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PII: S0960-8524(16)30691-5  
DOI: <http://dx.doi.org/10.1016/j.biortech.2016.05.051>  
Reference: BITE 16547

To appear in: *Bioresource Technology*

Received Date: 1 April 2016  
Revised Date: 13 May 2016  
Accepted Date: 14 May 2016



Please cite this article as: Xafenias, N., Kmezik, C., Mapelli, V., Cathodes enhance *Corynebacterium glutamicum* growth with nitrate and promote acetate and formate production, *Bioresource Technology* (2016), doi: <http://dx.doi.org/10.1016/j.biortech.2016.05.051>

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**Cathodes enhance *Corynebacterium glutamicum* growth with  
nitrate and promote acetate and formate production**

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**Abstract**

The industrially important *Corynebacterium glutamicum* can only incompletely reduce nitrate into nitrite which then accumulates and inhibits growth. Herein we report that cathodes can resolve this problem and enhance glucose fermentation and growth by promoting nitrite reduction. Cell growth was inhibited at relatively high potentials but was significant when potentials were more reductive (-1.20 V with anthraquinone-2-sulfonate as redox mediator or -1.25 V vs. Ag/AgCl). Under these conditions, glucose was consumed up to 6 times faster and acetate was produced at up to 11 times higher yields (up to 1.1 mol/mol-glucose). Acetate concentrations are the highest reported so far for *C. glutamicum* under anaerobic conditions, reaching values up to  $5.3 \pm 0.3$  g/L. Herein we also demonstrate for the first time formate production (up to  $3.4 \pm 0.3$  g/L) by *C. glutamicum* under strongly reducing conditions, and we attribute this to a possible mechanism of CO<sub>2</sub> bioreduction that was electrochemically triggered.

**Keywords:** acetate; biocathodes; *Corynebacterium glutamicum*; formate; nitrate reduction

## 1. Introduction

Within the framework of a more sustainable bio-based economy, microbially-driven electrochemical transformations have recently attracted a lot of attention for the production of chemicals and electricity, and for wastewater treatment. An essential feature of bio-electrochemical systems is the use of specialized bacteria in anodes and cathodes. When chemicals production is the main aim, then important bacteria are the ones that are already in place for industrial applications and therefore have an acknowledged biotechnological potential (Kracke et al., 2015). Only a few of these bacteria have been extensively studied by now in bioelectrochemical systems, and particularly gram-positive bacteria have just recently started to be examined for their potential use (Freguia et al., 2009; Wrighton et al., 2011). Such gram-positive bacteria which hold great industrial promise belong to *Corynebacterium glutamicum* species, a model species that has long been used and engineered for lysine and glutamate overproduction at industrial scale (Becker et al., 2011; Hirao et al., 1989).

Although a lot has been known regarding *Corynebacterium* spp., there is still a lot of potential for research. A good example comes with regards to our knowledge on its respiratory flexibility; for many years after its discovery in the late 1950s, *C.*

*glutamicum* has been regarded to be an aerobe. It was only recently demonstrated that it is in fact a facultative anaerobe which has the ability to grow anaerobically when alternative electron acceptors like nitrate are supplied (Nishimura et al., 2007; Takeno et al., 2007). This observation was remarkable, as it paved the way for a number of new potential applications with terminal electron acceptors other than oxygen (Takeno et al., 2007). However, important obstacles still need to be overcome in this matter, including

the fact that *C. glutamicum* is unable to reduce nitrate further than nitrite (Takeno et al., 2007), which then accumulates and inhibits growth. Overcoming these obstacles by finding ways to turn inhibitory nitrite into bioavailable ammonium could eventually improve anaerobic growth and metabolites production by *C. glutamicum* (Takeno et al., 2007).

*Corynebacterium* spp. possess cellular components that are useful for extracellular electron transfer and therefore have a potential for bioelectrochemical systems applications (Kracke et al., 2015). In a first biocathodes study, poised cathodes with *C. glutamicum*, neutral red as the redox mediator, and glucose as the carbon source, produced electricity that possibly contributed to increasing the yield of glutamic acid production by 10-15 % (Hongo and Iwahara, 1979a, 1979b). In a most recent and final study, a 20 % increase was reported in the lactic acid yield from glucose, when anthraquinone-2,6-disulfonate (AQDS) was the redox mediator in an oxygen-consuming cathode and under oxygen-deprivation conditions (where oxygen was supplied but rapidly consumed in the fermentation) (Sasaki et al., 2014). Research on the behavior of *Corynebacterium* spp. in biocathodes has not gone much farther though, also because of our limited knowledge on its capability to utilize alternative terminal electron acceptors potentially found in cathodes like nitrate, quinones, and humic acids (Takeno et al., 2007; Wu et al., 2012, 2010).

The aim of this study was to elucidate the potential of *C. glutamicum* to grow and respire under different biocathode conditions. Such knowledge is necessary for the successful application of *C. glutamicum* in bioelectrochemical systems. In this work we tested the metabolic capabilities of *C. glutamicum* in an anaerobic environment obtained via CO<sub>2</sub> sparging, and supplemented with glucose and nitrate for growth, and polarized

electrodes for providing reducing power. Our findings demonstrate for the first time the importance of reducing power provided in the form of polarized cathodes when nitrate is the terminal electron acceptor, and contribute to a better understanding of the anaerobic respiration mechanisms and the ecological niche of this gram-positive soil bacterium.

## 2. Materials and methods

### 2.1 Reactor construction

Dual-chamber, H-type borosilicate reactors with a working volume of 280 mL in each chamber were assembled as described elsewhere (Xafenias et al., 2015a). The two chambers were separated by a proton exchange membrane (Nafion<sup>®</sup> N117; Ion Power Inc., USA), pretreated as described in Xafenias et al. (Xafenias et al., 2015a). The dual-chamber type of reactors was selected in order to study the working electrode with the minimum interference possible from the counter electrode (Xafenias et al., 2015a). Graphite felt electrodes (SIGRATHERM; SGL Carbon Ltd., UK) with a  $3.8 \times 10^{-3} \text{ m}^2$  projected surface area were used as both the working electrode (WE) and the counter (CE) electrode, manufactured and pre-treated as described previously (Xafenias et al., 2015a). For investigating potential electron donors for *C. glutamicum*, cyclic voltammetry (CV) experiments were performed in a single-chamber voltammetry cell (VC-2; BASi, USA) with a working volume of 15 mL. A Ø 3 mm graphite rod (Alfa Aesar GmbH, Germany) with a  $5 \times 10^{-4} \text{ m}^2$  active surface area was used as the WE, and a Pt wire with a gold-plated connector was used as the CE.

## 2.2 Chemicals

High purity chemicals (Sigma-Aldrich) were used for media preparation. The working electrode medium consisted of a pH 7.0 phosphate buffer solution: 2 g/L  $\text{KH}_2\text{PO}_4$ , 6 g/L  $\text{K}_2\text{HPO}_4$ , 0.4 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 4 g/L  $\text{NH}_4\text{Cl}$ . This medium was amended to contain also 1 mL/L of a vitamin solution: 0.100 g/L biotin, 5 g/L thiamine·HCl and 5 g/L nicotinic acid, and 1 mL/L of a trace elements solution: 10 g/L  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 4 g/L  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ . Filter sterilized (0.2  $\mu\text{m}$  filter) glucose was added to the working electrode medium at the concentrations mentioned for each experiment. Nitrate ( $\text{NO}_3^-$ ) was added in the biocathode experiments as the electron acceptor for growth, at approximately 1.0 g  $\text{NO}_3^-$ -N/L using a 252.8 g/L  $\text{KNO}_3$  solution. Unless otherwise stated, ammonium ( $\text{NH}_4^+$ ) addition as  $\text{NH}_4\text{Cl}$  was omitted from the biocathodes because that was electrochemically produced by the reduction of  $\text{NO}_3^-$ . In order to achieve a more efficient nitrite ( $\text{NO}_2^-$ ) reduction,  $\text{NO}_3^-$  was normally replenished only when both  $\text{NO}_3^-$ -N and  $\text{NO}_2^-$ -N detected in the medium dropped below 10 mg/L. The medium described above was also used in the counter electrode chambers but lacking any glucose,  $\text{NO}_3^-$ , or vitamins. When needed, pH adjustments were made by addition of filter-sterilized (0.2  $\mu\text{m}$  filter) NaOH or HCl solutions. When mentioned, the redox mediator anthraquinone-2-sulfonate (AQ2S; 0.1 mM), which has a midpoint potential of  $E' = -0.45$  V at neutral pH (all electrode potentials mentioned are vs. Ag/AgCl), was used to facilitate nitrite reduction to ammonium ( $E' = +0.23$  V).

## 2.3 Setup and operation

After assembling the H-type reactors, they were filled with milli-Q water and autoclaved (121 °C, 20 min). After autoclaving, milli-Q water was replaced with

working and counter electrode solutions under sterile conditions in a laminar flow cabinet. Reference electrodes were then inserted (Ag/AgCl; 3 M NaCl, RE-5B, BASi, USA) after ethanol sterilization, within 1 cm distance from the WE. Autoclaved spargers were also inserted on top of the reactors for CO<sub>2</sub> sparging after filtering the gas through a sterile 0.2 µm air-filter. All bioelectrochemical experiments were conducted at room temperature (21±1 °C).

#### 2.4 *Corynebacterium glutamicum* cultivation

Lyophilized *Corynebacterium glutamicum* (DSM 20300; ATCC 13032) was purchased from the Leibniz Institute-DSMZ and resuscitated aerobically at 30 °C in liquid medium (pH 7.2) containing: peptone (10.0 g/L), yeast extract (5.0 g/L), glucose (5.0 g/L), NaCl (5.0 g/L). Glycerol stocks (50 % (v/v) glycerol) of the strain were then made and stored at -80 °C prior to use. For biomass growth 10 µL of the glycerol stock was added into 200 mL flasks and agitated aerobically at 200 rpm and 30 °C for 48 h. At the end of the incubation period cells were centrifuged and washed to remove all organics in suspension, as described elsewhere (Xafenias et al., 2015b). After disposing the washing solution the pellet was suspended in 10 mL of medium and the cells were inoculated in the working electrode chamber already containing 270 mL of the medium.

#### 2.5 Electrochemical monitoring and control

To control the applied potential and to monitor current produced, a three-electrode configuration was used. For monitoring and control during chronoamperometry experiments in the H-type reactors, three two-channel potentiostats (MLab; Bank Elektronik-Intelligent Controls GmbH, Germany) were used and current was recorded



every one minute. Monitoring and control during the CV experiments was performed using a single-channel potentiostat (PG580, Uniscan Instruments Ltd., UK). The CVs were performed at least three times, with a scan rate of  $5 \times 10^{-3}$  V/sec, at pH 7, under quiescent anaerobic conditions and in a  $N_2$  atmosphere.

## 2.6 Analytical methods and calculations

Samples were centrifuged at  $20,000 \times g$  and the supernatant was filtered through a  $0.2 \mu m$  filter before analysis. Glucose, acetate, succinate, formate, glycerol, and lactate were analyzed using a high-performance liquid chromatographer (HPLC; Dionex<sup>®</sup> Ultimate 3000, Dionex Corp., USA) equipped with a Rezex<sup>™</sup> ROA-Organic Acids  $H^+$  (8 %) column ( $300 \text{ mm} \times 7.8 \text{ mm}$ ; Phenomenex Inc., Denmark) operating at  $80^\circ C$ . A  $5 \text{ mM } H_2SO_4$  solution was used as the mobile phase at a flow rate of  $0.8 \text{ mL/min}$ . All target compounds were detected by a refractive index detector (RI-101; Dionex Corp., USA). All organic acids were additionally detected using a variable wavelength detector (VWD 3100; Dionex Corp., USA) operating at the fixed wavelength of  $210 \text{ nm}$ . Lysine was measured using an ion chromatographer (IC; Dionex<sup>®</sup> ICS-3000, Dionex Corp., USA) equipped with an electrochemical detector and a Dionex AminoPac<sup>™</sup> PA-10 column ( $250 \text{ mm} \times 2 \text{ mm}$ ; Dionex Corp., USA) operating at  $30^\circ C$ . A multi-step gradient mode was used, where He-degassed NaOH ( $250 \text{ mM}$ ) and Na-Acetate ( $1 \text{ M}$ ) were proportionally mixed with milli-Q  $H_2O$  under a total flow of  $0.25 \text{ mL/min}$  (Supplementary Table S1). High purity compounds (Sigma-Aldrich) were used to make the calibration curves and to quantify the compounds of interest. Concentrations of  $NO_3^-$ -N,  $NO_2^-$ -N, and  $NH_4^+$ -N were measured spectrophotometrically using test kits (HACH-

LANGE® LCK 340, LCK 342, and LCK 303 respectively) and a benchtop spectrophotometer (DR3900; HACH-LANGE® AB, Sweden).

### 3. Results and Discussion

#### 3.1 Current production and nitrate reduction

The glucose and nitrate supplemented cathodes were inoculated with *C. glutamicum* ATCC 13032 (except in the abiotic control) and chronoamperometry experiments started immediately after inoculation. Current produced for the duration of the experiments under different applied conditions is shown in Fig. 1. Cathodic (reducing) current generally increased with decreasing potential (Fig. 1a), and the presence of AQ2S as a redox mediator resulted in increased charge production by 2.8 times under the potential of -1.20 V (Fig. 1b). Higher current produced was also associated with the higher  $\text{NO}_3^-$  reduction rates (Fig. 2a) and the higher  $\text{NH}_4^+$  concentrations observed (Fig. 2c), compared with the ones under the potentials of -1.00, -1.10, and -1.20 V. When the potentials were even higher (-0.80, -0.60 and -0.40 V),  $\text{NO}_3^-$  reduction rates were up to 7 times lower (Fig. S1a,b,f), despite the fact that  $\text{NH}_4^+$  was externally supplied for cell growth and subsequent biological  $\text{NO}_3^-$  reduction. Considering these observations, a schematic representation of the fate of nitrogen in the system is given in Fig. 2d.

The  $\text{NO}_2^-$  levels in the -1.20, -1.20+ AQ2S and -1.25 V reactors where current production was higher increased at start when  $\text{NO}_3^-$  was abundant, but decreased in time after  $\text{NO}_3^-$  concentrations became lower than 0.29 g  $\text{NO}_3^-$ -N/L (Fig. 2a,b). However, in the -1.20 V reactors lacking AQ2S, accumulation of  $\text{NO}_2^-$  continued until it reached 0.45 g  $\text{NO}_2^-$ -N/L on the 4<sup>th</sup> day of operation. After this point the  $\text{NO}_3^-$ -N reduction rate

decreased from 0.21 g/L/d to only 0.04 g/L/d, while  $\text{NO}_2^-$ -N concentration started decreasing. This performance indicated that in the absence of added redox mediators, this potential was not low enough to support an ideal balance between  $\text{NO}_2^-$  production and reduction, and to avoid continuous accumulation of  $\text{NO}_2^-$ . Similarly, higher applied potentials were shown incapable of protecting the system from continuous accumulation of inhibitory  $\text{NO}_2^-$  (Fig. 2b and Fig. S1g). On the contrary,  $\text{NO}_2^-$  concentrations were lower in the -1.20 V+ AQ2S and the -1.25 V reactors (Fig. 2b). That was despite the fact that current produced in the -1.25 V reactors did not differ considerably from that produced in the -1.20 V reactors (Fig. 1b), showing that a larger portion of charge was utilized for  $\text{NO}_2^-$  reduction under -1.25 V than under -1.20 V. In addition, the -1.25 V reactors produced 2.2 times the current produced by their abiotic counterpart (Fig. 1); this was in relation to 1.05 g ( $\text{NO}_3^- + \text{NO}_2^-$ )-N/L reduced in the first instance but only 0.60 ( $\text{NO}_3^- + \text{NO}_2^-$ )-N/L in the second (Fig. 2a,b). Considering also that ( $\text{NO}_3^- + \text{NO}_2^-$ )-N removal was lower with *C. glutamicum* under higher applied potentials, it can be concluded that the combination of both the appropriate potentials and the presence of *C. glutamicum* led to higher denitrification rates.

With regards to  $\text{NH}_4^+$ , that was electrochemically produced under strongly reducing conditions and up to 0.73 g  $\text{NH}_4^+$ -N/L in the -1.20 V+ AQ2S case (Fig. 2c). On the other hand,  $\text{NH}_4^+$  was produced only up to the very limited levels of 0.03 g  $\text{NH}_4^+$ -N/L in the cases of -1.00 and -1.10 V, due to the inappropriately high electrode potentials and the incapability of *C. glutamicum* to convert  $\text{NO}_3^-$  further than  $\text{NO}_2^-$ . In the reactors operating at -1.20 V,  $\text{NO}_3^-$  reduction (up to 55 % more when compared to the -1.00 V reactors; Fig. 2a) resulted in 5-fold higher levels of  $\text{NH}_4^+$  electrochemically produced when compared to the -1.00 V reactors (Fig. 2c), although  $\text{NO}_2^-$  accumulated to a large

extent (Fig. 2b). Under abiotic conditions and -1.25 V electrode potential, only 0.03 g  $\text{NH}_4^+$ -N/L was produced; that was only 9 % of the  $\text{NH}_4^+$  produced under biotic conditions, stressing the importance of the biocatalyst for the conversion of  $\text{NO}_3^-$  into  $\text{NH}_4^+$ . A possible pathway for enhanced  $\text{NH}_4^+$  production could involve the indirect production of  $\text{NO}_2^-$  from  $\text{NO}_3^-$ , which is then electrochemically reduced under strongly reducing conditions (Fig. 2d). Further research is needed to determine the exact speciation and pathways involved, and also the exact biocatalytic role of the bacterium.

### 3.2 Poised cathodes can enhance glucose consumption and promote growth in the presence of nitrate

Glucose consumption in the bioelectrochemical cathodes is shown in Fig. 3a. Glucose was completely consumed within 6-8 days in the bioelectrochemical cathodes where current production was the highest in the cases of -1.25 V and -1.20 V+ AQ2S. On the contrary, more than 5.6 g/L of glucose still remained in all other reactors on the 10<sup>th</sup> and last day of operation. A comparison between reactors with AQ2S (-1.20 V+ AQ2S) and without AQ2S (-1.20 V) shows an 8 times faster glucose consumption in the first instance. Reactors -1.20 V, which accumulated  $\text{NO}_2^-$  at the highest rate, were also the slowest ones in consuming glucose; this is in accordance to previous observations showing the growth-inhibitory properties of  $\text{NO}_2^-$ -N (Takeno et al., 2007).

With regards to cell growth, an initial drop in the  $\text{OD}_{600}$  was observed in all cases (Fig. 3b), which could be attributed to the shift from the rich, aerated medium to the mineral anaerobic one, but also to biomass held on the electrode surface and therefore not represented in the  $\text{OD}_{600}$ . A clear influence of the electrode conditions was observed and growth was supported only under the conditions of -1.25 V and -1.20 V+ AQ2S. On the

contrary, growth was very weak in all other cases, including the supplementary experiments where  $\text{NH}_4^+$  was supplied since the beginning, but electrode potentials were not low enough to support reductive transformations (Fig. S1c). Also negligible growth was observed when anodes were the sole electron acceptors available ( $\text{NH}_4^+$  supplemented and  $\text{N}_2$  sparged; data not shown). These facts clearly showed that by applying low electrode potentials (-1.25 V) or by enhancing current production with the use of a redox mediator (-1.20 V+ AQ2S),  $\text{NO}_2^-$  reduction becomes more efficient, therefore resulting in a less inhibitory system that allows growth of *C. glutamicum* via glucose consumption.

Anaerobic  $\text{NO}_3^-$  reduction by *C. glutamicum* has been shown before (Nishimura et al., 2007; Takeno et al., 2007), though the metabolic end-products under different redox conditions are still to be defined. The accumulation of  $\text{NO}_2^-$  as the result of incomplete  $\text{NO}_3^-$  reduction has been identified as growth-inhibiting (Takeno et al., 2007) and it represents an important obstacle for sustaining anaerobic fermentations of *C.*

*glutamicum*. In order to overcome this obstacle we applied strong electro-reducing conditions in the fermentation, and these conditions were shown to cause higher glucose consumption rates and growth. These conditions also allowed us to have a more clear view of the metabolism under  $\text{NO}_3^-$  reducing conditions because growth was clearly observed, reaching levels that were higher than in any other case of anaerobic glucose fermentation that we tested.

### 3.3 Metabolic products alter according to the applied conditions

Lactate, acetate, succinate, and glycerol are typical end-product metabolites when *C. glutamicum* is subjected to oxygen deprivation conditions (Jojima et al., 2014; Okino et

al., 2005). The three organic acids were the main metabolic products found by the end of the fermentation in aerated flasks (Fig. S2), probably due to oxygen limitations. Lactate, acetate, succinate, glycerol, and formate could be detected under  $\text{NO}_3^-$  reducing conditions (Fig. 3c-g). Highest concentrations (Fig. 3c-g) and yields (Fig. 4; formate was excluded as its production does not derive from glucose) of these metabolites differed according to the conditions applied in the nitrate biocathodes, and a clear metabolic shift was observed. Lactate, the main fermentation product normally observed under oxygen-deprivation and growth-arrested conditions (Okino et al., 2005), was produced (up to 1.6 g/L) with a yield ranging from 0.2 to 0.3 mol/mol-glucose (0.1 to 0.2 C-mol/C-mol of glucose). Though lactate was the main metabolite produced at the higher electrode potentials, acetate became the main product when more reductive current was produced; acetate levels reached up to 5.3 g/L and yields up to 1.1 mol/mol-glucose (0.3 to 0.4 C-mol/C-mol of glucose) (Fig. 4a). To the best of our knowledge, these acetate concentrations and yields are the highest reported in the literature under oxygen deprivation or anaerobic conditions in batch fermentations of the wild type *C. glutamicum* (Inui et al., 2004; Okino et al., 2005; Wieschalka et al., 2012).

Succinate (Fig. 3f) was detected at concentrations ranging between 0.5-1.0 g/L regardless of the different electrochemical conditions applied. Clear differences in succinate yields were observed, as strongly reducing conditions resulted in lower yields of succinate (0.06-0.09 mol/mol-glucose; 0.04 to 0.06 C-mol/C-mol of glucose) than weakly reducing conditions did (0.12-0.17 mol/mol-glucose; 0.08 to 0.11 C-mol/C-mol of glucose).

Glycerol is a fermentation product produced during overflow of carbon metabolism in order to regenerate  $\text{NAD}^+$  in the absence of external electron acceptors (Jojima et al.,

2014). Here, we observed glycerol production in two cases (Fig. 3g), under the electrode potentials of -1.00 V and -1.10 V where  $\text{NO}_3^-$  consumption was the lowest. The maximum concentration of glycerol was 0.3 g/L and yields were up to 0.08 mol/mol-glucose (0.03 to 0.04 C-mol/C-mol of glucose). These yields are similar to the ones observed elsewhere (Jojima et al., 2014).

The overall effect of applying strongly reducing conditions (-1.25 V and -1.20 V+ AQ2S) when  $\text{NO}_3^-$  was the terminal electron acceptor is also shown in Fig. 4b where the carbon content of each fermentation product is considered (excluding carbon stored in biomass and  $\text{CO}_2$  produced). More carbon was stored in the fermentation metabolites produced under strongly reducing conditions (0.42-0.52 mol-C in products/mol-C in glucose consumed), compared to all other cases (0.27-0.33 mol-C in products/mol-C in glucose consumed). Moreover, a remarkable outcome was that under strongly reducing conditions 27-37 % of the glucose carbon converted into products was converted into acetate; this is compared to only 3-7 % in all other cases.

Metabolites produced without considerable bioelectrochemical interaction (under -0.40 V, -0.60 V and -0.80 V) are shown in Fig. S1d. The results from these experiments showed concentrations of the metabolites lactate, acetate, succinate and glycerol that were similar to the relatively low concentrations produced by the -1.00 V, -1.10 V, and the -1.20 V reactors; that was despite the fact that  $\text{NH}_4^+$  was externally supplemented to support growth.

While it has long been known that under oxygen limited conditions *C. glutamicum* ferments glucose to organic acids (Okino et al., 2005), the anaerobic metabolism of *C. glutamicum* remains largely unclear (Nishimura et al., 2011). Poised electrodes were

shown to play a role in *C. glutamicum* fermentations, possibly by directly or indirectly altering the intracellular redox status; when *C. glutamicum* was provided with a poised cathode (-0.60 V vs. Ag/AgCl) and 0.2 mM AQDS as a redox mediator under oxygen-limited conditions, an increase in the lactate yield was observed, as compared to the yields without the poised electrode or AQDS (Sasaki et al., 2014). In an earlier study, cathodic current was shown to have an effect on glutamic acid production; when *C. glutamicum* was supplied with neutral red as the redox mediator, the yield of glutamic acid from glucose increased by up to 15 % when compared to a non-electrochemical control (Hongo and Iwahara, 1979b). Both these studies demonstrated an effect of poised electrodes on glucose fermentations by *C. glutamicum*. However, to the best of our knowledge, no study had been performed until now showing the metabolic shifts caused by the applied electrochemical conditions when  $\text{NO}_3^-$  is the only electron acceptor available or when  $\text{CO}_2$  is supplied.

Formate was also observed at concentrations up to 3.5 g/L (Fig. 3e) when reductive current production was high and  $\text{NO}_3^- + \text{NO}_2^-$  reduction was more effective. On the other hand, concentrations were considerably lower in the cases of higher potentials (up to 0.2 g/L at -1.20 V and below 0.1 g/L in all other cases). Formate production from glucose is not possible by wild type *C. glutamicum* as it is with other *Corynebacterium* spp.

(Reddy and Kao, 1978), though formate production with methanol as carbon source has recently been reported (Witthoff et al., 2013). *C. glutamicum* lacks the formate-lyase pathway for formate production from sugars and therefore formate detected in our study cannot originate directly from glucose metabolism. However, *C. glutamicum* ATCC 13032 has a molybdenum-dependent formate dehydrogenase (FDH) catalyzing the oxidation of formate to  $\text{CO}_2$  (Witthoff et al., 2013, 2012). In *C. glutamicum*, FDH is



either associated with the membrane or present in the cytosol and uses a yet unspecified electron acceptor (Witthoff et al., 2012). It has been proven that some bacterial FDH can catalyze also the reverse electrochemical reaction, i.e. CO<sub>2</sub> reduction to formate and bioelectrochemically recycled redox factors, at electrode potentials lower than -0.64 V or when H<sub>2</sub> is supplied (Alissandratos et al., 2014, 2012; Bassegoda et al., 2014; Poehlein et al., 2012; Srikanth et al., 2014). In a recent study it was demonstrated that Mo-containing formate dehydrogenases, and particularly the Mo-center of the enzymes, are active in both formate oxidation to CO<sub>2</sub> and CO<sub>2</sub> reduction to formate when interacting with electrodes polarized under appropriate potentials (Bassegoda et al., 2014). In that study it was also shown that polarized electrodes were more successful in catalyzing CO<sub>2</sub> reduction into formate than reducing compounds like methyl viologen; this was attributed to the more sufficient driving force of the electrode that was capable to overcome redox barriers within the enzyme cluster. In our case a net formate consumption was observed after glucose depletion, possibly indicating an active mechanism of formate oxidation to CO<sub>2</sub> via FDH. Therefore we can speculate that the *C. glutamicum* FDH had a reversible role in our systems, i.e. reducing CO<sub>2</sub> into formate with a concurrent consumption of reducing power and production of the associated intracellular electron acceptors. Additionally, the fact that high formate production was observed in the -1.20 V+ AQ2S case and not in the -1.20 V one, helps us identify the role of AQ2S in the system. The formal potential of this particular redox mediator ( $E' = -0.45$  V at neutral pH) is higher than the formal potentials of the H<sup>+</sup>/H<sub>2</sub> ( $E' = -0.62$  V), the CO<sub>2</sub>/formate ( $E' = -0.64$  V), and the NAD<sup>+</sup>/NADH ( $E' = -0.53$  V) couple; therefore formate production enhancement via AQ2S would not be thermodynamically possible. It is therefore more likely that the role of AQ2S in the system was to promote reduction

of the nitrite inhibitor ( $E' = +0.23$  V), which in turn promoted growth and bioelectrochemical formate production. The electric charge needed for formate production according to the reaction  $\text{HCO}_3^- + 2\text{H}^+ + 2e^- = \text{HCOO}^- + \text{H}_2\text{O}$  is shown in Fig. 1b. As can be seen, formate represented only a portion of the total charge produced (up to 24 %). On the other hand, formate was not observed at higher potentials (-0.40 V, -0.60 V and -0.80 V; Fig. S1d), as the electrode potentials were not low enough to exceed the overpotentials imposed by the system for formate production from  $\text{CO}_2$  ( $E' = -0.64$  V). These supplementary results support the argument that a properly poised electrode was an important addition to the anaerobic cultivation, leading to the production of high titers of the metabolites acetate and formate.

In this study we demonstrated that effectively reducing conditions had a significant effect on altering the fermentation products, and that a better regulation of the  $\text{NO}_2^-$  levels could be a reason for the considerably increased glucose consumption and conversion into organic acids (mainly acetate and formate). The results showed that the yields of lactate, glycerol, and succinate (NADH-consuming) decrease, while those of acetate (NADH-producing) considerably increase under intense reducing conditions imposed by either reducing the electrode potential down to -1.25 V or by adding AQ2S at -1.20 V. This effect could be related to the coupling of  $\text{NO}_3^-$  and  $\text{CO}_2$  reduction to the regeneration of the intracellular electron acceptors, under more efficiently tuned  $\text{NO}_2^-$  levels. Under intense reducing conditions, acetate yield was up to 11 times higher and concentration was up to 17 times higher than in the cases where the potential was  $\geq -1.20$  V and without AQ2S added.

Strongly reducing conditions could have had implications on the redox status of the electrofermentations and the intracellular  $\text{NAD(P)}^+/\text{NAD(P)H}$  ratios. Altering the

intracellular reducing power by using cathodes has been demonstrated before in *Clostridium pasteurianum*, where reducing conditions increased the production of NADH-consuming products (Choi et al., 2014). However, the formation of acetate which is the main metabolic product from glucose that was observed under strong electro-reducing conditions, is producing NADH (Inui et al., 2004; Witthoff et al., 2013). Increased  $\text{NAD}^+/\text{NADH}$  ratios in *C. glutamicum* have been related to increased glucose consumption and acetate production rates under oxygen deprivation conditions (Inui et al., 2004; Tsuge et al., 2015). Therefore, we can speculate that when accumulation of inhibitory  $\text{NO}_2^-$  was better regulated, the reduction of  $\text{NO}_3^-$  could be coupled to  $\text{NAD}^+$  regeneration, as also happens in other bacterial species under anaerobic conditions (e.g. *Escherichia coli*) (Takeno et al., 2007).

Both acetate and formate produced are important intermediates that could be utilized as electron carriers for hydrogen production (formate), and as feedstock for acetoclastic methanogenesis (acetate); therefore, these findings can be useful to manipulate more complex systems where the soil bacterium *C. glutamicum* interacts with other microbial populations in the same habitat (e.g. methanogens). The exact electrochemical pathways that *C. glutamicum* is utilizing for the production of these two compounds are certainly worth further investigation.

### 3.4 Metabolites as potential electron donors

To further investigate whether the organic acids produced could be further metabolized and thus act as electron donors, cyclic voltammetry tests were conducted in the presence of AQ2S (0.1 mM) and the four organic acids produced formate, acetate, succinate, and lactate (10 mM). As can be seen in Fig. 5a, formate was the only organic acid tested that

produced cyclic voltammograms which differed considerably from the rest, including the abiotic control and the control under non-turnover conditions (with *C. glutamicum* but no substrate addition). In fact, *C. glutamicum* produced a substrate-related anodic electric signal only when formate was present, which can be clearly seen in Fig. 5b that demonstrates the average current produced between the forward and backward CV scans. As can be seen, the anodic (oxidative) current produced by the formate-fed *C. glutamicum* experiments, exhibited a considerably higher oxidative current when compared to the controls. This current difference caused by a current increase in the presence of formate, is indicated by the double arrow at high electrode potentials. This result shows that *C. glutamicum* is capable of using formate as the electron donor and an electrode as the final electron acceptor to produce anodic current, as also happens with other *Corynebacterium* species (e.g. *Corynebacterium* sp. strain MFC03) (Liu et al., 2010). Overall, these results demonstrate that formate is a very important electron carrier potentially utilized by *C. glutamicum* for bioelectrochemical conversions under anaerobic conditions.

### 3.5 Production of lysine

Small amounts of lysine were also produced in our systems, even when the sole nitrogen source provided into the system was  $\text{NO}_3^-$  (Fig. 6). While no lysine was detected in the aerated flasks (data not shown), lysine produced under  $\text{NO}_3^-$  reducing conditions (Fig. 6a) reached 32  $\mu\text{M}$ . Lysine concentrations increased with increasing current, up to the -1.20 V applied potential, also as a result of the increased  $\text{NH}_4^+$  produced, compared to the -1.00 V and the -1.10 V reactors. Maximum lysine produced was lower for the -1.25 V and the -1.20 V+ AQ2S reactors. However, it should also be

considered here that glucose and  $\text{NO}_3^-$  limitations occurred in these reactors, especially after the 4<sup>th</sup> day, which could have had implications on the maximum amount of lysine produced. At higher potentials and lower cathodic current produced, lysine was produced at similar levels (Fig. S1e), though  $\text{NO}_2^-$  accumulated and  $\text{NH}_4^+$  had to be externally added to the medium.

Lysine is an essential amino acid for both human and microbial metabolisms, and production has been demonstrated before under anaerobic conditions using the mutated strain *C. glutamicum* AK-1 (Takeno et al., 2007). The wild-type *C. glutamicum* ATCC 13032 has been shown to produce lysine but only at the  $\mu\text{M}$ -level and under aerobic conditions (Wittmann and Heinzle, 2002). Such levels (up to 5 mg/L) were also demonstrated in our study, under  $\text{NO}_3^-$  reducing conditions. Nevertheless, the production of such low levels of lysine would eventually have a relatively low effect on the overall microbial metabolism (e.g. via recirculation of the  $\text{NAD(P)}^+$  cofactors), when compared to the g/L (mM) levels of the other metabolites produced (e.g. acetate). Overall, our results demonstrate that when  $\text{NO}_3^-$  was the terminal electron acceptor, small amounts of extracellular lysine were produced, with electrochemically produced  $\text{NH}_4^+$  as the nitrogen source. Our results on lysine production provide information that could be useful in the study of anaerobic microbial consortia in bioelectrochemical systems where *C. glutamicum* is present; in those environments, even low levels of amino acids production can potentially play a role in the microbial symbiosis.

#### 4. Conclusions

Reducing power supplied by cathodes was demonstrated to be very useful for supporting anaerobic growth when nitrate was supplied as terminal electron acceptor in

*C. glutamicum* anaerobic fermentations. By electrochemically providing strongly reducing conditions we enhanced denitrification and glucose consumption by *C. glutamicum*. We also demonstrated a shift in the metabolism towards acetate production and we were able to reveal the potential of *C. glutamicum* for formate production, possibly via CO<sub>2</sub> reduction. Though the exact mechanisms are to be revealed, cathodes possess a great potential for exploiting the existing bacterial tools in order to alter the metabolism towards targeted bio-products.

### **Acknowledgements**

Funding in support of this work was provided in the frame of the collaborative project BioBUF, which is financed by the Swedish Research Council Formas, Sweden (Project N° 2013-11649-24470-114; Upgrading of Renewable Domestic Raw Materials to Value-added Bulk and Fine Chemicals for a Biobased Economy: Technology Development, Systems Integration and Environmental Impact Assessment).

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## Figure legends

**Fig. 1.** Current produced by the biocathodes and the abiotic control at different potentials (a) and total charge produced by the end of the operation period (b). In (b), the charge accumulated as formate via  $\text{CO}_2$  reduction, and also as  $\text{NH}_4^+$  (maximum charge assuming that all  $\text{NH}_4^+$  is electrochemically produced from  $\text{NO}_3^-$ ), are indicated. A steep drop in current produced by -1,20 V+ AQ2S after day 7 was due to electrical connection problems in one of the two reactors that was caused by the high current produced.

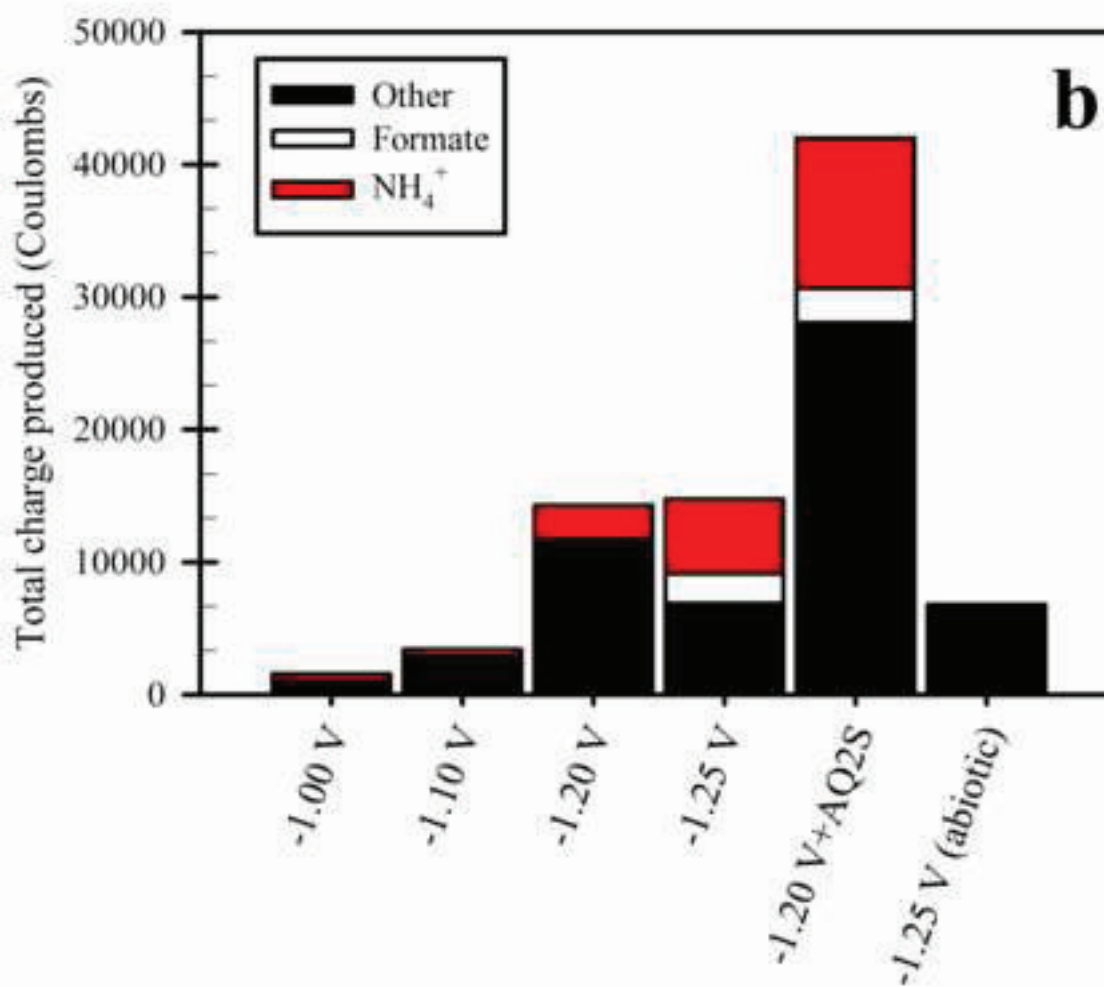
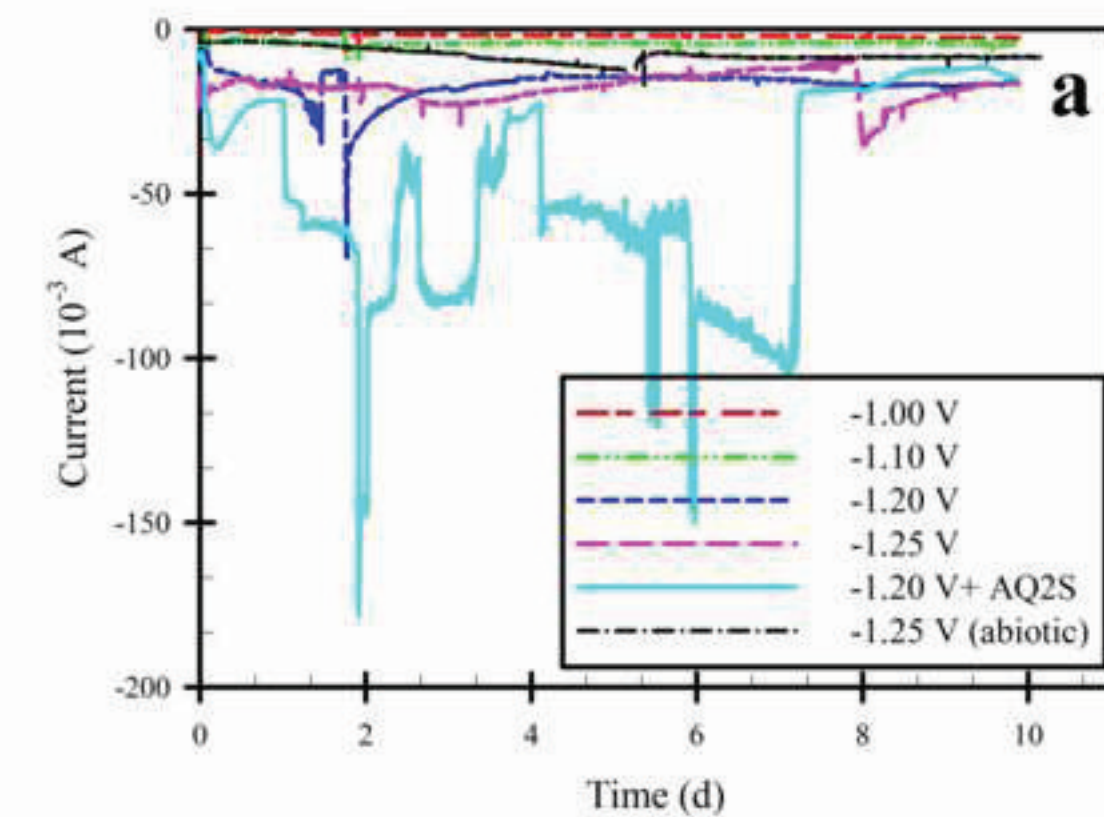
**Fig. 2.**  $\text{NO}_3^-$ -N (a),  $\text{NO}_2^-$ -N (b), and  $\text{NH}_4^+$ -N (c) concentrations in the bioelectrochemical cathodes and the abiotic control, supplied with  $\text{NO}_3^-$  as the only nitrogen source for  $\text{NH}_4^+$  production. Error bars indicate min and max values of the duplicate reactors. A simplified representation of the overall fate of  $\text{NO}_3^-$  in the bioelectrochemical cathodes is shown in (d); the role of *C. glutamicum* in the observed enhancement of the bioelectrochemical production of  $\text{NH}_4^+$  from  $\text{NO}_3^-$  requires further investigation.

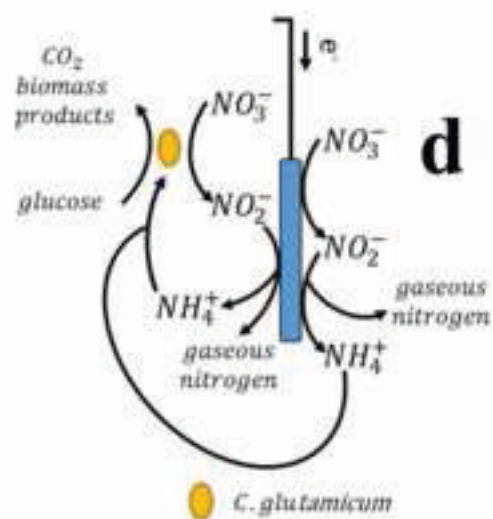
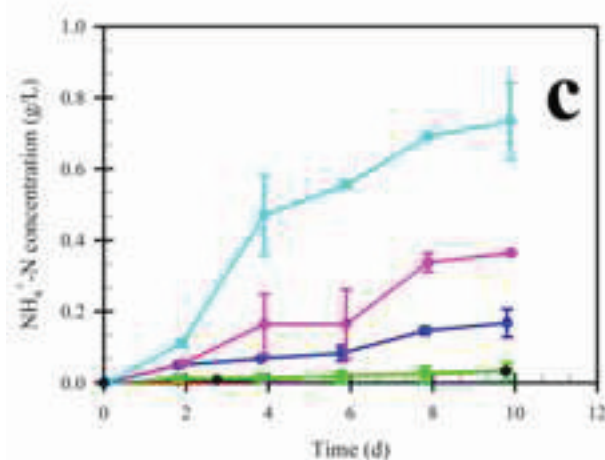
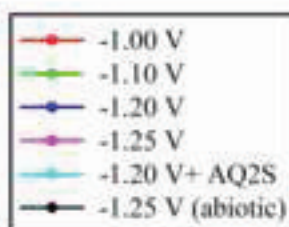
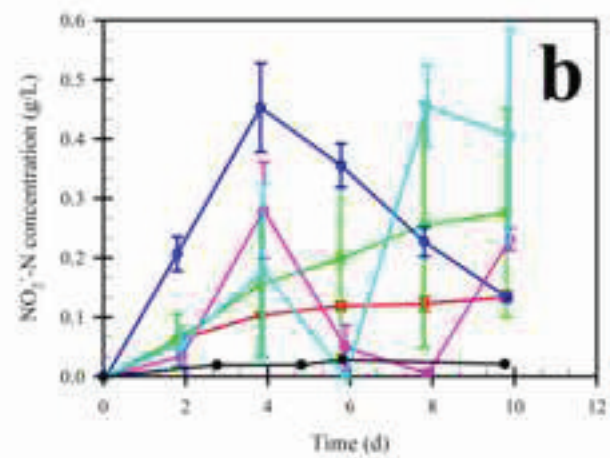
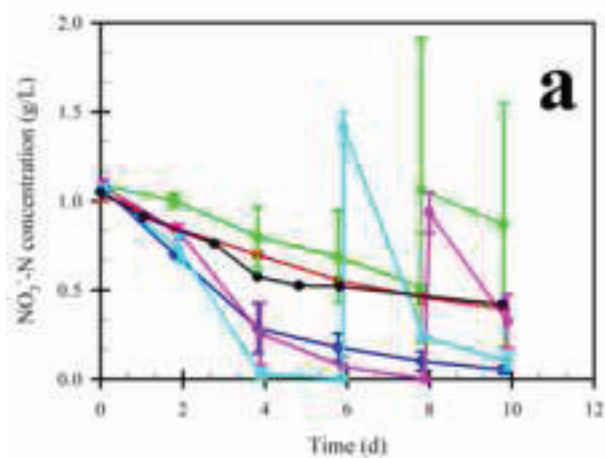
**Fig. 3.** Anaerobic glucose consumption, growth, and metabolites concentrations in the *C. glutamicum* biocathodes and the abiotic control where  $\text{NO}_3^-$  was supplied as the terminal electron acceptor for growth: glucose (a)  $\text{OD}_{600}$  (b), lactate (c), acetate (d), formate (e), succinate (f), glycerol (g). Considerably higher glucose consumption and growth were observed under strongly reducing conditions, together with the high acetate and formate concentrations produced. Error bars indicate min and max values of the duplicate reactors.

**Fig. 4.** Yields of products (a) and the carbon in products (b) produced from glucose; yields were calculated for the time point where glucose was no longer detected (-1.20 V+ AQ2S and -1.25 V), or for the end of the run (-1.00 V, -1.10 V and -1.20 V).

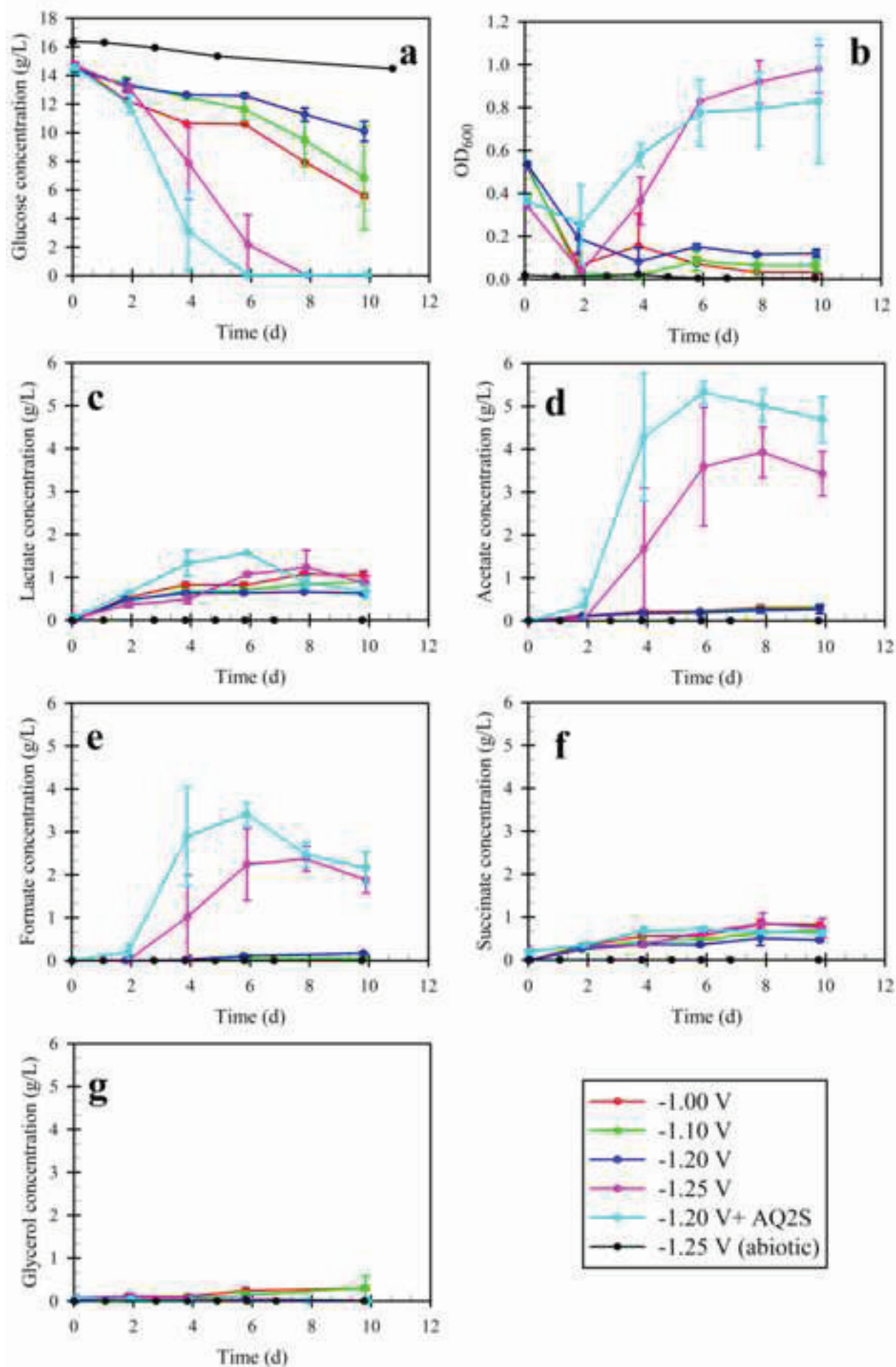
**Fig. 5.** Cyclic voltammetry tests conducted with *C. glutamicum*, 10 mM of various potential electron donors, and 0.1 mM of AQ2S as the redox mediator. CVs demonstrate the ability of *C. glutamicum* to readily produce current in the presence of formate but not in the presence of the other metabolic products produced from glucose under anaerobic conditions. The original CV tests are demonstrated in (a), while the average values between the forward and backward scans are demonstrated in (b). The double arrow in (b) indicates the oxidative current difference between the formate-fed experiment and the controls.

**Fig. 6.** Concentrations of lysine produced during fermentation in the bioelectrochemical cathodes.

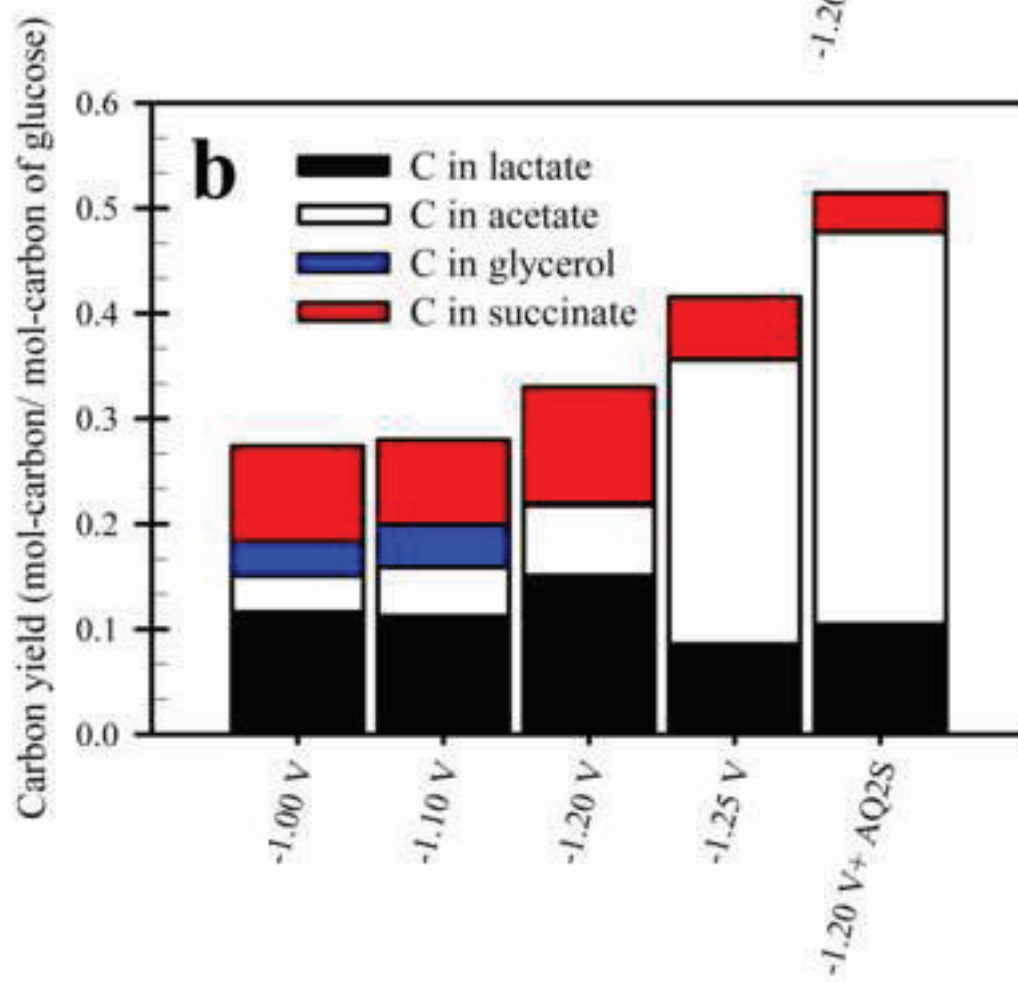
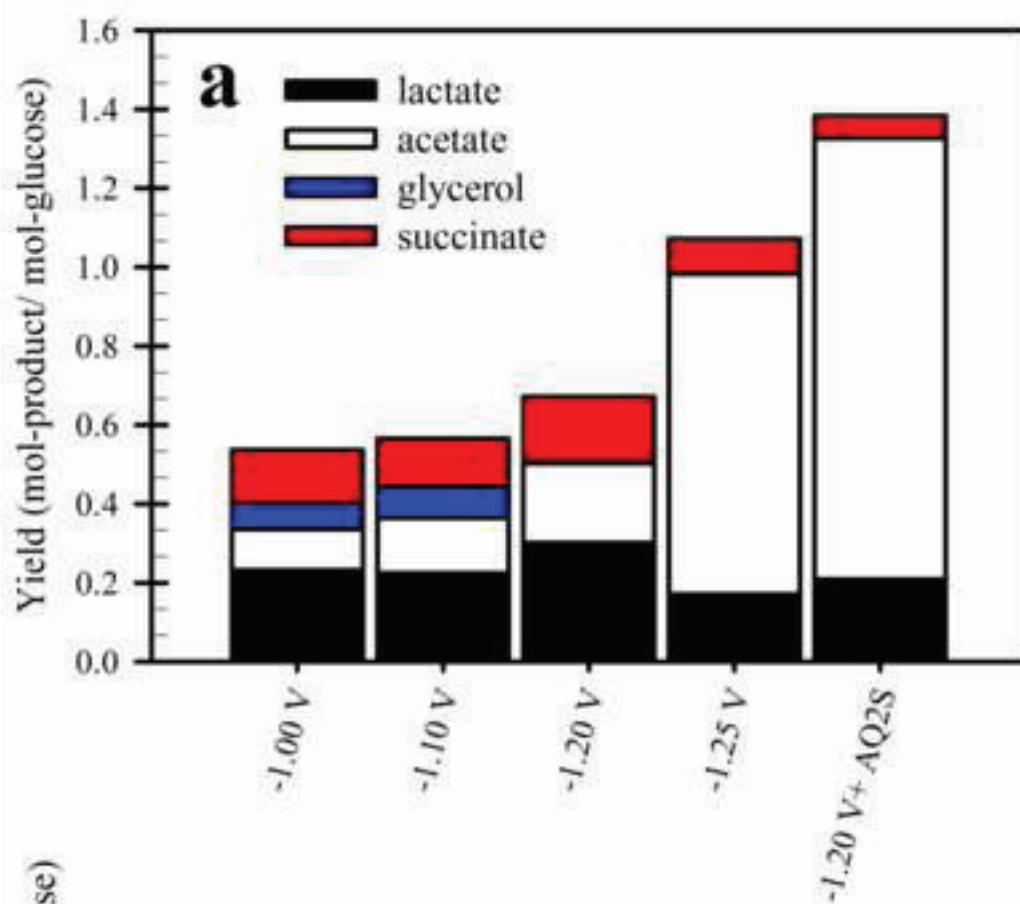


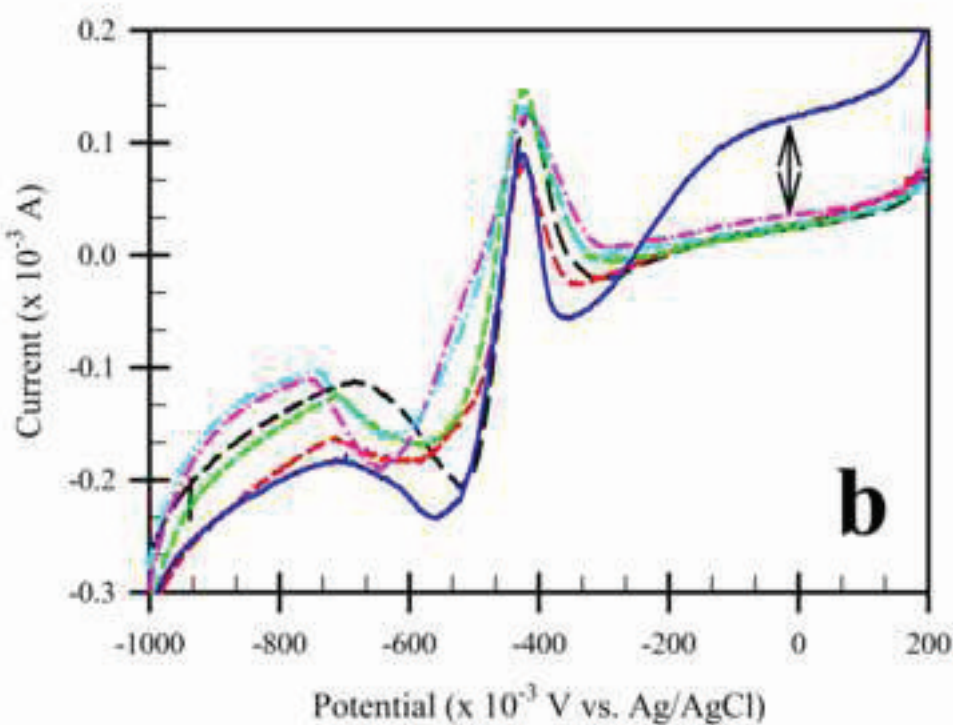
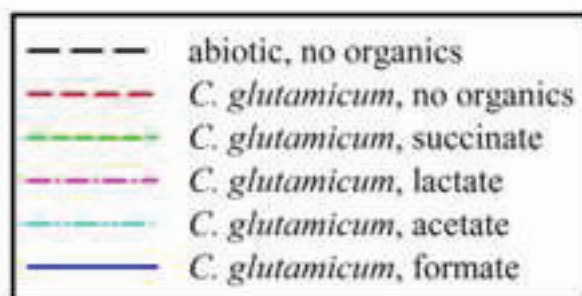
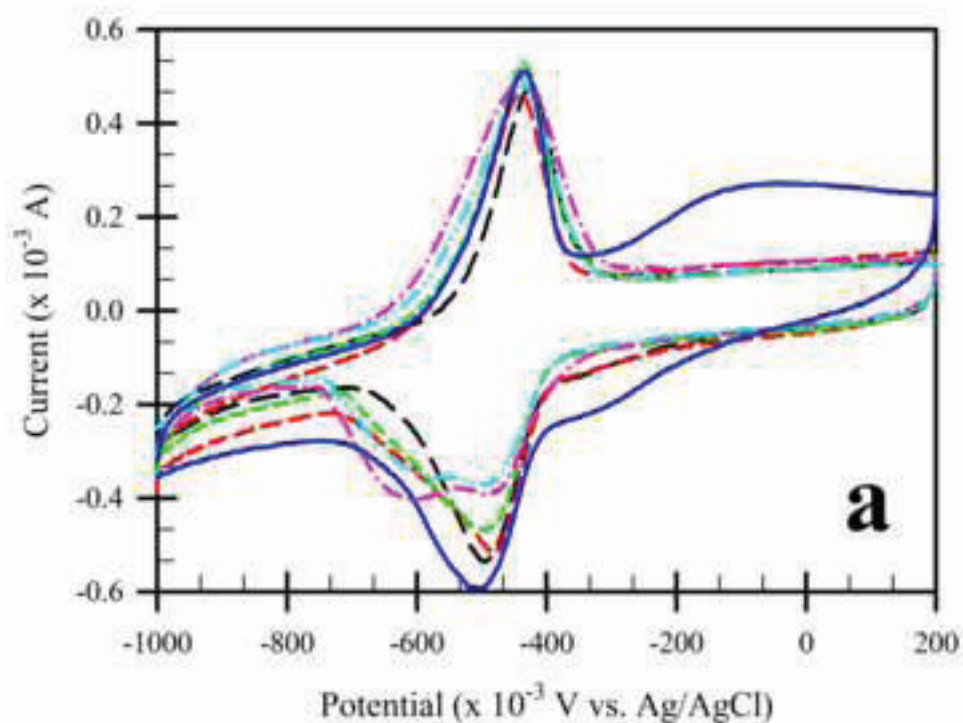


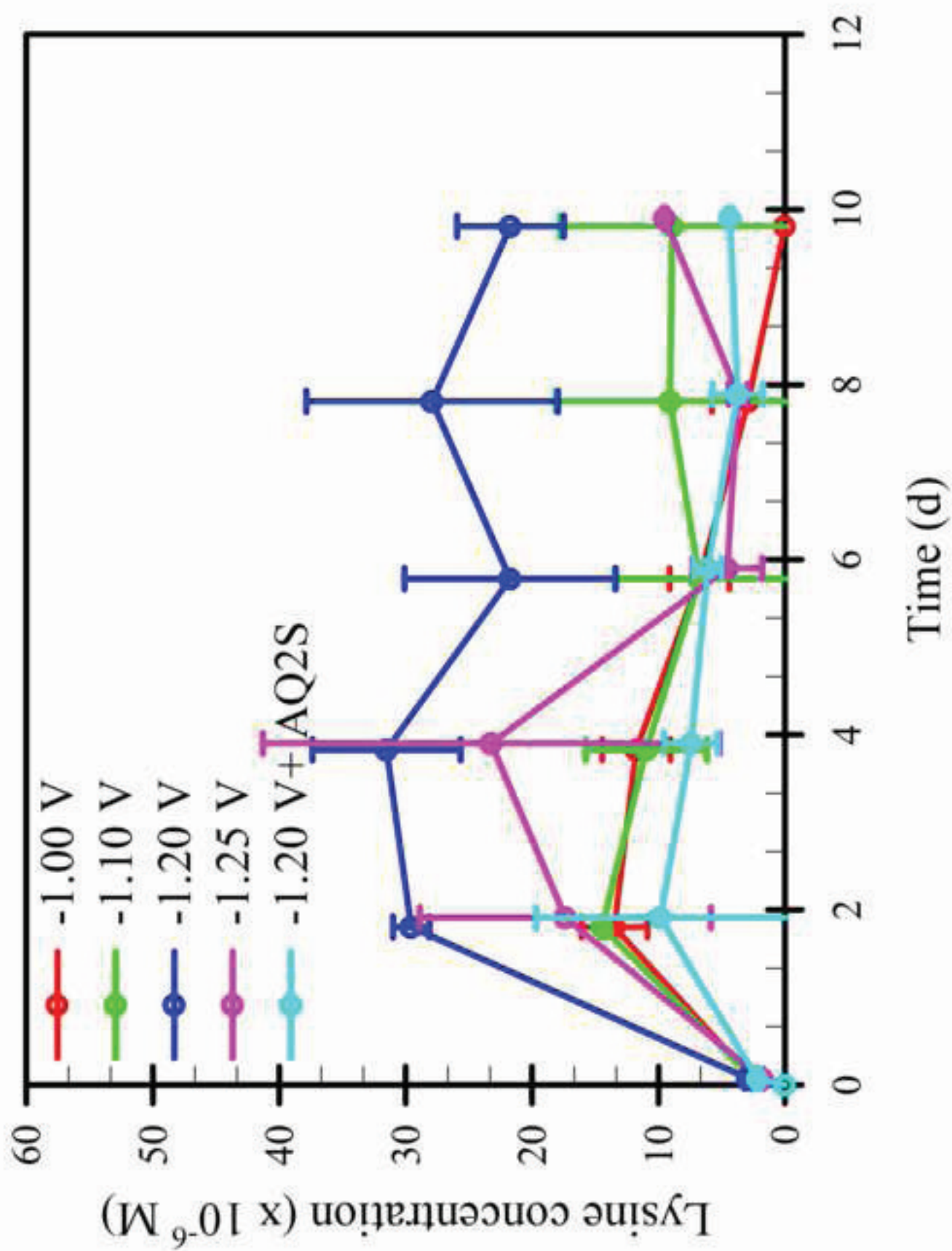












- Considerable growth was observed only under strongly reducing potentials
- High yields of acetate were achieved from glucose
- Increase in acetate yields occurred with a decrease in lactate and succinate yields
- Considerable amounts of formate were produced possibly via CO<sub>2</sub> reduction
- Denitrification was enhanced when both a cathode and *C. glutamicum* were present