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

Considering these challenges, the recent advances of bio-electrochemical systems (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 ($\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$, 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
nutrients recovery by microalgae-assisted MFCs from various wastewater (urine, domestic wastewater, etc.) (Elshobary et al., 2020). From the sustainability point of view, in-situ utilization of the recovered nutrients for microalgae cultivation could be a promising option with respect to the direct recapture of nutrients into microalgal cells. Efforts have been made on single- and two-chamber microalgal-MFCs (mMFCs) for in-situ removal and recovery of nutrients from wastewater (Lee et al., 2015, Cui et al., 2014). Though the single-chamber mMFC demonstrated simultaneous nitrogen and phosphorus recovery for microalgae cultivation, it placed the *C. vulgaris* directly into the wastewater (Zhang et al., 2011), rising the contamination risk of microalgal biomass and limiting the utilization of the biomass as food or feed. In this view, the two-chamber mMFCs can avoid direct contamination of biomass by using an ion-exchange membrane, which isolates the microalgae from wastewater. However, the applied selective ion-exchange membrane could only target at either ammonia or phosphorus recovery, leading to a nutrient loss and lowered efficiency of waste treatment (Pei et al., 2018, Xiao et al., 2012). Therefore, the development of efficient hybrid microalgae and BESs system that integrates clean microalgae cultivation and comprehensive wastewater treatment is urgent.

In this study, a novel three-chamber microalgal-BES reactor (mBES) was developed to achieve simultaneous clean cultivation of *C. vulgaris* for protein production, industrial organic wastewater treatment, and energy production. In this novel system, organic matters from the wastewater were oxidized by the electroactive bacteria in the anode, while the oxygen reduction (MFC mode) or H₂ evolution (MEC mode) occurred in the middle and the bottom chambers.
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$_3$COO$^-$) 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.](image)

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/cm², 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 $2.9 \pm 1.8 \times 10^5$ 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 ($22 \pm 1 ^\circ C$), and with continuous
stirring (VWR, US). The original potato juice wastewater (3.3 ± 0.1 g COD L⁻¹, pH 4.75 ± 0.2, acquired from KMC, Denmark) was directly added to the cathode chamber. Meanwhile, the pH of the potato juice wastewater was adjusted to pH 7 (with 50mM NaOH), and then added to the anode. All chambers achieved the same initial working volume of 200 mL. Each batch was running for 18 days and all the experiments were conducted in duplicate.

2.2 Sampling and analytical methods

The pH was detected using a PHM00 LAB pH meter for each chamber daily. The voltage across the resistor with varying ohm (10 or 1000 Ω) was collected by a model 2700 data acquisition system (Keithley Instruments, Inc, Cleveland, OH, USA). The chemical oxygen demand (COD) was detected every two days according to the water quality standard method (Federation and Association, 2005). The Kjeldahl method was applied for the total ammonia detection at day 0 and 18. Phosphorus and nitrogen in forms of ammonia, nitrate, nitrite were determined by the segmented flow analysis method (Scan++ system, Skalar analytical BV, the Netherlands), among which, phosphorus and ammonia were measured every another day, while nitrate and nitrite were measured at day 0 and 18. For the total phosphorus detection, samples were firstly digested with peroxodisulfate (50 g L⁻¹) under 121 °C for 60 min, pH was adjusted to 4 with HCl (for the detection of phosphorus precipitation), and then measured at day 18 by the segmented flow analysis method mentioned above. The cell size distribution of microalgae was determined by the laser diffraction method applying the Mastersizer 2000 coupled with a Hydro SM sample loader (Malvern Instruments, UK), and the
microscopy method was applied with a LEICA microscopy (DFC320, Germany) on
day 18. After pre-treatment with phosphoric acid, samples were detected for VFA
contents by a gas chromatographer (GC, TRACE 1300 of Thermo Scientific, US)
equipped with a flame ionization detector and HP free fatty acid phase (FFAP) column
at day 0 and 18.

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

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

It is to be observed that, after 18 days, at least 29.3% of COD was still left in each anode chamber, indicating the existence of non-biodegradable organic matter in the wastewater under the operation conditions, e.g., lignin, cellulose, and potato solid waste. For the MEC mode (MEC 0.5 V) and MFC mode with 10 Ω (MFC 10 Ω) groups, the biodegradable COD was rapidly consumed by bacteria in 4 days, leaving a residue of un-biodegradable COD (29.3%-35.2%, under the given conditions). This corresponded well with the rapid COD removal in the first 4 days and lowered COD removal after day 4 (Fig.2a). Unlike the anode chamber, the cathode chamber promoted a faster COD removal in the early phase (in 2-4 days) due to the occurrence of multiple processes including microorganisms activities (Zhang et al., 2019) and carboxylic acids migration processes. Due to the continuous aeration in the cathode chamber, the activities of aerobic bacteria could be promoted for the removal of COD, e.g., aerobic accumulation of phosphorus by polyphosphate accumulating microorganisms, and aerobic respiration by aerobic bacteria (Zhang et al., 2019). As shown in Fig. 2, with a decrease of COD and acetic acid in the cathode chamber, a significant increase of both COD and acetic acid were observed in the middle chamber (Fig. 2b, c, e, and f), indicating a migration of organic matter (especially carboxylic acids) from the cathode chamber via the AEM to the middle chamber. A part of the migrated organic matter, e.g., acetic acid, could serve as an organic carbon source to promote a mixotrophic growth of *C. vulgaris*. As 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
chambers of each mBES were detected. Besides, the nitrogen that was re-captured by the *C. vulgaris* in the middle chamber was also detected for a better understanding of nitrogen balance. In the anode chamber, with low circuit current (MFC 1000 Ω), ammonium concentration tends to increase in the first 4 days, followed by a stepwise decrease till the end of the batch run. In contrast, under higher circuit current (MFC 10 Ω and MEC 0.5 V), no significant increase of ammonium concentration was detected. There might be a dynamic equilibration between the removal and formation of ammonium. The digestion and hydrolysis of protein in the anode chamber might contribute to the raise of ammonium concentration. For the removal of ammonium, it is well known that anaerobic processes (in the anode chamber) rarely contributed to the removal of ammonium (Zhang et al., 2019). At non-obligate anoxic conditions and at low COD loads, anaerobic ammonium oxidation (anammox) mediated by bacteria in the anode chamber has been previously reported as one significant pathway of nitrogen removal (Strous et al., 1998). However, in this study, due to the high COD concentration of the potato juice wastewater and no detection of nitrite during the treatment, the anammox process was unlikely to occur in the anode chamber (Chen et al., 2016). Instead, the migration of NH$_4^+$ via CEM from the anode chamber to the middle chamber (Park et al., 2009) mainly contributed to the decrease of ammonium concentration in the anode chamber (Fig. 3a and b), which has also been reported by two-chamber MFC system (Zhang et al., 2019). The higher electric current was, the faster NH$_4^+$ migration would occur from the anode chamber via CEM to the middle chamber (Fig. S1, 3a). In the anode chamber of MFC 1000 Ω, MFC 10 Ω and MEC 0.5 V groups, the ammonium
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 achieved, respectively. Correspondingly, a total Kjeldahl nitrogen removal was 90.75 mg L\(^{-1}\) (69.5%), 120.5 mg L\(^{-1}\) (79.0%) and 113.5 mg L\(^{-1}\) (72.8%), respectively (Fig. 3a).

Meanwhile, in the middle chambers, a fluctuant amount of ammonium in the supernatant was observed, which was migrated from the anode chamber (Fig.3b). Actually, larger amounts of ammonium might be migrated than the detected amounts into the middle chamber considering the dynamic re-capturing by microalgae.

Additionally, the growth of microalgae rapidly elevated the pH (through inorganic carbon consumption by the photosynthesis, i.e., HCO\(_3^\)\(^-\), Besson and Guiraud, 2013) in the middle chamber to alkaline (pH 9-11) (Fig.S1 b), leading to a loss of ammonium through volatilization. At day 18, higher electric current promoted a significant higher nitrogen re-capture (61.0 mg L\(^{-1}\) in the MEC 0.5 V groups, p<0.05) than the other two groups (50.9 and 44.7 mg L\(^{-1}\) in the MFC 10 \(\Omega\) and MFC 1000 \(\Omega\) groups, respectively).

Consequently, \textit{C. vulgaris} has recaptured 39.1%, 33.4% and 34.3% of the total Kjeldahl nitrogen from anode in the MEC 0.5 V, MFC 10 \(\Omega\) and MFC 100 \(\Omega\) treatments, respectively.

In the aerated cathode chamber, the removal efficiency of nitrogen showed the same trend as the electric current. As the increase of electric current, the removal efficiency of ammonium and total Kjeldahl nitrogen was elevated from 36.59 mg L\(^{-1}\) (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%), respectively. The NH\(_4^+\) migration between the cathode and middle chamber through 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

![Graphs showing phosphorus concentration changes](image)

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

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 (2.01x10^7 mL^-1) than the MFC mode (MFC 10 Ω with 2.60x10^7 mL^-1 and MFC 1000 Ω with 2.82x10^7 mL^-1, Fig. 5a). However, the lower cell number resulted in
higher dry weight (1.11, 0.94, and 0.87 g L\(^{-1}\)) of the MEC 0.5V, MFC 10 \(\Omega\), and MFC 1000 \(\Omega\) 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 \(\Omega\) groups, (c) MFC 1000 \(\Omega\) groups, and (d) biomass controls and (e) the cell size distribution of \textit{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 \textit{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 \(\mu\)m was observed in the MEC 0.5 V (0.4mA), MFC 10 \(\Omega\) (0.2mA), MFC 1000 \(\Omega\) (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
autotrophic conditions due to the increased intracellular storage (Azaman et al., 2017, Li et al., 2020). In the present study, the increasing electric current accelerated the nutrients migration from both anode and cathode chambers to the middle chamber, leading to relatively higher availability of organic carbon and nutrients. The accumulated organic carbons, such as acetate (Fig.2e), may promote different levels of mixotrophic growth due to the varying concentrations, which might contribute to the enlargement of the *C. vulgaris* cell size. Practically, during microalgae production, harvesting may occupy 20-30% of the total cost (Grima et al., 2003). The enhanced cell size from the novel BESs cultivation process proposed in this study may increase the harvesting efficiency and reduce the costs, which could benefit the industrial utilization in view of practical application. Considering the biomass production of *C. vulgaris* (0.87-1.11 g L⁻¹), the system applied in this study could further be optimized due to its relatively lower biomass amount than that of direct cultivation in other wastewater (1.67-2.59 g L⁻¹, Salati et al., 2017).

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

As shown in Fig. 7, all three treatment groups achieved higher protein content than the groups using MWC+SE standard medium (protein control, p<0.05). The overall higher protein contents of the three treatment groups than the protein control were mainly due to the continuous nitrogen supplementary driven by the electric current in the three-chamber BES reactors. Under a certain nitrogen range, the rising nitrogen concentration may support a higher intracellular protein accumulation (Xie et al., 2017). Among the three treatment groups, the groups with lower electric current achieved higher protein content. Specifically, significantly higher contents of the total amino acid were achieved in the MFC 1000 Ω (552.1 mg g⁻¹ biomass) and MFC 10 Ω (397.6 mg g⁻¹ biomass) groups than the MEC 0.5 V (320.8 mg g⁻¹ biomass) and control (305.3 mg g⁻¹ biomass) groups (Fig. 7, p<0.05). Meanwhile, the results of amino acid profiles indicated the *C. vulgaris* biomass cultivated in the potato juice 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 (Table 1). 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

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Control</th>
<th>MFC 1000 Ω</th>
<th>MFC 10 Ω</th>
<th>MEC 0.5 V</th>
<th>Soybean meal&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threonine</td>
<td>1.72±0.13</td>
<td>2.90±0.01</td>
<td>2.00±0.26</td>
<td>1.67±0.01</td>
<td>2.06</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>Value 1 ± Standard Deviation</td>
<td>Value 2 ± Standard Deviation</td>
<td>Value 3 ± Standard Deviation</td>
<td>Value 4 ± Standard Deviation</td>
<td>Value 5 ± Standard Deviation</td>
</tr>
<tr>
<td>-------------------</td>
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<td>------------------------------</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.21 ± 0.03</td>
<td>0.64 ± 0.13</td>
<td>0.72 ± 0.24</td>
<td>0.36 ± 0.09</td>
<td>0.99</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.78 ± 0.06</td>
<td>1.45 ± 0.08</td>
<td>1.00 ± 0.33</td>
<td>0.80 ± 0.06</td>
<td>2.63</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.07 ± 0.04</td>
<td>4.84 ± 0.02</td>
<td>3.14 ± 0.35</td>
<td>2.44 ± 0.27</td>
<td>4.18</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.94 ± 0.03</td>
<td>2.01 ± 0.11</td>
<td>1.48 ± 0.22</td>
<td>1.13 ± 0.12</td>
<td>2.46</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.19 ± 0.05</td>
<td>2.10 ± 0.12</td>
<td>1.91 ± 0.74</td>
<td>1.03 ± 0.03</td>
<td>3.50</td>
</tr>
<tr>
<td>Valine</td>
<td>1.90 ± 0.13</td>
<td>4.11 ± 0.06</td>
<td>2.23 ± 0.13</td>
<td>2.26 ± 0.02</td>
<td>1.94</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.45 ± 0.16</td>
<td>1.46 ± 0.10</td>
<td>0.61 ± 0.01</td>
<td>0.71 ± 0.26</td>
<td>1.53</td>
</tr>
<tr>
<td>Proline</td>
<td>2.01 ± 0.13</td>
<td>2.84 ± 0.46</td>
<td>2.24 ± 0.19</td>
<td>1.96 ± 0.12</td>
<td>2.20</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.89 ± 0.03</td>
<td>2.00 ± 0.10</td>
<td>1.55 ± 0.27</td>
<td>0.94 ± 0.11</td>
<td>1.62</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.42 ± 0.10</td>
<td>5.76 ± 0.07</td>
<td>4.03 ± 0.33</td>
<td>3.51 ± 0.13</td>
<td>2.32</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.64 ± 0.27</td>
<td>4.41 ± 0.14</td>
<td>3.72 ± 0.07</td>
<td>2.78 ± 0.14</td>
<td>2.01</td>
</tr>
<tr>
<td>Serine</td>
<td>1.57 ± 0.08</td>
<td>2.92 ± 0.12</td>
<td>2.39 ± 0.31</td>
<td>1.80 ± 0.19</td>
<td>2.54</td>
</tr>
<tr>
<td>Glutamine + Glutamic acid</td>
<td>3.58 ± 0.21</td>
<td>6.63 ± 0.79</td>
<td>4.59 ± 0.44</td>
<td>4.44 ± 1.16</td>
<td>9.10</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>4.33 ± 0.38</td>
<td>7.75 ± 0.24</td>
<td>5.73 ± 0.61</td>
<td>4.23 ± 0.11</td>
<td>6.00</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.83 ± 0.03</td>
<td>3.38 ± 0.45</td>
<td>2.50 ± 0.10</td>
<td>2.01 ± 0.35</td>
<td>4.18</td>
</tr>
</tbody>
</table>

**Essential amino acid**

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$_3$COO$^-$) 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...
(Trapero et al., 2017). Indeed, a much lower rate of aeration is needed to maintain a certain dissolved oxygen (DO) concentration in BESs than that of AAS. Self-sufficient energy consumption could even be achieved with optimal operation of BESs (MFC mode), due to its electric energy recovery property (Lu and Li, 2012). Moreover, air-cathode could be applied instead of cathodic aeration for energy saving (Kim et al., 2016). This novel three-chamber BES could even utilize the secondary effluents from WWTPs (after aeration treatment) as continuous feed to the cathode, to upcycle the nutrients, and avoids aeration.

Recent advance of membranes has favored non-selective separators, e.g., porous membranes, as alternatives of ion-exchange membranes due to its low-cost. Such porous membrane has also been investigated in a two-chamber MFC for nutrients recovery (in anode) and microalgal cultivation (in cathode, Colombo et al., 2017). However, the potential difference significantly inhibits the natural diffusion of anions from anode to cathode, resulting in no removal and upcycling of phosphorus (Colombo et al., 2017). Even cations (NH$_4^+$, 80% removal) could be driven by potential difference to the cathode, the removal and migration efficiency was also limited due to the drawbacks of porous membranes, i.e., oxygen/substrate crossover and quick biofouling formation (Leong et al., 2013). In contrast, the three-chamber BESs developed in this study using the selective membranes achieved simultaneous migration and upcycling of anions (PO$_4^{3-}$, NO$_3^-$, CH$_3$COO$^-$) and cations (NH$_4^+$, up to 96%) with high efficiency.

In this proof of concept study, the wastewater was added in both chambers, but in future studies, continuous flow of wastewater from anode to cathode could be adopted to
simplify the process and maximize the utilization of organics in the anode chamber. Additionally, different types of wastewater could be simultaneously treated in different chambers according to their properties, e.g., wastewater with high DO, high anions and wide pH range for cathode, while wastewater with low DO and high cations for anode. The biofouling problem of both ion-exchange membranes and non-selective separators could be addressed by physical membrane cleaning and modified membranes, e.g., nano-composite ion-exchange membranes (Leong et al., 2013). Moreover, maximum flux of membrane could limit the migration efficiency even with increase of current (PO₄³⁻ and CH₃COO⁻ in this study). Further development of high flux, anti-biofouling and cost-effective membranes could contribute to the implementation of the proposed three-chamber BESs. The developed novel microalgal BES could contribute to a more efficient and safe production of clean microalgal biomass from various industrial wastewaters, as well as offer insights into the substrate migration process in a three-chamber BES.

4. Conclusions

The present study demonstrated a novel hybrid microalgal-BES system for the simultaneous treatment of industrial organic wastewater and clean cultivation of pure microalgae. The results demonstrated the removal efficiencies of COD and nitrogen (mainly in form of ammonium) were elevated in both anode and cathode chambers with increasing electric current. However, comparable removal efficiencies of phosphorus in the cathode chambers were achieved regardless of current changes. Meanwhile, the rising electric current significantly improved substrates recovery via CEM (NH₄⁺) and
AEM (CH$_3$COO$^-$, 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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References


Assisting cultivation of photosynthetic microorganisms by microbial fuel cells to

Cui, Y., Rashid, N., Hu, N., Rehman, M.S.U., Han, J.-I., 2014. Electricity generation
and microalgae cultivation in microbial fuel cell using microalgae-enriched anode

new source of bioactive compounds in food supplements. Curr. Opin. Food Sci. 7,
73–77.

Bio-electrochemical COD removal for energy-efficient, maximum and robust
nitrogen recovery from urine through membrane aerated nitrification. Water Res.
185, 116223.

microalgae-assisted microbial fuel cells for generating sustainable bioelectricity.
Int. J. Hydrogen Energy.

Fang, C., Boe, K., Angelidaki, I., 2011. Biogas production from potato-juice, a by-
product from potato-starch processing, in upflow anaerobic sludge blanket (UASB)
and expanded granular sludge bed (EGSB) reactors. Bioresour. Technol. 102,
5734–5741.

Federation, W.E., Association, A.Ph., 2005. Standard methods for the examination of


Kim, K.-Y., Yang, W., Evans, P.J., Logan, B.E., 2016. Continuous treatment of high


recovering potato protein from potato processing wastewater using the column with the spiral internal component. J. Food Eng. 114, 192–198.


Matassa, S., Batstone, D.J., Hülsen, T., Schnoor, J., Verstraete, W., 2015. Can direct conversion of used nitrogen to new feed and protein help feed the world?


Zhu, X., Treu, L., Kougioumtzoglou, S., Campanaro, S., Angelidaki, I., 2018. Converting...