Engineering in Life Sciences

Eng. Life Sci. **2017**, *17*, 781–791 www.els-journal.com

Anne Sydow[1]
Thomas Krieg[1]
Roland Ulber[2]
Dirk Holtmann[1]

[1] Biochemical Engineering, DECHEMA-Forschungsinstitut, Frankfurt, Germany

[2] Bioprocess Engineering, University of Kaiserslautern, Kaiserslautern, Germany

Research Article

Growth medium and electrolyte—How to combine the different requirements on the reaction solution in bioelectrochemical systems using *Cupriavidus necator*

Microbial electrosynthesis is a relatively new research field where microbial carbon dioxide fixation based on the energy supplied by a cathode is investigated. Reaction media used in such bioelectrochemical systems have to fulfill requirements of classical biotechnology as well as electrochemistry. The design and characterization of a medium that enables fast electroautotrophic growth of *Cupriavidus necator* in microbial electrosynthesis was investigated in detail. The identified chloride-free medium mainly consists of low buffer concentration and is supplied with trace elements. Biotechnologically relevant parameters, such as high-specific growth rates and short lag phases, were determined for growth characterization. Fast growth under all conditions tested, i.e. heterotrophic, autotrophic and electroautotrophic was achieved. The lag phase was shortened by increasing the $FeSO_4$ concentration. Additionally, electrochemical robustness of the reaction media was proven. Under reductive conditions, no deposits on electrodes or precipitations in the media were observed and no detectable hydrogen peroxide evolved. In the bioelectrochemical system, no lag phase occurred and specific growth rate of *C. necator* was 0.09 h^{-1}. Using this medium shortens seed train drastically and enables fast electrobiotechnological production processes based on *C. necator*.

Keywords: *Cupriavidus necator* / Design of experiments / Electroautotrophic growth / Media design / Microbial electrosynthesis

Additional supporting information may be found in the online version of this article at the publisher's web-site

Received: December 14, 2016; *revised:* February 8, 2017; *accepted:* February 27, 2017

DOI: 10.1002/elsc.201600252

1 Introduction

Electrochemical (EC) treatment of bacteria impacts cell viability in very diverse ways. Sublethal injuries and cell death may occur, from which various techniques are derived such as EC disinfection of waste water or electroporation [1–3]. Interestingly, input of electric energy into bacterial cultures can also stimulate the metabolism of microorganisms. Improvement of growth with a significant increase in optical density in the exponential phase, enhanced maximum cell dry weight as well as faster or improved production or enhanced bio-remediation have all been described [4–8]. Effects on cells mainly depend on current or potential applied [2, 6, 7, 9], duration of EC treatment [4, 9, 10], electrode material [7], cell type [7], vicinity of cells to the electrode/shielding equipment [7], medium ingredients [6, 11], temperature, and pH [6]. In 2010, microbial CO_2 fixation based on the energy supplied by a cathode was achieved and termed microbial electrosynthesis (MES) [12]. It serves as a potentially attractive method to store electrical energy provided by renewable resources, such as solar cells or wind turbines as chemical energy in case of energy surpluses. Initially,

Correspondence: Dr. Dirk Holtmann (holtmann@dechema.de), DECHEMA Forschungsinstitut, Theodor-Heuss-Allee 25, 60486 Frankfurt, Germany

Abbreviations: BES, bioelectrochemical system; **CDW**, cell dry weight; **CE**, counter electrode; **CP**, center point; **DoE**, design of experiments; **EC**, electrochemistry; **LB**, Luria–Bertani broth; **LSV**, linear sweep voltammetry; **MES**, microbial electrosynthesis; **MM**, minimal medium; **OCP**, open circuit potential; **OD**, optical density; **PHB**, poly(3-hydroxybutyrate); **Pt**, platinum; **SS**, stainless steel; **WE**, working electrode; μ, specific growth rate

mainly acetogenic microorganisms like *Sporomusa ovata* have been used for those applications [12]. In recent years, a large number of organisms that are able to take up electrons directly from the cathode or by electrochemically produced carriers have been investigated [13]. Due to limitations in genetic manipulation techniques and restricted growth rates for those mostly sensitive anaerobic organisms, product titers are typically low and production of nonnatural products is hard to achieve [13].

Cupriavidus necator, formerly *Ralstonia eutropha*, is a Gram negative bacterium that has been investigated intensively since the 1970s [14]. *C. necator* is of interest for the biotechnological industry as it has a very flexible metabolism, meaning it can grow on a variety of industrially relevant carbon and energy sources [15]. Organic substrates, such as organic acids and sugars, or even waste products, such as glycerol or waste lipids, can be used as carbon and energy sources [16]. Besides its heterotrophic nature, the organism can use energy supplied by hydrogen to fix carbon dioxide *via* the Calvin-Benson-Bassham cycle (lithoautotrophic metabolism). Oxygen can serve as electron acceptor, but under anaerobic conditions nitrate can be reduced [17]. Since the genome of *C. necator* has been sequenced and a multitude of genetic tools are available for this organism, genetic engineering of the strain became possible [15, 18]. Soluble products such as alcohols [19, 20], alka(e)nes [21], methyl ketones [22], and hydroxy acids [23] have been produced under heterotrophic and lithoautotrophic conditions. Defined media were optimized, especially for improvement of heterotrophic poly(3-hydroxybutyrate) (PHB) production and growth of *C. necator* strains [24–27]. Besides experimental optimization a lot of effort has been put into mathematic modeling to optimize production processes with *C. necator* in silico in order to decrease the number of laboratory experiments during process development [28].

The substrates for its lithoautotrophic lifestyle carbon dioxide and hydrogen can be obtained from waste streams and *via* splitting water by electrolysis, respectively. From a potential difference of 1.23 volts onwards, hydrogen, and oxygen can be produced simultaneously at the cathode and anode, respectively, enabling continuous feeding of those substrates. Mainly because of the lithoautotrophic metabolism under aerobic conditions, fast growth up to high densities and the availability of genetic tools, *C. necator* represents an ideal candidate to be used in bioelectrochemical systems. Electroautotrophic growth of this strain using the indirect electron transfer on electrochemically produced electron donors, such as hydrogen and formate, has been established for more than 50 years [29]. Since 2012, genetically modified strains were used under similar conditions to produce short-chain alcohols, such as isobutanol, isopentanol, and isopropanol, as well as PHB [19,30,31]. Using the same strain under different conditions, productivity under electroautotrophic conditions is trailing far behind heterotrophic conditions. Strain *C. necator* Re2133-pEG12 could produce approximately 1.9 g/L isopropanol on fructose as carbon source [20]. Under electroautotrophic conditions less than 0.6 g/L were achieved [31], indicating limitations of those processes.

Recent publications have mainly focused on development of electrode material to improve water splitting efficiency, on reaction efficiency, reducing production of toxic side products or on strain engineering [30, 31]. Investigation and optimization demands of an electrobiotechnological process have not been addressed to date. During our laboratory work we recognized that the media established for lithoautotrophic or heterotrophic processes were not suitable due to the production of toxic byproducts in the potential range needed for water splitting. On the other hand, media used so far in bioelectrochemical processes based on *C. necator* were not ideal for electroautotrophic production, as they were lacking in performance considerably compared to a "standard" lithoautotrophic process. In particular, the specific autotrophic growth rate decreased by 50% compared to established media.

In this work, the design and characterization of a new minimal medium is presented. A design of experiments approach was used to reduce the number of total conditions investigated and to take into account parameter interactions. By utilizing response surface methodology, buffer concentration, medium pH, and trace elements concentration were optimized to shorten lag time and increase specific growth rate. Finally, a detailed characterization of the electrical properties of the medium was performed.

2 Materials and methods

2.1 Microorganisms and media

The isopropanol producing strain *C. necator* Re2133 + pEG7c [20] was a kind gift from S. E. Guillouet from the INSA, Toulouse and was used in all experiments, unless otherwise stated. Here, *C. necator* $PHB^{-}4$ (DSM-541) was used as wild-type control. The strain Re2133 is derived from *C. necator* H16. It is incapable of producing PHB [32]. The plasmid pEG7c contains genes encoding for 3-ketothioloase A, acetoacetyl-CoA transferase, acetoacetyl decarboxylase, and alcohol dehydrogenase, localized under arabinose inducible P_{BAD} promoter. These genes enable redirection of acetyl-CoA to isopropanol under limitation of nitrogen with simultaneous excess of carbon source [20].

Luria-Bertani (LB) broth was used as complex medium. Different minimal media (MM) were investigated in this study (composition in Supporting Information Table S1). Production medium 1 [33] and 2 [34] were described for lithoautotrophic growth and heterotrophic isopropanol production with *C. necator*, respectively. Media used so far for electroautotrophic growth are based on the MM described by Schlegel and Lafferty [29]. Ingredient concentrations for the electroautotrophic medium used in this study are based on references [29] and [30]. For heterotrophic MM, D(-)-fructose was added to a final concentration of 4 g/L. For growth under nitrogen limiting conditions, final concentration of $(NH_4)_2SO_4$ was 0.36 g/L (c_N = 0.076 g/L). This facilitates growth up to a maximum cell dry weight (CDW) of approximate 2.5 g/L. Media was supplied with 200 μg/mL kanamycin to ensure pEG7c maintenance during cultivation.

Absorbance of bacterial cultures (optical density, OD) was measured by the Biowave Cell Density Meter CO8000 (Biochrom WPA, Cambridge, England) at $\lambda = 600$ nm. CDW was calculated with the following formula: CDW [g/L] = 1.248*OD (data not shown). pH was determined using a Five Easy Plus pH Meter (Mettler Toledo, Ohio, USA).

2.2 Seed train

All cultivations were performed at 30°C. Cultures were incubated at 180 rpm, except in the case of electroautotrophic growth. A 5 mL LB (in tubes) was inoculated from cryo stock and culture was incubated until stationary growth phase (OD ~ 5) was reached after approx. 48 h. Heterotrophic cultures were inoculated in 25 mL of the MM to be examined in 100 mL Erlenmeyer flask to a CDW of 0.06 ± 0.01 g/L. When a CDW of 2.5 ± 0.25 g/L was reached, cells were harvested, centrifuged at room temperature for 20 min at 3220 × g and washed in the corresponding autotrophic medium to remove remaining fructose. Autotrophic cultures were inoculated in 25 mL of the MM to be examined in 200 mL septum flasks to a CDW of 0.25 ± 0.3 g/L. Septum flasks were prepared by autoclaving, filling in MM, closing the septum and adding defined gas mixture. For the defined gas atmosphere, flasks were evacuated twice to a pressure of 80 mbar and pressurized with H_2/CO_2 (80:20) to 1.2 bars. Afterwards, flasks were evacuated to a pressure of 40 mbar and pressurized first with H_2/CO_2 (80:20) to 1.2 bars and afterwards with O_2 to 1.5 bars. Therefore, gas mixture composition was 64:16:20 ($H_2/CO_2/O_2$). Bioelectrochemical system (BES) was inoculated as septum flasks, except that autotrophic cultures served as precultures and washing was carried out with N-limited medium. During the whole seed train, the same basic MM was used for all experiments.

2.3 Design of experiments

Design of experiment (DoE) was done with the Design Expert 9 software (Stat-Ease Inc., MN, USA). Analysis of variance (ANOVA) was used to verify the models fitting the measured data best. Especially, the coefficient of determination R^2 and adjusted R^2 were used to verify the models fitting the measured data. Independent variables influencing the response were determined according to statistical tests and parameter significance was confirmed by testing at a 95% confidence level for the confidence interval.

Response surface central composite design was used to investigate the influence of the independent parameters on the responses CDW_{max} (highest measured CDW, factor F) and growth time until a CDW of 2.5 g/L was reached (factor E). Four independent variables were chosen: trace elements concentration (factor C), $FeSO_4$ concentration (factor D), buffer concentration (factor A), and pH (factor B). Trace elements solution stock was composed of (in 0.1 M HCl, in g/L): $FeSO_4{*}7H_2O$ 15, $MnSO_4{*}H_2O$ 2.4, $ZnSO_4{*}7H_2O$ 2.4, $CuSO_4{*}5H_2O$ 0.48, $Na_2MoO_4{*}2H_2O$ 1.8, $Ni_2SO_4{*}6H_2O$ 1.5, $CoSO_4{*}7H_2O$ $4.02{*}10^{-2}$. A central composite design was used to investigate influence of independent factors on the aforementioned responses. Three groups of design points were chosen for the practical design with the coded unit 0 as center point (CP), +1 and -1 as factorial points and $+\sqrt{2}$ and $-\sqrt{2}$ as axial points (Table 2).

For all runs of DoE, DoE verification, and detailed investigation of $FeSO_4$ concentration, CP medium supplemented with 4 g/L fructose served as preculture and CP medium was used for washing before inoculation of autotrophic medium. Exact value of response variable E was determined by fitting the measured data with the sigmoidal logistic function, type 3 of origin [35].

2.4 Conductivity measurement

Media conductivity was measured using an electrical conductivity meter LF 197-S (WTW, Weilheim, Germany). The probe was placed in a 30°C tempered vessel filled with MM and electrolytes, respectively.

2.5 Electrochemical setups

A three-electrode system was used throughout the study. All potentials are versus Ag/AgCl. Electrochemical systems were maintained at 30°C with an incubation hood. CO_2 was purged by a needle into the headspace at a flow rate of 10–15 cm³/min.

2.5.1 BES for electroautotrophic growth

For bioelectrochemical experiments, a modified Schott flask with three side arms was used as cultivation system. The main screw cap and the three side screw caps were supplied with septums. A platinum (Pt) plate contacted by a Pt wire served as working electrode (WE) (WE1: 8.1 × 2.2 × 0.01 cm = 35.8 cm^2, WE2: 4.05 × 2.2 × 0.01 cm = 17.9 cm^2, WE3: 1.6 × 0.7 × 0.01 cm = 2.3 cm^2) and a dimensional stable anode (Denora, Rodenbach, Germany, Ti expanded metal type 11159 with Ir-mixed metal oxide coating) as counter electrode (CE) to catalyze oxygen evolution. A Ag/AgCl reference electrode placed in a Luggin capillary filled with 0.5 M Na_2SO_4 was used to control the potential at –1.5 V with a ECM8 potentiostat (Gamry, Warminster, PA, USA). Due to water electrolysis, both gases H_2 and O_2 were produced at the cathode and anode, respectively. Exhaust gas was guided out of the system into a water column. By this, a continuous overpressure of 40 mbar was reached in BES. Culture was stirred at 150 rpm with a stir bar. System was equilibrated at least 16 h before inoculation. Cell voltages, currents, and current densities described are the mean values of whole operation times of n individual cultivations (for n see details in text).

2.5.2 Linear sweep voltammetry

MM was characterized by linear sweep voltammetry (LSV), which was performed using a potentiostat (Interface 1000, Gamry, Warminster, PA, USA) with a three electrodes system. Pt plates served as CE and WE (0.8 × 2.0 × 0.01 cm) and a Ag/AgCl as reference electrode. Medium was equilibrated with CO_2 for 16 h as described before. LSV were started at open circuit potential, which was measured for 60 sec before LSV. LSVs were measured until –2 V were reached. Scan rate was 100 mV/sec with a step size of 5 mV. Between every LSV, WE was cleaned by cyclic voltammograms in 0.5 M H_2SO_4 (Autolab PGSTAT12, Metrohm, Herisau, Switzerland) with a similar three electrode system. 200 CVs between +2.5 V and –1 V were performed at a scan rate of 2 V/s (step size 0.1 V).

Table 1. Electrobiotechnological processes need to take into account requirements of biotechnology and electrochemistry

Research field	Challenge or requirement	Experimental task or solution
Biotechnology	Fast (electroautotrophic) growth, i.e. reduced lag phase and a high-specific growth rate	Design of experiment to identify optimal medium, e.g. buffer molarities and pH value, including trace elements that are present in established MM
	Autotrophic growth solely based on CO_2 fixation	Replacing carbon containing ingredients, i.e. citrates and carbonates
Electrochemistry	Robust MM during cultivation, i.e. avoiding precipitations, chlorine gas, side reactions	Replacing chloride species by sulfate containing ingredients
	Restricted conductivity in biological media	Examination of different buffer concentrations

Challenges and experimental tasks or solutions are given. Details are given in Supporting Information Table S1.

2.6 Measurement of electrochemical hydrogen peroxide production

The setup was the same as for the BES, described before. Besides Pt, a stainless steel (SS, EN steel number 1.4404) was used as WE. Abiotic samples were analyzed by using the tube test Nanocolor® Peroxid 2 (Macherey-Nagel, Düren, Germany) according to manufacturer information.

2.7 Calculation of lag time and specific growth rate (μ)

Lag time (t_{lag}) and μ were determined by fitting the CDW data with the formula

$$x(t) = x_0 * \exp\left(\mu * \left(t - t_{lag}\right)\right)$$

(x_0 inoculated CDW in g/L, μ specific growth rate in h^{-1}, $x(t)$ CDW in g/L at time point t in h, t_{lag} lag time in h) using Origin.

2.8 Formate analysis

Formate concentration was quantified by HPLC (SCL 10-A, Shimadzu, Japan) equipped with a refractive index detector. A Rezex organic acid H+ (8%) analytical column was used (Phenomenex Inc., California, USA). Elution was preceded with 5 mM H_2SO_4 at a pump rate of 0.6 mL/min at 65°C. Concentrations were determined by using a calibration curve for the defined concentration range.

2.9 Statistical analysis

An unpaired two-tailed *t*-test was used to determine *p*-values. A 95% confidence interval was used to determine significance.

3 Results and discussion

3.1 Electrochemistry and biology have different demands on their reaction solution

MM for electroautotrophic growth and production processes need to consider biological and electrochemical requirements (Table 1). Established MM for *C. necator* are listed in Supporting Information Table S1 regarding composition of ingredients, element concentration, and growth relevant parameter. For two established production media, specific growth rate μ, lag time, and maximum CDW were 0.23–0.24 h^{-1}, 2.3–2.7 h, and 6.7–6.8 g/L for heterotrophic growth (Table 3). For autotrophic growth μ was decreased by approximately one half (to 0.12–0.14 h^{-1}). Lag time in media established for biotechnological processes is increased to 8.6 and 10.9 h for autotrophic growth. This results most probably from the shift from heterotrophic to lithoautotrophic metabolism [36, 37]. During the lag time, regulatory cascades are activated and enzymes for the energy

Table 2. Experimental range of independent variables studied, goal, and importance of variables

Factor	Name	Level (coded units)					Goal	Importance
		$-\sqrt{2}$	−1	0	+1	$+\sqrt{2}$		
A (mM)	Buffer concentration	15	30	65	100	115	In range	n.a.
B (-)	pH	6.3	6.4	6.6	6.8	6.9	In range	n.a.
C (% v/v)	Trace elements	0	0.03	0.1	0.17	0.2	In range	n.a.
D (μM)	$FeSO_4$ concentration	0	0	75	150	181	In range	n.a.
E (h)	Time until CDW 2.5 g/L is reached						Minimize	+++++
F (g/L)	CDW_{max}						Maximize	+

Independent variables A–D, response variables E–F. n.a. = not applicable.

Table 3. Specific growth rate (μ), lag time, and maximum cell dry weight (CDW_{max}) of *C. necator* Re2133 + pEG7c under heterotrophic (hetero) and autotrophic (auto) conditions in different minimal media (listed in Supporting Information Table S1)

	Production medium 1 [33]		Production medium 2 [34]		Electroautotrophic medium [29,30]		Medium of this study	
	Hetero (3)	Auto (2)	Hetero (3)	Auto (2)	Hetero (3)	Auto (5)	Hetero (3)	Auto (5)
μ (h^{-1})	0.24 ± 0.01	0.14 ± 0.00	0.23 ± 0.00	0.12 ± 0.03	0.23 ± 0.01	0.06 ± 0.01	0.25 ± 0.02	0.12 ± 0.00
lag time (h)	2.3 ± 1.2	10.5 ± 1.5	2.7 ± 0.3	8.6 ± 5.1	3.2 ± 0.7	10.9 ± 2.3	1.5 ± 1.8	2.8 ± 0.9
CDW_{max} (g/L)	6.7 ± 0.3	3.5 ± 0.2	6.8 ± 0.3	4.2 ± 1.4	5.5 ± 0.1	3.2 ± 0.9	5.0 ± 0.0	3.9 ± 0.0

Numbers in brackets represent quantity of individual cultures, mean ± SD or variance of individual experiments for n > 2 and n = 2, respectively.

conversion of hydrogen (hydrogenases, maturation machinery) and for fixation of carbon from CO_2 need to be expressed. Maximum autotrophic CDW is decreased to values between 3.24 and 4.24 g/L. The amount of gas available in septum flasks limits the production of higher biomass concentrations. During batch experiments, the pressure decreased from 1.5 bars to 0.5 to 0.6 bars as the gases supplied were consumed. Gas feeding during growth resulted in higher biomass concentrations (data not shown).

Although all biotechnological requirements (see Table 1) were fulfilled, electrochemical challenges occurred. A potential of –1.5 V was applied to split water. Immediately after polarizing the working electrode, a white precipitation occurred. The deposits became more pronounced after incubation over night (Supporting Information Fig. S1). Additionally, toxic chlorine gas was produced (olfactory determination), excluding the application of those media in BES. We concluded that deposits were chlorine containing species, as in both production media more than 0.1 g/L chlorine is included (Supporting Information Table S1).

MM used for electroautotrophic processes [29–31,34] are robust in BES. Biotechnological requirements, mainly availability of different trace elements and carbon content, are not taken into account (Table 1). For heterotrophic and autotrophic growth, values observed for μ, lag time and maximum CDW are comparable to established production media (Table 3), except for μ for autotrophic growth. With 0.06 h^{-1}, μ was just 50% of production media for autotrophic growth and 25% of heterotrophic growth in the same MM.

Discrepancy of autotrophic growth between the MM is also true for the *C. necator* strain DSM-541 (Supporting Information Fig. S2). Both strains need approximately twice the time to reach a CDW of 2.5 g/L in the electroautotrophic medium compared to the production media.

The results show clearly the need for an adapted reaction solution that fulfills electrochemical and biotechnological requirements of media in bioelectrochemical synthesis.

3.2 Design of a suitable reaction solution that fulfills all requirements

3.2.1 Design pretests

Solutions presented in Table 1 were taken into account, yielding in a carbon and chloride-free medium supplemented with important nutrients identified for autotrophic growth [38]. A phosphate buffered minimal medium supplemented with sulfate salts and trace elements, including $(NH_4)_2SO_4$ as nitrogen source, resulted (Supporting Information Table 1, "medium for pretest"). Besides different pH values, two buffer concentrations were investigated: 65.7 mM, which corresponds to the highest buffer concentration of the three MM compared before (Supporting Information Table 1); and twice that concentration, 131.4 mM, which should improve conductivity and pH stability.

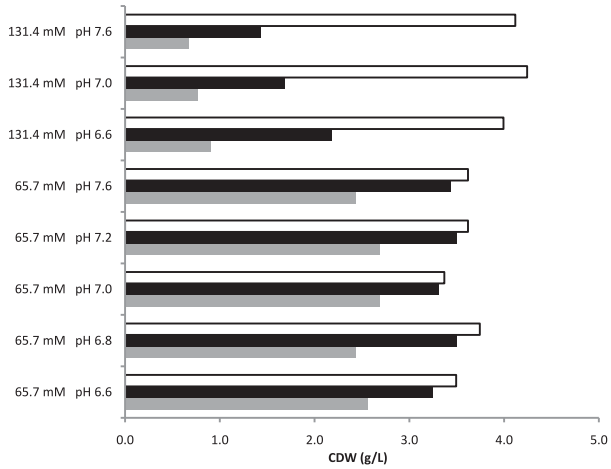

Figure 1. CDW of autotrophically grown *C. necator* Re2133 + pEG7c after 23 h (gray), 30 h (black), and 72 h (white). Cells were grown in MM with different pH and buffer molarities for screening purpose ($n = 1$).

Autotrophic cultures grown in media with low molarities and pH values exhibited shortest lag phases (Fig. 1). Effects of pH and buffer molarities on heterotrophic cultures were comparable (Supporting Information Fig. S3). In medium with low buffer concentration and low pH, pH decrease during autotrophic cultivation is very pronounced (Supporting Information Fig. S4). This is caused by the decreasing buffer capacity at lower buffer concentrations and by the proximity of the adjusted pH toward the optimal buffering range. To summarize; using low buffer concentration and low pH is advantageous regarding short process time. However, pH stabilization during the cultivation period is more efficient in high molarities and at higher pH values. It should be mentioned that besides the composition of the soluble media components also the H_2, CO_2, and O_2 transfer from the gas phase influences the growth characteristic of *C. necator*. Repaske et al. showed that the lag period can be shortened by

decreasing both the pO_2 and the pCO_2 [39]. Furthermore the authors have shown that the bicarbonate requirements changed at the different growth phases.

3.2.2 Media optimization for autotrophic growth using DoE

Response surface method designs allow the quantification of responses within specified ranges of investigated factors. It explores the relationships between several explanatory variables and one or more response variables. Specifically a circumscribed central composite design was chosen to optimize a fast growth under autotrophic conditions as mentioned before. The CP of buffer molarity corresponds to the molarity of production medium 2. The impact of trace elements was investigated (factor C), as there was a great difference between the three published MM. Iron is an important element for the production of [NiFe]-hydrogenases of *C. necator*. Therefore, $FeSO_4$ concentration, which is also part in the trace elements stock, was investigated separately (factor D). Experiments were performed in three blocks with a total of 32 experiments (Block 1 and 2 contained 8 noncenter points and two center points, respectively, block 3 contained 16 noncenter points and two center points) with different combinations of the independent variables A-D (Supporting Information Table S2).

3.2.3 Model results

A central composite design was used to determine the optimal levels for the maximization of culture density (F) and minimization of time until CDW 2.5 g/L is reached (E), corresponding to a short lag phase and/or high-specific growth rate (Supporting Information Table S2). The factor "time until CDW 2.5 g/L is reached" was defined, as the strain produced isopropanol under N limiting conditions and nitrogen source was depleted at that biomass concentration [20]. In future experiments, this nitrogen limitation should be reached as fast as possible.

Analysis by design expert software revealed a linear model for response variable E (Supporting Information Table S3). Data analysis revealed that the buffer concentration showed a quadratic behavior indicated by a p-value of 0.0088. A reduced quadratic model with an adjusted R^2 of 0.82 was chosen (Supporting Information Table S4). For F, a reduced two factor interaction model with an adjusted R^2 of 0.88 was fitting the data best (Supporting Information Table S6). Model significancies were calculated by ANOVA with a p-value of < 0.0001. Factor responses with a p-value > 0.055 were considered to fullfil the null hypothesis. p-values < 0.055 declined the null hypothesis and were used to fit the model. For the model calculated for maximal CDW (factor F), parameters C and D were included although p-values were > 0.1, as factor interactions in the hierarchic model became significant (AD, BC, and CD). In the current study, model F values of 27.5 and 32.7 for E and F, respectively, designate the models as significant. The ANOVA results before and after exclusion of insignificant parameters and interactions are shown in Supporting Information Table S3–6.

Based on experimental design and ANOVA calculation, the following final equations in terms of actual factors were calculated showing the interactions between the independent variables:

$$E = -14.087 - 0.068^{*}A + 5.994^{*}B + 15.705^{*}C - 0.023^{*}D + 8.712^{*}10^{-4*}A^2$$

$$F = -12.878 + 0.010^{*}A + 2.454^{*}B + 62.842^{*}C + 4.708^{*}10^{-3*}D - 5.199^{*}10^{-5*}AD - 9.528^{*}BC - 0.015^{*}CD.$$

Validity of experimental data was analyzed. The actual values are in good agreement with the predicted ones as shown in Predicted versus Actual plots. The residuals were normally disributed, the corresponding plots are shown in Supporting Information Fig. S5.

In Fig. 2, interpretation between explanatory and response variables as well as visualization of factor interactions by using two and three dimensional plots of the suggested model is shown graphically. In the two dimensional perturbation plots, response is plotted by changing only one factor over its range, while holding all other factors constant. Perturbations are shown with the center point as reference point. For minimizing process time (factor E), increasing $FeSO_4$ concentration and decreasing buffer concentration, pH, and traces concentration is advantageous. For maximizing CDW (factor F), buffer concentration and pH needs to be increased.

In the three dimensional response surface plots, interactions of variables can be observed. Factors A and D interfere. When buffer concentration is low, addition of various amounts of $FeSO_4$ has no impact on CDW_{max}. At high buffer concentrations, CDW_{max} increases with decreasing $FeSO_4$ concentration (negative correlation between D and F at high buffer concentrations). Furthermore, factors B and C interfere. When pH value is low, addition of trace elements has no impact on CDW_{max}. At high pH values, CDW_{max} increases with decreasing trace elements concentration (negative correlation between C and F at high pH values). In addition, factors C and D interfere. Without either C or D, the concentration of the other variable can be changed without altering CDW_{max}. When both trace element concentration and $FeSO_4$ concentration is increased, CDW_{max} decreases. Transition to the stationary growth phase occures at lower biomasses when the sum of C and D is high.

Models showed that the two examined response variables have very contrary demands. The solution of the problem highly depends on which response is defined at beeing more important (fast growth versus high CDW). In this work, we aimed for a short process time and optimized the medium for a fast growth on the cost of a lower maximum CDW (Table 2). In the model optimized medium, a maximum CDW of 4.1 g/L should be reached and 2.5 g/L biomass should be achieved after 19.8 h with a desirability of 79%. This medium was supplied with 181 μM $FeSO_4$, no trace elements and 40 mM buffer at pH 6.56 (6.78 under air saturation). To avoid any depletion of trace elements during subsequent cultivations or long-term experiments, a small amount of the trace elements solution stock was added routinely (5% of the value used in CP cultivations). This just increased slighty the response parameter time until CDW 2.5 g/L is reached (19.9 h).

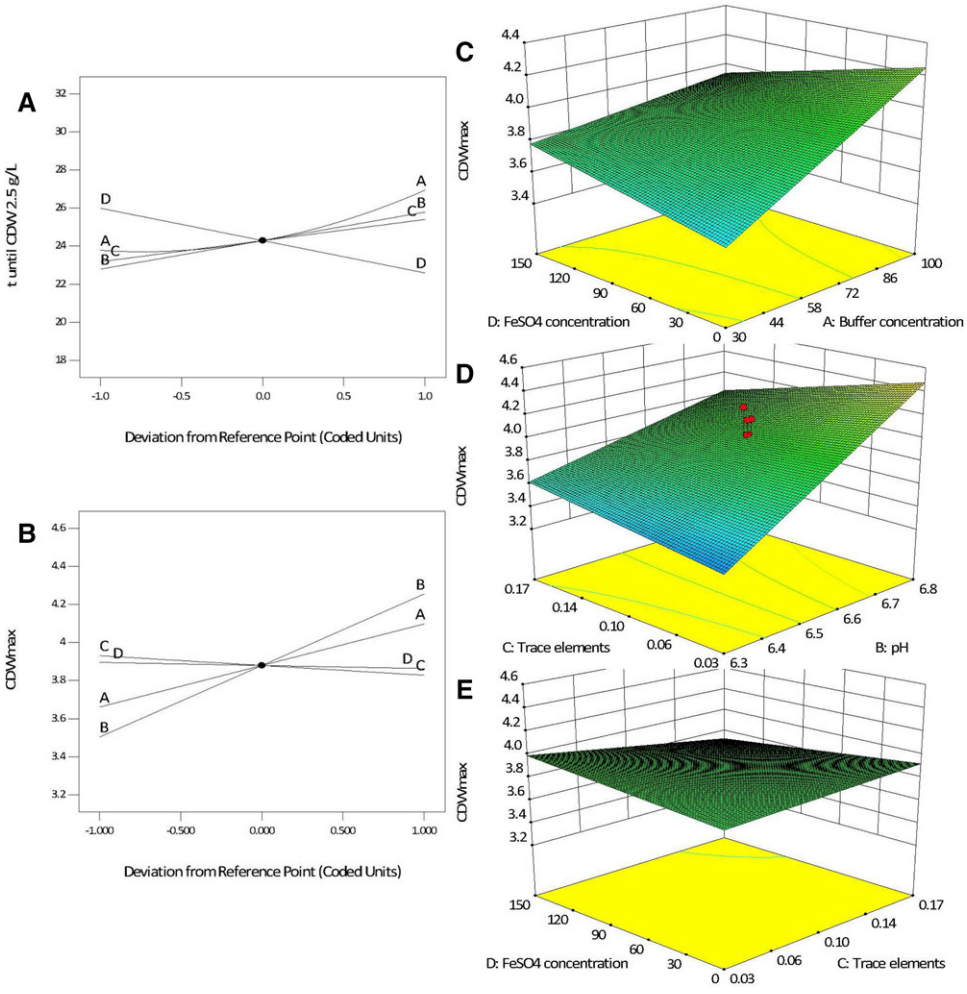

Figure 2. Perturbation plots (A, B) and 3D surface plots (C, D, E). Perturbation plots are shown for time until CDW 2.5 g/L is reached (A) and CDW_{max} (B). 3D surface plots are given for the three parameter interactions observed for response CDW_{max} (AD, BC, CD) in the range of +/- 1 Coded Units. (A) Buffer concentration (mM), (B) pH value (–), (C) Trace elements (% v/v), (D) $FeSO_4$ concentration (μM).

The predicted values for E and F (19.9 h and 4.1 g/L) were expected to be in the range of 18.6–21.2 h and 3.9–4.4 g/L, respectively, assuming a confidence interval of 95%. To verify the validity of the model used for optimization the calculated optimal medium was used in 10 cultures, whereby five individual heterotrophic precultrues were used. The model is valid as a CDW of 2.5 g/L was reached after 21.0 hours and maximal CDW was 4.1 g/L (data not shown).

3.2.4 *Detailed investigation of $FeSO_4$ concentration*

The predicted optimal value for the explanatory variable $FeSO_4$ (F) is equal to the axial point $+\sqrt{2}$ with 181 μM. As the model has just limited reliability at its edges, the influence of $FeSO_4$ concentration was investigated in detail. Although AD and CD factor interactions were observed, $FeSO_4$ concentration was investigated in an one-factor-at-a-time-varied experiment as degree of interaction was just minor (see equation for F above).

Concentrations up to 724 μM were investigated (Fig. 3). High amounts of $FeSO_4$ shortens the lag phase, so that CDW 2.5 g/L is reached earlier (81% using 724 μM $FeSO_4$ compared to calculated DoE optimum, statistically significance between 362 and 724 μM, $p = 0.013$). High $FeSO_4$ concentrations lower

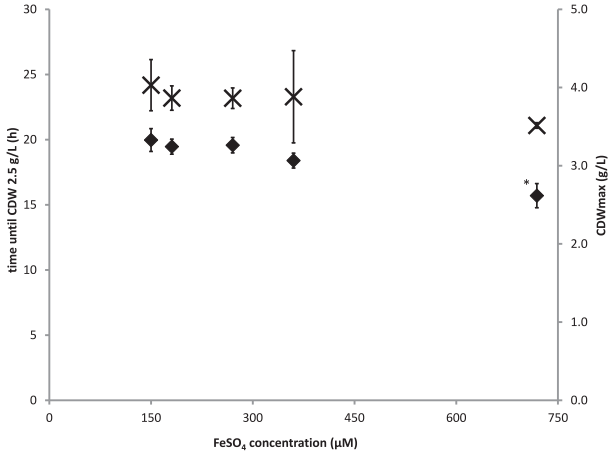

Figure 3. Maximum CDW (crosses) and time needed to achieve a CDW of 2.5 g/L (rhombuses) in MM of this study supplied with different $FeSO_4$ concentrations. One hundred fifty micromolar and 181 μM represent coded units +1 and $+\sqrt{2}$ of the DoE. One hundred eighty-one micromolar represents optimal concentration after DoE. $n = 3$, mean ± SD. Student's *t*-test; $^*p < 0.05$.

the maximal cell density (91% using 724 μM $FeSO_4$ compared to calculated DoE optimum). This is in agreement with the results of the DoE and also fits results published before [40], where a $FeSO_4$ concentration of at least 10 μM was necessary for nonlimited growth of the organism (Fig. 2).

A multitude of enzymes needed for lithoautotrophic growth of *C. necator* are iron dependent. Most especially, the hydrogenases contain iron in the [NiFe] active site and in the [Fe-S] clusters. Heme containing cytochromes are used as electron carriers [41] and regulatory machinery, e.g. regulatory hydrogenase, contains iron [42]. The element can regulate mechanisms used for energy conversion or CO_2 fixation, as shown for *Escherichia coli* and *Acidithiobacillus ferrooxidans*, respectively [43, 44]. Iron uptake (putative gene regions described by [45]) and thus accessibility of intracellular iron could be improved by higher extracellular concentrations. Although adding higher amounts of $FeSO_4$ to the MM designed in this study improved process time further, the predicted concentration (181 μM) was used in the standard medium for further experiments mainly taking into account the costs of the MM.

3.3 Growth comparison with established minimal media

The newly developed reaction medium was compared with the published media in regard to specific growth rate, lag time, and maximum CDW under heterotrophic and autotrophic conditions (Table 3). Growth on fructose was similar regarding specific growth rate (0.25 h^{-1} versus 0.23 to 0.24 h^{-1}). Lag phase could be slightly decreased to 1.5 h. Maximum CDW is decreased to 5.0 g/L, from initially 5.5 to 6.8 g/L. Under autotrophic conditions, specific growth rate of the production media could be restored (0.12 h^{-1}) and considerable reduction in lag phase to 2.8 h was investigated. Obviously, any medium ingredient(s) shorten the time needed for the adaptation toward autotrophic conditions, e.g. iron. Regarding a short-process time, the MM designed in this study is even better than the MM described so far for autotrophic growth [33] and for heterotrophic isopropanol production [34] and much better than the bioelectrochemically suitable medium used for electroautotrophic growth and isopropanol production before [29, 30]. Using the new designed MM can reduce time and thus costs of processes, where an electrochemically robust medium is needed by decreasing the time needed for the seed train.

The suitability of the designed medium for repetitive autotrophic batch cultures was proven to ensure that no depletion of any ingredient occurred within the seed train.

3.4 Electrochemical characterization of the optimized MM

3.4.1 Conductivity

One major limitation of MM described for growth of prokaryotes is the restricted conductivity in comparison to highly conductive electrolytes typically used in electrochemical systems, e.g. 0.5 M sodium sulfate or saturated potassium chloride. At 30°C, conductivities of these electrolytes were 64 and 373 mS/cm, respectively. Conductivities of the medium described for electroautotrophic production and the MM described in this study achieved conductivities of 6.3 and 6.1 mS/cm, respectively. In general, low conductivities will lead to higher inner resistances in an electrolyte, resulting in unwanted high ohmic losses in the media. By designing the MM by DoE, higher conductivities were tested. The MM used for the CP conditions of the DoE had a conductivity of 9.5 mS/cm. However, these values are still lower than the usual electrolytes used in electrochemical syntheses. MM with more positive-coded units of the explanatory variables would have had even higher conductivities due to the addition of former salts and ingredients. Nevertheless, for optimal growth of *C. necator* low conductive media with approximate 6 mS/cm seem to be optimal and thus the best compromise between biological and electrochemical demands. This in agreement with observations of Liu et al. who tried to overcome limitations in mass transport and attendant current by increasing buffer concentration to 108 mM. However, under these conditions bacterial production was affected negatively [31]. Evolutive adaption of the organism to media containing higher salt concentrations may be beneficial for future optimization.

3.4.2 Stability of the optimized MM

Potentials of –1.1 and –1.5 V versus Ag/AgCl at SS and Pt WE were applied for more than 7 days. No precipitates were observed, showing the electrochemical stability of the developed MM. In recent publications [19, 30, 31], reactive species such as hydrogen peroxide and hydroxyl radicals evolved due to the applied reductive potential. In our study, no H_2O_2 evolution was observed using Pt as WE material, independent of the potential applied (Supporting Information Fig. S6). Using SS as working electrode, H_2O_2 is produced electrochemically depending on the reductive potential. Concentration increased steadily during an operation period of 2 days, reaching 0.2 and 1.2 mg/L for –1.1 and –1.5 V, respectively. Torella et al. and Liu et al. [30, 31] observed production up to 10 mg/L after 10 h and 18 mg/L H_2O_2 after 60 min, respectively. Different amounts of H_2O_2 in recent publications vs. this study could be explained by different cell potentials and diverging electrode materials, which can favor alternative electrochemically reactions. Furthermore this could indicate better electrochemical robustness of the medium designed in this study. In our system, Pt was advantageous over SS regarding production of the toxic peroxide. Therefore, Pt was chosen as WE in further experiments. For sure, for large-scale applications the costs for electrode materials should also be taken into account.

3.4.3 Characterization of hydrogen evolution by linear sweep voltammetry (LSV)

Open circuit potential (OCP) of the MM designed in this study was approximately 0 V versus Ag/AgCl. Linear sweep voltammograms from OCP to –2 V versus Ag/AgCl were performed. Reductive current increased at approximately –600 mV, indicating hydrogen evolution (Supporting Information Fig. S7). This is in agreement with the minimal thermodynamic potential for water splitting (1.23 V) and reduction potential of –615 mV versus Ag/AgCl.

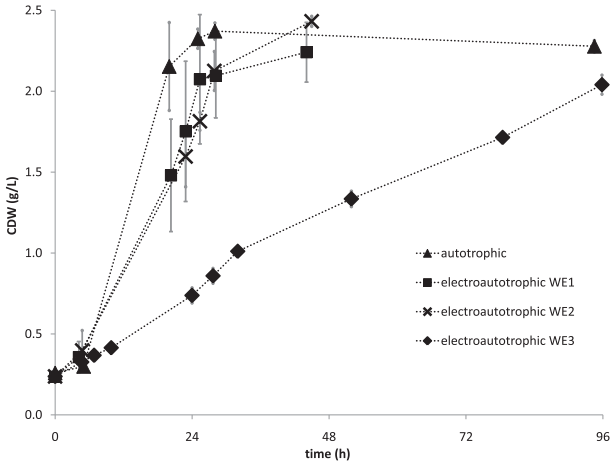

Figure 4. Autotrophic and electroautotrophic growth in the MM that has been designed in this study under *N* limiting conditions ($(NH_4)_2SO_4 = 0.36$ g/L). Medium was inoculated with heterotrophically grown cells and autotrophically grown cells for autotrophic and electroautotrophic conditions, respectively. $n = 2$ for autotrophic conditions, WE2 and WE3: mean ± values for individual experiments (lines with dots). $n = 6$ for WE1: mean ± SD.

3.4.4 *Abiotic evolution of formate*

The indirect electron transfer in MES relies on the production of small electron donors such as hydrogen and formate at the cathode. In a recent study, electrochemical reduction of CO_2 to formate was described [19], yielding in growth of *C. necator*. Therefore, formate evolution over a period of three days was investigated (Supporting Information Fig. S8). Just traces of formate were produced (approximately 0.1 mM after 24 h and <0.4 mM after 72 h). By comparing the growth in Fig. 4 and the measured formate production with literature data [46], sole biomass increase from formate consumption as well as inhibition of growth can be excluded. Thus in our system, carbon from CO_2 is mainly fixed directly by using hydrogen as electron donor.

3.5 Electroautotrophic growth characteristics

In the developed MM, cultures can reach nitrogen limitation after approximately 24 h on electrochemically produced hydrogen. Growth without lag phase after inoculation is observed (Fig. 4). In comparison to the autotrophic culture, lag time could be decreased as cells did not have to adapt to either carbon or energy source. Specific growth rate was slightly decreased in comparison to autotrophic growth (Table 3). A μ of 0.09 h^{-1} was observed in systems with high WE electrode areas (WE1 35.8 cm^2, WE2 17.9 cm^2). Using a lower electrode area (WE3 2.3 cm^2), after short exponential growth phase with $\mu = 0.045\ h^{-1}$, linear growth with $\mu = 0.018\ h^{-1}$ was observed. We assume that initial exponential growth is caused by an excess of redox equivalents stored in the cells from preculture, whereas hydrogen is the limiting factor during subsequent cultivation. The value of μ for WE1 and WE2 is trailing behind autotrophic conditions. Shortage of hydrogen availability is a unlikely reason for this, as two strongly different WE areas (35.8 and 17.9 cm^2) caused comparable μ (Fig. 4). A multitude of further parameter differs from autotrophic conditions and could decrease μ, e.g. gas composition, pressure, and agitation. Moreover, evolution of small amounts of ROS (besides H_2O_2) cannot be ruled out.

Applying –1.5 V versus Ag/AgCl, cell voltages of 3.3, 2.9, and 2.8 V and currents of approximate –45, –23, and –3.6 mA were obtained using WE1, WE2, and WE3, respectively. This goes along with a relatively constant current density of –1.26, –1.29, and –1.57 mA/cm^2. Schlegel and Lafferty [29] observed fastest growth, corresponding with no lag phase and $\mu < 0.015\ h^{-1}$ (calculated), at 4.2 V cell voltage at a Pt WE with surface area of approx. 16 cm^2 (600 mA, 37.5 mA/cm^2, calculated area, and current density), using a culture that had been preadapted to electroautotrophic conditions. Using an autotrophic preculture, lag phase was increased to 14 h (calculated). We observed no lag phase and approximately 6-times higher μ in our setup.

Growth observed by Torella et al. and Liu et al. was much slower, as C3 to C5 compounds producing strains reached CDW_{max} after 3 and 4 days (cell voltage 3.0 V with >10 mA/cm^2 and 2.0 V), respectively [30,31]. We could shorten growth phase drastically to one day by using comparable cell voltages (approximate 3 V). Current densities were much lower than the ones published before, which demonstrates high efficiency of hydrogen conversion in our setup. Therefore, multiple effects like reactor design and medium composition might have caused the observed advantages of this study.

4 Concluding remarks

The newly developed MM fulfills requirements of classical biotechnology and electrochemistry (see Table 1) and enables efficient CO_2 fixation on electrochemically produced hydrogen. For solving biotechnological challenges, a carbon-free medium supplemented with important trace elements was designed. Taking electrochemical demands into consideration, most importantly chloride containing salts were exchanged with sulfate ones. This prevented precipitation of medium ingredients on Pt working electrodes. In the robust medium, no hydrogen peroxide evolves up to an applied potential of –1.5 V versus Ag/AgCl. Most importantly, the MM enables fast autotrophic and electroautotrophic growth and production processes using *C. necator*. A shorter lag phase and a higher specific growth rate were realized under autotrophic conditions in comparison to the MM described for electroautotrophic production so far.

By response surface methodology, optimal buffer concentration was calculated as 40 mM and pH as 6.56. Furthermore, a high concentration of $FeSO_4$ (181 μM) and a low concentration of the trace element solution mix were advantageous. $FeSO_4$ concentrations up to 724 μM shortened process time further.

Using the new designed minimal medium is advantageous if an electrochemically robust medium is needed. Process time can be reduced and thus costs of processes. Under autotrophic conditions, μ was as high as observed in the two production media but more importantly twice as high as in media used in BES so far (0.12 versus 0.06 h^{-1}). Therefore, time needed for the seed train, could be reduced. This aims at a more economically feasible process. Furthermore, the MM enables fast production

processes under electroautotrophic conditions without lag phase and specific growth rates up to 0.09 h^{-1}. Our findings act as one further step toward efficient usage of *C. necator* in MES.

Practical application

Microbial electrosynthesis is an upcoming research field where ideally the greenhouse gas carbon dioxide is converted in valuable products. The biological conversion is driven by energy supplied at the cathode. Peak electrical energy that arises from renewable energy plants can be converted and stored in chemical energy by using biological reactions. The research field aims to promote efficiencies of renewable energies. This publication focuses on the development of a suitable reaction medium and finally on the improvement of the performance in microbial electrosynthesis. The designed medium fulfills requirements of biotechnological processes; in particular fast growth as well as of electrochemical processes, e.g. robustness under reductive conditions. Most of the results can be easily transferred to the design of reaction media for further bioelectrochemical production systems.

We thank S. E. Guillouet from the INSA, Toulouse for providing the strain C. necator Re2133 + pEG7c and for fruitful discussions. We also thank Jeffery Alan Wood from the University of Twente, Enschede for critical review and discussion. Furthermore, we 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).

The authors have declared no conflict of interest.

5 References

[1] Guillou, S., Besnard, V., El Murr, N., Federighi, M., Viability of *Saccharomyces cerevisiae* cells exposed to low-amperage electrolysis as assessed by staining procedure and ATP content. *Int. J. Fodd. Microbiol.* 2003, *88*, 85–89.

[2] Diao, H., Li, X., Gu, J., Shi, H. et al., Electron microscopic investigation of the bactericidal action of electrochemical disinfection in comparison with chlorination, ozonation and Fenton reaction. *Process Biochem.* 2004, *39*, 1421–1426.

[3] Chassy, B. M., Mercenier, A., Flickinger, J., Transformation of bacteria by electroporation. *Trends Biotechnol.* 1988, *6*, 303–309.

[4] She, P., Song, B., Xing, X.-H., Van Loosdrecht, M. et al., Electrolytic stimulation of bacteria Enterobacter dissolvens by a direct current. *Biochem. Eng. J.* 2006, *28*, 23–29.

[5] Nakanishi, K., Tokuda, H., Soga, T., Yoshinaga, T. et al., Effect of electric current on growth and alcohol production by yeast cells. *J. Fermeent. Bioeng.* 1998, *85*, 250–253.

[6] Tiehm, A., Lohner, S. T., Augenstein, T., Becker, D. et al., Entwicklung eines Sanierungsverfahrens für LCKW durch Kombination elektrolytischer und mikrobiologischer Prozesse. *Chem. Ing. Tech.* 2008, *80*, 967–974.

[7] Tiehm, A., Lohner, S. T., Augenstein, T., Effects of direct electric current and electrode reactions on vinyl chloride degrading microorganisms. *Electrochimica Acta* 2009, *54*, 3453–3459.

[8] Shin, H., Zeikus, J., Jain, M., Electrically enhanced ethanol fermentation by Clostridium thermocellum and Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* 2002, *58*, 476-481.

[9] Jeong, J., Kim, J. Y., Yoon, J., The role of reactive oxygen species in the electrochemical inactivation of microorganisms. *Environ. Sci. Technol.* 2006, *40*, 6117–6122.

[10] Nakayama, T., Wake, H., Ozawa, K., Kodama, H. et al., Use of a titanium nitride for electrochemical inactivation of marine bacteria. *Environ. Sci. Technol.* 1998, *32*, 798–801.

[11] Velizarov, S., Electric and magnetic fields in microbial biotechnology: possibilities, limitations, and perspectives. *Electro. Magnetobiol.* 1999, *18*, 185–212.

[12] Nevin, K. P., Woodard, T. L., Franks, A. E., Summers, Z. M. et al., Microbial electrosynthesis: feeding microbes electricity to convert carbon dioxide and water to multicarbon extracellular organic compounds. *MBio* 2010, *1*, e00103–e00110.

[13] Sydow, A., Krieg, T., Mayer, F., Schrader, J. et al., Electroactive bacteria—molecular mechanisms and genetic tools. *Appl. Microbiol. Biotechnol.* 2014, *98*, 8481–8495.

[14] Vandamme, P., Coenye, T., Taxonomy of the genus Cupriavidus: a tale of lost and found. *Int. J. Syst. Evol. Microbiol.* 2004, *54*, 2285–2289.

[15] Pohlmann, A., Fricke, W. F., Reinecke, F., Kusian, B. et al., Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. *Nat. Biotechnol.* 2006, *24*, 1257–1262.

[16] Spoljaric, I. V., Lopar, M., Koller, M., Muhr, A. et al., Mathematical modeling of poly[(R)-3-hydroxyalkanoate] synthesis by *Cupriavidus necator* DSM 545 on substrates stemming from biodiesel production. *Bioresour. Technol.* 2013, *133*, 482–494.

[17] Pfitzner, J., Schlegel, H., Denitrifikation bei Hydrogenomonas eutropha stamm H16. *Arch. Microbiol.* 1973, *90*, 199–211.

[18] Gruber, S., Hagen, J., Schwab, H., Koefinger, P., Versatile and stable vectors for efficient gene expression in *Ralstonia eutropha* H16. *J. Biotechnol.* 2014, *186*, 74–82.

[19] Li, H., Opgenorth, P. H., Wernick, D. G., Rogers, S. et al., Integrated electromicrobial conversion of CO2 to higher alcohols. *Science* 2012, *335*, 1596.

[20] Grousseau, E., Lu, J., Gorret, N., Guillouet, S. E. et al., Isopropanol production with engineered *Cupriavidus necator* as bioproduction platform. *Appl. Microbiol. Biotechnol.* 2014, *98*, 4277–4290.

[21] Crepin, L., Lombard, E., Guillouet, S. E., Metabolic engineering of *Cupriavidus necator* for heterotrophic and autotrophic alka(e)ne production. *Metab. Eng.* 2016, *37*, 92–101.

[22] Muller, J., MacEachran, D., Burd, H., Sathitsuksanoh, N. et al., Engineering of *Ralstonia eutropha* H16 for autotrophic and heterotrophic production of methyl ketones. *Appl. Environ. Microbiol.* 2013, *79*, 4433–4439.

[23] Ewering, C., Heuser, F., Benolken, J. K., Bramer, C. O. et al., Metabolic engineering of strains of *Ralstonia eutropha* and *Pseudomonas putida* for biotechnological production of 2-methylcitric acid. *Metab. Eng.* 2006, *8*, 587–602.

[24] Passanha, P., Kedia, G., Dinsdale, R. M., Guwy, A. J. et al., The use of NaCl addition for the improvement of polyhydroxyalkanoate production by *Cupriavidus necator*. *Bioresour. Technol.* 2014, *163*, 287–294.

[25] Darani, K. K., Farahani, E. V., Shojaosadati, S. A., Application of the Plackett-Burman statistical design to optimize poly (beta-hydroxybutyrate) production by *Ralstonia eutropha* in batch culture. *Iran J. Biotechnol.* 2003, *1*, 155–161.

[26] Vasheghani Farahani, E., Khosravi Darani, K., Shojaosadati, S. A., Application of the Taguchi design for production of Poly (β-hydroxybutyrate) by *Ralstonia eutropha*. *Iran J. Chem. Eng.* 2004, *23*, 131–136.

[27] Khanna, S., Srivastava, A. K., Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. *Process Biochem.* 2005, *40*, 2173–2182.

[28] Novak, M., Koller, M., Braunegg, G., Horvat, P., Mathematical modelling as a tool for optimized PHA production. *Chem. Biochem. Eng. Q.* 2015, *29*, 183–220.

[29] Schlegel, H., Lafferty, R., Submerskultur von Hydrogenomonas mit elektrolytischer Knallgaserzeugung im Kulturgefäss. *Zentrabl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. II* 1964, *118*, 483–490.

[30] Torella, J. P., Gagliardi, C. J., Chen, J. S., Bediako, D. K. et al., Efficient solar-to-fuels production from a hybrid microbial-water-splitting catalyst system. *Proc. Natl. Acad. Sci. USA* 2015, *112*, 2337–2342.

[31] Liu, C., Colon, B. C., Ziesack, M., Silver, P. A. et al., Water splitting-biosynthetic system with CO(2) reduction efficiencies exceeding photosynthesis. *Science* 2016, *352*, 1210–1213.

[32] Budde, C. F., Riedel, S. L., Willis, L. B., Rha, C. et al., Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from plant oil by engineered *Ralstonia eutropha* strains. *Appl. Environ. Microbiol.* 2011, *77*, 2847–2854.

[33] Tiemeyer, A. K., *Reaktionstechnische Untersuchungen zur chemolithoautotrophen Nitratatmung von Cupriavidus necator*, Universität München 2007.

[34] Lu, J., Brigham, C. J., Rha, C., Sinskey, A. J., Characterization of an extracellular lipase and its chaperone from *Ralstonia eutropha* H16. *Appl. Microbiol. Biotechnol.* 2013, *97*, 2443–2454.

[35] Seber, G., Wild, C., *Nonlinear Regression*, John Wiley & Sons, New York 1989.

[36] Bowien, B., Kusian, B., Genetics and control of CO2 assimilation in the chemoautotroph *Ralstonia eutropha*. *Arch. Microbiol.* 2002, *178*, 85–93.

[37] Lenz, O., Friedrich, B., A novel multicomponent regulatory system mediates H2 sensing in *Alcaligenes eutrophus*. *Proc. Natl. Acad. Sci. USA* 1998, *95*, 12474–12479.

[38] Repaske, R., Mayer, R., Dense autotrophic cultures of *Alcaligenes eutrophus*. *Appl. Environ. Microbiol.* 1976, *32*, 592–597.

[39] Repaske, R., Ambrose, C. A., Repaske, A. C., De Lacy, M. L., Bicarbonate requirement for elimination of the lag period of *Hydrogenomonas eutropha*. *J. Bacteriol.* 1971, *107*, 712–717.

[40] Repaske, R., Repaske, A. C., Quantitative requirements for exponential growth of *Alcaligenes eutrophus*. *Appl. Environ. Microbiol.* 1976, *32*, 585–591.

[41] Cramm, R., Genomic view of energy metabolism in *Ralstonia eutropha* H16. *J. Mol. Microbiol. Biotechnol.* 2008, *16*, 38–52.

[42] Kleihues, L., Lenz, O., Bernhard, M., Buhrke, T. et al., The H2 sensor of *Ralstonia eutropha* is a member of the subclass of regulatory [NiFe] hydrogenases. *J. Bacteriol.* 2000, *182*, 2716–2724.

[43] Pinske, C., Sawers, R. G., The importance of iron in the biosynthesis and assembly of [NiFe]-hydrogenases. *Biomol. Concepts* 2014, *5*, 55–70.

[44] Appia-Ayme, C., Quatrini, R., Denis, Y., Denizot, F. et al., Microarray and bioinformatic analyses suggest models for carbon metabolism in the autotroph *Acidithiobacillus ferrooxidans*. *Hydrometallurgy* 2006, *83*, 273–280.

[45] Schwartz, E., Henne, A., Cramm, R., Eitinger, T. et al., Complete nucleotide sequence of pHG1: a *Ralstonia eutropha* H16 megaplasmid encoding key enzymes of H 2-based lithoautotrophy and anaerobiosis. *J. Mol. Biol.* 2003, *332*, 369–383.

[46] Grunwald, S., Mottet, A., Grousseau, E., Plassmeier, J. K. et al., Kinetic and stoichiometric characterization of organoautotrophic growth of *Ralstonia eutropha* on formic acid in fed-batch and continuous cultures. *Microbial. Biotechnol.* 2015, *8*, 155–163.