Biochar based cathode enriched with hydroxyapatite and Cu nanoparticles boosting electromethanogenesis

G. Ghiara[a], S. Campisi[b], A. Goglio[c], F. Formicola[d], M. Balordi[e], A. Gervasini[b], S.P.M. Trasatti[a], F. Adani[c], A. Franzetti[d], P. Cristiani[e,*]

[a]Department of Environmental Sciences and Policy (ESP), Università degli Studi di Milano, Via C. Golgi 19, 20133, Milano, Italy

[b] Department of Chemistry, Università degli Studi di Milano, Via C. Golgi 19, 20133, Milano, Italy

[c] Department of Agriculture and Environmental Sciences (DiSAA), Università degli Studi di Milano, Via Celoria 2, Milano, Italy

[d] Department of Earth and Environmental Sciences (DISaT), University of Milan Bicocca, Piazza della Scienza 1, 20126, Milano, Italy

[e]RSE-Ricerca sul Sistema Energetico S.p.A., Via Rubattino 54, 20134 Milano, Italy

[*]corresponding author: pierangela.cristiani@rse-web.it

Abstract

Electromethanogenesis is an innovative technology addressing the need of storing renewable energy from unprogrammable sources. It allows for the electrochemical production of methane from CO2-rich wastes on microbial cathodes, in a logic of power-to-gas (BEP2G). The challenge of cost-effective and sustainable biocathodes enhancing the microorganism performance and yield of electromethanogenesis is approached in this work. For the first time, porous carbonaceous cathodes were functionalized with Cu nanoparticles and hydroxyapatite (HAP) and successfully experimented for supporting microbial CO2 reduction reaction (CO2RR) to methane. Tests were performed in a double chamber system under CO2 flow at 45 °C. Next Generation Sequencing of 16S RNA indicated that the microbial pool on the cathodes was mostly enriched in Metanobacteriaceae (hydrogenotrophic Archaea) and different fermenting bacteria, depending on the cathode type. High methane production on cathodes made of Cu 20%, HAP 10%, and carbon balance (20Cu/10HAP) was achieved, with a maximum of 866±199 mmol□m-2□d-1 (projected cathode area, Coulombic efficiency of 64%), corresponding to values comparable to the maximum in literature, but in shorter timespans (8 vs 30 days). The documented effect of pH stabilization in the cathodic chamber by HAP was one of the main parameters that concurred to the selectivity of CO2RR towards methane.

Keywords: Electromethanogenesis, HAP, CO_2RR, Bioelectrolyses, BES, Hydrogenotrophic Archaea.

1. INTRODUCTION

Electromethanogenesis [1, 2], consisting of the reduction of CO_2 in the form of methane (CH_4) *via* microbial electrolysis [3-4], is a valuable alternative to both biochemical methanogenesis and thermochemical methanation from the Sabatier process [5-6]. Among the innovative technologies for energy conversion, the peculiarity of this bioelectrochemical process is to address the need of storing the energy produced from unprogrammable sources in renewable fuel (hydrogen or methane), in a power-to-gas concept (BEP2G) [1, 5-9]. Although such processes address marginal energy resources, their study finds valuable meaning in promoting a green circular economy by producing new biomethane or upgrading biomethane and CO_2 from anaerobic digestion (AD) in the already operating biogas plants [7]. In Italy, about 1600 biogas plants were operative in 2019, making this country the 2nd country for biogas production in the EU and the 4th worldwide [10]. The potential of biomethane production has been estimated at 10 billion cubic meters by 2030, which means that about 8 billion cubic meters of CO_2 will be produced by 2030, i.e. about $15 \cdot 10^6$ tons of CO_2, assuming a biogas composition, as average, of 45 % CO_2 (v/v) and 55 % CH_4 [11]. CO_2 produced by biogas/biomethane plants does not contribute to greenhouse gases (GHG) because it comes from renewable biomass and therefore, its recovery as feedstock can reduce GHG emission balancing fossil-origin emitted CO_2 (C trading). For Italy, it is possible to calculate a theoretical recovery of $15 \cdot 10^6$ tons of CO_2 from biogas by 2030, i.e. 4 % of the total emitted CO_2 (348.4 Mtons, [10, 11]).

The mechanisms of CH_4 biochemical production follow specific metabolic pathways ascribed to selected species of microorganisms [12-16]. From a thermodynamic point of view, the reaction of CH_4 production is driven by the type of substrate and the energy source according to their values of Gibbs free energy at standard conditions (25 ° C and pH 7), as displayed by the following reactions:

$$4H_2 + HCO_3^- + H^+ \rightleftarrows CH_4 + 3H_2O \qquad (\Delta G° = -136\ kJ \cdot mol^{-1}) \qquad \text{equation 1}$$

$$CH_3OH + H_2 \rightleftarrows CH_4 + H_2O \qquad (\Delta G° = -113\ kJ \cdot mol^{-1}) \qquad \text{equation 2}$$

$$CH_3COO^- + H_2O \rightleftarrows CH_4 + HCO_3 \qquad (\Delta G° = -31\ kJ \cdot mol^{-1}) \qquad \text{equation 3}$$

In brief, it is possible to discern two main pathways for methanogenesis: i) hydrogenotrophic one, which involves the reduction of carbonate ions (equation 1), the most metabolically efficient process for energy generation [17, 18]; ii) acetoclastic pathway, which consumes acetate ions or methanol, (equations 2 and 3).

Both these pathways (more relevantly the acetoclastic one) occur during the anaerobic digestion (AD) in the biogas process producing methane. In the BEP2G process, the sole hydrogenotrophic microorganisms of the Archaea domain - the same as in the biogas plants - produce methane on a polarized electrode around -1V *vs* the Standard Hydrogen Electrode *(SHE)* [12] (equation 4):

$$CO_2 + 8H^+ + 8e^- \rightleftarrows CH_4 + 2H_2O \qquad \text{equation 4}$$

CH_4 forms, catalysed by microorganisms, directly on the biocathode or indirectly after the hydrogen evolution reaction [1]. Methanogenesis can be catalysed using only CO_2 and bicarbonate that can be coupled to both the oxidation of water and the abiotic, or microbial, oxidation of organic molecules [19-20]. In both cases, the end products of metabolic pathways are methane and water.

Combining microbial electromethanogenesis with conventional AD, the upgrading of biomethane produced in biogas plants (rich in CO_2 up to 45%) can be achieved [7]. Several other CO_2-rich industrial wastes would reach a technological upgrade towards a more circular economy by electromethanogenesis. However, important challenges concerning methane yield from this technology, still approached at the laboratory level only, are persisting [20-23]. Previous studies are mainly focused on the optimization of the chamber set-up (i.e., single, or double chamber) [2], applied cathode potential, choice of microorganisms, cathode and/or anode feeding, pH, and other process parameters [24-26]. To scale up BEP2G energy storage system to a possible economically and industrially competitive level, the study of performing materials for the cathode is generally agreed upon as mandatory. Electrodes must give large surfaces to maximize the interaction with microorganisms, minimizing charge transfer resistance and diffusive mass transport [26]. One of the main challenges for increasing the performance of electromethanogenesis, compared to the chemical electrolysis of water, consists of manufacturing electrodes with a geometric surface equal to at least one order of magnitude greater than the volume of the bioreactor (ratio of 10 $m^2 \cdot m^{-3}$) [27]. This indication is useful to counterbalance the low current density sustained by microorganisms. Metallic materials that can chemically support microorganisms for reaching a suitable performance of the electrodes in bioelectrochemical systems (BES) are very expensive (e.g. Pt group) or tend to suffer from microbial corrosion. Sometimes this last phenomenon can greatly invalidate the data of methane electrochemical production, as reported by literature [28]. The choice of a more cost-effective material drove recent research toward carbon of biological origin (biochar) [28-30], which assures high porosity, good conductivity, high biocompatibility, acceptable mechanical strength, and resilience. Biochar is generally characterized by a high specific surface (reaching 100 $m^2 \cdot m^{-3}$) with a porosity and pore size distribution that makes its surface entirely available for extracellular electron transfer by microbes [31, 32]. The performance of biochar can, therefore, in principle, approach that of an ideal electrode to produce CH_4, as confirmed by recent studies [33, 34].

To further enhance the chemical reduction reaction of CO_2 (CO_2RR), enriched layers of metallic catalysts can be applied on the surface of the cathode [35-37]. Among the active phases, Cu presented encouraging results (around 40%) towards CO_2RR at applied potentials of -1.2 V vs RHE [38] and Cu-based nanostructures have also been shown to generate CH_4, with different faradaic efficiencies

depending on the shape, size, and supports of copper nanoparticles (CuNPs) [39, 40]. However, studies indicate a low selectivity of Cu towards the CH_4 formation. Therefore, materials alternative to metals are currently explored [41, 42]. Relevantly, it was recently demonstrated that phosphates in the nanocrystalline form of hydroxyapatites (HAP) can show good absorption toward CO_2 [43, 44]. HAPs are green materials widely distributed in nature and the principal component of the bones [43,44]. In a nanocrystalline structure, they are characterized by acidic and basic sites that can catalytically trigger the CH_4 production [45, 46]. Particularly, the combined presence of CuNPs and HAP has a synergistic effect on the CO_2RR to CH_4 as CuNPs allows the direct reduction of CO_2 (transfers > 2 e-) to a wide range of so-called higher-order products (as CH_4, C-2 or C-3) [47].

In this work, for the first time to our knowledge, an innovative biochar-based material functionalized with CuNPs and HAP is studied and tested for enhancing the biocathode performance in electromethanogenesis. The addition of hydroxyapatite is supposed to introduce additional basic sites complementary to those exhibited by the biochar [43]. Furthermore, the presence of acidic sites is supposed to stabilize pH. Low-cost solid electrodes of biochar (biogenic carbon) were produced from the pyrolysis of maize stalk and then enriched with different wt. percentages of Cu nanoparticles and HAP (5Cu/5HAP and 10Cu/20HAP). Samples with only Cu and HAP were also produced and tested as control. The preparation of the electrode materials requested an innovative multi-step protocol here described in detail. The performance of CuNPs/HAP functionalized biochar is also compared with carbon cloth, as the best performance in terms of methane production from literature has been reported with this carbon material [19].

MATERIALS AND METHODS

Six different carbon-based cathodes, described and named as in Table 1, were experimented with double-chamber electrochemical cells.

Commercial CuNPs (SigmaAldrich, CAS 7440508, ø = 25 nm) and HAP produced at the University of Milan, according to a procedure described elsewhere [48], were used as additives of carbon powders achieved by pyrolysis of plant residues.

For carbon cloth, a commercial product (*SAATI*, Legnano, Italy) was used. The other carbonaceous materials were prepared in the lab, following a protocol consisting of three steps (Fig. S1, Supplementary material).

First step: preparation of the biochar. Biochar was produced from the pyrolysis of maize stalk. To obtain an electrically conductive material, the canes were positioned in a quartz tube inside a horizontal furnace (Carbolite) and pyrolyzed at controlled temperature. The pyrolysis procedure of the material was carried out according to the following protocol: two h at 25 °C, slow heating (5

°C·min^{-1}) up to 900 °C, one h residence time at 900 °C and cooling down to 25 °C. During the treatment, N_2 flowed constantly at 1 NL·h^{-1}. This type of procedure allows fast heating and maximum treatment efficiency, without any heat loss [30].

Second step: preparation of the functionalized powder. Four biochar based powders of different compositions were synthesized and labelled according to the weight percentage of each component of the carbon powder as described in Table 1.

Firstly, biochar was pulverized using an agate mortar. Then, the powder was mixed in a solution containing different concentrations of copper nanoparticles. To obtain the 20Cu powder approximately 85 of biochar was dispersed in ca. 40 ml of isopropyl alcohol (IPA). The pH of the suspension was adjusted to 10 by adding an aqueous solution of 0.1 M KOH. Approx. 25 of commercial CuNPs were dispersed in ca. 40 mL of IPA, adjusting the pH as indicated for the biochar suspension. Both mixtures were placed in ultrasounds for 30 minutes to obtain optimal suspensions. Then, the CuNPs suspensions was added one shot to the biochar suspension. The resulting mixture was stirred at room temperature for 48 h (Fig. S1, Supplementary material). After the immobilization step, the powder was vacuum filtered on a 0.45 m Nylon membrane, washed with MilliQ water and dried at 110 ° C for 45 minutes.

The same procedure was used to obtain the powder 5Cu, weighing around 95 mg of biochar and 7 mg of CuNPs, respectively.

To obtain the powder 10HAP, nanocrystalline hydroxyapatite was added by co-precipitation to the biochar powder. Approximately 90 mg of biochar were suspended in ca. 90 ml of MilliQ water. The pH of the suspension was brought to 7 by adding a 0.1 M aqueous solution of KOH. Similarly, ca. 15 mg of HAP were dispersed in ca. 20 mL of MilliQ water, adjusting the pH of the suspension to 7 by adding a 0.1 M KOH solution. Both mixtures were sonicated for 45 minutes. Then, the HAP suspension was added one shot to the biochar suspension. the resulting mixture was stirred for 48 h at room temperature. Finally, the powder was vacuum filtered on a 0.45m Nylon membrane, washed with MilliQ water, and dried at 110 ° C for three hours (Fig. S1, Supplementary material).

To obtain the composite 20Cu/10HAP the already mentioned procedure for HAP was applied, replacing the undoped biochar with the already synthesized composite 20Cu. Analogously, to produce the composite 5Cu/5/HAP, 5Cu was used, weighing around 100 mg of 5Cu powder and 7 mg of HAP.

Third step: application of the powder to the electrode. The final step involved enriching the biochar support with powder functionalized with the four composites obtained. The resulting tested composite material were labelled according to the percentages of the synthesized powder (Table 1). Similar to the composite powder alone, the percentage value of copper and hydroxyapatite both express the gravimetric ratios vs. biochar. For the application of the powder on the carbonaceous material, a dip

coating procedure was carried out. It consisted in immersing the previously pyrolyzed biochar in an aqueous solution (100 mL for 100 mg of powder) of the four composites (10HAP, 20Cu, 5Cu/5HAP 20Cu/10HAP obtained by co-precipitation as previously described. Solutions were first sonicated for ten minutes to achieve good mixing and the biochar was immersed in the solution for ten minutes (Fig. S1, Supplementary material). The electrode thus obtained was dried at 110 ° C for three h.

2.2 Experimental setup

Two apparatus of six double-chamber BESs were operated in parallel. A schematic of the electrochemical cell is reported in Figure 1a. Images of operating cells are shown in Figure 1b and 1c. Each apparatus was equipped with: i) a thermostatic tank containing BESs; ii) a controlled CO_2 flow system; iii) a current generator, equipped with six independent channels each one connected to the anode and cathode of each BES, designed by AMEL s.r.l. (Milano, Italy).

A reference electrode 3M Ag/AgCl was inserted in each cathodic compartment. A polarization between the anode and cathode was imposed in order to achieve -1.2 V between the cathode and reference (approximately -1 V vs SHE) of each BES.

The current circulating between the anode and the cathode was estimated by measuring voltage across an electrical resistance of 10 Ω connected in series with the anode (Fig. 1a) [49]. The recording of voltages was performed with an analog to digital acquisition board (Agilent 4930 A data-logger). The duration of the experiments was 200 h (8 days) and was replicated twice. For each material, also an abiotic condition was tested.

The double-chamber BESs were made of borosilicate (Pyrex) bottles and were operated at 45 ± 1° C. The anodic and cathodic chambers were separated by a proton exchange membrane (PEM NAFION 417) with a working surface of 7.0 cm^2. Each chamber was filled with about 0. 200 L of solution and was hermetically sealed with a rubber stopper, that hosted the gas dosing and vent pipes, the reference electrodes, the electrode current collectors, and the steel needles for gas sampling (Fig. 1a).

The anode was a Ti-mesh of a suitable area of 36 cm^2 connected to a Ti current collector (ø = 1 mm). The cathodes, made with one of the six different materials under investigation (Table 1), were fixed with an electrolytic copper wire (99.9%) acting as a current collector.

The anolyte was a 0.2 M solution of Na_2SO_4. The catholyte solution consisted of the inoculum retrieved from a biogas plant and stored at the University of Milan, Bicocca. The inoculum was supplied with 2.54 g $\cdot L^{-1}$ KH_2PO_4, 11.7 g$\cdot L^{-1}$ $Na_2HPO_4 \cdot 12H_2O$, 0.53 g$\cdot L^{-1}$ NH_4Cl, 0.1 g$\cdot L^{-1}$ Na_2SO_4, 5.0 g$\cdot L^{-1}$ $NaHCO_3$, and vitamins.

CO_2 was fluxed at each cathode through sparging at the beginning of the experiment for 40 minutes at a flux of 0.5 $NL \cdot h^{-1}$ (total of 14.8 mmol dosed). The apparatus also automated the CO_2 dosage and the sampling of the gases produced.

2.3 Material characterization techniques

To verify the morphology and the pore size of the pyrolyzed biochar and to characterize the biochar powder (and the final functionalized biochar electrode) different analytical methods were performed. A Scanning Electron Microscopy analysis (SEM) coupled with Energy dispersive X-Ray measurements (EDS) was carried out at the Department of Earth Sciences of the University of Milan using a SEM-EDS JSM-IT500 LV (JEOL S.p.a.). The analyses were conducted using a secondary electrons (SE) detector at different magnifications and with an accelerating voltage of 20 KeV and a WD of 10.5 mm. Image analysis was executed using the Software Fiji-ImageJ, version 1.49b [50] to estimate the volume percentage of the composite on the surface of the biochar.

X-Ray powder diffraction (XRPD) patterns have been collected using a Philips PW3020/PW1830 powder diffractometer operating with an X-ray source at 40 kV and 40 mA in the range between 5 °-60 °(2θ), step of 0.02 °2θ and scan rate of $0.6° \cdot min^{-1}$.

Electrochemical analyses were performed to determine the electrochemically active surface area (ECSA) of each material. To this purpose, electrically conductive disks of 1 cm^2 were prepared and immersed in an aerated solution of 0.1M KNO_3. A typical three electrode cell was employed with the cathode as the Working Electrode (WE) a 3M Ag/AgCl as a Reference Electrode (RE) and a platinum wire as a Counter Electrode (CE). Cyclic voltammograms (CV) were recorded at different scan rates (from 10 to 200 mV/s) with an Ivium Compactstat (Ivium Technologies, Netherlands). The scanned range was ± 25mV vs OCP and the double layer capacitance, C_{dl}, proportional to the ECSA, was determined. All CVs were conducted in duplicate.

2.4 Chemical analyses

The composition of the produced biogas (CH_4, H_2, and CO_2) was analysed using a gas chromatograph (Agilent 3000A micro-GC) with the software "SOPRANE". The sample for the GC was taken from the headspace of the cathode chamber with gas bags of 5 L connected to the gas outlet. Gas samplings were carried out at the end of the test. The amount of CH_4 produced was analysed as a concentration of gas sampled in a 5L gas bag and the methane value was normalized to the cathode projected area. Furthermore, the pH was monitored on a three-day basis using three narrow range pH indicator papers.

The Volatile Fatty Acids (VFAs) present in the initial inoculum solution and produced at end of the test were analysed by Ionic Chromatography (IC, Dionex 300), equipped with a S9-SC column and chemical suppressor AMMSIC. The sample intended for the analysis through IC (10 mL) was withdrawn using a sterile syringe and stored at 2°C before the measurement.

At the end of the test microbial communities grown on the surface of the electrodes were characterized through visual inspection and microscopy observation by scanning electron microscopy equipped with X-Ray detector (SEM-EDS).

2.5 Microbiological sampling and analysis

Small size pieces of the cathodes and 3 mL of cathodic media were sampled from each bioelectrochemical system before and after the test. Samples were stored at -26°C before analyses.

The sequencing of V5-V6 16S RNA genes of Bacteria and Achaea were carried out on the initial inoculum and on the sampled operated cathodes using MiSeq Illumina sequencing, to obtain the taxonomic characterization of the microbial communities. Total bacterial DNA was extracted using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) and the V5-V6 hypervariable regions of the 16S rRNA gene was amplified through a PCR with initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 50 s, 47°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min. The reaction was performed in 2·50 µL volume reactions with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 µM of each primer (783F: 5'-CAGGATTAGATACCC-3', 1027R: 5'-CGACRRCCATGCANCACCT-3'). Both primers included barcodes and sequencing adapter fused to the 5' end. The resulting amplicons were purified form 1% agarose gel using Wizard SV Gel and PCR Clean-up System kit (Promega Corporation, Madison,WI, USA). Resulting DNA was quantified using Qubit® (Life Technologies, Carlsbad, CA, USA). The obtained sequences were taxonomically classified by RDP classifier (confidence >80%).

3 RESULTS

3.1 Cathodes characterization

Pyrolyzed biochar

Firstly, maize stalk samples were weighted before and after pyrolysis to verify the success of the procedure. Fig. 2 shows representative pictures of a sample before and after the treatment.

The material after pyrolysis maintained the same mechanical structure and highlighted an increasing number of pores on the surface. A weight loss corresponding to approximately 75% of the initial weight was measured for all samples, consistent with the decomposition of the material in the absence of oxygen. During this process, the lignocellulosic components such as cellulose ($C_6H_{10}O_5$) n-

hemicellulose and lignin undergo depolymerization reactions, fragmentation, and cross-linking [51]. This treatment was carried out to increase the porosity of the material and the surface area by several orders of magnitude [51]. Porosity is formed because of the loss of water during the treatment. Generally, three types of pores can be distinguished according to the final application of the biochar: i) micropores (<2nm); ii) mesopores (2-50 nm); ii) macropores (> 50 nm). As displayed in Fig. 2, the material is mainly composed of macropores, with average dimensions around 50 ± 10 μm. After the pyrolysis treatment, the presence of macropores can enhance i) the diffusion of gases (CO_2 and H_2) inside the material, also allowing for a good wettability by the H_2O; ii) the settlement of microbes that catalyses the direct reduction reaction of CO_2 into CH_4 by extracellular electron transfer.

Functionalized biochar powder

Functionalized biochar powder samples (20Cu, 10HAP, 5Cu/5HAP and 20/10HAP) were qualitatively characterized by SEM-EDS. Fig. 3 shows two images of the two Cu/HAP powders at 2000x with relative EDS spectra.

From a first observation, it is possible to highlight the presence of micrometric crystals that are distributed on the surface of the biochar (large flakes). They arrange as agglomerates of different type and size and tend to grow around a nucleation centre. These structures bear the typical morphology of HAP adsorbed on the surface of a substrate [52]. Compositional spectra were carried out in some of these areas: spectra 1 and 2 of Fig. 3 indicate a high percentage of C, attributed to the biochar, and the signals of Cu, Ca, O, and P, confirming the presence of elements characteristics of HAP. It was not possible to quantify the overall percentage of the single components due to limits connected to the massive presence of C as substrate (as indicated by the different intensities of the elements in the analysed points). The elemental mapping (Fig. S2, Supplementary information) also shows a good and homogeneous distribution of the elements in the sample with the higher percentages of Cu and HAP (20/10). The composite with a lower presence of the active phase and the dopant (5Cu/5HAP) did not show a total overlap of the signals for the elements Cu, Ca, P and O.

Further investigations via XRD were carried out to qualitatively assess the phase composition of materials. The XRPD patterns of 5Cu/5HAP and 20Cu/10HAP (Fig. S3 of Supplementary material) were dominated by a broad peak at 2θ of 20˚–30˚ which can be ascribed to the (002) plane of aromatic layers that indicate the presence of small graphitic domains in the biochar structure [53]. In addition, typical reflections of bulk Cu (JCPDS 01-070-3038) at 2θ of 43.2° (111) and 50.0° (200) confirmed the presence of copper nanoparticle in the metal form, whereas the peak at 36.5° may be associated to the (111) plane of cubic Cu_2O (JCPDS 00-065–3288), thus suggesting a partial oxidation of Cu. On the other hand, the successful incorporation of hydroxyapatite in the composites was confirmed by the emergence of the most intense reflections of crystalline hydroxyapatite (26° and 32° in

agreement JCPDS 00-064-0738). The relative intensities of the graphite, Cu and HAP peaks for each XRD pattern also confirm a different percentage of the agents in the composite.

Composite electrode

Once the dip coating procedure was terminated, the final electrode was characterized through SEM-EDS. Fig. S4, Supplementary information displays pictures of the Cu/HAP functionalized cathodes (b, c, e and f) compared to the undoped biochar (a, d).

Analyses show a good dispersion of the functionalized biochar powders on the electrode surface and particularly both on the pores and on the cell walls. Consistent with the previous observations, a substrate of organic material (biochar), is found below the aggregates of Cu/HAP crystals, visible as white spots (red arrows) in Fig. S4, Supplementary information. The elemental maps (Fig. 4) show a good dispersion of the Cu/HAP composite on the electrode surface even if in much lower percentages than that obtained on the powder. By calculating the total volume percentage of functionalized powder *via* image analysis, it was possible to infer that it constituted less than the 10 % of the total surface for both the composite electrodes.

3.2 Electrochemical behaviour of the electrodes

The electrochemically active surface (ECSA) of the composite electrodes was determined in the range ± 25 mV vs OCP hypothesizing that no faradaic reaction (exchange of electrons) occurs (see Figure S5, supplementary information for the CV curves). This technique is based on the double layer delays in charging and discharging at the interface electrode/solution according to the potential scan-rate [54]. The measured parameter is defined as double layer capacitance (C_{dl}):

$$C_{dl} = \varepsilon A/d \qquad \text{equation 5}$$

where ε is the absolute permittivity of the dielectric material used, A is the area of the electrode and d is the distance between the plates in a pure capacitor (here the interface electrode/electrolyte) [55]. Assuming ε and d as constants, the C_{dl} is only affected by the area of the electrode (the larger the electrode, the higher the double layer capacitance). This parameter (C_{dl}) can be thus calculated applying different potential scan-rates (v) and measuring the resulting current output, defined as double layer charging current (i_c) (equation 6):

$$i_c = v \cdot C_{dl} \qquad \text{equation 6}$$

By the slope of the scan rate (v_s) *vs* the capacitive current (i_c) graph, the C_{dl} was obtained and ECSA was calculated from the following equation:

$$ECSA = C_{dl}/C_s \qquad \text{equation 7}$$

where C_s is the specific capacitance of the material. To our knowledge, the C_s of this specific biochar is still unknown, so a plausible value of 7.5 μF cm^{-2} was selected, based on studies on other carbon-based materials [56-59]. The same value of C_s was considered for all cases (Biochar, 20Cu, 10HAP,

5Cu/5HAP, 20Cu/10HAP) giving the low quantities of dopants in the final electrode (<10% volume percentage from the SEM). The results from the ECSA analyses indicated different electrochemically active surfaces according to the type of electrode considered, as visible from Table 2.

The pure biochar exhibits the highest ECSA and values up to two orders of magnitude lower are measured for the other composites. It appears that the active phase Cu, if present in high percentages in the synthesized powder (20 wt. %), greatly decreases the electrochemically active surface (10^{-2} cm^{-2} for biochar compared to 10^{-4} cm^{-2} for 20Cu). On the other hand, the presence of the sole dopant HAP mildly lowers the active surface of the electrode (one order of magnitude). In the composites with both Cu and HAP, the role of hydroxyapatite on the electrochemically active surface is less obvious and a synergistic effect of both Cu and HAP must be considered.

3.3 Bioelectrochemical tests

Methane production

The production of methane was affected by the nature and functionalization of the cathode. Fig. 5 reports the results of the GC analyses after 200 h, displaying the moles of CH_4, H_2 and CO_2 found in the gasbags, normalized per L and for the projected surface area of the cathode. CC and undoped biochar show a very different production of CH_4, observing a consistent increase for the latter (3 and 13 $\mu mol \cdot cm^{-2} \cdot L^{-1}$, respectively). Cu in the biochar (composite 20Cu) lowers the average production of CH_4, which results comparable to the CC. The presence of HAP leads to a 3-fold-increase compared to pure biochar (and 10 times *vs* CC). The combined effect of Cu and the HAP was evidenced in the cathodes functionalized with the highest percentages(20Cu/10HAP), whose methane production reached 70 $\mu mol \cdot cm^{-2} \cdot L^{-1}$. On the other hand, the 5Cu/5HAP functionalization was ineffective.

The CO_2 and H_2 moles were also measured to understand the chemical pathways of CO_2RR. Medium to high levels of CO_2 was found in all systems (ranging from 92 to 741 $\mu mol \cdot cm^{-2} \cdot L^{-1}$), except for the undoped biochar suggesting from a minimal to a possible total consumption of the reagent (small leaks from the BESs must not be excluded). Also, the consumption of the CO_2 could have occurred due to the precipitation of carbonates (pH > 10), shifting chemical equilibria towards the products of the reaction bicarbonate-carbonate. Likewise, H_2 present in the gasbags is variable according to the case (2 to 31 $\mu mol \cdot cm^{-2} \cdot L^{-1}$), except for the CC that shows no H_2. Its absence can be due to the occurrence of parasitic reactions taking place at the electrode interface, leading to the production of C-2 or C-3 compounds [60-63]. As for the CO_2 levels, possible minor leaks in the BESs must be considered

Coulombic Efficiency (CE) of methane

The production of CH_4 by hydrogenotrophic Archaea in the BESs is connected to the electrochemically formed H_2. The potential set at -1.2 V vs Ag/AgCl is thermodynamically ideal to produce H_2 according to many works that use different cathodes that enhance the selectivity towards the reaction [64-66]. As can be seen from Fig. S6 of Supplementary information, the measured current densities ($mA \cdot cm^{-2}$) are affected by the nature of the cathode. The 200 h trend (8 days) considers the average of two replicas and a standard deviation below 5% (error bars not visible in the graph). The maximum current densities were detected for the composite 20Cu and the undoped biochar (7.8 ± 3.1 $mA \cdot cm^{-2}$ and 7.4 ± 0.2 $mA \cdot cm^{-2}$, respectively) followed by 10HAP and CC (3.9 ± 0.1 $mA \cdot cm^{-2}$ and 2.6 ± 0.1 $mA \cdot cm^{-2}$, respectively), and equally by the 5Cu/5HAP and 10Cu/20HAP (1.2 ± 0.1 $mA \cdot cm^{-2}$). With the same applied potential, the presence of HAP increases the resistance of the material, lowering the current density of a 2 to a 7-fold-factor.

From the current densities values, the total number of moles of H_2 that could be electrochemically produced by the system was calculated following equation 8:

$$2H^+ + 2e^- \rightleftarrows H_2 \qquad \text{(cathode)} \qquad \text{equation 8}$$

Table 3 summarizes the results obtained in μmoles of H_2 *per* day and cm^2 of projected surface area, based on the assumption that the charge supplied was exclusively used for the H_2 evolution reaction. Assuming that for each mole of CH_4 four moles of H_2 are needed, the electrochemically produced moles of CH_4 were also calculated (equation 9):

$$4H_2 + H_2O + CO_2 \rightleftarrows CH_4 + 3H_2O \qquad \text{equation 9}$$

The ideal CH_4 μmoles average from 130 to 850 which is in line with recent literature work on bioelectrochemical systems [28, 67] using specific materials as cathodes to produce methane. Results of this experimentation were compared (mmol m^2 d^{-1}) to the highest CH_4 production available in literature using CC as a cathode [19] and to the general maximum production of methane in a double chamber BES [68]. Table 4 displays the average production of CH_4 for each system, the maximum theoretical CH_4 moles calculated from the electrochemical process, and the Coulombic efficiency (CE). The CE was calculated according to the following equation:

$$CE = \frac{8Fn_{CH_4}}{\int_{t_0}^{t_i} I(t)dt} \qquad \text{equation 10}$$

where 8 is the number of electrons exchanged in the reaction F is the Faraday constant, n is the number of moles of CH_4 electrochemically produced and I is the current density circulating for 200h.

406 Most of the BESs show a low methane coulombic efficiency (up to 10%) compared to the moles of
407 CH_4 ideally produced. The average CE varies according to the cathode, showing a minimum value
408 for the CC. Replacing CC with biochar in the cathodic chamber, a two-time increase of the methane
409 bioelectrochemically produced is noticed, suggesting a slightly higher selectivity for the reduction of
410 CO_2 into methane, despite the detection of H_2 in the gasbags. As previously stated, the addition of Cu
411 does not show a significant improvement of methane production which is reflected in a CE
412 comparable to the CC. On the other hand, the presence of HAP enhances the performances of the
413 BESs. Particularly, the composite with 20Cu/10HAP shows production of CH_4 comparable to the one
414 achieved by Gajaraj [19] and Jiang [68]. It is worth mentioning that the production of methane with
415 this new composite is more rapid than the other systems and a value comparable to the maximum
416 methane production available in literature was achieved in less than a quarter of the time (8 days *vs*
417 30 days or more), in a CO_2 sparging configuration. Also, a good CE (64%) was calculated, suggesting
418 a specific selectivity towards the direct reduction of CO_2 into methane in presence of bacteria [69].
419 *Solution analyses*
420 Analyses of the concentration of total Volatile Fatty Acids (VFAs) in the solution and pH were carried
421 out before and after each test to verify the occurrence of parasite reaction during the experimentation,
422 made more thermodynamically favoured than the conversion of CO_2 into methane. The microbial
423 transformation of carbon dioxide in organic acids as formates and acetates is one of the possible
424 CO_2RR pathways, specifically when high current densities are supplied [70, 71]. Results for the VFAs
425 concentrations are displayed in Figure 6. Their initial concentration on the pristine inoculum was
426 around 1100 mg L^{-1}, increasing at the end of the test up to more than one order of magnitude. Based
427 on the CE, unwanted or parasite reactions are all taking place in BESs. The CC based system seems
428 the less affected, while biochar cathodes in the BES induce a 10 to 50 folds increase of the VFAs in
429 the solution. As expected, the presence of Cu in the composite (20Cu) catalyses the transformation
430 of CO_2 into C-2 or C-3 hydrocarbons, visible from the high values of VFAs in the solution (50,774
431 mg L^{-1}). Samples with high HAP content (10HAP and 20Cu/10HAP) show comparable values to the
432 pure biochar, indicating that it can positively affect the reaction in terms of selectivity, hindering
433 possible parasitic reactions taking place. However, a low quantity in the mixture (5Cu/5HAP), is not
434 sufficient to contrast those reactions promoted by Cu.
435 The pH in the cathodic chamber was also monitored periodically as displayed in Table 5. Cells with
436 CC, undoped biochar and the 20Cu composite required periodical pH correction due to the H_2
437 produced, therefore pH was lowered with drops of a 0.1 M HCl solution to the initial value. At the
438 end of the test a deposition of white incrustations (not visible), possibly carbonates were visually
439 observed on all cathodes, confirming that the increase of the pH promoted unwanted precipitation on

the cathode material of salts from the solution. SEM analyses confirmed the morphology and composition of the precipitation, made primarily of Ca carbonates.

Small increases in pH were noted for the systems with HAP, as they remained more stable during the test, thermodynamically favouring methane production [2,72]. Such an optimal control of pH might be ascribed to the peculiar interface behaviour of HAP [47]. Indeed, when in contact with water, hydroxyapatite generates disordered hydrated layers located at the surface and undergoes partial dissolution with the release of its constituting ions (calcium cations, phosphates, and hydroxyl ions). Consequently, the pH at the interface of hydroxyapatite-biochar is buffered by the presence of these ions, in particular various species of phosphates, which impose a pH range between 5.8 and 7.

3.4 Identification of the microorganisms on the biocathodes

To identify the presence of electroactive microorganisms (Archaea and Bacteria) on the cathodes, preliminary SEM observations were carried out on the projected areas exposed to the solution. The micrographs in Fig. 7 show that microorganisms are differently attached on the selected electrodes following the roughness of the surface. Biofilm was observed on all cathodes, more significantly on CC, biochar, 10HAP, and 20Cu (Fig. 7a, b, c, d) while consortia of microorganisms, arranged in concretions were visible on the other composites 5Cu/5HAP and 20Cu/10HAP (Fig. 7e, f).

The high porosity of the biochar (macropores) also provides a larger surface for the adhesion of the microorganisms. A preliminary differentiation of microorganisms among the composites can be assessed by the different morphologies observed. The sample 10HAP shows a predominance of filamentous species attached to the surface and close to the HAP aggregates (Fig. 7c). Spherical microorganisms are abundant on the 20Cu biochar substrate, and their attachment follows a first covering of the surface by biofilm (Fig. 7d). A great density of microorganisms was found on the samples 5Cu/5HAP and 20Cu/10HAP, visible as agglomerates (in Fig. 7e, arrows). They bear different morphologies as spherical, rod-shaped, or filamentous (Fig. 7f, at high magnification) and are located predominantly close to and on top of the areas richer in HAP.

Fig. 8 and 9 respectively show the graphs of the main genera of Archaea and Bacteria domains from the Next Generation sequencing on V5-V6 16S RNA genes of cathodes samples carried out on the media, before and after the test on all operated cathodes at the end of the test.

Methanobacterium is the most abundant genus of Archaea for all systems, especially for composite cathodes, enriched with Cu and HAP (more than 90% of relative abundance). It is one of the first known hydrogenotrophic organisms producing methane directly from CO_2. *Methanobrevibacter*, another well-known hydrogenotrophic methanogen, was also found abundantly on carbon cloth (more than 25%) and noticed on the samples 10HAP and 20Cu. *Methanospirillum* was the second

abundant hydrogenotrophic methanogen on pure Biochar and was also found on the composites with both Cu and HAP.

The graph of bacteria genera in fig. 9 highlights a pronounced difference among the cathodes and from the initial inoculum, enriched with different microorganisms. A multitude of diverse bacteria genera is present in the n the initial anaerobic inoculum picked up from an already enriched pool of hydrogenotropic methanogens.

After the experiment, *Thermoanaerobacteraceae* was the most present genus on CC, followed by *Petrobacter*. The first is a large group of common bacteria whose metabolism is greatly variable, ranging from carbohydrate fermentation to chemolithoautotrophy. A decrease of genus variability is noticed for the undoped biochar, the 20Cu and the 10HAP, dominated by *Clostridia* and *Bacilli* (a group of anaerobic fermenters, which can ferment several organic substrates. It is not to exclude that the significant change of pH during the experimentation (from 6.5 to 10 as displayed in Table 5) could have induced more microorganisms' selectivity. In the system 10HAP *Proteiniborus* is also present. Notably, composite cathodes containing both Cu and HAP (5Cu/5HAP and 20Cu/10HAP) are enriched in *Tepidiphilus* phylotypes, which are very active in the transformation and biodegradation of alkanes [73]. On the 20Cu/10HAP sample, the abundance of *Tepidiphilus* was limited by the relevant enrichment of the genus *Proteiniborus*, anaerobic, mesophilic, protein-utilizing bacterial group, which can ferment organics to ethanol, acetic acid, and hydrogen. Comparative phenotypic, chemotaxonomic, and genetic analysis revealed significant similarities among strains of the genus *Tepidiphilus* and *Petrobacter* [74]. It is suggested that the presence of fermentative bacteria contributed to the low quantity of methane detected and that *Tepidiphilus* inhibited the methane production reaction, competing for the transformation of H_2 (system 5Cu/5HAP).

1. DISCUSSION

In bioelectrochemical systems, the pathways of CH_4 formation from CO_2 is still unexplored to a large extent due to the complexity associated to the transfer of 8 e^- and the number of microorganisms involved. The metabolic pathways that can lead to the transformation of CO_2 into methane are numerous, often involving a multi-step reaction with different intermediates. The reduction of CO_2 into CH_4 by hydrogenotrophic Archaea cannot take place without the presence of sufficient H_2, which theoretically is the rate-limiting step of the overall reaction (4:1 stoichiometric ratio). Opposite to hydrogen, the direct electron uptake from the cathode by these microorganisms requests a high electrochemically active surface as well as controlled local chemical-physical parameters to efficiently produce CH_4. The experimentation of innovative and green cathodes described in this work, based on biochar enriched with Cu and HAP in different quantities, aims to address these

requirements: i) selectivity towards the CO_2RR to methane; ii) electrochemically active surface area; iii) control of the pH conditions to an acceptable range for hydrogenotrophic methanogens microorganisms.

The support (biochar) was proven to have adequate properties to be used as a biocathode compared to the carbon cloth (CC) in terms of methane productivity: from 3 to an 8-fold increase in presence of 20Cu/10HAP. However, when we look at the coulombic efficiency of the process, only the 20Cu/10HAP exhibited a good performance (64% CE). The low production and efficiency of CH_4 at the CC, biochar and 20Cu cathodes, also compared to literature data [19, 68], can be explained by the modification of the pH during the experiment due to the uptake of H^+ ions from the solution that allowed to produce of carbonates, as visually experienced. The equilibrium bicarbonates-carbonates shifts towards the latter when pH increases up to 10, this way inducing the carbonate precipitation on the electrically active sites. This well-known phenomenon leads to the complete deactivation of the cathode, as previously well demonstrated for other types of microbial electrochemical systems [75, 76]. The excessive change of the pH led to the proliferation of fermentative microorganisms that: i) promoted alternate metabolic pathways other than the production of methane; ii) transformed the methane produced in organic acids. The competition of different microorganisms on the electrode and the production of different organics by-products could significantly have contributed to the reduction of methane yield compared to other cases.

On the other hand, the presence of HAP in the system from a certain amount (10 wt. %) guarantees higher values of methane production, possibly due to: i) its chemical effect on the pH; ii) selectivity towards methane production. It can be supposed that the presence of acidic HAP groups inhibited the increase of the local alkalinity, acting as a buffer [77]. Furthermore, the remarkable adsorption properties of HAP further increase the retention time of the methane production intermediates on the catalyst surface, thus suppressing the generation of CO and HCOO. Wai et al. [78] proved the crucial role of HAP in directing the selectivity, by promoting the formation of hydrogen-carbonate species from CO_2 chemisorption onto O^{2-} ions of the phosphate groups and by stabilizing the bidentate formate species which derive from further hydrogenation of hydrogen-carbonate species. These chemical catalysts mixed in sufficient quantities with graphitic biogenic carbon and deposited on the surface of the biochar cathodes formed a condition that allowed to improve the production of CH_4, being able to preferentially stabilize defined reaction intermediates [47]. The predominance of a different genus here, *Proteiniborus*, whose species generally does not metabolize carbohydrates, alcohols, or fatty acids, but grows in peptone-yeast broth producing ethanol, acetic acid, hydrogen, and carbon dioxide [79] can further indicate the prevalence of different syntropic metabolisms in this case, which more efficiently led to methane.

This is not the case for 5Cu/5HAP, which exhibit the same methane production as CC and the undoped biochar, despite the pH control. This low productivity is of difficult explanation also considering that the presence of basic sites of the HAP on the cathodic sites should exercise the function of reservoirs of adsorbed and pre-activated CO_2, further enhancing the CO_2 conversion to methane. Since the preparation of the composite was not completely successful in the case of 5CuNPs/5HAP/C, due to a partial and not total overlapping of the Cu and the HAP, it is possible to assume that the CO_2 produced at the cathode was preferentially catalysed in the areas rich in Cu, where the formation of C-2 and C-3 by-products (e.g. acetates, formates, carboxylates, methanol…) are also enhanced [80, 81]. The relevant presence of these organic compounds was indeed documented in the related electrolytes (Fig. 6). This assumption is also corroborated by the great abundance of methylotroph *Tepidiphilus* phylotypes on these cathodes, well known to degrade alkanes [73]. Hypothesizing the absence of alkanes, it can be assumed that the C-1 C-2 compounds produced on the cathode could have enhanced the bacteria metabolism that competed with the hydrogenotrophic production of methane by Archaea.

A final remark concerns the cathodic active area. As indicated by the results, the undoped biochar and the 20Cu produced 3 times higher current density than the CC (7.4 ± 0.2 *vs* 2.6 ± 0.1 $mA \cdot cm^{-2}$), thus ideally increasing 3 times the H_2 content (and consequently of CH_4) available at the electrode, probably due to a bigger specific projected area (macropores). However, for the composites, the presence of insulating HAP (resistivity of HAP is in the order of 10^9 $\Omega \cdot cm$ *vs* few $\Omega \cdot cm$ of the biochar [32, 82]) increased the electrode resistivity lowering the current density circulating in the cells at 3.9 ± 0.1 $mA \cdot cm^{-2}$ (10HAP) and 1.2 ± 0.1 $mA \cdot cm^{-2}$(5Cu/5HAP and 20Cu/10HAP) The electrochemical analysis confirms that this outcome is related to both this effect and the actual electrochemical active surface area (ECSA) of the electrode, lowered by a homogeneous dispersion of HAP nanoparticles on the surface of the material. The presence of Cu which should increase this last parameter (particularly for the sample 20Cu/10HAP) has no beneficial effect. This fact could be partially explained by i) the absence of a network of Cu nanoparticles on the surface that could better distribute the charge; ii) the covering of Cu nanoparticles by HAP that are located at the interface with the solution, partially suppressing the charge flow.

Although the supplementation of Cu and HAP as a composite material lowers the current density, thus the ECSA, this parameter less affects the production of methane compared to the composition of the electrode. Indeed, both the active phase and the dopant contribute to: i) the selectivity of the reaction; ii) faster kinetics; iii) a more efficient coulombic efficiency. From the perspective of a scale-up of the technology, further research will be dedicated to the production of a more conductive composite electrode enhancing the concentration of Cu and HAP at the interface with the solution,

allowing to create a network that could better transfer the charge for the bioelectrochemical production of methane.

5. Conclusion

Innovative composite biochar-based cathodes with Cu nanoparticles and Hydroxyapatite (20Cu/10HAP) are successfully tested in this work, for the first time in the literature, to enhance the electromethanogenesis process in double-chamber BES. The significant differences in methane yield and faradic efficiency observed between the different types of biocathodes can be ascribed to the crucial role that Cu nanoparticles and hydroxyapatite dispersed on the biochar surface express in terms of CO_2 reduction reaction catalysis toward C-1 and C-2 compounds and pH stabilization. Proliferation of hydrogenothropic archaea of *Metanobacterium* genus, in possible syntropy with different genera of fermenting bacteria depends on the cathode type and its functionalization. Porous biochar enriched with 20% Cu and 10% hydroxyapatite showed higher electrochemical performance and biomethane production compared with less enriched biochar, undoped biochar and carbon cloth cathodes. The productivity of methane (866±199 $mmol \cdot m^{-2} \cdot d^{-1}$cathode projected area) was comparable to the maximum obtained in literature in a double chamber BES (1103 and 1159 $mmol \cdot m^{-2} \cdot d^{-1}$cathode projected area respectively with a CC and a carbon felt electrode). Although these experiments had a lower faradaic efficiency (64% vs 90%) these outcomes were achieved in less than a quarter of the time (8 days *vs* 30 days or more), hypothesizing faster kinetics of production. It is important to underline that the performance of the cathode was determined by the external surface area only, while the internal porous surface of biochar remained almost non-functionalized and was not colonized. Addressing effort in building more uniformly functionalized biochar-based electrode, the methane productivity would be hence further increased.

6. FIGURE CAPTIONS

Figure 1. Schematic of a BES (a), and images of operating BES in the experimented set-up (b and c).

Figure 2. Macro and SEM-SE observations of maize stalk sample before and after pyrolysis.

Figure 3. SEM-SE micrographs with EDS spectra in point x: a) 5Cu/5HAP (S1 EDS); b) 20Cu/10HAP (S2 EDS).

Figure 4. SEM elemental mapping of electrodes: a) 5Cu/5HAP; b) 20Cu/10HAP. red=C; orange=P; yellow=Ca; green=Cu.

Figure 5. CO_2, H_2 and CH_4 concentration inside the gas bags after 200h (8 days) of test.

Figure 6. Total Volatile Fatty Acids measured in the cathodic compartment at the end of the test (200 h).

Figure 7. SEM micrographs of microorganisms on: a) CC; b) biochar; c) 10HAP; d) 20Cu; e) 5Cu/5HAP; f) 20Cu/10HAP.

Figure 8 Main genera of Archaea from the NG sequencing of cathodes samples. *Methanobacterium* is the most abundant genus for all cases.

Figure 9. Main genera of Bacteria from the NG sequencing of cathodes samples. The most abundant genus for each cathode type is highlighted. Inoculum: Other Genera; CC: Undefined_*Thermoanaerobacteraceae*; Biochar: *Clostridium XI*; 10HAP: *Bacillus*; 20Cu: *Proteiniborus*; 5Cu/5HAP: *Tepidiphilus*; 20Cu/10HAP: *Proteiniborus*.

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DATA AVAILABILITY

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

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