Bioelectrochemically assisted sustainable conversion of industrial organic wastewater and clean production of microalgal protein

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Abstract

Chlorella vulgaris, one of the single cell protein sources, is a promising alternative to address the ever-growing demand for food-quality protein. Efforts have been made to overcome the high production costs by using wastewater for the cultivation of *C. vulgaris*. However, direct use of wastewater poses threats to the safety of applying the obtained biomass for food and animal feed. This study applied a novel three-chamber microalgal-bio-electrochemical systems for simultaneous clean cultivation of *C. vulgaris* and treatment of industrial organic wastewater. Results demonstrated that the removal of COD (38.7-66.8%) and total Kjeldahl nitrogen (TKN, 49.8-69.0%) improved with the increase of electric current in both anode and cathode chambers. Meanwhile, comparable phosphorus removal rates of 34.2-48.5% were achieved in all operation modes. Through nutrients migration, the middle chamber recovered 34.4-39.4% TKN, 16.8-47.3% phosphorus, and acetate from the wastewater to support a mixotrophic growth of *C. vulgaris*. Moreover, increasing electric current promoted higher dry algal biomass weight (0.87-1.11 g L^{-1}), higher protein content (320.8-552.1 mg Protein g^{-1} Biomass), and larger cell size (enlarged up to 151.2%) than the control. Nevertheless, the ratio of protein content decreased with the increase of cell size due to the prior accumulation of other compounds under mixotrophic growth. This study provides a sustainable approach for the conversion from industrial organic wastewater to clean production of microalgal protein.

Keywords: Microalgal-bio-electrochemical system; Single cell protein; Wastewater treatment; Pure microalgal cultivation; Nutrients recovery

1. Introduction

An ongoing driving force for exploring alternative protein sources is continuously generated by the increasing demands for food and poultry feed (Godfray et al., 2010). In this view, even with concerns of extraction, purification, and protein quality, single cell protein (SCP) derived from microorganisms is still recognized as a promising protein source due to its multi-advantages over the conventional protein sources, *e.g.*, high protein content, high conversion efficiency and wide feedstocks for the conversion (Matassa et al., 2015). Among the sources of SCP, microalgae are regarded as a promising source of SCP, because they contain a broad spectrum of nutrients rather than only protein, including lipids, minerals and, vitamins (Becker, 2007). Studies demonstrate that the amino acid compositions of plenty of microalgal species are comparable with that of the reference composition recommended by the World Health Organization. Among them, *Chlorella vulgaris* (*C. vulgaris*) is one of the few microalgal species that have already been commercialized as food additives for human (Becker, 2007). In addition to the high protein content (up to 58% w/w dry weight biomass) (Spolaore et al., 2006), the bioactive compounds such as carotenoids and polyunsaturated fatty acids (PUFAs) (da Silva Vaz et al., 2016) in the cells of *C. vulgaris* may also contribute to the essential nutrients for food and feed application.

Though promising, the high production costs of microalgae cultivation, which is mainly contributed by the costs of nitrogen, phosphorus, and carbon substrate (Hülsen et al., 2018), enormously inhibit the wide application of microalgae as protein sources to compete with agricultural alternatives. Efforts have been made on using wastes, *e.g.*,

68 cheese whey (Salati et al., 2017), dairy wastewater, and poultry wastewater (Hülsen et
69 al., 2018), as a nutrient source to decrease the cultivation costs of *C. vulgaris* and
70 simultaneously achieve the waste remediation. Apart from the cost, the recovery of
71 nitrogen and phosphorus from waste is also significant considering the depleting
72 amount of natural reserve (Rittmann et al., 2011). Among the various sources of waste,
73 potato juice wastewater (resulting, e.g., from potato-starch production) is present in
74 large amount (Fang et al., 2011) and recognized as industrial organic wastewater rich
75 in organic matter (protein, starch, *etc*.), phosphorus, and nitrogen (Fang et al., 2011,
76 Zhu et al., 2018). Untreated discharge of such wastewater poses big threats to the
77 environment (Liu et al., 2013). However, the conventional wastewater treatment
78 approaches usually only target the removal of organic matters rather than the nutrients
79 (nitrogen and phosphorus) (Fang et al., 2011, Zhu et al., 2018). In this view, microalgae
80 may have advantages over the conventional approaches in nutrients re-capture and
81 upcycling. Microalgae have been broadly demonstrated to have a high capacity of
82 phosphorus and nitrogen recovery during wastewater treatment (Hülsen et al., 2018,
83 Salati et al., 2017). Nevertheless, the direct cultivation of microalgae in the wastewater
84 could contaminate the algal biomass with bacteria or chemical pollutants from the waste,
85 and thus, raises the safety concerns. Thus, the biomass of *C. vulgaris* obtained from the
86 wastewater, even with high protein content, is still being limited for the practical
87 utilization as food and feed.

88 Considering these challenges, the recent advances of bio-electrochemical systems
89 (BESs) for efficient nutrients recovery from wastewater may provide an alternative

solution. The membranes applied in BESs may separate microalgae from wastewater, meanwhile leaving access to migration of nutrients. Along with the development of sustainability, approaches such as adsorption, precipitation, biological uptake, and ion exchange have been extensively investigated for nutrients upcycling from wastewater (Rittmann et al., 2011). Among them, BESs and microalgae have attracted arising attention (Zhang and Angelidaki, 2015; Kelly and He, 2014). To date, most of BESs studies usually focus on single- and two-chamber MFCs and MECs with high-efficiency in nutrients recovery. It has been indicated that two-chamber BESs could successfully achieve either nitrogen (Kuntke et al., 2011) or phosphorus (Fischer et al., 2011) recovery at one time, while single-chamber BESs can achieve simultaneous nitrogen and phosphorus recovery (Zang et al., 2012).

Though BESs in both MFCs- and MECs-modes demonstrated successful recovery of nutrients from wastewater, further separation and up-concentration of the recovered nutrients are still challenges. For instance, the recovery of nitrogen from BESs requires extra equipment and chemicals to collect ammonia gas (Wu and Modin, 2013). Besides, the recovery of phosphorus via BESs requires extra chemicals (magnesium and ammonia) to form struvite ($NH_4MgPO_4{\cdot}6H_2O$, formed in alkaline conditions, phosphorus-fertilizer) precipitation for timely precipitates collection (Fischer et al., 2011). Therefore, efforts have been made on the development of efficient and cost-effective BESs approaches for nutrients upcycling, e.g., nitrate recovery from urine by a combination of MECs and membrane-aerated biofilm reactor (De Paepe et al., 2020), liquid fertilizer upcycling from urine by modified MFCs (Freguia et al., 2019), and

112 nutrients recovery by microalgae-assisted MFCs from various wastewater (urine,
113 domestic wastewater, etc.) (Elshobary et al., 2020). From the sustainability point of
114 view, in-situ utilization of the recovered nutrients for microalgae cultivation could be a
115 promising option with respect to the direct recapture of nutrients into microalgal cells.
116 Efforts have been made on single- and two-chamber microalgal-MFCs (mMFCs) for
117 in-situ removal and recovery of nutrients from wastewater (Lee et al., 2015, Cui et al.,
118 2014). Though the single-chamber mMFC demonstrated simultaneous nitrogen and
119 phosphorus recovery for microalgae cultivation, it placed the *C. vulgaris* directly into
120 the wastewater (Zhang et al., 2011), rising the contamination risk of microalgal biomass
121 and limiting the utilization of the biomass as food or feed. In this view, the two-chamber
122 mMFCs can avoid direct contamination of biomass by using an ion-exchange
123 membrane, which isolates the microalgae from wastewater. However, the applied
124 selective ion-exchange membrane could only target at either ammonia or phosphorus
125 recovery, leading to a nutrient loss and lowered efficiency of waste treatment (Pei et al.,
126 2018, Xiao et al., 2012). Therefore, the development of efficient hybrid microalgae and
127 BESs system that integrates clean microalgae cultivation and comprehensive
128 wastewater treatment is urgent.

129 In this study, a novel three-chamber microalgal-BES reactor (mBES) was
130 developed to achieve simultaneous clean cultivation of *C. vulgaris* for protein
131 production, industrial organic wastewater treatment, and energy production. In this
132 novel system, organic matters from the wastewater were oxidized by the electroactive
133 bacteria in the anode, while the oxygen reduction (MFC mode) or H_2 evolution (MEC

mode) occurs in the cathode. Therefore, the anode chamber was conducted in anoxic condition, meanwhile, the cathode chamber was aerated to supply oxygen as electron acceptor to accept electrons generated and transferred from anode (oxidation of organic matter). Due to the potential difference between the anode and cathode chambers, anions (e.g., phosphate, nitrate, and CH_3COO^-) and cations (e.g., ammonium) migrated respectively from the cathode and anode through the AEM and CEM into the middle chamber, where the accumulated nutrients were then recovered by *C. vulgaris* for production of biomass. The wastewater treatment performance and the migration of carbon, nitrogen, and phosphorus via ion-exchange membranes were investigated. The obtained biomass of *C. vulgaris* was analyzed for its amino acid profiles. This study offers insights into the development of an efficient and cost-effective approach for waste nutrients recovery and upcycling for the clean cultivation of microalgae. This work could contribute to address the conversion from industrial organic wastewater to high-quality microalgal SCP production.

2. Material and methods

2.1 Reactor setup and operation

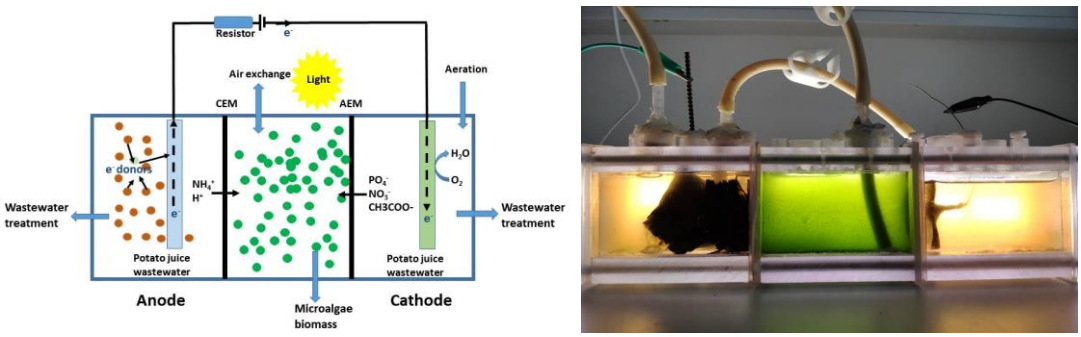


Fig.1 Schematic diagram and photo of the three-chamber mBES.

For the treatment groups, the three-chamber mBES was made of polycarbonate

material (Fig. 1). Each chamber has a working volume of 200 mL (5×5×8 cm). The anode and middle chamber were separated with a cation exchange membrane (CEM, CMI7001, Membrane International, NJ), while the middle and cathode chambers were separated with an anion exchange membrane (AEM, AMI 7001, Membrane International, NJ). Both membranes were soaked in NaCl solution (5% w/w) for 24 h and washed with distilled water before use. In the anode chamber, a carbon brush (length 6.9 cm, diameter 5.9 cm, Mill-Rose, USA) was pretreated at 450 °C for 30 min in a muffle furnace to avoid introduction of external microbes (Zhang and Angelidaki, 2015). Subsequently, in order to improve and stabilize the performance of the BESs, the anode was pre-enriched to form mature electroactive biofilm (Liu et al., 2010) using the potato juice wastewater (pH adjusted to 7 with 5 mol L^{-1} sodium hydroxide) as inoculum and substrate in the same three-chamber mBES before being used as the anode electrode. Meanwhile, a Ti-electrode mesh coated with Pt/C (0.5 mg/cm^2, 20 wt% Pt/C, JM) was applied as the cathode electrode. To provide electron acceptor to the cathode, approaches such as chemical catholyte (e.g., Ferricyanide), aeration, or air-cathode could be applied (Logan et al., 2007). However, given the efficiency and sustainability, continuous aeration at 15 mL min^{-1} was provided by a peristaltic pump (OLE DITCH, Instrument Makers APS, Denmark) to the cathode in this study. To study the effect of electric current intensity on the growth of microalgae and migration of substrates, the reactor was operated at two different modes (*i.e.*, microbial fuel cell, MFC; and microbial electrolysis cell, MEC). In the MFC mode, anode and cathode electrode were connected in series with a resistor (resistor of 1000 or 10 Ω), named as

MFC-1000 Ω and MFC-10 Ω, respectively. In the MEC mode, the two electrodes were connected in series with a 10 Ω resistor and a power supply (HQ PS3003, 102 Helmholt Elektronik A/S, Denmark) which provided 0.5 V constant voltage to the circuit, named as MEC-0.5 V.

A control of microalgal growth (named as biomass control) was conducted in an open-single-polycarbonate chamber with the same size of the treatment groups mentioned above. The modified WC medium with selenium (MWC+SE medium) without nitrogen, phosphorus, and carbon sources (Table S1) was used for this biomass control. Meanwhile, another control for protein profile comparison (named as protein control) was conducted with the same type of open-single-polycarbonate chamber as biomass control, however, with entire components of MWC+SE medium (Table S2). For the treatment groups, the modified MWC+SE medium (Table S1, all nitrogen, phosphorus, and carbon sources were removed from original MWC+SE medium) was used as the initial culture medium in the middle chamber. The microalgal species, *Chlorella vulgaris* K-1801(*C. vulgaris* K-1801), was obtained from NORCCA NIVA (Norway). Before inoculating into the control groups and the middle chambers of the treatment groups, *C. vulgaris* K-1801 was pre-cultured in MWC+SE standard medium until the late exponential phase. The control groups and the middle chambers of the treatment groups were inoculated with the *C. vulgaris* K-1801 to achieve an initial cell number of $2.9 \pm 1.8\times10^5$ cells mL^{-1}. All control groups and the middle chamber of treatment groups were provided with a 12: 12 light/darkness lumination of 5530 lux (white light, LED, Ledvance), at room temperature (22 ± 1 °C), and with continuous

197 stirring (VWR, US). The original potato juice wastewater (3.3±0.1 g COD L^{-1}, pH
198 4.75±0.2, acquired from KMC, Denmark) was directly added to the cathode chamber.
199 Meanwhile, the pH of the potato juice wastewater was adjusted to pH 7 (with 50mM
200 NaOH), and then added to the anode. All chambers achieved the same initial working
201 volume of 200 mL. Each batch was running for 18 days and all the experiments were
202 conducted in duplicate.

203 2.2 Sampling and analytical methods

204 The pH was detected using a PHM00 LAB pH meter for each chamber daily. The
205 voltage across the resistor with varying ohm (10 or 1000 Ω) was collected by a model
206 2700 data acquisition system (Keithley Instruments, Inc, Cleveland, OH, USA). The
207 chemical oxygen demand (COD) was detected every two days according to the water
208 quality standard method (Federation and Association, 2005). The Kjeldahl method was
209 applied for the total ammonia detection at day 0 and 18. Phosphorus and nitrogen in
210 forms of ammonia, nitrate, nitrite were determined by the segmented flow analysis
211 method (Scan++ system, Skalar analytical BV, the Netherlands), among which,
212 phosphorus and ammonia were measured every another day, while nitrate and nitrite
213 were measured at day 0 and 18. For the total phosphorus detection, samples were firstly
214 digested with peroxodisulfate (50 g L^{-1}) under 121 °C for 60 min， pH was adjusted
215 to 4 with HCl (for the detection of phosphorus precipitation), and then measured at day
216 18 by the segmented flow analysis method mentioned above. The cell size distribution
217 of microalgae was determined by the laser diffraction method applying the Mastersizer
218 2000 coupled with a Hydro SM sample loader (Malvern Instruments, UK), and the

219 microscopy method was applied with a LEICA microscopy (DFC320, Germany) on
220 day 18. After pre-treatment with phosphoric acid, samples were detected for VFA
221 contents by a gas chromatographer (GC, TRACE 1300 of Thermo Scientific, US)
222 equipped with a flame ionization detector and HP free fatty acid phase (FFAP) column
223 at day 0 and 18.

224 On day 18, microalgal pellets were collected for amino acid profile detection.
225 Specifically, after pre-treatment of centrifugation and freeze-drying, a 10 mg sample of
226 the dry biomass of *C. vulgaris* was hydrolyzed with the assistance of microwave (3000
227 SOLV, Anton-Paar, US) with 300 μL 6N HCl. The hydrolysis vessels were flushed with
228 Ar gas before hydrolysis and heated with a stepwise increase (5 °C min^{-1}) of
229 temperature to 130 °C and hold for 30 min. The hydrolyzed samples were further
230 analyzed for the concentrations of individual amino acid by Liquid chromatography
231 with tandem mass spectrometry (LC-MSMS, 1290 Infinity II 6470 QQQ, Agilent
232 Technologies). The column of InfinityLab Poroshell 120 HILIC-Z (100 mm × 2.1 mm,
233 2.7 μm, Agilent Technologies) was applied with mobile phases of 20 mM ammonium
234 formate in ultra-pure water (A, pH3) and 20 mM ammonium formate in acetonitrile (B,
235 pH3). Eluent A was increased from 0 to 30% in 10 minutes with a column flow speed
236 of 0.8 mL min^{-1} and a column temperature of 30 °C. The working parameters of MSMS
237 were: gas flow speed and temperature 7.0 L min^{-1} and 300 °C, sheath gas flow and
238 temperature 11 L min^{-1} and 400 °C, respectively, positive electrospray ionization,
239 nebulizer 45 psi, with an operation mode of dynamic MRM.

240 **3. Results and discussion**

3.1 COD removal and organic matter migration

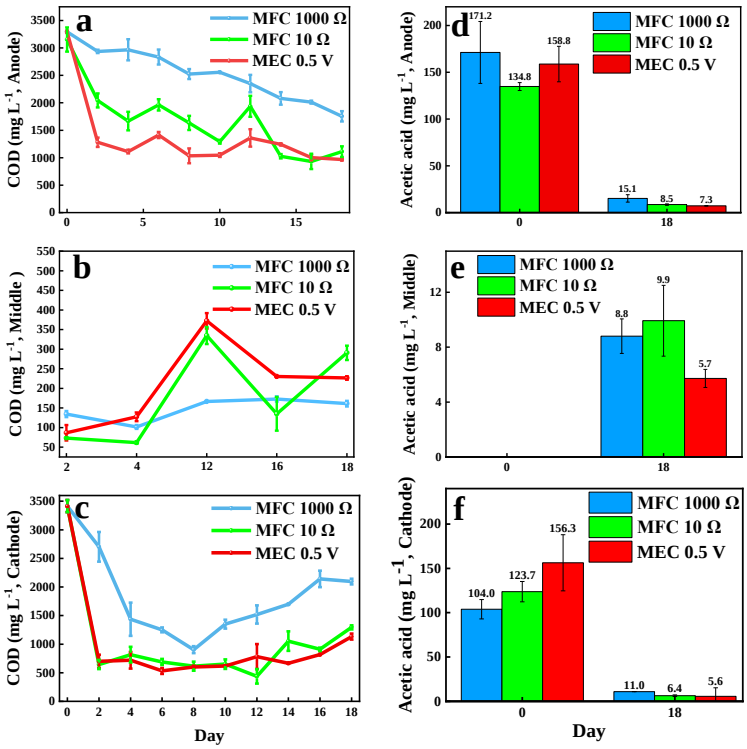


Fig.2 COD removal and/or migration in the anode chamber (a), middle chamber (supernatant) (b), and cathode chamber (c), and acetic acid removal and/or migration in the anode chamber (d), middle chamber (supernatant) (e), and cathode chamber (f).

Along with the light/darkness cycle, a fluctuant electric current with time was observed in each operation mode. An overall trend of the maximum current was achieved in the following order: MEC mode (0.46 mA, MEC 0.5 V) > MFC mode with 10 Ω (0.22 mA, MFC 10 Ω) > MFC mode with 1000 Ω (0.12 mA, MFC 1000 Ω) (Fig. S1). Under different operation modes, with the increase of electric current, a stepwise accelerated COD removal was generated in the anode chambers. In detail, at day 18, 1541 mg L^{-1} (46.8%), 2043 mg L^{-1} (64.8%) and 2329 mg L^{-1} (70.7%) of COD was removed by the MFC 1000 Ω, MFC 10 Ω, and MEC 0.5 V, respectively (Fig. 2a).

Meanwhile, in the cathode chambers, the removal of COD was relatively faster in the first 2-4 days (60-84.4% removal of COD), and then the COD concentration kept stable or even slightly raised. On day 18, the MEC 0.5 V and MFC 10 Ω systems generated a similar COD removal (66.8% and 62.1%, respectively), which were higher than that of the MFC 1000 Ω system (38.7%, Fig. 2c). The improved removal of COD in both anode and cathode chambers was probably due to the increased current, which accelerated electrons transfer between bacteria and electrode (Kim et al., 2016).

In the anode chamber, two groups of bacteria, namely electroactive and anaerobic fermentative bacteria, mainly contribute to the COD removal through the respiration activity (Zhuang et al., 2012). A previous study conducted under similar conditions with potato wastewater demonstrated that the dominant microorganism groups in the anode were *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Li et al., 2014). Among them, the exoelectrogens (microbes with ability of exocellular electron transfer, specific species in the mentioned three phyla, e.g., *Clostridium butyricum*, *Geobacter metallireducens*) may biologically oxidize organic matter (COD removal) during their anaerobic respiration, and transfer electrons to the anodic electrode (Logan, 2009) to generate current power. The formation of biofilm (exoelectrogens and fermentative bacteria) attached to the anode is essential and mainly responsible for such a bio-electrochemical process in the anode chamber (Baranitharan et al., 2015). Meanwhile, other anaerobic fermentative bacteria in the biofilm, e.g., *Bacteroides*, could also reduce COD in the anode by the fermentation process (Jia et al., 2013). A competition on substrate exists between exoelectrogens and other anaerobic fermentative microorganisms. Therefore,

276 the COD removal in the anode chamber is attributed to the complex processes of both
277 exoelectrogens and anaerobic fermentative bacteria (Zhang et al., 2015).

278 It is to be observed that, after 18 days, at least 29.3% of COD was still left in each
279 anode chamber, indicating the existence of non-biodegradable organic matter in the
280 wastewater under the operation conditions, e.g., lignin, cellulose, and potato solid waste.
281 For the MEC mode (MEC 0.5 V) and MFC mode with 10 Ω (MFC 10 Ω) groups, the
282 biodegradable COD was rapidly consumed by bacteria in 4 days, leaving a residue of
283 un-biodegradable COD (29.3%-35.2%, under the given conditions). This corresponded
284 well with the rapid COD removal in the first 4 days and lowered COD removal after
285 day 4 (Fig.2a). Unlike the anode chamber, the cathode chamber promoted a faster COD
286 removal in the early phase (in 2-4 days) due to the occurrence of multiple processes
287 including microorganisms activities (Zhang et al., 2019) and carboxylic acids migration
288 processes. Due to the continuous aeration in the cathode chamber, the activities of
289 aerobic bacteria could be promoted for the removal of COD, e.g., aerobic accumulation
290 of phosphorus by polyphosphate accumulating microorganisms, and aerobic respiration
291 by aerobic bacteria (Zhang et al., 2019). As shown in Fig. 2, with a decrease of COD
292 and acetic acid in the cathode chamber, a significant increase of both COD and acetic
293 acid were observed in the middle chamber (Fig. 2b, c, e, and f), indicating a migration
294 of organic matter (especially carboxylic acids) from the cathode chamber via the AEM
295 to the middle chamber. A part of the migrated organic matter, *e.g.*, acetic acid, could
296 serve as an organic carbon source to promote a mixotrophic growth of *C. vulgaris*. As
297 for microalgae, autotrophic growth significantly relies on the light intensity, shadow

effect caused by the increasing microalgal density may inhibit the further growth (Carvalho et al., 2011). In this study, the migrated acetic acid, which served as organic carbon source to support the mixotrophic growth of *C. vulgaris*, may reduce the growth limitation caused by the shadow effect in further scale-up cultivation. Notably, the microbial community could be significantly affected by the difference in wastewater quality and operation conditions. The present study discussed the microbial functions according to relevant references, but a comprehensive investigation on microbial community will further contribute to the understanding of COD, nitrogen and phosphorus removal. For a better understanding of carbon balance, methods such as isotope labelling could be carried out in the further investigation, so that the utilization efficiency of acetic acid could be tracked. Additionally, in order to improve the removal COD removal in the systems, pre-treatments such as sedimentation could be conducted to remove the non-biodegradable organics (e.g., lignin, cellulose and solid waste) before the BES process.

3.2 Nitrogen removal and recovery

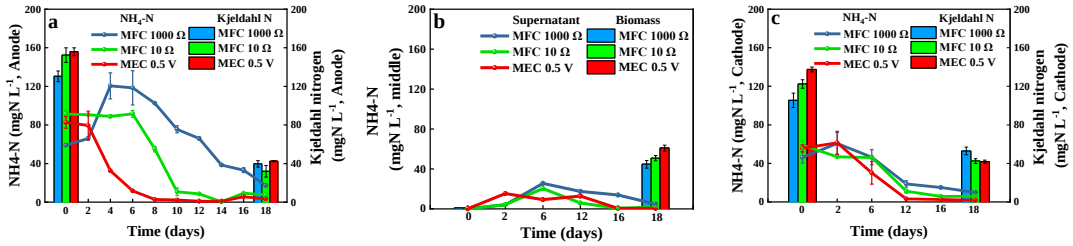


Fig.3 Nitrogen (in forms of total Kjeldahl nitrogen and ammonium) removal and/or recovery in the anode chamber (a), middle chamber (b), and cathode chamber (c)

The main nitrogen in the potato juice wastewater was in form of ammonium and protein. Therefore, the changes of ammonium and total Kjeldahl nitrogen from all three

318 chambers of each mBES were detected. Besides, the nitrogen that was re-captured by
319 the *C. vulgaris* in the middle chamber was also detected for a better understanding of
320 nitrogen balance. In the anode chamber, with low circuit current (MFC 1000 Ω),
321 ammonium concentration tends to increase in the first 4 days, followed by a stepwise
322 decrease till the end of the batch run. In contrast, under higher circuit current (MFC 10
323 Ω and MEC 0.5 V), no significant increase of ammonium concentration was detected.
324 There might be a dynamic equilibration between the removal and formation of
325 ammonium. The digestion and hydrolysis of protein in the anode chamber might
326 contribute to the raise of ammonium concentration. For the removal of ammonium, it
327 is well known that anaerobic processes (in the anode chamber) rarely contributed to the
328 removal of ammonium (Zhang et al., 2019). At non-obligate anoxic conditions and at
329 low COD loads, anaerobic ammonium oxidation (anammox) mediated by bacteria in
330 the anode chamber has been previously reported as one significant pathway of nitrogen
331 removal (Strous et al., 1998). However, in this study, due to the high COD concentration
332 of the potato juice wastewater and no detection of nitrite during the treatment, the
333 anammox process was unlikely to occur in the anode chamber (Chen et al., 2016).
334 Instead, the migration of NH_4^+ via CEM from the anode chamber to the middle chamber
335 (Park et al., 2009) mainly contributed to the decrease of ammonium concentration in
336 the anode chamber (Fig.3 a and b), which has also been reported by two-chamber MFC
337 system (Zhang et al., 2019). The higher electric current was, the faster NH_4^+ migration
338 would occur from the anode chamber via CEM to the middle chamber (Fig. S1, 3a). In
339 the anode chamber of MFC 1000 Ω, MFC 10 Ω and MEC 0.5 V groups, the ammonium

340 removal of 41.9 mg L^{-1} (71.0%), 83.6 mg L^{-1} (91.9%) and 78.64 mg L^{-1} (96.0%) were
341 achieved, respectively. Correspondingly, a total Kjeldahl nitrogen removal was 90.75
342 mg L^{-1} (69.5%), 120.5 mg L^{-1} (79.0%) and 113.5 mg L^{-1} (72.8%), respectively (Fig.3a).
343 Meanwhile, in the middle chambers, a fluctuant amount of ammonium in the
344 supernatant was observed, which was migrated from the anode chamber (Fig.3b).
345 Actually, larger amounts of ammonium might be migrated than the detected amounts
346 into the middle chamber considering the dynamic re-capturing by microalgae.
347 Additionally, the growth of microalgae rapidly elevated the pH (through inorganic
348 carbon consumption by the photosynthesis, i.e., HCO_3^-, Besson and Guiraud, 2013) in
349 the middle chamber to alkaline (pH 9-11) (Fig.S1 b), leading to a loss of ammonium
350 through volatilization. At day 18, higher electric current promoted a significant higher
351 nitrogen re-capture (61.0 mg L^{-1} in the MEC 0.5 V groups, $p<0.05$) than the other two
352 groups (50.9 and 44.7 mg L^{-1} in the MFC 10 Ω and MFC 1000 Ω groups, respectively).
353 Consequently, *C. vulgaris* has recaptured 39.1%, 33.4% and 34.3% of the total Kjeldahl
354 nitrogen from anode in the MEC 0.5 V, MFC 10 Ω and MFC 100 Ω treatments,
355 respectively.

356 In the aerated cathode chamber, the removal efficiency of nitrogen showed the
357 same trend as the electric current. As the increase of electric current, the removal
358 efficiency of ammonium and total Kjeldahl nitrogen was elevated from 36.59 mg L^{-1}
359 (79.0%) and 52.50 mg L^{-1} (49.8%) to 54.96 mg L^{-1} (96.9%) and 92.75 mg L^{-1} (69.0%),
360 respectively. The NH_4^+ migration between the cathode and middle chamber through
361 AEM should be limited. Thus, under aerated cathode conditions, ammonia was

probably removed either by the nitrification process (Sotres et al., 2016) or through the ammonium volatilization (Tao et al., 2014). In the cathode chamber, a decline of nitrate concentration (Fig.S2c) was observed, while nitrite was not detected. Meanwhile, only slight amount of nitrate was migrated to the middle chamber (0.5-1.8 mgN L^{-1}, Fig.S2b). Therefore, due to the rapid increase of pH in the cathode chamber (Fig.S3c), removal of ammonium was most likely taken place through the ammonia volatilization. Besides, the nitrate concentration was kept stable in the anode chamber, but decreased in the cathode chamber (Fig.S2 a and c). The nitrate migrated from the cathode chamber via the AEM to the middle chamber, resulting in an elevated nitrate concentration in the middle chamber (Fig.S2 b). However, the CEM between the anode and middle chambers inhibited the migration of nitrate. Therefore, to improve the nitrogen recapture rate, a recycling of the effluents from the anode chamber to the cathode chamber could be adopted in the future investigation.

3.3 Phosphorus removal and recovery

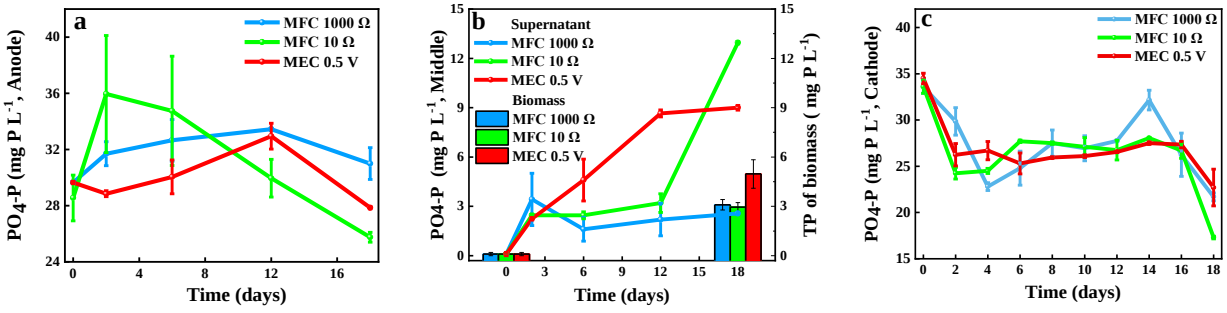


Fig.4 The change of phosphorus concentraition in the anode chamber (a), middle chamber (b) and cathode

Results indicated that no obvious changes in phosphorus concentration were observed in the anode chamber during the tests (Fig.4a). A previous study demonstrated

that phosphorus could be rarely removed (Park et al., 2009) through an anaerobic process in the anode chamber. Moreover, the CEM between the anode and middle chamber would not allow the migration of phosphorus across the two chambers. The phosphorus precipitation process was also not possible due to the neutral pH (Tao et al., 2014) in the anode chamber (Fig.S1a). Consequently, no phosphorus removal was achieved in the anode. In contrast, in the cathode chamber, higher currents promoted faster phosphorus removal (MEC 0.5 V ≥ MFC 10 Ω > MFC 1000 Ω, in two days, Fig. 4c), and larger amount of phosphorus-recovery into the middle chamber (15.9, 13.9 and 5.7 mg L^{-1} in MFC 10 Ω, MEC 0.5 V and MFC 1000 Ω group, Fig.4b). However, no obvious trend of total phosphorus removal (34.2-48.5%, cathode chamber) was found with the increase of electric current among different systems. This was mainly due to the effects of pH change on the multi-processes that might contribute to the phosphorus removal, including: (1) microorganisms activities of aerobic phosphorus uptake removal (Zhang et al., 2019); (2) phosphorus precipitates (Tao et al., 2014) regarding the alkaline cathode conditions (Fig.S1c); (3) phosphorus migration from cathode chamber via AEM to the middle chamber. Fig. S3 demonstrated that the increasing currents promoted faster pH rise. Moreover, the phosphorus precipitation caused by the rapidly increased pH may inhibit the phosphorus migration through the AEM (Tao et al., 2014), and reduce the bioavailability for other microorganisms. Therefore, higher currents promoted faster cathodic phosphorus removal via migration in shorter time, however, inhibited further phosphorus uptake (by microorganisms) and migration with rapid formation of phosphorus precipitates in long-term. Consequently,

after 18 days, no significant phosphorus removal was achieved among different systems, and more than 50% of phosphorus remained in the cathode chamber (Fig.4c). Therefore, the addition of pH buffer in the cathode chamber may further increase the phosphorus upcycling by microalgae, and the total removal efficiency. Moreover, a timely harvest of the *C. vulgaris* pellets from the middle chamber could also improve the recapture of the phosphorus.

3.4 *C. vulgaris* cultivation and cell size enlargement

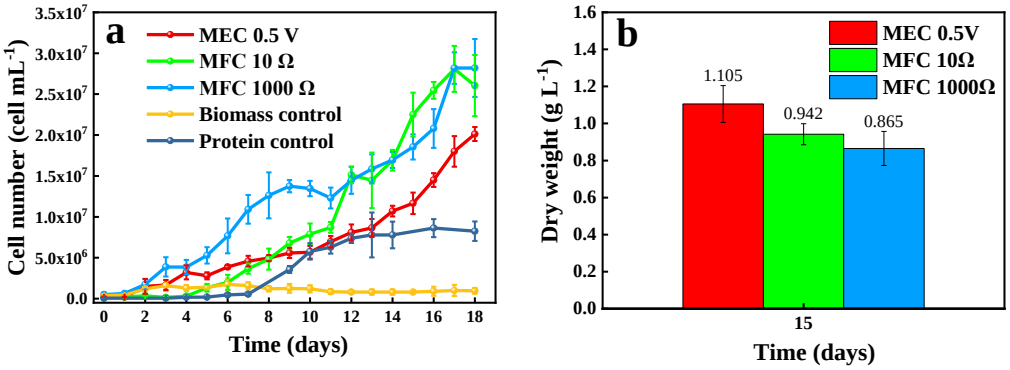


Fig.5 The growth of *C. vulgaris* of (a) cell number growth and (b) dry weight accumulation

During the running of BESs, nitrogen, phosphorus, and acetic acid were accumulated into the middle chamber to support a mixotrophic growth of the *C. vulgaris*. As shown in Fig.5, all groups demonstrated a much higher continuous microalgal biomass accumulation than the biomass control during the whole experiment period. With higher electric currents (MEC 0.5 V and MFC 10 Ω groups), a longer lag phase and reduced raise of the cell number of *C. vulgaris* K-1801 were observed in the first 11 days. At day 18, the MEC mode (MEC 0.5 V) obtained a lower cell number (2.01×10^7 mL^{-1}) than the MFC mode (MFC 10 Ω with 2.60×10^7 mL^{-1} and MFC 1000 Ω with 2.82×10^7 mL^{-1}, Fig.5a). However, the lower cell number resulted in

a higher dry weight (1.11, 0.94, and 0.87 g L^{-1} of the MEC 0.5V, MFC 10 Ω, and MFC 1000 Ω groups, respectively, Fig.5b), indicating the size change of the microalgal cells. Therefore, cell size distribution was further investigated with the laser diffraction and microscopy methods.

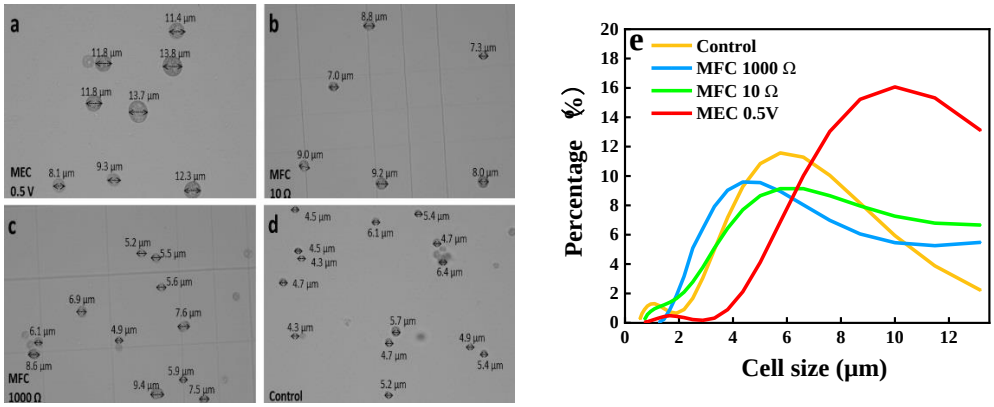
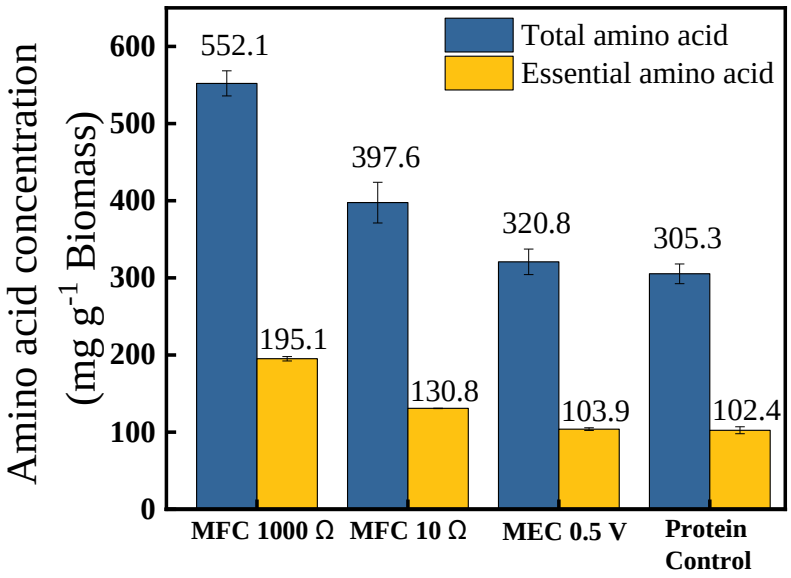


Fig.6 The cell sizes detected by the microscopy method in the (a) MEC 0.5 V groups, (b) MFC 10 Ω groups, (c) MFC 1000 Ω groups, and (d) biomass controls and (e) the cell size distribution of *C. vulgaris* in different groups according to the laser diffraction method groups at day 18.

The microscopy analysis showed an enlarged cell size of the *C. vulgaris* K-1801 with increasing electric current (Fig.6 a, b, c, and d). The laser diffraction results further showed the distribution of cell sizes and confirmed the increase of cell size with rising electric current (Fig. 6e). Specifically, the average cell size of 8.86, 6.38, 5.91 and 5.85 μm was observed in the MEC 0.5 V (0.4mA), MFC 10 Ω (0.2mA), MFC 1000 Ω (0.1mA) and biomass control groups, respectively. Previous studies have revealed that the organic carbon may dramatically affect the size of microalgae (Perez-Garcia et al., 2011). With the organic carbon sources, such as glucose, promoted a mixotrophic growth of microalgae, leading to a significant increase of cell size than those under

439 autotrophic conditions due to the increased intracellular storage (Azaman et al., 2017,
440 Li et al., 2020). In the present study, the increasing electric current accelerated the
441 nutrients migration from both anode and cathode chambers to the middle chamber,
442 leading to relatively higher availability of organic carbon and nutrients. The
443 accumulated organic carbons, such as acetate (Fig.2e), may promote different levels of
444 mixotrophic growth due to the varying concentrations, which might contribute to the
445 enlargement of the *C. vulgaris* cell size. Practically, during microalgae production,
446 harvesting may occupy 20-30% of the total cost (Grima et al., 2003). The enhanced cell
447 size from the novel BESs cultivation process proposed in this study may increase the
448 harvesting efficiency and reduce the costs, which could benefit the industrial utilization
449 in view of practical application. Considering the biomass production of *C. vulgaris*
450 (0.87-1.11 g L^{-1}), the system applied in this study could further be optimized due to its
451 relatively lower biomass amount than that of direct cultivation in other wastewater
452 (1.67-2.59 g L^{-1}, Salati et al., 2017).

453 **3.5 Protein production of *C. vulgaris***



454

455 Fig.7 Total and essential amino acid content of *C. vulgaris* in different groups at day 18

456 As shown in Fig.7, all three treatment groups achieved higher protein content
457 than the groups using MWC+SE standard medium (protein control, $p<0.05$). The
458 overall higher protein contents of the three treatment groups than the protein control
459 were mainly due to the continuous nitrogen supplementary driven by the electric
460 current in the three-chamber BES reactors. Under a certain nitrogen range, the rising
461 nitrogen concentration may support a higher intracellular protein accumulation (Xie
462 et al., 2017). Among the three treatment groups, the groups with lower electric current
463 achieved higher protein content. Specifically, significantly higher contents of the total
464 amino acid were achieved in the MFC 1000 Ω (552.1 mg g^{-1} biomass) and MFC 10
465 Ω (397.6 mg g^{-1} biomass) groups than the MEC 0.5 V (320.8 mg g^{-1} biomass) and
466 control (305.3 mg g^{-1} biomass) groups (Fig.7, $p<0.05$). Meanwhile, the results of
467 amino acid profiles indicated the *C. vulgaris* biomass cultivated in the potato juice
468 wastewater obtained a balanced amino acid profile, as it contained at least eight types

of the essential amino acids (EAAs, due to the acid hydrolysis pre-treatment of the protein, tryptophan was destroyed) (Boisen et al., 2000) and other non-essential amino acids. Notably, except for the histidine, all the rest seven types of EEAs significantly increased with the decreasing of electric current in the treatment groups than the control (Table1). However, the total ratios of total EEAs remained similar in the range of 32.5-35.4% (w/w). The decreasing content of protein and specific amino acid with the increase of electric current was mainly caused by the varying levels of mixotrophic growth. The higher electric current resulted in faster and larger organic carbon accumulation into the middle chamber (Fig.2b), supporting a higher level of mixotrophic growth for the *C. vulgaris*. As interpreted by the previous study, the microalgal cells predominate lipid and starch accumulation under mixotrophic conditions (Azaman et al., 2017). As a consequence, the protein content decreased with the prior increase of lipid and starch accumulation. The biomass obtained from treatment groups contained a balanced and comparable amino acid to those in the soybean meal (Winkler et al., 2011), demonstrating its potential for food or feed applications. Notably, the protein content in this study (30.5-55.2%) was still lower than that reported in reference (51-58%, Becker, 2007). Optimization of the BESs could still be required considering the protein content.

Table 1 Amino acid component and contents (g per 100g dry biomass) of *C. vulgaris* from the microalgal-MEC/MFC cultivation and MWC+SC media

Amino acid (g per100g dry biomass)	Control	MFC 1000 Ω	MFC 10 Ω	MEC 0.5 V	Soybean meal[a]
Threonine	**1.72±0.13**	**2.90±0.01**	**2.00±0.26**	**1.67±0.01**	**2.06**

Methionine	**0.21±0.03**	**0.64±0.13**	**0.72±0.24**	**0.36±0.09**	**0.99**
Isoleucine	**0.78±0.06**	**1.45±0.08**	**1.00±0.33**	**0.80±0.06**	**2.63**
Leucine	**2.07±0.04**	**4.84±0.02**	**3.14±0.35**	**2.44±0.27**	**4.18**
Phenylalanine	**0.94±0.03**	**2.01±0.11**	**1.48±0.22**	**1.13±0.12**	**2.46**
Lysine	**1.19±0.05**	**2.10±0.12**	**1.91±0.74**	**1.03±0.03**	**3.50**
Valine	**1.90±0.13**	**4.11±0.06**	**2.23±0.13**	**2.26±0.02**	**1.94**
Histidine	**1.45±0.16**	**1.46±0.10**	**0.61±0.01**	**0.71±0.26**	**1.53**
Proline	2.01±0.13	2.84±0.46	2.24±0.19	1.96±0.12	2.20
Tyrosine	0.89±0.03	2.00±0.10	1.55±0.27	0.94±0.11	1.62
Alanine	3.42±0.10	5.76±0.07	4.03±0.33	3.51±0.13	2.32
Glycine	2.64±0.27	4.41±0.14	3.72±0.07	2.78±0.14	2.01
Serine	1.57±0.08	2.92±0.12	2.39±0.31	1.80±0.19	2.54
Glutamine + Glutamic acid	3.58±0.21	6.63±0.79	4.59±0.44	4.44±1.16	9.10
Aspargine + Aspartic acid	4.33±0.38	7.75±0.24	5.73±0.61	4.23±0.11	6.00
Arginine	1.83±0.03	3.38±0.45	2.50±0.10	2.01±0.35	4.18
Essential amino acid					

a Data from Winkler et al., 2011

3.6 Insight into technical barriers and outlook

The present study demonstrated a novel three-chamber BESs, which could simultaneously achieve wastewater remediation, and upcycling of both anions (PO_4^{3-}, NO_3^-, CH_3COO^-) and cations (NH_4^+) for clean production of microalgal biomass with high efficiency. Though promising, common barriers of BESs, e.g., the energy consumption of aeration (cathode), high-cost and biofouling of ion-exchange membranes, are still challenging. Aeration has been widely used in wastewater treatment plants (WWTPs), such as aerated activated sludge (AAS) technology

497 (Trapero et al., 2017). Indeed, a much lower rate of aeration is needed to maintain a
498 certain dissolved oxygen (DO) concentration in BESs than that of AAS. Self-sufficient
499 energy consumption could even be achieved with optimal operation of BESs (MFC
500 mode), due to its electric energy recovery property (Lu and Li, 2012). Moreover, air-
501 cathode could be applied instead of cathodic aeration for energy saving (Kim et al.,
502 2016). This novel three-chamber BES could even utilize the secondary effluents from
503 WWTPs (after aeration treatment) as continuous feed to the cathode, to upcycle the
504 nutrients, and avoids aeration.

505 Recent advance of membranes has favored non-selective separators, e.g., porous
506 membranes, as alternatives of ion-exchange membranes due to its low-cost. Such
507 porous membrane has also been investigated in a two-chamber MFC for nutrients
508 recovery (in anode) and microalgal cultivation (in cathode, Colombo et al., 2017).
509 However, the potential difference significantly inhibits the natural diffusion of anions
510 from anode to cathode, resulting in no removal and upcycling of phosphorus (Colombo
511 et al., 2017). Even cations (NH_4^+, 80% removal) could be driven by potential difference
512 to the cathode, the removal and migration efficiency was also limited due to the
513 drawbacks of porous membranes, i.e., oxygen/substrate crossover and quick biofouling
514 formation (Leong et al., 2013). In contrast, the three-chamber BESs developed in this
515 study using the selective membranes achieved simultaneous migration and upcycling
516 of anions (PO_4^{3-}, NO_3^-, CH_3COO^-) and cations (NH_4^+, up to 96%) with high efficiency.
517 In this proof of concept study, the wastewater was added in both chambers, but in future
518 studies, continuous flow of wastewater from anode to cathode could be adopted to

519 simplify the process and maximize the utilization of organics in the anode chamber.
520 Additionally, different types of wastewater could be simultaneously treated in different
521 chambers according to their properties, e.g., wastewater with high DO, high anions and
522 wide pH range for cathode, while wastewater with low DO and high cations for anode.
523 The biofouling problem of both ion-exchange membranes and non-selective separators
524 could be addressed by physical membrane cleaning and modified membranes, e.g.,
525 nano-composite ion-exchange membranes (Leong et al., 2013). Moreover, maximum
526 flux of membrane could limit the migration efficiency even with increase of current
527 (PO_4^{3-} and CH_3COO^- in this study). Further development of high flux, anti-biofouling
528 and cost-effective membranes could contribute to the implementation of the proposed
529 three-chamber BESs. The developed novel microalgal BES could contribute to a more
530 efficient and safe production of clean microalgal biomass from various industrial
531 wastewaters, as well as offer insights into the substrate migration process in a three-
532 chamber BES.

533 **4. Conclusions**

534 The present study demonstrated a novel hybrid microalgal-BES system for the
535 simultaneous treatment of industrial organic wastewater and clean cultivation of pure
536 microalgae. The results demonstrated the removal efficiencies of COD and nitrogen
537 (mainly in form of ammonium) were elevated in both anode and cathode chambers with
538 increasing electric current. However, comparable removal efficiencies of phosphorus
539 in the cathode chambers were achieved regardless of current changes. Meanwhile, the
540 rising electric current significantly improved substrates recovery via CEM ($NH4^+$) and

AEM (CH_3COO^-, PO_4^{3-} and NO_3^-) into the middle chamber, promoted rapid and continuous growth of *C. vulgaris*. Notably, higher accumulation of nutrients (by higher current), especially acetate, led to a higher level of mixotrophic growth and resulted in lower cell numbers but larger cell size and more biomass of *C . vulgaris*. As a potential protein source, all treatments achieved higher protein content than that of the standard medium. However, the protein content declined with the rising current regarding the prior accumulation of lipid and starch under mixotrophic growth. Through this study, the industrial organic wastewater was efficiently treated and pure microalgal protein product, which is comparable with soybean meal, was achieved. The mBES system may offer insight into the development of low-cost microalgal protein production and biomass harvesting process (by the enlarged cell size). However, optimization could still be required given the relatively lower biomass and protein content than other studies that cultivated microalgae directly in wastewaters.

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