DISSERTATION

Development of a two-step bioprocess for CO₂ fixation from industrial flue gas and production of a fuel chemical

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Katharina Novak

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Abstract

In Austria, the main part of greenhouse gases such as CO_2 is produced by the industrial sector. The vision of a CO_2 neutral future will only be fulfilled if a significant reduction of CO_2 emissions goes hand in hand with a transition towards greener energy sources. Using CO_2 as a substrate is one possibility to close carbon cycles and to establish a circular economy.

This thesis aimed to show the feasibility of a two-step process for CO_2 fixation and production of a fuel chemical. In the first process step, CO_2 from an industrial source is fixed by the acetogenic microorganism *Acetobacterium woodii* and acetate is produced. The intermediate product acetate is then utilized by a genetically modified *Escherichia coli*, which produces the fuel chemicals isobutanol or 2,3-butanediol in the second process step. Considering the overall mass balance, the goal therefore was to achieve net CO_2 fixation for both steps while producing a fuel chemical from CO_2 via acetate. The development of such a process is challenged by several factors such as (i) components of the industrial flue gas inhibiting CO_2 fixation and growth, (ii) acetate toxicity, (iii) the low energy density of acetate and (iv) 2,3-butanediol and isobutanol production not being native to *E. coli*. To investigate the influence of these factors, each step of the process was investigated individually in this thesis.

Industrial blast furnace gas contains significant amounts of CO and CO_2, but lacks H_2, usually required for CO_2 fixation in *A. woodii*. CO_2 fixation in blast furnace gas is additionally complicated by the fact that CO is toxic and inhibits growth of *A. woodii*. The strategy therefore was to blend the industrial gas stream with H_2 as an energy source and to design a process where CO is limited in the liquid medium. Using CO-limited conditions, steady state data during continuous cultivations showed co-utilization of CO, CO_2 and H_2 and acetate production at a rate of 14 mmol acetate l^{-1} h^{-1}. Blending blast furnace gas with H_2 not only enabled CO_2 fixation in the industrial gas stream, but the ratio of flue gas stream to H_2 was also shown to influence gas uptake, metabolic fluxes and product formation, thus providing a potential control strategy for gas fermentations.

The assimilation of acetate in the second step is hindered by its toxicity as a weak acid, emphasizing the importance to select a suitable host. The robust and stress tolerant *E. coli* W was hypothesized to be a promising candidate for acetate utilization and upgrading into chemicals. Apart from being toxic, acetate also has a lower energy density compared to sugars. Therefore, acetate assimilation of *E. coli* W was investigated in batch and continuous cultures both as sole carbon source and during co-utilization with glucose to increase energy availability. Overexpression of acetyl-CoA synthetase, one of two pathways for acetate assimilation improved acetate uptake and co-utilization with glucose in batch

experiments. The suitability of *E. coli* W as efficient host for acetate utilization was confirmed by high uptake rates during batch and continuous cultivations.

Isobutanol is not naturally synthesized by *E. coli*, and recombinant production in this thesis was achieved by a systematic strain development approach relying on examination of different expression vectors and selection of a suitable host, *E. coli* W. Considering the chemical properties of isobutanol, a production process was developed, which allowed for the efficient utilization of both glucose on defined medium and the waste stream cheese whey, achieving a final isobutanol titer of 20 g l^{-1}. Low product yields on acetate in combination with isobutanol volatility hampered the development of isobutanol production from acetate. Therefore, 2,3-butanediol served as a platform to establish knowledge on chemical production from acetate. A chemically defined medium was designed, enabling the development of a production process where feeding of acetate as sole carbon source led to the formation of 1.16 g l^{-1} diols (2,3-butanediol and acetoin).

This thesis showed the feasibility of net CO_2 fixation and production of a fuel chemical in a novel two-step process. Combining time-resolved characterization of physiological parameters with rational process and screening design allowed to generate knowledge on both acetate production from an industrial gas stream and upgrading of acetate for microbial chemical production. While strain selection and engineering were key to improving acetate assimilation and conversion into a (fuel) chemical, steady state quantification and dynamic shift experiments enabled the determination of parameters limiting the microbial cell factory. Due to its flexible nature, the platform technology developed in this thesis could be an important cornerstone in industrial CO_2 fixation. Using blending of two independent gas streams as a general method for adjusting gas compositions to achieve net CO_2 fixation might enable reduction of industrial CO_2 emissions. Similarly, the strategies for upgrading the alternative feedstock acetate presented in this thesis carry potential for the establishment of a circular economy on the way towards a CO_2 neutral future.

Zusammenfassung

In Österreich kann der Großteil der ausgestoßenen Treibhausgase wie z.B. CO_2 auf den Energie- und Industriesektor zurückgeführt werden. Die Vision einer CO_2-neutralen Zukunft wird daher nur möglich sein, wenn die signifikante Reduktion der CO_2 Emissionen in Kombination mit dem Übergang zu grüneren Energiequellen umgesetzt wird. CO_2 als Substrat für mikrobielle Prozesse zu verwenden ist daher eine Möglichkeit, globale Kohlenstoffkreise zu schließen und eine Kreislaufwirtschaft zu etablieren.

Das Ziel dieser Arbeit war, die Realisierbarkeit eines zweistufigen Prozesses zur CO_2 Fixierung und Produktion einer Treibstoffchemikalie zu zeigen. Im ersten Schritt wird CO_2 in einem industriellen Abgasstrom fixiert und mit dem acetogenen Mikroorganismus *Acetobacterium woodii* in Acetat umgewandelt. Dieses Zwischenprodukt wird dann in eine zweite Stufe überführt, wo ein rekombinanter *Escherichia coli* Stamm die Treibstoffchemikalien 2,3-Butandiol bzw. Isobutanol produziert. In Bezug auf die Massenbilanz des Gesamtprozesses sollte daher eine netto CO_2 Fixierung über beide Stufen erreicht werden, während eine Treibstoffchemikalie aus CO_2 über Acetat produziert werden sollte. Die Herausforderung für die Entwicklung eines solchen Prozesses liegt in mehreren Faktoren: (i) Komponenten des Industrieabgases, welche die CO_2 Fixierung und Wachstum behindern können, (ii) die Toxizität von Acetat, (iii) die niedrige Energiedichte von Acetat und (iv) dass 2,3-Butandiol und Isobutanol nicht natürlich in *E. coli* produziert werden. Um diese Einflussfaktoren zu evaluieren, wurde in dieser Arbeit jeder Prozessschritt einzeln betrachtet und untersucht.

Industrielles Gichtgas enthält signifikante Mengen an CO und CO_2, es fehlt jedoch H_2, der als Energiequelle für die CO_2 Fixierung in *A. woodii* dient. Die CO_2 Fixierung im Industrieabgas wird zusätzlich dadurch erschwert, dass CO toxisch ist und das Wachstum von *A. woodii* inhibiert. Es wurde daher die Strategie gewählt, das Gichtgas mit H_2 als Energiequelle zu mischen und einen Prozess zu etablieren, wo CO im Flüssigmedium limitiert ist. Unter CO limitierten Bedingungen zeigten Daten aus dem Fließgleichgewicht eine gleichzeitige Verwertung von CO, CO_2 und H_2 und eine Acetat Produktivität von 14 mmol l^{-1} h^{-1}. Das Mischen von Gichtgas und H_2 ermöglichte nicht nur CO_2 Fixierung im Industrieabgas, sondern es zeigte sich, dass das Mischungsverhältnis von Gichtgas zu H_2 die Gasaufnahme, metabolische Flüsse und die Acetatproduktion beeinflusste und daher eine mögliche Kontrollstrategie für Gasfermentationen darstellt.

Die Assimilation von Acetat in der zweiten Prozessstufe wird durch dessen Toxizität als schwache Säure behindert, was die Wichtigkeit der Wahl eines geeigneten Wirtes hervorhebt. Die Hypothese war, dass der robuste und stress tolerante *E. coli* W ein vielversprechender Kandidat für die Acetatverwertung und Umwandlung in eine Chemikalie wäre. Neben der Toxizität hat Acetat im Vergleich zu Zuckern auch eine geringere Energiedichte. Daher wurde die Acetatassimilation sowohl in Batch als auch in kontinuierlichen Kulturen untersucht. Acetat lag dabei entweder als einzige Kohlenstoffquelle oder zusammen mit Glucose vor, um die Energieverfügbarkeit zu erhöhen. Die Überexpression von Acetyl-CoA Synthetase, eines zweier Enzyme für die Acetatassimilation, verbesserte die Acetataufnahme sowie die gleichzeitige Verwertung mit Glucose im Batch. Die

Eignung von *E. coli* W als effizienter Wirt für die Acetatverwertung wurde durch hohe Aufnahmeraten im Batch und kontinuierlichen Prozess bewiesen.

Isobutanol wird von *E. coli* nicht natürlich produziert und rekombinante Produktion wurde in dieser Arbeit durch systematische Stammentwicklung, im Konkreten durch Untersuchung verschiedener Expressionsvektoren sowie Auswahl eines geeigneten Wirts, *E. coli* W, erreicht. Durch Einbeziehung der chemischen Eigenschaften von Isobutanol wurde ein Produktionsprozess entwickelt, der die effiziente Verwertung von sowohl Glucose im definierten Medium als auch dem Abfallstoff Molke ermöglichte, wobei hier eine finale Isobutanol Konzentration von 20 g l^{-1} erreicht werden konnte. Niedrige Produktausbeuten in Kombination mit der Flüchtigkeit von Isobutanol verhinderten jedoch eine weitere Entwicklung der Isobutanolproduktion aus Acetat. Es wurde daher die 2,3-Butandiolproduktion als Plattform herangezogen, um Wissen zur Produktion einer Chemikalie aus Acetat zu generieren. Es wurde ein chemisch definiertes Medium entwickelt, dass es erlaubte einen Prozess zu etablieren, wo Acetat als einzige Kohlenstoffquelle zur Produktion von 1.16 g l^{-1} Diolen (Acetoin und 2,3-Butandiol) führte.

Diese Arbeit zeigte daher die Realisierbarkeit der netto CO_2 Fixierung zur Produktion einer Treibstoffchemikalie in einem neuen zweistufigen Prozess. Die zeitaufgelöste Charakterisierung von physiologischen Parametern zusammen mit rationalem Prozess- und Screeningdesign erlaubten es Wissen über Acetatproduktion aus einem Industrieabgas sowie Acetatassimilation für mikrobielle Produktion zu generieren. Während Stammauswahl und -engineering ein wesentlicher Erfolgsfaktor für die Aufwertung von Acetat sowie die Umwandlung in eine Treibstoffchemikalie waren, diente die Quantifizierung im physiologischen Gleichgewicht, sowie dynamische Veränderungen dazu, limitierende Faktoren zu bestimmen. Aufgrund seiner flexiblen Eigenschaften kann die Plattformtechnologie, die in dieser Arbeit etabliert wurde, einen wichtigen Grundstein für die weitere industrielle CO_2 Fixierung legen. Das Mischen zweier unabhängiger Gasströme kann als allgemeine Strategie zur Einstellung von Gas Zusammensetzungen für die CO_2 Fixierung dienen und damit die Reduktion von industriellen CO_2 Emissionen ermöglichen. In gleicher Weise haben die präsentierten Strategien für die Aufwertung der alternativen Rohstoffquelle Acetat Potenzial für die Etablierung einer Kreislaufwirtschaft am Weg zu einer CO_2 neutralen Zukunft.

Content

I. INTRODUCTION

1. Background

1.1. Climate change and CO_2 emissions

Extraction and combustion of fossil resources to produce fuels, chemicals and energy has led to an increase in atmospheric CO_2 concentrations to the highest levels in ~ 3 Mio. years (Wuebbles et al., 2017). Global climate change, triggered by these CO_2 emissions, is one of two energy-related challenges of the 21st century (Appel et al., 2013), which is why the global community set goals to reduce atmospheric CO_2 release in the Paris Agreement in December 2015 (Köpke and Simpson, 2020; United Nations, 2015). Limiting global warming to 1.5 °C will require global net CO_2 emissions to reach zero by 2050 (Rogelj et al., 2018). To that end, fossil fuels will have to be replaced by "above ground" carbon and carbon cycles will have to be closed by establishing a circular economy (Bengelsdorf and Dürre, 2017; Köpke and Simpson, 2020; Liew et al., 2016).

It is a promising start that since 1990, the European Union was able to reduce their greenhouse gas (GHG) emission by 23 %, thus surpassing the target of 20 % by 2020, but further measures will be required (*Energy Policy Review*, 2020). In Austria just like in the European Union, the major part of greenhouse gas is emitted by the energy- and industrial sector (*Energy Policy Review*, 2020; *Klimaschutzbericht*, 2019). One of the biggest industrial CO_2 emitters is the iron and steel industry, which accounts for 4 % to 7 % of anthropogenic CO_2 emission in the European Union and 16 % in Austria (*Klimaschutzbericht*, 2019; Pardo and Moya, 2013). A variety of exhaust gases containing CO_2, CO and methane are produced during the steel milling process (Molitor et al., 2016). Other industries producing flue gas streams containing CO_2 or CO are the ferroalloy industries, refineries and chemical plants (Heijstra et al., 2017; *Klimaschutzbericht*, 2019).

1.2. Renewable energy sources

The second energy-related problem of mankind in the 21st century is the depletion of fossil fuels (Appel et al., 2013). For a switch to non-fossil energy, strategies for storage, transport and the availability of these energy sources will have to be developed (Appel et al., 2013).

In Austria, 80 % of the energy produced, but only 33.5 % of the energy consumed is derived from renewable resources (*Energie in Österreich 2018 - Zahlen, Daten, Fakten*, 2018). With this share, Austria is one of the countries reaching their EU goal for 2020 (*Energie in Österreich 2018 - Zahlen, Daten, Fakten*, 2018; *Klimaschutzbericht*, 2019). The average share of renewable energy in the European Union was 19 % in 2019 an thus only slightly below the 20 % goal for 2020 (*Energy Policy Review*, 2020). Around 35 % of the total Austrian energy demand is required in the transportation sector, relying mainly on

hydrocarbon fuels since those are cheap, easily storable, and transportable with the existing infrastructure (*Energie in Österreich 2018 - Zahlen, Daten, Fakten*, 2018). The European Union had targeted a 10 % share of renewable biofuels in the transportation sector by 2020, but has reached only 8 % (*Energy Policy Review*, 2020; Official Journal of the European Union, 2009).

Renewable electricity generated from wind, hydro and solar power can be used directly as an energy source. However, storage is not easily feasible, leading to the evolvement of Power-to-X technologies (*Energy Policy Review*, 2020; Foit et al., 2017; Lund et al., 2015). To date, H_2 is generated mainly from natural gas, as this is a rather cheap method (Arregi et al., 2018; Yukesh Kannah et al., 2021). The production of H_2 from excess electrical energy via water electrolysis is a relatively mature technology and low-emission generation will improve as the availability of renewable energy increases and costs decline (Haas et al., 2018; Köpke and Simpson, 2020). Other methods for the production of renewable H_2 include thermochemical or biochemical utilization of biomass and conversion of solar energy as heat or via photolysis (Dou et al., 2019). Hydrogen is generally regarded a promising future electron carrier, since it is a clean, "zero-carbon" energy source (Das and Veziroglu, 2008; Hallenbeck and Ghosh, 2009; Yukesh Kannah et al., 2021).

To sum up, a strategy combining the use of renewable energy sources, efficient energy storage and low CO_2 emission industrial plants is required for the transition towards a "zero CO_2" future.

1.3. Alternative renewable feedstocks

With emerging development of microbial cell factories producing chemicals of interest, the interest in the use of alternative feedstocks has increased. These feedstocks should fulfil two main requirements: (i) they should be cost-effective in order to obtain a process competitive to production from fossil fuels (Lim et al., 2018) and (ii) competition with arable land should be avoided (Havlík et al., 2011; Scarlat et al., 2008). Potential streams for such feedstocks include lignocellulosic biomass as well as industrial, landfill and residual waste streams (Wolf et al., 2005).

Lignocellulosic biomass has to be pretreated heavily to access the fermentable sugars and a large proportion of carbon remains unused (Jönsson and Martín, 2016). Therefore, the gasification of biomass to syngas containing CO, CO_2, H_2 and N_2 is a promising alternative, since it allows for the utilization of nearly all available carbon (Liew et al., 2016). Traditionally, syngas is further converted into hydrocarbons via the Fischer-Tropsch synthesis operated at high pressure and temperature (Takors et al., 2018), but recently, microbial gas fermentation technology has recently emerged as a promising alternative (Köpke and Simpson, 2020; Liew et al., 2016; Takors et al., 2018).

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The use of waste streams for microbial chemical production is considered promising not only due to the presence of sugars and proteins, but also because it can help to reduce the ecological footprint (Matsakas et al., 2014; Novak and Pflügl, 2018). The utilization of these waste streams is complicated by anaerobic digestion reducing available sugars, heterogeneity and variations in composition (Pfaltzgraff et al., 2013). However, the utilization of cheese whey has been shown to enable recombinant protein production as well as butanol production (Hausjell et al., 2019; Qureshi and Maddox, 2005). The dairy industry produces large amounts of cheese whey and its disposal poses environmental burden due to a high organic content (Guimarães et al., 2010; Yadav et al., 2015). Cheese whey mainly consists of lactose, proteins and peptides and is therefore a promising alternative feedstock (Hausjell et al., 2019).

1.4. Biological CO_2 fixation

Costs for CO_2 emission certificates and criticism on the use of fossil fuels have increased industrial interest in the investigation and development of biological CO_2 fixation technology and microbial chemical production from C1 substrates (Takors et al., 2018).

The advantage of biological CO_2 fixation in comparison to physical processes such as direct mineral carbonation is that, in addition to the reduction of CO_2 concentrations, valuable chemicals can be produced (Mistry et al., 2019). Conversion of CO_2 requires the input of energy in the form of electrons, since carbon atoms in CO_2 are present in their most oxidized form (Appel et al., 2013; Gong et al., 2018; Liew et al., 2016). Energy availability can be a bottleneck in biological CO_2 fixation processes and possible sources include light and reduced compounds such as H_2, H_2S or other inorganic compounds (Gong et al., 2018; Nybo et al., 2015). CO_2 fixation was also investigated by using electricity as a direct energy source in a process termed microbial electrosynthesis (MES) (Philips, 2020; Rabaey and Rozendal, 2010).

Pathways for biological CO_2 fixation have evolved over billions of years and use diverse mechanisms and enzymes to process CO_2 (Appel et al., 2013). The most prominent pathway for CO_2 fixation is the Calvin-Benson-Bassham (CBB) cycle in plants, algae and cyanobacteria and accounts for 90 % of global carbon fixation (Bassham et al., 1950; Fuchs, 2011; Gong et al., 2018). There, CO_2 is converted into organic matter using light as an energy source (Razzak et al., 2017). The key enzyme for CO_2 fixation via the CBB cycle is RuBisCo (Ribulose bisphosphate carboxylase/oxygenase), one of the most abundant proteins on Earth (Raven, 2013). The performance of this enzyme is hampered by its low affinity towards CO_2 and its additional function as oxygenase leading to photorespiration instead of photosynthesis (Mistry et al., 2019). Engineering the process of photorespiration has therefore been a target for reducing carbon loss (Trudeau et al., 2018). Generally, the

photosynthetic efficiency is low, resulting in the loss of solar energy (Work et al., 2012). For instance, the energy conversion efficiencies for production of oil by microalgae are below 2 % (Santero et al., 2016). Despite these energetic constraints, cyanobacteria and algae were successfully established for CO_2 fixation and production of biofuels and chemicals such as polyhydroxybutyrate (PHB) (Brennan and Owende, 2010; Chisti, 2007; Kamravamanesh et al., 2017). Although photoautotrophic CO_2 allows for the synthesis of high-value chemicals, industrial competitiveness is challenged by slow cell growth, inefficient protein expression and low chemical productivity (Gong et al., 2018). Additional hurdles on the way towards industrial implementation of photoautotrophs for CO_2 fixation and production of valuable chemicals include physiological factors such as growth and production being inhibited at high CO_2 concentrations, low utilization of CO_2 and limited tolerance towards impurities in flue gas streams. The operation of photoautotrophic CO_2 fixation plants are challenged by the high capital and operating cost of such cultivation systems, the scale-up being limited by the poor permeability of light in microbial cultures and the high cost for downstream processing (Anand et al., 2020; Gong et al., 2018). One opportunity to increase competitiveness of CO_2 fixation is to make use of mixed-substrate conversion systems, where CO_2 is utilized together with an abundant co-substrate such as glucose, glycerol or methanol (Steiger et al., 2017).

CO_2 fixation was even established in heterotrophic organisms by introducing a heterologous CBB cycle (Gassler et al., 2020; Gleizer et al., 2019). In *Pichia pastoris*, CO_2 was used as the sole carbon source, while methanol solely served as an energy source since methanol assimilation was blocked (Gassler et al., 2020). Similarly, *E. coli* was turned into a full autotroph capable of using CO_2 as the sole carbon source and formate, which can be produced electrochemically as an energy source (Gleizer et al., 2019). In both studies, decoupling energy and carbon metabolism was crucial to obtain biomass formation from CO_2. In contrast to photosynthetic CO_2 fixation, which requires expensive and specific bioreactor equipment, processes using chemical, potentially renewable energy sources can be directly applied in standard large-scale bioreactor set ups (Cotton et al., 2020; Olah, 2013).

In addition to the CBB cycle, five other natural CO_2 fixation pathways have been discovered: the 3-hydroxypropionate bicycle and the 3-hydroxypropionate-4-hydroxybutyrate cycle also function aerobically, whereas the reductive tricarboxylic acid cycle, the dicarboxylate/4-hydroxybutyrate cycle and the Wood-Ljungdahl pathway contain oxygen-sensitive enzymes and work anaerobically (Gong et al., 2018, 2016). All CO_2 fixation pathways apart from the CBB cycle comprise the reduction of two molecules of CO_2 to acetyl-CoA (Fuchs, 2011). The Wood-Ljungdahl, also termed reductive acetyl-CoA pathway, is the only linear pathway

(Drake et al., 2008) and considered to be the oldest (Adam et al., 2018) and most efficient CO_2 fixation mechanism (Fast and Papoutsakis, 2012; Liew et al., 2016).

1.5. Acetogenic microorganisms

Acetogenic bacteria are a group of chemolithoautotrophic microorganisms capable of producing acetate from CO_2. During autotrophic growth, H_2 serves as an electron donor (Schuchmann and Müller, 2014). Acetogens can also utilize CO by performing an internal water-gas shift reaction, but the oxidation of CO is accompanied by CO_2 formation (Adam et al., 2018; Köpke and Simpson, 2020; Ragsdale and Pierce, 2008). In addition to autotrophic growth, acetogens can utilize a broad variety of organic substrates such as hexoses and pentose sugars, alcohols, and organic acids. This metabolic flexibility gives acetogenic bacteria an economical advantage, which is why they are omnipresent in nature (Drake et al., 2008; Ragsdale and Pierce, 2008; Schuchmann and Müller, 2016). Acetate is the main product of all acetogens, especially during growth on CO_2 and H_2, but other valuable chemicals such as ethanol, 2,3-butanediol, butanol, butyrate, hexanol and hexanoate can also be produced, preferably in the presence of CO (Bruant et al., 2010; Hess et al., 2015; Köpke et al., 2011; Liou et al., 2005; Phillips et al., 2015; Richter et al., 2016).

Acetogenic bacteria are very suitable for the utilization of gaseous C1 substrates (Köpke and Simpson, 2020; Takors et al., 2018). Potential gas sources include CO and CO_2 containing gas streams such as industrial waste gases or syngas (Köpke and Simpson, 2020). Due to the broad product spectrum in combination with a high product selectivity, gas fermentations can serve as an alternative to chemical synthesis, i.e. the Fischer-Tropsch synthesis. The main advantages of gas fermentations are lower temperature and pressure in combination with an increased tolerance towards variability in the gas composition and contaminants (Heijstra et al., 2017; Liew et al., 2016; Takors et al., 2018). Anaerobic conditions applied during gas fermentation reduce the flammability of gases and make biological contaminations less likely (Liew et al., 2016).

What all acetogenic organisms have in common is that they use the Wood-Ljungdahl pathway (WLP, **Figure 1**), also known as the reductive acetyl-CoA pathway for CO_2 fixation (Drake et al., 2008; Ragsdale and Pierce, 2008). The WLP consists of two branches, the methyl, and the carbonyl branch. In the methyl branch, one molecule of CO_2 is reduced to formate, which is then activated to formyl-tetrahydrofolate (THF) by consumption of one molecule of ATP. Electrons for the reduction of CO_2 to formate are either derived directly from H_2 or from a redox equivalent, depending on the acetogenic organism (Mock et al., 2015; Schuchmann and Müller, 2014). Formyl-THF is further reduced to methyl-THF by an enzyme cascade and the methyl group is transferred to a corrinoid iron-sulfur-containing

protein (CoFeSP). Another molecule of CO_2 is reduced to CO in the carbonyl branch and fused to the methyl-group to form acetyl-CoA by the CO dehydrogenase/acetyl-CoA synthase complex (CODH/ACS) (Ljungdhal, 1986; Ragsdale, 2008). During growth on CO alone, the CO_2 for the methyl branch is generated by oxidation of CO via CODH (Liew et al., 2016). During heterotrophic growth, the CO_2 released can be assimilated via the WLP thus increasing product yields (Drake et al., 2013; Fast et al., 2015; Jones et al., 2016). Acetyl-CoA is further converted into acetate by a phosphotransacetylase and an acetate kinase (Ljungdhal, 1986; Ragsdale, 2008). In this process, one molecule of ATP is produced via substrate level phosphorylation. (Schuchmann and Müller, 2014).

The WLP is the only pathway for CO_2 fixation where input of ATP is not required. A chemiosmotic mechanism is present in acetogens for ATP synthesis (Schuchmann and Müller, 2014). Especially in acetogens lacking cytochromes, the exact mechanism had not been clear until a few years ago, when the *Rhodobacter* nitrogen fixation complex (Rnf) was discovered in *A. woodii* (Müller et al., 2008). This multi-subunit oxidoreductase catalyzes electron transfer from reduced ferredoxin to NAD^+, thereby allowing for the translocation of sodium against the transmembrane potential. This electrochemical sodium gradient is then used to drive ATP synthesis via a membrane bound ATPase (Biegel et al., 2011, 2009; Biegel and Müller, 2010; Heise et al., 1992, 1991; Matthies et al., 2014; Westphal et al., 2018). The same mechanism with a proton instead of a sodium gradient was found in *Clostridium ljungdahlii*, *Clostridium autoethanogenum* and *Clostridium aceticum* (Hess et al., 2016; Köpke et al., 2010; Mock et al., 2015). Other acetogens such as *Morella thermoacetica* possess cytochromes and a membrane-bound energy converting hydrogenase (Ech) for building up the ion gradient (Huang et al., 2012). Acetogens are therefore classified according to their energy-conserving enzymes (Rnf or Ech) and the ion translocated (sodium or a proton) (Schuchmann and Müller, 2014).

The reduction of CO_2 in the WLP requires the input of electrons in the form of reducing equivalents. During autotrophic growth on CO_2 and H_2, the reducing power must be derived from H_2 (Schuchmann and Müller, 2014). Reducing power in the form of the reducing equivalents NADH and reduced ferredoxin is provided through electron transfer from H_2. The basis of this reaction is flavin-based electron-bifurcation. In short, electrons are transferred from H_2 to NAD^+ and ferredoxin, yielding NADH and reduced ferredoxin in equal amounts. The endergonic reduction of ferredoxin is enabled by the exergonic reduction of NAD^+ (Herrmann et al., 2008; Schuchmann and Müller, 2014, 2012). Several electron bifurcating enzymes have been identified and these include bifurcating hydrogenase complexes in *A. woodii*, *M. thermoacetica* and *C. autoethanogenum* (Huang et al., 2012; Schuchmann and Müller, 2012; Wang et al., 2013b, 2013a) the NADH-dependent reduced ferredoxin:NADP oxidoreductase (*Nfn*) in *C. autoethanogenum* (Mock et al., 2015). And the

methylene-THF reductase of *C. autoethanogenum* (Valgepea et al., 2018, 2017). The confurcating lactate dehydrogenase of *A. woodii* also uses a bifurcating mechanism to drive endergonic lactate oxidation (Weghoff et al., 2015).

Genes of the WLP pathway are highly conserved in all acetogens (Poehlein et al., 2015), while there are major differences in some key enzymes such as the electron-bifurcating hydrogenase, the electron-bifurcating *Nfn* and the aldehyde:ferredoxin oxidoreductase (AOR) (Mock et al., 2015; Poehlein et al., 2015; Schuchmann and Müller, 2014). These genetic variations in combination with deviations in energy conservation also account for different product spectra as well as the general growth behavior of acetogens (Bertsch and Müller, 2015a; Mock et al., 2015). In detail, product formation in acetogens depends on: (i) whether CO or CO_2 and H_2 are utilized, (ii) the ATP gain during the formation of acetyl-CoA or acetate, (iii) the amount of ATP required for or produced from the subsequent reaction towards a product and (iv) the presence of functional enzymes that catalyze the reaction to a product (Bertsch and Müller, 2015a). As an example, *A. woodii*, *C. aceticum* and *M. thermoacetica* produce mainly acetate under autotrophic conditions, whereas *C. carboxidivorans*, *C. ljungdahlii* and *C. autoethanogenum* are able to produce more reduced compounds such as ethanol, 2,3-butanediol and butanol (Liew et al., 2016). The latter organism has successfully been established as a commercial strain for ethanol production from steel mill flue gases by the company LanzaTech (Heijstra et al., 2017).

Synthetic biology and metabolic engineering carry the potential to improve the efficiency of gas fermentations as well as to extend the product spectrum (Jin et al., 2020; Latif et al., 2014; Liew et al., 2016). For example, non-native butanol production from CO was successfully established in *C. autoethanogenum* and *C. ljungdahlii* by expression of the pathway from *C. acetobutylicum* (Köpke et al., 2010; Köpke and Liew, 2011). Similarly, heterologous acetone biosynthesis from either H_2 and CO_2 was shown in *C. aceticum*, *C. ljungdahlii* and *A. woodii* (Banerjee et al., 2014; Hoffmeister et al., 2016; Schiel-Bengelsdorf and Dürre, 2012). Upgrading of acetone produced from CO to isopropanol was found to depend on the redox state in *C. ljungdahlii* and *C. autoethanogenum* (Köpke et al., 2012) and the production of several additional high value molecules using gas fermentation has been developed by LanzaTech (Heijstra et al., 2017). Metabolic engineering was not only used to extend the product spectrum, but also to improve the efficiency of native production, e.g. by overexpressing enzymes of the WLP of *C. ljungdahlii* in *A. woodii* (Straub et al., 2014). Deletion of the gene *budA* involved in 2,3-butanediol production eliminated this by-product and resulted in increased ethanol production in *C. autoethanogenum* (Koepke et al., 2014). A very efficient tool for strain engineering is the use of genome-scale metabolic models in combination with the collection of omics datasets (Humphreys and Minton, 2018; Latif et al., 2014; Vees et al., 2020). As an example, a genome-scale metabolic

reconstruction of *C. ljungdahlii* was used to propose targets for deletions and overexpression aiming for increased product formation (Chen and Henson, 2016). Metabolic models can also be used to improve productivity by optimizing cultivation conditions. A model based on thermodynamic and nutrient limitations as well as pH effects was proposed for instance for *C. autoethanogenum* (Richter et al., 2016). In the same organism, metabolic modelling improved CO_2 utilization by adapting the feed gas composition (Heffernan et al., 2020). As genetic engineering tools for acetogens emerge, the efficiency of gas fermentations will increase even further (Heijstra et al., 2017).

1.6. *Acetobacterium woodii*

A. woodii was first isolated from black sediment in an Oyster pond in Massachusetts (US) in 1977 (Balch et al., 1977) and is one of the best studied acetogens today, serving as a model-organism (Biegel et al., 2009; Braun and Gottschalk, 1981; Godley et al., 1990; Schuchmann and Müller, 2016, 2014; Westphal et al., 2018; Wiechmann et al., 2020). *A. woodii* is a homoacetogenic bacterium, meaning that its main product is acetate (Diekert and Wohlfarth, 1994). However, ethanol is produced under phosphate limitation or when acetaldehyde is the carbon source (Buschhorn et al., 1989; Trifunović et al., 2020). Propionic acid and 1-propanol is formed during growth on 1,2-propanediol (Schuchmann et al., 2015). Under sodium limiting conditions, formate becomes the main product (Müller, 2019). *A. woodii* is known to efficiently utilize CO_2 and H_2 (Kantzow et al., 2015; Schuchmann and Müller, 2012; Takors et al., 2018). In contrast to other acetogenic bacteria, CO cannot be used as the sole carbon and energy source and its utilization is only possible in the presence of CO_2 and H_2 or formate (Bertsch and Müller, 2015b; Müller, 2019). In addition to autotrophic growth, *A. woodii* can utilize a broad variety of organic substrates such as formate, methanol, ethanol, lactate, fructose, glucose, 2,3-butanediol, etc. (Bainotti and Nishio, 2000; Balch et al., 1977; Bertsch et al., 2016; Bertsch and Müller, 2015b; Braun and Gottschalk, 1981; Drake et al., 1997; Eichler and Schink, 1984; Godley et al., 1990; Hess et al., 2015; Kremp et al., 2018; Peters et al., 1998; Schuchmann et al., 2015; Schuchmann and Müller, 2016). The metabolic flexibility is further increased by the ability of *A. woodii* to simultaneously utilize organic substrates and CO_2 (i.e. mixotrophy) (Braun and Gottschalk, 1981; Demler, 2012a; Godley et al., 1990; Peters et al., 1998). During heterotrophic growth, H_2 occurs as an intermediate, and a new mechanism for intracellular H_2 cycling was recently described (Wiechmann et al., 2020). Alternative electron acceptors such as aromatic acrylates (e.g. caffeate) can be metabolized by *A. woodii* instead of CO_2 (Dilling et al., 2007; Tschech and Pfennig, 1984).

A. woodii was the first acetogen in which energy conservation was elucidated and it is classified as an Rnf and sodium dependent acetogen (Fritz and Müller, 2007; Müller et al., 2008; Schuchmann and Müller, 2014). A membrane-integral ATPase catalyzes the

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generation of 1 mol ATP per 3.3 mol sodium ions (Fritz and Müller, 2007; Müller et al., 2001) and the sodium gradient is driven by the Rnf complex, which catalyzes electron transfer from reduced ferredoxin to NAD^+ (Biegel et al., 2011, 2009; Biegel and Müller, 2010; Westphal et al., 2018). This complex was found to be the only coupling site between energy and redox metabolism of *A. woodii* and is essential for growth on H_2 and CO_2 as well as on low energy substrates (Schuchmann and Müller, 2014; Westphal et al., 2018). Therefore, acetogenic metabolism must be designed to have a high ratio of reduced ferredoxin to NADH (i.e. more reactions that reactions producing reduced ferredoxin) to achieve net ATP production. Reduced ferredoxin can be considered a high-energy intermediate and ATP generation depends on the reactions that produce reduced ferredoxin (Bertsch and Müller, 2015a; Schuchmann and Müller, 2014). During autotrophic growth, reduced ferredoxin is generated from H_2 via the electron-bifurcating hydrogenase, HydABCD, a tetrameric soluble enzyme that catalyzes the reduction of ferredoxin and NAD^+ by oxidation of H_2 (Schuchmann and Müller, 2012). Both reducing equivalents, NADH and reduced ferredoxin are required for CO_2 reduction in the methyl and carbonyl branch of the WLP, respectively (**Figure 1**). The reduction of CO_2 to formate in the first step of the methyl branch of the WLP is catalyzed by a hydrogen-dependent CO_2 reductase (HDCR) in *A. woodii*. Direct use of H_2 as an electron donor by this enzyme instead of a redox equivalent is energetically advantageous for the cell (Schuchmann and Müller, 2014, 2013). The HDCR is inhibited by CO, but this inhibition is completely reversible (Ceccaldi et al., 2017; Ragsdale and Ljungdahl, 1984). Finally, the reduction of 2 mol CO_2 with 4 mol H_2 results in the production of 1 mol acetate and the generation of 0.3 mol ATP (**Figure 1**) (Bertsch and Müller, 2015a; Schuchmann and Müller, 2014). This low ATP gain allows *A. woodii* to grow at low H_2 concentrations and is mainly responsible for the enhanced H_2 utilization characteristics of *A. woodii* compared to other acetogens (Mock et al., 2015).
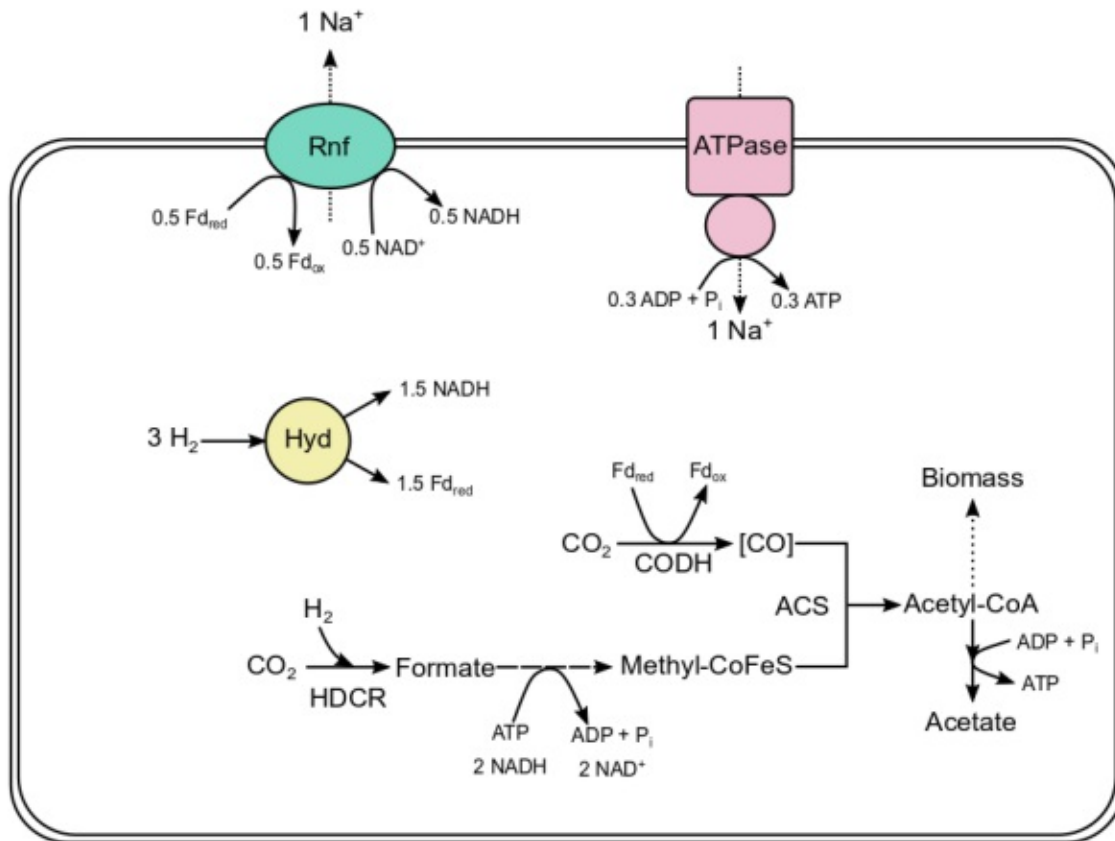
Figure 1 – Acetogenesis in *A. woodii*. The Wood-Ljungdahl pathway is shown with enzymes for redox balancing and energy conservation. Rnf = *Rhodobacter* nitrogen fixation complex, Hyd = Hydrogenase, HDCR = Hydrogen dependent CO_2 reductase, CODH = CO dehydrogenase, ACS = Acetyl-CoA synthase.

1.7. Using acetogenic bacteria for upgrading C1 gases to value-added chemicals

The production of chemicals from C1 gases has until now mainly relied on direct conversion in acetogenic bacteria. In these one-step processes, ethanol or other reduced products are directly produced in acetogenic bacteria, mainly from CO (Heffernan et al., 2020; Hermann et al., 2020; Valgepea et al., 2018). CO and CO_2 fixation in these processes typically yield mixed products or acetate as the sole product, depending on the acetogenic organism used. Although reduced chemicals are provided by the former scenario, the latter is characterized by its robustness towards varying feed gas composition and its superior controllability. Additionally, formation of acetate increases flexibility since production can aim for any other product in a subsequent step.

Alternatively, the establishment of a two-step process with two microbial systems with different properties can be advantageous compared to one-step production. Making use of a second organism, the product spectrum can be enhanced, or a substance can be produced with less side products. Especially, when aiming for the utilization of CO_2, the

production of more reduced products is difficult to achieve (Liew et al., 2016; Mock et al., 2015). Co-cultures or mixed cultures can be promising due to symbiotic relationships as well as increased substrate utilization and product yields, but might be hampered due to the difficulty in understanding and controlling these systems (Liew et al., 2016). A secondary process can combine the high product selectivity of an anerobic process with the high product diversity of an aerobic process (Hu et al., 2016; Köpke and Simpson, 2020). Recently, such processes have been presented: in one process, acetate produced from CO_2 and H_2 by *M. thermoacetica* was converted into lipids or isopropanol with *Yarrowia lipolytica* (Hu et al., 2016) and in another process, acetate from syngas fermentation was upgraded to isopropanol in *E. coli* (Yang et al., 2020).

1.8. Microbial upgrading of acetate in *E. coli*

E. coli is a biotechnological workhorse and belongs to the best-studied microorganisms with the focus on recombinant protein as well as bulk chemical production (Erian et al., 2018; Ferrer-Miralles et al., 2015; Kopp et al., 2017; Ohta et al., 1991; Wurm et al., 2018). Glucose remains the most-utilized substrate but the assimilation of other substrates such as pentoses, glycerol and acetate has also been studied (Alterthum and Ingram, 1989; Jeon et al., 2016; Kopp et al., 2017; Nagata, 2001; Trinh et al., 2008; Valgepea et al., 2010; Xu et al., 2017).

In *E. coli*, there are different routes for acetate uptake and production. Acetate assimilation is either mediated by the low-affinity *pta-ackA* node (phosphate acetyltransferase – acetate kinase) or the high-affinity irreversible acetyl CoA synthetase (*acs*) pathway (Oh et al., 2002; Wolfe, 2005). The second way enables *E. coli* to utilize even small amounts of acetate (Brown et al., 1977; Kumari et al., 1995) and its activity is tightly regulated on both a transcriptional and a post-translational level (Enjalbert et al., 2017; Starai et al., 2002; Starai and Escalante-Semerena, 2004; Wolfe, 2005). Another way for acetate formation is direct oxidation of pyruvate by pyruvate dehydrogenase (*poxB*) (Enjalbert et al., 2017).

Acetate has been upgraded to a variety of bulk chemicals and these have been summarized in the current opinion article (Novak and Pflügl, 2018). Among those promising chemicals, two should be further highlighted: isobutanol and 2,3-butanediol.

Isobutanol is a C4 chemical and considered an interesting fuel chemical, since its use has advantages over the utilization of shorter chain alcohols. Compared to ethanol, isobutanol has a higher energy density, is better miscible with gasoline and can be transported with the existing infrastructure (Atsumi et al., 2010; Tao et al., 2014). Isobutanol is also considered to be advantageous over n-butanol production, since it has a higher octane number, is less toxic to cells and requires less energy for downstream processing (Tao et al., 2014). Other applications include the use of isobutanol as precursors for esterification

of phthalic, adipic and dicarboxylic acids and to isobutyl acetate. These esters are used as common plasticizers and solvents (Hahn et al., 2000). Industrially, isobutanol is produced by carbonylation of propylene and further hydrogenation of isobutyraldehydes (Hahn et al., 2000). Isobutanol is not naturally synthesized by any organism, but its production has been shown feasible by combining valine biosynthesis with the Ehrlich pathway (Atsumi et al., 2008). Efficient isobutanol production from glucose was established on media containing significant amounts of complex media additives (Baez et al., 2011). Generation of isobutanol from acetate was generally shown to be feasible, although at very low product titers (Song et al., 2018).

Another promising platform chemical is the C4 compound 2,3-butanediol, which can be used as an anti-freezing agent or as a food-additive (Magee and Kosaric, 1987; Tran and Chambers, 1987). Usually produced from fossil resources, 2,3-butanediol serves as a precursor for asymmetric synthesis of different chemicals including methyl ethyl ketone, gamma-butyrolacetone and 1,3-butadiene (Celińska and Grajek, 2009; Xiu and Zeng, 2008). The heating value of 2,3-butanediol is 93 % of that of ethanol and 2,3-butanediol can therefore serve as a liquid fuel or a fuel additive (Tran and Chambers, 1987). So far, 2,3-butanediol has only been manufactured in very small quantities via chemical synthesis using butene separated from cracked gas (Song et al., 2019). After chemical production, 2,3-butanediol is present as three isomers and expensive separation and purification processes are required to obtain stereospecific 2,3-butanediol (Song et al., 2019; Yang and Zhang, 2019). Since microbial synthesis usually yields optically pure isoforms, interest in biological 2,3-butanediol production has recently risen (Celińska and Grajek, 2009; Song et al., 2019; Yang and Zhang, 2019). However, its high boiling point and high affinity towards water makes separation and purification of 2,3-butanediol from fermentation broth challenging (Xiu and Zeng, 2008). Therefore, several purification methods including steam stripping, solvent extraction, reverse osmosis and pervaporation have been investigated to reduce energy consumption and increase product recovery (Qureshi et al., 1994; Shao and Kumar, 2009; Sridhar, 1989; Wheat et al., 2011). During microbial production of 2,3-butanediol, acetoin serves as a precursor. Industrially, this chemical can be used as a flavor enhancer and a chemical building block (Xiao et al., 2009; Xiao and Lu, 2014). 2,3-butanediol and acetoin are naturally produced in several microorganisms including *C. ljungdahli*, *C. autoethanogenum*, *Klebsiellla oxytoca* and *Enterobacter cloacae* (Ji et al., 2010; Köpke et al., 2011; Li et al., 2015). Production of 2,3-butanediol has been established on glucose and other sugar sources (Erian et al., 2020, 2018; Li et al., 2010; Xu et al., 2014).

Apart from being the main product of gas fermentation with acetogens, acetate is also present in a variety of renewable feedstocks and waste streams and microbial upgrading of

acetate is therefore an emerging research field. To further highlight the potential of acetate as a promising alternative feedstock, current knowledge on microbial upgrading of acetate was summarized in a current opinion article.

Title of manuscript

Towards biobased industry: acetate as a promising feedstock to enhance the potential of microbial cell factories

Content description and contribution

In this current opinion article, the potential of acetate as an alternative feedstock is highlighted. Acetate is present in different low-cost feedstocks such as lignocellulosic hydrolysates, anaerobic sludge, and food waste. Additionally, acetate is produced in acetogenic bacteria by utilization of gaseous streams containing CO_2, H_2 and/or CO. The results from different publications on microbial production from acetate alone or in combination with another carbon source are summarized and discussed. The importance of the selection, manipulation, and improvement of suitable host organisms for acetate (co-) utilization is emphasized. Deeper insights into acetate uptake and metabolism should help drive the development of more efficient cell factories.

KN and SP performed the literature research and drafted the manuscript.

Citation

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CURRENT OPINION – Biotechnology & Synthetic Biology

Towards biobased industry: acetate as a promising feedstock to enhance the potential of microbial cell factories

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One sentence summary: Acetate is discussed as a promising feedstock to improve microbial cell factories for production of value-added chemicals and other compounds towards biobased industry.
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ABSTRACT

A broad range of different chemical and pharmaceutical compounds have been produced in microbial cell factories. To compete with traditional crude oil based production processes, the use of complex alternative raw materials such as lignocellulosic biomass, waste streams and utilization of CO_2 in gas fermentations has been suggested. All of these streams contain acetate, a cheap and potentially interesting carbon source for microbial production processes. Acetate (co-)utilization remains challenging, which is the reason for extensive research on the use of acetate for the production of value-added compounds. For industrial implementation of microbial conversion processes using acetate as a feedstock gaining a deeper insight into acetate metabolism of microorganisms is essential. Systems level analyses and manipulation of potential host organisms should be applied to achieve full utilization of this prospective substrate.

Keywords: acetate metabolism; metabolic engineering; microbial cell factories; acetate utilization; co-utilization of glucose and acetate; Escherichia coli

INTRODUCTION

Extensive research efforts are underway to develop microbial cell factories for the production of proteins and chemicals of interest (Ohta et al. 1991; Atsumi et al. 2010; Nielsen et al. 2010; Lee et al. 2012; Eggenreich et al. 2016). With the advent of recombinant DNA technologies, the speed of development of production platforms has been further increased. That way, biobased production of a wide variety of different compounds using an array of different host organisms has been achieved. As the focus for most of these studies was proof-of-principle, they rely on expensive pure carbon substrates and complex media compo-

nents. However, the biobased production of chemicals for everyday products as well as pharmaceutical products requires economically sensible production routes. In order to achieve competitiveness with crude oil based production, cheap substrates are necessary (Lim et al. 2018), which include but are not limited to hydrolysates from lignocellulosic biomass, industrial and landfill waste streams and streams containing acetate derived from industrially available CO_2 (Crank et al. 2005). These streams represent, either alone or in combination, mixed feed systems. These systems contain a number of different carbon sources including hexoses, pentoses and organic acids such as acetate (Ko et al. 2015; Jönsson and Martín 2016). Acetate represents an

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Table 1. Examples of potential acetate sources with approximate acetate concentrations of individual streams.

Source	Process	Acetate concentrations	Reference
	Potential sources of acetate		
Lignocellulosic biomass	Hydrolysis, typical process	1 - > 10 g/l	(Mills, Sandoval and Gill 2009)
Waste	Anaerobic digestion, typical process	<15 g/l	(Braun 2013)
Industrial CO_2 and H_2	Batch gas fermentation with A. woodii (30°C, 1200 rpm, 0.5 vvm. 1 bar, H_2:CO_2:N_2 = 40:17:43)	59.2 g/l	(Kantzow, Mayer and Weuster-Botz 2015)
	Continuous gas fermentation with A. woodii (30°C, 1200 rpm, 1 bar, 0.5 vvm. H_2:CO_2:N_2 = 60:25:15)	18–23 g/l	(Kantzow, Mayer and Weuster-Botz 2015)
Syngas/CO	Continuous gas fermentation with C. authoethanogenum (37°C, 510–1000 rpm, 0.06–0.15 vvm, varying CO, H_2, CO_2)	4–8 g/l (4–12 g/l ethanol)	(Valgepea et al. 2018)
CO_2 and electricity	Microbial electrosynthesis (–590 mV vs SHE, 100 % CO_2)	10.5 g/l	(Marshall et al. 2013)

interesting carbon substrate as it usually inhibits microbial production processes. Here, it is argued that the use of acetate as carbon substrate could significantly increase the performance of microbial cell factories and allow for more efficient and economic production of a wide range of products.

ACETATE—POTENTIAL SOURCES

To achieve economic production and to compete with fossil fuels, it is necessary to use low-cost feedstocks (Lim et al. 2018). Feed-stocks such as pure sugars from corn, sugar beet, etc., are often criticized, since they compete with food industry and occupy arable land (Sitepu et al. 2014). Hence, raw materials which do not compete with the food and feed industry will likely play a key role in future applications of microbial cell factories.

In this respect, plant biomass seems to be the only available feedstock for the foreseeable future that is able to both satisfy this requirement and is available in sufficient quantities. However, the use of biomass in most cases requires pretreatment and the use of lignocellulosic hydrolysates in microbial fermentations is often inhibited by an array of different compounds including organic acids such as acetate. To achieve full conversion of available carbon sources, acetate would need to be utilized in hydrolysate-based processes as well.

When acetate should serve as an additional carbon source for fermentation processes, several potential resources can be imagined.

Plant biomass

Extraction of carbohydrates from pretreated plant biomass provides streams containing a mixture of hexoses and pentoses (Ko et al. 2015; Jönsson and Martín 2016). Ideally, high concentrations of fermentable sugars such as glucose are obtained. However, inhibitory compounds such as organic acids (e.g., acetate) that are released during hydrolysis hamper microbial conversion (Jönsson and Martín 2016). Full conversion of all available carbon sources is crucial for successful application of such streams for microbial production processes (Duque, Cardona and Moncada 2015).

Food waste

Food waste can be imagined for utilization in microbial fermentations, because it contains both sugars and proteins, making it an attractive alternative feedstock (Matsakas et al. 2014). Additionally, reducing the large quantities of waste production by today's society would help improve the economical footprint. However, due to fast partial anaerobic digestion it usually also contains organic acids such as acetate as a result of partial anaerobic digestion. Valorization of these streams is further complicated by their heterogeneity as well as seasonal and process-related variations in composition and pH (Pfaltzgraff et al. 2013).

Gas fermentation

In addition to biomass-derived materials, gaseous substrates such as CO, CO_2 and H_2 that are available in large quantities from industrial sources represent an interesting alternative carbon source (Schiel-Bengelsdorf and Dürre 2012). A very interesting group of carbon-fixing organisms that have recently gained more attention are acetogenic bacteria, which can be used for production of different chemicals such as acetate (Schiel-Bengelsdorf and Dürre 2012), ethanol (Valgepea et al. 2018), 2,3-butanediol (Köpke et al. 2011), and butanol (Liou et al. 2005). In a subsequent microbial conversion process, acetate could be upgraded by production of high value-added compounds. This principle has already been used in an integrated two-phase process, where acetate produced from syngas is continuously converted into C_{16}-C_{18} triacylglycerides (Hu et al. 2016).

Each of these potential streams has distinct characteristics which need to be considered for microbial conversion, where fast substrate co-conversion into products at high yields is required for economic and sustainable processes. With this background in mind it is important to select, manipulate and improve suitable organisms for acetate (co-)utilization. Additionally, it might not be possible to directly use the described acetate streams, as further processing such as purification or detoxification might be of need.

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Table 2. Selected examples of microbial cell factories used for production of value-added compounds by (co-)utilization of acetate.

Strain	Source	Product	Reference
Microbial cell factories using acetate as sole carbon source			
E. coli BL21 pET-22b + (MNEI)	Acetate	180 mg/l monellin (protein)	(Leone et al. 2015)
E. coli W ΔiclR pCDF(cad), pET(acs), pCOGA5(gltA, aceA)	Acetate	3.6 g/l itaconic acid	(Noh et al. 2018)
E. coli MG1655 ΔsdhAB ΔmaeB ΔiclR gltA	Acetate	7.3 g/l succinate	(Li et al. 2016)
E. coli BL21 ΔfadE pYX26(tesA) pYX30(acs)	Acetate	1 g/l fatty acids	(Xiao et al. 2013)
R. toruloides AS 2.1389	Acetate	2.1 g/l fatty acids	(Huang et al. 2016)
Y. lipolytica Po1g pMT065 (acc, dga)	Acetate from syngas fermentation	18 g/l C_{16}-C_{18} triacylglycerids	(Hu et al. 2016)
Mixed culture of glycogen accumulating organisms	Acetate	41 % of dry cell weight polyhydroxyalkanoates	(Dai et al. 2007)
Microbial cell factories for acetate co-utilization			
E. coli BW25113/F' ΔadhE Δfrd ΔldhA Δpta ΔpflB Δfnr pAL953(ackA-pta) pAL603(alsS-ilvCD, kivd-adhA) pAL991(atf1)	Glucose and acetate	19.7 g/l isobutyl acetate	(Tashiro, Desai and Atsumi 2015)
S. cervisiae SR7(D452-2 Δald6 xyl1, xyl2, xks1) adhE	Glucose, xylose and acetate	42 g/l ethanol	(Wei et al. 2013)
E. coli BL21 ΔfadE pYX26(tesA) pYX30(acs)	waste streams from dilute acid hydrolysis of lignocellulosic biomass	0.43 g/l fatty acids	(Xiao et al. 2013)
E. coli BL21 ΔfadE pYX26(tesA) pYX30(acs)	effluent from anaerobic-digested sewage sludge	0.17 g/l fatty acids	(Xiao et al. 2013)

(CO-)UTILIZATION OF ACETATE AS CARBON SOURCE

Co-utilization of acetate and other carbon sources is of great interest for microbial production processes as the use of acetate in addition to carbohydrates could potentially assist in improving target product yields or to decrease the formation of byproducts (Wu et al. 2016). The current state of the art for production of chemicals with microbial systems is the use of cultivation media containing glucose as the sole source of carbon, rather than complex raw materials (Baez, Cho and Liao 2011; Xu et al. 2014). Even co-utilization of glucose and acetate in a defined background is still challenging, because of the toxicity and inhibitory effects of acetate on many microorganisms (Salmond, Kroll and Booth 1984; Roe et al. 1998; Russell and Diez-Gonzalez 1998) as well as the underlying regulatory networks of metabolism favoring glucose utilization (Stülke and Hillen 1999; Schmidt and Schaechter 2012; Enjalbert et al. 2017). As shown in Table 2, metabolic engineering has been shown to be helpful in addressing these problems by improving natural acetate utilization capacities in well-studied organisms such as Escherichia coli (Castaño-Cerezo et al. 2015; Noh et al. 2018; Novak et al. 2018). In addition to relying on model organisms, another potential strategy could be the implementation of extraordinary mechanisms found in efficient natural utilizers of acetate in model organisms such as E. coli and Saccharomyces cerevisiae. One such example would be the acetate uptake and metabolism of Azotobacter vinelandii in which glucose uptake is inhibited in the presence of acetate, thus resulting in a reversed diauxic growth pattern (George, Costenbader and Melton 1985).

Escherichia coli using glucose as the carbon source has been used for the production of a wide range of chemicals as well as proteins (Ohta et al. 1991; Atsumi et al. 2010; Nielsen et al. 2010; Ferrer-Miralles et al. 2015). The most important pathways of acetate utilization are the low affinity and reversible phosphate acetyltransferase (Pta) and acetate kinase (AckA) node as well as the high affinity acetyl-CoA synthetase (Acs) (Wolfe 2005; Enjalbert et al. 2017). The manipulation of these pathways was shown to have a positive effect on acetate (co-)utilization (Novak et al. 2018). Furthermore, regulation of acetate metabolism to explain phenomena such as overflow metabolism has been extensively studied (Valgepea et al. 2010; Enjalbert et al. 2017).

Recently, acetate as carbon source has gained more interest (Table 1). Tashiro, Desai and Atsumi (2015) could demonstrate potential benefits of co-utilization of glucose and acetate. It was shown that product yield of isobutyl-acetate can be increased by simultaneous use of glucose and acetate as in this case carbon loss by decarboxylation of pyruvate to acetyl-CoA can be circumvented, resulting in an increased product yield. Apart from this example, co-utilization of acetate for production of value-added products with E. coli is still in its early stages and the focus of attention has mainly been on improving co-utilization of glucose and acetate and understanding underlying mechanisms (Lin et al. 2006; Castaño-Cerezo et al. 2015; Ding et al. 2015).

Saccharomyces cerevisiae is another well-studied and well-used organism in biotechnology. Ethanol production using baker's yeast is among the best studied examples of microbial chemical production. Recent studies addressed the well-known phenomenon of glycerol formation during ethanol fermentation

by providing acetate as alternative to regenerate NADH, thus increasing ethanol yield by directing more carbon towards ethanol, both from glucose and acetate (Henningsen et al. 2015; Papapetridis et al. 2016).

CONCLUSION AND OUTLOOK

Its low cost makes acetate a promising feed-stock for biotechnological processes with different applications. Already today, it plays an important role as an intermediate product in anaerobic digestion and can be indirectly used for the production of heat from biogas. To provide alternative and potentially more valuable chemicals as final product of acetate (co-)utilization, deeper insights will have to be gained. Shedding light on mechanisms of acetate uptake and metabolism through systems biology tools, would allow for more efficient manipulation of microbial cell factories. Finally, the use of acetate as a carbon source could contribute towards (co-)utilization of complex raw materials, which can make microbial production processes more competitive and thus accelerate industrial implementation. All in all, acetate is worth to be considered as a potential substrate for the improvement of microbial cell factories.

FUNDING

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2. Goal of the thesis

For the transition towards a CO_2 neutral future, industrial plants will need to reduce their CO_2 emissions, and several microbial CO_2 fixation processes have been suggested to achieve this. The development of such systems is mainly challenged by the chemical inactivity of CO_2 and the need for an additional energy source. Moreover, the presence of CO in a CO_2 containing gas streams usually reduces the amount of fixed CO_2 and the overall carbon efficiency and leads to the formation of a mixed product spectrum in acetogens. In this thesis, a novel two-step process to achieve full and specific conversion of industrially produced CO_2 into a value-added chemical is presented.

This thesis therefore aims to show the feasibility of a two-step process for CO_2 fixation and production of a fuel chemical. In a first step, *A. woodii* converts CO_2 from an industrial source to acetate, which is subsequently fed to a second process for production of a fuel chemical by a genetically engineered *E. coli* (**Figure 2**). It was hypothesized that using an efficient CO_2/H_2 utilizer in the first step in combination with a genetically engineered *E. coli* strain in the second step would allow to specifically convert CO_2 into a valuable chemical. Acceptance criteria for achieving this goal were therefore defined as follows: (i) net CO_2 fixation in the whole process and (ii) production of 2,3-butanediol or isobutanol indirectly from CO_2 via acetate. Since the development of each process step is accompanied by different challenges, the individual steps were studied independently and limitations and influencing factors were defined.

The proposed process stands out due to its metabolic flexibility, as both individual modules can be exchanged when aiming at the use of different industrial gas streams or products. In contrast to traditional one-step approaches, the suggested two-step process is characterized by a robustness towards variations in feed-gas compositions. This robustness is, among other factors, caused by the production of acetate as the sole product rather than a mixed product spectrum. In contrast to other one-step and two-step processes in literature, an acetogenic microorganism very suitable for utilization of H_2 and CO_2 rather than CO in the first step allows for efficient CO_2 fixation, even in gas containing high amounts of CO. In contrast to state-of-the-art processes for microbial upgrading of acetate, this thesis focuses on the use of defined medium and renewable feedstocks for microbial chemical production. Additionally, focus is laid on determining effects mediated by complex media additives and reaching solid quantification of product formation. The two-step process in this thesis is the first for which production of a fuel chemical from CO_2 is quantified in the form of mass balances for the individual steps as well as the entire process. This elemental balancing is crucial to determine steps limiting the overall efficiency of the process and will help to improve process performance in the future.

3. Structure of the thesis

This thesis is structured into three parts, which deal with the individual steps of the two-step net CO_2 fixation process (**Figure 2**). **Part 1** summarizes the characterization and development of a continuous bioprocess for CO_2 fixation in industrial CO-containing blast furnace gas using *A. woodii*. The limitation of the production system and the influence of CO and H_2 concentrations on process performance could be evaluated by a combination of time-resolved and steady state quantification. Blending of blast furnace gas with H_2 did not only enable net CO_2 fixation and acetate production but can also be used to control gas uptake rates, metabolic fluxes, and product formation.

Part 2 links the first to the second process step by dealing with acetate (co-)utilization in *E. coli* W independently from product formation. Within this part, it is evaluated how acetate uptake can be improved, both when it is used as a sole carbon source as well as in combination with glucose. Efficient acetate uptake is a prerequisite for the second process step and can potentially be achieved by metabolic engineering of the acetate assimilation pathway. To this end, it was investigated how the overexpression of one acetate pathway influenced acetate utilization. Acetate assimilation was studied in batch and continuous cultivations to obtain a holistic impression of the metabolic mechanisms.

The establishment of fuel chemical production, i.e. isobutanol and 2,3-butanediol is described in **part 3**. The development of a suitable isobutanol production system is shown in part 3.1. Construction of different expression vectors in combination with screening of various strain backgrounds was the basis for further process intensification. Cost-competitiveness of microbial isobutanol production was evaluated, especially in the context of using a residual waste material as an alternative feedstock. The chemical properties of isobutanol (i.e. volatility, toxicity) had to be considered for a systematic investigation of different production strategies. The same properties, however, hampered the development of isobutanol production from acetate as described in part 3.2. The feasibility of chemical production from acetate is shown in part 3.3, where 2,3-butanediol was chosen as a promising product, since it is derived from the same metabolite in the central carbon metabolism as isobutanol without being toxic or volatile. Considerations about media composition were discussed and quantitative media optimization was crucial for the establishment of an appropriate process upgrading acetate to 2,3-butanediol.
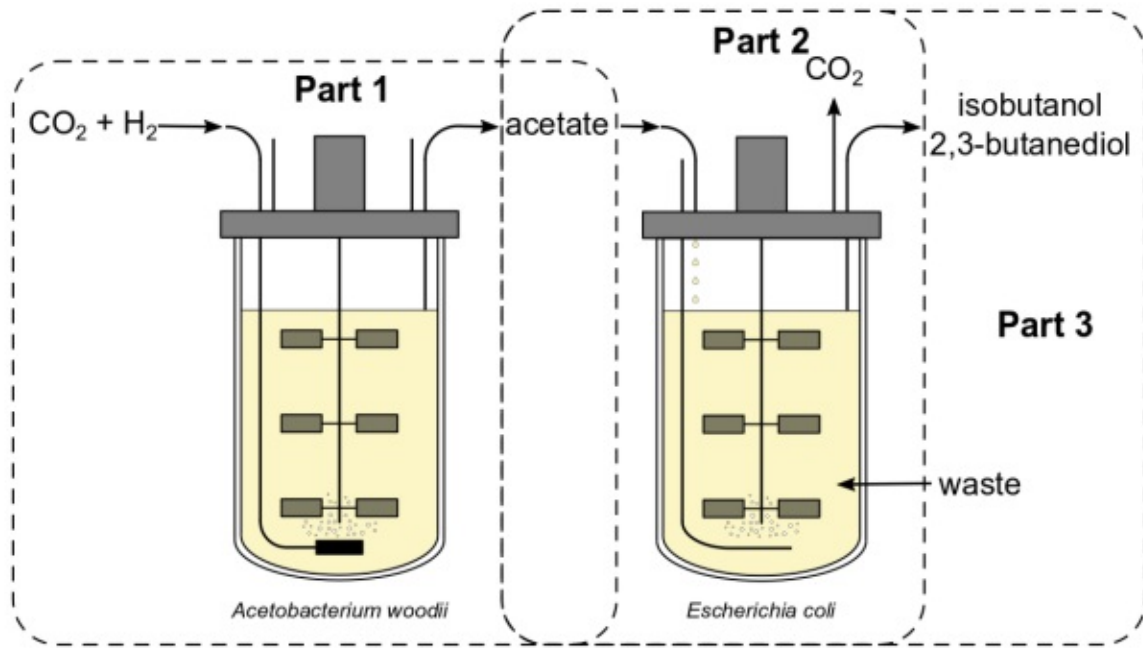
Figure 2 – Structure of the thesis. Part 1 deals with CO_2 fixation in *A. woodii*, part 2 with the improvement of acetate uptake in *E. coli* and part 3 with isobutanol and 2,3-butanediol production.

II. RESULTS

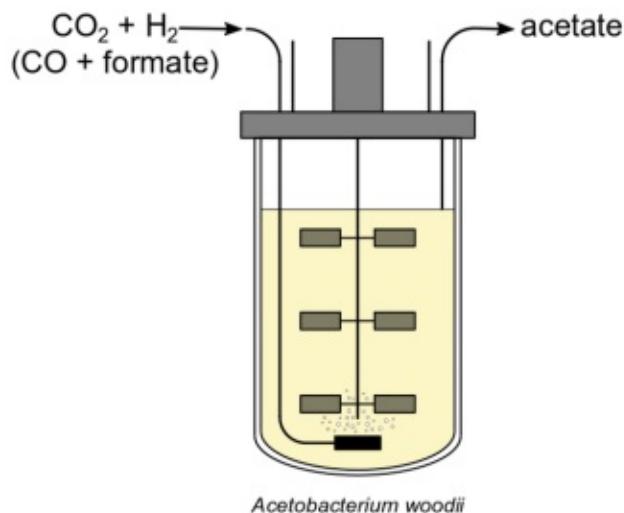## 1. CO_2 fixation and acetate production from industrial flue gas using *A. woodii*

Figure 3 – Overview part 1. This part deals with process development for CO_2 fixation in an industrial blast furnace gas stream using *A. woodii*.

Problem statement

Autotrophic CO_2 fixation requires an energy source. In *A. woodii* this is H_2 but blast furnace gas does not contain enough H_2. Additionally, CO present in blast furnace gas is known to reversibly inhibit hydrogenase enzymes in *A. woodii.* Therefore, the presence of CO was reported to inhibit CO_2 and H_2 utilization, leading to net CO_2 production and increased lag phases.

State-of-the-Art

A. woodii is known to efficiently utilize CO_2 and H_2 and was recently shown to be capable of utilizing CO (Bertsch and Müller, 2015b; Schuchmann and Müller, 2014). While CO utilization is well characterized in other acetogenic bacteria (e.g. *C. autoethanogenum* and *C. ljungdahlii*), it was still unknown how the presence of this toxic gas would influence process performance in batch and continuous cultivations. Generally, CO_2 fixation had so far not been shown in gases containing significant amounts of CO.

Scientific questions

How does the presence of CO and other inhibitors influence growth, acetate formation and gas uptake?

What are the process requirements for a continuous cultivation for CO_2 fixation using industrial blast furnace gas?

Hypotheses

The presence of CO results in a prolonged lag-phase and its presence in the liquid phase inhibits H_2 utilization. Blending CO containing blast furnace gas with H_2 and achieving limitation of CO in the liquid phase enables net CO_2 fixation and co-utilization of CO_2, H_2, and CO.

Approach

The effect of CO and H_2 partial pressures on gas uptake, growth and acetate formation was studied in batch and continuous cultivations. Using idealized gases with the same CO, CO_2 and H_2 or the same CO_2 and H_2 concentrations as the BFG-H_2 blend allowed to draw conclusions about the individual effects of CO and H_2 availability. A comparison of steady state and dynamic shift data made it possible to define the limitation of the system, which was important for the establishment of comparable H_2-limited cultures.

Content and contribution

We aimed on quantifying the influence of CO, CO_2 and H_2 availability on CO_2 utilization, growth, and acetate production to achieve net CO_2 fixation in industrial blast furnace gas. We hypothesized that the gas had to be blended with H_2 as an energy source to enable net CO_2 fixation and compared different gases containing CO, CO_2 and H_2. CO metabolism was studied both in time-resolved and continuous processes and a limitation of CO in the liquid phase was found to be key for the co-utilization of CO, CO_2 and H_2 in blast furnace gas. Blending of blast furnace gas with H_2 did not only enable CO_2 fixation, but could additionally be used as a strategy to control gas uptake rates, metabolic fluxes, and acetate production.

KN carried out the experiments together with CN and performed the analysis. SP and KN planned the study and wrote the manuscript.

Title of manuscript

Blending industrial blast furnace gas with H_2 enables *Acetobacterium woodii* to efficiently co-utilize CO, CO_2 and H_2

Citation

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Manuscript:

Blending industrial blast furnace gas with H₂ enables *Acetobacterium woodii* to efficiently co-utilize CO, CO₂ and H₂

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Abstract

In this study, we investigated the impact of gas composition (i.e. CO, CO_2 and H_2 partial pressures) on CO_2 utilization, growth, and acetate production in batch and continuous cultures of *A. woodii*. Based on an industrial blast furnace gas, H_2 blending was used to study the impact of H_2 availability on CO_2 fixation alone and together with CO using idealized gas streams. With H_2 available as an additional energy source, net CO_2 fixation and CO, CO_2 and H_2 co-utilization was achieved in gas-limited fermentations. Using industrial blast furnace gas, up to 15.1 g l^{-1} acetate were produced in continuous cultures. Flux balance analysis showed that intracellular fluxes and the ATP gain were dependent on the availability of H_2 and CO. Overall, H_2 blending was shown to be a suitable control strategy for gas fermentations and demonstrated *A. woodii* to be an interesting host for CO_2 fixation from industrial gas streams.

Key words

Chemostat, net CO_2 fixation, acetogenic gas fermentation, gas co-utilization, maximum specific H_2 uptake rate

Graphical Abstract
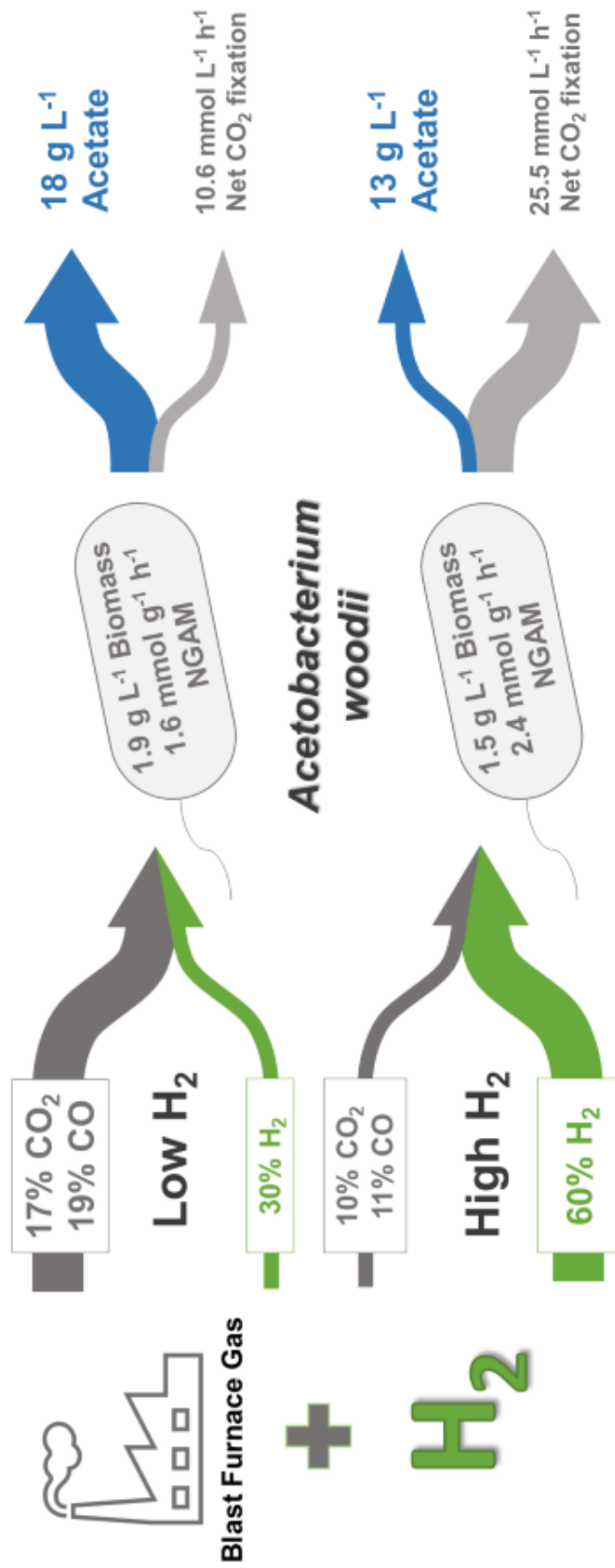
Introduction

Within the Paris Climate Agreement from December 2015, the global community set goals to abate atmospheric CO$_2$ release (Köpke and Simpson, 2020). The use of CO$_2$ as a substrate is one possibility to reduce emissions, close global carbon cycles and to establish a circular economy (Bengelsdorf and Dürre, 2017; Liew et al., 2016b; Vees et al., 2020). Using fossil fuels in combination with costly payments for CO$_2$ certificates, has increased industrial interest in investigation and development of biological CO$_2$ fixation and microbial processes for the production of platform and fuel chemicals based on one carbon substrates (Takors et al., 2018).

One of the biggest industrial CO$_2$ emitters is the iron and steel industry, which accounts for 4 % to 7 % of anthropogenic CO$_2$ emission in the European Union (Pardo and Moya, 2013). A variety of process gases are produced during the production process and blast furnace gas (BFG) is one example for a by-product gas from the steel milling process. This flue gas is formed during the reduction of iron ore to iron and contains high amounts of N$_2$, in combination with CO$_2$, CO and traces of H$_2$ (Hou et al., 2011; Molitor et al., 2016). However, hydrogen is an important energy source for acetogenic gas fermentations and a promising future electron carrier that can be obtained from renewable resources (Das and Veziroglu, 2008; Hallenbeck and Ghosh, 2009; Yukesh Kannah et al., 2021). Alternatively, H$_2$ might be derived by converting readily available heat energy from the steel milling process itself (Molitor et al., 2016).

Acetogenic bacteria have the ability to fix carbon via the Wood-Ljungdahl pathway and can utilize the gaseous compounds CO, CO$_2$ and H$_2$, rendering them promising microbial hosts for industrial CO$_2$ fixation (Bertsch and Müller, 2015a; Griffin and Schultz, 2012; Köpke and Simpson, 2020). Autotrophic CO$_2$ fixation in acetogens requires H$_2$ as an energy source (Schoelmerich and Müller, 2020; Schuchmann and Müller, 2014a).

Acetate is the main product of acetogens, but the product spectrum of different acetogenic strains is broad and includes ethanol, 2,3-butanediol, butanol, butyrate, and others (Liew et al., 2016b). Metabolic engineering was successfully used to increase product selectivity, enhance productivity or to extend the product spectrum of acetogens (Dai et al., 2012; Hoffmeister et al., 2016; Liew et al., 2017; Straub et al., 2014). Chemical production using acetogens therefore is a promising alternative to energy intensive chemical syntheses processes such as the Fisher-Tropsch synthesis (Griffin and Schultz, 2012; Munasinghe and Khanal, 2010). An industrial process for ethanol production from CO using *Clostridium autoethanogenum* has already been commercialized by the leading company LanzaTech Inc (Skokie, IL, USA).

Acetobacterium woodii is a homoacetogenic bacterium, which is known to efficiently utilize CO_2 and H_2 (Kantzow et al., 2015; Schuchmann and Müller, 2014a). In addition to autotrophic growth, *A. woodii* can utilize a broad variety of substrates including C1 components such as formate and methanol (Drake et al., 1997; Schuchmann and Müller, 2016). It was recently shown in this context that H_2 is an important intermediate during heterotrophic growth of *A. woodii*, since it is directly required for reduction of CO_2 in the WLP (Wiechmann et al., 2020). During growth on H_2 and CO_2, 0.3 mol ATP are formed per mol of acetate produced (Schuchmann and Müller, 2014a). This low ATP gain allows *A. woodii* to grow at a very low H_2 partial pressure and explains why cultivation on H_2 and CO_2 is much easier compared to *C. autoethanogenum* with an ATP gain around 1 ATP per acetate and a higher H_2 threshold (Mock et al., 2015). *A. woodii* was the first organism where energy conservation was fully elucidated (Schuchmann and Müller, 2014a) and additional metabolic properties continue to be described (Westphal et al., 2018; Wiechmann et al., 2020). The main product of *A. woodii* is the platform chemical acetate, which can either be used directly or can be further upgraded into other valuable products (Novak et al., 2020; Novak and Pflügl, 2018). The production of acetate poses an advantage over the production of a mixed product spectrum due to the controllability of the process (Schwarz et al., 2020). Growth and acetate production on CO_2 and H_2 was characterized in detail (Demler and Weuster-Botz, 2011; Kantzow et al., 2015) and the mechanism of CO utilization was described (Arantes et al., 2020; Bertsch and Müller, 2015b; Schwarz et al., 2020), but data on utilization of synthesis gas containing CO and CO_2 is scarce. The presence of CO was found to inhibit bacterial hydrogenases, in particular the hydrogen-dependent CO_2 reductase (HDCR) (Bertsch and Müller, 2015b; Ragsdale and Ljungdahl, 1984). This inhibitory effect leads to delayed growth in the presence of CO, which can be restored by the addition of formate, the product of the HDCR (Bertsch and Müller, 2015b). Although initially reported to grow on CO as the sole carbon and energy source, it was recently shown that *A. woodii* does not grow on CO alone (Bertsch and Müller, 2015b; Genthner and Bryant, 1987).

Another member of the genus *Acetobacterium*, *Acetobacterium wieringae* was recently shown to utilize CO (at 100 % CO, 170 kPa) as sole carbon and energy source to produce acetate (Arantes et al., 2020). A sub-strain of *A. wieringae* was found to possess better CO utilization properties than both wildtype *A. wieringae* and *A. woodii*, indicating the potential of metabolic adaptation to toxic CO concentrations (Arantes et al., 2020).

In contrast to *A. woodii*, CO utilization of several clostridial species has been broadly studied, owing to their enhanced CO utilization properties (Cotter et al., 2009; Mahamkali et al., 2020; Mohammadi et al., 2012; Valgepea et al., 2018, 2017). Special attention has been given to the utilization of synthesis gas, which contains large amounts of CO and H_2

and CO_2 in varying minor quantities (Takors et al., 2018). Correspondingly, most published work has focused on the utilization of CO alone or in combination with H_2.

Clostridium ljungdahli and *C. autoethanogenum* are better suited for growth on CO than on CO_2 and H_2, due to higher growth rates, increased ATP generation and the possibility to make reduced products such as ethanol (Hermann et al., 2020; Mahamkali et al., 2020). However, CO utilization is accompanied by CO_2 production. This formation can be reduced by the additional supply of H_2 to CO-grown *C. autoethanogenum* (Valgepea et al., 2018). Concurrently, the addition of H_2 shifted the product spectrum towards more reduced end products, highlighting that the feed gas composition can be considered a critical factor in enhancing the economics of gas-fermentation processes. A recent study showed that the addition of 2 % CO enhanced fermentation of CO_2 and H_2 in *C. autoethanogenum* (Heffernan et al., 2020). There, the addition of CO improved the flux towards pyruvate and reduced ferredoxin, thereby improving CO_2 fixation. However, utilization of syngas containing high amounts of CO usually results in the formation of CO_2 (Valgepea et al., 2018, 2017).

Due to the toxicity mechanisms of CO, a process focussing on CO_2 fixation in CO-containing gas (e.g. BFG) has to aim for limitation of CO in the liquid medium to achieve co-utilization of CO, CO_2 and H_2. This CO limitation can be achieved due to low solubility of CO in combination with high biomass and thereby high CO uptake rates (Bertsch and Müller, 2015a). While batch experiments can provide general process information, continuous cultivations are preferable since they enable studying cells under steady state conditions (Takors et al., 2018). The controlled and reproducible conditions of continuous cultures enable quantitative description of the metabolism (Adamberg et al., 2015).

In this study, we aimed to determine the effect of gas composition (i.e. CO, CO_2 and H_2 partial pressures) on CO_2 utilization, growth, and acetate production by *A. woodii*. Using the gas composition of industrial BFG as a starting point, we used idealized gases with and without CO and blended them with different concentrations of H_2. With these gas streams, we studied the influence of CO and H_2 partial pressures on growth and acetate formation in batch and continuous cultures. This approach allowed us to draw conclusions about effects mediated by both H_2 and CO availability. Moreover, we hypothesized that H_2 blending could also be a useful tool to enable co-utilization of CO, CO_2 and H_2 and net CO_2 fixation. Indeed, H_2 blending and limitation of the toxic gas CO in continuous cultures enabled *A. woodii* to efficiently grow and produce acetate from industrial BFG. Using a metabolic model, we could furthermore show the effect of gas composition on intracellular carbon, redox and energy metabolism. The gas fermentation strategy used here provides valuable information

on acetogenic metabolism and shows potential in upgrading industrial gas pollutants into value-added products.

Materials and methods

Organism and media

Acetobacterium woodii DSM 1030 was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and used for all cultivations.

The medium for all cultivations is based on media 135 suggested by DSMZ. Bicarbonate-buffered medium was used for the initial experiments in serum bottles investigating growth on BFG. This medium was later replaced by phosphate-buffered medium which was used in serum bottle experiments investigating the effect of formate and for all bioreactor cultivations. Bicarbonate-buffered medium contained per liter: 1 g NH_4Cl, 0.1 g $MgSO_4$ * $7H_2O$, 2 g yeast extract, 0.33 g KH_2PO_4, 0.45 g K_2HPO_4, 0.5 g cysteine-HCl * H_2O, 10 g $NaHCO_3$, 0.25 ml sodium resazurin (0.2 % w/v), 20 ml trace element solution DSMZ 141 and 10 ml vitamin solution DSMZ 141. In phosphate-buffered serum bottle medium $NaHCO_3$ was replaced by 1.76 g l^{-1} KH_2PO_4, 8.44 gl^{-1} K_2HPO_4 and 3.47 g l^{-1} NaCl. For mixotrophic precultures, 5 g l^{-1} fructose or formate were added from anoxic sterile stocks of 250 g l^{-1}. The trace element solution was adapted from medium DSMZ 141 and contained per liter: 1.5 g nitrilotriacetic acid, 3 g $MgSO_4$ * 7 H_2O, 0.5 g $MnSO_4$ * H_2O, 1 g NaCl, 0.1 g $FeSO_4$ * 7 H_2O, 0.152 g $Co(II)Cl_2$ * 6 H_2O, 0.1 g $CaCl_2$ * 2 H_2O, 0.18 g $ZnSO_4$ * 7 H_2O, 0.01 g $CuSO_4$ * 5 H_2O, 0.02 g $KAl(SO_4)_2$ * 12 H_2O, 0.01 g boric acid, 0.01 g Na_2MoO_4 * 2 H_2O, 0.033 g $Ni(II)SO_4$ * 6 H_2O, 0.3 mg Na_2SeO_3 * 5 H_2O and 0.4 mg Na_2WO_4 * 2 H_2O. The vitamin solution was prepared according to DSMZ medium 141 and contained per liter: 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 5 mg thiamine-HCl, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg D-Ca-pantothenate, 0.1 mg vitamin B12, 5 mg para-aminobenzoic acid and 5 mg lipoic acid. The pH of all media was adjusted to 7.0 using 5 M KOH. For bioreactor cultivations, the media composition was adapted: concentration of the phosphate buffer was reduced to 0.33 g KH_2PO_4 and 0.45 g K_2HPO_4, trace element and vitamin concentrations (with the exception of Ca-pantothenate which was added to a concentration of 1 mg l^{-1} in the final medium according to (Godley et al., 1990)) were doubled and the iron concentration was increased to 26.9 mg l^{-1} $FeSO_4$*7 H_2O in the final medium (Demler and Weuster-Botz, 2011).

Media was prepared using anaerobic techniques as described previously (Hungate, 1969). All chemicals were purchased from Roth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) or Merck (Merck KGaA, Darmstadt, Germany).

Gases and compositions

Premixed gas containing 80 % H_2 and 20 % CO_2 was obtained from Air Liquide Austria GmbH (Schwechat, Austria) and used for routine cultivations such as precultures. Industrial blast furnace gas (BFG) was filled into gas cylinders directly on site at the blast furnace of voestalpine Stahl GmbH (Linz, Austria). The gas composition represents the annual average of the online quantification by the multi-analysis-system Advance Optima AO2000 (ABB Asea Brown Boveri Ltd, Zürich, Switzerland).

BFG was blended with H_2 (Messer Austria GmbH, Gumpoldskirchen, Austria) to obtain H_2 concentrations of 30 % (low H_2 blend) and 60 % (high H_2 blend) using two independent mass flow controllers (Eppendorf AG, Hamburg, Germany and CRANE Instrumentation & Sampling, Spartanburg, SC, USA). Idealized gases with and without CO containing the same amount of CO_2, CO and H_2 as the corresponding BFG-H_2 blend were purchased from Messer Austria GmbH (Gumpoldskirchen, Austria).

Preparation of precultures

A. woodii was stored as anoxic stocks at -80°C in 125 g l^{-1} saccharose. For cultivations, these stocks were transferred into 125 ml serum bottles containing 50 ml medium with 5 g l^{-1} fructose. For mixotrophic growth, the atmosphere was exchanged to 80 % H_2 and 20 % CO_2 and an overpressure of 1.5 bar was applied. *A. woodii* was cultivated in a rotary shaker at 30 °C and 200 rpm (Infors AG, Bottmingen, Switzerland). For further cultivation in serum bottles, 2 ml of the mixotrophic culture was transferred anaerobically to fresh medium. Batch experiments in bioreactors were inoculated with an exponentially growing culture to reach an optical density at 600 nm (OD_{600}) of 0.1. For cultivations on gas without CO mixotrophic precultures were used, whereas cultivations on gas containing CO were inoculated with a preculture previously grown on 5 g l^{-1} formate and high H_2 blend of the BFG.

Experiments in serum bottles

Growth experiments in 125 ml serum bottles were carried out using 50 ml medium. During the initial experiments for growth on the industrial flue gas, bicarbonate-buffered medium was used and later replaced by phosphate-buffered medium for experiments with formate. The atmosphere in the head space was exchanged by the desired gas composition and pressurized with 1.5 bar. The industrial flue gas was diluted with H_2 using two mass flow controllers (Brooks Instrument, Matfield, USA). Upon gas consumption, the headspace was refilled with the corresponding gas at 1.5 bar. Samples of 2 ml were regularly taken and used for OD_{600} and high-performance liquid chromatography (HPLC) measurements.

Cultivations in bioreactors

Bioreactor cultivations were carried out in duplicates in a DASbox® Mini Bioreactor system (Eppendorf AG, Hamburg, Germany) with a working volume of 200 ml at a temperature of 30 °C. The pH was initially set to 7.0, monitored by a pH electrode EasyFerm Plus K8 120 (Hamilton, Reno, NV, USA) and controlled by the addition of 2 M phosphoric acid and 5 M KOH with a MP8 multi pump module (Eppendorf AG, Hamburg, Germany). The agitator speed was routinely set to 600 rpm and adjusted to 300 and 1200 rpm to investigate gas liquid mass transfer. The medium was continuously sparged with 0.25 vvm (3 sl h^{-1}) of the indicated gas. Microspargers made of sintered metal with a pore size of 10 μm (Sartorius Stedim Biotech GmbH, Göttingen, Germany) were used for improved gas transfer into the liquid phase. Prior to inoculation, the reactor medium was flushed with the appropriate gas for at least 3 hours. Feed medium and a 1 % (w/v) solution of antifoam (Struktol SB2020, Schill und Seilacher, Hamburg, Germany) were continuously added with the MP8 pump module at a rate of 10 ml h^{-1} and 0.2 ml h^{-1}, respectively. Culture broth was continuously removed by peristaltic pumps (Ismatec SA, Glattburg, Germany) via a dip tube and the cultivation volume was maintained at 197 ± 11 ml. The feed bottles were continuously flushed with 6 sl h^{-1} N_2 to maintain anaerobic conditions. Steady state conditions were examined after a minimum of three volume changes when biomass and acetate concentrations as well as gas uptake was constant. Samples were taken in regular intervals to measure OD_{600} and estimate biomass growth. The culture broth was centrifuged at 14,000 rpm (21,913 g) for 5 min and the supernatant was used for HPLC analysis of acetate and formate.

Biomass determination

Biomass concentrations were determined gravimetrically by transferring 5 ml of cultivation broth into pre-weighed glass tubes. The tubes were centrifuged at 4,800 rpm (2,396 g) for 10 min, washed with 5 ml of distilled water and centrifuged again. The biomass was dried for at least 72 h at 105 °C. Gravimetrical biomass determination was carried out at the end of the batch phase and at the cultivation end. The optical density at 600 nm (OD_{600}) was measured in a spectrophotometer (Genesys^{TM} 20, Thermo Fisher Scientific, Waltham, MA, USA) against a water blank. Gravimetrically determined biomass concentrations were correlated with OD_{600} values (biomass = 0.43 * OD_{600}) and this correlation was used to estimate the biomass concentration at all other time points.

HPLC analysis

Substrates and products in the liquid medium were determined with an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad, Hercules/CA, USA) in an Ultimate 3000 system (Thermo

Scientific, Waltham/MA, USA). 4 mM H_2SO_4 was used as a mobile phase and the column was operated at 60 °C at a flow of 0.6 ml min^{-1} for 30 min. 10 µl sample were injected onto the column (Erian et al., 2018). Detection was performed using a refractive index (Refractomax 520, Thermo Fisher Scientific, Waltham/MA, USA) and a diode array detector (Ultimate 3000, Thermo Fisher Scientific, Waltham/MA, USA). Chromeleon 7.2.6 Chromatography Data System (Thermo Fisher Scientific, Waltham/MA, USA) was used for control, monitoring and evaluation of the analysis. 450 µl of culture supernatant were mixed with 50 µl of 40 mM H_2SO_4 and centrifuged for 5 min at 14,000 rpm (21,913 g) at 4°C. The remaining supernatant was used for further analysis. Standards at defined concentrations of formate, acetate, ethanol and fructose were treated the same way. A 5-point-calibration was used for quantification.

GC analysis

The concentrations of H_2, CO_2 and CO were determined using a gas chromatograph (Trace Ultra GC, Thermo Fisher Scientific, Waltham/MA, USA). 100 µl of sample were injected at 100 °C with a split ratio of 20 on a ShinCarbon ST 100/120 packed column (Restek Corporation, Bellefonte/PA, USA). The oven was kept at 30 °C for 6.5 min, temperature was increased by 20°C min^{-1} to 250°C and kept constant for 1.5 min. Argon 5.0 (Messer Austria GmbH, Gumpoldskirchen, Austria) was used as a carrier gas at a flow rate of 2 ml min^{-1}. Samples were analyzed with a thermal conductivity detector. The filament temperature, cell block temperature and transfer temperature were set to 370 °C, 240 °C and 200 °C, respectively. The combination of two electrical valves enabled offgas measurement of each of the four reactors in 2-hour intervals. Chromeleon 7.2.10 Chromatography Data System (Thermo Scientific, Waltham/MA, USA) was used for control, monitoring and evaluation of the analysis. The gas composition of each gas was determined before and after its use in the fermentation process and gas uptake rates were determined by calculating the difference to these reference gas concentrations.

Rate calculations and elemental balancing

In batch experiments, volumetric acetate production rates r_{Ace} [mmol l^{-1} h^{-1}] were calculated for every sampling point according to the equation:

$$r_{Ace} = \frac{c_{Ace,t} - c_{Ace,(t-1)}}{\Delta t} \; , \tag{1}$$

where c_{Ace} represents the acetate concentration [mmol l^{-1}] at each time point and Δt the time difference [h] between measurements. Specific production rates q_{Ace} [mmol g^{-1} h^{-1}] were calculated according to:

$$q_{Ace} = \mu \, \frac{c_{Ace,t} - c_{Ace,(t-1)}}{c_{BM,t} - c_{BM,(t-1)}}, \tag{2}$$

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Where c_{BM} represents the biomass concentration [g l^{-1}] at each time point and μ the specific growth rate [h^{-1}].The highest value of these uptake rates calculated between individual sampling points is described as the maximum production rate. Total volumetric acetate production rates were calculated by considering the final acetate concentration after the fermentation and the total process time.

Gas uptake was measured in 2 h intervals and the volumetric gas uptake rates HUR, COUR, and CO_2UR [mmol l^{-1} h^{-1}] were calculated after normalization of gas composition using N_2 as inert gas. For the calculation of CO_2UR during continuous cultivations, the amounts of CO_2 and HCO_3^- accounting for saturation of fresh medium were considered using the solubility of CO_2, the Henderson-Hasselbalch equation assuming a pK_s of 6.2 and the feeding rate. The specific gas uptake rates q_{H2}, q_{CO} and q_{CO2} were obtained by dividing HUR, COUR and CO_2UR [mmol g^{-1} h^{-1}] by the biomass concentration.

In chemostats, r_{Ace} was calculated by multiplying the average acetate concentration from at least 3 data points from steady state conditions by the liquid dilution rate D. Specific rates for gas uptake and acetate production were obtained by dividing the volumetric rate by the average biomass concentration of steady state data points.

For elemental balancing, biomass was assumed to contain 50 % (w/w) carbon and 0.6 % (w/w) hydrogen. The degree of reduction (DoR) of biomass was assumed to be 4.15 mol electrons per mol of carbon (Rittmann et al., 2012). For hydrogen balancing, water production was assumed to correlate to substrate uptake with 0.5 mol water produced per mol hydrogen taken up, 0.5 mol water per mol formate and 1 mol water per mol CO (Bertsch and Müller, 2015b; Schuchmann and Müller, 2014b). The influence of water formation can be neglected for the calculation of DoR balances. All balances closed well without consideration of yeast extract.

Metabolic modeling and flux balance analysis

To model intracellular fluxes, we adapted a previously published *A. woodii* core model (Koch et al., 2019) consisting of 118 reactions (Supplementary File 1, Tables S1 and S2). The model considers energy conservation and redox balancing as previously described for *A. woodii* (Schuchmann and Müller, 2014). The biomass composition was assumed to be similar to *C. autoethanogenum* (Valgepea et al., 2017).

Flux balance analysis (FBA) was done using the *CellNetAnalyzer* toolbox (Klamt et al., 2007; von Kamp et al., 2017). For all simulations, the measured specific rates for biomass formation, substrate uptake rates (CO, CO_2, H_2) and acetate were used to constrain the model. These measurements bear redundancies with respect to carbon and redox balances in the metabolic model and, therefore, fluxes had to be corrected prior to the actual FBA

calculations to obtain a consistent system. This was done by minimizing the relative changes in the measured rates necessary to obtain a consistent flux scenario ("Check feasibility" function in *CellNetAnalyzer*). FBA requires the formulation of an objective function and most FBA studies use maximization of biomass synthesis. Since the growth rate was fixed to the experimentally observed value, we maximized instead the pseudo reaction that quantifies the non-growth associated ATP maintenance (NGAM) demand. In this way, we obtain an upper bound of ATP available for NGAM processes.

Results and Discussion

Gas composition

Industrial BFG typically contains high amounts of N_2, ~ 20 % CO and CO_2, and ~ 3 % H_2 (Hou et al., 2011). To determine the exact composition, the gas was filled into gas cylinders at the stack of the steel production site and analyzed for its main components (Table 1). The gas composition of the gas did not differ greatly from literature data (Hou et al., 2011; Molitor et al., 2016).

Some components of the BFG might inhibit growth of *A. woodii*. Among those inhibitory compounds, the high concentrations of 26 % CO were assumed to affect growth and production to the highest extent. Growth was not possible on BFG alone (Figure 2b), which confirmed that in contrast to other organisms, utilization of CO as sole carbon and energy source is not possible in *A. woodii* (Bertsch and Müller, 2015b). We hypothesized, that blending BFG with H_2 would enable growth, acetate formation and CO_2 fixation in the CO-containing gas. To this end, two H_2 blending ratios were chosen, low (30 %) and high (60 %) H_2. In accordance, CO and CO_2 partial pressures were higher in the low H_2 blend compared to the high H_2 blend. To investigate the effect of the presence of CO as well as other potential inhibitors in the industrial flue gas, performance of *A. woodii* was additionally investigated in idealized gases. Concentrations of CO, CO_2 and H_2 in idealized gases were identical to those in BFG blended with the same amount of H_2. In one approach, the gas only consisted of CO_2, N_2 and H_2, which allowed us to determine effects mediated by the presence of CO. In another approach, CO, CO_2, N_2 and H_2 were present and the effect of other potential inhibitors could be studied. Table 1 gives an overview of all gas compositions used in this study.

Table 1 – Composition of gases used in this study. Low and high H_2 gases were blended with 30 % H_2 and 60 % H_2, respectively· n.d. = not determined; errors for CO_2, H_2 and CO determination in gas mixtures are 2 % relative.

Blast furnace gas from industrial steel production site						
Compound	N_2		CO_2		CO	H_2
composition [%]	48.5		22.7		25.2	3.6
Gas mixtures						
Gas	calculated value			actual value		
	CO_2 [%]	H_2 [%]	CO [%]	CO_2 [%]	H_2 [%]	CO [%]
idealized, low H_2	16.5	30.0	-	16.6	30.3	-
idealized, high H_2	9.4	60.0	-	9.55	60.00	-
idealized with CO, low H_2	16.5	30.0	18.3	16.73	29.71	18.64
idealized with CO, high H_2	9.4	60.0	10.4	9.54	60.12	10.57
BFG, low H_2*	16.5	30.0	18.3	n.d.	n.d.	n.d.
BFG, high H_2*	9.4	60.0	10.4	8.8	62.18	9.81
Standard	20.0	80.0	-	20.0	80.0	-

Utilization of blast furnace gas requires adaptation phase

In a first step, the utilization of different gases by *A. woodii* was tested in serum bottle experiments. Growth and acetate production were compared in idealized gas and BFG at low and high H_2 blend. As a reference, performance was also monitored for a standard gas composition of 20 % CO_2 and 80 % H_2.

While growth and acetate production started immediately in idealized and standard gas mixtures, the culture containing CO displayed a lag phase of ~ 100 h (Figure 1a and 1c). This adaptation phase could be reduced by transferring cells from a culture growing on CO-containing BFG (stage 1) to fresh medium (stage 2, Figure 1b and 1d). This observation implies that *A. woodii* can be adapted to growth on BFG by sequential transfers. Maximum acetate production rates were similar in both cultivations, but the total volumetric acetate productivity could be increased in stage 2 cultivations due to the reduction of the adaptation phase (Table 2). An adaptation phase of *A. woodii* when grown on CO was described previously and the delay was hypothesized to be a result of the time required for expression of enzymes involved in CO metabolism (Bertsch and Müller, 2015b).

Production rates in ideal gases increase linearly with increasing hydrogen partial pressure (Table 2), which suggests limitation by the poorly soluble substrate H_2. In contrast, acetate production is reduced by 70 % in BFG at low compared to high H_2 blends. The final OD_{600} in these two experiments did not vary to the same extent, probably due to different CO

partial pressures in the gases. Average maximum and total acetate production on BFG at the high H_2 blend in the second stage only differ by 12 % at high H_2 blend. Conclusively, the efficient utilization of BFG is possible in *A. woodii* when cells are previously adapted to growth on CO-containing gas.
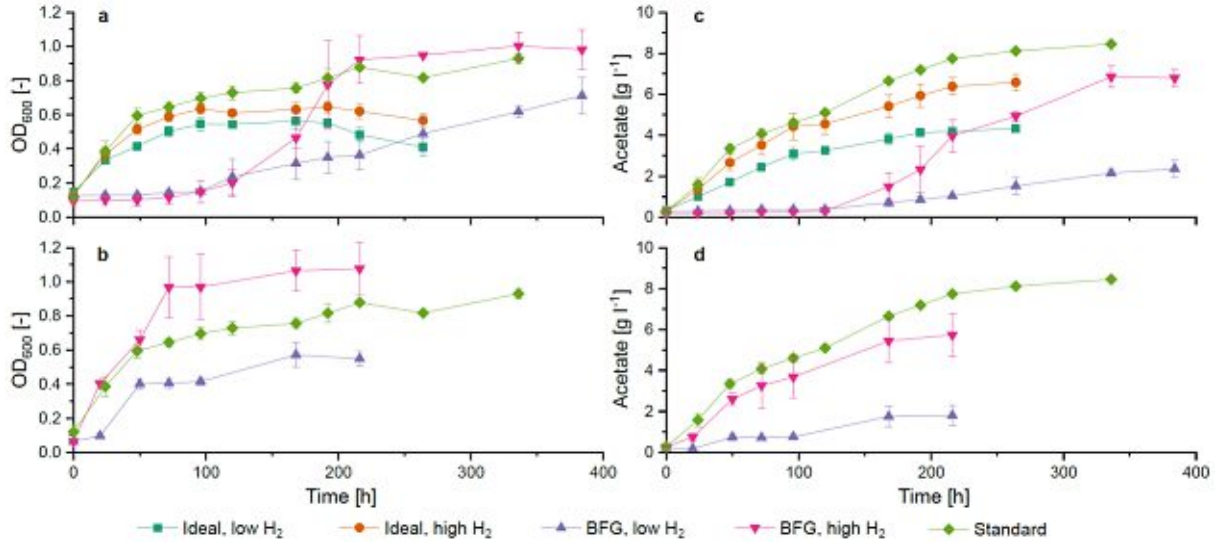
Figure 1 – Presence of CO delays growth and acetate production of *A. woodii* and adaptation to CO prevents this delay. Optical density (a, b) and acetate concentration (c, d) over time are shown. Industrial furnace gas (real) was diluted with H_2 and compared to ideal gas without CO and a standard mixture (80 % H_2, 20 % CO_2). In a and b serum bottles were inoculated from mixotrophically growing cells (Stage 1), in c and d adapted cells from the first stage were used for inoculation (Stage 2). Bicarbonate-buffered medium was used. Results represent means and standard deviations from biological triplicates in serum bottle experiments. The head space was refilled when the gas was consumed.

Table 2 – Maximal and total acetate productivity of *A. woodii* on gases of different compositions. Means and standard deviations of biological triplicates are shown in bicarbonate-buffered medium. After growth on CO-containing gas (stage 1), adapted cells were transferred to fresh medium (stage 2).

Gas	CO_2 [%]	H_2 [%]	CO [%]	$r_{Ace, max}$ [mmol l^{-1} h^{-1}]	$r_{Ace, total}$ [mmol l^{-1} h^{-1}]
idealized, low H_2	16.7	30.0	-	12.2 ± 0.1	7.9 ± 0.1
idealized, high H_2	9.5	60.0	-	21.8 ± 0.9	11.3 ± 0.9
BFG, low H_2, stage 1	16.7	30.0	18.5	4.1 ± 0.5	2.2 ± 0.5
BFG, high H_2, stage 1	9.5	60.0	10.6	27.2 ± 0.6	7.9 ± 0.6
BFG, low H_2, stage 2	16.7	30.0	18.5	7.6 ± 0.5	3.7 ± 1.0
BFG, high H_2, stage 2	9.5	60.0	10.6	24.7 ± 5.7	12.3 ± 2.5
standard	20.0	80.0	-	29.3 ± 0.2	13.8 ± 0.2

Formate addition reduces adaptation phase during growth on CO

The principle behind CO toxicity in acetogens like *A. woodii* is that CO inhibits hydrogenases, i.e. the hydrogen dependent CO_2 reductase (HDCR). This enzyme is responsible for the formation of formate from H_2 and CO_2 in the methyl branch of the Wood-Ljungdahl pathway. Inhibition of HDCR therefore prevents the formation of the methyl group for acetyl-CoA synthesis, thereby disabling growth. The methyl branch can alternatively be fed by the addition of formate, which finally allows for growth and CO utilization (Bertsch and Müller, 2015b).
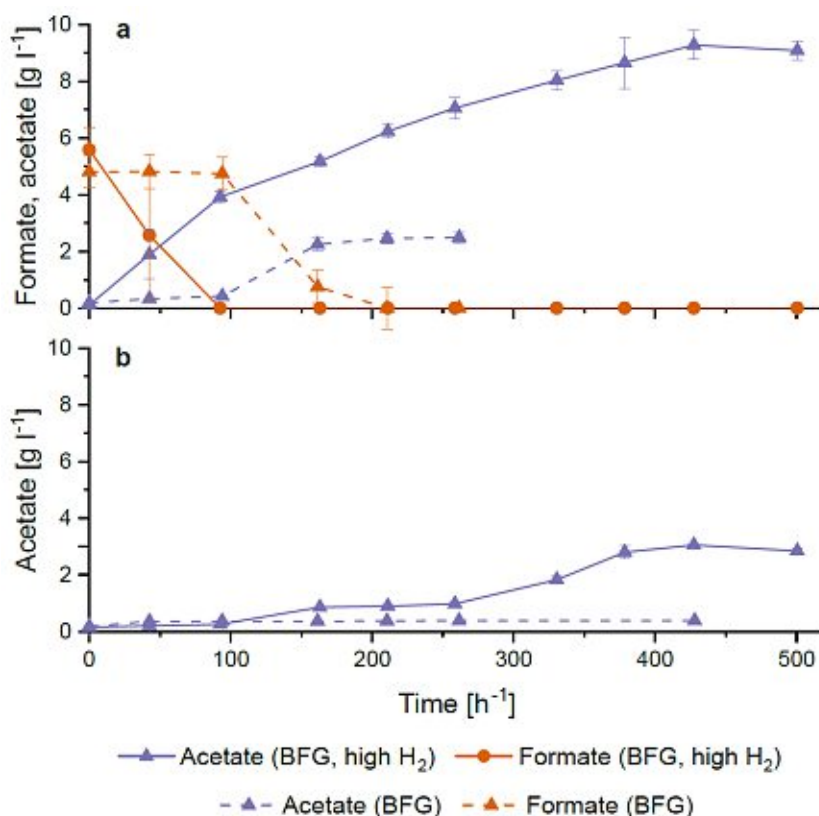
Figure 2 – Addition of formate circumvents adaptation phase of *A. woodii* when grown on CO-containing gas. *A. woodii* was grown on industrial furnace gas with 60 % H_2 in phosphate-buffered medium with (a) or without (b) the addition of 5 g l^{-1} formate. Acetate and formate concentrations are shown over time. Results represent means and standard deviations from biological duplicates in serum bottle experiments. The head space was refilled when the gas was consumed.

To investigate whether the addition of formate can improve growth and production characteristics on BFG, further experiments were carried out in serum bottles. BFG alone and the high H_2 blend BFG were used as carbon and energy source and cultures with and without formate were compared (Figure 2). Whereas cultivations without formate still displayed a growth delay of ~ 150 h, no lag phase was observed when cultures were provided with formate. Growth and acetate production even continued in BFG with H_2 after formate depletion, indicating co-consumption of CO, CO_2 and H_2. When CO was present as the sole carbon and energy source, acetate production ceased after formate depletion (Figure 2a). More acetate was produced in experiments where formate was added, potentially due to a pH effect. The consumption of formic acid leads to a pH increase thereby providing more capacity for acid (acetate) production, since *A. woodii* growth ceased at conditions of low pH. Acetate titers in the experiment without formate are not comparable to initial experiments since phosphate-buffered instead of bicarbonate-buffered medium was used. Apart from differences in buffer capacities, the presence of bicarbonate potentially also had an influence on enzymes of the WLP or enzymes involved in energy

conservation, as shown for *Thermoanaerobacter kivui* (Schwarz et al., 2020; Schwarz and Müller, 2020).

Conclusively, the addition of formate to cultures of *A. woodii* containing CO in the gas phase reduced the lag-phase and enabled growth on BFG blended with H_2 after formate depletion.

Batch cultivations show feasibility of CO, CO_2 and H_2 co-utilization

In contrast to experiments in serum bottles, continuously supplied gas in bioreactor cultivations enhances substrate availability and improves productivity. To study whether growth of *A. woodii* on CO-containing BFG is also feasible in bioreactor cultivations and to investigate acetate production and uptake characteristics of the individual gases, batch experiments were carried out. Since high H_2 blends were shown to result in superior production characteristics, this gas dilution was chosen for cultivations with ideal gas with and without CO. Formate was added in cultivations using CO-containing gas to facilitate efficient growth.

During growth on gas with only CO_2 and H_2, uptake rates of these two gases approximately represented a stoichiometric ratio of 2:1 (Figure 3a and 3b). Gas uptake rates increased steadily until reaching their maximum after 50 – 60 h. High and inhibiting acetate concentrations in the culture broth might have been a reason for the subsequent decrease of gas uptake. Although biomass formation ceased in the late batch phase, acetate was still produced.

When CO is present in the gas, it is first co-utilized with formate, resulting in the production of CO_2 and H_2 (Figure 3c and 3d). As soon as CO in the offgas dropped below 8.5 % CO (from the initial 10.6 %), CO_2 and H_2 uptake started. Subsequently, CO, CO_2 and H_2 co-utilization progressed and continued even after the depletion of formate. In accordance with cultivations on ideal gas without CO, the uptake rates of CO_2 and H_2 decreased after ~ 50 h of cultivation. Product inhibition by high acetate concentrations could be responsible for these decreasing gas uptake rates. While inhibition by acetate was previously observed at concentrations between 8 – 12 g l^{-1} (Kantzow et al., 2015), in this study impaired gas uptake rates were only observed when acetate titers exceeded 20 g l^{-1}.

The final biomass concentration is 3-fold higher in cultivations on gas containing CO, whereas the final acetate titer was increased by 16 % (Table 3). Our data from bioreactor cultivations therefore confirmed the previous observation that the utilization of CO is beneficial for biomass formation in *A. woodii* due to a 5-fold higher ATP yield (Bertsch and Müller, 2015b, 2015a). Volumetric H_2 uptake rates were comparable in both cultivations, which suggests a limitation during the exponential growth phase. Increased biomass

concentration during cultivation on CO, CO_2 and H_2 in combination with comparable H_2 uptake rates generally resulted in decreased specific rates on CO-containing gas.

The maximum specific growth rate (μ_{max}) was ~ 0.1 h^{-1} on both gases (Table 3) and is comparable to values previously reported (0.112 h^{-1} and 0.125 h^{-1} on H_2/CO_2 and formate-CO, respectively) (Bertsch and Müller, 2015b). CO only decreased growth rates when present at partial pressures above 50 %.
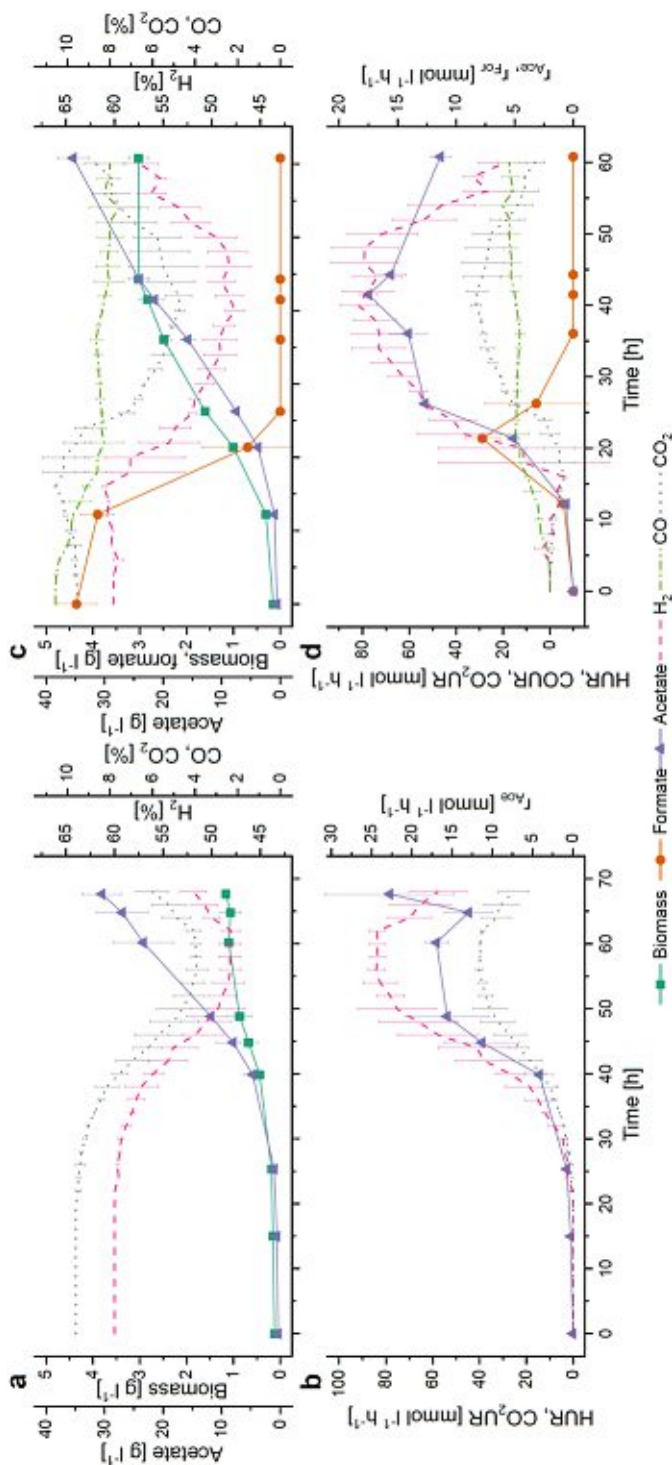
Figure 3 – Growth and acetate production is possible in batch cultivations of *A. woodii* at ideal gas composition even in the presence of CO. Medium was continuously sparged with ideal gas containing 60 % H_2 (a, b) and the ideal gas with CO and 60 % H_2 (c, d). Metabolite concentrations and gas composition (a, c) as well as volumetric rates (b, d) are shown for acetate, biomass, formate, CO_2, CO and H_2. The gas composition (in %) in a and c is based on the gas flow rate of the ingas. Results represent means and standard deviations from biological duplicates of bioreactor cultivations.

Table 3 – Fermentation characteristics of A. woodii cultivations in batches on ideal gas mixtures containing 60 % H_2 with and without CO. Maximum volumetric and specific production (acetate) and uptake (H_2, CO_2 and CO) rates are shown. Means and standard deviations were calculated from biological duplicates.

Gas	Titer [g l⁻¹]		Max. spec. growth rate [h⁻¹]	Maximum volumetric rates [mmol l⁻¹ h⁻¹]				Maximum specific rates [mmol g⁻¹ h⁻¹]				Balances [%]		
	Acetate	Biomass	μ_{max}	r_{Ace}	HUR	CO_2UR	COUR	q_{Ace}	q_{H2}	q_{CO2}	q_{CO}	Carbon	Hydrogen	DoR
Idealized gas without CO, high H_2	30.5 ± 3.3	1.16 ± 0.08	0.10 ± 0.03	22.8 ± 8.0	84.1 ± 4.2	40.3 ± 0.4	-	21.1 ± 2.0	82.9 ± 4.8	39.6 ± 0.3	-	112 ± 16	108 ± 3	108 ± 6
Idealized gas with CO, high H_2	35.4 ± 2.7	3.02 ± 0.22	0.13 ± 0.01	17.5 ± 0.8	79.8 ± 13.8	31.2 ± 4.6	19.8 ± 1.3	9.9 ± 0.4	41.0 ± 13.5	17.5 ± 1.9	11.8 ± 1.3	87 ± 1	101 ± 7	82 ± 6

Table 4 – Steady state data of A. woodii chemostat cultivations on ideal gas mixtures without CO under varying mass transfer. Volumetric and specific production (acetate) and uptake (H_2, CO_2 and CO) rates are shown. Means and standard deviations were calculated from biological duplicates. DoR = degree of reduction.

Idealized gas without CO	Stirrer speed [rpm]	Titer [g l⁻¹]		Volumetric rates [mmol l h⁻¹]			Specific rates [mmol g⁻¹ h⁻¹]			Yields [mol mol⁻¹]		Balances [%]		
		Biomass	Acetate	r_{Ace}	HUR	CO_2UR	q_{Ace}	q_{H2}	q_{CO2}	$Y_{Ace/CO2}$	$Y_{Ace/H2}$	Carbon	Hydrogen	DoR
Low H_2 blend	300	0.32 ± 0.02	3.7 ± 0.3	3.1 ± 0.1	9.0 ± 2.4	4.2 ± 1.6	9.6 ± 0.2	27.9 ± 9.6	12.3 ± 4.8	0.67 ± 0.29	0.36 ± 0.10	123 ± 35	104 ± 17	114 ± 36
	600	0.82 ± 0.02	13.3 ± 0.91	11.5 ± 0.2	52.3 ± 1.7	24.3 ± 0.9	14.2 ± 0.3	64.6 ± 1.3	30.1 ± 0.8	0.47 ± 0.02	0.22 ± 0.01	103 ± 4	99 ± 9	95 ± 4
	1200	0.92 ± 0.03	17.2 ± 0.1	17.0 ± 0.1	78.2 ± 5.2	36.3 ± 0.8	18.6 ± 0.7	85.6 ± 8.5	39.7 ± 3.1	0.47 ± 0.02	0.22 ± 0.01	100 ± 5	94 ± 3	93 ± 7
High H_2 blend	600	0.80 ± 0.01	15.6 ± 0.2	13.5 ± 0.9	64.0 ± 2.0	28.6 ± 1.0	16.9 ± 0.9	79.7 ± 1.2	35.6 ± 1.8	0.48 ± 0.05	0.21 ± 0.01	102 ± 10	93 ± 1	90 ± 3
	1200	1.20 ± 0.05	19.5 ± 0.2	20.6 ± 0.2	102 ± 10	46.2 ± 0.5	17.1 ± 1	85.2 ± 4.5	38.4 ± 2.1	0.45 ± 0.01	0.20 ± 0.02	96 ± 1	91 ± 5	87 ± 10

Continuous cultivations of *A. woodii* on CO_2 and H_2 are limited in gas liquid mass transfer of H_2

After demonstrating simultaneous CO, CO_2 and H_2 uptake in batch cultivations, we aimed to characterize gas uptake, growth, and acetate formation under steady state conditions in continuous cultivations of *A. woodii*. To that end, we sought to establish gas-limited cultures as a basis for solid characterization of the process performance. Due to the low solubility of gases, especially of H_2, gas fermentations are often limited in gas liquid transfer of H_2 (Mohammadi et al., 2011). To study H_2 limitation, we first investigated the influence of different H_2 partial pressures and gas liquid mass transfer rates independently from the presence of CO. Therefore, chemostats were performed on low (30 %) and high (60 %) H_2 blend idealized gas (without CO) at a dilution rate of 0.05 h^{-1} (~ 50 % of μ_{max}).

Acetate was produced at a rate of 15.6 mmol l^{-1} h^{-1} at 600 rpm in the high H_2 blend, which is ~ 20 % higher compared to the low H_2 blend. H_2 and CO_2 uptake rates were enhanced to the same extent by the improved availability of H_2 in the high H_2 blend.

Gas transfer is typically proportional to partial pressures and several factors can influence hydrogen solubility (Demler and Weuster-Botz, 2011). Apart from media additives like e.g. antifoam, increased biomass concentrations were reported to decrease H_2 solubility (Cotter et al., 2009). Subsequently, we examined whether cultivations at 600 rpm were limited in H_2 gas liquid mass transfer by increasing and decreasing the stirrer speed to 1200 and 300 rpm, respectively.

Increasing the stirrer speed from 600 rpm to 1200 rpm improved gas-liquid mass transfer as indicated by enhanced gas uptake rates (Figure 4). In both approaches at high and low H_2 blend, the enhanced volumetric power input increased volumetric H_2 uptake and acetate production rates to 150 %, while specific uptake rates stagnated or increased by 30 % in the high and low H_2 blend, respectively (Table 4). A decrease of the stirrer speed from 600 rpm to 300 rpm drastically reduced H_2 uptake and acetate production to ~ 20 %. Therefore, cultures were indeed H_2-limited at 600 rpm stirrer speed. Interestingly, the specific rates for H_2 uptake and acetate production reduced with decreasing mass transfer, indicating that carbon flux was directed towards biomass rather than acetate. Low gas uptake during conditions of reduced gas uptake led to more acetate and biomass being generated from yeast extract in the liquid medium.

In addition to a limitation of H_2 gas liquid mass transfer, continuous cultivations on H_2 and CO_2, could be limited by the following mechanisms: (i) limitation in the liquid medium, e.g. nitrogen source or a trace element, (ii) CO_2 limitation, (iii) a physiological limitation, i.e. by

reaching maximum uptake or production per cell or (iv) product inhibition. To exclude any other limitation the other four limitation possibilities were systematically examined.

To eliminate a limitation in the liquid medium, individual pulses at steady state conditions were applied to increase the yeast extract and trace element concentrations by 100 and 50 %, respectively. These pulses showed no effect on gas uptake rates, biomass, or acetate production (data not shown), indicating that cultivation performance was not restrained by any of the pulsed components. A limitation in gas liquid transfer of CO_2 is also not probable, since the CO_2 transfer rate is still 4.5-fold higher than the HTR under the conditions tested (pH_2 = 60 %, pCO_2 ~ 10 %, Supplementary File 2). Since both, the acetate concentration and the specific H_2 uptake and acetate production rates were higher at 1200 compared to 600 rpm, a physiological limitation or product inhibition is also unlikely.
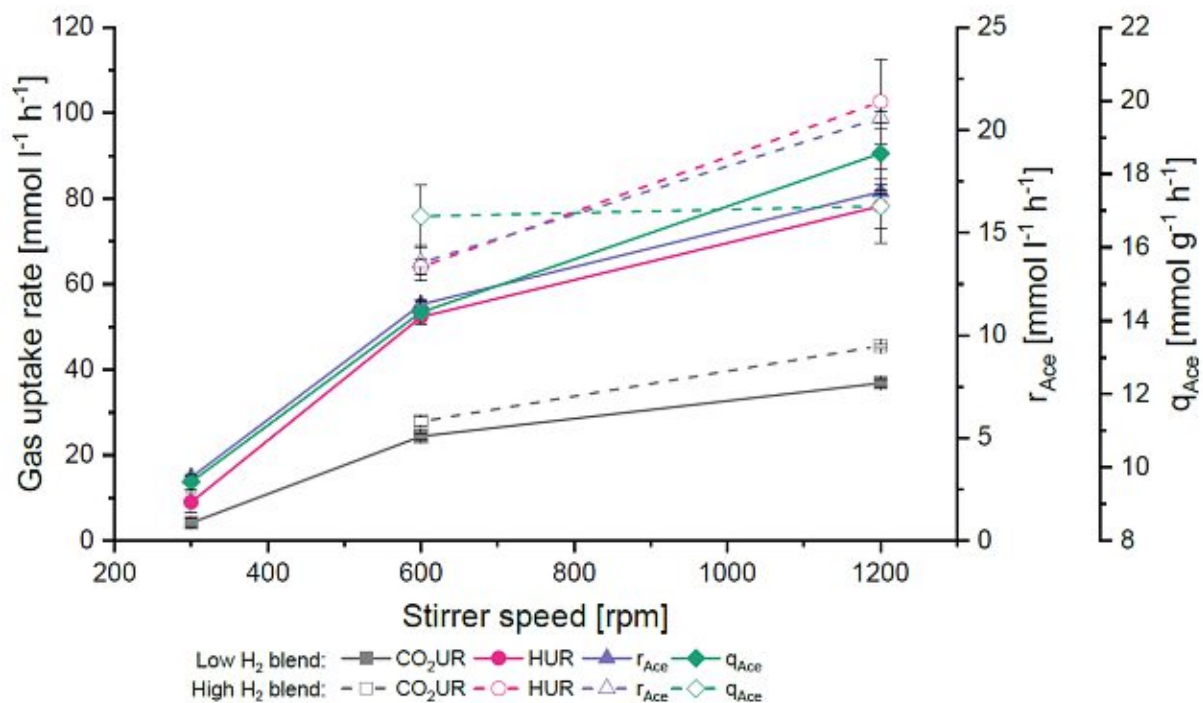
Figure 4 – Increased gas liquid transfer improves gas uptake and production rates during continuous cultivation of *A. woodii* on gas containing CO_2 and H_2. Mass transfer was improved by increasing stirrer speed. Bioreactors were sparged continuously with ideal gas diluted with 30 % H_2. Results represent means and standard deviations of biological duplicates in steady state during continuous cultivation.

Our results in chemostat experiments (13.5 mmol l^{-1} h^{-1}) are within 7 % to previous reports, where a slightly lower acetate productivity of 12.6 mmol l^{-1} h^{-1} (= 302.4 mmol l^{-1} d^{-1} = 18.2 g l^{-1} d^{-1}) was observed during continuous cultivation of *A. woodii* (Kantzow et al., 2015). There, acetate productivity was only increased when a cell retention system was used (higher dilution rates could be increased at constant acetate concentrations. In contrast to this study, H_2 uptake rates were not comparable in batch and chemostat experiments. Concomitantly, acetate concentrations reached ~ 22 g l^{-1} in continuous cultivations

(Kantzow et al., 2015). Since we observed product inhibition at acetate concentrations above 20 g l^{-1} in batch experiments of this study, it is possible that continuous cultivations described by Kantzow et al. (2015) were limited by product inhibition rather than by H_2 transfer. When aiming for gas-limited processes, the focus should therefore be laid on achieving acetate concentrations below 20 g l^{-1}. High acetate productivities in H_2-limited cultures could be reached by increasing the dilution rate and eventually establishing a cell retention process.

Conclusively, we showed that autotrophic cultivations with *A. woodii* on H_2 and CO_2 are very flexible regarding both gas composition and gas liquid mass transfer. Cultivations at 600 rpm at low and high H_2 blend were limited in H_2 availability, which makes these conditions suitable for further process characterization.

Dynamic gas liquid mass transfer shifts allow to approximate maximum qH$_2$

To determine the physiological limit of our system, we aimed to approximate the maximum possible specific H_2 uptake rate. This important characteristic of the host strain can contribute to a better understanding of the physiology of *A. woodii* and facilitate further process development. To this end, a dynamic experiment with a shift from lower to higher volumetric power input was performed. By this means, the hydrogen transfer rate was suddenly increased in a cultivation with low biomass (low stirrer speed). At low biomass concentrations, *A. woodii* cannot utilize the available H_2 and will grow at maximum q_{H2} until cells are no longer physiologically limited. Since uptake rates of H_2 and CO_2 as well as acetate are stoichiometrically dependent (H_2 : CO_2 ~ 2:1, H_2 : acetate ~ 4:1), different approaches can be used to approximate the maximum q_{H2}: directly via online measurement of HUR or indirectly via online measurement of CO_2 and offline determination of acetate.

After an increase of the stirrer speed at 280.2 h, specific H_2 and CO_2 uptake instantly increased, remained constant for ~ 20 h and subsequently decreased (Figure 5a). To correct for errors and variations in quantification of specific uptake rates, we plotted the volumetric rates over biomass concentration during growth at maximum q_{H2} (Figure 5b). Data was fitted with a linear regression, where the slope represents the corresponding maximum specific rate. These fitted specific rates indicate that the maximum specific H_2 uptake rate $q_{H2,max}$ is between 116 and 167 mmol g^{-1} h^{-1} (Table 5). The intercept of the regression has a negative value and if the gas uptake rate is set to 0 mmol l^{-1} h^{-1}, solving the linear regression for the biomass concentration (X) will result in the amount of biomass produced at no gas uptake. This biomass production can be attributed to the presence of yeast extract in the medium and was used to correct carbon balances of chemostat cultivations at low gas transfers (300 rpm). An approximation of the acetate production from

yeast extract was performed accordingly (Table 5 and Supplementary File 2, Figure and Table S1).
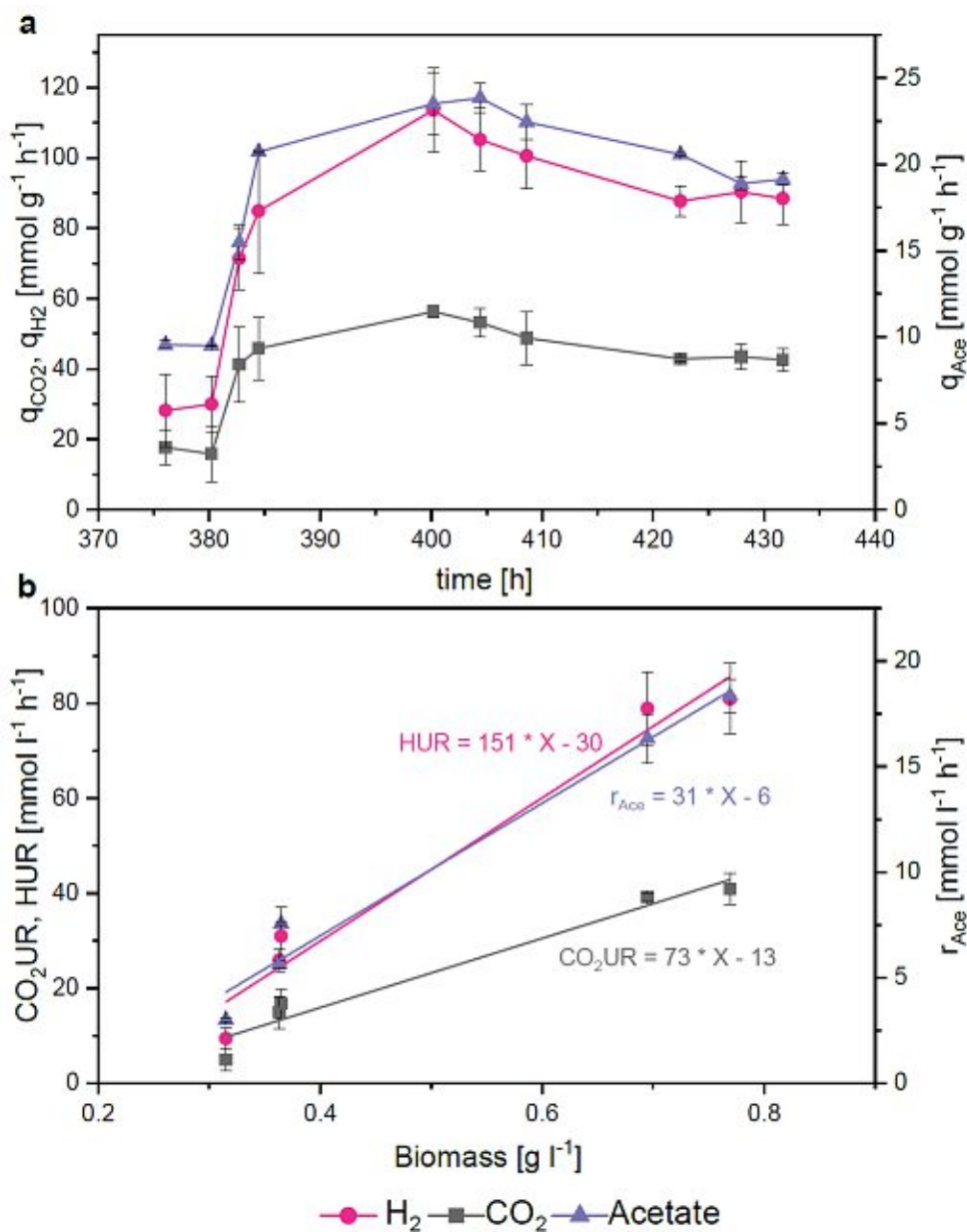
Figure 5 – Approximation of maximum q_{H2} using dynamic shift experiments in continuous cultivation of *A. woodii* on gas containing CO₂ and H₂. In a, specific uptake and production rates over time are shown. Cultivation was shifted from low mass transfer (300 rpm) to high mass transfer (1200 rpm) at 380.2 h. In b, volumetric CO₂ and H₂ uptake and acetate production are plotted against biomass concentration for samples from 380.2 h to 440.4 h (linear range). The data was fitted by linear regression and the corresponding line and equation is shown in the plot (X = biomass). The slope represents the maximum specific rate and when X is set to zero, the biomass concentration at no gas uptake (and thus only from yeast extract in the medium) can be obtained. Results represent means and standard deviations of biological duplicates during continuous cultivation.

Table 5 – Process characteristics obtained from mass transfer shift experiment. To increase gas transfer into the liquid phase, stirrer speed was increased from 300 to 1200 rpm. Data was obtained from linear regression of volumetric rates with biomass concentrations. Means and standard error of regression are shown. [1] calculation is shown in more detail in Supplementary File 2, Figure and Table S1.

Calculation for	Slope = specific gas uptake rate [mmol g^{-1} h^{-1}]	R^2	$q_{H2,\ max}$ [mmol g^{-1} h^{-1}]	Intercept [mmol l^{-1} h^{-1}]	Biomass at no gas uptake [g l^{-1}]
H_2	151 ± 16	0.966	151 ± 16	-30.4 ± 8.7	0.20 ± 0.08
CO_2	73 ± 9	0.957	146 ± 18	-13.3 ± 4.8	0.18 ± 0.09
Acetate	32 ± 3	0.974	128 ± 12	-5.6 ± 1.6	-

Comparing the approximated $q_{H2,max}$ to steady state data on ideal gas revealed that all specific rates obtained at steady state conditions were lower than the possible maximum. Therefore, a physiological limitation of *A. woodii* in any chemostat cultivation in this study is unlikely. Conversely, specific H_2 uptake could possibly be increased by 46 % using a reactor with improved gas liquid mass transfer.

A. woodii can efficiently utilize CO_2 in gases containing CO

After characterizing growth of *A. woodii* on CO_2 and H_2 containing gases, we aimed to study CO, CO_2 and H_2 utilization in BFG and determine the effects of CO on *A. woodii* metabolism. To this end, continuous cultivations were compared using idealized gas with CO at low and high H_2 blends and BFG at high H_2 blend.

The transition from batch to continuous cultures was found to be crucial for successful growth on CO-containing gas. The optimal starting point for the continuous cultivation was after formate had been depleted, but when CO, CO_2 and H_2 were still co-utilized and before CO_2 and H_2 uptake decreased at the end of the batch. Since formate was not added to the feed, all cultivations on CO-containing gas were carried out autotrophically similar to chemostats without CO.

A. woodii was able to co-utilize CO, CO_2 and H_2 in all gas compositions and even at higher CO partial pressures (low H_2 blend, Table 6). A comparison of steady state data confirmed observations from batch experiments, where biomass increased with increasing CO partial pressure (decreasing pH_2). Higher CO partial pressures decreased acetate titers, which is also reflected by the higher specific acetate productivity in the high H_2 blend compared to the low H_2 blend. Uptake rates of CO, CO_2 and H_2 differed for the low and high H_2 blend. While HUR was 1.8-fold higher in the high H_2 blend of idealized gas with CO, COUR was 1.7-fold lower. These ratios correspond to the differences in partial pressures of the two gases, suggesting that cultures were limited in both CO and H_2 gas liquid mass transfer.

CO and H_2 limitation were also experimentally demonstrated as decreasing the stirrer speed from 600 rpm to 300 rpm resulted in decreased gas uptake rates (data not shown). Surprisingly, H_2 uptake rates were lower in high H_2 blends when gases did not contain CO (Table 4 and 6). Apart from the influence of biomass concentration on H_2 and CO solubility (Cotter et al., 2009; Rittmann et al., 2012), the presence of other gases could potentially influence gas uptake or solubility. Since H_2 was the limiting factor in all chemostats, the uptake of CO_2 was tightly coupled to H_2 utilization. Because CO was utilized as an additional carbon and energy source, total carbon turnover was higher compared to gases without CO. Utilization of CO reduced CO_2 uptake, but still enabled net CO_2 fixation even in the low H_2 blend with a high partial pressure of CO.

Cultivations of *A. woodii* on industrial BHG resulted in similar gas uptake rates compared to idealized gas with CO and the same H_2 blending ratio. Biomass and acetate production were slightly decreased, and acetate productivity reached 86 % of that in idealized gas of the same composition. The specific acetate productivity remained constant due to identical H_2 and CO partial pressures. The slight difference in production rates on real and ideal gas with CO indicate that other inhibitors in the BFG could have a minor influence on the performance of *A. woodii*. Gas uptake rates during the cultivation on BFG varied to a greater extent than on idealized gas with CO. These variations can probably be attributed to the *in situ* blending of two independent gas streams, whereas premixed gases were used for all other bioreactor experiments. The fact that *A. woodii* was not negatively influenced by these fluctuations renders the organism suitable for industrial applications, since similar deviations of the gas composition might also occur in large-scale fermentations.

During growth on CO_2 and H_2 alone, specific H_2 uptake rates of *C. autoethanogenum* were ~ 60 % of those in *A. woodii* (Heffernan et al., 2020). Based on the comparison of specific CO and H_2 uptake rates, we could therefore confirm that the high efficiency of H_2 and CO_2 utilization by *A. woodii* compared to *C. autoethanogenum* and *C. ljungdahli* (Hermann et al., 2020; Schuchmann and Müller, 2014b; Takors et al., 2018). On the other hand, for gases with comparable CO and H_2 partial pressures, higher specific CO uptake rates were observed in *C. autoethanogenum*, while q_{H2} was only 60 % of the maximum reached using BFG in this study (Valgepea et al., 2018).

Table 6 - **Steady state data of A. woodii chemostat cultivations on gas mixtures containing CO.** Stirrer speed was set to 600 rpm. Volumetric and specific production (acetate) and uptake (H_2, CO_2 and CO) rates are shown. Means and standard deviations were calculated from biological duplicates DoR = degree of reduction.

Gas	Titer [g l^{-1}]		Volumetric rates [mmol l^{-1} h^{-1}]				Specific rates [mmol g^{-1} h^{-1}]				Yields [mol mol^{-1}]		Balances [%]		
	Biomass	Acetate	r_{Ace}	HUR	COUR	CO$_2$UR	q_{Ace}	q_{H2}	q_{CO}	q_{CO2}	$Y_{Ace/CO2}$	$Y_{Ace/H2}$	Carbon	Hydrogen	DoR
Idealized gas with CO, low H$_2$ blend	1.86 ± 0.07	13.4 ± 0.7	12.6 ± 0.7	40.8 ± 2.5	22.1 ± 0.9	10.6 ± 0.2	6.8 ± 0.6	21.9 ± 0.6	11.9 ± 0.4	5.5 ± 0.1	0.37 ± 0.01	0.29 ± 0.01	89 ± 3	86 ± 6	95 ± 8
Idealized gas with CO, high H$_2$ blend	1.54 ± 0.12	17.8 ± 1.5	16.6 ± 0.7	71.6 ± 2.3	13.4 ± 1.4	26.2 ± 0.2	10.8 ± 0.4	46.6 ± 2.2	8.7 ± 0.2	17.1 ± 1.2	0.43 ± 0.01	0.23 ± 0.01	93 ± 1	87 ± 1	87 ± 1
BFG, high H$_2$ blend	1.27 ± 0.06	15.1 ± 0.7	14.3 ± 0.4	72.2 ± 8.0	12.5 ± 8.1	20.8 ± 5.7	11.1 ± 0.8	51.2 ± 13.0	9.5 ± 6.3	16.1 ± 5.5	0.44 ± 0.04	0.23 ± 0.05	97 ± 8	86 ± 1	81 ± 3

The fact that CO utilization in *A. woodii* is less efficient compared to other acetogens has long been described. Potential reasons include inhibition of hydrogenases by CO. *A. woodii* might be affected by this inhibition to a larger extent compared to other acetogens because the organism reduces CO_2 directly with H_2 instead of redox equivalents (NAD(P)H and/or reduced ferredoxin) used by *C. autoethanogenum* and *C. ljungdahlii* (Bertsch and Müller, 2015b, 2015a; Mock et al., 2015; Ragsdale and Ljungdahl, 1984; Schuchmann and Müller, 2014a; Schwarz et al., 2020). In addition to hydrogenase inhibition, the efficiency of the CO dehydrogenase reaction has also been speculated to be responsible for efficient CO utilization (Liew et al., 2016a; Ragsdale et al., 1983). *T. kivui* could be adapted to growth on high concentrations of CO even though the organism also uses H_2 for CO_2 reduction to formate (Weghoff and Müller, 2016). Therefore, it was suggested that monofunctional CO dehydrogenases might play an important role in CO metabolism to quickly scavenge CO and prevent hydrogenase inhibition.

Fermentation of CO_2 and H_2 using *C. autoethanogenum* was found to be improved by adding small amounts of CO (Heffernan et al., 2020). While CO_2 and H_2 partial pressures were comparable to our study, CO concentrations in the gas were only 2 %. In this study, we achieved net CO_2 fixation by simultaneous uptake of CO_2 and H_2 with CO at higher CO and lower H_2 concentrations of 18 and 30 %, respectively. Comparable CO and H_2 partial pressures of 15 and 45 %, respectively promoted efficient CO utilization and a shift towards reduced metabolites in *C. autoethanogenum* but resulted in the production of small amounts of CO_2 (Valgepea et al., 2018).

Prior to this study, *A. woodii* had not been considered for fermentation of CO-rich industrial gases. Moreover, CO utilization at comparable gas compositions usually results in net CO_2 production (Hermann et al., 2020; Valgepea et al., 2018, 2017). Here, we show that *A. woodii* is highly flexible regarding co-utilization of CO, CO_2 and H_2 at different gas compositions allowing net CO_2 fixation. H_2 blending in combination with its efficient H_2 metabolism renders *A. woodii* a suitable host for CO_2 fixation from industrial gases even in the presence of CO.

Intracellular flux distributions of *A. woodii* are dependent on H_2 and CO availability

Steady state data for growth of *A. woodii* obtained from chemostat cultivations on idealized gas with and without CO were used to model intracellular flux distributions. An *A. woodii* core model was used for FBA simulations. The measured specific rates for biomass formation, substrate uptake rates (CO, CO_2, H_2) and acetate were used to constrain the model. Since these measurements bear redundancies with respect to carbon and redox balances in the metabolic model, fluxes were corrected prior to the actual FBA calculations by minimizing the relative changes in the measured rates necessary to obtain a consistent

flux scenario. In typical FBA studies, maximization of biomass synthesis is used as objective function. Since the growth rate was fixed to the experimentally observed value, we instead maximized the pseudo reaction that quantifies the non-growth associated ATP maintenance (NGAM) demand. In this way, we obtain an upper bound of ATP available for NGAM processes. Figure 6 summarizes the simulation results for intracellular fluxes of the carbon, redox and energy metabolism. Experimental data of biomass formation as well as specific uptake (CO, CO_2 and H_2) and acetate excretion rates proved to be very consistent, since in all scenarios considered only small corrections for two measured fluxes were necessary (Figure 6). In terms of ATP production, the specific flux through the ATPase increased by ~ 20 % for high H_2 compared to low H_2 for H_2/CO_2 fermentations. ATP generation was further increased in the presence of CO regardless of the H_2 partial pressure. In detail, low and high CO partial pressures increased flux through the ATPase by an additional 20 % compared to the corresponding H_2/CO_2 without CO. Likewise, the NGAM was lowest for the condition using low H_2 without CO (0.9 mmol g^{-1} h^{-1}) and highest for the high H_2 in combination with CO (2.4 mmol g^{-1} h^{-1}). Generally, higher maintenance ATP demands in the presence of high H_2 or CO could be a result of additional protein synthesis required. Furthermore, higher acetate concentrations were observed when high H_2 was used, which could also increase the ATP demand to maintain the proton gradient across the membrane.

Model simulations showed that at lower CO and high H_2 partial pressures (i.e. high H_2 blend), CO was only assimilated in the carbonyl branch, thus only serving as a carbon, but not as an electron source (Figure 6b). CO being solely utilized as a carbon source is an interesting observation, as CO usually serves as the main electron source during acetogenic growth on CO or syngas and CO_2 produced is only partially utilized in the methyl branch (Hermann et al., 2020; Valgepea et al., 2018, 2017). On the contrary, higher CO and lower H_2 partial pressures (i.e. low H_2 blend) resulted in CO additionally being consumed in the methyl branch after oxidation to CO_2 by CO dehydrogenase (CODH). CO was therefore used both as a carbon and electron source, which resulted in increased availability of reducing equivalents, i.e. reduced ferredoxin. This change in metabolic activity finally led to differences in co-factor distribution and energy conservation. However, the conversion of CO to CO_2 reduced net CO_2 fixation in a H_2-limited process (Table 6). If the goal was to utilize CO_2 only via the methyl and CO only via the carbonyl branch and the flux through CO dehydrogenase linearly correlated with CO partial pressures, the BFG would have to be blended with 44 % H_2 (linear regression of CODH flux and pCO). In this study, we achieved net CO_2 fixation in gases with at CO/H_2 ratio of 0.18 to 0.62. Based on the CO_2 uptake rates for these ratios, linear extrapolation shows that CO_2 utilization would be possible up to a CO/H_2 ratio of 0.92 (linear regression of CO_2UR and CO/H_2 ratio). In other words, blending industrial BFG with H_2 as an energy source can be used to control individual

gas uptake rates and shift *A. woodii* metabolism towards a desired direction (ATP and biomass versus acetate formation). This strategy could also be applied to other gas fermenting organisms to control product spectra and shift production towards a desired target compound.
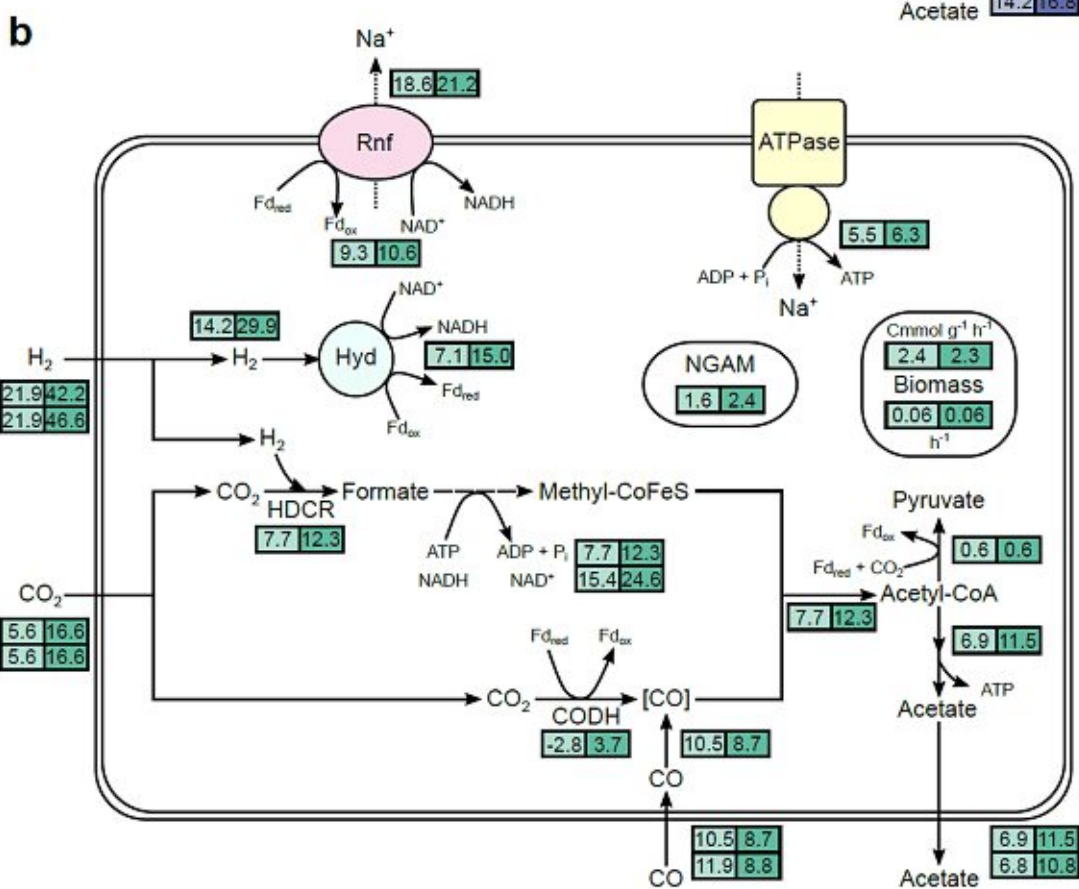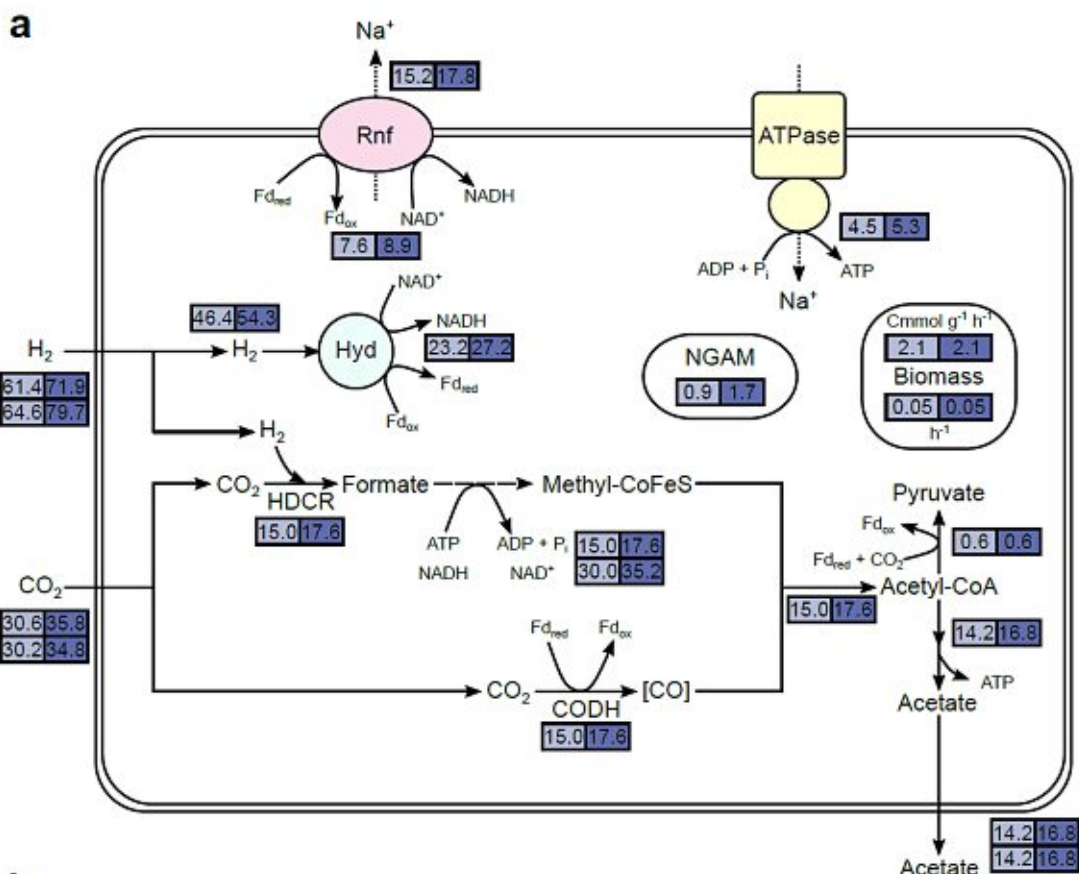
Figure 6 – Metabolic flux distributions of *A. woodii* chemostat cultivations depend on gas composition. Flux distributions for **a**, cultivations with idealized gas without CO with low H_2 (30 %) (light blue) and high H_2 (60 %) (dark blue) and **b**, cultivations with idealized gas with CO with low H_2 (30 %) (light green) and high H_2 (60 %) (dark green) are shown. Experimentally determined specific rates for acetate production, biomass formation and gas uptake rates were used as input data and constraints for FBA simulations with maximization of the ATP maintenance pseudo reaction (NGAM) as the objective function. All fluxes are shown in mmol g^{-1} h^{-1} (Biomass box: upper values in Cmmol g^{-1} h^{-1}, lower values in h^{-1}) and represent means of biological duplicates. Upper values for uptake and production rates represent experimental values, lower values represent values used for FBA simulation. Model input values may slightly deviate from measured experimental data because stoichiometric inconsistencies were corrected (see Methods). NGAM = non growth-associated ATP maintenance, HDCR = hydrogen dependent CO_2 reductase, CODH = CO dehydrogenase, Hyd = electron-bifurcating hydrogenase, Rnf = *Rhodobacter* nitrogen fixation complex, Fd_{red} = reduced ferredoxin, Fd_{ox} = oxidized ferredoxin.

Conclusion

In this study, we determined the effect of gas composition and gas-liquid mass transfer on CO_2 utilization, growth, and acetate production in *A. woodii*. Blending of industrial BFG with H_2 was a useful tool to enable co-utilization of CO, CO_2 and H_2 and net CO_2 fixation at different CO and H_2 partial pressures for the first time. Additionally, this strategy allowed to control CO_2 fixation, gas uptake and metabolic fluxes in continuous cultures. The flexibility with respect to gas composition makes *A. woodii* a very promising host for the reduction of industrial CO_2 emissions, even in CO-containing gases.

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Competing interests

Voestalpine Stahl GmbH has interest to reduce its CO_2 emissions by recycling the carbon via gas fermentation for further use in products.

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Author contributions

KN: investigation, data curation, formal analysis, conceptualization, writing – original draft, visualization

CN: investigation, writing – review & editing

IK: resources, investigation

NK: resources, investigation, writing – review & editing

SK: formal analysis, resources - software, writing – review & editing

SP: conceptualization, formal analysis, resources, writing – original draft, supervision, project administration, funding acquisition

All authors have read and accepted the manuscript.

Supplementary Material
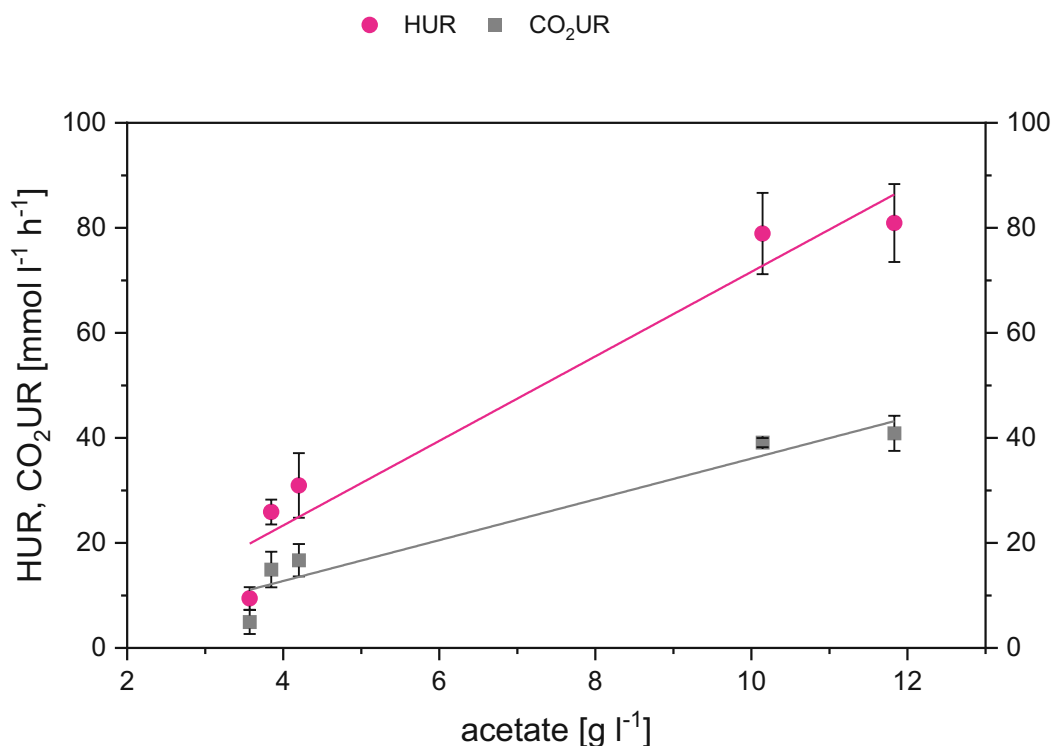
Figure S1 – Approximation of acetate production from yeast extract by regression of H_2 and CO_2 uptake rate over acetate concentration. Cultivation was shifted from low mass transfer (300 rpm) to high mass transfer (1200 rpm) and gas uptake rates are plotted against acetate concentration for samples from 380.2 h to 440.4 h (linear range). The data was fitted by linear regression.

Table S1 – Coefficients for linear regression in Figure S1. The slope represents the maximum specific rate and when X is set to zero, the biomass concentration at no gas uptake (and thus only from yeast extract in the medium) can be obtained. Results represent means and standard deviations of biological duplicates during continuous cultivation.

calculation for	slope [mmol g^{-1} h^{-1}]	R^2	intercept [mmol l^{-1} h^{-1}]	acetate at 0 gas uptake [g l^{-1}]
H_2	8.1 ± 1.1	0.947	-8.9 ± 8.4	1.1 ± 1.2
CO_2	3.9 ± 0.6	0.934	-2.8 ± 4.5	0.7 ± 1.3

Calculation of maximum CO_2 transfer rate in ideal gas with 60 % H_2 and 10 % CO_2

$HUR_{max} \sim 80$ mmol l^{-1} h^{-1}

Limitation: $HTR = HUR_{max}$

$$k_La\,(CO_2) = k_La\,(H_2)\sqrt{\frac{D_{i,CO_2}}{D_{i,H_2}}} = k_La\,(H_2) * 0.768 \quad \text{(Demler, 2012b)}$$

$HTR = k_La\,(H_2)\,(c^*(H_2) - c(H_2))$

$CO_2TR = k_La\,(CO_2)\,(c^*(CO_2) - c(CO_2))$

Limitation: $c \sim 0$ mmol l^{-1}

$c^*\,(H_2) = 0.8$ mmol l^{-1} * 0.6 = 0.48 mmol l^{-1}

$c^*\,(CO_2) = 28.6$ mmol l^{-1} * 0.1 = 2.86 mmol l^{-1}

$$k_La\,(H_2) = \frac{HUR_{max}}{c^*(H_2)} = 166 \text{ h}^{-1}$$

$k_La\,(CO_2) = 128$ h^{-1}

$CTR_{max} = k_La(CO_2) * c^*(CO_2) = 358$ mmol l^{-1} h^{-1}

Table S2 – Parameters for calculation of maximum CO_2 transfer rate.

parameter	definition	unit
HUR	hydrogen uptake rate	mmol l^{-1} h^{-1}
HTR	Hydrogen transfer rate	mmol l^{-1} h^{-1}
k_La	specific mass transfer coefficient	h^{-1}
D_i	diffusion coefficient	$m^2\,s^{-1}$
c^*	gas solubility	mmol l^{-1}
c	gas solubilized	mmol l^{-1}
CO_2TR	CO_2 transfer rate	mmol l^{-1} h^{-1}

68

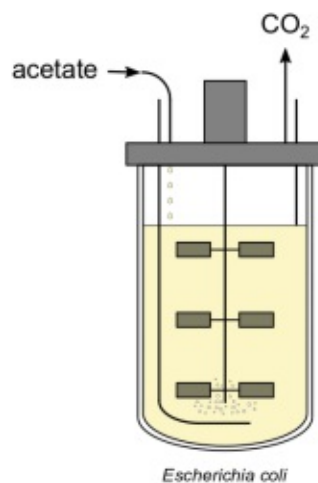## 2. Characterization and engineering of acetate (co-)utilization in *E. coli* W

Figure 4 – Overview part 2. Within this part, acetate uptake in *E. coli* W is studied.

Problem statement

Several factors limit the use of acetate as a promising alternative carbon source: (i) acetate is toxic due to its properties as a weak acid, (ii) acetate has low energy content, which limits product formation and (iii) co-utilization of acetate and glucose is restricted by carbon catabolite repression and the occurrence of a diauxic growth pattern.

State-of-the-Art

In *E. coli*, two pathways are responsible for acetate uptake, the irreversible acetyl-CoA synthetase (*acs*) and the reversible phosphate acetyltransferase / acetate kinase node. The activity of Acs is controlled at two levels: in addition to transcriptional regulation, Acs is inactivated by post-translational acetylation. A point-mutation in *acs* was shown to result in an insensitivity to post-translational acetylation in *Salmonella enterica* (Starai, 2005). Detailed information of Acs activity was not available, especially in the context of characterizing co-utilization with glucose.

Scientific questions

Is *E. coli* W a suitable host for acetate utilization?

Can acetate (co-)utilization be improved by the overexpression of an acetyl-CoA synthetase?

Hypotheses

E. coli W has been reported to be a robust, stress-tolerant and low-acetate excreting strain, which is why it can be a suitable strain for acetate (co-)utilization. Overexpression of acetyl-

CoA synthetase, one of two pathways for acetate assimilation, improves acetate uptake and co-utilization of acetate and glucose.

Approach

For the evaluation of the performance of *E. coli* W, volumetric and specific acetate uptake rates acquired in batch and continuous cultures on chemically defined medium were considered. *E. coli* W strains expressing native acetyl-CoA synthetase or an acetylation-insensitive acetyl-CoA synthetase were compared to a control strain. The effects on acetate uptake as well as acetate and glucose co-utilization were studied in both, batches and chemostats. The quantification of individual uptake rates also allowed to draw conclusions about diauxic growth.

Content and contribution

The effect of acetyl-CoA synthetase expression was studied at different levels: native *acs* expression in a control strain, overexpression of *acs* and overexpression of an acetylation-insensitive *acs*. Acetate (co-)utilization was investigated on defined media in batch and accelerostat cultivations. The overexpression of an acetylation-insensitive *acs* led to a decreased lag-phase in batches with acetate as the sole carbon source and improved acetate-glucose co-utilization. In chemostats, overexpression of acetylation-insensitive *acs* did not show any positive effects on culture performance since *E. coli* W was found to already be a very suitable host for acetate utilization.

KN carried out the experiments together with LF and AME, planned the study with SP and LF, and KN and SP drafted the manuscript.

Title of manuscript

Characterizing the effect of expression of an acetyl-CoA synthetase insensitive to acetylation on co-utilization of glucose and acetate in batch and continuous cultures of *E. coli* W

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Characterizing the effect of expression of an acetyl-CoA synthetase insensitive to acetylation on co-utilization of glucose and acetate in batch and continuous cultures of *E. coli* W

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Abstract

Background: Due to its high stress tolerance and low acetate secretion, *Escherichia coli* W is reported to be a good production host for several metabolites and recombinant proteins. However, simultaneous co-utilization of glucose and other substrates such as acetate remains a challenge. The activity of acetyl-CoA-synthetase, one of the key enzymes involved in acetate assimilation is tightly regulated on a transcriptional and post-translational level. The aim of this study was to engineer *E. coli* W for overexpression of an acetylation insensitive acetyl-CoA-synthetase and to characterize this strain in batch and continuous cultures using glucose, acetate and during co-utilization of both substrates.

Results: *Escherichia coli* W engineered to overexpress an acetylation-insensitive acetyl-CoA synthetase showed a 2.7-fold increase in acetate uptake in a batch process containing glucose and high concentrations of acetate compared to a control strain, indicating more efficient co-consumption of glucose and acetate. When acetate was used as the carbon source, batch duration could significantly be decreased in the overexpression strain, possibly due to alleviation of acetate toxicity. Chemostat cultivations with different dilution rates using glucose revealed only minor differences between the overexpression and control strain. Accelerostat cultivations using dilution rates between 0.20 and 0.70 h^{-1} indicated that *E. coli* W is naturally capable of efficiently co-utilizing glucose and acetate over a broad range of specific growth rates. Expression of acetyl-CoA synthetase resulted in acetate and glucose accumulation at lower dilution rates compared to the control strain. This observation can possibly be attributed to a higher ratio between *acs* and *pta-ackA* in the overexpression strain as revealed by gene expression analysis. This would result in enhanced energy dissipation caused by an imbalance in the Pta-AckA-Acs cycle. Furthermore, *yjcH* and *actP*, genes co-transcribed with acetyl-CoA synthetase showed significant down-regulation at elevated dilution rates.

Conclusions: *Escherichia coli* W expressing an acetylation-insensitive acetyl-CoA synthetase was shown to be a promising candidate for mixed feed processes using glucose and acetate. Comparison between batch and

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continuous cultures revealed distinct differences in glucose-acetate co-utilization behavior, requiring additional investigations such as multi-omics analysis and further engineering towards even more efficient co-utilization strains of *E. coli* W.

Keywords: E. coli W, Acetyl-CoA-synthetase, Acetate (Acs), Metabolic engineering, Protein acetylation, Biomass composition, Mixed feed system, Continuous cultures, Gene expression analysis, Acetate permease (ActP)

Background

Escherichia coli is among the best-studied organisms today and a workhorse of biotechnology used for the production of recombinant proteins [1–3] and fuel and bulk chemicals including ethanol [4, 5], isobutanol [6, 7] and 2,3-butanediol [8–10]. In particular, *E. coli* W has been described as good production host for industrial applications due to high stress tolerance [11, 12], fast growth up to high cell densities on various substrates including sucrose [13–15] and low acetate excretion [14].

Most studies utilize glucose as the carbon source, making glucose the best studied substrate for *E. coli* [12, 16]. However, other substrates such as pentoses [12, 17, 18], glycerol [19] and acetate [20, 21] have also been studied.

Acetate comprises an interesting alternative carbon source as it is a cheap industrial waste product contained in a broad variety of materials [22]. For instance, acetate is produced by anaerobic digestion of biomass from waste [23], during syngas fermentations [22, 24] and preparation of lignocellulosic hydrolysates [25]. Examples of acetate utilization for production of chemicals using *E. coli* W include succinic acid [26], itaconic acid [27] and isobutanol [28].

Co-utilization with glucose, a sugar abundantly available in a variety of potential substrate streams, would be an interesting option to increase competitiveness of an industrial process.

Escherichia coli produces acetate via different pathways, with the main route being the phosphate acetyltransferase (Pta) and acetate kinase (AckA) node. Others include direct oxidation of pyruvate to acetate and CO_2 by pyruvate dehydrogenase (PoxB). Acetate uptake is mediated either by the low affinity Pta-AckA node or high affinity Acs node, enabling *E. coli* to efficiently scavenge even small amounts of acetate excreted during glucose catabolism [29, 30]. Acetyl-CoA is a major branching point in central metabolism and a precursor for several pathways such as the tricarboxylic acid (TCA) cycle, fatty acid and amino acid synthesis, the glyoxylate bypass and ethanol production [31].

However, *E. coli* is not able to co-utilize glucose and acetate efficiently due to carbon catabolite repression, favoring glucose utilization in the presence of more than one carbon substrate [32, 33]. Furthermore, most *E. coli* strains show acetate secretion upon growth on glucose, a phenomenon usually described as overflow metabolism. Different studies have pointed towards limited respiratory or proteomic capacity of *E. coli* as the potential reason for acetate accumulation [31, 34–36]. Moreover, Acs plays a key role in acetate excretion, as it was reported that *acs* is down-regulated at high specific growth rates [37]. Generally, *acs* is activated by cAMP-CRP, and co-transcribed together with two other genes, a putative inner membrane protein (*yjcH*) and an acetate permease (*actP*) [33]. ActP has previously been described as a cation/acetate symporter, and knock-out strains lacking *actP* grow poorly on acetate as the sole carbon source [38].

Studies using *E. coli* and *Salmonella enterica* have found that in addition to transcriptional control via carbon catabolite repression [31, 33] activity of Acs is also controlled by posttranslational modification. Protein acetyltransferase, *patZ*/Pat, was found to be responsible for acetylation of Acs, rendering the enzyme inactive. In detail, Leu-641 is recognized by Pat, resulting in acetylation of Lys-609 of Acs, and consequently in inactivation of the enzyme [39, 40]. It could be demonstrated that a mutation at Leu-641 in Acs made the enzyme insensitive to acetylation [41]. Acetylation of Acs by Pat can be reversed by NADH-dependent CobB [39, 40]. Generally, *patZ* expression is regulated by cAMP-CRP [42] and during exponential growth phase on glucose *patZ* expression is up-regulated [43]. However, more detailed information on acetylation and activity of Acs is scarcely available, especially in the context of co-utilization of glucose and acetate.

Previous findings showed that *acs* down-regulation during glucose cultivations leads to acetate accumulation [37], deletion of *patZ* leads to more efficient growth on acetate as the sole carbon source in *E. coli* BL 21 [43] and decreased acetate accumulation in glucose limited continuous cultures [44]. To that end, the hypothesis behind the current work was that expression of an acetyl-CoA synthetase insensitive to acetylation (*acs*_L641P) from a constitutive promoter would have a similar effect, enabling efficient co-utilization of glucose and acetate at high concentrations. The aim of this work was to study the effect of overexpression of an acetylation insensitive acetyl-CoA synthetase on a mixed feed system of glucose and acetate in *E. coli* W. To that end, three different strains were constructed,

namely ACS_L641P (expressing an acetyl-CoA synthetase insensitive to acetylation from a constitutive promoter), ACS (expressing native acetyl-CoA synthetase from a constitutive promoter) and VC (a control strain carrying an empty vector) which were first characterized in batch cultivations using glucose and acetate, glucose or acetate. The behavior of the strains was further characterized under glucose and acetate-limited conditions using continuous chemostat and accelerostat (A-stat) cultivations. Gene expression analysis during A-stat cultivations using glucose and acetate were performed for acetate metabolism related genes to get insight into the effect of overexpression of an acetyl-CoA synthetase insensitive to acetylation.

Results

Escherichia coli W was chosen for this study because it shows reasonable resistance towards acetate [14], which was also evaluated in batch cultures where growth on up to 2% (w/v) acetate as the sole source of carbon was observed in shake flask cultures, while other *E. coli* strains such as BL21 and K-12 MG1655 did not show growth (data not shown). Sequence comparison of acetyl-CoA synthetase (Acs) from *Salmonella enterica* subsp. *enterica* LT 2 with the enzyme from *Escherichia coli* W revealed that residues Lys-609 and Leu-641 are conserved and the two enzymes show an overall identity of 95% of the amino acids (Additional file 1: Figure S1).

It was previously shown that Lys-609 is the site of acetylation activity by Pat rendering the enzyme inactive. This acetylation can be reversed by NADH-dependent CobB [39, 40]. A random mutation at the residue Leu-641 in Acs made the enzyme insensitive to acetylation, thereby disabling posttranslational modification in presence of high glucose concentrations [41]. To that end, two strains were constructed for expression of either *acs* or *acs*_L641P under control of the

constitutive promoter J23114 (Anderson constitutive promotor library).

Batch cultivations on glucose and acetate

The main hypothesis of this study was that expression of *acs*_L641P from a constitutive promoter should enable *E. coli* W to co-utilize glucose and acetate as both transcriptional and posttranslational control of *acs* by carbon catabolite repression would be circumvented in this case. Additionally, expression of *acs* without the L641P mutation, thus still sensitive to acetylation, from a constitutive promoter was studied. This construct should only be controlled on a transcriptional level but no longer on a posttranslational level.

Batch cultivations on defined media supplemented with 1% (w/v) glucose and 1% (w/v) acetate were carried out with three strains: ACS (strain expressing *acs* from promoter J23114), ACS_L641P (strain expressing *acs*_L641P from promoter J23114) and VC (a strain carrying an empty vector as a control).

Since the aim was to study co-utilization of glucose and acetate, all values mentioned in this paragraph and shown in Tables 1 and 2 are for the exponential phase (cultivation time ~ 4 h until depletion of glucose) where both glucose and acetate were present in the media.

μ, q_{GLC}, q_{ACE}, q_{CO2}, q_{NH3}, q_{O2} for the batch cultivations are shown in Table 1, $Y_{X/S}$, $Y_{CO2/S}$, $Y_{O2/S}$, $Y_{CO2/X}$ and the carbon recovery are shown in Table 2. As shown in Fig. 1, all three strains displayed a lag phase of around 4 h. Upon entering exponential growth phase, comparable specific growth and glucose uptake rates for all three strains were observed (Table 1) and at the time glucose was depleted biomass concentrations of 5.71 ± 0.52, 6.22 ± 0.64 and 5.80 ± 0.42 g l^{-1} for ACS_L641P, ACS and the VC, respectively, were observed. At this point the residual acetate concentration for ACS_L641P was significantly lower compared to ACS and VC (3.20 ± 1.23, 7.21 ± 1.74 and 5.20 ± 2.30 g l^{-1}, respectively).

Since the biomass concentration was comparable for all strains, a lower acetate concentration at the time

Table 1 Growth rate, specific glucose, acetate and base uptake as well as CO$_2$ production rates and growth rates for batch processes on glucose + acetate, glucose and acetate during exponential growth phase

	Glc + Ace VC	ACS	ACS_L641P	Glucose VC	ACS_L641P	Acetate VC	ACS_L641P
μ [h^{-1}]	0.23 ± 0.05	0.20 ± 0.03	0.27 ± 0.04	0.72 ± 0.01	0.68 ± 0.07	0.19 ± 0.03	0.18 ± 0.04
q_{GLC} [mmol g^{-1} h^{-1}]	2.85 ± 0.29	2.71 ± 0.81	3.20 ± 0.38	7.24 ± 0.18	6.90 ± 0.89	–	–
q_{ACE} [mmol g^{-1} h^{-1}]	1.76 ± 0.26	1.91 ± 0.58	4.72 ± 0.26	–	–	12.42 ± 2.13	12.36 ± 1.96
q_{CO2} [mmol g^{-1} h^{-1}]	8.23 ± 1.96	5.96 ± 2.26	16.33 ± 0.88	16.16 ± 0.11	23.98 ± 0.78	12.79 ± 0.31	17.03 ± 3.32
q_{NH3} [mmol g^{-1} h^{-1}]	3.55 ± 0.58	3.29 ± 0.74	1.44 ± 0.86	5.35 ± 0.97	6.30 ± 0.41	–	–

Mean values and errors as standard deviation are displayed

Table 2 Yields and carbon recovery for batch processes on glucose + acetate, glucose and acetate, $Y_{X/S}$, $Y_{CO2/S}$, $Y_{O2/S}$

	Glc + Ace VC	ACS	ACS_L641P	Glucose VC	ACS_L641P	Acetate VC	ACS_L641P
$Y_{X/S}$ [Cmol Cmol^{-1}]	0.37 ± 0.06	0.37 ± 0.05	0.29 ± 0.03	0.57 ± 0.09	0.44 ± 0.02	0.34 ± 0.01	0.26 ± 0.02
$Y_{CO2/S}$ [Cmol Cmol^{-1}]	0.54 ± 0.01	0.56 ± 0.02	0.65 ± 0.04	0.47 ± 0.07	0.57 ± 0.02	0.77 ± 0.02	0.71 ± 0.03
$Y_{O2/S}$ [Omol Cmol^{-1}]	0.99 ± 0.16	0.96 ± 0.27	1.26 ± 0.11	0.58 ± 0.13	0.93 ± 0.35	0.85[a]	1.12 ± 0.07
C-recovery [%]	91 ± 7	92 ± 7	94 ± 7	104 ± 16	101 ± 4	111 ± 3	97 ± 5

Mean values and errors as standard deviation are displayed

$Y_{X/S}$ biomass yield, $Y_{CO2/S}$ CO$_2$ production yield, $Y_{O2/S}$ oxygen uptake yield

[a] Calculation with only one value, due to O$_2$ offgas signal perturbations

point where glucose was depleted conversely indicates that ACS_L641P takes up acetate with a higher specific rate. Indeed, a 2.7-fold increase was observed for q_{ACE} of ACS_L641P, whereas q_{ACE} for ACS remained unchanged compared to VC (Table 1). Interestingly, a similar increase of twofold for q_{CO2} of ACS_L641P was observed, while similar to q_{ACE}, the specific carbon dioxide production rate of ACS was comparable to that of VC.

In addition to a higher specific acetate uptake and carbon dioxide production rate, ACS_L641P displayed a 2.5-fold lower specific base consumption rate, indicating that due to higher acetate consumption less ammonia per biomass was required to adjust the pH because of glucose catabolism related acidification.

The different behavior of ACS_L641P with respect to carbon uptake and production compared to ACS and VC can also be observed in the yields at the end of the glucose phase. Compared to the vector control, ACS_L641P showed a 21% decrease in $Y_{X/S}$ while $Y_{CO2/S}$ was increased by 20% (Table 2).

Batch cultivations on glucose or acetate

To further characterize the effect of expression of acetylation insensitive acetyl-CoA synthetase in *E. coli* W, the behavior of ACS_L641P and VC was studied during cultivations on either glucose or acetate as the sole source of carbon.

The cultivations using glucose as the carbon source showed no significant differences in specific growth and glucose uptake rate for ACS_L641P and VC (Table 1). However, ACS_L641P displayed a 48% increase in q_{CO2}, and in addition, showed a 23% decrease in $Y_{X/S}$ and a 21% increase in $Y_{CO2/S}$ (Table 2). These observations may indicate changes in the metabolism of glucose by expression of ACS_L641P.

For the cultivations using acetate as the carbon source similar values in specific growth and acetate uptake rate for the two strains were observed (Table 1). Despite similar acetate uptake rates, ACS_L641P showed a 33% increase in q_{CO2}. Moreover, a significantly longer lag

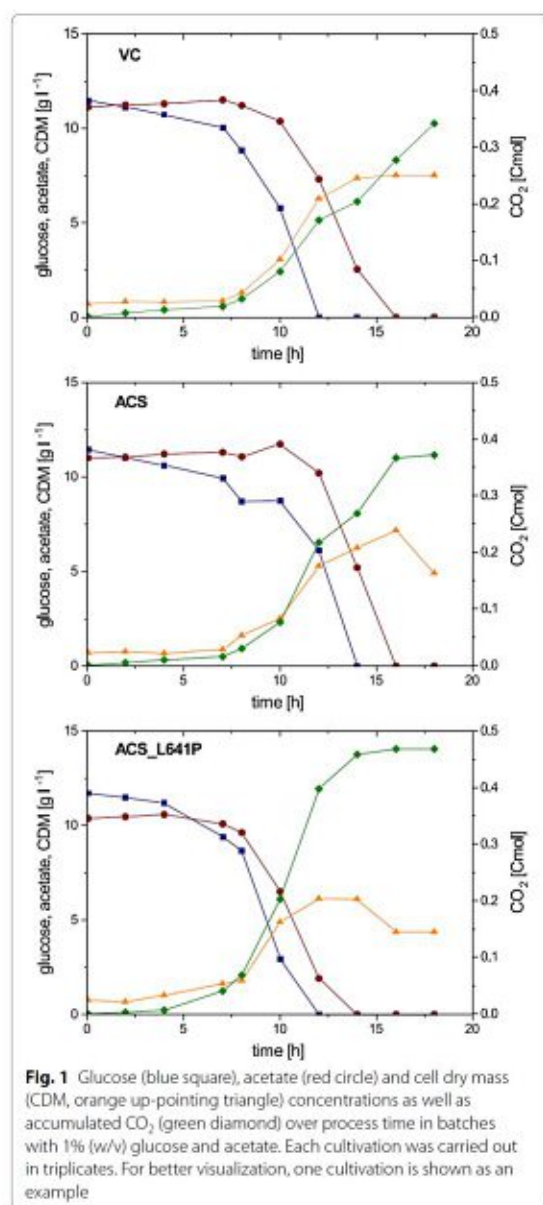phase and total batch duration was observed for VC compared to ACS_L641P (Fig. 2).

Continuous cultivations on glucose or glucose and acetate

Based on the findings of the different batch cultivations, a series of continuous cultivations was carried out to study the effect of acetyl-CoA synthetase overexpression under carbon-limited conditions. It is known that upon glucose limitation carbon catabolite repression is less severe, and *acs* expression is induced under these conditions [45]. To that end, the question was if the behavior in C-limited continuous cultures on glucose or co-utilizing glucose and acetate would be different to what has been observed during batch cultures with carbon surplus conditions.

Chemostat cultivations on glucose

Despite the fact that only small differences were observed for ACS_L641P and VC in batch cultures on glucose, chemostat cultivations at different dilution rates were performed. The aim of this experiment was to study if there were any growth rate dependent effects caused by the expression of *acs*_L641P in the catabolism of glucose observable. Furthermore, it was sought to compare the results obtained for other *E. coli* strains which are less robust against acetate stress. To that end, one chemostat cultivation for each strain was performed at different dilution rates ranging from 0.1 to 0.75 h^{-1} using 2% (w/v) glucose as the carbon source.

As shown in Fig. 3, both, ACS_L641P and VC display similar values for q_{GLC} which is in accordance with the findings for the batch cultures on glucose. However, q_{CO2} for ACS_L641P and VC also showed comparable values for all dilution rates where no acetate or glucose accumulation was observed, which is in contrast to the results of the batch cultures. In detail, both strains displayed an increase in $Y_{X/S}$ with increasing dilution rates, while $Y_{CO2/S}$ decreased (Table 3), i.e. more biomass and less CO$_2$ is produced per substrate. Due to this fact, biomass concentrations were 20% higher at dilution rate 0.50 h^{-1} compared to 0.10 h^{-1} for both

Fig. 1 Glucose (blue square), acetate (red circle) and cell dry mass (CDM, orange up-pointing triangle) concentrations as well as accumulated CO_2 (green diamond) over process time in batches with 1% (w/v) glucose and acetate. Each cultivation was carried out in triplicates. For better visualization, one cultivation is shown as an example

ACS_L641P and VC. At a dilution rate of 0.63 h^{-1} ACS_L641P started to accumulate acetate and glucose. Upon accumulation of acetate and glucose, q_{GLC} of ACS_L641P increased to higher levels than would be the result of the increased dilution rate. VC started to accumulate acetate at a dilution rate of 0.66 h^{-1}, but no glucose accumulation was observed at this growth

rate. However, further increasing the dilution rate to 0.82 h^{-1} also led to glucose accumulation for VC.

A-stat cultivations on glucose and acetate

During batch characterization, increased specific acetate uptake rates for ACS_L641P were obtained when glucose and acetate were co-utilized. Based on this finding, it was hypothesized that ACS_L641P should be able to co-utilize glucose and acetate more efficiently compared to VC also in continuous cultures co-utilizing both substrates. To investigate this hypothesis, accelerostat (A-stat) cultivations (continuous cultures with a constantly increasing dilution rate) [46] starting at a dilution rate of 0.20 h^{-1} were performed. The dilution rate was increased at a rate of 0.01 h^{-2} until a dilution rate of 0.70 h^{-1} using 1% (w/v) glucose and 0.5% (w/v) acetate as carbon sources. Based on the batch cultures, it was speculated that ACS_L641P would accumulate acetate at higher dilution rates compared to VC as higher dilution rates in C-limited cultures with constant biomass concentrations correspond to higher specific substrate uptake rates.

For both strains, the specific rates q_{GLC}, q_{ACE} and q_{CO2} as well as biomass concentrations constantly increased with increasing dilution rate until cell wash out started to occur (Fig. 3b). Acetate accumulation in ACS_L641P and VC started at a dilution rate of 0.59 and 0.66 h^{-1}, respectively. Glucose was accumulated at 0.67 h^{-1} in ACS_L641P, whereas no glucose accumulation was observed for VC until the end of the experiment (D = 0.71 h^{-1}).

With respect to $Y_{X/S}$ and $Y_{CO2/S}$, a similar behavior as for the chemostat cultivations with glucose as the carbon source was observed. Specifically, both strains display a shift from CO_2 to biomass at high dilution rates, resulting in 15% increased biomass yield at a dilution rate of 0.55 h^{-1} compared to the initial dilution rate of 0.20 h^{-1} for the VC. The ratio between biomass and CO_2 production for ACS_L641P did not significantly change as a function of the dilution rate. Upon glucose and acetate accumulation $Y_{X/S}$ and $Y_{CO2/S}$ sharply decrease due to reduced carbon source consumption for ACS_L641P. However, for VC only the decrease of $Y_{CO2/S}$ could be observed upon accumulation of acetate, while $Y_{X/S}$ did not decrease.

To further investigate the performance of the two strains, ACS_L641P and VC, gene expression analysis was performed for several genes of the acetate metabolism. Two dilution rates were investigated, 0.20 and 0.65 h^{-1}. Since the outcome of the experiment did not confirm the hypothesis that ACS_L641P should be able to more efficiently co-utilize glucose and acetate at high dilution rates (corresponding to high specific uptake rates) gene expression analysis might be able to shed light

Fig. 2 Glucose (blue square), acetate (red circle) and cell dry mass (CDM, orange up-pointing triangle) concentrations as well as accumulated CO_2 (green diamond) over process time in batches with 1% (w/v) glucose or 1% (w/v) acetate. Each cultivation was carried out in triplicates. For better visualization, one cultivation is shown as an example
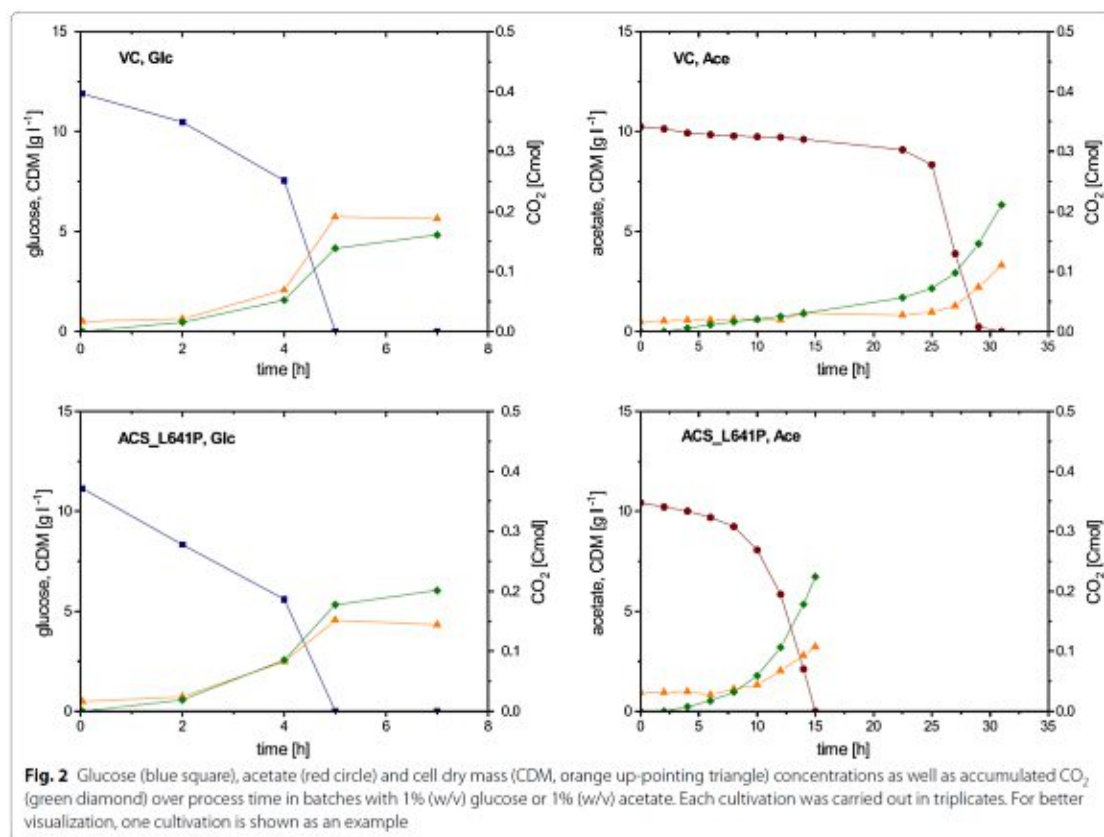
on what might be the reason for the observed behavior of the two strains.

The expression levels of the eight genes investigated were each compared between different dilution rates (e.g. vector control at 0.20 h^{-1} vs. 0.65 h^{-1}) as well as between strains (e.g. VC vs. ACS_L641P at D = 0.65 h^{-1}). Figure 4 shows the results of the gene expression analysis depicted in a simplified metabolic network (standard errors and *p* value are given in Additional file 2: Table S1).

Most prominently, ACS_L641P showed 10.4- and 19.8-fold increased expression levels for *acs* at dilution rates 0.20 and 0.65 h^{-1}, respectively, compared to VC. Furthermore, the expression level of *acs_L641P* in ACS_L641P did not significantly decrease upon increased dilution rates, while a twofold downregulation for *acs* was observed in VC. Hence, the apparently increased relative expression level of *acs* in ACS_L641P at 0.65 h^{-1} compared to the VC is a consequence of down-regulation of *acs* in the VC and seems to be unrelated to the acetate concentration or dilution rate. At a dilution rate of 0.65 h^{-1}, a sharp downregulation of *yjcH* and *actP* was

observed for ACS_L641P compared to VC (less than 5% of original expression level). Generally, expression levels of *yjcH* and *actP* dropped significantly when comparing dilution rate 0.65 h^{-1} to the initial dilution rate of 0.20 h^{-1} for both strains (~ fourfold decrease for VC at 0.65 h^{-1} vs. 0.20 h^{-1} and < 5% expression level for ACS_L641P at 0.65 h^{-1} vs. 0.20 h^{-1}). Genes involved in acetate metabolism (*pta*, *ackA*, *poxB*) showed lower expression levels at high dilution rates for both strains, although for *poxB* the decrease in expression rate at high dilution rates was less severe for ACS_L641P compared to VC.

The expression levels for the transcriptional regulators *crp* and *cra* decreased with increasing dilution rates, with the effect being slightly more prominent for ACS_L641P than for VC (Fig. 4).

Discussion

The aim of the present study was to investigate if and how efficient co-utilization of glucose and acetate in *E. coli* can be achieved. To that end, it was studied how expression of an acetylation insensitive acetyl-CoA synthetase
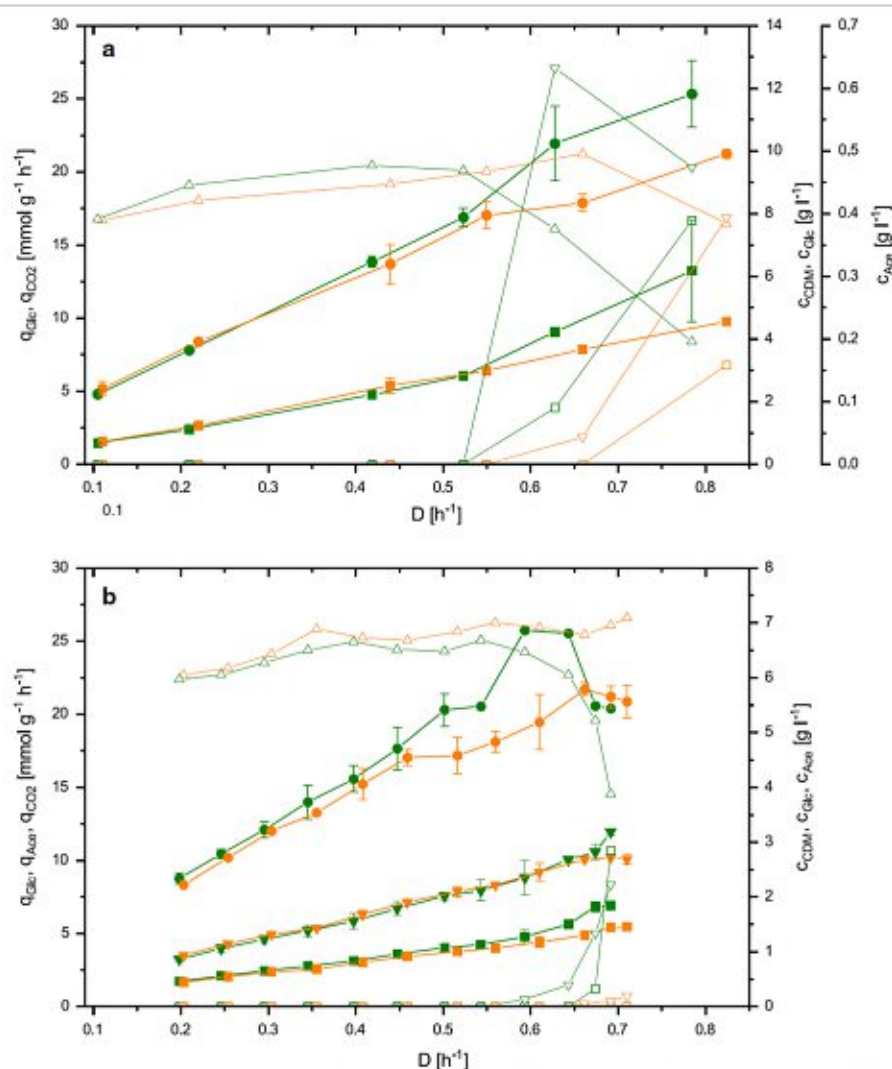
Fig. 3 Specific production rates q_{GLC} (filled square), q_{ACE} (filled down-pointing triangle), q_{CO2} (filled circle) of ACS_L641P (green) and the VC (orange) with corresponding glucose (white square), acetate (white down-pointing triangle) and cell dry mass (white up-pointing triangle) concentrations over dilution rate in a glucose chemostat (**a**) and a glucose + acetate mixed-feed A-stat (**b**). Error bars represent the standard deviation of three samples taken during steady state for chemostat cultivations, and the standard deviation of two biological replicates for A-stat cultivations. Due to perturbations in the CO_2 offgas measurements during the last five samples of the mixed-feed A-stat, q_{CO2} was obtained from a single cultivation, thus no error bars are displayed

from a constitutive promoter affects co-utilization of glucose and acetate in *E. coli* W, both under high carbon conditions in batch cultures and carbon-limiting conditions of continuous cultures.

During aerobic batch cultivations using glucose and acetate as carbon sources it was found that the strain

expressing *acs*_L641P displayed a 2.7-fold increased specific acetate uptake rate, whereas no change in q_{ACE} was observed for ACS compared to VC. These findings seem to confirm the hypothesis that activity of Acs alone during metabolism of high concentrations of glucose is sufficient to enable more efficient co-utilization

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Table 3 Yields for chemostat cultivations on glucose and A-stat cultivations on glucose + acetate, $Y_{X/S}$, $Y_{CO2/S}$

	ACS_L641P		VC	
	$Y_{X/S}$ [Cmol Cmol^{-1}]	$Y_{CO2/S}$ [Cmol Cmol^{-1}]	$Y_{X/S}$ [Cmol Cmol^{-1}]	$Y_{CO2/S}$ [Cmol Cmol^{-1}]
Chemostat Glucose				
D [h^{-1}]				
0.100	0.455 ± 0.027	0.544 ± 0.023	0.453 ± 0.015	0.557 ± 0.065
0.200	0.562 ± 0.014	0.546 ± 0.001	0.531 ± 0.009	0.528 ± 0.004
0.400	0.562 ± 0.013	0.485 ± 0.007	0.527 ± 0.050	0.425 ± 0.001
0.500	0.553 ± 0.026	0.465 ± 0.005	0.551 ± 0.022	0.445 ± 0.006
0.600	0.443 ± 0.016	0.403 ± 0.034	0.536 ± 0.013	0.379 ± 0.004
0.750	0.393 ± 0.103	0.327 ± 0.057	0.541 ± 0.006	0.363 ± 0.001
A-stat Glucose + acetate				
D [h^{-1}]				
0.200	0.451 ± 0.004	0.520 ± 0.042	0.457 ± 0.009	0.489 ± 0.009
0.250	0.458 ± 0.011	0.507 ± 0.042	0.466 ± 0.006	0.488 ± 0.010
0.300	0.474 ± 0.007	0.507 ± 0.044	0.485 ± 0.015	0.500 ± 0.010
0.350	0.492 ± 0.014	0.521 ± 0.072	0.520 ± 0.009	0.506 ± 0.010
0.400	0.503 ± 0.022	0.515 ± 0.068	0.508 ± 0.022	0.493 ± 0.009
0.450	0.492 ± 0.011	0.507 ± 0.068	0.504 ± 0.003	0.488 ± 0.009
0.500	0.490 ± 0.06	0.517 ± 0.037	0.516 ± 0.024	0.447 ± 0.009
0.550	0.505 ± 0.024	0.488[a]	0.528 ± 0.007	0.445 ± 0.009
0.600	0.493 ± 0.041	0.464[a]	0.522 ± 0.037	0.433 ± 0.008
0.650	0.458 ± 0.015	0.469[a]	0.514 ± 0.004	0.440 ± 0.009
0.680	0.416 ± 0.015	0.410[a]	0.501 ± 0.001	0.400 ± 0.011
0.700	0.405 ± 0.012	0.414[a]	0.514 ± 0.012	0.393 ± 0.009

Mean values and errors as standard deviation are displayed

$Y_{X/S}$ biomass yield, $Y_{CO2/S}$ CO$_2$ production yield

[a] Calculation with only one value, due to CO$_2$ offgas signal perturbations

of acetate. It would appear that under high glucose and acetate concentrations, where only Pta-AckA but not Acs are active, expression of *acs* from a constitutive promoter and insensitive to acetylation is sufficient to partially overcome control mechanisms by glucose-mediated carbon catabolite repression, thus presenting a proof-of-principle. However, acetate uptake for VC and ACS are quite significant. This could potentially be explained by previous reports showing that *E. coli* can both produce and assimilate acetate during glucose metabolism via Pta-AckA, and that the direction of the pathway depends only on extracellular concentrations of acetate [33].

Another phenomenon observed for ACS_L641P during all cultivations using glucose and acetate was an approximate 20% increase in $Y_{CO2/S}$ and therefore lower $Y_{X/S}$, i.e. more CO$_2$ and less biomass were produced in ACS_L641P compared to ACS and VC.

Steady energy input for gene expression and protein production [37] was ruled out as the reason for this shift in yields, as the comparison of ACS and ACS_L641P showed that ACS did not display the same shift.

Therefore, two other explanations can be argued to be responsible for the different behavior of ACS_L641P, namely either energy requirements by the activity of Acs in ACS_L641P or a different metabolic flux pattern.

Regarding energy, it can be stated that the net consumption of the Pta-AckA-Acs cycle is 1 ATP (2 ATP utilized by Acs, 1 ATP produced by Pta-AckA) [33, 37]. If simultaneous assimilation and dissimilation is assumed entirely through this cycle, 16% of the overall ATP needs of a cell would be required for the recycling of acetyl-CoA [33, 47]. Increasing the activity of Acs in ACS_L641P by overexpression would likely result in a higher overall activity of the Pta-AckA-Acs cycle. As little or no activity of *acs* would be expected in either ACS or VC, this enhanced Pta-AckA-Acs cycle activity in ACS_L641P would require more energy, which in turn would not be available for biomass formation, thus lowering $Y_{X/S}$ and increasing $Y_{CO2/S}$. This is further supported by a previous report that during exponential growth on glucose, *patZ*, the gene coding for protein acetyltransferase (Pat) is expressed at high levels, thus resulting in Acs acetylation

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Fig. 4 Acetate metabolism in *E. coli* and results of the gene expression analysis. Green arrows indicate overexpression of *acs* with Leu-641 residue changed to proline under control of a constitutive promoter. Solid black lines show glucose catabolism and acetate formation pathways, dashed lines indicate reverse reactions. Grey boxes show fold change of expression levels: A, ACS_L641P vs. VC at dilution rate 0.2 h^{-1}, B, ACS_L641P vs. VC at dilution rate 0.65 h^{-1}, C, 0.65 h^{-1} vs. 0.2 h^{-1} for VC, D, 0.65 h^{-1} vs. 0.2 h^{-1} for ACS_L641P. *acs*, acetyl-CoA synthetase, *phdc*, pyruvate dehydrogenase complex, *poxB*, pyruvate oxidase, *pta*, phosphotransacetylase, *ackA*, acetate kinase, *yjcH*, putate membrane protein, *actP*, acetate permease, *cra*, catabolite repressor activator, *crp*, cAMP receptor protein. The fold changes given represent the mean of two biological and three technical replicates. For visualization reasons, standard errors and p-values are not shown, but can be seen in Additional file 2: Table S1

and inactivation [43] and therefore only ACS_L641P would display Acs activity but not VC and ACS.

Regarding a different metabolic flux pattern, metabolic flux analysis showed that *E. coli* shows little glyoxylate cycle activity during glucose metabolism, while high fluxes through the glyoxylate shunt and only small fluxes for the TCA cycle were observed during metabolism of acetate [48]. The anaplerotic glyoxylate cycle negatively controlled by isocitrate lyase regulator (IclR) during glucose excess [34]. As a consequence, the glyoxylate shunt is likely to be inactive during co-utilization of glucose and acetate. Strain ACS_L641P catabolizes acetate to a higher extent in the presence of glucose compared to ACS and VC. This additional acetate would therefore be channeled into the TCA cycle rather than the glyoxylate cycle, resulting in a phenotype producing more CO_2 (2 mol CO_2 in the TCA cycle compared to no CO_2 in the glyoxylate cycle).

When grown on acetate as sole carbon source, the lag phase of VC was eightfold longer than that of ACS_L641P, thus resulting in an overall increased total batch duration. However, μ as well as q_{ACE} are not significantly different in the exponential phase for both strains. The long lag-phase might be caused by the toxicity of

acetate as a weak acid, causing a decrease in intracellular pH, an increase in osmotic pressure and interference with methionine biosynthesis [21, 49–52]. ACS_L641P is likely to overcome acetate toxicity by more efficient consumption due to overexpression of *acs*_L641P compared to VC [31]. Increased growth on acetate was also shown in an *E. coli* BL21 *patZ* knock-out strain [43], in both cases likely due to more active Acs (i.e. non-acetylated). When acetate is used as the sole carbon source, *acs* should not be repressed by carbon catabolite repression and thus expression rates in ACS_L641P and VC are expected to be more similar compared to mixed substrate fermentations, where *acs* is repressed in the VC. This fact can explain that there is no significant difference in q_{ACE}, which also corresponds to what has been observed previously [27]. Acetate consumption might also be limited by transport or subsequent metabolic reactions, which is further supported by the fact that inactivation of iclR increased acetate consumption in *E. coli* [27].

It was reported that *E. coli* W shows higher growth rates on acetate compared to other *E. coli* strains (BL21-DE3, K-12 W3110, and K-12 MG1655), and that protein acetylation of Acs by Pat is likely strain specific, as different expression levels for *patZ* were observed for

E. coli BL21 and a K-12 strain in glucose batch cultivations [43]. In cultivations with 10 g l^{-1} acetate, a growth rate of 0.46 h^{-1} and a specific acetate uptake rate of 3.66 mmol g^{-1} h^{-1} were reached [27]. In this study, lower growth rates and higher consumption rates of 0.19 h^{-1} and 12.4 mmol g^{-1} h^{-1}, respectively, were observed.

Only very low amounts of acetate (less than 0.5 g l^{-1}) accumulated at the end of the aerobic batches on glucose in ACS_L641P and VC, which corresponds well with previous reports for *E. coli* W describing a highly oxidative metabolism [14] and represents a distinct difference to other strains accumulating higher amounts of acetate such as K-12 BW25113 [31].

Chemostat cultivations on glucose with ACS_L641P and VC showed ambiguous results for the two strains. In ACS_L641P, accumulation of acetate and cell wash out (D = 0.63 h^{-1}) occurred almost simultaneously and at lower dilution rates compared to VC. This observation is different to previous reports where A-stat cultivations on glucose with *E. coli* K-12 MG1655 showed acetate accumulation between growth rates of 0.27 and 0.54 h^{-1}, and above the latter threshold, glucose was accumulated and cells were washed out [37]. In the present study, the phase of acetate accumulation was much shorter and both accumulation as well as wash out were observed at higher dilution rates.

Considering that cell wash out started to occur in ACS_L641P at a dilution rate similar to the mean specific growth rate observed during batch cultures (0.68 h^{-1} vs 0.72 h^{-1} for ACS_L641P and VC, respectively), these findings are somewhat surprising. During glucose metabolism, the Pta-AckA-Acs cycle is thought to be responsible for balancing of the intracellular acetyl-CoA and acetyl-P pools [31]. Natural imbalance of the cycle at the expense of Acs results in accumulation of acetate during glucose excess cultures [45]. It was speculated that higher expression levels of *acs*_L641P in ACS_L641P, creating an imbalance in favor of Acs in comparison to the other genes of the Pta-AckA-Acs cycle would possibly enable ACS_L641P to more efficiently cycle acetate, thus leading to delayed accumulation of acetate compared to the VC. This hypothesis is supported by previous reports where the coordinated activation of Acs by inactivation of Pat and the TCA cycle by deletion of *arcA* led to a delayed onset of overflow metabolism and an overall significantly decreased accumulation of acetate in accelerostat cultures using glucose [44].

As the findings were contrary to this hypothesis, accumulation of acetate at lower dilution rates in ACS_L641P could potentially be a consequence of the lack of additional TCA cycle activity an *arcA* knock-out strain would display. Furthermore, earlier onset of acetate accumulation could be due to increased energy dissipation as a result of higher Pta-AckA-Acs cycle activity owing to overexpression of *acs*_L641P in ACS_L641P.

The latter could also explain what was observed for the A-stat cultivations co-utilizing glucose and acetate. Similar to the glucose chemostats, acetate accumulation in A-stats occurred at lower dilution rates for ACS_L641P compared to VC. Moreover, $Y_{X/S}$ and $Y_{CO2/S}$ did not differ significantly for both strains, which is in contrast to the results obtained from the batch cultures on glucose and acetate.

However, it was reported that compared to glucose surplus batch cultivations during glucose limited chemostat cultivations transcription of *acs* is up-regulated [43, 45]. As a consequence, a more similar behavior for ACS_L641P and VC compared to batch cultures on glucose and acetate appears reasonable at low specific acetate uptake rates, while it was assumed that additional Acs activity by overexpression in ACS_L641P would allow for more efficient acetate uptake (higher q_{ACE}) at high dilution rates.

Despite this assumption, earlier acetate accumulation and cell wash out for ACS_L641P compared to VC in glucose-acetate A-stat cultivations could have been caused by a severe imbalance of the Pta-AckA-Acs cycle due to *acs*_L641P overexpression. In cultures co-utilizing glucose and acetate, both the Pta-AckA node as well as Acs could be responsible for acetate uptake, where 1 or 2 mol ATP per mol of acetate would be required for uptake, respectively.

Based on the results of the gene expression analysis, the ratio between *acs* and *pta-ackA* in ACS_L641P is much higher compared to VC. Hence, acetate flux via Acs rather than the Pta-AckA node could occur already at lower dilution rates for ACS_L641P compared to VC, resulting in higher ATP consumption for acetate uptake.

Another interesting finding of the gene expression analysis was that *yjcH* and *actP* were significantly down-regulated at higher dilution rates. This effect was more severe for ACS_L641P than for VC, and could potentially be the reason for earlier acetate accumulation in ACS_L641P, if acetate transport at high dilution rates is less effective or limiting. To shed light on this, flux analysis using labeled acetate could be used to determine the source of acetate accumulation (feed medium vs. excretion of intracellular acetate). Additionally, overexpression of *actP* could help to uncover transport limitations.

Finally, cell wash out and acetate accumulation in ACS_L641P could be caused by energy demand for gene expression and protein production compared to VC, which would be expected to be more severe at high dilution rates.

However, it must be emphasized that in this study *E. coli* W was shown to be naturally very efficient in

co-utilization of glucose and acetate, and that the strategy pursued here could have led to different results in notorious acetate excreting *E. coli* strains.

Conclusion

In this study it was shown that *E. coli* W is a promising candidate for processes relying on efficient acetate uptake or low acetate excretion. In detail, the overexpression of an acetylation-insensitive acetyl-CoA synthetase, for the first time significantly increased (2.7-fold) the specific acetate uptake rate in a mixed batch system using glucose together with high concentrations of acetate. Additionally, shorter batch durations during cultures using high concentrations of acetate were observed for the overexpression strain, likely due to *acs* related alleviation of acetate toxicity. Further characterization in chemostat and A-stat cultures showed that *E. coli* W is naturally capable of efficiently co-utilizing glucose and acetate in C-limited A-stat cultivations as no significant differences were found between the overexpression strain and a control strain with respect to acetate uptake. To that end, further work is required to gain a deeper understanding of metabolism in continuous cultures co-utilizing glucose and acetate. Metabolic flux analysis could shed light on the intracellular fluxes for glucose and acetate and help identify targets for further engineering. Among others, acetate transport could be manipulated by overexpression of *actP* for enhanced acetate uptake or genome engineering to deregulate the TCA cycle (via deletion of *arcA*) and glyoxylate cycle (via deletion of *iclR*) could further improve co-utilization of glucose and acetate in *E. coli* W.

Methods

Bacterial strains and media

Escherichia coli W (DSM 1116 = ATCC 9637 = NCIMB 8666) was obtained from DSMZ (Braunschweig, Germany) and used for all cultivations in this study. *Escherichia coli* BL21 (DE3) was obtained from New England Biolabs (MA, USA) and used as host for plasmid assembly and propagation.

Lysogeny broth (LB) containing per litre liquid medium: soy peptone, 10 g, yeast extract, 5 g, sodium chloride, 10 g, and LB agar additionally containing per litre: agar agar, 15 g, was used for all cloning and plasmid propagation steps. $2 \times$ LB medium was used for all precultures (soy peptone and yeast extract concentration doubled).

For all bioreactor cultivations defined medium containing per litre: KH_2PO_4, 13.3 g, $(NH_4)_2HPO_4$, 4.00 g, citric acid, 1.70 g, $MgSO_4 \cdot 7H_2O$, 1.2 g, Fe(III)citrate, 0.100 g, EDTA, 0.0084 g, $Zn(CH_3COO)_2 \cdot 2 H_2O$, 0.013 g, $CoCl_2 \cdot 6 H_2O$, 0.0025 g, $MnCl_2 \cdot 4 H_2O$, 0.015 g, $CuCl_2 \cdot 2$

H_2O, 0.0012 g, H_3BO_3, 0.0030 g, $Na_2MoO_4 \cdot 2 H_2O$, 0.0025 g as described previously was used. As carbon source either 1% (w/v) glucose + 1% (w/v) acetate, 1% (w/v) glucose or 1% (w/v) acetate was used. The medium for the continuous process was equivalent to the batch medium and contained either 2% (w/v) glucose or 1% (w/v) glucose + 0.5% (w/v) acetate. For the continuous culture with glucose and acetate as carbon sources, 3.24 g l^{-1} NH_4Cl were added to the feed medium.

Liquid and solid media were supplemented with 50 µg ml^{-1} kanamycin or 100 µg ml^{-1} ampicillin as necessary.

Plasmid and strain construction

The *acs* gene coding for acetyl-CoA synthetase was PCR amplified from genomic DNA of *E. coli* W using Q5 High-Fidelity DNA Polymerase (New England Biolabs, MA, USA) and primers FS2_acs_fw and FS3_acs_rev (Table 4). All primers in this study were purchased from Integrated DNA Technologies (IA, USA). To introduce the L641P mutation into acs and to add the fusion sites (FS) required for GoldenMOCS cloning, two PCR reactions amplified *acs* until position 641 using primers acs_fw and ACS_L641P_rev. In a second PCR reaction, FS sites and the rest of the coding sequence was added using primers FS2_acs_fw and FS3_acs_L641P_rev.

For all cloning steps in this study GoldenMOCS, a Golden Gate based cloning system, was used [53, 54]. The two PCR fragments were used for assembly into backbone 1 (BB1) of the GoldenMOCS as described previously and clones were verified for correct assembly and PCR amplification via restriction digests and Sanger sequencing (Microsynth AG, Switzerland) using primers seq_fw and seq_rev, respectively (Table 4).

BB2 assembly was used to arrange *acs/acs_L641P* in a single expression cassette under control of the

Table 4 List of used primers in this work

Name	Sequence (5'-3')
acs_fw	atgAGCCAAATTCACAAACAC
acs_L641P_rev	CTTCAAGCGGCTTCTC
FS2_acs_fw	GATCGGTCTCACatgAGCCAAATTCACAAACAC
FS3_acs_rev	GATCGGTCTCAAAGCttaCGATGGCATCGCG
FS3_acs_L641P_rev	GATCGGTCTCAAAGCttaCGATGGCATCGCGAT AGCCTGCTTCTCTTCAAGC<u>G</u>GCTTCTC
seq_fw	GCAGTCCAGTTACGCTG
seq_rev	CGTGGACCGATCATACG
acs_seq_in_fw	GCAGTATTCCGCTGAAG
acs_seq_in_rev	GGTAGCGCCTTCCAG

Inserted mutations are underlined

Table 5 Generated plasmids and used strains in this work

Name	Source
Plasmids	
BB1_pIDTSmart(Kan^R)_FS1_114p_FS2	Sarkari et al. [54]
BB1_pIDTSmart(Kan^R)_FS2_amilCP_FS3	Sarkari et al. [54]
BB1_pIDTSmart(Kan^R)_FS3_BBa_B1001_FS4	Sarkari et al. [54]
BB1_pIDTSmart(Kan^R)_FS2_acs_FS3	This work
BB1_pIDTSmart(Kan^R)_FS2_acs_L641P_FS3	This work
BB2_pUC(Amp^R)_LinkerA_FS1_FS4_LinkerB	Sarkari et al. [54]
BB2_pUC(Amp^R)_LinkerA_114p_acs_LinkerB	This work
BB2_pUC(Amp^R)_LinkerA_114p_acs_L641P_LinkerB	This work
BB3_pUC(Kan^R)_LinkerAD	Sarkari et al. [54]
BB3_pUC(Kan^R)_LinkerA_114p_acs_BBa_B1001_LinkerB	This work
BB3_pUC(Kan^R)_LinkerA_114p_acs_L641P_BBa_B1001_LinkerB	This work
Strains	
Escherichia coli BL21 (DE3)	New England Biolabs
Escherichia coli W DSM 1116	DSMZ

BB backbone; *114p* BBa_J23114, constitutive promoter from Anderson promoter library; *FS* fusion site; *BBa_B1001* artificial terminator

constitutive promoters BBa_J23114 (114p) of the Anderson promoter library and BBa_B1001 as terminator (Table 5).

BB3 assemblies were carried out to change the antibiotic resistance cassette to kanamycin (Table 5). All BB2 and BB3 plasmids were checked for correct assembly by restriction digests.

BB3 plasmids carrying either a functional *acs*/*acs_L641P* cassette or an empty BB3 were transformed into chemically competent *E. coli* W using the heat shock method.

Preculture preparation

Glycerol stocks (stored at -80 °C in 10% (w/v) glycerol) were streaked onto LB agar plates containing 50 µg ml^{-1} kanamycin and incubated overnight at 37 °C. Subsequently, 250 ml LB medium was inoculated with a single colony and incubated in 1 l shake flasks for 14 h at 37 °C and 200 rpm. The cells were grown until they reached an OD$_{600}$ of ~4, pelleted and washed twice with 80 ml sterile, 0.9% (w/v) NaCl solution (4800 rpm, 30 min, room temperature) and resuspended in 20 ml 0.9% (w/v) NaCl solution. The OD$_{600}$ of the resuspended culture was determined and a volume appropriate to inoculate the bioreactor with an OD$_{600}$ of 1 (corresponding to a CDW of approx. 0.59 g l^{-1}) was transferred to the bioreactor.

Bioreactor cultivations

Batch cultivations were performed in four parallel DAS-GIP Benchtop Bioreactors for Microbiology (Eppendorf AG, Hamburg, Germany) with an initial OD$_{600}$ of 1 and an initial batch volume of 1 l. The temperature for all cultivations was 37 °C. To maintain aerobic cultivation conditions all reactors were stirred with 1400 rpm and gassed continuously with pressurized air at 2 vvm ($=120$ l h^{-1}). The dissolved oxygen concentration was monitored using a VisiFerm DO 225 (Hamilton, Reno, NV, USA) and remained above 30% throughout all cultivations. A pH electrode (Mettler-Toledo GmbH, Giessen, Germany) was used for monitoring the pH value and a constant pH of 7 was maintained by addition of NH$_4$OH (12.5% v/v) and 5 M HCl. CO$_2$ and O$_2$ concentrations were measured using the off-gas analysis module GA4 Eppendorf AG, Hamburg, Germany). Samples were taken immediately after inoculation, then at least every 2 h during batch phases as well as directly after the observed phase and batch end.

For the continuous culture 200 ml medium was inoculated with an OD$_{600}$ of 1 in four parallel DASBOX Mini Bioreactors (Eppendorf AG, Hamburg, Germany). The reactors were stirred with 1400 rpm; the pH was set to 6.8 (to avoid media precipitation) and measured by a pH electrode (Mettler-Toledo GmbH, Giessen, Germany). NH$_4$OH (12.5% v/v) and 5 M HCl were added to correct the pH. To assure aerobic cultivation conditions, air was added at 2 vvm ($=24$ l h^{-1}) and the dissolved oxygen concentration, which was monitored by a VisiFerm DO 225 probe (Hamilton, Reno, NV, USA), was kept above 30% by the addition of pure oxygen. Offgas analysis (CO$_2$ and O$_2$ concentrations) was carried out using the off-gas analysis module GA4 (Eppendorf AG, Hamburg, Germany).

For chemostat cultures, feed medium with 2% (w/v) glucose was used and dilution rates of 0.10, 0.20, 0.40,

0.50, 0.60 and 0.75 h^{-1} were tested. After three volume changes, at least three samples were taken with a minimum interval of 2 h between samples. The average of these triplicates was used for all further calculations.

In the accelerostat (A-stat), feed medium with 1% (w/v) glucose and 0.5% (w/v) acetate was used. After the initial batch, the dilution rate was set to 0.20 h^{-1} (F = 40 ml h^{-1}). After more than three volume changes (= 15 h), a steady state was assumed and a sample was taken. Subsequently, the dilution rate was increased linearly with 0.01 h^{-2} (2 ml h^{-2}) and samples were taken every five hours until the dilution rate reached 0.70 h^{-1}.

Biomass determination

Samples from bioreactor cultivations taken at regular intervals were used for gravimetric determination of the cell dry weight (CDW) (in triplicate for batch, duplicates for chemostat and A-stat cultures). Briefly, 4 ml culture broth was centrifuged (4500 rpm, 10 min, 4 °C) and washed with deionized water in pre-weighed test glasses. The pellet was dried for at least 72 h at 105 °C. OD$_{600}$ was measured in a spectrophotometer (Genesys™ 20, Thermo Scientific, Waltham, Massachusetts, USA) against a blank of water.

HPLC analysis

The substrate and metabolite concentrations of the culture broth were measured by HPLC with an Agilent system (1100 series, Agilent Technologies, Santa Clara/CA, USA) using an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules/CA, USA) with a refractive index detector (Agilent 1100 series G1362A, Agilent Technologies, Santa Clara/CA, USA) and an UV detector (Agilent 1100 series G1315A, Agilent Technologies, Santa Clara/CA, USA). The column was operated at 60 °C with a flow of 0.6 ml min^{-1} for 30 min and with 4 mM H$_2$SO$_4$ as a mobile phase. The HPLC run was controlled and monitored using ChemStation for LC 3D systems (Agilent Technologies, Santa Clara/CA, USA). For sample preparation, 450 μl cell-free supernatant was mixed with 50 μl 40 mM H$_2$SO$_4$ and 10 μl sample was injected for analysis. 5-point calibration curves treated in the same way as the samples were used to determine substrate and metabolite concentrations in the samples.

Biomass composition

To determine biomass composition E. coli W was grown for 7 h at 37 °C and 200 rpm in defined medium supplemented with 1% (w/v) glucose. Cells were pelleted (4500 rpm, 30 min, 4 °C) and washed three times with sterile filtered, deionized water, transferred to 50 ml tubes and lyophilized at −55 °C and 0.02 mbar (Martin Christ, alpha 1–4 LD plus, Osterode am Harz, Germany)

for 24 h. The pellet was subsequently milled and biomass composition with respect to carbon, hydrogen, nitrogen, oxygen, phosphorous and sulphur was determined in triplicate (University of Vienna, Vienna, Austria). From the results the elementary composition of the biomass was determined to be C$_{1.000}$H$_{1.676}$O$_{0.439}$N$_{0.234}$P$_{0.018}$S$_{0.005}$, i.e. the carbon content of E. coli W dry biomass is 46.1% (w/w).

Gene expression analysis

Immediately after samples (at 0.2 and 0.65 h^{-1} for ACS_L641P and VC) were taken from A-stat cultivations, 100 μl samples were aliquoted and centrifuged in a desk centrifuge for 30 s, 16,000g at 4 °C. The supernatant was discarded and the cell pellet was snap frozen in liquid nitrogen. The samples were stored at −80 °C until further use.

RNA from frozen sample was isolated using the PureLink RNA Mini Kit (Ambion by life technologies, ThermoFisher Scientific, USA) according to the manufacturer's recommendation. RNA was eluted in RNase free MQ water. Subsequently, genomic DNA was digested using RNAse free DNAse (ThermoFisher Scientific, USA) together with RiboLock RNase inhibitor (ThermoFisher Scientific, USA) in a 20 μl reaction, using 2 μl of purified RNA. The DNA-free purified RNA was quantified using a Nanodrop 1000 (ThermoFisher Scientific, USA).

The RNA was reverse transcribed using the RevertAid H Minus First Strand cDNA kit (ThermoFisher Scientific, USA) according to the manufacturer's protocol using random hexamer primers (20 μl reaction volume).

Gene expression levels were determined by gene-specific quantitative real-time PCR using Luna Universal qPCR Master Mix (New England Biolabs, USA). The primers for the qPCR were designed using the PrimerQuest tool (Integrated DNA Technologies, USA) and are listed in Additional file 2: Table S3. The genes for a 16S ribosomal rRNA gene, rrsG, and a DNA replication terminus site-binding protein, tus, were used as housekeeping genes for normalization. The qPCR reaction was performed on a qTower 2.2 (Analytik Jena AG, Germany) system using the program specified in Additional file 3. Determination of primer efficiency was performed by establishing a standard curve from a dilution series of cDNA (dilution steps 5, 10, 20, 50 and 100) for the housekeeping genes rrsG and tus. For the individual genes, each qPCR reaction was performed in triplicates for each condition.

Data evaluation was performed as described previously [55]. In brief, the mean C$_t$ values were determined by calculating the average of the triplicate measurements for each gene and condition. The ΔC$_t$ values were calculated by subtracting the average mean C$_t$ value of the two

housekeeping genes from the mean C_t value of the gene of interest. $\Delta\Delta C_t$ is constituted by the difference between the C_t value of the sample of interest (ACS_L641P at 0.2 and 0.65 h^{-1}, respectively, and VC at 0.65 h^{-1}) and the reference sample (VC at 0.2 h^{-1}). The relative fold changes shown were calculated by averaging the fold changes of the two biological replicates using Relative quantity $= 2 - \Delta\Delta C_t$. The deviation given in Additional file 2: Table S1 is the standard error of the two biological and three technical replicates.

Data evaluation
Data were analyzed according to Additional file 3.

Additional files

Additional file 1: Figure S1. Sequence alignment of Acs of E. coli W and S. enterica LT 2. Residue Lys-609 highlighted by green box represents site of acetylation by Pat, residue Lys-641 highlighted by red box indicates recognition site of Pat for acetylation.

Additional file 2: Table S1. Mean fold change values including standard errors for the four comparisons A, B, C, D (named in the same way as in Figure 1 of the manuscript). Values highlighted in green represent significantly different expression levels (p-value 0.05). Table S2. qPCR program used for gene expression analysis. Lid temperature was set to 95 °C. Table S3. List of primers for gene expression analysis.

Additional file 3. Statistical/data evaluation.

Abbreviations
Ra_{inert}: inert gas ratio (–); y: mole fraction (–); y_{wet}: O_2 concentration in off-gas diluted by water content (without bioreaction) (–); ex_{H_2O}: water content in off-gas (–); V_m: molar volume of gas at norm conditions (0 °C and 1 atm) (nl mol^{-1}); x: concentration of biomass in the fermentation broth (mmol l^{-1}); s: concentration of substrate in the fermentation broth (mmol l^{-1}); X: total amount of biomass in the fermentation broth and sampling (Cmol); S: total amount of substrate in the fermentation broth and sampling (Cmol); n_{CO_2}: accumulated total amount of carbon dioxide (Cmol); n_{O_2}: accumulated total amount of oxygen (Omol); n_{ci}: amount of mole carbon from component i (Cmol); t: time (h); q_{CO_2}: specific CO_2 production rate (mmol g^{-1} h^{-1}); q_{NH_3}: specific base consumption rate (mmol g^{-1} h^{-1}); r_{ci}: volumetric uptake/production rate (Cmol l^{-1} h^{-1}); $Y_{i/S}$: subtrate yield (Cmol $Cmol^{-1}$); q_{ci}: specific uptake/production rate (Cmol $Cmol^{-1}$ h^{-1}).

Authors' contributions
LF and AME constructed the strains, KN, LF, AME, and PF carried out the cultivation experiments, AME and SP designed the gene expression analysis experiment, AME carried out the gene expression analysis experiment, KN, LF, CH and SP analyzed the data, SP conceived the study, KN, LF, AME and SP wrote the manuscript. All authors read and approved the final manuscript.

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3. Strain and process development for production of fuel chemicals from acetate and renewable resources in *E. coli* W
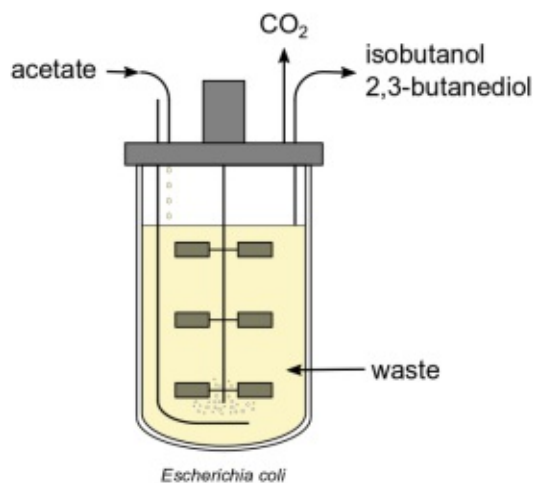

Figure 5 – Overview part 3. In this part, the production of two fuel chemicals from renewable resources is investigated.

3.1. Process and strain development for isobutanol production on glucose defined medium and cheese whey

Problem statement

So far, most studies investigating microbial isobutanol production have used high amounts of complex media additives, which renders these processes economically infeasible. Development of efficient isobutanol production is, however, hampered by the toxicity of isobutanol.

State-of-the-Art

Isobutanol is not natively produced in any organism and a synthetic pathway was recently introduced in *E. coli* (Atsumi et al., 2008). Utilization of glucose as carbon source resulted in the production of high isobutanol concentrations by relying on the addition of a complex media additives (Baez et al., 2011). The combination with a strong induction system that requires expensive inducers renders these processes largely uneconomical. The use of lignocellulosic hydrolysates was proposed as a cheap alternative, but this resulted in low isobutanol productivities (Akita et al., 2015). Cost competitiveness can be achieved by using defined medium or a waste source.

Scientific questions

How can cost-effective isobutanol production be established as a basis for further utilization of alternative raw materials?

Can isobutanol be produced from an alternative raw material such as cheese whey?

Hypotheses

For the cost-effective production of isobutanol the following requirements need to be met: (i) use of a constitutive promotor system, (ii) the use of a robust *E. coli* strain with an optimized strain background, (iii) the selection of appropriate process conditions, (iv) the use of defined media and (v) the use of a cheap alternative feedstock. Due to the availability of different nutrients, utilization of lactose and proteins in cheese whey enables the production of isobutanol at high titers and productivities.

Approach

To achieve cost-effective isobutanol production, a construct library expressing each gene individually using constitutive promotors was screened on defined media for the best producing strain. Considering the strain background and testing different aeration strategies should improve the product yield. Further process intensification aimed for increased titers and productivities. Process development for isobutanol production on defined medium was transferred to production from cheese whey and allowed for efficient production.

Content and contribution

In this paper, an isobutanol production process on defined medium and cheese whey was developed. The publication first describes strain and construct screening on chemically defined medium. To this end, each gene of the pathway was individually expressed under a constitutive promotor and different strain backgrounds were investigated. Upon selection of the most promising strain and construct combination, different aeration strategies were examined. Further process development enabled high rate isobutanol production on defined medium. Finally, the cost-competitiveness of the process could be increased by establishing efficient isobutanol production on the alternative raw material cheese whey.

KN carried out the bioreactor cultivations. KN and SP conceived the study, analyzed the data, and wrote the manuscript.

Title of manuscript

Metabolic engineering of *Escherichia coli* W for isobutanol production on chemically defined medium and cheese whey as alternative raw material

Citation

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BIOENERGY/BIOFUELS/BIOCHEMICALS - ORIGINAL PAPER

Metabolic engineering of *Escherichia coli* W for isobutanol production on chemically defined medium and cheese whey as alternative raw material

Katharina Novak[1] · Juliane Baar[1] · Philipp Freitag[1] · Stefan Pflügl[1]

Abstract

The aim of this study was to establish isobutanol production on chemically defined medium in *Escherichia coli*. By individually expressing each gene of the pathway, we constructed a plasmid library for isobutanol production. Strain screening on chemically defined medium showed successful production in the robust *E. coli* W strain, and expression vector IB 4 was selected as the most promising construct due to its high isobutanol yields and efficient substrate uptake. The investigation of different aeration strategies in combination with strain improvement and the implementation of a pulsed fed-batch were key for the development of an efficient production process. *E. coli* W $\Delta ldhA$ $\Delta adhE$ Δpta $\Delta frdA$ enabled aerobic isobutanol production at 38% of the theoretical maximum. Use of cheese whey as raw material resulted in longer process stability, which allowed production of 20 g l^{-1} isobutanol. Demonstrating isobutanol production on both chemically defined medium and a residual waste stream, this study provides valuable information for further development of industrially relevant isobutanol production processes.

Keywords Chemically defined medium · Promotor fine-tuning · Constitutive promotor · Pulsed fed-batch · Isobutanol adaptation

Introduction

Second-generation biofuels, which are produced from lignocellulosic biomass or waste streams, are considered as strategically important sustainable fuels due to their renewability, biodegradability and low emissions of greenhouse gases [1]. The production of higher molecular weight alcohols such as n-butanol and isobutanol poses advantages over ethanol production. Both alcohols have a higher energy content closer to gasoline, can be transported using existing infrastructure and their lower vapor pressures improves mixing with gasoline [2, 3]. Isobutanol has a higher octane number than n-butanol, is less toxic to cells and requires less energy for downstream processing [2]. Biotechnologically, the isobutanol pathway is less complex and not acetyl-CoA dependent, which results in lower side-product formation compared to n-butanol production [4].

Isobutanol is a metabolite not naturally synthesized by any organism. However, its synthesis is possible through a combination of the valine biosynthesis and the Ehrlich pathway [5]. The production of isobutanol has been demonstrated in several organisms, including *Escherichia coli* [5, 6], *Saccharomyces cerevisiae* [7, 8], *Corynebacterium glutamicum* [9], *Clostridium thermocellum* [10] and even autotrophic organisms like *Ralstonia eutropha* [11] and *Synecococcus elongatus* [12]. Advances in microbial isobutanol production have recently been reviewed [4].

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Synthesis of isobutanol from pyruvate relies on a five-enzyme pathway (Fig. 1). Acetolactate synthase (AlsS or BudB) converts two molecules of pyruvate to 2-acetolactate, which is further processed to 2,3-dihydroxyvalerate by ketol-acid reductoisomerase (IlvC). Dihydroxy-acid dehydratase (IlvD) catalyzes the reaction to 2-ketoisovalerate and α-ketoisovalerate decarboxylase (KdcA) converts it to isobutyraldehyde. Finally, isobutanol is produced by an alcohol dehydrogenase (AdhA). Two of these enzymes, *ilvC* and *ilvD*, are native to *E. coli* [5]. Acetolactate synthase from *Bacillus subtilis* (AlsS) was used for efficient isobutanol production [13]. Acetolactate synthetase is also the first enzyme in the 2,3-butanediol production pathway of natural producers. For example, *budB* codes for acetolactate synthase from *Enterobacter cloacae* subsp. *dissolvens* [14, 15]. *E. coli* cannot naturally produce isobutanol because it lacks an α-ketoisovalerate decarboxylase [16]. Overexpression of α-ketoisovalerate decarboxylase (*kdcA*) from *Lactococcus lactis* was shown to result in high isobutanol yields [13]. *E. coli* has a native alcohol dehydrogenase *yqhD*, but overexpression of *adhA* from *L. lactis* is advantageous due to utilization of NADH rather than NADPH as a cofactor [17]. A mutant of *adhA* was shown to have higher affinity towards isobutyraldehyde. In a mutated form of *ilvC*, the cofactor was exchanged from NADPH to NADH. These mutations enabled anaerobic isobutanol production at 100% of the theoretical yield [18].

Since isobutanol is toxic to microorganisms [3], procedures for product removal from the culture broth are important to obtain high titers [19]. Until now, the highest isobutanol titer of 50 g l^{-1} was achieved in a fed-batch fermentation of *Escherichia coli* applying in situ product
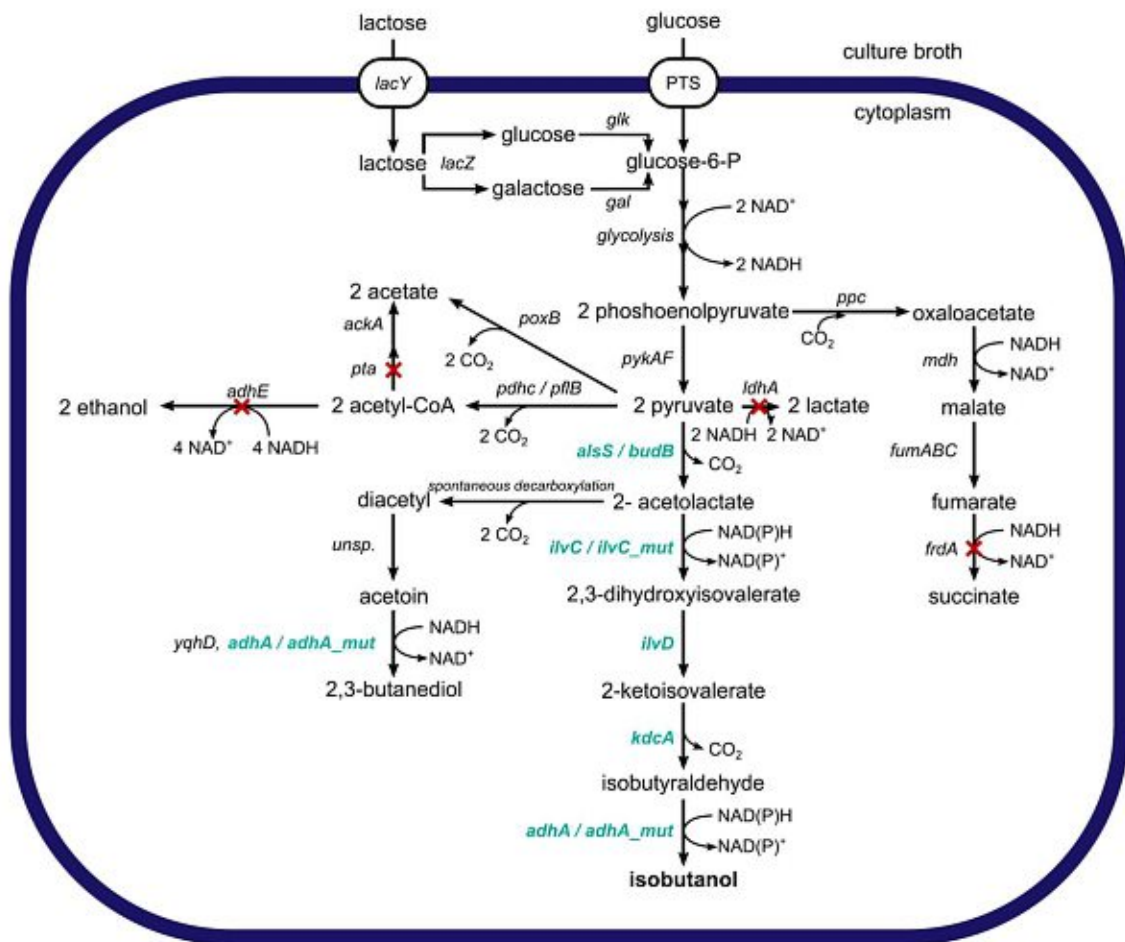
Fig. 1 Metabolic network of *Escherichia coli* for isobutanol production, substrate uptake and by-product formation. Bold green genes were overexpressed. Red crosses indicate deleted genes in *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA*. *Unsp.* indicates unspecific reactions (color figure online)

removal by gas stripping [6]. The elimination of competing pathways (Fig. 1) for acetate, lactate, succinate and ethanol formation was shown to increase isobutanol yields and thus enabled production at higher titers [5, 6]. However, production was only achieved by the addition of complex media components such as yeast extract (Table 1). When defined medium is used, all cellular components must be synthesized de novo, whereas different precursors can be metabolized when complex media components such as yeast extract are added. The drawback and limitations of the use of complex media components have recently been intensively investigated and described [20]. In short, utilization of yeast extract modulates growth parameters like the specific growth rate μ due to the depletion of components throughout the cultivation [20, 21]. It leads to a lack in reproducibility due to variable composition [22] and to differences in cellular metabolism, e.g., in acetate excretion and protein expression [23].

Low material costs are pivotal for industrial production of cheap compounds such as fuel alcohols. The production cost of biobutanol has been reported to be greatly affected by the feedstock price, which accounts for 60–65% of the total production cost [24]. The cost of the production medium can be decreased by the omittance of expensive induction and complex media compounds as well as by the utilization of an alternative raw material. An overview over substrates that have been used for isobutanol production is depicted in Table 1. The use of lignocellulosic hydrolysates as cheap raw material has only led to the production of low amounts of isobutanol in *E. coli* and *Shimwellia blattae* [25, 26]. Lignocellulose is a promising raw material, but its pre-treatment poses some disadvantages such as high energy input and the accumulation of inhibitory side products [27]. In contrast, the use of cheese whey has been shown to increase growth rate, biomass yield and specific product titers during recombinant protein expression in *E. coli* [28]. In *Clostridium acetobutylicum*, the utilization of cheese whey enabled the production of 5.6 g l^{-1} butanol [29]. Annually, 180–190 million tons of cheese whey are produced worldwide during cheese or curd production [30]. High volumes and high organic content, mainly attributed to lactose, pose environmental burden on whey disposal [31]. To this end, microbial production of fuel chemicals from cheese whey could be a promising alternative for the reduction of media costs.

The aim of this study was to construct a recombinant *E. coli* strain for isobutanol production and establish an efficient, cheap, and easily feasible production process. The requirements to achieve this goal were defined as the following: (i) the use of constitutive promoters for fine-tuning of gene expression and to avoid the use of expensive inducers, (ii) the use of a robust *E. coli* strain with an optimized strain background, (iii) the improvement of titer, yield and production rate by the selection of appropriate process conditions, (iv) the use of defined medium to avoid expensive media components and (v) the utilization of cheese whey as an alternative raw material. Using this approach, a production system was established that produced isobutanol from glucose, lactose and cheese whey.

Materials and methods

Bacterial strains and media

For all general cloning steps and plasmid propagation, *Escherichia coli* BL21(DE3) (New England Biolabs, MA, USA) and *E. coli* Top 10 (kind gift of Prof. Michael Sauer, BOKU, Vienna, Austria) were used. *E. coli* W (DSM 1116 = ATCC 9637 = NCIMB 8666) from DSMZ (Braunschweig, Germany), *E. coli* W $\Delta ldhA$ $\Delta adhE$ Δpta $\Delta frdA$ (kind gift of Prof. Michael Sauer, BOKU, Vienna, Austria) and *E. coli* K12-BW25113 (Yale CGSC, New Haven, US) were used for cultivations.

Lysogeny broth (LB) containing 10 g l^{-1} soy peptone, 5 g l^{-1} yeast extract and 10 g l^{-1} sodium chloride was used for all cloning and plasmid propagation steps. Soy peptone and yeast extract concentrations were doubled (giving 2x-LB) for bioreactor precultures. For cultivation on plates, 15 g l^{-1} agar was added to LB medium.

SOC medium containing 10 g l^{-1} NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose, 20 g l^{-1} tryptone and 5 g l^{-1} yeast extract at pH 7.0 was used for transformant recovery.

All experiments in shake flasks and serum bottles as well as all bioreactor cultivations were carried out in chemically defined medium adapted from Riesenberg et al. [32], containing 13.3 g l^{-1} KH$_2$PO$_4$, 4.0 g l^{-1} (NH$_4$)$_2$HPO$_4$, 1.7 g l^{-1} citric acid (autoclaved), 1.2 g l^{-1} MgSO$_4$ * 7 H$_2$O, 0.10 g l^{-1} Fe(III)citrate, 0.0084 g l^{-1} EDTA, 0.013 g l^{-1} Zn(CH$_3$COO)$_2$ * 2 H$_2$O, 0.0025 g l^{-1} CoCl$_2$ * 6 H$_2$O, 0.015 g l^{-1} MnCl$_2$ * 4 H$_2$O, 0.0012 g l^{-1} CuCl$_2$ * 2 H$_2$O, 0.0030 g l^{-1} H$_3$BO$_3$ and 0.0025 g l^{-1} Na$_2$MoO$_4$ * 2 H$_2$O (sterile filtered). The carbon source was added from a 10 × concentrated stock. Glucose was used at 8 g l^{-1} in isobutanol adaptation experiments, at 20 g l^{-1} in the strain screening experiments and at 50 g l^{-1} in batches and fed-batches. An initial lactose concentration of 50 g l^{-1} was used in pulsed fed-batches.

The feed medium contained 800 g l^{-1} glucose and MgSO$_4$ * 7 H$_2$O (5.0 g l^{-1}), Fe(III)citrate (0.42 g l^{-1}), EDTA (35 mg l^{-1}), Zn(CH$_3$COO)$_2$ * 2 H$_2$O (54.0 mg l^{-1}), CoCl$_2$ * 6 H$_2$O (11 mg l^{-1}), MnCl$_2$ * 4 H$_2$O (63 mg l^{-1}), CuCl$_2$ * 2 H$_2$O (5.0 mg l^{-1}), H$_3$BO$_3$ (13 mg l^{-1}), Na$_2$MoO$_4$ * 2 H$_2$O (11 mg l^{-1}) or 250 g l^{-1} lactose and MgSO$_4$ * 7 H$_2$O (1.6 g l^{-1}), Fe(III)citrate (0.13 g l^{-1}), EDTA (11 mg l^{-1}), Zn(CH$_3$COO)$_2$ * 2 H$_2$O (17.0 mg l^{-1}), CoCl$_2$ * 6 H$_2$O (3.3 mg l^{-1}), MnCl$_2$ * 4 H$_2$O (20 mg l^{-1}), CuCl$_2$ * 2 H$_2$O (1.6 mg l^{-1}), H$_3$BO$_3$ (3.9 mg l^{-1}) and Na$_2$MoO$_4$ * 2 H$_2$O

Table 1 State-of-the-art of heterotrophic isobutanol production processes and their performance parameters as described in literature

Host organism	Substrate	Complex media additives	Overexpressed genes	Host engineering	Titer	Isobutanol production rate	% of max. theor. yield	Production system	References
Escherichia coli	Glucose	None	budB, ilvC_mut, ilvD, kdcA, adhA_mut	ΔldhA ΔadhE Δpta ΔfrdA	16 g l⁻¹	0.25 g l⁻¹ h⁻¹	38%	Fed-batch (aerobic)	This study
Escherichia coli	Cheese whey	None	budB, ilvC_mut, ilvD, kdcA, adhA_mut	ΔldhA ΔadhE Δpta ΔfrdA	20 g l⁻¹	0.29 g l⁻¹ h⁻¹	39%	Fed-batch (aerobic)	This study
Escherichia coli	Glucose	5 g l⁻¹ yeast extract	alsS, ilvCD, kivd, adhA	ΔadhE, ΔfrdBC, Δfnr-ldhA, Δpta, ΔpflB	22 g l⁻¹		86%	Shake flasks (micro-aerobic)	[5]
Escherichia coli	Glucose	25 g l⁻¹ yeast extract	alsS, ilvCD, kivd, adhA	ΔadhE, ΔfrdBC, Δfnr-ldhA, Δpta, ΔpflB	50 g l⁻¹	0.7 g l⁻¹ h⁻¹	86%	Fed-batch (aerobic)	[6]
Escherichia coli	Glucose	10 g l⁻¹ yeast extract	alsS, ilvD, kivD, ilvC_mut, adhA_mut	ΔldhA, ΔadhE, Δfrd, ΔpflB, ΔilvC, Δpta	13.4 g l⁻¹	0.09 g l⁻¹ h⁻¹ OD⁻¹	103%	Bottles (anaerobic)	[18]
Escherichia coli	Cellobiose	5 g l⁻¹ yeast extract	alsS, ilvCD, kivD, adhA, bglC	ΔadhE, ΔfrdBC, Δfnr-ldhA, Δpta, ΔpflB	7.6 g l⁻¹	0.16 g l⁻¹ h⁻¹	28%	Shake flasks	[37]
Escherichia coli	Glucose and xylose	5 g l⁻¹ yeast extract	alsS, adhA, kivD, ilvCD	ΔldhA ΔadhE ΔpflB ΔackA-pta	8.4 g l⁻¹	0.18 g l⁻¹ h⁻¹	66%	Shake flasks	[26]
Escherichia coli	Hydrolysate from cedar	None	alsS, adhA, kivD, ilvCD	ΔldhA ΔadhE ΔpflB ΔackA-pta	3.7 g l⁻¹	0.04 g l⁻¹ h⁻¹	14%	Shake flasks	[26]
Escherichia coli	Sucrose	5 g l⁻¹ yeast extract	alsS, ilvC, ilvD, kivD		1.7 g l⁻¹		47%	Shake flasks	[25]
Shimwellia blattae	Wheat straw hydrolysate	5 g l⁻¹ yeast extract	alsS, ilvC, ilvD, kivD		3 g l⁻¹		25%	Shake flasks	[25]
Corynebacterium glutamicum	Glucose	5 g l⁻¹ yeast extract	ilvBNCD, kivd, adhA, pntAB	ΔaceE, Δpqo, ΔilvE, ΔldhA, Δmdh	13 g l⁻¹	0.33 g l⁻¹ h⁻¹	48%	Fed-batch (micro-aerobic)	[9]
Saccharomyces cerevisiae	Glucose	6.7 g l⁻¹ yeast nitrogen base	ILV2, ILV3, ILV5, KDC, ADH	ΔPDC1	0.14 g l⁻¹		1.6%	Shake flasks	[7]
Saccharomyces cerevisiae	Glucose	6.7 g l⁻¹ yeast nitrogen base	kivd, ADH6, ILV2, ILV5c, ILV3c, ILV2c, sMAE1	lpd1Δ	1.6 g l⁻¹		3.8%	Shake flasks	[8]
Bacillus subtilis	Glucose	5 g l⁻¹ yeast extract, 10 g l⁻¹ peptone	alsS, ilvCD, kivd, adh2		2.6 g l⁻¹	0.09 g l⁻¹ h⁻¹		Microaerobic shake flask fed-batch	[38]
Clostridium thermocellum	Cellulose	4.5 g l⁻¹ yeast extract	kivd, ilvBN, ilvCD	Δhpt	5.4 g l⁻¹		41%	Bottles	[10]

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(3.3 mg l^{-1}). Feed medium was pulsed to the cultures upon substrate depletion to restore a concentration of 50 g l^{-1}.

Liquid and solid media were supplemented with 50 µg ml^{-1} kanamycin or 100 µg ml^{-1} ampicillin as necessary.

Glycerol stocks for the storage at − 80 °C of all strains were prepared by mixing 700 µl of liquid overnight culture with 300 µl sterile glycerol (75%).

Preparation of medium containing sour whey

Spray-dried sour whey powder was kindly provided by NÖM AG (Niederösterreichische Molkerei AG, Baden, Austria). For the batch medium, 67.5 g sour whey powder was dissolved in 1 l distilled water and heated to 70 °C for 20 min. After cooling down, 13.3 g l^{-1} KH_2PO_4, 4.0 g l^{-1} $(NH_4)_2HPO_4$ and 1.7 g l^{-1} citric acid were added, and the pH was adjusted to 6.8. The medium was centrifuged at 14 000 rpm and 21 °C for 10 min, sterile filtered and supplemented with trace elements at the same concentration as the defined medium.

The whey feed was prepared by dissolving 337.5 g sour whey per 1 l dissolved water, followed by heating, centrifugation and sterile filtration. $MgSO_4 * 7 H_2O$ (1.6 g l^{-1}), Fe(III)citrate (0.13 g l^{-1}), EDTA (11 mg l^{-1}), $Zn(CH_3COO)_2 * 2 H_2O$ (17.0 mg l^{-1}), $CoCl_2 * 6 H_2O$ (3.3 mg l^{-1}), $MnCl_2 * 4 H_2O$ (20 mg l^{-1}), $CuCl_2 * 2 H_2O$ (1.6 mg l^{-1}), H_3BO_3 (3.9 mg l^{-1}) and $Na_2MoO_4 * 2 H_2O$ (3.3 mg l^{-1}) were added as for the defined feed media [14].

Construction of plasmids and strains

For all cloning steps in this study GoldenMOCS, a Golden Gate-based cloning system, was used [33, 34] and all primers and gBlocks were purchased from Integrated DNA Technnologies (IA, USA).

The genes *alsS* from *Bacillus subtilis* and *budB* from *Enterobacter cloacae subsp. dissolvens* DSM 16,657 were amplified as described elsewhere [14]. The genes *kdcA*, *adhA* and *adhA_mut* from *Lactobacillus lactis* and *ilvD* and *ilvC_mut* from *E. coli* W were purchased as gBlocks from IDT (IA, USA). The genes were flanked with fusion site 2 (FS2) and fusion site 3 (FS3). A colony PCR with Q5 High-Fidelity DNA Polymerase (New England Biolabs, MA, USA) was used to amplify *ilvC* from *E. coli* W and fusion sites 2 and 3 were added with the primers.

The PCR fragments and gBlocks were used for individual BB1 (backbone 1) assemblies [34]. The correct plasmid assembly was verified by restriction digest and Sanger sequencing (Microsynth AG, Switzerland) using the primers seq_fw and seq_rev and additional primers as indicated (Supplementary Material, Table S1).

Subsequently, each gene was assembled in BB2 with a constitutive promotor from the Anderson constitutive promotor library (J23109 or J23114) [35] and a synthetic terminator (B1001). These individual expression cassettes were finally used for BB3 assembly resulting in plasmids containing the full pathway consisting of five genes on one plasmid. Different promotor and gene combinations were used to construct a library of eight different vectors (Fig. 2). After BB2 and BB3 assembly, restriction digest was performed to verify for correct integration.

Adaptation to higher isobutanol concentrations

To enable *E. coli* W to grow in the presence of higher isobutanol concentrations, the strain was adapted by cultivation on increasing isobutanol concentrations. The initial isobutanol concentration was 5 g l^{-1} and was increased in steps of 1 g l^{-1} up to 10 g l^{-1}. Then, isobutanol concentrations of 12 g l^{-1} and from 15 g l^{-1} to 23 g l^{-1} steps of 2 g l^{-1} were applied. As soon as growth was observed for a certain condition, the cells were transferred to a higher concentration and glycerol stocks were prepared. The cells were grown at 37 °C and 200 rpm in 100 ml Erlenmeyer flasks with 20 ml defined medium containing 8 g l^{-1} glucose.

Preparation of precultures

All strains and constructs were stored at − 80 °C in 23% glycerol. For cultivations, they were streaked onto LB agar plates containing 50 µg ml^{-1} kanamycin and incubated overnight at 37 °C. A single colony was used for inoculation of 500 ml shake flasks containing 50 ml of LB medium or 2xLB medium for serum bottles or bioreactor cultivation, respectively. The preculture was incubated overnight at 37 °C and 230 rpm. The cells were centrifuged at 4800 rpm for 10 min at room temperature and washed with 25 ml of sterile 0.9% (w/v) NaCl. After resuspension in 5 ml 0.9% (w/v) NaCl, the optical density at 600 nm (OD_{600}) was measured and the appropriate volume of preculture to reach an initial OD_{600} of 1 was transferred to the bioreactor. The same procedure was used for shake flask and serum bottle experiments, but the initial OD_{600} was 0.5.

Strain and construct screening

For exact isobutanol quantification, the construct screening was carried out in 120 ml serum bottles sealed with butyl rubber septa to avoid loss by evaporation. The bottles were filled with 20 ml defined medium with a glucose concentration of 20 g l^{-1}. The bottles were incubated at 37 °C and 180 rpm. Samples were taken after 24 h and 48 h for OD_{600} and HPLC measurements.

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Cultivations in bioreactors

Bioreactor cultivations were performed in duplicate in a DASbox® Mini Bioreactor system (Eppendorf AG, Hamburg, Germany). The working volume was 200 ml and all cultivations were carried out at 30 °C. The pH was maintained at 6.8 by addition of 12.5% (v/v) NH_4OH with a MP8 Multipumpmodule (Eppendorf AG, Hamburg, Germany) and monitored by a pH electrode EasyFerm Plus K8 120 (Hamilton, Reno, NV, USA). The concentration of dissolved oxygen was monitored by a VisiFerm DO 120 probe (Hamilton, Reno, NV, USA). The agitator speed was kept constantly at 500 or 800 rpm for microaerobic cultivations of *E. coli* W $\Delta ldhA$ $\Delta adhE$ Δpta $\Delta frdA$ ($\Delta 4$) IB4 and *E. coli* W (W) IB4, respectively, and adapted from 800 to 2000 rpm in aerobic cultivations. The gassing rate was set to 0.2 vvm (2.4 sl h^{-1}) to avoid isobutanol stripping in batches. During aerobic cultivations, air was mixed with oxygen to maintain a dissolved oxygen concentration above 30%. For isobutanol stripping in pulsed fed-batches, the gassing rate was increased to 1 vvm (12 sl h^{-1}) after the first batch. To collect isobutanol from the reactor off-gas, the gas stream was flushed through cooled wash bottles on ice containing 500 ml distilled water and 5 g l^{-1} citric acid. For calculation of the absolute isobutanol concentrations, amounts in the reactor were added to the amounts in the wash bottles. Off-gas analysis for O_2 and CO_2 was carried out using the gas analyzer module GA4 (Eppendorf AG, Hamburg, Germany).

Samples of 4 ml were taken regularly and the optical density at 600 nm was measured to estimate biomass growth. The samples were centrifuged at 14 000 rpm for 5 min and the supernatant was used for HPLC analysis of substrate and product concentrations.

Determination of biomass

Cell dry weight (CDW) was determined gravimetrically in duplicates from bioreactor samples at the end of the batch phases. To this end, 4 ml of culture broth was centrifuged at 4800 rpm and 4 °C for 10 min, washed with 4 ml deionized water and centrifuged again. The pellet was dried in pre-weighed glass tubes for at least 72 h at 105 °C. The optical density at 600 nm (OD_{600}) was measured in a spectrophotometer (Genesys™ 20, Thermo Scientific, Waltham, Massachusetts, USA) against a water blank. The correlation between OD_{600} and cell dry weight was used to estimate the cell concentration for all time points except end of batch and feed phases.

HPLC analysis

Sugars, organic acids, and alcohols were determined using an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules/CA, USA) in an Ultimate 3000 system (Thermo Scientific, Waltham/MA, USA). 4 mM H_2SO_4 was used as a mobile phase at 60 °C and a flow of 0.6 ml min^{-1} for 40 min and the injection volume was 10 μl. Metabolites were detected using a refractive index (Refractomax 520, Thermo Scientific, Waltham/MA, USA) and a DAD detector (Ultimate 3000, Thermo Scientific, Waltham/MA, USA). Chromeleon 7.2.6 Chromatography Data System (Thermo Scientific, Waltham/MA, USA) was used for control, monitoring and evaluation of the analysis.

For sample preparation, 450 μl of cell-free culture supernatant was mixed with 50 μl of 40 mM H_2SO_4 and centrifuged for 5 min at 14 000 rpm at 4 °C. The supernatant was used for analysis and standards were treated the same way. A 5-point calibration was used for substrate and metabolite concentrations in the samples.

Results

Strain construction and screening

The goal of this study was to establish an *E. coli* system for isobutanol production in chemically defined medium and alternative raw materials such as cheese whey. To that end, we created a construct library expressing each gene individually under a constitutive promotor. Additionally, different strain backgrounds were tested to find the best construct–strain combination for efficient isobutanol production.

For the assembly of the isobutanol production pathway (Fig. 1), acetolactate-synthase, ketol-acid reductoisomerase, dihydroxy-acid dehydratase, α-ketoisovalerate decarboxylase and alcohol dehydrogenase were constitutively overexpressed using promotors of different strength from the Anderson constitutive promotor library [35]. This enabled the expression fine-tuning of each gene in an independent expression cassette. Two different types of constitutive promotors were used, the medium strength BBa_J23114 (114p) and the weaker promotor BBa_J23109 (109p). Plasmid assemblies were found to be challenging, as some promotor–gene combinations did not yield positive clones indicating the burden posed to the cell by expression of this pathway. A library containing eight different genetic constructs was created.

Investigating the influence of the strain background on isobutanol production, construct IB2 was tested in two different strains: *E. coli* W and K12-BW25113. To avoid evaporation and for the exact determination of product concentrations, serum bottles were used.

E. coli W showed growth and isobutanol production on chemically defined medium containing 20 g l^{-1} glucose (Fig. 2). In contrast, no isobutanol was produced in *E. coli* K12-BW25113. This strain showed growth, but glucose
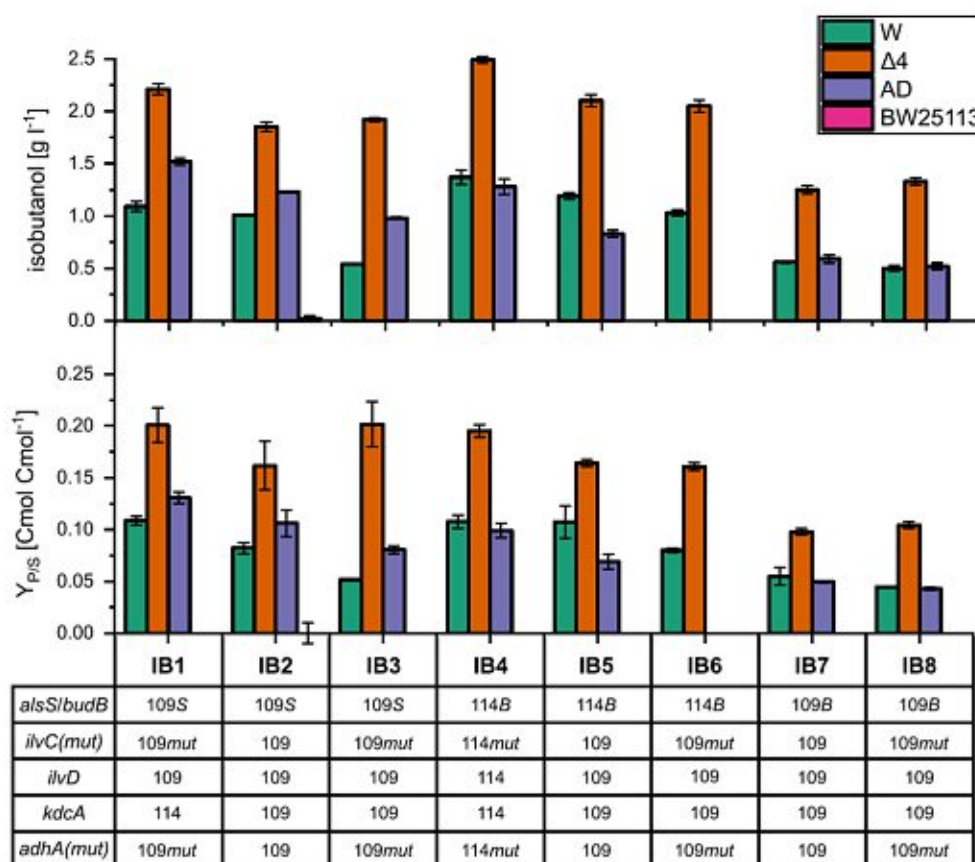
Fig. 2 Results of strain and construct screening for isobutanol production in *Escherichia coli* BW25113, *E. coli* W (W), *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* (Δ4) and *E. coli* W adapted to high isobutanol concentrations (AD) on minimal medium with 20 g l⁻¹ glucose. Results are given as means and standard deviations of biological triplicates. The indication of overexpressed genes are as follows: *alsS* (S) from *Bacillus subtilis* or *budB* (B) from *Enterobacter cloacae* subsp. *dissolvens* are acetolactate synthases, *ilvC* from *E. coli* W serves as ketol-acid reductoisomerase, *ilvC_mut* (mut) indicates a mutated form using NADH rather than NADPH as a cofactor, *ilvD* is dihydroxy-acid dehydratase from *E. coli* W, *kdcA* from *Lactococcus lactis* is α-ketoisovalerate decarboxylase and *adhA* from *L. lactis* is the alcohol dehydrogenase with the mutated form *adhA_mut* (mut) that displays higher catalytic activity. Constitutive promotors of the Anderson constitutive promotor library are indicated by 109 (J23109, weaker promotor) and 114 (J23114, stronger promotor). AD IB6 was not positively transformed and thus not tested

utilization was low and high amounts of acids were secreted (Supplementary Material, Table S2).

Subsequently, the influence of isobutanol pathway gene expression was studied. For an improved strain background, all eight constructs were screened in three *E. coli* W-derived strains: the parental *E. coli* W, *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* (Δ4) and *E. coli* W adapted to high isobutanol concentrations (AD). The deletion of by-product formation pathways in Δ4 should increase the driving force towards product formation and isobutanol cytotoxicity should be overcome using the adapted strain. After 30 sequential transfers to increased isobutanol concentrations, the adapted strain was able to grow in the presence of 21 g l⁻¹ isobutanol

(Supplementary Material, Table S3). *E. coli* W AD showed improved growth at higher isobutanol concentrations than the parental strain and this effect was more propagated at lower temperatures of 30 °C (data not shown). The screening showed that isobutanol was produced regardless of the construct composition, indicating the suitability of the constitutive expression system. Because genes were expressed as individual cassettes, we could study the influence of different promotors and genes on isobutanol production (Fig. 2). For instance, the expression of *alsS* rather than *budB* as acetolactate synthase (IB3 vs. IB8) led to higher product concentrations and yields. A stronger promotor for *budB* increased the isobutanol yield (IB5 versus IB7 and IB6 versus IB8) to

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the level reached with weaker expression of *alsS* (IB6 versus IB3). Apart from the acetolactate synthase, constructs using either the wild-type or mutated versions of *ilvC* and *adhA* showed similar isobutanol yields (IB5 versus IB6 and IB7 versus IB8). Additionally, an increased promotor strength of *kdcA* led to similar or increased isobutanol yields compared to a weaker promotor in *E. coli* W Δ4 and *E. coli* W, respectively (IB1 versus IB3).

The knockout of the mixed acid fermentation pathways (Δ4) resulted in decreased by-product formation, which in turn led to an increase of the isobutanol production and yield for all constructs (Supplementary Material, Table S2). For most constructs, there was no difference in the product yield between the *E. coli* W strain which was adapted to high isobutanol concentrations (AD) and the W strain. However, in the construct IB5, the obtained isobutanol yield was even 36% lower in the AD strain compared to the W strain.

For the selection of a suitable production strain, total isobutanol production and yield were evaluated (Fig. 2). The highest yield in combination with the highest titer and highest glucose uptake was achieved in *E. coli* W Δ4 IB4 expressing all genes from the medium strong 114p promotor and carrying *budB* as acetolactate synthase. Additionally, both *ilvC* and *adhA* were present in the mutated forms and utilized NADH as a cofactor.

Isobutanol production in aerobic and microaerobic batch cultivations

Microorganisms typically produce alcohols in the absence of oxygen under anaerobic or microaerobic conditions. In the strain screening experiments, microaerobic conditions in sealed serum bottles were successfully used for isobutanol production. Anaerobic conditions led to a growth defect in *E. coli* W Δ4 and accumulation of high amounts of acids in *E. coli* W. To investigate the effect of oxygen availability on isobutanol production under controlled conditions, *E. coli* W IB4 and Δ4 IB4 were tested in batch experiments. Based on the initial construct screening, microaerobic conditions (dissolved oxygen concentration of 0% in exponential phase) were compared to aerobic conditions. For all bioreactor cultivations, isobutanol stripping was monitored by a retention system.

Figure 3 shows the comparison of isobutanol production under aerobic and microaerobic conditions in *E. coli* W and Δ4. Under aerobic conditions, *E. coli* W mainly produced biomass and CO_2 and only minor amounts of isobutanol (0.9 ± 0.1 g l^{-1}), but under microaerobic conditions isobutanol production increased to 4.9 ± 0.4 g l^{-1}. In contrast, the aerobic and microaerobic cultures of *E. coli* W Δ4 achieved significantly higher isobutanol titers of 7.7 ± 0.2 g l^{-1} and 6.6 ± 0.4 g l^{-1}, respectively. Moreover, deletion of mixed acid fermentation pathways resulted

in a decreased biomass yield in *E. coli* W Δ4 (Fig. 4a), as shown before [14]. Nevertheless, the specific glucose uptake and isobutanol production rate of *E. coli* W Δ4 was significantly higher compared to *E. coli* W (Table 2). Additionally, *E. coli* W Δ4 produced significantly less by-products under all conditions compared to *E. coli* W and the isobutanol yield increased by 80% to 0.25 Cmol Cmol^{-1}. However, all by-products combined still accounted for 12% of the total carbon in aerobic cultures of *E. coli* W Δ4 (Fig. 4b). Despite deletion of phosphate acetyl transferase (*pta*), acetate was still a major by-product during aerobic cultivation of this strain. Additionally, isobutyraldehyde, diacetyl, 2,3-butanediol and acetoin associated with the isobutanol production pathway were detected as unspecific by-products. Interestingly, also pyruvate accumulated in significant amounts (5.0 ± 0.4 g l^{-1}) during aerobic cultivation of *E. coli* W Δ4, while the wild-type strain did not secrete pyruvate.

Isobutanol production in pulsed fed-batch cultivations

Upon successful production of isobutanol in batch experiments, we aimed to further increase product titers using fed-batch cultivations. Initially, we sought to establish a fed-batch cultivation with a linear feeding profile. However, a stable process could not be achieved and high variations in isobutanol and biomass concentration were observed (data not shown). Therefore, we performed pulsed fed-batches for process intensification, which have been successfully used for microbial production of platform chemicals such as 2,3-butanediol [14]. Aerobic conditions were selected for the pulsed fed-batches as initial batch cultivations had yielded the highest isobutanol titers and yields for *E. coli* W Δ4. All subsequent pulsed fed-batches were carried out with this strain under aerobic conditions. In the first batch phase, low gassing rates were applied. To prevent cell death caused by isobutanol toxicity in the subsequent phases, the volatile compound was stripped by the increase of gassing rates (Supplementary Material, Tables S5, S6 and S7).

Glucose pulses resulted in the production of 15.6 ± 0.5 g l^{-1} isobutanol (Fig. 5a). In the later phases (batch 2 and 3), the carbon flux shifted from biomass to isobutanol production and formation of CO_2 (Supplementary Material, Table S5). Batch 2 showed the highest isobutanol production rate (0.25 g l^{-1} h^{-1}), whereas batch 3 showed the highest isobutanol yield (52% of the theoretical maximum). In batch 3, glucose uptake and isobutanol production rates decreased. (Supplementary Material, Table S5).

With the final production of 15.6 g l^{-1} isobutanol, the process mode of a pulsed fed-batch was found to be suitable for cheap, reproducible, and easily feasible production.
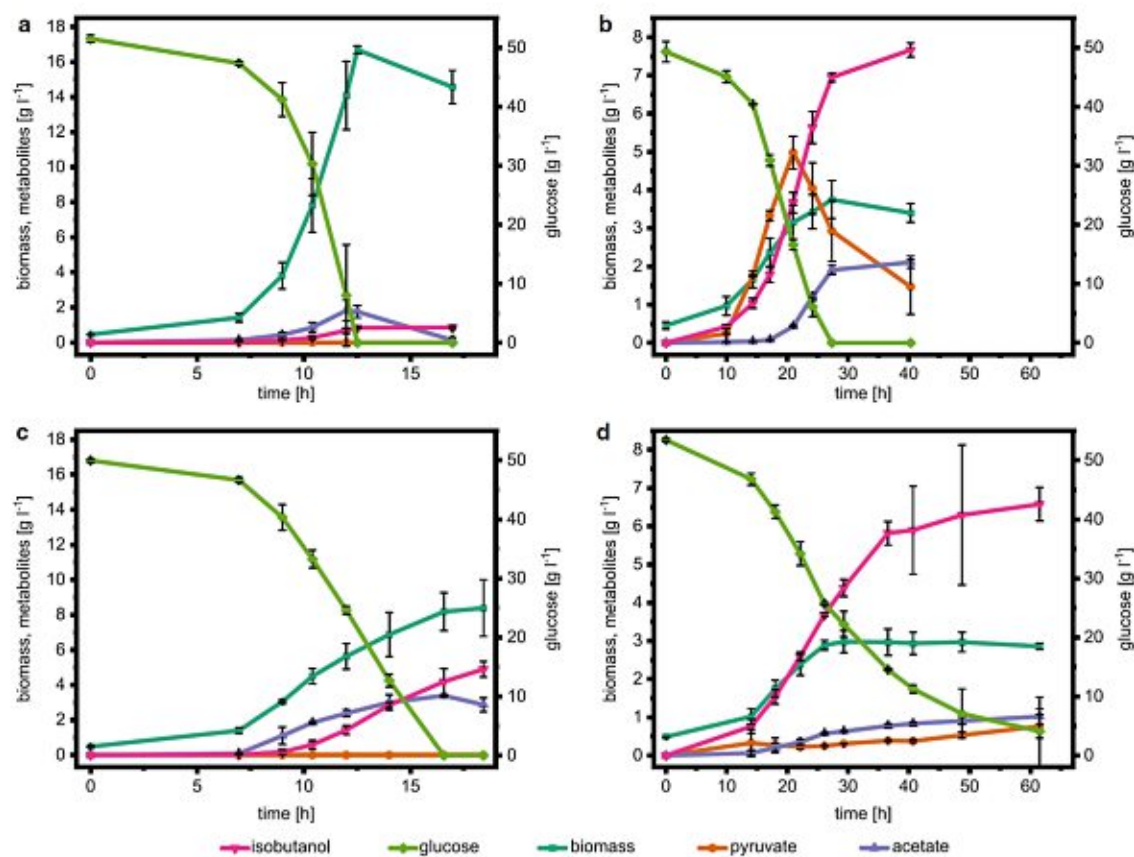
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Fig. 3 Substrate and metabolite concentrations in batch experiments on defined medium with 50 g l⁻¹ glucose of **a** *E. coli* W IB4 under aerobic conditions, **b** *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* IB4 under aerobic conditions, **c** *E. coli* W IB4 under microaerobic conditions and **d** *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* IB4 under microaerobic conditions. Means of biological duplicates are shown and error bars represent standard deviations

Production of isobutanol from lactose and cheese whey

The production cost for fuel alcohols could further be decreased using alternative raw materials. Therefore, we investigated whether spray-dried sour whey is a suitable substrate for isobutanol production.

For comparison of the process performance, a reference process was carried out on defined medium with lactose as carbon source (Fig. 5b). Using pure lactose instead of glucose as carbon source decreased the final isobutanol titer by 10% (Table 3). In contrast, the fed-batch using cheese whey showed a higher isobutanol titer of 19.6 ± 1.8 g l⁻¹ representing an increase of 26 and 40% compared to defined medium with glucose and lactose, respectively (Table 3). In contrast to synthetic media, constant substrate uptake and isobutanol production are ensured over a longer time

period, resulting in an overall higher isobutanol productivity (Table 3). The isobutanol yield reached 0.26 Cmol Cmol⁻¹, which is 39% of the theoretical maximum. In contrast to the defined medium, lactose utilization was accelerated in the cheese whey process. In the second batch of the whey process, lactose uptake peaked at 1.97 g l⁻¹ h⁻¹, a twofold increase compared to the defined lactose medium (Supplementary Material, Tables S6 and S7). Moreover, lactose and lactate co-utilization was observed in whey based medium (Fig. 5c). Lactate co-utilization can increase NADH availability, which is beneficial for isobutanol production. The availability of additional substrates in cheese whey resulted in a slightly increased biomass yield compared to synthetic medium (Supplementary Material, Tables S5, S6 and S8).

Pyruvate accumulated in the first batch and was subsequently consumed in the subsequent process phases. Cultures using lactose as carbon source completely consumed
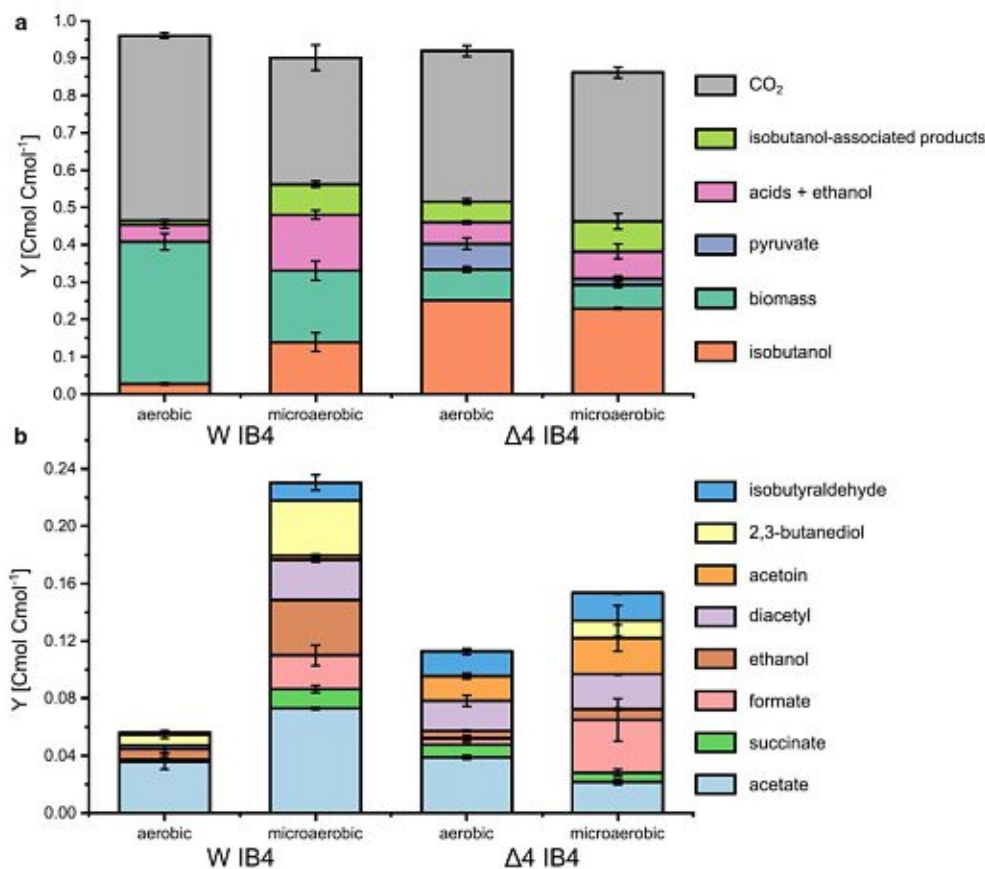
Fig. 4 Product yields [Cmol product Cmol^{-1} glucose] in batch experiments of *E. coli* W IB4 (left) and *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* IB4 (right) under aerobic and microaerobic conditions in minimal medium with 50 g l^{-1} glucose. In **a**, isobutyraldehyde, 2,3-butanediol, acetoin and diacetyl are summarized as isobutanol-associated products and acetate, formate, succinate and ethanol are summarized as acids + ethanol. Detailed by-product yields are shown in **b**. Acetol (hydroxyacetone) and 1,2-propanediol were also analyzed but not detected. Means of biological duplicates are shown and error bars represent standard deviations

Table 2 Maximum volumetric (r_{Iso}) and specific (q_{Iso}) isobutanol production and maximum volumetric (r_S) and specific (q_S) glucose uptake rates of *E. coli* W IB4 (W) and *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* IB4 (Δ4) in batch experiments on chemically defined medium with 50 gl^{-1} glucose

Strain	Condition	r_{Iso} (g l^{-1} h^{-1})	q_{Iso} (g g^{-1} h^{-1})	r_S (g l^{-1} h^{-1})	q_S (g g^{-1} h^{-1})	OUR (mmol l^{-1} h^{-1})	q_{O2} (mmol g^{-1} h^{-1})
W	Aerobic	0.33 ± 0.04	0.02 ± 0.00	15.7 ± 0.3	1.29 ± 0.01	263 ± 40	17.1 ± 1.6
	Microaerobic	0.75 ± 0.01	0.11 ± 0.01	6.1 ± 0.6	1.50 ± 0.22	49.9 ± 21	9.5 ± 1.2
Δ4	Aerobic	0.62 ± 0.04	0.19 ± 0.01	3.7 ± 0.1	1.70 ± 0.08	21.3 ± 0.4	8.7 ± 2.5
	Microaerobic	0.27 ± 0.04	0.15 ± 0.01	2.1 ± 0.4	1.01 ± 0.04	9.8 ± 0.8	3.9 ± 1.3

For aerobic conditions, dissolved oxygen was maintained above 30%. Microaerobic conditions were maintained at constant stirrer speed of 800 rpm (W) and 500 rpm (Δ4) and dissolved oxygen dropped to 0% in the exponential phase. Mean values and standard deviations were calculated from biological duplicates

pyruvate, whereas a portion of pyruvate remained in the culture broth when glucose was the substrate. Since pyruvate accumulation is generally associated with metabolic stress [36], it is possible that higher specific substrate uptake rates for glucose increased metabolic burden compared to lactose cultures (Supplementary Material,

Fig. 5 Pulsed fed-batches of *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* IB4 under aerobic conditions on **a** minimal medium with 50 g l⁻¹ glucose, **b** minimal medium with 50 g l⁻¹ lactose and **c** cheese whey medium. Substrate uptake and metabolite as well as CO_2 formation are shown. Upon depletion of the carbon source, new medium was pulsed to obtain substrate concentrations of 50 g l⁻¹. Means of biological duplicates are shown and error bars represent standard deviations
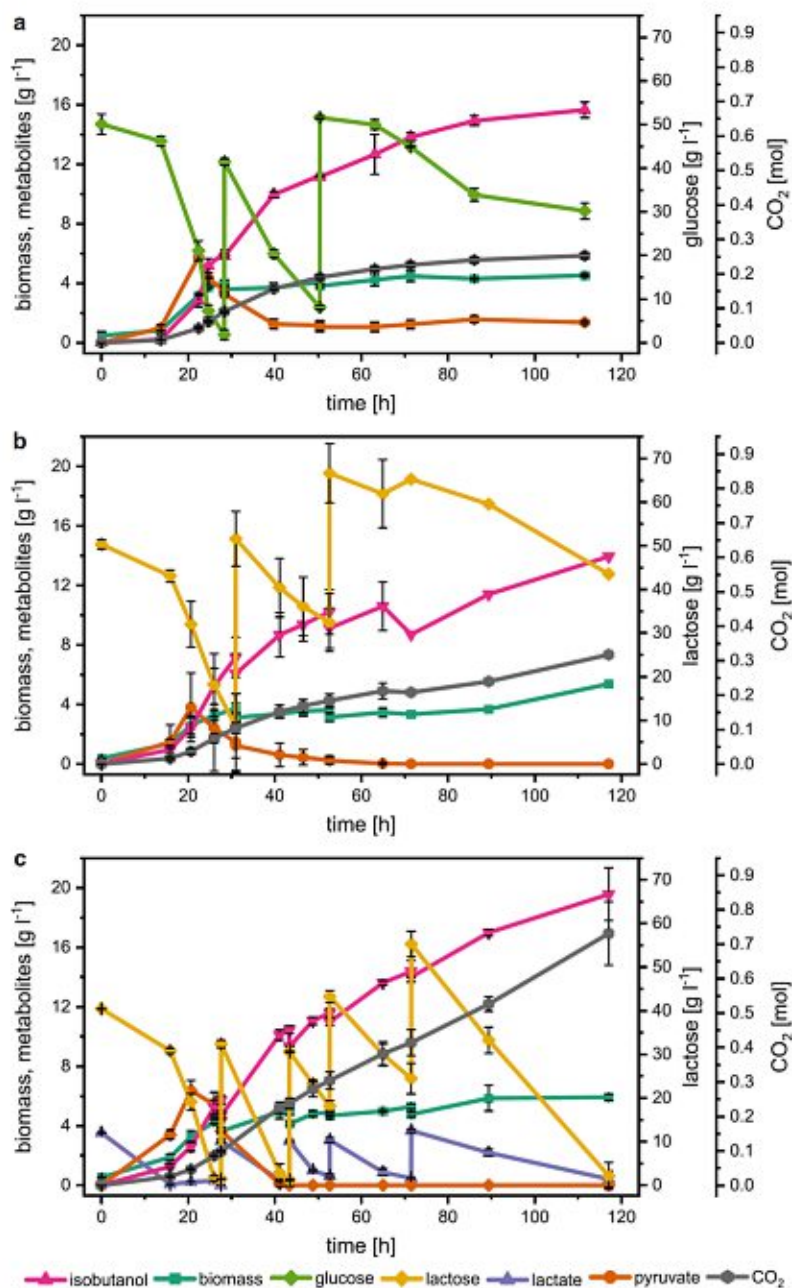
Tables S5 and S7). Comparable to batch experiments, the by-products acetate, succinate, diacetyl, acetoin and isobutyraldehyde were detected in different amounts in the culture broth (Supplementary Material, Table S4).

Discussion

The comparison of two different strain backgrounds revealed the suitability of *E. coli* W strains for isobutanol production

Table 3 Mean total volumetric (r_{Iso}) and specific (q_{Iso}) isobutanol production, volumetric (r_S) and specific (q_S) glucose uptake rates, isobutanol ($Y_{Iso/S}$) biomass ($Y_{X/S}$) and CO_2 ($Y_{CO2/S}$) yield and total carbon recoveries of *E. coli* W $\Delta ldhA$ $\Delta adhE$ Δpta $\Delta frdA$ IB4 in glucose, lactose and cheese whey pulsed fed-batch experiments

	Glucose	Lactose	Cheese whey
Isobutanol (g l^{-1})	15.6±0.5	14.0	19.6±1.8
r_{Iso} (g l^{-1} h^{-1})	0.14±0.01	0.12	0.17±0.02
q_{Iso} (g g^{-1} h^{-1})	0.04±0.01	0.04	0.04±0.01
r_S (g l^{-1} h^{-1})	0.92±0.03	0.70	1.32±0.02
q_S (g g^{-1} h^{-1})	0.27±0.01	0.24	0.31±0.01
$Y_{Iso/S}$ (Cmol Cmol^{-1})	0.25±0.02	0.30	0.26±0.04 0.24±0.04*
$Y_{X/S}$ (Cmol Cmol^{-1})	0.045±0.002	0.073	0.063±0.009 0.057±0.008*
$Y_{CO2/S}$ (Cmol Cmol^{-1})	0.56±0.05	0.58	0.53±0.05 0.48±0.04*
C recovery (%)	102±6	102	90±1.5

Mean values and standard deviations were calculated from duplicate experiments. As one lactose cultivation failed after batch 2, the parameters are calculated from one replicate. For the cheese whey process, yields were calculated considering lactose or the sum of lactose and lactate (*) as substrate

using chemically defined medium. The superior performance of this strain can probably be attributed to a high stress tolerance and fast growth [39]. *E. coli* K12-BW25113 has been reported as an efficient isobutanol producer on complex media [5, 6, 37], but isobutanol production on defined medium failed in this study. This discrepancy in the performance of a strain on defined and complex medium suggests that the right screening platform (i.e., chemically defined medium) should be chosen for further strain and process development [14].

Especially on minimal medium, a balance between enzyme expression and cell fitness has to be established for microbial production of platform chemicals [14]. Pathway construction as individual cassettes without inducible promotors was suitable for isobutanol production and revealed the influence of single gene expression levels. For the first enzyme in the pathway, acetolactate synthase, the tenfold higher activity of AlsS compared to BudB [40] probably accounted for increased isobutanol yields. We aimed to further increase acetolactate expression by using *alsS* with a stronger promotor, but this assembly was technically not feasible due to metabolic burden by enhanced enzyme expression. Stronger expression of *alsS* might not necessarily lead to improved isobutanol production, since a construct expressing *budB* rather than *alsS* from the same promotor was found to yield higher 2,3-butanediol production [14]. These results indicate that keeping a balance between strain fitness and product formation is especially important on defined medium. The expression of *kdcA* and *adhA* is a

potential bottleneck, since the intermediate product isobutyraldehyde is both very toxic and volatile [41]. That the stronger expression of *kdcA* improved isobutanol production suggests that the high affinity towards isobutyraldehyde in the subsequent enzyme AdhA_mut [18] allowed for efficient conversion of the toxic intermediate in the best producing strain. Considering product yield and isobutanol titer, IB4 with medium strong expression levels for all genes was found to be the most suitable construct.

In accordance with other studies, adaptation of *E. coli* W to isobutanol did increase the tolerance concentration up to which growth was possible significantly. However, the production characteristics were not improved in the adapted strain [3, 42, 43]. In contrast, the toxicity of isobutanol led to the inhibition of biomass formation in the non-adapted strain, which was one key factor to enable efficient isobutanol production. Similarly, increasing the driving force of pyruvate availability by deletion of competing pathways led to higher alcohol production [5, 44–46]. Therefore, the combination of inhibited growth by toxic isobutanol production and the availability of pyruvate were crucial for efficient isobutanol production, while adaptation of *E. coli* did not improve product formation.

The driving force can also be improved by increased NADH availability. Since this availability is directly related to oxygen supply in a cell, different aeration strategies can greatly influence product formation [44, 47]. In *E. coli* W, isobutanol production could be improved by microaerobic cultivation, whereas the reduction of oxygen supply led to decreased substrate utilization and production rates in *E. coli* W Δ4. Considering microbial production of other alcohols, the reduction of oxygen availability was successful for 2,3-butanediol and n-butanol production [14, 15, 44], whereas isobutanol and isopropanol could be produced under aerobic conditions [6, 48].

That the deletion of mixed acid fermentation pathways in *E. coli* W Δ4 increased aerobic isobutanol production is somewhat surprising, as these pathways are usually repressed under aerobic conditions. In this scenario, pyruvate accumulates, which indicates metabolic stress [36] and is associated with isobutanol production. Isobutanol toxicity is, among other factors, based on quinone inhibition, which activates the aerobic respiration control protein ArcA [36]. By repressing aerobic enzymes such as pyruvate dehydrogenase, ArcA activation leads to pyruvate accumulation [49] and increased NADH/NAD$^+$ ratios [36]. Additionally, deletions of *ldhA* and *pta* have been shown to increase pyruvate formation [50, 51]. In other words, the combination of increased NADH availability, reduced by-product formation and higher pyruvate availability led to increased isobutanol titers and yields under aerobic conditions in *E. coli* W Δ4 [3, 36]. Since the described toxicity mechanism is unique

for higher alcohols, the effect of aerobic production is not directly transferable to other alcohols.

In accordance with our findings, reduced acetate accumulation by deletion of *pta* was previously shown and an additional knockout of pyruvate oxidase *poxB* was not reported to increase product formation [6]. The formation of other by-products is probably a result of lacking specificity of the individual isobutanol pathway enzymes or utilization of pathway intermediates as substrates by native *E. coli* enzymes [52, 53] (Fig. 1). In detail, diacetyl is produced by spontaneous decarboxylation of acetolactate and is converted to acetoin and 2,3-butanediol [54] by native or overexpressed enzymes with indistinct substrate patterns (Fig. 1). Requiring NADH as a cofactor, the formation of 2,3-butanediol from acetoin is one observation that reflects the redox status of the cell. Similarly, NADH availability influences the conversion of isobutyraldehyde to isobutanol. A low activity of AdhA might have caused isobutyraldehyde accumulation, but AdhA_mut was reported to have a high affinity toward its substrate [18]. In accordance, the toxic intermediate was mainly found in the wash bottles, which suggests that its high volatility caused stripping from the culture.

Aerobic cultivation of *E. coli* W Δ4 yielded the highest isobutanol of 38% of the theoretical maximum which is comparable to approximately 36% previously reported for defined medium [5]. Systems relying on complex media components using yeast extract concentrations of up to 25 g l^{-1} showed higher yields [5, 6, 18]. Moreover, the addition of yeast extract has been shown to enhance isobutanol productivity (2.8-fold increase, Table 1) [6]. For comparable defined production systems, data are only available for nontoxic diol production. Using the same strain background (*E. coli* W Δ4) for 2,3-butanediol production, a fivefold higher production rate was reported [14]. That 76% of the theoretical yield was reached suggests limited production due to isobutanol toxicity in this study.

The availability of additional nutrients in cheese whey resulted in an increased final product titer, which is beneficial for further cost-effective downstream processing [55]. The production of 19.6 g l^{-1} isobutanol is the highest titer obtained on alternative raw materials. Isobutanol yield reached 39% of the theoretical maximum, which is a 1.5- to 2.8-fold increase compared to lignocellulosic hydrolysates [25, 26]. These promising results were obtained by keeping cells at high performance for an extended time period and thereby increasing overall productivity. We speculated that a strategy combining different factors is key for successful isobutanol production. One factor might be the optimum concentration of isobutanol in the fermentation broth to favor product over biomass formation due to isobutanol toxicity. By applying pulses rather than a constant feeding profile, the process is operated at the maximal possible uptake and production rate, which might also improve product formation. Similarly, high glucose concentrations at the beginning of every pulse could also slightly inhibit cell growth and favor isobutanol production. A comparison of state-of-the-art processes for isobutanol production is shown in Table 1.

In this work, investigation of isobutanol production on defined medium and cheese whey as an alternative carbon source provided valuable information for further investigation on the way to potential industrial applications. In Table 4, we calculated commercial indicators for different production scenarios from this study and literature reports [6]. Additionally, we estimated the production cost if yeast extract is replaced by an alternative nutrient source. To this end, the amounts of utilized carbon source and media additives were calculated based on reported yields and product titers. These amounts were used to estimate the media cost [56] and the minimum price at which isobutanol has to be sold to cover these costs. Since cheese whey and corn steep liquor are waste products, these media were assumed not to generate costs. On the contrary, the costs for safe disposal of whey are difficult to estimate [30, 58], but can range from

Table 4 Comparison of estimated media cost and minimal selling price for microbial isobutanol production

Medium	C-Source	Complex media additive	Isobutanol titer (g l^{-1})	Isobutanol yield (g g^{-1})	Media cost ($/m^3$)	Minimum isobutanol selling price ($/kg)	References
Defined	103 g l^{-1} glucose	–	16	0.15	48.6	3.1	This study
Cheese whey	254 g l^{-1} spray-dried cheese whey	–	20	0.08	0.00	0.0	This study
Complex	176 g l^{-1} glucose	25 g l^{-1} yeast extract	51	0.29	1644	32	[6]
Alternative complex	176 g l^{-1} glucose	250 g l^{-1} corn steep liquor	51	0.29	83	1.7	Theoretical

Prices for glucose and yeast extract were obtained from Rodrigues et al. (2007) and converted to US$ at the current exchange rate of 1.18 US$ per € [56]. Corn steep liquor and cheese whey were assumed to cost 0.00 $ since they are waste products. The alternative complex medium is based on yields reported by Baez et al. [6], but yeast extract was assumed to be replaced by corn steep liquor as successfully shown by Saha (2006) [57]. The calculation of the minimum selling price is greatly simplified, since only the main media components were used for calculation. Additional costs such as for energy, downstream processing or other media additives were not considered

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0.6 to 4.4 (US) cent per pound of cheese processed [59]. Costs for media components, bioreactor operation and downstream processing have not been considered, as they do not depend on the substrate utilized for isobutanol production and would therefore add similar but hard to estimate costs to all scenarios.

Table 4 shows that the addition of yeast extract greatly influences the total media cost. Its replacement by other raw materials such as corn steep liquor (CSL) might be a promising alternative. However, Saha (2006) reported that CSL had to be used at a concentration of 50 g l^{-1} to achieve a similar effect as with 5 g l^{-1} yeast extract [57]. The high concentrations that need to be applied could limit the use of this media additive. Alternatively, yeast extract could also be purchased at lower prices from breweries, where yeast is a main by-product [60, 61]. However, yeast biomass is from fermentation processes is frequently used as an animal feedstock. It seems likely that the availability of yeast biomass for a fermentation process yielding a low-price product such as isobutanol is therefore limited in comparison to the higher price that can be obtained when sold as an animal feedstock. Additionally, variations in yeast extract quality could affect process performance and different brewing processes were shown to influence the nutrient composition [60]. Due to additional costs related to the use of a complex media additive, it is more cost-effective to omit additional media components. We suggest using a cheap raw material (e.g., cheese whey) as a carbon source, thereby avoiding costs for glucose or other sugars that increase the total production cost.

The selling price for isobutanol was reported to be around 1750 $/t in 2015 [62]. Comparing this price to the calculated theoretical selling prices shows that only the use of cheese whey production could result in a cost-competitive process.

Further reduction of fermentation cost can be achieved by the omittance of expensive inducers. This reduction can either be achieved by induction systems that rely on cheaper inducers [26] or by the use of constitutive expression as reported in this study.

Typically, plasmid-based expression requires the use of a cost-intensive selection marker such as kanamycin. Genome integration could therefore be a promising goal for future research.

Conclusion

In this study, isobutanol was efficiently produced in a chemically defined medium due to the choice of a suitable strain background and expression system. Individual expression of each gene under a constitutive promotor allowed for the selection of a suitable construct. The use of the robust *E. coli* W in combination with strain improvement and the investigation of different aeration strategies were key for the development of an efficient production process. Using cheese whey as an alternative raw material in pulsed fed-batches enabled longer process stability and higher isobutanol titers. In future, investigation of other cheap raw materials and waste streams can contribute to the development of cost-effective processes. In this study, isobutanol production on both chemically defined medium and a residual waste stream was demonstrated, which provides valuable information for further development of industrially relevant isobutanol production processes.

Data availability The datasets used and/or analysed during the current study, if not shown in the text or additional files, are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

Ethical approval Not applicable.

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3.2. Isobutanol production from acetate (monographic chapter)

Problem statement

Several factors can hamper the efficiency of isobutanol production from acetate and these include the toxicity and low energy density of acetate as well as the inhibiting effect of isobutanol on microorganisms.

State-of-the-Art

Studies on product formation from acetate have mainly focused on the use of complex media and most of them also investigated the production of chemicals derived from acetyl-CoA (Xu et al., 2017; Yang et al., 2020, 2019) or TCA cycle intermediates (Li et al., 2016; Noh et al., 2018). Isobutanol production from acetate was shown to be successful, but only very low titers of 120 mg l^{-1} were achieved in medium containing high amounts of yeast extract (Song et al., 2018).

Scientific question

Which environmental factors mainly influence isobutanol production from acetate?

Hypothesis

Isobutanol production from acetate depends on strain background and media composition.

Approach

The production of isobutanol was evaluated in screening experiments on defined and complex medium. Different strain backgrounds were compared regarding their production characteristics.

Content

In this chapter, isobutanol production from acetate was investigated. The comparison of three different strain backgrounds revealed that deletion of the mixed acid fermentation pathways in *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* enabled growth and diol production on complex, but not on defined medium. However, pulsed fed-batches indicated that isobutanol was not produced from acetate, but only from yeast extract. This hypothesis was further supported in experiments comparing isobutanol production in complex medium with and without acetate. However, a reliable statement about isobutanol production from acetate could not be made since quantification was complicated due to its volatility and low product titers. Therefore, further media development and research will focus on 2,3-butanediol production from acetate. This should lead to the establishment of a platform process that can be transferred to isobutanol production from acetate in future.

Abstract

The abundantly available carbon source acetate carries potential for upgrading into value-added chemicals such as isobutanol. This chapter aims on establishing isobutanol production from acetate in *Escherichia coli* W. To this end, three different strain backgrounds were evaluated with respect to their isobutanol production capabilities. It was shown that deletion of the mixed acid fermentation pathways in *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* enabled growth and isobutanol production on complex, but not on defined medium. Pulsed fed-batches using acetate complex medium showed great variations in isobutanol titers along the fermentation process. Apart from showing that quantification of minimal amounts of isobutanol was especially complicated in continuously sparged bioreactors, this observation resulted in the hypothesis that isobutanol is only produced from the complex media additive yeast extract and not from acetate. Although this hypothesis was further supported by comparing complex medium with and without acetate, an exact statement was not possible due to unreliable quantification of the volatile product. Since 2,3-butanediol is also derived from pyruvate and was developed using the same expression system, but is neither volatile nor toxic, a platform process for 2,3-butanediol production from acetate will be developed.

Background

Global concerns about climate change have recently increased interest in sustainable solutions for a transition towards a biobased industry, which is why focus has been laid on microbial chemical production (Conijn et al., 2014). Isobutanol is one example for a promising chemical since its use as a fuel has advantages over ethanol. It has, for example, a higher energy density which is closer to gasoline and can therefore be mixed with gasoline and transported with the existing infrastructure (Atsumi et al., 2010; Tao et al., 2014).

Isobutanol cannot be produced naturally by any organism, but its synthesis is possible via the combination of valine biosynthesis and the Ehrlich pathway via a five-enzyme pathway (Atsumi et al., 2008). Isobutanol production was first developed in *E. coli* (Atsumi et al., 2008), but has been demonstrated in other organisms as well (Atsumi et al., 2009; Blombach et al., 2011; Lee et al., 2012; Lin et al., 2015). Advances in microbial isobutanol production have recently been reviewed (Chen and Liao, 2016). Product removal by gas stripping was shown to be important for the production of high titers, since isobutanol is toxic to microorganisms (Atsumi et al., 2010; Mariano et al., 2011). Due to its volatility, a suitable method for detection of product stripped from the fermentation medium must be applied to achieve reliable quantification.

Isobutanol production has until now been successfully shown from different sugar sources such as glucose, sucrose and cellobiose (Baez et al., 2011; Desai et al., 2014; Felpeto-Santero et al., 2015). In this thesis, isobutanol production was additionally shown on cheese whey as an alternative raw material (Results 3.1, (Novak et al., 2020a)). Acetate is another alternative carbon source, since it is present in a variety of cost-effective resources such as food waste and lignocellulosic hydrolysates and it is a product of gas fermentations (Novak and Pflügl, 2018). Isobutanol production from acetate was previously shown in *E. coli* and maximum titers of 120 mg l^{-1} isobutanol were achieved. While isobutanol is derived from pyruvate, other studies mainly investigated acetate utilization for acetyl-CoA (Xu et al., 2017; Yang et al., 2020, 2019) or TCA cycle derived products (Li et al., 2016; Noh et al., 2018). In addition to variations in physiology, the main difference between chemicals produced from acetyl-CoA and pyruvate is the decreased theoretical yield for pyruvate derived C4 chemicals.

Most studies for microbial chemical production using acetate as a carbon source relied on the addition of complex media additives for growth and production. However, there are some major drawbacks and limitations related to the use of complex media additives and these have recently been investigated and described (Maser et al., 2020).

The goal of this chapter is to investigate the influence of media composition and strain background on isobutanol production from acetate. Therefore, isobutanol production of

different strain backgrounds was evaluated in complex and defined medium. Key factors that influence productivity were determined as a basis for further investigation.

Methods

Bacterial strains and media

E. coli W (DSM 1116 = ATCC 9637 = NCIMB 8666) from DSMZ (Braunschweig, Germany), *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* and *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* Δ*pykA* (kind gifts of Prof. Michael Sauer, BOKU, Vienna, Austria) were used for cultivations.

Lysogeny broth (LB) containing 10 g l^{-1} soy peptone, 5 g l^{-1} yeast extract and 10 g l^{-1} sodium was used for all precultures in the shake flask experiments. Yeast extract and soy peptone concentrations were doubled for bioreactor precultures (giving 2x LB). 15 g l^{-1} agar were added to LB medium for cultivation on plates.

The basic medium for all experiments is chemically defined medium as described in Results parts 2 and 3.1 (Novak et al., 2018, 2020a). In experiments on complex medium, 5 or 10 g l^{-1} yeast extract were added as indicated. The feed medium contained 200 g l^{-1} acetate and was pulsed to the reactor to restore an acetate concentration of 10 g l^{-1}. Liquid and solid media were supplemented with 50 µg ml^{-1} kanamycin.

Plasmid construction

Plasmids for isobutanol production are described in Results part 3.1. To be specific, the construct IB4 expressing *budB*, *ilvC_mut*, *ilvD*, *kdcA* and *adhA_mut* from constitutive medium-strong promotor 114p was used (Novak et al., 2020a).

Precultures and shake-flask experiments

All strains and constructs were stored at -80°C in 20 % (w/v) glycerol and cultivated at 37°C on LB agar plates containing 50 µg ml^{-1} kanamycin overnight. Preculture cultivation and inoculation was performed as described in Results part 3.3 (Novak et al., 2020b). Shake-flasks of 500 ml containing 20 ml medium were inoculated at an optical density at 600 nm (OD$_{600}$) of 0.5. The main cultures were incubated at 30°C and 230 rpm and samples were taken regularly for OD$_{600}$ and HPLC analysis.

Bioreactor cultivations

Bioreactor cultivations were carried out as described in Results part 3.3 with the difference that the pH was set to 7.0 at all times and that washed cells previously grown on 2xLB were used for inoculation at an initial OD$_{600}$ of 1.0. Processing of samples and biomass as well as HPLC analysis for sugars and acids was performed as described in Results part 3.3 (Novak et al., 2020b).

Substrate and product quantification

Isobutanol and acetate concentrations were determined using an HPLC method previously described (Novak et al., 2020a). In bioreactor cultivations, total isobutanol was quantified by adding stripped (gaseous) and liquid isobutanol. To this end, isobutanol stripped from

the fermentation broth was collected in cooled wash-bottles as described in Part 3.1 (Novak et al., 2020a). In shake flasks, stripped isobutanol was not quantified.

Results and Discussion

As a first step, isobutanol production was screened in different strain backgrounds. Previous construct screenings on glucose revealed that construct IB4 expressing all genes (budB, *ilvC_mut*, *ilvD*, *kdcA* and *adhA_mut*) from the medium strong promotor 114p resulted in the highest isobutanol yield (Novak et al., 2020a). To this end, construct IB4 was used for strain screening on acetate and isobutanol production was compared in *E. coli* W (W), *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* (Δ4) and *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* Δ*pykA* (Δ5).

Figure 6 shows the production of 0.15 g l^{-1} isobutanol in *E. coli* W Δ4 on complex medium, while almost no isobutanol was produced in *E. coli* W and *E. coli* W Δ5. Growth of *E. coli* W Δ4 and *E. coli* W Δ5 was not promoted on defined medium, while *E. coli* W was able to grow but did not produce any isobutanol.

Conclusively, isobutanol was only produced in *E. coli* W Δ4 on complex medium at a yield of 0.031 ± 0.002 g isobutanol per g acetate. It had not been shown before that the deletion of mixed-acid fermentation pathways enabled product formation from acetate. Strategies successfully applied to enhance microbial chemical production from acetate include blocking of the TCA cycle (Yang et al., 2019) and activation of the glyoxylate shunt (Lee et al., 2018). Similarly, research has focused on engineering acetate uptake pathways for the production of isopropanol, acetone or phloroglucinol from acetate (Xu et al., 2017; Yang et al., 2020, 2019).

The main difference between C3 products and isobutanol as a C4 compound is their metabolic precursor. While acetone, isopropanol, phloroglucinol and 3-hydroxypropionic acid are directly derived from acetyl-CoA, isobutanol is derived from pyruvate. In contrast to acetyl-CoA derived products, where production mainly requires the activity of acetate assimilation genes, a combined activity of the TCA and the glyoxylate cycle as well as gluconeogenesis is crucial to achieve isobutanol production from acetate. Furthermore, the maximum theoretical yields for acetyl-CoA derived products is 2-fold higher compared to pyruvate-derived products.

To further investigate isobutanol production from acetate and to achieve higher titers, *E. coli* W Δ4 was grown in bioreactor cultivations. After substrate depletion in a batch experiment, acetate was pulsed to the culture. In accordance with our other experiments where acetate was used as a sole carbon source, acetate consumption rates differed in biological duplicates (**Figure 7**). However, cells were able to consume a total of ~34 g l^{-1} acetate in both approaches. Biomass growth was achieved mainly in the first batch and carbon flux shifted towards CO_2 production during the subsequent pulses. The time-dependent course of isobutanol production shows that only very low concentrations of isobutanol were produced. Additionally, the quantification of isobutanol was unreliable since

the amounts detected varied significantly (two individual duplicates in **Figure 7**). The established system for the capture and quantification of stripped isobutanol was very suitable when high titers were achieved in glucose grown cultures (Novak et al., 2020a) but seems to be unsuitable for low product concentrations. Additionally, et seems that isobutanol is only produced in the first batch, but not in the following periods.
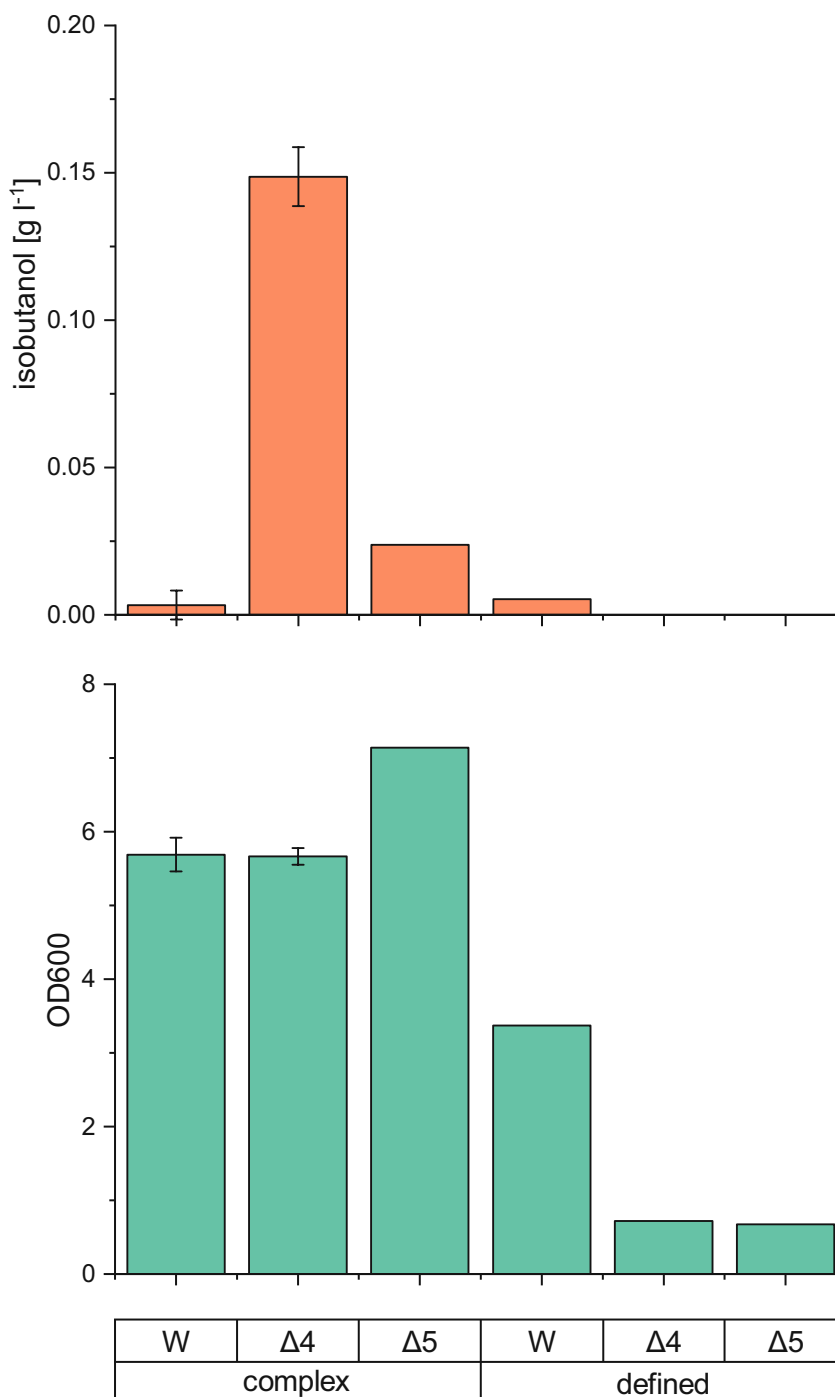

Figure 6 – Growth and isobutanol production of different strains on complex and defined medium. *E. coli* W (W), *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* (Δ4) and *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* Δ*pykA* (Δ5) with construct IB 4 were cultivated in shake-flasks on defined medium with 5 g l⁻¹ acetate and on medium additionally containing 10 g l⁻¹ yeast extract. Mean values and standard deviation were calculated from biological triplicates.
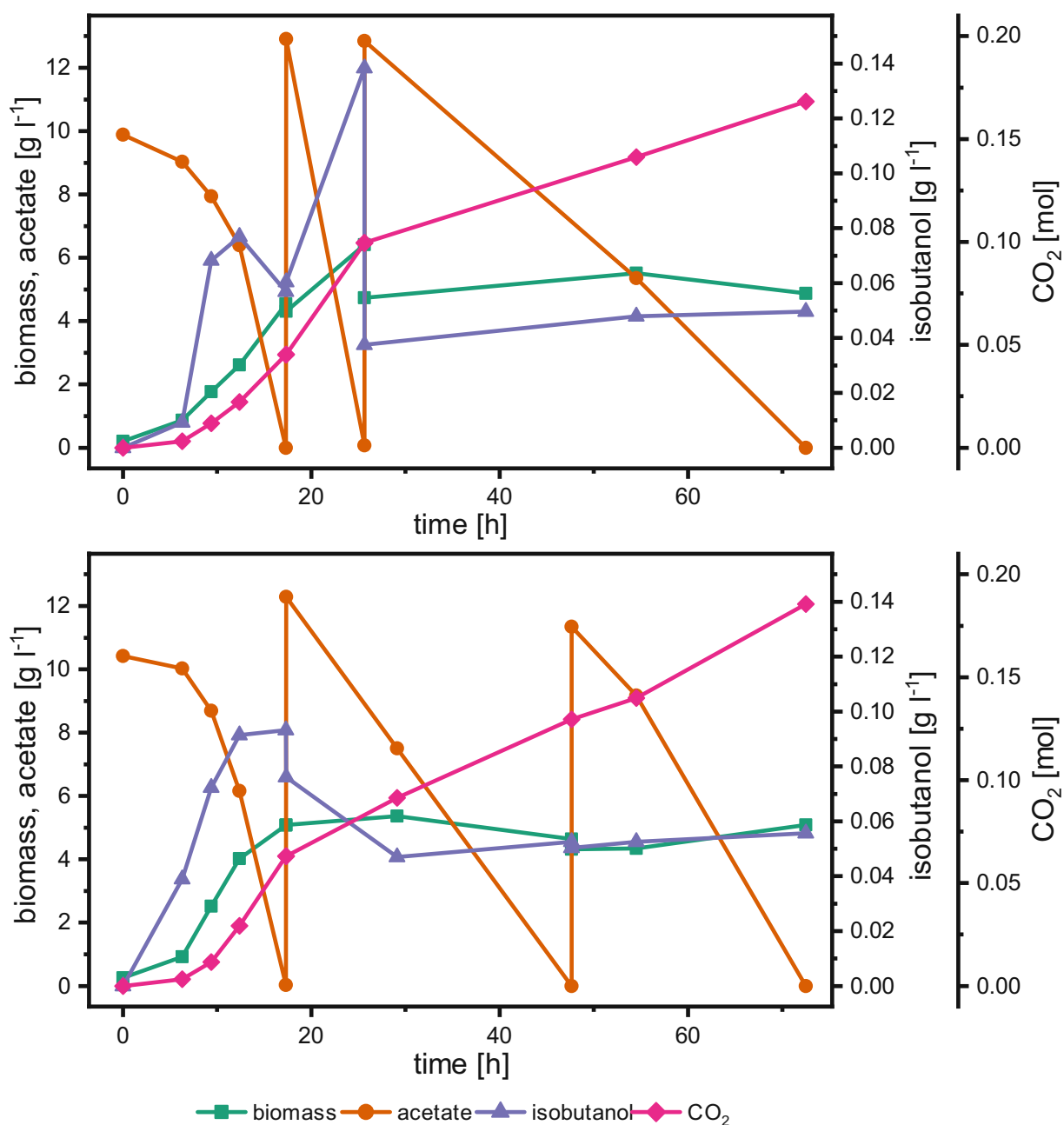
Figure 7 – Pulsed fed-batches for isobutanol production from acetate in complex medium.
E. coli W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* (Δ4) IB4 was grown on complex medium containing 10 g l^{-1} yeast extract and pulsed with a concentrated acetate stock to restore a concentration of 10 g l^{-1}. Individual duplicates are shown due to the variance in acetate uptake.

The fact that isobutanol was not produced during the feeding period, but only in the batch led to the assumption that isobutanol is not produced from acetate, but from the complex media additive. Therefore, we evaluated "background production" from yeast extract and product formation from acetate by comparing experiments with and without the addition of acetate (**Figure 8**).
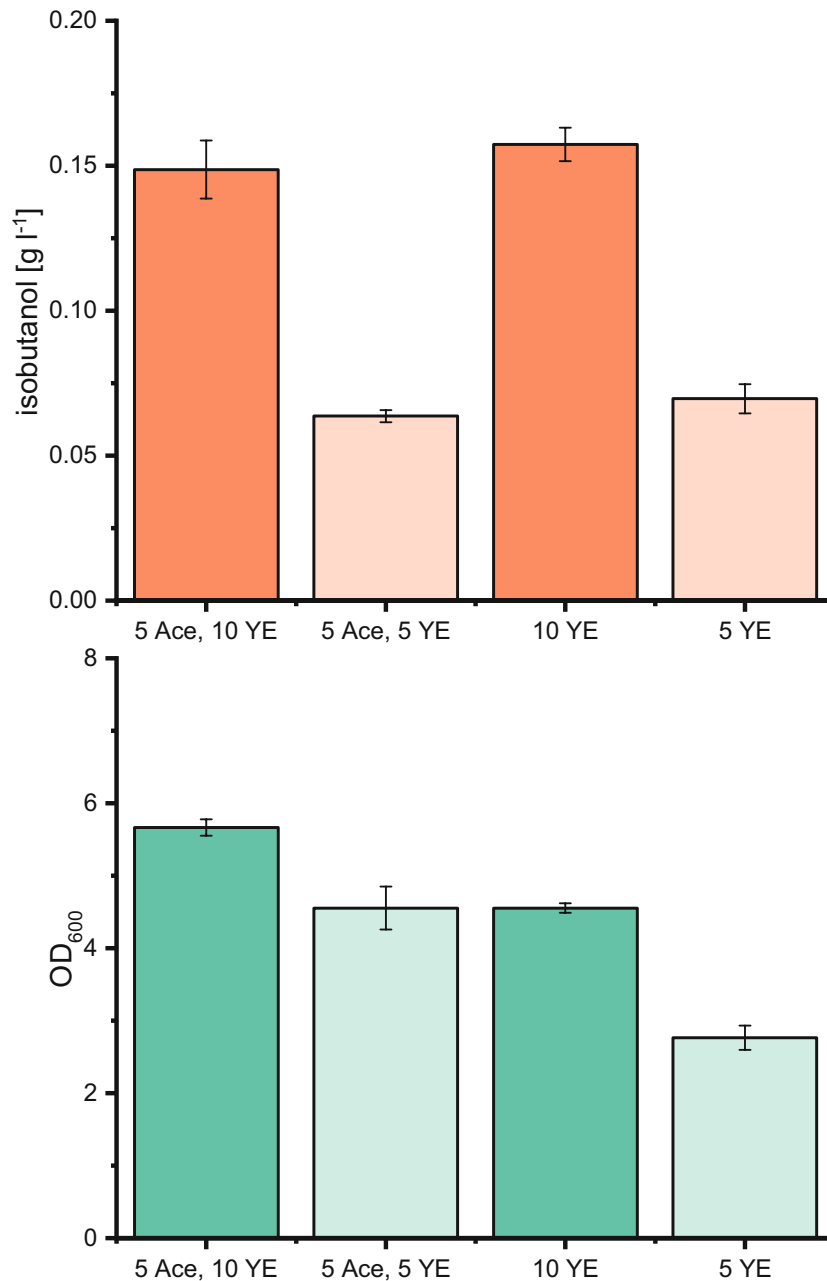
Figure 8 – Growth and isobutanol production on complex and defined medium. *E. coli* W *ΔldhA ΔadhE Δpta ΔfrdA* (Δ4) with construct IB 4 were cultivated in shake-flasks on medium with 5 g l^{-1} acetate (5 Ace) or without acetate and 5 or 10 g l^{-1} yeast extract (5 YE = light bars, 10 YE = dark bars). Mean values and standard deviation were calculated from biological triplicates.

Comparison of isobutanol production in medium with 10 g l^{-1} yeast extract with and without acetate revealed that the same amount of isobutanol was produced in both experiments. Similarly, growth on medium containing 5 g l^{-1} yeast extract with and without acetate resulted in the same isobutanol production, which leads to the conclusion that isobutanol was not produced from acetate, but only from the complex media additive yeast extract. Isobutanol production from yeast extract also explains why isobutanol was only produced in the batch phase during pulsed fed-batch experiments (**Figure 7**). The addition of yeast extract was therefore not suitable to achieve the goal of isobutanol production from acetate.

This conclusion stands in contrast to previous studies, where 50 % of the 80 mg l^{-1} isobutanol produced could be attributed to acetate alone (Song et al., 2018).

Since complex media was not suitable to achieve isobutanol production from acetate, a defined medium should be developed by adding media components that support growth and product formation from acetate. To develop this medium, it was clear that various screening experiments had to be carried out in shake flasks. However, the volatility of isobutanol makes product quantification during shake flask cultivation difficult. During the screening experiments on glucose (3.2), this problem was circumvented using sealed serum bottles (Novak et al., 2020a). Since this form of cultivation does not allow for air exchange during the cultivation, aerobic conditions could not be ensured. However, acetate uptake is only possible under aerobic conditions, which is why growth on acetate was not possible in serum bottles. Product quantification is further hampered by low titers probably caused by the toxicity of isobutanol. In other words, media development for isobutanol production from acetate will be difficult due to toxicity and volatility of the product formed and was not followed further in this work.

As an alternative, 2,3-Butanediol is another promising platform chemical and its production in *E. coli* W has previously been developed in the research group using the same expression system as for isobutanol production (Erian et al., 2018). 2,3-Butanediol like isobutanol is derived from pyruvate but is neither toxic nor volatile, which would allow to reach higher product titers. Although 2,3-butanediol and isobutanol differ in their chemical and physical properties, both can be used as fuel additives (Atsumi et al., 2008; Tran and Chambers, 1987). Therefore, 2,3-butanediol production from acetate can serve as a platform to acquire process knowledge on the production of pyruvate-derived C4 fuel chemicals from acetate. Media and process development for 2,3-butanediol production from acetate is described in Chapter 3.3 and in the future, results could be applied to isobutanol production from acetate.

Conclusion

In this chapter, it has been shown that the knock-out of mixed acid fermentation pathways in *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* (Δ4) allows for isobutanol production on acetate complex medium. On complex medium however, isobutanol is not produced from acetate but from the media additive yeast extract. Therefore, further investigation and the development of a defined medium is needed to achieve product formation from isobutanol. Since process development and extensive screenings are hampered by the volatility and the toxicity of isobutanol, a platform process will be designed for the pyruvate derived chemical 2,3-butanediol.

3.3. Creating platform knowledge for microbial upgrading of acetate: 2,3-butanediol as a case study

Problem statement

Neither the use of the original defined medium nor the use of complex medium was successful to establish isobutanol production from acetate. 2,3-butanediol, which is derived from the same metabolic intermediate, will therefore serve as a case study for microbial upgrading of acetate.

State-of-the-Art

A broad spectrum of chemicals has so far been produced from the promising alternative raw material acetate and among these are also alcohols like ethanol, isopropanol and isobutanol (Lee et al., 2016; Song et al., 2018; Yang et al., 2020). Most studies focused on the production of chemicals derived from acetyl-CoA in combination with the use of complex media additives. However, the impact of these additives has not been quantified, and the amount of product derived from acetate remains unclear. Chemical production from acetate has not been established on defined medium so far.

Scientific questions

Which environmental factors mainly influence 2,3-butanediol production from acetate?

How can the production of 2,3-butanediol from acetate be established?

Hypotheses

Since 2,3-butanediol is derived from the same metabolic intermediate as isobutanol (pyruvate), influencing factors are similar. Therefore, 2,3-butanediol production from acetate also depends on the strain background and the media composition. Since complex media do not promote 2,3-butanediol production from acetate, a defined medium had to be designed. Additionally, top-down media screening in combination with process intensification should improve production.

Approach

As for isobutanol, 2,3-butanediol production was compared in different strains on complex and defined medium. The "background production" from the complex media additive alone was monitored by comparing media with and without acetate. A defined medium for 2,3-butanediol production from acetate was designed and screened for the influence of individual components on growth and production. Different process modes with various feeding strategies were compared regarding their efficiency for 2,3-butanediol production.

Content and contribution

In this publication, the importance of applying material balances to attribute product formation to acetate or the complex media additive is emphasized. Following this strategy,

we found that 2,3-butanediol was not produced from acetate was on complex medium. To this end, a newly defined medium was developed, and the reduction of media components finally revealed that aspartate / asparagine was responsible for enabling production of diols (2,3-butanediol and acetoin) from acetate. Co-utilization of aspartate and acetate was not required. Finally, an efficient production process was developed where aspartate was only used as a kick-start and feeding was performed on acetate as the sole carbon source.

KN carried out the experiments together with RK and KN analyzed the data. KN and SP conceived the study and wrote the manuscript.

Title of manuscript

Microbial upgrading of acetate into 2,3-butanediol and acetoin by *E. coli* W

Citation

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RESEARCH

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Microbial upgrading of acetate into 2,3-butanediol and acetoin by *E. coli* W

Katharina Novak, Regina Kutscha and Stefan Pflügl[*] ©

Abstract

Background: Acetate is an abundant carbon source and its use as an alternative feedstock has great potential for the production of fuel and platform chemicals. Acetoin and 2,3-butanediol represent two of these potential platform chemicals.

Results: The aim of this study was to produce 2,3-butanediol and acetoin from acetate in *Escherichia coli* W. The key strategies to achieve this goal were: strain engineering, in detail the deletion of mixed-acid fermentation pathways *E. coli* W ΔldhA ΔadhE Δpta ΔfrdA 445_Ediss and the development of a new defined medium containing five amino acids and seven vitamins. Stepwise reduction of the media additives further revealed that diol production from acetate is mediated by the availability of aspartate. Other amino acids or TCA cycle intermediates did not enable growth on acetate. Cultivation under controlled conditions in batch and pulsed fed-batch experiments showed that aspartate was consumed before acetate, indicating that co-utilization is not a prerequisite for diol production. The addition of aspartate gave cultures a start-kick and was not required for feeding. Pulsed fed-batches resulted in the production of 1.43 g l^{-1} from aspartate and acetate and 1.16 g l^{-1} diols (2,3-butanediol and acetoin) from acetate alone. The yield reached 0.09 g diols per *g* acetate, which accounts for 26% of the theoretical maximum.

Conclusion: This study for the first time showed acetoin and 2,3-butanediol production from acetate as well as the use of chemically defined medium for product formation from acetate in *E. coli*. Hereby, we provide a solid base for process intensification and the investigation of other potential products.

Keywords: Acetate utilization, Chemically defined medium, Aspartate, Acetate toxicity, *E. coli* W, Alternative raw materials, NADH availability, Amino acid and vitamin supplementation, Yeast extract

Background

Global rising energy demand, uncertainty about crude oil availability and concerns about climate change have recently increased the interest in renewable energy sources and microbial fuel and chemical production [1]. One promising platform chemical is 2,3-butanediol which can be used as food additive, anti-freezing agent [2] or as a precursor for butanone formation [3]. Similarly, acetoin is used as a flavor enhancer [4] or chemical building block [5]. Although 2,3-butanediol can be produced naturally by, e.g., *Klebsiella oxytoca* or *Enterobacter cloacae*, some hosts are pathogenic and require complex and expensive media additives [6]. Therefore, 2,3-butanediol production has recently been developed in *Escherichia coli* [6–8]. Since *E. coli* is not a natural producer, a heterologous pathway consisting of three genes has to be overexpressed: acetolactate synthase, acetolactate decarboxylase and butanediol dehydrogenase (Fig. 1) [9]. So far, 2,3-butanediol has been produced from glucose [7, 8, 10] and alternative sugar sources such as sugar beet molasses [6].

Acetate is a promising alternative raw material, since it can be derived from a variety of cost-effective resources

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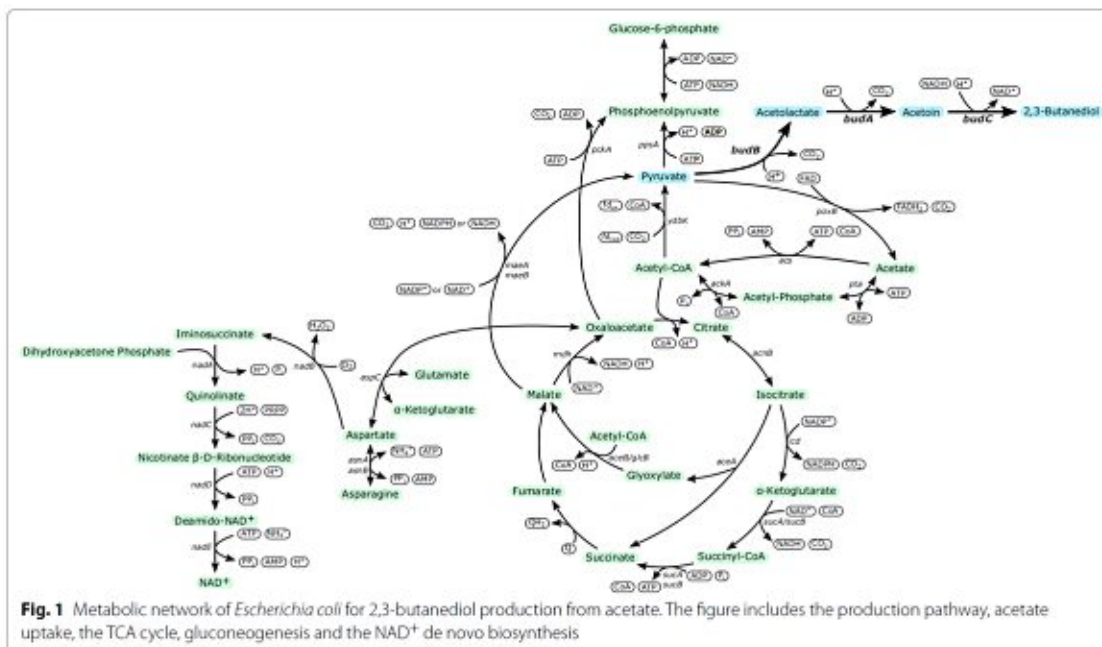
Fig. 1 Metabolic network of *Escherichia coli* for 2,3-butanediol production from acetate. The figure includes the production pathway, acetate uptake, the TCA cycle, gluconeogenesis and the NAD⁺ de novo biosynthesis

such as food waste and lignocellulosic hydrolysates [11]. Moreover, acetate can also be produced in gas fermentations relying on C1 compounds which enables production in a cascaded process with two steps [12]. A broad spectrum of chemicals has already been produced from acetate such as polyhydroxyalkanoates [13], 3-hydroxypropionic acid [14] and succinate [15], and also alcohols like ethanol [16], isopropanol [17] and isobutanol [18]. So far, the vast majority of products that have been produced from acetate are derived from acetyl-CoA. The metabolic intermediate from where a product pathway branches plays an important role in microbial chemical production, as it influences the maximal theoretical yield that can be achieved. Three carbon compounds such as isopropanol, acetone and 3-hydroxypropionic acid, which are derived from acetyl-CoA or C3 products from the TCA cycle, like succinate, display a theoretical yield of 0.5 mol mol^{-1}. In contrast, the theoretical yield of C4 alcohols produced from pyruvate like isobutanol and 2,3-butanediol is reduced to 0.25 mol mol^{-1} at the expense of higher CO_2 emissions.

Acetate uptake in *E. coli* is possible via two routes: the high-affinity, irreversible acetyl-CoA synthetase (*acs*) or the low-affinity, reversible acetate kinase–phosphate acetyl transferase (*ackA–pta*) system [19]. Overexpression of either of these pathways has been shown to increase acetate uptake and product formation [14, 17,

20, 21]. Other strategies that resulted in increased product formation were the deletion of *icdA*, which led to blocking of the TCA cycle and increased acetone synthesis [21] or deletion of *iclR*, which activated the glyoxylate shunt and improved 3-hydroxypropionic acid production in combination with *acs* overexpression [14]. Generally, the glyoxylate shunt is highly active when acetate is used as sole carbon source, whereas the TCA cycle is downregulated [19].

The main route for gluconeogenesis during growth on acetate is the formation of phosphoenolpyruvate (PEP) from oxaloacetate via phosphoenolpyruvate carboxykinase (*pckA*) or alternatively from malate via the malic enzymes and phosphoenolpyruvate synthase (*ppsA*) (Fig. 1) [19, 22]. A combined overexpression of *pckA*, the malic enzymes and *acs* showed improved isobutanol production from acetate [18].

Product formation from acetate has so far only been achieved by the addition of complex media components such as yeast extract. However, there are some major drawbacks and limitations related to the use of complex media additives [23]. It was recently shown that the availability of casamino acids led to differences in cellular metabolism, especially acetate metabolism in glucose-grown cultures [24]. Complex media additives are usually also cost-intensive and media cost was shown to be one of the core expenses in bioreactor cultivations [25, 26].

Media cost was mainly influenced by the feedstock price for peptones [25], which suggests that the omittance of complex media additives is beneficial for industrial applications. Additionally, Yang et al. [17] reported that these components can disguise the true product yield, since isopropanol production reached 112% of the maximal theoretical yield. Concluding, to maintain reproducibility, discover true physiological effects and reduce media costs, research should focus on the use of chemically defined medium for the investigation of acetate utilization.

Peptides and amino acids represent a major part of available substrates in yeast extract. Regarding acetate, different amino acids are known to protect cells against acid stress in the form of low pH. While the first acid resistance (AR) system is repressed in the presence of glucose, the activity of the other three systems is dependent on the availability of arginine (AR2), glutamate (AR3) or lysine (AR4) [27, 28]. Acetate has also been shown to inhibit the biosynthesis of methionine [29]. Apart from the toxicity of acetate, the low availability of NADH during acetate assimilation is not only a challenge in terms of energy conservation, but also for efficient production of reduced metabolites such as alcohols [30]. Aspartate is a precursor of the de novo NADH biosynthesis (Fig. 1) and its addition increased product formation in *Clostridium acetobutylicum* [31]. Carrying an amino group, amino acids can also have a stabilizing effect on the intracellular pH [32, 33]. Conclusively, amino acids play a major role during the assimilation of acetate.

The goal of this study was the production of 2,3-butanediol from acetate. To reach this goal, we investigated the influence of the strain background on product formation and developed a chemically defined medium containing amino acids and vitamins which enabled diol production from acetate. The stepwise reduction of media additives revealed that the addition of aspartate as sole amino acid is sufficient to trigger diol production from acetate. Moreover, using pulsed fed-batches we could prove that aspartate addition was only necessary in the batch phase, while acetate could be used as a sole carbon source for diol production during the feeding period.
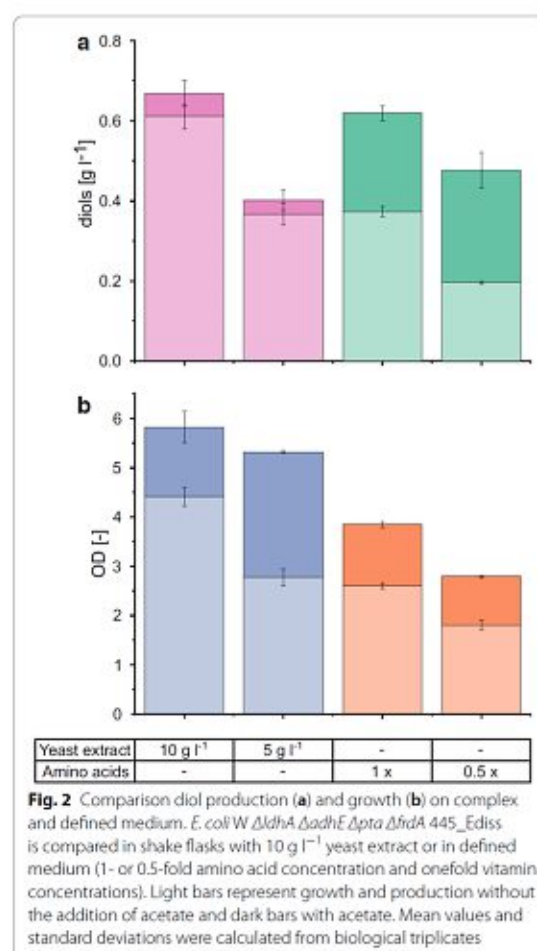
Results

No production from acetate on complex medium containing yeast extract

In a previous study, 2,3-butandediol production from glucose was successfully established on chemically defined medium [6]. The two most promising strains, *Escherichia coli* W 445_Ediss (W) and *E. coli* W ΔldhA ΔadhE Δpta ΔfrdA 445_Ediss (Δ4) were therefore chosen to investigate product formation from acetate. While *E. coli* W was able to utilize acetate in the original defined medium,

E. coli W Δ4 was unable to grow. Both strains, however, grew well on the same medium when yeast extract was added. While *Escherichia coli* W only produced low amounts of 2,3-butanediol, product formation was 3.4-fold higher in *E. coli* W Δ4 (Additional file 1: Table S1). Given that aerobic conditions were used for this experiment, we did not expect that deleting mixed-acid fermentation pathways significantly influences 2,3-butanediol and acetoin formation from acetate. Conclusively, 2,3-butanediol and acetoin can be produced in *E. coli* W Δ4 on complex medium containing yeast extract.

To determine the amount of diols produced from acetate, we omitted acetate in one approach and increased the yeast extract concentration in another approach (Fig. 2). This comparison clearly revealed that

| Yeast extract | 10 g l⁻¹ | 5 g l⁻¹ | - | - |
| Amino acids | - | - | 1 x | 0.5 x |

Fig. 2 Comparison diol production (**a**) and growth (**b**) on complex and defined medium. *E. coli* W ΔldhA ΔadhE Δpta ΔfrdA 445_Ediss is compared in shake flasks with 10 g l⁻¹ yeast extract or in defined medium (1- or 0.5-fold amino acid concentration and onefold vitamin concentrations). Light bars represent growth and production without the addition of acetate and dark bars with acetate. Mean values and standard deviations were calculated from biological triplicates

2,3-butanediol and acetoin are almost exclusively formed from yeast extract, and not from acetate.

Based on these results, we investigated whether product formation from acetate is possible by designing a chemically defined medium.

Development of defined medium enables diol production from acetate

Since the original chemically defined medium did not allow for growth and 2,3-butanediol production on acetate, the medium was expanded by specific components. These media components were based on literature reports on mechanisms behind 2,3-butanediol production as well as acetate toxicity. To this end, we selected several compounds as media additives: (i) glutamate, as intracellular glutamate pools are decreased in the presence of acetate [34] and glutamate is responsible for the acid resistance 2 (AR2) system [28]; (ii) arginine, which mediates the AR3 system; (iii) lysine, which mediates the less efficient AR4 system [27]; (iv) methionine due to the inhibition of the methionine biosynthesis in the presence of acetate [29]; (v) aspartate and nicotinic acid, since they are precursors of the NADH biosynthesis and NADH availability is low during acetate assimilation [31], and (vi) thiamine, which is a cofactor of acetolactate synthase, the first enzyme in the 2,3-butanediol production pathway. Other vitamins were added as reported to be beneficial for the growth of *E. coli* [35]. Finally, a chemically defined medium containing 5 different amino acids and 7 vitamins was designed.

In contrast to the original defined medium, the newly designed medium enabled growth and diol production from acetate. Diol formation was compared in experiments with and without the addition of acetate, which enabled us to quantify the amount of product formed from acetate. Figure 2 shows the production of 0.25 g l^{-1} diols from 5 g l^{-1} acetate. In other words, only the use of the newly designed defined medium allowed for acetoin and 2,3-butanediol production from acetate.

In addition to the design of a defined medium, the deletion of by-product formation pathways in *E. coli* W Δ4 was key to enable diol production from acetate. One of those deletions, the knock-out of *pta* also concerns acetate utilization and might therefore influence product formation from acetate. Generally, *E. coli* takes up acetate via two routes: the high-affinity, irreversible acetyl-CoA synthetase (*acs*) or the low-affinity, reversible acetate kinase–phosphate acetyl transferase (*ackA-pta*) system [19]. Therefore, we investigated whether the absence of one of the uptake systems, *pta*, can influence growth and product formation during acetate uptake. To this end, diol formation of *E. coli* W Δ4 was compared to *E. coli* W Δ*ldhA* Δ*adhE* (Δ2) in Table 1.

Table 1 Comparison of diol production (2,3-butanediol and acetoin) in strains with and without deletion of *pta*

Strain Compared experiments	*E. coli* W Δ4 1 AA ± 5 Ace	*E. coli* W Δ2 1 AA ± 5 Ace
Total diols [g l^{-1}]	0.62 ± 0.03	0.65 ± 0.01
Diols from medium [g l^{-1}]	0.37 ± 0.01	0.41 ± 0.01
Diols from acetate [g l^{-1}]	0.25 ± 0.04	0.23 ± 0.02
Y [g diols g^{-1} acetate]	0.053 ± 0.009	0.045 ± 0.005

E. coli W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* 445_Ediss (Δ4) is compared to *E. coli* W Δ*ldhA* Δ*adhE* 445_Ediss (Δ2) in shake flasks with defined medium and the onefold amino acid concentration and the onefold vitamin concentrations. Means and standard deviations were calculated from biological triplicates
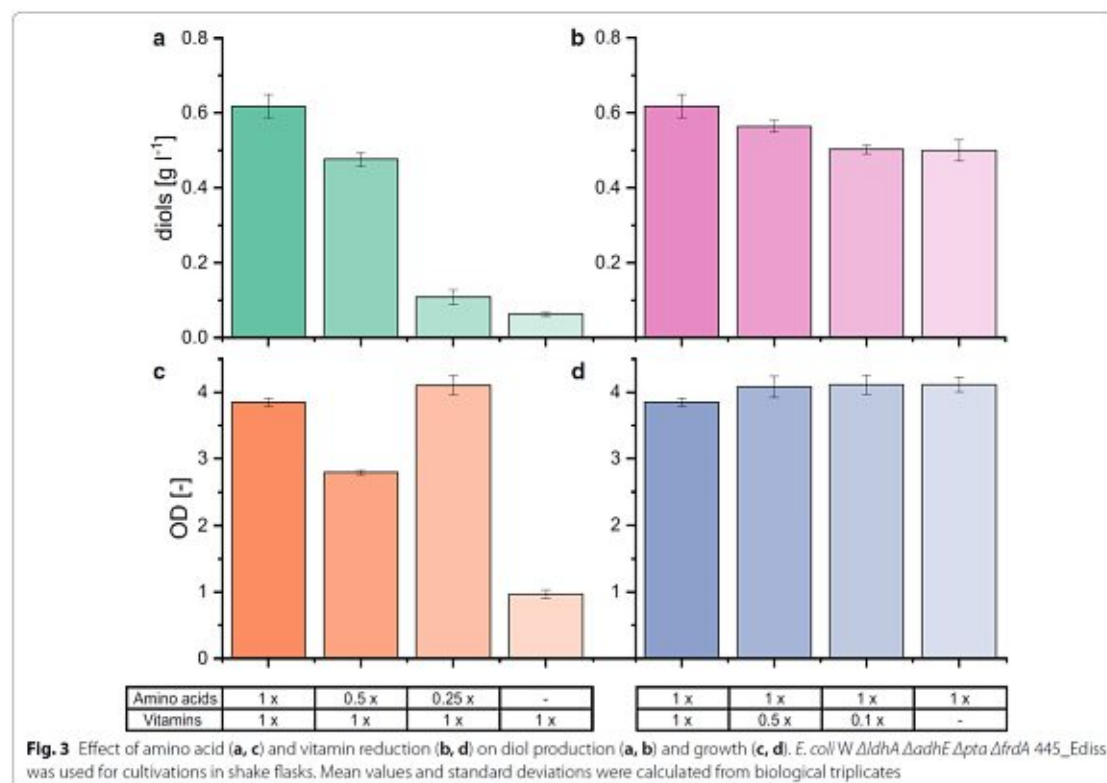
Product formation in *E. coli* W Δ4 is comparable to *E. coli* W Δ2, which indicates that the deletion of *pta* does not influence the production of 2,3-butanediol. Therefore, all further experiments were carried out with *E. coli* W Δ4.

Can the media additives be reduced?

To better understand the mechanisms behind acetate uptake for diol production, our goal was to investigate which of the components in the designed medium are responsible for the observed effect and thereby essential. Therefore, we reduced or omitted the additives in a stepwise manner (Fig. 3). The reduction of the amino acid mix by 50% did not show an effect on growth and diol production, but reduction to 25% of the original composition resulted in decelerated growth and loss of diol production. Growth was not possible on medium without the addition of amino acids (Fig. 3a). In contrast, the reduction of the vitamin concentration only slightly decreased the final product titers and completely omitting vitamins still enabled the production at 83% of the diol concentration with the onefold vitamin concentration.

Asparagine and aspartate trigger diol production from acetate

To further investigate the influence of single components of the designed medium on diol formation from acetate, we systematically removed several media additives from the chemically defined medium. Therefore, the five amino acids were grouped: asparagine and glutamate as the main amino acids present in yeast extract were compared to the mixture of arginine, lysine, and methionine (Fig. 4). While the overall concentration of amino acids was similar in both approaches, only the asparagine–glutamine group resulted in product formation comparable to the defined medium containing all amino acids. By adding asparagine or glutamate at the onefold concentration as sole amino acids, we could show that the presence of asparagine and glutamate as sole amino acid resulted

Fig. 3 Effect of amino acid (**a**, **c**) and vitamin reduction (**b**, **d**) on diol production (**a**, **b**) and growth (**c**, **d**). *E. coli* W ΔldhA ΔadhE Δpta ΔfrdA 445_Ediss was used for cultivations in shake flasks. Mean values and standard deviations were calculated from biological triplicates

in diol concentrations of 0.44 g l^{-1} and 0.17 g l^{-1}, respectively. Supplementing arginine, lysine, and methionine individually at higher concentrations (corresponding to the mass of asparagine), enabled growth on acetate but did not trigger diol production (Additional file 1: Table S2).

Diol formation from acetate was quantified similarly to previous experiments by comparing media with and without acetate. Additionally, we tested whether diol formation from acetate can also be achieved by the addition of aspartate instead of asparagine. To quantitatively verify the amount of diol produced from acetate, different amounts of amino acids were used in experiments with a constant acetate concentration (Table 2).

When asparagine or aspartate are used instead of the amino acid mixture, product formation from acetate was equal, whereas the overall product formation from the medium is decreased. Equal yields in all approaches indicate that all other amino acids except asparagine simply increased the media background, but did not significantly improve product formation from acetate. The use of aspartate instead of asparagine led to comparable diol yields and is therefore equally suitable as a media additive. Reduction of the aspartate concentration led to decelerated acetate uptake and growth. A decrease to 50% of the initial concentration drastically reduced growth and 2,3-butanediol production (Table 3).

Elucidating the mechanism behind the aspartate/asparagine effect

To further investigate how the addition of asparagine or aspartate enables product formation from acetate, we hypothesized about possible physiological mechanisms behind this effect. Since the addition of other amino acids did not result in product formation from acetate, asparagine or aspartate might act as a precursor for other important metabolic processes. It is possible that aspartate boosts gluconeogenesis by conversion to oxaloacetate and phosphoenolpyruvate (Fig. 1). Alternatively, asparagine or aspartate could support the flux through the citrate or glyoxylate cycle. According to these hypotheses, other TCA cycle intermediates should also promote growth and diol production from acetate. Therefore, aspartate in the medium was replaced by oxaloacetate, succinate, and malate (Table 4).
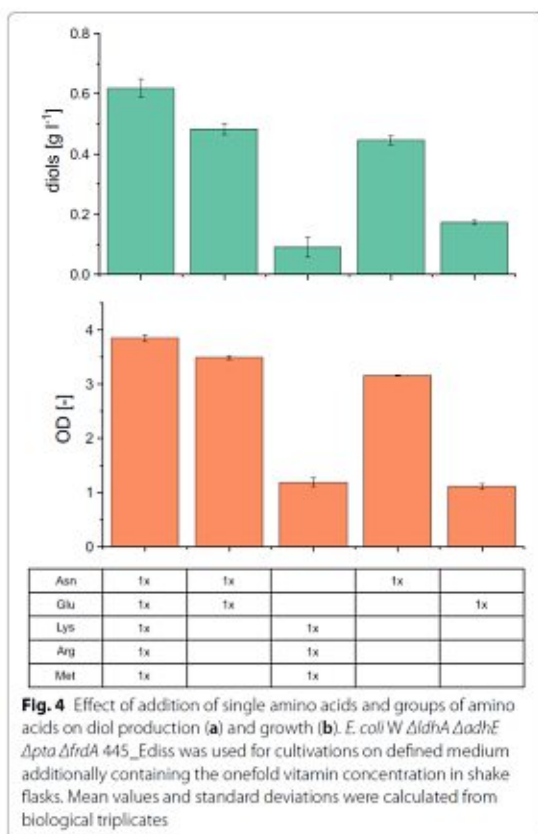
Fig. 4 Effect of addition of single amino acids and groups of amino acids on diol production (**a**) and growth (**b**). *E. coli* W ΔldhA ΔadhE Δpta ΔfrdA 445_Ediss was used for cultivations on defined medium additionally containing the onefold vitamin concentration in shake flasks. Mean values and standard deviations were calculated from biological triplicates

None of these alternative media supplements supported growth and diol production from acetate. It is likely that the mechanism behind aspartate triggering diol production is different. Therefore, we speculated that aspartate mediates a form of acid resistance. This hypothesis is supported by the observation that a decrease of the initial acetate concentration from 5 g l^{-1} to 2 g l^{-1} enabled growth and production without the addition of amino acids or vitamins. However, cultivations at low substrate concentrations are hardly feasible due to the low product titer which additionally complicates quantification.

Co-utilization or start-kick?

After narrowing down the possible mechanisms mediated by aspartate, it was important to find out how aspartate uptake influences acetate uptake and diol production in a time-resolved manner. To this end, the kinetics of aspartate and acetate uptake as well as product formation were studied in bioreactor cultivations, which also reduced distortions due to pH increase caused by acetate uptake from the medium in shake flasks.

Generally, acetate-grown cultures displayed a high degree of variation between individual cultivations. These variations could be reduced but not completely prevented by: (i) the adaptation of cells to defined medium in the preculture and (ii) the transfer of the preculture to the reactor in the exponential phase. These measures resulted in reproducible results in terms of product concentrations and yields.

The batch cultivations as well as all further cultivations were carried out on defined medium containing 5 g l^{-1} acetate, 0.88 g l^{-1} (8 mol%) aspartate and the

Table 2 Comparison of diol production (2,3-butanediol and acetoin) in *E. coli* W Δ4 on different defined media

Experiments	1 AA ± 5 Ace	0.5 AA ± 5 Ace	2 Asn ± 5 Ace	1 Asn ± 5 Ace	1 Asp ± 5 Ace
Total diols [g l^{-1}]	0.62 ± 0.03	0.48 ± 0.02	0.42 ± 0.02	0.35 ± 0.01	0.35 ± 0.01
Diols from medium [g l^{-1}]	0.37 ± 0.01	0.20 ± 0.01	0.19 ± 0.01	0.08 ± 0.01	0.10 ± 0.01
Diols from acetate [g l^{-1}]	0.25 ± 0.04	0.28 ± 0.02	0.24 ± 0.02	0.27 ± 0.01	0.26 ± 0.02
Y [g diols g^{-1} acetate]	0.053 ± 0.009	0.062 ± 0.005	0.049 ± 0.005	0.052 ± 0.002	0.052 ± 0.004

E. coli W ΔldhA ΔadhE Δpta ΔfrdA 445_Ediss was used for cultivation. Media containing the five amino acid mixture (AA) is compared to medium containing 0.88 g l^{-1} (onefold) or 1.76 g l^{-1} (twofold) asparagine (Asn) or aspartate (Asp) and the onefold vitamin concentration. The diol concentration from acetate is calculated by the subtraction of the concentration attributed to the amino acid(s). Mean values and standard deviations were calculated from biological triplicates

Table 3 Diol production in medium containing different amounts of aspartate in *E. coli* W Δ4

Aspartate reduction	100% (8 mol%)	50% (4 mol%)	25% (2 mol%)	10% (0.8 mol%)
OD [—]	2.92 ± 0.08	0.84 ± 0.03	0.66 ± 0.02	0.62 ± 0.01
Total diols [g l^{-1}]	0.35 ± 0.01	0.12 ± 0.01	0.06 ± 0.01	0.03 ± 0.01

E. coli W ΔldhA ΔadhE Δpta ΔfrdA 445_Ediss was used for cultivation. Media contained 5 g l^{-1} acetate and the indicated percentage of 0.88 g l^{-1} aspartate. The ratio of aspartate to acetate is given in % of moles. Cultures were inoculated at OD$_{600}$ = 0.5. Mean values and standard deviations were calculated from biological triplicates
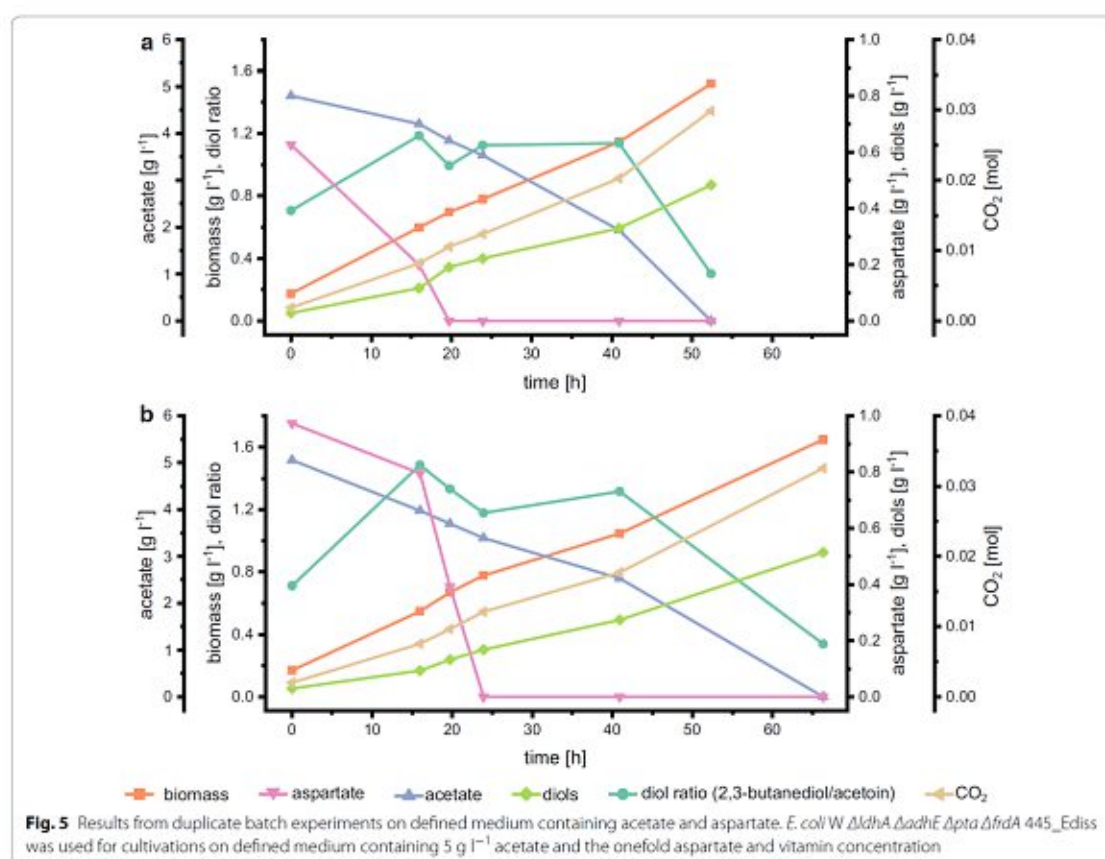
Table 4 Comparison of diol production (2,3-butanediol and acetoin) in *E. coli* W Δ4 from different TCA intermediates

Compared experiments	1 Mal ± 5 Ace	1 Suc ± 5 Ace	1 OAA ± 5 Ace	1 Asn ± 5 Ace
OD [−]	0.95 ± 0.04	0.99 ± 0.06	0.53 ± 0.03	3.16 ± 0.01
Total diols [g l^{-1}]	0.12 ± 0.01	0.14 ± 0.01	0.02 ± 0.01	0.35 ± 0.01
Diols from medium [g l^{-1}]	0.05 ± 0.01	0.07 ± 0.01	0.03 ± 0.01	0.08 ± 0.01
Diols from acetate [g l^{-1}]	0.06 ± 0.01	0.07 ± 0.02	−0.01 ± 0.01	0.27 ± 0.01

E. coli W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* 445_Ediss was used for cultivation. Media containing 0.88 g l^{-1} malate (Mal), succinate (Suc), oxaloacetate (OAA) and asparagine (Asn) with the onefold vitamin concentration were compared. The diol concentration from acetate is calculated by the difference of experiments with and without acetate or by the subtraction of the concentration attributed to the amino acid(s). Mean values and standard deviations were calculated from biological triplicates

onefold concentration of vitamins. Although experiments in shake flasks did not require vitamin addition for 2,3-butanediol and acetoin formation, we observed that omitting vitamins in bioreactor cultivations led to a 50% decreased product yield and only acetoin rather than 2,3-butanediol production (data not shown). Therefore, vitamins were added at the onefold concentration in all further experiments.

Figure 5 shows that growth and diol production from acetate and aspartate is possible in batch experiments. Growth occurred in two phases: in the first one, acetate was partially, and aspartate completely consumed while in the second phase the remaining acetate was utilized. This diauxic growth pattern indicates that aspartate is only needed to give a start-kick for growth and that co-utilization is not required for product formation from

Fig. 5 Results from duplicate batch experiments on defined medium containing acetate and aspartate. *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* 445_Ediss was used for cultivations on defined medium containing 5 g l^{-1} acetate and the onefold aspartate and vitamin concentration

acetate. Although we maintained reproducibility by adapting the preculture, cultures still varied in substrate uptake as well as production rates.

Product yields were higher than in shake-flask experiments and reached 26% of the theoretical maximum in the acetate phase (Table 5).

Table 5 Process performance parameters of *E. coli* W Δ4 in batches with aspartate and acetate

Parameter	Aspartate phase	Acetate phase	Total
$Y_{\text{diols/Ace}}$ [Cmol Cmol^{-1}]	0.18±0.09	0.13±0.03	0.14±0.01
$Y_{\text{diols/Ace}}$ [g g^{-1}]	0.14±0.07	0.09±0.02	0.10±0.01
$Y_{\text{diols/S}}$ [Cmol Cmol^{-1}]	0.11±0.05	0.13±0.03	0.12±0.01
$Y_{X/S}$ [Cmol Cmol^{-1}]	0.36±0.09	0.29±0.05	0.31±0.01
$Y_{\text{CO2/S}}$ [Cmol Cmol^{-1}]	0.71±0.18	0.84±0.11	0.78±0.01
Carbon balance [%]	105±9	112±10	117±1

E. coli W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* 445_Ediss was used for cultivation. Yields are either calculated per acetate consumed ($Y_{\text{diols/Ace}}$) or per acetate and aspartate consumed ($Y_{\text{diols/S}}$, $Y_{X/S}$, $Y_{\text{CO2/S}}$, X=biomass, Ace=acetate, S=substrate (acetate + aspartate)). The medium contained the onefold aspartate (0.88 g l^{-1}) and vitamin concentration. Mean values and standard deviations were calculated from biological duplicates. The theoretical yield is 0.5 Cmol diols per Cmol acetate

Efficient diol production in pulsed fed-batches

Finally, we aimed to gain deeper insight into the mechanisms of the two-substrate system and to increase product titers and production rates. Therefore, we tested whether the addition of aspartate was necessary only during the batch or also in the feeding period of a pulsed fed-batch. To this end, pulses with a mixture of aspartate and acetate were compared to pulses where acetate was used as sole carbon source. To obtain comparability, all experiments were pulsed until they had consumed the same amount of acetate. It seems that aspartate was depleted before acetate during every pulse (Fig. 6). In all cultivations, a mixture of 2,3-butanediol and acetoin was produced, and the product spectrum shifted towards acetoin in later cultivation phases. Production of acetoin rather than 2,3-butanediol is probably caused by insufficient NADH supply during acetate utilization.

Pulsing aspartate in addition to acetate led to a 20% increase in the final titer (Table 6). Overall product yields ($Y_{\text{diols/S}}$) were identical in approaches with and without aspartate. Since the absolute amount of acetate pulsed is equal in both experiments and they just differ in aspartate, which was added in one approach, it seems like the increased product titers are mainly caused by the additional carbon source in the form of aspartate. Figure 6 shows that aspartate is only needed for a start-kick in the first batch and that pulses with acetate as the sole
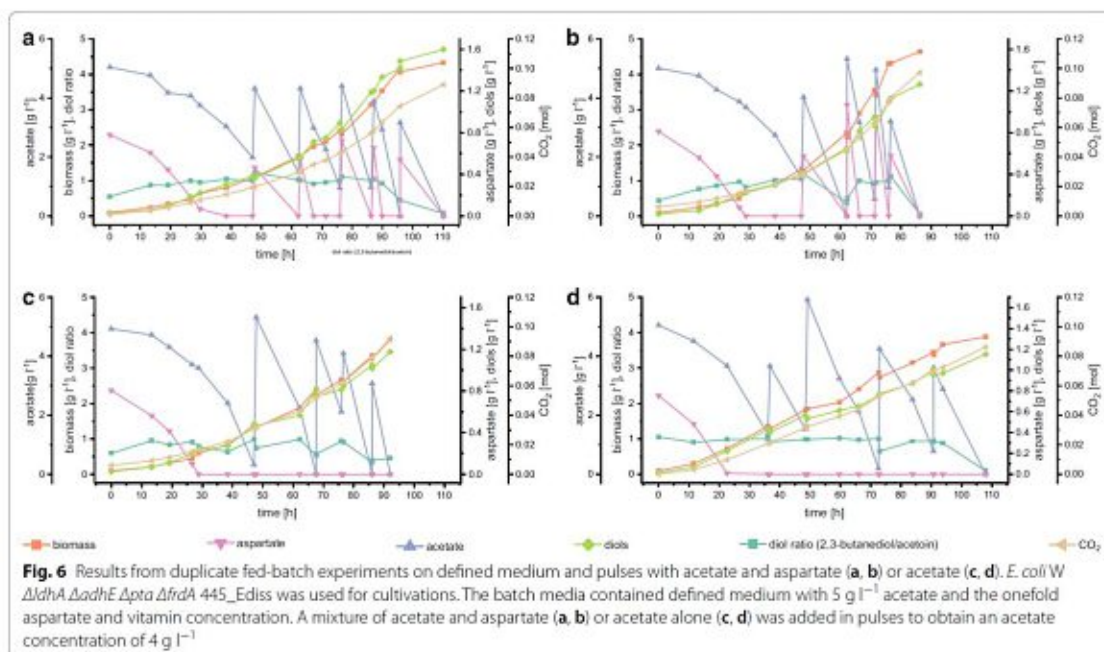

Fig. 6 Results from duplicate fed-batch experiments on defined medium and pulses with acetate and aspartate (**a**, **b**) or acetate (**c**, **d**). *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* 445_Ediss was used for cultivations. The batch media contained defined medium with 5 g l^{-1} acetate and the onefold aspartate and vitamin concentration. A mixture of acetate and aspartate (**a**, **b**) or acetate alone (**c**, **d**) was added in pulses to obtain an acetate concentration of 4 g l^{-1}

Table 6 Process performance parameters of *E. coli* W Δ4 in pulsed fed-batches with aspartate and acetate or solely acetate

	Acetate + aspartate pulses	Acetate pulses
Titer, yields and carbon balance		
Diols [g l^{-1}]	1.43 ± 0.24	1.16 ± 0.02
$Y_{diols/Ace}$ [g g^{-1}]	0.075 ± 0.06	0.067 ± 0.02
$Y_{diols/Ace}$ [Cmol Cmol^{-1}]	0.103 ± 0.009	0.091 ± 0.003
$Y_{diols/S}$ [Cmol Cmol^{-1}]	0.087 ± 0.008	0.087 ± 0.003
$Y_{X/S}$ [Cmol Cmol^{-1}]	0.24 ± 0.01	0.24 ± 0.01
$Y_{CO2/S}$ [Cmol Cmol^{-1}]	0.64 ± 0.02	0.69 ± 0.01
Carbon balance [%]	110 ± 1	117 ± 1
Volumetric and specific rates		
r_{Ace} [g l^{-1} h^{-1}]	0.21 ± 0.03	0.20 ± 0.03
r_{diols} [g l^{-1} h^{-1}]	0.015 ± 0.001	0.013 ± 0.001
q_{Ace} [g g^{-1} h^{-1}]	0.25 ± 0.01	0.24 ± 0.04
q_{diols} [g g^{-1} h^{-1}]	0.018 ± 0.003	0.016 ± 0.002

E. coli W ΔldhA ΔadhE Δpta ΔfrdA 445_Ediss was used for cultivation. The sum of 2,3-butanediol and acetoin is indicated as diols. Yields are either calculated per acetate consumed ($Y_{diols/Ace}$) or per acetate and aspartate consumed ($Y_{diols/S}$, $Y_{X/S}$, $Y_{CO2/S}$, X = biomass, Ace = acetate, S = substrate (acetate + aspartate)). Rates (volumetric rate r and specific rate q (per biomass)) calculated for the time course of the whole process. The media contained the onefold aspartate and vitamin concentration. Mean values and standard deviations were calculated from biological duplicates. The theoretical yield is 0.5 Cmol diols per Cmol acetate

carbon source are successfully used for diol production. Moreover, acetate uptake and diol production rates do not differ between experiments with only acetate or the two-substrate system, which indicates that the addition of aspartate does not improve acetate uptake and diol production. Similarly, the addition of aspartate does not increase biomass yields or specific uptake and production rates.

Conclusively, aspartate addition is only necessary for a start-kick in the first batch phase and acetate can be used as the sole carbon source to produce 2,3-butanediol and acetoin during the feeding period without negatively affecting productivity or product yields.

Low diol production in continuous culture

Continuous bioprocessing is a very promising tool to investigate physiological mechanisms and we sought to investigate diol production from acetate in substrate-limited cultures at steady-state conditions. Continuous cultivation is also an opportunity to increase productivity. To this end, the influence of co-feeding acetate and aspartate on product formation was evaluated in chemostat experiments.

Although both carbon sources were completely consumed, diol production decreased to

0.003 ± 0.002 g l^{-1} h^{-1}. Co-utilization of acetate and aspartate resulted in the production of 0.009 ± 0.007 g diols per g substrate. This corresponds to only 15% and 11%, respectively, of what was reached in the pulsed fed-batches.

Consequently, continuous cultures under the conditions chosen are not suitable for production of 2,3-butanediol from acetate.

Discussion

In this study, we showed that a knock-out strain lacking mixed-acid fermentation pathways can produce acetoin and 2,3-butanediol from acetate. Since a complex medium containing yeast extract did not allow for production from acetate, we designed a new chemically defined medium which enabled acetoin and 2,3-butanediol formation from acetate. By further reduction of this medium, we could reduce the additives to aspartate, which was only needed to give cultures a start-kick.

Microbial chemical production from acetate is an emerging field and commonly relies on the use of complex media additives [17, 18, 20, 21]. Therefore, it was surprising that the addition of yeast extract did not allow 2,3-butanediol and acetoin formation from acetate, but only from yeast extract. In another study investigating isobutanol production from acetate product formation in complex acetate medium was also compared to a control without acetate, and it was found that yeast extract accounted for about 50% of the total product formation with 40 mg l^{-1} isobutanol being produced from acetate alone [18]. Our results underline the importance of examining product formation from the "background medium" without the designated carbon source. Evaluating the "background production" is therefore especially important when low titers are to be expected, which would be the case for substrates displaying toxicity or a low-energy content such as acetate. Generally, the use of complex media comes with drawbacks and limitations, which have recently been intensively investigated and described [23]. For instance, the addition of amino acids resulted in differences of cellular metabolism, especially of acetate metabolism [24]. Although no data are available for acetate-grown cultures, changes in acetate metabolism are a possible explanation for the deviations between complex and defined medium observed in this study.

The fact that the deletion of mixed-acid fermentation pathways is relevant for product formation on acetate as the sole carbon source has not been reported before. A major focus in research on acetate utilization was laid on genetic engineering of the acetate assimilation system. It was previously shown that the overexpression of

acs could reduce the lag phase during cultivations using acetate as sole carbon source and this indicates that acetate uptake can be improved at the expense of enhanced energy demand [36]. The great importance of *acs* for microbial production from acetate is also suggested by the comparison of two studies: while the overexpression of *pta–ackA* only increased acetone and isopropanol yields by 8 and 13%, respectively [17, 21], the production of 3-hydroxypropionic was improved by 75% by overexpressing *acs* [14]. With acetate as the sole carbon source, acetyl-CoA synthetase (*acs*) is the main route for acetate uptake, while *pta–ackA* is down-regulated [37]. This expression pattern probably accounts for enhanced effects by *acs* rather than *pta–ackA* overexpression. Similarly, the low expression of *pta* during acetate assimilation might be the reason that we did not find any differences in product formation between *E. coli* W Δ4 with *pta* knocked-out and *E. coli* W Δ2. We hypothesized that low activity of the reversible *pta–ackA* pathway can positively influence growth and product formation, since it prevents acetic acid cycling in the *acs–pta–ackA* node and avoids wasting of energy [38]. However, the activity of *pta–ackA* is important for providing acetyl-phosphate, which is essential for proper growth of *E. coli* [38]. In detail, acetyl-phosphate plays a role in degradation of misfolded proteins [39], phosphate and nitrogen assimilation [40, 41], survival during starvation [42] and gene expression [43]. It seems like acetyl-phosphate formation from acetate via *ackA* is sufficient to maintain all cellular functions, since growth and production did not differ between *E. coli* W Δ4 (*pta* deleted) and *E. coli* W Δ2. The activity of *acs* was also reported to depend on post-translational acetylation [44, 45] and its extent might vary within different media and process conditions. While the deletion of *pta* did not affect diol production, it seems that deletions of the by-product formation pathways for lactate and ethanol (*ldhA* and *adhE*) are important for product formation, since *E. coli* W Δ2 and Δ4 produced equally well, whereas *E. coli* W failed to produce diols from acetate. Comparing this study to recent literature [14, 15, 17, 18] shows that the major difference is the metabolic intermediate, from which the product is derived. While the precursor for other products such as acetone, isopropanol, 3-hydroxypropionic acid and phloroglucinol is acetyl-CoA [14, 17, 20, 21], 2,3-butanediol is derived from pyruvate. Therefore, to produce 2,3-butanediol from acetate, acetate must first be converted into pyruvate. To provide pyruvate, acetate is converted into acetyl-CoA, which is transformed to oxaloacetate or malate via the TCA cycle and from there PEP or pyruvate is formed. All factors that influence this conversion and thereby increase the intracellular pyruvate pool might be key for product formation. In this context, pyruvate

availability is not only important for anabolic reactions, when cells are grown on acetate, but also for 2,3-butanediol formation.

The main routes for gluconeogenesis (*pckA* and malic enzymes + *ppsA*) branch from oxaloacetate or malate and are highly active during growth on acetate [37]. Because aspartate is a precursor for oxaloacetate, the addition of aspartate could enable growth and product formation by supporting gluconeogenesis via *pckA*. This hypothesis is in accordance with the observation that overexpression of *pckA* improved phloroglucinol synthesis [20]. In *C. glutamicum*, aspartate addition inhibited PEP carboxylase, which catalyzes the reverse reaction of *pckA* [46]. Combined overexpression of *acs*, *pckA* and the malic enzymes increased isobutanol production from acetate [47]. However, since the addition of oxaloacetate did not enable growth and product formation from acetate in our study, an influence of aspartate on gluconeogenesis via *pckA* is unlikely. Similarly, an influence of aspartate on the citric acid or glyoxylate cycle is questionable, since the addition of neither succinate nor malate triggered growth or product formation from acetate. An activation of the glyoxylate shunt was previously shown to improve acetate assimilation and biomass formation, but not 3-hydroxypropionic acid formation [14].

Alternatively, aspartate has been described to trigger acid resistance in different microorganisms and based on a variety of mechanisms. In *Yersinia pseudotuberculosis* aspartate increased acid resistance through expression of aspartase, which released NH_4^+ for the stabilization of the intracellular pH [33]. Aspartate also increased the NADH de novo biosynthesis and has been shown to increase product formation in *Clostridium acetobutylicum* [31]. Besides these two mechanisms, aspartate increased acetate formation in *Acetobacter pasteurianus* by reducing acid stress due to (i) enhanced glutathione production, which protects cells against low pH and stress; (ii) increased nucleic acid synthesis and DNA repair and (iii) improved membrane integrity due to increased fatty acid synthesis [32]. It is possible that one or several of the described mechanisms account for the effect that aspartate triggered diol production from acetate in this study. The fact that aspartate is a precursor for NADH synthesis is especially interesting in this context, because NADH availability is important for the conversion of acetoin to 2,3-butanediol. However, increased NAD^+ biosynthesis mediated by aspartate availability does not necessarily mean that it is present as NADH. All experiments showed a mixture of 2,3-butanediol and acetoin in acetate-grown cultures. When vitamins were omitted, total diol production decreased, and the reaction

shifted towards acetoin. The addition of vitamins to batch cultures might be important for diol formation, since thiamine is a cofactor for acetolactate synthase or since nicotinic acid is a precursor for NAD^+ biosynthesis. Conclusively, these observations indicate insufficient NADH supply and possibly a critical redox status of the cell, which was at least partially circumvented by the addition of aspartate.

Acid resistance systems that are dependent on the availability of an amino acid have been reported to rely on glutamate, arginine and lysine, but not on aspartate [27, 28]. Therefore, it is surprising that the addition of these components did not enable growth on acetate. Similarly, the addition of methionine did not support growth, although its biosynthesis is known to be inhibited during growth on acetate [29].

Aspartate being consumed before acetate might suggest that aspartate simply gives cultures a start-kick by boosting biomass formation to a certain level. However, utilization of other substrates (succinate, glutamate, etc.) also increased initial biomass formation without leading to acetate utilization and/or product formation.

Pulsed fed-batches were successfully applied for efficient diol production. The production of 1.43 g l^{-1} diols is the highest titer obtained for any pyruvate-derived metabolite from acetate. Yang et al. [17] produced 1.5 g l^{-1} isopropanol from acetate via acetyl-CoA at a yield above the theoretical maximum (due to the addition of complex media additives). When products are derived from the TCA cycle, higher titers were achieved, i.e., 3.6 g l^{-1} itaconic acid and 7.5 g l^{-1} succinate were produced from acetate in *E. coli* [48, 49].

Low productivities in continuous cultures could suggest that co-utilization of aspartate and acetate might negatively affect diol production or that substrate limitation decreases product formation as reported before for isobutanol production from glucose [50]. However, how 2,3-butanediol production from acetate can be improved in continuous cultures requires further investigations.

The importance of this study lies in the use of chemically defined medium for microbial chemical production from acetate. Since the addition of aspartate is only needed for a start-kick during the first batch, the designed process is especially promising for further research focusing on industrial applications.

To further optimize the system, acetate uptake and production should be improved in combination with an enhancement of the product yield, e.g., by optimizing pyruvate availability. Better understanding of the mechanisms behind the importance of aspartate addition can provide targets for genetic engineering and to completely avoid media additives in the future. Those mechanisms could, e.g., be elucidated in transcriptome

and metabolome studies. Overexpression of the NADH biosynthesis pathway could potentially bypass the addition of aspartate and improve product formation on acetate. Future research might also shed light on the effect of the deletions in *E. coli* W Δ2 and Δ4 on product formation and acetate toxicity and provide information for the development of a continuous production process.

Conclusion

In this study, we showed for the first time (i) 2,3-butanediol and acetoin production from acetate; (ii) product formation from acetate in *E. coli* on chemically defined medium and (iii) triggering of diol production by addition of aspartate. Yeast extract was demonstrated to be an unsuitable media additive for 2,3-butanediol and acetoin production from acetate. Therefore, a chemically defined medium was designed and reducing the media additives for the first time showed that the addition of aspartate alone enabled product formation from acetate. Furthermore, aspartate is only needed to give the culture a start-kick and acetate can subsequently be used as the sole carbon source for product formation.

Concluding, we could create a picture of conditions influencing production performance of a microbial system utilizing acetate. For instance, we observed that reproducibility could be improved by adapting precultures in defined medium and inoculating during the exponential phase. Therefore, the combined outcome of this study provides a sound basis for further optimization and investigation of the production of 2,3-butanediol and other platform chemicals from acetate.

Methods
Bacterial strains and media

E. coli W (DSM 1116 = ATCC 9637 = NCIMB 8666) from DSMZ (Braunschweig, Germany), *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* and *E. coli* W Δ*ldhA* Δ*adhE* (kind gifts of Prof. Michael Sauer, BOKU, Vienna, Austria) were used for cultivations.

Lysogeny broth (LB) containing 10 g l^{-1} soy peptone, 5 g l^{-1} yeast extract and 10 g l^{-1} sodium chloride was used for all precultures in the shake-flask experiments. 15 g l^{-1} agar was added to LB medium for cultivation on plates.

All chemicals were purchased from Roth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) if not stated otherwise. The basic medium for all experiments is chemically defined medium adapted from Riesenberg et al. [51], containing 13.3 g l^{-1} KH_2PO_4, 4.0 g l^{-1} $(NH_4)_2HPO_4$, 1.7 g l^{-1} citric acid (autoclaved) 1.2 g l^{-1} $MgSO_4 \cdot 7 H_2O$, 0.10 g l^{-1} Fe(III)citrate, 0.0084 g l^{-1} EDTA, 0.013 g l^{-1} $Zn(CH_3COO)_2 \cdot 2 H_2O$, 0.0025 g l^{-1} $CoCl_2 \cdot 6 H_2O$ (Merck KGaA, Darmstadt, Germany), 0.015 g l^{-1} $MnCl_2$

$\cdot$ 4 H_2O, 0.0012 g l^{-1} $CuCl_2$ $\cdot$ 2 H_2O, 0.0030 g l^{-1} H_3BO_3, 0.0025 g l^{-1} Na_2MoO_4 $\cdot$ 2 H_2O (sterile filtered). Acetate was added as a carbon source at concentrations of 5 g l^{-1}. In experiments on complex medium, 5 or 10 g l^{-1} yeast extract were added as indicated. For the defined medium an amino acid mix and vitamins were added from sterile filtered stocks. Amino acids were added from separate stocks at a final concentration of 1 g l^{-1} asparagine $\cdot$ H_2O (Merck KGaA, Darmstadt, Germany), 1 g l^{-1} monosodium glutamate $\cdot$ H_2O, 0.3 g l^{-1} arginine, 0.5 g l^{-1} lysine $\cdot$ H_2O (Merck KGaA, Darmstadt, Germany) and 0.5 g l^{-1} methionine (Merck KGaA, Darmstadt, Germany) in the medium. Asparagine was later replaced by monosodium aspartate $\cdot$ H_2O (Merck KGaA, Darmstadt, Germany) at concentrations of 1.15 g l^{-1} as indicated. For experiments with TCA cycle intermediates, stocks of 0.88 g l^{-1} succinate, malate (Merck KGaA, Darmstadt, Germany) and oxaloacetate were used.

The vitamin stock solution was adapted according to Pfeifer et al. [35] and the final concentration in the medium was 4.5 mg l^{-1} thiamine hydrochloride, 0.53 mg l^{-1} riboflavin, 6.8 mg l^{-1} calcium D-pantothenate, 7.5 mg l^{-1} nicotinic acid (Merck KGaA, Darmstadt, Germany), 1.75 mg l^{-1} pyridoxine hydrochloride (AppliChem GmbH, Darmstadt, Germany), 0.075 mg l^{-1} biotin (Merck KGaA, Darmstadt, Germany) and 0.05 mg l^{-1} folic acid (Merck KGaA, Darmstadt, Germany). These concentrations accounted for the 1 × concentration and amounts were reduced in some experiments as indicated.

The feed medium contained 100 g l^{-1} acetate. If indicated, 28.8 g l^{-1} monosodium aspartate $\cdot$ H_2O was added to the feed. Feed medium was pulsed to the cultures to restore an acetate concentration of 4 g l^{-1}. Low-pulse volumes potentially accounted for inaccuracies in carbon balances.

Liquid and solid media were supplemented with 50 µg ml^{-1} kanamycin.

Construction of plasmids and strains

The genes *budA*, *budB* and *budC* from *Enterobacter cloacae* subsp. *dissolvens* were overexpressed for 2,3-butanediol production. Plasmid 445_Ediss previously constructed and transformed into *E. coli* W and *E. coli* W Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* was used in this study [6]. This plasmid was additionally transformed into *E. coli* W Δ*ldhA* Δ*adhE* by electroporation.

Preparation of precultures

All strains and constructs were stored at − 80 °C in 20% (w/v) glycerol. For cultivations, they were streaked onto LB agar plates containing 50 µg ml^{-1} kanamycin and incubated overnight at 37 °C. A single colony was used for inoculation of 500 ml shake flasks with 50 ml LB medium. The preculture was incubated overnight at 37 °C and 230 rpm. Cells were centrifuged at 4800 rpm (2396*g*) for 10 min at room temperature and washed with 25 ml of sterile 0.9% (w/v) NaCl. After resuspension in 5 ml 0.9% (w/v) NaCl, the optical density at 600 nm (OD_{600}) was measured and for experiments in shake flasks, the appropriate volume to reach an initial OD_{600} of 0.5 was transferred. For bioreactor experiments, the culture was adapted to growth on acetate in defined medium. To this end, the preculture was sequentially transferred twice into 500 ml shake flasks with 20 ml defined medium containing the 1 × concentration of aspartate and vitamins. The optical density was measured in regular intervals and bioreactors were inoculated with 10 ml of the culture in the exponential growth phase.

Media and strain screening in shake flasks

Screening of different media compositions and strains was carried out in 500-ml shake flasks containing 20 ml medium as indicated. The flasks were incubated at 30 °C and 230 rpm. Samples were taken every 24 h for OD_{600} and HPLC measurements.

Cultivations in bioreactors

Bioreactor cultivations were carried out in duplicates in a DASbox® Mini Bioreactor system (Eppendorf AG, Hamburg, Germany) at a working volume of 200 ml and a temperature of 30 °C. The pH was initially set to 7.2 and after initial growth has started, it was changed to 7.0. To avoid precipitations in the continuous cultures, the pH was set to 6.8. The pH was monitored by a pH electrode EasyFerm Plus K8 120 (Hamilton, Reno, NV, USA) and controlled by the addition of 5 M phosphoric acid with a MP8 multi-pump module (Eppendorf AG, Hamburg, Germany). The concentration of dissolved oxygen was monitored by a VisiFerm DO 120 probe (Hamilton, Reno, NV, USA). The agitator speed was kept at 800 rpm and the medium was sparged with 0.2 vvm (2.4 sl h^{-1}) air. Gassing rates and stirrer speed were increased to maintain a dissolved oxygen concentration above 30%. Off-gas analysis for O_2 and CO_2 was carried out using the gas analyzer module GA4 (Eppendorf AG, Hamburg, Germany).

Samples were taken regularly to measure the optical density at 600 nm and estimate biomass growth. The samples were centrifuged at 14,000 rpm (21,913*g*) for

5 min and the supernatant was used for HPLC analysis of substrate and product concentrations.

For monitoring the acetate concentration during fed-batch cultures, the supernatant was diluted 1:10. Acetate was measured at-line using a Cedex Bio HT Analyzer (Roche, Switzerland).

Biomass determination

Cell dry weight was determined gravimetrically in duplicates from bioreactor samples at the end of each bioreactor experiment. In short, 4 ml of culture broth was centrifuged in a glass tube at 4800 rpm (2396g) and 4 °C for 10 min, washed with 4 ml 0.9% (w/v) NaCl and centrifuged again. The biomass was dried in pre-weighed glass tubes for at least 72 h at 105 °C. The optical density at 600 nm (OD_{600}) was measured in a spectrophotometer (Genesys™ 20, Thermo Scientific, Waltham, Massachusetts, USA) against a water blank. The correlation between biomass and OD_{600} was used to estimate the cell dry weight calculation of all other samples.

HPLC analysis

Organic acids, alcohols and amino acids were determined using an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules/CA, USA) in an Ultimate 3000 system (Thermo Scientific, Waltham/MA, USA). The mobile phase was 4 mM H_2SO_4 and the column was operated at 60 °C and a flow of 0.6 ml min^{-1} for 30 min. The injection volume was 10 µl. Detection was performed using a refractive index (Refractomax 520, Thermo Scientific, Waltham/MA, USA) and a DAD detector (Ultimate 3000, Thermo Scientific, Waltham/MA, USA). Chromeleon 7.2.6 Chromatography Data System (Thermo Scientific, Waltham/MA, USA) was used for control, monitoring and evaluation of the analysis.

For the measurement of organic acids and alcohols, 450 µl of culture supernatant were mixed with 50 µl of 40 mM H_2SO_4 and centrifuged for 5 min at 14,000 rpm (21,913g) at 4 °C. The remaining supernatant was used for further analysis.

For the measurement of amino acids and particularly aspartic acid, 250 µl of culture supernatant was mixed with 50 µl of 1 M sodium nitrite and 10 µl of 12 M HCl. The solution was heated to 45 °C for 90 min and the reaction was stopped by adding 50 µl 2 M NaOH [52]. The derivatized amino acid solution was directly transferred to an HPLC tube and measured at the conditions described above.

Standards were treated like samples and a 5-point calibration was used for quantification.

Supplementary information

Additional file 1: supplementary tables. **Table S1**. Comparison of growth and production in *E. coli* W and *E. coli* W ΔldhA ΔadhE Δpta ΔfrdA (Δ4). **Table S2**. Diol production from acetate and other amino acids in *E. coli* W ΔldhA ΔadhE Δpta ΔfrdA.

Authors' contributions
KN: conceptualization, formal analysis, investigation, writing—original draft, visualization. RK: investigation, visualization. SP: conceptualization, writing—review and editing, project administration, funding acquisition, resources. All authors have read and accepted the manuscript.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication.
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

III. CONCLUSIONS

Apart from showing the feasibility of CO_2 fixation and production of a fuel chemical in a novel two-step process, the main achievements in this thesis were: (i) to highlight the potential of acetate as a promising feedstock for microbial conversion in a current opinion article, (ii) to show net CO_2 fixation and production of acetate from an industrial blast furnace gas stream blended with H_2, (iii) the establishment of *E. coli* W as a promising host for acetate (co-) utilization and improving this capability by overexpression of an acetylation insensitive acetyl-CoA synthetase, (iv) the development of a system for isobutanol production on both defined medium and a waste stream and (v) the promotion of 2,3-butanediol production from acetate by quantitative media development and strategic process design.

Those achievements were possible due to a holistic scientific approach combining different advanced bioprocessing methods. These included the time-resolved characterization of physiological parameters and rational process design, which allowed to generate knowledge on acetate production from an industrial flue gas stream and utilization of acetate for microbial chemical production. Similarly, strain selection and engineering were key success factors to improving acetate assimilation and conversion into a fuel chemical. This thesis focused especially on the application of advanced cultivation methods such as chemostats and changestats, applying dynamic shifts. Those cultivation approaches allowed to study cellular metabolism under defined steady state conditions and were the basis for in-depth understanding of parameters limiting the microbial cell factories.

1. CO_2 fixation and acetate production from industrial flue gas using *A. woodii*

What are the process requirements for a continuous cultivation for CO_2 fixation using industrial blast furnace gas?

The development of a CO_2 fixation process using industrial blast furnace gas is mainly challenged by the presence of inhibitors and toxic CO in the gas stream. It has been shown that two criteria have to be met to achieve CO_2 fixation in blast furnace gas containing CO: (i) the gas stream has to be blended with H_2 and (ii) the CO concentration in the liquid medium has to be limiting. Additionally, the establishment of a H_2-limited culture was crucial to obtain comparable and reproducible results. Other limitations such as product inhibition by acetate, media, and physiological limitation could be excluded by determination critical levels for these limitations.

How does the presence of CO and other inhibitors influence growth, acetate formation and gas uptake?

The presence of CO delayed initial growth and led to a prolonged adaptation phase. In accordance to previous observations, omitting this phase was possible by adding formate is (Bertsch and Müller, 2015b). After the depletion of formate and when CO fell below a certain threshold, CO, CO_2 and H_2 were co-utilized. However, this co-utilization required blending of the blast furnace gas with H_2. Without the addition of H_2, growth on the flue gas was not possible. Blending with H_2 was not only required as an energy source during CO_2 fixation, but also because *A. woodii* cannot utilize CO as the sole carbon and energy source (Bertsch and Müller, 2015b). CO limitation is especially important for process design and crucial for the transition from batch to continuous cultures. The presence of CO did not affect the maximum specific growth rate of *A. woodii*, but CO utilization shifted the carbon flux towards biomass instead of acetate formation compared to CO_2 utilization alone. In CO and H_2 limited cultures, the utilization of these two gases was directly related to their respective partial pressures. Subsequently, CO_2 was either assimilated in both branches of the Wood-Ljungdahl pathway (WLP) in the high H_2 – low CO blend or only used in the methyl branch in the low H_2 – high CO blend. Net CO_2 fixation was achieved in both gas blends independently from the CO_2 and H_2 partial pressure. Since the different fluxes through the branches of the WLP also changed the redox status and energy conservation of the cell, blending of CO containing gases can be used as a strategy to control product formation in gas fermentations.

Determination of process requirements was possible due to an approach combining the characterization of physiological parameters in a time-resolved manner and the application of continuous bioprocessing. To be specific, the quantification of data during reproducible steady state conditions generated knowledge on how the gas composition (i.e. CO, H_2, CO_2 concentrations) of industrial flue gas influenced CO_2 fixation, growth, and acetate production. Furthermore, dynamic shifts during steady state conditions allowed to draw conclusions about the limitation of the system.

We have shown that *A. woodii* is an acetogenic host very suitable for net CO_2 fixation in gases containing CO due to its excellent H_2 utilization characteristics even at high CO partial pressures. This observation was surprising, since this strain had so far not been considered for efficient fermentation of gases containing CO. Organisms in which growth on CO is mainly established include *C. autoethanogenum* and *C. ljungdahlii*, but in these, CO utilization is usually accompanied by CO_2 production rather than net fixation (Hermann et al., 2020; Valgepea et al., 2018, 2017). Simultaneous uptake of CO and CO_2 could therefore only be achieved at low CO partial pressures of 2 %, but not at concentrations comparable to this thesis (Heffernan et al., 2020; Valgepea et al., 2018). In contrast to the latter two,

efficient CO utilizing acetogens, which produce a mixture of acetate and ethanol, *A. woodii* exclusively forms acetate on H_2 and CO_2. Ethanol production is not possible since the formation via acetaldehyde dehydrogenase is energetically not feasible and the organism lacks a functional acetaldehyde:ferredoxin oxidoreductase (Bertsch and Müller, 2015a; Mock et al., 2015). Despite the lower energy density of acetate compared to ethanol and the associated limitations in its application, the production of acetate as the sole product can be superior to a mixed product spectrum, since complexity is reduced, while controllability increases (Schwarz et al., 2020). Potential fluctuations in the feed gas composition usually occurring in industrial plants should therefore not greatly influence the performance of such a production system.

Outlook

The performance of gas fermentations in general and the examined cultivation in detail is limited by gas-liquid mass transfer. Implementing a reactor system with increased transfer rates could therefore improve gas utilization (Takors et al., 2018). These fermentation systems include air-lift and bubble column reactors already in use or trickle-bed biofilm reactors (Heijstra et al., 2017; Takors et al., 2018). However, increasing gas uptake rates result in increased acetate productivity and potentially enhanced acetate concentrations in the liquid medium. In this thesis, acetate concentrations above 20 g l^{-1} were found to inhibit CO_2 and H_2 uptake, which is higher compared to previous observations (Kantzow et al., 2015). In case acetate in the liquid medium exceeds this critical amount, higher gas liquid mass transfer rates might not necessarily lead to higher production rates, since the limitation of the cultivation system is changed from H_2 limitation to product inhibition. Inhibition could be avoided by constantly removing acetate, as achieved in continuous processes at high dilution rates. Continuous production could therefore be intensified by the implementation of a cell retention system.

2. Characterization and engineering of acetate (co-)utilization in *E. coli* W

Is *E. coli* W a suitable host for acetate utilization?

The utilization of acetate is generally hampered by its toxicity as a weak acid as well as its low energy density. Due to the latter characteristic, co-utilization with glucose or other substrates is an interesting alternative to increase product formation. However, acetate assimilation in the presence of glucose is obstructed by carbon catabolite repression (CCR), which leads to diauxic growth (Valgepea et al., 2010). Therefore, the selection of a suitable host is crucial. *E. coli* W was known to be a robust and stress-resistant strain (Arifin et al., 2014; Nagata, 2001) and its suitability for acetate assimilation was confirmed in this thesis. This strain especially stood out, since acetate assimilation and co-utilization of acetate and

glucose in batches was possible at high concentrations compared to other organisms. Additionally, acetate was utilized at high specific uptake rates in chemostat cultivations.

Can acetate (co-)utilization be improved by the overexpression of an acetyl-CoA synthetase?

Co-utilization of glucose and acetate in batch experiments was still characterized by diauxic growth. Since acetyl-CoA synthetase (*acs*), one of two pathways for acetate assimilation, is known to be down-regulated during CCR, we have shown that overexpression of this enzyme improved co-utilization. To be specific, the expression of *acs* insensitive to post-translational modification showed two main effects in batch cultivations: (i) it decreased the lag-phase of cultivations with acetate as the sole carbon source and (ii) it improved acetate utilization during glucose assimilation and thus reduced diauxic growth. However, there were only minor differences between the overexpression and a control strain in chemostat cultures, probably since *acs* is anyways the main route for acetate uptake during glucose limited conditions (Castaño-Cerezo et al., 2015; Renilla et al., 2012). Gene expression analysis has shown that overexpressing *acs* potentially caused an imbalance in the acetate assimilation node, which resulted in enhanced energy dissipation and wash-out at lower dilution rates.

The scientific basis for the in-depth characterization of acetate assimilation in both the wildtype and the overexpression strain of *E. coli* W was a combination of time-resolved and steady state quantification of physiological parameters. In detail, the effect of overexpression of *acs* on carbon catabolite repression under excess carbon conditions could be shown by investigating uptake and production rates in batch experiments and the described effects could be explained by studying the activity of acetate metabolism in accelerostat cultivations.

Outlook

A deeper understanding of acetate metabolism is required for the establishment of microbial cell factories efficiently utilizing acetate. Most research has so far been based on glucose-grown cells and has been aiming on preventing acetate formation triggered by overflow metabolism. Therefore, further research could focus on studying acetate metabolism in acetate-grown cells and describing the effect mediated by *acs* overexpression in this context.

2.1. The transition of acetate from the first to the second process step

As the intermediate product, acetate would have to be transferred from fermentation with *A. woodii* in the first step to *E. coli* cultivation in the second step. Acetate concentrations in the first process step remained below 20 g l^{-1} and these low titers would be detrimental for downstream processing (DSP). However, for further biological processing, high acetate

titers are not required due to the inhibitory effect of this substrate. Since the costs for DSP largely contribute to the total process costs (Takors et al., 2018), direct utilization in a second process is preferred to an intermediate purification step. Due to the composition of the harvest of the first step, purification would not be needed except for removing biomass content.

2.2. Applying knowledge about acetate utilization and toxicity on fuel chemical production in the second process step

Considering the effects of *acs* overexpression on acetate and glucose (co-)utilization, we evaluated whether this engineering strategy could also be applied for fuel chemical production from acetate in the second step of the two-step process. *Acs* overexpression improved process performance in batch experiments by reducing the lag-phase on acetate as sole carbon source and improving acetate and glucose co-utilization. However, no positive effect was observed in substrate limited chemostats and overexpression of *acs* seemed to cause an imbalance in the acetate assimilation node resulting in increased energy dissipation. Two main factors therefore led to the decision against overexpression of *acs* in the second step of fuel chemical production from acetate: (i) the availability of ATP is especially important during recombinant enzyme expression and decreased ATP supply would probably reduce production efficiency and (ii) natural up-regulation of *acs* occurs during glucose starvation and when acetate is used as the sole carbon source (Castaño-Cerezo et al., 2015; Oh et al., 2002; Renilla et al., 2012). These conditions should be favored during process development in the second step. Therefore, the strategy of overexpressing *acs* was not chosen when we aimed for isobutanol and 2,3-butanediol production from acetate.

Another possible strategy for manipulation of acetate assimilation would be based on the consideration of cellular mechanisms against acetate toxicity. Comparing acetate concentrations in cultivations of *A. woodii* and *E. coli* reveals that growth of the former organism remained largely unaffected by titers up to 20 g l^{-1}, whereas acetate concentrations of 10 g l^{-1} already drastically increased the lag-phase of the latter one. The toxicity of acetate as a weak acid can be attributed to several mechanisms which include decreasing the intracellular pH and increasing the osmotic pressure (Menzel and Gottschalk, 1985; Roe et al., 1998; Russell, 1992). The decrease of the intracellular pH has to be compensated by pumping protons via a membrane ATPase at the expense of ATP consumption (Verduyn et al., 1992). Since generation of ATP is coupled to a sodium rather than a proton transmembrane gradient, *A. woodii* might be less prone to this specific mechanism of acetate toxicity. Similarly, because acetate is the sole product of *A. woodii*, this organism might have evolved other mechanisms to deal with high concentrations of

weak acids. The investigation of acid resistance in *A. woodii* might therefore provide valuable information as a basis for the improvement of acetate tolerance in *E. coli*.

3. Strain and process development for production of fuel chemicals from acetate and renewable resources in *E. coli* W

For the conversion of renewable resources such as acetate and cheese whey, the selection of a proper strain in combination with metabolic engineering were key scientific success factors. Rational process and screening design enabled efficient production of (i) isobutanol from the residual waste stream cheese whey and (ii) 2,3-butanediol from acetate on defined medium. For both fuel chemicals, applying a pulsed fed-batch was successful to increase productivity and final product titers. 2,3-butanediol production from acetate could further be improved by quantitative media optimization and screening.

3.1. Process and strain development for isobutanol production on glucose defined medium and cheese whey

How can cost-effective isobutanol production be established as a basis for further utilization of alternative raw materials?

Isobutanol is not produced naturally in any organism and microbial production is challenged by its toxicity and volatility. We hypothesized that for economic production, expensive inducers as well as complex media additives had to be omitted. Individual expression of each gene using constitutive promotors enabled expression fine tuning and initial screening experiments showed great variations in isobutanol production in *E. coli* BW25113 and *E. coli* W with different constructs. *E. coli* BW25113, which was an established host for isobutanol production on complex media using inducible expression (Atsumi et al., 2008; Baez et al., 2011; Desai et al., 2014), was found to be unsuitable for isobutanol production on chemically defined medium. Yields could be increased by strain improvement via deletion of mixed acid fermentation pathways (i.e. pathways for ethanol, acetate, succinate, and lactate). It was surprising that the deletion of mixed acid-fermentation pathways enabled efficient isobutanol production under aerobic conditions, since aerobic isobutanol production – in accordance with our general expectations - was very low in the wild type strain. Aerobic isobutanol production was likely possible due to pyruvate accumulation mediated by a combination of isobutanol toxicity mechanisms (i.e. quinone inhibition) and the deletion of mixed acid-fermentation pathways. In conclusion, while other studies focused on the addition of complex media components and the use of expensive inducers, a more economic isobutanol production was established due to (i) the use of chemically defined medium and the omittance of complex media additives, (ii) the replacement of expensive inducers by constitutive expression, (iii) selection of a suitable expression system (construct and strain) in screening experiments and (iv) the improvement of process performance by choosing appropriate process conditions. In the future, genome integration

could be a goal to further reduce the cultivation costs associated with the use of antibiotics for plasmid-based expression or alternatively, growth can be coupled to isobutanol production.

Can isobutanol be produced from an alternative raw material such as cheese whey?

The cost-competitiveness of microbial isobutanol production could further be increased using cheese whey as an alternative feedstock. Utilization of this waste stream not only reduced raw material costs, but also increased process stability due to increased productivity and titers. Other waste streams that have so far been used for isobutanol production include biomass hydrolysates such as cedar and wheat straw (Akita et al., 2015; Felpeto-Santero et al., 2015). Apart from heavy pretreatment, which is required to access fermentable sugars of this feedstock, observed isobutanol titers and yields were low (Akita et al., 2015; Felpeto-Santero et al., 2015). Cheese whey was therefore established as a promising feedstock for microbial isobutanol production.

Outlook

While achieving low-cost isobutanol production from glucose and cheese whey, there is still potential to increase production performance by improving the isobutanol yield. In a very similar expression system aiming to produce 2,3-butanediol instead of isobutanol ~2-fold higher product yields were achieved, which shows the theoretical potential of fuel chemical production in *E. coli* W (Erian et al., 2018). This discrepancy raised the question why isobutanol production was less efficient than 2,3-butanediol formation. Technical challenges during construction of the expression vector suggested that metabolic burden for pathway expression might play a role. Additionally, isobutanol and 2,3-butanediol are produced via a five-enzyme and three-enzyme pathway, respectively implying that more cellular resources are needed for gene expression. Finally, isobutanol is a toxic chemical, whereas 2,3-butanediol has not been reported to inhibit cellular growth and product formation. To conclude, stronger expression might help to increase isobutanol yields, but a better understanding of isobutanol toxicity and the underlying mechanisms is required to engineer more robust and tolerant strains for efficient isobutanol production. Focusing on the use of cheese whey, inducible expression might increase isobutanol yields and lactose might be an alternative inducer to expensive IPTG (Isopropyl-β-D-thiogalactopyranoside).

3.2. Isobutanol production from acetate

Which environmental factors mainly influence isobutanol production from acetate?

It was observed that the influence of strain background and media composition on isobutanol production was even more pronounced on acetate than on glucose. When acetate was used as a carbon source, production was only achieved in a strain where mixed-acid fermentation pathways were deleted, but not in the wild-type strain. However,

this knock-out strain was not able to grow on defined medium and the addition of yeast extract was required. However, quantitative development of a production process was hampered by the volatility and toxicity of isobutanol in combination with acetate toxicity: screening experiments had to be carried out under fully aerobic conditions resulting in evaporation of isobutanol and problems in correct quantification. Additionally, acetate toxicity made the use of low substrate concentrations necessary, resulting in low product concentrations and inaccurate product quantification. Because of that, it was decided to develop fuel chemical production from acetate using 2,3-butanediol as a product, which is also derived from pyruvate as isobutanol but is neither toxic nor volatile. The acquired platform knowledge could in the future be used to establish isobutanol production from acetate and understand how a system inhibited by two components (i.e. acetate and isobutanol) works.

3.3. Creating platform knowledge for microbial upgrading of acetate: 2,3-butanediol as a case study

Which environmental factors mainly influence 2,3-butanediol production from acetate?

Similar to isobutanol production, 2,3-butanediol formation from acetate was mainly influenced by the strain background and media composition. Again, 2,3-butanediol was only produced in *E. coli* W where mixed-acid fermentation pathways were deleted. This strain did not grow on the original defined medium but required the addition of yeast extract. By quantitively assessing the "background production" from this complex media additive we have shown that 2,3-butanediol is not produced from acetate but only from yeast extract.

How can the production of 2,3-butanediol from acetate be established?

The key success factor for establishing 2,3-butanediol production from acetate was the development of a new defined medium. Extensive media screening revealed that the component promoting growth and production from acetate was aspartate / asparagine and that this effect is possibly mediated by improved NAD^+ availability. By studying the kinetics of acetate and aspartate uptake, we have shown that aspartate is only needed to "kick-start" *E. coli*" and co-utilization was not required for 2,3-butanediol production. This observation finally enabled the development of a production process using acetate as the sole carbon source for feeding.

In order to establish a two-step process, we initially investigated continuous 2,3-butanediol production from acetate. Surprisingly, production was not successful in this process mode since either no 2,3-butanediol was formed when acetate and aspartate were co-fed or acetate accumulated when acetate was used as the sole carbon source. The reason for this observation has remained unclear and it can only be speculated that direct co-utilization or substrate limitation would not promote production. As a basis for further research, e.g.

labelling experiments determining the intracellular flux distributions of both substrates during co-utilization might therefore shed light on underlying mechanisms leading to the described observation. Alternatively, the acquisition of transcriptome and especially metabolome data could identify potential bottlenecks.
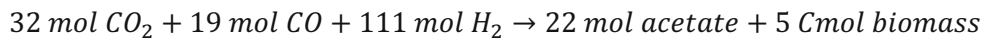
Outlook

While the feasibility of fuel chemical production from acetate was shown in this thesis and achieved 2,3-butanediol titers were among the highest reported values for chemical production from acetate, final product titers remained too low for efficient down-stream processing. 2,3-butanediol production could potentially be increased in a cell retention system but enhanced engineering will be needed to increase product yields. Metabolic engineering could improve product formation at several sites of metabolism, and additional knowledge will be required to evaluate these sites. For example, more detailed information on the effect mediated by aspartate / asparagine might reveal engineering targets and could eventually allow to omit this media additive. Alternatively, metabolic modelling could identify limitations of the production system and an increase in pyruvate and NADH availability during acetate assimilation might improve 2,3-butanediol production. Alternatively, the regeneration of NADH could be coupled to the production of 2,3-butanediol (Bastian et al., 2011; von Kamp and Klamt, 2017). Without prior knowledge, random mutagenesis or adaptive laboratory evolution could be applied as alternatives to metabolic engineering (Seong et al., 2020). Product titers might also increase by co-feeding e.g. glucose, although this approach did not result in 2,3-butanediol production from acetate in this thesis.

Deletion of mixed-acid fermentation pathways to enable chemical production from acetate under aerobic conditions was previously unknown. It would be interesting to elucidate the underlying mechanism behind this effect and evaluate in which way it is similar to chemical production from glucose in this thesis. Additional research, including acquisition of omics data and evaluation of metabolic fluxes, might shed further light on how the activity of the acetate assimilation node can influence acetate utilization, product formation and acetate toxicity.

4. Considerations on the way towards the integration of the final two-step process
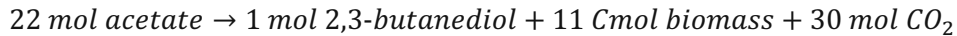
This thesis has shown the feasibility CO_2 fixation and fuel chemical production via acetate in a two-step process. In *A. woodii*, acetate was produced from CO_2, CO and H_2 according to the following equation:
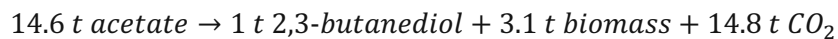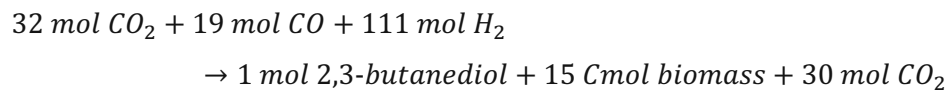
$$32 \ mol \ CO_2 + 19 \ mol \ CO + 111 \ mol \ H_2 \rightarrow 22 \ mol \ acetate + 5 \ Cmol \ biomass$$

or

$$15.6 \ t \ CO_2 + 6.0 \ t \ CO + 2.5 \ t \ H_2 \rightarrow 14.6 \ t \ acetate + 1.2 \ t \ biomass$$
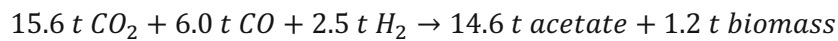
In the second step, *E. coli* converted acetate into 2,3-butanediol according to:

$$22 \ mol \ acetate \rightarrow 1 \ mol \ 2,3\text{-}butanediol + 11 \ Cmol \ biomass + 30 \ mol \ CO_2$$

or

$$14.6 \ t \ acetate \rightarrow 1 \ t \ 2,3\text{-}butanediol + 3.1 \ t \ biomass + 14.8 \ t \ CO_2$$

The overall production in the two-step process can therefore be described by the following equation:

$$32 \ mol \ CO_2 + 19 \ mol \ CO + 111 \ mol \ H_2$$
$$\rightarrow 1 \ mol \ 2,3\text{-}butanediol + 15 \ Cmol \ biomass + 30 \ mol \ CO_2$$

or

$$15.6 \ t \ CO_2 + 6.0 \ t \ CO + 2.5 \ t \ H_2 \rightarrow 1 \ t \ 2,3\text{-}butanediol + 4.3 \ t \ biomass + 14.8 \ t \ CO_2$$
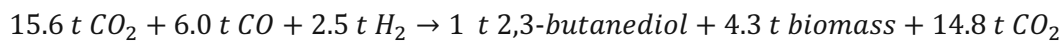
The mass balance of the overall process shows that the two acceptance criteria have been met: net CO_2 fixation was achieved, and a fuel chemical (2,3-butanediol) was produced. Elemental balancing of CO_2 fixation and 2,3-butanediol production allows to draw conclusions about steps limiting the efficiency of the two-step process. The overall process yielded production of 0.031 mol 2,3-butanediol per mol CO_2 (corresponding to 0.064 g 2,3-butanediol per g CO_2). These yields are still low but further strain engineering and process development could help to increase conversion efficiency. In the second process, 70 % of the carbon flux are diverted towards CO_2, which reduces the overall efficiency of the two-step process. CO_2 formation can never by completely prevented, since it is partially coupled to product formation (formation of 4 mol CO_2 per mol 2,3-butanediol produced, corresponds to 50 % of carbon loss). However, the second process step could still be improved by diverting the acetate flux towards the product rather than CO_2 production. The integration of genes for intracellular recycling of CO_2 might further improve the carbon flux towards the product.

Due to the flexible process design, the knowledge gained in this thesis can serve as a platform for net CO_2 fixation and conversion into different products. In the first process step, CO_2 could also be fixed in a different industrial flue gas stream or by another gas fermenting organism. Similarly, the product of the second step could be exchanged and alternative chemicals could be obtained either by genetic engineering or by native production.

Bioprocessing strategies discussed in this thesis include time-resolved quantification of physiological parameters and the acquisition of steady state data in chemostats and changestats. These methods carry the potential to optimize processes for net CO_2 fixation and can help to establish a circular economy on the way towards a CO_2 neutral future.

Other processes for CO_2 fixation proposed in literature include both one-step as well as two-step processes using a broad variety of microorganisms capable of fixing CO_2 via different mechanisms. While photosynthetic CO_2 fixation allows for the production of complex, high-value chemicals, the efficiency of such processes are typically hampered by low CO_2 utilization rates in combination with slow growth and low productivities (Gong et al., 2018). The use of algae and cyanobacteria for CO_2 fixation in industrial blast furnace gas would additionally be challenged by growth being inhibited at high CO_2 concentrations and limited tolerance towards impurities (Anand et al., 2020). So, while photoautotrophic microorganisms are highly suitable for other applications, their use would be restricted within the scope of this thesis. In contrast, acetogenic bacteria show higher energy efficiencies and improved CO_2 utilization characteristics, making them very suitable for CO_2 fixation in industrial flue gases. Compared to traditional one-step cultivations, the suggested process in this thesis is especially robust towards varying feed gas compositions. Additionally, net CO_2 fixation is achieved even at high CO concentrations by using an acetogenic microorganism, which efficiently utilizes CO_2 and H_2. The production of acetate as the sole product rather than a mixture of different compounds further increases the overall robustness of the process. In contrast to literature reports on microbial upgrading of acetate, this thesis focused on the determination of effects mediated by complex media additives and aimed on achieving solid quantification of 2,3-butanediol production on defined medium. In contrast to other two-step processes, a total mass balance for CO_2 fixation and 2,3-butanediol production is presented in this thesis. Elemental balancing was especially important to determine where the efficiency of the overall production process was limited and to identify potential targets for future improvements. To sum up, the novel two-step process shown in this thesis stands out by its robustness towards variations in flue gas composition and the production of 2,3-butanediol from acetate.

The industrial applicability for CO_2 fixation with the process development in this thesis is still restricted by different economic factors. Although it is a good sign that industrial flue gas can be used directly without the need for prior purification procedures, the low prices for CO_2 emission certificates limit the innovation drive towards biological CO_2 fixation technology. The availability of H_2 will be another important factor for cost-competitiveness of the process. The cost of H_2 still contributes most to the total cost of the CO_2 fixation process. H_2 is mainly produced from natural gas and a massive expansion of this technology might enable cheaper production. However, H_2 generation from natural gas is not a "green"

technology and is accompanied by CO_2 release (Arregi et al., 2018; Yukesh Kannah et al., 2021). Therefore, technologies for renewable H_2 production are increasingly gaining interest and their development is supported by the EU (*Energy Policy Review*, 2020). With an expansion of this technology, renewable H_2 production will become more cost competitive. Recycling of CO_2 produced in the second step or CO_2 not utilized in the first step might additionally increase the efficiency of the proposed two-step production system. Although not yet cost-competitive to chemical production from fossil fuels, a combination of political decision-making, emerging research and process intensification might drastically increase the potential of the two-step process developed in this thesis within the next decades.

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List of figures

Appendix

List of publications

Matsakas, L., Novak, K., Enman, J., Christakopoulos, P., Rova, U. Acetate-detoxification of wood hydrolysates with alkali tolerant *Bacillus sp*. as a strategy to enhance the lipid production from Rhodosporidium toruloides. Bioresour Technol. 2017 Oct;242:287-294. doi: 10.1016/j.biortech.2017.04.002

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Novak, K., Neuendorf, C.S., Kofler, I., Kieberger, N., Klamt, S. and Pflügl, S. Blending industrial blast furnace gas with H_2 enables *Acetobacterium woodii* to efficiently co-utilize CO, CO_2 and H_2. Currently under Revision in Bioresource Technology

Conferences and workshops

K. Novak, L. Flöckner, A. Erian, P. Freitag, C. Herwig, S. Pflügl. Overexpression of an acetylation-insensitive acetyl-CoA synthetase in E. coli W and its effect on glucose and acetate co-utilization in batch and continuous cultures. Poster: 10th ÖGMBT Annual Meeting, Vienna; 17.09.2018 - 20.09.2018; in: "Abstract Book", (2018), p. 84.

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