Influence of ferric oxides with varying crystallinity on the enrichment of halophilic exoelectrogens: An application for power generation in microbial fuel cells

Arshia Fathima a, Muhammad Zarul Hanifah bin Md Zoqratt b, Shu Yong Lim b, Fong Yoke Ling b, Meng Nan Chong a,*

a Department of Chemical Engineering, School of Engineering, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor, Malaysia
b Genomics Facility (MUMGF), School of Science, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor, Malaysia

ARTICLE INFO

Keywords:
Bioelectrochemical systems
Microbial electrochemical systems
Dissimilatory metal-reducing bacteria
Wastewater resource recovery
Electroactive microbes

ABSTRACT

Pre-enriching electroactive microbes (EAM) capable of respiration using solid terminal electron acceptors (TEA) is a prospective strategy that promotes faster start-up of bioanodes in microbial fuel cells (MFC) for energy-efficient treatment of saline wastewater. Previous studies demonstrated a reduction in the start-up times of bioanodes with ferric oxides as TEA, though the enriched EAM generated a wide range of current densities as opposed to those using graphene oxide (GO) as TEA. Additionally, the influence of ferric oxides with varying crystallinity on the anode-respiring activity of halophilic EAM is understudied. This study elaborates a systematic investigation into the influence of inoculum sources, different crystallinity of ferric oxides and their redox potentials on the selective enrichment of GO-respiring EAM for developing bioanodes with high current-generating capacity at shorter start-up times. A holistic assessment of the bioanodes, including analysis of their electrochemical performance and generated power densities, revealed that enrichment of GO-respiring EAM with amorphous ferricydrate as TEA fostered the growth of high-performing biofilms at shorter start-up times (48–72 h) compared to crystalline goethite used as TEA (~96 h). These biofilms produced power densities ranging from 142 ± 10 mW/m² to 276 ± 76 mW/m² with corresponding Coulombic efficiencies of 16 ± 6 % to 30 ± 6 %. Finally, the implications of promising halophilic EAM consortia were explored for saline MFC as a prospective energy-neutral wastewater treatment technology.

1. Introduction

Microbial fuel cells (MFC) are prospective energy-efficient technologies for simultaneous wastewater treatment and resource recovery from saline industrial effluents that pose significant pollution hazards, including landfill leachates and food processing wastewater [1]. Compared to the conventional biological wastewater treatment technologies that are adversely impacted by the total dissolved solids (TDS) [2], MFC are robust systems capable of effectively treating wastewater with salinities ranging from 1 to 25 w/v% TDS [2]. At the anodes, MFC employ electroactive microbes (EAM) to harvest electrons directly from the oxidation of organic wastes, while the coupled cathodic reactions enable resource recovery, including power generation [1]. Recent findings on halophilic EAM, which thrive under saline conditions, have bolstered the development of MFC for sustainable treatment of saline industrial effluents [2]. However, the scale-up of these MFC is challenged by the lack of an efficient anodic electroactive biofilm (EABF) whose growth and performance stability are influenced by multiple parameters.

The widely employed enrichment strategies of growing EABF on
polarised anodes directly from the inoculum source or from a pre-established EABF are energy-intensive and time-consuming [3]. Additionally, the growth of halophilic EABF directly from sludge inoculum exhibited considerably long start-up times, which are defined as the time taken to reach peak values of stabilised voltage or current, ranging from 24 d [4] to a few months [5, 6]. Thus, there is a pressing need to devise an effective strategy to establish robust and efficient EABF with shorter start-up times for practical implementation, which would also ensure its resilience when treating industrial wastewater under fluctuating operational conditions [7]. One of the feasible strategies is through pre-enrichment of inoculum sources to increase the abundance of dissimilatory metal-reducing bacteria (DMRB) [3]. In order to gain energy for their metabolic activities, DMRB can respire via extracellular electron transfer (EET) on solid terminal electron acceptors (TEA), including Fe (III) oxides often found in their natural habitats [8]. The energy gained by DMRB is influenced by the redox potentials of Fe (III) oxides that depend on their particle size, phase, crystallinity, concentration and solution pH [9]. Varying the redox potentials of TEA also modulated the expression of EET pathways in the exoelectrogens [10], though the maximum energy harvested by EAM is thermodynamically constrained by their intracellular electron transporters [11]. Previous studies have shown that the crystallinity of ferric oxides also consigned a selection pressure that altered the composition of EAM consortia in the presence of suitable carbon sources [12, 13].

The amorphous ferrihydrite had higher bioavailability than crystalline goethite and thus, ferrihydrite facilitated a higher abundance of DMBR in the community [12]. In contrast, the reduction of goethite was attributed to synergistic interactions between the fermentative bacteria and the enriched sulfate-reducing DMBR that were also capable of respiring on the Fe (III) oxide [12]. Additionally, the bioavailability of Fe (III) oxides regulated the proportion of exoelectrogens enriched in the consortia and their dominant EET mechanism employed to reduce these TEA [13]. However, the halotolerance and anode-respiring capacity of these enriched EAM have not been investigated, with limited studies reporting the enrichment of halophilic DMBR that were also capable of generating high current densities in the dual-chamber MFC [3]. At present, there is also a lack of understanding on how the current generation capacities of the halophilic EAM consortia are influenced by their exposure to different ferric oxides with varied redox potentials as TEA.

Whilst the pre-enrichment of DMBR with Fe (III) oxides could shorten start-up times of MFC [3], it does not ensure the selection of efficient anode-respiring EAM as opposed to those high-current generating exoelectrogens (>1.8 A/m²) that engaged in synergistic interactions with non-EAM via carbonaceous electron shuttles [14]. A halophilic EAM consortium was also isolated using graphene oxide (GO) as TEA from seawater-based inoculum [15], where the reduced GO acted as an electron shuttle to enhance interspecies electron transfer [16]. Although this EAM consortium generated high currents [15], there have been few attempts to pre-enrich halophilic DMBR that are capable of synergistic interactions with non-EAM and possess high anode-respiring activity in the saline MFC.

Thus, this paper presents an investigation on the potential of pre-enriching halophilic DMBR-based consortium in the presence of both GO and Fe (III) oxides as TEA in order to develop robust EABF generating high currents within shorter start-up times of MFC. A systematic investigation was carried out on the source of inoculum and the crystallinity of Fe (III) oxides, including amorphous ferrihydrite and crystalline goethite, governing the pre-enrichment of EAM consortia. Developing a facile and pragmatic strategy to enrich efficient halophilic EAM consortia is anticipated to foster the development of MFC as a sustainable and energy-neutral technology for saline wastewater treatment.

2. Materials and methods

2.1. Pre-enrichment of halophilic electroactive inoculum

Widely and locally available activated sludge (AS) from municipal wastewater treatment plant and the anaerobic seed sludge from palm oil mill ponds (AP) were used as the sources of inoculum, with mixing of the sludge sources also evaluated in order to increase the diversity of EAM in the enriched consortium [7]. GO was synthesised using the modified Hummer’s method with water-enhanced oxidation adopted from the literature to obtain GO with a higher abundance of hydrophilic groups [17].

Halophilic EAM consortia were selectively enriched using the adopted “Put and Wait” strategy [18] using different inoculum sources, namely AS, AP or mixed sludge (ASP) that was comprised of AS and AP sludge in equal ratios. Briefly, 50 mL selective growth media, composed of phosphate buffer solution with GO as the TEA, was mixed with the inoculum source for its enrichment and incubated without shaking at 30 °C for 7 d (Fig. 1). The subsequent formation of solid reduced graphene oxide (RGO) signified the degree of acceptable enrichment of the EAM consortia. Further enrichment of the RGO-sludge was done by transferring it to fresh growth media supplemented with or without ferric oxides as the additional TEA (Fig. 1). The enriched RGO-sludge flocs were then added to the anolyte during the setup of the MFC for the growth of anodic EABF.

The source sludge and sterilised selective media were refrigerated at 4–8 °C until their use to diminish microbial activity. The adapted composition of selective growth media containing GO [18] was as follows: 0.278 g/L FeSO₄·7 H₂O, 0.5 g/L NH₄Cl, 0.5 g/L KNO₃, 9.5 g/L NaCl, 0.1 g/L CaCl₂·2H₂O, 0.1 g/L MgSO₄·7H₂O, 0.2 g/L KH₂PO₄, 2.5 g/L NaHCO₃, 0.65 g/L GO, 0.1 v/v% ATCC Trace Mineral solution and 5 v/v% LB Broth (Miller). These media were then supplemented with concentrated NaCl solution to achieve 2–6% salinity and 20 mM CH₃COONa as the electron donor. For ferric oxides as TEA, the growth media were supplemented with 1 mM Fe^{2+} ions sourced from either freshly produced amorphous ferrihydrite obtained by adding aqueous stock solution (100 mM) of anhydrous FeCl₃ (300 mM) to the selective media [10] or by adding the aqueous stock solution (100 mM) of synthesised crystalline goethite (α-FeOOH) [19]. This supplementation of ferric oxides was based on the positive energy gains that were theoretically estimated from the differences in Nernst redox potentials between electron donor and TEA [20] at their respective concentration in the selective media at 30 °C. The estimated Nernst redox potentials are −298 mV vs SHE (standard hydrogen electrode) for acetate [21], −380 mV vs SHE for GO [18], −117 mV vs SHE and −303 mV vs SHE for the Fe^{3+} ions from ferrihydrite and goethite, respectively [9]. Based on these positive energy gains (Table S.1), the dissolution rates and bioavailability [13], amorphous ferrihydrite and crystalline goethite were chosen as TEA for this study over other ferric oxides.

As the objective of this pre-enrichment strategy was to decrease the start-up times of saline MFC, RGO-sludge enriched with only GO as the TEA was considered as the base case for this study. The following sample names are henceforth referred to as RGO-sludge samples used as anodic inoculum:

- Samples enriched with only GO as the TEA – AS-RGO, AP-RGO, ASP-RGO
- Samples enriched with GO and amorphous ferrihydrite as the TEA – AS-Fe, AP-Fe, ASP-Fe
- Samples enriched with GO and crystalline α-FeOOH as the TEA – AS-α-Fe, AP-α-Fe, ASP-α-Fe

2.2. Genomic analysis of microbial communities in pre-enriched inoculum

The RGO-sludge samples were collected post-incubation for 16S rRNA gene sequencing analysis and were stored at −80 °C until further
processing. Genomic DNA was extracted from these samples using a QIAGEN DNeasy PowerSoil Pro DNA isolation kit as per the manufacturer’s instructions. This extraction was followed by the amplification of the V3-V4 region of the extracted 16S rRNA gene sequence and library preparation according to the 16S Metagenomic Sequencing Library Preparation protocol [22]. High throughput sequencing was then performed on the Illumina Miseq platform with subsequent annotation of taxonomy for clustered and classified sequences using the SILVA database [23].

2.3. Experimental setup of saline microbial fuel cell

A H-cell MFC with an empty bed volume of 250 mL was used for the experiments with a 4 cm tube housing the Nafion 212 (5 cm²) membrane that isolated the 6 mm-thick carbon felt anode (3 x 3 cm²) from Pt/C coated carbon cloth cathode (5 x 5 cm²). Saline PBS was used as the electrolyte (230 mL) for both chambers, where the catholyte was continuously aerated with an air pump while the anolyte was initially purged with N₂ gas and then sealed to maintain anaerobic conditions. The composition of PBS electrolyte was adopted from the literature [24] with 20 g/L NaCl added to increase its salinity. This electrolyte was composed of 0.87 g/L K₂HPO₄, 0.68 g/L KH₂PO₄, 0.25 g/L NH₄Cl, 0.453 g/L MgSO₄, 0.1 g/L KCl and 0.04 g/L CaCl₂ with 0.5 mL of ATCC trace minerals solution added during start-up of the MFC. A 7-decade resistor circuit board (±1% tolerance) was used as the applied external resistance (Rext) that was connected across the anode and cathode using titanium wires.

The MFC was initially started at 1000 Ω for 20–24 h with subsequent growth of EABF monitored with chronoamperometry (CA) using a potentiostat (Autolab PGSTAT 204) at a constant anode potential at +0.2 V vs Ag/AgCl (3 M KCl) for 72 h. This oxidation potential was applied to provide an energy gain similar to that obtained with GO as the TEA [18]. The MFC was then operated at a static Rext of 1000 Ω, which was selected based on similar MFC setups in literature [6,25], until the complete characterisation of its performance. Initially, 30 mM CH₃COONa was added to the anolyte as the electron donor, followed by its supplementation with 20 mM CH₃COONa every 3 d based on the decline in cell voltage at the static Rext. This supplementation was determined to be sufficient for maintaining the concentration of electron donor between 30 and 40 mM based on the preliminary tests on residual acetate concentration in the MFC anolyte inoculated with ASP-RGO. The anolyte was partially replaced (~50%) after 7 d to enhance its self-buffering capacity via accumulation of in situ generated bicarbonate [26] along with the addition of RGO-sludge at 4.35 v/v% taking into account the sludge washout during anolyte replacement. Weekly replacements of the catholyte were performed to stabilise its pH and prevent precipitation formation on the cathodes. The entire experiment duration for each MFC lasted over 20 d, which included 14 d for the growth of EABF and stabilisation of generated cell voltage of the MFC.

2.4. Electrochemical characterisation and performance analysis of microbial fuel cell

The polarisation curve was obtained using the single-cycle method [27] after 2 weeks of biofilm growth by varying Rext from OCV to 25 kΩ, 10 kΩ, 1000 Ω, 500 Ω and 250 Ω, and subsequently monitoring the cell voltage across Rext with a USB voltage logger (Lascar Electronics) and also measuring it with a multimeter. Based on the preliminary tests with ASP-RGO (Fig. S1), each Rext was connected to the MFC for 90 min to allow for stabilisation of the generated cell voltage. Applied Rext lower than 250 Ω were not used to obtain polarisation curves because of significant power overshoot observed during preliminary studies for ASP-Fe at lower acetate concentration (Fig. S2 (a) and (e)). Additionally, the corresponding MFC voltage at 250 Ω dropped down to 0 V without being recovered over time (Fig. S2 (b)), which did not allow for further analysis of anodic Coulombic Efficiency (CE). Thus, the analysis for peak power density was captured within the range of Rext from OCV to 250 Ω, where current and power densities were calculated based on the projected surface area of the anode (18 cm²) [21]. The linear portion of the obtained voltage curves from polarisation tests was used to estimate the internal resistance of the MFC [21].

After obtaining the polarisation curves, the anodic CE [21] was calculated by operating the MFC at high current density conditions that are known to correspond with maximum chemical oxygen demand (COD) removal rates [28]. Thus, the anodic CE [21] was calculated based on the current generated at the lowest investigated Rext of 250 Ω for ~90 h using a USB voltage logger (Lascar Electronics). The corresponding acetate consumption was monitored by residual COD in the anolyte samples that were filtered through a 0.45 μm syringe filter. The COD was measured using Hach COD Low Range Vials as per Hach COD Method 8000. The MFC was then operated at the peak power density for another 48 h following which the electrochemical characterisation was performed using a potentiostat. Standard errors for power densities, COD removal and Coulombic efficiencies are reported in this study based on standard deviations obtained from experimental duplicates [29].

The ohmic resistance of the MFC and electrochemical performance of the anode were analysed with electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV), respectively. The EIS of the full cell setup was measured using the 2-electrode configuration, where the cathode was set as the working electrode (WE) and the anode was connected as the counter electrode and reference electrode (RE). During this measurement, a cell voltage of 0.75 V was applied, and the frequency ranged from 100 kHz to 1 Hz at a sinusoidal perturbation of 10 mV amplitude, with the subsequent fitting of the obtained Nyquist plots using the Randles circuit. The applied cell voltage for EIS was either at OCV or close to OCV for each MFC in order to analyse the ohmic resistance of the system [30]. The CV analysis of the anode was conducted in the 3-electrode configuration under substrate-depleted conditions in the MFC with the anode as WE, cathode as counter electrode and Ag/AgCl
(3 M KCl) as RE. The anode potential was scanned from +0.6 V to −0.6 V vs RE at a rate of 10 mV/s over 2 cycles with well-mixed conditions of anolyte maintained via magnetic stirring. The range for scanning anode potentials in CV was selected based on previous literature, where CV was used to characterise redox species involved in EET for pure strains of model exoelectrogens, namely Geobacter sp. and Shewanella sp. [31]. For comparative analysis, the second CV cycle was reported here with normalisation of the current density by peak anodic current density of the respective anodic EABF. For all the electrochemical characterisation, no iR compensation was applied as the overall voltage losses due to the uncompensated solution resistance at the low currents generated (<2–3 mA) were minimised by using saline PBS electrolyte with high conductivity of 36 mS/cm.

3. Results and discussion

3.1. Microbial composition of pre-enriched inoculum

Pre-enrichment of raw sludge sources using selective media with GO and ferric oxides as TEA caused significant shifts in their microbial community structure. The abundant classes Gammaproteobacteria and Clostridia accounted for 40–80 % of the microbes in all of the enriched inoculum as compared to the raw sludge sources (< 20 %) (Fig. 2). Additionally, the selective media reduced the abundance of Methanoseta class in the enriched inoculum, which would diminish substrate competition at the anode by the methanogens [7]. The presence of EAM was analysed at the genus level to evaluate the potential of these enriched consortia as anodic inoculum for saline MFC. Whilst all of the enriched inoculum displayed similar composition at the class level, these consortia presented varied composition and abundance of the electroactive species at the genus level (Fig. 3).

At the genus level, the enriched EAM consortium presented with a mixture of denitrifying bacteria, sulfur-reducing bacteria and fermenters for all of the investigated enrichment conditions using selective media containing only GO or both GO and ferric oxides as TEA (Fig. 3). Upon enrichment, the relative abundance of the electroactive denitrifying microbes in the enriched consortia, including the weak exoelectrogen Pseudomonas sp. [32] and the electroactive Thauera sp. [33], was considerably higher than that of the raw sludge sources (< 1 % abundance). For the enriched consortia, the relative abundance of Pseudomonas sp. ranged from 12.1 % to 42.5 %, while that of Thauera sp. ranged from 0.3 % to 10.3 %.

Fig. 2. Overview of the dominant classes for the microbial community in the enriched inoculum as compared to the raw sludge sources (AS, AP and ASP). Complete information on the sample names used in this figure is provided in Section 2.1.
On the other hand, the increased abundance of the *Trichloromonas* sp. (1.2–10.1%) under the *Desulfuromonadia* class indicated the enrichment of a high-performing exoelectrogenic consortium. The enriched *Trichloromonas* genus, including *Trichloromonas acetexigens* (basonym *Desulfuromonas acetexigens*) and *Trichloromonas michiganensis* (basonym *Desulfuromonas michiganensis*), is related to the sulfur-reducing *Desulfuromonas* sp. [34]. Previous studies have isolated *Desulfuromonas* sp. from marine habitats with GO as TEA [15,35], where these strong exoelectrogens produced high currents similar to that of the model exoelectrogen *Geobacter* sp. [32,36]. Thus, the presence of these strong
exoelectrogens in the enriched EAM consortia could promote the development of an efficient EABF with enhanced anode-respiring activities. The exoelectrogens, *Pseudomonas* sp. and *Trichloromonas* sp., together accounted for 15–50 % of the enriched inoculum, where a lower abundance of these species was noted for AS-based consortia (22 ± 7 %) as compared to the AP-based (29 ± 10 %) and ASP-based (35 ± 10 %) consortia. Besides increasing the diversity of microbes and possible syntrophic interactions among its member species, this outcome determined that mixing of sludge sources during pre-enrichment would enhance the electrochemical activity of the resultant EABF as discussed in the following sections.

In addition to the above EAM species, an increased abundance was observed in the enriched consortia for DMRB, including *Exiguobacterium* sp. [37], *Geospirillum* sp. [38] and *Alkaliphilus* sp. [39], as well as the exoelectrogen *Desulfovibrio* sp. [33,40]. Enrichment of the fermenter *Petrimonas* sp. [33] and the electroactive acetate-producing *Acetobacter* sp. [41] was also noted in the enriched consortia. Potential synergistic interactions among the non-EAM, exoelectrogens and other EAM species present in these pre-enriched anodic inoculum are anticipated to enhance the efficiency of saline industrial wastewater treatment in the MFC [7,33]. However, the presence of these supporting microbial species varied among the duplicates of enriched consortia (Fig. 3), which was attributed to the phylogenetic variations occurring in the raw sludge sources over time [42]. Nonetheless, previous studies have reported the production of similar power densities from phylogenetically diverse microbial consortia [42,43]. The reproducible functionality of these diverse consortia can be ascribed to the presence of dominant exoelectrogens that significantly contribute towards the electricity generation in the MFC as well as further enrichment of the inoculum at the anodes of MFC [3]. Similarly, the presence of the exoelectrogens *Pseudomonas* sp. and *Trichloromonas* sp. in the enriched consortia being investigated in this study enabled similar functionality across the duplicates that resulted in consistent performance at the anodes of the MFC as discussed in the following sections. Compared to the pure strains of exoelectrogens used in previous studies [32], this study utilised inoculum from pre-enriched environmental samples that could present variabilities in robustness and performance, including electricity generation and COD removal efficiency, as discussed in the following sections.

### 3.2. Effect of pre-enriched mixed sludge inoculum on subsequent power generation

The mixed cultures at anodes of MFC, comprised of EAM and fermentative bacteria, can generate higher and more stable currents than pure EAM strains [7]. These consortia have increased resilience to sudden variations in the anolyte during MFC operation and thus, they are more suitable for pragmatic scale-up of the MFC [44]. Whilst being effective for the degradation of complex organics, the mixed inoculums have demonstrated varied performance in MFC when generating currents from the oxidation of non-fermentable organics, such as acetate [7,45]. For a given reactor configuration, including reactor and electrode design, this variable performance of MFC can be attributed to anodic oxidation kinetics and substrate competition by the non-EAM [7] as observed in this study (Fig. 4).

The peak power densities generated by ASP-RGO (201.4 ± 23.6 mW/m²) were similar to those obtained with AS-RGO (236.1 ± 36.1 mW/m²) and AP-RGO (243.6 ± 28.6 mW/m²). These results indicated that combining inoculum sources did not considerably affect the energy generation capacity of the halophilic EABF (Fig. 4). Additionally, a slight decrease in current density generated by ASP-RGO was noted during polarisation measurements at Rext of 250 Ω, which could be attributed to slower rates of substrate oxidation and consequent rate of electron transfer to the anode [27]. Nevertheless, the MFC with ASP-RGO exhibited increased performance stability over time under high current conditions at Rext of 250 Ω as deduced from the relatively high anodic CE values corresponding to its COD removal efficiency (Section 3.3). Furthermore, it also had a lower internal resistance than AP-RGO (Section 3.4). This observed performance of ASP-RGO corresponds with previous studies reporting improved efficiency for mixed cultures formed by combining inoculums from different sludge sources, where enhanced electron transfer among its species led to improved anodic performance [46]. Thus, ASP-RGO showed higher potential for robust, stable and potentially scalable performance over time in the MFC.

The inclusion of ferric oxides along with GO in the selective media could facilitate the enrichment of halophilic DMRB having enhanced anode-respiring capacity as discussed in the following Section 3.3. Although the theoretical energy gained during cellular respiration with ferrihydrite and goethite is lower than that obtained with GO as TEA, these varied redox potentials would influence the adopted EET pathways in the EAM [11]. For instance, the expression of EET pathways in pure strains of *Geobacter* sp. was regulated by its exposure to ferric oxides with varied redox potentials that influenced energy gained by the exoelectrogens [10]. Based on available energy gain, the exoelectrogens regulate various components involved in the EET process for energy harvesting and conservation, including intracellular electron carriers and extracellular network of electron transporters [47]. Hence, exposure to ferric oxides with varied redox potentials influences the EET process that will significantly affect the efficiency of currents generated in the MFC. Therefore, a systematic investigation was conducted on the
selective enrichment of halophilic EAM using GO and ferric oxides as TEA and their consequent energy production in the MFC for all of the sludge sources.

3.3. Influence of ferric oxides as TEA on current and energy production

The inclusion of ferric oxides as TEA in the selective media accelerated the reduction of GO to solid RGO-sludge by the enriched EAM and thus, these TEA decreased the enrichment cycle times by 30% as compared to those with GO as the sole TEA. However, the resultant EABF developed from these pre-enriched inoculums differed in their performance (Figs. 5 and 6) that correlated with their source of sludge as well as the crystallinity of the ferric oxide TEA used during the selective enrichment of EAM.

Generally, the ferrihydrite-enriched inoculum generated peak power densities similar to those enriched without ferric oxides as TEA,
irrespective of the source of sludge used for their pre-enrichment (Fig. 5). Additionally, these EABF exhibited faster start-up during CA with polarised anodes than all of the other anodic biofilms, with their start-up times required to reach peak currents ranging from 24 to 50 h (Fig. 6 (a), (c), (e)) after an initial lag phase of ~24 h. In contrast to goethite, higher available energy gains with ferrihydrite as TEA (Table S.1) and its increased bioavailability [13] had facilitated enhanced anode-respiring activities in the enriched EAM consortia. However, AS-Fe and AP-Fe demonstrated higher performance variability among their replicates, where decreased microbial activity of raw sludge sources was suspected of having affected the pre-enrichment of EAM and hence, determined its eventual performance in the MFC. Furthermore,
competing biological reactions, such as substrate consumption by the
non-EAM [48], degraded the anodic performance over time as observed
by the lower CE values of AP-Fe that were disproportionate to its high
COD removal efficiency (Fig. 6(d)). These effects were minimised in the
mixed sludge-based ASP-Fe though its peak power density was slightly
lower than that of ASP-RGO (Fig. 5(f)). Nevertheless, both of these EABF
harnessed similar Coulombs of charge from acetate oxidation at R_ext of
250 Ω, irrespective of their COD removal efficiencies influencing their
CE values (Fig. 6(f)). Therefore, ASP-Fe is a better candidate than AS-Fe
and AP-Fe to develop a robust and efficient EABF in the saline MFC.

On the other hand, the goethite-enriched AS-α-Fe and AP-α-Fe
underperformed in the MFC with power overshoot observed under high-
current conditions at the R_ext of 250 Ω (Fig. 5(b) and (d)). Additionally,
these EABF had slower growth rates than those developed from AS-Fe
and AP-Fe (Fig. 6(a) and (c)). Furthermore, the low COD removal ef-
ciciencies and CE values (Fig. 6(b) and (d)) alluded to their reduced
capacity to generate currents due to the slow redox kinetics for acetate
oxidation reaction and electron transfer, which also caused power
overshoot in the MFC [27]. The catabolic activities of EAM were ther-
modynamically curbed by the low bioavailability of goethite for cellular
respiration [12], which was compounded by its low redox potentials
that potentially diminished the energy gained by microbes [11] and
hence, resulted in decreased EET activity for electricity production.

Contrarily, ASP-α-Fe generated the highest power densities among

![Fig. 7. Electrochemical performance of the MFC inoculated with pre-enriched AS sludge ((a) and (b)), AP sludge ((c) and (d)), and mixed (ASP) sludge ((e) and (f)) after 20 d of operation. Left panel: CV of the established EABF. Right Panel: Nyquist plot depicting internal resistance of the MFC. Complete information on the sample names used in this figure is provided in Section 2.1.](image-url)
the EABF, surpassing even those obtained with ASP-RGO and ASP-Fe (Fig. 5(f)). The diverse microbial species available in the mixed sludge source (ASP) enabled the reduction of crystalline goethite, where synergistic interactions among fermentative bacteria and DMRB possibly facilitated the solubilisation of goethite despite its low bioavailability [12]. Therefore, these synergistic interactions in the microbial consortium supported the enrichment of EAM consortium. The higher current density of ASP-a-Fe also indicated enhanced EET to the anode as goethite was reportedly reduced by exoelectrogens possessing a higher affinity for Fe (III) under its limited availability and employing direct EET mechanisms for its reduction [13]. These mechanisms were later confirmed using CV analysis (Section 3.4).

Nonetheless, the growth of non-EAM competing for substrates in the anodic chamber caused high variability of COD removal efficiency accompanied by nearly consistent CE values among the replicates of ASP-a-Fe. These non-EAM could dominate over time in MFC when provided with conducive growth conditions, such as high COD loading and increased complexity of wastewater, which would eventually diminish the energy generated in the MFC and undermine its scalability [48]. Thus, ASP-Fe would be more suitable than ASP-a-Fe for developing an efficient saline MFC. A common observation among the MFC was increased variance in their performance under high-current conditions at \( R_{\text{ct}} \) of 250 \( \Omega \). Although neutral pH conditions were maintained, the EABF might have limited mass transport due to the low buffer capacity of the 10 mM PBS used as the anolyte. Previous studies with slightly alkaline anolytes demonstrated significantly enhanced energy production in the MFC [6]. Thus, future investigations could consider simultaneously increasing the alkalinity and buffer capacity of the anolyte by adding bicarbonate as the buffering agent [49].

### 3.4. Electrochemical performance of halophilic electroactive biofilms

The CV analysis of the established EABF was performed under non-turnover conditions (Fig. 7 (a), (c), (e)), i.e. nearly depleted concentration of acetate in the anolyte, in order to gain insights into the potential EET pathways of the EABF [31]. As the COD removal efficiencies reached up to 80 %, the oxidation of residual acetate in the anolyte gave rise to the anodic peaks between +0.2 V to +0.4 V vs Ag/AgCl (+0.403 V to +0.603 V vs SHE) for the EABF. These potentials are ascribed to the possible complex formation between membrane-bound mediators and cytochromes involved in the oxidation reaction [50]. Meanwhile, the redox peaks between −0.4 V and +0.2 V vs Ag/AgCl (−0.203 V to +0.403 V vs SHE) indicated the presence of potential redox couples in the EABF that engaged in EET to the anode interface. Taking into consideration the pH-dependant shifts and redox activity of multitheme molecules in the cytochromes [50,51], the observed CV peaks coincided with those reported for outer membrane cytochromes involved in direct EET with corresponding redox potentials ranging from +0.1 V to −0.420 V vs SHE [31].

Additionally, the varied potentials of redox couples observed in CV could be attributed to the dynamic expression of EET pathways used by EAM for optimal energy harvesting from the reduction of TEA during their enrichment [11]. Whilst these potentials also overlapped with that of mediators involved in indirect EET, such as flavins and phahenzines [50], the requisite secretion of these mediators to sustain the observed high current densities would limit indirect EET activity [32]. Thus, the EABF demonstrated direct EET as the dominant pathway for anode respiration. Future complementary studies, including proteomics investigation, spectroscopic analytical techniques and genomic analysis of the anodic EABF, are needed to establish the identity of redox couples involved during EET [31].

The internal resistance of the MFC (Table 1), which is comprised of concentration overpotentials and ohmic resistance, was estimated from the linear portion of the polarisation curves [21]. Similar to previously observed trends for power densities, the internal resistance of the MFC with ferricydrite-enriched inoculum was close to MFC with inoculum enriched using GO as the only TEA, irrespective of the source of sludge used for their pre-enrichment. However, the accuracy of the internal resistance estimated in this study needs to be improved by collecting additional data points at a broader range of applied \( R_{\text{ct}} \) used in the single-cycle polarisation tests. Further investigations to identify the major contributors to the internal resistance can also be performed using the method of electrode slope analysis [25].

Conversely, the ohmic resistance of the MFC was evaluated using EIS analysis (Table 1 and Fig. 7 (b), (d), (f)), where the average solution resistance of 31.5 ± 2.4 \( \Omega \) was equivalent to the ohmic losses accounted by the tube length, electrolyte conductivity and membrane area in the H-cell setup [25]. A reduction in the ohmic resistance was observed for MFC with ferricydrite-based EABF, where AS-Fe (84 \( \Omega \)), AP-Fe (87 \( \Omega \)) and ASP-Fe (102 \( \Omega \)) had smaller charge transfer resistance \( R_{\text{ct}} \) than AS-RGO (148 \( \Omega \)), AP-RGO (205 \( \Omega \)) and ASP-RGO (150 \( \Omega \)), respectively. In contrast, the goethite-based EABF had higher ohmic losses than the ferricydrite-based EABF with ASP-a-Fe and AP-a-Fe having \( R_{\text{ct}} \) of 106 \( \Omega \) and 314 \( \Omega \), respectively, which also correlated with their decreased energy production capacities. Similarly, the high-performing ASP-a-Fe had the lowest \( R_{\text{ct}} \) (54 \( \Omega \)) corresponding to its highest power generation capacity among the EABF. Nevertheless, ASP-Fe is a better candidate than ASP-a-Fe for developing effective and efficient saline MFC based on the holistic consideration of the resistance losses and performance stability along with the desired high performance and energy conversion efficiency denoted by anodic CE as discussed previously. At lower frequencies in the Nyquist plots, the occurrence of straight lines at −50–60° angles to the abcissa for AS-Fe, ASP-RGO, ASP-Fe and ASP-a-Fe suggest mass transport losses at the electrodes of MFC. Additional EIS analyses at varied applied cell potentials in 2-electrode and 3-electrode configurations are required to quantify these resistance losses and identify the significant electrode contributing to it for further optimisation of the system [30].

### 3.5. Implications for scalable energy production in saline MFC

In this study, the MFC with ASP-Fe produced energy up to 1.1 ± 0.1 W/m² at an influent COD loading of 3400 ± 280 mg COD/L with an energy conversion efficiency of 16.3 ± 6.3 %. This MFC had a similar performance to the previously reported MFC treating highly saline mustard tube wastewater with simultaneous power generation of 1.45 W/m² and CE of 17.8 ± 1 % [52]. Moreover, the lower power densities reported in this MFC compared to 35 W/m² [5,53] was not feasible due to differences in the reactor configurations, including high anode surface areas [53] and smaller anode volumes [5,53]. Additionally, high power densities reported in saline MFC with ferricydane catholytes [4,53] are unsuitable for gauging their scalability due to

### Table 1

Summary of the analysed internal resistance and ohmic resistance of the saline MFC with anodic EABF developed using pre-enriched inoculum.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( R_{\text{int}} ) (( \Omega ))</th>
<th>( R_{\text{ct}} ) (( \Omega ))</th>
<th>CE (% \text{int})</th>
<th>( \mu \text{Mo}^{-1}\text{m}^{-2}\text{s}^{-1} ) (N value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-RGO</td>
<td>343.72</td>
<td>27.5</td>
<td>148</td>
<td>140 (0.679)</td>
</tr>
<tr>
<td>AS-Fe</td>
<td>293.06</td>
<td>28.2</td>
<td>84</td>
<td>38.6 (0.815)</td>
</tr>
<tr>
<td>AS-a-Fe</td>
<td>546.77</td>
<td>39.4</td>
<td>106.5</td>
<td>110 (0.690)</td>
</tr>
<tr>
<td>AP-RGO</td>
<td>311.56</td>
<td>46.5</td>
<td>205</td>
<td>91.6 (0.719)</td>
</tr>
<tr>
<td>AP-Fe</td>
<td>332.56</td>
<td>28.2</td>
<td>86.87</td>
<td>201 (0.640)</td>
</tr>
<tr>
<td>AP-a-Fe</td>
<td>445.17</td>
<td>24.1</td>
<td>314.19</td>
<td>54.7 (0.737)</td>
</tr>
<tr>
<td>ASP-RGO</td>
<td>383.83</td>
<td>33.5</td>
<td>150</td>
<td>27.1 (0.835)</td>
</tr>
<tr>
<td>ASP-Fe</td>
<td>448.17</td>
<td>25.2</td>
<td>102</td>
<td>46.1 (0.770)</td>
</tr>
<tr>
<td>ASP-a-Fe</td>
<td>196.61</td>
<td>30.7</td>
<td>54.5</td>
<td>42.0 (0.780)</td>
</tr>
</tbody>
</table>

Notes: The internal resistance \( (R_{\text{int}}) \) was calculated from the linear portion of the polarisation curves [21]. On the other hand, EIS in 2-electrode configuration at open-circuit conditions was used to analyse the ohmic resistance of the H-cell MFC [30]. The constant phase element (CPE) in the Randles circuit used to fit the Nyquist plots gives the overall capacitance of the electrodes in the MFC [25]. Complete information on the pre-enriched inoculum and their associated sample names used in this table is provided in Section 2.1.
dissimilar reduction kinetics from that of aqueous oxygen reduction reaction, which may also overestimate its performance [25]. Furthermore, oxygen as the TEA at the cathode is more feasible than ferricyanide for practical implementation of the MFC [21].

This study also demonstrated a feasible and scalable strategy to develop efficient anodes with pre-enriched halophilic ASP-Fe inoculum for simultaneous wastewater treatment and energy production in saline MFC. A direct comparison of anodic EABF from previous studies is not possible due to the variations in the inoculum, MFC setup and anolyte conditions across the experiments. Additionally, optimisation of the anolyte composition, such as buffer concentration, substrate loading and pH [6] and start-up strategy using either lower $R_{ext}$ or dynamic $R_{ext}$ [54], is required to overcome the observed power overshoot in the saline MFC and enable investigation of generated power densities and CE at $R_{ext}$ lower than 250 $\Omega$ used in the current study. Nonetheless, shorter start-up times of 48–50 h, including the initial lag phase of 20–24 h at $R_{ext}$ of 1000 $\Omega$, were observed in this study for the EABF grown from pre-enriched inoculum as compared to those previously reported EABF grown directly from raw sludge having start-up times ranging from 24 d [4] to a few months [5,6]. The faster start-up of the resultant EABF also allows quicker turnaround and restoration of the anodes upon potential performance losses caused by sudden electrolyte fluctuations during the operation of the MFC [7,44]. However, potential inhibitory effects on the microbial cells arising from toxic compounds and hypersaline conditions must be considered to enhance the performance of MFC using industrial effluents.

This study used widely available waste sludge sources to enrich halophilic EAM that may tolerate salinities up to 2 w/v% as demonstrated by previous studies [5]. This tolerance would limit their performance in hypersaline solutions as antagonistic effects at salinities above 2 w/v% would inhibit their activity and consequently, degrade the performance of MFC [1,5]. Robust EAM with salinity tolerance above 2 w/v% can be enriched from inoculums originating from a hypersaline environment, such as seawater [55] and salt marsh sediments [56], as these halophilic microbes have developed physiological mechanisms to survive under extreme salt stress [2,55]. With increased salinity tolerance, the scope of halophilic EAM could be expanded to applications involving resource recovery, such as desalination [2] and metal recovery [57], and thereby, boost the economic viability of saline MFC.

4. Conclusions

This work presented a viable strategy to develop an efficient halophilic DMRB consortium by using energy gained from the reduction of solid TEA, including GO and ferric oxides, as a criterion for their selective enrichment from waste sludge sources. Besides increasing the diversity of microbes available for the enrichment of EAM, combining different sludge sources as inoculum facilitated synergistic interactions in the enriched EAM consortium. These interactions improved the efficiency of harvesting electricity from the oxidation of acetate and stabilised the performance of the resultant EABF under high current conditions. The crystallinity of ferric oxides affected its redox potentials and the resultant energy gain for EAM, which dictated their EET pathways and electrochemical performance in the MFC. The holistic performance assessment of developed EABF identified ASP-Fe, enriched using GO and amorphous ferrhydrite as TEA, to be the most suitable halophilic DMRB consortium for high-performing MFC as compared to crystalline goethite as TEA. The resultant EABF met the desired requisites for enhanced energy production with minimal performance losses arising from ohmic resistance to transfer electrons, high CE with minimal competing reactions from non-EAM, and increased performance stability over time. Thus, the enrichment of efficient halophilic EAM consortia using the developed selective media provided a pragmatic and scalable strategy to enhance the performance of sustainable saline MFC.

Funding

This work was supported by the Graduate Research Merit Scholarship offered by Monash University Malaysia to Ms. Arshia Fathima.

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.

Acknowledgements

The authors are grateful for the financial aid provided by the School of Engineering and Monash University Malaysia. Ms. Arshia Fathima is also thankful for the full postgraduate scholarship awarded by Monash University Malaysia for her to pursue her Ph.D. research degree. The authors also express their gratitude to all of the team members at Genomics Facility (MUMGF), Monash University Malaysia for their collaborative support on this project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2023.104458.

References
