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Enhancement of anaerobic lysine production in *Corynebacterium glutamicum* electrofermentations

Nikolaos Xafenias *, Cathleen Kmezik, Valeria Mapelli

Division of Industrial Biotechnology, Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg 41296, Sweden

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ABSTRACT

It has been suggested that application of electric potential can affect lysine producing fermentations, although experimental evidence is lacking. To study this hypothesis we used the lysine producer *Corynebacterium glutamicum* ZW04, and we exposed it to 12 different conditions regarding anaerobic gas environment, applied electrode potential (cathodic, open circuit, anodic), redox mediator and nitrate presence. The gas environment was found to play a major role, with CO_2 leading to double the lysine concentrations and yields when compared to N_2. Electrode potentials also played a major role, with reductive conditions doubling the titers and increasing the yields of lysine up to 4 times. Addition of the redox mediator anthraquinone-2-sulfonate (AQ2S) under the presence of CO_2 and reductive conditions led to additional doubling of the titers, although the yields were not altered considerably. This study demonstrates for the first time that cathodic electrode conditions combined with CO_2 and AQ2S as a redox mediator can significantly improve both the yields and the titers of lysine production of a *C. glutamicum* lysine producing strain, reaching levels that have only been achieved under aerobic conditions.

1. Introduction

Lysine is an amino acid with considerable industrial importance and market value, important in both human and microbial nutrition. Lysine is nowadays produced aerobically by industrial strains of *Corynebacterium glutamicum*, in an aeration-intensive and sensitive process [1]. *C. glutamicum* is a facultative anaerobe that can utilize alternative electron acceptors in addition to oxygen (e.g. nitrate and humic acids) [1–4], and possesses a capability for marginal growth in the absence of an external electron acceptor, in particular when CO_2 is supplied [5]. Anaerobic lysine production could be an attractive alternative option to the aerobic process [1], and from a microbial ecology point of view this is a very interesting topic as *C. glutamicum* are soil bacteria often present in mixed microbial systems [6]. Even though the proof-of-concept has been demonstrated for some lysine producing strains of *C. glutamicum* [1], an efficient strategy for anaerobic lysine production has not yet been developed.

C. glutamicum has been extensively studied and engineered for producing lysine [7], aiming, among others, in modifying the central carbon metabolism, the terminal pathways, and the redox co-factor regeneration systems which are playing a major role [6]. In particular, regeneration of NADPH is a crucial obstacle as 4 mol of NADPH are required to produce 1 mol of lysine from glucose [8]. Intracellular redox balance could be potentially achieved by electrochemically assisted fermentations (electrofermentations), a concept where microbes use electrodes to dispose of or receive electrons from [9]. Although this is a relatively old concept, it has recently regained interest mainly because of "green" electricity expansion and the lower electricity prices that make the grid an attractive source of electrons. Incorporating electrodes as energy source to lysine fermentations could result in cost savings and better market conditions [10], which is an additional reason why we need to learn more about the conditions needed for this strategy to succeed.

Kracke and Krömer recently demonstrated by elementary mode analysis that both anode and cathode electrodes can increase lysine yields from glucose [11]. While anodic electron sinks are expected to result in a proton gradient that will eventually drive ATP synthesis, cathodes are expected to have a higher impact by producing more reduced redox factors (NADPH) [11]. *Corynebacterium* spp. are the main lysine producers and also capable of extracellular electron transfer [12], and therefore they are potential candidates for electrofermentations. In fact this concept has been demonstrated by Hongo and Iwahara, who first showed that there is a potential for increasing glutamate yields from glucose by 10–15% when neutral red is mediating electrons from cathodes used as additional energy sources [13,14]. Recently, Sasaki et al. reported an increase of the lactate yield from glucose by 20% when anthraquinone-2,6-disulfonate was added as a redox mediator in an oxygen-supplemented cathode [15]. In our previous study with a wild type strain of *C. glutamicum* we showed a glucose consumption up to 6 times faster under strongly reductive conditions in the presence of the redox mediator anthraquinone-2-sulfonate (AQ2S) [3].

* Corresponding author.
E-mail address: xafenias@chalmers.se (N. Xafenias).

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In this study we aimed at identifying and understanding the conditions under which lysine production by *C. glutamicum* can be electrochemically supported with the use of polarized electrodes. To achieve this aim we tested *C. glutamicum* ZW04, a lysine overproducing strain [16,17] engineered in a similar way as reported elsewhere for the generation of the AHP-3 strain [18–20]: *lysC* (T311I), hom (V59A), and *pycA* (P458S) [16]. To study lysine production under anaerobic electrofermentation conditions we subjected this strain to 12 different conditions in relation to the gas environment (N_2 and CO_2), the electrode potentials (reductive, open circuit, oxidative), the presence/absence of a soluble electron acceptor (with and without nitrate), and the presence/absence of a soluble redox mediator (with and without AQ2S). This study demonstrates for the first time the importance and different roles of CO_2, reductive potentials, and redox mediator conditions in lysine electrofermentations. This knowledge is necessary to expand the use of bioelectrochemical systems to the production of fine chemicals, and to consider "green" electricity produced from sustainable sources as an alternative electron source.

2. Materials and methods

2.1. Reactor assembling and electrochemical control

H-type bioelectrochemical reactors of a total 720 mL volume were constructed by joining two 360 mL borosilicate bottles together (Adams and Chittenden Scientific Glass, USA). A Nafion® N117 (Ion Power Inc., USA) proton exchange membrane was placed in between each pair of bottles to separate the working electrode (WE) from the counter electrode (CE) chamber. Pretreatment of the proton exchange membrane and assembling of the reactor was performed as previously described [3]. Each bioelectrochemical reactor consisted of a three-electrode setup where the WE and CE were made of graphite felt (SIGRATHERM, SGL Carbon Ltd., UK) with a total projected surface area of 38 cm^2 (5.0 cm × 3.0 cm × 0.5 cm). These were pretreated and bonded to graphite rods (4.5 cm × ∅ 0.3 cm; 40,765, Alfa Aesar®) and Ti wires as described elsewhere [3]. WE, CE, and reference electrodes (RE; Ag/AgCl: 3 M NaCl, RE-5B, BASi, USA) were connected with two-channel potentiostats (MLAB, Bank Elektronik-Intelligent Controls GmbH; Germany) to apply the desired potentials and record the current produced, every 1 min.

2.2. Chemicals

High purity chemicals purchased from Sigma Aldrich (Sweden) were used in this study and all solutions were made using milli-Q water. Pre-cultures of *Corynebacterium glutamicum* were grown in a rich, autoclaved (121 °C, 20 min) growth medium (pH 7.2) consisting of (g/L): bacto peptone (10.0), yeast extract (5.0), glucose (5.0) and NaCl (5.0). The medium used for the WE solution (pH 7.0) was adapted from another study [21] and consisted of (g/L): glucose (10.0), NaCl (1.0), $CaCl_2 \cdot 2H_2O$ (0.06), $MgSO_4 \cdot 7H_2O$ (0.20), $(NH_4)_2SO_4$ (15.0), K_2HPO_4 (10.7), KH_2PO_4 (5.25), $FeSO_4 \cdot 7H_2O$ (20.0×10^{-3}), biotin (0.5×10^{-3}), thiamine·HCl (1.0×10^{-3}), 3,4-dihydroxybenzoic acid (30.0×10^{-3}; dissolved in 950×10^{-6} L of milli-Q water mixed with 50×10^{-6} L of 4 M NaOH), $FeCl_3 \cdot 6H_2O$ (2.0×10^{-3}), $MnSO_4 \cdot H_2O$ (2.0×10^{-3}), $ZnSO_4 \cdot 7H_2O$ (0.5×10^{-3}), $CuCl_2 \cdot 2H_2O$ (0.2×10^{-3}), $Na_2B_4O_7 \cdot 10H_2O$ (0.2×10^{-3}), and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.1×10^{-3}). Prior to mixing the media components, stock solutions were prepared and sterilized separately to avoid precipitation: salt solution (NaCl, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$), $(NH_4)_2SO_4$ solution, buffer solution (K_2HPO_4, KH_2PO_4; pH 7.0) and $FeSO_4 \cdot 7H_2O$ solution (pH 1.0) were autoclaved separately. The vitamin solution (biotin and thiamine·HCl), the 3,4-dihydroxybenzoic acid solution, and the trace elements solution ($FeCl_3 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuCl_2 \cdot 2H_2O$, $Na_2B_4O_7 \cdot 10H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; pH 1.5) were filter sterilized (0.2 μm) separately and mixed when the autoclaved solutions had cooled down. An autoclaved cell washing solution (pH 7.0) was also used and consisted of: 5.8 g/L NaCl, 6.0 g/L K_2HPO_4, and 2.0 g/L KH_2PO_4. This was also used as a CE solution. When mentioned, a nitrate solution (252.0 g/L KNO_3) was added to the WE medium to give concentrations of $557 \pm 72 \times 10^{-3}$ g-NO_3^- N/L. Also the redox mediator AQ2S ($E' = -0.45$ V at pH 7.0 [3], supplemented in the oxidative form) was added in the WE chamber when mentioned, at a final concentration of 0.1 mM (equivalent to an electric charge of 5C), because this particular compound was shown to enhance glucose fermentation by *C. glutamicum* [3]. Adjustments of pH were made using filter sterilized (0.2 μm) HCl and NaOH solutions, whichever appropriate.

2.3. Setup and operation

Twelve different conditions were tested in duplicate (24 reactors in total) to test the effect of the gas environment (CO_2 or N_2), the electrode potential (reductive, open circuit, oxidative), the presence of nitrate as electron acceptor, and the presence of AQ2S as redox mediator. Because the focus of this work is on lysine production and lysine was poorly produced under a N_2 gas environment independently of the electrochemical conditions, all subsequent experiments were performed under CO_2 sparging. The reactors were assembled and filled with milli-Q water prior to autoclaving. After autoclaving the setting up of the reactors was done under sterile conditions in a laminar flow cabinet; the milli-Q water was discarded, the WE chamber was filled with 270 mL of the WE medium, and the CE chamber was filled with 280 mL of the CE solution. Ethanol-sterilized reference electrodes were inserted into the WE chamber in close proximity to the WE. Autoclaved spargers were inserted into the WE chamber and immersed into the solution. Reactors were sparged with filter-sterilized (0.2 μm) CO_2 or N_2 gas depending on the setup. Mixing was performed by magnetic flees on magnetic stirrer plates. The pH in the WE and CE was re-adjusted manually 2–3 times a day to an average daily value of 7; in the case of N_2 sparging and reductive conditions, adjustments were made 4–6 times a day. All experiments were performed at room temperature (20 ± 1 °C).

2.4. Corynebacterium glutamicum ZW04 cultivation

Glycerol stocks (50% v/v glycerol) of *Corynebacterium glutamicum* ZW04 were made as described elsewhere [3] and kept at −80 °C prior to cultivation. For pre-cultures 10×10^{-6} L of stock culture was added in rich growth medium in Erlenmeyer flasks and incubated under aerobic conditions in shakers (KS 4000 I control, IKA®) at 180 rpm and 30 °C. After two days the cells were harvested by centrifugation (Allegra™ 25R Centrifuge, BECKMAN COULTER™) of the fermentation broth for 20 min (5000 ×*g*, 4 °C). Cells were then washed as outlined in a previous study [22] prior to inoculation of the WE reactor chambers. Cells were then suspended in 10×10^{-3} L of WE medium and added into 270 mL of medium already in the WE chamber, resulting in an initial OD_{600} of 1.2.

2.5. Analytical methods and calculations

Sampling was performed daily by extracting 1.0×10^{-3} L of working chamber solution using sterile syringes. Samples were first analyzed for pH and OD_{600} and then centrifuged (5 min, 21,100 ×*g*) and filtered (0.2 μm) prior to further analyses. A high-performance liquid chromatographer (HPLC; Dionex® Ultimate 3000, Dionex Corp., USA) equipped with a Rezex™ ROA-Organic Acids H^+ column (8%, 300 mm × 7.8 mm, Phenomenex Inc., Denmark), a refractive index detector (RI-101; Dionex Corp., USA) and a variable wavelength detector (VWD 3100; Dionex Corp., USA) was used to monitor glucose and organic acid concentrations (lactate, acetate, succinate). Lysine concentrations were determined electrochemically with an ion chromatographer (IC; Dionex® ICS-5000, Dionex Corp., USA) equipped with a Dionex AminoPac™ PA-10 column (250 mm × 2 mm; Dionex Corp., USA). HPLC and IC methods

were performed as described previously [3]. Nitrate and nitrite were measured spectrophotometrically (DR3900; HACH-LANGE® AB, Sweden) using test kits (HACH-LANGE® LCK 340 and LCK 342 respectively) and following the instructions of the manufacturer.

3. Results and discussion

3.1. Charge production, pH and nitrate reduction

Total charge produced over the 7 experimental days represents the total amount of electrons transferred from (cathodic) and to (anodic) the electrode and is shown in Fig. 1. Charge consumed under cathodic and produced under anodic conditions is shown in Fig. 1a and Fig. 1b respectively, and the formula used for the calculations is presented in Supplementary Material. Total cathodic charge consumed was between 15,500C and 35,000C. This corresponded to an average cathodic current between 26 and 58 $\times 10^{-3}$ A (between 6.8 and 15.3 A/m^2), with the highest charge observed in the case of CO_2 sparging conditions. Addition of AQ2S did not considerably affect charge consumption, despite the favourably negative electrode potentials for AQ2S reduction. This indicated that other parameters (e.g. fermentation conditions) had a greater effect on the cathodic current produced. Addition of nitrate did not alter cathodic charge considerably, however this was approximately 5 times the charge produced abiotically within a 10 d period in our previous work [3] (−1.25 V, 1.1 g NO_3^--N/L initial, 38 cm^2 electrodes). Anodic charge produced was 2 orders of magnitude lower than cathodic charge, corresponding to currents between 0.18 and 0.63 $\times 10^{-3}$ A (between 0.1 and 0.2 A/m^2). Replacing CO_2 with N_2 resulted in a 43% decrease of anodic charge, while addition of nitrate as a competing electron acceptor ($E' = 0.43$ V at pH 7.0 for the incomplete reduction to nitrite; [23]) caused a decrease in charge production by 17%. Addition of AQ2S as a redox mediator in a CO_2 gas environment on the other hand doubled the electron transfer rate, and anodic current increased from 0.32 ± 0.12 $\times 10^{-3}$ A to 0.63 ± 0.12 $\times 10^{-3}$ A (from 0.1 ± 0.0 to 0.2 ± 0.0 A/m^2). This clearly indicates the ability of *C. glutamicum* ZW04 to utilize this redox mediator for organic carbon oxidation coupled to electrode reduction, as also observed for a wild type strain [3].

The different electrochemical conditions applied had a slight effect on the pH (increasing in cathodes and decreasing in anodes), however this was mitigated by the pH adjustments made and the buffering capacity of CO_2 (Fig. S1). In the 8 reactors operating under cathodic conditions the pH was 7.2 ± 0.6; this is compared to 6.9 ± 0.4 in the 8 reactors under open circuit and 6.9 ± 0.5 in the 8 reactors under oxidative conditions.

Nitrate supplemented to *C. glutamicum* as a soluble electron acceptor under the three electrochemical conditions was also monitored. Nitrate reduction was faster under strongly reducing conditions, with only 2% of nitrate left on the 4th day of operation (0.6 g N-NO_3^-/L consumed; Fig. S2a). This is compared to 31% and 20% of nitrate left under open circuit and oxidative conditions respectively and is attributed to the reductive current produced which also consumed part of the reduced nitrate. In abiotic experiments in our previous work, the same amount of nitrate (0.6 g N-NO_3^-/L) was reduced only after 10 d of operation [3] (−1.25 V, 1.1 g NO_3^--N/L initial, 38 cm^2 electrodes). Despite the fact that nitrate addition did not clearly produce a higher cathodic current, it is clear that reductive current was responsible for the reduction of nitrite, the product of nitrate reduction by *C. glutamicum* (Fig. S2b). While 87–93% of NO_3^--N was recovered as NO_2^--N under open circuit and oxidative conditions, no NO_2^--N was detected under reductive conditions by the end of the 7 d operation period.

3.2. Glucose consumption enhancement

Glucose consumption under the different conditions applied is shown in Fig. 2. When CO_2 was supplied and in the absence of AQ2S or nitrate, glucose consumption did not differ much when either cathodic or open circuit conditions were applied (Fig. 2a). However, glucose consumption under anode conditions was more than double that under cathodic and open circuit conditions and reached 9.1 ± 0.2 g/L on the 7th day. Under a N_2 atmosphere (Fig. 2b) or when nitrate was supplemented as an additional electron acceptor in a CO_2 environment (Fig. 2c) glucose consumption was from 3.0 ± 0.7 to 6.5 ± 0.7 g/L within the 7 d time span. A great effect was observed when AQ2S was supplemented in the fermentation under reductive and open circuit conditions (Fig. 2d), where 10 g/L of glucose was consumed within 3 d (−1.25 V) and 4 d (open circuit) respectively. Rapid glucose consumption in the presence of a cathode electrode and AQ2S as a redox mediator is in accordance with our previous findings with a wild-type strain [3], thus stressing the importance of this redox mediator in the electron transfer chain of *C. glutamicum*. No such effect was observed when AQ2S was used as a redox mediator for anodic current production, in accordance with the rapid drop of the biomass concentration observed in that case (Fig. S3).

3.3. Enhancement of lysine production

Lysine concentrations under CO_2 environment ranged from 75 ± 21 $\times 10^{-3}$ g/L under open circuit to 154 ± 27 $\times 10^{-3}$ g/L under reductive conditions (−1.25 V) (Fig. 3a). Concentrations were significantly lower when N_2 was used, and the maximum concentration observed in all N_2 cases was 12 ± 4 $\times 10^{-3}$ g/L (Fig. 3b). When nitrate was present at start, lysine production appeared independent of the electrode conditions and reached a maximum value of 112 ± 11 $\times 10^{-3}$ g/L in all cases. Adding AQ2S under oxidative or open circuit conditions did not have a tremendous effect as it had on glucose consumption under open circuit conditions (Fig. 2d, Fig. 3d); in these cases the maximum lysine

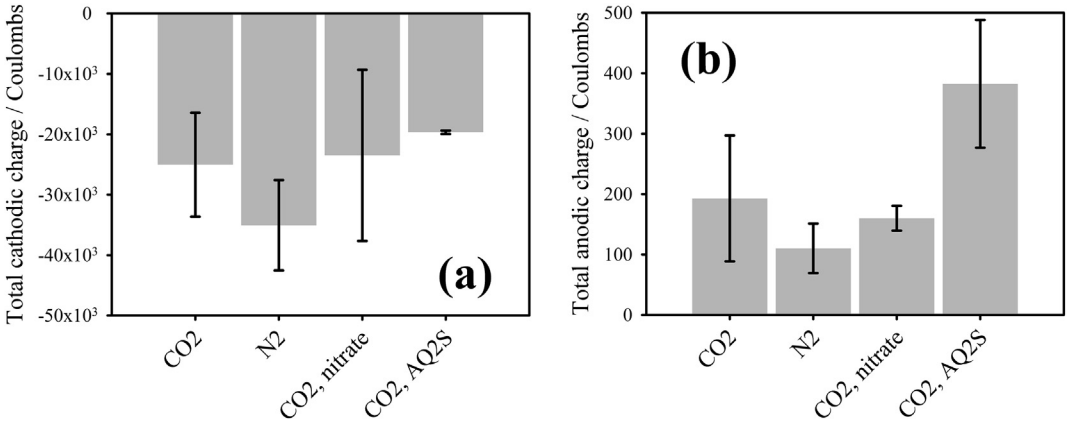

Fig. 1. Charge produced by the biocathodes (a) and the bioanodes (b) under −1.25 V and +0.45 V respectively. Error bars indicate min and max values obtained from the replicates.

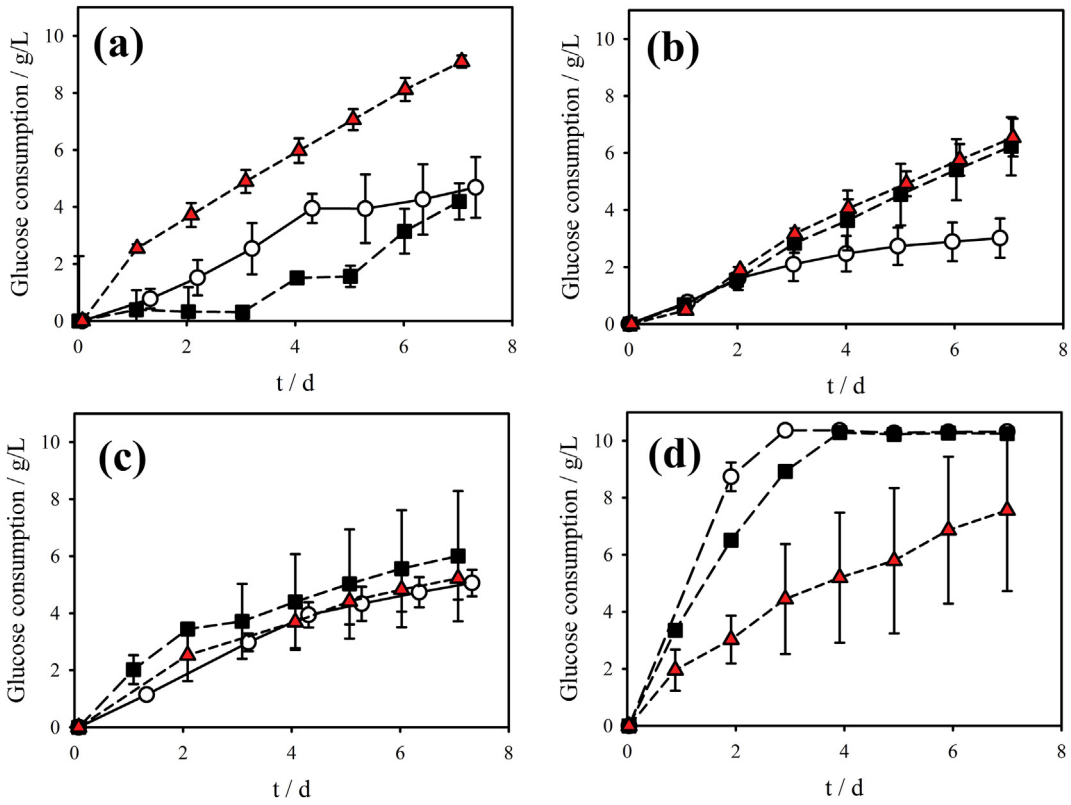

Fig. 2. Glucose consumption; under reductive (−1.25 V; white circles), open circuit (black squares), and oxidative (+0.45 V; red triangles) electrode conditions (a–d) with CO_2 (a), N_2 (b), CO_2 and nitrate (c), CO_2 and AQ2S (d). Error bars indicate min and max values obtained from the replicates.

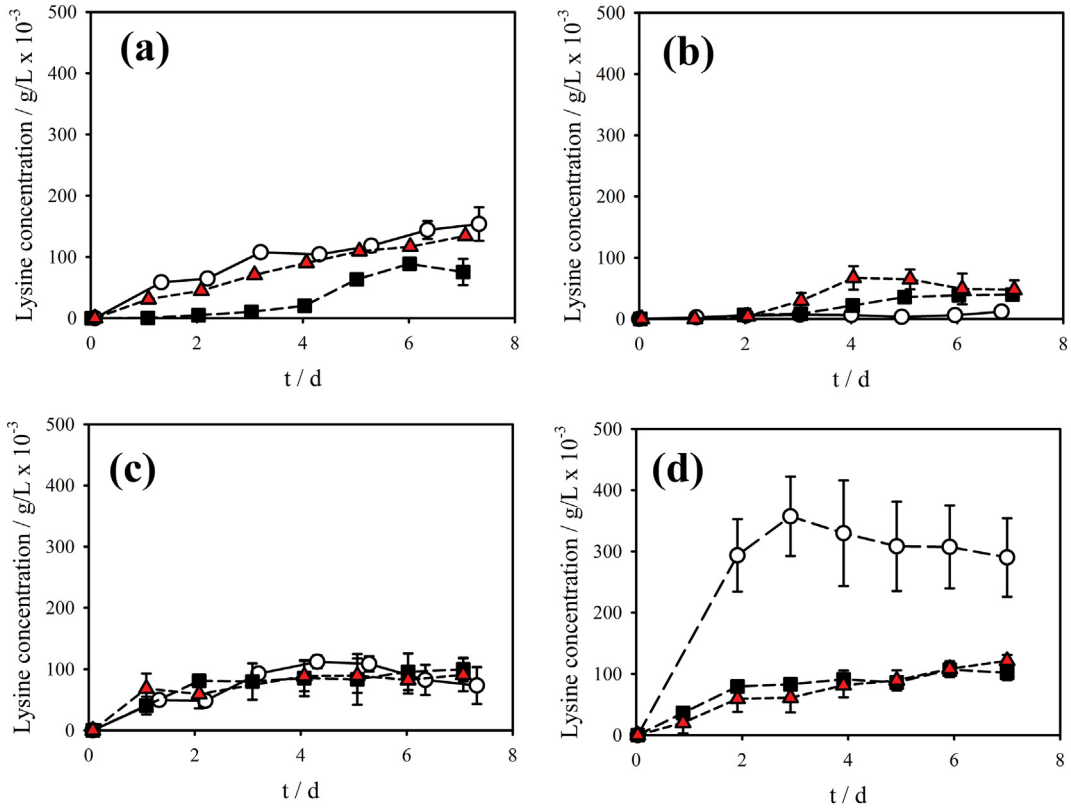

Fig. 3. Lysine production; under reductive (−1.25 V; white circles), open circuit (black squares), and oxidative (+0.45 V; red triangles) electrode conditions (a-d) with CO_2 (a), N_2 (b), CO_2 and nitrate (c), CO_2 and AQ2S (d). Error bars indicate min and max values obtained from the replicates

produced ranged from 102 ± 12 to 121 ± 9 × 10^{-3} g/L. On the other hand, the maximum lysine concentrations observed tripled when AQ2S and reductive electrode conditions were applied under a CO_2 environment, reaching a maximum of 357 ± 65 × 10^{-3} g/L (2.4 ± 0.4 × 10^{-3} M). This maximum concentration was observed on the 3rd day, upon complete consumption of glucose. The same lysine concentration range was reached when fermentations were initially supplied with higher concentrations of glucose (27–55 g/L) under all other applied conditions staying the same (CO_2, AQ2S, −1.25 V). In those cases the maximum lysine concentrations ranged from 297 ± 5 to 341 ± 35 × 10^{-3} g/L (Fig. S4).

Lysine yields relative to glucose consumed are presented in Fig. 4a. The lowest lysine yields were observed under N_2 sparging and ranged from 5 ± 1 to 9 ± 3 × 10^{-3} mol-lysine/mol-glucose consumed. The highest lysine yields were produced by *C. glutamicum* under cathodic conditions, and in a CO_2 environment. In the absence of AQ2S, yields reached 40 ± 10 × 10^{-3} mol-lysine/mol-glucose consumed, while when AQ2S was supplied these were slightly increased to 43 ± 8 × 10^{-3} mol-lysine/mol-glucose consumed; in contrast to its effect on the concentrations, addition of AQ2S did not seem to affect the yields considerably. All other conditions produced lysine yields which were lower than 24 × 10^{-3} mol-lysine/mol-glucose consumed, a value that is 40–56% lower than the maximum values observed. Even though nitrate addition did not offer a particular advantage in neither the lysine concentrations or the yields, the percentage of carbon present in lysine compared to the carbon in all organic molecules detected was the highest (5.5%) when reducing conditions were applied.

In a previous study where the ZW04 strain [16] was exposed to aerobic conditions, 13 g/L glucose and 30° C, lysine concentrations and yields reached 570 ± 0 × 10^{-3} g/L and 72 ± 4 × 10^{-3} mol-lysine/mol-glucose consumed, respectively. Similar concentrations (512 ± 0 × 10^{-3} g/L) and yields (62 ± 3 × 10^{-3}C-mol/C-mol) were obtained when 10 g/L sucrose were supplemented to the same strain under the same temperature and aeration conditions in another study [17]. The maximum concentrations and yields observed in our study were 31–38% and 31–40% lower respectively, which can be attributed, apart from the anaerobic conditions applied, also to the lower (by 68%) biotin concentrations used in our study. As *C. glutamicum* is a biotin auxotroph and lysine production relies on biotin-dependent enzymes [16,24], achieving the maximum lysine concentrations possible will require further medium optimization.

Organic acids were co-produced during the anaerobic fermentation as is also discussed in the next paragraph. It is interesting to note the fact that the part of soluble organic carbon ending up in lysine, compared to the total soluble organic carbon ending up in organic products, was higher under a CO_2 environment and particularly under reductive conditions (Fig. 4b; for the calculation formula see Supplementary Material). Under a CO_2 environment, lysine-C was 4.0–5.5% in the case of cathodic electrode conditions; this is compared to only 1.1–2.6% under open circuit and 1.8–2.9% under oxidative electrode conditions. A remarkable case again is that of N_2 addition, where lysine-C was considerably lower, ranging from 0.9 to 1.5%.

Lysine is essential for both humans and microbes, therefore our findings can be useful both for industrial lysine production and for studying the effect on mixed microbial systems. Even though anaerobic production could have important benefits, this has only been scarcely reported and with a limited number of strains (e.g. *C. glutamicum* AK-1; [1]). This is the first study reported with *C. glutamicum* ZW04 producing lysine under anaerobic conditions, even though the same strain has been studied recently for its tolerance to adipic acid, in relation to the potential production of adipic acid using lysine as a precursor [25].

3.4. Production of organic acids

Maximum concentrations of organic acids lactate, succinate and acetate, which are metabolites typically produced by *C. glutamicum* under oxygen deprivation conditions, are shown in Fig. 5. Lactate was the main metabolite observed in all cases (Fig. 5a), followed by succinate (Fig. 5b) and small amounts of acetate (Fig. 5c). The highest maximum concentrations of lactate observed were 6.7 ± 1.0 g/L (open circuit, CO_2, AQ2S) and 7.1 ± 0.3 g/L (−1.25 V, CO_2, AQ2S). However, the maximum lactate yield was observed under N_2 sparging and oxidative conditions, and was 1.9 mol-lactate/mol-glucose consumed (Fig. S5a). This is compared to the lactate yields of up to 1.6 mol-lactate/mol-glucose consumed by a wild-type strain in the study of Sasaki et al., when cathodic conditions showed the highest yields under oxygen deprivation [15]. There is a distinct difference from our observations with the ZW04 though, as the lactate yield increased under N_2 sparging according to the sequence −1.25 V < open circuit < +0.45 V.

Succinate was produced at concentrations up to 3.3 ± 0.1 g/L (open circuit, CO_2, AQ2S) and yields up to 0.59 mol-succinate/mol-glucose consumed (open circuit, CO_2; Fig. S5b). A noteworthy fact here is that under N_2 sparging succinate was produced at considerably lower

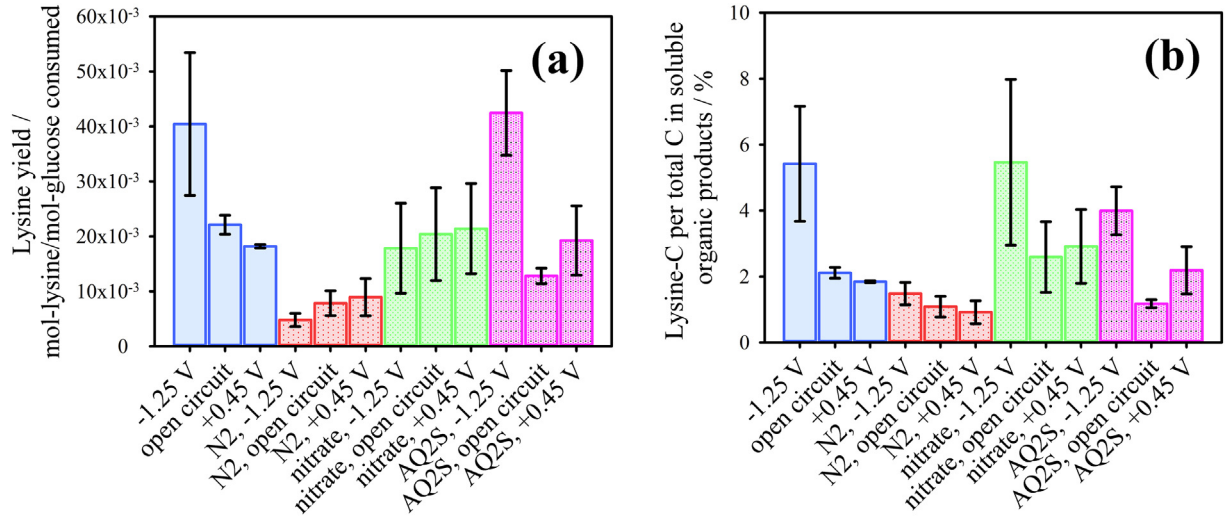

Fig. 4. Lysine yields (a) and % of lysine carbon (b) under the different gas, electrode potential, and redox mediator conditions. Blue bars; CO_2, red bars; N_2, green bars; CO_2 + NO_3^-, purple bars; CO_2 + AQ2S. Error bars indicate min and max values obtained from the replicates. Yields were calculated for the last day of the experiments except when glucose was depleted before that time; for the latter, yields were calculated for the day when no glucose was detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

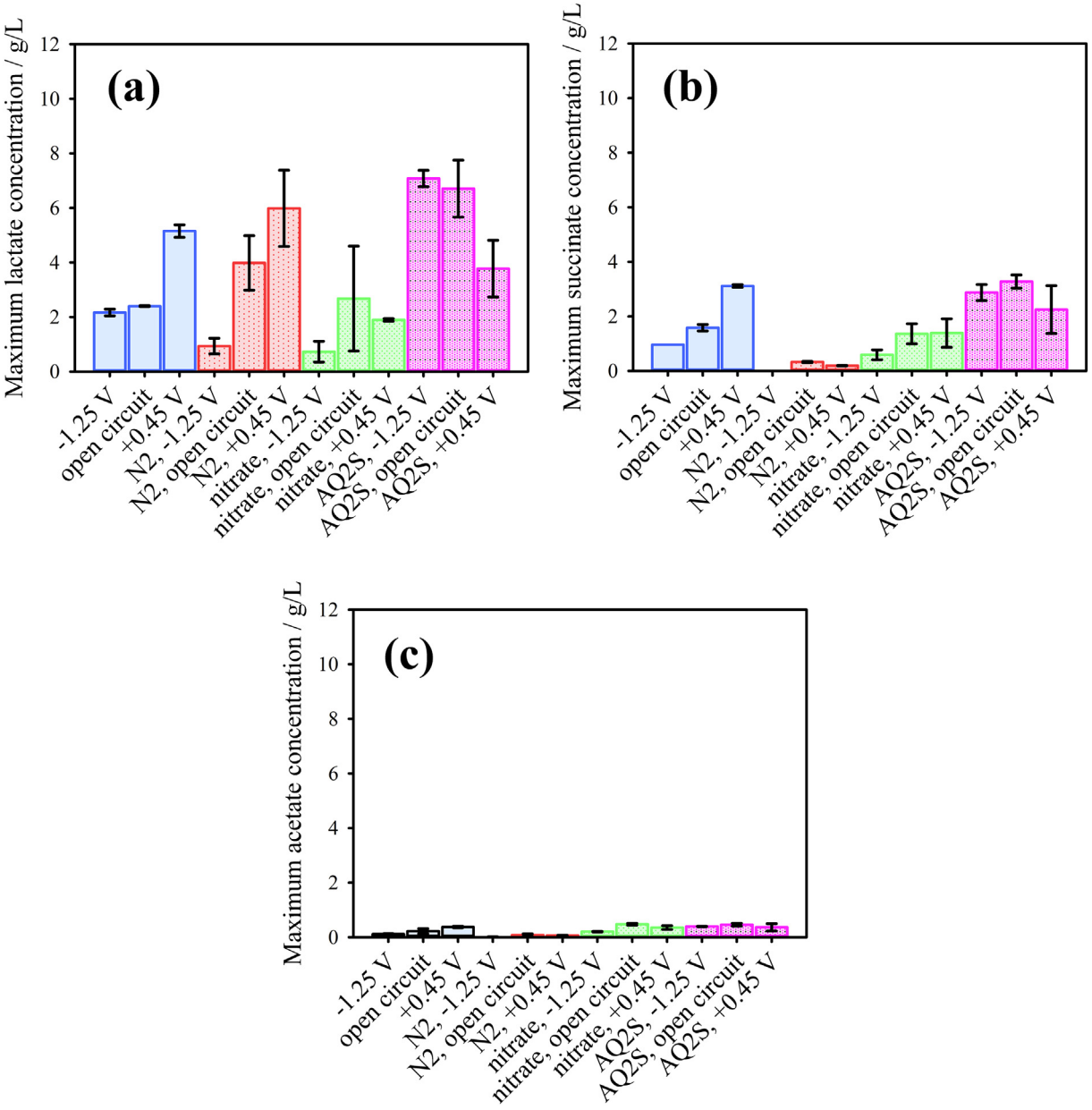

Fig. 5. Metabolites production (maximum values) under the different gas, electrode potential, and redox mediator conditions; lactate (a), succinate (b), and acetate (c). Blue bars; CO_2, red bars; N_2, green bars; $CO_2 + NO_3^-$, purple bars; CO_2 + AQ2S. Error bars indicate min and max values obtained from the replicates. Note the different y-axis scale used in (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentrations of up to 0.33 ± 0.02 g/L and yields of up to 0.08 mol-succinate/mol-glucose consumed. Acetate was produced at concentrations up to 0.61 ± 0.13 g/L and yields up to 0.24 mol-acetate/mol-glucose consumed (Fig. S5c). Likewise, when N_2 was supplied instead of CO_2, yields were considerably lower and not more than 0.04 mol-acetate/mol-glucose consumed. Overall, the total carbon yield for the 3 organic acids (calculation formula presented in Supplementary Material) in the N_2-sparged reactors was up to 0.97 mol-C in organic acids/mol-C in glucose consumed (+0.45 V, N_2; Fig. S5d). In addition, lower concentrations of organic acids were produced under all cathodic conditions in the absence of AQ2S, when compared to the open circuit and oxidative conditions (Fig. S5a–d). The relatively lower values of the cumulative organic acid yields observed in some cases (Fig. S5d) can be attributed to the production of other carbon by-products not targeted in our analysis (e.g. amino acids, CO_2 and biomass), cell stress and the use of carbon for production of maintenance energy. This is also in accordance with the charge balance (Fig. S6), where in most cases charge supplemented from glucose was higher than the charge stored in organics. An exception was the case of AQ2S, −1.25 V though, where the charge stored in organics was 2% (673C) higher than that supplemented as glucose. This would have been the result of using an additional source of electrons (e.g. cathode); although relatively small, this extra charge could have caused a metabolic shift towards more reduced products [26].

3.5. Pathways involved and the role of CO_2, reducing conditions and AQ2S

The metabolic pathways involved in lysine and organic acids production by *C. glutamicum* are shown in Fig. 6. As can be seen, both CO_2 and NADPH play a crucial role in the anaerobic production of lysine. On one hand, CO_2 is incorporated into oxaloacetate by a pyruvate carboxylase; on the other hand, high availability of NADPH is also crucial, with 4 mol of NADPH involved in the conversion of oxaloacetate to lysine [6,19]. The major contributors to the NADPH supplies of *C. glutamicum* are glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase,

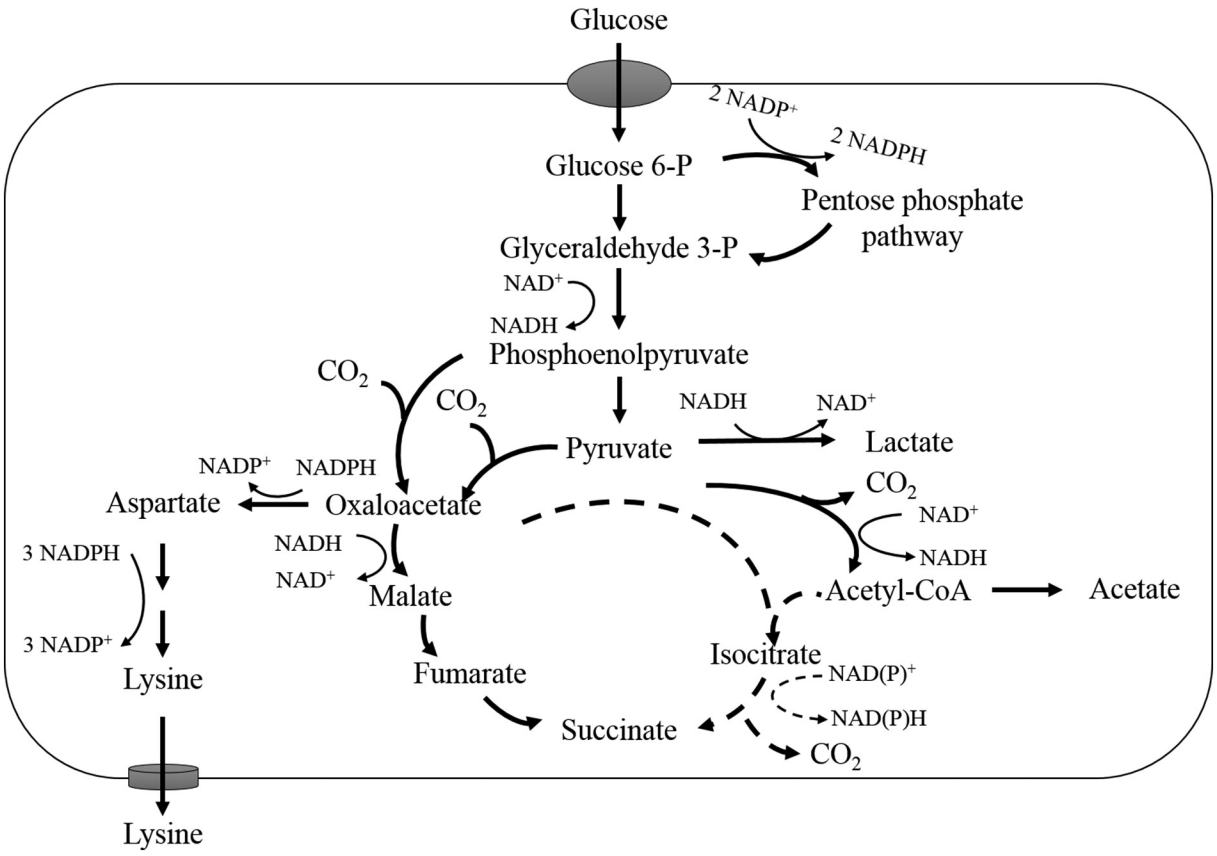

Fig. 6. Overview of the main pathways involved in co-production of lysine and organic acids during anaerobic glucose fermentation by *C. glutamicum*. Solid lines represent active pathways for lysine and organic acids co-production under anaerobic conditions [6,18,27]. NAD^+/NADH; the oxidized and reduced forms of Nicotinamide Adenine Dinucleotide, respectively. $NADP^+$/NADPH; the oxidized and reduced forms of Nicotinamide Adenine Dinucleotide Phosphate, respectively.

and isocitrate dehydrogenase [19]; however, reducing electrode conditions could have also played a role in the intracellular redox balance, as evidenced by the increased lysine yields. Even though strongly reducing conditions alone (under N_2 sparging) did not lead to higher lysine yields, the function of the cathode electrode can be considered responsible for increasing the carbon flow towards lysine when CO_2 is also present. Lysine yields were relatively lower when either of the two conditions (CO_2, −1.25 V) were applied alone, strongly indicating the existence of a synergetic effect that promoted anaerobic lysine production. On one hand, supplementation of reductive power is important, otherwise higher succinate yields are obtained and the amount of total organic carbon that ends up in the three main organic acids increases. On the other hand, supplementation of CO_2 is also important, as it redistributes the carbon flux towards oxaloacetate production via carboxylation of phosphoenolpyruvate and pyruvate [27]. This is significant from a utilization point of view, as CO_2 is an unwanted by-product of many industrial processes (e.g. fermentations) and could potentially be recycled and reused to enhance lysine production.

Reducing conditions in the presence of CO_2 increased the organic carbon directed to lysine, and this was observed both with and without the presence of the redox mediator AQ2S. Also considering that *C. glutamicum* are not known for utilizing molecular H_2 (e.g. produced by the cathodes) with the use of hydrogenases, this indicates that *C. glutamicum* can incorporate reducing power supplemented by an electrode without the need of an artificially added redox mediator. This is in accordance with the fact that *C. glutamicum* could produce anodic current even in the absence of AQ2S, although AQ2S had an active role and doubled the anodic electron transfer in that case. Because of the relatively high redox potential of AQ2S compared to that of the relevant redox cofactors ($E' = -0.45$ V vs. −0.57 V for $NADP^+$/NADPH reduction under physiological conditions [3,12]), it cannot be considered responsible for directly contributing to intracellular redox power by donating electrons to $NADP^+$. However, AQ2S seemed to have a vital, indirect role by accelerating glucose fermentation and increasing the lysine titers when CO_2 and cathodic conditions were applied. The exact mechanisms of AQ2S are definitely worth of investigating further, together with the role of redox mediators with redox potentials lower than those of the redox factors. This is because the latter could potentially have a combined effect of transferring the redox power into the cells and also increasing the glucose fermentation rates likewise to AQ2S. Methyl viologen ($E' = -0.65$ V) is such an example, although toxicity issues will also have to be considered in this case.

4. Conclusions

This study demonstrated for the first time the potential for improving lysine fermentations in bioelectrochemical reactors. By applying a combination of reducing power, AQ2S as a redox mediator and CO_2 as a favourable gas environment, we observed a remarkable effect on glucose consumption while lysine yields and concentrations were comparable to those of previous studies with the same strain and oxygen as an electron acceptor. This strengthens the position of bioelectrochemical systems as a way to enhance conventional fermentations and paves the way for bioprocess improvements based on the use of raw electricity.

5. Author contributions

Nikolaos Xafenias conceived the idea and designed the study, performed the experiments, acquired, analyzed and interpreted the data, drafted and critically revised the article. Cathleen Kmezik performed the experiments, acquired the data, drafted and critically revised the article. Valeria Mapelli analyzed and interpreted the data and critically

revised the article. All authors have approved the final version of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bioelechem.2017.06.001.

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Nikolaos Xafenias is a Dipl. Ing. Environmental Engineer and has received his PhD degree from the University of Southampton, UK. He currently works as a Researcher at Chalmers University of Technology in Sweden where he is engaged in various projects related to the production of valuable chemicals and fuels from low-value/waste process residues and by-products. His main focus is on bioelectrochemical systems and more precisely on microbial electrosynthesis and electrochemically assisted fermentations. He has recently been granted the "Future Research Leaders" grant of the Swedish Research Council Formas to work on the production of microbially catalyzed electrofuels from CO_2.

Cathleen Kmezik works as a PhD student at the Chalmers University of Technology (Sweden). She holds a Master's degree in Biotechnology obtained at the Technical University of Braunschweig in Germany. Her focus lies on the electrosynthesis of lysine with Corynebacteria species in bioelectrochemical systems. Recently, she started expanding her expertise by studying the abilities of polysaccharide degrading bacteria of the genus Bacteroides for the production of short-chain fatty acids from lignocellulose.

Valeria Mapelli has a PhD degree in Industrial Biotechnology from the University of Milan-Bicocca (Italy) and a Docent title from Chalmers University of Technology (Gothenburg, Sweden). After her PhD graduation she has been working at the Danish Technical University (Kgs. Lyngby, Denmark) and at Chalmers University of Technology in the field of microbial and bioprocess engineering. She started the research activities on BES in the Division of Industrial Biotechnology at Chalmers University of Technology envisioning their application in wider biorefinery systems. She currently works at the company Sacco Srl (Italy) and is guest researcher at Chalmers University of Technology.