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Bioremediation of *Pyropia*-processing wastewater coupled with lipid production using *Chlorella* sp.

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HIGHLIGHTS

- *Pyropia*-processing wastewater (PPW) was directly reutilized by *Chlorella* sp.
- *Chlorella* cells could grow well in concentrated PPW.
- *Chlorella* strains displayed great potential in nutrient removal from undiluted PPW.
- *Chlorella* strains exhibited higher biomass and lipid productivity in undiluted PPW.
- PPW would be an alternatively cost-effective medium for microalgae cultivation.

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ABSTRACT

Pyropia-processing wastewater (PPW) contains diverse organic nutrients and causes environmental pollution. To explore the nutrient removal efficiency and growth performance of *Chlorella* sp. on PPW, the cultures were conducted in different culture substrates. Results showed that, after 7 days of incubation, the removal rates of total nitrogen (TN), total phosphorus (TP) and phycobiliprotein (PP) all reached more than 90% by cultivating *Chlorella* sp. C2 and *C. sorokiniana* F-275 in PPW. The chemical oxygen demand (COD) removal efficiencies could be over 50%. Meanwhile, the increments of biomass in two tested *Chlorella* strains were 1.39 and 4.89 times higher than those of BG11 and BBM substrates and the increases in lipid productivity were 1.34 and 10.18- fold, respectively. The C18:3 fatty acid proportions were markedly reduced by 27.89% and 29.10%. These results suggest that *Chlorella* sp. could efficiently reduce various nutrients in PPW and simultaneously accumulate higher biomass with higher biodiesel characteristics.

1. Introduction

Pyropia is the most valuable cultivated seaweed in the world (Kim et al., 2017; Venkatraman and Mehta, 2019). According to the statistical data in 2017 by Food and Agriculture Organization of the United Nations (FAO), the annual value of *Pyropia* was approximately $0.95 billion. The strains of *Pyropia yezoensis*, *P. haitanensis* and *P. tenera* are the major economic species, which are mainly cultivated in China, Korea and Japan, accounting for 99.99% of total production in the world. *Pyropia*-based products are made by primary and secondary processing. The primary processing procedures are mainly composed of rinse, cut, purification, cake formation of *Pyropia*, dehydration and drying. The wastewater used in this research is generated from the primary processing procedures of *P. yezoensis*, which causes environmental pollution since massive organic substances, such as polysaccharides and phycobiliprotein, are separated from *Pyropia* cells and accumulate in

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wastewater. According to our survey, nowadays, there are approximately 1000 machines used for the primary processing of *Pyropia* in China. The generated wastewater of each machine is about 100–150 tons per day. It takes more than 4 months each year for *Pyropia*-processing. These data indicate that there is a large amount of *Pyropia* wastewater being produced every year, which is a noteworthy environmental issue. Moreover, the wastewater treatment based on microalgae has been intensively considered as an increasingly promising method to mitigate environmental contamination (Li et al., 2019).

Microalgae could biosynthesize substantial amounts of valuable active substances such as lipids, polyunsaturated fatty acids, pigments, proteins as well as carbohydrates, which has been regarded as an importantly renewable bio-resource feedstock with attracted extensive concerns (Georgianna and Mayfield, 2012; Ho et al., 2020; Hu et al., 2018; Yarnold et al., 2019). To date, some microalgae species have been utilized to produce functional foods, health products, pharmaceuticals, cosmetics, animal feeds, and so on (Katiyar et al., 2017; Mata et al., 2010). The large-scale culture of microalgae requires massive consumptions of water and nutrients, which results in the increased production cost, severely limiting the development of microalgae industry. This also triggers that the production of microalgal biomass cannot meet the market demand at present. It was confirmed that the combination of microalgae cultivation and wastewater treatment is an efficient way to reduce the production cost of microalgae culture (Nagarajan et al., 2020); meanwhile, the environmental pollution caused by wastewater discharge can be friendly alleviated, even solved (Li et al., 2019).

Many studies reported that some algae could grow and accumulate higher biomass and high-value compounds in certain wastewaters, in addition to their great performance in nutrients removal (Arif et al., 2020; Guldhe et al., 2017; Yin et al., 2020). For instance, the improved biomass and lipid contents of *Arthrospira platensis* were gained in dairy farm wastewater (Hena et al., 2018). The strain of *Tetraselmis* sp. CTP4 exhibited 100% removal rates of total nitrogen and phosphorus with high biomass productivity when cultured in urban wastewater (Schulze et al., 2017). The enhanced growth and lipid productivity of *Micratinium reisseri* was observed in diluted municipal wastewater with mine wastewater, the nutrient absorption of which reached 35.9 mg TN/L and 5.4 mg TP/L (Ji et al., 2014). Arashiro et al. (2020) pointed out that *Nostoc* sp., *A. platensis* and *Porphyridium purpureum* could efficiently remove COD, inorganic nitrogen and PO_4^{3-}-P in food-industry wastewater and simultaneously accumulate massive phycobiliproteins. However, there are no reports to research *Pyropia*-processing wastewater treatment based on microalgae cultivation.

It was found that *Chlorella* strains have strong adaptabilities to various environments, and displayed great potentials for reusing waste resources for biomass production (Chen et al., 2018; Cheng et al., 2020; Li et al., 2019). Su et al. (2011) reported that the removal efficiencies of *C. pyrenoidosa* on COD, TN, NH_4^+-N and TP in soybean processing wastewater could reach 77.8%, 88.8%, 89.1% and 70.3% after culturing for 120 h in fed-batch. Meanwhile, *C. pyrenoidosa* could obtain 0.64 g/L/d of biomass productivity and 0.40 g/L/d of lipid productivity. *C. pyrenoidosa* also grew well in co-substrates of anaerobic digested starch wastewater and alcohol wastewater, where the highest biomass and lipid productivities were 0.63 and 0.13 g/L/d with the COD, TN and TP removal rates of 75.78%, 91.64% and 90.74% (Yang et al., 2015). Ramsundar et al. (2017) found that *C. sorokiniana* could remove 44.03%, 94.29% and 83.30% of COD, NH_4^+-N and PO_4^{3-}-P in anaerobic tank centrate of municipal wastewater under mixotrophic culture, and attained 77.14 and 24.91 mg/L/d of biomass and lipid productivities. Gao et al. (2018) pointed out that *Chlorella* sp. accumulated higher biomass and lipid productivities (77.7 and 20.4 mg/L/d) in aerated seafood processing wastewater with 4.98 and 1.91 mg/L/d of nitrogen and phosphorus removal rates.

The pre-experiment results revealed that the markedly improved growth of *Chlorella* cells was observed in untreated PPW. To further probe the nutrient removal rate and utilization of microalgae on PPW, the water quality parameters of PPW and the lipid accumulations of tested algal cells were analyzed in this study. This research aims to illustrate the feasibility of microalgae bioremediation on PPW, and explore an alternative low-cost measure to cultivate microalgae for high biomass and lipid production approached from recycling PPW resource.

2. Materials and methods

2.1. Pyropia-processing wastewater and pretreatment

Pyropia-processing wastewater (PPW) was collected from *Pyropia*-processing plant at Lianyungang city of Jiangsu province in China. The wastewater was first centrifuged at 4000 g for 10 min to remove *Pyropia* residues. Then, the supernatant was applied to culture microalgae, which was sterilized for 30 min with UV light before inoculating.

2.2. Algal strains and cultivation conditions

Two microalgal strains of *Chlorella* sp. C2 and *C. sorokiniana* F-275, provided by Institute of Hydrobiology, Chinese Academy of Sciences, were used in this research. The tested strains were separately cultivated in Blue-Green medium (BG11) (Stanier et al., 1971) and Bold's Basal medium (BBM) (Stein, 1973) with a 200 mL of working volume in 250 mL Erlenmeyer flask. Microalgal cells were grown at 25 ± 3 °C with a light intensity of 45 μmol/m^2/s and a 14/10 h light/dark cycle in illumination incubators. Each flask was manually shaken three times a day.

To explore the nutrient removal efficiencies of microalgae on PPW, and the growth performance of *Chlorella* sp. in PPW, the algal cells were cultivated in five different culture substrates, which were BG11, BBM, PPW, and the co-substrates of PPW supplemented with BG11/BBM nutrients (PPW + BG11/BBM), respectively. The cultures in BG11/BBM were considered as the control. The initial inocula of both microalgae were ~0.05 g/L of biomass. Each set of culture conditions was performed in triplicate.

2.3. Measurement of nutrients in PPW and culture supernatant

The culture supernatants of different substrates after cultivating two *Chlorella* strains were obtained by centrifugation at 6800 g for 10 min. The contents of chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), ammonium nitrogen (NH_4^+-N) and other basic mineral elements in PPW and all culture supernatants were analyzed according to the Chinese State Environmental Protection Agency Standard methods (SEPA, 2002). The phycobiliprotein (PP) contents of PPW and culture supernatants were assessed by measuring the absorbance at 562 nm, 615 nm and 652 nm with a Microplate Reader (Thermo Multiskan GO, America) (Bennett and Bogorad, 1973). The calculated equations were as following (1)–(4).

$$\text{Phycocyanin}(\text{PC}, \text{mg/mL}) = 0.187 \times \text{OD}_{615} - 0.089 \times OD_{652} \quad (1)$$

$$\text{Allophycocyanin}(\text{APC}, \text{mg/mL}) = 0.196 \times \text{OD}_{652} - 0.041 \times \text{OD}_{615} \quad (2)$$

$$\text{Phycoerythrin}(\text{PE}, \text{mg/mL}) = 0.104 \times \text{OD}_{562} - -0.253 \times PC - 0.088 \times APC \quad (3)$$

$$\text{Phycobiliprotein}(\text{PP}, \text{mg/L}) = (\text{PC} + \text{APC} + \text{PE}) \times 1000 \quad (4)$$

The total sugar content of PPW was determined with a modified Phenol-sulfuric acid method (Dubois et al., 1956). One mL of PPW supernatant was put into a 15 mL glass test tube, and mixed with 0.5 mL of 6.0% (v/v) phenol solution and 2.5 mL of concentrated sulfuric acid. Then the mixture was placed into a water bath at 45 °C for 30 min. The mixture absorbance at 490 nm was recorded by Multiskan GO Microplate Reader. The contents of amino acids in PPW were determined

using an amino acid analyzer (Hitachi L-8900, Japan).

2.4. Analysis of microalgae growth

Growth kinetics of microalgae in different culture substrates were displayed as dry cell weight (DCW), which was measured by a gravimetric method. The cultures was collected on 0.45 μm mixed fiber filter membrane with a vacuum pump, and then dried at 65 °C for 24 h with constant weight to calculate DCW. Meanwhile, the specific growth rate (μ) was calculated by the equation (5).

$$\mu(/d) = (lnX_2 - lnX_1)/(t_2 - t_1) \quad (5)$$

where X_1 and X_2 are the DCW at time t_1 and t_2, respectively.

The contents of chlorophylls in microalgal cells were measured by a modified methanol method (Lichtenthaler and Wellburn, 1983). A sample of 1 mL cells was centrifuged at 6800 g for 10 min, removing the supernatant, and then added the same volume of concentrated methanol with extracting for 24 h at 4 °C in darkness. Absorbance of methanol-extracts at 653 and 666 nm was recorded by Multiskan GO Microplate Reader.

2.5. Determination of lipid content and fatty acid composition in microalgae

Neutral lipid content in treated cells was evaluated by a modified Nile red staining method (Kimura et al., 2004). The detailed processes were as described by Zheng et al. (2017). Fluorescence intensities of samples were measured by a Microplate Reader (Tecan Infinite M1000 Pro, Switzerland). The excitation and emission wavelengths were 480 and 575 nm, respectively.

All microalgal cells grown at stationary phase were harvested with centrifugation at 6800 g for 10 min, and then dried by a vacuum freeze dryer (Labconco FreeZone Plus, America) for 48 h. The total lipid contents in cultures were determined by a modified Chloroform - methanol method (Bligh and Dyer, 1959). An aliquot of 50 mg dried cells was used to extract total lipids. Each sample was mixed with 3 mL chloroform - methanol (2:1, v/v) solution in a 15 mL centrifuge tube and sonicated for 20 min in an ice bath. Then the supernatants were collected with centrifugation at 6800 g for 10 min. Above extracted processes were repeated two times for completely extracting lipids. The collected supernatants were placed into an oven at 65 °C till to constant weight. The total lipid contents of all samples were expressed as DCW percentages (% DCW).

To analyze the fatty acid profile of cultures with a gas chromatography (GC) method, fatty acids were primarily converted into the fatty acid methyl esters (FAMEs) with 2% (v/v) H_2SO_4-CH_3OH solution. About 25 mg dried microalgal cells were mixed with 2 mL H_2SO_4-CH_3OH in a 15 mL glass centrifuge tube and incubated at 80 °C for 1 h in a water-bath with a magnetic stirring (Kumari et al., 2011). Then each 1 mL of ultrapure water and chromatographic n-hexane was added into the glass tube to isolate the FAMEs. The n-hexane phase was transferred to a 2 mL GC sampling vial after centrifugation at 1700 g for 5 min to determine fatty acid constituents by GC (Agilent Technologies 7890A, America) equipped with a flame ionization detector (FID). A sample of 1 μL FAMEs was injected into a GC column (CP7489 CP-Sil 88 for FAME 100 m × 0.25 mm × 0.2 μm), where hydrogen was considered as the carrier gas with a 25 mL/min flow rate. The injector and detector temperatures were separately 250 °C and 240 °C. The temperature control procedure was set as the initial temperature of 190 °C maintaining for 0 min, then rising to 220 °C with a 4 °C/min rate and remaining at 220 °C for 35 min. Chromatograph peaks of all samples were identified by the retention time of a Supelco 37 component FAME mix (Sigma - Aldrich). The content of each fatty acid was quantified according to the percentage of each peak area accounting for the total FAMEs peak areas. All samplings were carried out in triplicate.

2.6. Statistical analysis

All data were expressed as means ± standard deviation (SD) and evaluated the significance by one-way analysis of variance (ANOVA) at a 5% level with SPSS version 16.0 (SPSS Inc., America). All figures were created by Origin 2020b (OriginLab Corporation, America).

3. Results and discussion

3.1. Characteristics of PPW

The physicochemical parameters of *Pyropia*-processing wastewater (PPW) were shown in Table 1. PPW was composed of diverse organic substances and mineral elements. The contents of total sugar and amino acid reached 94.47 and 287.36 mg/L, respectively. Phycobiliprotein content was also analyzed, which was around 22.58 mg/L. It can be seen that calcium (Ca) was the most abundant mineral element in PPW, which was about 253.25 mg/L, followed by magnesium (Mg, 149.75 mg/L). The contents of total nitrogen (TN), total phosphorus (TP) and potassium (K) were 47.92, 3.90 and 83.50 mg/L, respectively. PPW also contained a few trace elements, such as Fe and Zn. Compared with the nutrient components of BG11 and BBM media, the mineral nutrients in PPW could almost meet the demands of microalgae grown in BG11 and BBM media. Besides, the pH value of PPW was about 6.80, which is appropriate for most microalgae growth. A higher concentration of chemical oxygen demand (COD) with ~523.08 mg/L was observed in PPW. It would be ascribed to the fact that PPW contained massive organic substances such as sugars and proteins. Various studies reported that there was better growth performance for some microalgae when exogenous organic carbon and nitrogen sources were supplemented in culture solution (Zhang et al., 2015; Zheng et al., 2016, 2017). Above results indicate that PPW might be a potential medium for microalgal biomass production.

3.2. Growth changes of Chlorella sp. Cultivated in PPW

Two tested *Chlorella* strains showed similar growth responses to different culture substrates. As shown in Fig. 1, the biomass and biomass productivity of *Chlorella* cells cultivated in PPW-included media were markedly higher than those of the cultures in BG11/BBM. The maximal biomass and biomass productivity of microalgal cells were acquired under the conditions of PPW + BG11/BBM, followed by PPW substrate. Compared with the cultures in BG11, the biomass and biomass productivity of *Chlorella* sp. C2 cultivated for 7 days were increased by 1.39 and 1.93 times under the substrate of PPW, and 1.74 and 2.41- fold in PPW + BG11 (Fig. 1a and c). The biomass (536.11 mg/ L) and biomass productivity (68.76 mg/L/d) in PPW + BG11 medium were 14.34%and 16.23% higher than those of PPW. In *C. sorkiniana* F-275, under PPW and PPW + BBM media, the biomass were promoted by 4.89 and 5.52 times in contrast with BBM cultures (Fig. 1b), and biomass productivity enhanced by 11.55 and 13.06- fold (Fig. 1d). The biomass (552.78 mg/ L) and biomass productivity (71.98 mg/L/d) in PPW + BBM were improved by 10.86% and 12.04% compared with PPW cultures. Interestingly, the optimal values of biomass and biomass productivity in

Table 1
Physicochemical characteristics of PPW.

Parameters	Content	Parameters	Content
pH	6.8 ± 0.25	K (mg/L)	83.50 ± 22.25
Total sugars (mg/L)	94.47 ± 7.90	Ca (mg/L)	253.25 ± 43.71
Amino acids (mg/L)	287.36 ± 62.91	Mg (mg/L)	149.75 ± 18.08
Phycobiliprotein (mg/L)	22.58 ± 2.44	Fe (mg/L)	1.45 ± 0.32
COD (mg/L)	523.08 ± 36.14	Mn (mg/L)	0.21 ± 0.16
TN (mg/L)	47.92 ± 12.88	Cu (mg/L)	< 0.05
TP (mg/L)	3.90 ± 0.78	Zn (mg/L)	1.50 ± 0.34
NH_4^+-N (mg/L)	5.56 ± 2.23	B (mg/L)	< 0.2

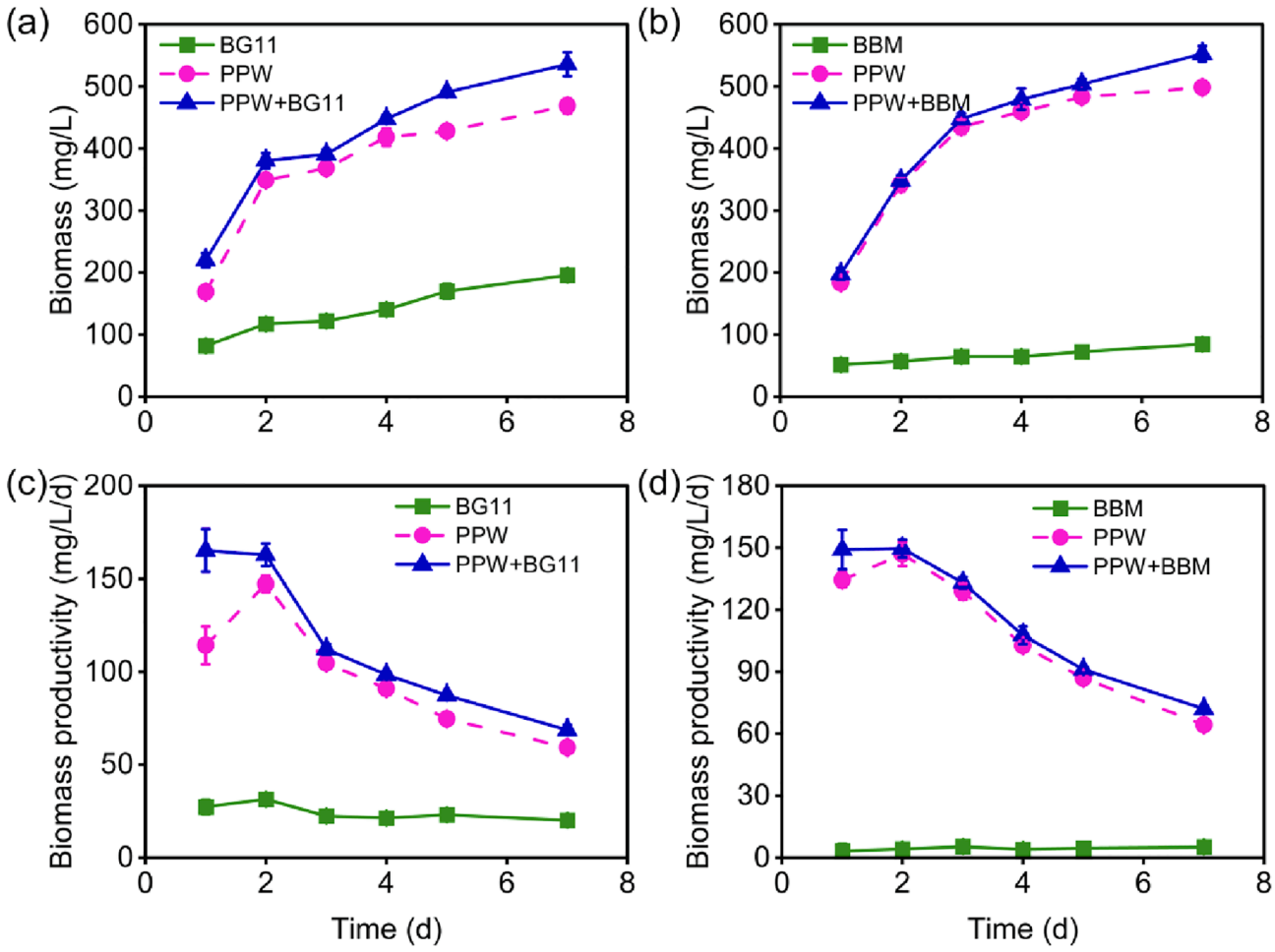

Fig. 1. Growth kinetics of two tested *Chlorella* strains under different culture conditions. (a) and (b) are the changes of biomass; (c) and (d) are the variations of biomass productivity. BG11, BBM, PPW, PPW + BG11 and PPW + BBM represent that *Chlorella* strains were cultured in BG11 medium, BBM medium, *Pyropia*-processing wastewater and the co-substrate of PPW supplemented with BG11 or BBM medium nutrients, respectively; The same as below.

C. sorkiniana F-275 were slightly higher than those of *Chlorella* sp. C2. This result reveals that supplement of BBM medium components in PPW might be conducive to gain higher biomass for *Chlorella* strains.

Similarly, the highest specific growth rates of two *Chlorella* strains were achieved in co-substrates of PPW + BG11/BBM, which were dramatically higher than that of BG11/BBM (Table 2). But, there were no significant differences between PPW and PPW + BG11/BBM. During the culture time, the increments of specific growth rate of *Chlorella* sp. C2 cultured with PPW and PPW + BG11 substrates were 0.68–1.77 and 0.79–2.43 times higher than those of BG11 cultures (Table 2). For *C. sorkiniana* F-275, they were improved by 3.23–20.41 and 3.41–21.64 times in comparison with the cultures in BBM (Table 2). The observed discrepancy between two algal strains on specific growth rate would be triggered by the fact that the growth of *C. sorkiniana* F-275 in BBM was

Table 2
The changes of specific growth rate and chlorophylls of two tested *Chlorella* strains in different culture substrates.

Parameter	Time (d)	*Chlorella* sp. C2			*C. sorokiniana* F-275		
		BG11	PPW	PPW + BG11	BBM	PPW	PPW + BBM
Specific growth rate (/d)	1	0.41 ± 0.05c	1.13 ± 0.06b	1.39 ± 0.05a	0.06 ± 0.07b	1.32 ± 0.02a	1.40 ± 0.05a
	2	0.38 ± 0.03b	0.93 ± 0.01a	0.97 ± 0.02a	0.08 ± 0.03b	0.97 ± 0.02a	0.98 ± 0.01a
	3	0.27 ± 0.02b	0.64 ± 0.01a	0.65 ± 0.01a	0.09 ± 0.02b	0.73 ± 0.01a	0.74 ± 0.01a
	4	0.24 ± 0.01b	0.51 ± 0.01a	0.53 ± 0.00a	0.07 ± 0.01b	0.56 ± 0.00a	0.57 ± 0.01a
	5	0.23 ± 0.01b	0.41 ± 0.00a	0.44 ± 0.00a	0.08 ± 0.00b	0.46 ± 0.00a	0.47 ± 0.00a
	7	0.18 ± 0.01b	0.31 ± 0.00a	0.33 ± 0.01a	0.08 ± 0.00b	0.33 ± 0.00a	0.35 ± 0.00a
Chlorophyll *a* + *b* (mg/L)	1	3.15 ± 0.25b	5.29 ± 0.34a	5.06 ± 0.05a	3.92 ± 0.21b	5.19 ± 0.24a	5.97 ± 0.42a
	2	3.95 ± 0.19c	8.36 ± 0.23a	7.32 ± 0.60b	4.17 ± 0.21b	8.82 ± 0.64a	9.29 ± 0.46a
	3	4.74 ± 0.12b	10.11 ± 0.22a	10.35 ± 0.50a	4.42 ± 0.26b	9.44 ± 0.52a	9.24 ± 0.47a
	4	4.90 ± 0.07b	10.29 ± 0.05a	10.92 ± 0.50a	4.48 ± 0.26b	9.68 ± 0.45a	9.36 ± 0.40a
	5	4.83 ± 0.06b	11.22 ± 0.31a	11.15 ± 0.41a	4.24 ± 0.25b	9.75 ± 0.36a	9.57 ± 0.45a
	7	5.27 ± 0.05b	11.42 ± 0.17a	11.36 ± 0.15a	4.74 ± 0.30b	10.58 ± 0.60a	10.28 ± 0.55a

BG11 and BBM represent that *Chlorella* strains were cultured in BG11 or BBM medium, which were considered as the control; PPW represents algal cells were cultivated in *Pyropia*-processing wastewater; PPW + BG11 and PPW + BBM indicate microalgal cells were cultured in the co-substrate of PPW supplemented with BG11 or BBM medium nutrients; The different letters in each column represent the differences are significant at $p < 0.05$; The same as below.

significantly slower than that of *Chlorella* sp. C2 in BG11.

As shown in Table 2, the changes of chlorophylls in *Chlorella* sp. under different culture substrates also exhibited the marked increase trends with the increasing culture time. Chlorophyll $a+b$ contents in PPW and PPW + BG11/BBM substrates were obviously higher than that of BG11/BBM. However, the differences were non-significant for the cultures between PPW and PPW + BG11/BBM. The maximum chlorophyll $a+b$ content (11.42 mg/L) in *Chlorella* sp. C2 was obtained at the end of incubation under the condition of PPW, followed by PPW + BG11 (11.36 mg/L). They were improved by 1.17 and 1.16 times compared with BG11 medium. Similarly, the optimum content of chlorophyll $a+b$ (10.58 mg/L) in *C. sorkiniana* F-275 was also gained on the 7th day of cultivation in PPW substrate, subsequently PPW + BBM (10.28 mg/L). They were raised by 1.23 and 1.17- fold in contrast with the cultures of BBM.

The above results indicate that *Chlorella* strains could grow well and accumulate higher biomass when cultivated in PPW substrate. Supplementing PPW with the nutrients of BG11/BBM medium could slightly enhance the growth of *Chlorella* cells. This should be attributed to the rich nutrients of PPW, particularly organic substances such as sugars and proteins. PPW was also abundant in mineral elements as described in Section 3.1. It was confirmed that mineral elements have importantly positive impacts on the improved microalgae growth and high-value compounds production (Guldhe et al., 2017; Nagarajan et al., 2020). The sugars in PPW might be mostly porphyran, which is a sulfated polysaccharide mainly contained various galactose residues and ester sulfate (Zhang et al., 2004). Galactose is one of constituents of microalgal cell wall, and also is a carbon source to stimulate microalgae growth (Bernaerts et al., 2018; Zhang et al., 2014). It was also found PPW contained diverse amino acids, where the contents of aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), alanine (Ala) and leucine (Leu) were more than 20 mg/L (data not shown). Asp was the most abundant amino acid in PPW, which was 38.20 mg/L, followed by Glu (35.83 mg/L). Amino acids can be assimilated by some algae as nitrogen sources, which exhibit great effects on the growth and active substance accumulation of microalgae (Nagarajan et al., 2020). Zhang et al. (2015) pointed out that the marked improvement on biomass of *C. pyrenoidosa* was observed by addition of exogenous Asp and arginine (Arg) in medium, which could significantly improve the cellular protein content under the condition of nitrogen deficiency. The content of Arg in PPW was also analyzed in this research, which was 14.95 mg/L (data not shown). These results suggest PPW is an alternatively cost-effective medium for the production of microalgae biomass.

3.3. Nutrients removal efficiencies of Chlorella sp. On PPW

Growing two tested *Chlorella* strains in different culture substrates, the significant reductions of COD concentrations in PPW and PPW + BG11/BBM were observed (Fig. 2a and b). And the COD removal rates exhibited marked increase tendencies during the culture time (Fig. 2c and d). By cultivating *Chlorella* sp. C2, the lowest COD concentration (236.75 mg/L) in PPW was observed on the 7th day of culture (Fig. 2a). The removal rate of COD was 52.90% (Fig. 2c). Unexpectedly, the COD concentration cannot be further reduced by addition of BG11

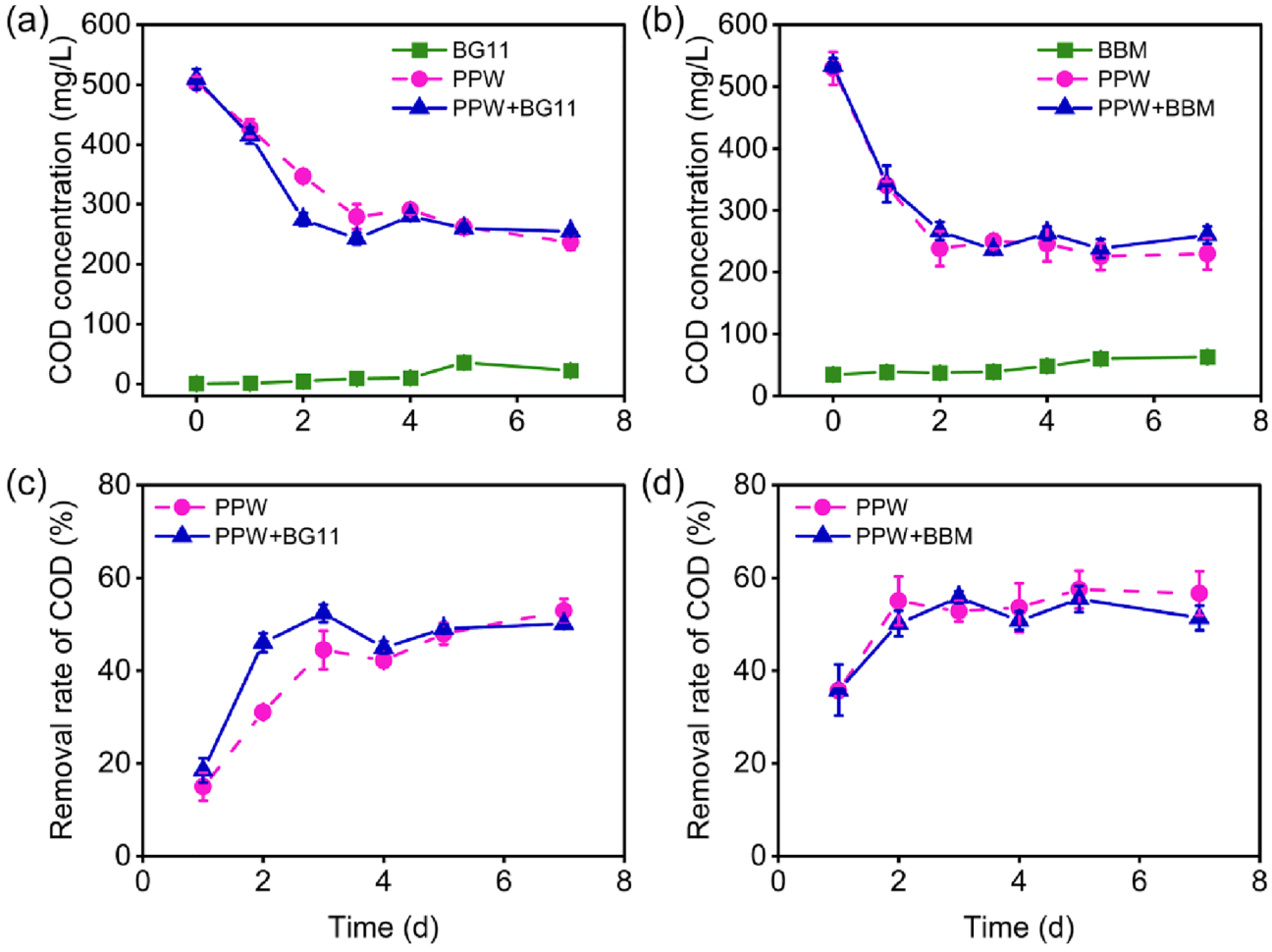

Fig. 2. The removal efficiencies of two tested *Chlorella* strains on chemical oxygen demand (COD) in different culture substrates. (a) and (b) indicate the changes of COD concentrations; (c) and (d) represent the variations of COD removal rates.

medium nutrients in PPW. The minimal COD concentration (242.61 mg/L) in PPW + BG11 was achieved at the third day of incubation, the removal efficiency of which was 52.33%. By culturing *C. sorokiniana* F-275, the minimum COD concentration (225.07 mg/L) in PPW was recorded after 5 days of cultivation with 57.47% of COD removal rate (Fig. 2b and d). The reduction cannot be further enhanced by supplementing exogenous nutrients. The minimal COD concentration (236.06 mg/L) in PPW + BBM was obtained on the third day of incubation, the removal efficiency of which was 55.80% (Fig. 2d).

Interestingly, for two *Chlorella* strains, the rapid decreases of COD in PPW-contained substrates were primarily occurred in the first three days. It was most probably that *Chlorella* cells exhibited higher specific growth rates within the first three days, which triggered that substantial amounts of organic nutrients in culture substrates were fast consumed. Based on above results, it can be concluded that two tested *Chlorella* strains could significantly reduce the COD concentration of PPW. There was no obvious difference between PPW and PPW + BG11/BBM on removal efficiency of COD. The marked improvement of COD removal rate cannot be obtained by supplement of BG11/BBM constituents. It might be attributed that the nutrients of PPW could satisfy the requirements of *Chlorella* cells growth. Alternatively, *Chlorella* strains cannot utilize some organic nutrients of PPW. Nagarajan et al. (2020) reported that microalgae cannot assimilate most organic carbon nutrients in certain wastewaters under photoautotrophic mode. It needs more work to improve the COD removal efficiency and explain the mechanism of microalgae on absorbing the organic nutrients in PPW.

The contents of total nitrogen (TN) in different culture substrates after cultivating two tested *Chlorella* strains were inclined to obviously decrease with the increasing incubation time (Fig. 3a and b). The TN removal rates of *Chlorella* cells on different culture solution showed obvious increase trends during the incubation time (Fig. 3c and d). After cultivating for 7 days, the TN concentrations in PPW could be dramatically decreased to 0.27 and 2.00 mg/L by *Chlorella* sp. C2 and *C. sorokiniana* F-275, respectively. The TN removal efficiency of *Chlorella* sp. C2 on PPW was 99.42%, slightly higher than that of *C. sorokiniana* F-275 (95.87%). Surprisingly, it was found that the TN contents in PPW could be reduced to 1.83 and 8.36 mg/L on the third day of culture by *Chlorella* sp. C2 and *C. sorokiniana* F-275. The removal rates separately reached 96.10% and 82.69%. This result reveals that *Chlorella* strains could rapidly eliminate TN in PPW. However, at the end of culture, the TN removal rates of *Chlorella* sp. C2 on BG11 and PPW + BG11 substrates were 20.37% and 39.65%, respectively. For *C. sorokiniana* F-275, the TN removal rates on BBM and PPW + BBM were 30.68% and 72.88%. It should be ascribed that the growth of *Chlorella* strains cultivated in BG11 and BBM were much slower than the cultures in PPW-included substrates. And the nitrogen contents in BG11 and BBM might exceed the demands of two tested algae growth. It was more beneficial for *Chlorella* strains to assimilate nitrogen nutrition in the presence of carbon sources. Similar issue was also found by some previous studies (Li et al., 2019; Perez-Garcia et al., 2011a).

Similarly, the contents of total phosphorus (TP) in different culture substrates exhibited obvious decrease tendencies by cultivating two tested *Chlorella* strains during the incubation time (Fig. 4a and b). Higher TP removal efficiency on PPW was achieved by *Chlorella* cells

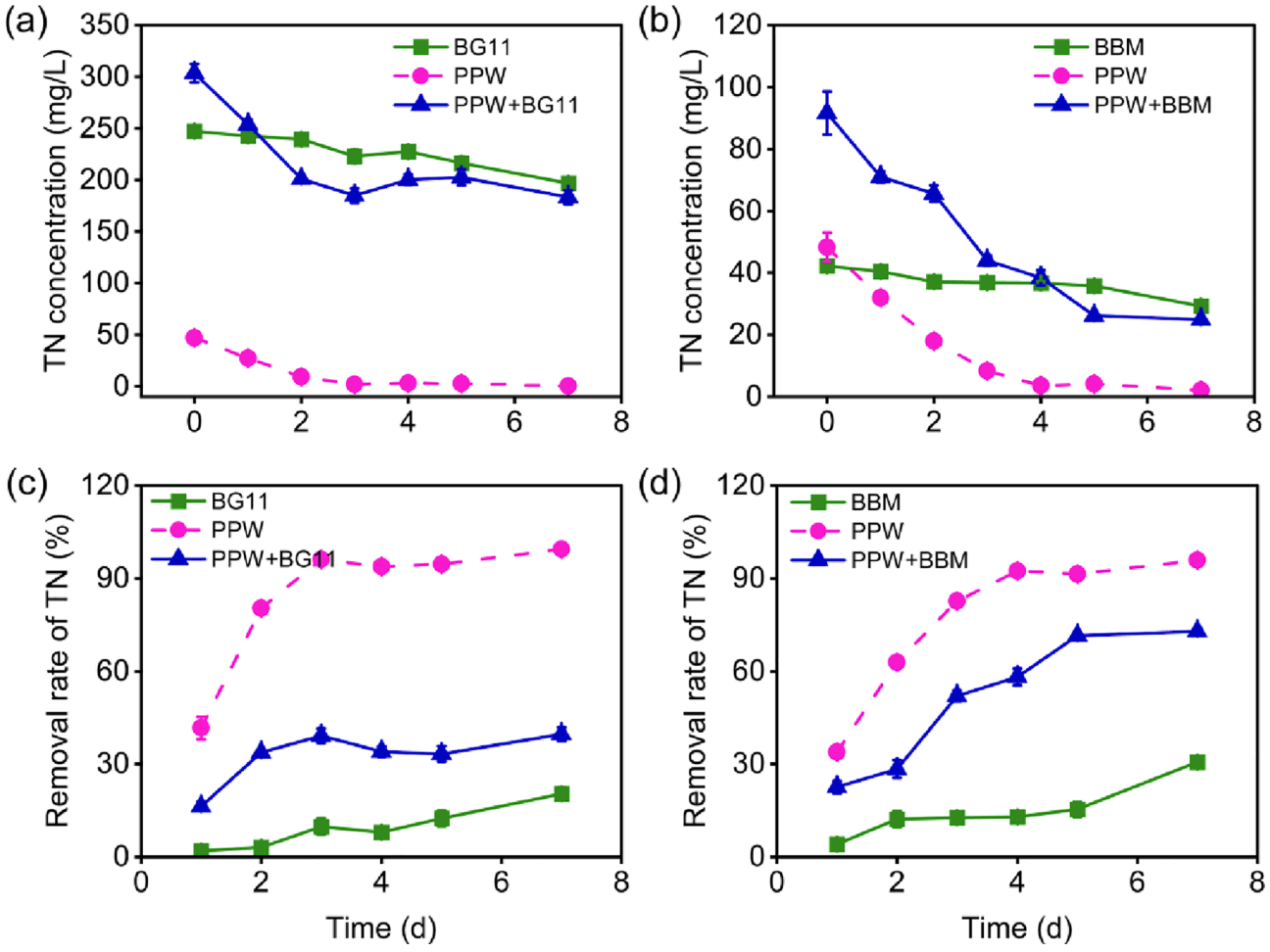

Fig. 3. The removal efficiencies of two tested *Chlorella* strains on total nitrogen (TN) in different culture substrates. (a) and (b) represent the changes of TN concentrations; (c) and (d) exhibit the variations of TN removal rates.

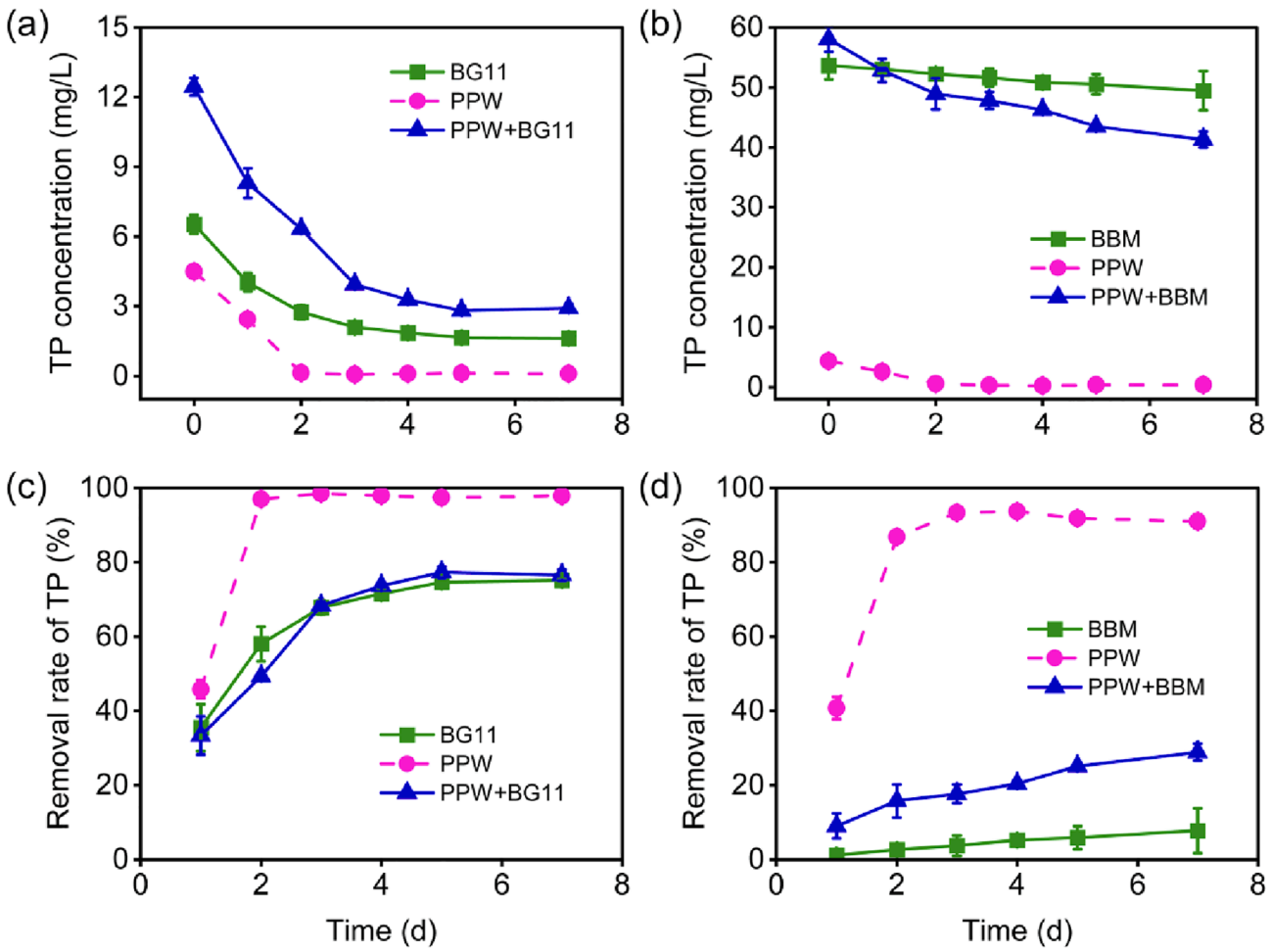

Fig. 4. The removal efficiencies of two tested *Chlorella* strains on total phosphorus (TP) in different culture substrates. (a) and (b) show the changes of TP concentrations; (c) and (d) represent the variations of TP removal rates.

(Fig. 4c and d). After 7 days of culture, the TP concentration in PPW could be reduced to 0.10 and 0.39 mg/L by *Chlorella* sp. C2 and *C. sorokiniana* F-275. The TP removal rates were 97.84% and 91.06%, respectively. Surprisingly, after cultivating two algal strains in PPW for 2 days, the TP contents could be reduced to 0.13 and 0.58 mg/L with the removal rates being 97.05% and 86.82%, respectively. However, lower removal efficiencies of TP were observed in BG11/BBM-contained substrates. The TP removal rates of *Chlorella* sp. C2 on BG11 and PPW + BG11 were 75.17% and 76.53% after incubating for 7 days. *C. sorokiniana* F-275 could remove only 7.78% and 28.90% of TP in BBM and PPW + BBM, respectively. This difference was largely caused by higher phosphorus concentrations in BBM and PPW + BG11/BBM substrates, which exceeded the demands of cells growth. And lower specific growth rates were observed in BG11 and BBM media compared with PPW (Table 2).

According to above related results, it can be clearly concluded that two *Chlorella* strains could efficiently remove the nitrogen and phosphorus nutrients of PPW. The TN and TP removal efficiencies of *Chlorella* sp. C2 on PPW were slightly higher than those of *C. sorokiniana* F-275. Generally, the assimilation and chemical processes are the primary manners for microalgae to eliminate nitrogen and phosphorus compositions in various wastewaters (Gao et al., 2018; Nagarajan et al., 2020). Based on the PPW characteristics (Table 1) and the growth performance of *Chlorella* cells in PPW (Fig. 1 and Table 2), the absorption should be the main way to remove nitrogen and phosphorus nutrients in this research. Moreover, Beuckels et al. (2015) found that nitrogen concentration in wastewaters displayed important impact on phosphorus removal rate by microalgae. Higher nitrogen levels in wastewaters exhibited higher phosphorus elimination rate. And when the nitrogen content was 40 mg/L in wastewater, the phosphorus removal could reach 6 mg/L for *Chlorella* sp. and *Scenedesmus* sp.. Similar conclusion was obtained in this study.

Due to containing phycobiliprotein (PP), especially phycoerythrin, PPW is a red wastewater, which is one of the main reasons resulting in environment contamination. Interestingly, by cultivating two tested *Chlorella* strains, the PP contents in different culture substrates exhibited marked reduction trends with the increase of cultivation time (Fig. 5a and b). The PP removal rates showed significant increase tendencies (Fig. 5c and d). After culturing for 7 days, the concentrations of PP in PPW and PPW + BG11were dramatically decreased to 1.75 and 1.91 mg/L by *Chlorella* sp. C2 (Fig. 5a). The PP removal efficiencies were 92.60% and 91.32% (Fig. 5c). By cultivating *C. sorokiniana* F-275, the concentrations of PP in PPW and PPW + BBM were significantly reduced to 1.76 and 2.05 mg/L, where the PP removal rates were 92.38% and 91.13% (Fig. 5 b and d). Surprisingly, the PP concentration in substrates could be rapidly decreased to ~2.50 mg/L and ~5.50 mg/L on the third day of incubation by *Chlorella* sp. C2 and *C. sorokiniana* F-275, respectively. The PP removal rates individually reached over 90% and 80%. It was reasonable to conclude that two tested *Chlorella* strains could efficiently assimilate PP nutrient in PPW. This result illustrates that *Chlorella* strains could effectively remove PP constituents in PPW. There were no significant discrepancies in PP removal rates of *Chlorella* cells between PPW and PPW + BG11/BBM substrates. It was probably because that both two *Chlorella* strains grown fast in PPW and PPW + BG11/

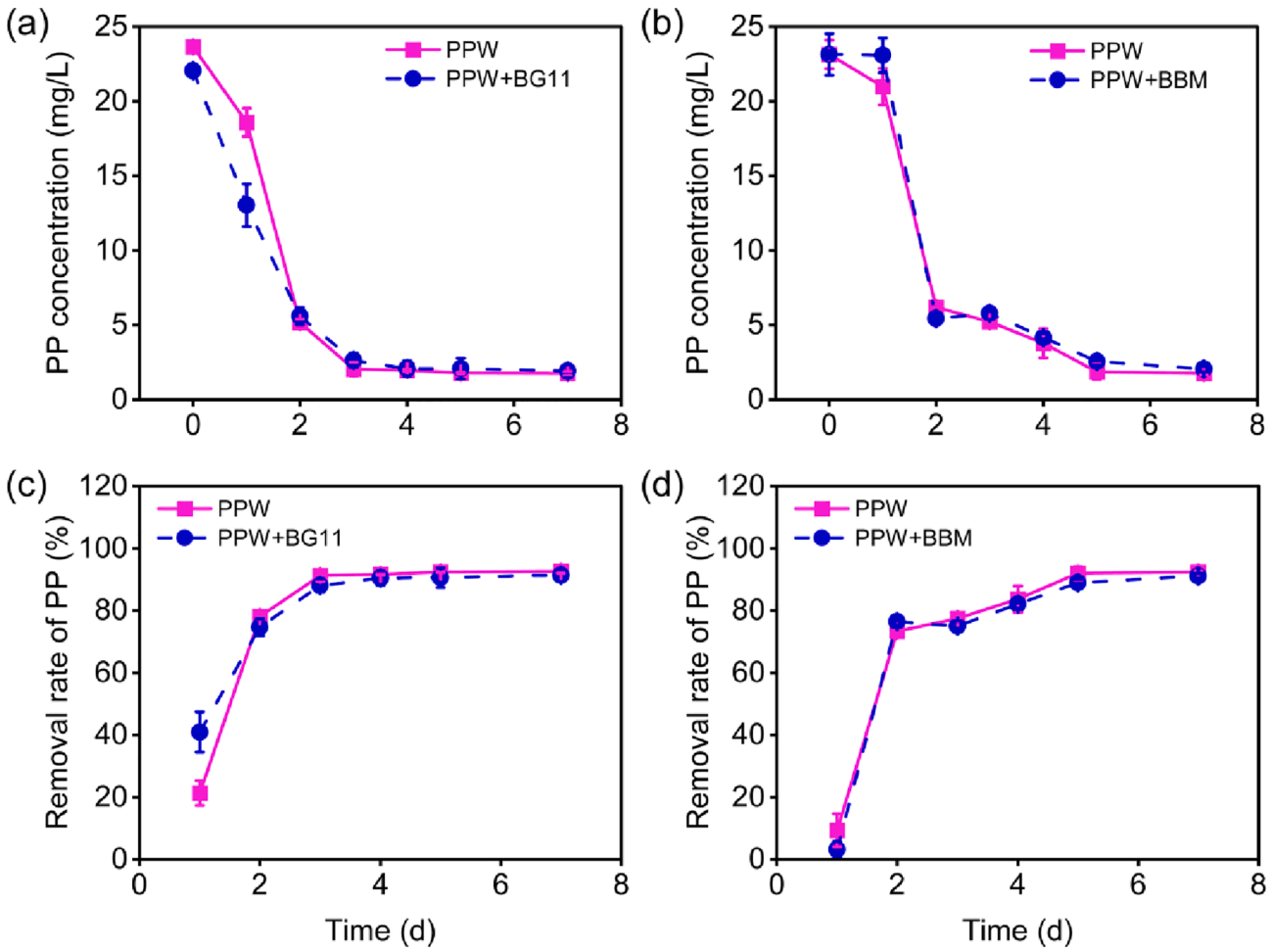

Fig. 5. The removal efficiencies of two tested *Chlorella* strains on phycobiliprotein (PP) in different culture substrates. (a) and (b) indicate the changes of PP concentrations; (c) and (d) display the variations of PP removal rates.

BBM, which could absorb PP as a nutrient to stimulate the growth of algal cells. As shown in Table 2, the specific growth rates of two *Chlorella* cells in these four substrates were almost the same.

Previous studies reported that phycobiliprotein, except for being a light harvesting pigment protein, could be considered as nitrogen storage compound to provide nutrients for cyanobacteria growth under the nutrient-deprived conditions (Carr, 1988; Collier and Grossman, 1994). Based on this experiment results, it was speculated that phycobiliprotein was primarily degraded into diverse amino acids under the related protease (Collier and Grossman, 1994; Sokolenko, 2005), and further catalyzed into oxo-acids, NH_3 and H_2O_2 by amino acids oxidase (Vallon et al., 1993). Then, oxo-acids and NH_3 participate in carbon and nitrogen metabolism. Moreover, it was confirmed that microalgae are more inclined to preferentially utilize ammonium compared with other nitrogen sources such as nitrate and nitrite (Perez-Garcia et al., 2011b). Above issues might explain that why *Chlorella* strains could efficiently remove the phycobiliprotein in PPW. The specific mechanism of *Chlorella* cells on phycobiliprotein removal needs more work to be done.

3.4. Lipid production and fatty acid compositions of Chlorella sp. Grown in PPW

Microalgal lipids can be considered as the feedstock of biodiesel, food, chemicals, pharmaceuticals and cosmetics, which have been extensively concerned in the world (Bellou et al., 2014). To illustrate the lipid characteristics of *Chlorella* microalgae cultivated in PPW, neutral lipids, total lipids and fatty acid compositions in cells were investigated under different culture conditions. The neutral lipid content was measured by the Nile red staining method and displayed as the fluorescence intensity per culture volume in this research. The changes of neutral lipid in two *Chlorella* strains exhibited increasing tendencies during the cultivation time (Fig. 6a and b). The accumulations of neutral lipid in cultures were markedly promoted in the presence of PPW. The maximum neutral lipid contents in both algal cells were obtained in PPW substrate. The fluorescence intensity in *Chlorella* sp. C2 cultivated for 7 days was increased by 1.60 times compared with the cultures in BG11 and 13.64% by contrast with PPW + BG11 (Fig. 6a). In *C. sorokiniana* F-275, after 7 days of incubation, the fluorescence intensity was 2.33-fold higher than that of BBM, and improved by 57.40% in comparison with PPW + BBM (Fig. 6b). This result implies that PPW performs a significantly positive effect on stimulating *Chlorella* strains to generate neutral lipids. It might be ascribed to the abundant carbon sources in PPW. Higher carbon–nitrogen ratio might be more conducive to improve the neutral lipid accumulation of *Chlorella* cells (Li et al., 2019; Zheng et al., 2017).

As it can be seen from Fig. 6c and d, lipid productivities in cultures could be dramatically improved when cultivated in PPW. But, unfortunately, PPW cannot enhance the total lipid contents in two tested *Chlorella* cells. The optimum lipid productivity in *Chlorella* sp. C2 was attained by co-substrate of PPW + BG11, which increased by 1.87 times compared with BG11, and 22.25% in contrast with PPW. The maximum value of lipid productivity in *C. sorokiniana* F-275 was recorded under the condition of PPW, which was 10.18 times higher than that of BBM and slightly higher than that of PPW + BBM. This result indicates that

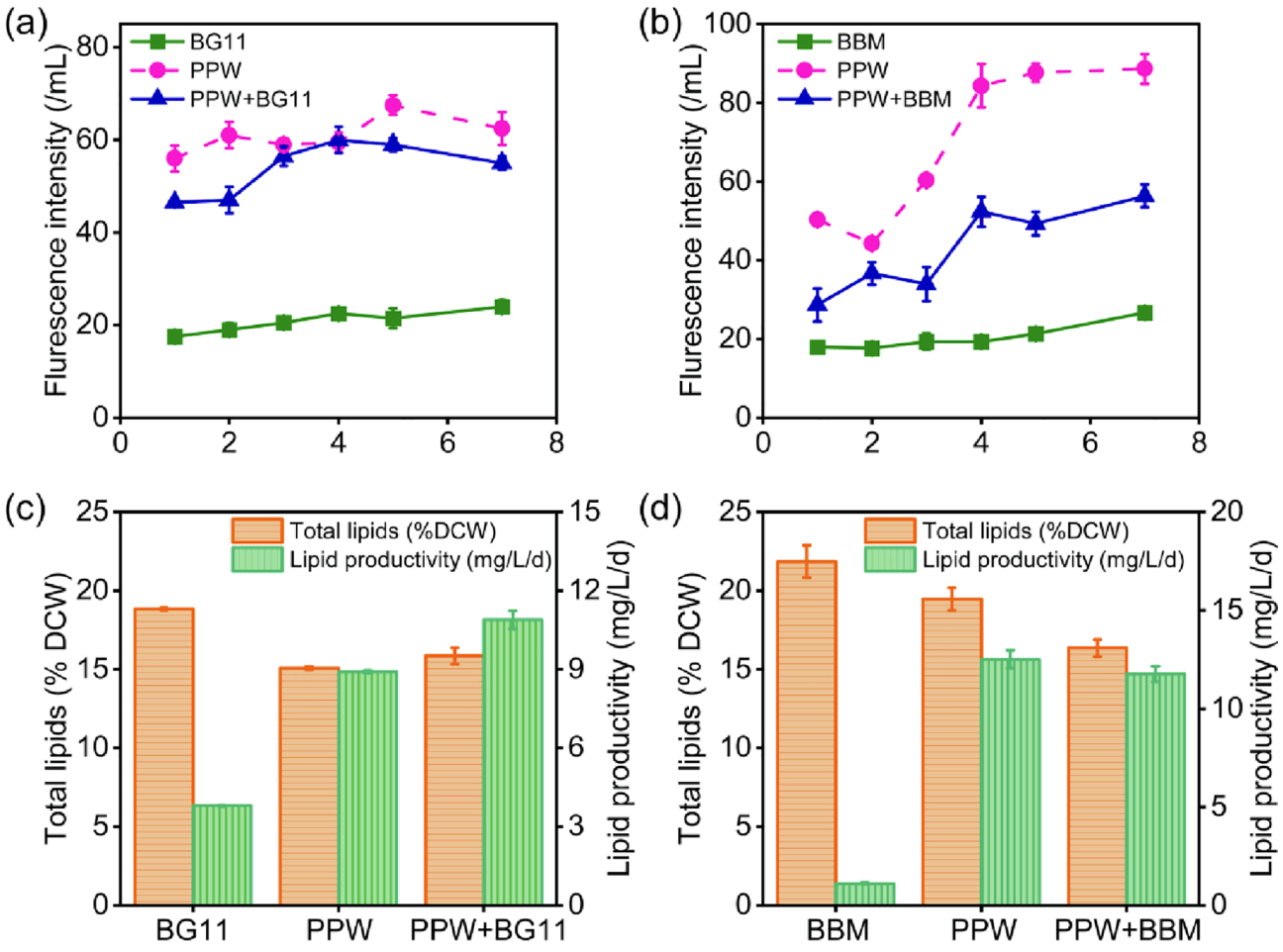

Fig. 6. The changes of neutral lipid and total lipid in two tested *Chlorella* strains under different culture conditions. (a) and (b) represent the variations of neutral lipids; (c) and (d) indicate the changes of total lipids.

PPW could increasingly enhance the lipid productivities of *Chlorella* strains. When extra nutrients are supplemented in PPW, lipid productivities in the two algal strains exhibit different responses. The reason why the total lipids of *Chlorella* cells cannot be improved by PPW while lipid productivities increased might be that the absorbed nutrients by algae from PPW were largely used for cells growth since the cultures in PPW exhibited higher biomass than that of BG11/BBM (Fig. 1).

As shown in Table 3, the main fatty acids were composed of C16:0, C18:1, C18:2 and C18:3 in two tested *Chlorella* strains, the proportions of which were more than 10% of total FAMEs. Significant changes of fatty acid compositions in *Chlorella* cells were observed in different culture substrates. Compared with the cultures in BG11/BBM, the C18:3 ratio in cells was markedly decreased when *Chlorella* strains were cultivated in PPW-involved substrates. The reductions were 27.89% in *Chlorella* sp. C2 and 29.10% in *C. sorokiniana* F-275 under the condition of PPW. Meanwhile, the proportions of C18:1 and C18:2 fatty acids in *Chlorella* sp. C2 were significantly increased by 8.92% and 52.53%. In *C. sorokiniana* F-275, the ratios of C16:0, C18:0, C18:1 and C18:2 fatty acids were obviously enhanced by 14.11%, 30.52%, 28.08% and 23.07%, respectively. When the cultures were cultivated in co-substrates of PPW + BG11/BBM, the ratios of C18:3 in *Chlorella* sp. C2 and *C. sorokiniana* F-275 were reduced by 8.51% and 13.96%.The C18:1

Table 3
The fatty acid compositions of two tested *Chlorella* strains under different culture conditions (% of total FAMEs, n = 3).

Fatty acids (%)	*Chlorella* sp. C2			*C. sorokiniana* F-275		
	BG11	PPW	PPW + BG11	BBM	PPW	PPW + BBM
C14:0	1.58 ± 0.07a	1.53 ± 0.10a	1.51 ± 0.07a	2.64 ± 0.06a	1.50 ± 0.12b	2.10 ± 0.05a
C14:1	2.50 ± 0.19b	2.73 ± 0.09b	2.63 ± 0.05b	3.15 ± 0.06a	2.19 ± 0.15b	3.51 ± 0.33a
C16:0	22.17 ± 0.17a	22.28 ± 0.45a	22.33 ± 0.18a	21.74 ± 0.58b	24.81 ± 0.34a	22.04 ± 0.33b
C16:1	4.76 ± 0.14b	5.34 ± 0.20a	4.95 ± 0.18ab	5.96 ± 0.07a	4.90 ± 0.36b	5.85 ± 0.12a
C18:0	5.23 ± 0.15c	6.22 ± 0.24a	5.89 ± 0.07b	4.86 ± 0.21c	6.34 ± 0.09a	5.70 ± 0.21b
C18:1	17.91 ± 0.18b	19.51 ± 0.22a	19.30 ± 0.07a	13.12 ± 0.48c	16.81 ± 0.41a	15.64 ± 0.35b
C18:2	11.60 ± 0.19b	17.69 ± 0.17a	12.06 ± 0.12b	17.34 ± 0.23c	21.34 ± 0.19a	18.32 ± 0.19b
C18:3	34.26 ± 0.38a	24.70 ± 0.50c	31.34 ± 0.24b	31.09 ± 0.10a	22.12 ± 0.15c	26.84 ± 0.25b
ΣSFA	28.97 ± 0.06b	30.03 ± 0.70a	29.73 ± 0.10ab	29.23 ± 0.34b	32.64 ± 0.30a	29.85 ± 0.37b
ΣMUFA	25.17 ± 0.14b	27.58 ± 0.26a	26.87 ± 0.23ab	22.23 ± 0.47b	23.90 ± 0.58ab	24.99 ± 0.78a
ΣPUFA	45.86 ± 0.19a	42.39 ± 0.68b	43.40 ± 0.33b	48.54 ± 0.18a	43.46 ± 0.33c	45.16 ± 0.43b

ΣSFA: the total of saturated fatty acids; ΣMUFA: the total of monounsaturated fatty acids; ΣPUFA: the total of polyunsaturated fatty acids.

proportion was markedly improved by 7.76% and 19.18%. This result implies that PPW could significantly affect the proportions of fatty acid constituents in *Chlorella* strains. The decrease of C18:3 ratio in *Chlorella* sp. C2 was conducive to enhance the biosynthesis of C18:1 and C18:2 fatty acids under the condition of PPW. For *C. sorokiniana* F-275, PPW facilitates the synthesis of C16:0, C18:0, C18:1 and C18:2 fatty acids. However, the addition of BG11/BBM nutrients in PPW cannot further improve the fatty acid profiles of two *Chlorella* strains for biodiesel production.

It was confirmed that fatty acid constituents directly affect the quality of microalgal biodiesel (Knothe, 2008). Higher saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) are more beneficial to enhance oxidative stability, cetane number and combustion characteristics of biodiesel (Gao et al., 2019; Knothe, 2008). This research found that when two *Chlorella* cells were grown in PPW, the decreases of polyunsaturated fatty acids (PUFA) were inclined to stimulate SFA and MUFA accumulations (Table 3). And neutral lipids in cultures were also significantly promoted (Fig. 6a and b), which were the primary feedstock of microalgal biodiesel production (Damiani et al., 2010). Hence, it was reasonable to conclude that cultivating *Chlorella* strains in PPW could obviously improve the biodiesel properties of algal cells.

4. Conclusions

This is the first study indicating that PPW could be directly applied to culture microalgae which could grow well in undiluted PPW without any nutrient supplementation. Higher reductions of COD, TN, TP and PP were achieved by cultivating *Chlorella* strains in PPW. Meanwhile, the desired increases of biomass and lipid productivities could be obtained. These results indicate that culturing *Chlorella* cells in PPW observably alleviates the environmental pollution caused by wastewater discharge. PPW would be an alternative medium for microalgal biomass production. The present study will be greatly important in PPW recycle and low-cost large-scale culture of *Chlorella* strains.

CRediT authorship contribution statement

Shiyan Zheng: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Project administration, Funding acquisition. **Shanyi Chen:** Validation, Formal analysis, Investigation, Data curation. **Shangyun Zou:** Formal analysis, Investigation. **Yiwen Yan:** Formal analysis, Investigation. **Guang Gao:** Resources, Writing - review & editing. **Meilin He:** Resources, Writing - review & editing. **Changhai Wang:** Supervision, Conceptualization. **Hui Chen:** Resources, Methodology. **Qiang Wang:** Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no know competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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