processes

MDPI

Article

Efficient Bio-Oxidation of Cellobiose with Engineered *Gluconobacter oxydans* to Provide Highly Concentrated Cellobionic Acid

Emmeran Bieringer [1], Lisa Pütthoff [2], Arne Zimmermann [1], Mariana de Souza Góes [1], Uraz Yilmaz [1], Armin Ehrenreich [2], Wolfgang Liebl [2] and Dirk Weuster-Botz [1,*]

[1] Chair of Biochemical Engineering, Technical University of Munich, Boltzmannstraße 15, 85748 Garching, Germany
[2] Chair of Microbiology, Technical University of Munich, Emil-Ramann-Straße 4, 85354 Freising, Germany
* Correspondence: dirk.weuster-botz@tum.de

Abstract: Cellobionic acid (CBA) can be obtained through the oxidation of cellobiose, the monomer of cellulose. CBA serves as a plant-based alternative to its stereoisomer lactobionic acid, which is used in the pharmaceutical, cosmetic, and food industries. *Gluconobacter oxydans* is a well-established whole-cell biocatalyst with membrane-bound dehydrogenases (mDH) for regio-specific oxidations. As *G. oxydans* wildtype cells show low cellobiose oxidation activities, the glucose mDH from *Pseudomonas taetrolens* was overexpressed in *G. oxydans* BP9, a multi mDH deletion strain. Whole-cell biotransformation studies were performed with resting cells of the engineered *G. oxydans* in stirred tank bioreactors. Initial biomass specific cellobionate formation rates increased with increasing cellobiose concentrations up to 190 g L^{-1}, and were constant until the solubility limit. The maximal volumetric CBA formation rates and the oxygen uptake rates increased linearly with the concentration of engineered *G. oxydans*. This enables the estimation of the maximum biocatalyst concentration limited by the maximum oxygen transfer rate of any bioreactor. Thus, 5.2 g L^{-1} *G. oxydans* was sufficient to produce 502 g L^{-1} CBA with >99% yield in a simple aerobic batch process. The highly concentrated CBA will reduce downstream processing costs considerably after cell separation.

Keywords: *Gluconobacter oxydans*; whole-cell biocatalysis; cellobionic acid; bio-oxidation; aldobionic acid; multideletion strain

Citation: Bieringer, E.; Pütthoff, L.; Zimmermann, A.; de Souza Góes, M.; Yilmaz, U.; Ehrenreich, A.; Liebl, W.; Weuster-Botz, D. Efficient Bio-Oxidation of Cellobiose with Engineered *Gluconobacter oxydans* to Provide Highly Concentrated Cellobionic Acid. *Processes* **2024**, *12*, 1464. https://doi.org/10.3390/pr12071464

Academic Editor: Pedro Fernandes

Received: 14 June 2024
Revised: 10 July 2024
Accepted: 11 July 2024
Published: 13 July 2024

1. Introduction

Cellobionic acid (4-O-ß-D-glucopyranosil-D-gluconic acid) belongs to a subgroup of sugar acids called aldobionic acids [1]. Sugar acids are derived from the oxidation of mono- or disaccharides [2]. Aldobionic acids consist of one monosaccharide (e.g., glucose), chemically linked through a glycosidic bond to a polyhydroxy acid (e.g., gluconic acid) [3]. The most well-known aldobionic acid is lactobionic acid (LBA), which can be obtained by oxidizing lactose [4]. LBA finds many applications in the pharmaceutical, food, cosmetics, and chemical industries due to its moisturizing, biodegradable, non-toxic, and antioxidant properties [5–7]. Even though biocatalytic methods exist to produce this aldobionic acid, industrial production is carried out through chemical synthesis, which brings disadvantages like using toxic high-cost metal catalysts and unwanted byproducts [8,9]. As cellobionic acid (CBA) is a stereoisomer of LBA, it is likely that they share a range of applications due to their similar physical and chemical properties [10–12]. Additionally, CBA would be a plant-based vegan alternative, as it is obtained through the oxidation of cellobiose, which is the structural repeating unit of cellulose. Cellobiose can be obtained by degradation of cellulose with cellobiohydrolase, an enzyme produced by numerous bacteria and fungi.

Until now, the production of CBA has been poorly studied. Besides chemical synthesis, which again needs unfavorable conditions like toxic catalysts, high temperatures, and

high pressure, there are already some biological approaches. Biotransformation with *Gluconobacter frateurii* NBRC3285 achieved 0.86 g L^{-1} CBA within 24 h in shake flasks with initial cellobiose concentrations of 9.5 g L^{-1} [13]. The cellulase-secreting fungus *Neurospora crassa* expresses a cellobiose dehydrogenase, thus enabling the degradation of cellulose into cellobiose before oxidizing it to CBA [14,15]. By deletion of the beta-glucosidase (bgl) gene, 7.7 g L^{-1} cellobiose was produced from 20 g L^{-1} cellulose in shake flasks [16]. The addition of the enzyme laccase and the redox mediator 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) enabled the production of CBA from cellulose [14]. The deletion of two transcription factors and the gene of the cellobionate phosphorylase in combination with the overexpression of a laccase in *N. crassa* resulted in the production of CBA from cellobiose or cellulose without the addition of enzymes for cofactor regeneration [12,17]. By using ground lignocellulosic wheat straw, 17 g L^{-1} CBA was produced with this engineered *N. crassa* from cellobiose in shake flasks within 10 d [18]. Recently, it was reported that *Pseudomonas taetrolens* with homologous overexpression of a membrane-bound glucose dehydrogenase enabled the production of 200 g L^{-1} CBA from cellobiose in shake flasks within 11 h [11]. It was further shown that the engineered *P. taetrolens* was able to produce CBA by oxidation of cellobiose in a hydrolysate obtained by the treatment of wastepaper with commercial cellulolytic enzymes with low beta-glucosidase activities [19].

The oxidation of cellobiose was also shown for the acetic acid bacterium *Gluconobacter oxydans*. Responsible for CBA formation by *G. oxydans* are membrane-bound glucose dehydrogenase (mGDH) and alcohol dehydrogenase (mADH) [20]. This is not surprising as *G. oxydans* can incompletely oxidize a huge range of different substrates, like sugars and alcohols, with membrane-bound dehydrogenases (mDHs) [21–23], and has already been established as a high performing whole-cell biocatalyst for oxidations [24]. Industrial examples are the oxidation of D-sorbitol to L-sorbose, a step within the production of vitamin C based on the Reichstein–Grüssner process [25,26], or the oxidation of glycerol to form dihydroxyacetone [21,22], which finds application as additive in self-tanning lotions in cosmetics [23].

To avoid side reactions and to ensure high loadings of the membrane with a specific mDH of interest, a multideletion strain was designed with nine membrane-bound dehydrogenases being deleted (*G. oxydans* BP9) [20]. As an example, resting cells of *G. oxydans* BP9 with solely expressing the native membrane-bound polyol dehydrogenase were able to produce 242 g L^{-1} of L-erythrulose from meso-erythritol in stirred tank bioreactors achieving full conversion within 25 h in the batch process [20,24].

As the membrane-bound glucose dehydrogenase of *P. taetrolens* is so far the most active mDH known for the oxidation of cellobiose, and *G. oxydans* is an industrially well-established biocatalyst for oxidations, we decided to overexpress this enzyme in the multi-deletion strain *G. oxydans* BP9 to provide a new biocatalyst for the whole-cell production of CBA from cellobiose. Unfortunately, CBA is freely soluble in water. As a consequence, whole-cell biotransformations should be performed with resting cells in simple aqueous buffer solutions until full conversion of cellobiose, and up to high concentrations of CBA to enable cost-efficient isolation of CBA. Full conversion and high product concentrations can be achieved in batch processes. Highly active bio-oxidation catalysts need sufficient oxygen supply. Thus, we planned to perform batch studies in scalable stirred tank bioreactors with resting cells of *G. oxydans* BP9 overexpressing the mGDH of *P. taetrolens* to identify suitable reaction conditions for the efficient production of CBA. Furthermore, the heterologous expression of this mGDH and successful production of CBA could be a step forward to establish the multi-deletion strain *G. oxydans* as a platform for bio-oxidations. As *G. oxydans* is known for its high tolerance against osmotic pressure, we aimed to even increase the so-far-reported maximal CBA concentrations reached with *P. taetrolens*. Increasing product concentrations is important to reduce costs in the downstream processing of CBA, which contributes to overall production costs.

2. Materials and Methods

2.1. Materials

D-cellobiose with a purity of ≥98% was purchased from Biosynth Ltd. (Compton, UK). Ammonium salt of cellobionic acid with a purity of ≥98% was obtained from Synthose (Concord, CA, Canada). Fructose, isopropanol, ethanol and RNAse A were from AppliChem GmbH (Darmstadt, Germany). T&C Lysis Solution and MPC protein precipitation reagent were purchased from Biozym Scientific GmbH (Hessisch Oldendorf, Germany). All other chemicals were ordered from Carl Roth (Karlsruhe, Germany).

2.2. Development of Gluconobacter oxydans BP9.1 pta-mGDH

2.2.1. Bacterial Strains and Culture Methods

Escherichia coli (*E. coli*) TOP10, purchased from Invitrogen by Thermo Fisher Scientific (Darmstadt, Germany), and *E. coli* HB101 carrying plasmid pRK2013 were grown in LB medium (Lennox) containing 50 µg mL^{-1} kanamycin at 37 °C and 180 rpm. *Pseudomonas taetrolens* DSM 21104 was purchased from the DSMZ (Braunschweig, Germany) and cultivated in 30 g L^{-1} CASO-bouillon at 30 °C and 180 rpm. *Gluconobacter oxydans* BP.9 derived from ATCC 621H (DSM 2343) was constructed by Peters et al. and was grown in complex medium containing 3 g L^{-1} tryptone and 5 g L^{-1} yeast extract at 30 °C and 180 rpm [20]. The pH was set to 6 using HCl. An amount of 9 g L^{-1} fructose was added, sterile, to the autoclaved medium. In the case of agar plates, 15 g L^{-1} agar-agar was added. For the selection of plasmids, 50 µg mL^{-1} kanamycin was added. For the counterselection of *E. coli*, 60 µg mL^{-1} cefoxitin was added as well. The BP.9 strain was further optimized by introducing the previously deleted *upp* gene back into the genome. The obtained strain was named BP9.1.

2.2.2. Isolation of Genomic DNA

Genomic DNA of *P. taetrolens* was extracted with the MasterPure DNA Purification Kit from Epicentre (Madison, WI, USA) using 1 mL of a 2-day old liquid culture.

2.2.3. Construction of Expression Plasmid

The primers that were used to amplify the *P. taetrolens* mGDH gene and the plasmid backbone are listed in Table 1. They were designed with CloneManager 9 software (Sci-Ed Software, Cary, NC, USA) and synthesized by Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Sequencing was performed by Azenta/Genewiz (South Plainfield, NJ, USA). Molecular biology manipulations were undertaken according to standard procedures [27]. The plasmid pADH, previously named pMM3b, was constructed by Mientus et al. [28] and prepared from *E. coli* TOP10 with the NucleoSpin Plasmid kit from Macherey-Nagel (Düren, Germany). Amplification of the backbone and the gene was performed via PCR using Phusion Plus polymerase by Thermo Fisher Scientific (Darmstadt, Germany). Clean-up of the products was performed with NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel. Cloning was performed via the SLiCE method by Zhang et al. [29]. After heat-shock transformation in *E. coli* TOP10, clones were verified with colony-PCR and checked by sequencing. The plasmid obtained for expression of the *P. taetrolens* mGDH gene was designated pADH-pta_mGDH.

Table 1. Primers for the amplification of the gene of the mGDH from *P. taetrolens* and the amplification of the pADH backbone.

Name	5′–3′ Sequence	Application
pta_pADH_for	CCATGATTACGCCAAGCGTTATTTCTCTTTAGGATCGGGCAG	Amplification of pta-mGDH from *P. taetrolens* with overhangs
pta_pADH_rev	ACAAAAAGGACAGTTGGATCATGAGTACGCAAGCGAAAGG	
pADH_pta_for	CTGCCCGATCCTAAAGAGAAATAACGCTTGGCGTAATCATGG	Amplification of pADH plasmid backbone with overhangs
pADH_pta_rev	CCTTTCGCTTGCGTACTCATGATCCAACTGTCCTTTTTGT	

2.2.4. Transformation of *G. oxydans*

The expression plasmid carrying the *P. taetrolens* mGDH gene was transferred to *G. oxydans* BP9.1 by conjugation, using tri-parental mating. *E. coli* HB101 carrying pRK2013 was used as the helper strain, and *E. coli* TOP10 carrying the mGDH expression plasmid pADH-pta_mGDH was used as the donor strain. Both *E. coli* strains were grown to an OD_{600} of 1.0. Cells from 800 µL of each strain were pelleted and washed two times with 5 mL LB medium. *G. oxydans* BP9.1 was grown in 20 mL complex medium to an OD_{600} of 0.6–0.9, and 5 mL was pelleted together with the *E. coli* strains. The pellet was resuspended in a small volume of supernatant retained in the tube and densely dropped on an agar plate without antibiotics. After 24 h at 30 °C, the cell spot was collected and resuspended in 300 µL liquid medium and plated on selection plates containing 50 µg mL^{-1} kanamycin and 60 µg mL^{-1} cefoxitin. Positive clones were verified with colony-PCR and checked by sequencing. The strain used for further study was designated as *G. oxydans* BP9.1 pta-mGDH.

2.3. Maintenance

Cells were maintained in 35% (*v*/*v*) glycerol at −80 °C. To prepare the stock, cells were cultivated for at least 48 h in 500 mL shake flasks without baffles with 50 mL pre-culture medium (see Section 2.4) at 30 °C and 180 rpm (Multitron, Infors HT, Bottmingen, Switzerland). After combining the cells with glycerol, the mixture was aliquoted in sterile 1.5 mL reaction tubes. Before freezing at −80 °C, the tubes were incubated for 1 h at room temperature.

2.4. Pre-Culture Preparation

The pre-culture of *G. oxydans* BP9.1 pta-mGDH was prepared in sterile shake flasks without baffles using a modified complex medium containing 5 g L^{-1} yeast extract, 3 g L^{-1} peptone from casein, and 10 g L^{-1} fructose (FYP-medium) [30]. Then, 3 M HCl was used to adjust pH 6.0. Kanamycin and cefoxitin were added, each with 50 mg L^{-1}. Before the addition of the antibiotics, the medium was autoclaved at 121 °C for 20 min.

The pre-culture was prepared in two consecutive steps [24]: In the first step, 500 mL shake flasks without baffles filled with 50 mL of FYP medium were inoculated with 500 µL of a cryogenic stock solution of *G. oxydans* BP9.1 pta-mGDH (see Section 2.3). The shake flask was incubated for 72 h at 30 °C with a shaking frequency of 180 rpm (Multitron, Infors HT, Bottmingen, Switzerland) until the stationary growth phase was reached. The second pre-culture was prepared in a 1 L shake flask with 180 mL of FYP-medium, inocculated with 10% (*v*/*v*) of the first pre-culture. The shake flask was incubated for 24 h at 30 °C with a shaking frequency of 180 rpm. Afterwards, the *G. oxydans* BP9.1 pta-mGDH cells were separated by centrifugation (3260× *g*, 20 min, Rotixa 50 RS, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). The pellets of up to six 1 L shake flasks were resuspended in 50 mL of phosphate saline buffer containing 8.0 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.42 Na_2HPO_4, and 0.27 g L^{-1} KH_2PO_4 (pH 7.4) and unified in one sterile centrifugation tube. The pellet was resuspended after centrifugation (3260× *g*, 20 min) in the medium for biomass production. These cell suspensions were filled into sterile syringes (BD Discardit II, Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a sterile single-use canula (Sterican 0.8 × 120 mm, B. Braun, Melsungen, Germany) for the inoculation of the stirred tank bioreactor.

2.5. Production of Engineered *G. oxydans* BP9.1 pta-mGDH Cells

The biomass production was conducted in fully controlled 7.5 L stirred tank bioreactors (Labfors, Infors-HT, Bottmingen, Switzerland) with 4 L medium in batch processes of 24 h. The stirred tank bioreactor was equipped with probes for pH (405-DPAS-SC-K8S, Mettler-Toledo International Inc., Columbus, OH, USA) and for dissolved oxygen (DO) (InPro6820, Mettler-Toledo International Inc., Columbus, OH, USA). The pH was kept constant at pH 6.0 by controlled titration with 25% (*v*/*v*) NH_4OH. The gas phase (sterile air) was dispersed by three six-blade Rushton turbines assembled at the axis of the stirred tank bioreactor. The DO concentration was kept at >30% air saturation by increasing the stirrer speed from

500 to 800 rpm at a constant air flow of 2.0 vvm. The control script was provided by the control software (Iris 5.0, Infors-HT, Bottmingen, Switzerland). To compensate evaporation effects during the batch cultivations, the stirred tank bioreactor was equipped with an off-gas cooler.

A modified complex medium according to Buchert et al. [28] was used for biomass production with 50 g L^{-1} D-glucose, 3 g L^{-1} yeast extract, 5 g L^{-1} peptone from casein, 5 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4 \cdot \times$ 7 H_2O, 1 g L^{-1} KH_2PO_4, 1.5 g L^{-1} K_2HPO_4, and 0.1% (v/v) antifoam 204 (Sigma-Aldrich, Taufkirchen, Germany). The stirred-tank bioreactor was autoclaved with the medium without D-glucose, $MgSO_4 \cdot \times$ 7 H_2O, antifoam 204, and antibiotics at 121°C for 20 min. 250 g L^{-1} D-Glucose, 400 g L^{-1} $MgSO_4 \cdot \times$ 7 H_2O, and 10% (v/v) antifoam 204 were each sterilized separately and were added after autoclaving. Kanamycin and cefoxitin were added before inoculation to achieve concentrations of 50 mg L^{-1} kanamycin, and 2.5 mg L^{-1} cefoxitin, respectively, and the pH of the medium was titrated manually to pH 6.0 by adding 12.5% (v/v) H_3PO_4.

Batch processes were inoculated with the *G. oxydans* BP9.1 pta-mGDH cells (OD_{600} ~ 0.2). After reaching the stationary phase after 24 h, the cell suspension was harvested by centrifugation in four 1 L sterile centrifugation flasks (3260× *g*, 20 min). The pellets were then resuspended and washed with 50 mL of phosphate saline buffer (pH 7.4) and unified in one sterile tube. After another centrifugation step (3260× *g*, 20 min), cells were resuspended in biotransformation medium. These cell suspensions were filled into sterile syringes (BD Discardit II, Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a sterile single-use canula (Sterican 0.8 × 120 mm, B. Braun, Melsungen, Germany) for the inoculation of the stirred tank bioreactor.

2.6. Biotransformation in Stirred Tank Bioreactors

Biotransformation experiments were carried out in four fully controlled parallel 1 L stirred-tank bioreactors (DASGIP® Bioblock, DASGIP® reactor SR0700ODSS, Eppendorf AG, Hamburg, Germany) with resting cells of *G. oxydans* BP9.1 pta-mGDH. Temperature, pH, DO concentration, and exhaust gas concentrations of O_2 and CO_2 were measured online. Sterile air was dispersed with two six-blade Rushton turbines assembled at the axis of the stirred tank bioreactors. The stirred tank reactors were equipped with off-gas coolers to reduce evaporation of water. Biotransformations were performed with resting cells suspended in phosphate buffer with antifoam according to Burger et al. [24] with 1.0 g L^{-1} KH_2PO_4, 1.6 g L^{-1} Na_2HPO_4, 1.4 g L^{-1} $(NH_4)_2SO_4$, 0.2 g L^{-1} $MgSO_4 \cdot \times$ 7 H_2O, 0.1 g L^{-1} $CaCl_2$ × 2 H_2O, and 0.1% (v/v) antifoam 204. Varying concentrations of D-cellobiose and *G. oxydans* BP9.1 pta-mGDH cells were added.

Prior to autoclaving, the pH probes (405-DPAS-SC-K8S, Mettler-Toledo International Inc., Columbus, OH, USA) were calibrated at pH 4 and pH 7. The stirred tank bioreactors filled with aqueous cellobiose solutions were autoclaved at 121 °C for 20 min. After autoclaving, 70 mL of a 10-fold sterile stock solution of KH_2PO_4, Na_2HPO_4, and $(NH_4)_2SO_4$ was added to the medium. $MgSO_4 \cdot \times$ 7 H_2O, $CaCl_2$ × 2 H_2O, and antifoam 204 were also added from separately sterilized stock solutions with concentrations of 250 g L^{-1}, 250 g L^{-1}, and 10% (v/v), respectively. Finally, kanamycin was added to achieve a concentration of 50 mg L^{-1}. Prior to the addition of the *G. oxydans* BP9.1 pta-mGDH cells, the DO probes (VisiFerm DO ECS 225 H2, Hamilton AG, Bonaduz, Switzerland) and the channels of the exhaust gas analyzer (EasyLine, ABB, Zurich, Switzerland) were calibrated by stripping the bioreactor with air until reaching 100% air saturation as well as with nitrogen gas to reach 0% air saturation. Initially, the pH was set manually to pH 6.0 by adding 12.5% (v/v) H_3PO_4. During the biotransformation experiments, pH 6.0 was controlled automatically by adding 25% (v/v) NH_4OH. If possible, the DO concentration was kept above 30% air saturation by increasing the stirrer speed from 500 rpm to 1200 rpm, increasing the gassing rate from 2.0 vvm to 2.8 vvm air, and if necessary, applying the DO control function of the control software (Dasgip Control 4.0, Eppendorf AG, Hamburg, Germany). The temperature was

controlled to 30 °C. The initial reaction volume was 0.7 L after adding the *G. oxydans* BP9.1 pta-mGDH cells for the biotransformation studies.

2.7. Determination of Oxygen Uptake Rates

Oxygen uptake rates (OUR) were estimated by quantifying the concentrations of O_2 and CO_2 in the exhaust gas of the bioreactors. Assuming an equal in-gas and off-gas flow rate, OUR was calculated with Equation (1) with air flow rate F_{Air}, reaction volume V_R, molar volume of 22.414 L mol^{-1} (V_{mol}), inlet and outlet fraction of O_2 and CO_2 (y_{CO_2} and y_{O_2}) and factor θ (Equation (2)).

$$OUR = \frac{F_{Air}}{V_R \cdot V_{mol}} \cdot \left(y_{O_2}^{in} - \theta \cdot y_{O_2}^{out} \right), \quad (1)$$

$$\theta = \frac{1 - y_{CO_2}^{in} - y_{O_2}^{in}}{1 - y_{CO_2}^{out} - y_{O_2}^{in}}, \quad (2)$$

2.8. Offline Analytics

To estimate the cell dry weight (CDW) concentrations based on OD_{600} measurements during biomass production or in the biotransformation experiments, samples were taken from the stirred tank bioreactors. A single beam photometer (Genesys 10S UV–VIS, Thermo Scientific, Neuss, Germany) was applied for the measurement of the optical density (OD_{600}) at 600 nm in 1 cm single-use cuvettes. A linear correlation factor of 0.36 g L^{-1} was used to estimate CDW concentrations of growing cells. A correlation factor of 0.48 g L^{-1} was applied to samples from the biotransformation studies [24].

High-performance liquid chromatography (HPLC) analysis (LC-2030C Plus, Shimadzu Corp., Kyoto, Japan) was used to quantify the substrate and product concentrations. Samples from the biotransformation processes were centrifuged in 1.5 mL reaction tubes (14,500× *g*, 10 min, Espresso, Thermo Fisher Scientific Inc., Waltham, MA, USA) to separate the cells from the liquid phase. The supernatant was filtered to remove the remaining cells through a 0.22 µm pore size exclusion filter (Chromafil RC20/15 MS, Macherey-Nagel GmbH & Co.KG, Düren, Germany). Samples were stored in sterile reaction tubes at 4 °C before HPLC analysis. The HPLC was equipped with a refractive index (RI) detector (RID-20A, Shimadzu Corp., Kyoto, Japan) followed by an ultraviolet (UV) detector (SPD-20A/20AV, Shimadzu Corp., Kyoto, Japan). Additionally, 5 mM H_2SO_4 was used as mobile phase. The elution was isocratic with a constant flow rate of 0.6 mL min^{-1}. An anion-exchange column (Aminex HPX-87H, Bio-Rad Laboratories Inc., Hercules, CA, USA) was applied as stationary phase. The column temperature remained constant at 60 °C and the operating pressure was 43 bar. An injection volume of 10 µL was applied. The chromatograms were recorded within 30 min. Standards with 1–30 g L^{-1} cellobiose and cellobionic acid were prepared separately to gain reference measurements for calculating the concentrations in the samples. If necessary, samples were diluted with deionized and purified water. The anion exchange column was not able to separate cellobiose and cellobionic acid, resulting in the same retention time. Both substrate and product were detected with the RI detector. Cellobionic acid was solely detected with the UV detector. The corresponding peak area of cellobionic acid measured with the RI detector was estimated based on calibration data to calculate the cellobiose concentration.

2.9. Determination of Volumetric and Cell-Specific Product Formation Rates

Progress curves of product concentrations obtained in bio-oxidation experiments were fitted to Equation 3 with the product concentration c_P, the model parameter k, and the process time t using the SciPy package (Version 1.10.1) in a python environment (Version 3.10).

The function scipy.optimize.curve_fit was used for parameter fitting ($c_{P,max}$, k), which is based on the method of least squares.

$$c_P = c_{P,max} \cdot \left(1 - e^{-k \cdot t}\right) \quad (3)$$

To obtain volumetric product formation rates, Q_P, as a function of process time, the identified model parameters $c_{P,max}$ and k were used with the derivative of Equation (3):

$$Q_P = \frac{dc_P}{dt} = c_{P,max} \cdot k \cdot e^{-k \cdot t} \quad (4)$$

Initial cell-specific cellobionate formation rates q_P were then calculated by dividing the initial volumetric product formation Q_{P0} rate with the initial biocatalyst concentration c_{X0}.

$$q_P = \frac{Q_{P0}}{c_{X0}} \quad (5)$$

3. Results and Discussion

3.1. Production of Cellobionate with G. oxydans BP9.1 pta-mGDH

Figure 1 shows the results of the biotransformation of 98 g L^{-1} cellobiose with resting cells of *G. oxydans* BP9.1 pta-mGDH at an initial concentration of 2.6 g L^{-1} CDW. The cell concentration decreased slightly to 2.2 g L^{-1} within a process time of 50 h. The oxidation of cellobiose and the formation of CBA started immediately at the beginning of the biotransformation with a maximum of the OUR. The pK_a of cellobionic acid was 3.28 ± 0.35 [31]. Accordingly, CBA is mainly present as cellobionate in an aqueous medium at pH 6. The oxidation rate decreased rapidly within 15 h. Complete conversion of cellobiose was reached after ~21 h with a product yield $Y_{P/S}$ of >99% (mol/mol). The stirred tank bioreactor was able to control the DO concentration at 30% air saturation by increasing the agitation rate to 780 rpm at a constant gassing rate of 2.0 vvm. After a process time of 5.2 h, the stirrer speed reached the initial value of 500 rpm and the DO concentration increased until 95% air saturation at ~15 h. For the oxidation of 98 g L^{-1} cellobiose to cellobionic acid, a total amount of 143 mM O_2 was needed. Integrating the measured oxygen consumption in the biotransformation process up to a process time of 60 h, a total oxygen conversion of 175 mM O_2 was observed. This means that 18% of the consumed O_2 was not recovered in the product. Most probably, this O_2 fraction was consumed for maintenance metabolism by the resting *G. oxydans* cells.

The high product yield of >99% can be attributed to the multideletion strain *G. oxydans* BP9.1 pta-mGDH overexpressing solely the glucose dehydrogenase of *P. taetrolens*, which avoids any competing oxidation activities by the deleted native mDHs of *G. oxydans*. High product yields were already shown with other bio-oxidation examples based on deletion strains of *G. oxydans* [32–34].

3.2. Variation of the Initial Cellobiose Concentration

Figure 2 shows the progress curves of the cellobionate concentrations and the volumetric product formation rates (Q_P) at varying initial cellobiose concentrations with 1.4 g L^{-1} *G. oxydans* BP9.1 pta-mGDH. Increasing the initial cellobiose concentrations from 25 g L^{-1} to 290 g L^{-1} resulted in prolonged process times until the final product concentrations were achieved. The maximum soluble substrate concentration was 290 g L^{-1} cellobiose. With the exception of the highest initial cellobiose concentration, all other batch processes showed full cellobiose conversion and cellobionate production with $Y_{P/S}$ > 99% (mol/mol) within a process time of 50 h. Incomplete oxidation of cellobiose was solely observed at initial 290 g L^{-1} with the formation of 250 g L^{-1} cellobionate.

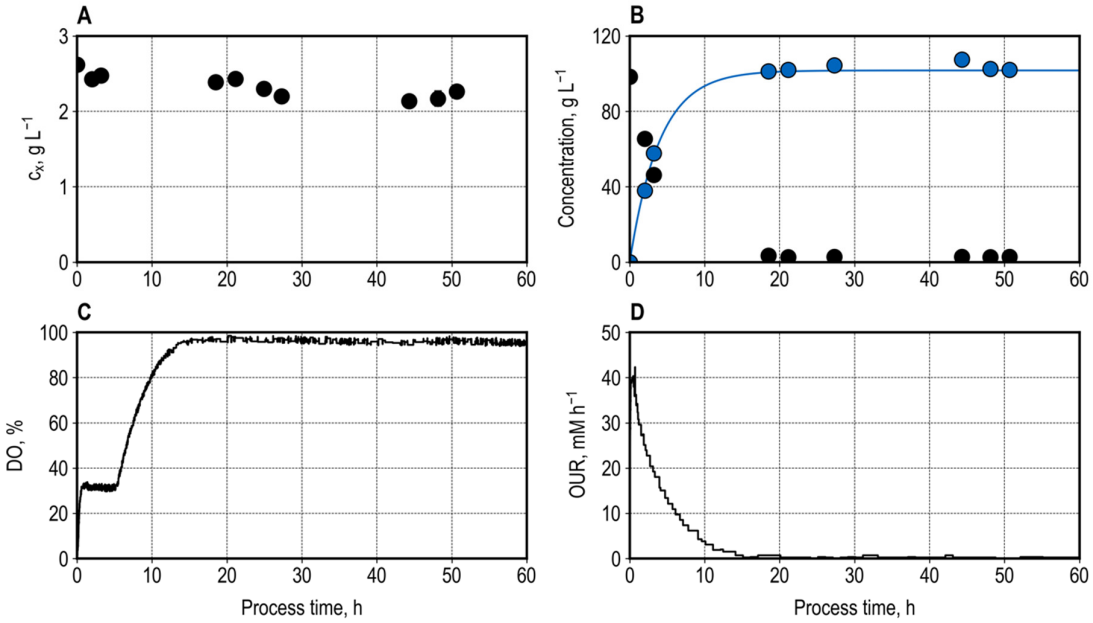

Figure 1. Biotransformation of 98 g L^{-1} cellobiose with resting cells of *G. oxydans* BP9.1 pta-mGDH for the production of CBA in a stirred tank bioreactor: (**A**) CDW concentration, (**B**) cellobiose (•) and cellobionate (•) concentrations, (**C**) DO concentration, and (**D**) OUR as function of process time (V_R = 0.7 L, pH 6.0, T = 30 °C, F_{air} = 2.0 vvm, DO > 30% air saturation by controlling the agitation rate n = 500–780 rpm).

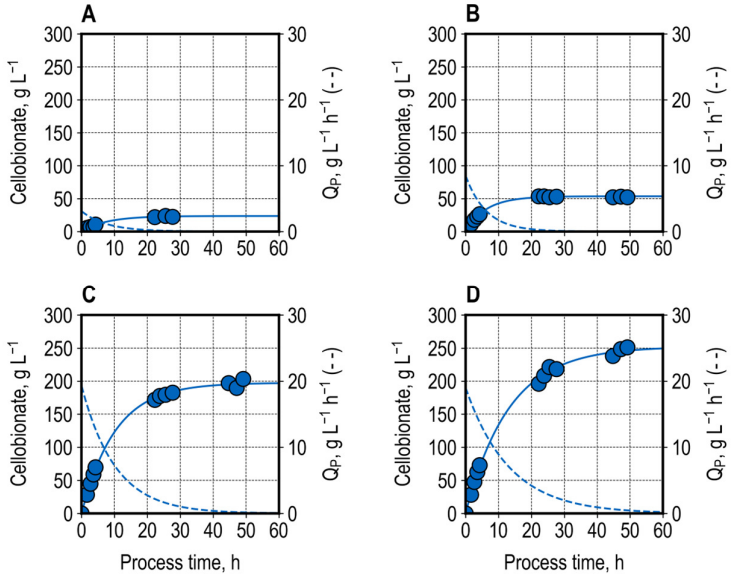

Figure 2. Biotransformations of cellobiose with resting cells of *G. oxydans* BP9.1 pta-mGDH for the production of CBA in stirred tank bioreactors at varying initial cellobiose concentrations: Cellobionate concentrations (•) with interpolated concentrations (solid line) and volumetric product formation rates (--): (**A**) 25 g L^{-1} cellobiose, (**B**) 50 g L^{-1} cellobiose, (**C**) 190 g L^{-1} cellobiose, and (**D**) 290 g L^{-1} cellobiose (c_{X0} = 1.4 g L^{-1}, pH 6.0, T = 30 °C, F_{air} = 2.0 vvm, V_R = 0.7 L, DO > 30% air saturation ensured by increasing agitation rate n = 500–1030 rpm).

The initial cell-specific cellobionate formation rates are shown in Figure 3 as a function of the initial cellobiose concentrations. Increasing initial cellobiose concentrations of c_{S0} = 25 g L^{-1}, 50 g L^{-1}, 98 g L^{-1}, 190 g L^{-1}, or 290 g L^{-1} resulted in increased initial product formation rates of 2.1 g g^{-1} h^{-1}, 4.9 g g^{-1} h^{-1}, 9.7 g g^{-1} h^{-1}, 12.3 g g^{-1} h^{-1}, and 12.6 g g^{-1} h^{-1}, respectively. The maximum initial cellobionate formation rate was

achieved with an initial cellobiose concentration of 190 g L^{-1}. A further increase in the initial substrate concentration up to the solubility limit (290 g L^{-1}) resulted in the same initial cellobionate formation rate within the estimation error. Substrate inhibition was not observed up to the solubility limit, as no decrease in the initial cellobionate formation rates at high initial substrate concentrations was measured. With the reaction conditions applied, 12.3–12.6 g cellobionate g^{-1} CDW h^{-1} is the maximum cellobionate formation rate of *G. oxydans* BP9.1 pta-mGDH. High initial cellobiose concentrations of up to 190 g L^{-1} are necessary to enable this maximum biomass specific cellobionate formation rate.

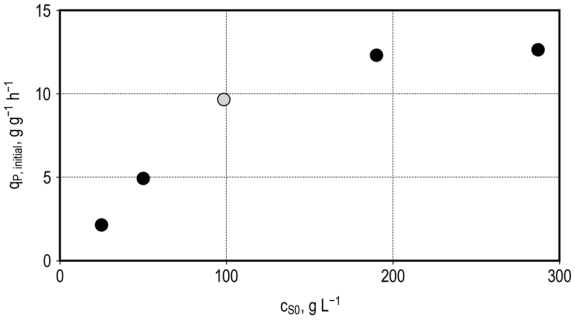

Figure 3. Initial biomass specific cellobionate formation rates as function of the initial cellobiose concentrations in biotransformations with resting cells of *G. oxydans* BP9.1 pta-mGDH in stirred tank bioreactors. The initial cell concentrations were 1.4 g L^{-1} (black) and 2.6 g L^{-1} (grey) (pH 6.0, T = 30 °C, F_{air} = 2.0 vvm, V_R = 0.7 L, DO > 30% air saturation ensured by increasing agitation rate *n* = 500–1030 rpm).

3.3. Variation of the Initial Concentrations of G. oxydans BP9.1 pta-mGDH

Higher initial *G. oxydans* BP9.1 pta-mGDH concentrations should enable shorter batch process times until full conversion of cellobiose. The initial biomass concentrations were varied between 0.5 and 2.9 g L^{-1}, applying 100 g L^{-1} cellobiose in bio-oxidation studies in stirred tank bioreactors (Figure 4). Oxygen limitation was not observed. At biocatalyst concentrations < 0.5 g L^{-1}, no complete conversion of cellobiose was observed within 48 h. With further increasing initial CDW concentrations, the volumetric cellobionate formation rates increased, and the process times until full conversion were shortened accordingly. The maximum volumetric productivity (space-time yield) of 9.8 g L^{-1} h^{-1} was measured with the highest CDW concentration (c_{X0} = 2.9 g L^{-1}) until full conversion was achieved. In all batch biotransformations, a selectivity or yield at full cellobiose conversion of $Y_{P/S}$ > 99% (mol/mol) was measured.

The maximum initial volumetric cellobionate formation rates are plotted against the initial concentrations of *G. oxydans* BP9.1 pta-mGDH in Figure 5A. As expected, the initial product formation rates increased linearly ($Q_{P,max}$ = 11.4 c_{X0}) with increasing CDW concentrations with a relative standard deviation of 3.4%.

Figure 5B shows the corresponding initial biomass-specific cellobionate formation rates as a function of the initial biomass concentrations. The biomass-specific cellobionate formation rates were constant independently of the biomass concentration (11.7 g g^{-1} h^{-1}, relative standard deviation of 6.6%). The reason for the significantly higher initial cellobionate formation rate of 21.0 g g^{-1} h^{-1} at the lowest biomass concentration (0.2 g L^{-1}) is unknown (this batch process was not reproduced due to the low conversion of cellobiose within 48 h).

As the oxidation of cellobiose needs oxygen, the maximum oxygen uptake rates (OUR_{max}) measured in the stirred tank bioreactor increased with increasing initial *G. oxydans* BP9.1 pta-mGDH concentrations (OUR_{max} = 19.1 c_{X0}), as shown in Figure 5C (relative standard deviation of 4.7%).

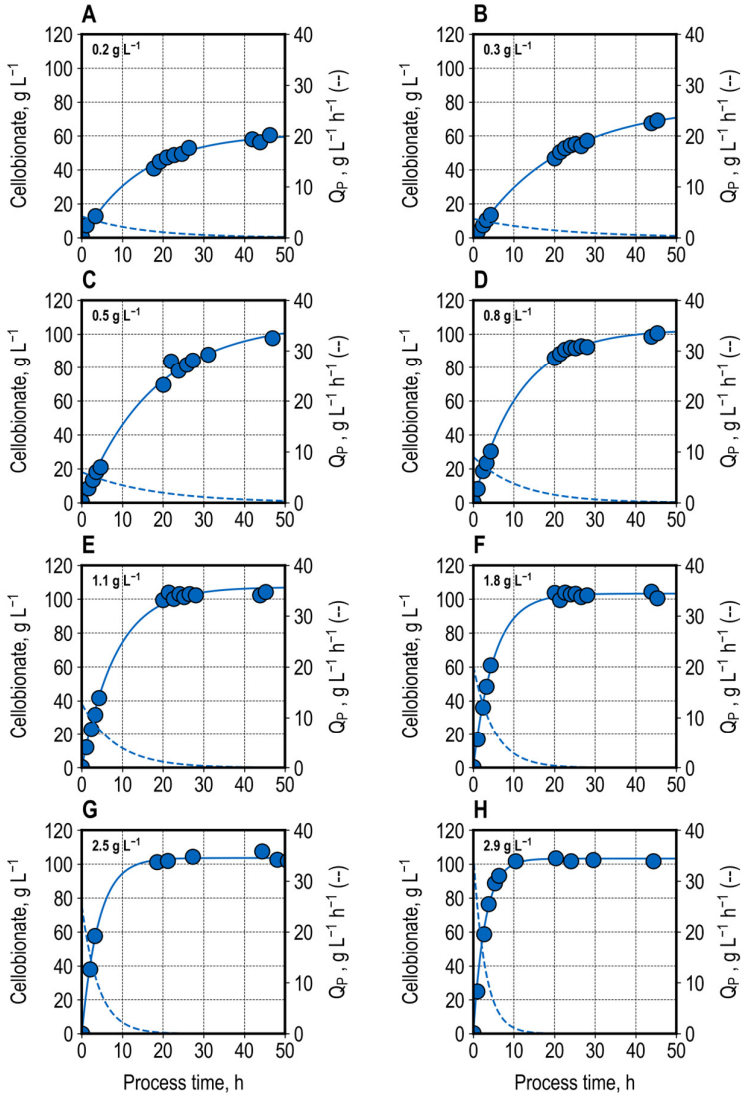

Figure 4. Biotransformations of cellobiose with resting cells of *G. oxydans* BP9.1 pta-mGDH for the production of CBA in stirred tank bioreactors with varying initial biomass concentrations (0.2–2.9 g L^{-1}) (**A**–**H**): Cellobionate concentrations (•) with interpolated concentrations (solid line) and volumetric product formation rates Q_P (--) (c_{S0} = 100 g L^{-1}, pH 6.0, T = 30 °C, F_{air} = 2.0 vvm, V_R = 0.7 L, DO > 30% air saturation ensured by increasing agitation rate *n* = 500–860 rpm).

The linear correlation of OUR_{max} with the initial concentration of *G. oxydans* BP9.1 pta-mGDH can be used to adapt the initial biomass concentration to the maximum oxygen transfer rate (OTR_{max}) of any bioreactor. The stirred tank bioreactor applied here enabled an OTR_{max} of 109 mM h^{-1} at the maximum agitation rate of 1200 rpm. This should be sufficient to supply enough oxygen to at least 5.4 g L^{-1} *G. oxydans* BP9.1 pta-mGDH cells if an initial cellobiose concentration of 100 g L^{-1} is applied in a batch bio-oxidation process. Increasing the initial cellobiose concentration to 190 g L^{-1} (solubility limit) and more will result in a 5.1% increase in the maximum specific cellobionate formation rate of the cells and thus in the OUR_{max} (Figure 3).

3.4. Providing Highly Concentrated Cellobionate

As shown before, the initial biomass-specific cellobionate formation rates of *G. oxydans* BP9.1 pta-mGDH were at maximum at cellobiose concentrations up to the solubility limit (290 g L^{-1}). Increasing the cellobiose concentration in batch processes will keep the solved cellobiose concentration at the solubility limit as long as there are cellobiose solids in the

bioreactor. The initial biomass concentration of *G. oxydans* BP9.1 pta-mGDH is limited by the OTR_{max} of the stirred tank bioreactor (109 mM h^{-1}). The maximum biomass-specific cellobionate formation rate of 12.6 g g^{-1} h^{-1} at 290 g L^{-1} cellobiose solved in the bioreactor (Figure 3) and the corresponding OUR_{max} (see above) results in an initial 5.2 g L^{-1} of CDW. Thus, two batch bio-oxidation processes were performed with 5.2 g L^{-1} *G. oxydans* BP9.1 pta-mGDH, and 500 g L^{-1} cellobiose in the stirred tank bioreactor (Figure 6).

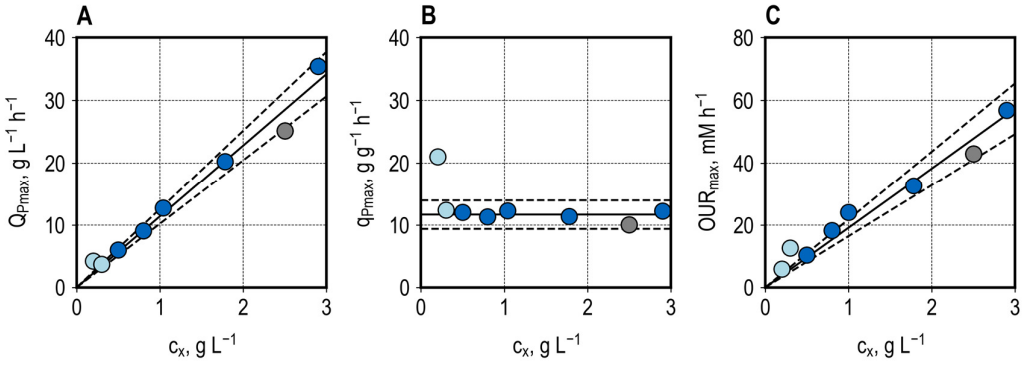

Figure 5. Maximal initial volumetric (**A**) and biomass-specific (**B**) cellobionate formation rates, and maximal oxygen uptake rates (**C**) in biotransformations with resting cells of *G. oxydans* BP9.1 pta-mGDH in stirred tank bioreactors. Obtained results of the batch processes shown in Figure 4 are indicated in dark blue. The results of the batch processes without full conversion of cellobiose are indicated in light blue. Product formation rates derived from Figure 3 are indicated in grey. The dashed lines show the range (+/−) of three standard deviations (c_{S0} = 100 g L^{-1}, pH 6.0, T = 30 °C, F_{air} = 2.0 vvm, V_R = 0.7 L, DO > 30% air saturation ensured by increasing agitation rate n = 500–860 rpm).

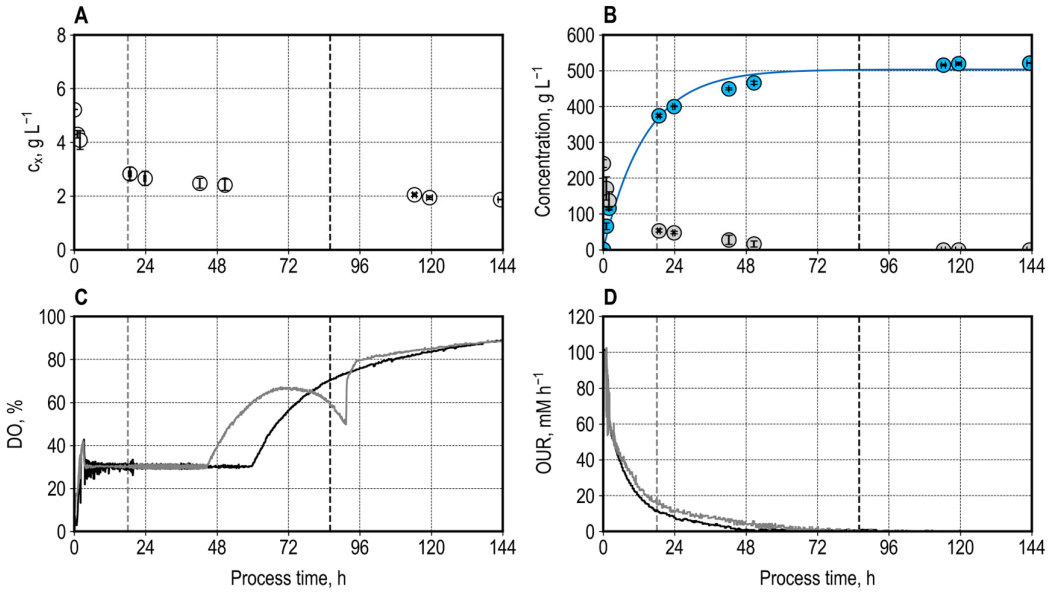

Figure 6. Biotransformation of 500 g L^{-1} cellobiose with resting cells of *G. oxydans* BP9.1 pta-mGDH for the production of CBA in a stirred tank bioreactor: (**A**) CDW concentration, (**B**) cellobiose (grey) and cellobionate (blue) concentrations, (**C**) DO concentration, and (**D**) OUR as function of process time. The batch process was reproduced once, min–max values are shown in (**A**,**B**), and both processes are shown in black and grey in (**C**,**D**). The grey dashed line indicates the total dissolution of the cellobiose particles. The black dashed line shows the estimated full conversion of cellobiose to cellobionate (c_{S0} = 500 g L^{-1}, pH 6.0, T = 30 °C, V_R = 0.7 L, DO > 30% air saturation ensured by increasing agitation rate n = 500–1200 rpm and air flow rate F_{air} = 2.0–2.8 vvm).

The biomass concentration rapidly decreased to 4.1 g L^{-1} in the first two hours (Figure 6A). This may be caused by the delayed reaction of the DO controller of the stirred tank bioreactor resulting in DO concentrations clearly below 30% air saturation in the first hours of the bio-oxidation process (Figure 6C). The following linear decrease in the concentrations of *G. oxydans* BP9.1 pta-mGDH has already been observed before (e.g., Figure 1A). DO was kept constant at 30% air saturation until a process time of 44 h or 60 h, before DO started to increase due to the reduced bio-oxidation activities of the cells. The difference in the DO-signals can be explained by a failure of the DO probe in one of the batch processes, which was recognized during the process and solved at about 90 h (rapid increase in the DO concentration, grey line in Figure 6C).

The cellobiose concentration in the stirred tank bioreactor was always below the solubility limit. Initially, a maximum cellobiose concentration of 240 ± 11 g L^{-1} was measured followed by a rapid decrease. Complete dissolution of the cellobiose particles in the bioreactor was observed after ~18 h. Total conversion of cellobiose was estimated to be achieved after ~86 h according to the interpolated cellobionate concentrations (Figure 6B). Finally, 501.9 ± 3.7 g L^{-1} cellobionate was produced with a space–time yield of 5.84 ± 0.04 g L^{-1} h^{-1} at a harvest time of 86 h with a yield of Y_{PS} > 99% (mol/mol).

The initial OUR was 103 ± 2.7 mM h^{-1}, approaching the OTR_{max} of the stirred tank bioreactor of 109 mM h^{-1}. As with the other bio-oxidation processes, the OUR decreased until zero after ~86 h, when cellobiose was fully oxidized to cellobionate.

One explanation for the rapid decrease in cell density in the first two hours could be the high sensitivity of *G. oxydans* to oxygen limitation. As the energy conservation of *G. oxydans* is connected to substrate oxidation by using oxygen as terminal electron acceptor, a low concentration of oxygen could be harmful to the cells and could cause cell lysis [35,36].

The final cellobionate concentration of 501.9 g L^{-1} achieved with this simple batch process exceeds the so-far-reported maximum cellobionate concentration (200 g L^{-1}) by 251% [11,37]. *G. oxydans* is known for its tolerance to high osmotic pressure. This could explain the increased cellobionate concentration compared to the production of cellobionic acid with *Pseudomonas taetrolens*. The significantly higher product concentrations could lead to drastic reduction in the costs in downstream processing, which is the biggest advantage in the production of CBA with the *G. oxydans* multideletion strain expressing a mGDH of *P. taetrolens*. Compared to the reported concentrations of maltobionic acid, a stereoisomer to CBA, the final product concentrations (204–232 g L^{-1}) were improved by 216–246% [38,39]. Although final product concentrations and biomass-specific production rates are quite high, the costs in the production of *G. oxydans* in batch processes prior to the biotransformation would be a drawback. In preliminary tests growth-associated biotransformations to produce CBA resulted in significantly lower productivities. Anyway, the local and temporal separation of production of biocatalysts and biotransformation results in high flexibility for the potential industrial use of this process. However, one solution to reduce the costs for the biocatalyst could be biomass recycling in repeated batch biotransformations. This could increase the space-time yields of batch processes and thereby reduce the costs of the whole process. The ability of the cells to perform repeated batch biotransformations is currently under study.

4. Conclusions

The multideletion strain *G. oxydans* BP9.1, overexpressing a membrane-bound glucose dehydrogenase from *P. taetrolens*, enabled stoichiometric CBA selectivities and yields at full conversion of >99% (mol/mol) in aerobic whole-cell bio-oxidations of cellobiose with resting cells. The highest biomass-specific cellobionate formation rates were measured at initial cellobiose concentrations of above 190 g L^{-1} cellobiose and remained constant up to the solubility limit. Thus, simple batch processes are beneficial for the bio-oxidation of cellobiose with the engineered *G. oxydans* strain, as the substrate concentrations remain high for a prolonged process time, especially if cellobiose is added above the solubility limit. The high cell-specific bio-oxidation capacity of the engineered *G. oxydans* cells in combination

with the possibility to adapt the initial biomass concentration to the maximum oxygen transfer rate (OTR_{max}) of the bioreactor reduces the costs for the biocatalyst, which is usually one of the major cost factors in biotransformations. The resulting high cellobionate concentrations of >500 g L^{-1} in combination with full cellobiose conversion, which can easily be achieved in simple batch processes with resting cells in an aqueous buffer, will reduce downstream processing costs considerably.

Author Contributions: Conceptualization, E.B. and D.W.-B. (biochemical engineering), A.E. and W.L. (strain design and genetic engineering); methodology and investigation, E.B. and A.Z. (CBA production); E.B. and M.d.S.G. (varying biocatalyst concentration); E.B. and U.Y. (varying substrate concentration); L.P. (microbiology); writing—original draft preparation, E.B., D.W.-B. and L.P. (engineering of *G. oxydans*); writing—review and editing, D.W.-B., A.E. and W.L.; visualization, E.B.; supervision, project administration, and funding acquisition, A.E., W.L. and D.W.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by German Federal Ministry of Food and Agriculture (BMEL), research project PolyBioOx (2220NR129A and 2220NR129B).

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

Acknowledgments: The support of Emmeran Bieringer, Lisa Pütthoff, and Arne Zimmermann by the TUM Graduate School (Technical University of Munich, Germany) is acknowledged.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

References

1. Bieringer, E.; García Vázquez, U.; Klein, L.; Moretó Bravo, N.; Tobler, M.; Weuster-Botz, D. Bioproduction and Applications of Aldobionic Acids with a Focus on Maltobionic and Cellobionic Acid. *Bioprocess. Biosyst. Eng.* **2023**, *46*, 921–940. [CrossRef] [PubMed]
2. Mehtiö, T.; Toivari, M.; Wiebe, M.G.; Harlin, A.; Penttilä, M.; Koivula, A. Production and Applications of Carbohydrate-Derived Sugar Acids as Generic Biobased Chemicals. *Crit. Rev. Biotechnol.* **2016**, *36*, 904–916. [CrossRef] [PubMed]
3. Yu, R.J.; Van Scott, E.J. Alpha-Hydroxyacids and Carboxylic Acids. *J. Cosmet. Dermat.* **2004**, *3*, 76–87. [CrossRef]
4. Oh, Y.-R.; Jang, Y.-A.; Lee, S.S.; Kim, J.-H.; Hong, S.H.; Han, J.J.; Eom, G.T. Enhancement of Lactobionic Acid Productivity by Homologous Expression of Quinoprotein Glucose Dehydrogenase in *Pseudomonas Taetrolens*. *J. Agric. Food Chem.* **2020**, *68*, 12336–12344. [CrossRef] [PubMed]
5. Sarenkova, I.; Ciprovica, I. The Current Status and Future Perspectives of Lactobionic Acid Production: A Review. In Proceedings of the Annual 24th ISC Research for Rural Development, Jelgava, Latvia, 16–18 May 2018; Volume 1, pp. 233–239.
6. Cardoso, T.; Marques, C.; Dagostin, J.L.A.; Masson, M.L. Lactobionic Acid as a Potential Food Ingredient: Recent Studies and Applications. *J. Food Sci.* **2019**, *84*, 1672–1681. [CrossRef]
7. Alonso, S.; Rendueles, M.; Díaz, M. Bio-Production of Lactobionic Acid: Current Status, Applications and Future Prospects. *Biotechnol. Adv.* **2013**, *31*, 1275–1291. [CrossRef]
8. Enteshari, M.; Martínez-Monteagudo, S.I. One-Pot Synthesis of Lactose Derivatives from Whey Permeate. *Foods* **2020**, *9*, 784. [CrossRef]
9. Mäki-Arvela, P.; Tokarev, A.V.; Murzina, E.V.; Campo, B.; Heikkilä, T.; Brozinski, J.-M.; Wolf, D.; Murzin, D.Y. Kinetics of Lactose and Rhamnose Oxidation over Supported Metal Catalysts. *Phys. Chem. Chem. Phys.* **2011**, *13*, 9268–9280. [CrossRef] [PubMed]
10. Oh, Y.-R.; Jang, Y.-A.; Hong, S.H.; Eom, G.T. High-Level Production of Maltobionic Acid from High-Maltose Corn Syrup by Genetically Engineered *Pseudomonas Taetrolens*. *Biotechnol. Rep.* **2020**, *28*, e00558. [CrossRef]
11. Oh, Y.-R.; Song, J.K.; Eom, G.T. Efficient Production of Cellobionic Acid Using Whole-Cell Biocatalyst of Genetically Modified *Pseudomonas Taetrolens*. *Bioprocess. Biosyst. Eng.* **2022**, *45*, 1057–1064. [CrossRef]
12. Hildebrand, A.; Szewczyk, E.; Lin, H.; Kasuga, T.; Fan, Z. Engineering *Neurospora Crassa* for Improved Cellobiose and Cellobionate Production. *Appl. Environ. Microbiol.* **2015**, *81*, 597–603. [CrossRef] [PubMed]
13. Kiryu, T.; Kiso, T.; Nakano, H.; Murakami, H. Lactobionic and Cellobionic Acid Production Profiles of the Resting Cells of Acetic Acid Bacteria. *Biosci. Biotechnol. Biochem.* **2015**, *79*, 1712–1718. [CrossRef] [PubMed]
14. Hildebrand, A.; Kasuga, T.; Fan, Z. Production of Cellobionate from Cellulose Using an Engineered *Neurospora Crassa* Strain with Laccase and Redox Mediator Addition. *PLoS ONE* **2015**, *10*, e0123006. [CrossRef] [PubMed]
15. Zhang, R.; Xu, C.; Shen, Q.; Kasuga, T.; Wu, W.; Szewczyk, E.; Ma, D.; Fan, Z. Characterization of Two Cellobiose Dehydrogenases and Comparison of Their Contributions to Total Activity in *Neurospora Crassa*. *Int. Biodeterior. Biodegrad.* **2013**, *82*, 24–32. [CrossRef]

16. Wu, W.; Hildebrand, A.; Kasuga, T.; Xiong, X.; Fan, Z. Direct Cellobiose Production from Cellulose Using Sextuple *Beta*-Glucosidase Gene Deletion *Neurospora Crassa* Mutants. *Enzym. Microb. Technol.* **2013**, *52*, 184–189. [CrossRef] [PubMed]
17. Lin, H.; Hildebrand, A.; Kasuga, T.; Fan, Z. Engineering *Neurospora Crassa* for Cellobionate Production Directly from Cellulose without Any Enzyme Addition. *Enzym. Microb. Technol.* **2017**, *99*, 25–31. [CrossRef] [PubMed]
18. Zhou, M.; Tao, L.; Russell, P.; Britt, R.D.; Kasuga, T.; Lü, X.; Fan, Z. The Role of Lignin in the Conversion of Wheat Straw to Cellobionic Acid by *Neurospora Crassa* HL. *Ind. Crops Prod.* **2022**, *188*, 115650. [CrossRef]
19. Yoo, Y.; Oh, Y.-R.; Eom, G.T. Valorization of Cellulose in Waste Paper into Value-Added Cellobionic Acid by Genetically Engineered *Pseudomonas Taetrolens*. *Ind. Crops Prod.* **2022**, *186*, 115186. [CrossRef]
20. Peters, B.; Mientus, M.; Kostner, D.; Junker, A.; Liebl, W.; Ehrenreich, A. Characterization of Membrane-Bound Dehydrogenases from *Gluconobacter oxydans* 621H via Whole-Cell Activity Assays Using Multideletion Strains. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 6397–6412. [CrossRef] [PubMed]
21. Bories, A.; Claret, C.; Soucaille, P. Kinetic Study and Optimisation of the Production of Dihydroxyacetone from Glycerol Using *Gluconobacter oxydans*. *Process Biochem.* **1991**, *26*, 243–248. [CrossRef]
22. Hekmat, D.; Bauer, R.; Fricke, J. Optimization of the Microbial Synthesis of Dihydroxyacetone from Glycerol with *Gluconobacter oxydans*. *Bioprocess. Biosyst. Eng.* **2003**, *26*, 109–116. [CrossRef] [PubMed]
23. La China, S.; Zanichelli, G.; De Vero, L.; Gullo, M. Oxidative Fermentations and Exopolysaccharides Production by Acetic Acid Bacteria: A Mini Review. *Biotechnol. Lett.* **2018**, *40*, 1289–1302. [CrossRef] [PubMed]
24. Burger, C.; Kessler, C.; Gruber, S.; Ehrenreich, A.; Liebl, W.; Weuster-Botz, D. L-Erythrulose Production with a Multideletion Strain of *Gluconobacter oxydans*. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 4393–4404. [CrossRef] [PubMed]
25. Reichstein, T.; Grüssner, A. Eine Ergiebige Synthese Der L-Ascorbinsäure (C-Vitamin). *Helv. Chim. Acta* **1934**, *17*, 311–328. [CrossRef]
26. Pappenberger, G.; Hohmann, H.-P. Industrial Production of L-Ascorbic Acid (Vitamin C) and d-Isoascorbic Acid. In *Biotechnology of Food and Feed Additives*; Zorn, H., Czermak, P., Eds.; Springer: Berlin/Heidelberg, Germany, 2014; pp. 143–188. ISBN 978-3-662-43761-2.
27. Green, M.R.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*, 4th ed.; Cold Spring Harbor Laboratory Press: New York, NY, USA, 2012; ISBN 978-1-936113-41-5.
28. Mientus, M.; Kostner, D.; Peters, B.; Liebl, W.; Ehrenreich, A. Characterization of Membrane-Bound Dehydrogenases of *Gluconobacter oxydans* 621H Using a New System for Their Functional Expression. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 3189–3200. [CrossRef] [PubMed]
29. Zhang, Y.; Werling, U.; Edelmann, W. SLiCE: A Novel Bacterial Cell Extract-Based DNA Cloning Method. *Nucleic Acids Res.* **2012**, *40*, e55. [CrossRef] [PubMed]
30. Kostner, D.; Peters, B.; Mientus, M.; Liebl, W.; Ehrenreich, A. Importance of codB for New codA-Based Markerless Gene Deletion in *Gluconobacter* Strains. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 8341–8349. [CrossRef]
31. Cellobionic Acid | 534-41-8. Available online: https://www.chemicalbook.com/ChemicalProductProperty_EN_CB51365060.htm (accessed on 9 July 2024).
32. Ano, Y.; Shinagawa, E.; Adachi, O.; Toyama, H.; Yakushi, T.; Matsushita, K. Selective, High Conversion of D-Glucose to 5-Keto-D-Gluoconate by *Gluconobacter Suboxydans*. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 586–589. [CrossRef] [PubMed]
33. Merfort, M.; Herrmann, U.; Bringer-Meyer, S.; Sahm, H. High-Yield 5-Keto-d-Gluconic Acid Formation Is Mediated by Soluble and Membrane-Bound Gluconate-5-Dehydrogenases of *Gluconobacter oxydans*. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 443–451. [CrossRef] [PubMed]
34. Elfari, M.; Ha, S.-W.; Bremus, C.; Merfort, M.; Khodaverdi, V.; Herrmann, U.; Sahm, H.; Görisch, H. A *Gluconobacter oxydans* Mutant Converting Glucose Almost Quantitatively to 5-Keto-d-Gluconic Acid. *Appl. Microbiol. Biotechnol.* **2005**, *66*, 668–674. [CrossRef] [PubMed]
35. Prust, C.; Hoffmeister, M.; Liesegang, H.; Wiezer, A.; Fricke, W.F.; Ehrenreich, A.; Gottschalk, G.; Deppenmeier, U. Complete Genome Sequence of the Acetic Acid Bacterium *Gluconobacter oxydans*. *Nat. Biotechnol.* **2005**, *23*, 195–200. [CrossRef] [PubMed]
36. Deppenmeier, U.; Ehrenreich, A. Physiology of Acetic Acid Bacteria in Light of the Genome Sequence of *Gluconobacter oxydans*. *Microb. Physiol.* **2009**, *16*, 69–80. [CrossRef] [PubMed]
37. Oh, Y.-R.; Eom, G.T. Efficient Production of Cellobionic Acid from Cellobiose by Genetically Modified *Pseudomonas Taetrolens*. *Biochem. Eng. J.* **2022**, *178*, 108282. [CrossRef]
38. Malvessi, E.; Carra, S.; Pasquali, F.C.; Kern, D.B.; da Silveira, M.M.; Ayub, M.A.Z. Production of Organic Acids by Periplasmic Enzymes Present in Free and Immobilized Cells of *Zymomonas Mobilis*. *J. Ind. Microbiol. Biotechnol.* **2012**, *40*, 1–10. [CrossRef] [PubMed]
39. de Souza, R.C.; da Silva, L.M.; Carra, S.; Flores, M.; Puton, B.M.; Malvessi, E.; Valduga, E.; Zeni, J. High-Sodium Maltobionate Production by Immobilized *Zymomonas Mobilis* Cells in Polyurethane. *Bioprocess. Biosyst. Eng.* **2022**, *45*, 1465–1476. [CrossRef] [PubMed]