The different behaviour of *Thermotoga neapolitana* in the anodic and cathodic compartment of a bioelectrochemical system

Pierangela Cristiani[1*], *Laura* Malavola[2], *Silvia* Franz[3], *Massimiliano* Bestetti[2], *Giuliana* D'Ippolito[3], *Nunzia* Esercizio[3], Mariamichela Lanzilli[3], Angelo Fontana[3]

[1]Ricerca sul Sistema Energetico - RSE S.p.A., 20134 Milano, Italy
[2]Politecnico di Milano, Department of Chemistrty Materials and Chemical Engineering, 20131 Milano, Italy
[3]Institute of Biomolecular Chemsitry (ICB), National Research Council (CNR), Pozzuoli, 80078, Na, Italy

Abstract. *Thermotoga neapolitana* is a hyperthermophilic bacterium that can metabolize glucose and several organic wastes in hydrogen and lactate at a temperature of 80°C. Their high performance in producing hydrogen at so high a temperature as 80°C suggests a potential energy application of them where hydrogen is an important element of the process. In this view, experimentation of a *T.neapolitana* strain is carried out in double-chamber electrochemical systems. The aim is to explore the interaction of these bacteria with the anode and the cathode, stressing their capability to survive in presence of a polarized electrode which can drastically change the pH of the media. A culture enriched of 5 g/L of glucose, under CO2 pressure (80 °C) was used to fill both the anodic and cathodic compartments of the electrochemical system, applying a voltage of 1.5 V between the anode and the cathode. The test lasted ten days. Results clearly indicate that bacteria colonize both electrodes, but the glucose metabolism is completely inhibited in the anodic compartments. On the contrary, metabolism is stimulated in the cathodic compartment. Bacteria are alive on the electrodes in the pH interval of 3 - 9.

1 Introduction

The use of single strains in hyperthermophilic conditions is a possible innovative way to make electrochemical systems simpler to manage and efficient. Hyperthermophilic microorganisms are characterized by an extremely productive metabolism that allows them to survive in challenging conditions for life, such as a temperature of 80°C up to more than 100°C, and under a scarcity of energetic organic nutrients. *Thermotoga neapolitana* is a microorganism that produces hydrogen and lactate by metabolizing glucose at a temperature of 80°C. Previous works, [1, 2] found a great affinity of T. neapolitana to form biofilm on different materials. In principle, they can be exploited to catalyze the hydrogen evolution reaction in a microbial electrolytic cell, with the aim to improve the overall yield of the system, as recently attempted [3]. Nevertheless, the previous tests were performed in single-chamber electrochemical systems, using an alternated polarization between electrodes [2]. Those conditions could not allow differentiating the behaviour of the bacterial colony on anode or cathode. Aiming at exploring separately the interaction of the hyperthermophilic bacteria with the anode and the cathode electrodes, new tests of a *T. neapolitana* strain in double-chamber electrochemical systems have been carried out. Carbon cloth (CC) and Boron Doped Diamond (BDD) are used for electrodes. A culture enriched of 5 g/L of glucose, under CO_2 pressure, at 80 °C, is used to fill both the anodic and cathodic compartments of the electrochemical system, applying a voltage of 1.7 V (or more) between the anode and the cathode in two tests that lasted nine days each one. The metabolism of glucose to acetic and lactic acid was monitored by periodic chemical analyses in the first test. The pH of the solution of the anodic and cathodic compartments was measured in the second test, where higher polarization and BDD anodes were used to monitor the resistance of bacteria to the pH variation induced by the electrochemical reactions. The results are here reported and discussed.

2 Materials & Methods

2.1.1 Media and Inoculum

250 ml of culture broth ATCC 1977 for anaerobic microorganisms, with trace element solution (DSM medium 141) was used as media. 5 g/L of glucose (28 mM) was added before the inoculation. A stream of CO_2 gas was sparged in the culture to create anaerobic condition until the resazurin in the solution was colourless, as described for previous tests [3]. An aliquot of 7 ml of pre-acclimate batch culture (at 80 °C for 1 day) was inoculated in each electrochemical bioreactor at the beginning of the test. All transfers and sampling of cultures were performed with sterile syringes and needles. *Thermotoga neapolitana* subsp. capnolactica (DSM33033), a lab strain derived from *T. neapolitana* DSMZ 4359T [2] was tested.

2.1.2 Set-up and operational conditions

The double chamber electrochemical reactor was set up using a small glass bottle of 250 ml connected by the polymeric membrane Nafion 417. The setup of electrochemical bioreactors is shown in Figure 1.

Two electrochemical reactors were first operated with identical electrodes (anode and cathode) of carbon cloth, in duplicate. A subsequent test was carried out using Boron Doped Diamond instead of carbon cloth at the anode, to limit the current and the consequent pH decay during the polarization. Only pH variation was measured during the second test (not the metabolism's products).

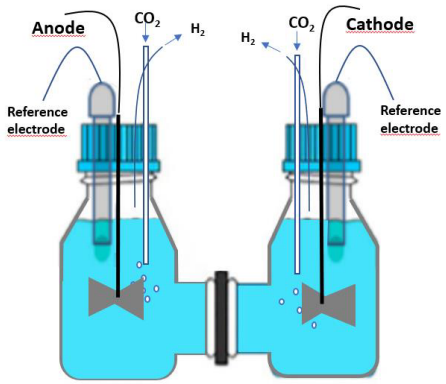


Figure 1. Schematic of double chamber electrochemical reactor used for the tests.

In the first test that lasted eight days, the same polarization of 1.7 ± 0.1 V was applied between the anode and cathode of each polarized electrochemical bioreactor.
4 more bottles were operated in the same condition of the reactor semi-cells, as follows: 2 bottles without electrodes (REF1, REF2), and 2 bottles containing one carbon cloth electrode (unpolarized) operating at the open circuit potential (OCP1, OCP2).
In a second test, the polarization was increased for A3, A4 to 6.5 V, to stimulate overproduction of hydrogen and also inverted for BDD1, BDD2 and C5, C6, to very if bacteria survived in that extreme polarization condition.
In Table 1 a summary of the tested conditions is reported.

Table 1. Experimental condition and labels.

First test	**Material**	**Label**
Reactors without electrodes	-	REF1 REF2
Unpolarized electrodes (OCP)	CC	OCP1 OCP2
Cell voltage 1.7 ± 0.1 V	Anode: CC Cathode: CC	A1, A2 C1, C2
Second test	**Material**	**Label**
Reactors without electrodes	-	REF3 REF4
Cell voltage 1.7 ± 0.1 V (6 days) + 6.5 V (2 days)	Anode: CC Cathode: CC	A3, A4 C3, C4
Cell voltage 1. 7 ± 0.1 V (6days) Inverted polarization (4days)	Anode: BDD Cathode: CC	BDD1 BDD2 C5, C6

Each electrochemical reactor is equipped with an Ag/AgCl (saturated) reference electrode (AMEL Srl, Italy, mod. 373/12). The bottle without electrodes is equipped with a red-ox (platinum combined electrode (AMEL Srl, Italy) for measuring the redox potential of the solution. A tube sealed on the cap and with the tip immersed down in the solution allows the sparging of CO2 gas into the solution at the beginning of the test and after the sampling/refilling operations in all the chambers/bottles. A syringe needle is used for the collection of produced gas, connected to a gas bag of 1 L.
0.25 L of culture medium ATCC 1977 was introduced in every reactor. T. neapolitana was inoculated at the start of the test.
The operation for each test lasted nine days, counted from the inoculum of bacteria.

2.1.3 Electrodes

Carbon cloth (CC, SAATI, Italy) is used for setting up the cathodes; the anodic materials were: i) the same CC as used for the anodes or ii) Boron Doped Diamond BDD (BDD/Nb, by NeoCoat®, Netherlands).
A carbon cloth piece of 10 × 10 cm is wrapped around and tightly fixed to a titanium wire to assemble CC electrodes. They geometrically occupy almost 1/5 of the liquid volume when immersed almost completely in the reactor (Fig1).
BDD electrodes is a plate of 5 x 2.5 x 0.1 cm. The BDD plate was suspended in the solution by a titanium collector.

2.1.4 Chemical analyses

To quantify the level of the metabolic activity, the medium was sampled during the experiment through sterile syringes and analysed.
The generated H_2 in the bioreactors was collected in a gasbag of 3L. Samples from the gasbags were collected by a gas-tight syringe at room temperature before the analysis. H_2 analysis was performed by gas-chromatography using a gas-chromatographer (Focus GC, Thermo Scientific) equipped with a thermo-conductivity detector (TCD) and fitted with a 3 m molecular sieve column (Hayesep Q). Nitrogen was used as gas carrier.
Acetic acid (AA), lactic acid (LA), and glucose concentration was determined by the dinitrosalicylic acid method calibrated on a standard solution of 1 g/L glucose [4]. Organic acids were measured by ERETIC 1H NMR as described by Nuzzo et al. (2019). All experiments were performed on a Bruker DRX 600 spectrometer equipped with an inverse TCI CryoProbe. Peak integration, ERETIC measurements, and spectrum calibration were obtained by the specific subroutines of Bruker Top-Spin 3.1 program.

2.1.4 SEM observations

A scanning Electron Microscopy SEM (Zeiss EVO 50, Carl Zeiss Jena GmbH, Jena, Germany) was used to observe the surface of electrodes. Samples were gentle rinsed with water after collection and dried in hover for 15 minutes. Then stored at 4°C until the analyses.

Observations were carried out at high vacuum and high voltage (20keV).

3 RESULTS AND DISCUSSION

The glucose metabolism during 9 days of testing is monitored during the first test. The hydrogen production and pH variation, under different polarization conditions, are estimated with the second test.
BDD, which is known to control the formation of reactive oxidant substances (ROS) at potential >1 V [4] is used in comparison with carbon cloth anodes in the second test.

3.1 First test: glucose metabolism

The results of the chemical analyses (glucose, acetate, lactate, and alanine concentrations) sampled after 30h from each cell are reported in Tables 2-4. A decrease in glucose concentration with a subsequent increase in acetate indicates that bacteria are carrying out their normal glucose metabolism. The data after 30 h indicated that the glucose metabolism in the cathodic compartment started first and overperformed vs the other condition, in the order: Cathode > Reference > OCP > Anode.

Table 2. Data of the glucose consumption, during the first test. Information on H_2 and Alanine production is also reported.

	Glucose consume (G)		**H_2**		**Alanine (Ala)**	
	mM	dev	mM	dev	mM	dev
Cathode	23.67	0.47	88*	-	1.81	0.00
REF	8.41	2.23	88*	-	1.29	0.04
OCP	2.28	3.03	-	-	0.61	0.87
Anode	1.84	1.35	-	-	1.02	0.11

*Estimated value, as gasbag exploded because an overproduction of H_2 during the night.

Table 3. Data of the acid production from the glucose metabolism during the first test.

	Acetic acid (AA)		**Lactic acid (LA)**		**LA/AA**	
	mM	dev	mM	dev	mM	dev
Cathode	25.91	0.60	3.99	0.82	0.15	0.04
REF	8.12	1.75	2.86	0.94	0.35	0.04
OCP	4.40	5.16	1.11	1.34	0.24	0.03
Anode	0.67	0.05	0.39	0.04	0.59	0.02

Table 4. Ratios of the glucose metabolism for acetic acid, lactic acid, and alanine.

	AA/G		**LA/G**		**Ala/G**	
	mM	dev	mM	dev	mM	dev
Cathode	1.09	0.00	0.17	0.04	0.08	0.00
REF	0.97	0.05	0.34	0.02	0.16	0.04
OCP	3.71	2.67	0.83	0.52	0.14	0.20
Anode	0.51	0.40	0.30	0.24	0.79	0.64

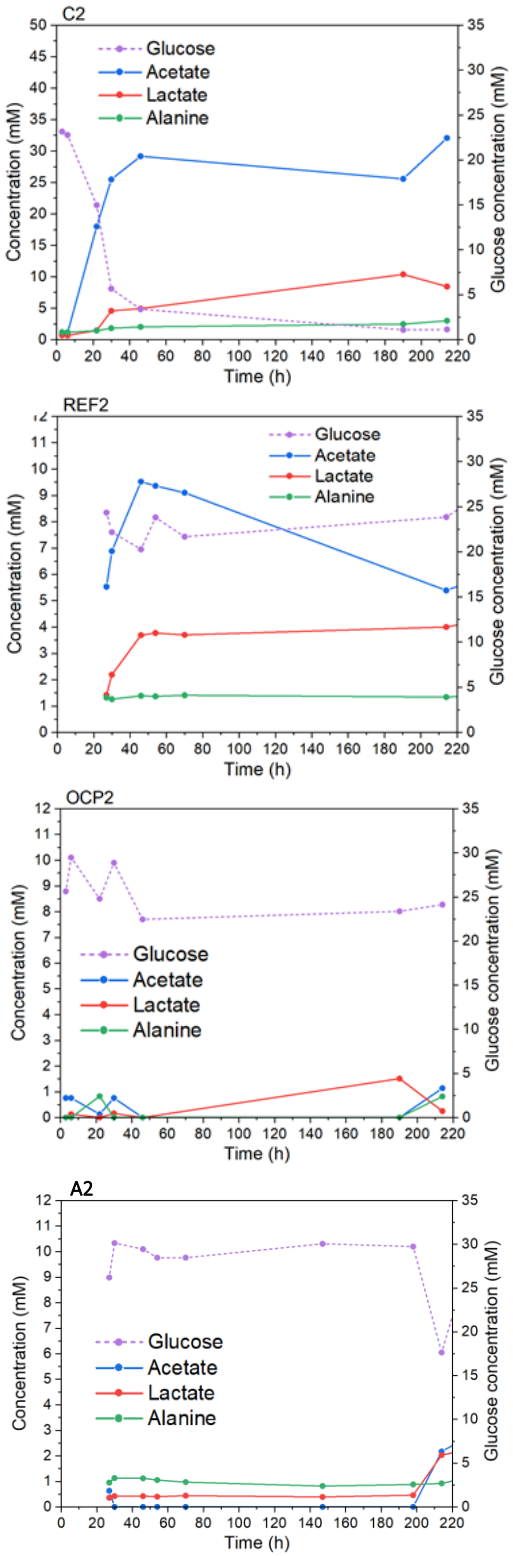


Figure 2. Trends of the metabolism products and glucose consumption in C2, REF2, OCP2, and A2, during the first test.

The trends of the chemical product and glucose consumption for the total first test (220 h, 9 days), in one of the replicates of all compartment types, are reported in the graphics of Figure 2 (replicates are similar). After 190 hours, the solution in the compartment was refilled with an aliquot of fresh media enriched with glucose. This

caused little increase in glucose concentration in compartment OCP2 and REF2.
The solution in the cathodic reactors and references become murky during the first 30 h, indicating that bacteria grew in number in the bulk of the medium.
The anodic compartments and the OCP ones, indeed, remained transparent and clear, so we assume that all the bacteria were attached to the electrode in a stasis form (Figure 3). The condition of stasis was broken in OCP cells during the rest of the test as solution went numb, while persisted in the anodic compartments until stress caused by the performing of a strong (4 cycles) of cyclic polarization probably stimulated the detachment of some bacterial cells from the anode, sudden inducing consume of glucose and acids production (Figure 2, A2).

3.1.1 Cathodic chamber

It is clearly observable from Figure 2 that in the semi-reactors where a negative polarization was present the concentration of metabolism products is way more significant than in other cells: indeed, 50 h after the inoculum, the concentration of acetate in C1 and C2 is about 30 mM (lower than 0,5 mM in the anodic compartments and in OCP2, and roughly 10 mM in all other cells).
Moreover, between 50 and 140 h, the concentration of acetate decreases from 30 to 25 mM in cell C2, while the lactate concentration increases by the same amount. This aspect, particularly, deserve more future investigation since it points to a possible induction of the capnophilic lactic fermentation pathway (1) [3,4] as a particularity of the cathodic compartment.

$$CH_3CO_2H + CO_2 + 4H^+ + 4e^- \leftrightarrow 2CH3CH(OH)CO_2 + H^+ + H_2O \quad (1)$$

It is a remarkable result as the same phenomenon was already noticed in previous experimentations with alternated polarized electrodes, where was not possible to address it to the anode or to the cathode specifically.

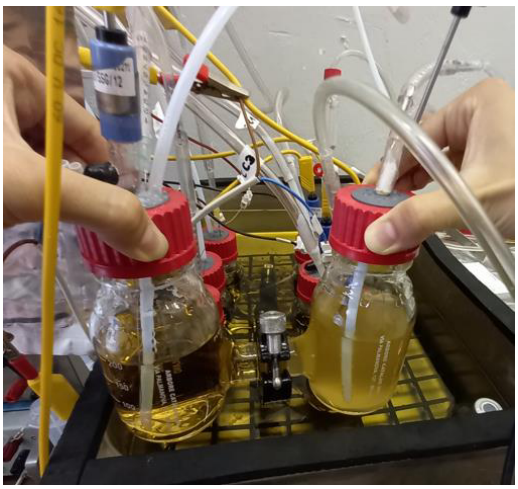


Figure 3. Image of anodic compartment (A2, on the left) and cathodic compartment (C2, on the right) after 30 h of test.

3.1.2 Anodic chamber

In the anodic compartments, the metabolism was almost negligible for all the tests. Nevertheless, a higher concentration of aniline than acetate is here detected, meaning that under anodic conditions the metabolism of microorganisms is stressed.
This fact was remarked performing cyclic voltammetry which strong polarized (with alternated polarity) the anodic electrodes, after refreshing the solution on day 6. After this, in both anodic compartments, the concentration of acetate and lactate suddenly increases, indicating that microorganisms detached from the electrode and actively start the glucose consumption similarly as in the case of the OCP2. This fact is highlighted because it proves that *T. neapolitana* survived, although in a sort of stasis, on the anode.

3.2 Second test: hydrogen production

Unfortunately, it was not possible to estimate the produced hydrogen from the volume of the gasbags (1 L) during the first test. This, because the gas bags connected to C1, C2, REF1, and REF2 broke the day after the inoculum before the sampling. Therefore, it is assumed that the production of gas in those reactors has been massive during night-time. The gasbags connected to the other cells, on the contrary, have shown an unvaried volume. The hydrogen production in these last reactors (anodes and OCPs) was indeed almost negligible during the test.
The produced hydrogen is better measured during the second test, in the cathodic compartments C3 – C6. After 5 days of the second test. The gas measured in the gasbag (1 L) was hydrogen 80 - 95% with 5-15% of CO_2.
The amount of hydrogen produced during the electrochemical process can be calculated from Faraday's law (2).

$$\Delta n_i = \frac{v_i I t}{v_e F} \quad (2)$$

In the equation, Δn_i is the amount of species reacted in the time interval in mol, v_i is the stoichiometric number of the species i, ν_e is the stoichiometric number of electrons implied, I is the current circulation, t is the time interval and F is the Faraday constant (approximatively 96500 C/mol). From this calculation, in the case of reactors with anode and cathodes of CC, the amount of hydrogen electrochemically produced in 5 days, sustained by a continue current of 6.3 mA (1.7 V), is about 14 mmol, corresponding to 316 mL in standard conditions, from the Avogadro's law of gases. This is the electrochemical condition that characterizes the first as well as the second experiment with CC electrodes (the current in the case of BDD anodes was inferior). The gas collected from the gasbags is about 1 L, which ~0.1 L consists of dosed CO_2. Therefore, only a maximum of about 500-600 mL of hydrogen is produced by microorganisms, which corresponds to about 22 mmol in

standard conditions (88 mM). The dosed glucose in 250 mL of solution is ~ 7 mmol. Considering that glucose wouldn't be completely consumed, this estimation agrees with the theoretical molar ratio of H_2/Glucose (4/1) from the Embden–Meyerhoff pathway [5] (2),

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_3O_2^- + 2H^+ + 4H_2 + 2\ CO_2 \quad (2)$$

and with a maximum of 3.85 ± 0.07 achieved from previous experiments in bioreactors [6]. It can be consequently concluded that bacteria were productive at best in the cathodic compartments of the tested systems.

3.2.1 pH variation

The pH measured in the different compartments of tested systems during the second test is reported in Figure 4. The pH variation in the references, without electrodes, declined just a little to 5. Similar behaviour has the pH of C5 and C6 coupled to BDD anodes.

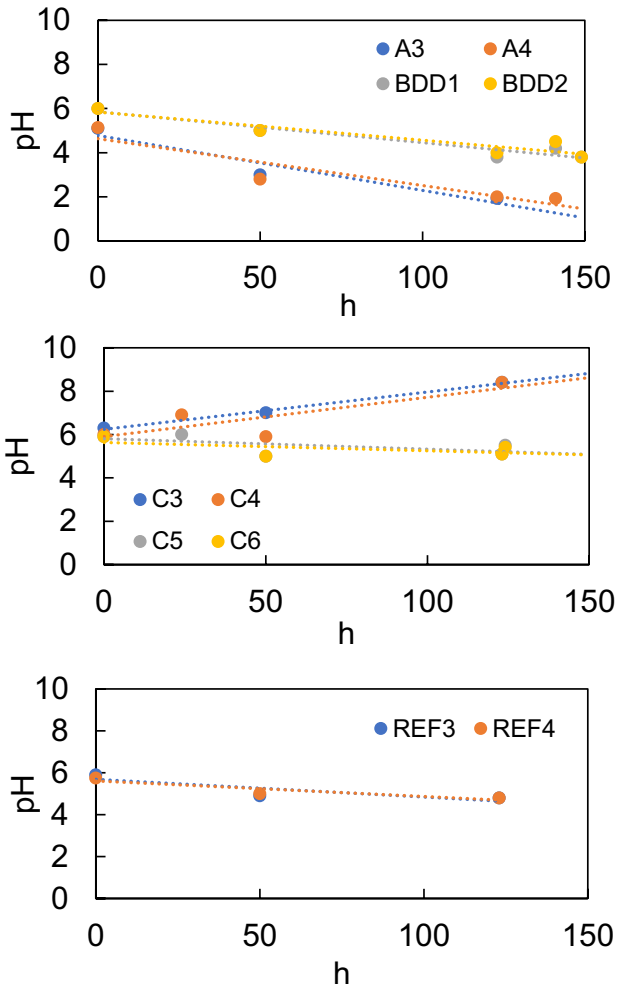
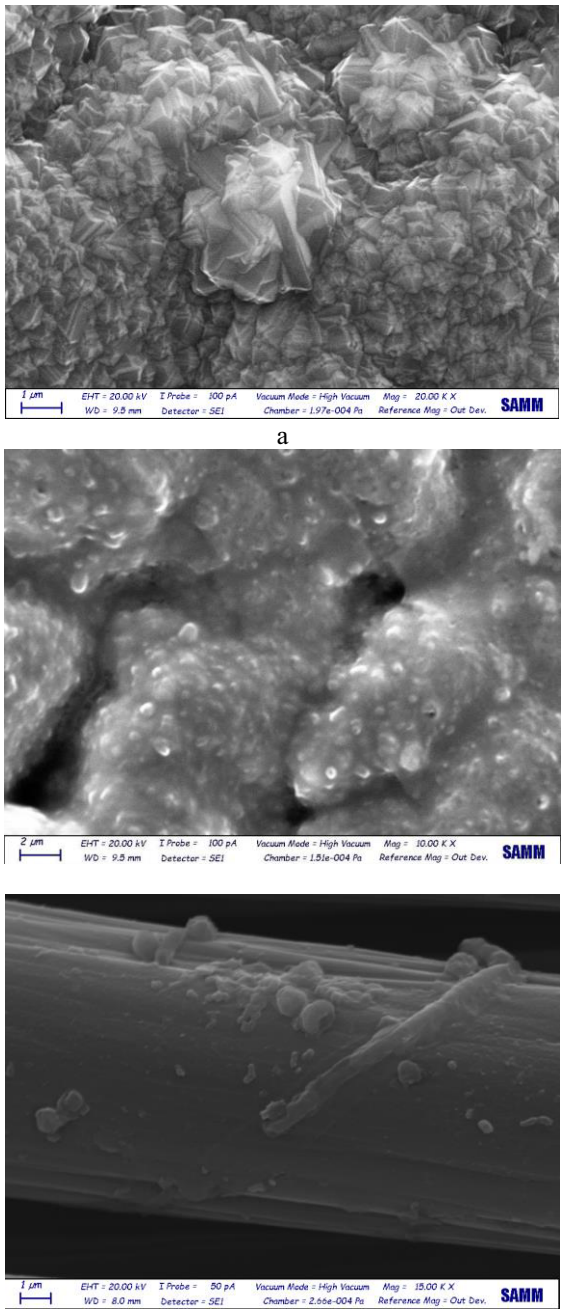


Figure 4. pH variation in the compartments of different bioreactors during the second test (see Table 1).

On the contrary, the pH raised over 9 in the cathodic compartments C3 and C4, and fell down to 2 for A3 and A4, due to the high imposed polarization in those cells. The pH in the anodic compartments of BDD electrodes, only declines to 3.8. To verify if bacteria survived the pH decline in BDD anodes, at the end of the test (150 h) the polarity between the electrodes was reversed. After few hours from the inversion, the solution of the inverted BDD compartment become mouldy, clearly indicating the presence of alive bacteria detaching from the electrode.

3.2.2 SEM images of the electrodes

SEM micrographs of the biofilm on the BDD surface pristine and covered of bacteria at the end of the test is reported in Figure 5a and 5b, respectively.



a

Figure 5. Micrographs of the biofilm on: the BDD surface pristine (a), BDD1 covered of bacteria at the end of the test (b) and CC cathode (C3) at the end of the second test.

The two images, in comparison, clearly evidenced that biofilm uniformly cover the surface, hindering the polycrystalline structure of the BDD material. Similarly, carbon cloth anode was covered by an uniform biofilm (data not reported). More uneven biofilm characterized the cathodes, where bacteria (single units or aggregates) are more evident (Figure 5c).

4. Conclusions

The data produced in this work confirmed that it is possible to drive the metabolism of hyperthermophilic bacteria T. neapolitana in polarized bioelectrochemical reactors. *Thermotoga neapolitana* confirms its strong affinity for polarized electrodes as well as for conductive materials such as carbon cloth and BDD.
The bacteria metabolism of glucose is clearly stimulated in the cathodic compartment where electrodes are negatively polarized, while it is almost completely inhibited in the anodic compartments, where a positive polarization attracts bacterial cells, forcing them in a condition of stasis.
On BDD, bacteria create a biofilm where they can survive in spite of a pH degree down to 3.
Further investigations on more cost-effective materials than BDD are proposed, which might mitigate the pH change induced by polarization, such as pH variation, preserving the bacteria vitality, as BDD does.
The enhancement of hydrogen production on cathodes stimulated by hyperthermophilic bacteria such as T. neapolitana, can find several industrial and energy applications. One example of a possible application that deserve to be investigated is the technologies of the power-to-gas, where hydrogen is the primary resource with CO_2 for methane production.

5 Acknowledgements

This work was financed by the Research Fund for the Italian Electrical System in compliance with the Decree of March 19th 2018.

References

1. G., Squadrito, G. d'Ippolito, M. Tucci, M. Vastano, N. Esercizio, A. Sardo, M. Lanzilli, A. Fontana, P. Cristiani. Bioresource Technology, **319** (2021) 124078.

2. 6.G Squadrito, P Cristiani, G d'Ippolito, M Tucci, N Esercizio, A Sardo, M. Lanzilli, A. Fontana. Data in brief **33**, (2020), 3.

3. G. d'Ippolito, L. Dipasquale, F.M. Vella, I. Romano, A. Gambacorta, A. Cutignano, A. Fontana. Int. J. Hydrogen Energy **35**, (2010), 2290.

4. P. Bernfeld, Amylases a and b. Methods Enzymol. **1** (1995), 1995149.

5. RS. Ronimus, HW. Morgan, Archaea, **1,** 3, (2003), 199.

6. N. Pradhan, G. D'Ippolito, L. Dipasquale, G. Esposito, A. Panico, P.N.L. Lens, A. Fontana. Biomass Bioenergy **125**, (2019), 17.