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Research paper

Balancing pH for enhanced bacterial community performance in microbial fuel cells: implications for bio-electricity generation and pollutant reduction

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Microbial fuel cells (MFCs) represent a promising technology to generate bio-electricity and synchronously reduce wastewater pollutants. The presence of exoelectrogens in wastewater is critical for bio-electricity and pollutant reduction, but the performance of exoelectrogens at different pH levels remains unknown. This study aims to bridge this gap by offering an integrated approach to understanding the performance of exoelectrogens under varying substrate pH, particularly in bio-electricity generation and pollutant reduction in sugarbeet processing wastewater (SBWW). Three pH levels (ranging from acidic to alkaline) were studied and MFC's electricity output was measured. Later, current density, power density, and coulombic efficiency (CE) were calculated. Both pre- and post-experiment substrate samples were analysed with inductively coupled plasma (ICP). Furthermore, 16S rRNA gene analysis, DNA amplification, sequencing library preparation, and bioinformatics workflows on post-experiment samples of the substrate and anode samples were conducted. A diverse community of microorganisms was identified, especially Alphaproteobacteria, Gammaproteobacteria, and Deltaproteobacteria (*Geobacter*). Bacteroidetes and *Desulfovibrio* were the major exoelectrogens responsible for electricity generation. Among the three pH levels tested, the most alkaline pH level (9.5±0.1) outperformed the others, achieving a 54% higher power density, 21% greater current density, and a 40% higher CE compared to the acidic pH level (6.5±0.1). Around 50–99% of pollutants were removed from the SBWW. The study revealed that *Gammaproteobacteria* thrive and perform better in alkaline environment.

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INTRODUCTION

Effluents from food industry are rich in dissolved organic matter, especially simple carbohydrates, and they need an expensive compliance treatment process before discharging to adjoining water sources (Zheng et al., 2013). The processing effluents of the sugarbeet industry are no exception. An older sugarbeet processing industry typically uses 25–45 L of water per 100 kg of fresh beet water per day, with wastewater production at a nearly equal rate (Vaccari et al., 2005). There is a need to treat the wastewater before discharging it (Rahman et al., 2018). This sugarbeet processing wastewater (SBWW) can be a valuable substrate for generating bioelectricity using microbial fuel cells (MFC), since it is rich in organic matter and contains some nutrients. MFC is a promising process that can generate bioelectricity by oxidizing wastewater organic matter using exoelectrogens (Logan, 2008). MFC can be a viable treatment option for treating SBWW to reduce pollutants as well as generate bioelectricity. Exoelectrogens are electrochemically active bacteria that act as biocatalysts in MFC by oxidizing organic substrates in an anaerobic anode compartment, transferring electrons, and consequently helping to generate electricity from wastewater (Rahimnejad et al., 2015). In addition, MFC-treated wastewater can also be recycled.

Studies suggest that performance of MFC depends on various parameters such as anode–cathode material, substrate types, surface area of electrodes, electrode spacing, proton exchange membrane types, pH, reactor volume, O_2 level in cathode chamber, etc. (Rahimnejad et al., 2015; Sharma and Li, 2010). Among other parameters, pH plays a considerable role in electricity production. For example, a higher pH difference between anolyte and catholyte in an MFC led to a more negative anolyte and positive catholyte potential, causing a higher achievable cell voltage. Higher anolyte pH (>7.0) favoured higher power production, while lower pH (<6.0) produced less electricity in MFC (Behera and Ghangrekar, 2009; Ren et al., 2007). Acidic pH (≤5.0) notably reduced electricity generation and power output in MFCs compared to neutral pH (7.0), as acidic conditions led to lower voltage outputs and slower chemical oxygen demand (COD) removal (Zhang et al., 2011).

Research indicates that the performance of MFC is closely linked to the pH of the anolyte. For instance, in studies where weakly acidic to strongly alkaline anodic pHs (ranging from 6.0 to 10.0) were used, Puig et al. (2010) achieved the highest power production (0.66 W/m^3), effective organic matter removal (77% COD removal) and ammonium reduction at pH 9.5. Similar observations were noted when researchers adjusted both the anodic (alkaline) and cathodic (acidic) pH values. Operating with an anodic pH of 10.0 and a cathodic pH of 2.0 suppressed methanogenesis, resulting in the MFC achieving a higher open circuit voltage (1.04 V) and maximum power density (29.9 W/m3) compared to operation at neutral pH (Zhuang et al., 2010). Studies on MFC performance investigating variations in anolyte and/or catholyte pH have shown that alkaline anodic conditions were

conducive to higher electricity generation and pollutant reduction. However, very few studies have conducted microbial analysis specifically focusing on anodic pH variations. Similarly, limited studies have specifically examined the performance of microbial communities while simultaneously assessing the effectiveness of treatments for bioelectricity generation and pollutant reduction. The efficacy of MFC significantly depends on the activity of exoelectrogens. Not all microbial communities have electrochemical activity, and therefore it is necessary to identify what kind of microorganisms are present in the substrate and contribute to bioelectricity generation. Microbial community analysis of the exoelectrogens associated with MFC showed a great range of bacteria are capable of anodophilic electron transfer, i.e., the capacity for electron transfer to an anode (Logan et al., 2006). Further bacterial taxonomy studies focusing on electricity generation from MFC noted that Proteobacteria (Jung and Regan, 2007) and Bacteroidetes (Zhang et al., 2009) are the two broad bacterial phyla that are electrochemically active and account for electricity generation in MFC. Especially, different classes, families, genera and species of Proteobacteria, Bacteroidetes and Firmicutes are thought to be particularly active in electricity generation (Table 1).

Studies have emphasized the importance of anolyte pH on the efficiency of MFCs. Investigating MFC performance in relation to electricity generation and COD removal, Zhang et al. (2011) suggested that anolyte pH could be the reason for decreased MFC performance, potentially due to changes in anodic microbes and biofilms. Although separate studies on the performance of MFCs have been conducted focused either on wastewater treatment (Wu at al., 2020; Jung and Regan, 2007), power generation (Cheraghipoor et al., 2021; Rahman et al., 2018), or microbial consortia (Zohri et al., 2019; Wu et al., 2018), to the best of our knowledge, very few studies have concurrently investigated microbial performance, pollutant reduction, and electricity generation in the same experiment with a specific focus on all three aspects, especially with regard to microbial analysis of anodic pH variations. This study aims to provide an integrated approach to microbial consortia, electricity generation, and reduction of pollutants from SBWW using MFC at different pHs. Therefore, the objectives of the MFC study were: (i) to quantify bacterial consortia (using 16S rRNA gene analysis) associated with electricity generation and pollutant reduction in SBWW; and (ii) to understand the efficacy of substrate pHs on the performance of exoelectrogens in electricity generation and pollutant reduction from SBWW.

MATERIALS AND METHODS

MCF fabrication and experimental setup

For this research, 9 dual-chamber MFCs were fabricated with clear translucent polyvinyl chloride (PVC) material (Fig. 1A). To separate the anode and cathode chamber, Nafion (N117-30, Fuel Cell Earth LLC, Woburn, MA, USA) was utilized as a cation exchange membrane (CEM) (Fig. 1B), whereas carbon cloth (EC40-40, Fuel Cell Earth LLC, Woburn, MA. USA) was used as an electrode and hung up vertically and parallel to CEM, using 22 AWG copper wires through the top lid. A low-flow rate air-stone (Small Fish Tank Air Stone, Top Fin, Phenix, AZ) was placed inside the cathode chamber connected with a fish tank water pump (Air-1000 pump, Top Fin, Phenix, AZ) to supply oxygen.

Table 1. Electrochemically active bacterial community

Phylum	Class	Order	Family	Genus	Species
Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Desulfuromonadaceae	*Desulfuromonas*	*Desulfuromonas acetoxidans*[1]
		Desulfobacterales	Desulfobulbaceae	*Desulfobulbus*	*Desulfobulbus propionicus*[2]
		Desulfuromonadales	Geobacteraceae	*Geobacter*	*Geobacter sulfurreducens*[3] *Geobacter metallireducens*[4]
		Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio*[5]	
	Gammaproteobacteria	Alteromonadales	Shewanellaceae	*Shewanella*	*Shewanella putrefaciens*[6]
		Enterobacteriales	Enterobacteriaceae	*Enterobacter*[7]	
		Betaproteobacteriales	Burkholderiaceae	*Rhodoferax*	*Rhodoferax ferrireducens*[8]
	Alphaproteobacteria[9]				
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae[10]		
			Marinilabiliaceae	*Cytophaga*[11]	
Firmicutes	Clostridia	Clostridiales	Peptococcaceae	*Desulfotomaculum*	*Desulfotomaculum reducens*[12]

[1]Alves et al. (2011); [2]Holmes et al., (2004); [3]Guadarrama-Pérez et al. (2023); [4]Sindhuja et al. (2018); [5]Kumar et al. (2020); [6]Nath et al. (2021); [7]Toczyłowska-Mamińska et al. (2015); [8]Li et al. (2009); [9]Wang et al. (2012); [10]Albarracin-Arias et al. (2021); [11]Rashid et al. (2013); [12]Dalla Vecchia et al. (2014)

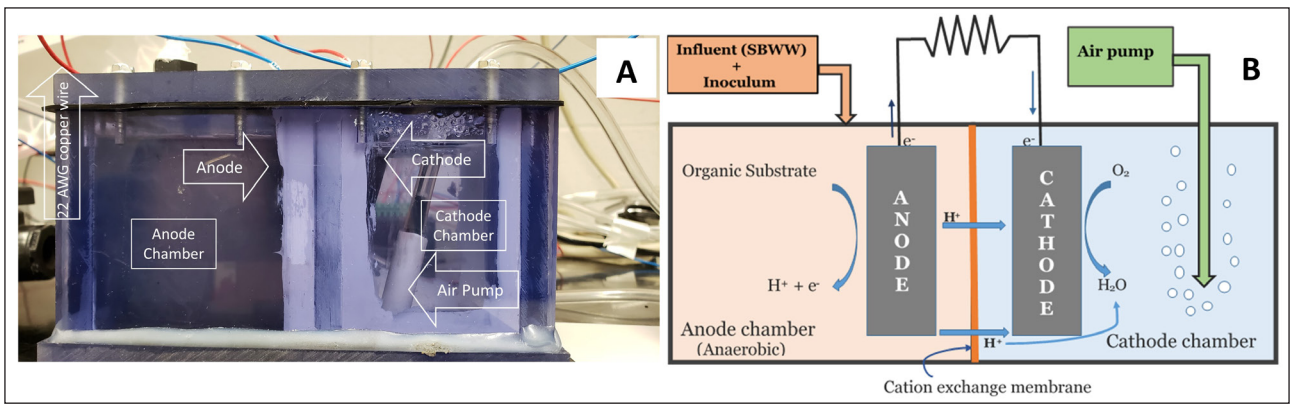

Figure 1. A: Dual-chambered MFC used in the study; B: Schematic diagram of a dual-chambered MFC

Wastewater was collected from American Crystal Sugar Co. (46° 54' 10.5192" N, 96° 45' 31.9356" W), Moorhead, MN, USA, and was utilized on the same day of collection. Following Khanaum et al. (2020), by diluting SBWW and adding 5% inoculum that was collected from the Fargo City Wastewater Treatment Plant (46° 55' 21.284" N, 96° 47' 15.234" W) Fargo, ND, USA, the substrate/anolyte was prepared. As catholyte, 50 mM monobasic potassium phosphate (VWR, West Chester, PA, USA) solution was used. The working volume of each MFC compartment was 320±40 mL. Nitrogen gas was purged into the anode chamber prior to placing the top lid, to remove oxygen before setting up the experiment, and an anaerobic condition was always maintained during the MFC experiment. A 0.5-L SKC Tedlar sample bag (SKC Inc., PA, USA) was attached to an anode chamber with a steel pipe and Teflon tube to collect headspace gas and to prevent any pressure buildup on the CEM.

Electrodes were connected to the datalogger (CR 1000X, Campbell Scientific LLC, UT, USA) to record operating temperature and voltage potential every 15 min. During the operation, when the electricity generation was relatively high, the voltage output was recorded by applying various external loads (e.g., 10, 22, 47, 100, 220, 330, 470, 680, 1 000, 2 200, 3 300, 4 700 Ω resistances) to calculate current density and power density. Polarization curves and power curves were developed using those data. According to Ohm's law, internal and external resistances remain the same at maximum power density. Hence, power density (PD) and current density (CD) were calculated by dividing the obtained power and current by the surface area (m^2) of the anode.

Initial parameters

The experiment was conducted with 3 substrate pH levels (pH#1 = 6.5±0.1; pH#2 = 8.6±0.1; pH#3 = 9.5±0.1) with 3 replicates. Pre- and post-treatment parameters (Table 2), such as electrical conductivity (EC), pH, and chemical oxygen demand (COD) of the substrate and catholyte were measured. Additionally, selective elemental analysis was done in a commercial lab. For measuring the EC and pH, a Hanna multi-parameter bench meter (HI 4522; Hanna, Woonsocket, RI, USA) was utilized. The Hach high range COD reagent and method (Hach-8000, Loveland, CO) were employed to determine COD content of SBWW. For measuring COD, 2 mL of the sample was added to the Hach high-range COD reagent vial and digested at 150°C for 2 h at the Hach block digester (DRB 200, Loveland, CO). After cooling samples at room temperature, a colorimetric test was done in the Hach spectrophotometer (Hach 2800, Loveland, CO). Elemental analyses on pre- and post- treated samples were done with an inductively coupled plasma (ICP) analyser at Agvise Laboratories, Northwood, ND, USA.

Microbial analysis

DNA extraction

16S rRNA gene sequencing for microbiome analysis was done separately to analyse the diversity of the microbial consortium in water and cloth on the post-experiment anode samples. DNA extraction was performed using a Qiagen DNeasy PowerSoilTM kit with some modifications. Briefly, anode samples were submerged in 20 mL antiseptic mouthwash in a sterile 50 mL centrifuge tube and vortexed for 1 min to disperse and lyse the microbes. A 250 µL aliquot of mouthwash was then added to the PowerSoil tube containing beads and lysis buffer. Water samples (25 mL for each replicate) collected from anodes were centrifuged at 10 000 x *g* for 30 min at 4°C. The sediment was resuspended in 1 mL mouthwash and vortexed for 1 min. A 250 µL aliquot was then added to a PowerSoil tube containing beads and lysis buffer. Water samples and cloth samples were handled identically from that point forward. A Mini Beadbeater (Biospec Products) was used at maximum speed for 3 min for the lysis step. The PowerSoil protocol was adhered to thereafter. A negative control DNA extraction was also done to ensure that there was no microbial DNA contamination from the mouthwash or the kit reagents and supplies. At that point, polymerase chain reaction (PCR) was conducted using 27F and U1492R primers to amplify the 16S rRNA region. Amplification was confirmed by running the PCR products on an agarose gel. No amplification was detected from the negative control.

DNA amplification and sequencing

DNA libraries were prepared using the 16S Barcoding Kit from Oxford Nanopore Technologies (SQK-RAB204). Each 10 µL DNA sample was combined with 14 µL nuclease-free water, 25 µL LongAmp Taq 2X master mix (NEB, M0287), and a unique barcode (1 µL). Amplification was accomplished in an Eppendorf thermal cycler (model 5341, Enfield, CT, USA) using the following conditions: initial denaturation for 1 min at 95°C, 25 cycles of 20 s at 95°C, 30 s at 55°C, 2 min at 65°C, and final extension for 5 min at 65°C. Samples were then cooled down to 4°C until DNA clean-up could be completed.

DNA clean-up was completed using AMPure XP beads as defined by the 16S Barcoding Kit protocol. DNA quantities were determined using a Quant-IT Picogreen dsDNA fluorescent quantitation kit (Life Technologies) on a BioTek Synergy Hybrid plate reader. Samples were pooled to obtain a final DNA concentration of 50–100 ng in equal mass amounts. Pooled samples contained up to 12separately barcoded samples for sequencing using an Oxford Nanopore minION. The flow cell (Oxford Nanopore, FLO-MIN106D) was primed and loaded according to the manufacturer's protocol. Each pooled library was run for approximately 4 h.

Bioinformatics workflow

Raw fast5 formatted reads were base-called and demultiplexed using Guppy v3.4. EPI2ME 16S workflow (https://epi2me.nanoporetech.com, rev 2.1.0) was used for QC and initial characterization. For QIIME2 analysis, sequences were pre-processed using MetONTIIME (Maestri et al., 2019) and QIIME2 (Bolyen et al., 2019) was used to filter and analyse the resulting table containing relative abundances of operational taxonomic units (OTU). The SILVA v138 database was downloaded and utilized as a reference database for taxonomic identification (Quast et al., 2012).

Table 2. Initial parameters of the substrate and catholyte used in the study

pH levels	pH	Substrate		Catholyte		Study period (days)
		EC (mS)	COD (mg/L)	EC (mS)	pH	
pH#1	6.5±0.1	1.2±0.02	2050±89	5.5±0.02	7.19±0.03	39
pH#2	8.6±0.1					
pH#3	9.5±0.1					

Microbial data analysis

Alpha and beta diversity of microbial communities were analysed for the impact of different pHs as well as sample type (the anode – carbon cloth and substrate). All analyses were performed in QIIME2 on samples that were filtered for a minimum sequencing depth of 15 000. Using this filter, 3 samples were discarded from diversity analysis. Dissimilarity of microbial communities was measured using Bray-Curtis distances (Bray and Curtis, 1959). Permanova (Anderson, 2001) was performed on Bray-Curtis distance using the adonis() function in R/vegan (Oksanen et al., 2013). Visualizations were generated in QIIME2 View (https://view.qiime2.org/). A Kruskal-Wallis test was done to determine significant differences of means among various treatments (pHs) and between the anode and substrate samples.

Polarization and power curves calculation

Polarization and power curves were prepared to observe the relationship between voltage potential vs. current density and power density vs. current density, respectively. Moreover, coulombic efficiency (CE) was calculated by the following equations (Rahman et al., 2018):

$$CE = CP/CT \times 100 \quad (1)$$

where CP = total coulombs calculated by integrating the current over time, CT = maximum possible coulombs if all substrate removal produced current.

$$CP = \int I \, dt \quad (2)$$

$$CT = FbV\Delta COD/M \quad (3)$$

where F = Faraday's constant (96 485 C/mol of electrons), b = number of electrons exchanged per mole of oxygen (b = 4), V = liquid volume (mL), ΔCOD = COD concentration difference over time (g/L), and M = molecular weight of oxygen (M = 32).

Data analysis

Duncan's multiple range test (DMRT) analysis was performed in Statistical Analysis System (SAS) software (SAS Development manager 9.4, SAS Institute Inc. NC, USA). The statistical difference among treatment means was done in a completely randomized design with a threshold p-value of 0.05. The null hypothesis tested that the means of bioelectricity generation and pollutant reduction among various pH levels were equal. All statistical analyses were conducted in SAS using PROC Means command. Analysis of variance with Duncan's test was done to determine significant differences among treatments. Additionally, the R studio (V4.2.3) was employed to create all tables and charts of the bioelectricity generation and pollutant removal section.

RESULTS AND DISCUSSION

The results of this study were divided into three major parts: (i) 16S rRNA gene analysis of the anode and substrate samples; (ii) bioelectricity generation from SBWW; and (iii) pollutant removal from SBWW at 3 different substrate pH levels (hereafter pH#1 (6.5±0.1), pH#2 (8.6±0.1), and pH#3 (9.5±0.1)), while the anode–carbon cloth and substrate (effluent) will be referred to as 'cloth' and 'water' respectively.

Microbiome analysis of anode samples

In this study, 16S rRNA gene analysis was performed for all 9 post-experiment cloth samples. A total of 918 723 sequence reads were obtained from the analysis produced by the minION raw data processor. Operational taxonomic units were classified to the genus level (Fig. 2) and alpha and beta diversity were compared between pH and cloth and water samples of the anodes.

The phylum-level bacterial plot (Fig. 2) was very lucid and well depicted, where the branch thickness refers to the abundance of different bacterial phyla present on cloth and water samples of the study. According to the relative abundance plot for cloth, Proteobacteria was the most prevalent microbiome on the cloth, followed by Bacteroidetes, Verrucomicrobia, Firmicutes, Acidobacteria, Nitrospirae and Plantomycetes. The abundance of Proteobacteria was also observed in water samples, followed by Firmicutes, Plantomycetes, Bacteroidetes, Epsilonbacteraeota, Actinobacteria and Acidobacteria. According to the findings presented in Fig. 2, there were evident disparities observed in the bacterial community composition between the cloth and water samples. Additionally, both the cloth and water samples showed distinct bacterial communities at different pH levels in pH#1 (6.5±0.1) as compared to pH#2 (8.6±0.1) and pH#3 (9.5±0.1), as illustrated in the first 6 samples (in both cloth and water) in Fig. 2. Specifically, Verrucomicrobia was predominant in the cloth samples, whereas Firmicutes was abundant in the water samples.

Proteobacteria dominated at all three pH levels. Yet, beyond that, the bacterial community varied depending on the pH level. For instance, in pH#1 (6.5±0.1) treatment Verrucomicrobia, Bacteroidetes, Actinobacteria were abundant; whereas in pH#2 (8.6±0.1) Bacteroidetes, Epsilonbacteraeota, Actinobacteria, Lentisphaerae were abundant; and in pH#3 (9.5±0.1) Bacteroidetes, Firmicutes, Actinobacteria, Plantomycetes were abundant. Since all MFCs employed the same substrate, the proportion of the abundances of Proteobacteria and Bacteroidetes, two major phyla, in the substrate confirmed the observed variations in the bacterial communities across different pH levels. Whilst, due to the acidic to alkaline state, the composition of the remaining phyla may vary. Nonetheless, it was evident that the bacterial communities present in the pH#1 (6.5±0.1) samples were markedly distinct from those observed in the pH#2 (8.6±0.1) and pH#3 (9.5±0.1) samples.

The phylum Proteobacteria was dominant in both cloth and water samples (Fig. 2). However, water samples exhibited higher proportions of Firmicutes and Planctomycetes, whereas cloth samples showed elevated levels of the phyla Verrucomicrobia and Bacteroidetes. Additionally, Firmicutes and Proteobacteria were more prevalent in pH#1 (6.5±0.1) of water samples, while Proteobacteria and Planctomycetes were more abundant in pH#2 (8.6±0.1) and pH#3 (9.5±0.1) of water samples. This indicates a pH-dependent effect on different phyla, with a more alkaline anolyte promoting the thriving of Proteobacteria and Planctomycetes, whereas a weakly acidic to neutral anolyte favoured Verrucomicrobia and Bacteroidetes.

Class-level bacterial analysis indicated that the most profuse bacterial class observed in the study was Gammaproteobacteria followed by Bacteroidia, Alphaproteobacteria, Deltaproteobacteria. Among them, Gammaproteobacteria, Deltaproteobacteria, and Alphaproteobacteria were three electrochemically active classes (Jung and Regan, 2007; Kim et al., 2006) belonging to Proteobacteria – the major abundant phylum of the study. The second major phylum in the study was Bacteroidetes, with its significant electrochemically active class – Bacteroidia (Zhang et al., 2009). Moreover, the study observed an abundance of *Geobacter* – a current-generating bacterial genus in the Deltaproteobacteria class (Jung and Regan, 2007; Kim et al., 2006), at all pH levels. According to the study, Gammaproteobacteria appeared more in pH#3 (9.5±0.1) than pH#2 (8.6±0.1) and pH#1 (6.5±0.1), indicating that Gammaproteobacteria may thrive in alkaline environments.

Alpha diversity is a measure of the taxonomic diversity within samples. The cloth anodes contained fewer observed OTU than

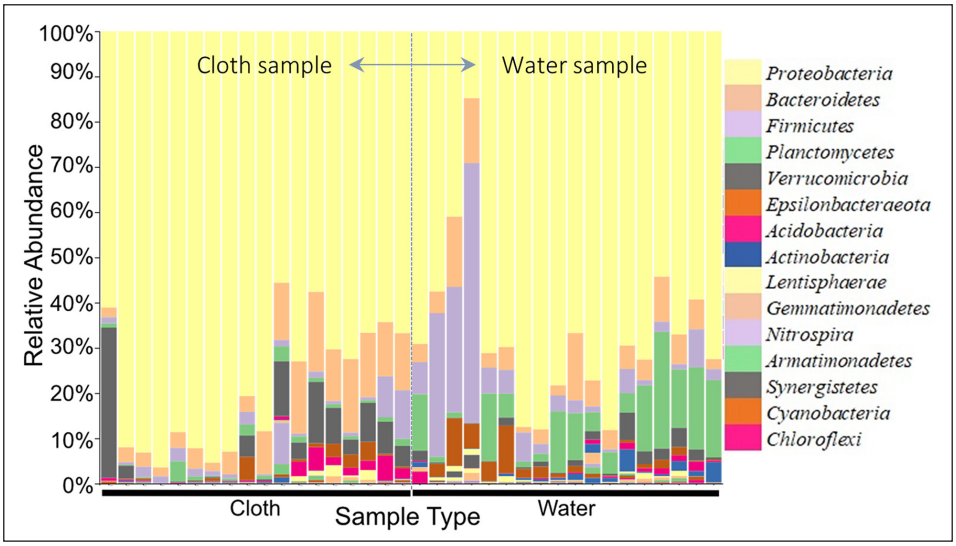

Figure 2. Bacterial phyla produced by minION raw data processor (the order of the taxa in bars and legend are from highest to lowest overall abundance)

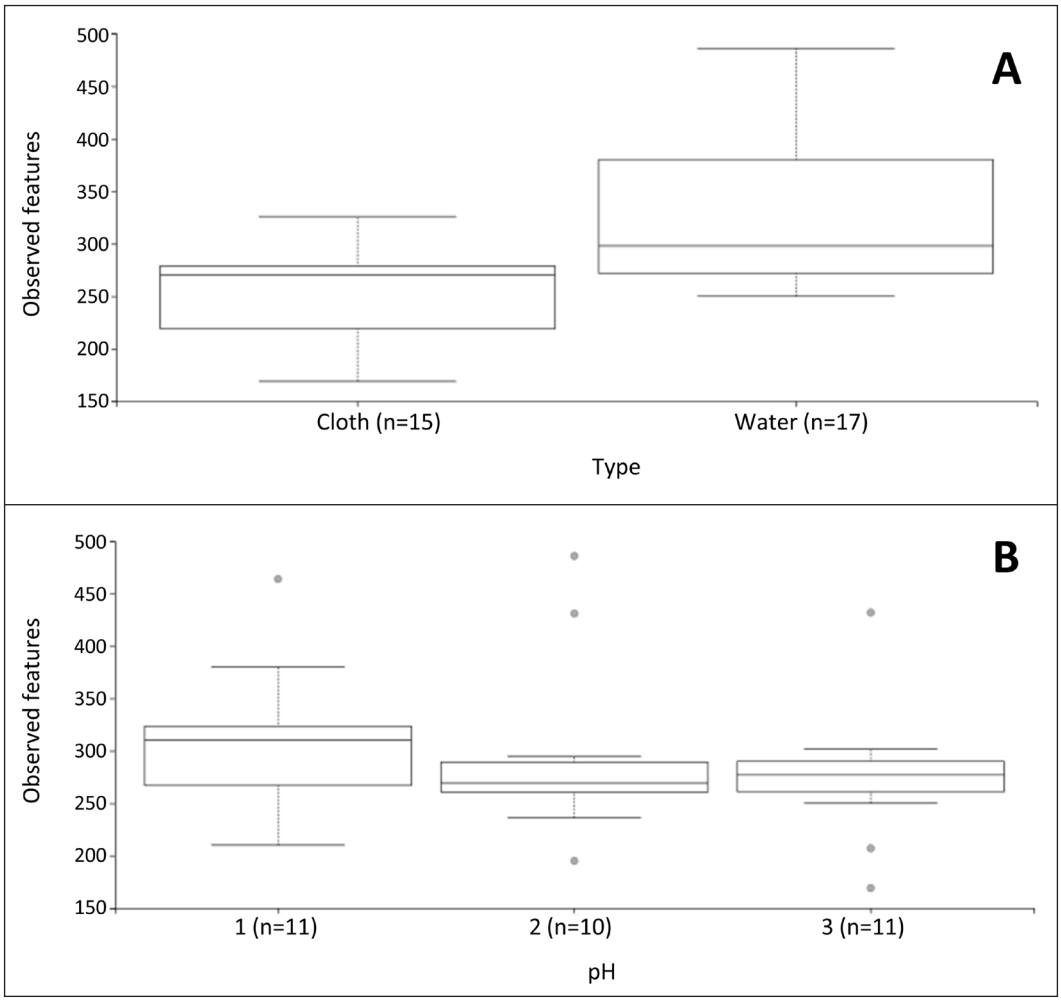

Figure 3. Alpha diversity boxplots for (A) two different types, (B) three different pHs

the water samples, and this taxonomic richness was significantly different (Fig. 3; Kruskal-Wallis test, $p < 0.05$). Evenness and the Shannon-Weaver index were not significantly different between cloth and water. Alpha diversity did not differ between the three different pH levels, though observed OTU quantities were lower at pH#2 (8.6±0.1) and pH#3 (9.5±0.1) than at pH#1 (6.5±0.1) (Fig. 3).

Community composition differed significantly between pH levels and between sample types (cloth vs. water; Fig. 4). Composition differences based on Bray-Curtis dissimilarity between cloth and water microbiomes explained approx. 13% of the variation between communities based on Permanova analysis ($p < 0.05$), and pH explained approx. 6% of variation in community composition ($p < 0.05$). Consistent with the Alpha diversity

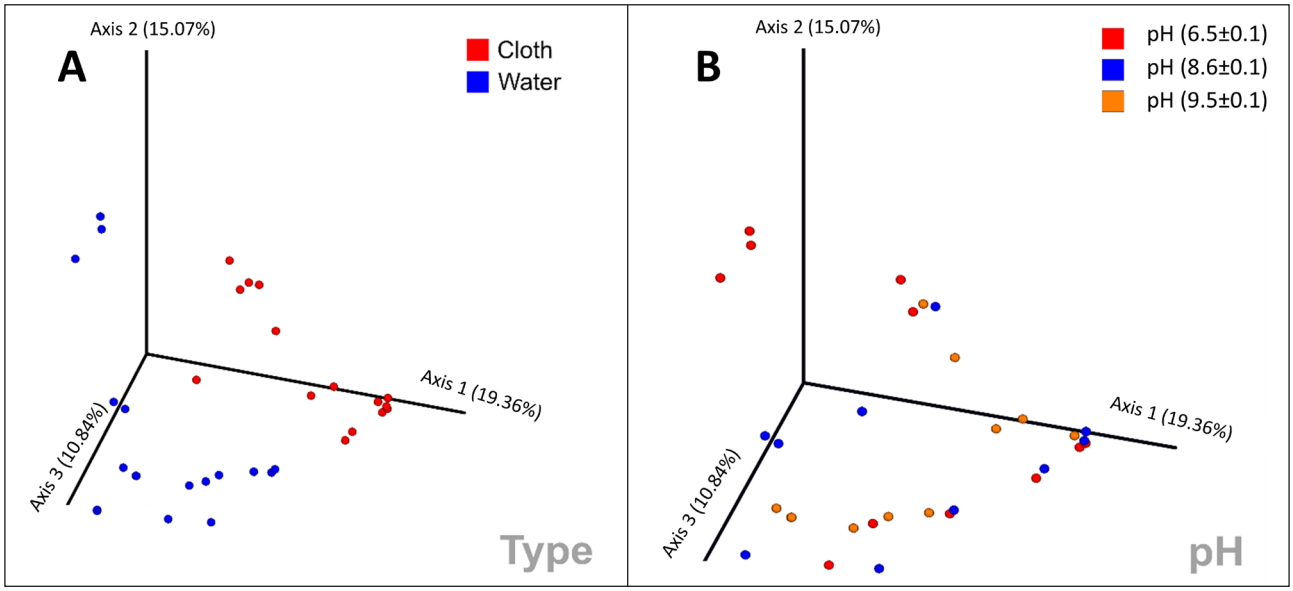

Figure 4. PCoA plots showing the relatedness of bacterial communities between (A) two types, (B) three different substrate pH levels

Table 3. Daily average generated voltage in mV (mean±SD) at three pH levels

Day	pH#1 (6.5±0.1)	pH#2 (8.6±0.1)	pH#3 (9.5±0.1)	Day	pH#1 (6.5±0.1)	pH#2 (8.6±0.1)	pH#3 (9.5±0.1)
2	39.1±12.1	128.7±6.2	100.8±12.4	21	113.1±56.7	196.9±6.1	341.9±197.9
3	132.4±63.5	292.5±14.2	278.6±87.3	22	110.7±55.7	191.1±6.1	269.2±129.4
4	204.6±103.9	393.1±41.2	373.8±99.3	23	110.3±53.2	187.3±5.6	194.8±64
5	342.3±166.7	639.6±69.3	442±98.4	24	111.2±49.6	184±4.5	176.5±51.2
6	452.2±208.7	760.7±8.9	533.6±99.6	25	111.5±46.6	180.9±4.2	168±47
7	508.9±231.6	779.2±6.4	606.1±127.9	26	110.6±44.5	177.7±4.4	163.4±45.7
8	519.8±236.9	791.5±5.9	586.4±193.4	27	109±42.8	174.5±4.5	158.8±45.2
9	528.1±241.2	798.9±5.6	576.6±214.3	28	107.5±41.5	172.6±4.3	155.6±46.1
10	505.5±230.1	810.9±2.4	580.8±233	29	105.9±40.4	170±4.8	152.4±48.1
11	385±225.4	811.7±4.6	580.7±239	30	104.1±39	166.8±5.7	149±50.9
12	354.9±230	809.6±7.5	577.9±239.5	31	102±38	163.8±5.9	147.9±54.6
13	346.5±227.3	807.6±6.8	573.5±238.9	32	99.4±36.6	160.9±6.1	147.9±57.5
14	331.7±224.3	764.1±35.1	576.7±241.9	33	96.4±35	158.4±6.1	144.2±56
15	319.3±223.1	531.6±94.1	575.8±242.6	34	92.5±33.6	155.9±6.1	141.3±54.4
16	222.1±130.4	314.5±9.5	509.2±218.1	35	89.5±32.5	152.7±6.2	138.9±52.7
17	146.1±64	258.1±10.3	412.5±215.1	36	88±31.4	148.8±6.7	137±51
18	130.1±57.9	234.6±11.2	386±221.6	37	87±31.1	144.6±7.1	136.1±49.6
19	123.1±58.1	218.9±9.5	376.5±223.3	38	85.4±31.2	140.4±6.4	135.1±48.1
20	117±57.3	205.3±6.7	369.4±222	39	84.9±30.8	138.2±5.2	134.2±47.1

analyses, dissimilarity among water samples was greater than dissimilarity among cloth samples.

Electricity generation at various pH levels

Daily average voltage generation from 3 substrate pH levels is shown in Table 3. It is evident that pH#2 (8.6±0.1) and pH#3 (9.5±0.1) outputs are significantly different to pH#1 (6.5±0.1). Electricity generation with pH#1 (6.5±0.1) was lower than for the other two pHs levels. At pH#2 (8.6±0.1), higher electricity was generated on Days 6–10 and the highest output (528.1±241.2 mV) was observed on Day 9. After that, the electricity generation decreased sharply until Day 19, and remained steady at around 100 mV after that. Both pH#2 (8.6±0.1) and pH#3 (9.5±0.1) treatments had the highest electricity production, of 811.7±4.6 mV and 606.1±127.9 mV, on Days 10 and 7, respectively.

The study suggests that substrate pH played a substantial role in exoelectrogen performance. Organic matter content, the driving force of exoelectrogen functioning, was similar for all three substrate levels. Despite this, pH#1 (6.5±0.1, i.e., acidic substrate) generated lower electricity than others, indicating that exoelectrogens were less operative in acidic environments and performed better under higher substrate pH. Other studies have supported this finding, where higher power generation at higher substrate pH was observed (Ren et al., 2007; Behera and Ghangrekar, 2009).

Polarization curves and power curves were created using the data observed, by applying various external resistances. Figure 5 illustrates the polarization curves and power curves for all three substrate pH levels; pH#3 (9.5±0.1) produced higher voltage and current density than the other two pH levels. The cell voltage and current density were inversely proportional – cell voltage gradually decreased with an increase in current density. When external resistance increases, the reactor produces a lower current density because current is inversely proportional to the resistance. Similarly, lower external resistance produces higher current density. The highest cell voltage of 382.7±26.6 mV was obtained at 4 700 Ω external resistance at pH#3 (9.5±0.1), while a similar external resistance at pH#1 (6.5±0.1) and pH#2 (8.6±0.1) produced 318.3±44.6 mV and 327.4±19.4 mV, respectively.

Even though pH#2 (8.6±0.1) generated the highest current density (1 215.90±71.8 mA/m^2), pH#1 (6.5±0.1) and pH#3 (9.5±0.1) both reached similar current densities, of 1 010.20±18.48 mA/m^2 and 1 194.5±76.1 mA/m^2, respectively. Current density at pH#1 (6.5±0.1) was lower compared to other pH levels, at 1 010.2± 85.4 mA/m^2. Other studies have reported a similar relationship between cell voltage and current densities (Ullah and Zeshan, 2020). Overall, pH#2 (8.6±0.1) and pH#3 (9.5±0.1) generated significantly higher voltage and current density, with 78% and 61% more voltage, respectively, than pH#1 (6.5±0.1). Using a similar configuration, e.g., laboratory-scale double-chambered MFCs and glucose as the anolyte with pH 7.0, Ullah and Zeshan (2020) observed a maximum voltage, power density, and current density of 262 mV, 310 mW/m^2, and 378 mA/m^2, respectively.

Moreover, pH#3 (9.5±0.1) outperformed the other pH levels in both polarization curves and power curves, achieving 54% and 35% higher power density, as well as 21% and 10% higher current density, compared to pH#1 (6.5±0.1) and pH#2 (8.6±0.1), respectively. The higher power density was observed from 47– 470 Ω external resistances. While using 100 Ω external resistance for pH#3 (9.5±0.1), the highest power density was 47.1±3.4 mW/m^2 and the current density was 387.4±28.8 mA/m^2. High current density creates considerable demands on the oxygen transportation mechanism. At this point, partial air starvation was likely to occur, thus resulting in low-performing cells and accelerating the end-of-life of the MFC due to insufficient power production (Hartnig and Roth, 2012).

Furthermore, pH#3 (9.5±0.1) also exceeded pH#1 (6.5±0.1) and pH#2 (8.6±0.1) in terms of coulombic efficiency (CE), achieving 40% and 50% more CE than the respective values for pH#1 (6.5±0.1) and pH#2 (8.6±0.1). CE is the fractional recovery of electrons from the substrate (organic matter) relative to the electrons that are supposed to be produced theoretically (Logan et al., 2006). Figure 5C compares the CE among the three pH levels, where pH#3 (9.5±0.1) performed better than the other two substrate pH levels. The maximum CE of 4.3% was obtained at pH#3 (9.5±0.1) on Day 14, whereas, on Day 13 pH#1 (6.5±0.1) and pH#2 (8.6±0.1) produced their highest CE of 3.9% and 3.7%, respectively. Using an anolyte pH of 9.03 (similar to pH#3 in this experiment), Manesh et al. (2024) achieved a maximum CE of 1.63%, while Wang et al. (2018) reported a maximum CE of 8.12±0.04% when assessing the effect of temperature on pollutant removal and electricity generation in a double-chambered MFC.

The results of the current study were in line with those from previous research (He et al., 2005); however, power density and CE observed in the current study were lower. The reason may be due to the differences in substrate characteristics, electrode spacing, and MFC sizes. Overall, the current study established that pH#3 (9.5±0.1) treatment outperformed electricity generation among the three substrate pH levels tested in this study.

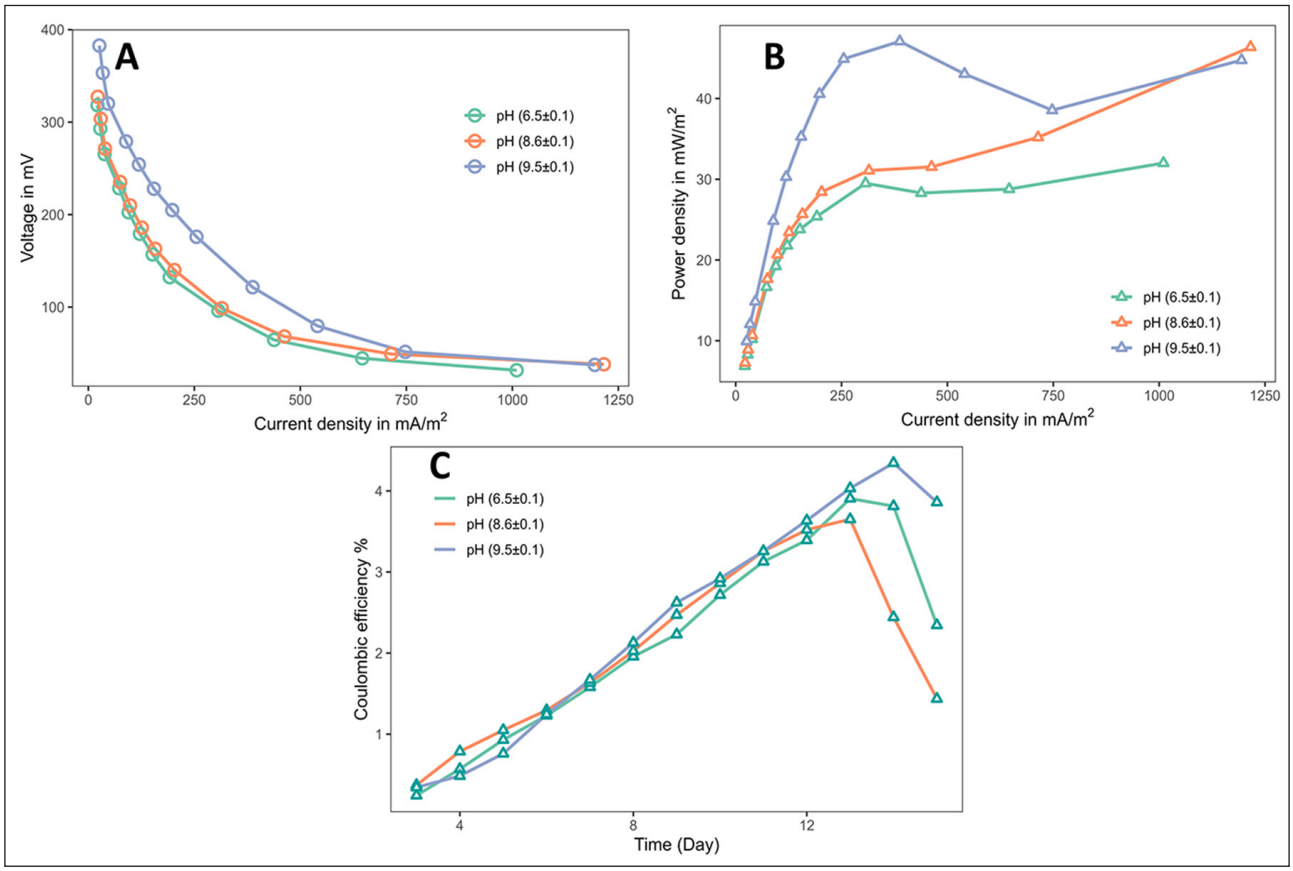

Figure 5. (A) Polarization curve, (B) power curve, and (C) coulombic efficiency curves at three different pH levels

Pollutant removal from SBWW at varying pH levels

The study confirmed that MFC is highly effective in COD reduction from SBWW. The initial COD of the substrate was 2 050±89 mg/L for all three pH levels. At the end of the study, about 99.5, 99.8, and 99.0% of substrate COD were reduced at pH#1 (6.5±0.1), pH#2 (8.6±0.1), and pH#3 (9.5±0.1), respectively. This result is supported by that of other studies using a similar substrate (e.g. Ahn and Logan, 2010; Rahman et al., 2018). However, the current study suggested that substrate pH has no specific effect on substrate COD removal. Compared to other published studies, the current study demonstrated greater COD removal from the substrate (99%). While working with a dual-chamber MFC, Rahman et al. (2018) noted 89.5% substrate COD removal (initial substrate 1 000 mg/L) and Ahn and Logan (2010) achieved 88% substrate COD removal (initial substrate COD 800–900 mg/L).

This study also noted a significant reduction in total organic carbon (TOC) from SBWW (Fig. 6A). Among three substrate pH levels, pH#3 (9.5±0.1) showed a maximum of 87% TOC removal from SBWW, followed by pH#2 (8.6±0.1) and pH#1 (6.5±0.1), with 81% and 83% TOC removal, respectively. The higher the substrate pH, the higher the TOC reduction in the substrate, which indicates the connection between substrate pH and the bacterial community, which did not perform well in an acidic substrate. As a result, less TOC was removed from the acidic substrate. In general, electrochemically active bacteria decompose organic carbon matter in wastewater and produce electrons and protons, which generate electricity in MFC, resulting in reduced TOC from SBWW. The results of this study suggest that MFC with a higher substrate pH could be a good approach to reducing TOC in SBWW.

A significant reduction in total nitrogen (TN), sulfate-sulfur (Fig. 6B) and iron (Table 4) were noted in this study. TN removal was observed at pH levels 2 and 3, resulting in reductions of 49% and 22%, respectively. This TN reduction was most likely due to the ammonium ion fluxes through the CEM because more cations needed to be transported towards the cathode through CEM which produced a higher current. This finding agrees with that of Rozendal et al. (2006) that the ammonium fluxes through the CEM increased with increasing current. Moreover, the current study also confirmed that iron can be removed significantly from SBWW. Iron was removed entirely at pH#1 (6.5±0.1) and pH#2 (8.6±0.1), while 93% of iron was removed at pH#3 (9.5±0.1). Interestingly, no sulfate-sulfur reduction was noted for pH#1 (6.5±0.1), but for the other two pH levels the reductions were 44% and 50%, respectively. Despite the presence of sulfate-sulfur reducing bacteria – *Desulfovibrio* (Cooney et al., 1996) – in the substrate, they did not perform in an acidic substrate at pH#1 (6.5±0.1). TN and sulfate-sulfur reduction rate indicated a relationship between bacterial performance and substrate pH level. Bacterial activity might slow down in an acidic environment and be boosted at higher pH; consequently, lower sulfate-sulfur reduction and no TN reduction occurred at pH#1 (6.5±0.1).

In addition, the study demonstrated that MFC could be used as an effective tool for reducing the hardness of SBWW. The hardness of water is defined as the amount of dissolved minerals, especially calcium (Ca) and magnesium (Mg). Salt and hardness removal from wastewater is an expensive process. In this study, Ca, Mg, and hardness of SBWW significantly reduced for all three pH levels (Table 4). Explicitly, 99.8%, 99.4% and 99.9% hardness were

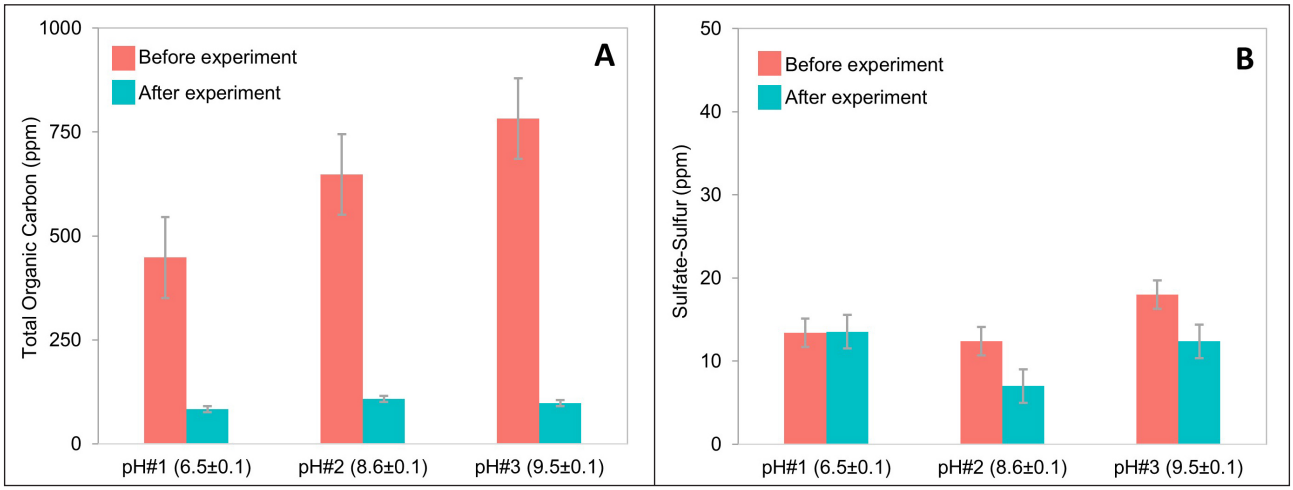

Figure 6. (A) Influent-effluent TOC, (B) sulfate-sulfur reduction at three pH levels

Table 4. Summary of significant differences in pre- and post-experimental parameters across all three substrate pH levels

Parameter	pH#1 (6.5±0.1)		pH#2 (8.6±0.1)		pH#3 (9.5±0.1)	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
COD (mg/L)	1 980.0±104.4*	80.0±9.5*	1 940.0±98.5*	75.0±3.6*	2 110.0±81.9*	96.7±20.8*
TOC (ppm)	448.1±35.3*	83.1±18.0*	647.9±118.0*	108.2±36.3*	782.1±230.8*	98.3±3.0*
TN (ppm)	37.9±1.5	40.6±15.6	59.5±8.1*	30.3±3.9*	51.3±18.0	40.0±24.4
S (ppm)	13.4±4.4	13.2±4.2	12.4±2.8	7.0±6.1	18.0±0.0*	9.4±5.4*
Hardness**	313.7±92.1*	0.7±0.6*	181.0±21.4*	1.0±0.0*	230.7±133.0*	0.3±0.6*
Ca (ppm)	113.7±32.3*	0.1±0.1*	65.0±7.9*	0.2±0.1*	80.7±46.0*	0.1±0.2*
Mg (ppm)	7.0±2.6*	0.0±0.1*	286.8±169.8*	0.0±0.1*	6.8±4.2*	0.0±0.1*
Fe (ppm)	0.4±0.2*	0.0±0*	0.3±0.0*	0.0±0.0*	0.5±0.1*	0.0±0.1*

*$p < 0.05$, i.e. significant difference among means in paired *t*-test for pre- and post-experiment parameters; **mg equivalent $CaCO_3$/L

reduced from SBWW at pH#1 (6.5±0.1), pH#2 (8.6±0.1), pH#3 (9.5±0.1), respectively. However, no significant differences in hardness removal from SBWW were observed among pH levels.

Furthermore, along with various bacterial communities, iron-reducing bacteria – *Geobacter* (Jung and Regan, 2007) and Gammaproteobacteria (Kim et al., 2006) were observed in the substrate. However, due to the very low concentration of iron in the substrate, the effect of iron-reducing bacteria was inconclusive for this study.

CONCLUSION

This study noticed a diverse community of bacteria using 16S rRNA gene analysis. Among them, three classes in the Proteobacteria phylum – Gammaproteobacteria, Deltaproteobacteria (especially, *Geobacter* genus) and Alphaproteobacteria – and one class in the Bacteroidetes phylum – Bacteroidia – were present to generate electricity from wastewater. Iron-reducing *Geobacter* and Gammaproteobacteria and sulfate-sulfur reducing *Desulfovibrio* bacteria were also present. Furthermore, the study found that when working with anodes or substrates, special attention should be paid to their selection, as microbiomes can differ significantly from substrate to anode. The substrate would be a preferable choice for those who wish to concentrate on the microbiome investigation because it displayed a wider diversity of bacterial communities than the anode.

The study also showed that changes in pH levels exerted a modest effect on the variation in the bacterial communities at phyla level. This suggested a pH-dependent effect on different phyla, with a more alkaline anolyte promoting the thriving of Proteobacteria and Planctomycetes, whereas a weakly acidic to neutral anolyte favoured Verrucomicrobia and Bacteroidetes.

Overall, the highest power density (47.1±3.4 mW/m^2), current density (1 194.5±76.1 mA/m^2), and CE (4.3 %) was observed at pH#3 (9.6±0.1). Similarly, pH#2 (8.6±0.1) produced more electricity and reduced pollutants, but pH#1 (6.5±0.1) never performed well in electricity generation. This study confirmed that exoelectrogens were less functional in an acidic environment (pH 6.5) and functioned better at higher substrate pHs (pH 8.6 and 9.6).

In terms of pollutant removal from the substrate, regardless of substrate pH, around 99% of COD, 99% of hardness, and 93% of iron were observed to be removed in this study. However, bacterial activity slowed down in an acidic environment and increased with increased substrate pH. As a result, the highest reductions in TOC (83%), TN (22%), and sulfate-sulfur (50%) were noted at pH#3 (9.5±0.1).

In conclusion, pH#3 (9.5±0.1) outperformed the other pH levels in terms of electricity generation and pollutant removal from the substrate. Electrochemically active Gammaproteobacteria, Deltaproteobacteria (especially *Geobacter* genus), Alphaproteobacteria and Bacteroidia; iron-reducing *Geobacter* and Gammaproteobacteria; and sulfate-sulfur reducing *Desulfovibrio* operated well at higher substrate pH (9.5±0.1) compared with lower pHs (6.5±0.1 and 8.6±0.1). Although no significant effect of substrate pH on COD, hardness, and iron reduction was observed, the effect of substrate pH on TOC, TN and sulfate-sulfur reduction was noticeable. Thus, substrate pH plays a significant role in microbial community composition, pollutant removal, and bioelectricity generation from wastewater.

AUTHOR CONTRIBUTIONS

Mosammat Mustari Khanaum: conceptualization, methodology, investigation, set up experimental design, data analysis, visualization, writing – original draft. Peter Bergholz: microbial analysis, writing and review – microbial section. Md. Saidul Borhan: set up experimental design, writing – review and editing. Shafiqur Rahman: writing – review and editing, supervision, funding acquisition.

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DECLARATION OF COMPETING INTEREST

None.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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