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C. saccharoperbutylacetonicum N1-4 ELECTROACTIVITY AND CO_2 FIXATION UNDER DIFFERENT ELECTROCHEMICAL CONDITIONS

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ABSTRACT

The capability of certain microbial strains to uptake electrons and fix CO_2 can be exploited to capture greenhouse gas and convert it into products of interest through a process called microbial bioelectrosynthesis. This study evaluated the capability of *C. saccharoperbutylacetonicum* N1-4 to utilize bicarbonate for growth, by both supplying electrons in a single-cell reactor and using a graphite-felt assembly as electrodes. The medium was supplemented with 4 g/L bicarbonate and 200 µM NADH. An open circuit experiment was carried out in a medium with bicarbonate and no complex nitrogen sources. The applied potential was -600 $mV_{Ag/AgCl}$. The impedance spectroscopy and cyclic voltammetry techniques were used to characterize and monitor the reduction and oxidation peaks. The conditions that promote the highest observed specific growth rate (0.87±0.18 h^{-1}), were -600 mV, 4 g/L HCO_3^- and NADH. The growth rates of 0.57±0.01 h^{-1} were observed at 4 g/L HCO_3^- without any potential input, and 0.51±0.10 h^{-1} at a potential of -600 mV in the presence of NADH. The results showed that an environment that provides exogenous electrons and an externally applied potential promotes the capability of *C. saccharoperbutylacetonicum* N1-4 to reduce CO_2, as evidenced when biomass concentration and specific growth rate were increased.

Keywords: Bioelectrosynthesis, *C. saccharoperbutylacetonicum*, bioelectrochemical Reduction, Carbon Dioxide, Biocathode, NADH.

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INTRODUCTION

The capability of certain strains to fix CO_2 can be used as a means to capture and convert this important greenhouse gas so that it can be used as a carbon source for fermentation processes. Approximately 95% of the feedstocks used in the chemical industry worldwide are based on non-renewable and unsustainable fossil fuels, and even the bio-based process generates 1 ton of CO_2 per ton of ethanol.[1] Recently, using CO_2 as a feedstock [2–5], the bioelectrosynthesis (electrofermentative process using CO_2 as a carbon source) has become a potential biotechnological route for the production of biofuels or organic compounds.[6,7] Bioelectrosynthesis (BES) technology is based on the delivery of electrons to the fermentation medium by an external source of electrons and DC potential. Its main objective is the utilization of compounds of a low degree of reduction as a carbon source to synthesize compounds of greater value. Both providing exogenous electrons to a fermentation culture and using specific microorganisms with concurrent CO_2 assimilation can improve the production of reduced organic metabolites. Therefore, if the extracellular oxidation-reduction potential (ORP) can be modified by supplying electrons or an electron carrier (NADH), a shift to electron flow and protons toward a reductive process will be achieved. The first reported microbial species, capable of converting CO_2 into acetic acid, was *C. aceticum*. Most recently, through a fermentation procedure, by supplying redox potential (electrons) in an electrochemical process with acetate synthesis, other species such as *Acetobacterium woodii*[8,9], *Sporomusa ovata*[10], *C. ljungdahii*[11-13], *Clostridium* spp [3], and *Clostridium acetobutylicum*[14-15] have shown the capability to reduce gaseous or aqueous CO_2. Although *C. saccharoperbutylacetonicum* N1-4 (Csb N1-4) is close to some of the anaerobic species mentioned above, this is an H_2 producer and a solventogenic Clostridium for Acetate-Butanol-Ethanol synthesis, which

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presents a metabolic profile that makes it a good candidate for bicarbonate reduction (HCO_3^-) (the aqueous form of CO_2). However, no reports are available about the capability for CO_2 fixation and solvent electrosynthesis. So this study aimed to evaluate the capability of Csb N1-4 to reduce bicarbonate for growth by supplying electrons and reducing equivalents in a single-cell reactor, assessing its electrochemical performance to follow the catalytic activity.

EXPERIMENTAL

Bacterial Cultivation and Inoculum

A strain of *C. saccharoperbutylacetonicum* N1-4 Biosafety Level 1 was used throughout this study. Csb N1-4 cells were grown anaerobically at 30 ºC with a syngas mixture (H_2, CO_2, N_2). The microbial strain was activated as follows: a cryovial stock (-80 ºC) was inoculated in a 10 mL RCM medium and incubated for 24 h. Likewise, a 30 mL fresh RCM medium was added to the culture and incubated for a further 24 h. Similarly, 10 mL of this culture was inoculated in a 35 mL TY medium. After 24 h, the culture was centrifuged at 3000 rpm for 5 min, and the resulting pellet was resuspended in 25 mL of fermentation medium. This suspension was transferred to the cathodic chamber of the BES. All procedures were carried out at 30ºC under anaerobic conditions.

C. saccharoperbutylacetonicum N1-4 Culture

The catholyte medium was composed as follows (g/L): Yeast Extract, 2; Tryptone, 6; $(NH_4)_2SO_4$, 2.57; $MgSO_4.7H_2O$, 0.3; KH_2PO_4, 0.5; $FeSO_4.7H_2O$, 0.01; 10 mL trace element solution (DSMZ 141) and 10 mL vitamin solution (DSMZ 141). The initial pH of the medium was adjusted to 6.5 with 1 M NaOH or 1 M HCl. In bioelectrosynthesis cultures, the medium was supplemented with sodium bicarbonate as a carbon source and NADH as required (Table-1.) An open circuit experiment was performed in a medium with HCO_3^- and no complex nitrogen sources (tryptone or yeast extract).

Table-1: Csb N1-4 Culture Medium Composition

Batch	CNS	HCO_3^- (4 g/L)	Potential (-600 $mV_{Ag/AgCl}$)	NADH (200 µM)
M1	+	+	+	+
M2	+	+	+	-
M3	+	+	-	+
M4	+	-	+	+
M5	+	-	+	-
M6	-	+	-	-

Csb N1-4 was grown for 48 h at 30ºC, and samples were taken between 24 and 48 h. The cells were harvested by centrifugation at 3000 rpm for 5 min and resuspended in 100 mL of fresh medium between each batch. The catholyte was frequently flushed with filter-sterilized CO_2 gas and continuously stirred at 150 rpm by using a magnetic stirrer.

Experimental Procedure

Electrofermentation Reactor Setup

The single-cell reactor was constructed by using a 100 mL glass bottle, with an anode and cathode made of carbon foil (5 cm x 5 cm), as shown in Fig.-1. Nickel wire was used to connect the carbon foil electrodes to the external power supply. The electrical performance of the single-cell reactor was characterized by Impedance Spectroscopy (IS) and by using a PalmSens 4.0, under the following conditions and parameters: Scan type; and fixed potential. Frequency type; scan. Minimum frequency 5.0 Hz and Maximum frequency 50 Hz; 100 data points.

Detection Method

Electrochemical Characterization of Microbial Cultures

The electrochemical analysis of the microbial culture was carried out with a potentiostat/Galvano stat (PalmSens 4.0) and controlled by the PStrace 5.7 software. BES culture samples were centrifuged (3000 rpm, 5 min, 4°C) and the supernatant (7 mL) was used for the electrochemical analysis. Cyclic voltammetry

(CV) was used as a three-electrode system: a platinum plate working electrode (WE), a nickel counter-electrode (CE), and an Ag/AgCl reference electrode (ER). Cyclic voltammograms were run in the range of potentials -0.7 mV to +0.7 mV vs $V_{Ag/AgCl}$ at a scan rate of 10 mV/s. The recorded scans were taken after 4 initial cycles and showed an average of three measurements with a maximum standard deviation of 2.5%. [16]

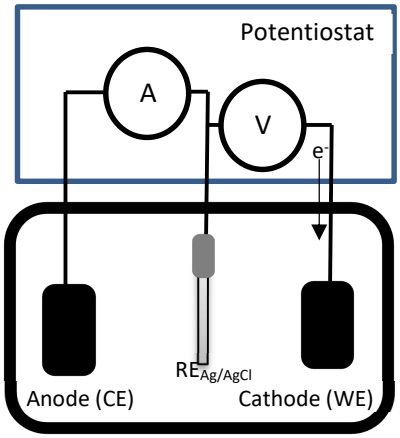

Fig.-1: Potentiostat Configuration in a Single-Cell Reactor

RESULTS AND DISCUSSION

The single-cell reactor impedance measurement and the CV performed to analyze the behavior of the systems at different HCO_3^- concentrations are shown in Fig.-2. The equivalent circuit model obtained by fitting the impedance data analysis used to describe the electrochemical properties of the reactor showed a charge transfer between electrodes with a resistance of 1360 Ω (Fig.-2A). The Levenberg-Marquardt algorithm (X^2 = 1.42x10-^{6}) was used to validate the fitted equivalent circuit model. The obtained Cyclic voltammograms (CVs) show that both the reduction and the oxidation currents decrease with decreasing HCO_3^- concentration (Fig.-2B). In terms of current peaks, the CV's show an anodic peak with a potential between E^o=50 mV and 219 mV (vs Ag/AgCl) at the four concentrations, and two cathodic peaks with potentials between E^o= -10 mV to -160 mV for the first peak, and E^o= -610 mV to -680 mV for the second peak, indicating electron transfer reactions.

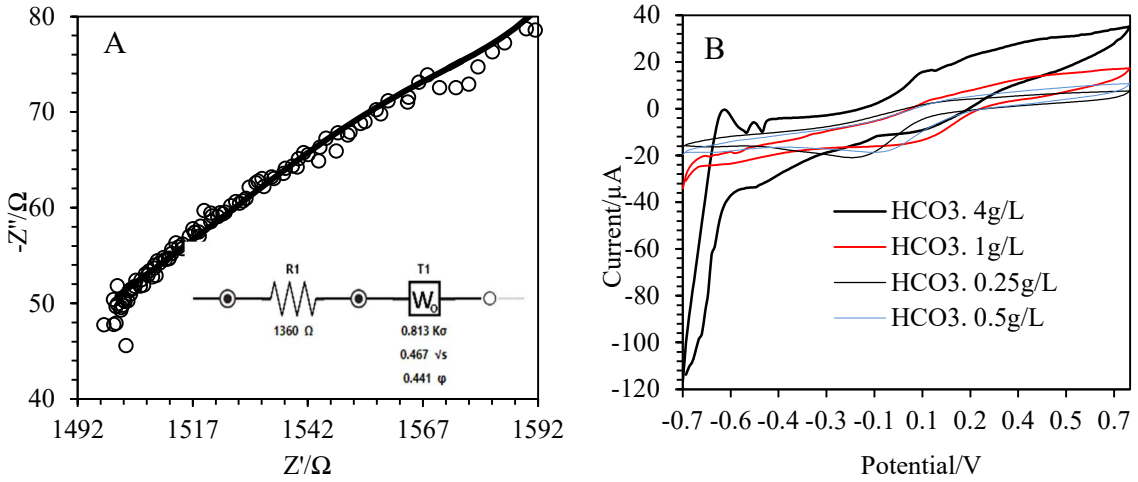

Fig.-2: Impedance Spectra and Cyclic Voltammetry. A. The Complex Plane Plot; B. Cyclic Voltammograms of HCO_3^- Solutions. Impedance Spectra, Shown as the Imaginary Part (-Z") Versus the Real Part of the Impedance (-Z') ● Measurement and ▬Fitting

The cyclic voltammograms were obtained under different BES medium conditions to study the Csb N1-4 reduction of HCO_3^-, (Fig.-3). HCO_3^- was one of the compounds added to the medium at a high concentration (4 g/L). The other was tryptone, added at 6 g/L. The CVs-HCO_3^- solutions were used as a qualitative

experiment based on the known capability of Csb N1-4 to use HCO_3^- as a carbon source, and to consume and synthesize molecules with redox properties that affect CV's behavior. The CV's-BES culture supernatant showed similar CV's-CO_2 reduction waveforms to those reported in electrocatalytic studies with formate dehydrogenase[17] and carbon monoxide dehydrogenases.[18] In those studies, the cathodic current decreased with CO_2 reduction. A similar behavior was observed with HCO_3^- reduction, which indicates that Csb N1-4 can metabolize the aqueous form of CO_2. All reduction and oxidation currents decreased until the exponential growth stopped (24 h), except in the medium with HCO_3^-, in which the reduction current was about half at 24 h and continued to decrease until 48 h Fig.-3. Although no growth was observed in the HCO_3^- culture, the pH and cathodic current increased and decreased respectively, which suggests that CHO_3^- is being reduced. In general, at 48h reduction currents did not show significant changes.

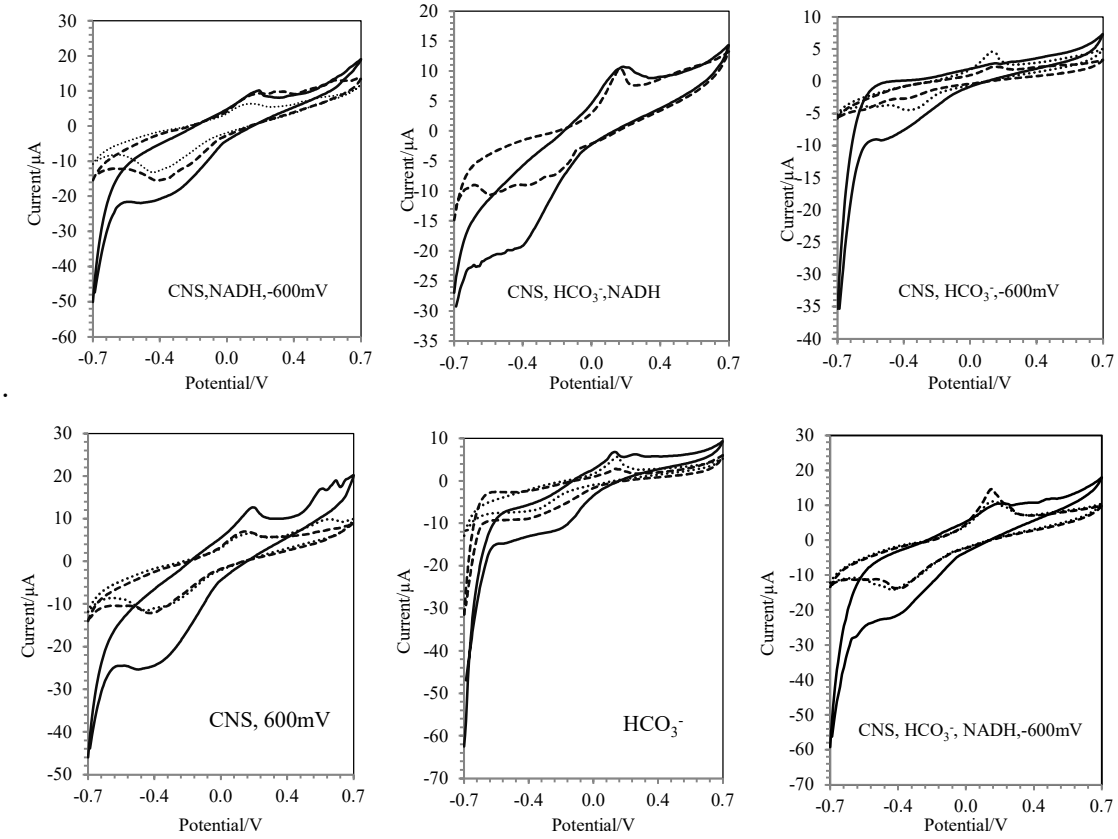

Fig.-3: Electrocatalytic Voltammograms at 0 (-), 24(---) and 48h (…) for Csb N1-4 in Different Media BES Cultures

Potential peaks of oxidation were observed, and it did not show important variations with a mean potential E^o = 130±20 mV (vs Ag/AgCl), nor did reduction peak with a first E^o=-370±90 mV and a second mean potential E^o = -560±50 mV. Similarly, the peak intensity (Ip) did not show important variations, except that Ipa and Ipc in BES cultures with HCO_3^- and NADH are higher than the peaks in other cultures, indicating a high electrocatalytic ability towards the oxidation of NADH at lower potential.[19] The relationship between Csb N1-4 growth and cathodic currents is shown in Fig.-4. The decrease in cathodic current is higher during the first 24 hours, which coincides with the exponential growth phase. The cathodic current reduction was also observed in BES cultures with HCO_3 medium where no growth was detected (Fig.-4A), which suggests that Csb N1-4 can reduce HCO_3^- although it requires a complex nitrogen source to grow. Growth was highest in the presence of complex nitrogen sources, HCO_3^-, and a potential of -600 $mV_{Ag/AgCl}$ (Fig.-4B), and lowest when Csb N1-4 was grown with CNS and the applied potential.

An increase in pH was recorded, even in BES cultures where no growth was observed (Fig.-5). pH increased in all cultures independently of exponential or stationary phase, except in a medium with CNS, HCO_3^- and NADH, where a maximum pH of 6.5 was reached at 24h (Fig.-5A). If the decrease in cathodic current was caused by HCO_3^- reduction, it is important to note that the decrease in the catalytic activity of Csb N1-4

implies that electrons and protons (H^+) are flowing across the inner cell membrane, resulting in an increase in external pH. The relationship between the potential and pH increase was positive. However, when NADH was added, a decrease in the pH was observed, which means that the addition of NADH has an effect on the pH of the culture. This effect decreases the pH, regardless of whether the potential is applied and/or bicarbonate is added.

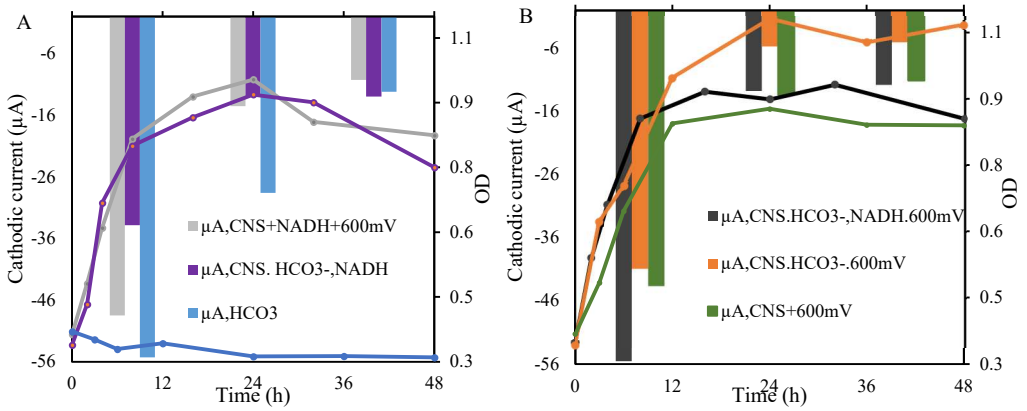

Fig.-4: Cathodes Current at 0, 24, and 48 h (bars) with BES Culture Growth (OD-lines) at a Different Composition Medium

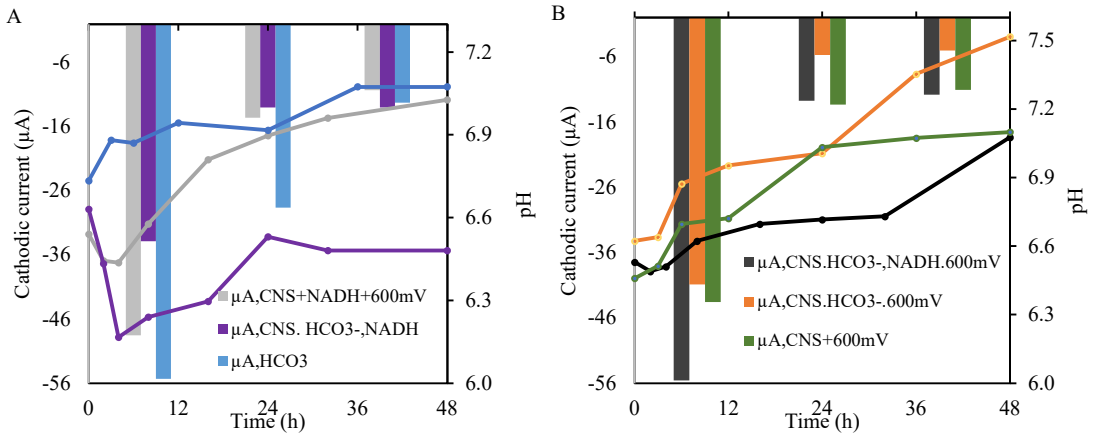

Fig.-5: Cathodes Current (bars) with BES Culture pH Evolution (lines) at Different Media Composition

When HCO_3^- was added as a carbon source, the cathodic current was higher than in the cultures where only potential was applied. Interestingly, the cathodic current reduction decreased when NADH was added without applying any potential (Table-2). The increase of the reducing equivalent could favor HCO_3^- reduction. Csb N1-4 shows metabolic activity even where no growth is observed, which means that the reduction occurred in all culture conditions. Although Csb N1-4 showed no growth in the absence of complex nitrogen sources, the HCO_3^- present in the medium is important for metabolic activities, as the changes in OD and pH were evidenced. Csb N1-4 grew (μ_{max} =0.27±0.4 h-1) in a culture with a CNS without HCO_3^-, NADH, and the applied potential.

Table-2: Variation of Csb N1-4 Growth and pH in Relation to Cathodic Current Decreased at 24 h

Cod	CNS	HCO_3^- (4g/L)	Potential (600mV)	NADH (200µM)	$OD_{(24h)}$	μ_{max} (h^{-1})	$pH_{(24h)}$	μA_{0h}-μA_{24h}
M1	+	+	+	+	0.90±0.01	0.87±0.2	6.7±0.03	-42.8
M2	+	+	+	-	1.08±0.04	0.36±0.03	6.8±0.09	-35.1
M3	+	+	-	+	0.92±0.01	0.60±0.1	6.5±0.03	-20.8
M4	+	-	+	+	0.95±0.01	0.51±0.1	6.9±0.02	-33.9

M5	+	-	+	-	0.87±0.04	0.37±0.1	7.1±0.15	-30.2
M6	-	+	-	-	No grew	-	6.9±0.03	-26.7

$OD_{average,\ 0h}$: 0.35±0.01; $pH_{average,\ 0h}$: 6.58±0

Under biological conditions, the redox reaction of H_2 and NADH occurs between -420 mV and -320 mV, respectively. The Pourbaix diagram shows the region in which H_2-mediated reduction of CHO_3^- is favorable.[4,17] The conditions of pH>6.5 and redox potential <-400 mV were observed in the cultures. The pH_{24h} and potential conditions were superimposed on the Pourbaix diagram (Fig.-6), showing which Csb N1-4 culture with imposed potential would favor HCO_3^- reduction since it shifts to pH conditions in the range below H_2 evolution, where it could mediate the bioelectrochemical reduction of HCO_3^-. In the Csb N1-4 cultures, in which the potential was applied, the cathodic current decrease was higher than in the conditions where the potential was not applied.

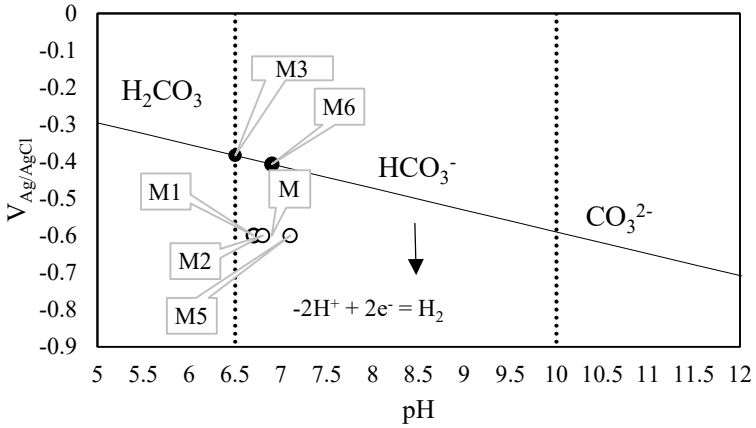

Fig.-6: pH-Dependent Reduction Potential HCO_3^- in Csb N1-4 BES Cultures. $pH_{average,\ 0h}$: 6.58±0.09. Relationship between pH and Electropotential $E_{H^+/H_2} = E^0{}_{H^+/H_2} - 0.059pH$

CONCLUSION

C. *saccharoperbutylacetonicum* N1-4 is able to reduce bicarbonate for growth in a single-cell reactor where the potential and reducing equivalents were applied. The electrocatalytic activity of the strain during its growth was confirmed by electrochemical measurements. All in all, this study shows that Csb N1-4 culture with imposed potential can increase the cathodic current reduction even in cultures where no growth was observed, creating pH conditions that favor bioelectrochemical HCO_3^- reduction.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONs

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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