From Microbial Succinic Acid Production to Polybutylene Bio-Succinate Synthesis

Niklas Tenhaef, Alina Hermann, Moritz Fabian Müller, Jonas Görtz, Jan Marienhagen, Marco Oldiges, Wolfgang Wiechert, Michael Bott, Andreas Jupke, Laura Hartmann, Sonja Herres-Pawlis, and Stephan Noack*

DOI: 10.1002/cite.202200163

Dedicated to Prof. Dr. Christian Wandrey on the occasion of his 80th birthday

A simplified and scalable one-pot process for the anaerobic production of succinic acid using a metabolically engineered *Corynebacterium glutamicum* strain is demonstrated. With targeted bioprocess optimization, succinic acid titer of 78 g L^{-1} and yield of 1.41 $mol_{SA}mol_{GLC}^{-1}$ were achieved. Succinic acid was recovered from the neutral fermentation broth by electrochemically induced crystallization and applied for polybutylene bio-succinate synthesis using a biocompatible zinc catalyst. Except for a slight color change, the final biopolymer was comparable to the polymer from commercial precursors.

Keywords: Anaerobic production, *Corynebacterium glutamicum*, Electrochemically induced crystallization, Polybutylene succinate, Succinic acid

Received: August 09, 2022; *revised:* December 16, 2022; *accepted:* January 26, 2023

1 Introduction

Succinic acid (SA) is a well-established platform chemical in the areas of food industry, consumer products and polymers, but also for the production of important chemical intermediates such as tetrahydrofuran, adipic acid or 1,4-butanediol (BDO) [1]. The polymerization of SA and BDO yields polybutylene succinate (PBS), a biodegradable polymer used as disposables, in biomedicine and in agriculture [2].

Within the last decades, many potential bioprocesses for microbial production of SA (bio-SA) have already been described [3–5]. Large-scale commercialization of bio-SA has been achieved, but in some cases failed due to high production costs, i.e., lack of inexpensive feedstocks as well as efficient upstream and downstream technologies [6, 7].

Among the various microbes tested for bio-SA production [8, 9], the Gram-positive soil bacterium *Corynebacterium glutamicum* shows exceptional performance under aerobic and anaerobic process conditions, both as wild-type and metabolically engineered variants [10–13]. With one of the currently best performing strains, *C. glutamicum* BOL-3/pAN6-*gap*, bio-SA titer of 144 g L^{-1} and yield of 1.67 $mol_{SA}mol_{GLC}^{-1}$ could be achieved [10]. However, the underlying process was based on several steps and the use of different cultivation systems, i.e., a phase for aerobic biomass preparation in shake flasks, followed by media exchange and finally anaerobic bio-SA production in sealed flasks or bioreactors. While such a setup can be easily applied on a lab-scale, it is neither practical nor cost-effective for large-scale production.

For the purification of bio-SA, different technologies have been proposed and implemented at industrial scale [14–16]. Depending on the pH of the fermentation process, bio-SA is separated from the fermentation broth via reactive extraction, electrodialysis or ion exchange and subsequently

[1,2]Niklas Tenhaef, [2,3]Alina Hermann, [1,2]Moritz Fabian Müller, [2,4]Jonas Görtz, [1,2,5]Jan Marienhagen, [1,2,5]Marco Oldiges, [1,2,6]Wolfgang Wiechert, [1,2]Michael Bott, [2,4]Andreas Jupke, [2,7]Laura Hartmann, [2,3]Sonja Herres-Pawlis, [1,2]Stephan Noack https://orcid.org/0000-0001-9784-3626 (s.noack@fz-juelich.de)

[1]Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany.

[2]Bioeconomy Science Center (BioSC), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany.

[3]Institute of Inorganic Chemistry, RWTH Aachen University, 52074 Aachen, Germany.

[4]Aachener Verfahrenstechnik – Fluid Process Engineering (AVT.FVT), RWTH Aachen University, 52074 Aachen, Germany.

[5]Institute of Biotechnology, RWTH Aachen University, Worringer Weg 3, 52074 Aachen, Germany.

[6]Computational Systems Biotechnology (AVT.CSB), RWTH Aachen University, 52074 Aachen, Germany.

[7]Institute of Organic and Macromolecular Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany.

crystallized. In production processes with fermentations at neutral pH, as is the case in the setup with *C. glutamicum*, the pH management is crucial for an economically and ecologically feasible process. In addition to the crystalline product, neutral salt emissions result from pH-adjusting additives. Morales et al. concluded that reactive extraction is the most promising purification method, despite the need for pH adjustment [16]. Novel electrochemical separation techniques [17–20] applied in this work overcome the disadvantage of neutral salt emissions by using water splitting electrolysis to control pH by acidification for downstream processing and base addition during fermentation when the catholyte is recycled. Compared to electrodialysis, the electric energy per kg product is significantly reduced by approx. 55 % [18].

PBS is produced via catalytic polycondensation, in which metal catalysts are typically used. These complexes are mostly titanium, antimony or tin catalysts, like titanium(IV) isopropoxide, antimony(II) butoxide or tin(II) octoate [21–23]. In particular, titanium-based catalysts exhibit high activity in the polycondensation reaction but at the same time these catalysts lead to undesirable degradation reactions and can cause yellowing of the material [24, 25]. Antimony or tin catalysts are not suitable alternatives due to their toxic properties [26–28], which necessitates the development and application of other harmless and active catalysts for the polycondensation.

In this study, the successful development of a one-pot production process for bio-SA is demonstrated that finally overcomes the aforementioned limitations associated with intermediate biomass transfer and media exchange. The strain *C. glutamicum* BOL-2 was used, which is the direct predecessor of the variant BOL-3 mentioned above. Apart from an additional formate dehydrogenase, this strain carries the same genetic modifications to increase flux into the reductive branch of the tricarboxylic acid cycle and to minimize by-product formation of lactate and acetate. Furthermore, we show that the electrochemically purified bio-SA can be used for polycondensation with commercial BDO using a biocompatible zinc catalyst to finally obtain polybutylene bio-succinate (PBbioS, Fig. 1).

2 Material and Methods

2.1 Chemicals, Bacterial Strain, Growth Media, and Cryo-Conservation

If not stated otherwise, all chemicals were of analytical grade and purchased from Sigma (Steinheim, Germany), Merck (Darmstadt, Germany) or Roth (Karlsruhe, Germany). In this study, strain *C. glutamicum* BOL-2 [10] was used. The strain was stored as cryostock. For generation of new cryostocks, a shake flask cultivation in defined CGXII medium containing 20 g L^{-1} D-glucose as substrate was performed overnight. Cells were harvested from exponential phase by centrifugation (4000 g, 10 min), resuspended in a sterile solution containing 0.9 wt % sodium chloride and 20 vol % glycerol and subsequently stored at –80 °C. Defined CGXII medium [29] used in this study contained per liter of deionized water: 20 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 1 g KH_2PO_4, 13.25 mg $CaCl_2 \cdot 2H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$,

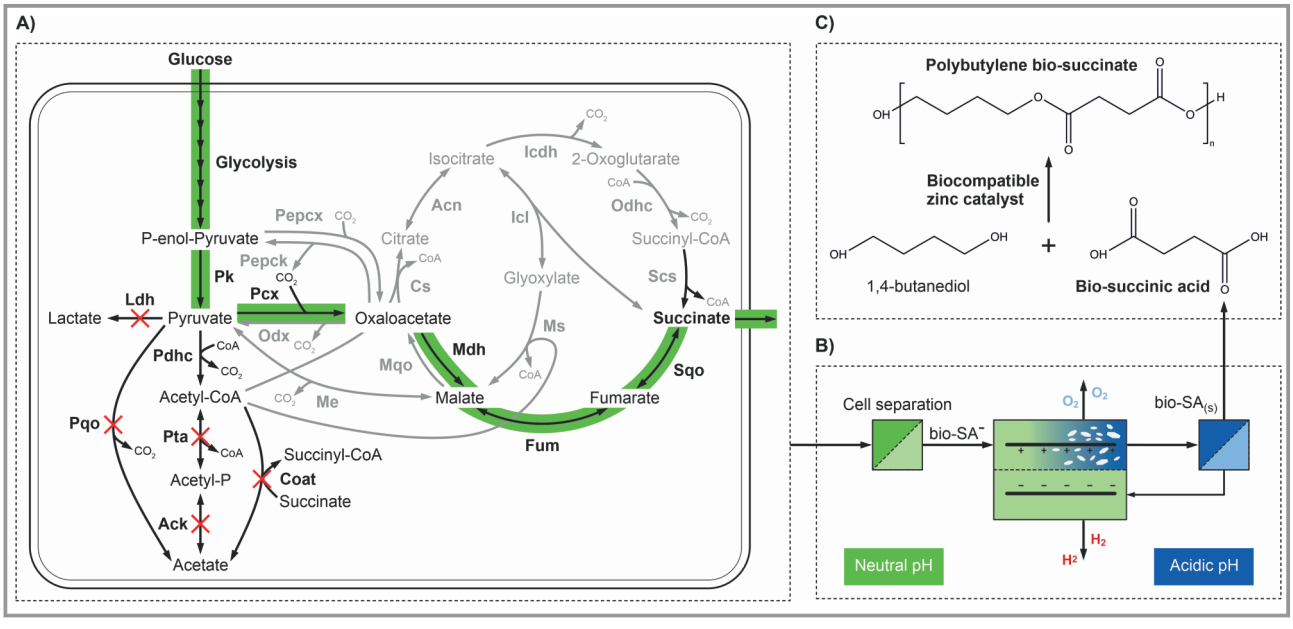

Figure 1. From D-glucose to PBbioS. A) Metabolic routes for anaerobic bio-SA production with *C. glutamicum* BOL-2. Gene deletions for blocking reactions leading to unwanted by-products are marked with red crosses. Other reactions with negligible rates during production are shown in gray. B) Setup for electrochemically induced crystallization of bio-SA from the neutral fermentation broth. C) Polycondensation reaction using the purified bio-SA in combination with a biocompatible zinc catalyst.

10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $MnSO_4 \cdot H_2O$, 0.02 mg $NiCl_2 \cdot 6H_2O$, 0.313 mg $CuSO_4 \cdot 5H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg biotin, and 30 mg 3,4-dihydroxybenzoate. When shake flasks were used, 5 g urea and 42 g 3-(N-morpholino)-propanesulfonic acid were added. D-glucose was added in varying amounts dependent on the respective experiment.

2.2 Biomass Preparation

For precultures in shake flasks, flasks with three baffles and a volume of 1 L, filled with 100 mL medium each, were used. Flasks were shaken in an incubator at 250 rpm and 30 °C. Inoculation was done from cryo-cultures to a starting optical density (OD_{600}) of 0.1. Depending on the experiment, biomass generation for bio-SA production was either done in shake flasks or in a bioreactor. For the shake flask process, four 1-L shake flasks with three baffles, each filled with 200 mL of defined CGXII medium containing 40 g L^{-1} D-glucose, were used. The flasks were inoculated to an OD_{600} of 0.1 from an exponential growing shake flask culture.

Bioreactor processes were done in a parallel cultivation platform (Eppendorf/DASGIP, Jülich, Germany) using 1-L glass vessels. The vessels were filled with 800 mL of defined CGXII medium and inoculated from an exponential growing shake flask culture to a final OD_{600} of 0.1. A pH of 7 was maintained by feeding 5 M H_3PO_4 and 5 M NH_4OH on demand. Temperature and air flow were set to 30 °C and 0.5 vvm, respectively. Online measurements were taken for pH (405-DPAS-SC-K80/225, Mettler Toledo), dissolved oxygen (DO) concentration (Visiferm DO 225, Hamilton) and exhaust gas composition (GA4, DASGIP AG). Depending on the experiment, fully aerobic or oxygen-limited process conditions were to be achieved: Aerobic process conditions were maintained by controlling the stirrer speed (400–1200 rpm) to reach an DO of at least 30 %. The oxygen-limited process conditions were achieved either by controlling the stirrer speed and aiming for an DO of 5 % or by setting a constantly increasing fixed stirrer speed, starting at 400 rpm and raising by 30 rpm h^{-1}. This gradient was deliberately chosen to keep the cells oxygen-limited, but still provide an increasing oxygen supply during growth. Growth was monitored by OD_{600} measurement in regular intervals. Additionally, in bioreactor process, DO signal and off-gas analytics were monitored to determine exhaustion of D-glucose and therefore end of batch growth phase.

2.3 Production of Bio-SA

Bio-SA was produced using the same bioreactor setup as described above, adapted to oxygen-deprived conditions by fully closing the gas inlet. If the biomass was prepared in shake flasks, the content of all four flasks were transferred manually into one bioreactor. If the biomass was already prepared in the bioreactor, the process was directly continued with the bio-SA production phase as "one-pot-process". If a feed pulse was used, 555 mmol D-glucose and 250 mmol $NaHCO_3$ were added to the bioreactor as powder. The gas inlet was then closed and the stirrer rate set to 400 rpm to avoid cell sedimentation. Temperature was maintained at 30 °C and pH at 7 by feeding 5 M NH_4OH via a closed-loop control strategy. After stop of the production phase, supernatant for purification of bio-SA was obtained via centrifugation (Beckman Coulter Avanti JXN26, Rotor JLA 8.1000) for 45 min at 12 500 g and 10 °C. The supernatant was stored at –28 °C.

2.4 Biomass and Supernatant Analysis

OD_{600} was measured using a UV-spectrophotometer (UV-1800, Shimadzu) and 1-mL cuvettes. 0.9 % (w v^{-1}) NaCl solution was used as a reference. Absorption of samples was ensured to be between 0.05 and 0.3 a. u. by dilution. If not stated otherwise, measurements were done in analytical triplicates. Supernatant samples were prepared by centrifugation of culture suspension in a tabletop centrifuge and subsequent passing of the supernatant through a cellulose acetate syringe filter (0.2 µm, DIA-Nielsen, Düren, Germany). Concentrations of D-glucose and bio-SA were measured by high performance liquid chromatography (Agilent 1100 Infinity, Agilent Technologies, Santa Clara, CA) using isocratic ion exchange on an Organic Acid Resin HPLC Column 300×8 mm (CS Chromatography, Düren, Germany) as stationary phase and 0.1 M H_2SO_4 as mobile phase with a flow rate of 0.6 mL min^{-1}. The column temperature was 45 °C and the injection volume 10 µL. Components were detected with a refractive index detector in case of D-glucose and an UV light absorption at 215 nm with a diode array detector in case of bio-SA. For external calibration, standards of D-glucose and bio-SA (supplied by Sigma-Aldrich, Steinheim, Germany) were applied in the linear dynamic range. Estimation of measurement errors was done by parametric bootstrapping as previously described [30]. Data was processed and visualized using Python 3.8, together with the packages numpy [31], pandas [32], and matplotlib [33].

2.5 Electrochemical Purification of Bio-SA

The supernatant from fermentation was filtered (0.2 µm, Filtrox, Sankt Gallen, Switzerland) to remove any remaining cells from the biotechnological production process and spiked with commercial SA (99 %) from ThermoFisher (Kandel, Germany) to 900 mmol L^{-1} to simulate a previous concentration step. Subsequently, 300 mL of the aqueous solution was filled into the anodic chamber and 300 mL of 0.5 mol Na_2SO_4 (99 %, Sigma Aldrich, Darmstadt, Germany) solution in the cathodic chamber of an electrolysis

cell. The chambers are divided by a cation exchange membrane. The anode consists of a ruthenium oxide-coated titanium sheet and a nickel (99 %) sheet was used as cathode. A constant current of 0.3 A was applied to the system over the course of 29 h. Due to water splitting, the pH in the anode decreases and the bio-SA is protonated. This protonated form is less soluble than the deprotonated species and thus bio-SA was crystallized. Finally, the crystals were vacuum-filtered through a paper filter (8–12 µm) and dried in a vacuum oven at 40 °C and 0.1 bar.

2.6 Synthesis of PBS

Commercially available SA (99 %) and BDO (99 %) were obtained from Alfa Aesar. The zinc catalyst was resynthesized according to literature [34]. In a Schlenk flask, equipped with Dean Stark and reflux condenser, BDO (2.2530 g, 25 mmol, 2.209 mL, 1 eq), SA (2.9530 g, 25 mmol, 1 eq) and zinc catalyst (52.0 mg, $5.0 \cdot 10^{-2}$ mmol, 1 mol %) were added. SA was previously weighed out exactly to 0.001 g with a milligram precision scale and the diol was measured with a syringe and simultaneously counterweighed on a precision scale. The mixture was heated to 200 °C for 1 h under dry nitrogen flow. The polycondensation was continued under high vacuum for further 2 h at 200 °C. The crude product was dissolved in an appropriate amount of chloroform (ca. 10 mL) and then precipitated in methanol (200 mL). The product was filtered through a Büchner funnel and dried under vacuum.

2.7 Nuclear Magnetic Resonance Spectroscopy

NMR spectra were recorded at room temperature on a Bruker Avance II (400 MHz). The NMR signals were calibrated to the residual signals of the deuterated solvent ($CDCl_3$: $\delta(^1H) = 7.26$ ppm).

2.8 Differential Scanning Calorimetry

DSC analysis was conducted with a Mettler-Toledo DSC3 STAR^e System differential scanning calorimeter equipped with an autosampler and calibrated with indium and zinc. Samples were weight into 40 µL aluminum crucibles with punctured lids. The experiments were performed by heating the samples from –40 °C to 200 °C with a heating rate of 10 °C min^{-1} in nitrogen atmosphere. Heating and cooling scans were repeated, at least in triplicate, to ensure reproducibility.

3 Results and Discussion

3.1 Microbial Production of Succinic Acid

3.1.1 Effect of Biomass Preparation on Anaerobic Bio-SA Production

Previous studies showed that *C. glutamicum* BOL-2 (Fig. 1B) produced high titers of bio-SA when cells were precultured aerobically in shake flasks, followed by centrifugation and resuspension in sodium chloride solution containing D-glucose and $NaHCO_3$, and agitated under anaerobic conditions in a closed bottle or bioreactor [10].

Aiming for a simplified process, we tested the following two variants for biomass preparation: A) cells were grown in shake flasks, then transferred to a bioreactor without medium exchange, followed by supplementation with D-glucose and $NaHCO_3$ and a switch to anaerobic production conditions, or alternatively B) cells were directly grown in a bioreactor, then (without medium exchange) supplemented with D-glucose and $NaHCO_3$ and switched to anaerobic production conditions (one-pot setup). Both cultivation experiments were conducted using *C. glutamicum* BOL-2 and defined CGXII medium that initially contained 222 mM D-glucose.

In both setups, cells began to grow comparatively fast until OD_{600} signals diverged at t = 12 h, indicating impaired growth in the shake flask most likely due to oxygen limitation at higher cell densities (Fig. 2). For phase II (bio-SA production), a total volume of 800 mL cell suspension from shake flask precultures was transferred into an empty bioreactor. Both bioreactors were then supplemented with 555 mmol D-glucose and 250 mmol $NaHCO_3$ and subsequently sealed to prevent oxygen inlet. In the experimental setup with shake flask precultures, D-glucose was almost completely consumed during the production phase, and an bio-SA titer of 445 mM was reached within 144 h (Tab. 1). In contrast, D-glucose consumption in the one-pot setup was negligible and no bio-SA was formed (Fig. 2).

Two conclusions can be drawn: First, a medium change from defined cultivation media to sodium chloride solution as in the previous study [10] is not necessary to achieve high bio-SA titers. Second, standard biomass preparation in a bioreactor batch approach is unsuitable for bio-SA production. Shake flasks usually provide lower maximum oxygen transfer rates (OTR_{max}) than bioreactors. While a typical lab-scale bioreactor setup can deliver OTR_{max} > 100 mmol $L^{-1}h^{-1}$, cultivation in shake flasks under conditions similar to those used for this study yielded $OTR_{max} \approx$ 25 mmol $L^{-1}h^{-1}$ [35]. It is likely that the cells adapt their metabolism to the lower oxygen availability, which seems to be essential for subsequent bio-SA production.

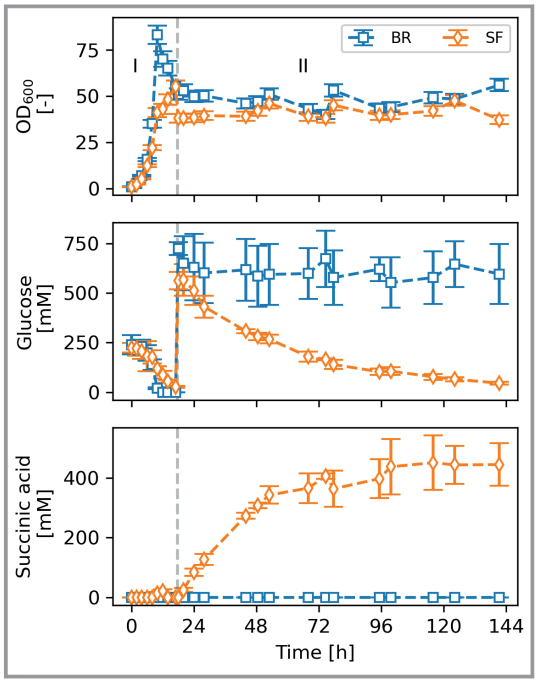

Figure 2. Effect of biomass preparation on anaerobic bio-SA production with *C. glutamicum* BOL-2. In phase I, cells were grown until t = 17.75 h either in shake flasks (SF setup) or directly in the bioreactor (BR setup). In the SF experiment, five 1-L shake flasks containing 200 mL CGXII medium were used and the obtained cell suspension was then transferred to an empty bioreactor. The BR experiment was performed in a 1-L bioreactor containing 800 mL CGXII medium. When switching to bio-SA production (phase II), equal amounts of 555 mmol D-glucose and 250 mmol $NaHCO_3$ were added to the cells. Both bioreactors were kept oxygen-free in phase II by sealing the oxygen inlet. Mean values and standard deviations were estimated from analytical triplicates.

3.1.2 Oxygen-Limited Growth Is Key for Anaerobic Bio-SA Production in One-Pot

To investigate this hypothesis, bioreactor cultivation experiments were performed under oxygen-limited conditions during cell growth (Fig. 3 and Tab. 1). These conditions were achieved by setting the DO controller acting on the stirrer rate to 5 %, resulting in DO values between 0 and 5 % during cell growth. Notably, this reduction in oxygen abundance did not result in a lower biomass yield compared to the non-limited bioreactor process (cf. Fig. 2). In the subsequent production phase, bio-SA accumulation to titers of 384 and 392 mM was achieved.

Our results show that oxygen availability during biomass preparation is a key factor in the cell's ability to produce bio-SA during the subsequent anaerobic phase of the process. This finding is in accordance with recent detailed studies on the metabolic adaptation of *C. glutamicum* during transition from aerobiosis to anaerobiosis [36, 37]. By applying such conditions, a one-pot process could be realized.

Most interestingly, substrate consumption and formation of bio-SA stopped at t = 75 h, leaving a residual D-glucose amount of 189 and 134 mmol, which was about 30 % of the amount added (Fig. 3). There are several plausible reasons for this behavior: First, the feeding regime, which included the addition of high amounts of substrate as a pulse, may have resulted in osmotic shock [38]. Nevertheless, the biomass concentration remained stable throughout the production phase, indicating that no cell lysis occurred. Second, the amount of the co-substrate $NaHCO_3$ may have been too low for complete conversion of the available D-glucose. Third, a change in pH might have affected CO_2 abundance, which has already been identified as a critical process parameter in this context [10].

Table 1. Key performance indicators for anaerobic bio-SA production with *C. glutamicum* BOL-2 under various conditions. Volumetric productivity was calculated for the first 48 h of the production phase. Specific yield always refers to the amount of glucose converted as the sole source of carbon and energy.

Mode of biomass generation	pH production phase	Feed mode	Added D-glucose [mmol]	Residual D-glucose [mmol]	Bio-SA titer [mmol]	Bio-SA titer [mmol L^{-1}]	Bio-SA yield [mol mol^{-1}]	Bio-SA vol. productivity [mmol $L^{-1}h^{-1}$]
Shake flask	7.0	Pulse	555	44.8	441.8	445.3	0.87	7.6
Bioreactor				520.6	0.0	0.0	0.00	0.0
Bioreactor, oxygen limited		Continuous	544	122.7	380.9	359.0	0.90	4.8
				145.1	362.6	349.3	0.91	4.8
		Pulse	555	189.0	362.1	383.9	0.99	7.9
				133.5	368.4	392.1	0.87	8.7
				173.3	518.4	505.8	1.36	8.3
	7.2			82.1	538.5	524.5	1.14	9.1
	7.5			56.4	685.1	667.7	1.37	9.2
	7.8			76.4	672.6	656.4	1.41	7.4

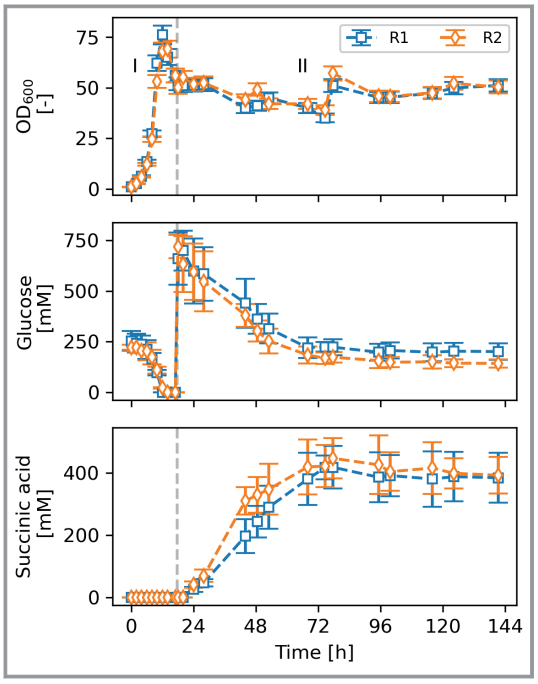

Figure 3. Effect of oxygen limitation during biomass preparation on anaerobic bio-SA production with *C. glutamicum* BOL-2. In phase I, cells were grown until t = 17.75 h in the bioreactor under oxygen limiting conditions. When switching to bio-SA production (phase II), 555 mmol D-glucose and 250 mmol $NaHCO_3$ were added to the cells. Both bioreactors (replicates R1 and R2) were kept oxygen-free in phase II by sealing the oxygen inlet. Mean values and standard deviations were estimated from analytical triplicates.

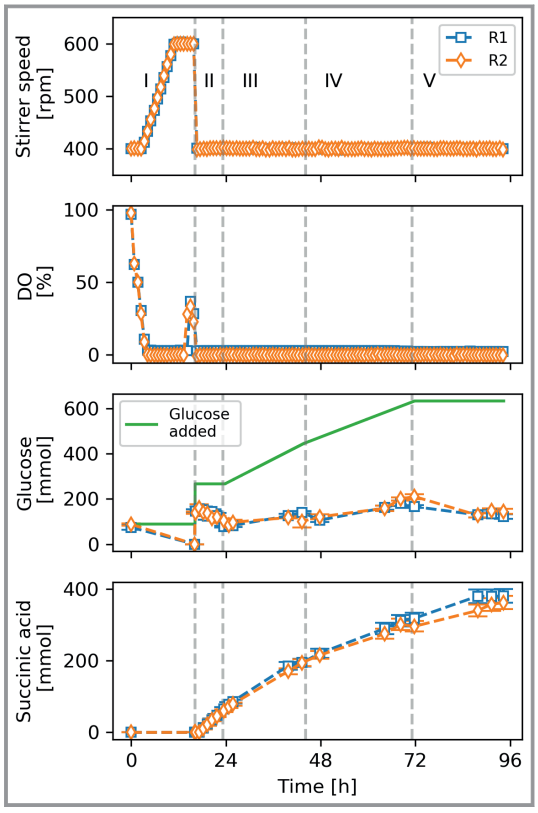

Figure 4. Effect of a continuous feeding mode on anaerobic bio-SA production with *C. glutamicum* BOL-2. In phase I, cells were grown in the bioreactor under oxygen limiting conditions until t = 16.17 h. When switching to bio-SA production (phase II), 178 mmol D-glucose and 250 mmol $NaHCO_3$ were added to the cells. At t = 23.17 h, a feed pump delivering 1.61 g h^{-1} D-glucose was activated (phase III). At t = 44.17 h and t = 71.17 h, the feeding rate was adjusted to 1.21 and 0 g h^{-1} D-glucose (phase IV and V), respectively. Both bioreactors (replicates R1 and R2) were kept oxygen-free in phase II by sealing the oxygen inlet. Mean values and standard deviations were estimated from analytical triplicates.

3.2 Effect of Feed Regime and pH on Anaerobic Bio-SA Production

To test continuous feeding, a bioprocess with oxygen-limited biomass preparation similar to the previous process was performed (Fig. 4 and Tab. 1). For bio-SA production, a smaller amount of 178 mmol D-glucose was added as a pulse along with 250 mmol $NaHCO_3$ and the reactor was immediately sealed (phase II). Subsequently, D-glucose was continuously fed via peristaltic pumps, and pumping rates were adjusted to keep the D-glucose concentration in the medium nearly constant (phase III-V). The continuous mode also led to production of bio-SA in phase II, reaching titers of 359 and 349 mM, respectively. Nevertheless, bio-SA formation decreased at the end of cultivation and volumetric productivity was greatly reduced compared to pulsed feeding (Tab. 1).

Finally, the effect of pH on anaerobic bio-SA production was investigated. Four additional bioprocesses with oxygen-limited biomass preparation and pulse feeding were performed, which were similar to the second process, but maintaining pH at different values during the production phase (Fig. 5 and Tab. 1). A pH set point of 7, which was also used in all previous experiments, resulted in an bio-SA titer and yield of 506 mM and 1.36 $mol_{SA}mol_{GLC}^{-1}$, respectively. These significantly higher key performance indicators (KPIs) were surprising, and it can only be surmised that oxygen limitation was more pronounced in phase I, leading to better metabolic adaptation and ultimately higher production levels. Higher pH settings further increased KPIs, with the highest bio-SA titer of 668 mM at pH 7.5 and the highest bio-SA yield of 1.41 $mol_{SA}mol_{GLC}^{-1}$ at pH 7.8. The amount of residual D-glucose was also reduced, but no condition resulted in complete substrate conversion, leaving room for further improvement. For example, further addition of CO_2, either as $NaHCO_3$ or ideally in gaseous form, could maximize substrate conversion.

4 PBbioS from Electrochemically Purified Bio-SA

In the following, it was investigated whether the produced bio-SA can be used to synthesize the same polyconden-

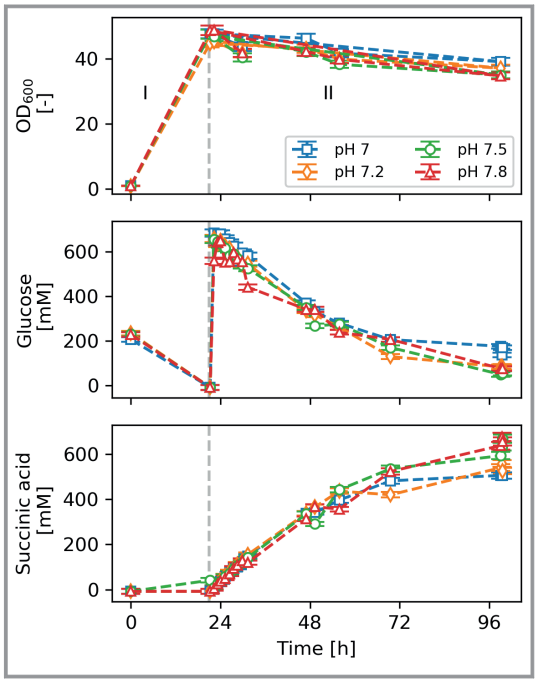

Figure 5. Effect of pH on anaerobic bio-SA production with *C. glutamicum* BOL-2. In phase I, cells were grown until t = 21 h in the bioreactor under oxygen limiting conditions. When switching to bio-SA production (phase II), 555 mmol D-glucose and 250 mmol $NaHCO_3$ were added to the cells, and pH was adjusted to the target values shown. All four bioreactors were kept oxygen-free in phase II by sealing the oxygen inlet. Mean values and standard deviations were estimated from analytical triplicates.

sation product with BDO as commercially SA. Therefore, the broth containing bio-SA was first purified by an electrochemical-introduced crystallization (Fig. 1B).

Initially, the spiked cell free supernatant, obtained by centrifugation and filtration from the bio-SA production was filled into the anodic chamber of the electrolysis cell and the starting pH was measured to be 7.4. Over the course of the experiment, the pH was lowered to 1.8 due to an applied constant current of 0.3 A. After 20.5 h at a pH of 3.8, crystal nucleation was observed. The final concentration was measured to be 620 mmol L^{-1} bio-SA, which resulted in 9.9 g of crystalline bio-SA and a theoretical yield of 31 %. The pH in the cathode was increased from 2 to 13.8 and thus, could be used for pH buffering of fermentation, as discussed by Kocks et al. [39]. To further improve the yield, additional process steps to increase the concentration of the bio-SA like reactive extraction [18, 40] or evaporation [41] have to be applied.

The obtained crystalline bio-SA was further examined for its use in the polycondensation reaction. In this metal catalyzed reaction a biocompatible zinc catalyst was used, which is important for an overall sustainable production process (Fig. 1C). A zinc bisguanidine complex was chosen due to its additional robustness to impurities, stability at high temperatures (up to 200 °C) and its enormously high activity in the ring opening polymerization [34]. The polycondensation was performed at a 1:1 ratio of SA and BDO with 0.1 mol % catalyst at 200 °C. For a direct comparison commercial SA and bio-SA were each polycondensated with BDO. In Fig. 6, slight visual differences between the starting materials and obtained products from SA and bio-SA are depicted. By introducing a decolorization step for the bio-SA, e.g., by using activated carbon [41], a comparable white PBbioS product should be obtainable.

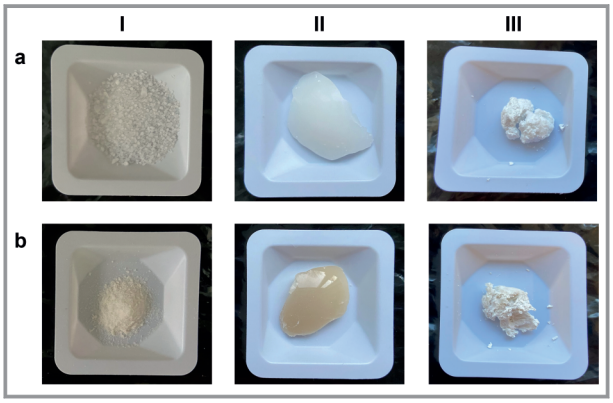

Figure 6. Comparison between commercially SA (Ia) and electrochemically purified bio-SA (Ib). PBS crude product (II) and precipitated product (III) from commercially BDO in combination with commercially SA (a) and bio-SA (b), respectively.

Both products were analyzed in more detail using proton nuclear magnetic resonance (^{1}H NMR) and differential scanning calorimetry (DSC). ^{1}H NMR analysis showed identical spectra for both products, synthesized with commercial SA or bio-SA (Fig. 7). It was demonstrated that PBS was successfully formed with both starting materials. Thermal properties of the obtained products were analyzed by DSC measurements. Semi-crystalline materials with post-crystallization during the heating phase were obtained regardless of the used SA (Fig. 8). Heating and cooling curves of the two PBS samples are almost identical with a melting temperature (T_m) of about 110 °C. The obtained T_m values correspond to the literature data for PBS (T_m = 113 °C) [42, 43].

5 Conclusions

One-pot processes are advantageous because of their scalability and easier process control. For anaerobic bio-SA production with *C. glutamicum*, oxygen limitation during biomass preparation in form of a microaerobic transition phase proved to be essential for one-pot operation. Other process parameters such as D-glucose feeding and pH were adjusted, resulting in the highest bio-SA titer of 668 mmol L^{-1} (78 g L^{-1}) and productivity of 9.2 mmol $L^{-1}h^{-1}$ at pH 7.5, while the highest yield of 1.41 $mol_{SA}mol_{GLC}^{-1}$ (70.5 % of the theoretical maximum) was obtained at pH 7.8. However, none of the nine conditions tested resulted in

complete D-glucose conversion, leaving room for further process optimization. This is also true for bio-SA recovery by electrochemically induced crystallization, where an intermediate concentration step, e.g., by reactive extraction, would be required to increase the yield. Finally, the purified bio-SA could be used for the synthesis of a PBbioS product using a biocompatible zinc catalyst for polycondensation with BDO.

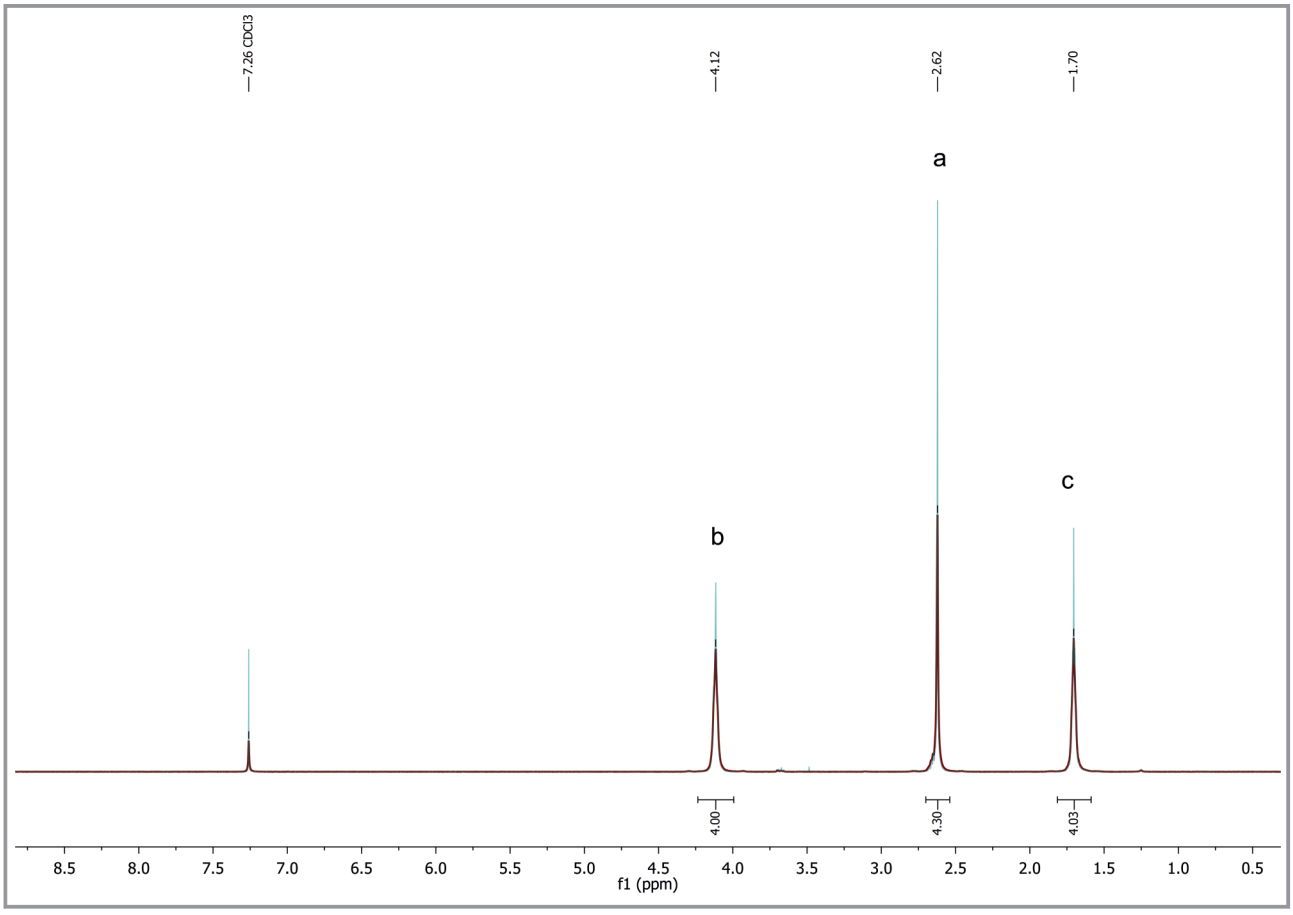

Figure 7. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of the precipitated product from commercially SA (red) and bio-SA (blue).

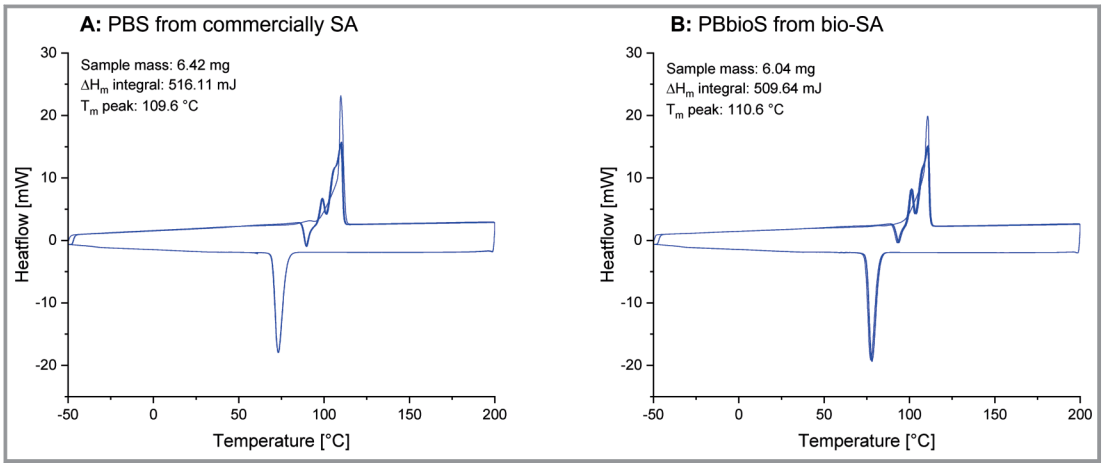

Figure 8. DSC data of the precipitated product from commercially SA (left) and bio-SA (right).

The authors acknowledge the financial support of the Bioeconomy Science Center as part of the projects HyImPAct ("Hybrid processes for Important Precursor and Active pharmaceutical ingredients"), R2HPBio ("Renewables to high-performance bioplastics by sustainable production ways") and BioPlastiCycle ("Transitioning bioplastics to the circular economy"). The scientific activities of the Bioeconomy Science Center were financially supported by the Ministry of Innovation, Science and Research within the framework of the NRW Strategieprojekt BioSC (no. 313/323-400-002 13). The colleagues from IBG-1 would like to express their sincere thanks to Prof. Christian Wandrey for his continuous support. The presented study can be seen as a demonstration of his well-known slogan: "In the end, there must be a bottle on the table!". Well, in our case the final product was too sticky for a bottle. Moreover, we thank Stephanie Scheelen and Dr. Monir Tabatabai for performing the DSC measurements. Open access funding enabled and organized by Projekt DEAL.

Abbreviations

bio-SA	bio-based succinic acid
BDO	1,4-butanediol
DO	dissolved oxygen
DSC	differential scanning calorimetry
^{1}H NMR	proton nuclear magnetic resonance
OD_{600}	optical density at 600 nm wavelength
PBbioSA	polybutylene bio-succinate
PBS	polybutylene succinate
SA	succinic acid

References

[1] J. J. Bozell, G. R. Petersen, *Green Chem.* **2010**, *12 (4)*, 539–554.
[2] S. A. Rafiqah et al., *Polymers* **2021**, *13 (9)*, 1436.
[3] J. Wang, A.-P. Zeng, W. Yuan, *Curr. Opin. Environ. Sci. Health* **2022**, *25*, 100313.
[4] D. M. Oliveira, T. R. Mota, O. Ferrarese-Filho, W. D. dos Santos, in *Production of Top 12 Biochemicals Selected by USDOE from Renewable Resources* (Eds: A. K. Chandel, F. Segato), Elsevier, Amsterdam **2022**.
[5] E. Mancini et al., *Chem. Eng. Res. Des.* **2022**, *179*, 401–414.
[6] X. Li, E. Mupondwa, *Renewable Sustainable Energy Rev.* **2021**, *137*, 110587.
[7] R. Dickson et al., *Energy Environ. Sci.* **2021**, *14 (6)*, 3542–3558.
[8] M. Jiang et al., *Bioresour. Technol.* **2017**, *245*, 1710–1717.
[9] B. Litsanov, M. Brocker, M. Oldiges, M. Bott, *Bioprocessing of renewable resources to commodity bioproducts*, Wiley, Hoboken **2014**, 435–472.
[10] B. Litsanov, M. Brocker, M. Bott, *Appl. Environ. Microbiol.* **2012**, *78 (9)*, 3325–3337.
[11] A. Briki et al., *Eng. Life Sci.* **2020**, *20 (5–6)*, 205–215.
[12] S. Okino et al., *Appl. Microbiol. Biotechnol.* **2008**, *81 (3)*, 459–464.
[13] D. Radoš et al., *Appl. Environ. Microbiol.* **2014**, *80 (10)*, 3015–3024.
[14] K.-K. Cheng et al., *Appl. Microbiol. Biotechnol.* **2012**, *95 (4)*, 841–850.
[15] M. L. Jansen, W. M. van Gulik, *Curr. Opin. Biotechnol.* **2014**, *30*, 190–197.
[16] M. Morales et al., *Energy Environ. Sci.* **2016**, *9 (9)*, 2794–2805.
[17] M. Gausmann et al., *Sep. Purif. Technol.* **2020**, *240*, 116489.
[18] M. Gausmann et al., *ACS Sustainable Chem. Eng.* **2021**, *9 (28)*, 9336–9347.
[19] C. Kocks, C. M. Krekel, M. Gausmann, A. Jupke, *Crystals* **2021**, *11 (9)*, 1090.
[20] C. Kocks, J. Görtz, M. Gausmann, A. Jupke, *Chem. Ing. Tech.* **2020**, *92 (9)*, 1175–1176.
[21] N. Jacquel et al., *J. Polym. Sci., Part A: Polym. Chem.* **2011**, *49 (24)*, 5301–5312.
[22] L. P. Ferreira, A. N. Moreira, J. C. Pinto, F. G. de Souza Jr., *Polym. Eng. Sci.* **2015**, *55 (8)*, 1889–1896.
[23] A. Díaz, R. Katsarava, J. Puiggalí, *Int. J. Mol. Sci.* **2014**, *15 (5)*, 7064–7123.
[24] T. Shah, J. Bhatty, G. Gamlen, D. Dollimore, *Polymer* **1984**, *25 (9)*, 1333–1336.
[25] U. K. Thiele, *Int. J. Polym. Mater.* **2001**, *50 (3–4)*, 387–394.
[26] M. C. Tanzi et al., *J. Mater. Sci.: Mater. Med.* **1994**, *5 (6)*, 393–396.
[27] R. J. Lewis, *Sax's Dangerous Properties of Industrial Materials, Vol. 3, Wiley, New York **1996***.
[28] P. Westerhoff, P. Prapaipong, E. Shock, A. Hillaireau, *Water Res.* **2008**, *42 (3)*, 551–556.
[29] C. Keilhauer, L. Eggeling, H. Sahm, *J. Bacteriol.* **1993**, *175 (17)*, 5595–5603.
[30] J. Hemmerich et al., *Biotechnol. J.* **2019**, *14 (9)*, 1800428.
[31] C. Harris et al., *Nature* **2020**, *585*, 357–362.
[32] W. McKinney, in *Proc. of the 9th Python in Science Conference*, Austin, TX, June **2010**. DOI: https://doi.org/10.25080/Majora-92bf1922-00a
[33] J. Hunter, *Comput. Sci. Eng.* **2007**, *9 (3)*, 90–95.
[34] A. Hermann et al., *Angew. Chem., Int. Ed.* **2020**, *59 (48)*, 21778–21784.
[35] K. Meier et al., *Biochem. Eng. J.* **2016**, *109*, 228–235.
[36] A.-K. Kaboré et al., *Process Biochem.* **2017**, *52*, 10–21.
[37] J. Lange et al., *Genes* **2018**, *9 (6)*, 297.
[38] S. Ochoa, *Biochem. Eng. J.* **2016**, *105*, 177–188.
[39] C. Kocks, D. Wall, A. Jupke, *Materials* **2022**, *15 (23)*, 8412.
[40] A. Eggert et al., *Sep. Purif. Technol.* **2019**, *215*, 463–472.
[41] M. Okabe, D. Lies, S. Kanamasa, E. Y. Park, *Appl. Microbiol. Biotechnol.* **2009**, *84 (4)*, 597–606.
[42] J. Xu, B. H. Guo, *Biotechnol. J.* **2010**, *5 (11)*, 1149–1163.
[43] C.-H. Chen et al., *Colloid Polym. Sci.* **2010**, *288 (7)*, 731–738.

