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A combined light regime and carbon supply regulation strategy for microalgae-based sugar industry wastewater treatment and low-carbon biofuel production to realise a circular economy

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ABSTRACT

The replacement of fossil fuels with clean and renewable biofuels is of both research and market interest for realising a circular economy. However, microalgae-based biofuels have shown promise as alternative low-carbon biofuels to other crop-based biofuels, some key obstacles in their production remain to be addressed, such as high costs and low lipid productivity. In this study, a *Chlorella* sp. CSH4 was cultivated using a combined light regime and carbon supply regulation strategy to enhance sugar industrial wastewater bioremediation, biomass accumulation and lipid production. Blue light irradiance of 200 μmol photons m^{-2} s^{-1} together with 10 g/L glucose and 9.2 g/L glycerol supply was found to effectively enhance the biomass accumulation and pollutant-removal capacity of *Chlorella* sp. during the growth phase and its lipid production during the stationary phase. Furthermore, the biodiesel properties of the lipid retrieved from *Chlorella* sp., as demonstrated by its fatty acid profile, were found to be suitable for commercial application. Possible mechanisms were explored to explain how this combined strategy caused this microalga to exhibit highly efficient biomass and lipid production together with efficient pollutant removal. Moreover, upscaled semi-continuous treatment using both sugar industry wastewater and negligible carbon sources (e.g., food waste hydrolysate and crude glycerol) with a mass balance analysis was conducted to initially validate the feasibility of applying our combined strategy for microalgae-based wastewater treatment. In sum, this study demonstrated the feasibility of cultivating a microalga using a combined strategy comprising a light regime and carbon supply regulation to achieve both wastewater treatment and low-carbon biofuel production.

1. Introduction

The current supply of non-renewable fossil fuels cannot meet rapidly increasing demands due to worldwide economic development and population growth [1]. In addition to greenhouse gas emissions generated by the burning of fossil fuels exacerbating the climate crisis, these emissions also contain pollutants that pose a risk to humans [2]. As such, there is an urgent need for the production of clean and renewable fuels, termed biofuels, as an alternative to fossil fuels.

Biofuels can be generated from various feedstocks, including edible crops and non-edible plants. Moreover, microalgae, which are plant-like organisms, are considered as a feasible and sustainable feedstock for manufacturing low-carbon renewable biofuels without compromising environmental and food safety [3,4]. In addition, it is advantageous that

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the cultivation of microalgae in harsh conditions accumulates their biomass and lipid [5]. The feasibility and potential application of algae-based biofuel production has been explored and investigated recently [6,7]. However, the extremely high costs associated with the production of algae-based biofuels is a major bottleneck that hinders further development in this industry. Microalgal lipid production capacity must be increased, and associated costs decreased to achieve economic production of algae-based biofuels. Therefore, tremendous efforts have been made to investigate the optimisation of algae-based biofuel production [8–10].

The sugar industry produces large volumes of wastewater with high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), which can cause severe environmental pollution [11]. Typically, sugar industry wastewater is required to be treated via chemical or physical approaches to remove organic pollutants. However, the practical application of such wastewater treatment systems in sugar refineries is hindered by their high costs and tendency to generate secondary pollution. In addition, such treatments squander organic pollutants present in sugar industry wastewater – such as carbohydrates, minerals, and nitrogen sources – that otherwise could be recycled and reused. For example, these organic pollutants can be used in microalgal cultivation to generate valuable products [12–14]. Accordingly, the microalgae-mediated treatment of wastewater has gained considerable attention as it can both benefit the sugar industry, by enabling biorefinery and valorisation processes, and minimise the environmental damage caused by untreated wastewater discharge [15]. Nevertheless, the removal of pollutants from wastewater via microalgae-based processes is not highly efficient, which hampers their widespread application.

The critical feature that distinguishes microalgae-based treatment systems from other microorganism-based treatment systems is that microalgae use both light and carbon as energy sources [16,17]. In addition, as microalgal growth and metabolism – including the pathways involved in pollutant biodegradation – are typically driven by light regimes, this means that light is both a primary energy source and a key environmental factor influencing microalgal metabolism [18]. This feature is an exceptional advantage, as light is a highly controllable environmental condition, and light intensity affects microalgal growth and consequently its pollutant biodegradation performance [19]. For instance, under red light irradiation, *Chlorella vulgaris* removed heavy metals, including copper and zinc, from marine sediments [20]. Similarly, under purple light irradiation of 5,800 lx, phosphate and ammonium salts were effectively removed from municipal sewage by *Spirulina platensis* in semi-batch cultivation mode [21]. Furthermore, microalgae can utilise various carbon sources for mixotrophic cultivation under different light conditions; that is, microalgae function as photoautotrophs when supplied with inorganic carbon and as heterotrophs when supplied with organic carbon. For example, *Chlorella* sp. GY-H4 was found to utilise glucose for biomass and lutein accumulation under mixotrophic conditions [22]. It was found that, compared with photoautotrophic cultivation, mixotrophic cultivation of microalgae with an organic carbon source enhanced their pollutants removal performance [23]. Another study reported that approximately 63.85% of COD, 91.54% of total nitrogen (TN), and 83.25% of total phosphorus (TP) from palm oil refinery wastewater were consumed by *Chlorella sorokiniana* supplied with glycerol under mixotrophic conditions [24].

Studies have reported that a light source and inorganic carbon supply can enhance the pollutant removal efficiency and valuable product yield of microalgae [25,26]. However, the feasibility of a combined strategy for microalgal cultivation – i.e., a strategy comprising a light regime and organic carbon supply regulation, which would enable both microalgae-based wastewater treatment and low-carbon biofuel production to realise a circular economy – remains unclear. Moreover, the synergistic roles of the light regime and carbon supply in wastewater treatment and biofuel production are unexplored.

To address these research gaps, we regulated the light regime and carbon supply conditions during *Chlorella* sp.-mediated treatment of wastewater, to explore the effects of each of these factors. Thereafter, we explored the metabolic changes induced in *Chlorella* sp. by a combination of a light regime and carbon supply regulation, to elucidate the possible mechanisms by which the light regime and carbon supply affected this *Chlorella* sp. Moreover, we examined the feasibility of our combined regulation strategy in the semi-continuous cultivation of this *Chlorella* sp. Our findings provide novel insights into the simultaneous achievement of wastewater treatment and low-carbon biofuel production by microalgae cultivated using a combined strategy comprising a light regime and regulated carbon supply.

2. Materials and methods

2.1. Wastewater collection and treatment

The two types of wastewaters used in this study were collected from discharge points in the sugar manufacturing factory of Zixiang Sweet Co., Ltd. (Hangzhou, China) during (i) sugar feedstock washing and (ii) ion-exchange processes. These two types of wastewater were mixed at a volume ratio of 1:4 to generate sugar industry wastewater, which was then filtered through a 10 μm membrane (Pall, USA) to remove impurities and subsequently stored at −20 °C until it was further processed. The filtered wastewater had a BOD of 183.76 mg/L, a COD of 647.56 mg/L, an ammonia–nitrogen (NH_4^+-N) concentration of 61.57 mg/L, TN of 75.88 mg/L, and TP of 5.82 mg/L. In addition, the turbidity of filtered wastewater is good, suggesting that collected sugar wastewater did not cause any hinderance against light penetration. The frozen wastewater was thawed when required for further microalgae-based wastewater treatment.

2.2. Microalgal strain and its cultivation under a light regime and carbon supply

Chlorella sp. (Strain No.: CSH4) was preserved in Jinan University (Guangzhou, China) and maintained in an artificial climate incubator in a 500 mL Duran laboratory bottle containing 400 mL of filtered wastewater. The *Chlorella* culture with 10 days cultivation period was cultured at 20 ± 0.5 °C under a 12-h:12-h light/dark cycle supplied with the aerated air flow (containing 21% of O_2 and 0.04% of CO_2) at 3 L/min, with a specific wavelength of illumination provided in the light phase using a light-emitting diode. Specifically, *Chlorella* sp. was cultivated without a supply of organic carbon in diluted wastewater (BOD, 36.75 mg/L; COD, 129.51 mg/L; NH_4^+-N, 12.32 mg/L; TN, 15.18 mg/L; TP, 1.16 mg/L) under blue or red light irradiance at 50, 100, 200, 400, or 800 μmol photons m^{-2} s^{-1}. In addition, *Chlorella* sp. was cultivated with a supply of organic carbon in diluted wastewater (BOD, 36.75 mg/L; COD, 129.51 mg/L; NH_4^+-N, 12.32 mg/L; TN, 15.18 mg/L; TP, 1.16 mg/L) under cold white light irradiance at 200 μmol photons m^{-2} s^{-1}, and were also supplied with glucose concentrations of 5, 10, 20, 30, and 50 g/L or glycerol at concentrations of 5, 10, 50, 100 (equal to 9.2 g/L), and 200 mM. Moreover, in the combined strategy, *Chlorella* sp. was cultivated in the original mixed wastewater (BOD, 183.76 mg/L; COD, 647.56 mg/L; NH_4^+-N, 61.57 mg/L; TN, 75.88 mg/L; TP, 5.82 mg/L) under optimised light spectra (blue light at 200 μmol photons m^{-2} s^{-1} was provided throughout the entire treatment period of 10 days) and carbon supply (glucose was supplied at the beginning of the wastewater treatment (day 1), and glycerol was supplied in the middle of the wastewater treatment (day 7)) conditions. The cells were inoculated every 10 days in the original mixed wastewater at a volume ratio of 1:5, and harvested at specific time points for further experiments by centrifuging a sample aliquot at 5,000 rpm for 10 min.

2.3. Analyses of microalgal growth and photosynthesis

Chlorella sp. cultured in the sugar industry wastewater was first

observed to check cell integrity. The cell density was measured by a direct-counting approach using an Neubauer haemocytometer under an optical microscope (Nikon, Japan). Thereafter, microalgal biomass quantification was performed by gravimetric analysis. Specifically, 50 mL of the *Chlorella* culture was harvested and transferred onto a pre-weighed 0.22 μm membrane, which was then dried at 60 °C for 24 h to a constant weight and evaluated gravimetrically to calculate the dry cell weight (DCW). The specific growth rate of *Chlorella* sp. during the exponential growth phase was calculated using Equation (1):

$$\mu\,(\mathrm{day}^{-1}) = \frac{\ln N - \ln N_0}{t - t_0} \tag{1}$$

where N and N_0 indicate the DCW (g/L) at time t and t_0, respectively, during the exponential growth phase.

The photosynthetic parameters of *Chlorella* sp., namely the photochemical efficiency of photosystem II (Fv/Fm), the photosystem II-based electron transport rate (ETR), and non-photochemical quenching (NPQ), were measured on a PhytoPAM Phytoplankton Analyser (Walz, Germany) according to the manufacturer's instructions. The activities of two representative photosynthetic enzymes, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and carbonic anhydrase (CA), were measured following standard protocols [27,28].

2.4. Analyses of microalgal primary metabolites

The carbohydrate content of *Chlorella* sp. was analysed using the classical phenol–sulfuric acid method, with detection at 483 nm via UV/visible (UV/Vis) spectrophotometry (Shimadzu, Japan). The absorbance was plotted on a glucose (Sigma-Aldrich, USA) standard curve to obtain the carbohydrate content. The total protein of the *Chlorella* culture was first extracted using a cell lysis buffer (Beyotime, China) supplemented with a phenylmethanesulfonyl fluoride protease inhibitor (Beyotime, China). The protein was then quantified using a commercial bicinchoninic acid kit (Beyotime, China) according to the manufacturer's instructions, with detection at 562 nm by UV/Vis spectrophotometry (Shimadzu, Japan). The absorbance was plotted on a bovine serum albumin standard curve to obtain the total protein content.

The relative neutral lipid (NL) content was evaluated using the Nile red fluorometric assay. Briefly, 3 mL of the *Chlorella* culture was mixed with 30 μL of Nile red solution at in volume ratio of 100:1, and the resulting solution was incubated at room temperature in the dark for 30 min. Thereafter, the stained cells, unstained cells, stained culture medium, and unstained culture medium were successively transferred into a 96-well plate for fluorometric determination using a Synergy H1 Hybrid Multi-Mode Reader (Bio-Tek, USA) at an excitation wavelength of 488 nm and an emission wavelength of 592 nm. The total lipid content of *Chlorella* sp. was extracted using the classical organic solvent method and quantified gravimetrically. Additionally, the extracted total lipid was fractionated into NL, phospholipid (PL), and glycolipid (GL) phases using a solid-phase extraction (SPE) column with pre-packed silica cartridges (500 mg, 6 cc Sep-Pak, Waters, USA), according to the standard procedure [29], and each lipid fraction was taken to dryness under a stream of N_2 and then quantified gravimetrically.

The fatty acids of *Chlorella* sp. were extracted and transesterified to fatty acid methyl esters (FAMEs) following a standard protocol [29]. Briefly, 500 μL of toluene was added to transfer wet algae pellet (approximately 5 mg) into a Teflon-lined screw-cap tube, and subsequently added with 1 mL of fresh NaOH/MeOH (0.5 N). The mixed solvent was incubated at 80 °C for 20 min after vortex. After cooling for 5 min, 1 mL of fresh AcCl/MeOH (1:10, *v/v*) was slowly added for further incubation of 20 min. Afterwards, 1 mL of 6% K_2CO_3, 500 μL of hexane, and 10 μL of methyl nonadecylate (C19:0, Aladdin, China) were added into the above mixture for 1 min of vortex. The upper phase after centrifugation at 2,000 rpm for 10 min was collected for following gas chromatograph–mass spectrophotometer (GC–MS) analysis. For GC–MS procedure, 1 μL of each sample was injected into Agilent 7000C Triple Quadrupole GC/MS System (Agilent, USA) equipped with a J&W HP-5 ms Ultra Inert GC Column (15 m × 0.25 mm × 0.25 μm, Agilent, USA). The helium was considered as carrier gas with a constant flow of 1 mL/min. The injector temperature was set at 250 °C. The column temperature was held at 65 °C for 3 min, and increased to 180 °C by 10 °C/min and subsequently held at 180 °C for 2 min. Afterwards, 1 °C/min of increment from 180 °C to 185 °C was performed with hold at 185 °C for 5 min. At last, the temperature was increased to 300 °C by 5 °C/min, and held there for 8 min. The scanning range of m/z was 50–550 with scanning time of 300 ms. The acquired data was analysed against with the MS spectrum from the National Institute of Standards and Technology (NIST) Standard Reference Database. The peak area of each FAME was normalised to the internal reference of C19:0 to quantify the content of each FAME. The content of each FAME was summed together to obtain the total fatty acid content. The percentage of each FAME was calculated against the total content of all FAMEs.

2.5. Evaluation of the properties of microalgae-based biodiesel

To evaluate the quality of the obtained microalgae-based biofuel, its physicochemical properties were evaluated based FAMEs data. The representative parameters – the saponification value (SV), the iodine value (IV), the cetane number (CN), the degree of unsaturation (DU), the long-chain saturation factor (LCSF), the high heating value (HHV), the cold-flow plugging point (CFPP), the kinematic viscosity (kV) and the oxidative stability (OS) – were calculated using Equations (2)–(10):

$$\mathrm{IV} = \sum 254\mathrm{DB} \times \frac{\%\mathrm{FC}}{\mathrm{M}} \tag{2}$$

$$\mathrm{SV} = \sum 560\frac{\%\mathrm{FC}}{\mathrm{M}} \tag{3}$$

$$\mathrm{CN} = 46.3 + \frac{5458}{\mathrm{SV}} - (0.255 \times \mathrm{IV}) \tag{4}$$

$$\mathrm{DU}\,(\%) = \mathrm{MUFA} + (2 \times \mathrm{PUFA}) \tag{5}$$

$$\mathrm{LCSF} = (0.1 \times \mathrm{C16}) + (0.5 \times \mathrm{C18}) \tag{6}$$

$$\mathrm{HHV} = 49.43 - 0.041\,(\mathrm{SV}) - 0.015\,(\mathrm{IV}) \tag{7}$$

$$\mathrm{CFPP} = (3.417 \times \mathrm{LCSF}) - 16.477 \tag{8}$$

$$\ln(\mathrm{kV}) = -12.503 + 2.496 \times \ln\left(\sum \mathrm{M}\right) - 0.178 \times \sum \mathrm{DB} \tag{9}$$

$$\mathrm{OS} = \frac{117.9295}{(\mathrm{wt\%\,C18:2} + \mathrm{wt\%\,C18:3})} + 2.5905 \tag{10}$$

where M represents the molecular weight of each fatty acid, DB represents the number of double bonds in the fatty acid component, %FC represents the percentage of each fatty acid in the total fatty acid profile, and MUFA and PUFA represent the molecular weights of monounsaturated fatty acid and polyunsaturated fatty acid, respectively.

2.6. Analyses of microalgae-based biodegradation of pollutants and nutrients from wastewater

To monitor the pollutant biodegradation performance of *Chlorella* sp., the pollutants (the BOD, COD, NH_4^+-N, TN, and TP) in the sugar industry wastewater were analysed every 3 days from the beginning of *Chlorella* sp. inoculation (day 1) up to the end of its cultivation (day 10). The wastewater samples were filtered through a 0.22 μm membrane and then diluted to appropriate concentrations for pollutant analyses. The BOD and COD were analysed using Hach BOD/COD instruments (Hach, USA) combined with the Hach BOD/COD reagent (Hach, USA),

according to the manufacturer's specifications. NH_4^+-N was determined on a UV/Vis spectrophotometer (Shimadzu, Japan) at 420 nm following the standard protocols published by Sate Environmental Protection Administration of China (SEPA, HJ535–2009). TN was measured using a Total Phosphorus and Total Nitrogen Analyser (Hach, USA) at 220 nm and 275 nm, following the standard instructions of SEPA (HJ636-2012). TP was determined using a Total Phosphorus and Total Nitrogen Analyser (Hach, USA) at 700 nm following the standard specifications of SEPA (GB11893-89). The removal efficiency (P_{RE}) and removal rate (P_{RE}) of pollutants in the wastewater samples were determined using Equations (11)–(12):

$$P_{RE} = \frac{P_i - P_e}{P_i} \times 100\% \quad (11)$$

$$P_{RR} = \frac{P_i - P_e}{t} \quad (12)$$

where P_i represents the concentration of the pollutant (mg/L) in the wastewater samples at the beginning of the microalgae-based treatment, P_e represents the concentration of the pollutant (mg/L) in the wastewater samples at the end of the microalgae-based treatment, and t represents the total treatment time.

Glucose consumption was monitored using a high-performance liquid chromatography system (HPLC; Waters, UK) equipped with an Aminex HPX-87H column and a refractive index detector (Waters, USA), following the standard protocol [30]. The glucose concentration was calculated with reference to the peak area of a glucose standard. Glycerol consumption was detected using an HPLC system following the protocol reported by Liang et al. [31].

2.7. Analyses of microalgal membrane permeability and mitochondrial membrane potential

Microalgal membrane permeability was analysed using the propidium iodide (PI) fluorometric assay. Briefly, the 2 mL of *Chlorella* culture was mixed with a PI solution (Sigma-Aldrich, USA) at a final PI concentration of 10 μM and this mixture was incubated at room temperature in the dark for 20 min. Subsequently, microalgal cells were collected by centrifugation at 4,000 rpm for 10 min and then transferred into 2 mL fresh medium for PI fluorescence determination using a Synergy H1 Hybrid Multi-Mode Reader (Bio-Tek, USA) at an excitation wavelength of 535 nm and an emission wavelength of 617 nm. The microalgal mitochondrial membrane potential was determined using a cationic carbocyanine dye. Specifically, the mitochondrial membrane potential probe JC-1 dye solution (Abcam, UK) was added to the microalgal culture to give a final concentration of 10 μM, and the resulting mixture was incubated at room temperature in the dark for 20 min. Thereafter, the cells were collected by centrifugation and transferred into fresh medium for fluorometric determination using a Synergy H1 Hybrid Multi-Mode Reader (Bio-Tek, USA) at an excitation wavelength of 475 nm and an emission wavelength of 590 nm. The adenosine triphosphate (ATP) content of *Chlorella* sp. was measured using a Plant ATP enzyme-linked immunosorbent assay kit (Bangyi Biotech, Shanghai, China), according to the manufacturer's instructions.

2.8. Analyses of the microalgal antioxidant system

The reactive oxygen species (ROS) content of the *Chlorella* culture was monitored by staining with a solution of the cell-permeable probe 2′,7′-dichlorodihydrofluorescein diacetate (Beyotime, China) at a volume ratio of 100:1, followed by fluorometric determination using a Synergy H1 Hybrid Multi-Mode Reader (Bio-Tek, USA) at an excitation wavelength of 488 nm and an emission wavelength of 500–600 nm. The activities of intracellular antioxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT)) of the *Chlorella* culture were determined using the corresponding assay kits (Beyotime, China), according to the manufacturer's instructions. The malondialdehyde (MDA) content of the *Chlorella* culture was detected using the classic thiobarbituric acid method combined with the corresponding assay kit (Beyotime, China), with the absorbance measured at 532 nm using a Synergy H1 Hybrid Multi-Mode Reader (Bio-Tek, USA). The reduced nicotinamide adenine dinucleotide phosphate (NADPH) content of *Chlorella* sp. was determined by colorimetric determination using a NADPH assay kit (AAT Bioquest, USA), according to the manufacturer's instructions.

2.9. Transcript analyses of genes encoding enzymes involved in lipid metabolism

The abundances of transcripts of genes encoding enzymes involved in fatty acid and triacylglycerol biosynthesis – acetyl-CoA carboxylase (encoded by *ACCase*), malonyl-CoA:ACP transacylase (encoded by *MCAT*), 3-oxoacyl acyl carrier protein reductase (encoded by *FabG*), glycerol-3-phosphate acyltransferase (encoded by *GPAT*), 1-acylglycerol-3-phosphate acyltransferase (encoded by *LPAT*) and acyl-CoA: diacylglycerol acyltransferase (encoded by *DGAT*) – were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. This elucidated the potential mechanistic roles of the light regime and carbon supply in the combined *Chlorella* sp.-based wastewater treatment and low-carbon biofuel production process. Briefly, the total RNA of the *Chlorella* culture was extracted using the Plant Total RNA Isolation Kit (Sangon, China) and then transcribed into cDNA using HiScript II Q RT SuperMix for qPCR (Vazyme, China), according to the manufacturer's instructions. The RT-qPCR assay was performed in an 8-strip qPCR tube containing cDNA, primers, and AceQ qPCR SYBR® Green Master Mix (Vazyme, China) at a premixed volume of 20 μL, and quantified on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The RT-qPCR data were normalised to the 18 s rRNA reference gene using the $2^{-\Delta\Delta Ct}$ method. All of the primers used in this study are listed in Table S1.

2.10. Semi-continuous treatment of the sugar industry wastewater using Chlorella sp. cultivated using a combined light regime and carbon supply regulation strategy

The sugar industry wastewater treatment with *Chlorella* sp. cultivated using a combined light regime and carbon supply regulation strategy was upscaled to a 2.0-L benchtop bioreactor (Sartorius, Germany). The stirring speed was maintained at 300 rpm and the aerated air flow (containing 21% of O_2 and 0.04% of CO_2) at 3 L/min. The initial wastewater volume of 1.6 L was subjected to blue light irradiance at 200 μmol photons m^{-2} s^{-1}. Food waste obtained from canteen located in Guangzhou, China was hydrolysed to generate food waste hydrolysate as a negligible glucose source, following a literature method [22]. The turbidity of filtered food waste hydrolysate is good, suggesting that diluted food waste hydrolysate with wastewater as culture medium did not cause any hinderance against light penetration. Crude glycerol was purchased from Shengliang Chemical (Guangzhou, China), and contained glycerol, water, methanol, ash and sodium chloride at volume percentages of 87.79%, 8.27%, 0.75%, 0.31%, and 0.22%, respectively. At the beginning of the wastewater treatment process (day 1), food waste hydrolysate was added to the wastewater to give a final glucose concentration of 10 g/L. Subsequently, during the middle of the wastewater treatment process (day 4), crude glycerol was added to the wastewater to give a final glycerol concentration of 9.2 g/L (equal to 100 mM). Then, on day 6 (at the end of one round of the treatment process), 85% of the processed wastewater was discarded and replaced with the sugar industry wastewater supplemented with food waste hydrolysate and crude glycerol, as described above. The complete wastewater treatment process lasted 30 days and comprised five semi-continuous rounds.

2.11. Statistical analysis

All experiments were performed in triplicate, and the results are expressed as means ± standard deviations (SDs). Data were analysed by GraphPad Prism 8.0 (GraphPad, USA). The Shapiro–Wilk test and Levene's test were used to determine data normalcy and variance homogeneity, respectively. Differences between groups were analysed by the Kruskal–Wallis one-way analysis of variance, followed by Duncan's multiple range test. P values of < 0.05 were considered to indicate statistical significance, indicating with different lowercase letters on the bars of columns. Differences between the individual control and treatment groups were determined by unpaired Student's t-tests at a statistical significance of $P < 0.05$ (indicated by *) or $P < 0.01$ (indicated by **).

3. Results and discussion

3.1. Effect of the light regime and carbon supply on the sugar industry wastewater treatment performance of Chlorella sp.

The mechanistic roles of a light regime and a carbon supply in the microalgae-based biodegradation of pollutants have been found to differ [32]. In addition, *Chlorella* sp. can be potentially tolerable to various pollutants, implying the possibility for sugar industry wastewater treatment. Therefore, optimal light regime and carbon supply parameters must be determined for efficient wastewater treatment using a given species of *Chlorella* sp. CSH4. As microalgae selectively absorb blue and red light [33], we irradiated *Chlorella* sp. with a range of spectrum intensities (50, 100, 200, 400, and 800 μmol photons m^{-2} s^{-1}) of blue light and red light, and evaluated the effects on the wastewater treatment performance. Results revealed that exposure to blue or red light at<400 μmol photons m^{-2} s^{-1} enhanced the biodegradation performance of *Chlorella* sp. compared with the control conditions (exposure to white light at 200 μmol photons m^{-2} s^{-1} or to darkness) (Table S2). In particular, the *Chlorella* cultures exposed to 200 μmol photons m^{-2} s^{-1} of blue light exhibited the highest pollutant-removal efficiencies: 99.6% for BOD, 98.3% for COD, 96.9% for NH_4^+-N, 93.3% for TN removal, and 99.1% for TP (Figure S1A). This suggests that this blue light treatment significantly enhanced the ability of *Chlorella* sp. to remove pollutants from sugar industry wastewater treatment. The TN and COD removal efficiencies of microalgae exposed to blue light in open raceways were shown to be upregulated with the growth rate [34]. Similarly, microalgal species isolated from wastewater were found to generate more biomass and lipids when cultivated under blue light than when cultivated under red light [35]. Accordingly, the biomass and NL contents of the *Chlorella* cultures treated with various intensities of blue light and red light were evaluated. As expected, a blue light intensity of 200 μmol photons m^{-2} s^{-1} was found to be the optimal for biomass and NL accumulation, both of which were enhanced simultaneously in *Chlorella* sp. (Figure S1B-C). This is consistent with the finding that blue light influenced enzyme activation, gene transcription control and energy generation in microalgae, leading to the simultaneous accumulation of biomass and lipid [36]. Thus, these results demonstrate that blue light at 200 μmol photons m^{-2} s^{-1} is the optimal light regime to enhance *Chlorella* sp.-mediated biodegradation of pollutants in sugar industry wastewater while simultaneously increasing this microalgal biomass and lipid accumulation.

Regarding carbon supply, the treatment of the *Chlorella* cultures with 10 g/L glucose at the beginning of wastewater treatment has been shown to significantly enhance their pollutant removal efficiency (Table S3, Figure S1D). Similar to these previous studies [22,37], we found that the microalgal biomass was highest in the 10 g/L glucose condition, suggesting that this condition significantly enhanced biomass accumulation and thus pollutant biodegradation (Figure S1E). In contrast, we found that glycerol supply reduced the pollutant removal efficiencies of the *Chlorella* cultures by contributing to the BOD and COD of the wastewater (Table S3). However, glycerol supplementation nevertheless caused a remarkable increase in the NL content, which reached a maximum in the 100 mM (equal to 9.2 g/L) glycerol condition (Figure S1F). These results are in agreement with those obtained for engineered *Phaeodactylum tricornutum*, which showed that glucose supply promoted cell growth while glycerol supply improved NL content [30]. Thus, the carbon supplied to the *Chlorella* cultures was the main contributor to their accumulation of biomass and NL.

In a relevant study, a stepwise carbon-feeding strategy was developed, which consists of supplying glucose at the early phase of microalgal cultivation, to support cell growth, followed by supplying glycerol at the late phase of microalgal cultivation, to support lipid accumulation [30]. In addition, organic carbon and light of a certain intensity have been shown to synergistically influence the biochemical and physiological performances of microalgae [38,39]. Therefore, we investigated the effects of a combined light regime (blue light intensity of 200 μmol photons m^{-2} s^{-1}) and carbon supply (10 g/L glucose and 9.2 g/L glycerol) regulation strategy on the sugar industry wastewater treatment performance of *Chlorella* sp. As shown in Table 1, all pollutant residues were detected to be significantly downregulated at the end point of wastewater treatment (day 10) under the combined conditions, suggesting that this combined strategy maximised the pollutant-degradation capabilities and efficiencies of *Chlorella* sp. (Figure S2). In addition, we found that the removal rate of pollutants from the sugar industry wastewater by the *Chlorella* culture subjected to these combined conditions was significantly greater than that achieved by the *Chlorella* cultures subjected to other conditions (Fig. 1A-F). Optimum light intensity at 216 μmol photons m^{-2} s^{-1} associated with 9.1% of CO_2 supply were performed to achieve the complete removal of pollutants by *C. vulgaris*, resulting in increasing microalgal growth [25]. Similarly, in this study, a higher growth rate with a higher biomass content (2.61 g/L) and a higher NL content (2.00-fold of DCW) were recorded in *Chlorella* sp. subjected to the combined conditions relative to that subjected to other conditions (Fig. 1G-H). Taken together, these findings demonstrate that cultivating *Chlorella* sp. using a combined light regime and carbon supply regulation strategy dramatically enhanced its sugar refinery wastewater pollutant-removal efficiency and its accumulation of biomass and lipids.

3.2. Cultivation of Chlorella sp. using the combined strategy regulated its photosynthesis and carbon metabolism to achieve biomass accumulation and pollutant removal in the growth phase

The cultivation of *Chlorella* sp. using the combined strategy resulted in a stable increase in biomass, leading to a significant increase in the growth rate throughout the treatment period (from day 1 to 7) (Fig. 1G, Figure S3). This suggests that the efficient pollutant removal by the *Chlorella* cultures was mainly due to their increased biomass. This is consistent with the fact that increased microalgal biomass contributes to nutrient removal from raw swine wastewater [40]. Similarly, increased biomass accumulation induced by CO_2 improved the tolerance of *Tribonema* sp. to toxic pollutants, thereby effectively enhancing its pollutant-removal capabilities [41]. Accordingly, we attempted to determine the relationship between biomass accumulation and pollutant removal by *Chlorella* sp. cultivated using the combined light regime and carbon supply regulation strategy. We first determined the photosynthetic performance of the *Chlorella* cultures to explore how the combined strategy affected their growth during the wastewater treatment period. As shown in Fig. 2A-C, the photosynthetic parameters (the Fv/Fm, ETR, and NPQ) of the *Chlorella* culture treated with blue light and supplied with glucose were significantly enhanced compared with the *Chlorella* cultures subjected to other treatments (blue light, glucose, and glycerol treated or untreated). This suggests that the combined strategy significantly enhanced photosynthesis in *Chlorella* sp., and thereby increased its pollutant-removal efficiency. This finding is similar to a previous observation that enhanced microalgal photosynthesis led to a rapid

Table 1
Wastewater pollutant-removal performance of *Chlorella* sp. using various strategies.

Composition	Combined strategy	Blue light	Glucose	Glycerol	Control	Untreated oWW
COD (mg/L)	10.29 ± 0.93	136.02 ± 8.87	108.16 ± 7.75	277.15 ± 23.04	363.74 ± 27.11	647.56 ± 39.25
NH_4^+-N (mg/L)	0.74 ± 0.08	33.74 ± 2.61	15.72 ± 2.01	27.69 ± 1.96	43.15 ± 2.83	61.57 ± 3.74
TN (mg/L)	1.33 ± 0.20	41.53 ± 5.39	18.74 ± 1.38	33.46 ± 2.37	51.26 ± 5.09	75.88 ± 8.02
TP (mg/L)	0.27 ± 0.04	1.96 ± 0.86	1.05 ± 0.09	1.82 ± 0.33	2.91 ± 1.26	5.82 ± 0.33
glucose (g/L)	N.D.	N.D.	0.16 ± 0.02	N.D.	N.D.	2.37 ± 0.14
glycerol (g/L)	0.13 ± 0.02	N.D.	N.D.	0.14 ± 0.03	N.D.	N.D.

oWW, original mixed wastewater; N.D., not detected; BOD, biochemical oxygen demand; COD, chemical oxygen demand; NH_4^+-N, ammonia–nitrogen; TN, total nitrogen; TP, total phosphorus.

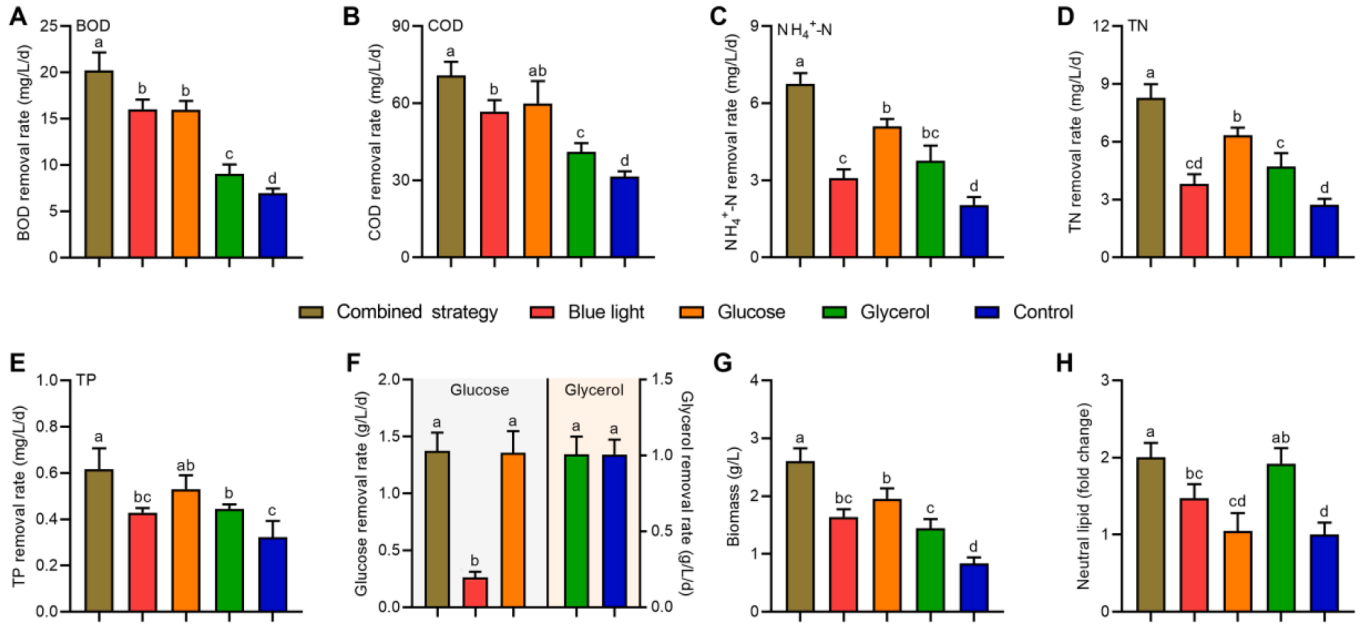

Fig. 1. Sugar industry wastewater pollutant-removal rate and biomass and neutral lipid content of the *Chlorella* cultures under various cultivation conditions. (A) Biochemical oxygen demand (BOD) removal rate; (B) chemical oxygen demand (COD) removal rate; (C) ammonia–nitrogen (NH_4^+-N) removal rate; (D) total nitrogen (TN) removal rate; (E) total phosphorus (TP) removal rate; (F) glucose and glycerol removal rate; (G) biomass content of the *Chlorella* cultures after 10 days of treatment; and (H) neutral lipid content of the *Chlorella* cultures after 10 days of treatment. Each error bar represents the standard deviation of three samples. Different lowercase letters on the column bars indicate significant differences at $P < 0.05$.

growth of a fungi–bacteria–microalgae system, and enhanced its pollutant removal performance [42]. A positive relationship between microalgal biomass and pollutant removal was observed in this study, suggesting that higher biomass led to a higher rate of pollutant assimilation, and thus a higher pollutant-removal efficiency [43].

Studies have demonstrated that supplying a high flow of CO_2 to microalgae-based wastewater treatment systems improves their pollutant removal rates and efficiencies, suggesting that high CO_2 utilisation enhances their pollutant-removal performance [44,45]. Thus, we monitored the CO_2 utilisation efficiency of the *Chlorella* cultures during the treatment period. As expected, the CO_2 utilisation efficiency of *Chlorella* sp. cultivated using the combined strategy was remarkably enhanced (Fig. 2D), indicating that the enhanced biodegradation efficiency of pollutants in wastewater might be due to enhanced CO_2 utilisation. Furthermore, studies have demonstrated that elevated CO_2 concentration increase the rate of photosynthesis in microalgal cultures, which enhances their biomass accumulation [46,47]. CA is a key molecule in the carbon fixation system of microalgae, and thus plays an important role in assisting RuBisCO in photosynthetic carbon-concentrating processes that enable highly efficient CO_2 capture [48]. Similarly, high RuBisCO activity is needed for achieving enhanced CO_2 fixation by increasing CA activity in the diatom *P. tricornutum* [49]. As shown in Fig. 2E-F, the activities of RuBisCO and CA were higher in the *Chlorella* culture cultivated using the combined strategy than in the cultures cultivated using other conditions. This may be because more CO_2 entered into the microalgal cells due to the enhanced RuBisCO and CA activities in the photosynthetic carbon-concentrating processes, which improved the photosynthetic performance and thus enhanced biomass accumulation.

To assess the effect of microalgal carbon flux redirection during the wastewater treatment period, we analysed the *Chlorella* cultures to determine their primary metabolites. The maximum carbohydrate content (29.5%) occurred on day 4 in the *Chlorella* culture cultivated using the combined light regime and carbon supply regulation strategy (Fig. 3A). This is consistent with the fact that CO_2 fixation can be promoted by providing a sufficient supply of carbon to support carbohydrate biosynthesis [50]. Similarly, the addition of carbon sources into a culture medium caused intracellular carbohydrate accumulation in microalgae, which triggered biomass conversion in the growth phase [51]. Thus, we speculate that the combined light regime and carbon supply strategy regulated photosynthesis and carbon flux in *Chlorella* sp., which facilitated its carbohydrate biosynthesis and thus resulted in its accumulating biomass and demonstrating efficient pollutant removal.

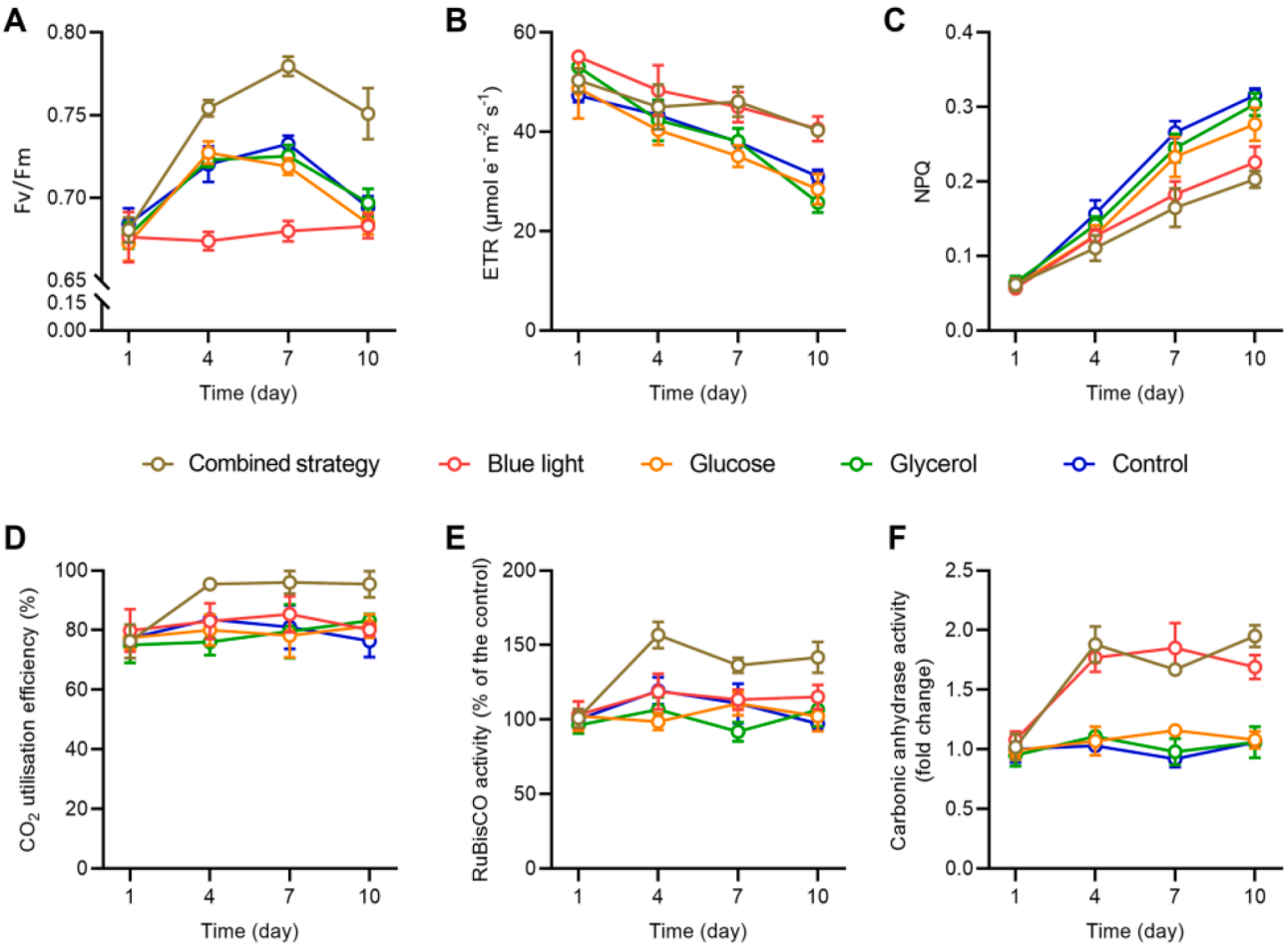

Fig. 2. Analyses of the photosynthetic performance of the *Chlorella* cultures cultivated in sugar industry wastewater under various conditions. (A) Maximum photochemical efficiency of photosystem II (F_v/F_m); (B) electron transport rate (ETR); (C) non-photochemical quenching (NPQ); (D) CO_2 utilisation efficiency; (E) activity of ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO); (F) activity of carbonic anhydrase (CA). Error bars indicate the standard deviations of three samples.

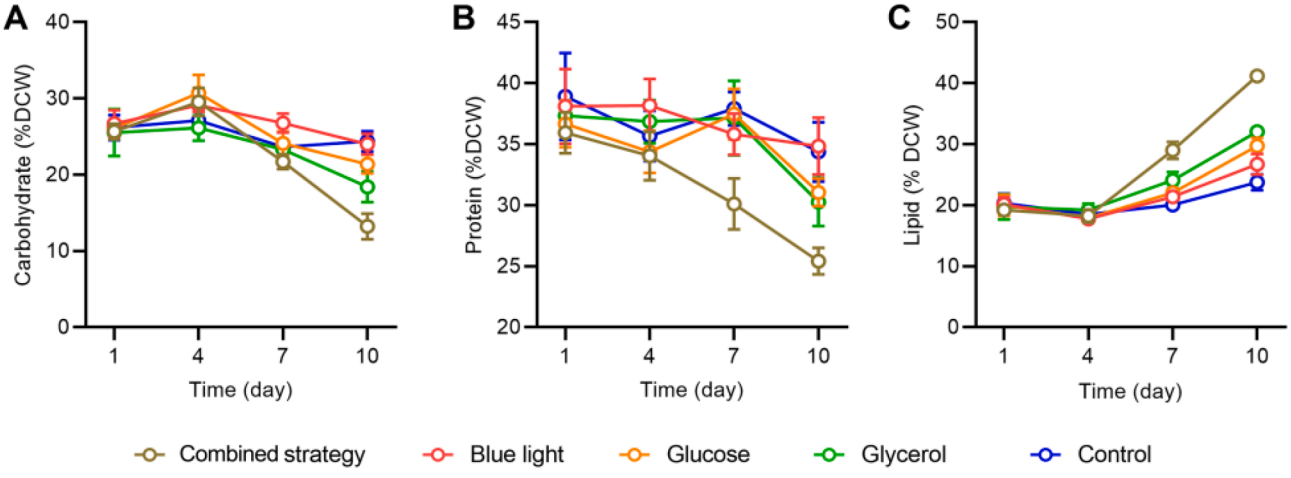

Fig. 3. Primary metabolites of the *Chlorella* cultures cultivated in sugar industry wastewater under various conditions. (A) Carbohydrate content; (B) protein content; and (C) lipid content. Error bars indicate the standard deviations of three samples.

3.3. Combined strategy for the cultivation of Chlorella sp. for wastewater treatment redirected its metabolic flux towards lipogenesis to yield biofuels in the stationary phase

We found that compared with the *Chlorella* cultures cultivated using other strategies, the lipid content of the *Chlorella* culture cultivated using the combined strategy was significantly increased after the stationary phase (Fig. 3C), which led to lipid accumulation. This suggests that the combined strategy recalibrated the carbon flux of *Chlorella* sp. towards lipogenesis, as indicated by lipid accumulation of up to 41.2% of DCW

observed at the end of wastewater treatment. Previous reports have similarly demonstrated the synchronous wastewater bioremediation and lipid accumulation for biodiesel production by microalgae [13,14]. Furthermore, the contents of carbohydrate and protein, two other primary carbon metabolites, were remarkably and gradually decreased (Fig. 3A-B) at day 7 and 10, confirming that the combined light regime and carbon supply regulation strategy redirected carbon metabolism from carbohydrate and protein bioconversion to lipogenesis [52,53].

Lipids, consisting mainly of NL, PL, and GL, are the primary structural components of cellular and organelle membranes, and NL also acts as a major energy storage compound. To elucidate the mechanism by which the combined strategy affected the lipid metabolism of the *Chlorella* culture, we analysed the fractionated lipid profiles. This revealed that after 7 days of cultivation using then combined strategy, the NL and PL contents of the cells were considerably increased, whereas their GL content was decreased (Figure S4). GL is a crucial component of the chloroplast membrane [54], and thus a decrease in GL content can perturb the formation and function of the chloroplast membrane, and thereby decrease photosynthetic performance; this is what we observed in our data. Furthermore, PL forms the membrane of lipid droplets, which are the major organelle for NL storage in microalgae. Studies have also revealed that the NL and PL contents were remarkably increased in *P. tricornutum* used for biofuel production [55,56]. These data indicate that the cultivation of *Chlorella* sp. using our combined strategy led to a remarkable increase in the microalgal cells' NL and PL contents and a decrease in their GL content, which enhanced their production of biofuels.

Studies have demonstrated that microalgal lipid production occurs via fatty acid biosynthesis [57,58]. Thus, we next aimed to ascertain the fatty acid profile of *Chlorella* sp. cultured in wastewater using the combined light regime and carbon supply regulation strategy. As expected, the total fatty acid content of this culture was clearly enhanced, suggesting that the combined strategy caused microalgal fatty acid accumulation during wastewater treatment (Fig. 4A). The fatty acid composition, particularly the proportions of C16:0, C18:0, C16:1, C18:1, and C18:3 fatty acids, was also observed to be significantly influenced by the combined strategy (Fig. 4B-F, Table S4). In particular, compared these fatty acid proportions in *Chlorella* sp. cultivated using other strategies, the proportions of C16:0, C18:1, and C18:3 in *Chlorella* sp. cultivated using the combined strategy were increased by 9.03%, 41.37%, and 39.22%, respectively, whereas those of C16:1 and C18:0 were decreased by 78.48% and 52.26%, respectively.

The fatty acid profile of a potential microalgae-based biofuel dictates its properties [59]. For instance, compared to biofuels with a low cetane number (CN), biofuels with a high CN generate less white smoke and pollutant emissions during complete combustion in engines, thereby offering optimal machine performance [60]. It was also reported that C16:0 and C18:1 are the most desirable fatty acids that contribute to a high CN [61]. Similarly, we found that in the *Chlorella* culture cultivated using the combined strategy, C16:0 and C18:1 were the major fatty acids that contributed to its high CN value (50.19), suggesting that the biodiesel properties of this microalgae-based biofuel were good [62]. Furthermore, the unsaturation level of fatty acids generally dictates the cold flow properties of biofuels, which reflects the operability of biofuels in cold conditions [63]. Increasing the proportion of C18:3 fatty acid in microalgae-based biofuels was found to improve their cold flow properties and oxidation stability [64]. We also observed an increase in the C18:3 fatty acid content of lipids from the *Chlorella* culture cultivated

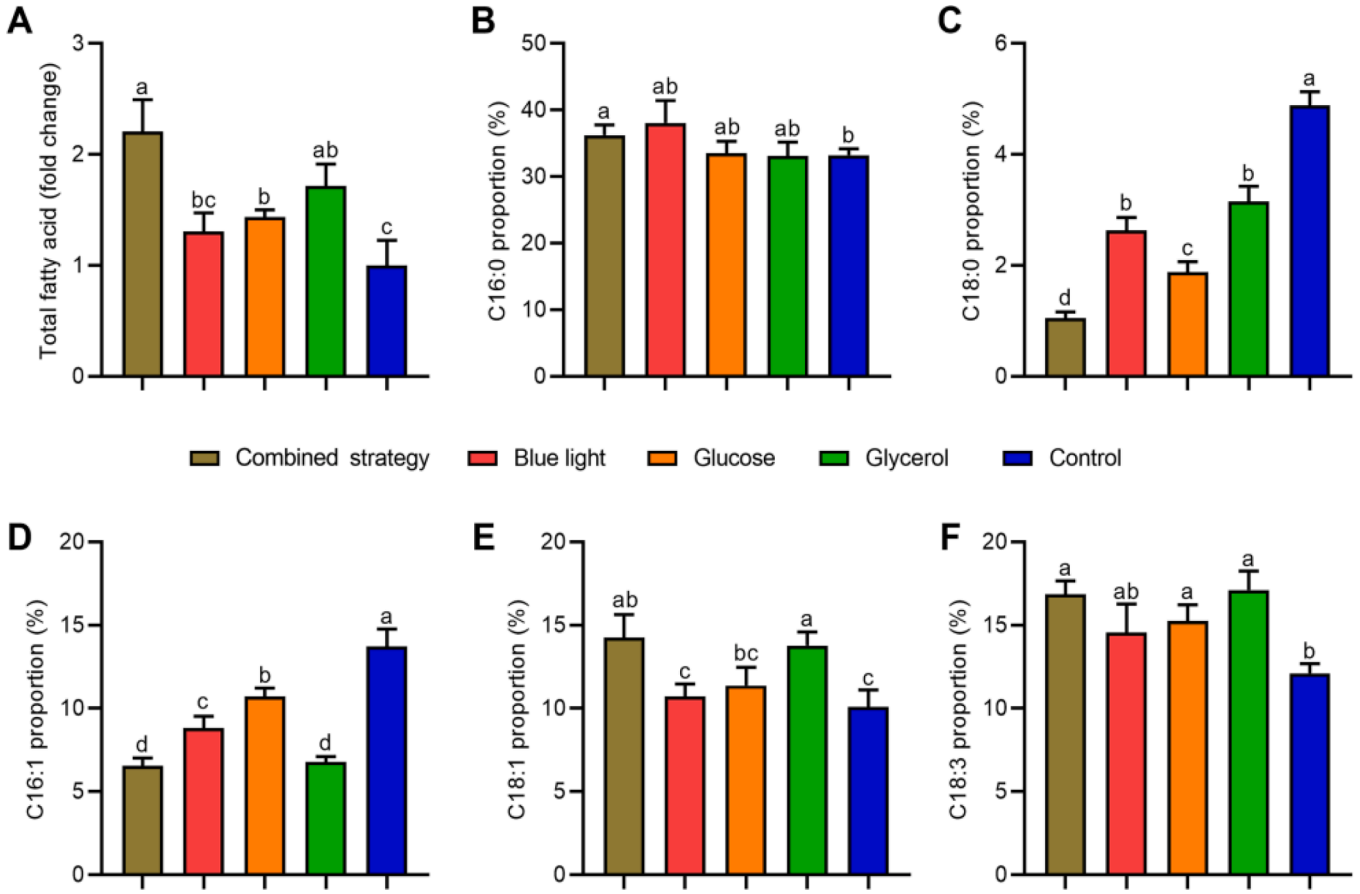

Fig. 4. Analyses of the fatty acid profiles (on day 7) of *Chlorella* cultivated in sugar industry wastewater using various treatment strategies. (A) Total fatty acid content; (B) C16:0 proportion; (C) C18:0 proportion; (D) C16:1 proportion; (E) C18:1 proportion; (F) C18:3 proportion. Each error bar represents the standard deviation of three samples. Different lowercase letters on the column bars indicate significant differences at $P < 0.05$.

using the combined strategy, compared to the C18:3 fatty acid content of lipids from *Chlorella* cultures cultivated using other strategies. Overall, based on the fatty acid profiles shown in Table 2, the biodiesel properties of the lipids from the *Chlorella* culture cultivated using the combined strategy meet most of the biodiesel standards of China, the US, and Europe, indicating that are suitable for commercial application.

3.4. Possible mechanisms underlying biomass and lipid production with high-efficiency pollutant removal by Chlorella sp. cultivated using the combined strategy

Our investigations of the biochemical changes in *Chlorella* sp. cultivated in sugar industry wastewater using the combined regulation strategy revealed that biomass accumulation and pollutant removal dominated in the growth phase, whereas lipid production dominated in the stationary phase. As the cellular membrane of microalgae is their primary defensive barrier against environmental pollutants [65], we investigated the membrane permeability of the *Chlorella* cells. This showed that the membrane permeability of those cultivated using the combined strategy was slightly greater than that of those cultivated using other strategies at 7-day (Figure S5A). This increased permeability might enhance the entry of environmental pollutants into the microalgal cells for biodegradation in the growth phase. In addition, as membrane permeability is responsible for the function of mitochondria and chloroplasts [66], we hypothesised that the enhanced membrane permeability might contribute to these functions. To test this, we investigated the mitochondrial membrane potential and the ATP content of the microalgal cells. We found that relative to cultivation using other strategies, the mitochondrial membrane potential was slightly enhanced and the ATP content was significantly increased during the entire cultivation period using the combined strategy (Figure S5B-C).

It was suggested that an increased mitochondrial membrane potential might be attributable to more energy being generated in the mitochondria for cell metabolism [67]. In addition, excessive ATP content in microalgae was found to improve nutrient removal from swine wastewater [68]. In the current and compared to the *Chlorella* cultures cultivated using other strategies, the cultures cultivated using the combined strategy exhibited increased photosynthetic performance and redirection of carbon flux towards carbohydrate accumulation, which led to biomass accumulation. Regarding carbon flux, a study showed that excess CO_2 could reduce NH_4^+-N toxicity and thus promote the efficiency of removal of both nitrogen and phosphorus from wastewater by microalgae [69]. Similarly, Delgadillo-Mirquez et al. demonstrated the positive correlation between nutrient removal and microalgal growth, as indicated by microalgae exhibiting greater nitrogen and phosphate removal performance from wastewater when they accumulated greater biomass [70]. Moreover, the nitrogen and phosphate uptake were proportional to the wastewater COD, as the organic carbon sources supported microalgal growth [71]. Overall, in the current study, we demonstrated that the cultivation of *Chlorella* sp. using a combined treatment strategy enhanced cell membrane permeability, allowing more wastewater pollutants to enter into cells for biodegradation aided by mitochondria and chloroplasts during the growth phase. This enhanced biomass accumulation and consequently improved the pollutant removal capacity.

An increased mitochondrial membrane potential is considered to cause mitochondrial hyperpolarisation, which leads to ROS generation [72]. As such, some ATP energy is assigned to maintain cellular antioxidant capacities to alleviate the oxidative damage caused by these ROS [73]. Moreover, it is well known that wastewater contains various impurities such as excessive nitrogen, phosphorus and organic pollutants, which will perturb the dynamic balance of biological process by overproduction of ROS in microalgae, thus leading to unexpected stress behaviours [74]. Therefore, we next evaluated the microalgal antioxidant system of *Chlorella* cultivated using the combined strategy. We found that ROS generation was higher on day 7 and lower on day 10 (Fig. 5A), implying that ROS overproduction was caused by the increased mitochondrial membrane potential on day 7. Compared with the control strategy, after day 7, the combined strategy led to significant upregulation of antioxidant enzymes (GPx, SOD, and CAT), a decrease in the MDA content (Fig. 5B-E) and an increase in the NADPH content (Fig. 5F).

A study demonstrated that melatonin treatment of *Monoraphidium* sp. QLY-1 could decrease ROS generation and increase the activity of antioxidant enzyme, leading to lipid accumulation [75]. Given the considerable lipid production by the *Chlorella* culture cultivated using the combined strategy, we examined the transcript abundances of key lipogenic genes to elucidate the mechanistic roles of the light regime and carbon supply in lipogenesis in the stationary phase. Specifically, on day 7, we investigated the expression pattern of key genes involved in triacylglycerol and fatty acid biosynthesis. This showed that the abundances of transcripts of these genes were all enhanced in the *Chlorella* cultures cultivated using the combined strategy, but not in those cultivated using the control strategy (Figure S6). This suggests that the combined strategy stimulated the overexpression of lipogenic genes during the stationary phase, resulting in lipid production together with a decrease in the ROS content. Taken together, our results suggest that using the combined strategy for cultivating *Chlorella* in wastewater minimised its oxidative stress and enhanced its lipid production during the stationary phase (Figure S7).

3.5. Semi-continuous wastewater treatment facilitated by microalgae-based pollutant removal and biofuel production

Recently, a semi-continuous cultivation strategy, considered suitable for pilot or up-scale cultivation, has been implemented for simultaneous wastewater treatment and valuable product generation by microalgae [76–78]. In this strategy, a portion of the previously grown microalgal cultures is retained in bioreactors as seed cultures for the next round of wastewater treatment, which also brings fresh nutrients. Therefore, in

Table 2
Comparison of biodiesel properties of the lipids extracted from the *Chlorella* cultures obtained from empirical measurements and calculations, with reference to recognised standards.

Biodiesel properties	Combined strategy	Blue light	Glucose	Glycerol	Control	Commercial biodiesel	ASTM D7651-08	EN 14,214	GB252-2011
DU (wt%)	87.34	82.19	87.27	89.09	82.33	–	–	–	–
LCSF (wt%)	5.21	6.23	6.25	6.02	6.89	–	–	–	–
CFPP (°C)	−0.12	3.10	3.17	2.45	5.17	−5	+5	−5 to −15	–
IV (g I_2/100 g)	103.09	99.11	104.23	106.92	97.49	130	120 (max)	120 (max)	–
SV (mg KOH)	201.50	201.88	201.68	200.85	202.15	–	370 (max)	–	–
CN	50.19	51.04	49.91	49.42	51.37	45–55	47 (min)	51 (min)	≥49
kV (mm^2/s)	4.77	4.60	4.85	5.02	4.56	4	1.9–6.0	3.5–5.0	1.6–9.0
HHV (MJ/kg)	39.62	39.67	39.60	39.59	39.68	37.3	40	≤5 /≤-20	–
OS (h)	5.40	5.77	5.64	5.38	6.25	4	–	≥6	–

DU, degree of saturation; LCSF, long-chain saturation factor; CFPP, cold-flow plugging point; IV, iodine value; SV, saponification value; CN, cetane number; kV, kinematic viscosity; HHV, high heating value; OS, oxidative stability.

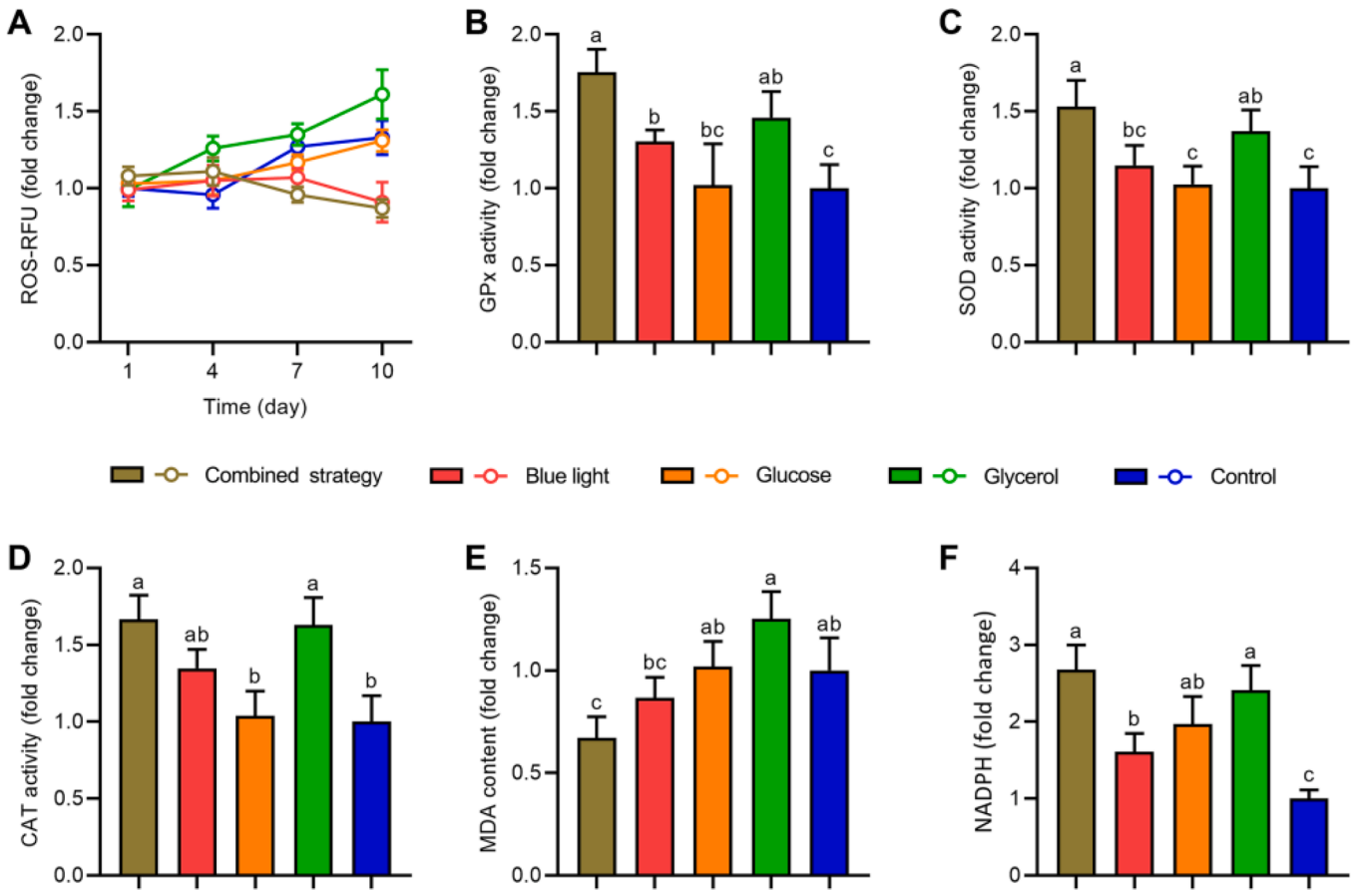

Fig. 5. Analyses of the antioxidant system (on day 7) of *Chlorella* cultured in sugar industry wastewater using various strategies. (A) Relative reactive oxygen species (ROS) content during the cultivation period; (B) activity of glutathione peroxidase (GPx); (C) activity of superoxide dismutase (SOD); (D) activity of catalase (CAT); (E) malondialdehyde content; and (F) relative content of NADPH. Each error bar represents the standard deviation of three samples. Different lowercase letters on the column bars indicate significant differences at $P < 0.05$.

this study, a novel semi-continuous cultivation strategy was used to culture *Chlorella* using the combined strategy, and this culture's wastewater treatment performance and biomass and lipid accumulation were evaluated. As shown in Fig. 6A, the average biomass concentration in wastewater culture under these semi-continuous conditions was 6.8–7.9 g/L with a productivity of 1.1–1.3 g/L/d. After one round of these conditions, 85% of the total culture volume was harvested and replenished with fresh wastewater. The biomass retained from the previous round thrived on the nutrients from fresh wastewater, despite no organic carbon sources being supplied. The average lipid production was 2.9–3.2 g/L with a productivity of 0.48–0.54 g/L/d (Fig. 6B). After four rounds under these semi-continuous conditions, the efficiency of pollutant removal from wastewater gradually decreased until the end of the cultivation period (Fig. 7). Nonetheless, the wastewater treatment efficiency met the wastewater discharge standards of China (GB8978-1996). In summary, 51.18 g of biomass with 20.94 g of total lipids was harvested after five rounds of semi-continuous cultivation in wastewater using a blue-light irradiance of 200 μmol photons m^{-2} s^{-1} and with sequential supplementation of 10 g/L glucose (70.4 g in total) and 9.2 g/L glycerol (64.84 g in total), and a treated water output of 7.04 L was obtained. These results indicate that semi-continuous cultivation of this *Chlorella* sp. in wastewater enhanced its biomass accumulation and lipid production, and its pollutant-removal efficiency.

A mass balance analysis can be performed to determine the

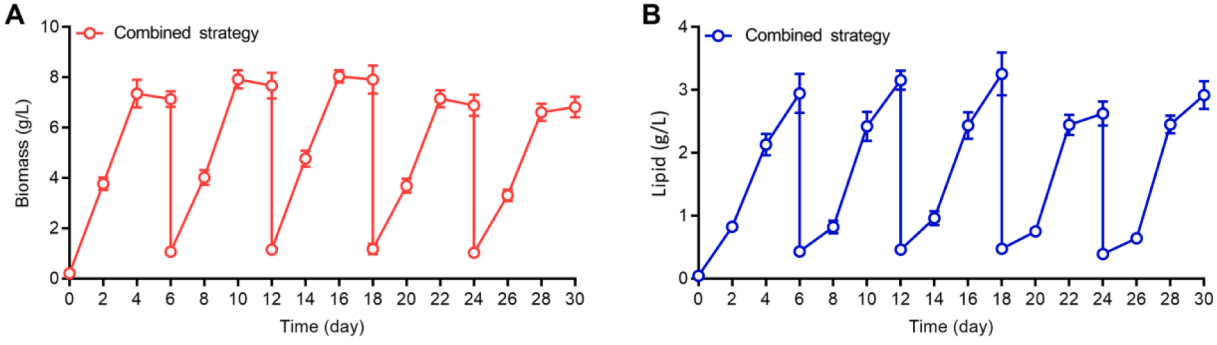

Fig. 6. Biomass and lipid accumulation in the *Chlorella* cultures during 30 days of semi-continuous cultivation in wastewater. (A) Biomass content; and (B) lipid content. Each error bar represents the standard deviation of three samples.

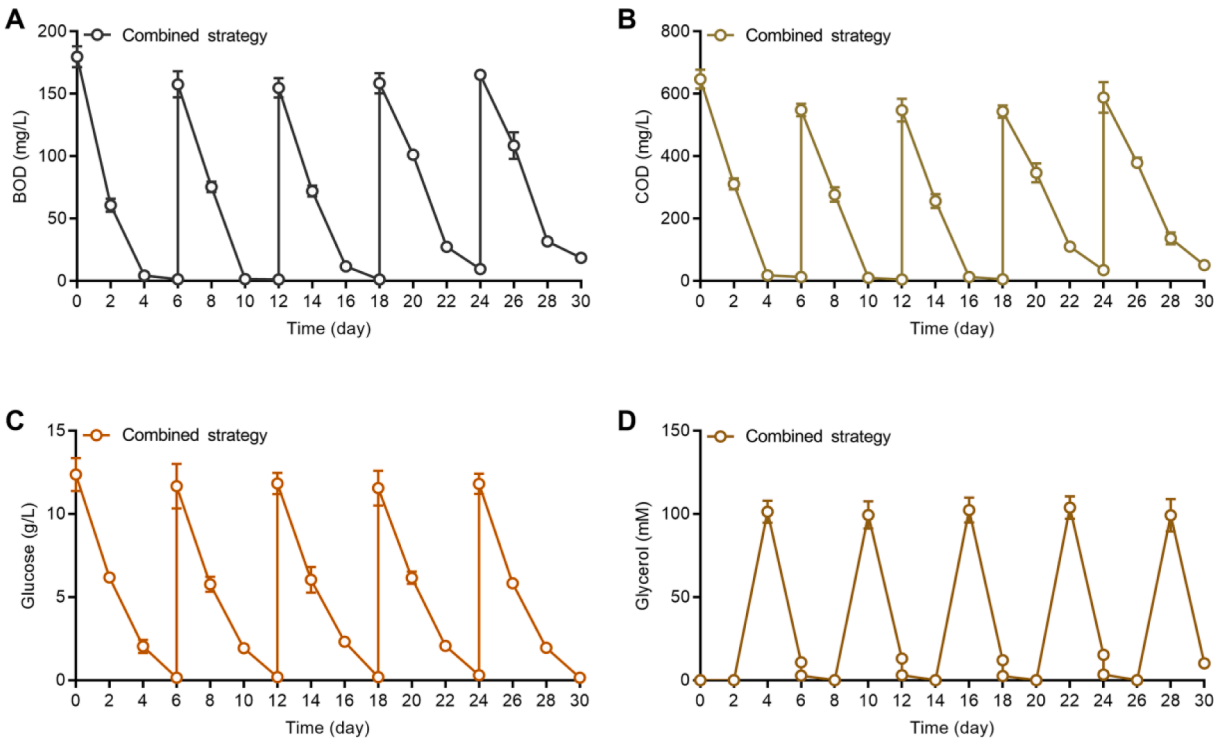

Fig. 7. Pollutant removal by the *Chlorella* cultures during 30 days of semi-continuous cultivation in wastewater. (A) Biochemical oxygen demand (BOD); (B) chemical oxygen demand (COD); (C) glucose consumption; and (D) glycerol consumption. Each error bar represents the standard deviation of three samples.

feasibility of upscaled processes [79]. Thus, to estimate the mass balance from waste to products, we performed process flow analyses of stream inputs and outputs during the wastewater treatment by *Chlorella* sp. cultivated using the combined strategy. As shown in Fig. 8, the entire process flow involves the steps of wastewater preparation, food waste hydrolysate preparation, and wastewater treatment. Wastewater was prepared by combining two types of wastewater including (i) sugar feedstock washing wastewater and (ii) ion-exchange process wastewater in a 1:4 vol ratio. Food waste hydrolysate was prepared by combining food waste, tap water, and commercial enzymes.

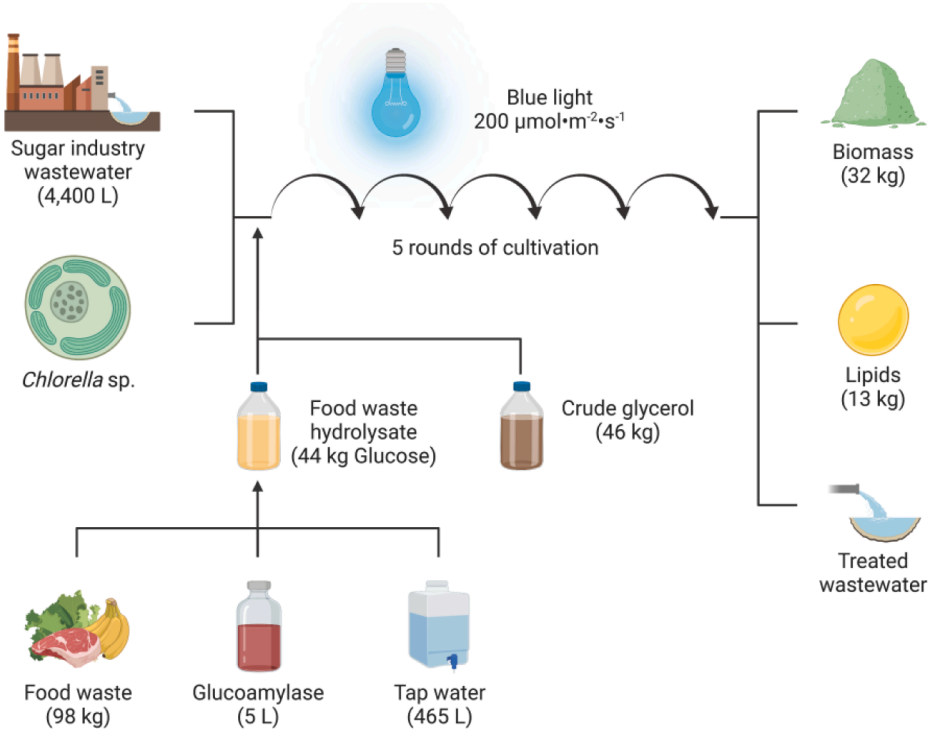

Fig. 8. Process flow diagram for sugar industry wastewater treatment and low-carbon biofuel production by *Chlorella* sp. cultivated using a combined light regime and carbon supply regulation strategy.

Based on our laboratory-scale data from this study, we estimated that ten 100-L-scale bioreactors would be required for semi-continuous cultivation and wastewater treatment over 1 month, to provide the preliminary data for further techno-economic analysis. To that end, 4,400 L of wastewater was collected for treatment, and 98 kg of food waste was collected for hydrolysis. The initial inoculum of *Chlorella* sp. (0.2 g/L of initial biomass) was used for semi-continuous cultivation and wastewater treatment, which yielded 32 kg of biomass and 13 kg of total lipids. This suggests that this microalgae-based wastewater treatment approach is feasible, and enables sustainable biofuel production that meets the principles of 'waste to wealth' [22,37]. However, entire process flow and economic analyses could not be performed in this study. This kind of systematic assessment is needed in future work, to evaluate the economic, environmental, and social feasibility of this microalgae-based approach for wastewater treatment and biofuel generation.

4. Conclusions

In this study, we examined the abilities of *Chlorella* sp. cultivated using a combined light regime and carbon supply regulation strategy to remove pollutants from sugar industry wastewater. Our results show that this strategy effectively enhanced microalgal biomass accumulation and pollutant removal capacity during the growth phase and lipid production during the stationary phase. Comparison to commercial biodiesel standards confirmed that the fatty acid profile of the lipids extracted from the *Chlorella* culture generated using this combined strategy was suitable for commercial application. By performing biochemical and molecular analyses, we elucidated possible mechanisms underlying the biomass and lipid production and the high-efficiency pollutant removal by *Chlorella* sp. cultivated using the combined strategy. Furthermore, semi-continuous cultivation of *Chlorella* sp. and processing of sugar industry wastewater was performed to explore the feasibility of implementing the combined cultivation strategy in upscaled bioreactors. In summary, this study provides novel insights into a microalgae-based method for high-efficiency wastewater treatment combined with low-carbon biofuel production, using a combined light regime and carbon supply regulation strategy.

CRediT authorship contribution statement

Xiang Wang: Conceptualization, Methodology, Data curation, Writing – original draft, Visualization. **Zi-Hao Qin:** Methodology, Data curation, Visualization. **Ting-Bin Hao:** Methodology, Data curation. **Guang-Bin Ye:** Methodology, Validation. **Jin-Hua Mou:** Resources, Investigation. **Srinivasan Balamurugan:** Resources, Validation. **Xiao-Yun Bin:** Resources, Visualization. **Joseph Buhagiar:** Data curation, Writing – review & editing. **Hong-Mei Wang:** Data curation. **Carol Sze Ki Lin:** Conceptualization, Writing – review & editing, Project administration. **Wei-Dong Yang:** Project administration. **Hong-Ye Li:** Data curation, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2022.137422.

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