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Effects of the applied voltage on electroactive microbial biofilm viability and hydrogen production in a recalcitrant organic waste-fed single-chamber membrane-free microbial electrolysis cell performance

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ABSTRACT

The microbial electrolysis cell (MEC) is a future clean technology with a wide range of applications in energy recycling fields, particularly for hydrogen production. Applied voltage governs the electrochemically active microbial activities in MEC and further affects hydrogen production. Therefore, this study investigated the impacts of the applied voltages on MEC performance and anodic biofilm viability during the biotransformation of straw waste biomass into green hydrogen energy in an anaerobic environment. The results revealed that the COD removal efficiency and bioH_2 yield increased with the augmentation of the applied voltage and beyond 0.8 V started decreasing. Among the performed protocols MEC0.8 depicted the best performance with a maximum H_2 production of 6.017 mmoles H_2/g-COD which was ~28.2 % and ~9.8 % higher than that of MEC0.5 and MEC1.0 respectively. Moreover, it also achieved a maximum COD removal of 73.4% and a coulombic efficiency (CE) of 68.4%. Consistently, the anodic biofilm viability and bacterial cell shape in the MECs performed with 0.5 V and 0.8 V were less affected but largely damaged in a high potential operated protocol (MEC1.0), suggesting that the mixed electroactive consortia were so sensitive to high potentials. Furthermore, MEC1.0 displayed a high charge transfer resistance of 16.06 Ω which was ~22.1 %, and ~73.7 % higher than that of MEC0.8, and MEC0.5 respectively. Based on our novel study's results, the optimal applied voltage for the best performance of MEC was 0.8 V; and this information will be a pillar for future MEC operations prior to maintaining its industrial practice.

1. Introduction

Bioelectrochemical system (BES) is an emerging technology that uses electrochemically active bacteria clinging to the working electrode to catalyze electrochemical reactions [1]. BESs have been broadly investigated and deemed a future energy-producing approach for both wastewater treatment and green manner of high-value chemicals including H_2, CH_4, etc. [2–6]. Among the BES systems, microbial electrolysis cell (MEC) has been applied in various energy recycling and it has been particularly used for upgrading green hydrogen production at a low electric energy consumption compared to the electric-based water-splitting process [7,8]. The applied voltage to MEC drives liberated electrons during substrate (waste biomass) oxidation from the anodic electrode to the cathodic one and noticeably assists biological hydrogen production at the latter electrode [4,9]. Generally, the MECs cathode is performed under anaerobic conditions to enable hydrogen evolution.

It is believed that applying only an electric potential of 0.14 V to an operating microbial electrolysis cell alleviates the endothermic impediment of −0.414 V vs standard hydrogen electrode (SHE) to enable hydrogen production. The remaining overpotential of −0.279 V is

Abbreviations: **MEC**, Microbial electrolysis cell; **MFC**, Microbial fuel cell; **BES**, Bioelectrochemical system; **MEC0.5**, Microbial electrolysis cell supplied by 0.5V; **MEC0.8**, Microbial electrolysis cell supplied by 0.8V; **MEC1.0**, Microbial electrolysis cell supplied by 1.0V; **COD**, Chemical oxygen demand; **CE**, Coulombic efficiency; **TEM**, Transmission electron microscopy; **EIS**, Electrochemical impedance spectroscopy; **CV**, Cyclic voltammetry; **CLSM**, Confocal laser scanning microscopy; **EET**, Extracellular electron transfer; **EAB**, Electrochemically Active bacteria; **HPR**, Hydrogen production rate; **HP**, Hydrogen production.

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provided by the bio-oxidations reactions carried out by the electroactive microbial consortium on the anode [4,10]. MEC is recently taking special researchers' attention since it's an eco-friendly and efficient approach that could concomitantly achieve wastewater treatment and green hydrogen energy production. However, due to the shortcomings of the MEC technology including low hydrogen volumetric efficiency, and a challenging storage system [11], some researchers started working on how to upgrade this technology by finding out the optimum operating conditions. Recently, several studies have been conducted to improve hydrogen production via MEC technology, and most of them mainly focused on controlling the reactor type, electrode type, reaction conditions, etc. For instance, in 2015, Carmona-Martinez et al. produced 201.1 ± 7.5 L $H_2/m^2_{Cathode}$·d from acetate-containing saline wastewater by performing a bench scale 4 L dual chambered-microbial electrolysis cell reactor employing stainless steel and graphite felt as a cathodic and anodic electrode respectively [12]. Jeremiasse et his colleagues [13] lowered the cathode overpotential and achieved a maximum of 50 m^3 H_2 m^{-3} MEC d^{-1} by increasing Ni cathode surface areas in the MEC system. More recently, San-Martin et al. [14,15] attained a biohydrogen production as high as ~0.4 m^3 m^{-3} d^{-1} in a membrane-free MEC reactor using municipal solid waste at a pH controlled at 5.5.

Nevertheless, the effects of applied voltage on the microbial metabolic activities that control the overall MEC performance have not been fully tackled. It has been reported that a high applied voltage could harmfully affect the living cells, therefore, it is hypothesized that applying a high voltage to MEC could strongly inhibit the electrochemically active bacterial (EAB) growth, resulting in bioelectrochemical reactions failure [16–19]. For instance, in 2002, Luo et al. [20] reported that the high electric current results in augmenting the bacterial cell wettability that leads to overall cell apoptosis, but on the other side Yang and his colleagues [21] reported that a low electric current of 10 mA could promote *Pseudomonas aeruginosa* metabolic activities, and enhance the substrate utilization to reach a removal rate of 6.9 %. Therefore, these contradictory arguments left a key confusing point to know exactly the optimum applied voltage that could be employed in the bioelectrohydrogenesis process to efficiently promote electrochemically active bacterial growth or metabolic activities for enhanced biohydrogen production.

This current study aims to find out the optimum applied potential for magnifying MEC performance, by applying different voltages on the novel single-chambered MEC fed with acid-pretreated agricultural straw wastes for hydrogen production. Confocal laser scanning microscopy (CLSM) was performed to evaluate the impact of applied voltages on anodic biofilm viability, and transmission electron microscopy (TEM) was used to assess the ultra-structure of the microbial cells employed as biocatalysts. The electrochemical analysis characterized by electrochemical impedance spectrometry (EIS) and cyclic voltammetry (CV) were also performed to investigate the effects of applied potentials on both the MEC charge transfer resistance and the biofilm electrochemical behavior as key parameters to evaluate MEC performance. Hence, this current study provides raw theoretical and practical knowledge for future investigations of MEC application toward its industrial implementation.

2. Methodology

2.1. MEC reactor configuration

A single-chambered MEC reactor made of acrylic material was constructed and utilized in this investigation. The reactor diameter was 170 mm and a height of 200 mm with a working volume of 3L. The same chamber contained both anode and cathode electrodes. The anode electrode consisted of carbon felt (6.5 cm × 9.5 cm, without waterproofing) whereas the cathode consisted of stainless steel mesh (30 mesh) covered by carbon cloth (17 cm × 49 cm), (W0S1002, CeTech, China); and were both connected linked with copper wire for free electron charge circulation (see Fig. 1).

2.2. MEC reactor start-up and operation.

2.2.1. MEC start-up

The MEC was started by using synthetic wastewater consisting of acetate (1 g/L) as a model readily biodegradable substrate and 100 mmoles of phosphate buffer solution including $NaH_2PO_4{\cdot}2H_2O$ (5.54 g/L), $Na_2HPO_4{\cdot}12H_2O$ (23.08 g/L) together with KCl (0.26 g/L); NH_4Cl (0.62 g/L) for promoting the electrochemical active bacterial growth as previously reported by various investigators [22]. The overall process was solely biocatalyzed by the thermal-pretreated activated sludge originally obtained from the Yanqi campus sewage treatment plant, University of Chinese Academy of Sciences, Beijing, China. DC Power Supply (GPE-4323C, Beijing Good Will Instrument Co., Ltd, China) was used to supply the needed potential voltage which was directly applied to the MEC reactors as described by the manufacturer's instruction, and the pH was adjusted to 7.2 (E-201F, Jingke, China) while the bioreactor operating temperature was set to 55 °C.

2.2.2. MEC reactor operation.

The microbial electrolysis reactors were performed in batch-based conditions, and the hydraulic retention time (HRT) was approximately 11 days. Once the MEC effluent characteristics and biogas composition output remained stable, the MEC was directly performed with the agricultural wheat straw biomass (20 g/L) as a source of carbon. The latter was collected from Wei County, Hebei Province, China, and prior to using it as a potential substrate readily accessible by the biocatalysts, was substantially pretreated as instructed by Li et al [23]. Since the required potential voltage for water electrolysis is 1.23 V and the previously reported current that could limit the microbial growth is 20 mA or 10 mA/cm^3 [20,21], we, therefore, applied different potential voltages of 0.5 V, 0.8 V, and 1 V to the operating MEC reactors to access the impact of employed external potential on the performance of the agricultural wastes-fed MEC. Prior to collecting stable and promising results, each selected voltage was used for at least five batches of MEC performance, and fortunately, the last two operating batches resulted in providing similar data and trends. Then steady data were collected to plot the used figures in this report. More details on the experimental design are summarized in Table 1.

2.3. Electrochemical analysis

To evaluate the effects of the applied electric potential on charge transfer kinetics during the electrohydrogenesis process, the electrochemical impedance spectroscopy (EIS) test of each MEC operating reactor was performed and discussed. The electrochemical working station (model-660E_HK20071025, Beijing Huake Putian Technology Co., Ltd, China) was used to analyze the electrochemical impedance spectrometry over a frequency span of 100 kHz to 10 mHz [24,25]. Moreover, the electroactive microbial biofilm characteristics developed on the anodic electrode during the electrohydrogenesis process were examined by screening cyclic voltammetry (CV) of each MEC reactor based on the conducted protocol. Prior to starting the CV, the externally connected circuit of the MEC was disconnected for at least 2 h, and then CV curves were scanned by an electrochemical working station (model-660E_HK20071025, Beijing Huake Putian Technology Co., Ltd, China) at a speed of 10 mV/s from 0 to −0.8 V [26]. The overall system was equipped with 3 electrodes, including a MEC anode that was utilized as a working electrode, a MEC cathode as a counter electrode and Ag/AgCl served as a reference electrode to examine the anodic potential [26,27].

The current intensity (I) was instantly recorded by a paperless recorder (EDR7100, Hangzhou Yikong Technology, Co., Ltd., China), and the resulting current density (Cd) was considered as I/A, a formula derived from Ohm's law (herein, A represents the surface area of the employed anode electrode, which was approximately 61.75 cm^2).

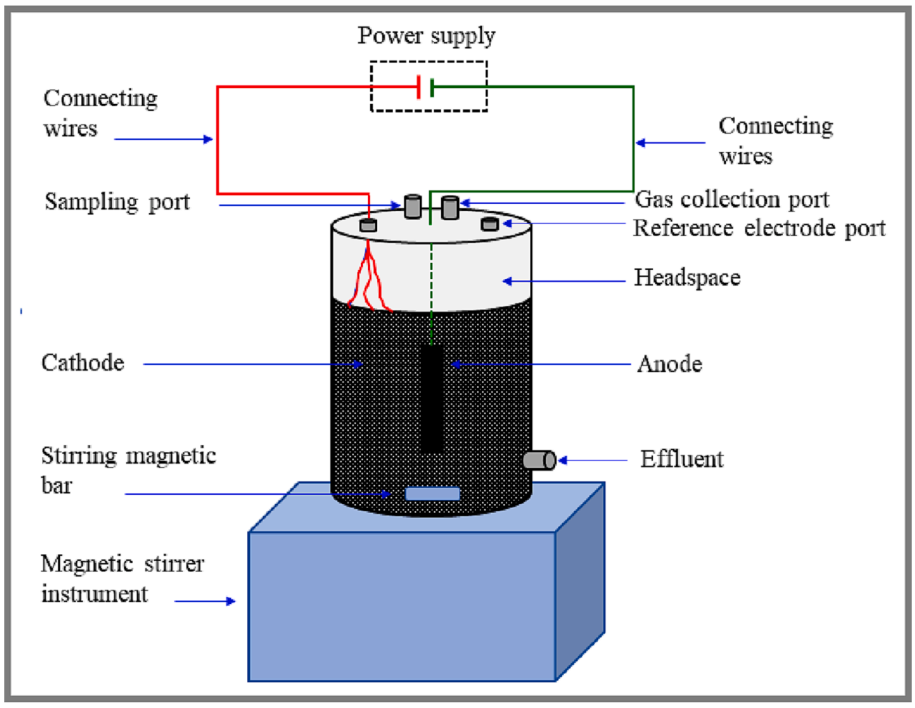

Fig. 1. Schematic diagram of the designed MEC bioreactor used in this investigation.

Table 1
A summary of the following experimental protocols; used electrode type and MEC medium composition.

MEC reactors	Applied voltage	pH	T°	Substrates	Electrode types		MEC medium
				Straw waste	Anode	Cathode	
MEC0.5	0.5 V	7.0 ± 0.3	55 °C	20 g/L	Carbon felt	Stainless steel mesh with carbon cloth	$NaH_2PO_4{\cdot}2H_2O$ (5.54 g/L), $Na_2HPO_4{\cdot}12H_2O$ (23.08 g/L)
MEC0.8	0.8 V	7.0 ± 0.3	55 °C	20 g/L	Carbon felt	Stainless steel mesh with carbon cloth	KCl (0.26 g/L); NH_4Cl (0.62 g/L)
MEC1.0	1.0 V	7.0 ± 0.3	55 °C	20 g/L	Carbon felt	Stainless steel mesh with carbon cloth	12.5 mL mineral salts, and 25 μL of vitamin solution

MEC0.5, MEC0.8, and MEC1.0 stand for MEC operated under 0.5 V, 0.8 V and 1.0 V respectively. Whereas T° stands for temperature.

2.4. Confocal microscopy for MEC anodic biofilm viability test

The effects of applied voltage on the MEC anodic biofilm viability were assessed by performing confocal microscopy. Prior to preparing the sample, a small part of the MEC bioanode approximately 2 cm^2 was aseptically plunged into the basal medium to get rid of unwanted restudies. The obtained section was then stained as described in a LIVE/DEAD® BacLight™ bacterial viability kit (Molecular Probes, L7012, Thermo Fisher, China) following the manufacturer's instructions. The viability of the pretreated bacterial cells was then analyzed employing a confocal laser scanning microscopy (CLSM, FV1200, Olympus, China) at excitation wavelengths of 488 nm for live cells (SYTO-9) and 546 nm for dead cells (PI), respectively [28–30].

2.5. Ultra-structure observation of the microorganisms

To evaluate the effects of the applied electric potential on the electroactive microbial cell morphology and estimate the overall cell viability, the microorganisms employed as biocatalysts to accelerate the bio-oxidation of the substrate at various applied potentials were collected from the MEC reactors and observed by performing transmission electron microscopy (TEM; HT-7700Exalens, HATACHI Ltd., Japan). Prior to collecting samples, microbial cells were obtained at the end of the MEC operations and immediately got rid of residues by washing the samples using phosphate buffer solutions (PBS) at least times. The resulting biomass slurry was then immersed in 2.5% $C_5H_8O_2$ (glutaraldehyde) for at least 12 h at 4 °C. The sample pretreatment methods toward the cell ultrastructure observations were achieved following the previously published report of Yu et al. [30].

2.6. Chemical and biogas analysis

The analysis of COD was conducted based on standard methods [31]. The gas production was quantified by the gas bag linked to the reactors. The composition of the generated biogas was evaluated by performing gas chromatography (GC, SP7800, Beijing Jing Ke Ruida, China) connected with a thermal conductivity detector (TCD) and a 2.0-m GDX-103 (60/80 mesh) column. The GC operational temperatures of the column oven, injection port, and detector were 120 °C, 80 °C, and 160 °C respectively. The used carrier gas was nitrogen at a flow rate of 25 mL/min and a 50 μL Agilent syringe was employed to inject the sampled gas in GC.

2.7. Calculation

The effects of the applied voltage on MECs performance were investigated in all protocols (MEC0.5, MEC0.8, and MEC1.0) and compared based on the calculated cumulative hydrogen production;

cathodic hydrogen recovery (r_{cat}), and coulombic efficiency (CE); and all other calculations were based on the consumed COD as previously reported by Douglas et al. [32]. The cathodic hydrogen recovery (rCat, %), (which is defined as the recovered hydrogen moles from generated biogas relative to that obtained based on the regularly detected electric current) was calculated as follows (Eq. (1):

$$r_{Cat} = \frac{n_{H2}}{n_{CE}} \tag{1}$$

Herein n_{H2} is the number of H_2 moles generated at the cathode electrode. It is obtained referred to the cumulative volume of bioH_2 produced during the MEC performance and the ideal gas law. The CE (%) was calculated as $CE = n_{ce}/n_{th}$ where n_{ce} (moles) stands for the number of bioH_2 moles recovered based on the instantly recorded electric current (A) during MEC performance, and n_{th} represents the total theoretical number of hydrogen moles estimated referring to the ratio of the consumed chemical oxygen demand (COD), and the latter was calculated following equation (2) (Eq. (2).

$$n_{th} = \frac{b_{H2/s} v_L \Delta S}{M_S} \tag{2}$$

In equation (2), $b_{H2/S} = 4$ mol/mole, which represents the possible utmost stoichiometric fermentative-H_2 production from the used electron donor, v_L stands for the total volume of medium (fermentation broth) in the MEC-reactors, which is approximately 3L in the current experiment. ΔS, generally given in gram COD/L (g-COD/L), represents the amount of consumed substrate which is estimated based on the variation in initial COD (at the beginning of the experimental batch cycle) and final COD (at the end of the batch cycle), whereas, *Ms* stands for the molecular weight of the substrate [32]. In the aforementioned formula for calculating cathodic hydrogen recovery, n_{CE} is given by equation (3) (Eq. (3)).

$$n_{CE} = \frac{\int_{t=0}^{t} Idt}{2F} \tag{3}$$

In equation (3), I stands for recorded current (A) which was measured from the applied voltage across the resistor, and the number 2 is generally used to turn moles of liberated electrons during substrate oxidation into moles of hydrogen gas. F stands for Faraday's constant which is equal to 96,485C/mol electrons, and dt (s) represents the data recording interval period of 20 min. Both hydrogen production (HP) and hydrogen production rate (HPR) were evaluated and calculated as described in Equation (4) and Equation (5) (and 5b) respectively, whereas the COD removal efficiency was measured according to Equation (6).

$$\text{HP} = \frac{\text{Cumulative H}_2\text{ production(mmol) or (L)}}{\Delta\text{COD}} \tag{4}$$

$$HPR = \frac{Cumulative\ H_2\ production\ (mmol)\ or\ (L)}{H_2\ evolution\ time\ (D)\ \times\ bioreactor\ volume\ (L)} \tag{5}$$

Or

$$HPR = \frac{Cumulative\ H_2\ production\ (mmol)\ or\ (L)}{H_2\ evolution\ time\ (D)\ \times\ cathode\ surface\ area\ (m^2)} \tag{5b}$$

$$COD\ removal\ effeciency\ (\%) = \frac{COD_{Initial} - COD_{Final}}{COD_{Initial}} \times 100 \tag{6}$$

Herein, $COD_{Initial}$ is the COD concentration (mg/L) of the influent and COD_{Final} is the COD concentration (mg/L) of the effluent as previously described by Gini et al. [33,34].

The MEC performance characterized by the energy recovery efficiency, was evaluated based on the consumed energy during the bioelectrochemical reactor operation and the resulting energy obtained from the cumulative produced H_2 gas [35]. The former was calculated as described by Yan et al. [36], and the latter is calculated referring to the generated number of H_2 moles and the energy produced when one mole H_2 is burnt (heat combustion for H_2 is 285.83 Kj/mole) [35,37], The overall energy recovery efficiency was estimated as demonstrated in Equation (7).

$$\eta(\%) = \frac{H_2\ output(mol) \times H_2\ energy\ content\left(J.mol^{-1}\right)}{Electric\ energy\ input\ (J)} \times 100 \tag{7}$$

2.8. Statistical analysis

Origin software together with Microsoft Excel were concomitantly employed to evaluate the elemental descriptive statistical analysis. However, since the MEC was operated under different experimental protocols using various applied voltages as described in section 2.2.2, a *t*-test was directly performed at $p < 0.05$ level of significance to know whether the differences were statistically significant among operating protocols.

3. Results and discussion

3.1. MEC performance based on biohydrogen production

To access the effects of applied electric potential on the MEC performance during the biotransformation of agricultural wastes biomass (wheat straw) into bioH_2, three reactors were connected to 0.5 V, 0.8 V and 1 V respectively; and run as stated in section 2.2.2. The electrochemical voltage supplied to the employed electrodes accelerates the bio-oxidation of the electron donor at the anode (Eq. (8)) and bioH_2 generating reaction (Eq. (9)) at the cathode. It's notewealthy that the bioH_2-producing reaction on plain carbon electrodes was reported to be so slow, therefore a high overpotential is required to pilot bioH_2 production [38].

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 9H^+ + 8e^- \tag{8}$$

$$9H^+ + 8e^- \rightarrow 4H_2 \tag{9}$$

In the present study, the results revealed that the biohydrogen production was increasing along with the augmentation of the supplied electric potential to +0.8 V, beyond which it decreased (see Fig. 2a). However, such observation rebuts the massive investigations that reported a linear augmentation of bioH_2 evolution with the increase of applied voltage [39,40]. This decrease in hydrogen production for the MEC operated under the applied voltage beyond 0.8 V is attributed to the fact that the energy level of the liberated electrons became high enough to drive other chemical reactions at the cathode surface including oxygen evolution reaction, that competed with the reductions of the protons (H^+) to hydrogen gas, thus leading to a decrease in the overall production of hydrogen gas [41].

Moreover, hydrogen peroxide is likely produced at high applied voltages, which can inhibit hydrogenase enzymes that are responsible for hydrogen production, as well as other enzymes involved in microbial metabolism [42,43]. The applied high voltage can also cause oxidative stress, which can lead to cell damage and reduce microbial growth and activities, thus limiting the oxidation of the substrate which results in reducing the availability of protons (H^+) and decrease the overall hydrogen production [6]. Herein, among other protocols, MEC0.8 depicted the best performance and produced a maximum cumulative biohydrogen production of $1.444 \times 10^3 \pm 11.3$ mL which was 34.97 % and 25.69 % higher than that of MEC0.5 and MEC1.0 respectively (see Fig. 2a).

Consistently, the overall hydrogen yield produced in the MEC0.8 protocol was as high as 6.017 mmole H_2/g-COD (approximately 134.8 mL/g-COD) whereas the other protocols namely MEC0.5 and MEC1.0 achieved only 4.33 mmole H_2/g-COD (97 mL H_2/g-COD), and 5.426 mmole H_2/g-COD (121.55 mL/g-COD) respectively. The MEC0.8

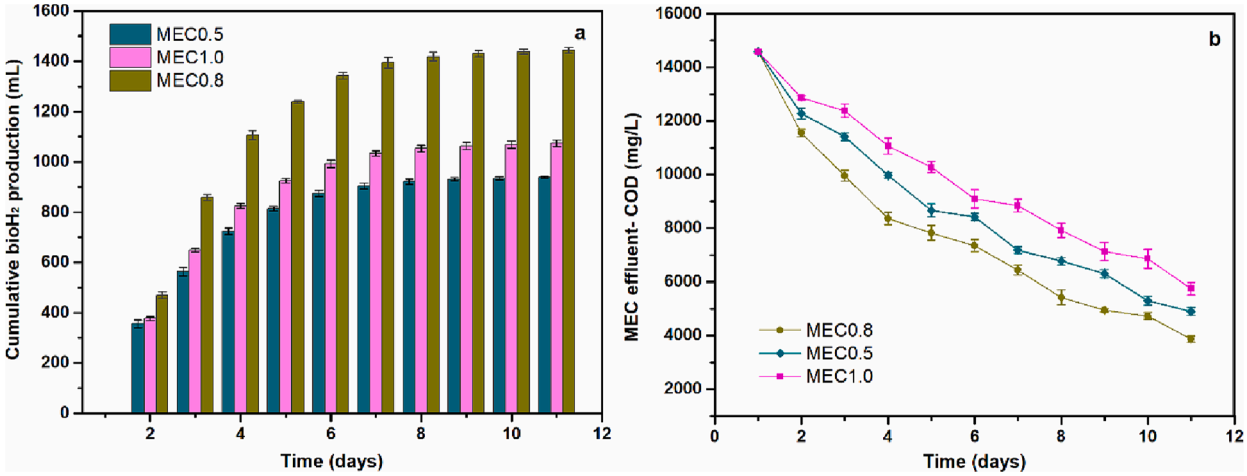

Fig. 2. Biohydrogen production in performed MECs reactors using agricultural straw biomass and consumed COD analysis at different applied potentials. Cumulative bioH_2 production (a) and COD removal analysis (b).

protocol also reported a high HPR of ~1.95 mmol·L^{-1}·D^{-1} (approximately 21.269 ± 0.011L·m^{-2} cathode·D^{-1}) which was comparatively higher than the previously reported ones. For example in 2020 Swee and his colleagues [41] investigated the impact of applied cell voltage on the performance of a microbial electrolysis cell and reported only a maximum of 6.0 ± 1.5 L m^{-2} cathode day^{-1} though they even used acetate as an electron donor, which is considered a model easily biodegradable substrate in bioelectrochemical cells. The same researchers in 2022, also achieved a maximum H_2 production rate of 14.6 ± 0.8 L m^{-2} cathode day^{-1} in a continuous mode of MEC operation[44]. The key factors negatively affecting HPR include long hydraulic retention time (HRT) and the use of inefficient catalysts to speed up the hydrogen-producing metabolic reactions. Therefore, the authors suggest enhancing both hydrogen production and hydrogen production rate by reducing the MEC operating period and finding a potential bioengineered microbial strain that could be used as a potential biocatalyst to accelerate the H_2-producing microbial activities.

Moreover, the MEC0.8 protocol achieved a maximum coulombic efficiency of 68.4 % which was quite close to that of the MEC1.0 protocol (69.5 %), but significantly higher than that of MEC0.5 (40.28 %) ($p < 0.05$) (See Fig. 6b). This high CE value is attributed to the high rate of the microbial metabolic activities indicated by the high rate of substrate utilization rate indicated by its high COD removal efficiency in this protocol. Meaning that the high substrate-oxidation rate results in liberating plenty of charges (electrons or coulomb) and protons (H^+) (that get recovered as H_2 gas at the cathode) thus reading to high CE. The CE is likely to increase with the increase of the applied cell voltage, as the anode potential increases and thus provides more energy for microbial metabolism [40]. However, the excessive increase of the applied voltage results in inhibiting microbial growth thus impeding substrate utilization (metabolic activities slow down) and leading to low CE as well.

It is noteworthy that the achieved maximum CE in this present work was slightly higher than the previously reported works that investigated the microorganism-catalyzed-MEC performance using biodegrading-resistant feedstocks as a source of carbon for hydrogen production. For instance, Xu et al. [45] reported a coulombic efficiency of 63 % for a membraneless MEC fully catalyzed by microorganisms during H_2 production from wastewater, and Tom and his colleagues [46] achieved only 60 % of CE in research investigating how to increase both current density and coulombic efficiency in porous bio-anodes in microbial electrolysis cells by enhancing mass and charge transport. Furthermore, the MEC0.8 protocol again achieved a maximum cathodic hydrogen recovery efficiency (Rcat) of 460.4 %, which was approximately 1.16 and 1.44 times higher than those of the MEC0.5 and MEC1.0 protocols, respectively. These results further showed the remarkable impact of the applied voltage in governing the MEC reactor's performance and indicated 0.8 V as a promising optimum voltage that could lead to the best results.

Moreover, compared to MEC0.5 and MEC1.0, the substrate-bioconversion efficiency characterized by the consumed COD concentration was reported to be maximum in the MEC0.8 protocol. The latter protocol reported a maximum COD removal as high as 73.4 %, which was 9.7 % and 17.5 % for MEC0.5 and MEC1.0 respectively (see Fig. 2b). The reason behind such low performance both in hydrogen production and substrate utilization for MEC0.5 could probably be the low applied voltage that didn't yet reach its optimum to allow complete bio-oxidation of the substrate whereas the MEC1.0 protocol possibly inhibited or suppressed the electrochemically active bacterial growth owing to the overdose of the applied voltage, thus hindering the MEC performance. It is believed that the high applied potential leads to decreasing bacterial growth in MEC, which includes impeding the metabolic activity of electrochemically active bacteria (particularly bioH_2-producing microorganisms). Such behavior indeed lowers substrate utilization (COD removal efficiency) and thus limits the overall bioH_2 production [6]. Overall, the present study results for MEC performance were comparable but slightly higher than the previously published reports investigating the feasibility of enhancing hydrogen production from complex substrates using MEC technology. For example, in 2020, Varanasi et al. [28] investigated the feasibility of maximizing H_2 production from water hyacinth by coupling dark fermentation and MEC, and still achieved only an overall hydrogen yield of 67.69 mL H_2/g $COD_{consumed}$ (approximately 3.02 mmol H_2/g-$COD_{consumed}$), with COD removal efficiency of 70.33 % which were lower than this current study's results.

3.2. *Viability of electrochemically active microbial consortium on anodic biofilm*

To better understand the effects of the applied potential voltage on the MEC biocatalyst growth, the viability of the anodic biofilm at different applied potentials (0.5 V, 0.8 V, and 1.0 V), utilizing agricultural wheat straw biomass as an electron donor in performed MEC reactors was assessed using confocal laser scanning microscope (CLSM). The results showed that the high applied voltage inhibits the mixed electroactive microbial growth, and lower hydrogen production (see Fig. 3). CLSM images indicated that the cell viability in formed microbial biofilm was notably decreasing along with the augmentation of the supplied voltages (see Fig. 3), implying that the used microorganisms to catalyze the bioelectrochemical reactions were so sensitive to excessive

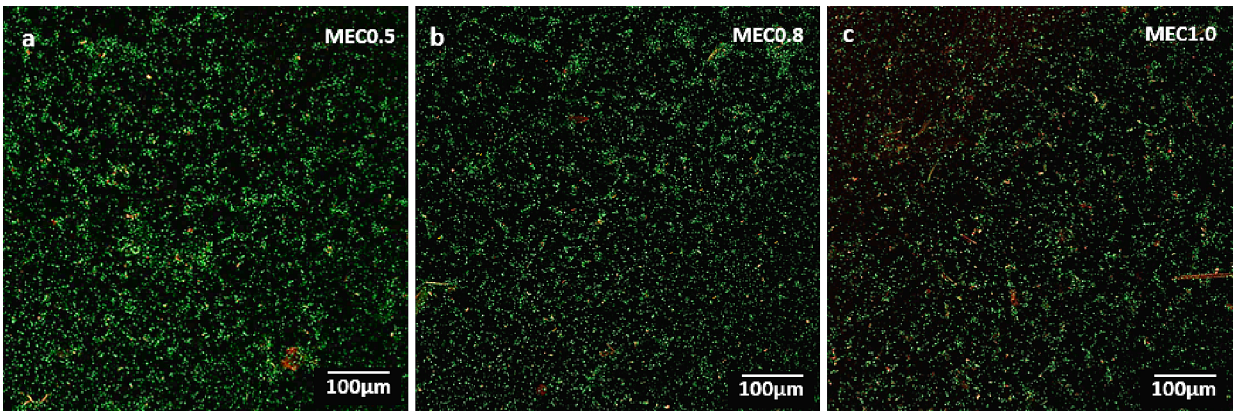

Fig. 3. Confocal laser scanning microscope images (100 X) of the formed bacterial biofilms at different applied potentials (MEC0.5, MEC0.8, and MEC1.0 protocols). After staining bacterial cells with Live/Dead backlight viability strain, live microbial cells turned green whereas the dead cells turned red. (a) MEC operated under 0.5 V (MEC0.5); (b)MEC operated under 0.8 V (MEC0.8); and (c) MEC operated under 1.0 V (MEC1.0).

supplied currents. It is therefore believed that the mediocre hydrogen yield observed in the protocols operated under high applied voltage beyond +0.8 V was related to the low bioactivity initiated at the MEC anode since the microbial biofilm and associated EAB communities are the major elements that govern the overall electrogenesis process and conspicuously control the charge transfer pathways from the oxidizing site (anode electrode) to reducing site (cathode) where the resulting protons are reduced into H_2 gas.

Though, some researchers reported changes in microbial composition with different applied voltages [47,48]; the electroactive microbial biofilm viability has been scarcely evaluated. Therefore, this novel current study shows that the viability of the formed microbial biofilm is a key parameter that needs to be considered and evaluated to optimize bioH_2 production in MEC technologies.

In the current investigation, it is also believed that applying high voltage to the MEC performance did not only inhibit the electroactive microbial growth but also killed most of the cells starting by largely destroying their cell membranes, thus leading to a reduction in H_2 production. To validate the aforementioned hypothesis, the transmission electron microscopy (TEM) test was performed for the microbial cell samples collected from three operating protocols namely MEC0.5, MEC0.8, and MEC1.0 V to ultra-visualize the electroactive bacterial cell structure, and indicate how higher applied potentials damaged the cell structures.

The results showed that the individual cells in the lower-potential operated protocols were less affected (see Fig. 4a & b), however, the higher operating protocol (MEC1.0) demonstrated that both the cytomembrane (CM) and cell walls (CW) were largely damaged (see Fig. 4c). Specifically, the cell damage rate was escalating along with the increase of applied potentials as fellows: MEC0.5 < MEC0.8 < MEC1.0 which was consistent with the low MEC performance operated under 1.0 V (see Fig. 4). Based on the afore-discussed results, we assume that the applied potentials mainly affect the used microorganisms in two ways; (1) the optimum applied current promotes microbial growth and substrate decomposition; and (2) the extreme current results in suppressing the electroactive microorganisms.

3.3. The electrochemical characteristics and current profile

As afore-discussed, the negative effects of higher voltage on MEC performance are generally initiated by the decrement of bacterial metabolic activities. To illuminate what exactly happened within the MEC reactors operated under high voltage, and better delineate how the applied voltage could be a key parameter governing the electrochemical reactions within the MEC reactors; the EIS evaluation was directly conducted at open circuit potential for the reactors operated at various applied potentials (MEC0.5, MEC0.8, and MEC1.0). Their results were directly compared to assess the effects of the applied potentials on the overall MEC performance in the bio-transformation of the straw wastes biomass straw wastes into the biological hydrogen energy process. The EIS of MEC0.5, MEC0.8, and MEC1.0 were respectively conducted by employing a three-electrode system namely anode (working electrode),

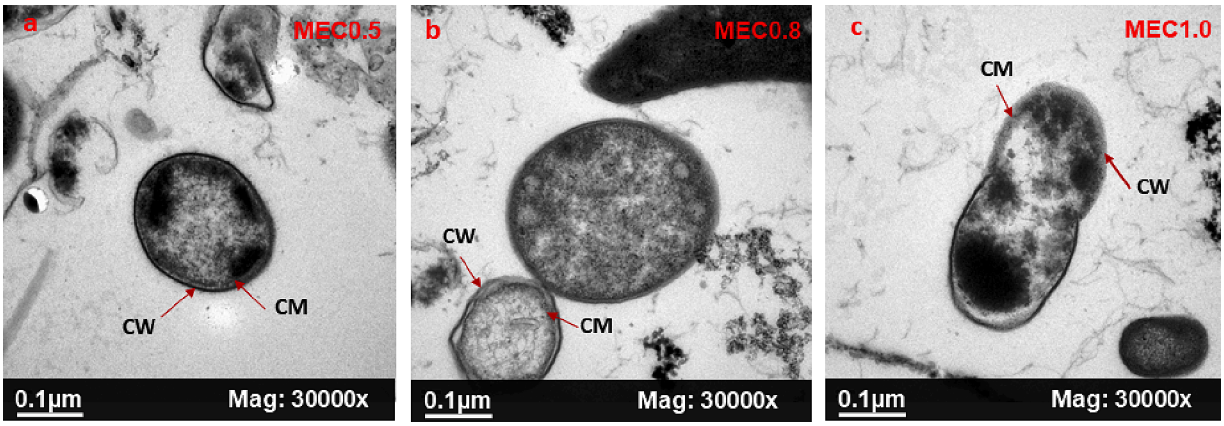

Fig. 4. Transmission electron microscopic (TEM) images of anodic-bacterial cells collected from MECs operated under different applied potentials. (a)MEC operated under 0.5 V (MEC0.5); (b)MEC operated under 0.8 V (MEC0.8); and (c) MEC operated under 1.0 V. CM stands for cytomembrane whereas CW represents bacterial cell wall.

cathode (counter electrode), and Ag/AgCl (a reference electrode).

The Nyquist plot results from the investigated protocols depicted well-structured semi-circles for all figures highlighting the charge transfer process which is normally estimated from the measured diameter of the semi-circle in the Nyquist plot [49,50] (see Fig. 5a-c). The smaller the radius of the semi-circle, the smaller the EIS is, and the higher the MEC reactor performance. Consistently, the results in Fig. 5a-c. clearly showed that the internal resistance of the performed reactor decreased along with the increase of the applied voltage until 0.8 V, but dramatically increased in MEC reactors operated at high applied potential beyond 0.8 V. However, within the same operating protocol, the internal resistance was also increased along with the reactor operating time (See Fig. 5a-c).

Comparatively, the obtained charge transfer resistances for MEC1.0 was the highest, and it was estimated as 16.06 Ω which was approximately 22.1 %, and 73.7 % higher than that of MEC0.5, and MEC0.8 protocols, respectively (see Fig. 5a-c), and these differences were significantly different since their associated *p*-values were both <0.05 ($p < 0.05$). The large EIS for MEC1.0 is attributed to the negative effects of high potentials on the electroactive microbial growth, which further suppressed many microbial cells and thence gave rise to the high aggregation of the abundant un-oxidized recalcitrant biopolymers from the electron donor (agricultural straw wastes) and the microbial dead cells.

Fortunately, these findings were consistent with both the detected TEM images (Fig. 4) and CLSM images (Fig. 3) that clearly indicated that the microbial cells in MEC1.0 were remarkably damaged, whereas the cells in MEC0.8 and MEC0.5 were less affected. Moreover, the detected high internal resistance for MEC1.0, also proves the worse performance of this protocol in times of COD removal and biohydrogen production compared to MEC0.8, since high internal resistance resulted in terminating the free movement of generated electrons and protons to anode and cathode respectively [50].

Moreover, in this current study, the current densities generated from MECs operated under different applied voltages were analyzed to better understand their effects on the electrochemically active bacterial growth (microbial community responsible for electrochemical metabolic activities including the bioelectrohydrogenesis process). The results indicated that the current density was increased as the applied electric potential was increasing (see Fig. 5d). Compared to MEC0.5, and MEC0.8; the MEC operated under high voltage (MEC1.0) displayed a high current density of 9.06 A/m^2 which was approximately 12.4 % and 35.7 % higher than the former protocols respectively. The generated electric current in this study was comparable with the maximum electric current (greater than40 mA) achieved by Hussain et al. while investigating the long-term MEC performance using different feedstocks including the acetate-based substrate and brewery wastewater [51]. However, such a high current density in MEC1.0 was reported in inhibiting microbial growth, altering bacterial shapes, and even destroy the bacterial cells [20]; which is consistent with the worse MEC1.0 performance characterized by low biofilm viability (Fig. 3) and high bacterial death rate (Fig. 4) within this protocol.

For instance, in 2001, Cao and his colleagues reported that a current

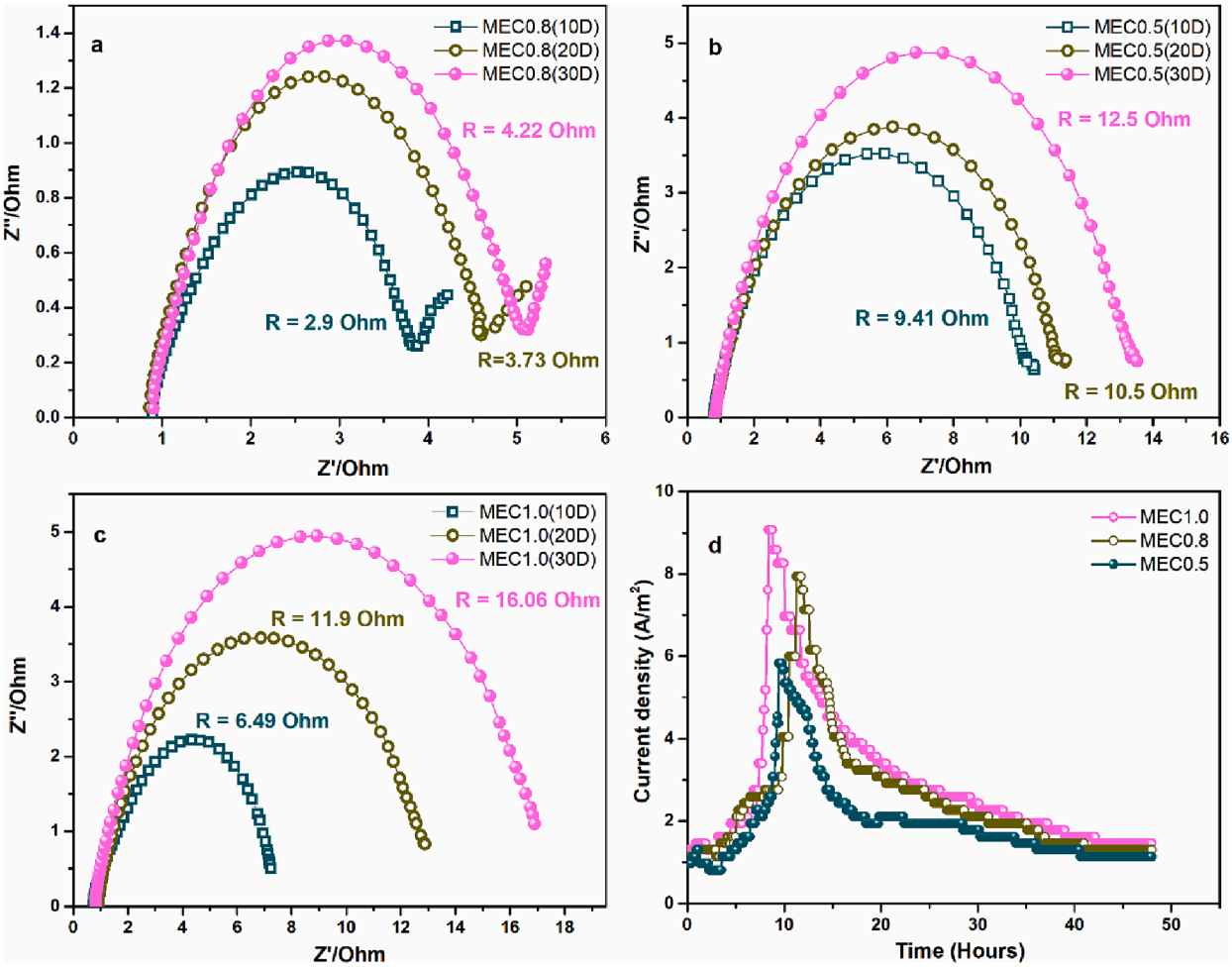

Fig. 5. The Nyquist plot from the EIS analysis of single chambered-MEC operated under various applied potentials and scanned at a different operating time. (a) MEC operated under 0.8 V (MEC0.8); (b) MEC operated under 0.5 V (MEC0.5); and (c) MEC operated under 1.0 V (MEC1.0). 10D; 20D and 30 stand for curves scanned on the 10th, 20th, and 30th day of the MEC operating period. (d) Current density generated during the MEC performance at different supplied voltages.

density of at least 2 A/m^2 inhibited the nitrifying bacterial metabolism, and the nitrification decreases by more than 60% to 10 A/m^2 [6,52]. Overall, these results imply that, though applying a proper voltage on MEC promotes microbial growth; ensures the bacterial biofilm viability, and particularly empowers the electroactive microbial community to efficiently bio-catalyze the electrochemical reactions for enhanced bioH_2 production, however, the voltage beyond 0.8 V on MEC operations does inhibit metabolic activities or kill microbial cells that lead to increase the reactor internal resistance resulting to impede the free circulations of the anions and cations within the reactor; thus leads to low MEC performance. Interestingly, such an investigation involving the impact of MEC internal resistance on the overall performance was not yet explored in detail.

Furthermore, the electrochemical behaviors characterized by cyclic voltammetry were performed in all protocols to investigate how the applied potentials affected the formed robust anodic biofilm for electrochemical reactions (redox reactions) characterization (Fig. 6a). The results showed that the oxidation current trends were increasing along with the augmentation of the supplied electric potential from 0.5 V to 0.8 V, but afterward slightly started decreasing. The reasons behind the low performance in times of electrochemical reactions in high voltage-operated protocols (MEC1.0), is probably due to the over-dose applied voltage that kills the abundant electrochemically active bacterial communities, which consequently increases the MEC reactor's internal resistance that couldn't allow easy circulation of the generated electrons charges to an anodic electrode and protons to the cathode where they got reduced into bioH_2 gas. Consistently, these CV results further support the high charge transfer resistance, low biohydrogen production, and low anodic biofilm viability results displayed in the MEC reactors operated at a high applied voltage (1.0 V). Thus, high applied voltage undoubtedly inhibits the MEC's operating reactions and the optimum voltage for best MEC performance is 0.8 V.

3.4. Energy recovery analysis

To access the cost-effectiveness of the MEC performance for H_2 production in times of energy efficiency, the overall energy recovery (η) of the performed protocols (MEC0.5 V, MEC0.8 V, and MEC1.0 V) were evaluated and compared. As it is discussed in section 2.7, it was evaluated based on the electric energy input (consumed energy during the MEC performance from the external power supply) and the energy generated from the produced H_2 production. The results indicated that the energy input was increasing along with the increase of the applied voltage, whereas the maximum energy obtained from the produced hydrogen gas was recorded for MEC0.8 V as high as 18.425 × 10^{3}J which was approximately 1.34 folds and 1.53 folds those calculated for MEC1.0 and MEC0.5 V respectively. However, the calculated overall energy recovery efficiency increased by 163.069 % from the MEC performed at a high applied voltage (MEC1.0) to the MEC operated at a low voltage (MEC0.5) which was comparable with the previous reports of Call and Logan et al., and Cui et al. [32,53]. The MEC0.8 reported a maximum energy recovery of 861.93 % depicting that energy recovered from H_2 gas was much higher than the consumed energy to produce it, and this finding was much higher than the previous work of Lim et al. [41] who reported an overall energy recovery efficiency of from both bioanode and external power in terms of hydrogen production at the cathode of only 29.4 ± 9.0 %. These results were comparable with the study of Cui et al. [53] who reported in 2021 a significant increase in the energy recovery rate when reducing the applied voltage as well. Referring to these findings, it is believed that this current MEC performance during the H_2-energy production from wastes biomass has a high energy recovery benefit.

Though the current study's results are promising, there are some limitations and experimental challenges that need to be addressed in future studies prior to the commercialization of this technology: (1) Notwithstanding the use of electroactive microorganisms as biocatalysts is more advantageous over costly metal-based catalysts [7,54], the maturation of formed biofilm is still a long and sophisticated process, thence, an easy way to boost microbial biofilm evolution for sustainable use in microbial electrolysis cell technology is sorely needed; (2) Moreover, the authors also suggest finding a potential bioengineered microbial strain that could be used as a potential alternative biocatalyst to accelerate hydrogen-producing metabolic reactions for enhanced H_2 production.

4. Conclusions

This novel study investigated the effects of applied voltage on the performance and electroactive microbial growth of MEC. The results indicated that the substrate utilization and bioH_2 yield both increased along with the applied voltage, and beyond 0.8 V started decreasing. The maximum hydrogen production of 6.017mmoles H_2/g-COD with a cathodic H_2 recovery efficiency of 460.4% was achieved in the MEC0.8 protocol. Consistently, the anodic biofilm viability and bacterial cell shape in the low voltage-operated reactors (MEC0.5 & MEC0.8) were less affected but largely damaged at high voltages (MEC1.0); suggesting

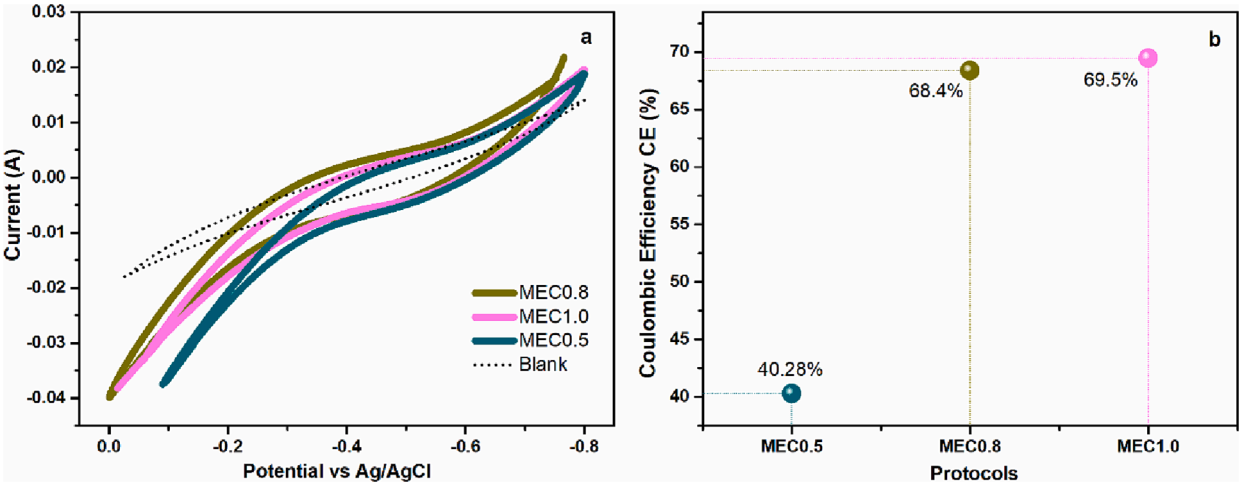

Fig. 6. Electrochemical behaviors characterized by cyclic voltammetry of the wheat straw-fed MECs operated under various applied potentials (a), and Coulombic efficiency (CE) analysis (b). The dark yellow curve stands for MEC operated under 0.8 V (MEC0.8); the magenta curve represents MEC operated under 1.0 V (MEC1.0); the Dark cyan curve stands for MEC operated under 0.5 V (MEC0.5), and a short dot black curve represents a blank scan.

that the mixed electroactive consortia were so sensitive to high voltage. Moreover, compared to MEC0.5 and MEC0.8, MEC1.0 reactors displayed a high charge transfer resistance of ~16.06 Ω which was ~22.1 %, and ~73.7 % higher than that of the former protocols, respectively. Based on these experimental results, the over-dose voltage notably inhibited microbial growth, thus decreasing bioH_2 production. Thence, this study suggests the optimal applied voltage for the best MEC performance as 0.8 V for future MEC operations.

CRediT authorship contribution statement

Fabrice Ndayisenga: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Zhisheng Yu:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing. **Bobo Wang:** Software, Validation. **Dandan Zhou:** Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgments

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