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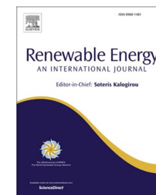

Bioelectrochemical protein production valorising NH_3-rich pig manure-derived wastewater and CO_2 from anaerobic digestion

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ABSTRACT

Microbial electrosynthesis (MES) cell use is an innovative approach for single-cell proteins (SCP) production. Coupling MES with the valorization of CO_2 from anaerobic digestion and nitrogen from livestock effluents has beneficial environmental effects, reducing greenhouse gas emissions and nitrogen overloading. In addition, the reducing power needed can come from surplus renewable energy. In this study, MES with a biochar-functionalized cathode was tested at varying polarizations, i.e. non polarized, −0.6 V and −1.0 V vs Ag/AgCl, and biogas-derived CO_2 and recovered ammonia from pig slurry was supplied.

Negative polarization switched the microbial community from heterotrophic, typical of unpolarized MES, to a mix of both heterotrophic and autotrophic/electrotrophic communities at −0.6 V and to mainly autotrophic/electrotrophic at −1.0 V. The more negative polarization allowed the highest CO_2 and N capture, i.e. 39 ± 2 % of the supplied CO_2, and 6.7 ± 0.8 % supplied N. Microbial biomass characterization indicated a protein content on dry matter basis of 33.1 ± 1.3 % (unpolarized), 43.2 ± 0.6 % (−0.6 V) and 69.1 ± 1.0 % (−1.0 V). The amino acids profiles investigated showed a high nutritional value of the produced biomass, not far from those of conventional protein sources used for producing feed/food.

1. Introduction

Alternative protein sources, including single cell proteins (SCPs), insects, algae and mycoprotein are emerging as substitutes for traditional protein sources such as plant- and animal-derived proteins [1,2]. SCPs, i.e., dried microbial biomass, offers several advantages over traditional protein sources: they include environmental sustainability, climate-independency, reduction in water use (i.e., 5 m^3 ton^{-1} for SCP versus 2364 m^3 ton^{-1} for soybean), lower land requirements (3000 tons ha^{-1} for SCP compared to 3–4 tons ha^{-1} for soybean), enhanced efficiency in nutrient utilization and higher protein content on a dry weight basis (d.w.) [1,3]. Bacteria offer distinct advantages over other microorganisms, such as the ability to grow on diverse substrates, rapid growth rates, high protein content (up to 80 % d.w.), and the high quality aminoacidic profile [1,4,5]. SCP-based feeds, particularly those derived from methane-oxidizing (MOB) and hydrogen oxidizing bacteria (HOB) fermentation, have already been approved by the European Commission and they are already commercially available under various trade names, e.g., Uniprotein® and FeedKind® Terra (https://feedkind.com/what-is-feedkind/; https://www.unibio.dk/end-product/protein/; [5,6]). However, SCP production by gas fermentation is limited by the low solubility of gases used as electron donors, such as H_2 or CH_4 [7,8]. To overcome this issue, microbial electrosynthesis systems (MES) have emerged as a potential solution offering the possibility of revolutionizing SCP production. Firstly, it eliminates the need for costly electrolysers. Additionally, it addresses the issue of limited hydrogen solubility by enabling in-situ hydrogen production or by furnishing direct reducing power in the form of electrons via the cathode [9].

Electron transfer from the cathode to microorganisms occurs via direct or indirect pathways. Electroactive microorganisms, known as electrotrophs in the context of MES, directly uptake electrons by specialized structures such as electroactive pili or proteins like cytochromes; alternatively, they can indirectly uptake electrons through electron-carrier molecules such as H_2 or formate produced on the

Abbreviations: AOB, ammonia oxidizing bacteria; GHG, greenhouse gas; HOB, hydrogen oxidizing bacteria; MES, microbial electrosynthesis cell; MFC, microbial fuel cell; MOB, methane oxidizing bacteria; PEM, proton exchange membrane; PM, particulate matter; SCP, single cell protein.

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cathode surface [10,11].

The possibility to couple bioelectrochemical protein production with the recovery and use of production side streams, e.g. CO_2 from unavoidable stationary point emitters, such as anaerobic digestion, industrial and power plants, and nitrogen-rich wastewaters, aligns with circular economy principles, making that approach even more appealing [12]. As the number of biogas plants in the EU continues to grow, there is a pressing need to valorise CO_2 from biogas upgrading, which accounts for 25–50 % of biogas produced ([13,14]; Y. [15,16]).

Additionally, the increase of the world population and its dietary shift towards more animal products, is leading to an increased demand for industrial livestock products [17]. Most of the ammonia (NH_3) emission, that contributes to PM 2.5 formation, in the EU, is caused by the agricultural sector. Moreover, better management of livestock effluent, i.e. N removal from animal slurries, is required to reduce nitrogen leaching and dispersion in the environment [18–20].

Bioelectrochemical microbial protein production presents an opportunity to address these challenges by capturing CO_2 from various unavoidable streams, such as biogas, and to recover NH_3 from livestock effluent [16,21]. Furthermore, if the supplied electricity is sourced from renewables, it can contribute to storing a renewable energy surplus in the form of protein (power-to-protein) [22,23].

Literature contains reports of different approaches in producing protein from CO_2 and ammonia ([7,9,24]; X. [25]). One approach consisted in testing MES under galvanostatic conditions, i.e. constant current, using both pure [24] or mixed culture [9]. Another approach consisted of operating under potentiostatic conditions i.e., constant working electrode potential [7]. A third approach kept constant the cell potential difference [25].

In this study, a potentiostatic approach was chosen to test different cathode polarizations for the production of SCP by MES, using biogas-derived CO_2 and NH_3 recovered from pig slurry as the C and N sources, within the context of a circular economy framework. This approach represents a novelty compared to the state of the art because energy is directly furnished to MES to reduce CO_2 and NH_3, avoiding the necessity to produce electron donor molecules such as H_2 and CH_4 to be then used as reducing power.

2. Materials and methods

2.1. Experimental operations

The inoculum consisted of a pre-acclimated microbial community sampled from a wastewater treatment plant (Peschiera Borromeo, Milan, Italy), fed with 80 % v/v of H_2 and 20 % v/v of CO_2 for 30 d and coming from previous experimentation.

Before the experiment, two-step acclimation was carried out to better adapt the inoculum to the MES system. First, the inoculum was acclimated in 125-mL serum bottles using a medium as described by Patil et al. [26]. The inoculum was fed every day for two weeks with a gas mixture composed of 80 % v/v of H_2 and 20 % v/v of CO_2.

In the second step of acclimation, which lasted two weeks, the same serum bottles were used, but the medium was modified by replacing distilled water with pig manure-derived wastewater (referred to as "PM medium"). Acclimated inoculum coming from the first-step acclimation was mixed with fresh PM medium in equal parts. The gas feeding regimen was the same as described before. Following that, second-step acclimated inoculum was transferred to the MES bioreactor with fresh PM medium for the third acclimation step. In this stage, the inoculum was fed with a gas mixture with a lower concentration of hydrogen (20:80 v/v H_2:CO_2), and electric current was supplied by means of a cathode. All the experimental phases were carried out at 28 °C.

2.2. Pig manure-derived wastewater

The last two steps of acclimation and the MES experiment were conducted using a modified medium containing pig manure-derived wastewater sampled from a plant treating pig slurry (OB-Slureless full-scale plant located in Cascina Tezze, Bergamo, Lombardy, Italy). This “plug and play” technology treats any kind of livestock manure; in this specific plant, swine manure from a nearby pig farm is treated continuously. Five steps are required to obtain clean permeate that can be safely discharged into the environment: (a) pig slurry mechanical separation using screw press, (b) vibrating screening on the liquid fraction, and (c) three reverse osmosis processes. Detailed information regarding the pig manure treatment plant can be found by Herrera et al. [19]. The wastewater used in the acclimation and experiments was sampled from the liquid fraction after the vibrating screen treatment; chemical characteristics are summarized by Gualtieri et al. [27]. Ammonium and total nitrogen concentration were measured using Nanocolor® test kits (Macherey-Nagel): ammonium 100 (4–80 mg L^{-1} NH_4^+-N) and total nitrogen TN_b 220 (5–220 mg L^{-1} N). Due to volatility of NH_3 and high pH of the wastewater, the ammonia concentration was determined just prior the beginning of the MES experiment.

Ammonia concentration in the raw pig manure-derived wastewater was of 440 mg L^{-1} NH_4^+-N, representing more than 90 % of the total nitrogen, COD was of 1167 ± 301 mg O_2 L^{-1}.

2.3. Bioreactor configuration

H-type double-chamber bioreactors were used. Anodic and cathodic chambers had a working volume of 100 mL. The anolyte consisted of a solution with conductivity of 6.679 mS cm^{-1}. The cathodic chamber was inoculated with 50 mL of pre-acclimated inoculum and 50 mL of PM medium.

The two chambers of each bioreactor were separated by a Nafion® 117 (DuPont™) proton exchange membrane (PEM). The membranes were activated in H_2SO_4 1 mol L^{-1} for 2 h (80 °C) and then rinsed in distilled water.

The anodes and cathodes were made of carbon cloth (SAATI C1, Appiano Gentile, Italy) with dimensions of 3 × 7 cm. The cathodes were functionalized to increase electroactive surface area by applying a suspension composed of 3 g of biochar from wood chip pyrolysis, 1 mL of polytetrafluoroethylene (PTFE) solution (60 % dispersion in water, Sigma-Aldrich) and 50 mL of distilled water. The cathodes were heat-treated 10 times for 30 min in a muffle at 340 °C. Each bioreactor was fed with 99 % CO_2 (SIAD, Bergamo, Italy) every day.

2.4. Cathode polarization

Two cathode polarizations were tested using potentiostats (NEV4, Nanoelectra S.L., Spain), i.e., - 0.6 vs Ag/AgCl and - 1.0 V vs Ag/AgCl. An unpolarized control (open circuit bioreactor) was prepared to monitor heterotrophic biomass production related to the degradation of residual organic carbon present in the treated swine-derived wastewater. Polarizations were chosen based on the H_2 evolution reaction potential at pH 7 as shown in Equation (1) [28], to assess the H_2 generation effect on the biomass growth.

$$2H^+ + 2e^- \rightarrow H_2 \quad E0' = -0.613 \text{ vs Ag/AgCl} \tag{1}$$

Henceforth, all the potential will be expressed versus an Ag/AgCl reference electrode.

2.5. Electrochemical and chemical analysis

Cyclic voltammetry analysis with potential range between −1.0 V and +1.0 V, a ramp of 1 mV s^{-1} and cathode as working electrode were performed to monitor the biofilm activity and development. Catholytes from each cell were analysed for ammonia concentration by using Nanocolor® test kits (Macherey-Nagel): ammonium 100 (4–80 mg L^{-1} NH_4^+-N) and total nitrogen TN_b 220 (5–220 mg L^{-1} N).

2.6. Biomass analysis

At the end of the experiment, microbial biomasses were sampled for CHN analysis, protein quantification and aminoacidic profile, and 16S rRNA next generation sequencing. Biomasses were recovered by centrifuging the catholyte. Biomasses for chemical analyses were previously lyophilized (Scanvac CoolSafe, LaboGene). The recovery of the cathodic biofilm for (a) and (b) analysis was not possible due to difficulties caused by the easy detachment from the cathode of the functionalizing agent (biochar).

2.6.1. Carbon and nitrogen balance

The lyophilized biomasses were analysed for CHN using a PerkinElmer CHN 2004 (Waltham, MA, USA).

The biomass yield (Y) was calculated by subtracting the control biomass production (heterotrophic biomass) from the biomass production of the polarized bioreactors and dividing the difference by the fed carbon grams (equation (2).

$$Y\left(g\ d.w.\ g^{-1}\ C\right) = \frac{W - W_b}{V_{CO_2} * V_m^{-1} * MM_C} \quad (2)$$

where W is the biomass weight recovered from polarized bioreactor, W_b is the biomass weight from control bioreactor, V_{CO2} is the total CO_2 supplied, V_m is the molar volume at 28 °C and MM_C is the molar mass of carbon (12.011 g mol^{-1}).

Thereafter, the carbon capture efficiency ($\%C_{capt.}$) was calculated by subtracting the control biomass production from the biomass production of a polarized bioreactor, and dividing the difference by the fed carbon moles (equation (3)):

$$\%C_{capt.} = \frac{(W - W_b) * C * MM_C^{-1}}{V_{CO_2} * V_m^{-1}} \quad (3)$$

where W is the biomass weight recovered from the polarized bioreactor, W_b is the biomass weight from control bioreactor, C is the percentage of carbon in the biomasses from CHN analysis, MM_C is the molar mass of carbon (12.011 g mol^{-1}), V_{CO2} is the total CO_2 supplied and V_m is the molar volume at 28 °C.

Nitrogen capture efficiency ($\%N_{capt.}$) was calculated as indicated in Equation (4):

$$\%N_{capt.} = \frac{W * N * MM_N^{-1}}{W_{NH_3} * MW_{NH_3}^{-1}} \quad (4)$$

where W is the biomass weight, N is the percentage of nitrogen in the biomasses from CHN analysis, MM_N is the molar mass of nitrogen (14.007 g mol^{-1}), W_{NH3} are the grams of supplied NH_3, and MM_{NH3} is the molar mass of NH_3 (17.031 g mol^{-1}).

2.6.2. Protein content and aminoacidic profile

The protein content (% d.m.) was determined by multiplying the N content detected by using the Dumas method (UNI EN 16634-2:2016) by the coefficient of 6.25 and by summing the single amino acid content of a sample. Amino acidic profiles were assessed by digesting the samples in acidic conditions (HCl 6 mol L^{-1}), derivatizing the amino acids with OPA and FMOC and finally determining the profiles using HPLC-DAD 1100 series (Agilent, Santa Clara, US).

2.7. 16S rRNA next generation sequencing

DNA extraction was performed on i. the original inoculum from a previous MFC experiment at Time 0; ii. the inoculum after acclimation with homoacetogenic medium at Time 0 and with PM medium again at Time 0 (before the MES acclimation); iii. the catholytes at Time 1 (after acclimation in MES); iv. the same cathodic solutions fed with 100 % CO_2 at Time 2 (end of the experiment); and v. the biofilm grown on the cathodes at Time 2.

Solution samples (~40 mL) were pelleted at 13,000 rpm for 20 min. From each pellet and biofilm, DNA was extracted using the DNeasy® PowerSoil® Kit (Qiagen, Germany) according to manufacturer's instructions after an initial thermal treatment (5 cycles of 10 min at −20 °C and 10 min at 65 °C). DNA yield and purity was quantified on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) while quality was determined through gel electrophoresis 1 % (w/v) 1 × TAE agarose gels. DNA was stored at −80 °C until analyses.

The NGS was performed at Novogene Co. Ltd (Cambridge, UK). Sequencing targeted the V3 and V4 regions of the bacterial 16S rRNA gene using primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) (Youngseob et al., 2005). The generated DNA libraries were sequenced with an Illumina NovaSeq PE250 and the generated nucleotide sequences are available at the NCBI SRA repository (BioProject accession number: PRJNA1091668). The sequences resulting from the NGS were quality checked through the FastQC software and analysed using DADA2 for R as per https://benjjneb.github.io/dada2/tutorial.html (Callahan et al., 2016). For taxonomic assignment, the SILVA database v. 138.1 was used as reference (McLaren and Callahan, 2021).

All statistical analyses were performed on R studio (version 4.3.1) as in (Clagnan et al., 2022). Linear Discriminant Analysis Effect Size (LEfSe) were executed as per https://usegalaxy.eu/.

2.8. Statistical analysis

All experiments and analyses were performed using three replicates. Mean and standard deviation (SD) values were determined following standard procedure through R Studio (Posit team, 2023, Boston, MA). Determination of significant differences among the parameters analysed at a level of significance of $p < 0.05$ was carried out by analysis of variance (ANOVA) and Tukey's post-hoc test using R Studio.

3. Results

3.1. Carbon and nitrogen balances

The biomass yields registered at the end of the experiment were of 1.3 ± 0.2 g d.w. g^{-1} C and 0.2 ± 0.0 g d.w. g^{-1} C for −1.0 V and −0.6 V, respectively. The biomass weight in all bioreactors was underestimated due to difficulties in sampling the biofilm from the cathode because of the presence of biochar that may have been inadvertently removed from the electrode.

The more negative cathode polarization led to higher CO_2 capture efficiency, i.e. 39 ± 2 % CO_2 dosed, compared to −0.6 V, i.e., 4.1 ± 0.2 % CO_2 dosed. Considering the N capture efficiency, again, the more negative polarization led to higher values compared to −0.6 V bioreactor, i.e., 6.7 ± 0.8 % N dosed versus 2.4 ± 0.2 % N dosed, respectively. Mostly N was lost as volatilized ammonia, but unfortunately no measures were taken. This point needs to be considered in future experimentations to quantify N losses and find solutions to prevent them.

The microbial biomass C/N ratio found in the −0.6 V MES (4.2 ± 0.3) and control MES (6.3 ± 0.8) were in line with those reported in the literature (Table 1). On the other hand, because of high nitrogen content in the microbial biomass developed in −1.0 V MES, the biomass C/N was very low, i.e., 3.2 ± 0.5 (Table 1).

3.2. Protein and aminoacidic profile analysis

The protein content calculated starting from biomass nitrogen content in the −1.0 V MES biomass was of 69.1 ± 1.0 % d.w. (Table 1) (65.6 ± 4.2 % d.w. calculated as sum of each single amino acid-AA, Table 2). On the other hand, the protein content in the biomass from the −0.6 V and control MES were of 43.2 ± 0.6 % d.w. (43.3 ± 2.1 % d.w. calculated as sum of each single AA) and of 33.1 ± 1.3 % d.w. (33.6 ± 2.4 % d.w. calculated as sum of each single AA), respectively (Table 1).

Table 1
Elemental composition, Carbon-to-Nitrogen ratio and protein content of the microbial biomass produced in this work, and comparison with literature data.

Condition or process	Elemental formula	C/N	Protein (%)	Reference
−0.6 V MES	$CH_{2.05}N_{0.24}$	4.2 ± 0.3b[a]	43.2 ± 0.6b[a]	This study
−1.0 V MES	$CH_{1.85}N_{0.32}$	3.2 ± 0.5a[a]	69.1 ± 1.0c[a]	
Control MES	$CH_{1.87}N_{0.16}$	6.3 ± 0.8c[a]	33.1 ± 1.3a[a]	
Single chamber MES	n.r.[b]	n.r.	87 ± 8	[9]
Cupriavidus necator H16 (DSM 428) fermentation	$CH_{1.74}N_{0.19}$	5.4	52 ± 1 50 ± 1	[29]
Single chamber MES	n.r.	n.r.	53 ± 8 64 ± 4	[7]
Double chamber MES	n.r.	n.r.	48.9 ± 6.9	[25]
	n.r.	n.r.	56.8 ± 7.5	
	n.r.	n.r.	63.3 ± 9.6	
Nocardioides nitrophenolicus KGS-27 (single chamber MES)	n.r.	n.r.	32.7 ± 1.7	[24]
Rhodococcus opacus DSM 43205 (single chamber MES)	n.r.	n.r.	36.9 ± 1.1	
Paracoccus denitrificans Y5 fermentation	$CH_{2.05}N_{0.23}$	4.3	63.65	[30]
Paracoccus versutus D6 fermentation	$CH_{1.86}N_{0.20}$	5.0	63.05	
Methanotrophic fermentation	$CH_{1.89}N_{0.12}$	8.3	28.75[c]	(Khoshnevisan et al., 2019)

[a] Mean ± SD (n = 3); mean followed by the same letter are not statistically different ($p < 0.05$; ANOVA, Tukey test).
[b] Not reported.
[c] Calculated multiplying N content (%) by 6.25.

The protein productivity in −1.0 V MES (i.e., 2.2 ± 0.1 mg L^{-1} d^{-1} or 417.8 ± 19.9 mg m^{-2} d^{-1}) was more than double than that obtained in the −0.6 V MES (i.e., 0.8 ± 0.0 mg L^{-1} d^{-1} or 145.5 ± 0.3 mg m^{-2} d^{-1}), and control bioreactors (i.e. 0.5 ± 0.0 mg L^{-1} d^{-1} or 98.2 ± 4.9 mg m^{-2} d^{-1}).

Proteins were characterized and seventeen amino acids (AA) were identified in each sample, apart for cysteine (Cys), which was below the detection limit in the control sample (Table 2), and Asparagine (Asn), glutamine (Gln) and tryptophan (Trp) which were not analysed due to analytical problems. All the analysed essential amino acids were present in the polarized MES biomass, confirming that microbial protein can be considered a good alternative source of protein for feed/food. The cathode polarization fostered the amino acid production, with the biomass from the more negative cathode polarization having a higher quantity of each amino acid compared to the control bioreactor. However, comparing qualitatively the amino acid profiles (Fig. 1a), no significant differences between the control and polarized samples were observed, apart for histidine which was higher in the polarized bioreactors. The essential amino acid (Fig. 1b) content, referred to the analysed amino acids, were of 34.6 ± 0.2 %, 32.6 ± 1.3 % and 26.2 ± 4.4 % for −1.0, −0.6 V and control, respectively.

3.3. 16s rRNA

The 16S rRNA analysis showed that the starting inoculum underwent changes in terms of most abundant phyla and genera during the acclimation, as expected. Detailed information about starting and acclimated inoculum are reported in the supplementary material.

More interesting are the differences observed for control vs. the other two treatments (Fig. 2b). Beta diversity (species diversity among

Table 2
Amino acid content of the three different samples expressed in mg 100 g-1 d.w.

Amino Acids	−0.6 V	−1 V	Control
	Essential amino acids (mg 100 g^{-1} d.w.)[a]		
His	3344 ± 356b	4865 ± 381c	500a
Ile	871 ± 54a	1635 ± 290b	813 ± 206a
Leu	3280 ± 71b	5380 ± 495c	2270 ± 226a
Lys	1100 ± 28b	1825 ± 205c	711 ± 100a
Met	751 ± 273a	1046 ± 302a	1200 ± 42a
Phe	1700 ± 240b	2615 ± 49c	538 ± 409a
Thr	2500 ± 230 ab	2980 ± 354b	2090 ± 266a
Val	555 ± 74a	2340 ± 113b	867 ± 585a
Non-essential amino acids (mg 100 g^{-1} d.w.)[a]			
Ala	1490 ± 141a	2055 ± 148b	1445 ± 233a
Arg	8775 ± 417a	12,100 ± 141b	7970 ± 1,018a
Asp	6400 ± 509a	10,125 ± 1,520b	5250 ± 1,160a
Cys	321 ± 22a	450 ± 40b	Udl[b]
Glu	5840 ± 339b	8290 ± 14c	4790 ± 141a
Gly	1855 ± 997a	3215 ± 1,124a	1990 ± 184a
Pro	439 ± 237a	502 ± 224a	313 ± 162a
Ser	2210 ± 127b	3520 ± 339c	1345 ± 7a
Tyr	1840 ± 14a	2670 ± 57b	1745 ± 375a

[a] Mean ± SD (n = 3) except for His; mean followed by the same letter are not statistically different ($p < 0.05$; ANOVA, Tukey test).
[b] Under the detection limit.

different treatments obtained by comparing the number of species unique to each ecosystem) of prokaryotic communities, NMDS and PERMANOVA analyses showed that different cathode polarizations led to a differentiation within the communities. In the control bioreactor after the MES acclimation phase (Time 1), *Pseudomonas* (7 %) was maintained as the main genus (>5 %) and accompanied by *Lentimicrobium* (39 %), an anaerobic bacterium capable of denitrification [38] and acetic acid production [16], and *Parapusillimonas* (12 %), a facultative anaerobic bacterium that can assimilate a wide range of carbohydrate, organic acids, and amino acids, capable of degrading recalcitrant organic matter too [39,40]. At −1.0 V, the main genera were *Brevundimonas* (15 %), nitrifying/denitrifying electrotrophic bacteria, already reported to be present in biocathode MES [41,42];, *Aquamicrobium* (12 %), ammonia-oxidizing bacteria (AOB) resistant to high concentrations of N–NH_4^+ [43], already reported in biocathode of MES for autotrophic nitrogen removal [44];, *Pseudomonas* (10 %), a genus that comprises autotrophic denitrifiers [44] and HOB [45]; *Paracoccus* (10 %), genus able to grow auto-, hetero- and mixotrophically and able to use H_2 as electron donor and CO_2 as carbon source, i.e., HOB [30], reported as electroactive [46], associated with denitrification [47]; with the addition of *Alcaligenes* (8 %), a genus known to comprise facultative autotrophic and HOB [48] and electrotroph species [49]; and *Tissierella* (6 %), reported in electroactive biofilms of fermentative BES [50]. At −0.6 V, electroactive genera able to uptake directly electrons from the cathode, such as *Brevundimonas* (24 %), *Paracoccus* (9 %) and *Pseudomonas* (7 %), were found. Additionally, *Leucobacter* (15 %), a facultative anaerobic heterotrophic bacterium [51], *Aquamicrobium* (9 %) and *Arcobacter* (7 %) were present (Fig. 2a).

At the end of the experimental period (Time 2), *Lentimicrobium* (8 %) was maintained as a core genus in the cathodic solution of control MES, with the addition of *Leucobacter* (18 %), *Sphaerochaeta* (13 %) and *Rhodococcus* (10 %). These last two genera are characterized by an anaerobic chemoorganotrophic growth, the former with fermentative metabolism [52] and the latter able to use various carbon and energy sources [53]. At −0.6 V, the main genera were similar to those found at Time 1, i.e., *Leucobacter* (12 %), *Paracoccus* (10 %) with the addition of *Lentimicrobium* (10 %), *Rhodococcus* (8 %), a versatile genus capable of heterotrophic growth and electroactivity [42], and *Fastidiosipila* (6 %): this genus has previously been reported in biocathodes [50] and it is correlated with proteolytic activity and generation of VFA as well [54,55]. At −1.0 V, main genera were similar to those found at Time 1 with *Alcaligenes* (12 %), *Tissierella* (9 %), *Fastidiosipila* (18 %) and *Paracoccus*

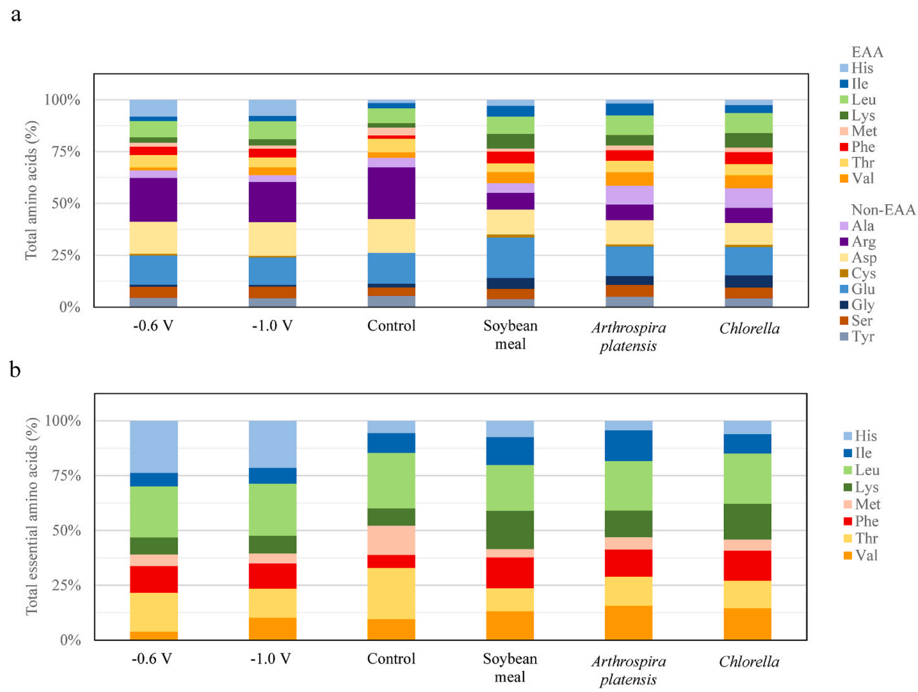

Fig. 1. Amino acid profile (a) and essential amino acid profiles (b) of the three samples, i.e., unpolarized control and MES with cathode polarized at −0.6 V and −1.0 V compared to other protein sources, i.e., soybean meal [31–35,66], Arthrospira platensis [34,36] and Chlorella [34,37].

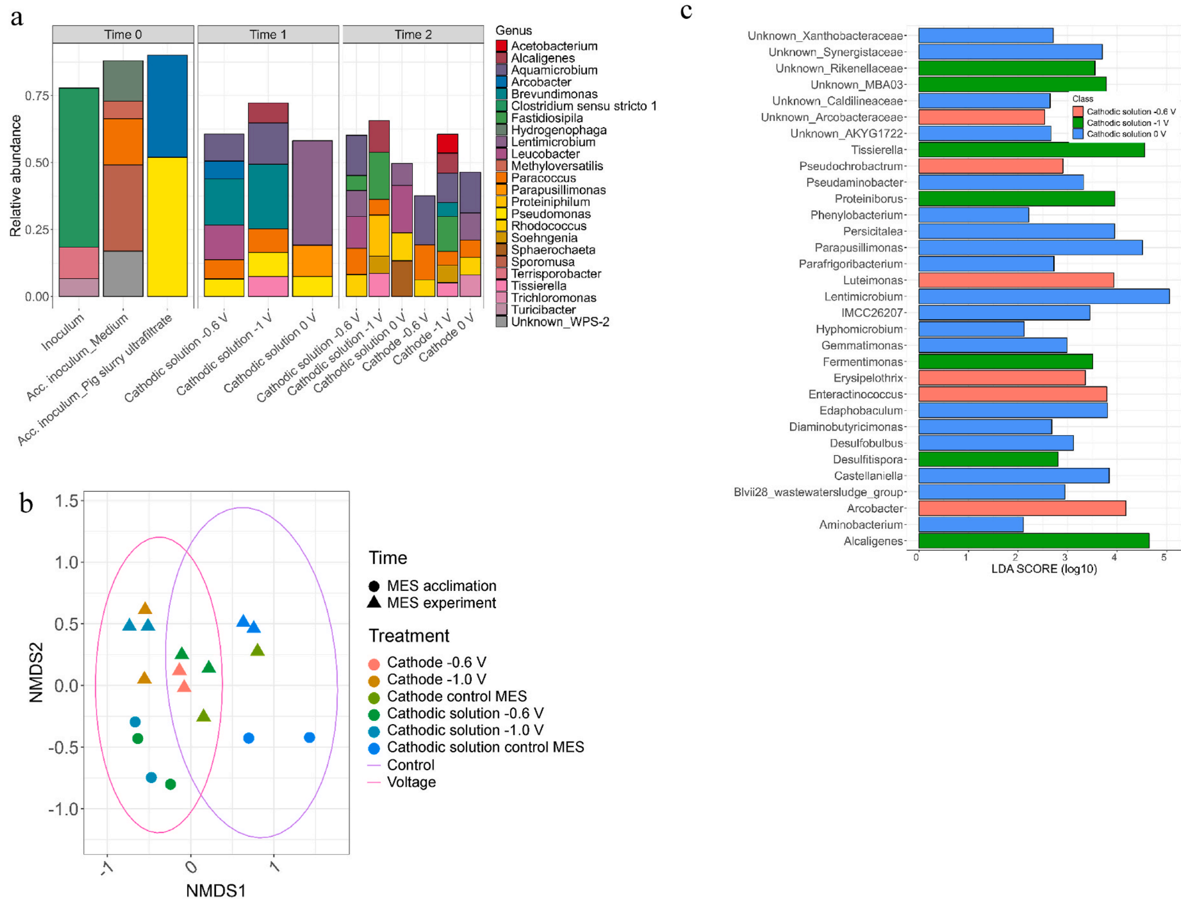

Fig. 2. a) Prokaryotic community composition at genus (cut-off >5 %) level (relative abundance of the average values of two replicas are shown for each bar). b) Non-metric multidimensional scaling (NMDS) ordination plots of the samples. c) Linear discriminant analysis (LDA) effect size analysis (LEfSe) at genus level for the prokaryotic communities.

(6 %). *Proteiniphilum* (15 %), a fermentative acetogen able to use CO_2 as carbon source and H_2 as electron donor [56,57] and *Soehngenia* (6 %) were also retrieved. At the end of the experiment, the cathodic biofilm was also sampled. The biofilm community in the control MES was characterized by a high abundance of *Aquamicrobium* (15 %), *Lentimicrobium* (10 %), *Trichloromonas* (8 %), *Rhodococcus* (7 %) and *Paracoccus* (6 %); at −0.6V by *Aquamicrobium* (18 %), *Paracoccus* (13 %) and *Rhodococcus* (6 %) while at −1.0 V of *Fastidiosipila* (13 %), *Aquamicrobium* (11 %), *Alcaligenes* (8 %), *Acetobacterium* (7 %), that comprises acetogenic bacteria able to use H_2 as electron donor and fix CO_2 by means of Wood-Ljungdahl pathway [58] and to directly uptake electrons from the cathode [59], *Soehngenia* (7 %), *Paracoccus* (5 %), *Brevundimonas* (5 %) and *Tissierella* (5 %) (Fig. 2a).

A LefSE analysis was further carried out on the whole dataset to highlight the genera significantly enriched for each specific treatment, i.e., control, −0.6 and −1.0 V within the cathodic solutions (Fig. 2c). As expected, the polarization showed an enrichment in species linked to NH_3 removal, organic matter degradation and electroactivity. Additional information is reported in the supplementary materials.

In summary, the communities adapted to each specific treatment with the highest difference between the control MES and the two treatments with polarized cathodes. The communities evolved towards genera linked to degradation and electroactivity with *Alcaligenes* and *Tissierella* playing the leading role for the treatment at −1.0 V.

4. Discussion

Bioelectrochemical production of SCP is a valuable biotechnological process to produce protein-rich biomass for feed and food use by applying small amounts of electricity and valorising side streams such as CO_2 from biogas and NH_3 from N-rich wastewater such as the one coming from livestock farming.

In this study, the importance of cathode polarization was highlighted by testing two polarizations, one below and one above the H_2 evolution reaction potential, i.e., −0.6 V and −1.0 V [28].

Results indicated that higher biomass and higher protein production occurred at −1.0 V MES because to the higher current density compared to −0.6 V MES. This resulted in a higher amount of reducing power in the form of electrons or hydrogen formed abiotically on the electrode surface. The average current densities measured were of -0.029 ± 0.008 mA cm^{-2} and of -0.018 ± 0.017 mA cm^{-2}, for −1.0 V and −0.6 V MES, respectively.

While current circulation in an MES is associated with electron transfer and electrochemical reactions, the direct relationship between current circulation and protein production may not be linear. However, a higher amount of reducing power resulting from an efficient electron transfer process can promote the selection of microbial species, such HOB, and their metabolism related to protein synthesis.

The more negative cathode polarization, i.e., −1.0 V, stimulated the microbial biomass yield (1.3 ± 0.2 g d.w. g^{-1} C) compared to the cathode polarization at −0.6 V (0.2 ± 0.0 g d.w. g^{-1} C). The higher productivity found at −1.0 V can be explained by the higher availability of reducing power, supplied in the form of electrons and/or abiotic H_2 generated on the surface of the cathode. Zhang et al. [48] highlighted the positive correlation between the increase in the amount of electron donor, i.e., H_2, and CO_2 fixation efficiency of hydrogen oxidizing bacteria (HOB). As described by Pous et al. [7], Pous et al. [9] and Manchon et al. [60], higher current densities (more negative cathode polarization) were correlated with the increase of biomass production. In this study, polarization at more negative values led to the selection of a microbial community (Fig. 2) able to capture and reduce CO_2 to protein (in the presence of an N source).

The acclimation in the MES bioreactor without polarizing the cathode, i.e., unpolarized control MES, led to the selection of facultative anaerobic bacteria able to assimilate a wide range of carbohydrate, organic acids, and amino acids, and capable of degrading recalcitrant organic matter, as suggested by 16S rRNA analyses. Therefore, it can be assumed that in the control bioreactor, the microbial biomass grew exclusively thanks to the heterotrophic consumption of organic carbon present in the PM medium.

However, the microbial community in the −1.0 V MES was completely different, being rich mainly in autotrophic and electrotrophic genera. In particular, the differences between the microbial community of control, i.e. heterotrophic microbial community, and −1.0 V MES, i.e. prevalently autotrophic microbial community, indicated that the electric current supplied was responsible for an electrotrophic metabolism with autotrophic CO_2 fixation into biomass, leading, thanks to the high presence of ammonia, to high protein production. The presence of electroactive microorganisms at the −1.0 V MES cathode was supported by the cyclic voltammetry, in which an oxidation and a reduction peak can be seen at −0.440 V and −0.530 V, respectively (Fig. S2). The current peaks indicated redox activity on the cathode, likely ascribed to electroactive microorganisms, i.e., proteins such as cytochrome *c* or secreted mediator [61].

At more positive cathode polarization, i.e., −0.6 V, a metabolic transition between control and −1.0 V MES can be assumed, observing that the microbial community combined both heterotrophic bacteria capable of organic matter degradation and the presence of electroactive genera able to uptake electrons from the cathode. This fact agreed with the CO_2 fixation rate. The higher reducing power available in the −1.0 V MES allowed microorganisms to reduce more CO_2, stimulating also the nitrogen uptake from the catholyte, resulting in enhanced NH_3 uptake ability compared to more positive polarization (−0.6 V) and control MESs. This was corroborated by the higher protein biomass production and low C/N ratio observed at −1.0 V MES. Pous et al. [9] reported that higher ammonia assimilation into biomass occurred at higher current density induced by negative cathode polarization, i.e., −1.0 V. A similar indication came from Yang et al. [25], who observed an increasing ability of NH_4^+ removal and a relative higher protein content in the microbial biomasses, with the increase of the cell potential difference, i.e. higher current intensity (expressed as positive value). Again, Wang et al. [62] observed higher protein content in *Alcaligenes faecalis* after lowering the cathode potential, and Zhang et al. [48] found higher protein concentration was produced by increasing the amount of electron donor (H_2).

Total protein content obtained in this study, i.e. 43.2 ± 0.6 % d.w. (−0.6 V MES) and 69.1 ± 1.0 % d.w. (−1.0 V MES), were in line with previous work performed by MES which reported for SCP a protein content between 33 % and 64 % d.w. (Average of 50.8 ± 12.2 % d.w.; $n = 7$) [7,24,25] but, also, with microbial protein produced through fermentation, i.e. range of 29 %–83 % d.w. (Average of 56.8 ± 15.7 % d.w.; $n = 14$) [29,30,63–65].

More interesting was the comparison of protein content obtained in this work with that of the most frequently used protein source, i.e. soybean meal, i.e. 49.7 ± 2.6 % d.w. ($n = 12$), which is widely used in livestock diets [31–35,66,67], with the data obtained at the more negative polarization being much higher. Protein contents of this study were, also, comparable with the protein content found in alternative and innovative protein sources such as cyanobacterial SCP (*Arthrospira plantensis*), i.e. 64.1 ± 6.5 % d.w. ($n = 7$) [34,36,68–71] and the microalgal SCP (*Chlorella*), i.e. 47.5 ± 9.4 % d.w. ($n = 5$) [34,37].

Total protein content alone is not enough to qualify the ability of a biomass to substitute for a common protein source, since the amino acid profile must also be considered. The amino acid profile obtained for protein produced in this work (Fig. 1a), reveals lower relative amounts of glutamic acid, isoleucine, lysine, proline and valine, alongside higher quantities of arginine and histidine, than soybean proteins. Similarly, when comparing the amino acid profile to alternative protein sources, such as Spirulina and *Chlorella*, lower amounts of alanine, isoleucine, lysine, proline, and valine were found, while higher amounts of arginine, aspartic acid and histidine were observed. Most importantly, comparing the percentage of essential amino acids on total amino acids

(Fig. 1b), slightly lower amounts were present in the microbial biomass from the −1.0 V biomass compared to soybean meal and algae biomass, with values of 34.6 ± 0.2 % versus 37.5 ± 1.5 % ($n = 12$) and 37.4 ± 2.9 % ($n = 7$), respectively.

For a comprehensive analysis of SCP production, the energetic cost was calculated, starting from the power output of the potentiostats. The obtained values in this study were of 158 ± 15 Wh g^{-1} of fixed C, or 49 ± 13 Wh g^{-1} d.w that was slightly higher than the theoretical energy requirements for water electrolysis and HOB cultivation, i.e., SCP production via gas fermentation (29.24 Wh g^{-1} d.w.) [23]. Similar values (37 ± 14 Wh g TSS^{-1}) were achieved in galvanostatic conditions by Pous et al. [9], while a much higher energy amount was used by the same authors in potentiostatic condition (303 Wh g TSS^{-1}) [7].

5. Conclusion

This study revealed that different cathode polarizations resulted in a shift of the microbial community from heterotrophic (no polarization applied) to electroactive and autotrophic bacteria (polarized MES, i.e., −1.0 V and −0.6 V), which allowed autotrophic CO_2 fixation activity (39 ± 2 % CO_2 $_{dosed}$ and 4.1 ± 0.2 % CO_2 $_{dosed}$, at −1.0 V and −0.6 V, respectively) producing, in presence of N, proteins that were of interest from both quantitative (total content on dry weight basis, i.e. 69.1 ± 1.0 %, 43.2 ± 0.6 % and 33.1 ± 1.3 %, at −1.0 V, −0.6 V and unpolarized control, respectively) and qualitative (AA profile and essential AA content, being these latter of 34.6 ± 0.2 % AA, 32.6 ± 1.3 % AA and 26.2 ± 4.4 % AA at −1.0 V, −0.6 V and unpolarized control, respectively) points of view. These results suggested the potential use of microbial SCP as a substitute for traditional protein sources.

The use of secondary streams as feed, i.e. CO_2 from biogas upgrading and ammonia solution from pig slurry treatment, and electricity from renewable sources, can represent a virtuous approach by which surplus electricity produced can be stored into chemical bonds producing valuable protein for feed/food use (power-to-protein) by simultaneously valorising production side streams.

SCP production exploiting MES is in early stage of research and this work contributed adding useful data to further increase the Technology Readiness Level (TRL) to 3/4. Additional research is required to improve the bioelectrochemical system for protein production. Tuning MES input, including CO_2, NH_3-rich wastewater, and electric current is required to better align with the optimal conditions for the bacteria involved in protein synthesis. Furthermore, efforts should focus on enhancing the efficiency and output of the MES system to reach productivity levels suitable for industrial applications. Achieving this goal requires improvement in bioreactor design (more industrially scalable design), optimization of the microbial biomass recovery, which entails improving cathode structure and preventing degradation and detachment of electrode components like biochar. Moreover, it is obviously crucial to evaluate health risks and safety aspects associated with the use of SCP produced from mixed culture for food and feed.

CRediT authorship contribution statement

Gabriele Soggia: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Andrea Goglio:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pierangela Cristiani:** Writing – review & editing, Validation, Resources, Conceptualization. **Ivan Luciani:** Formal analysis. **Elisa Clagnan:** Writing – original draft, Supervision, Investigation, Formal analysis. **Fabrizio Adani:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2024.120761.

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