A two-stage biological gas to liquid transfer process to convert carbon dioxide into bioplastic

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ABSTRACT

The fermentation of carbon dioxide (CO_2) with hydrogen (H_2) uses available low-cost gases to synthesis acetic acid. Here, we present a two-stage *bio*logical process that allows the *gas to l*iquid transfer (Bio-GTL) of CO_2 into the biopolymer polyhydroxybutyrate (PHB). Using the same medium in both stages, first, acetic acid is produced (3.2 g L^{-1}) by *Acetobacterium woodii* from 5.2 L gas-mixture of CO_2:H_2 (15:85 *v/v*) under elevated pressure (≥2.0 bar) to increase H_2-solubility in water. Second, acetic acid is converted to PHB (3 g L^{-1} acetate into 0.5 g L^{-1} PHB) by *Ralstonia eutropha* H16. The efficiencies and space-time yields were evaluated, and our data show the conversion of CO_2 into PHB with a 33.3% microbial cell content (percentage of the ratio of PHB concentration to cell concentration) after 217 h. Collectively, our results provide a resourceful platform for future optimization and commercialization of a Bio-GTL for PHB production.

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1. Introduction

Carbon dioxide (CO_2) is an abundant, nontoxic, recyclable and relatively pure by-product of many industrial processes, making it a cheap and attractive precursor for industrial applications (Alper and Yuksel Orhan, 2017). The bioprocessing of CO_2 through microbial fermentation with the use of hydrogen (H_2) as an energy source is a promising approach to produce acetic acid in a sustainable and environmentally-friendly way (Hu et al., 2016), because H_2 can be produced at low cost from the gasification of renewable and sustainable resources (such as biomass and domestic and agricultural waste) (De Tissera et al., 2017; Hu et al., 2016). This process can be performed by acetogens, a class of anaerobic microorganisms that synthesize acetic acid and cell carbon from CO_2 and H_2 using the Wood–Ljungdahl pathway (WLP) (Supplementary information, Fig. S1) (Hawkins et al., 2013).

Compared to conventional chemical methods, biological acetic acid production has several advantages: a) it uses abundant low-cost gases such as CO_2 and H_2, b) it operates at low processing temperatures that offer significant energy and cost savings compared to alternative thermo-chemical approaches (e.g., the Fischer-Tropsch process, FTP) (Liew et al., 2013; Mohammadi et al., 2011; Munasinghe and Khanal, 2010), c) it profits from the high specificity of the enzymes involved

in the microbial pathway that lead to improved yields of acetate production (Liew et al., 2013), and d) it avoids cost intensive and toxic reaction steps (Fogler, 2010; Stephanopoulos et al., 1998).

Acetic acid has a broad spectrum of applications as a solvent and as a key raw material for many products, including polymers, paints, adhesives, paper coatings, and textile treatment (Cheung et al., 2011). These applications result in a global acetic acid demand of 6.5 million metric tons per year (Mt a^{-1}), of which only about 1.5 Mt. a^{-1} come from recycled sources (Cheung et al., 2011). Indeed, the efficient recovery of acetic acid remains challenging and has received considerable attention recently (Blanch, 2009; Jipa et al., 2009; Kertes and King, 2009).

To circumvent the cost-intensive extraction of acetic acid from liquid media, a two-stage *bio*logical process that transfers *gas to l*iquid (Bio-GTL) can be used (Li et al., 2012). This process is a promising technology that combines two microbial production steps. First, the microbial conversion of gaseous substrates (e.g., CO_2 and H_2) into an intermediate product (e.g., acetic acid). In the second step this intermediate is biologically converted to bioproducts of higher value (Hu et al., 2016; Lagoa-Costa et al., 2017). One of the most interesting end products of Bio-GTL is polyhydroxybutyrate (PHB), a biodegradable biopolymer (Kulpreecha et al., 2009) that can be used in many applications, including packaging, biomedicine, and agriculture (Rehm, 2006). However, the production costs of conventional chemical PHB are high because of the use of expensive carbon sources (Kulpreecha et al., 2009). Bio-GTL could provide a cheaper and more sustainable process for the PHB

production, by using CO_2 and H_2 with acetate as an intermediate compound.

The use of Bio-GTL to produce PHB from CO_2 was only reported once by a recent paper (Lagoa-Costa et al., 2017). In that study, in the first stage, the anaerobic syngas fermentation was carried out by *Clostridium autoethanogenum* to produce ethanol and acetic acid, followed by a second aerobic stage that consequentially converts the produced acetic acid into PHB with a mixed microbial culture.

Here, we present a two-stage Bio-GTL to convert CO_2 into PHB using H_2 as an energy source. We focus on using two metabolically different strains. Stage one uses the acetogen *A. woodii* to convert CO_2 into acetic acid. In stage two, *R. eutropha* H16 converts acetate into PHB. Compared to the method by Lagoa-Costa et al. our approach has two advantages: (i) the increase in gas-to-liquid mass transfer by applying high pressure conditions without excessive gas consumption and (ii) the reduction of the experimental time. To increase the gas-to-liquid mass transfer for CO_2 capture during stage one, we use a high-pressure stirred-tank reactor at 2.0 and 5.5 bar pressure and monitor the production of acetic acid. In the second stage, we screen for the maximum growth and acetic acid uptake in parallel bioreactor fermentations on a milliliter-scale setup, under a wide range of acetic acid concentrations and pH values. We then integrate the kinetics (production of acetic acid and conversion rates of acetate into PHB) and thermodynamics (energy efficiencies), as well as the conditions with highest energy efficiency into a Bio-GTL using one optimized medium for both microorganisms (Fig. 1). Our results provide the foundation for a novel approach for a sustainable bioplastics production from CO_2.

2. Materials and methods

2.1. Microorganisms

A. woodii (DSM 1030) and *R. eutropha* H16 (DSM 428) strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Fresh cultures were prepared from aliquots and stored at −80 °C.

2.2. Media and culturing

Anaerobic pre-cultures of *A. woodii* were grown heterotrophically to an early stationary phase at 30 °C, 160 rpm in shaking flasks (Incu-Shaker Mini™, Benchmark, New Jersey, USA) placed in a N_2 glovebox (InerTec AG, Grenchen, Switzerland) in medium 'A1' consisting of NH_4Cl 1.0 g L^{-1}; KH_2PO_4 0.33 g L^{-1}; K_2HPO_4 0.45 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.16 g L^{-1}; yeast extract 2.0 g L^{-1} (Sigma-Aldrich, Missouri, USA); $NaHCO_3$ 10 g L^{-1}; cysteine-HCl·H_2O 0.5 g L^{-1}; $Na_2S \cdot 9H_2O$ 0.5 g L^{-1}; nitrilotriacetic acid 30 mg L^{-1}; $MnSO_4 \cdot H_2O$ 10 mg L^{-1}; NaCl 20 mg L^{-1}; $FeSO_4 \cdot 7H_2O$ 2.0 mg L^{-1}; $CoSO_4 \cdot 7H_2O$ 3.6 mg L^{-1}; $CaCl_2 \cdot 2H_2O$ 2.0 mg L^{-1}; $ZnSO_4 \cdot 7H_2O$ 3.6 mg L^{-1}; $CuSO_4 \cdot 5H_2O$ 0.2 mg L^{-1}; $KAl(SO_4)_2 \cdot 12H_2O$ 0.4 mg L^{-1}; H_3BO_3 0.2 mg L^{-1}; $Na_2MoO_4 \cdot 2H_2O$ 0.2 mg L^{-1}; $NiCl_2 \cdot 6H_2O$ 0.5 mg L^{-1}; $Na_2SeO_3 \cdot 5H_2O$ 6×10^{-3} mg L^{-1}; biotin 4×10^{-5} mg L^{-1}; folic acid 4×10^{-5} mg L^{-1}; pyridoxine-HCl 0.2 mg L^{-1}; thiamine-HCl·$2H_2O$ 0.1 mg L^{-1}; riboflavin 0.1 mg L^{-1}; nicotinic acid 0.1 mg L^{-1}; D-Ca-pantothenate 0.1 mg L^{-1}; cyanocobalamine 2×10^{-6} mg L^{-1}; p-aminobenzoic acid 0.1 mg L^{-1}; lipoic acid 0.1 mg L^{-1}; fructose 10 g L^{-1}. The initial pH was 7.0 and the inoculum was 1 mL of frozen cells (cryo stock, prepared by 0.9 mL early stationary phase cultures reserved in 0.1 mL DMSO) in 200 mL medium 'A1'. Batch processes for autotrophic fermentation of *A. woodii* were performed in a high-pressure reactor using 1 L medium 'A2' (initial pH 7.0) in which the concentrations of yeast extract, vitamins and trace elements were doubled to avoid growth limitation and the $NaHCO_3$ concentration was reduced from 10 g L^{-1} to 5 g L^{-1} as mentioned in the literature (Demler and Weuster-Botz, 2011; Kantzow et al., 2015). Later, medium 'A2' was modified to 1 L medium 'A3' by increasing the yeast extract concentration from 4 g L^{-1} to 6 g L^{-1}, which was used for autotrophic fermentation with an initial pH of 7.0. The inoculum for medium 'A2' and 'A3' were harvested from 200 mL pre-

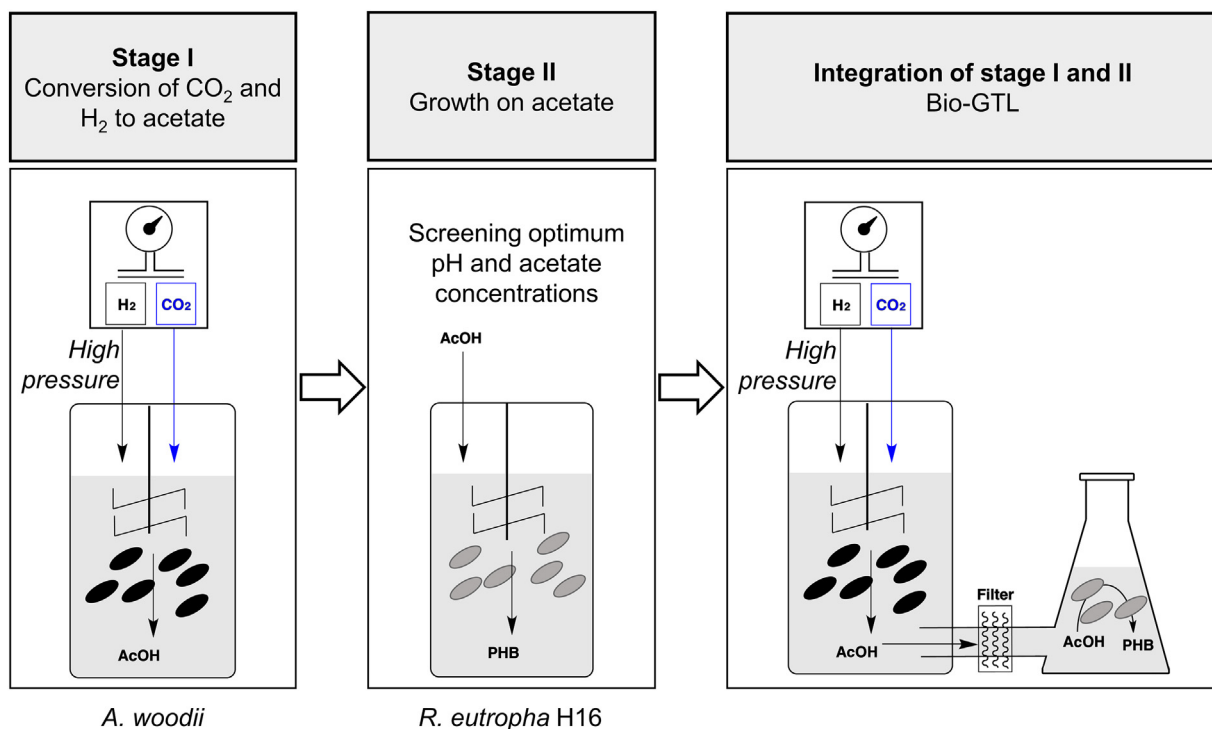**Fig. 1.** Schematic overview of the experimental procedures. In stage I: the fermentation of the gas mixture $CO_2:H_2$ was done using *A. woodii* in a high-pressure stirred-tank reactor to increase the gas-to-liquid mass transfer. Stage II: Screening the effect of different concentrations of acetic acid on growth and acetate uptake in *R. eutropha* in a milliliter-scale setup while varying pH to determine toxic levels of acetate. Integrating of the kinetics and thermodynamics, and the conditions with highest energy efficiency into a Bio-GTL using one optimized medium for both microorganisms.

cultures in early stationary phase by centrifugation (Eppendorf centrifuge 5430 R, Eppendorf, Hamburg, Germany; 4500 ×g, 10 min, 4 °C). The pellet was dissolved in 10 mL of medium 'A2'. The inoculum was anaerobically transferred with a syringe into the reactor. To increase the gas-to-liquid mass transfer for CO_2 capture, 2.0 bar and 5.5 bar absolute pressure were applied on medium 'A2'. Medium 'A3' was applied under 5.5 bar only. The metabolic products were identified, quantified (by HPLC and GC–MS, as described later) and the energy efficiency of CO_2 conversion to acetic acid was calculated. Pre-cultures of *R. eutropha* H16 were grown overnight (the inoculum was a loop-full from cryo stock) at 30 °C, 140 rpm (Genesys 20, Thermo Spectronic, Neuss, Germany) in 1 L rich medium (peptone 5 g L^{-1}, meat extract 3 g L^{-1}). 10% (v/v) of the overnight culture were transferred in 1 L (end volume) minimal medium 'R1' (initial pH 7.0) consisting of $(NH_4)_2SO_4$ 3 g L^{-1}; KH_2PO_4 1.5 g L^{-1}; Na_2HPO_4 4.45 g L^{-1}; $MgSO_4$ 0.097 g L^{-1}; $CaCl_2 \cdot 6H_2O$ 0.02 g L^{-1}; $FeSO_4 \cdot 7H_2O$ 0.02 g L^{-1}; $MnCl_2 \cdot 4H_2O$ 24 µg L^{-1}; $ZnSO_4 \cdot 7H_2O$ 528 µg L^{-1}; $Na_2MoO_4 \cdot 2H_2O$ 150 µg L^{-1}; $CuSO_4 \cdot 5H_2O$ 240 µg L^{-1}; $CoCl_2 \cdot 6H_2O$ 90 µg L^{-1}; H_3BO_3 864 µg L^{-1}; $NiCl_2$ 24 µg L^{-1}; 30 g L^{-1} fructose as carbon source. The cultures were grown for 15 h at 30 °C, 140 rpm (Genesys 20, Thermo Spectronic, Neuss, Germany). The influence of different acetic acid concentrations on cell growth was screened in single-use stirred-tank bioreactors on a milliliter scale (bioREACTOR 48, 2mag AG, Munich, Germany). Inoculum from 'R1' medium was inoculated to a final OD_{600} 1.6–1.8 in 12 mL 'R2' medium consisting of $(NH_4)_2SO_4$ 1.83 g L^{-1}; KH_2PO_4 1.47 g L^{-1}; K_2HPO_4 2.46 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.27 g L^{-1}; $CaCl_2 \cdot 2H_2O$ 0.03 g L^{-1}; $FeSO_4 \cdot 7H_2O$ 0.02 g L^{-1}; $MnCl_2 \cdot 4H_2O$ 24 µg L^{-1}; $ZnSO_4 \cdot 7H_2O$ 528 µg L^{-1}; $Na_2MoO_4 \cdot 2H_2O$ 150 µg L^{-1}; $CuSO_4 \cdot 5H_2O$ 240 µg L^{-1}; $CoCl_2 \cdot 6H_2O$ 90 µg L^{-1}; H_3BO_3 864 µg L^{-1}; $NiCl_2$ 24 µg L^{-1}. The batch fermentation was carried out with 30 g L^{-1} fructose as a starting carbon source for 8 h at 30 °C. The pH was varied in the following range: pH 6.5, pH 7.0, pH 7.5, pH 8.0. pH control was performed using 12% (w/w) NH_4OH and 0.5 N H_3PO_4. After 8 h, different concentrations of acetic acid were supplied in the form of sodium acetate (pKa 4.75) to reach the following final concentrations: 2.5 g L^{-1}, 5.0 g L^{-1}, 7.5 g L^{-1}, 10 g L^{-1}, 15 g L^{-1}, 20 g L^{-1} and incubated for an additional 10 h. The pH control was performed using 0.5 M KOH and 0.5 M H_3PO_4 under nitrogen-limited conditions. The optical density (OD_{600}) was measured at the end of each run (Genesys 20 (Thermo Spectronic, Neuss, Germany)). The integration study with *R. eutropha* H16 was performed in 50 mL medium obtained from *A. woodii* after high-pressure fermentation. Prior to integration, *A. woodii* was filtered from the medium using bottle top filters 0.2 µm, VWR international, PA, USA. *R. eutropha* H16 was incubated in shaking flasks at 30 °C, 140 rpm (initial pH 7.5). The *R. eutropha* H16 inoculum was pre-grown in medium "R1" over-night. Prior to integration, to remove media "R1" residues, the cells were centrifuged (Eppendorf centrifuge 5430 R, Eppendorf, Hamburg, Germany; 4,500 ×g, 10 min, 4 °C) and washed once with phosphate buffer (30 mM, pH 7.5). The amount of inoculum was chosen to reach a final OD_{600} of 1.5 in the experimental vessel.

2.3. High-pressure reactor

Autotrophic fermentation of *A. woodii* was performed in a high-pressure stirred-tank "ecoclave 075" type 1B/1.6 L reactor without baffles (Büchi-Glas-Uster, Uster, Switzerland). Dimensions and system operating conditions were 60 × 45 × 100 cm width/depth/height; −1/+6 bar min./max. pressure; −20/+200 °C min./max. Temperature controlled with a stainless-steel 2mag PT100 external temperature sensor (2mag-USA, Florida, USA). A mixed-gas tank of $H_2:CO_2$ (85:15 v/v) was connected to the system and controlled by a pressure regulator (bpc 2, Büchi pressflow controller; Büchi-Glas-Uster, Uster, Switzerland). The pH control system consisted of dosing pumps (ProMinent® The delta®, Pennsylvania, USA) for maximum backpressure of 25 bar (pumps controlled by frequency) and an analog pH glass sensor (Orbisint CPS11; Endress + Hauser, Switzerland). Since

the pH decreased due to acetic acid production, 0.1 M KOH was used to adapt the pH. To eliminate any traces of oxygen in the medium, the sterile medium was stripped with argon for 10 purges, followed by 10 purges of the gas mixture (0.5 mg L^{-1} resazurin was used as indication of oxygen absence). The reactor vessel was autoclaved and afterwards filled with 1 L filtered medium via a peristaltic pump (Thermo Scientific Masterflex® PS, Illinois, USA). The agitation speed was set to 500 rpm using a stirrer shaft Dm10 × 294 with turbine Dm45 (Büchi-glas-uster, Uster, Switzerland), and the temperature was controlled to 30 °C. The fermentation was performed in batch mode, where the gas from the mixed-gas tank was fed in the reactor to a set pressure using the bpc 2 (Büchi pressflow controller; Büchi-Glas-Uster, Uster, Switzerland). When the pressure of the gas dropped due to the uptake by the microorganisms, the drop was recorded in L by a press flow gas controller (bpc 6002; Büchi-glas-uster, Uster, Switzerland) and the same value was fed to the reactor from the tank to build the pressure again.

2.4. Parallel fermentations in milliliter-scale stirred-tank bioreactors

Parallel fermentation experiments were performed as described previously (Meo et al., 2017) on a milliliter-scale in sterile single-use stirred-tank bioreactors with an initial volume of 10 mL at 30 °C (bioREACTOR, 2mag AG, Munich, Germany). Dissolved oxygen (DO) and pH were monitored by fluorimetric sensors immobilized at the bottom of each single-use bioreactor (Kusterer et al., 2008) using fluorimetric readers (MCR 8*2 v5, PreSens GmbH, Regensburg, Germany). The pH of each bioreactor was adjusted with 12% (w/w) NH_4OH and 0.5 M H_3PO_4 during the first 8 h, then with 0.5 M NaOH (nitrogen limitation conditions to promote PHB production) and 0.5 M H_3PO_4 by a liquid handling system (Freedom EVO®, Tecan GmbH, Crailsheim, Germany) controlled by the software fedbatchXP (DasGip – an Eppendorf company, Jülich, Germany). The minimal volume of base addition was set to 10 µL (0.1% of the reaction volume). Substrate feeding was done manually. Samples were withdrawn automatically with the liquid handling system at preset process times and were used for the determination of bacterial growth and of concentrations of acetic acid. The oxygen transfer rate was kept sufficiently high by using gas-inducing stirrers (Hortsch and Weuster-Botz, 2010) operated at an agitation speed of 2800 rpm and a headspace aeration of 0.1 L sterile air min^{-1} $reactor^{-1}$. Liquid volume loss by evaporation was avoided by using sterile air that was saturated with water at room temperature (25 °C), and the headspace cooling was adjusted to 20 °C (Meo et al., 2017).

2.5. Fermentation product identification and quantification

High-performance liquid chromatography (HPLC, Agilent 1200 series, California, USA) was used to quantify the fermentation products. The HPLC was equipped with ICE-Coregel 87 H3 column (Transgenomic, Minnesota, USA), and the eluent was 0.008 M H_2SO_4 solution at a flow rate of 0.8 mL min^{-1}. A UV-VIS detector was used at $\lambda = 214$ nm and 35 °C. The acetic acid and formic acid were spiked on the HPLC to identify their retention times. The unknown metabolic products were identified by detecting their retention times and collecting the fractions using an HPLC equipped with a fraction collector (HPLC, Agilent 1200 series, G1364B Fraction Collector, California, USA). The fractions were freeze-dried (VirTis benchtop 6 K, New York, USA), treated with methoxyamine and then derivatized using trimethysilanol (TMS) reagent (BSTFA, Thermo Fisher Scientific, Massachusetts, USA). The fractions were analyzed using gas chromatography coupled with mass spectrometry, (GC/MS 7890/5975C; Agilent Technologies, California, USA) equipped with a DB-5 capillary column (30 m, 0.25 mm ID and 0.25 µm film thickness, Agilent Technologies, California, USA). The injection volume was 1 µL. The column ran with an initial temperature of 50 °C for 1 min, a ramp rate of 10 °C min^{-1} up to 290 °C for 35 min (total run time 60 min). The flow rate was 1.5 mL min^{-1}. The GC/MS raw

data file of each sample was de-convoluted using the AMDIS software (Mallard and Reed, 1997) and each compound detected was identified by the NIST 11 library database (Agilent Technologies, California, USA).

2.6. PHB quantification with GC-FID

Cultures (2 mL) were centrifuged (Eppendorf centrifuge 5430 R, Eppendorf, Hamburg, Germany; 6,500 ×g, 50 min, 4 °C) and then washed with double distilled water (ddH$_2$O) to decrease residual salts from the medium. Subsequently, the cells were re-suspended in water, shock-frozen in liquid N$_2$ and then lyophilized. Afterwards, methanolysis was performed by dissolving the dried cells in 2 mL of 6% (v/v) sulfuric acid in methanol solution containing 100 mg L^{-1} of sodium benzoate as an internal standard. Then 2 mL of chloroform were added to the mixture and heated for 3 h at 100 °C in a tightly closed pressure tube. After methanolysis, the samples were cooled on ice for 10 min and 1 mL of ddH$_2$O per 2 mL of CHCl$_3$ (1:2 ratio) was added (Oehmen et al., 2005). The mixture was vortexed for 1 min and the phases were separated by centrifugation at 4500 ×g for 5 min, at 20 °C. The organic phase was collected, neutralized with NaHCO$_3$ and dried over Na$_2$SO$_4$. The resulting mixture of 3-hydroxy-butanoyl methylester (3HBM) and further intracellular components in CHCl$_3$ were characterized and quantified by GC/FID (Agilent Technology GC system 7890A/5975 inter XL EI, CI MSD with a triple axis detector, California, USA), using the purchased Poly[(R)-3-hydroxybutyric acid] from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA) as a reference. The GC/FID was equipped with a DB-WAX (60 m × 0.25 μm × 0.5 μm, Agilent Technologies, California, USA) column and ran a temperature profile starting at 50 °C for 1 min then increasing 15 °C min^{-1} up to 240 °C for 5 min (total run time 18.6 min). The injection volume was 1 μL and the flow rate was 1.7 mL min^{-1}.

2.6.1. Theory/calculation
The cell-specific acetate formation/production rate (q_p) is defined as:

$$q_P = (1/cx) * dc_P/dt$$

cx: biomass concentration, g L^{-1}, **c$_p$**: product concentration, g L^{-1}, **q$_p$**: specific product formation rate, g(product) g(biomass)$^{-1}$ h^{-1}.
The space-time yield:

$$P_{end}/process\ time;\ \textbf{P}_{end} : end\ product$$

Energy efficiency (η):
H$_2$ to acetate by A. woodii ($\eta_{H2\ to\ AcOH}$)
Combustion energy of 1 mMole H$_2$:

$$H_2 + ½\ O_2 \rightarrow H_2O$$
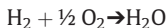
$$\Delta_r G'^{\circ} = -264.5\ kJ\ mol^{-1}$$

Combustion energy of 1 mMole acetate:

$$CH_3COOH + 2\ O_2 \rightarrow 2\ H_2O + 2\ CO_2$$
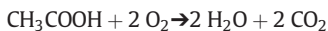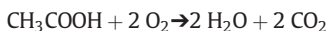
$$\Delta_r G'^{\circ} = -873.2\ kJ\ mol^{-1}$$

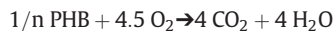$\eta_{H2\ to\ AcOH} = $ Combustion energy of acetate/Combustion energy of H$_2$

acetate to PHB by R. eutropha ($\eta_{AcOH\ to\ PHB}$).
Combustion energy of 1 mMole acetate:

$$CH_3COOH + 2\ O_2 \rightarrow 2\ H_2O + 2\ CO_2$$

$$\Delta_r G'^{\circ} = -873.2\ kJ\ mol^{-1}$$

Combustion energy of 1 mMole PHB:

$$1/n\ PHB + 4.5\ O_2 \rightarrow 4\ CO_2 + 4\ H_2O$$

$$\Delta_r G'^{\circ} = -\textbf{1987}\ kJ\ mol^{-1}$$

$\eta_{AcOH\ to\ PHB} = $ Combustion energy of PHB/Combustion energy of acetate

Growth

$Y_x/S;\ \textbf{Y}_x$: Active biomass, **S** : substrate Y_x/S

PHB storage

$Y_{PHB}/S;\ \textbf{Y}_{PHB}$: Produced PHB, **S** : substrate Y_x/S

3. Results and discussion

3.1. Stage I

3.1.1. Stage Ia: CO$_2$ to acetic acid by A. woodii and the influence of pressure
We assessed the fermentation of *A. woodii* in a high-pressure stirred tank reactor using a H$_2$:CO$_2$ gas mixture of 85:15 (v/v) at 2.0 bar and 5.5 bar to increase solubility of gases with pressure to improve growth and acetic acid production (Groher and Weuster-Botz, 2016; Kantzow and Weuster-Botz, 2016; Liew et al., 2013). We used a sealed-high pressure reactor that prevented the loss of gases introduced to the reactor, recorded the gas consumption and calculated the energy efficiency. We monitored the growth and acetic acid production of *A. woodii* in 'A2' medium at pH 7.0. At 2.0 bar pressure (H$_2$ partial pressure 1.7 bar, equal to 1.3 mM H$_2$), a total of 5.2 L gas was consumed. The cell dry weight (CDW) increased from 0.22 g L^{-1} to 0.40 g L^{-1} during the stationary phase (212 h of culturing). Acetic acid production was 3.20 g L^{-1}, with a space-time yield of 0.36 g L^{-1}d^{-1} and a maximum cell-specific acetate formation rate ($q_{acetate}$) of 0.09 g$_{AcOH}$ g$_{CDW}^{-1}$ h^{-1} (2.1 g$_{AcOH}$ g$_{CDW}^{-1}$d^{-1}) during the exponential growth phase. The energy efficiency ($\eta_{H_2\ to\ AcOH}$) was 55% (Fig. 2).

The final acetate concentrations might seem low (3.20 g L^{-1} after 212 h of culturing) compared to 59.2 g l^{-1} after a processing time of approximately 77 h reported by (Kantzow and Weuster-Botz, 2016). These differences can be explained by the different approaches used. In our approach, the gas mixture (85 H$_2$:15 CO$_2$ v/v) was delivered by pressure regulation (when the gas is consumed the pressure drops, and the bpc 2 rebuilds the pressure), which prevents gas losses and allows a detailed measurement of gas consumption. Conversely, Kantzow et al. used a gas mixture that contains N$_2$ of H$_2$:CO$_2$:N$_2$ (60:25:15 v/v) with a constant flow rate of 30 L h^{-1} at 1 bar pressure. This allows a higher gas-to-liquid mass transfer rate, resulting in higher total yield, but also a loss of about 25% (v/v) gas. At 5.5 bar pressure (H$_2$ partial pressure 4.67 bar, equal to 3.6 mM H$_2$), a total of 8.9 L gas was consumed. The CDW increased from 0.30 g L^{-1} to 0.40 g L^{-1} during the stationary phase (140 h of culturing). Acetate production (5.60 g L^{-1}), space-time yield (0.96 g L^{-1}d^{-1}) and the maximum acetate formation rate, $q_{acetate}$ (0.50 g$_{AcOH}$ g$_{CDW}^{-1}$ h^{-1}) (12 g$_{AcOH}$ g$_{CDW}^{-1}$d^{-1}) were also substantially higher at 5.5 bar than at 2.2 bar. However, the energy efficiency ($\eta_{H_2\ to\ AcOH}$) at 5.5 bar pressure was lower (22%) (Fig. 2). Table 1 compares our results of acetic acid production from CO$_2$ under different pressure conditions to Lagoa-Costa et al. (Lagoa-Costa et al., 2017).

When comparing the approach of Lagoa-Coate et al. to our approach, increasing the pressure of the gas mixture reduced the process time significantly, while increasing acetate production, space time yield and **q$_{acetate}$**. The use of pressure allows a moderate gas consumption without losses. Moreover, in our experiments we observed a decrease in energy efficiency ($\eta_{H2\ to\ AcOH}$) when increasing the pressure from 2.0 bar to
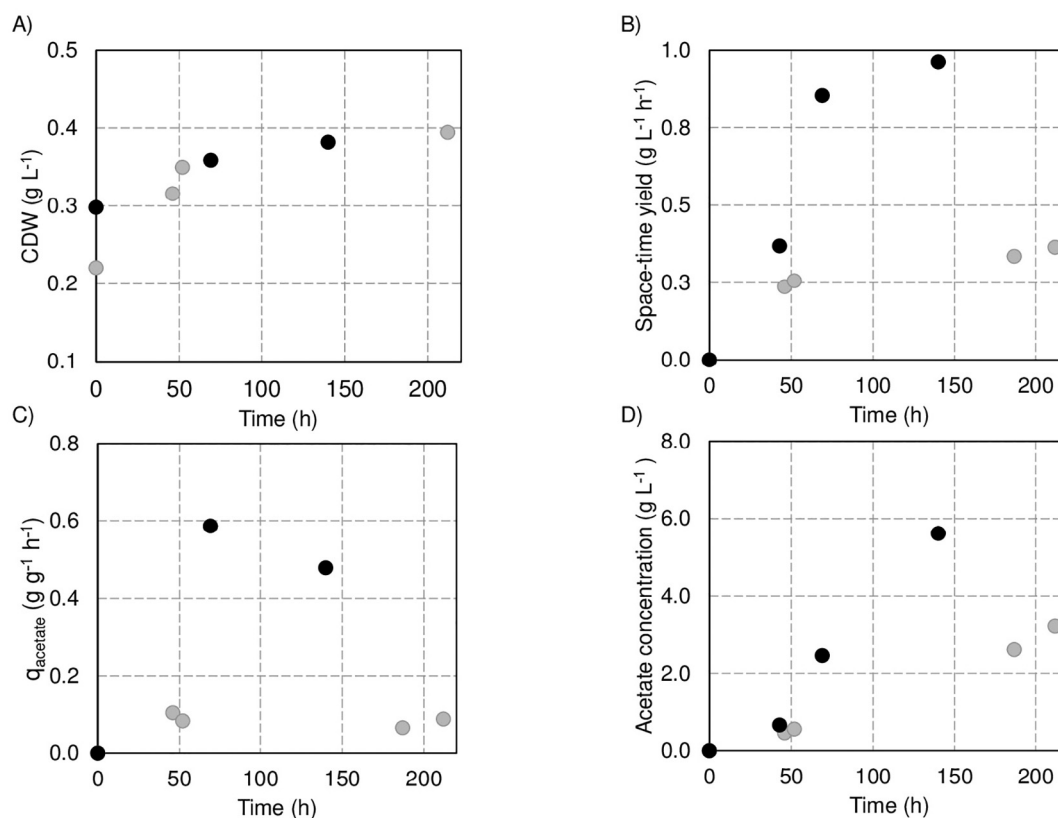
Fig. 2. Autotrophic processes of *A. woodii* in a high-pressure, stirred-tank bioreactor under 2.0 and 5.5 bar pressure, represented by grey lines and black lines, respectively. (**A**) Concentration of CDW (cell dry weight), (**B**) space-time yield, (**C**) $q_{acetate}$ (specific acetate formation rate) and (**D**) acetate concentration (AcOH), as a function of process time.

5.5 bar, and at 5.5 bar the energy efficiency was further reduced with increasing nitrogen content. The reduced energy efficiency at 5.5 bar may result from the applied high partial pressure of CO_2 that causes a shift in the thermodynamic equilibrium, leading to the accumulation of CO in the carbonyl branch of the WLP (Hawkins et al., 2013; Schuchmann and Muller, 2014) (Supplementary information, Fig. S1). CO inhibits the ability of *A. woodii* to use H_2 because the hydrogen-dependent carbon dioxide reductase HDCR is sensitive to CO (Bertsch and Muller, 2015; Schuchmann and Muller, 2012; Schuchmann and Muller, 2013), which results in the inhibition of acetate production from $H_2:CO_2$ (Bertsch and Muller, 2015). Diekert et al. confirmed that at 1.7 bar of $H_2:CO_2$ (80:20 v/v) atmosphere in batch cultures, *A. woodii* generates CO when forming acetic acid (Diekert et al., 1986). Additionally, the increased H_2 partial pressure in the gas phase or its accumulation in the fermentation medium may alter or inhibit the production of NADH, which consequentially redirects the carbon flow away from cell growth

and acetic acid production by *A. woodii* in autotrophic batch processes (Nie et al., 2008).

3.1.2. Stage Ib: Optimization of the acetic acid production rate in enriched medium at 5.5 bar

To increase the energy efficiency ($\eta_{H_2\ to\ AcOH}$) at 5.5 bar, we increased the yeast extract content from 4.0 g L^{-1} to 6.0 g L^{-1} (medium 'A3') to prevent nutrient limitations and promote higher cell activity, which should result in increased utilization of H_2 and CO_2. The consumption of 30 L gas mixture ($H_2:CO_2$, 85:15 v/v) and the production of 4.50 g L^{-1} acetic acid was observed in the enriched medium (medium 'A3') in the stationary phase (96 h of cultivation). The space-time yield was 1.12 g L^{-1} d^{-1} and the maximum acetate formation rate, $q_{acetate}$, was 0.6 g_{AcOH} g_{CDW}^{-1} h^{-1} compared to 0.96 g L^{-1} d^{-1} and g L^{-1} and 0.50 g_{AcOH} g_{CDW}^{-1} h^{-1}, respectively, with 4 g L^{-1} yeast extract (medium 'A2') at 5.5 bar. However, the energy efficiency ($\eta_{H_2\ to\ AcOH}$) was 5.4% in medium 'A3' compared to 22% in medium 'A2' (Table 1). Also, substantially more gas (30 L compared to 8.9 L of $CO_2:H_2$ mixture) was consumed, but less acetic acid was produced.

Detailed analysis of the medium revealed that *A. woodii* produced other substances besides acetic acid, such as formic acid, uracil, pyroglutamate and traces of lactate (Fig. 3), when grown with increased yeast extract. The differences in product formation in the enriched medium 'A3' likely resulted from the additional vitamins and nitrogen supplemented from the high content of yeast extract. These vitamins are essential cofactors for the WLP (Leclerc et al., 1998) and the additional nitrogen might promote build-up of the nitrogen-containing products uracil and pyroglutamate. We hypothesize that the high concentration of yeast extract in combination with the increased pressure led to a high production of NADH, which altered the carbon flow. The excess of NADH would then lead to the accumulation of acetyl-CoA in the WLP where acetyl-CoA is not only converted to acetic acid, but also to pyruvate. We further hypothesize that the pyruvate is then utilized in

Table 1
Bacterial growth under different pressures of $CO_2:H_2$ gas and different media. Our method is compared to the approach of Lagoa-Costa et al. (Lagoa-Costa et al., 2017).

|	Lagoa-Coate et al.	This paper		
Pressure (bar)	Atmospheric	2.0	5.5	5.5
Media		A2	A2	A3
Strain	*C. autoethanogenum*	*A. woodii*		
Acetate production (g L^{-1})	2.66	3.20	5.60	4.50
Space-time yield (g L^{-1} d^{-1})	0.121	0.36	0.96	1.12
$q_{acetate}$ (g_{AcOH} g_{CDW}^{-1} h^{-1})	0.02	0.09	0.50	0.60
Gas mixture	$CO:CO_2:H_2:N_2$ (30:10:20:40 v/v)	$CO_2:H_2$ (15:85 v/v)		
Gas utilized (L)	5240	5.2	8.9	30
Process time (h)	523	212	140	96
Energy efficiency % ($\eta_{H2\ to\ AcOH}$)	N/A	55	22	5.4
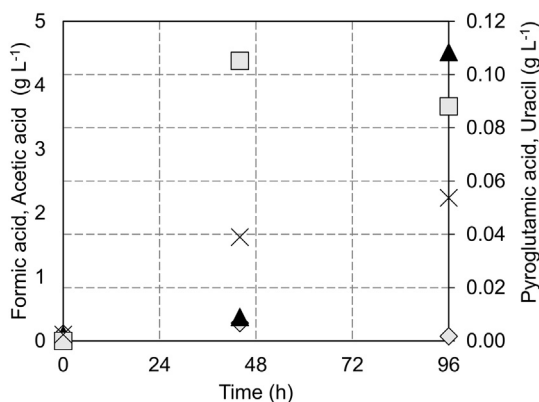
Fig. 3. Metabolite production of *A. woodii* grown under 5.5 bar in medium 'A3'. Acetic acid (AcOH, black triangles), formic acid (grey diamonds), pyroglutamic acid (grey square) and uracil (black x).

the TCA cycle that produces more NADH, which, in turn, is used to synthesize uracil and pyroglutamic acid using the excess of nitrogen (Supplementary information, Fig. S2).

3.2. Stage II: Conversion of acetic acid to PHB by R. eutropha H16

To produce PHB from acetic acid, *R. eutropha* H16, a well-studied model strain for PHB production, was chosen. We first assessed the effect of acetate concentration on aerobic cell growth, to avoid the toxicity at higher concentrations of acetate and/or other byproducts. Therefore, *R. eutropha* H16 growth was investigated in parallel in 10 mL stirred-tank bioreactors under varying concentrations of acetate and pH. *R. eutropha* H16 were grown in miniaturized stirred-tank reactors using 'R2' medium supplemented with 30 g L^{-1} fructose at different pH levels of pH 6.5, pH 7.0, pH 7.5 and pH 8.0. After 8 h of growth, acetic acid was added to reach the following concentrations: 2.5 g L^{-1}, 5.0 g L^{-1}, 7.5 g L^{-1}, 10 g L^{-1}, 15 g L^{-1} and 20 g L^{-1}. After 10 h (total 18 h) of batch cultivation, OD$_{600}$ was measured (Supplementary information, Fig. S3). We observed that at pH 7.5, no severe toxic effect was detected when the acetate concentration was up to 7.5 g L^{-1} and the cell density increased to an OD$_{600}$ of 9.8. By increasing the acetate concentration up to 20 g L^{-1} at pH 7.5, the growth increased to an OD$_{600}$ of 4.4 after 10 h. Despite the small increase, pH 7.5 allowed the bacteria to maintain better growth compared to pH 6.5, pH 7.0 and pH 8.5. These results showed that the ideal process pH is 7.5 with a maximum acetic acid concentration of 7.5 g L^{-1}, where a consumption rate of 0.8 g L^{-1} h^{-1} OD$_{600}^{-1}$ acetate was measured. However, at a lower pH of 6.5, a severe toxic effect of acetic acid started at relatively low concentrations of 5.0 g L^{-1}, which resulted in a decline in growth (Supplementary information). This severe effect is also visible at pH 7.0 with higher acetic acid concentrations (up to 10 g L^{-1}). At pH 8.0, the decline in growth was visible at 15 g L^{-1} acetic acid.

Short-chain fatty acids (SCFA) such as acetic acid tend to split into anions above their pKa (Chung et al., 1997; Visser and Postma, 1973). Acetic acid has a pKa of 4.76. Therefore, the higher the solution pH, the more acetic acid is split into anions. Although free anions can slowly enter the cytoplasm where they might adversely affect cell metabolism, cells can pump anions out of their cytoplasm. However, this mechanism of detoxification through anti-porters requires energy and results in a decreased growth rate and PHB production with increased anion concentrations (Axe and Bailey, 1995). At pH values below the pKa values of the SCFAs, SCFAs accumulate in their un-dissociated form, which can dissolve into the lipid bilayer of the cell membrane and act as uncoupling agents (Visser and Postma, 1973). Conversely, dissolved intact SCFAs allow protons to pass through the cell membrane by acting as proton carriers, resulting in uncoupled electron transport from the ATP

synthase (Baronofsky et al., 1984). This electron transport and proton pumping continues at a rapid rate, but no proton gradient is generated, and ATP can no longer be synthesized. The lack of ATP is initially compensated by utilization of acetyl-CoA in the TCA cycle, which inhibits the production of PHB and results in activity inhibition and eventually cell death over time (Chung et al., 1997).

Under the conditions used, pH 7.5 appears to most favorably balance the different effects of intact or dissociated acetic acid. Notably, at alkaline conditions between pH 8.0 to 8.5, 99.9% of acetic acid remained in its dissociated form (Yu and Wang, 2001), but this pH range had an inhibitory effect on *R. eutropha* H16 growth (Wang and Yu, 2000). These findings underscore that pH control is important because it affects the ionization of the active components of microbial cells (enzymes, enzyme complexes, or other ionizable substrate receptors). For optimal growth and activity, these components must be in their appropriate ionic forms to bind their substrates (Tan et al., 1998). Our results show the benefit of a slightly alkaline pH, while feeding acetic acid (20 g L^{-1}) with the highest cell yield at pH 7.5 and an OD$_{600}$ of 4.4 (after 10 h). In our study, the comparatively high tolerance for acetic acid can be explained by the use of bioreactors. The controlled environment in bioreactors (e.g., no oxygen limitation in combination with pH control) potentially allow optimized cell growth because of the higher pumping capacity of the cells.

3.3. Bio-GTL microbial process to convert the produced acetate from CO$_2$ fermentation into PHB

The here presented approach focused on the use of two metabolically different strains (*A. woodii* and *R. eutropha*). The development of a medium that supports both metabolic modes (gas fermentation and PHB production) would markedly enhance cost-effectiveness and ease of handling, and hence provide important improvements compared to the approach proposed previously by Lagoa-Costa et al., 2017 (Lagoa-Costa et al., 2017). We were indeed able to establish and optimize such a medium that can first be used for *A. woodii*, and then be kept for *R. eutropha* after simply filtering out *A. woodii* and adjusting the pH of the medium. For this protocol, in stage 1, *A. woodii* fermentation was carried out at 2.0 bar (the pressure that provided the highest energy efficiency) in medium A2. At the end of this reaction, this medium A2 contained 3.20 g L^{-1} acetate. We then filtered out *A. woodii*, adjusted the pH to 7.5 and inoculated with *R. eutropha* H16, which was pre-grown to an OD$_{600}$ of 1.5 (0.27 g L^{-1}) in medium R1 and washed once with phosphate buffer (30 mM, pH 7.5). *R. eutropha* H16 grew to a final OD$_{600}$ of 9.3 (active biomass 0.41 g L^{-1}) after 5 h of culturing with an uptake of 3.0 g L^{-1} acetate and a q$_{acetate}$ 1.46 g$_{AcOH}$ g^{-1}$_{CDW}$ h^{-1}. PHB production was 0.5 g L^{-1} and a q$_{PHB}$ of 0.24 g$_{PHB}$ g^{-1}$_{CDW}$ h^{-1} and the PHB content (percentage of weight) in the microbial cell was 33.3%. The PHB content was defined as the percentage of the ratio of PHB concentration to cell concentration (Ahn et al., 2000).

Despite the increase in biomass, due to the high nitrogen content from the acetogen media, *R. eutropha* had a higher q$_{PHB}$ and a PHB content compared to what was reported by Lagoa-Coate et al. (Lagoa-Costa et al., 2017). It appeared that the medium A2 successfully induced PHB production with a high PHB content by simply adjusting the pH to 7.5 (Table 2). Using a single medium that only requires pH adjustment is an important step towards a continuous setup in which both, the CO$_2$ based production of acetic acid and the conversion of acetic acid to PHB are connected to each other.

4. Conclusion

We developed a Bio-GTL process to convert CO$_2$ into PHB in two stages, but in one medium, using acetic acid as intermediate. The high-pressure reactor used in our study for CO$_2$ fermentation delivered the gas to the stirring tank only when pressure dropped as a result of microbial consumption. This system prevented loss of gas, which is

Table 2
Conceptual overview of the Bio-GTL microbial process reported in this paper in comparison to Lagoa-Costa et al. (Lagoa-Costa et al., 2017).

	Lagoa-Coate et al.	This paper
Microbial strain	*C. autoethanogenum*	*R. eutropha* H16
Culturing	Fed-batch	Shaking flask
Total acetate uptake (g L^{-1})	4.0 (8 pulses, 0.5 g L^{-1}/pulse)	3.0 with direct inoculation
Active biomass (mmol L^{-1})	0.01	16
Growth (C$_{CDW}$ mmol C$_{AcOH}$ mmol^{-1})	0	0.32
PHB storage (C$_{PHB}$ mmol C$_{AcOH}$ mmol^{-1})	0.275	0.116
q$_{PHB}$ (C$_{PHB}$ mmol C$_{CDW}$ mmol^{-1} h^{-1})	0.042	0.068
PHB cell content (%)	24%	33.3%

advantageous compared to previous approaches where large gas volumes flow through the stirring tank (Lagoa-Costa et al., 2017). The process could be coupled with photovoltaics to electrochemically produce H$_2$ for CO$_2$ fermentation resulting in a sustainable photovoltaic/Bio-GLT approach for the production of bioplastic.

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Author contributions

I.S.A, B.K., S.W.G., and C.B., conducted the experiments, I.A, B.K., S.W.G., and J.E. conceived and designed the experiments. I.A., B.K., S.W.G., and J.E. analyzed the data. I.A, B.K., S.W.G., C.B., R.K., D.W., J.E. and S.T.A. wrote and/or revised the manuscript.

Author agreement/declaration

All authors reviewed and approved the final version of the manuscript being submitted.

Conflict of interest disclosure

The authors declare no financial or commercial conflicts of interest.

Appendix A. Supplementary data

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References

Ahn, W.S., Park, S.J., Lee, S.Y., 2000. Production of poly(3-hydroxybutyrate) by fed-batch culture of recombinant Escherichia coli with a highly concentrated whey solution. Appl. Environ. Microbiol. 66 (8), 3624–3627.

Alper, E., Yuksel Orhan, O., 2017. CO2 utilization: developments in conversion processes. Petroleum 3 (1), 109–126.

Axe, D.D., Bailey, J.E., 1995. Transport of lactate and acetate through the energized cytoplasmic membrane of Escherichia-Coli. Biotechnol. Bioeng. 47 (1), 8–19.

Baronofsky, J.J., Schreurs, W.J.A., Kashket, E.R., 1984. Uncoupling by acetic-acid limits growth of and acetogenesis by clostridium-thermoaceticum. Appl. Environ. Microbiol. 48 (6), 1134–1139.

Bertsch, J., Muller, V., 2015. CO metabolism in the acetogen Acetobacterium woodii. Appl. Environ. Microbiol. 81 (17), 5949–5956.

Blanch, H.W., 2009. Extraction chemistry of fermentation product carboxylic acids introduction. Biotechnol. Bioeng. 103 (3), 431.

Cheung, H., Tanke, R.S., Torrence, G.P., 2011. Acetic acid. Ullmann's Encyclopedia of Industrial Chemistry.

Chung, Y.J., Cha, H.J., Yeo, J.S., Yoo, Y.J., 1997. Production of poly(3-hydroxybutyric-co-3-hydroxyvaleric)acid using propionic acid by pH regulation. J. Ferment. Bioeng. 83 (5), 492–495.

De Tissera, S., Köpke, M., Simpson, S.D., Humphreys, C., Minton, N.P., Dürre, P., 2017. Syngas Biorefinery and Syngas Utilization. In: Advances in Biochemical Engineering/Biotechnology. Springer, Berlin, Heidelberg, pp. 1–34 https://doi.org/10.1007/10_2017_5.

Demler, M., Weuster-Botz, D., 2011. Reaction engineering analysis of hydrogenotrophic production of acetic acid by Acetobacterium woodii. Biotechnol. Bioeng. 108 (2), 470–474.

Diekert, G., Schrader, E., Harder, W., 1986. Energetics of Co formation and Co oxidation in cell-suspensions of acetobacterium-woodii. Arch. Microbiol. 144 (4), 386–392.

Fogler, H.S., 2010. Essentials of Chemical Reaction Engineering. Prentice Hall, New Jersey, United States.

Groher, A., Weuster-Botz, D., 2016. General medium for the autotrophic cultivation of acetogens. Bioprocess Biosyst. Eng. 39 (10), 1645–1650.

Hawkins, A.S., McTernan, P.M., Lian, H., Kelly, R.M., Adams, M.W.W., 2013. Biological conversion of carbon dioxide and hydrogen into liquid fuels and industrial chemicals. Curr. Opin. Biotechnol. 24 (3), 376–384.

Hortsch, R., Weuster-Botz, D., 2010. Milliliter-scale stirred tank reactors for the cultivation of microorganisms. Adv. Appl. Microbiol. 73 (73), 61–82.

Hu, P., Chakraborty, S., Kumar, A., Woolston, B., Liu, H.J., Emerson, D., Stephanopoulos, G., 2016. Integrated bioprocess for conversion of gaseous substrates to liquids. Proc. Natl. Acad. Sci. U. S. A. 113 (14), 3773–3778.

Jipa, I., Dobre, T., Stroescu, M., Stoica, A., 2009. Acetic acid extraction from fermentation broth experimental and modelling studies. Rev. Chim. 60 (10), 1084–1089.

Kantzow, C., Weuster-Botz, D., 2016. Effects of hydrogen partial pressure on autotrophic growth and product formation of Acetobacterium woodii. Bioprocess Biosyst. Eng. 39 (8), 1325–1330.

Kantzow, C., Mayer, A., Weuster-Botz, D., 2015. Continuous gas fermentation by Acetobacterium woodii in a submerged membrane reactor with full cell retention. J. Biotechnol. 212, 11–18.

Kertes, A.S., King, C.J., 2009. Extraction chemistry of fermentation product carboxylic acids. Biotechnol. Bioeng. 103 (3), 432–445.

Kulpreecha, S., Boonruangthavorn, A., Meksiriporn, B., Thongchul, N., 2009. Inexpensive fed-batch cultivation for high poly(3-hydroxybutyrate) production by a new isolate of Bacillus megaterium. J. Biosci. Bioeng. 107 (3), 240–245.

Kusterer, A., Krause, C., Kaufmann, K., Arnold, M., Weuster-Botz, D., 2008. Fully automated single-use stirred-tank bioreactors for parallel microbial cultivations. Bioprocess Biosyst. Eng. 31 (3), 207–215.

Lagoa-Costa, B., Abubackar, H.N., Fernandez-Romasanta, M., Kennes, C., Veiga, M.C., 2017. Integrated bioconversion of syngas into bioethanol and biopolymers. Bioresour. Technol. 239, 244–249.

Leclerc, M., Elfoul-Bensaid, L., Bernalier, A., 1998. Effect of yeast extract on growth and metabolism of H2-utilizing acetogenic bacteria from the human colon. Curr. Microbiol. 37 (3), 166–171.

Li, H., Opgenorth, P.H., Wernick, D.G., Rogers, S., Wu, T.Y., Higashide, W., Malati, P., Huo, Y.X., Cho, K.M., Liao, J.C., 2012. Integrated electromicrobial conversion of CO2 to higher alcohols. Science 335 (6076), 1596.

Liew, F.M., Köpke, M., Simpson, S.D., 2013. Gas Fermentation for Commercial Biofuels Production. INTECH Open Access Publisher.

Mallard, W., Reed, J., 1997. Automated Mass Spectral Deconvolution and Identification System AMDIS-USER GUIDE. US Department of Commerce Technology Administration, National Institute of Standards and Technology (NIST), Gaithersburg, MD, 20899.

Meo, A., Priebe, X.L., Weuster-Botz, D., 2017. Lipid production with trichosporon oleaginosus in a membrane bioreactor using microalgae hydrolysate. J. Biotechnol. 241, 1–10.

Mohammadi, M., Najafpour, G.D., Younesi, H., Lahijani, P., Uzir, M.H., Mohamed, A.R., 2011. Bioconversion of synthesis gas to second generation biofuels: a review. Renew. Sustain. Energy Rev. 15 (9), 4255–4273.

Munasinghe, P.C., Khanal, S.K., 2010. Syngas fermentation to biofuel: evaluation of carbon monoxide mass transfer coefficient (k(L)a) in different reactor configurations. Biotechnol. Prog. 26 (6), 1616–1621.

Nie, Y.Q., Liu, H., Du, G.C., Chen, J., 2008. Acetate yield increased by gas circulation and fed-batch fermentation in a novel syntrophic acetogenesis and homoacetogenesis coupling system. Bioresour. Technol. 99 (8), 2989–2995.

Oehmen, A., Keller-Lehmann, B., Zeng, R.J., Yuan, Z.G., Keller, E., 2005. Optimisation of poly-beta-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems. J. Chromatogr. A 1070 (1–2), 131–136.

Rehm, B.H., 2006. Genetics and biochemistry of polyhydroxyalkanoate granule self-assembly: the key role of polyester synthases. Biotechnol. Lett. 28 (4), 207–213.

Schuchmann, K., Muller, V., 2012. A bacterial electron-bifurcating hydrogenase. J. Biol. Chem. 287 (37), 31165–31171.

Schuchmann, K., Muller, V., 2013. Direct and reversible hydrogenation of CO2 to formate by a bacterial carbon dioxide reductase. Science 342 (6164), 1382–1385.

Schuchmann, K., Muller, V., 2014. Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. Nat. Rev. Microbiol. 12 (12), 809–821.

Stephanopoulos, G., Aristidou, A.A., Nielsen, J., 1998. Metabolic Engineering: Principles and Methodologies. Academic Press, Massachusetts, United States.

Tan, Y.H., Wang, Z.X., Marshall, K.C., 1998. Modeling pH effects on microbial growth: a statistical thermodynamic approach. Biotechnol. Bioeng. 59 (6), 724–731.

Visser, A.S., Postma, P.W., 1973. Permeability of azotobacter-vinelandii to cations and anions. Biochim. Biophys. Acta 298 (2), 333–340.

Wang, J.P., Yu, J., 2000. Kinetic analysis on inhibited growth and poly(3-hydroxybutyrate) formation of *Alcaligenes eutrophus* on acetate under nutrient-rich conditions. Process Biochem. 36 (3), 201–207.

Yu, J., Wang, J.P., 2001. Metabolic flux modeling of detoxification of acetic acid by *Ralstonia eutropha* at slightly alkaline pH levels. Biotechnol. Bioeng. 73 (6), 458–464.