Krieg, T., Phan, L.M.P., Wood, J.A., Sydow, A., Vassilev, I., **Krömer, J.O.**, Mangold, K.-M., Holtmann, D. (2018):
Characterization of a membrane-separated and a membrane-less electrobioreactor for bioelectrochemical syntheses
Biotechnol. Bioeng. **115** (7), 1705 – 1716

http://dx.doi.org/10.1002/bit.26600

Bioprocess Engineering **Biotechnology and Bioengineering**
DOI 10.1002/bit.26600

Characterization of a membrane separated and a membrane-less electrobioreactor for bioelectrochemical syntheses†

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†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/bit.26600]

Additional Supporting Information may be found in the online version of this article.

Received December 3, 2017; Revision Received February 22, 2018; Accepted March 19, 2018

Abstract

Bioelectrochemical systems (BESs) have the potential to contribute to the energy revolution driven by the new bio-economy. Until recently, simple reactor designs with minimal process analytics have been used. In recent years, assemblies to host electrodes in bioreactors have been developed resulting in so-called “electrobioreactors”. Bioreactors are scalable, well-mixed, controlled and therefore widely used in biotechnology and adding an electrode extends the possibilities to investigate bioelectrochemical production processes in a standard system. In this work, two assemblies enabling a separated and non-separated electrochemical operation, respectively, are designed and extensively characterized. Electrochemical losses over the electrolyte and the membrane were comparable to H-cells, the bioelectrochemical standard reaction system. An effect of the electrochemical measurements on pH measurements was observed if the potential is outside the range of -1,000 to +600 mV vs. Ag/AgCl. Electrobiotechnological characterization of the two assemblies was done using *Shewanella oneidensis* as an electroactive model organism. Current production over time was improved by a separation of anodic and cathodic chamber by a Nafion® membrane. The developed electrobioreactor was used for a scale-up of the anaerobic bioelectrochemical production of organic acids and lysine from glucose using an engineered *Corynebacterium glutamicum*. Comparison to a small-scale custom-made electrobioreactor indicates that anodic electro-fermentation of lysine and organic acids might not be limited by the BES setup but by the biocatalysis of the cells. This article is protected by copyright. All rights reserved

Keywords: Electrobioreactor, bioelectrochemical synthesis, membrane-less, unbalanced fermentation, computational fluid dynamics

Introduction

The transition to a bio-economy opens up possibilities for renewably producing existing products in established markets but also opens exciting opportunities for new ones. A future bio-economy should not be based only on renewable raw materials but also has to assure circular utilization of resources and interlinking (bio)chemical transformations with the storage and utilization of electric energy (Holtmann et al., 2014). Bioelectrochemical systems (BES) can play a role in the upcoming transition to a bio-economy. BES can be in particular valuable to store renewable energy in organic compounds, extending the toolbox of biotechnology in combination with electrochemistry (Moscoviz et al., 2016). The basis for these systems is the ability of microorganisms to interact with electrodes.

Electroactive microorganisms, which can be used for example in waste water treatment, can transfer excess electrons to anodes breaking down organic compounds (Rozendal et al., 2008). Electrodes can also be used to enable unbalanced fermentations, where the redox balance of substrate and product are uneven. Excess electrons are shuttled outside of the cell to anodes if the product is more oxidized than the substrate or if the product is more reduced than the substrate electrons are supplied by a cathode. Specifically, the first case has been shown for the conversion of glycerol to ethanol and lactate or glucose to acetoin in engineered electroactive *Shewanella oneidensis* (Bursac et al., 2017; Flynn et al., 2010) and *Escherichia coli* strains (Förster et al., 2017), respectively, or enabling anodic respiration in the strict aerobic *Pseudomonas putida* (Hintermayer et al., 2016; Zeppilli et al., 2016). Anaerobic production of organic acids and lysine has been shown recently using an engineered *Corynebacterium glutamicum* strain (Vassilev et al., 2018). In terms of cathodic processes, acetogenic bacteria such as *Sporomusa* sp. and *Clostridium* sp. (Nevin et al., 2011) and chemolithotrophic bacteria such as *Cupriavidus necator* (Grousseau et al., 2014; Krieg et al., 2018; Sydow et al., 2017; Torella et al., 2015) were used to convert carbon dioxide to acetate and isopropanol, respectively, and a new MET called microbial electrosynthesis (MES) was

introduced. Bioconversions starting from other substrates can also be enhanced by electron supply, e.g. production of 1,3-propanediol from glucose in *Clostridium pasteurianum* by alternating the $NADH/NAD^+$ ratio (Choi et al., 2014). One of the biggest bottlenecks to date is the low productivity of BES. Electrode reactions are heterogeneous processes and their efficiency is directly correlated to the electrochemically active surface area, which is usually small compared to the reactor volume. The main requirements for the technical use of BESs have been described by Krieg et al. (2014): high specific electrode surface to volume ratio, biocompatible surfaces, scalable systems, low-cost and long-term stability of all components and the absence or reduction of consumables such as membranes and electrodes.

To date several systems have been used for BES (Krieg et al., 2014), the most prominent are flat-plate or cube-type reactors and divided H-type reactors, which are most commonly used for laboratory studies (Call and Logan, 2008; Lohner et al., 2014; Park et al., 1999). Furthermore, custom-made reactors on the small-scale are used for BESs in wide variety depending on the respective research group (Förster et al., 2017; Kipf et al., 2013). Disadvantages of these reactor types are: i) difficulty to scale-up, ii) comparison of the obtained data due to various designs, iii) insufficient mixing in many reactors and iv) integration of controls due to restricted space (e.g. temperature, pH, pO_2 etc.). In contrast, commercial available standard bioreactors are well characterized, easily scalable and used for several decades in biotechnological processes being available in most laboratories. Recent work using electrobioreactors with process monitoring showed better performances due to improved mixing and/or lower statistical variations compared to three neck flasks without controls (Hintermayer et al., 2016; Rosa et al., 2016).

Consequently, modifying bioreactors can help to increase the performance of electrobiotechnological processes enabling controlled environments for basic studies. However, a deeper characterization of this reactor concept is still missing. The following tasks were defined for this study: i) electrochemical characterization of the system and comparison

to "H-cells", the standard system used for BES, ii) comparison of the flow in standard bioreactors and bioreactors hosting electrodes (electrobioreactors), iii) bioelectrochemical characterization of a separated vs. a non-separated electrobioreactor using the model electroactive microorganism *S. oneidensis* and iv) scale-up from 350 mL to 2 L of an anaerobic bioconversion using *C. glutamicum lysC* for a bioelectrochemical production of chemicals.

Materials and methods

Strains, media and culture conditions

Strains used in this study were *Shewanella oneidensis* MR-1 (ATCC 700550, obtained from the American Type Culture Collection, Manassas, VA, USA) and the lysine-producing strain *C. glutamicum lys*C, which was kindly provided by Prof. Christoph Wittmann (Institute of Systems Biology, Saarland University) (Kim et al., 2006). This mutant has a single nucleotide exchange (S301Y) in the gene encoding the aspartokinase, resulting in the biosynthesis of lysine without a feedback inhibition.

Lactate *Shewanella* basal medium (LSBM) contains the following compounds per liter: K_2HPO_4 225 mg, KH_2PO_4 225 mg, NaCl 460 mg, $(NH_4)_2SO_4$ 225 mg, $MgSO_4$ x 7 H_2O 117 mg, HEPES 23.8 g, Na-lactate (50 % solution) 22.4 g (100 mM), 5 mL vitamin solution and 5 mL trace mineral solution. Prior adding the vitamin and trace mineral solution the pH was adjusted to 7.2 using NaOH pellets and a 1 M NaOH solution. The vitamin solution contained per liter: biotin 2 mg, folic acid 2 mg, pyridoxine hydrochloride 10 mg, thiamin hydrochloride 5 mg, riboflavin 5 mg, nicotinic acid 5 mg, DL-calcium pentothenate 5 mg, cyanocobalamine 0.1 mg, 4-aminobenzoic acid 5 mg and lipoic acid 5 mg. The trace mineral solution had the following composition per liter: nitrilotriacetic acid ($C_6H_9NO_6$) 1.5 g, $MgSO_4$ x 7 H_2O 3 g, $MnSO_4$ x 2 H_2O 0.5 g, NaCl 1 g, $FeSO_4$ x 7 H_2O 100 mg, $CoCl_2$ 100 g, $CaCl_2$ x 2 H_2O 100 mg, $ZnCl_2$ 130 mg, $CuSO_4$ x 5 H_2O 10 mg, $AlK(SO_4)_2$ 10 mg, H_3BO_3 10 mg, Na_2MoO_4 x 2 H_2O 25 mg, $NiCl_2$ 24 mg and Na_2WO_4 x 2 H_2O 25 mg. LSBM main solution

was sterilized at 121°C for 20 min, vitamin solution and trace mineral solution were sterile filtered and stored at 4°C until further use.

For cultivation of *C. glutamicum* a defined mineral medium was used, containing in per liter: glucose 11 g, KH_2PO_4 3.7 g, K_2HPO_4 15.5 g, $(NH_4)_2SO_4$ 10.6 g, $MgSO_4$ 260 mg, $CaCl_2$ 17 mg, 3,4-dihydroxybenzoic acid 32 mg and 1 mL vitamin solution. The vitamin solution contains per liter: cobalamin 0.11 mg, thiamine 0.32 g, pyridoxal phosphate 0.02 mg, biotin 0.11 g and 1 ml trace element solution, which contains per liter: $FeSO_4 \cdot x\ 7\ H_2O$ 10.6 g, $MnSO_4 \cdot x\ H_2O$ 10.6 g, $ZnSO_4 \cdot x\ 7\ H_2O$ 2.1 g, $CuSO_4 \cdot x\ 5\ H_2O$ 210 mg, $NiCl_2 \cdot x\ 6\ H_2O$ 21.3 mg and $Na_6Mo_7O_{24} \cdot x\ 2\ H_2O$ 21.3 mg (Krömer et al., 2004).

For biological experiments with *S. oneidensis*, a tube containing 5 mL Lysogeny broth (LB) medium was inoculated with *S. oneidensis* cells as a preculture from cryo stocks and cultivated in an orbital shaking incubator (Minitron HT, Infors, Bottmingen, Switzerland) at 180 rpm and 30°C for 8 hours. Three 1,000 mL Erlenmeyer flasks containing 200 mL LSBM each were inoculated and the cells were grown over night to an optical density (OD_{600nm}) of 1.0 to 1.2 under same conditions and harvested by centrifugation at 3,220 rcf for 30 minutes at room temperature (Hau et al., 2008).

Colonies of *C. glutamicum* were grown on lysogeny broth agar plates at 30 °C and transferred afterwards into 500-mL baffled shake flasks containing 100 mL of cultivation medium for aerobic overnight cultivation in an orbital shaking incubator (Minitron, Infors, Bottmingen, Switzerland) at 200 rpm and 30°C. When the cells had reached an OD_{600nm} between 5 and 6 (log phase) the cells were harvested by centrifugation (10,000 rcf, room temperature, 3 min).

Design and construction of electrode assemblies to enable non-separated and separated MET in conventional bioreactors

For first tests baffles of a 3.7 L KLF 2000 fermenter (Bioengineering, Wald, Switzerland) were equipped with a Polytetrafluoroethylene (PTFE) mounting hosting the working and the counter electrode (assembly 1, Figure 1 A). Silicon rubber foil (d=0.1 mm, size: width=2.5

cm, length=12.5 cm, Schulz & Souard, Frankfurt am Main, Germany) was used to isolate the electrodes from the stainless steel baffles (Figure 1 A). Carbon fabric (ACC-5092-15, Kynol, Hamburg, Germany) was desorbed at 100°C for 24 h, wetted in isopropanol for 1 h prior use, washed three times with ddH_2O and various layers were placed around the baffles of the bioreactor and clamped onto it using the PTFE mounting fixed by four screws at each side. One layer of carbon fabric equals a geometric surface area of 120 cm² with a mass of 2.42 ± 0.05 g, BET (Brunauer-Emmett-Teller) surface area is 1,450 m^2 g^{-1} (calculated from the I_2 adsorption, which was 1,500 mg g^{-1}, using a correlation graph provided by the supplier) or approximately 3,500 m^2 per layer. Carbon fabric was electrochemically contacted by weaving a platinum wire (d=0.5 mm) through the layer(s) winding it afterwards to the platinum wire of the contact fitting. A fitting with a diameter of 0.5 mm was used to insert a glassy Luggin capillary with silicone rubber to ensure tightness through the reactor lid for the reference electrode (Figure 1 C). Prior to sterilization, the port was closed by a clamped tube for sterilization at 121°C for 30 minutes. The Luggin capillary was sterilized using 70 % ethanol for approx. 5 min and added to the bioreactor after hitting the operating temperature of 30°C using a flame to ensure sterility. The pH was controlled with an electrode (405-DPAS-SC-K8S 325 mm, Mettler-Toledo, Gießen, Germany) and an external addition of 2 M NaOH or 2 M HCl as required maintaining the set pH.

< Insert Figure 1 here >

In assembly 2, working and counter electrode were separated by a membrane (Nafion® 117, Dupont, Wilmington (DE), USA, A=20.3 cm²). The assembly was made of Polyether-ether-ketone (PEEK) and designed and manufactured to host the working electrode, the membrane and the counter electrode in separate compartments (Figure 1 B).

PEEK was chosen due to its excellent mechanical, chemical and temperature stability, which allows sterilization at 121°C. The counter electrode compartment consisted of a cylindrical part, with a silicone sealed lid to prevent catholyte and anolyte from mixing. One layer of

carbon fabric was folded in the middle two times, placed into the counter electrode compartment and contacted by a platinum wire as described before. Fittings (SS-8-HRN-4, Swagelock, Schönefeld, Germany) were added for the perfusion of the cathode with catholyte from a stock bottle placed outside of the reactor, which was open to air. Identical fittings were used to contact the working and counter electrode by integration of a glass cylinder (d=6 mm) with a platinum wire (d=0.5 mm, manufactured from Fischer Labortechnik GmbH, Frankfurt am Main, Germany) melted into the glass cylinder. Various layers of pretreated carbon fabric were placed at the working electrode side into the gadget, in which the carbon fabric layers were clamped one above the other using a bar fixed with screws analogue to assembly 1. The reactor was set-up with the assembly 1 or assembly 2 for the non-separated and separated cultivation, respectively (Figure 1, A-C).

Characterization of the electrobioreactor using *Shewanella oneidensis* MR-1 wild type

The reactor was set-up with the assembly 1 or assembly 2 for the non-separated and separated cultivation, respectively, as described before (Figure 1, A-D). LSBM omitting trace element and vitamin solution was added to a working volume of 2.5 L and 2 L for the non-separated (assembly 1) or the separated system (assembly 2), respectively, and then sterilized at 121°C for 20 min. After cooling down, the reference electrode was sterilized using 70 % ethanol (aq.) and all electrodes were connected to a potentiostat and a potential of +400 mV was applied for 12 to 24 h prior inoculation with cells to equilibrate the medium and enable constant start conditions. All potentials mentioned in this study are vs. a Ag/AgCl reference electrode, which equals to +597 mV vs. the standard hydrogen electrode. Vitamins and trace element solutions were added 30 min before inoculation of the electrobioreactor. For inoculation, *S. oneidensis* preculture was harvested and cell pellets were resuspended in 25 mL of “equilibrated medium”, which was taken sterile from the pre-equilibrated electrobioreactor and inoculated into the reactor to the desired optical density. Optical density was converted into cell dry weight according the following equation: c_{CDW} (g L^{-1}) = OD_{600nm} ·

0.057 g L^{-1} (R^2 = 0.956). The potential was maintained by a potentiostat (Interface 1000, Gamry Instruments, Warminster, PA, USA) and current density was measured over a period of 50 to 75 h. The cathode chamber of assembly 2 was filled with a potassium phosphate buffer (PPB, consisting of 15 g L^{-1} KH_2PO_4 and 30 g L^{-1} K_2HPO_4). A scheme of the electrobioreactor is given in Figure 1 D.

Investigation of pH measurement affected by electrochemical potential and positioning of the pH electrode

Assembly 1 was used to investigate the influence of electrochemical measurements on the pH measurement. Assembly 1 was inserted, 2 L of LSBM was added and graphite rods were used as working and counter electrodes in a classical three-electrode setup with a surface area of 28.6 cm^2 each (Supplemental Figure 1). Measurements of pH were performed with the same positioning of the electrode as in the bioreactor and added to the setup. First, pH was measured before a potential was applied as a blank and to validate that the pH values are constant without electrochemical measurements. Next, the diaphragm of the pH electrode was moved to face towards the electrochemical field and the measurement at a certain potential was started (Supplemental Figure 1). The pH value was taken when the signal was constant (typically less than 5 min). Afterwards, the diaphragm of the pH electrode was faced away from the electrical field and again pH was measured as described. The procedure was repeated in a potential range of -1,500 mV to +900 mV. Circuits of the electrochemical and the pH measurement were isolated and the experiments were repeated to prevent possible circulating currents using an isolation transformer (LTT 003, VEB Technisch-Physikalischen Werkstätten, Thalheim, Germany).

Electrochemical characterization of the bioreactor assembly

Assembly 2 was characterized using the same experimental setup as described for the pH measurement effects (Supplemental Figure 2). However, three reference electrodes were placed in the system: in front of the working electrode, in front of the membrane and behind

the membrane directly in front of the counter electrode (distance to the corresponding component ~ 5 mm). Electrochemical losses were analyzed galvanostatically with currents in the range of 5 mA to 75 mA (0.14 mA cm^{-2} to 2.6 mA cm^{-2}) for positive and negative currents. Current was applied to the system until a steady state potential in front of the working electrode was reached (~ 30 to 120 min). The potential drops between the reference electrodes were measured using a Keithley 2000 Multimeter (Keithley Instruments, Cleveland, OH, USA). H-cells were characterized the same way using graphite rods (d=6 mm, length=10 cm with 5 cm being in the electrolyte) as working and counter electrodes with a surface area of 9.7 cm^2. LSBM containing 100 mM lactate, the medium omitting lactate (SBM) and 0.5 M Na_2SO_4 as an ideal electrolyte were characterized.

Computational fluid dynamic analysis on the turbulence and mixing times by the applied insert

Flow in both the conventional and designed electrobioreactor with assembly 2 was simulated using the mixer module in COMSOL Multiphysics® 5.2 (Boston, MA, USA) (Supplemental Table 1 for geometric values of the bioreactors) to estimate mixing due to turbulence (eddy viscosity) and to estimate if possible deadzones will arise. A simplified geometry of assembly 2 was drawn and meshed which is shown in Supplemental Figure 3. The Reynolds number of the reactor systems is calculated with the equation $\mathrm{Re} = \frac{\rho\, n\, d^2}{\mu}$ where ρ is the density of the fluid, d is the diameter of the stirrer, n is the stirrer rotation speed and μ the dynamic viscosity (Khang and Levenspiel, 1976). With ρ = 972 kg m^{-3}, d = 45 mm, n = 400 rpm and $\mu = 10^{-3}$ Pa s, the Re is calculated to be 13130 and consequently the flow is assumed to be turbulent (Potter et al., 2012). Velocity fields in both bioreactor configurations were determined through solving the incompressible Navier-Stokes equations with the k-ε model for turbulence in a frozen rotor framework and the conventional bioreactor was compared to the designed electrobioreactor with assembly 2. Mesh independence and resolution was confirmed by

successful refinement of the mesh and relative changes of the solution were checked, as well as the wall lift-off (Kuzmin and Mierka, 2006). Mixing times were compared in the two configurations and diffusion times t_D at different lengths x were calculated with Fick's second law, which is $t_D = \frac{x^2}{2\,D_T}$, where D_T is the estimated turbulent kinematic viscosity from simulations.

Anaerobic production of lysine and organic acids with *Corynebacterium glutamicum*

The procedure was described in detail elsewhere (Vassilev et al., 2018). Briefly, the electrobioreactor was equipped with assembly 2 and operated as described above.

$K_3[Fe(CN)_6]$ was added as a mediator to enable electron transport (1.5 mM). The electrobioreactor was inoculated to a start OD_{600nm} of ~ 4. The pH was controlled at 7.2 ± 0.2 as described before by adding HCl or NaOH to the medium as required. The potential of the working electrode was set to a value of +500 mV. The cathode chamber of assembly 2 was filled with a potassium phosphate buffer (PPB, consisting of 15 g L^{-1} KH_2PO_4 and 30 g L^{-1} K_2HPO_4).

Analytics

Lactate, succinate and acetate were measured by HPLC (Prominence 20 series, Shimadzu Deutschland GmbH, Duisburg, Germany) equipped with a Rezex ROA-Organic Acid 8% H+ 300x7.8 mm (Phenomenex, Aschaffenburg, Germany) column via a photo diode array detector (SPD-M20A, Shimadzu Deutschland GmbH, Duisburg, Germany) at the detection wavelength of 209 nm. Sulfuric acid (5 mM) was used as mobile phase with a flow rate of 0.6 mL min^{-1} at 60 °C over a total run time of 30 min. Quantification was done using a calibration curve with external standards in the range of 0 to 200 mM lactate, succinate and acetate, respectively. Lysine and alanine were analyzed on an equivalent HPLC system equipped with a Kinetex® 2.6 µm XB-C18 100 Å 30x2.1 mm column (Phenomenex, Aschaffenburg, Germany) and coupled to a triple-quadrupole mass spectrometer (LCMS-8040, Shimadzu

Deutschland GmbH, Duisburg, Germany) with an electrospray ionization source (Shimadzu Deutschland GmbH, Duisburg, Germany). Column temperature was 30 °C, the mobile phase consisted of solvent A, H_2O + 0.0025 % ammonia, and solvent B, acetonitrile, combined following a binary gradient method (0 min 5 % B, 1.5 min 5 % B, 1.8 min 95 % B, 3.2 min 95 % B, 3.3-4.5 min 5 % B) with a run time of 4.5 min and a total flow rate of 0.3 mL min^{-1}. Electrospray ionization was performed in the positive mode while the interface voltage was set at 4.5 kV. To analyze lysine and alanine, the mass spectrometer was run in the selected ion monitoring mode at m/z 147.1 for the detection of protonated lysine and m/z 90.1 to detect protonated alanine, respectively. A linear calibration curve of external standards in the range of 0 to 200 µM was used for quantification of the amino acids.

Results and discussion

Bioreactor cultivation of *Shewanella oneidensis* in the non-separated assembly 1

First, characterization and proof of principle of electrode integration into a conventional reactor system was done by a simple modification of vortex breakers. The first runs were carried out at a constant potential of +400 mV without inoculation of any cells into the reactor to prove suitability in the used bioreactor and evaluate the capacitive current caused by the high BET (Brunauer–Emmett–Teller) surface of the electrodes. A current of 5.2 mA (corresponding to a current density of 43.1 µA cm^{-2}) was measured after 24 h and without inoculation of cells current maintained this level for over 100 h. After 100 h medium was sampled under sterile conditions and plated onto an LB-agar plate. No colonies arose after 7 d incubation at 30°C. It was concluded from this result that the electrobioreactor was sterile over this time period. In the next step the bioreactor hosting assembly 1 was inoculated with different concentrations of *S. oneidensis* cells (5.1, 6.8 and 14.3 mg CDW L^{-1}) in order to investigate the current measured in the electrobioreactor. Immediately after inoculation a current was measured, showing that the electrobioreactor hosting assembly 1 can be used for BESs. Surprisingly, no dependency between the current production and the inoculated

biomass concentration was observed (Supplemental Figure 4). It was concluded that the working electrode surface was limiting the current production in the system. The working electrode surface was therefore increased by inserting additional layers of carbon fabric into assembly 1. A linear dependency between the maximum current and the working electrode surface area was observed up to a geometric surface area of 960 cm² (Figure 2). The current density between 1 and 8 layers (120 to 960 cm²) was at a constant level of 157 ± 11 µA cm^{-2}, decreasing to 110 µA cm^{-2} at 12 layers of fabric (1,440 cm²). Experiments with 120 cm² were done in duplicate and showed rather small deviations (19.6 mA vs. 17.1 mA measured peak current). It can be concluded that the experimental conditions are robust and one experiment per electrode surface area was performed. Current efficiencies increased from 38 % using 1 layer of carbon fabric up to around 73 % using more layers of carbon fabric (Figure 2). These values are rather high compared to reported current efficiencies for *S. oneidensis* based microbial fuel cells, which are usually around 10 % (Rosa et al., 2016; Rosenbaum et al., 2011). However, Rosa et al. used an aerobic growth phase for biomass generation and Rosenbaum et al. used a continuous process in H-cells. Here, the electrobioreactor was purged with nitrogen and the inoculum was washed in pre-equilibrated (nitrogen purged) medium. The rather high electroactive surface area in the experiments led to a fast degradation of lactate (no lactate was measured after 24 to 56 h after inoculation, data not shown).

< Insert Figure 2 here >

Further increasing the working electrode surface area up to 1,440 cm² did not improve the maximum current, meaning most likely the biomass concentration was now limiting current production instead of the working electrode area. To exclude the possibility due to limitation of the counter electrode surface area, it was increased by four fold. No increase of current was observed under these conditions (Supplemental Figure 5).

The designed assembly is advantageous and can host flexible electrode surface areas by simply applying different layers of carbon fabric and can be tailored for the respective

electrobiotechnological process. However, the ability to separate the working from the counter electrode is often needed in BESs to prevent cross-reactions (e.g. oxygen produced at the anode diffusing into the anaerobic cathode chamber). Therefore a new electrode holder was developed and constructed to host a membrane separating the two electrodes (assembly 2, Figure 1 B and C). A Nafion® 117 membrane was chosen to separate the system, as it is widely used in BES (Leong et al., 2013).

Characterization of assembly 2 for separated electrochemistry in bioreactors

Evaluation of electrochemical losses in the system

Like all electrochemical systems, also BESs suffer from electrochemical losses (Clauwaert et al., 2008). To compare assembly 2 with conventional reactor concepts for BESs, electrochemical losses were investigated using an idealized and simplified electrochemical system using graphite stick electrodes and 0.5 M Na_2SO_4 as electrolyte (Figure 3). Current was applied to the system and potentials were measured using reference electrodes placed next to the electrodes and the membrane to measure losses due to electrolyte and membrane (Supplemental Figure 2). The potential losses via the membrane and the electrolyte increase with the applied current in a linear (Ohmic) fashion. As expected, independent of negative or positive current applied, the potential losses are comparable to each other. These results show that the biggest loss in BESs is due to overpotentials at the two electrodes. This can be reduced by applying catalysts, for cathodes: e.g. i) platinum ii) electroactive bacteria catalyzing oxygen reductions or for anodes: e.g. i) dimensional stable anodes ii) electroactive bacteria catalyzing oxygen evolution or in general the supply of electrons (Dulon et al., 2006; Huang et al., 2011; Zhou, 2013).

< Insert Figure 3 here >

Negligible losses are measured when using 0.5 M Na_2SO_4 as an electrolyte due to its high conductivity (55 mS cm^{-1}, Wolf, 1966). For BES, growth media with far lower conductivity need to be used and most likely, the potential losses via bulk solution resistance do matter. To

test this hypothesis LSBM and SBM were used to characterize the system. The electroactive surface of a graphite rod is fairly low and would not be used in a technical setup. Therefore, a carbon fabric electrode with a higher electrode surface area was used for further characterization to be closer to a technical application. Electrochemical characterization reveals, that the growth media show higher potential losses in comparison to 0.5 M Na_2SO_4 (Figure 4, conductivity of the used buffer system is 10 mS cm^{-1}). Especially the medium omitting lactate shows high potential losses compared to a medium with 100 mM lactate and the "ideal" electrolyte Na_2SO_4. Comparable losses were measured in H-cells, which are widely used in BES (Supplemental Figures 6 and 7).

< Insert Figure 4 here >

Current densities reported for unbalanced fermentations are in the range of 55 µA cm^{-2} for a *S. oneidensis* based microbial fuel cell in a modified bioreactor (Rosa et al., 2016). For cathodic processes where current is consumed and acetate or butyrate is produced using pure or mixed cultures, current densities range from several µA cm^{-2} up to 3.7 mA cm^{-2} (de Campos-Rodrigues and Rosenbaum, 2014; Ganigué et al., 2015; Giddings et al., 2015; Jourdin et al., 2014). However, a *Pseudomonas putida* based anodic respiration process using an external mediator showed higher current densities (up to 12 mA cm^{-2}), where overpotentials at the electrodes play a greater role compared to the processes described before and need to be accounted for the electrochemical measurements (Hintermayer et al., 2016). In general, it can be concluded, that electrochemical losses over the electrolyte and the membrane can be neglected in most setups due to their low current densities. This postulate can be confirmed looking at current densities of BESs, which are limited due to high overpotentials at the electrodes and slow migration of protons or other ions via membranes (Clauwaert et al., 2008; Zhou, 2013). These results indicate that the electrobioreactor is suitable for BESs using the two designed and constructed assemblies.

Computational fluid dynamic analysis on the turbulence and mixing times by the applied insert

Applying an insert into a bioreactor may influence the velocity fields and potentially generate dead zones. To exclude this, the velocity fields were solved in the conventional bioreactor and turbulent kinematic viscosity was calculated and compared (Figure 5). Maximum values were 1.1 x 10^{-4} m^2 s^{-1} and minimum values were 2 x 10^{-6} m^2 s^{-1}, respectively, indicating that the conventional bioreactor is well mixed. Assuming a stirrer speed of 400 rpm, which was used in this study, a substance is transferred a distance of 12.7 cm (which is the vessel diameter) at the least mixed zone (nearby the shaft) in 66 min, 1.2 min in the best mixed zone and 2.1 min in average, respectively, in a conventional bioreactor. By using the electrobioreactor maximum values were 2.9 x 10^{-4} m^2 s^{-1} and minimum values were 1.5 x 10^{-6} m^2 s^{-1}, respectively. Assuming a stirrer speed of 400 rpm a substance is transferred a distance of 12.7 cm (which is the vessel diameter) at the least mixed zone (nearby the shaft) in 89 min, 0.5 min in the best mixed zone and 1.2 min in average, respectively, in the electrobioreactor. This implies a fairly minimal change in the mixing intensity between configurations. Values in the electrobioreactor hosting assembly 1 as an insert are to be expected in the same range due to geometric similarity. It can be concluded, that both systems are well mixed and no significant dead zones are caused by inserting electrodes into the bioreactor.

< Insert Figure 5 here >

Influence of electrochemistry on the pH measurements

During first tests of assembly 1, differences between pH values (up to 0.2 units) measured with the reactor probe vs. external measured values occurred frequently and were consequently investigated. Figure 6 shows the measurement of the pH with the diaphragm facing towards the electric field. Between -1,000 mV and +600 mV a shift of the measured pH values can be observed, increasing from negative potentials to positive potentials. This shift is not observed if the diaphragm of the pH electrode does not face towards the electric

field, which makes a pH measurement possible without interference (Supplemental Figure 1). Outside of this potential range, a measurement was not possible without interference, independent of the pH electrode diaphragm positioning. An electronic separation of the pH measurement system was done using an isolating transformer to isolate the circuits of the two measurement systems (electrochemical and pH measurement) but showed similar results. Parasitic currents may be responsible for this behavior. Functionality of the pH probe was ensured with calibrations prior to every cultivation and potential values of the probe did not drift over 20 cultivations.

< Insert Figure 6 here >

The identified operation range of -1,000 to +600 mV vs. Ag/AgCl is fitting most METs. However, METs using higher or lower potentials are excluded (e.g. H_2 evolution for indirect electron transfer) (Sydow et al., 2017). Therefore, other pH measurement techniques need to be considered for these applications like optical measurements, which already have been described for pH control in bioreactors (Hanson et al., 2007; Kusterer et al., 2008).

Comparison of bioreactor cultivations of *Shewanella oneidensis* in the separated assembly with the non-separated bioreactor

Besides abiotic characterizations, also an electrobiotechnological process using the model electroactive bacterium *S. oneidensis* was also done in both assemblies to compare the performance between a separated and a non-separated operation. *S. oneidensis* was inoculated after the working electrode was poised at a potential of +400 mV over night to eliminate capacitive currents. An increased current density was observed (Figure 7) and highest current densities of up to 154 ± 13 µA cm^{-2} were achieved in the non-separated electrobioreactor vs. the separated electrobioreactor, which had current densities up to 96 ± 14 µA cm^{-2}. Higher internal resistances were not responsible for the difference between the separated and non-separated electrobioreactor, as the electrochemical characterization showed only low electrochemical losses due to the membrane (Figure 3). The highest current densities in

electrobioreactors with *S. oneidensis* previously reported were 55 µA cm^{-2} (Rosa et al., 2016). Cells were first cultivated aerobically and gassing with nitrogen started after 24 h of cultivation, which may have led to rather small current efficiencies due to oxygen in the system in the reported electrobioreactor. Current profiles shown in Figure 6 may be explained by mass transport limitations via the Nafion® 117 membrane, which limit the performance (Chae et al., 2008). The authors reported that the membrane size should be approximately the size of the electrode to reduce the internal resistance. One drawback of an increased size is that oxygen diffusion in facilitated. In this study a membrane size of 20.3 cm^2 was used due to stability constraints. Membranes with larger surface areas bulged and interfered with the stirrer.

< Insert Figure 7 here >

Besides maximum current densities, the specific charge of the reactor systems was calculated (Figure 8). Both setups showed comparable specific charges transferred. Despite showing a lower maximum current density, the separated electrobioreactor with the designed and characterized assembly 2 was the best performing system in terms of long-term stability (meaning constant current densitiy over time), indicated also by the intersection point of current densities shown in Figure 7. Current efficiencies were highest with 65±18 % in the non-separated electrobioreactor and 27±8 % in the separated electrobioreactor. The differences may be explained by chemical short circuits in the non-separated electrobioreactor and/or limitations caused by the membrane. Substances may react at the different electrodes leading to false positive current measurements (Harnisch and Schröder, 2009).

< Insert Figure 8 here >

The difference between the two setups can not be explained by oxygen diffusion from the cathode chamber through the Nafion® membrane to the anode chamber, which has been reported before (Leong et al., 2013). Theoretical total oxygen flux through the membrane was 444 µmol during the measured time of 75 h and was calculated using $j = K_{O2} \cdot c_{O2} \cdot A_M$ with a

mass transfer coefficient of oxygen K_{O2} of 2.8 10^{-4} cm s^{-1}, an oxygen concentration c_{O2} in the catholyte of 0.235 mM at 30°C calculated by Henry's law and a membrane surface are A_M of 25 cm^2 (Chae et al., 2008). It can be concluded, that oxygen diffusion through the membrane accounts for only 1 % of the consumed lactate over the process time. Additional experiements were performed in a conventional H-cell (Supplemental Figure 8). Electrode distances between H-cells and the electrobioreactors were in a similar range (12 cm vs. 15 cm) as indicated in Figure 4 and Supplemental Figure 7. Maximum current densities and the trajectory of the current output were comparable for the non-separated H-cell vs. the electrobioreactor with assembly 1 (non-separated). Maximum current output was higher for the separated H-cells vs. the electrobioreactor with assembly 2 (separated). Current efficiencies were 16.5 % for the separated H-cell vs. 2.5 % for the non-separated H-cell. It has to be noted that the separated H-cell stopped after approximately 50 h and the electrobioreactor with assembly 2 produced at a constant level for 70 h and is the system of choice for electrobiotechnological processes. It can be concluded, that the H-cell can be used as a screening tool for development. However, from a technical point of view a H-cell type reactor can not be scaled-up .

Bioelectrochemical anaerobic production of lysine and organic acids using *Corynebacterium glutamicum*

To show the broad applicability of the developed reactor systems a second biological system was investigated in assembly 2. Previous works showed that anaerobic conversion of glucose to lactate, succinate and lysine with an engineered *C. glutamicum* is enhanced by the application of an anode as an external electron acceptor and 1.5 mM ferricyanide used as a mediator (Vassilev et al., 2018). These investigations were done in self-made electrobioreactors with a working volume of 350 mL (Lai et al., 2016; Vassilev et al., 2018). The counter electrode was separated by a cation exchange membrane and products were in the

bulk phase of the electrobioreactor. A scale-up of this process was realized from 350 mL to 2.4 L with the designed electrobioreactor using assembly 2. Mediator concentrations and specific electrode surface areas of the used carbon cloth electrodes were kept constant at 0.07 cm^2 cm^{-3} and specific membrane area was 10.2 cm^2 dm^{-3} (vs. 8.1 cm^2 dm^{-3} in the previous work by Vassilev et al., 2018.

Current production started immediately after inoculation and reached current densities of up to 115 µA cm^{-2}, which is in the same range compared to the *S. oneidensis* experiments (Figure 9 A). Glucose was consumed over a period of 25 h and concentrations of up to 70 mM lactate, 10 mM succinate and 2 mM lysine were produced in the system (Figure 9 B). Specific production rates were 1.7 mmol g_{CDW}^{-1} h^{-1} lactate, 0.25 mmol g_{CDW}^{-1} h^{-1} succinate and 0.047 mmol g_{CDW}^{-1} h^{-1} lysine, which are in the same range as in the 350 mL system (1.5 mmol g_{CDW}^{-1} h^{-1} lactate, 0.25 mmol g_{CDW}^{-1} h^{-1} succinate and 0.076 mmol g_{CDW}^{-1} h^{-1} lysine) previously reported by Vassilev et al., 2018.

These results prove that the designed electrobioreactor with assembly 2 is suitable for bioelectrochemical production of chemicals. Despite using a defined and well characterized modified electrobioreactor with optimal mixing conditions of the medium vs. a custom made electrobioreactor with non-defined mixing by a magnetic mixer, no increased production rates were measured. This indicates that anodic electro-fermentation of lysine and organic acids might not be limited by the BES setup but rather by the biocatalysis of the cells.

< Insert Figure 9 here >

Conclusion

While microbial fuel cells for energy conversion require reactors with cheap materials and easy designs, more sophisticated designs are required for the microbial synthesis of fine and bulk chemicals (Krieg et al., 2014). Autoclavable reactors and the integration of electrodes into controlled standard systems used in biotechnology should be preferred (Krieg et al., 2014). Two assemblies were designed based on previous studies: a membrane-less system for

a proof of principle a bioreactor enabling simple “one-pot” reactions and a more sophisticated design where the counter electrode can be separated from the working electrode by a membrane. Computational fluid dynamics show that the insertion of assemblies to host electrodes does not create dead zones, the mixing is not affected and can be compared to a conventional bioreactor. The designed electrobioreactors were characterized electrochemically and no differences in terms of electrochemical losses compared to H-cells occurred, which are commonly used in studies of BESs. One advantage of using bioreactors is that the pH can be controlled over the process. The data presented in this study show, that the pH signal is not influenced by the electrochemical measurement in a potential range between -1,000 mV and +600 mV vs. Ag/AgCl. Current densities of up to 154 ± 13 µA cm^{-2} were measured in the non-separated electrobioreactor (assembly 1), while the separated electrobioreactor (assembly 2) had current densities up to 96 ± 14 µA cm^{-2}. To date, the highest current densities in a electrobioreactor achieved with *S. oneidensis* were 55 µA cm^{-2} (Rosa et al., 2016). Finally, the anaerobic bioelectrochemical production of lysine and organic acids using *C. glutamicum* was performed in the reported electrobioreactor. Comparable results to another electrobioreactor were achieved providing that the designed electrobioreactor is suitable for bioelectrochemical production of chemicals (Vassilev et al., 2018).

Two different assemblies were developed and characterized in detail and can now be applied in a variety of applications of BES. The decision if a membrane-separated or a membrane-less assembly should be applied must be weighed up carefully against the effectiveness and the costs of a complete system. Finally, it must be mentioned that in our investigations the influence of the membrane as separator on the performance of the BES was less than expected.

Acknowledgements

The authors want to thank the DECHEMA workshop team, especially Yvonne Hohmann, Janno Bender and Heinrich Kopietz for fruitful discussions, the design and the construction of the two assemblies for the electrobioreactor. The authors wish to acknowledge Prof. Christoph Wittmann for kindly providing the *C. glutamicum lys*C strain to us. Igor Vassilev would like to thank the University of Queensland graduate school for awarding him the travel grant GSITA, which enabled this collaboration work. Thomas Krieg, Anne Sydow, Klaus-Michael Mangold and Dirk Holtmann want to thank the BMBF for funding the project "Microbial electrosynthesis - An integrated research project for the utilization of electric energy in microbial production processes" (FKZ 031A226).

Conflict of interest statement

The authors declare that there is no conflict of interest.

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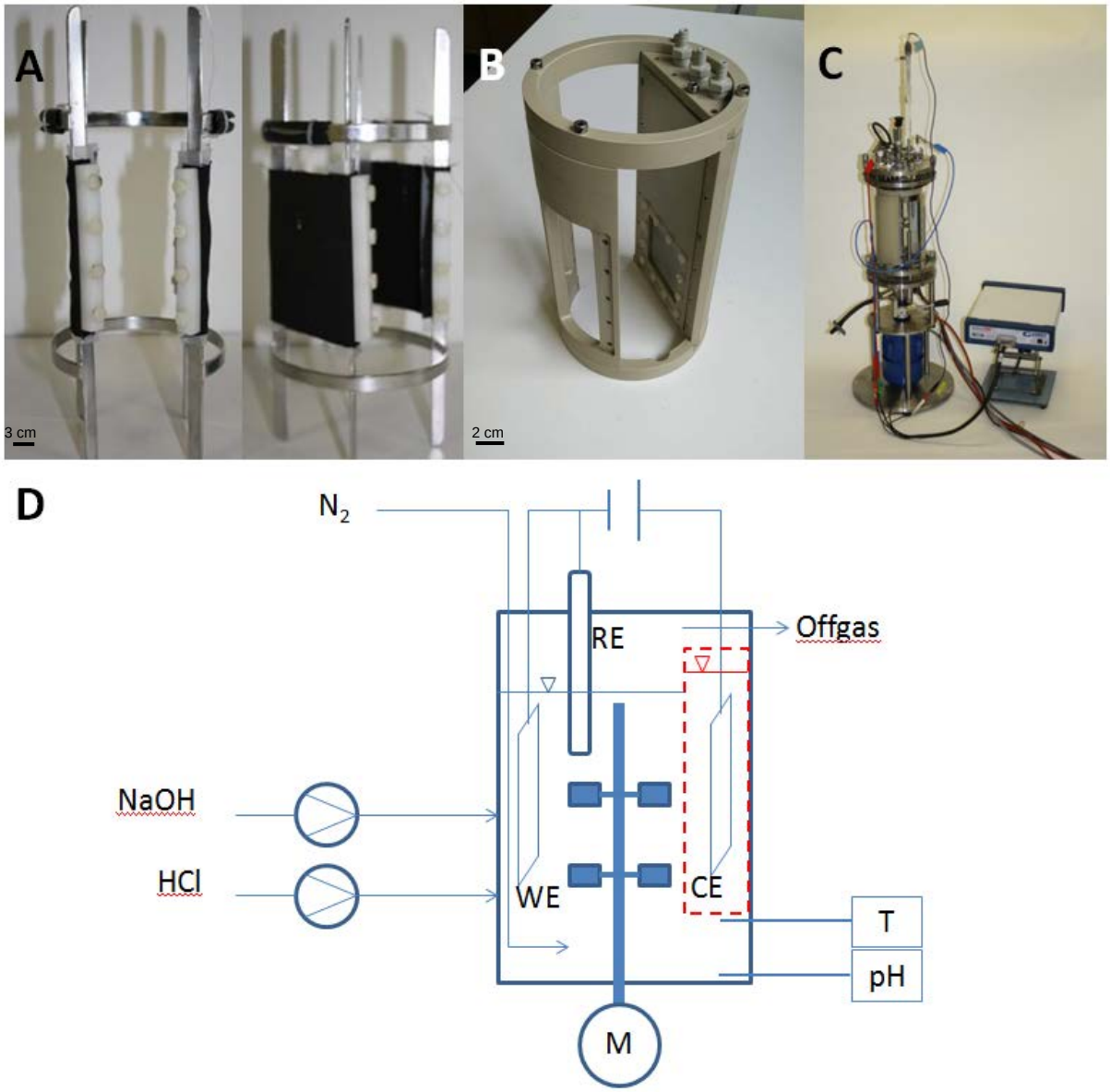

Figure 1: Different possibilities to equip a conventional bioreactor with electrodes. A: modified baffles with carbon fabric electrodes for non-separated MET processes (assembly 1). B: designed assembly 2 to allow separated MET processes (assembly 2). C: Equipped electrobioreactor with assembly 2, pH-electrode, thermo element, stirring unit and potentiostat. D: scheme of the electrobioreactor with controls and possible separation of the counter electrode (dashed red line) with temperature (T), pH control and stirring unit (motor M).

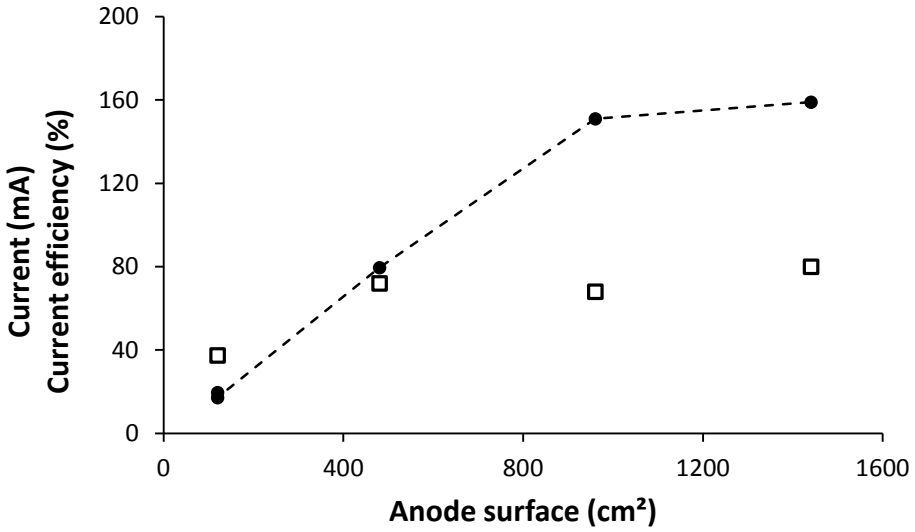

Figure 2: Closed dots show the maximum current productions of 11.5 ± 4.3 mg/L *S. oneidensis* using assembly 1 having no separation between the working and counter electrode and using 1 to 12 layers (120 to 1440 cm² of carbon fabric) of the working electrode carbon fabric poised at +400 mV vs. Ag/AgCl. Open squares are the corresponding current efficiencies.

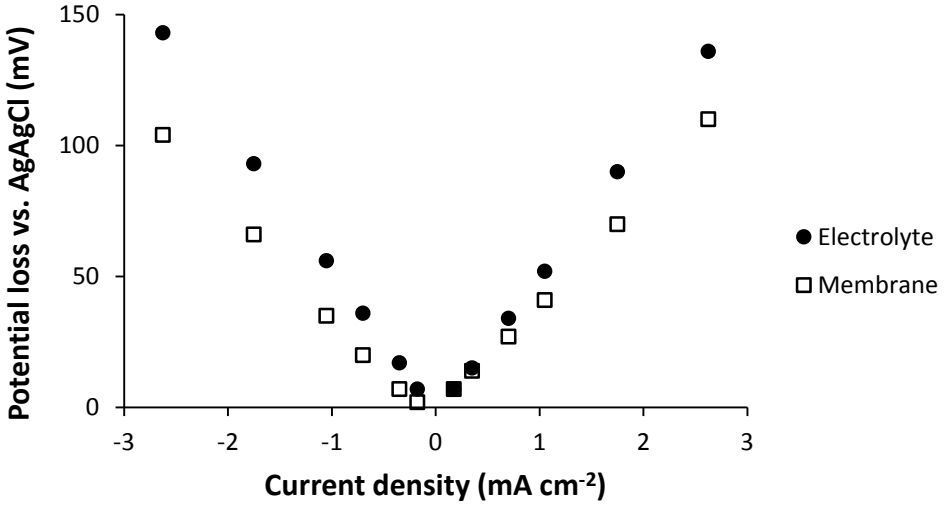

Figure 3: Electrochemical losses over the electrolyte 0.5 M Na_2SO_4 and the membrane (Nafion® 117) in the developed reactor assembly 2. Graphite sticks with a surface area of 28.6 cm² were used as working and counter electrodes.

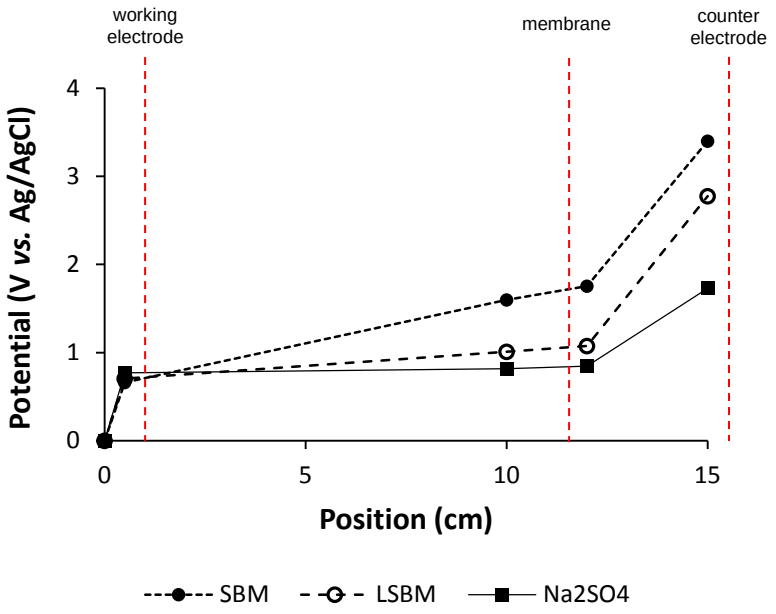

Figure 4: Electrochemical losses of the membrane (Nafion® 117) and different electrolytes (SBM, LSBM and 0.5 M Na_2SO_4) at a current density of 1.05 mA cm^{-2} in the developed reactor assembly. Carbon fabric (129 cm^2) contacted with a platinum wire (d=0.5 mm) was used as working and counter electrode.

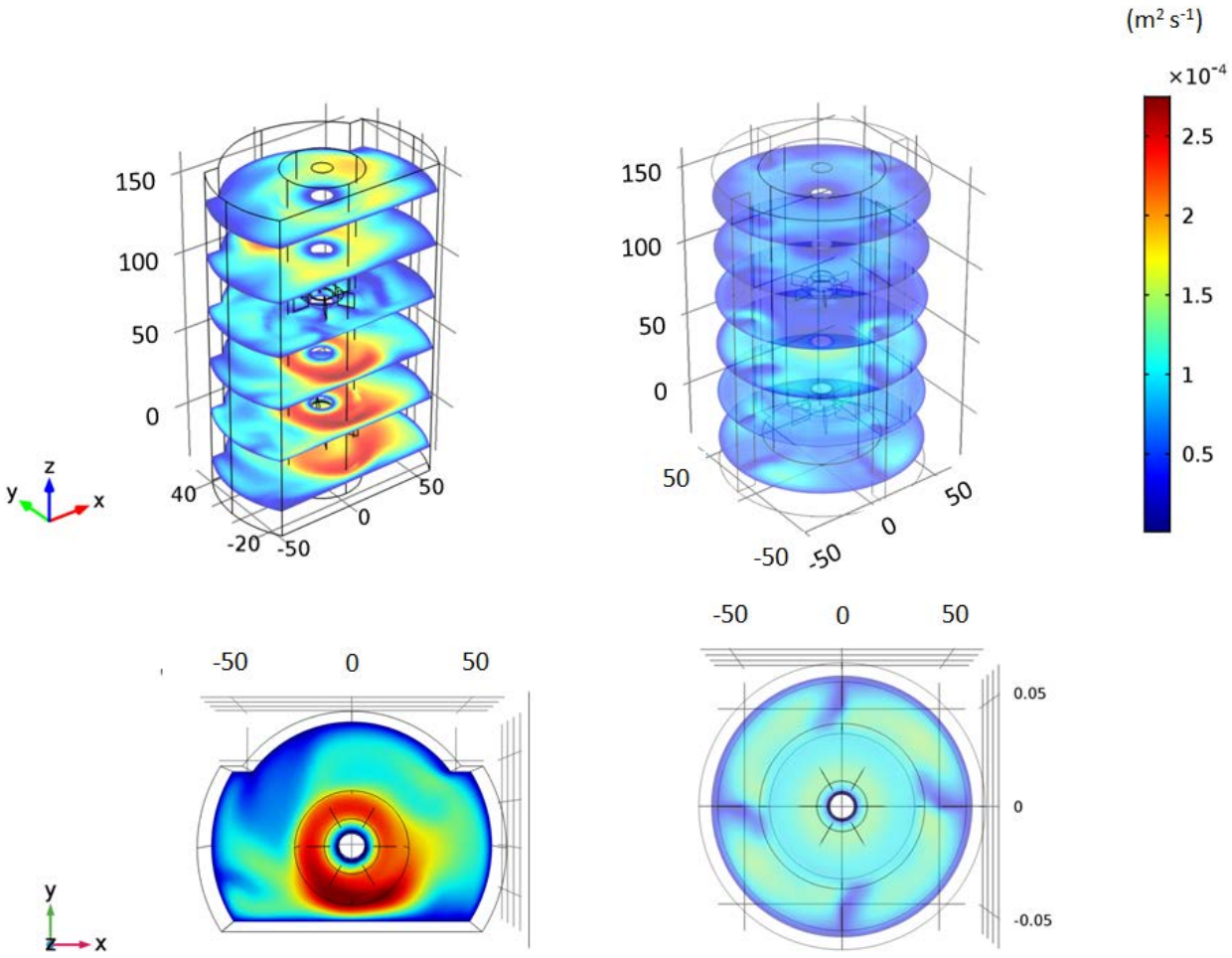

Figure 5: Comparison of the turbulent kinematic viscosity in the bioreactor equipped with assembly 2 (left image) and a standard bioreactor (right image) simulated by computational fluid dynamics in COMSOL®. Dimensions in x, y and z direction are given in mm. The figures show slices in xy-planes at different heights throughout the different reactor configurations, with the magnitude of turbulent kinematic viscosity plotting on each slice.

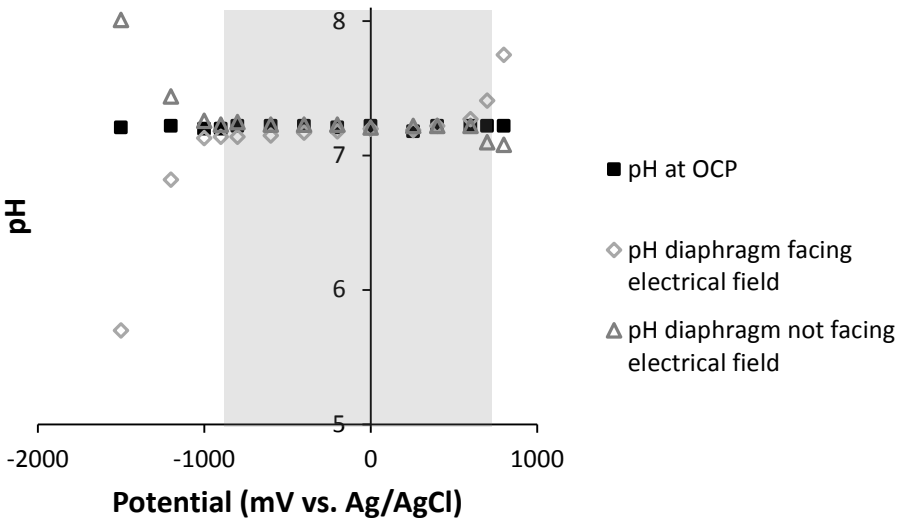

Figure 6: pH values during an electrochemical measurement and at the open circuit potential (OCP) with different positions of the pH electrode diaphragm towards the electric field. Graphite sticks with surface area of 28.6 cm² were used as working and counter electrodes at varied potentials. Operating area is marked in grey.

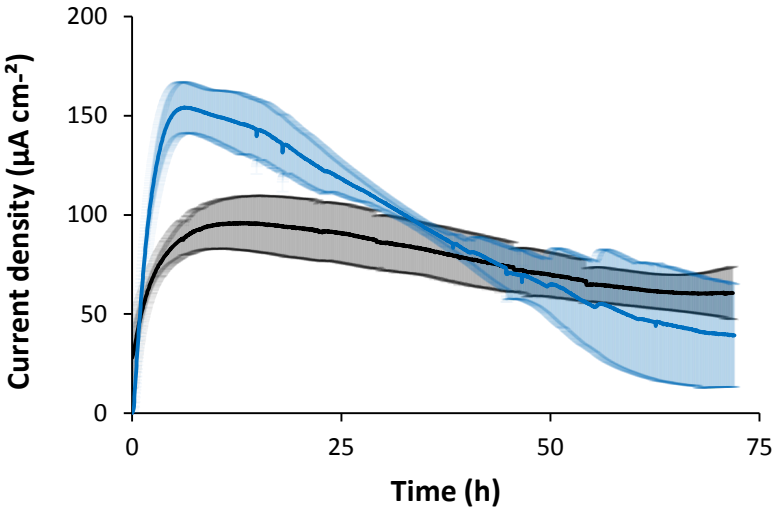

Figure 7: Current production of 12.5 ± 2.8 mg L^{-1} *S. oneidensis* in the separated (black) and non-separated (blue) electrobioreactor, respectively, using one layer of the working electrode carbon fabric poised at +400 mV vs. Ag/AgCl. The experiments were performed with repeats n = 3 in the electrobioreactor with assembly 1 and assembly 2.

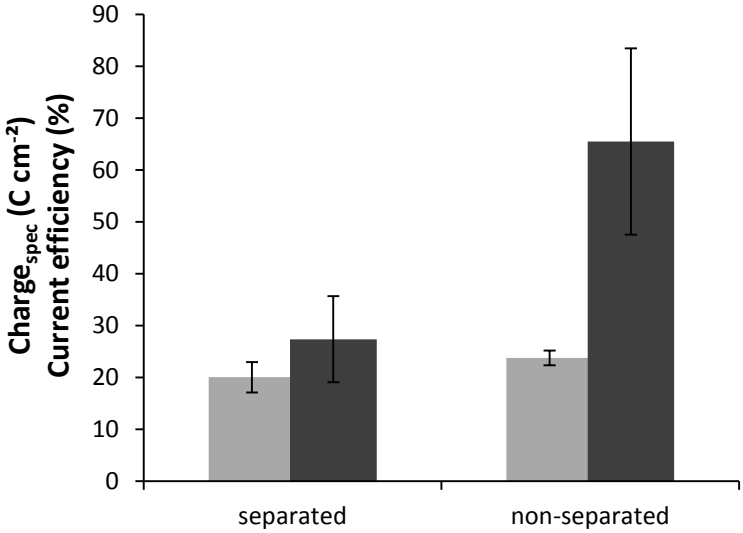

Figure 8: Transferred specific charge (light grey) and current efficiencies (dark grey) of 12.5 ± 2.8 mg L^{-1} *S. oneidensis* in the separated and non-separated electrobioreactor, respectively, using one layer of the working electrode carbon fabric (120 cm^2) poised at +400 mV vs. Ag/AgCl. Carbon fabric contacted with platinum wire (d=5mm) was used as an electrode material. The experiments were performed in n = 3 in the electrobioreactor with assembly 1 and assembly 2.

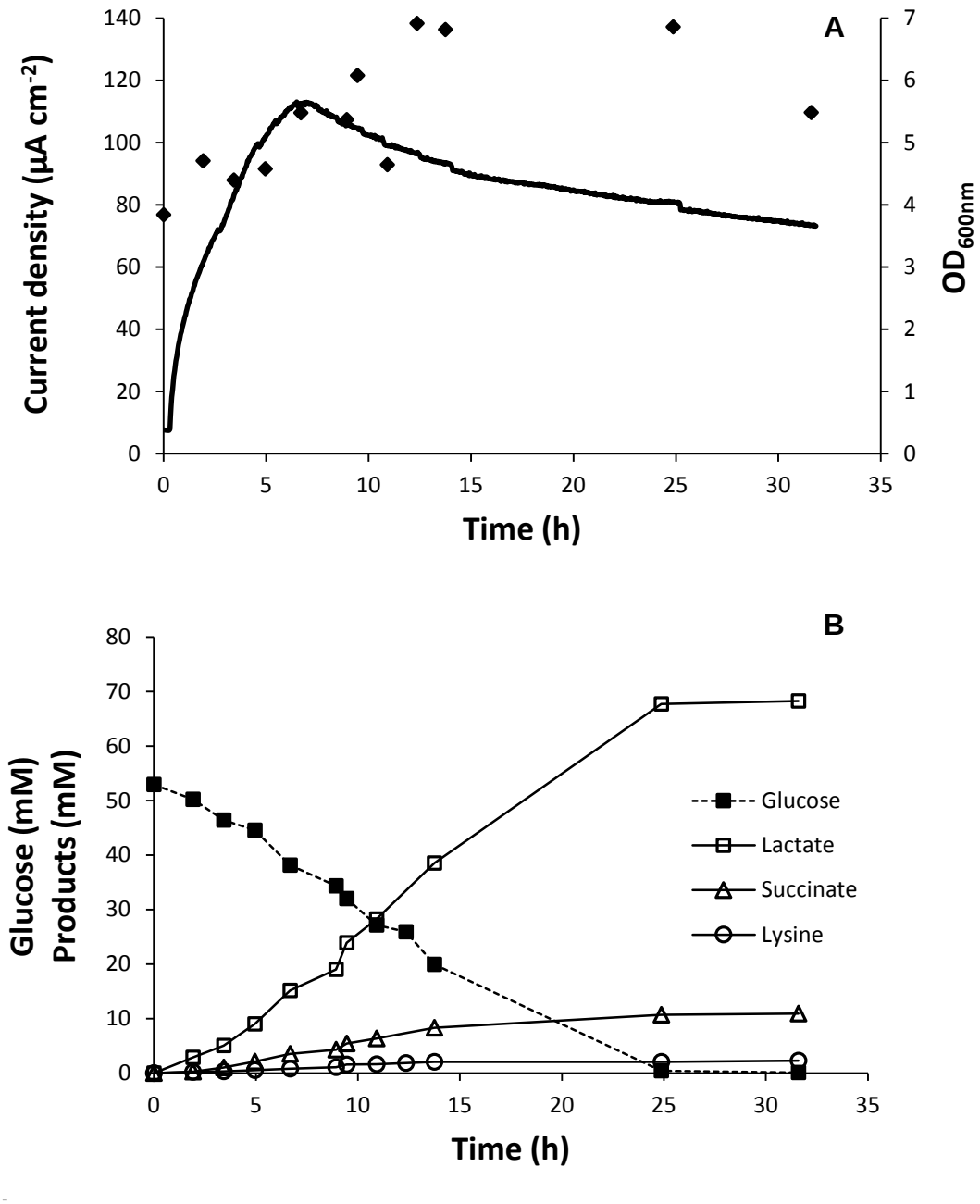

Figure 9: Electrochemically influenced conversion of glucose to lactate, succinate and lysine in *Corynebacterium glutamicum*. A: Current density (line) and OD_{600nm} (diamonds) during the conversion. B: Concentrations of the substrate glucose and the products lactate, succinate and lysine.