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Electrochemical startup increases 1,3-propanediol titers in mixed-culture glycerol fermentations

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ABSTRACT

In this study we investigated the use of electric potential to bioelectrochemically ferment glycerol, a cheap by-product of biodiesel production, into valuable 1,3-propanediol (1,3-PDO). The 1,3-PDO production rates were increased up to 6 times in electrofermentations, compared to non-electrochemical fermentations, and high concentrations up to 42 g 1,3-PDO/l were achieved in fed-batch mode. Extensive growth of the well-known 1,3-PDO producers *Clostridiaceae* (55–57%) was observed when an appropriate potential (−1.1 V vs. SHE) was constantly applied since the start. Potential propionate producers (*Veillonellaceae*) were also among the dominant families (20–21%); however, surprisingly enough, propionate production was not observed. On the contrary, *Clostridiaceae* were absent, *Veillonellaceae* dominated (56–72%), and propionate was produced when electric potential was not sufficient for current production since the beginning. In all cases, glycerol consumption ceased and electrocatalytic activity was lost when we replaced the biofilm electrodes with electrodes lacking a biofilm, clearly demonstrating that glycerol electrofermentation was mostly supported by the bacteria located in the biofilm. In the non-electrochemical systems the performance and the titers achieved were poor; only 18 g 1,3-PDO/l was achieved in more than twice the time, and lactate producing *Lactobacillaceae* became dominant.

1. Introduction

The biorefinery is an alternative to the use of fossil fuels where energy and commodity chemicals are sustainably produced using alternative chemistry processes [1]. Recently, this new concept has attracted a lot of attention from policy makers, research institutes, and the industry [1]. One major process that emerged from this paradigm shift in energy production is the production of biodiesel, which has already exceeded the production of 6 billion liters globally [1]. Biodiesel is produced from the transesterification of triglycerides, using methanol and sodium hydroxide as a catalyst [2]. Apart from the unreacted methanol, a major by-product of the process is glycerol; approximately 1 l of glycerol is produced per 10 l of biodiesel [2], which has resulted in increasing amounts of glycerol produced every year. On the other hand, industrial demands for glycerol did not increase accordingly and glycerol's market price dropped substantially, forcing the closure of a number of glycerol producing plants [3].

A number of value-added products can be produced during glycerol fermentation, like for example hydrogen, ethanol and succinate [4]. Amongst them is 1,3-propanediol (1,3-PDO), a product with an expanding market and a continuously increasing demand of over 50,000 tons per year, which has attracted a great commercial interest because of its extensive use in the chemical industry (e.g. for polymer synthesis, cosmetics, solvents, as an antifreeze, and in lubricants) [4,5].

Bioelectrochemical systems (BES), which employ microbial "catalysts" on electrodes to facilitate electrochemical reactions, have been tested for improving the rates and yields of glycerol conversion. However, the number of studies with glycerol electrofermentations in the cathode remains limited [4,6–8]. Selembo et al. [8] were the first ones to employ polarized anodes and cathodes in single-chamber, batch operating glycerol fermentations, and managed to increase the hydrogen yields produced by conventional glycerol fermentations. Later on, Dennis et al. [7] studied the metabolites produced during continuous, bioelectrochemically-altered glycerol fermentations, in association with the microbial population shifts. Interesting microbial correlations were obtained, showing the relationship between the metabolic products and the microbial population shifts. However, 1,3-PDO production was not the main metabolic product in this study, and the application of

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electrical current did not affect 1,3-PDO production in a positive way. The first study which clearly demonstrated an increased 1,3-PDO production was that of Zhou et al. [4] who used batch biocathodes to study the carbon and electron fluxes during bioelectrochemically enhanced glycerol fermentations. In a more recent study, Choi et al. [6] used pure cultures of *Clostridium pasteurianum* to demonstrate a successful shift in the microbial metabolism toward enhanced 1,3-PDO production when electrical potential is supplied. Improved 1,3-PDO production was demonstrated in both these last two studies; however, it was not the authors' aim to maximize 1,3-PDO concentrations and the systems operated at relatively low 1,3-PDO concentrations (up to 7.22 g/l in Choi et al.).

Extracting 1,3-PDO at low concentrations from the fermentation streams will be costly and ineffective, but on the other hand high 1,3-PDO concentrations can have an inhibitory effect on the microbial populations [9,10]. Production of 1,3-PDO at relatively high concentrations is possible by pure cultures of bacteria like *Enterobacteriaceae* [11] and *Clostridiaceae* [12]. However, using pure cultures will imply considerably higher costs related to avoiding contamination in the bioreactors, and therefore using mixed cultures could be beneficial [7,13,14]. Another argument in favor of using mixed or co-culture populations is that the symbiotic relationships that evolve can have a positive effect not expressed by the individuals [15]; in bioelectrochemical systems, this can result in higher electrical current produced [16], which could be beneficial for enhancing 1,3-PDO production. Despite these arguments, product specificity will be hard to achieve when using mixed cultures, and industrialization of the electrofermentation technology will most likely require defined pure or co-cultures of bacteria. In any case though, identifying the bacteria or bacterial combinations that will be used in this relatively new for the industry technology, is a question that could be approached by investigating mixed bacterial cultures. This will be particularly important to understand the needs and capabilities of these bioelectrochemically-modified environments, and therefore to further optimize their performance.

Up until now, the bacterial species that thrive in mixed culture electrofermentations where 1,3-PDO is the main metabolite produced at high concentrations have not been disclosed. In addition, the effect of electric potential on the performance and bacterial composition has only fairly been studied. In this study we aimed at bioelectrochemically enhancing 1,3-PDO production from glycerol, and we did this by studying glycerol-fermenting biocathodes under different electrochemical conditions and in fed-batch mode. After increasing 1,3-PDO to the highest concentrations reported in a glycerol electrofermentation study, we attempted a deeper insight into the process by investigating how the application of different electrochemical conditions affected the bacterial population, in relation to the different metabolites produced. Finally, cyclic voltammetry analysis allowed us to better acknowledge the effect of the applied conditions on the electrocatalytic activity of the glycerol fermenting biofilm.

2. Materials and methods

2.1. Reactor construction

Dual-chamber, H-type borosilicate reactors with a working volume of 260 mL in each chamber were employed to study the effect of cathodic current evolution on glycerol fermentations. The chambers were separated using a cation exchange membrane (CMI-7000, Membranes International Inc., USA) and reactors were assembled as described in Xafenias and Mapelli [17]. The electrodes were made of graphite felt (SIGRATHERM; SGL Carbon Ltd., UK) and were constructed and pre-treated as described elsewhere [18], with the exception that 0.8 mm titanium wires were used instead of copper, to reinforce the electrodes' resistance to corrosion. New electrodes were prepared for each experiment, with a total projected surface area of 38 cm^2 unless otherwise indicated. In order to avoid the counter electrode (CE) being current limiting, a larger electrode surface area was used in the CE chamber by immersing the 38 cm^2 electrode into a chamber containing 30 pieces of graphite felt with dimensions of 1.5 cm × 2.0 cm × 0.5 cm each. Biological non-electrochemical (NE) experiments were carried out in single borosilicate bottles lacking any electrodes.

2.2. Media and inoculum

A phosphate-buffered mineral medium [19] was used (pH 7.3 which drops to pH 6.6 when CO_2 saturated) in both the working electrode (WE) and the CE chambers of the H-type reactors, and in the control NE reactors. Biotechnology grade glycerol (99%; Amresco Inc., USA) was pulse-fed to give maximum glycerol concentrations of 11.0 ± 1.7 g glycerol/l in the WE chambers of the bioelectrochemical reactors and in the control reactors as noted, whenever concentration was lower than the average value of 0.8 g glycerol/l. When mentioned, pH adjustments were made by manual addition of 5 M NaOH.

The mixed microbial consortium used to inoculate the reactors originated from the anaerobic mesophilic (37 °C) sludge treatment process of Gothenburg's wastewater treatment plant (Gryaab AB, Sweden), and was stored for a period of 6 months at 4 °C prior to use. The bacterial composition of inoculum from the same source has been analyzed previously [17].

2.3. Setup and operation

Three different bioelectrochemical setups were studied, all with polarized WE immersed in the inoculated glycerol-containing medium. In order to maintain anaerobic conditions and to balance the pH rise caused by cathodic current production, the medium was continuously sparged with CO_2. In the first setup (fixed potential; FP), duplicates of electrodes polarized at potentials of −1.10 V operated for 15 days, after which both electrodes and part of the suspension were removed for microbial community analysis. In the second setup (fixed potential-increased electrode surface area; FP-ISA), the WE was in contact with 20 pieces of graphite felt (1.5 cm × 2.0 cm × 0.5 cm) to test the effect of higher current produced under the same potential of −1.10 V. The third setup (varying potential; VP) was a control setup with electrodes, which tested in duplicates whether decreasing the electrode potential from −0.80 V to −1.10 V stepwise would improve the system's performance. In this setup the electrodes were polarized at a starting potential of −0.80 V for the first 19 days and from then on at −0.90 V for 10 days, −0.95 V for 8 days, −1.00 V for 3 days, −1.05 V for 3 days, and −1.10 V for 4 days. At the end of operation the WE of the FP-ISA and the VP reactors were replaced with new ones lacking a biofilm and the experiments were prolonged for another 7 days. This was to test whether current produced under the same potential but with no established biofilm would alter the performance of the system. Additionally, two non-electrochemical setups, one sparged with CO_2 (NE-CO_2) and the other one with N_2 (NE-N_2), were inoculated from the same source and had the same medium as the electrochemical reactors, but ran in the absence of electrodes. Adding electrodes in open circuit would not represent an appropriate control of the FP reactors because of the absence of electrostatic interactions with the planktonic biomass and the medium; in this aspect, the VP reactors were considered more appropriate, and the NE reactors represented a more conventional fermentation system without any electrodes. All reactors employed in this study were covered with aluminum foil to

exclude light and kept at 21 ± 1 °C to test the performance at room temperature.

2.4. Electrochemical monitoring and control

To control the applied potential and to monitor current produced, a three-electrode configuration was used where Ag/AgCl reference electrodes (3 M NaCl; RE-5B, BASi, USA) were placed within 1 cm distance from the WE (+0.20 V; all electrode potentials mentioned are vs. SHE). A multichannel potentiostat (MLab; Bank Elektronik-Intelligent Controls GmbH, Germany) was used for monitoring and control, while performing chronoamperometry and cyclic voltammetry (CV) experiments. Current was recorded every one minute during chronoamperometry experiments and every one second during CV experiments. The CV experiments were performed in three cycles, at a scan rate of 1 mV/s, and under quiescent conditions (no stirring applied).

2.5. Analytical methods and calculations

Samples were centrifuged at 21,100 × *g* before analysis. Glycerol and the potential fermentation products formate, succinate, lactate, acetate, propionate, butyrate, iso-butyrate, 1,3-propanediol, 1,2-propanediol, 2,3-butanediol, 1-butanol, and 2-butanol, were then measured in the supernatant, using a high-performance liquid chromatographer (HPLC; Dionex Ultimate® 3000, Dionex Corp., USA). The HPLC was equipped with a Rezex ROA-Organic Acids H^+ (8%) column (300 mm × 7.8 mm; Phenomenex Inc., Denmark) operating at 80 °C. A 5 mM H_2SO_4 solution was used as the mobile phase at a flow rate of 0.8 mL/min, and all target compounds were detected by a refractive index detector (RI-101; Dionex Corp., USA). High purity compounds (Sigma Aldrich, Sweden) were used to make the calibration curves and to quantify the compounds of interest.

2.6. Bacterial community analyses

Bacterial community analyses were conducted on both samples taken from the biocathodes, and on suspension samples. Samples from the set of the FP biological duplicates (−1.10 V) were taken at a time point (15th day) that represented relatively high 1,3-PDO production rates and yields, to identify the bacteria responsible for this performance. Samples from the VP biological duplicates (−0.80 to −1.10 V) were taken at a time point (47th day) when the potential and current production were similar to those of the FP reactors and right before replacing the electrodes with new ones. Suspension samples from the NE control reactors were taken at the end of operation (68th day), when 1,3-PDO concentration was not considerably increasing. The sampling procedure, genomic DNA extraction, 16S rDNA amplification, cloning, transformation, and partial sequencing of cloned 16S rDNA (GATC Biotech AG, Germany) were performed as described in Xafenias and Mapelli [17]. The nucleotide sequences derived from this study have been deposited to GenBank under the accession numbers KP822548–KP822571.

3. Results

3.1. Glycerol consumption, 1,3-PDO and other metabolites production

3.1.1. Fixed electrode potential reactors (FP and FP-ISA)

In the FP reactors electrodes were continuously polarized at −1.10 V and current was produced at levels of $-28.5 \pm 5.2 \times 10^{-3}$ A (Fig. 1; the more negative the values the higher the cathodic current). Current was produced as soon as the experiments started (Supplementary Fig. S1), causing an initial pH increase to 6.9

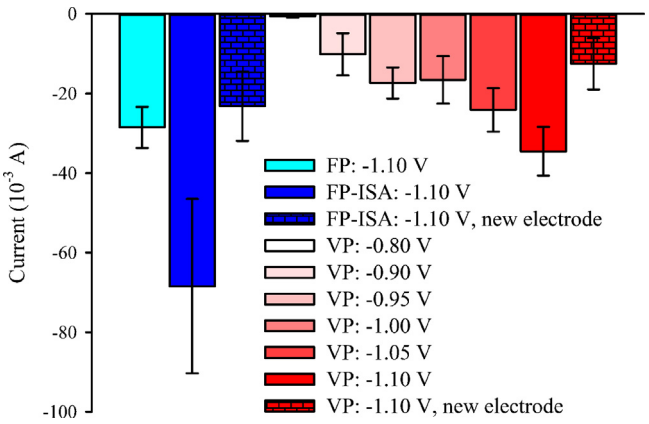

Fig. 1. Average current produced at different applied potentials during chronoamperometry experiments in the fixed-potential reactors (FP; −1.10 V), in the fixed-potential reactor with increased electrode surface area (FP-ISA; −1.10 V), and in the varying-potential reactors (VP; −0.80 to −1.10 V). Error bars represent standard deviations of all current values recorded every minute and throughout the duration of each applied potential. By convention, cathodic current is assigned a negative sign.

(Fig. 2a). After 3 days of startup and by the 15th day, when the electrodes were taken out for bacterial community analysis, 16.2 g glycerol was consumed at the average rates of 1.47 g/d (days 4–15; Fig. 2c). When an increased electrode area was used (FP-ISA), a 2-fold higher current was produced during the first 15 days ($-54.2 \pm 10.3 \times 10^{-3}$ A; Supplementary Fig. S1), however glycerol consumption did not differ considerably compared to the FP systems (Fig. 2c). This indicated that the system had reached a limit over which current was escaping the fermentation as hydrogen gas and was not further utilized for glycerol conversion. With an increased electrode surface area, 17.3 g glycerol was consumed at 1.54 g/d after startup, between days 4 and 15. Yet, only 10.3 g glycerol were consumed during the last 14 days of operation (days 15–29), corresponding to a decreased rate of 0.76 g/d.

Production of 1,3-PDO was 7.3 g 1,3-PDO (0.65 g/d) in the FP reactors and 7.9 g 1,3-PDO (0.71 g/d) in the FP-ISA reactors after startup, between days 4 and 15 (Fig. 2e). These rates were considerably higher than in all other performed experiments and corresponded to a yield of 0.46 g 1,3-PDO/g glycerol in all FP and FP-ISA reactors (Table 1). Nevertheless, prolonging the FP-ISA experiment for another 14 d (days 15–29) produced only 2.5 g 1,3-PDO (0.18 g 1,3-PDO/d; 0.24 g 1,3-PDO/g glycerol). Despite the continuous consumption of glycerol and current production, the concentration of 1,3-PDO reached a plateau on day 29 (Fig. 2e), with the highest 1,3-PDO concentration achieved of 42.10 g/l. A summary of the yields on glycerol, and 1,3-PDO production rates including maximum values observed, is reported in Table 1.

Besides 1,3-PDO, which contained a major part of the glycerol carbon converted (Supplementary Fig. S2), other organic compounds produced are shown in Fig. 3. In all FP-ISA (Fig. 3a) and FP (Fig. 3b) reactors, butyrate and acetate were produced at high concentrations as the main by-products, while lactate and succinate were produced to a lesser extent. Interestingly, the production of butyrate generally followed the observed production trend of 1,3-PDO (Figs. 2e and 3a). On the other hand, both acetate and lactate were initially produced relatively fast (up to 0.5 g acetate during days 4–7 (0.19 g/d) and 0.8 g lactate during days 5–8 (0.27 g/d)), but then product concentrations either increased at lower rates (acetate), or decreased (lactate) possibly due to microbial consumption (e.g. for butyrate, H_2, and CO_2 formation by *Clostridium* spp. [20]).

Another important operational factor is the pH. During operation, the pH dropped down to a minimum of 5.7–6.0 in all FP and

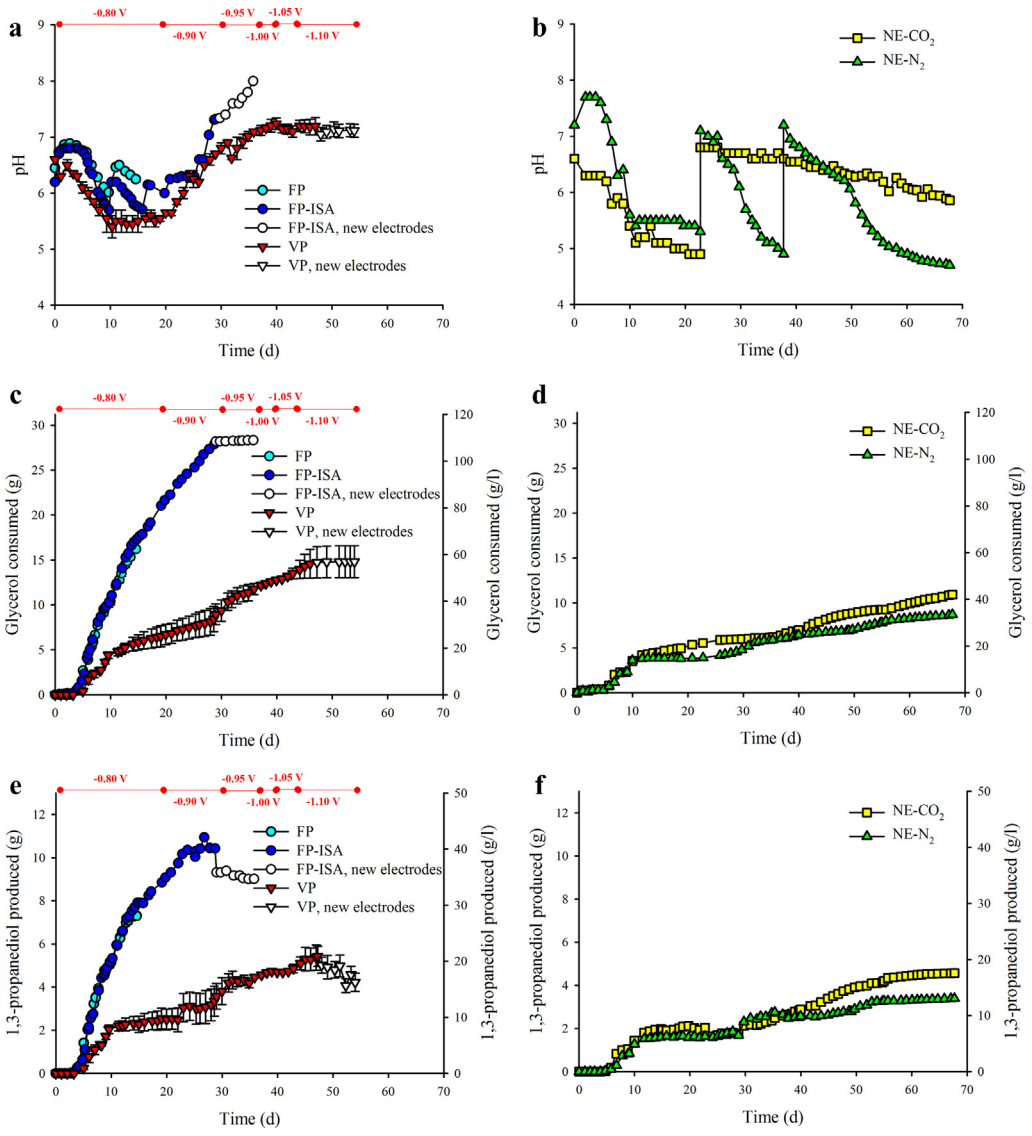

Fig. 2. The pH, glycerol consumption, and 1,3-PDO production changes through time. pH in the bioelectrochemical (a) and the non-electrochemical reactors (b). Cumulative glycerol consumption in the bioelectrochemical (c) and the non-electrochemical reactors (d). Cumulative 1,3-PDO production in the bioelectrochemical (e) and the non-electrochemical reactors (f). Error bars in (a), (c), and (e), indicate min and max of the biological replicates. Red bars on top of (a), (c), and (e) indicate the duration of each applied potential in the VP reactors (red triangles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of the yields and rates of 1,3-PDO production.

	FP	FP-ISA	VP	NE-CO_2	NE-N_2
Yield, g 1,3-PDO/g glycerol consumed (operating period, d)	0.46 (4–15)	0.46 (4–15) 0.24 (15–29)	0.44 (5–10) 0.30 (10–28) 0.32 (28–47)	0.45 (5–10) 0.42 (10–68)	0.40 (5–10) 0.39 (10–68)
Maximum yield, g 1,3-PDO/g glycerol consumed (day observed)	0.55 (7)	0.51 (6)	0.50 (8)	0.47 (56)	0.48 (30)
1,3-PDO production rate, g/d (operating period, d)	0.65 (4–15)	0.71 (4–15) 0.18 (15–29)	0.39 (5–10) 0.06 (10–28) 0.11 (28–47)	0.26 (5–10) 0.06 (10–68)	0.24 (5–10) 0.04 (10–68)
Maximum 1,3-PDO production rate, g/d (day observed)	1.84 (7)	1.81 (6)	0.60 (10)	0.64 (30)	0.66 (7)

FP: fixed-potential; FP-ISA: fixed-potential, increased electrode surface area; VP; varying-potential; NE-CO2: non-electrochemical, CO2- sparged; NE-N2: non-electrochemical, N2-sparged.

FP-ISA reactors on day 10 (Fig. 2a). Adding an alkali to increase the pH was avoided because that would also increase the salinity and alkalinity of the systems and would therefore alter the electrochemical properties and the osmotic pressure of the medium. However, the systems exhibited a remarkable pH self-regulating capability which consumed protons and was able to increase the pH by a maximum of 0.5 pH units within 1 day, without the need for extra chemicals addition (day 10; Fig. 2a). As a result of more efficient protons consumption in the biocathodes, the pH remained at fairly stable levels until the maximum 1,3-PDO concentration was

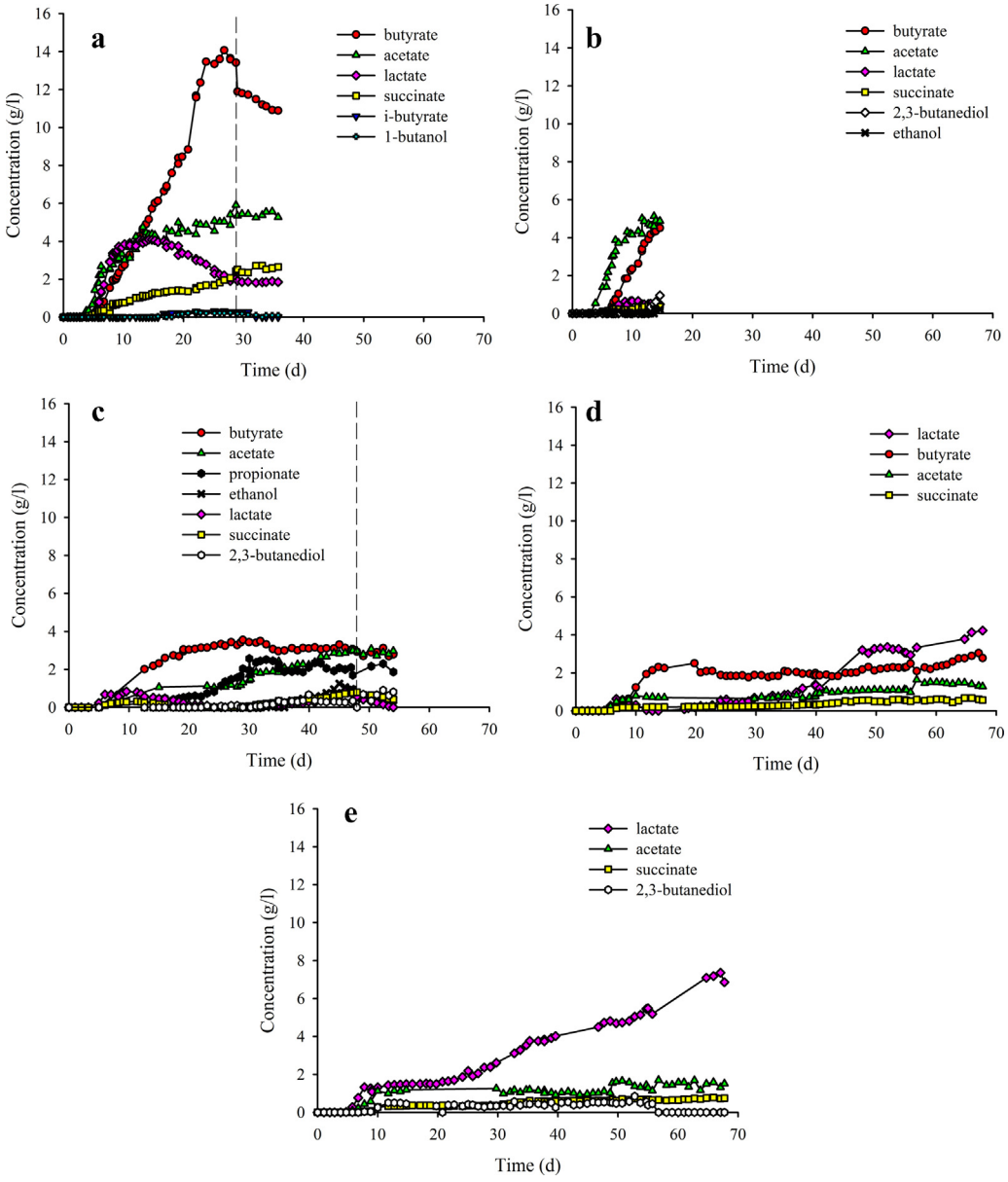

Fig. 3. Metabolites produced with time in the fixed-potential reactor with increased electrode surface area (FP-ISA) (a), the fixed-potential reactors (FP) (b), the varying-potential reactors (VP) (c), the CO_2-sparged non-electrochemical reactor (NE-CO_2) (d), and the N_2-sparged non-electrochemical reactor (NE-N_2) (e). The dashed lines in (a) and (c) indicate the time points where the electrodes were replaced with new ones lacking a biofilm.

reached (Fig. 2e). After that point, the pH increased at high levels as a result of protons consumed at higher rates than produced.

The importance of the biofilm established on the electrodes was demonstrated on day 29, after replacing the FP-ISA electrodes with new ones lacking a biofilm. Glycerol fermentation ceased immediately (Fig. 2c) and concentration remained relatively constant at 7.76 ± 0.18 g/l for the following 7 days. That was despite the fact that current ($-24.5 \pm 8.8 \times 10^{-3}$ A; Fig. 1) and hydrogen (0.1% hydrogen and no methane were detected in the headspace; Supplementary Fig. S3) were still produced, though at lower levels (1/6 of the hydrogen detected in the presence of the biofilm). After replacing the electrodes with new ones, the concentrations of 1,3-PDO and butyrate (and only these) were further reduced by the suspended microbes (1.2 g/l and 1.0 g/l respectively, during the following 7 days), demonstrating a process shift and the importance of the biofilm, in particular for producing these two metabolites.

3.1.2. *Varying electrode potential reactors (VP)*

In the VP reactors, the electrodes were initially polarized at −0.80 V. At this potential current was produced at the very low levels of -0.6×10^{-3} A (Fig. 1), and the startup period was prolonged to 4 days. Also glycerol was consumed at lower rates in this case (Fig. 2c), with average values of 0.87 g/d (4.6 g glycerol consumed between days 5 and 10). Contrary to the pH profiles of the FP reactors, the pH in the VP reactors dropped to 5.4 by day 10 and remained stable under the −0.80 V applied potential (Fig. 2a). After day 10, the glycerol consumption rates were lowered and remained fairly constant to only 0.20 g/d (3.7 g consumed between days 10 and 28), despite the fact that the electrode potential was lowered on day 19 to −0.90 V, resulting in increased current ($-10.2 \pm 5.3 \times 10^{-3}$ A; Fig. 1) and pH (6.6 on day 28). Further lowering the potential stepwise to values down to −1.10 V increased current levels up to $-34.5 \pm 6.1 \times 10^{-3}$ A (at −1.10 V) but pH levels

only up to 7.2. This change increased the average glycerol consumption rates to 0.34 g/d (6.6 g glycerol consumed between days 28 and 47).

In the same reactors, no remarkable difference was observed compared to the NE controls regarding 1,3-PDO production under −0.80 V applied potential (Fig. 2e and f), demonstrating that simply the addition of electrodes under a non-sufficient potential did not have a positive effect (e.g. by simply acting as a growth surface). During days 5–10, 2.1 g 1,3-PDO (0.39 g 1,3-PDO/d) were produced, yielding in 0.44 g 1,3-PDO/g glycerol during this time period (Table 1). However, 1,3-PDO production rates dropped quickly to only 0.06 g/d for the time period of days 10–28 (1.1 g 1,3-PDO produced), yielding in only 0.30 g 1,3-PDO/g glycerol. That was despite the higher current produced from day 19 to day 28 under −0.90 V. Further lowering the potential stepwise down to −1.10 V only increased the average 1,3-PDO production rates to 0.11 g/d (2.1 g 1,3-PDO produced between days 28 and 47) and the yields to 0.32 g 1,3-PDO/g glycerol. No linear relationship with the more current produced was observed, and only 20.63 g 1,3-PDO/l were achieved after 47 days. In addition, a remarkable point here is that when current started to be produced at higher levels in the VP reactors, this happened with a concurrent increase in propionate, which was not observed in the FP or the FP-ISA reactors (Fig. 3c).

Similarly to the FP-ISA reactors, when the biofilm was removed on the 47th day and electrodes were replaced with new ones lacking a biofilm, glycerol fermentation ceased (Fig. 2c) and glycerol concentration remained constant at 1.32 ± 0.09 g/l for the additional 7 days of operation. In addition, 1,3-PDO concentrations were decreased by the suspended microbes, at the average rates of 0.16 g/d during the same time period (1.1 g 1,3-PDO between days 47 and 54; Fig. 2e). That was despite the fact that the pH was not affected and current was still produced, though at lower levels ($-12.5 \pm 6.4 \times 10^{-3}$ A).

3.1.3. Non-electrochemical reactors (NE-CO_2 and NE-N_2)

The startup period was prolonged even more and glycerol fermentation started 5 days after inoculation in the NE reactors (Fig. 2d). Glycerol was consumed by 3.1 g (0.60 g/d) and 3.3 g (0.63 g/d) between days 5 and 10 in the CO_2 and the N_2 reactor respectively, somewhat lower than in the VP reactors for the same time period. Similarly to the VP reactors, these rates dramatically decreased afterwards and concentrations remained relatively stable until day 23. The pH in these reactors dropped quickly, reaching the values of 5.4 and 5.6 in the CO_2 and the N_2 sparged reactors respectively, on day 10 (Fig. 2b). In order to test whether increasing the pH to neutral would have a positive effect on the performance, the pH in both NE reactors was increased to neutral on day 23 and additionally on day 38 in the N_2 sparged reactor. For that reason, experiments were run for a longer period (68 days) in these cases; however, the rate of glycerol consumption did not change considerably, and between days 23 and 68 that was only 0.12 g/d (5.4 g glycerol consumed) and 0.11 g/d (4.8 g glycerol consumed) for the CO_2 and the N_2 sparged reactors, respectively. Overall, this resulted in only 10.94 g (NE-CO_2) and 8.70 g (NE-N_2) of glycerol consumed during the whole 68 days of operation.

The production of 1,3-PDO was also considerably lower in the NE reactors; from day 5 to day 10 only 0.24 (NE-N_2) to 0.26 (NE-CO_2) g 1,3-PDO/d were produced (1.3 and 1.4 g 1,3-PDO produced, respectively; Table 1), and production stopped almost completely from day 10 to day 23, when the pH was manually increased to neutral. Afterwards, and by the end of operation, 1,3-PDO was only produced at average rates varying from 0.04 (NE-N_2) to 0.06 (NE-CO_2) g/d (1.8 and 2.6 g 1,3-PDO produced respectively, from day 23 to day 68), achieving the relatively low concentrations of 13.04 g/l (NE-N_2) and 17.60 g/l (NE-CO_2). Overall, the average yields achieved in these reactors by the end of operation did not differ considerably and ranged from 0.39 (NE-N_2) to 0.42 (NE-CO_2) g 1,3-PDO/g glycerol.

The NE-CO_2 control initially produced other identified compounds at similar amounts to the VP reactors (Fig. 3c and d). However, contrary to the VP reactors where the pH increase was caused by current production, increasing the pH on day 23 by adding NaOH in the NE-CO_2 reactor resulted in increasing lactate production. Regarding the N_2 sparged reactor, no butyrate was detected and lactate was at all times the main metabolite produced after 1,3-PDO (Figs. 2f and 3e).

3.2. Bacterial community enrichment

The examination of the microbial communities can explain the presence of the particular metabolites in each case, as is further discussed in Section 4. As shown in Fig. 4, the bacterial communities that emerged under the diverse conditions differed considerably. Firmicutes was the dominant phylum present in all reactors, though very diverse bacterial families were detected according to the conditions applied. In the FP reactors, 55% of the biomass extracted from the electrode was classified as *Clostridiaceae* (mainly *Clostridium* spp.), followed by 20% *Veillonellaceae* (mainly *Propionispira* spp. and *Zymophilus* spp.). The bacterial composition of the suspension did not differ much and consisted mainly of *Clostridiaceae* (57%; mainly *Clostridium* spp.), *Veillonellaceae* (21%; all *Zymophilus* spp. and *Propionispira* spp.), and *Bacteroidaceae* (13%; all *Bacteroides* spp.). In the VP reactors, *Veillonellaceae* (72%; mainly *Zymophilus* spp. and *Propionispira* spp.) and *Enterobacteriaceae* (18%; *Enterobacter* spp., *Klebsiella* spp., *Raoultella* spp., *Citrobacter* spp., *Leclercia* spp. identified) were the major members of the biofilm population. The bacterial population in suspension of these reactors consisted mainly of *Veillonellaceae* (56%; mainly *Zymophilus* spp. and *Propionispira* spp.), *Porphyromonadaceae* (14%; *Barnesiella* spp. and *Dysgonomonas* spp. identified), and *Enterobacteriaceae* (12%; *Citrobacter* spp., *Enterobacter* spp., *Yokenella* spp., *Leclercia* spp. identified). The populations in the NE control reactor supplied with CO_2 were very different; *Lactobacillaceae* were dominant (50%; mainly *Lactobacillus* spp.), followed by *Shewanellaceae* (19%; all *Shewanella* spp.) and *Clostridiaceae* (10%; mainly *Clostridium* spp.). In the NE control reactor supplied with N_2, only Firmicutes were identified and *Lactobacillaceae* (*Lactobacillus* spp.) represented 98% of all identified bacteria.

3.3. Cyclic voltammetry

As a tool, cyclic voltammetry (Fig. 5) can provide useful information regarding the catalytic activity and the role of the biofilm in the process. Both the VP (Fig. 5a) and the FP reactors (Fig. 5b) produced approximately the same current at −1.10 V. However, in the case of the VP reactors, where the potential was gradually lowered and the electrode was mainly populated by *Veillonellaceae*, cathodic (reductive) current was mostly produced at potentials lower than −0.94 V. On the other hand, irrespectively of the available electrode surface area, the FP reactors (which were populated by both *Clostridiaceae* and *Veillonellaceae*) could produce considerable cathodic current already from −0.65 V, and that was further accelerated at potentials below −0.94 V. Replacing the electrodes with new ones lacking a biofilm had a detrimental effect on the process as it increased the system overpotentials and current was then produced only at potentials below −0.94 V.

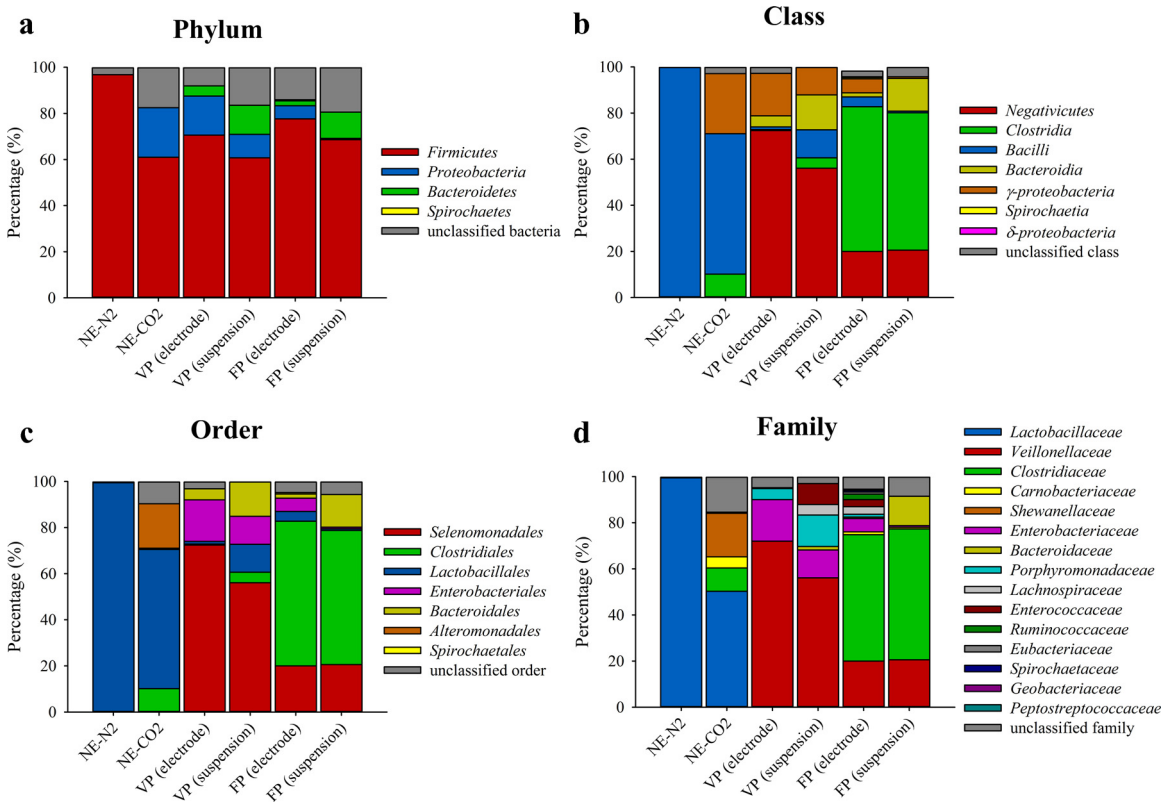

Fig. 4. Microbial analysis of the four experimental setups: suspension of the non-electrochemical control with N_2 (NE-N_2), suspension of the non-electrochemical control with CO_2 (NE-CO_2), electrode and suspension of the varying-potential (VP) reactors, electrode and suspension of the fixed-potential reactors (FP and FP-ISA). Phylum (a), class (b), order (c), and family (d). Class, order, and family represent percentages of classified bacteria only.

4. Discussion

4.1. Mixed culture biocathodes can produce high titers of 1,3-PDO

The concentration of 42 g 1,3-PDO/l achieved in this study is one of the highest reported in studies with mixed microbial populations and the highest reported in bioelectrochemically assisted fermentations (Table 2). This result is remarkable, also considering that it was achieved at ambient temperatures and using simple mineral media without addition of complex or expensive substances like yeast extract, vitamins, or amino acids, which are not readily available in industrial effluents. In comparison to other published studies, the rates reported in our study are well comparable with the calculated 0.55 g/d reported by Zhou et al. [4] (−0.9 V vs. SHE, 100 cm^2 cathode, 3 h of batch operation with 0.83 g/l glycerol, 20–25 °C). Similarly, the yields can be compared with the calculated 0.13 g 1,3-PDO/g glycerol produced in Dennis et al. [7] (−1.3 V vs. SHE, 750 cm^2 cathode, continuous operation with 8.25 g/l glycerol), and the 0.34 g 1,3-PDO/g glycerol calculated in Selembo et al. [8] (0.5 V applied voltage, 7 cm^2 cathodes with Pt catalyst, batch operation with 0.87 g/l glycerol, 30 °C). As also discussed later on, the improved 1,3-PDO production observed in our study could be explained by the application of the appropriate conditions that helped to enrich a bacterial consortium consisting also of species known for their ability to produce cathodic current and 1,3-PDO.

In the non-electrochemical study of Temudo et al. [21] it was reported that mixed population cultures Exhibit 1,3-PDO yields as low as 0.12 g 1,3-PDO/g glycerol. Nevertheless, this could have been more the effect of the unfavorable alkaline conditions applied, rather than inoculum limitations; when a more favorable acidic pH 6.2 was applied in another study, maximum yields of 0.58 g 1,3-PDO/g glycerol were achieved (batch operation, 3 g/l glycerol, 30 °C) [3]. However, it has to be noted that low concentrations of 1,3-PDO (Table 2) were reported in this latter study (though it was not the authors' aim to maximize 1,3-PDO concentration), and if tested, higher 1,3-PDO concentrations could have inhibited further 1,3-PDO production and decreased the reported 1,3-PDO yields [9]. In comparison to the yields achieved by pure bacterial cultures of species identified in our study, the maximum yields obtained herein can be considered competitive (0.45 g 1,3-PDO/g glycerol for *Klebsiella pneumoniae*, 0.47 g 1,3-PDO/g glycerol for *Klebsiella oxytoca*, 0.55 g 1,3-PDO/g glycerol for *Citrobacter freundii*, and 0.57 g 1,3-PDO/g glycerol for *Clostridium butyricum* strains [11,22,23]).

4.2. Biocathodes can tolerate high organic acids concentrations

Apart from 1,3-PDO, organic acids like butyric and acetic acids are known to inhibit microbial growth during glycerol fermentation [9]. These acids are more hazardous in their undissociated form, which is mainly present at low pH values [9]. Therefore, the generally higher operational pH of the FP and the FP-ISA reactors could have allowed tolerance of higher concentrations of these inhibitory byproducts. In addition, the higher pH generally occurring in the vicinity of the cathode electrodes, compared to the pH in the bulk [24], would have generated a more tolerable environment for the bacteria on the cathode, because of the lower concentrations of the undissociated acids very close to the cathodes. Effective tolerance against inhibitory fermentation by-products is essential and can become an important advantage of electrofermentations when compared to conventional fermentations.

Table 2
Operating conditions reported in glycerol fermenting studies with mixed microbial inoculum.

Mixed inoculum source	Max 1,3-PDO concentration reported (g/l)	Operation, reactor type	Medium	Operating temperature (°C)	Source
Anaerobic sludge from the sludge treatment of a municipal wastewater treatment plant	42	Fed-batch, bioelectrochemical (biocathodes)	Mineral medium with trace elements and 11 g/l glycerol	21	This study
Anaerobic sludge from the sludge treatment of a municipal wastewater treatment plant	18	Fed-batch, non-bioelectrochemical (borosilicate bottle reactors)	Mineral medium with trace elements and 11 g/l glycerol	21	This study
Anaerobic sludge from various biogas plants	70	Fed-batch, non-bioelectrochemical (stirred-tank foil bioreactors)	Mineral medium with trace elements, vitamins, amino acids, and 25 g/l glycerol	37	[13]
Granular sludge from an up-flow anaerobic sludge blanket reactor treating brewery wastewater	13	Continuous, non-bioelectrochemical (expanded granular sludge blanket reactors)	Mineral medium with trace elements, amino acids, yeast extract, and 25 g/l glycerol	37	[14]
A mixture of distillery wastewater inoculum and potato starch processing acidification tank inoculum	<7	Continuous, non-bioelectrochemical (continuously stirred reactors)	Mineral medium with trace elements and up to 24 g/l glycerol	30	[21]
Wheat soil	<2	Batch, non-bioelectrochemical (serum bottle reactors)	Mineral medium with trace elements, vitamins, and up to 6 g/l glycerol	30	[3]
Sewage sludge fermenter	1	Continuous, bioelectrochemical (biocathodes)	Mineral medium with trace elements, vitamins, and 8 g/l glycerol	–	[7]
Domestic wastewater	<1	Batch, bioelectrochemical (single chamber microbial electrolysis cells)	Mineral medium with trace elements, vitamins, and up to 3 g/l glycerol	30	[8]
Anaerobic glucose fed pH-stat reactor	<1	Batch, bioelectrochemical (biocathodes)	Mineral medium with trace elements and 1 g/l glycerol	20–25	[4]

4.3. Biofilm related mechanisms are responsible for enhanced glycerol electrofermentations

The effect of replacing the biofilm electrodes with new ones clearly showed that glycerol fermentation was very much relying on the biofilm formed on the electrode surface. Both hydrogen and NADH, which can be produced at potentials below −0.30 V under physiological conditions [25], are potential electron donors for glycerol reduction (for a more detailed analysis of the possible glycerol metabolic pathways by mixed cultures see Zhou et al. [4]). This means that both critical potentials identified by the CVs (−0.65 V and −0.94 V) were potentially related to glycerol reduction. However, the fact that reductive current production started at a higher electrode potential in the FP reactors, indicates that the biofilm composition responsible for the higher glycerol conversion rates also exhibited higher electroactivity (Figs. 2c and 5b).

Gram-positive *Clostridium* spp., the major species found in the FP reactors, are known along with *Klebsiella* spp., *Lactobacillus* spp., and *Citrobacter* spp., for their fermentative metabolism of glycerol that produces 1,3-PDO as sink of the excess of reducing equivalents [26,27]. Additionally, *Clostridium* spp. have been noted for their presence in denitrifying microbial fuel cell biocathodes [28]. In the study of Choi et al. [6], a cathode electrode shifted the metabolic pathways of glycerol fermenting *C. pasteurianum*, resulting in increased 1,3-PDO yields when compared to the yields in the absence of electricity (0.30 vs. 0.16 g 1,3-PDO/g glycerol). Other notable members of the *Clostridium* genus related to our study are *C. ljungdahlii*, *C. acetobutylicum*, and *C. butyricum* [29], because these species are capable of bioelectrochemical reduction of CO_2 (*C. ljungdahlii*) [30], and of glycerol fermentation to 1,3-PDO (*C. acetobutylicum* and *C. butyricum*) [10,12]. In particular, *C. butyricum* has been known to produce high 1,3-PDO concentrations, even by fermenting crude glycerol [31–33]. For these two very important properties of *Clostridiaceae* (i.e. glycerol fermentation to 1,3-PDO and cathode utilization), it could be expected that members of this family thrived in our glycerol electrofermenting reactors and our results confirmed this hypothesis. It should be pointed out that in the absence of glycerol, acetate was the main product produced as the result of CO_2 reduction (Supplementary Fig. S4), probably because other families of the Clostridiales order were favored (e.g. *Acetobacterium* spp.), as our previous research with anaerobic sludge from the same source has shown [17]. It is also interesting to note at this point that in the study of Dennis et al. [7], *Clostridium* spp. were detected only after 6 weeks of biocathode operation and were mostly associated with valerate and not 1,3-PDO production. Also, terminating current supply did not affect 1,3-PDO production in that study (contrary to the production of valerate and propionate, and the consumption of glycerol), even though *Clostridium* spp. were present at that time in the biofilm. In our case, also due to the different conditions applied (e.g. pH was sustained

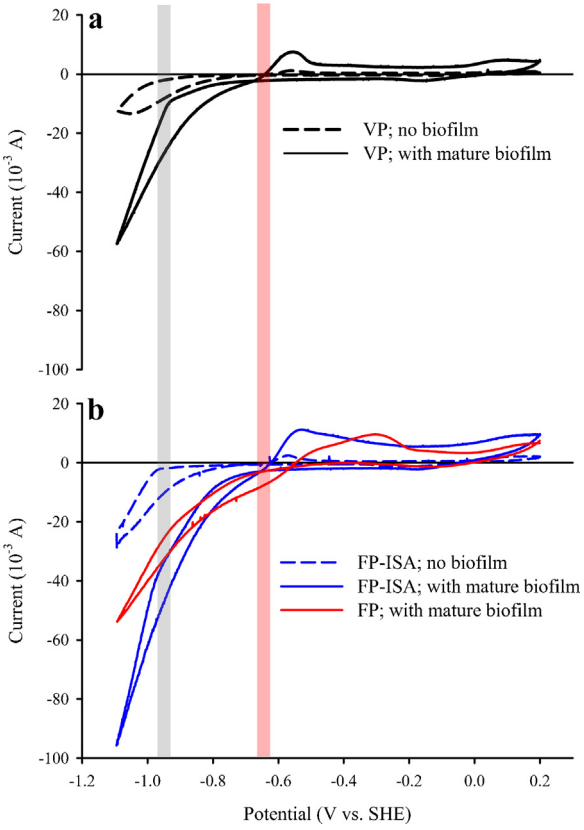

Fig. 5. Cyclic voltammetry profiles (1 mV/s, starting from +0.20 V) in the varying-potential (VP) (a) and in the fixed-potential (FP and FP-ISA) (b) reactors, both before and after replacing the biofilm electrodes with new electrodes lacking a biofilm. The vertical bars indicate the critical potentials where the cathodic catalytic current produced by the biofilm electrodes accelerates.

at 5.5 in that study and galvanostatic rather than potentiostatic mode was selected), different species and symbiotic relationships evolved, leading to the production of high 1,3-PDO concentrations. In a mixed cultures study without electrodes, *Clostridium* spp. were associated with either low (<6) or high (>7) pH values, while *Klebsiella* spp. were dominant between pH 6 and 7 [34]. Again, our biocathode results differ from these conclusions, most probably because of the different design (electrofermentations vs. conventional fermentations) and the conditions under which the biofilm was formed. In addition, the fact that *Clostridiaceae* were either scarce or not found in our VP and NE experiments where 1,3-PDO production was poor, indicates that the appropriate electrochemical conditions applied during startup are important for their successful enrichment in the electrofermentation process.

Apart from *Clostridiaceae*, another abundant family in our glycerol biocathodes was the *Veillonellaceae* family, and most notably the anaerobic, Gram-negative *Zymophilus* spp. and *Propionispira* spp. [35,36]. Members of this family have been observed before on microbial fuel cell anodes (*Anaeroarcus* spp. and *Anaeromusa* spp.) [37] and in biocathodes, where *Sporomusa* spp. were capable of synthesizing acetate from carbon dioxide [30,38]. In our work the *Veillonellaceae* family was found in all cases where electric current was produced, but was not detected in the absence of electric potential. It is also worth mentioning that this family was not found in the reactors of our previous work where we used an inoculum that originated from the same operation plant, and was supplied with electric potential and CO_2, but no glycerol [17]. Presumably, this would be because *Zymophilus* spp. and *Propionispira* spp. anaerobically utilized the electrode or hydrogen as the energy source, but required an organic carbon source for growth. The presence of *Veillonellaceae* can also explain the increased propionate production in the VP reactors [39], where *Veillonellaceae* co-existed with *Enterobacteriaceae* (a family with members which are capable of glycerol fermentation [11]). In the biocathode study of Dennis et al. [7], the conditions applied allowed *Veillonellaceae* (*Pectinatus* spp.) to be enriched earlier on the biofilm than other glycerol fermenters like *Clostridiaceae*, producing propionate as a major metabolite. It is important to stress the fact that in our study propionate was not produced in the FP reactors where *Clostridiaceae* were successfully established on the biofilm. Although *Veillonellaceae* accounted for an important percentage in the biomass, propionate production was not observed and that was probably due to a synergetic effect between the different microbial communities that resulted in altering the end metabolic products. The overall effectiveness of this bacterial combination, compared to that of the individual members, should be further addressed in future studies, using both pure and co-culture cultivations of *Clostridium* spp., *Zymophilus* spp., and *Propionispira* spp. identified in this study. Successful addressing of the role of microbial symbiosis will be a key point to effectively apply electrofermentations in industrial scale.

Lactobacillus spp., the major species found in the NE reactors (but not in the bioelectrochemical ones) are lactic acid producing species which can also metabolize glycerol into 1,3-PDO [40]. Lactic acid production by *Lactobacillus* spp. should have been beneficial for the growth of *Shewanella* spp., which can incompletely oxidize lactate to acetate under anaerobic conditions [41]. *Lactobacillus* spp., together with *Clostridium* spp., were present in the NE-CO_2 reactor, and this is in agreement with another study which operated a mixed anaerobic consortium to produce 1,3-PDO from glycerol [14]. However, neither *Shewanella* spp. nor *Clostridium* spp. were found in the N_2 sparged control setup, where *Lactobacillus* spp. were the only dominant ones.

In conclusion, this study demonstrated that when an appropriately reductive potential is supplied in mixed culture glycerol fermentations, the bacterial population alters and high 1,3-PDO titers can be achieved. The biofilm plays a key role, and establishing the right conditions early in the process can assist to control the bacterial growth in favor of 1,3-PDO production. One of the big challenges that future research will have to address is related to the impurities present in crude glycerol (e.g. metals and methanol), and how these affect the stability of the biofilm composition and therefore 1,3-PDO electrofermentations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2015.06.020

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