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Effect of cathode properties on the thermophilic electrosynthesis of PolyHydroxyAlkanoates by *Kyrpidia spormannii*

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ABSTRACT

The recent discovery of the Knallgas bacterium *Kyrpidia spormannii* EA-1, able to produce polyhydroxyalkanoates (PHA) on a cathode, is of great interest to produce bioplastics from electricity and CO_2 waste streams. However, little is known on how to improve its electroautotrophic growth and performance in PHA production. We investigated the effect of cathode properties on biofilm formation and PHA synthesis, focusing on the choice of cathode material, the surface modification of a graphite cathode with different treatments or by electrodeposition of metal catalysts, and the distance between anode and cathode. The results show higher performance of iron-based electrodes, isopropanol and sonication treatment, and close distance between electrodes, with up to a 3-fold increase of PHA production, reaching a production of 117 $mg \cdot day^{-1} \cdot m^{-2}$, and a 10-fold increase in cell density of the biofilm (10.7 Log_{10} $cells.cm^{-2}$).

1. INTRODUCTION

In order to manage the climate change crisis and its consequences, new sustainable technologies need to be developed to change our impact on the environment. Microbial electrosynthesis systems (MES) are promising technologies for a sustainable production of organic commodities, such as biofuel, bioplastics or chemical building blocks. In these systems, electrotrophic microorganisms act as biocatalyst on the cathode of an electrochemical system, consuming electrons and/or reducing equivalents or energy carriers such as H_2 and fixing CO_2 (i.e., from industrial waste streams) into biomass and extracellular products. Microbial biocatalysts include so far mainly acetogens and methanogens (Dykstra and Pavlostathis, 2017), and the range of organic products from MES is quite limited to short fatty acids and their relative alcohols (Vassilev et al., 2019). Chain elongation steps were studied to increase the value of end products, up to caproate (Jourdin et al., 2018), but remains challenging in terms of efficiency and value of the end product. The applicability of MES for chemical synthesis from CO_2 waste streams would benefit from a wider range of products, improved production rates, and higher energetic efficiencies. Microbial catalysts, electrode materials, and reactor design are the key components which influence the functioning of such processes.

New microbial biocatalysts with alternative metabolic pathways have been investigated, either by the metabolic engineering of new pathways in already described electrotrophs (Kracke et al., 2018) or through the enrichment of environmental communities (Alqahtani et al., 2019; Pillot et al., 2020) and the isolation of pure cultures (Reiner et al., 2020). This latter strategy has notably led to the isolation of a microaerophilic Knallgas bacterium, *Kyrpidia spormannii* EA-1, able to produce and accumulate PolyHydroxyAlkanoates (PHA) while growing on a cathode (Reiner et al., 2020). PHAs are aliphatic, biodegradable, and biosourced polyesters that have different pendant groups at the beta position of the repeating unit, allowing customized properties, showing similarities to synthetic thermoplastic polymers (Saranya Devi et al., 2012), and can be used as bioplastics. Conventional production of PHA involves the fermentation of purified carbohydrates, such as glucose, to achieve high yields and low impurities. Autotrophic production of PHA from CO_2 waste streams is also being developed but requires explosive H_2:O_2 gas mixes (Islam Mozumder et al., 2015). Despite its advantages, PHA are still commercially far behind because of their high cost for raw materials and downstream processing (Kourmentza et al., 2017). To acquire the

commercial viability and sustainability, alternative production technologies must be investigated, such as sustainable MES.

The use of *K. spormannii* in a MES might overcome the commercial limitation of PHA by reducing the necessity of purified carbohydrates as substrates, using renewable electricity while fixing anthropogenic CO_2 waste. However, efforts on the improvement of efficiency and production rates are required. Recent studies focused on the in-situ monitoring and quantification of *Kypridia*'s biofilm on cathode in a novel flow cell setup (Hackbarth et al., 2020), or the improvement of *Kyrpidia spormannii*'s capabilities through undirected mutagenesis (Jung et al., 2021). Our previous study aimed to optimize *Kyrpidia spormannii* biofilm formation on a cathode by optimizing the growth conditions (cathode potential, pH of the media, O_2 supply). The screening of cathode potential also showed a preference for *K. spormannii* for relatively positive potential, and the growth of dense biofilm at a potential close (up to -325 mV vs. SHE) to the standard potential of H_2 evolution (calculated at -195 mV vs. SHE at 60 °C, pH 6.5, and p_{H2} equal to the Michaelis constant Km=327 nM of hydrogenase from *Kyrpidia* species (Hogendoorn et al., 2020)) and the onset potential between -325 and -380 mV vs. SHE measured by cyclic voltammetry (Pillot et al., 2022). These results suggest the presence of a potential second electron transfer mechanism beside the H_2 mediated electron transfer, as previously mentioned (Jung et al., 2021). This is in opposition to the commonly studied acetogens showing higher performances at more negative potential, linked with higher H_2 evolution (Izadi et al., 2020). However, despite these considerations, the PHA production by *K. spormannii* remains relatively low and requires more optimization. In this context, a main aspect for improvement of the electrosynthesis process is the electrode material, which has not been systematically studied so far in conjunction with *Kyrpidia spormanii*.

In most MES systems, the reaction at the anode is not of primary interest, as it involves mostly water electrolysis into O_2. In electrosynthesis processes with anaerobic acetogens, the two electrodes are often separated with an ion exchange membrane in a two chamber MES, in particular to avoid oxygen transfer from the anode to the cathodic biofilm. However, in the case of microaerophilic biofilms, such as *Kyrpidia* biofilms, the O_2 produced at the anode can be used as electron acceptor for the biofilm at the cathode. Thus, the use of a one chamber MES is advantageous, but the distance between the electrodes must be optimized to achieve sufficient O_2

transfer to the cathode while minimizing the negative impact of reactive oxygen species or chlorine, which are potentially produced in anodic side reactions.

On the cathode side, different materials (mainly carbon-based), surface modifications, and designs were tested to enhance the microbial electrosynthesis, leading to different improvements over the last decade (Aryal et al., 2017). All these improvements are so far focused on the acetate production, with the exception of one study on the effect of magnetite nanoparticles anchored graphene cathode on the electrosynthesis of polyhydroxybutyrate (PHB) by *Rhodopseudomonas palustris* (Rengasamy et al., 2021). However, as previously mentioned, the cathode potential screening of *K. spormannii* and previous study suggest a potentially different extracellular electron transfer mechanism than with acetogens, where higher H_2 evolution seems to have a negative effect on biofilm formation and PHA production. This alternative pathway would potentially involve sulfur oxidation proteins (Jung et al., 2021). Therefore, different effects of cathode properties might be considered on the electrosynthesis of PHA by *Kyrpidia* strains than previously reported on acetogens.

In this context, we investigated these effects on the electrode-associated growth and electrosynthesis of PHA considering four different aspects: the cathode material, the surface modification by chemical/physical treatment and by electrodeposition of metals, and finally the distance between anode and cathode. All these conditions were selected to cover different degrees of hydrophilicity, catalytic activity, overpotentials, affinity for H_2 evolution, and finally reactivity with O_2. Eleven treatments (physical, chemical, and electrochemical) were tested on their ability to increase hydrophilicity, and narrowed down to 5 effective treatments to test in the MES. As PHA is not a product of the energy metabolism, compared to acetate in acetogens, a two-step process is required for its intracellular accumulation on the electrode, a growth phase and a stress/nutrient-limitation phase. We focused in this study on the growth phase and decided to compare the biofilm formation, and in a limited extend the PHA production, in each case after 3 days of experiment, as it resulted in the stabilization of current consumption previously correlated with biofilm growth (Hackbarth et al., 2020). The results obtained in the present study were compared to control experiments with plain graphite plates.

2. MATERIAL AND METHODS

2.1 Bacterial strain and culture media

K. spormannii EA-1 cultures were obtained as cryostock from the Applied Biology group of Johannes Gescher at the Karlsruhe Institute of Technology (Germany) and sub-cultured at 4%(v/v) inoculum in 100 ml serum bottles closed with a rubber stopper and filled with ES-medium before inoculation of the Microbial Electrochemical System (MES). The ES medium was prepared as previously described (Pillot et al., 2022) with the following content (Carl Roth, Germany) per litre: 0.53 g NH_4Cl, 0.15 g of NaCl, 0.04 g of KH_2PO_4, 0.2 g of yeast extract, 1 ml of 0.1 M $CaCl_2$, 0.12 ml of 1 M $MgSO_4$, and 1 ml of Wolfe's Mineral Elixir (Wolin et al., 1963) and was adjusted to pH 6.5.

2.2 Microbial Electrochemical System

The tests of the different cathodes were performed in a 6-electrode-pairs battery glass reactor, previously described (Erben et al., 2021, Supplementary Information). The cathode was always a 2.25 cm^2 exposed surface of the different material or treated graphite plate, the anode (counter electrode) was a 15×15 mm Ir-Ta mesh (Platinode® Mixed metal oxide Anode 177, umicore, Schwäbisch Gmünd, Germany) and the common reference electrode for all electrode pairs was a Saturated Calomel Electrode (SCE, calculated offset of -215 mV vs. SHE at 60 °C, Sensortechnik Meinsberg, Germany). The employed cathode materials were either graphite (MR40, Müller & Rössner, Troisdorf, Germany), stainless steel plate (SS) (0.5mm thickness, type: 1.4301 IIIc, Metall-Disch, Freiburg, Germany), Electrospun carbon nanofiber electrode (ESCE) (PAN-derived (polyacrylonitrile) carbon fibers, 296±88 nm fiber diameter) produced as previously described (Erben et al., 2021), or copper (CDA C11000, Metall-Disch, Freiburg, Germany). All the cathode materials were cleaned by sonication in isopropanol using an ultrasonic bath for 10 min and further sonicated in DI water for another 10 mins to remove any debris before use in the experiments. The isopropanol step was skipped for the surface modification tests. Electrodes were connected to a potentiostat (IPS Elektroniklabor, PGU-MOD-500mA, Münster, Germany) by titanium wires and polarized at -625 mV vs. SHE. The media were filled in the systems and autoclaved. The systems were agitated with a magnetic stirrer at 150 rpm. The gas mixture (N_2:CO_2:O_2 at 77.5:20:2.5) was purged continuously in the system using flow meters (Analyt-MTC, Germany) and the oxygen

concentration was monitored by an oxy-meter (Oxy-4 Mini, PreSens, Germany), to provide a well-controlled oxygen concentration independent of O_2-production at the anode. The MES were placed in an incubator (Incudrive H, Schuett Biotec, Germany) at a constant temperature of 60 °C. When the conditions were stabilized after 4h, the system was inoculated at 2%(v/v).

For the experiments on the effect of the distance between anode and cathode, specific electrode holders made of polypropylene (PP) were inserted in a modified 6-electrode-pairs battery glass reactor, and the distance was adjusted adding PP spacers of 3mm thickness (See Supplementary Information).

The current density was monitored and plotted over time. The maximum current density (J_{max}) was obtained as the average of the stabilized current density from 1.5 days to 3 days (see Supporting Information). The time of growth ($t_{90\% Jmax}$) was obtained when the current reached 90% of the J_{max} previously calculated. All experiments were performed in triplicates with random position inside the reactor for each condition, and all following measurements are expressed in average with their respective sample standard deviation.

2.3 Electrode modifications

To improve the interaction of *K. spormannii* with a graphite electrode, a number of surface modifications were employed. For the cold air plasma treatment, clean graphite electrode plates were treated on both sides for a duration of 60 s using a cold plasma jet apparatus (piezobrush PZ2 with PZ2 Nearfield Nozzle, Relyon plasma GmbH, Germany). To increase the surface roughness, the graphite plates were prepared by rubbing the surface with sand paper of varying grain sizes. The graphite electrodes were sanded for 10s horizontally and 10s vertically by manually applying a constant pressure with P120 and P500 (Matador, Wasserfest, Germany) on both sides, and subsequently sonicated in a water bath for 5 min to remove any unwanted debris. For the sonication treatment, the carbon electrodes were immersed in DI water and sonicated for 10mins in an ultrasonic bath (Branson 5200 Ultrasonic Cleaner, USA). The UV treatment was performed by subjecting the graphite electrodes to UV light between an exposure time of 30-45 min using an UV lamp (Germicidal G30T8, 30W) at a distance of 10 cm. For the CO_2 activation treatment, the pre-cleaned graphite electrodes were subjected to high temperature treatment (~900 °C) in a furnace (CWF 1200, Carbolite Gero, UK) under CO_2 atmosphere, as previously described for electrospun carbon nanofiber electrode fabrication (Erben et al., 2021). The flame oxidation of

precleaned graphite electrodes was conducted using a Bunsen burner employing natural gas as fuel. After 60 s in the flame, the graphite plate was removed from the flame and allowed to cool to ambient temperature. For the chemical treatments the electrodes were treated separately with two different solvents: following immersion in 10 ml acetone or isopropanol for 10 mins, the electrodes were rinsed with DI water for 10 min. The electrochemical oxidation of the graphite plate was performed in a mixture of H_2SO_4 and HNO_3 at 1.5 $mol \cdot L^{-1}$ and 4 $mol \cdot L^{-1}$ respectively, with a current set at 1.25 $mA \cdot cm^{-2}$ for 30 mins with the previously mentioned potentiostat.

2.4 Metal electrodeposition

A fast method for metal electrodeposition was developed to test the effect of metal catalysts on the biofilm growth of *Kyrpidia spormannii*. The graphite electrodes were first cleaned by sonication in DI water as previously described, and then placed in a two-electrode electrochemical cell, composed of a beaker containing a PP frame holding the working and counter electrodes made of graphite plates at 1cm distance. The electrochemical cell was placed in an ultrasonic bath (Branson 5200 Ultrasonic Cleaner, USA) and sonication was performed during all electro-deposition to increase deposition uniformity. The two electrodes were connected to a Gamry Interface 1000 System (Gamry Instruments, USA) to perform a chronoamperometry with a -10V difference potential between working and counter electrodes for 300 s. The electrolytes were composed of a 0.1 M PBS containing either 25mM of $CuSO_4$, $CrKS_2O_8$, $AgNO_3$, $FeCl_3$ (Carl Roth, Germany) or H_2PtCl_6 (Merck, USA), for their respective metal deposition.

2.5 Measurements of the hydrophilicity of the electrode surface

Contact angle measurements were performed for each of the modified graphite electrodes according to the sessile drop method, used to optically measure the contact angle between the liquid and the surface (Sharma et al., 2019). The measurement was performed with a contact angle measurement system G2 (KRUSS, Germany), using a 5 μL drop of DI water.

2.6 Fluorescence microscopy

Fluorescence microscopy was performed at the end of the experiments on the biofilm growing on the cathode. Bacterial cells were fixed, stained with DAPI and Nile Red (Carl Roth, Germany), and observed on a Zeiss Microscope Axioscope 5/7 (Zeiss, Germany), as previously described (Pillot et al., 2022).

2.7 PHA quantification

After the experiment, the cathodes containing the biofilm used for microscopy were sonicated for 10 min in 10 ml DI water to detach the biofilm from the electrode surface. The cell suspensions obtained were treated by alkaline hydrolysis and HPLC system (Alliance, Waters) as previously described (Pillot et al., 2022). The associated coulombic efficiency was calculated as previously described (Pillot et al., 2022).

2.8 Biofilm quantification through qPCR

The 16S rRNA gene of the cell suspensions previously obtained was quantified by qPCR an Eco 48 Real Time PCR System (PCRmax, United Kingdom), using the primers Alyc630F (5'-GAGAGGCAAGGGGAATTCC-3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3') as previously described (Pillot et al., 2022).

3. RESULTS AND DISCUSSION

3.1 Effect of cathode material

In the present work, four different materials: graphite, copper, stainless steel (SS), and an electro spun carbon nanofiber electrode (ESCE) were characterized with respect to their effect on *Kyrpidia* biofilm growth and PHA production. The chosen materials are amongst the commonly employed materials in microbial electrode investigation in the past (Wei et al., 2011). One of the criteria for the selection of these materials was their surface hydrophilicity. Indeed, previous study on microbial anode optimization has shown that only the hydrophilicity of the electrode, measured through the contact angle of a water droplet, was correlated with higher current production (Sharma et al., 2019). The contact angle measurements are presented in Figure 1. The graphite, copper, and SS plates exhibited contact angles of 30°, 30° and 65°, respectively. The ESCE shows almost zero contact angle because of its porosity, draining the water by capillarity as soon as the droplet is placed on the material. As the hydrophilicity is inversely proportional to the contact angle, graphite and copper exhibit higher hydrophilicity than SS, which has a contact angle 2-fold higher, suggesting a better cell attachment to the two first materials. The absorption of the droplet into the ESCE suggests a high hydrophilicity of the material, even if it cannot be compared with the plate materials.

Furthermore, to investigate if the hydrophilicity increases the biofilm growth, the materials were used as cathode for the development of *K. spormannii* biofilms. The comparison was performed simultaneously on the four materials in the same reactor in triplicates over two batches, sharing the same reference electrode and experimental conditions. These conditions were previously optimized for the growth and PHA production of this bacterial strain (-625 mV vs. SHE, 2.5% O_2 and pH6.5, Pillot et al., 2022). Current monitoring, biofilm density and PHA quantification on each material were assessed and are presented in Figure 2. In each condition, the current density reached a plateau after different periods of time and stabilized around this maximum current density until the end of the experiments after 3 days (see also Supporting Information). As previously mentioned, the stabilization of current consumption by *Kyrpidia spormannii* has been correlated by optical coherence tomography with a full coverage of the electrode surface (Hackbarth et al., 2020). The maximum current density J_{max} (Figure 2, dark-green histograms in the first horizontal panel), reported for the four different materials, shows that the highest current density was attributed to the graphite cathode, with an average and sample standard deviation of 0.44 ± 0.02 $mA \cdot cm^{-2}$ over the triplicates, followed by copper electrode with 0.40 ± 0.10 $mA \cdot cm^{-2}$, SS material with 0.37 ± 0.10 $mA \cdot cm^{-2}$, and ESCE with 0.25 ± 0.08 $mA \cdot cm^{-2}$. However, according to the sample standard deviations, these differences are not significant. The time required for the growth of the biofilm, proxied by the time $t_{90\% Jmax}$ to reach 90% of the maximum current density (Figure 2, light-green histograms in the first horizontal panel), shows faster establishment of biofilms on SS with 0.16 ± 0.08 days, compared to graphite with 0.34 ± 0.24 days, ESCE with 0.77 ± 0.92 days, or copper with 1.42 ± 0.89 days. Thus, no specific correlation can be established between the maximum current density and the time to reach this maximum. We can observe a high sample standard deviation of the time of growth on graphite, copper and ESCE, while relatively small on SS. This could indicate an instability of the material properties or an adaptation step required by the bacteria to adhere on the surfaces, not necessary for SS. Iron compounds (Fe^{2+}, Fe^{3+}, FeS, …) are often retrieved in hydrothermal vents structures (Sander and Koschinsky, 2011), as the one where *K. spormannii* EA-1 was isolated from, and could suggest a specific adaptation to these surfaces. Also, stainless steel and copper, which are known to exhibit catalytic activity e.g. for hydrogen evolution, do not seem to induce more abiotic reactions as their current densities are lower compared to the graphite electrode (Figure S2). Besides, higher current densities were expected for ESCE, as its porosity and structure produce a

higher catalytic surface for the development of the biofilm (Erben et al., 2021), but our results seem to indicate a different limiting parameter in this condition as it exhibits the lowest current density. This could be linked to limited O_2 or nutrient diffusion inside the electrode.

To investigate the relation between current density and biofilm formation, we quantified the cell density in the biofilm at the end of the experiment by two methods, namely, fluorescence microscopy using DAPI staining and qPCR of the 16S rDNA of *Kyrpidia*. Figure 3 reveals the visual observation of the cathode materials before and after MES experiment. The surfaces of all electrodes (Figure 3, right panel) were covered by biofilm (blue signal, stained with DAPI) and contained PHA granules (red signal, Nile red stained) within the cell. Microscopic quantification of the biofilm (Figure 2, dark-blue histograms in the first horizontal panel) does not show a significant difference between Graphite, SS and ESCE with 10.4 ± 0.1, 10.5 ± 0.1 and 10.6 ± 0.1 Log_{10} cells·cm^{-2}, respectively, and slightly lower on copper with 10.0 ± 0.2 Log_{10} cells·cm^{-2}. On the other hand, the qPCR technique (Figure 2, light-blue histograms in the first horizontal panel) shows a higher difference between the materials, with 10.1 ± 0.2 Log_{10} cells·cm^{-2} on graphite, followed by 9.9 ± 0.2, 8.8 ± 0.6 and 7.7 ± 0.5 Log_{10} cells·cm^{-2} for ESCE, SS and copper, respectively. This big difference between microscopy and qPCR results on SS and copper may be explained by the release of metallic ions (Fe^{3+}, Cu^{2+}) during the sonication, that are known to inhibit the DNA polymerase involved in qPCR (Kuffel et al., 2020). This difference may also be explained by the partial death of the biofilm by the production of oxides, such as copper oxides, that are known to be toxic for the cells (Naz et al., 2020). The DNA would then be released in the media while cell debris are still attached on the electrode and observed with microscopy. However, in our experiments the cathodes are poised at a reducing potential of -625mV vs. SHE, so no oxidation of the metallic surface and release of toxic copper ions is expected under these conditions. In the same way, the microscopic observation shows higher biofilm on ESCE than on qPCR, probably due to the physical entrapment of dead cell debris and biomass in the 3D structure of the material. Further investigation beyond the scope of the present manuscript is required to understand the effect of the materials on this difference of quantification between microscopy and qPCR techniques.

Along with the biofilm growth, we investigated the effect of the material on PHA production by *K. spormannii*. The PHA quantification and corresponding coulombic efficiency are presented in Figure 2 (red and yellow histograms respectively, in the first horizontal panel). PHA

production (quantified after 3 days) was significantly higher on SS with 34.8 ± 8.1 $\mu g \cdot cm^{-2}$, followed by 21.0 ± 1.0 $\mu g \cdot cm^{-2}$ on copper, 20.5 ± 1.7 $\mu g \cdot cm^{-2}$ on graphite, and 15.0 ± 0.6 $\mu g \cdot cm^{-2}$ on ESCE. The coulombic efficiency was higher on SS with 2.7 ± 0.6%, followed by 2.3 ± 0.2% on copper, 2.2 ± 0.7% on ESCE and 1.3 ± 0.6% on graphite. These results indicated that the major part of the electrons transferred from the electrode are not used to produce PHA, but rather fixed in the biomass and/or transferred to the electron acceptor O_2. In order to conclude on the best choice for PHA production, a performance indicator was calculated by simply multiplying the produced PHA amount by its relative coulombic efficiency. This indicator (Figure 4) shows a clear distinction between the stainless-steel electrode (2-fold increase) and the rest of the materials. Thus, the stainless-steel material seems to enhance PHA accumulation into the cells compared to the other materials. Our results indicate that this effect is not linked to the favored abiotic H_2 evolution on SS compared to carbon-based or copper materials, as no higher current density was observed in abiotic conditions (Supporting Information). This observation is in line with our previous results on potential screening (Pillot et al., 2022) that H_2 evolution might not be involved in *Kypridia*'s external-electron-transfer mechanism. To investigate this hypothesis, the use of other catalysts enhancing H_2 evolution were evaluated with respect to PHA production and biofilm formation by *Kyrpidia,* as reported in the following section.

3.2 Effect of graphite modification with catalytically active metals

As the stainless steel seems promising for PHA production, we investigated the effect of other metallic catalysts through electrodeposition on graphite plate, to further evaluate if the impact of the stainless steel on biofilm formation and PHA accumulation is linked to an increased H_2 evolution or some other catalytic effect or affinity.

When polarized at -625mV vs. SHE and after the inoculation of the reactor with *K. spormannii EA-1*, a current density is observed on all the electrodeposited cathodes. The Cr, Ag, and Fe electrodes showed similar maximum current densities as the control, with, respectively 0.24 ± 0.08 $mA \cdot cm^{-2}$, 0.39 ± 0.03 $mA \cdot cm^{-2}$, 0.30 ± 0.07 $mA \cdot cm^{-2}$ and 0.35 ± 0.16 $mA \cdot cm^{-2}$. The Cu and Pt exhibited higher current densities with 0.55 ± 0.06 $mA \cdot cm^{-2}$ and 0.63 ± 0.13 $mA \cdot cm^{-2}$. This is not surprising, as platinum is unanimously considered one of the most efficient catalysts not only for the electrochemical oxygen reduction reaction (Wang et al., 2019) but also for the H_2

evolution by water electrolysis (Serov et al., 2021). Then at our working potential, the abiotic O_2 reduction or H_2 evolution is potentially enhanced, as shown in the significantly higher current density on Pt and Cu electrodeposited electrode in abiotic tests in the same experimental conditions (Supplementary Information).

When looking at the time of growth, the shorter time is observed on the control, with 0.28 ± 0.12 days, followed by the Pt electrode with 0.42 ± 0.02 days, the Cr electrode with 0.61 ± 0.19 days, the Fe and Ag with 0.91 ± 0.32 days and 0.91 ± 0.18 days and finally the Cu with 1.41 ± 0.31 days. Thus, the electrodeposition of metals on the surface of the electrode seems to slow down, up to a factor 5, the colonization of the catalytic surface by *K. spormannii*. This could indicate an adaptation step necessary for Kyrpidia to adhere to metals, or the presence of ionic inhibitors (metal ions), requiring a detoxification of the electrode prior colonizing it. Also, the production of radicals ($O_2\cdot^-$, $HO_2\cdot$, H_2O_2 or $\cdot OH$) during oxygen reduction, potentially enhanced by autoxidation of the metals (spontaneous oxidation of metals in contact with oxygen, e.g. during bioreactor preparation, autoclaving, and periods without poised electrode potential), could also require a detoxification step prior to colonize the electrode, but little data is available in the literature to evaluate this potential effect in our conditions (Aust et al., 1985; Nørskov et al., 2004). In a similar way, the potential abiotic reduction of CO_2 into formate, notably enhanced by copper, could not have interfered with the biofilm attachment by producing an alternative electron donor, because *Kypridia* are not known to consume formate or be inhibited by formate, and the same effect is observed on iron that is known to be more selective to H_2 production (Lim et al., 2014).

To assess the colonization of the biofilm, microscopic observations and qPCR quantification give an overview of the final cell density of the biofilm. On the microscopic observation, the control experiment and the Cu electrode have similar cell density, with 9.6 ± 0.3 and 9.9 ± 0.2 Log_{10} cells·cm^{-2}, respectively. The Cr electrode shows lower cell density, with 9.2 ± 0.2 Log_{10} cells·cm^{-2}, while Ag, Fe and Pt show slightly higher cell density with 10.1 ± 0.1, 10.2 ± 0.3 and 10.3 ± 0.2 Log_{10} cells·cm^{-2}, respectively. The qPCR quantification gives another picture, with 9.1 ± 0.6 Log_{10} cells·cm^{-2} on the control, relatively low cell density on Cu with 6.6 ± 0.2 Log_{10} cells·cm^{-2}, and 8.9 ± 1.9, 9.7 ± 1.1, 9.9 ± 1.0 and 9.8 ± 1.3 Log_{10} cells·cm^{-2} on Cr, Ag, Fe and Pt, respectively. As previously mentioned, these significant differences with microscopic observations and high standard deviation can be explained by the release of metallic ions after the

experiment and during the biofilm suspension step, inhibiting the DNA polymerase of the qPCR (Kuffel et al., 2020). Then, we can assume, based on microscopic observations that Ag, Fe, and Pt electrodeposition tend to increase by almost 1 order of magnitude the cell density on the cathode.

With respect to PHA production, the Cu, Cr, Ag, and Pt electrodes, with, respectively 19.0 ± 3.4, 18.5 ± 5.5, 14.7 ± 0.2 and 18.0 ± 5.5 $\mu g \cdot cm^{-2}$, don't show significantly higher PHA accumulation than the control with 17.1 ± 3.0 $\mu g \cdot cm^{-2}$. However, the Fe electrodes exhibit 35.0 ± 4.5 $\mu g \cdot cm^{-2}$, which is twice the value of the control electrodes. The coulombic efficiency follows approximatively the same trend, with similar values for Cu, Ag, and Pt, with 1.0 ± 0.2%, 1.2 ± 0.1%, 1.0 ± 0.4%, respectively, compared to 1.6 ± 0.6% in the control experiment. We obtained a slightly higher coulombic efficiency for Cr with 2.5 ± 0.4%, and the highest value for Fe with 3.5 ± 0.9%. When looking at the performance indicator, the electrodes modified with the iron catalyst exhibit again the best performance (Figure 4), suggesting a specific effect of iron species on the production of PHA by *Kyrpidia spormannii*. Considering the fact that *K. spormannii* grow in a dense biofilm at potentials more positive than the standard potential of hydrogen evolution and that lower performance is obtained with catalysts with higher affinity for H_2 evolution, the effect observed on iron-based materials does not seem to be linked with H_2 evolution. It rather suggests a direct contact mechanism for electron transfer that may be enhanced on a metallic surface mimicking the environment where the strain was isolated. Further investigation is required to elucidate the underlying mechanisms.

3.3 Effect of graphite surface modification by physico-chemical treatments

Besides the investigation of the composition of the catalytic surface, we tested the effect of different surface modifications, either through physical, chemical or electrochemical treatments. These modification methods were selected based on literature review and feasibility in our laboratory. Prior to test in MES, eleven treatments were tested on their ability to increase the hydrophilicity of the electrode surface as a selection factor, as already discussed (See Effect of cathode material) from the study of Sharma et al. (2019). These surface modifications are known to have different effects on the chemistry or roughness of catalytic surfaces. Cold air plasma is known to have multiple effects on catalytic material, such as the reduction, deposition, combination, and decomposition of active components, or the modification, doping, etching, and exfoliation of the catalytic materials, exposing more active sites (Di et al., 2021). UV irradiations

produce a photochemical oxidation of C-C bonds leading to formation of carboxylic acid and ketone (Peng et al., 2020). The CO_2 activation of graphite increase the roughness of the surface by oxidizing the constituting carbon into $CO_{(g)}$, producing down to nano-porous structures (Warczok and Utigard, 2000). Sanding the surface increases the macro- and meso-porosity of the surface. The sonication dislocates graphite residues by breaking Van der Waals bonds. The other treatments oxidize the surface layer by chemical or electrochemical reactions. Graphite electrodes were used as an inert base to ease the comparisons and avoid the release of potential ions by oxidizing the material such as corrosion of the stainless steel.

To assess the hydrophilicity and stability of the treatments, the contact angle of a water droplet was measured after treatment, and after 3, 6 and 8 days immerged in ES media at 60 °C, after autoclaving of the electrode, and after polarization of the electrode in ES media for 4 days. The measurements for each modification are presented in Figure 1. We can observe that even if the control has a relatively high contact angle of 108° at the beginning of the experiment, it stabilized after 3 to 6 days when immersed in ES at 60 °C, at an average value of 75°. This average value is observed over the 8 days of immersion in ES with the treatments with P120 sanding, P500 sanding, soaking in acetone, soaking in acetone and then in isopropanol, or UV treatment in combination with sonication cleaning. Sonication alone decreases the contact angle just after the process, but has no more effect after a few days in ES media. On another hand, the soaking in isopropanol alone (42-62°), the electrochemical oxidation in H_2SO_4/HNO_3 (34°), the treatment with cold plasma (14-26°), the flame oxidation (6-12°) and the CO_2 activation at 900 °C (13-16°) decrease significantly the contact angle, and remains stable over the 8 days in ES medium. To further assess this stability, the electrodes were autoclaved in ES medium at 121 °C for 30 min and polarized at -625 mV vs. SHE for 4 days. In most cases the contact angle decreased further after these conditions, to a minimum of 1° for the cold plasma treatment after autoclaving. These drastic decreases of contact angle indicate higher hydrophilicity of the surfaces. These 5 last treatments were then selected to evaluate their effect on biofilm development and PHA production compared to a control without treatment.

The maximum current densities obtained are presented in Figure 2. The control experiment shows a J_{max} of 0.34 ± 0.01 mA·cm^{-2}, with similar values obtained on isopropanol-, cold plasma-, and flame oxidized-treated electrodes. Higher values were obtained on CO_2 activated and electrochemically oxidized electrodes with 0.52 ± 0.04 and 0.48 ± 0.07 mA·cm^{-2}. The time of

growth ($t_{90\%\ Jmax}$) followed a different pattern, with 0.30 ± 0.03 days on the control, and higher values on all treated electrodes to reach a maximum of 0.88 ± 0.41 days with cold plasma treatment. This indicates that the most hydrophilic surface, as on the cold plasma treated electrode, does not lead to a faster biofilm growth or a higher current density. It might, however have an impact on biofilm cell density.

The corresponding microscopic and qPCR analyses are presented in Figure 2 (3rd horizontal row of graphs). The microscopic quantification shows a slight improvement of biofilm density upon isopropanol, electrochemical oxidation, and flame oxidation treatments with 10.7 ± 0.2, 10.6 ± 0.1, 10.7 ± 0.3 Log_{10} cells·cm^{-2}, respectively, compared to 10.4 ± 0.1 Log_{10} cells·cm^{-2} in the control. The cold plasma does not show any effect on cell density, while a slight decrease is observed on CO_2 activated electrodes, with 9.8 ± 0.2 Log_{10} cells·cm^{-2}. The trend is different looking at the qPCR quantification results, with the control at 9.5 ± 0.1 Log_{10} cells·cm^{-2}, and only the cold plasma treatment showing an increase up to 9.81 ± 0.3 Log_{10} cells·cm^{-2}. The other treatments exhibit a decrease, down to a minimum of 8.3 ± 0.1 Log_{10} cells·cm^{-2} with CO_2 activation. This difference between microscopy and qPCR is surprising in this case, as no metallic ions should be released by the treatment. Moreover, the release of chemicals, such as traces of isopropanol, should be below any inhibition threshold. Thus, we can hypothesize the death of a part of the biofilm on the treated electrode, showing cell debris on microscopy, but less DNA kept on the surface of the electrode. However, this effect would require further investigation to fully understand the underlying mechanism.

Besides the biofilm density, the PHA production is slightly increased by the treatments. In the control experiment, 16.3 ± 2.9 µg·cm^{-2} of PHA were produced, that is increased to 19.1 ± 1.1, 20.3 ± 2.6 and 20.5 ± 0.8 µg·cm^{-2} by electrochemical oxidation, cold plasma and flame oxidation, respectively. The best results were obtained with the isopropanol treatment, with 22.6 ± 0.9 µg·cm^{-2} and CO_2 activation with 23.8 ± 2.0 µg·cm^{-2}. However, the coulombic efficiency decreased to values between 1.79% with Isopropanol to 1.28% with electrochemical oxidation, compared to the control value at 1.9 ± 0.2%. Thus, looking at the performance indicator, we cannot see a significant increase by the treatments, except a slight increase with isopropanol treatment. This observed improvement of isopropanol may be linked to the higher hydrophilicity observed after polarisation (Figure 1), but the reason for this higher hydrophilicity is still unclear. As this ~~last~~ method is probably the easiest and cheapest to perform, in particular with respect to large-scale electrodes, it

could allow to increase by a factor 1.3 the production of PHA and by a factor 2 the biofilm density (based on microscopy results), prior to a subsequent nutrient limitation step to stimulate PHA accumulation (Islam Mozumder et al., 2015).

3.4 Effect of electrode distance

The distance between the anode and cathode is of importance when looking at proton transfer and electron acceptor (in this case oxygen) availability. Indeed, the anodic reaction, feeding the system with electrons and protons, is necessary to allow the proper functioning of the MES. Additionally, in our condition, the major reaction on the anode, favoured by the Ir-Ta coated mesh, is the water electrolysis into O_2. This O_2 can then be directly used by *Kyrpidia* as electron acceptor in addition to the gas feed. In all the previous experiments, the anode was placed back-to-back to the cathode, with a longer travel path for the ions and O_2 to diffuse around the electrode holder. In this setup, the anode has only little influence on the cathode reaction, compared to the O_2 supply from the gas sparger (see Supplementary Information). We then tested the effect of the distance of the electrodes at a much lesser distance, by this time arranging them at 6 different distances and using the control graphite plate as the cathode. Results on current density, biofilm formation and PHA production are presented in Figure 2 in the last horizontal panel.

The current density increased from 0.17 ± 0.02 mA·cm^{-2} at the smaller distance of 6 mm, to a maximum of 0.41 ± 0.05 mA·cm^{-2} at 15 mm distance and then decreased down to 0.31 ± 0.03 mA·cm^{-2} at 21 mm. A same trend is observed for the time of biofilm growth with 0.49 ± 0.08 days at 6 mm, to a maximum of 1.05 ± 0.16 days at 15 mm, and down to 0.87 ± 0.14 days at 21mm. Thus, the best distance for the current density is at a medium distance of 15 mm. This might be the optimal balance between proton transfer across the electrolyte and the O_2 diffusion from the gas feed and the anode under our experimental conditions. However, as some of this current can come from the abiotic reduction of O_2, is this optimal balance also influencing biofilm formation and PHA production?

Looking at the biofilm density after 3 days, no significant effect can be observed, either by microscopic observation or qPCR. Indeed, in all cases the biofilm density amounts to around 9.83 to 10.23 Log_{10} cells·cm^{-2} with standard deviation from 0.1 to 0.67 Log_{10} cells·cm^{-2}. Then, the current density difference observed previously has no impact on the biofilm density at the end of the experiment. However, according to the time to reach the maximum current density, it might

have an impact on the biofilm growth rate, but this cannot be experimentally assessed when the biofilm is only observed at the end of the experiment.

Finally, the PHA production shows a different behaviour with significant differences, inversely proportional to the current density and time of growth. Indeed, the maximum production after 3 days is observed at 6 mm distance with 21.8 ± 1.6 $\mu g \cdot cm^{-2}$, decreasing down to 13.9 ± 0.9 $\mu g \cdot cm^{-2}$ at 12 mm and increasing again up to 20.3 ± 1.5 $\mu g \cdot cm^{-2}$ at 21mm. The coulombic efficiency follows a similar trend with a maximum of 3.2 ± 0.2% at 6 mm, decrease down to 1.3 ± 0.2% at 15 mm, and increasing up to 2.1 ± 0.2% at 21 mm. When looking at the performance indicator, higher values are obtained for closer distance (6 and 9 mm), then for further distances (18 and 21 mm) and finally for intermediate distances. Thus, the PHA production is favoured when the current density is low, corresponding to a small or large distance between the electrodes. Considering the layout of the reactor and electrode frame (Supporting Information), the renewal of media and O_2 from gas feed is limited with close electrodes, and O_2 from the anode is diffused slower with long distance. This could lead to a limitation of electron acceptor for the biofilm, probably producing a slight nutrient limitation step as commonly performed in PHA production with heterotrophic bacteria.

3.5 Overall assessment of PHA production

The overall PHA production performance was increased by 5 folds using an iron-modified graphite electrode together with previously optimized growth conditions (Pillot et al., 2022), as compared to the isolation conditions of *K. spormannii* EA-1 (-531mV vs. SHE, 0.5% O_2, pH 3.5) (Reiner et al., 2020). This suggests that using a large surface-area of iron-based electrodes, such as stainless-steel wool, and a limitation of nutrients (N-source, P-source, or O_2) would most probably increase further PHA production. Based on the cell density obtained under optimized conditions, the average weight of a bacterial cell, and a percentage of 90% PHA in dry mass obtained from heterotrophic cultures (Kourmentza et al., 2017), we could expect a production of 10.5 mg of PHA per cm^2 of cathode, or a production rate of 35 $g \cdot m^{-2} \cdot day$. This result could be compared to the previous photoelectrosynthesis of PHB reported at 91.3 $mg \cdot l^{-1}$ after 7 days (calculated here at 10 $g \cdot m^{-2} \cdot day$) with a poised electrode at +100mV vs. SHE (Rengasamy et al., 2021), but the dependency of this production to the poised electrode is, in our opinion, unclear. Indeed, the effect of the light source is not fully explained, and a high planktonic growth is observed. Furthermore,

the low current density (12 $\mu A \cdot cm^{-2}$) as well as the associated high coulombic efficiency (calculated here according to available data at 13520%) suggest an alternative energy source, probably by anoxygenic photosynthesis. Our theoretical production rate could also be compared to the maximum production of PHA of 3.9 $g \cdot day^{-1} \cdot L^{-1}$ (based on reactor volume) reported by heterotrophic bacteria in liquid culture (Kourmentza et al., 2017), but one has to consider the volume/surface difference of unit and the supplementary costs of material for MES compared to liquid culture. Finally, PHA electrosynthesis is still in its infancy compared to organic acids electrosynthesis, with still a lot of effort required to reach a potential industrial application.

4. CONCLUSION

This study aimed to optimize the cathode properties (Material, surface modification, and electrode distances) for the biofilm formation and PHA production by *Kyrpidia spormannii* EA-1 from CO_2 waste streams. The best results were obtained on iron-based materials, such as stainless steel and Fe electrodeposited electrodes, with a treatment with isopropanol and sonication, and with cathode and anode situated as close as possible. These modifications allowed to increase by 5-fold the PHA production performance of *K. spormannii* compared to the strain's isolation conditions. Finally, hydrogen evolution seems to have little effect on the growth, suggesting a potential alternative electron transfer mechanism.

5. CONFLICTS OF INTEREST

There are no conflicts to declare.

6. ACKNOWLEDGEMENTS

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E-supplementary data can be found in the online version of the paper.

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