Cultivation of energy microalga *Chlorella vulgaris* with low–toxic sludge extract

Xiurong Chen, Shanshan Wang, Xiaoli Sun and Quanling Lu

ABSTRACT

Chlorella vulgaris was cultivated in different proportions of activated sludge extracts, which was from the treatment of synthetic wastewater containing tetrachlorophenol. The growth period of *C. vulgaris* could be shortened for about 10 days when sludge extract was mixed into BG11 culture substrate, and the growth of *C. vulgaris* was promoted during the period of adaptation and logarithmic period. In the stable and decay period, when the proportion of sludge extract increased to 50%, cell proliferation was inhibited. There was an evident positive correlation between the total and average amount of starch polysaccharide with sludge concentration. When *C. vulgaris* was cultivated with pure sludge extracts, the total amount of starch and polysaccharide was up to 103 and 125 mg/L. Therefore, the low-toxic sludge extracts were more beneficial to the accumulation of carbohydrates. In the 100% sludge extracts culture medium, chlorophyll-a in *C. vulgaris* was accumulated to 30.2 mg/L on the 25th day. Through the analysis of algal cells' ultrastructures, it was shown that the photosynthesis was strengthened greatly with low-toxic sludge extracts. The results show that the rich heterotrophic carbon source in the sludge extract can be used as an excellent medium for *Chlorella*. It provides new ideas for the harmless utilization of surplus sludge as a resource. At the same time, the use of nutrients in the sludge extract to cultivate *Chlorella* is of great significance to low-cost algae cultivation.

Key words | carbohydrates, *Chlorella vulgaris*, full life cycle, low-toxic sludge extract, photosynthesis

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HIGHLIGHTS

- Low-toxic sludge extract-based algae cultivation system was used in this study.
- Sludge extract was more favorable for *C. vulgaris* growth during the period of adaptation and logarithmic period.
- Day 20 was optimal to obtain maximal algal biomass.
- The effect of sludge extract on the organelles of *C. vulgaris* was analyzed by the sub-microstructure of cells.

INTRODUCTION

At present, the exploitation and burning of fossil fuels not only cause damage to land and water resources but also release large amounts of greenhouse gases and destroy ecological balance (Hook & Tang 2013). It is reported that carbon dioxide released from fossil fuel burning is the main cause of global climate change. Currently, more than 80% of the world's energy consumption comes from fossil fuels, CO_2 emissions of over 12 billion tons per year (Cebrucean Cebrucean & Ionel 2014; Chen & Geng 2017). Therefore, the development of bio-energy is an important choice to solve energy and environmental problems. Today, the most widely used bio-energy is bio-ethanol, followed by biodiesel. Bioethanol is considered to be a clean renewable fuel, due to its biodegradability, non-toxic and low pollution or no contaminants to the environment, and

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it is an ideal alternative to gasoline (Hong *et al.* 2014; Tan & Lee 2014; Ullah *et al.* 2014; Wu *et al.* 2014). In recent years, microalgae have been widely concerned as a raw material of bio-ethanol due to the high production efficiency, short reproductive cycle, and high carbohydrate content, from the wastewater absorbing nitrogen, phosphorus, and another nutrient, not in competition with humans for freshwater resources (Kim *et al.* 2014). Sirajunnisa and Surendhiran indicated that algae can produce polysaccharides and could be converted into bioethanol by fermentation (Sirajunnisa & Surendhiran 2016). Seung Phill *et al.* showed that when the algae's intracellular starch content arrives at about 43.6% of dry base, total carbohydrate content arrived at about 59.7% of dry base, approximately 235 mg of ethanol was produced from 1.0 g of algal biomass (Choi *et al.* 2010). Therefore, in the fermentation of the bioethanol industry, the accumulation of starch and polysaccharide is an important research direction.

Nowadays, the microalgae are cultivated mainly by adding nitrogen and other nutrients into river water and tap water or micro-polluted water after pretreatment. According to statistics, in the process to produce bio-energy with microalgae as raw materials, microalgae culture costs accounted for about 70% of the total cost, and 40% of the cost of cultivation was from the water and nutrient consumption (Grobbelaar 2010; Ji *et al.* 2014). Therefore, the high cost of microalgae culture has gradually become a bottleneck limiting large-scale microalgae production capacity (Pienkos & Darzins 2009). At present, based on the characteristics of wastewater rich in nitrogen and phosphorus, researchers have proved that it is feasible to cultivate algae in sewage. Mata *et al.* analyzed the potential of using the microalgae *Scenedesmus obliquus* for brewery wastewater treatment and biomass production and the results found that a maximum of 0.9 g of dry biomass per liter of culture was obtained after 9 days in the conditions of aeration, exposure to a 12 h period of daily light, and with the intensity of 12000Lux (Mata *et al.* 2012). Chiu-Mei Kuo *et al.* used aquaculture wastewater aerated with boiler flue gas to culture *Chlorella* sp. GD, the results indicating that the microalgal biomass productivity after 7 days of culture was 0.794 $g\,L^{-1}\,d^{-1}$ (Kuo *et al.* 2016). In the widely biological wastewater treatment process, a lot of sludge extract could be produced in the sludge dehydration process. When the influent wastewater contained high concentrations of organic matter, due to the incomplete degradation of the substrate and its inhibiting stress to bacteria, the activated sludge would be bound to accumulate a lot of organic matter (Chen *et al.* 2015; Zhao *et al.* 2015, 2016). Meanwhile, the content of nitrogen and phosphorus in sludge extract was about 7–9 times that of nitrogen and phosphorus in wastewater. The conventional treatment method is to return the sludge to the front of the treatment for further treatment. However, the content of organic matter in the sludge is as high as 60–80% (Tang *et al.* 2010), which could cause a serious impact on the water treatment process. Therefore, based on the characteristics of sludge extract being rich in nitrogen and phosphorus, it can be used as an alternative microalgae medium. Then, not only low-cost cultivation but also the extraction of resources can be achieved without the impact of refractory organic matter, high levels of nitrogen, and phosphorus on the water treatment process.

4-chlorophenol is a kind of refractory toxic organic compound, which widely exists in industrial production. Chlorophenols are representative POPs due to their acute toxicity and resistance to biodegradation (Park *et al.* 2012a). The sludge taken from the laboratory is in the stabilization stage in the biological treatment of the chlorophyll wastewater. At this time, the water phase has been detected to not contain chlorophenol, the sludge toxicity has decreased and stabilized at a relatively low level (the relative inhibitory rate of the luminous bacteria was about 31%). This research aimed to investigate the accumulation of starch and polysaccharide in the whole life cycle of *C. vulgaris* by adjusting the ratio of low-toxic sludge extract and BG11 medium. The results could be used to provide a theoretical basis for the low-cost cultivation of energy microalgae.

MATERIALS AND METHODS

Cultivation of *C. vulgaris* using BG11

C. vulgaris (FACHB-31) was obtained from the Wuhan Institute of Aquatic Biology, Chinese Academy of Sciences. According to Wang's method (Wang *et al.* 2017), *C. vulgaris* was preserved in BG11 medium and pre-cultured in a light incubator (SPX-250B-G, Shanghai Boxun Industry & Commerce Co., Ltd, Shanghai, China). The conditions were as follows: temperature, $25 \pm 1\,^{\circ}C$; light intensity, 4000 Lx; and light-dark ratio, 14:10.

Sludge extracts

Cultivation of *C. vulgaris* with sludge extracts

Activated sludge was used to treat synthetic wastewater, which contained 50 mg/L of tetrachlorophenol in a

sequencing batch reactor (SBR) with an 8-h hydraulic retention time and a 20-day sludge retention time. According to Zhao's method (Zhao *et al.* 2014), sludge was disrupted by a 300 W ultrasonic processor (Fs-300, Shanghai Sonxi Co., Ltd, China) for 1s with 1s intervals; after 10 min of disruption, high-speed centrifugation (12,000 r/min, 5 min) was used to obtain supernatant as the sludge extract. The sludge extract was mixed with BG11 medium in different proportions (1: 0, 4: 1, 1: 1: 4, 0: 1) as the mixed medium. The initial composition of the mixed medium is shown in Table 1.

Energy consideration

In order to evaluate the energy balance in the ultrasonic crushing process of sludge, according to M. Al Ramahi's method, a detailed energy assessment was done to evaluate the economic viability of the disintegration technique. The equations employed to perform energy calculations are explained in detail as follows (Al Ramahi 2020):

$$E = P \times T/(V \times TS)$$

where E is the input energy (kJ/kg TS), P is the input power (kW), T is the treatment time (sec), V is the volume of the sample (L) and TS is total solids in (kg/L).

The input energy of ultrasonic sludge crushing is 45 KJ/kg TS, the SCOD release is 521 mg/L, and the temperature increases to 53 degrees Celsius. Therefore, ultrasonic crushing of sludge occurs in an ice water bath.

Analytical methods

C. vulgaris cell density

The algae liquid was made from a sample in the blood counting chamber for two minutes, and the number of cells was determined by direct counting under the optical microscope (BA200, Shanghai Boxun Industry & Commerce Co., Ltd, China).

Table 1 | Initial medium components (mg/L)

Sludge extracts	BG11	TN	TP	TOC
0%	100%	227 ± 8	6.56 ± 0.21	14.5 ± 0.4
25%	75%	185 ± 4	10.7 ± 0.2	65.6 ± 2.2
50%	50%	152 ± 6	17.0 ± 0.4	137 ± 6
75%	25%	130 ± 5	21.2 ± 0.5	194 ± 7
100%	0%	119 ± 5	26.4 ± 0.4	268 ± 9

Chlorophyll-a

Samples of *C. vulgaris* were centrifuged (5,000 rpm, 10 min), rinsed twice by deionized water, and the pellet was resuspended in 5 mL of 100% methanol and disrupted in an ultrasonic cleaner (ANA1860, Yingsum Ultrasonic Equipment Co., Ltd, China) at 135 W with an ice bag in a dark place for 30 min. After chlorophyll-a extraction, samples were centrifuged (5,000 rpm, 10 min); the absorbance of the supernatant was measured at 653 and 666 nm. Chlorophyll-a concentration was calculated according to Ritchie's and Zheng's method (Zheng *et al.* 2012).

$$C_a = [15.65 \times OD666 - 7.34 \times OD653] \times \text{Dilution Ratio} \quad (1)$$

Polysaccharide

25 mL algae were centrifuged at 5,000 r/min for five minutes. The pellet was resuspended in 5 mL PBS. Then it was disrupted by a 300 W ultrasonic processor for 1 s with 1 s intervals for 20 min, allowing the intracellular substances to move out of the cell and enter the liquid phase. Centrifugation (5,000 r/min, 5 min) was conducted to obtain the supernatant. The total polysaccharide was determined by the sulfuric acid anthrone method. The total polysaccharide reacted with anthrone-sulfuric acid reagent in a boiling water bath and absorbance was measured at 620 nm (Chang & Murillo 2017).

$$\text{Polysaccharide yield} = \frac{[149.98 \times OD620 - 0.4719]}{[\text{Sampling Volume} \times \text{Dilution Ratio}]} \quad (2)$$

$$M_P = C_P/C_D \quad (3)$$

where M_p stands for the concentrations of polysaccharide per 10^9 cell, mg/10^9 cell; C_p represents the concentration of polysaccharide per milliliter of algae, mg/L; C_D refers to the amount of *Chlorella* per milliliter of algae, 10^6 cell/mL.

Starch

With the same pre-treatment of *Chlorophyll-a*, after *chlorophyll-a* extraction, samples were centrifuged (5,000 rpm, 10 min). The pellet was dissolved in 80% calcium nitrate solution. In a boiling water bath for 10 min, samples were centrifuged at low speed (4,000 rpm, 4 min), adding 0.01 mol/L I-KI reagent to the supernatant. The absorbance

was measured at 620 nm.

$$\text{Starch yield} = \frac{[319.79 \times \text{OD620} + 0.0839]}{[\text{Sampling Volume} \times \text{Dilution Ratio}]} \quad (4)$$

$$M_S = C_S/C_D \quad (5)$$

where M_s stands for the concentrations of starch per 10^9 cell, mg/10^9 cell; C_s represents the concentration of starch per milliliter of algae, mg/L; C_D refers to the amount of chlorella per milliliter of algae, 10^6 cell/mL.

Transmission electron microscopy studies

For the TEM observation, samples were centrifuged at 5,000 r/min for 5 minutes and then pre-fixed with 2.5% glutaraldehyde. Fixed samples were washed three times with phosphate buffer, and then post-fixed with 1% osmium peroxide for 1 h, dehydrated in a graded series of ethanol solution, and embedded in Epon 812. The embedded samples were sectioned with a Leica slicer. Samples were further monitored by transmission electron microscopy (JEM-1400EX, TEX) (Wang *et al.* 2005).

Liquid phase index analysis

After centrifugation, the supernatant was gathered to analyze N and P. The supernatant was diluted 10 times. The utilization rate of the different forms of N, P, could be calculated by Equation (6).

$$r = (C_0 - C_t)/C_d \quad (6)$$

where r stands for the utilization rate of N, P, 10^6; C_0 represents the initial N, P concentration for each growth period, mg/L; C_t refers to the concentration of N, P at the end of each growth period, mg/L; C_d expresses the average algal cell density in different growing periods.

The organics in the sludge extract were characterized by TOC (TOC-V, Shimadzu, Japan). The molecular-weight distribution of the organics in the sludge extract was measured by ultrafiltration with different bore diameter membranes (10,000, 5,000 and 500 Da) according to Zhao's method (Zhao *et al.* 2015).

Statistical analysis

The results were expressed as the means ± standard error of the mean. IBM SPSS 22.0 (IBM Corporation, Somers, NY, USA) was used for statistical analysis (statistical significance $P < 0.05$). Analysis of variance (ANOVA/MANOVA) was used to determine the significance of the differences between the groups. The Pearson correlation test was performed to determine the correlations between the parameters.

RESULTS AND DISCUSSION

Growth characteristics of *Chlorella vulgaris*

Cell density

According to the dilution ratio, the sludge extract from the degradation of tetrachlorophenol was mixed with pure BG11 medium to cultivate *Chlorella vulgaris,* and the influence of the dilution ratio on the growth characteristics of *Chlorella vulgaris* was studied. The variation in cell density based on cell number, and the growth stage distribution, as a direct measure for the growth of *C. vulgaris*, were compared (see Figures 1 and 2) to investigate the effects of sludge toxicity.

It can be seen from Figure 1 that the *C. vulgaris* growth trend with 25% sludge extracts was the same as BG11. When the proportion of sludge extract increased from 50% to 100%, the stable period of *C. vulgaris* was continuously brought forward from the fifteenth day to the fifth day. Compared with BG11, the decline stage also appeared on the 25th day and the 30th day. It can be seen that the difference in the proportion of the sludge extract could lead to the earlier appearance of every growth stage.

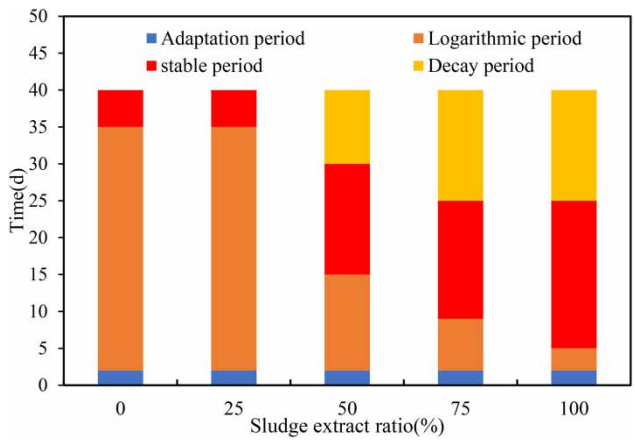

Figure 1 | The variation of growth stage distribution of *C. vulgaris* cultured in sludge extracts diluted by BG11 with different dilution ratios.

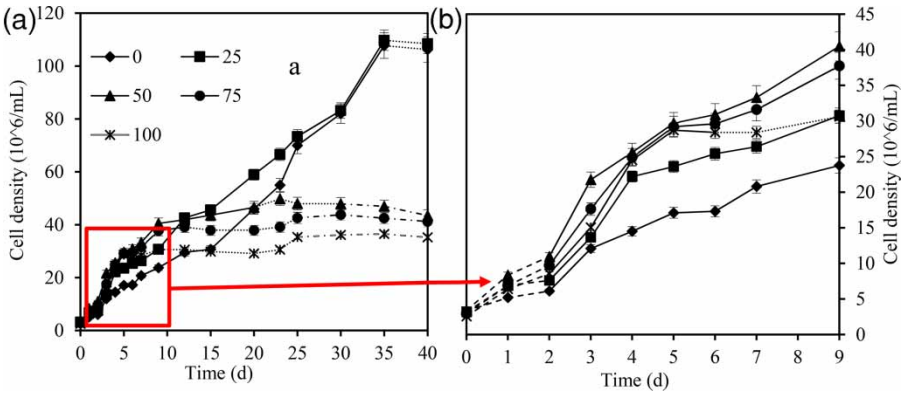

Figure 2 | The variation of cell density of *C. vulgaris* cultured in sludge extracts diluted by BG11 with the different dilution ratios. (a) The whole-cell density; (b) cell density on days 0–9).

It can be seen from Figure 2 that the change in cell density that can be observed is as follows: when the proportion of sludge extract is about 25%, the effect of promoting the *Chlorella* cells' proliferation occurred, and the maximum cell density was 109.8×10^6 cell/mL. When the ratio of the sludge extract was above 50%, the cell proliferation was inhibited, the maximum cell density decreased from 49.8×10^6 cells/mL to 36.5×10^6 cells/mL. The whole growth cycle consisted of an adaptation period of 0–2 days, and the cell density of *Chlorella* in this period has not changed too much, so the effect of sludge extracts on *Chlorella* was not obvious. The logarithmic period was 2–35 days, the *Chlorella* in BG11 grew rapidly, the cell density increased from 6.10×10^6 cells/mL to 107.8×10^6 cells/mL. From the different growth stages, the growth of *C. vulgaris* was promoted during the adaptation and logarithmic periods. In the stable and decay period, when the proportion of sludge extract increased to 50%, cell proliferation was inhibited greatly.

The analysis believes that some studies have shown that polyculture is the cultivation method that obtains the highest biomass yield (Chen *et al.* 2019). *Chlorella* mainly synthesizes biomass in the BG11 medium in autotrophic mode. Twenty five percent of the sludge extract promotes the growth of chlorella because the sludge extract contains rich organic carbon sources and nutrients. In the log phase, it can promote the growth of *Chlorella*, but there is still an inhibitory effect that makes the biomass similar to BG11. With the increase in the proportion of sludge extract, the inhibitory effect on the growth of *Chlorella* is enhanced. This may be due to the increase in sludge toxicity as the proportion of sludge extract increases, thereby inhibiting the growth of *Chlorella*. The heterotrophic metabolism promotion effect of 25% sludge extract is the best, and the biomass of chlorella in 100% sludge extract is at a lower level than other media, indicating that high-concentration nutrient conditions inhibit algae growth.

Starch

C. vulgaris stores carbohydrates mainly in the form of starch. The changes in starch content of chlorella cultured with different dilution ratios were studied, and the influence of sludge toxicity on the biochemical composition of chlorella was investigated. The results are shown in Figure 3.

Figure 3 shows that the concentration of total starch was lowest at the adaption stage among various growth stages, the average starch content decreasing by 0.26 μg/10^6 cells. By analysis, it is proven that due to the need for rapid synthesis of ribosomes, enzymes, ATP, and other adjustments to adapt to the extent of the sludge extracts, the activity of microalgae cell metabolism was strengthened, so *Chlorella* needed to degrade its starch-released energy to maintain the activities of algal cells. During the period of adaptation, namely before the 9th day, the total starch content in each group increased slowly, and the starch content in the unit cells was stable. Because of the logarithmic phase and the rapid proliferation of algal cells, it was found that the increase of starch content was caused by the increase of *Chlorella* cells. The average starch content and total starch increased at an accelerated speed from the 9th day to the 20th day and reached a peak value on the 20th day. With the increase in the proportion of the extract, the total amount of starch increased from 47.1 mg/L to about 102 mg/L, and the average starch content increased from 0.65 μg/10^6cells to 2.67 μg/10^6cells on the 20th day. Since the growth of cells in this stage was stable, cell density no longer significantly proliferated, so *C. vulgaris* cells achieved peak accumulation of metabolites in this period (Fabregas *et al.* 2001). During the period of 25–30 d, the groups with

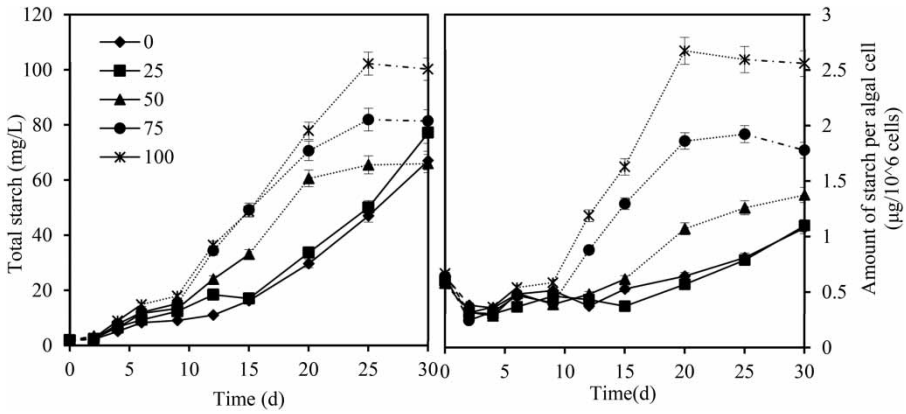

Figure 3 | The variation of total starch content and cell content of *C. vulgaris* with time under different sludge extract concentrations.

sludge extract ratios of more than 50% were in the decay period, and the total starch and average starch content were decreased, while the BG11 group and 25% sludge extracts group were still in the logarithmic period. So the amount of starch content of the two groups continued to accumulate, and the BG11 group accumulation rate was faster. At the end of the culture, the total amount of starch in the 100% sludge extracts group and BG11 group was similar, at about 100 mg/L.

The above changes in starch content can indicate that exogenous organics were absorbed by *C. vulgaris* to improve the accumulation of starch (Wang *et al.* 2015). By contrast, starch accumulation in BG11 was relatively slow due to the constant consumption in cell proliferation.

Polysaccharide

The changes in the polysaccharide content of cultured and domesticated *Chlorella* under different dilution ratios were investigated to provide a theoretical basis for the sludge extract to cultivate *Chlorella* to obtain high-efficiency biomass. The results are shown in Figure 4.

Figure 4 shows the changing polysaccharide content with time. Over the same period, the average polysaccharide content increased with the increase in the proportion of extract. After analysis, it was considered that the water quality was more suitable than BG11 for accumulation of polysaccharides. In the whole culture stage, the total amount of each group showed a slight increase at 0–2 d. The polysaccharide content at this time was mainly from the water quality contribution of the sludge extracts. However, because of the failure to make full use of the organic compounds in the mixed medium, the rate of intracellular polysaccharide consumption was greater than the rate of synthesis, which caused the decrease of polysaccharides. In a period of 2–15 days, all groups entered the logarithmic growth stage, the photosynthesis was strengthened with the increase of *C. vulgaris* chlorophyll content. The average

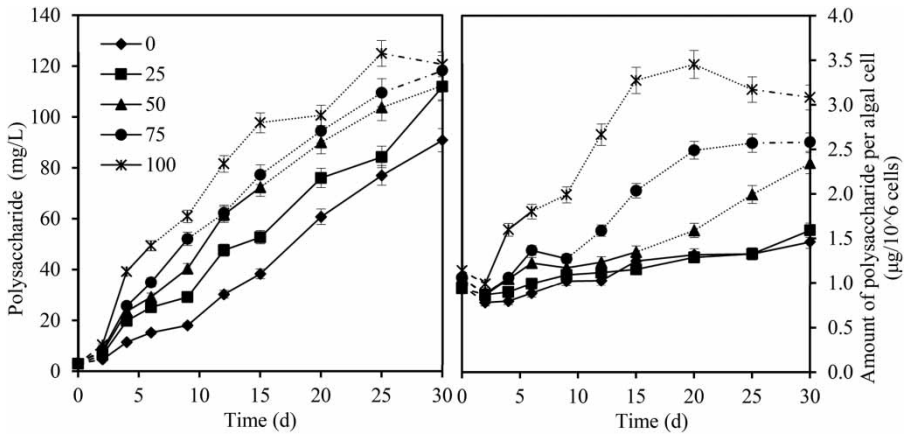

Figure 4 | The variation of total polysaccharide content and cell content of *C. vulgaris* with time under different sludge extract concentrations.

content of polysaccharides increased slowly in the beginning and then increased rapidly until the peak value at the 20th day (logarithmic period) (Liang *et al.* 2009). The peak values of average polysaccharide in BG11 group and 100% sludge extracts were respectively 1.32 μg/10^6cells and 3.45 μg/10^6cells. After 20 days, each group had entered the decline period, and combined with Figure 1 shows that the proportion of high sludge extracts could promote *C. vulgaris* to enter the decline period early. However, the photosynthetic rate decreased rapidly during the decline period (Ghosh *et al.* 2001), polysaccharide synthesis rate was less than the consumption and decomposition rate, so it was found that the polysaccharide content in the 75% sludge extracts group tended to be balanced, while it decreased in 100% sludge extracts group and continued to increase in the other groups.

Photosynthesis of *C. vulgaris*

From the above results, it was found that the algal cell density, total polysaccharide content, total starch content, average polysaccharide, and starch content were significantly different at various periods and groups; especially, the increasing ratio of sludge extracts was beneficial to the accumulation of starch and polysaccharide. Since starch and polysaccharide are the products of algal photosynthesis, the control of the sludge extract group and BG11 group via changes in photosynthetic activity is essential to determine the starch and polysaccharide content in each group.

Chlorophyll a content

The presence of additional organic substrate could significantly improve the photosynthetic rate (Zhang *et al.* 2016). Figure 5 illustrated that the total *chlorophyll* content of each group increased with the increase of the proportion of sludge extracts, especially when sludge extracts increased from 25% to 50% during 0–25 days. Furthermore, the *chlorophyll* content increment arrived maximum (the peak value was 17.1 mg/L) in the period of 4–15 days (logarithmic period). The increase of *chlorophyll* content in 100 and 75% sludge extract was also significant. The results showed that the density of algal cells increased rapidly and the intracellular matter began to accumulate rapidly during the logarithmic period. The total chlorophyll-*a* content correlated positively with that of the sludge extracts, which is due to the water quality enhancing the synthesis of photosynthesis or photosynthetic activity. After 25 days, the total *chlorophyll* content of the 75 and 100% sludge

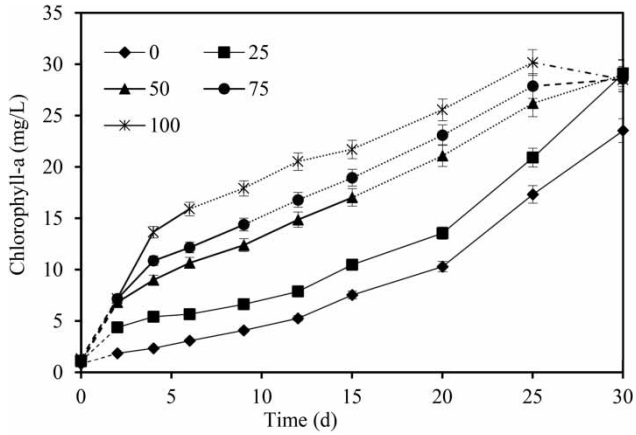

Figure 5 | The variation of Chlorophyll-a Content in *C. vulgaris* with time under different sludge extracts concentration.

extracts groups decreased because the high extraction groups entering the decline period advanced, and the other groups continued to rise. *Chlorophyll* is considered to be a photosynthesis machine, which could accept and convert the optical signal to start the water photolysis process (Voitsekhovskaja & Tyutereva 2015). Therefore, the difference in *chlorophyll* content can be shown as the difference between photosynthesis product accumulation (starch and polysaccharide).

Submicroscopic structure

To further verify the effect of the sludge extracts on the key organelles of photosynthesis in algal cells, the submicroscopic structure of BG11 and 100% sludge extracts were investigated, as shown in Figure 5. It can be seen from Figure 6 that when the *C. vulgaris* was cultured with 100% sludge extracts, the chloroplast became larger; the thylakoid slice was intact and more obvious than that in the BG11 group. Since the chloroplast is a place for photosynthesis, the thylakoid is a place for photoreaction, which contains the photosynthetic pigment and enzymes needed for photosynthesis (Dekker & Boekema 2005). It indicated that the photosynthesis of *C. vulgaris* in the sludge extracts was more pronounced, which is consistent with the results obtained in 2.2.1. In the cultivation of highly nutrient sludge extracts, it was observed that the cell volume became larger and the cytoplasm was rich, and there was no separation of the cytoplasmic membrane. Moreover, protein nuclear volume was also significantly larger, protein core was considered as the storage area of the early products of photosynthesis, with a large number of early products formed, it is constantly transformed into starch (Meyer

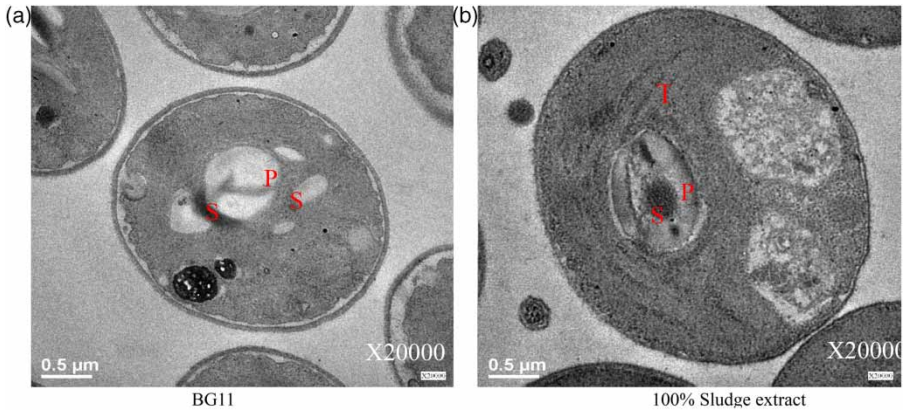

Figure 6 | Effect of sludge extracts on submicron structure of *C. vulgaris*. S - starch granules; P – pyrenoids; T - thylakoid.

et al. 2017; Zhan *et al.* 2018). Therefore, the increase of the protein core can be regarded as the increase of the early products of photosynthesis, which will lead to the increase of starch synthesis.

Effects of nutrient elements

C, N, P are the necessary nutrient elements for the growth of Chlorella, C provides nutrients for chlorella heterotrophs, N is an important component of *chlorophyll*, phosphate participate in various metabolic activities during the process of microalgae reproduction, and plays an important role in material metabolism, signal transduction, photosynthesis and membrane structure formation (Lippemeier *et al.* 2003; Martinez *et al.* 1999). Different concentrations and different forms of C, N, P will inevitably have an impact on *C. vulgaris*. NFY Tam *et al.* (Tam & Wong 1996) studied the effects of ammonia nitrogen concentration on the growth of *C. vulgaris*. It was found that the content of chlorophyll and protein in *C. vulgaris* was higher at higher ammonia nitrogen concentration. Pancha *et al.* (2014) found that the nitrogen limitation and sequential nitrogen starvation conditions significantly decrease the photosynthetic activity as well as crude protein content in the organism. Elsheek and Rady (El-Sheek & Rady 1995) found that the low concentration of P could affect the photosynthesis and chlorophyll content of algal. He *et al.* (He *et al.* 2011) found phosphorus, as an essential element for the growth and development of algae, directly participates in the various processes of photosynthesis. Phosphorus limitation will affect the level of photosynthetic phosphorylation, hinder metabolic synthesis, and decrease the photosynthetic rate. Therefore, this paper also compares the effects of different concentrations of nutrients in the sludge and BG11 on the metabolism of *Chlorella*.

Effects of different N and P concentrations on the metabolism of *C. vulgaris*

From Figure 7, it can be seen that in BG11 the nitrogen form was only nitrate nitrogen, the initial concentration was about 252 mg/L, and the utilization trend of nitrate decreased slowly. During the adaptation period and stable

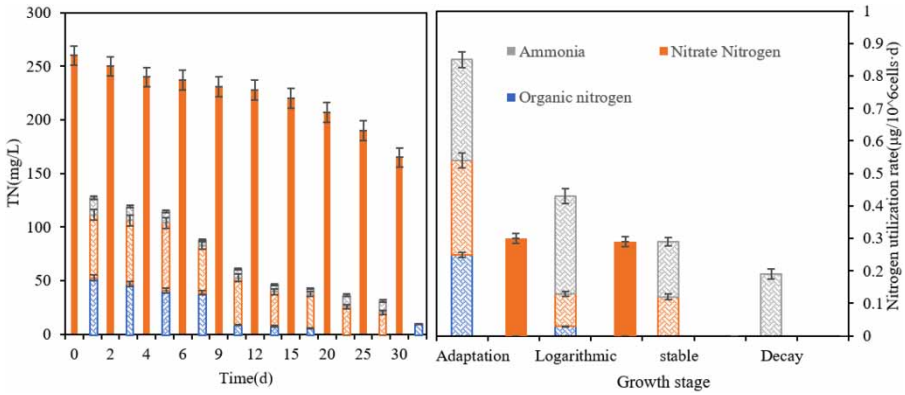

Figure 7 | Comparison of utilization and utilization rate of N by *C. vulgaris* in BG11 (solid line: BG11; dotted line: 100% sludge extract).

period, the nitrogen utilization rate was similar to about 0.29 μg/(10^6 cells·d). The nitrate-nitrogen concentration of BG11 on the 30th day was about 191 mg/L, which was reduced about 61 mg/L with regard to the beginning. In the 100% sludge extracts, the nitrogen forms were more diverse, the total nitrogen concentration was about 120 mg/L, the organic nitrogen concentration was about 18.5 mg/L, and the nitrate-nitrogen and ammonia nitrogen concentration were about 1:1. The initial concentration of total nitrogen decreased about 118 mg/L compared with BG11 but contained about 51.1 mg/L ammonia nitrogen. From the content point of view, in the first 9 days, nitrate-nitrogen content was almost unchanged, ammonia nitrogen and organic nitrogen content decreased by 43.9 mg/L and 8.38 mg/L respectively. From the utilization rates, the utilization rate of three kinds of nitrogen was similar in the adaption period, the utilization rate of TN was 0.89 μg /(10^6 cells·d), which was mainly due to the low density in the adaptation period. In the logarithmic period, the utilization rate of TN decreased, which may be due to the rapid increase of cell density, and the utilization rate of ammonia nitrogen was significantly greater than that of nitrate-nitrogen (Kamiya 1995). This is because the reduction of ammonia nitrogen can be directly used to save more organisms' energy (Kim *et al.* 2013), so *C. vulgaris* used ammonia nitrogen as priority, and some organic nitrogen could directly provide nutrients such as amino acids to *Chlorella vulgaris*. After 9 days, the content of organic nitrogen increased, and the supply of ammonia nitrogen was less. Then, the *C. vulgaris* began to consume the nitrate nitrogen, so the utilization rate of nitrate-nitrogen began to rise. At the 30th day, the nitrogen consumption in the water phase was complete; only about 11.1 mg/L of organic nitrogen was detected in the water phase.

The presence of phosphorus in the sludge and BG11 is mainly orthophosphate, as can be seen from Figure 8, the initial phosphorus concentration in BG11 was about 6.37 mg/L, and gradually decreased to about 0.27 mg/L by the 30th day. The initial concentration of orthophosphate in the sludge was about 18.3 mg/L and dropped rapidly to about 13.3 mg/L in the first 6 d, then tended to be stable, and finally decreased by about 6.52 mg/L after 15 days. The results indicated that *Chlorella vulgaris* could regulate the uptake capacity of P; in different initial concentrations of P, *Chlorella vulgaris* could accumulate the P quickly and use P slowly in sludge extracts (Ren *et al.* 2017). In the whole life cycle of microalgae, the utilization of orthophosphate in BG11 was about 6.10 mg/L, while the utilization of orthophosphate in sludge extract was about 13.1 mg/L. From the utilization rates, the utilization rate of P in sludge extracts was greater than BG11 in all growth stages. In the whole life cycle of the 100% sludge extract group, the utilization rate of P was the fastest during the adaption period, the logarithmic phase was second, the utilization rate of P was about 0.02 μg/(10^6 cells·d) in the stable period, which was similar to the decline period. The results show that the rich nutrients in the sludge extract can promote the growth of chlorella.

Effects of TOC on the nutritional way of *Chlorella vulgaris*

The TOC in the sludge extract is the carbon source for the assimilation and absorption of *Chlorella* and the main source of the initial accumulation of biomass of *Chlorella*. By comparing the changes of TOC content in the BG11 group and the 100% sludge extract, the difference in the utilization of organic matter in the two media by *Chlorella* was investigated, and the results are shown in Figure 9.

There was nearly no organic carbon source in BG11, so the concentration of TOC in BG11 was only about 38 mg/L and was unchanged in the first 9 days. Due to the secretion of organic matter, the concentration of TOC was increased

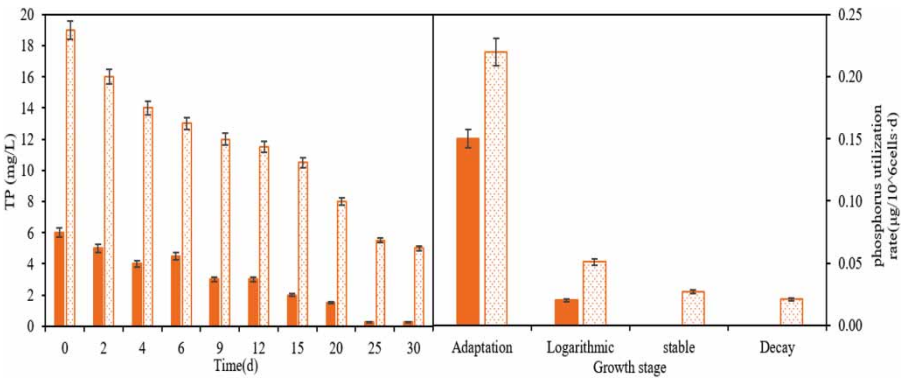

Figure 8 | Comparison of utilization and utilization rate of P by *C. vulgaris* in BG11 (Solid line: BG11; dotted line: 100% sludge extract).

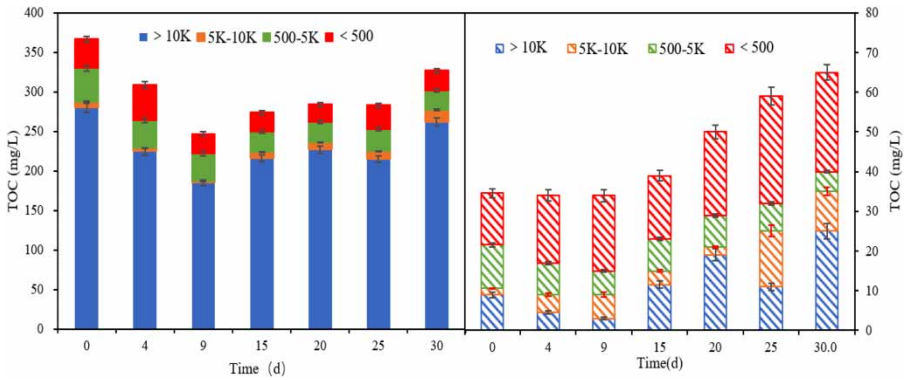

Figure 9 | Variation of total TOC and different molecular weight of TOC (solid line: 100% sludge extract; dotted line: BG11).

in the later stages. The concentration of TOC in the 100% sludge extract group was very high, reaching about 365 mg/L in the beginning, while it showed a decreasing trend in the first 9 days, and the decrease was about 117 mg/L on the 9th day. The results showed that the growth of *C. vulgaris* in BG11 was mainly autotrophic, while in the sludge extracts it was mixotrophic; under the condition of mixotrophy, the growth of *C. vulgaris* could be both autotrophic and heterotrophic, so the concentration of organic matter in the sludge was decreased, and its growth rate was regarded as the sum of autotrophic and heterotrophic (Li *et al.* 2014; Ji *et al.* 2015). After 15 days, TOC showed an upward trend, mainly due to the excessive synthesis of nutrients causing the amino acids and polysaccharides, proteins, and other organic matter transferred to the water phase, the other part being due to *Chlorella* decline. From the view of different molecular weight, the different molecular weights in the sludge extracts decreased, the most obvious being the organic matter of molecular weight >10 k, which decreased from 300 to 200 mg/L. By analysis, it is believed that this was due to the maximum concentration of organic matter >10 k in the sludge extracts leading to the priority degradation of these organic compounds to supplement the energy, and reduce the biomass loss caused by pure respiration in the dark time. Therefore, polysaccharide and starch production increased (Andrade & Costa 2007; Park *et al.* 2012b).

Combined with the growth of *Chlorella* and the changing trend of TN, TP, and TOC content in the medium, the sludge extract was selected to culture *Chlorella vulgaris* on the 15th day of the stable period for analysis. From 0 to 15 days, the utilization rates of TN, TP, and TOC were 15.4%, 52.6%, and 21.6% respectively, and the starch content increased by 48.2 mg/L, polysaccharide content increased by 97.4 mg/L, and chlorophyll-a content increased by 22.5 mg/L. It shows that the N, P, and C elements in the sludge extract are partially absorbed by *Chlorella vulgaris*, and converted into starch and polysaccharides in the algae cells for the growth of *Chlorella vulgaris*.

CONCLUSIONS

The growth period of *C. vulgaris* could be shortened about 10 days when the culture medium was alternated from BG11 to 100% sludge extracts. Through the analysis of algal cells' ultrastructures, it was shown that the cells of *Chlorella* cultivated with pure sludge extracts were much larger than those in the culture that contained BG11, and the thylakoid membrane was more clear. The total amount of starch and polysaccharide was up to 103 and 125 mg/L, respectively. The results indicated that the sludge extract may be applied as a culture medium for *C. vulgaris*.

This will effectively solve the treatment and disposal of the remaining sludge, the main by-product in the biological treatment of sewage, and realize the reduction and resource utilization of the remaining sludge. However, there are also huge challenges and limitations. The cost of processing the cultivated *Chlorella* is too high. At the same time, the cultivation of *Chlorella* is currently only suitable for laboratory research, and the means to apply *Chlorella* to practice are also limited. Therefore, in future work, we will study the resource utilization of cultivated *Chlorella*, such as biodiesel and biofertilizer.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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