−	4.61	2.33	1.24	1.26
N−P+	5.87	2.74	1.29	1.33
N−P−	4.55	2.33	1.62	4.54

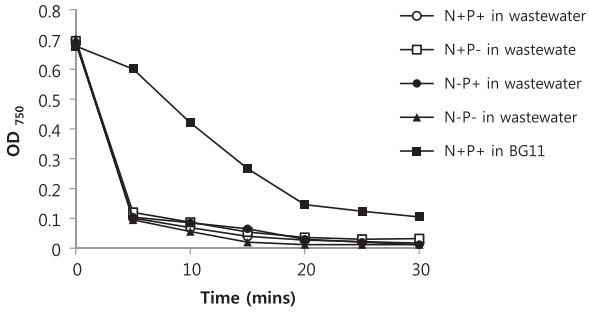

Fig. 5. Comparison between sedimentation ability of *Chlorella* sp. ADE4 in BG 11 and in wastewater under different starvation conditions.

that after 40 h, starved *Chlorella* sp. ADE4 did not enhance their cell aggregation. On the contrary, the high sedimentation observed from this study was mainly caused from cell attachment between microalgae and other particles or micro-organisms in real wastewater, despite filtration. Lee et al. previously reported that bacteria play a key role in flocculation by increasing floc size, resulting in the sedimentation of microalgae [32].

4. Conclusions

The results of this study showed that N starvation of *Chlorella* sp. ADE4 for 40 h increased biomass production and enhanced the removal of N and P in synthetic wastewater, while there was no significant change in real wastewater. *Chlorella* sp. ADE4 showed rapid N and P removal efficiencies in wastewater. The average removal efficiency of TN was 57%, and complete uptake of P took place after 48 h of treatment. It was found that more EPS, both bound and soluble EPS, were extracted under starvation conditions. Moreover, it was determined that microalgae *Chlorella* sp. ADE4 in N-deficient conditions likely store N outside their cells in the form of EPS instead of inside the cells. However, EPS contents of *Chlorella* sp. ADE4 after 40 h of starvation did not affect their sedimentation.

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