From waste to value: opportunities to use genetically engineered anaerobic bacteria for industrial purposes and the importance of isolation as well as reclassification of bacteria

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Abstract

Anaerobic bacteria have broad substrate range and product spectrum, thus these organisms are suitable as chassis strains for industrial purposes. Many anaerobic bacterial organisms use sugar as a carbon and energy source, however, the so-called autotrophic organisms can use C1 gases such as CO_2 and CO as carbon source, and therefore they can be used for waste valorization. In this work, the autotrophic organism *Acetobacterium woodii* was genetically manipulated to increase the recombinant production of acetone using synthetic waste gas as a substrate. For the improvement, genes of *Clostridium acetobutylicum* and *Clostridium aceticum* were plasmid-based expressed in *A. woodii*. Surprisingly, not only acetone was produced, but also isopropanol was synthesized with the recombinant *A. woodii* strains grown in stirred tank bioreactors with continuous gas supply. In bottle growth experiments, isopropanol production could only be seen with fructose as substrate. As a conversion of acetone to isopropanol must have been occurred, a native secondary alcohol dehydrogenase must be present in *A. woodii*. For the identification of the secondary alcohol dehydrogenase two potential genes were tested. However, the gene for the responsible enzyme was not identified. Nevertheless, the obtained results can serve as a basis for future investigations in the field of waste valorisation from waste gases. Another example for waste valorization described in this work is the fermentation of glycerol to 1,3-propanediol (1,3-PDO) with genetically modified *Clostridium beijerinckii* strains. 1,3-PDO production form glycerol was obtained by plasmid-based expression of the 1,3-PDO gene cluster from *Clostridium diolis* DSM 15410 (reclassified as *C. beijerinckii*) in *C. beijerinckii* DSM 6423. An improvement of 1,3-PDO production was achieved by buffering the minimal medium, leading to an increase of 1,3-PDO by 82 %. A further enhancement of 1,3-PDO was obtained by using the *thlA* promoter from *C. acetobutylicum*. Expression of the 1,3-PDO gene cluster controlled by P_{thlA} and cultivation in buffered minimal media resulted in an 1,3-PDO increase by 167 %, compared to the results of the first experiment without any improvements. With the development of new methods to classify bacterial strains such as digital DNA-DNA hybridization, average nucleotide identity, and/or

average amino acid identity many strains will be reclassified. This will help not only finding bacteria strains with similar features, but also strains which are more closely related and therefore more suitable for genetic modification processes as performed for the 1,3-PDO production with *C. beijerinckii*. In this study the clostridial strains *C. aurantibutyricum* and *C. rosemum* were reclassified based on digital DNA-DNA hybridization, average nucleotide identity as well as morphological investigations, fatty acid analyses, and substrate utilization spectrum. These strains belong now to the species *C. felsineum.* However, the search and isolation of new bacteria species are also part of future tasks to find new promising bacteria species for industrial purposes. As shown in this study a new agarolytic bacterial species was isolated from algae in the harbor of Susak, characterized and could be described as a novel member of agarolytic organisms. The agarase capability of these organisms are of interest as this offers the possibility to utilize agar obtained from algae as sustainable substrate.

1. Introduction

1.1 Opportunity of using anaerobic bacteria for industrial purposes

Anaerobic bacteria comprise a broad range of microorganisms able to live without oxygen. The so-called obligate anaerobes are organisms that cannot survive in presence of oxygen. The probably best examined anaerobic bacteria belong to the genus *Clostridium*. This group harbours a wide range of different bacteria species with very diverse features. Often, the genus *Clostridium* is associated with the pathogenic species such as *Clostridium difficile*, *Clostridium tetani* and *Clostridium botulinum*. Especially, *C. tetani* and *C. botulinum* produce one of the most effective neurotoxins and cause the well-known diseases tetanus and botulism, respectively [Shone and Hambleton, 1989]. Besides the harmful species, many clostridial strains are of medical interest in anticancer treatments [van Mellaert et al., 2006]. Due to low blood flow, deoxygenated tissue is only found in tumours, spores of *Clostridium* target the anoxic areas of tumours when injected into the bloodstream. Here they proliferate and promote cytotoxicity and tumour destruction [Pyne et al., 2014; van Mellaert et al., 2006; Malmgren and Flanigan,1955]. In addition to the medical field, clostridial species play an important role in industrial biotechnology. Besides the standard fermentation products (e.g. organic acids, carbon dioxide (CO_2), and hydrogen (H_2)) some clostridial species produce solvents, i.e. acetone, ethanol, isopropanol, 1,3-propanediol and butanol, naturally [Kubiak et al., 2012]. These products have industrial potential as bulk solvents and potential biofuels [Kök, 2015; Dürre, 2008]. One of the first large-scale production of commodity chemicals was exploited with clostridia. In the first part of the last century, the acetone-butanol-ethanol (ABE) fermentation was used as major source of these industrial solvents [Jones and Wood, 1986]. Already in the 1900s, commercial production of solvents, especially butanol, using solvent-producing bacteria became a focus of interest [Poehlein et al., 2017]. However, the production aim switched to acetone by the beginning of World War I, since acetone was necessary as solvent for the production of the explosive cordite [Gabriel, 1928]. In 1915, the

famous patent for the production of acetone and butanol using an anaerobic bacterium was granted to Charles Weizmann [Jones and Wood, 1986]. Later the organism was named *Clostridium acetobutylicum* [McCory et al., 1926] and is now the best studied clostridial species [Nölling et al., 2001]. In the 1950s, solvent production from cheap crude oils rendered the ABE fermentation process increasingly unprofitable resulting in closure of the large plants in the UK, US, and Japan [Poehlein et al., 2017]. However, a possible revival of the industrial ABE-fermentation to produce butanol as biofuel is on the horizon, due to environmental and political concerns surrounding the utilization and production of petroleum - based fuels [Pyne et al., 2014].

Anaerobic bacteria species are not only able to produce a variety of different products with industrial importance, but their substrate spectrum is also immense, ranging from gases to agricultural waste. In more detail, the so-called autotrophic organisms are able to use methanol and/or gases such as, CO_2 or carbon monoxide (CO), as well as waste for the production of e.g. acetate, ethanol, butanol, butyrate, and 2,3-butanediol [Straub et al., 2014; Leang et al., 2013]. Many autotrophic organisms belong again to the genus *Clostridium* (e.g. *Clostridium ljungdahlii*; *Clostridium autoethanogenum*, and *Clostridium aceticum*) [Najafpour and Younesi, 2006; Dürre and Eikmanns, 2015]. However, there are many more autotrophic bacteria, which do not belong to the genus *Clostridium* with gas fermenting properties (e.g. *Eubacterium limosum*, *Acetobacterium woodii*, and *Morella thermoacetica*) [Bengelsdorf et al., 2018]. The possibility to use these autotrophic anaerobic organisms for valorisation of waste gases is very advantageous. Therefore, this subject is discussed in more detail in an additional paragraph (1.2). Beside glucose and other common sugars, some clostridial species are able to utilise waste products of the agricultural and dairy industry such as starch, xylan, lactose, cellulose, and cellulose [Dürre, 1998]. Thus, clostridial species serve as a good chassis for waste valorisation from waste products of the agricultural and dairy industry. Furthermore, the fermentation of cheap glycerol from the biodiesel production by clostridial species is of great interest [Yazdani and Gonzalez, 2007]. The fermentation of glycerol to 1,3-propandiol using anaerobic

organisms as an example for waste vaporization is discussed in more detail in chapter 1.3.

1.2 Waste valorization using waste gases

Climate change caused by greenhouse gas emission is one of the main challenges in our near future. In 2015, the United Nations therefore adopted an agreement (Paris Agreement) which entered into force in November 2016. Long term goal was to limit global warming below 2 °C above pre-industrial levels and to reduce the emission of waste gases as soon as possible to achieve net-zero by 2050 [UNFCC; Rajamani and Werksman, 2018; Seto et al., 2021]. A study by the German Advisory Council on Global Changes showed that to reach the goal of limiting global warming to 2 °C, a reduction of carbon dioxide equivalents of industrial countries from approximately 12 t $CO_{2,eq}$·per year per inhabitant to less than 1 t $CO_{2,eq}$·per year per inhabitant by the year 2050 is necessary [Dobslaw and Ortlinghaus, 2020]. An important contribution to achieve the goals of the Paris Agreement are the developments of new bio-based processes for the synthesis of crucial chemicals without greenhouse gas emissions [de Jong et al., 2012]. Another approach is the removal of waste gases from the atmosphere using carbon capture or CO_2-fixing technologies [Kajla et al., 2022; Dürre, 2017; Al-Mamoori et al., 2017]. However, this will not only be environmentally friendly but an opportunity to use waste gases for waste valorisation by synthesising value-adding bulk chemicals. This process can be accomplished by using anaerobic autotrophic acetogenic bacteria such as *Clostridium, Acetobacterium, Eubacterium,* and *Morella* strains [Fuchs, 1986; Savage 1987]. The so-called acetogens are able to use industrial waste gases also called synthesis gases (syngas), CO_2, or carbon monoxide, and methanol using the Wood-Ljungdahl-Pathway for energy conversion. The resulting central intermediate of this pathway is acetyl-CoA [Drake et al., 2008]. Most acetogens convert the produced acetyl-CoA to acetate.

However, *C. autoethanogenum*, *Clostridium ragsdalei,* and *C. ljungdahlii* cannot only synthesize acetate, but also 2,3-butanediol and ethanol naturally. Other *Clostridium* species produce butyrate and formate in addition to acetate [Schiel-Bengelsdorf and Dürre, 2012]. With new methods for genetic modification of acetogens the product spectrum using waste gases could be expanded [Bengelsdorf et al., 2008]. The product spectrum was improved for the first time in 2010 by plasmid-based expression of the butanol production pathway from *C. acetobutylicum*. The plasmid was incorporated into *C. ljungdahlii* cells, resulting in recombinant butanol formation form syngas [Köpke et al., 2010].

Another example is the heterologous production of acetone using recombinant *A. woodii* cells and CO_2 + H_2 as carbon and energy source, this was achieved by heterologous expression of *thlA, ctfA/B,* and *adc* genes from *C. acetobutylicum* [Hoffmeister et al., 2016]. In the first synthesis step, 2 mol acetyl-CoA are converted into one mol acetoacetyl-CoA performed by a thiolase (ThlA) [Wiesenborn et al., 1988]. An acetoacetyl-CoA:acetate/butyrate-CoA transferase (CtfA/B) is used for the conversion of acetoacetyl-CoA to acetoacetate [Wiesenborn et al., 1989]. In the last step, an acetoacetate decarboxylase (Adc) synthesizes acetone and CO_2 from acetoacetate [Petersen and Bennett, 1990; Gerischer and Dürre,1990]. Since the acetoacetyl-CoA:acetate/butyrate-CoA transferase has a very high K_m value [Wiesenborn et al., 1989], the exchange of the *ctfA/B* gene with the one of *Clostridium aceticum* may lead to an acetone production improvement [Arslan et al., 2022]. Similar as described for *C. ljungdahlii* the recombinant *A. woodii* strain not only produced acetone, but also the basic chemical isopropanol [Jones et al., 2016; Arslan et al., 2022]. Thus, fermentation of waste gases not only has advantages for the environment, but also leads to waste valorisation. A rough overview of feasible gas sources for waste gas fermentation and possible value-added products is presented in Figure 1. Furthermore, the company LanzaTech, founded in 2005, already has a commercial ethanol-production plant operating since 2018 [Teixeira et al., 2018; Köpke and Simpson, 2020]. In cooperation with ArcelorMittal, a steel producing company, LanzaTech is building a new plant in Belgium with a capacity of 62,000 Mt per year [Stoll et al., 2020]. Even more facilities are under construction with the aim to

produce additional natural and recombinant products [Simpson et al., 2019; Nesbitt, 2020]. This shows the opportunity to use acetogens to capture and re-use waste gases to produce fuels and chemicals [Karlson et al., 2021]. However, a limiting factor is the constraining repertoire of tools for metabolic engineering of the desired production strains.

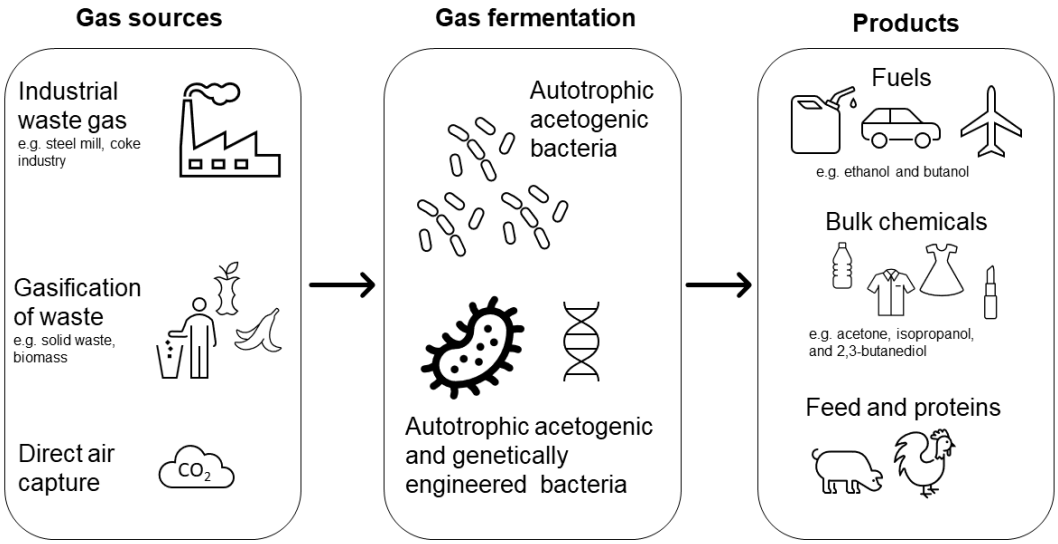

Figure 1: Overview of feasible gas sources for waste gas fermentation using autotrophic acetogenic bacteria and possible value-added products.

1.3 Waste valorization using glycerol

Another way to reduce greenhouse gases is to develop sustainable bio-based processes for the replacement of petroleum-based synthesis routes [Liu et al., 2010; Piemont and Gironi, 2012]. Furthermore, new bio-based processes can also be used for waste valorization. One of the best investigated production processes is the production of 1,3-propanediol from glycerol. 1,3-PDO is an important bulk chemical with growing demand, due to its broad utilization spectrum. 1,3-PDO is used as an organic solvent in the food, cosmetics, and pharmaceutical industry. Main application is found in the polymer industry, where 1,3-PDO is used as

precursor or intermediate, especially, as a key monomer to produce polytrimethylene terephthalate, a bio-degradable polymer [Zhu et al., 2022; Kaur et al., 2012; Webb et al., 2012]. Therefore, the rising demand in the last few years is not surprising. With a compound annual growth rate of 14.2 % as of 2018, an optimistic market expectation for 1,3-PDO is of US$ 1,442.77 million in 2027 [da Silva Ruy et al., 2020]. Unfortunately, 1,3-PDO is mainly produced via two chemical routes: the ethylene oxide hydroformylation and the acrolein hydration-hydrogeneration. However, both processes are based on petroleum-based precursors [Przystałowska et al., 2015; Hao et al., 2006; Cameron et al., 1998]. Furthermore, these two chemical routes have many disadvantages such as technical difficulties, high investment, substrate toxicity, as well as environmental issues [Zhu et al., 2022; Zhu et al., 2021]. Thus, the first essential development of a bio-based 1,3-PDO production was realized in the early 2000s by DuPont and Genencor. For the sustainable 1,3-PDO production, genetically engineered *E. coli* strains are used with glucose as substrate [Nakamura and Whited, 2003]. In 2006, the process was commercialized by DuPont Tate & Lyle in Loudon (USA), with a production capacity of 63,500 t per year [Dürre, 2020; Biotechnology Innovation Organization, 2016; Kurian, 2005]. However, the biobased synthesis of 1,3-PDO can still be improved. For example, the production will be more profitable by using cheaper substrates. This is a significant factor, since substrates costs account for 50 %- 60 % of the total production cost of 1,3-PDO [Hermann and Patel, 2007]. As a cheap substrate, glycerol or even crude glycerol can be used for the bio-based synthesis of 1,3-PDO [Clombug and Gonzalez, 2013; Yazdani and Gonzalez, 2007]. During the last years, the biodiesel industry expanded leading to a higher availability of crude glycerol [Anand and Saxena, 2012]. Glycerol is the main by-product throughout the biodiesel production in which 1 t of raw glycerol is produced for every 10 t of biodiesel [Alphy et al., 2021]. As result, the price of raw glycerol is dropping [Yang et al., 2012]. Thus, the utilization of glycerol-fermenting microorganisms for the synthesis of 1,3-PDO could be a good and profitable alternative to the common chemical production routes.

Multiple microorganisms can ferment glycerol to 1,3-PDO including *Klebsiella pneumonia*, *Citrobacter freundii*, and *Enterobacter agglomerans* [Biebl et al, 1998;

Barbirato et al., 1995; Homann et al., 1990]. Unfortunately, some of these organisms are pathogenic and the associated restrictions for industrial purpose are a big disadvantage for a commercial 1,3-PDO production [Zhu et al., 2021; Leja et al., 2011; Homann et al., 1990]. However, several *Clostridium* species are also able to reduce glycerol to 1,3-PDO such as *Clostridium pasteurianum*, *C. butyricum*, *C. diolis* (reclassified as *C. beijerinckii* [Kobayashi et al., 2020]), and few *C. beijerinckii* strains [Gungormusler et al., 2011; Biebl and Spröer, 2002; Biebl, 2001; Biebl et al., 1992; Heyndrickx et al., 1991]. Two distinct reduction pathways of glycerol to 1,3-PDO are known depending on the enzymatic equipment of the different clostridium strains. For the first conversion step from glycerol to 1,3-PDO in *C. pasteurianum*, glycerol is dehydrated to 3-hydroxypropionaldehyde (3-HPA) via a coenzyme B_{12}-dependent glycerol dehydratase (DhaB, DhaC, DhaE). In the next step, 3-HPA is reduced via a NADH-linked 1,3-PDO dehydrogenase (DhaT) to 1,3-PDO [Luers et al., 1997]. In contrast, *C. butyricum* and *C. beijerinckii* utilize a coenzyme B_{12}-independent glycerol dehydratase (DhaB1, DhaB2) for the conversion of glycerol to 3-HPA, following the reduction of 3-HPA to 1,3-PDO via a NADH-linked 1,3-PDO dehydrogenase (DhaT) [Sedlar et al., 2021; Raynaud et al., 2003]. However, in clostridia glycerol is also oxidized for production of biomass, energy conversion, as well as for the production of by-products [Kubiak et al 2012; Celińska 2010]. To improve 1,3-PDO production, sufficient NADH supply is an important factor. One strategy to increase NADH levels is the use of co-substrates for the fermentation of glycerol to 1,3-PDO [Zhu et al., 2021]. For co-substrate provision, the utilization of agricultural waste products is desired. Lignocellulosic agricultural waste mainly contains glucose, xylose, and arabinose when hydrolyzed. Furthermore, it is an under-utilized renewable biomass with high abundance and low costs [Anand et al., 2023]. With this approach, two waste products could be used to generate value-added products. The deletion of genes for the by-product production is also a possibility to further increase the 1,3-PDO production. This would lead again to higher NADH accumulation, the excess NADH can be used by the NADH-linked 1,3-PDO dehydrogenase. Consequently, with this approach not only more 1,3-PDO will be produced [Zhu et al, 2021], but the accumulation of the toxic intermediate 3-HPA

is reduced, which could result in better growth [Zheng et al., 2006]. To overcome such bottlenecks in 1,3-PDO production, there is a need to enhance the currently limited tools for metabolic engineering of production strains. An overview of a sustainable 1,3-PDO production as well as industrial fields in which 1,3-PDO is utilized can be seen in Figure 2.

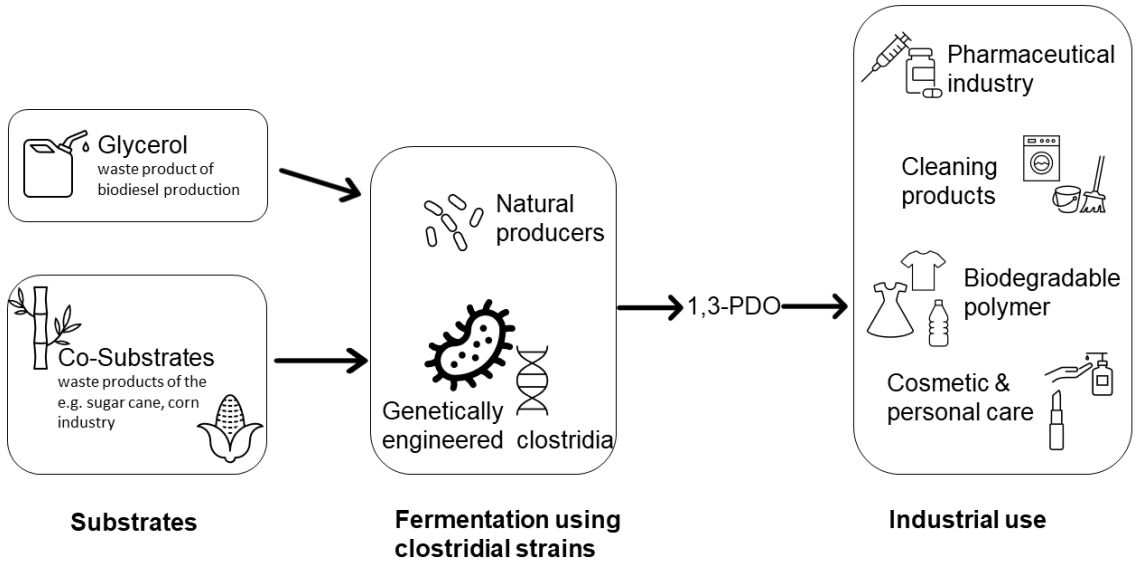

Figure 2: Overview of a sustainable 1,3-PDO production using clostridial strains and industrial fields in which 1,3-PDO is utilized.

1.4 Metabolic engineering of anaerobes

For the construction of bacterial chassis strains for waste-valorization, metabolic engineering tools are necessary. Furthermore, with those tools and techniques the bio-based production of bulk chemicals can be studied and improved, resulting in the development of commercially profitable bio-based synthesis. A lot of manipulation tools and molecular techniques already exist for model organisms such as *Escherichia coli* and *Corynebacterium glutamicum*. For example, plasmid-based gene expression is well established for these model organisms with reliable electrotransformation protocols as well as the required plasmid systems [Nešvera

and Pátek, 2011; Davison, 2002; Kirchner and Tauch, 2003]. Furthermore, multiple genome editing tools are available [Ren et al.,2020; Wang et al., 2021]. Gene expression and protein interactions can easily be studied using e.g. green fluorescent protein (GFP) labeled proteins [Nešvera and Pátek, 2011; Southward and Surette, 2002].

In contrast, the ability to genetically engineer anaerobes is limited and represents the main bottleneck for their industrial use. Especially for Gram-positive bacteria such as clostridial strains and acetogens, electrotransformation is less effective than for Gram-negative strains, due to the extensive exterior network of peptidoglycan of Gram-positive cells. This peptidoglycan network leads to a physically restricted passage of DNA into the bacteria cell [Trevors et al., 2012]. Thus, cell-wall-weakening agents (e. g. DL-threonine) are often used to overcome the thick peptidoglycan network [Pyne et al., 2013; Leang et al., 2013; Aune and Aachmann, 2010]. Additionally, a major barrier for effective genetically manipulation of clostridial strains is the presence of restriction and modification systems [Roslan et al., 2018; Dong et al 2010]. Restriction modification systems are some of the best known and common bacterial defense systems [Oliveira et al., 2016]. The system consists of a methyltransferase that actively discriminates between self and foreign DNA, by transferring methyl groups to particular sequences of the host genome. Furthermore, restriction modification systems contains an endonuclease that recognizes and cleaves DNA, when its specific recognition sequence is unmethylated. Thus, due to the methylation pattern, the cells can differentiate between host DNA and foreign DNA, which will be degraded [Oliveira et al., 2016; Vasu and Nagaraja, 2013; Meselson et al., 1972]. Such defense systems have been reported in *C. acetobutylicum* [Mermelstein et al., 1992], *C. pasteurianum* [Richards et al., 1988], and *Clostridium cellulolyticum* [Jennert et al., 2000]. However, the restriction modification systems can be outmaneuvered using an appropriate methylation pattern to protect the plasmid DNA. Examples include transformation of *C. acetobutylicum,* using *in vivo* methylation in *E. coli* using phage Φ3T I methyltransferase from *Bacillus subtilis* [Mermelstein and Papoutsakis, 1993]. Also, in case of *C. pasteurianum,* a methylated *in vivo* methylation of plasmid DNA must be performed prior to

transformation. Here, an *E. coli* strain producing the methyltransferase M.FnuDII from *Fusobacterium nucleatum* is used [Pyne et al., 2013]. In case of *C. cellulolyticum,* the commercial methyltransferase M.MspI can be used for *in vitro* methylation of plasmid DNA necessary for successful transformation [Jennert et al., 2000].

Even though transformation of anaerobes and especially clostridial species is difficult, many new molecular techniques were developed in the past years such as host-vector systems, gene reporter systems, electrotransformation protocols, antisense RNA gene knockdown, chromosomal gene integration as well as knockout methods [Pyne et al., 2014]. The first electrotransformed clostridial strains were *C. beijerinckii* (formerly *C. acetobutylicum*) [Oultram et al., 1988] and *C. perfringens* [Allen and Blaschek, 1988] in the 1980s. Another major improvement for the genetical manipulation of clostridial strains was the development of a modular system for *Clostridium-E. coli* shuttle vectors by Heap et al. (2009). The next important development was the ClosTron technology in the mid-2000s. With this technique, genes can be knocked out or integrated into the genome of the host strain, allowing a notably faster, more broadly applicable, and less labour-intensive approach for direct mutagenesis for clostridial species [Kuehne and Minton, 2012; Heap et al., 2010; Heap et al., 2007]. With the development of the CRISPR-Cas method for genetical manipulation of bacteria, a similar CRISPR-Cas technique was desired for acetogens and clostridial species [Molitor et al., 2016; McAllister and Sorg, 2019]. In 2013, the first CRISPR-Cas-based genome editing in bacteria was reported [Jiang et al., 2013; Jiang and Marraffini, 2015]. To date, heterologous as well as endogenous CRISPR-Cas systems are available for acetogenic and solventogenic anaerobes [Zhou et al., 2021; Pyne et al., 2016; Nagaraju et al., 2016]. Furthermore, nowadays strong fluorescent reporter proteins have been established in acetogenic and solventogenic anaerobes. For this purpose, the fluorescence-activating and absorption-shifting tag (FAST) was used [Flaiz et al., 2022]. Before, this common method in molecular biology was limited, since oxygen is needed for chromophore maturation of e.g. GFP [Zimmer, 2002]. The new FAST method was already employed to perform promoter activation studies in *Clostridium*

saccharoperbutylacetonicum [Baur et al., 2022]. However, even with the newly established and published methods for genetic manipulation of anaerobes, there are still further improvements necessary.

1.5 Isolation and reclassification of bacteria

The establishment and development of genetical manipulation tools for bacteria play a major role to develop new and rendering existing biobased processes more suitable. However, the search, isolation, and characterization of new species as well as reclassification of already described bacterial species is also of high importance. By finding and characterizing novel bacterial strains, new biological production pathways as well as products can be identified and potentially used for industrial application [Horikoshi, 1995]. Further, newly identified strains can be employed for the utilization of new feedstocks. One example is the use of algae biomass, especially agar, as feedstock for the industrial production of oligomeric sugars and biofuels [Li et al., 2022]. Bioethanol as product is of huge interest as the production is environmental friendly, sustainable and is produced with less greenhouse gas emissions [Li et al., 2014]. Algae cell walls mainly consist of agar, a mixture of the two linear polysaccharides agarose and porphyran [Park et al., 2020]. However, only few terrestrial bacteria species can hydrolyze agarose [Chi et al., 2012]. Therefore, the search for agarolytic marine bacteria and their enzymes, the so-called agarases, gained interest for industrial utilization of algae as feedstock.

On the other hand, the reclassification of already described species based on genomic-based methods is necessary. With the development of easier, faster, and cheaper DNA sequencing methods such as Illumina sequencing and Nanopore sequencing bacterial taxonomy reached a new aera [Paul et al., 2019; Mahato et al., 2017]. The beginning of bacterial taxonomy was in the late 19th century and was based on morphology, physiology and biochemistry [Schleifer, 2009]. In the

early 1960s, the idea to classify bacteria by comparison of their genome was supported by the increasing knowledge of the properties of DNA and the development of molecular techniques [Rosselló-Mora and Amann 2001]. The first genome-based taxonomical method was the wet-lab DNA-DNA-hybridization, however, this technique is time-consuming, labour intensive, expensive, and describes only a rough indirect comparison of two genomes [McCarthy and Bolton, 1963; Hayashi Sant'Anna et al., 2019]. Another widely used method is the 16S rRNA gene sequence analysis. The 16S rRNA gene is a critical component of cell function, therefore mutations in this gene cannot be compensated resulting in a highly conserved sequence. The rate of changes can be used to mark evolutionary distance and relatedness of organisms [Clarridge III, 2004]. Nowadays, a variety of different methods and tools are used to classify new and already described bacterial strains. The so-called modern taxonomic approaches should employ morphology, physiology, and biochemistry characteristics as well as genetic features such as digital DNA-DNA-hybridisation (dDDH), 16S rRNA gene sequence analysis, average nucleotide identity, and average amino acid identity [Mahato et al., 2017]. Reclassification based on genetical features provides the opportunity to group bacteria strains due to their phylogenetic relationships and therefore, harbouring similar products, pathways, and substrate patterns. By that, bacterial taxonomy gets clearer and more manageable. Genetic characterization and classification contribute to a better understanding and identification of gene clusters for substrate utilization, central metabolism, and product formation. Thus, it can serve as important basis in the development of novel bio-based industrial processes.

2. Results & Discussion

All results and corresponding discussions acquired during this thesis are included in the following publications and manuscripts.

2.1. Engineering *Acetobacterium woodii* for the production of isopropanol and acetone from carbon dioxide and hydrogen

Arslan, K., **Schoch, T.**, Höfele, F., Herrschaft, S., Oberlies, C., Bengelsdorf, F., Veiga, M. C., Dürre, P., & Kennes, C. (2022). Engineering *Acetobacterium woodii* for the production of isopropanol and acetone from carbon dioxide and hydrogen. *Biotechnology Journal*, *17*, 2100515.

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Biotechnology Journal

RESEARCH ARTICLE

Engineering *Acetobacterium woodii* for the production of isopropanol and acetone from carbon dioxide and hydrogen

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Abstract

The capability of four genetically modified *Acetobacterium woodii* strains for improved production of acetone from CO_2 and hydrogen was tested. The acetone biosynthesis pathway was constructed by combining genes from *Clostridium acetobutylicum* and *Clostridium aceticum*. Expression of acetone production genes was demonstrated in all strains. In bioreactors with continuous gas supply, all produced acetic acid, acetone, and, surprisingly, isopropanol. The production of isopropanol was caused by an endogenous secondary alcohol dehydrogenase (SADH) activity at low gas-feeding rate. Although high amounts of the natural end product acetic acid of *A. woodii* were formed,14.5 mM isopropanol and 7.6 mM acetone were also detected, showing that this is a promising approach for the production of new solvents from C1 gases. The highest acetic acid, acetone, and isopropanol production was detected in the recombinant *A. woodii* [pJIR750_ac1t1] strain, with final concentrations of 438 mM acetic acid, 7.6 mM acetone, and 14.5 mM isopropanol. The engineered strain *A. woodii* [pJIR750_ac1t1] was found to be the most promising strain for acetone production from a gas mixture of CO_2 and H_2 and the formation of isopropanol in *A. woodii* was shown for the first time.

KEYWORDS
Acetobacterium woodii, acetogen, isopropanol, syngas, Wood-Ljungdahl pathway

1 | INTRODUCTION

Acetone is the simplest and most important ketone and it is a widely used common solvent in industry. Besides, as a raw material, acetone is used in the manufacture of a variety of products such as methyl methacrylate and bisphenol A.[1] The industrial production of acetone through fermentation has a long history, dating back to the early years of the twentieth century. *Clostridium acetobutylicum* is one of, if not the most extensively, studied native species for its ability to produce acetone, together with butanol and ethanol, from different carbon sources through the Embden-Meyerhof-Parnas pathway.[2] This ABE (acetone-butanol-ethanol) process can use many different complex feedstocks, for example, biomass or waste, although it generally requires several steps, for example, saccharification, fermentation, and/or downstream processing, which affect its economics.[3] During World War I and World War II, microbial acetone was manufactured to a large extent for meeting the high demand for the production of smokeless powder. Later, in the 1950s, acetone demand started decreasing. With

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the development of the petrochemical industry, the profitability of oil-based solvent production was high and microbial acetone production faced a huge decline.[4–6] Currently, most of the worldwide acetone supply is based on the cumene process, in which petrochemicals, benzene and propylene, are converted to acetone as the by-product, with phenol as the main product.[7]

Nowadays, microbial bulk chemicals production is again gaining importance due to the need for sustainable and cleaner industrial processes and the growing concern about their environmental impact. A major challenge faced by our planet is the build-up of greenhouse gases (GHGs), such as carbon dioxide, in the atmosphere, leading to global warming and climate change. The primary contributor to these GHGs is the widespread use of fossil resources upon which our society depends for the production of chemicals, fuels, energy, and other materials.[8] Therefore, scientific efforts are underway, searching for new technologies to shift our feedstock dependence from fossil-based carbon to above ground carbon in order to implement a sustainable and circular economy.[9] In that sense, acetogenic bacteria, able to grow on CO_2 and/or CO, represent a promising microbial platform that can combine GHG reduction with fuels and bulk chemicals production.[2] Exhaust emissions from certain heavy industrial processes, such as steel making, may contain gases such as CO_2, CO, and H_2 in different ratios,[10,11] as well as some other minor compounds and impurities.[12] CO, CO_2, and H_2 gas mixtures (i.e., syngas) can also be generated from a variety of renewable or waste-derived feedstocks such as lignocellulosic biomass.[2] The Wood-Ljungdahl pathway (WLP) is considered to be the most efficient and oldest pathway used by acetogenic bacteria for fixing CO or CO_2 gases.[13,14] In this pathway, C1 units are converted into acetyl-CoA, the precursor of metabolic end products and biomass. The historical development and the underlying biochemistry of this pathway have been detailed elsewhere.[15–17] Acetic acid is the main end product of many acetogenic bacteria; however, some strains are naturally able to produce a range of other commodities such as ethanol, butyrate, butanol, 2,3-butanediol, hexanoate (i.e., caproate), and hexanol.[2,18–22] In order to broaden the spectrum of products generated from syngas or to improve the productivity of naturally occurring commodities, metabolic engineering approaches have recently been used and new pathways have been incorporated into acetogenic bacteria.[23] A few studies and successful transformation protocols have recently been developed for the production of ethanol, acetate, poly-3-hydroxybutyrate, and acetone in different acetogenic hosts such as *Clostridium ljungdahlii*, *C. coskatii*, *C. autoethanogenum* and *Acetobacterium woodii*.[24–29]

A. woodii is a model organism of acetogenic bacteria with its genome already sequenced and its energy conservation pathway elucidated.[14,30] Acetone production in recombinant *A. woodii* strains using genes from *C. acetobutylicum* has already been shown.[25] However, some of the enzymes of *C. acetobutylicum* have shortcomings for an envisaged production in heterologous hosts (e.g., the very high K_m value of CoA-transferase[31]). Therefore, the aim of the present study was to improve acetone production by construction and screening of novel recombinant *A. woodii* strains. Autotrophic bioreactor experiments, with continuous CO_2+H_2 gas feed, were performed with four different recombinant strains to compare the growth and metabolites production of four *A. woodii* transformants.

2 | EXPERIMENTAL SECTION

2.1 | Microorganisms and their cultivation

Bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains (DH5α and XL1-Blue MRF') were cultivated under aerobic conditions at 37°C in LB medium.[32] For solid medium, LB medium with 1.5% agar (w/v) was used. The media were supplemented with appropriate antibiotics for cloning purposes. *A. woodii* DSM 1030 and *C. aceticum* DSM 1496 were grown in modified DSMZ medium 135[25] under anaerobic conditions at 30°C. For cultivation of *C. acetobutylicum* DSM 792, CG medium was used,[33] and *C. beijerinckii* DSM 6423 was grown in 2xYTG medium.[34] For solid medium, CG medium with 1.5% agar w/v was used. The strains were cultivated under anaerobic conditions at 37°C. For recombinant strains construction and screening, liquid and solidified media were supplemented with the appropriate antibiotic.

2.2 | Isolation of genomic and plasmid DNA

Bacterial genomic DNA was isolated using the MasterPure GramPositive DNA Purification Kit (Epicentre, Madison, WI). A total 2-ml samples of late exponential cultures were centrifuged (18,000 g, 2 min, 4°C) and further processed according to the manufacturer's instructions. Isolation of plasmid DNA from *E. coli* strains was performed with the Zyppy Plasmid Miniprep Kit (ZYMO Research Europe GmbH, Freiburg, Germany). A total 2- or 4-ml samples of overnight culture were centrifuged (18,000 g, 1 min, RT) and further processed according to the manufacturer's instructions.

2.3 | Construction of acetone production plasmids

Standard molecular cloning techniques were performed according to established protocols.[32] Four different recombinant *A. woodii* strains ([pJIR750_ac1t1], [pMTL84151_act_{bIA}], [pJIR750_act_{bIA}], [pJIR750_ac2t1]) were used in this work.

The two newly constructed acetone production plasmids ([pJIR750_ac1t1] and [pJIR750_ac2t1]) are based on pJIR750_act_{bIA}.[25] Several modifications were performed for further optimization of acetone production in *A. woodii*. An overview of the newly constructed plasmids and their relevant characteristics is shown in Table 1. Primers used in this study are listed in Table 2. Genomic DNA served as template for amplifying via PCR. Restriction enzymes were purchased from Thermo Fischer Scientific. Purification of DNA fragments from agarose gel or solutions after PCR was performed using the Zymoclean Gel DNA Recovery Kit (ZYMO Research Europe GmbH, Freiburg, Germany) and the DNA Clean & Concentrator Kit

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli DH5α	F⁻, ϕ80d*lacZ*ΔM15, Δ(*lacZYA-argF*), U169, *recA*1, *end*A1, *hsd*R17 (rK⁻, mK⁺), *pho*A, *sup*E44, λ-, *thi*-1, *gyr*A96, *rel*A	Life Technologies, Carlsbad, CA, USA
E. coli XL1-Blue MRF'	Δ(*mrcA*)183, Δ(*mrcCB-hsdSMR-mrr*)173, *end*A1, *sup*E44, *thi*-1, *recA*1, *gyrA*96, relA1, *lac*, [F' *proA*B*lacI*^{q} ZΔM 15 Tn10 (Tet^R)]	Agilent Technologies, Santa Clara, CA, USA
A. woodii DSM 1030	wild type	DSMZ GmbH, Brunswick, Germany
C. acetobutylicum DSM 792	wild type	DSMZ GmbH, Brunswick, Germany
C. aceticum DSM 1496	wild type	DSMZ GmbH, Brunswick, Germany
C. beijerinckii DSM 6423	wild type	DSMZ GmbH, Brunswick, Germany
pDrive	ColE1 ori⁻, f1 ori, Ap^R(*bla*), Km^R (*aph*), *lac*POZ'	QiagenGmbH, Hilden, Germany
pJIR750	Cm^r, pMB1*ori*⁻, *lacZ*, pIP404 *ori*⁺	Bannam and Rood (1993)
pMTL84151	Cm^r, ColE1*ori*⁻, *lacZ*, pCD6 *ori*⁺, *traJ*	Heap et al. (2009)
pANS1	p15A ori⁻, Sp^R(*spcE*), Φ3T I	Böhringer, 2002
pIMP1_catP	ColE1 *ori*⁻, pIM13 *ori*⁺, Ap^r (*bla*), Em^r (*ermC*), Cm^r (*catP*)	Linder, unpublished
pMTL84151_act$_{thlA}$	pMTL84151, P$_{thlA}$, *adc*(CA_P0165), *ctfA*/*ctfB*(CA_P0163/CA_P0164), *thlA* (CAC2873) from*C. acetobutylicum*	Hoffmeisteret al. (2016)
pJIR750_act$_{thlA}$	pJIR750, P$_{thlA}$, *adc*(CA_P0165), *ctfA*/*ctfB*(CA_P0163/CA_P0164), *thlA* (CAC2873) from*C. acetobutylicum*	Hoffmeisteret al. (2016)
pJIR750_ac1t1	pJIR750_act$_{thlA}$including native ribosomal binding sites upstream of*adc* aswell as*ctfA*	This study
pJIR750_ac2t1	pJIR750, P$_{thlA}$, *ctfA*/*ctfB* (CACET_c04240/CACET_c04250) from*C. aceticum*	This study
pIMP1_Padc	pIMP1_catP, P$_{adc}$ from*C. acetobutylicum*	This study
pIMP1_Padc_01070	pIMP1_catP, P$_{adc}$ and genewith locus tag Awo_c01070 from*A. woodii*	This study
pIMP1_Padc_17360	pIMP1_catP, P$_{adc}$ and genewith locus tag Awo_c17360 from*A. woodii*	This study
pIMP1_Padc_SADH	pIMP1_catP, P$_{adc}$, *sadh* (CLOBI_40010) from*C. beijerinckii*	This study

TABLE 2 Primers used in this study

Primer	Sequence (5'-3')[1]	Application and plasmid
adc_KpnI_fwd adc_EcoRI_rev	GGTACCAGGAAGGTGACTTTTATG GAATTCTTACTTAAGATAATCATATATAACT	Amplification of *adc* from *C. acetobutylicum*for pDrive+adc$_{RBS}$
ctfA_BamHI_fwd ctfB_KpnI_rev	GGATCCAGGAGGGATTAAAATGAAC GGTACCCTTTCTAAACAGCCATGGGTCTAAG	Amplification of *ctfA*/*ctfB* from *C. acetobutylicum*for pDrive+ctfA/ctfB$_{RBS}$
ctfA/B_C.acet_fwd ctfA/B_C.acetSacII_rev	CATAAAAGTCACCTTCCTGGTACCTTATAATTCCATAACCTTTAAATCATC GCAGAAAAGTGCTAGAAAGGATCCGCGGAGGTGGTAGGTATGAGCC	Amplification of *ctfA*/*ctfB* from *C. aceticum* for pJIR750_ac2t1
Padc_fwd Padc_rev	TGCGATTAAGCTTGGCTGCAGTTATGCAGAATTTTTAGGAAGTG AGTGAATTCCCGGGGATCCGTCGACCCTTCCTAAATTTAATAATGTT-TAGC	Amplification of P$_{adc}$ from *C. acetobutylicum*for pIMP1_Padc
Awo_c01070_fwd Awo_c01070_rev	ATTATTAAATTTAGGAAGGGTCGACAGATGAAAGCGGTATGTATG AGTGAATTCCCGGGGATCCGTTAATTCTCCCCCCATGG	Amplification of Awo_c01070 from *A. woodii* for pIMP1_Padc_01070
Awo_c17360_fwd Awo_c17360_rev	ATTATTAAATTTAGGAAGGGACCGAAGATGAACAATTTTAAC ACGGCCAGTGAATTCCCGGGGATCCTTATAATGCGCGTTGATAAAG	Amplification of Awo_c17360 from *A. woodii* for pIM1_Padc_17360
SADH_CLOBI_fwd SADH_CLOBI_rev	ATTATTAAATTTAGGAAGGGTCGACAGATGAAAGGTTTTGCAATGC ACGGCCAGTGAATTCCCGGGGATCCTTATAATATAACTACTGCTTTAAT-TAAGTC	Amplification of SADH-encoding genefrom *C. beijerinckii* for pIMP1_Padc_SADH

(ZYMO Research Europe GmbH, Freiburg, Germany), respectively. The procedure was carried out as described in the manufacturer manuals.

Plasmid pJIR750_ac1t1 was obtained via a classical ligation approach. The genes *adc*(CA_P0165) and *ctfA/ctfB* (CA_P0163/CA_P0164) from *C. acetobutylicum* including the corresponding ribosomal binding sites were amplified by PCR (ReproFast polymerase; Genaxxon, Ulm, Germany) and ligated into the vector pDrive (QIAGEN GmbH, Hilden, Germany) resulting in the plasmids pDrive+adc_{RBS} and pDrive+ctfA/$ctfB_{RBS}$, respectively. pDrive+adc_{RBS} was digested using the restriction enzymes *Kpn*I and *Eco*RI, and pDrive+ctfA/$ctfB_{RBS}$ with *Bam*HI and *Kpn*I. The resulting fragments were ligated with the linearized plasmid pJIR750_act_{thlA} (digested with *Bam*HI and *Eco*RI), resulting in pJIR750_ac1t1. The construction of the second acetone production plasmid pJIR750_ac2t1 was performed via the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs Inc., Ipswich, MA, USA). The *ctfA/ctfB* genes from *C. aceticum* (CACET_c04240/CACET_c04250) were used to replace the corresponding genes from *C. acetobutylicum*. The genes were amplified from genomic DNA of *C. aceticum* via PCR using CloneAmp HiFi PCR Premix (Takara Bio Inc., Kusatsu, Shiga Prefecture, Japan) and ligated into the linearized pJIR750_ac1t1 plasmid (digested with *Bam*HI and *Kpn*I). *A. woodii* was transformed as described previously.[25]

2.4 | Cloning and analysis of candidate genes for secondary alcohol dehydrogenase (SADH) activity

For identification of the SADH-encoding gene in *A. woodii*, three plasmids were constructed. The plasmids pIMP1_Padc_01070 and pIMP1_Padc_17360 harbor the potential SADH-encoding genes with locus tags Awo_c01070 and Awo_c17360 from *A. woodii*, respectively. For a positive control, pIMP1_Padc_SADH was constructed using the NADPH-dependent isopropanol dehydrogenase gene (CLOBI_40010) from *C. beijerinckii* DSM 6423.[34,35] First, the *adc* promoter was amplified using genomic DNA from *C. acetobutylicum* and cloned into the linearized backbone of pIMP1_catP (digested with *Sal*I and *Pst*I) resulting in pIMP1_Padc. For plasmid pIMP1_Padc_01070, the amplified gene from *A. woodii* was ligated into the plasmid pIMP1_Padc (linearized with *Sal*I). Awo_c17360 was amplified and ligated into the linearized pIMP1_Padc (using *Bam*HI and *Sal*I), obtaining the plasmid pIMP1_Padc_17360. For the construction of pIMP1_Padc_SADH, theSADH-encoding gene was amplified from genomic DNA from *C. beijerinckii* DSM 6423 and inserted into pIMP1_Padc (digested with *Bam*HI and *Sal*I). Constructed plasmids and their relevant characteristics are shown in Table 1, primers used are listed in Table 2.

C. acetobutylicum was transformed with the constructed plasmids. Prior to transformation, the three plasmids were methylated in *E. coli* XL1-Blue MRF' [pANS1].[29] Transformation was performed using a modified protocol of Leang et al.[27] For competent *C. acetobutylicum* cells, 50 ml CG medium[33] were inoculated to an OD_{600} (optical density at 600 nm) of 0.1. Further preparations were performed in an anaerobic cabinet. Cells were harvested via centrifugation (3500 g, 10 min, 4°C) and the supernatant was discarded. *C. acetobutylicum* cells were washed twice with pre-cooled SMP buffer[27] (3500 g, 10 min, 4°C) and the cell pellet was suspended in 600 µl SMP buffer. For electrotransformation, 25 µl of competent cells were transferred into a pre-cooled electroporation cuvette (1 mm; Biozym Scientific GmbH, Hessisch Oldendorf, Germany), containing 5 µg of methylated plasmid DNA. After applying the pulse (0.625 kV, 600 Ω, and 25 µF; Gene PulserXcell, Bio-Rad Laboratories GmbH, München, Germany), *C. acetobutylicum* cells were transferred into 2 ml CG medium. Regeneration took place for at least 4 h at 37°C. Then, 200 µl of transformed cells were plated onto CG medium agar containing the appropriate antibiotic. Colonies were picked, transformation was verified, and the recombinant strains *C. acetobutylicum* [pIMP1_Padc_01070], *C. acetobutylicum* [pIMP1_Padc_17360]and *C. acetobutylicum* [pIMP1_Padc_SADH] were obtained.

For examination of involvement of the candidate SADH-encoding genes from *A. woodii* in isopropanol production, a growth experiment was performed with 200 mM glucose as substrate and the strains *C. acetobutylicum* [pIMP1_Padc_01070], *C. acetobutylicum* [pIMP1_Padc_17360] *C. acetobutylicum* [pIMP1_Padc_SADH] as well as *C. acetobutylicum* wild type. A total 50 ml anaerobic CG medium in 125-ml Müller-Krempel flasks (Müller & Krempel AG, Bülach, Switzerland) were sealed airtight and autoclaved (15 min at 121°C and 1.2 bar pressure). Before inoculation, the medium was supplemented with anoxic sterile solutions of the antibiotic thiamphenicol (25 µg ml^{-1}) and glucose (200 mM). The different *C. acetobutylicum* strains were cultivated under anaerobic conditions at 37°C. Prior to the proper growth experiment (biological triplicates), adaptation was performed by transferring the strains in fresh medium once. During the growth experiments, OD_{600} and pH were monitored, and samples were taken for product analysis.

2.5 | Growth conditions of batch experiments

For batch experiments, *A. woodii* strains were inoculated in modified DSMZ medium 135.[25] For autotrophic growth experiments, *A. woodii* strains were cultivated in 50 ml medium in 500-ml Müller-Krempel flasks. After medium preparation, the flasks were sealed airtight, and the gas phase was exchanged with $N_2 + CO_2$ (80% + 20%; MTI Industriegase AG, Elchingen, Germany). The flasks were autoclaved for 15 min at 121°C and 1.2 bar pressure. After autoclaving, the medium was supplemented with thiamphenicol (25 µg ml^{-1}) and the gas atmosphere of the flasks was changed to 1 bar $H_2 + CO_2$ (67% + 33%; MTI Industriegase AG, Elchingen, Germany). During autotrophic growth experiments, the gas phase was refilled at 0.3 bar pressure. The strains were cultured at 30°C and under shaking conditions (150 rpm). Adaptation was performed by transferring *A. woodii* strains into fresh medium once prior to the growth experiment. During autotrophic growth experiments, OD_{600}, pH, and pressure were monitored. Furthermore, samples were taken for analysis of end products.

6. | Bioreactor studies

1. | Preparation of inocula for bioreactor fermentation

A. woodii cells were cultivated in a medium with the following composition (per liter distilled water): NH_4Cl, 0.20 g; yeast extract, 2 g; KH_2PO_4, 1.76 g; K_2HPO_4, 8.44 g; $MgSO_4 \times 7 H_2O$, 0.33 g; $NaHCO_3$, 10 g; L-Cysteine-HCl, 0.30 g; $Na_2S \times 9 H_2O$, 0.30 g; resazurin (stock solution concentration, 1 g L^{-1}); 1 ml; vitamins solution, 2 ml; trace metals solution, 2 ml; and thiamphenicol, 7.5 mg.

The composition of the vitamins solution was (per liter distilled water): biotin, 25 μg; folic acid, 25 μg; pyridoxine-HCl, 50 μg; thiamine-HCl, 50 μg; riboflavin, 50 μg; nicotinicacid, 50 μg; D-Ca-pantothenate, 50 μg; vitamin B_{12}, 25 μg; *p*-amino benzoic acid, 50 μg and lipoic acid, 25 μg. The composition of the trace metals solution was (per liter distilled water): nitriloacetic acid, 12.8 g; $MnCl_2 \times 4 H_2O$, 0.1 g; NaCl, 5 g; $FeCl_2 \times 4 H_2O$, 2 g; $CoCl_2 \times 6 H_2O$, 0.2 g; $ZnCl_2$, 70 mg; $CuCl_2 \times 2 H_2O$, 2 mg; H_3BO_3, 6 mg; $Na_2MoO_4 \times 2 H_2O$, 36 mg, and $NiCl_2 \times 6 H_2O$, 24 mg. A total 100-ml glass serum bottles, with 40 ml working volume, were used to grow the inoculum. Serum bottle preparation procedure can be found in detail elsewhere.[36] The pH of each bottle was adjusted to 7.5 with either a 2 M NaOH solution or a 2 M HCl solution. $MgSO_4$, fructose, and vitamins were added from anoxic sterile stock solutions to their final concentrations before inoculation. Thiamphenicol (prepared as a solution of 2:1 water:N-N,dimethylformamide) was used as antibiotic.

2.6.2 | Bioreactor fermentations with continuous gas supply

The *A. woodii* strains were cultivated in the same medium as described in the previous section (Section 2.6.1), with gases as the only substrates. The experiments were carried out in 2-L Eppendorf BIOFLO 120 stirred tank bioreactors (Eppendorf AG, Hamburg, Germany) with around 1.3 L working volume for all assays, the details of the bioreactor preparation and the inoculation was given elsewhere.[36]

The bioreactor was equipped with four baffles and a six blade Rushton turbine. A gas mixture, consisting of 40% N_2, 25% CO_2, and 35% H_2, was fed through a microsparger into the reactor at a flowrate of 10 ml min^{-1}. That gas flow rate was maintained constant during the whole experiment by means of a mass flow controller (Aalborg GFC 17, Müllheim, Germany). A BIOFLO 120 condenser was connected to the bioreactors to avoid or minimize any possible gas losses, for example, acetone, using tap water at room temperature (below 20 °C). No gas losses were detected. The pH value of the fermentation broth was monitored on-line with a pH sensor (Mettler Toledo, Columbus, Ohio, USA) and maintained at 7.5 by using either 1 M HCl or 1 M NaOH solutions during all experiments. All fermentation processes were run at a temperature of 30 °C, which was maintained constant by means of a water jacket or a heating blanket. The medium was continuously agitated at 250 rpm.

2.7 | Analytical methods

Several 2-ml liquid samples were withdrawn periodically from the bioreactors during the experiments to carry out analytical tests. The cell concentration was determined by using a spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain, or, Ultrospec 1100 Pro, Amersham Biosciences Europe GmbH, Freiburg, Germany, respectively) at a wavelength of 600 nm (OD_{600}). Fructose, acetic acid, acetone and isopropanol concentrations in bioreactor experiments were determined with a high performance liquid chromatograph (HPLC) HP-1100 (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector and a refractive index detector, at 50 °C. The samples were centrifuged (ELMI Skyline Ltd CM 70M07, Riga, Latvia) at 7000 rpm for 5 min at room temperature and the supernatant was filtered through a 0.22-μm filter. Then, 20 μl of those samples were injected into the Agilent Hi-Plex H Column (300 × 7.7 mm) (Agilent Technologies, Santa Clara, CA, USA) at 45 °C, and a 5 mM H_2SO_4 solution was used as mobile phase with a flow rate of 0.80 ml min^{-1}.

In case of batch experiments, 2-ml samples were withdrawn via syringes from the culture broth and subsequently centrifuged (17,950 g, 30 min, 4 °C). Acetate concentration was measured by an Agilent 1260 Infinity Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector and diode array detector. Twenty microlitres of supernatant were injected onto a CS organic acid precolumn (40 × 8 mm) followed by a CS organic acid column (150 × 8 mm; CS-Chromatographie Service GmbH, Langerwehe, Germany). The column was heated to 40 °C, a mobile phase consisting of 5 mM H_2SO_4 with a flow of 0.7 ml min^{-1} was used. Acetone and isopropanol concentrations were determined using a Clarus 680 gas chromatograph (Perkin Elmer Inc., Waltham, MA, USA) equipped with a flame ionization detector, heated to 300 °C, and a flowrate of synthetic air at 450 ml min^{-1}. H_2 was used as carrier gas (45 ml min^{-1}). Prior to analysis, 480 μl supernatant were acidified with 20 μl 2 M HCl, 1 μl of acidified supernatant was injected onto an Elite-FFAP column (30 m × 0.32 mm; Perkin Elmer Inc., Waltham, MA, USA), injector was heated to 225 °C. For analysis, the following temperature profile was used: 40 °C for 4 min, 40 °C to 250 °C by 40 °C min^{-1}, 250 °C for 1 min.

3 | RESULTS

3.1 | Metabolic engineering and analysis of recombinant strains

For an improved acetone production in *A. woodii* two plasmids were constructed. The first modification of the initial plasmid pJIR750_act$_{thl}$ was the insertion of the native ribosome binding site (RBS) of the acetoacetate decarboxylase (*adc*) (CA_P0165) and CoA transferase (*ctfA*) genes (CA_P0163), resulting in plasmid pJIR750_ac1t1. Another improvement strategy was to exchange *ctfA*/*ctfB* from *C. acetobutylicum* by other clostridial CoA transferase genes, because CtfAB from *C. acetobutylicum* has a very high K_m value

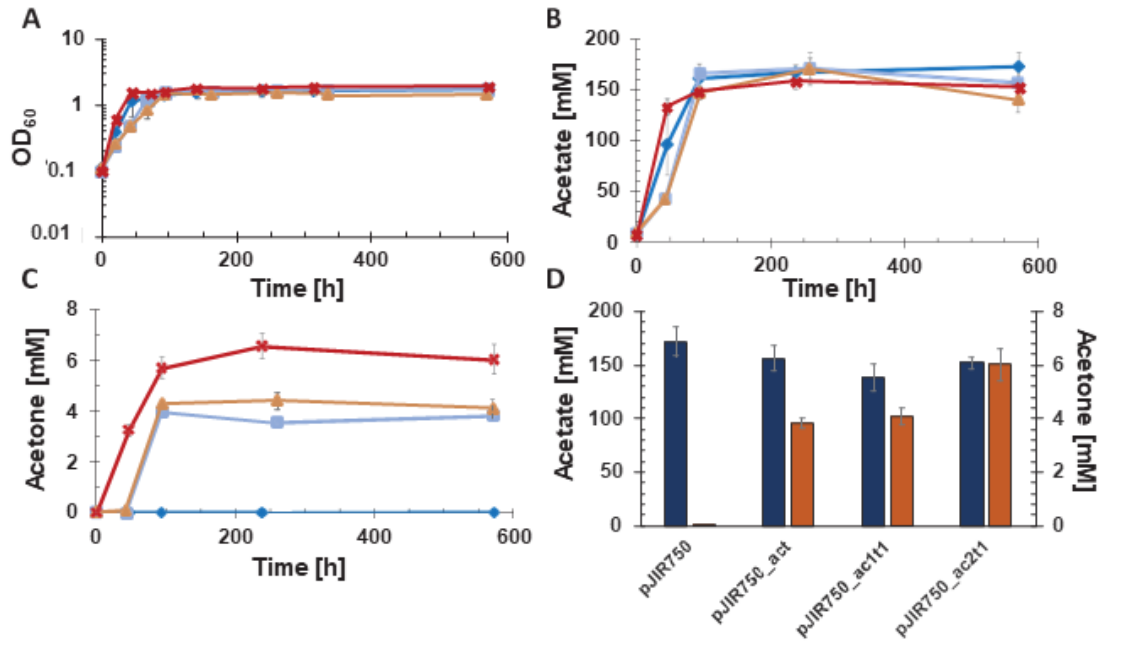

FIGURE 1 Autotrophic batch fermentations of recombinant *A. woodii* strains. *A. woodii* [pJIR750], *A. woodii* [pJIR750_act$_{thlA}$], *A. woodii* [pJIR750_ac1t1], and *A. woodii* [pJIR750_ac2t1] (◆, ■, ▲, and ×) in serum bottles. All data represent the mean with SDs of three biological replicates. (A) Optical density measured at 600 nm; (B) acetate production; (C) acetone production; (D) end concentrations of acetate (blue) and acetone (orange) (SD = Standard Deviation)

for acetate (1200 mM, pH7.5).[31] *Clostridium aceticum* carries two genes with high homology to *C. acetobutylicum ctfA* (CACET_c04240, 86% identity to *ctfA*) and *ctfB* (CACET_c04250, 89% identity to *ctfB*).[37] Enzymatic analysis of crude extracts of *C. aceticum* revealed K_m values for acetate of 44 mM (Lineweaver-Burk plot) and 43 mM (Eadie-Hofstee plot).[37] After subcloning and expression of the two *C. aceticum* genes in *E. coli*, a K_m value for acetate of 18.6 mM was determined (the *E. coli* parent showed a K_m value of 4.2 mM).[38] Thus, this enzyme was better suited than the *C. acetobutylicum* one and was therefore used for construction of pJIR750_ac2t1.

The two newly constructed acetone production strains were examined in a heterotrophic (data not shown) and an autotrophic uncontrolled batch experiment in serum bottles (Figure 1). In the latter experiment, CO_2+H_2 were used as substrates. For comparison, the previously described recombinant strains *A. woodii* [pJIR750_act$_{thlA}$] and *A. woodii* [pJIR750] [25] were used as control. *A. woodii* [pJIR750_ac2t1] and *A. woodii* [pJIR750] showed faster growth than the other two recombinant strains, after 94 h of incubation all tested strains reached the stationary phase at an OD_{600} of 1.6 (Figure 1). Products were mainly produced during the exponential growth phase. *A. woodii* [pJIR750] showed the highest acetate concentration (171.7 mM) and no re-uptake of acetate during the late stationary growth phase. In contrast, acetate concentration in the culture medium of *A. woodii* [pJIR750_act$_{thlA}$], *A. woodii* [pJIR750_ac1t1], and *A. woodii* [pJIR750_ac2t1] decreased during the late stationary phase with final concentrations of 156.1 mM, 138.9 mM, and 152.0 mM, respectively (Figure 1). Acetone was produced by all recombinant strains harboring an acetone production plasmid. In case of the vector control strain *A. woodii* [pJIR750], no acetone was detected. The highest acetone concentration was produced by *A. woodii* [pJIR750_ac2t1] (6.5 mM). Lower acetone concentrations (4.0 mM and 4.3 mM, respectively) were obtained for *A. woodii* [pJIR750_act$_{thlA}$] and *A. woodii* [pJIR750_ac1t1]. Acetone concentrations of culture supernatants of *A. woodii* [pJIR750_act$_{thlA}$], *A. woodii* [pJIR750_ac1t1], and *A. woodii* [pJIR750_ac2t1] dropped slightly during the stationary phase (final concentrations of 3.8 mM, 4.1 mM, and 6.0 mM, respectively) (Figure 1). *A. woodii* [pJIR750_ac2t1] showed a 57.9% higher acetone production than *A. woodii* [pJIR750_act$_{thlA}$], thus meeting the expectations based on the lower K_m value of the alternative CoA transferase. No isopropanol was produced during the autotrophic uncontrolled batch experiment in serum bottles.

3.2 | Bioreactor fermentations with recombinant strains under autotrophic conditions

The bioconversion profile of the engineered strains, under autotrophic conditions, was studied in automated stirred tank reactors (STR), with constant pH regulation and with the same continuous gas supply for each strain. The fermentations were stopped once the concentration

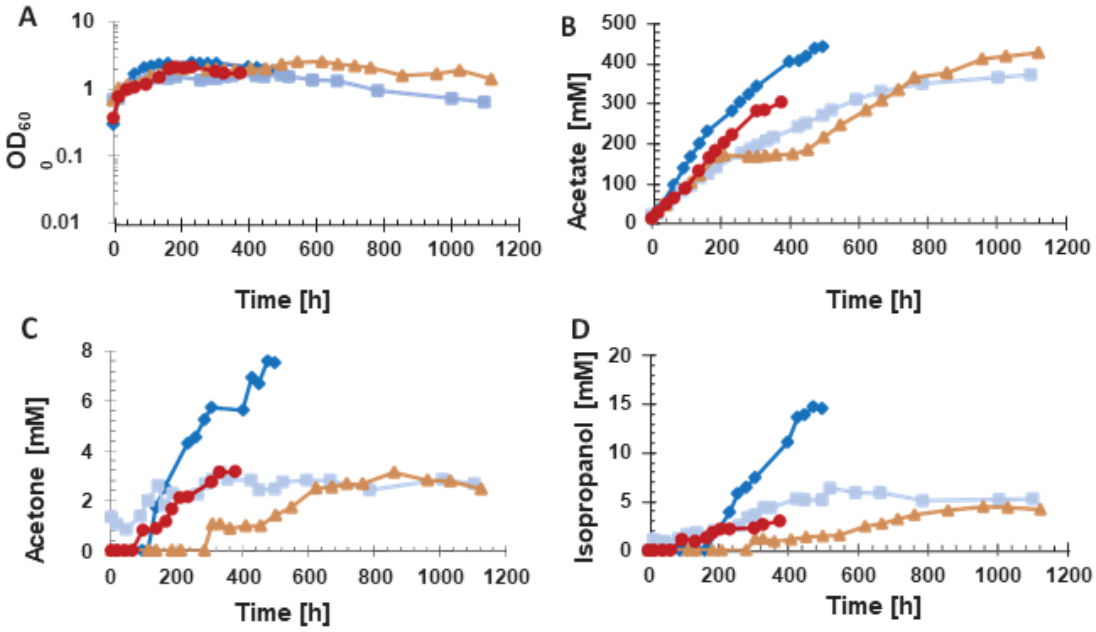

FIGURE 2 Autotrophic batch fermentations of recombinant *A. woodii* strains using stirred tank bioreactors with continuous gas supply. *A. woodii* [pJIR750_ac1t1], *A. woodii* [pJIR750_ac2t1], *A. woodii* [pJIR750_act$_{thlA}$], and *A. woodii* [pMTL84151_act$_{thlA}$] (◆, ■, ▲, and●). (A) Optical density measured at 600 nm; (B) acetate production; (C) acetone production; (D) isopropanol production

of all metabolites reached (near) stable values. In addition to the two newly constructed strains *A. woodii* [pJIR750_ac1t1] and *A. woodii* [pJIR750_ac2t1]) and their parent *A. woodii* [pJIR750_act$_{thlA}$], strain *A. woodii* [pMTL84151_act$_{thlA}$] was included in these experiments, as this strain produced acetone in a shorter period of time than the other ones.[25]

Figure 2 shows the growth and products formation profiles of the recombinant *A. woodii* strains. Bacterial growth and acetic acid production in *A. woodii* [pJIR750_ac1t1] started right after inoculation without any lag phase. A high biomass OD_{600} of 2.4 was achieved 140 h after inoculation and it remained close to that same value up to t = 300 h, reaching a maximum OD_{600} of 2.54 during this period. Later, it decreased continuously, but slowly, until reaching an OD_{600} of 1.8 at the end of the experiment (t = 500 h), though the strain continued producing all three metabolites up to a few hours before stopping the bioreactor. Acetone production in that strain did not start upon inoculation but faced some delay of a few days and only started being detected 135 h after the reactor start-up, when the acetic acid concentration had already reached around 165–200 mM (10–12 g L^{-1}). Surprisingly, also isopropanol appeared in the fermentation medium, about 70 h after acetone production had started and when the acetic acid concentration had already reached 280 mM. At the end of the experiment, the final concentrations of acetic acid, acetone, and isopropanol in the fermentation broth were 438 mM, 7.6 mM, and 14.5 mM, respectively. This results in an acetone:acetic acid ratio of 0.017 and an acetone:isopropanol ratio of 0.52.

The second recombinant strain, *A.woodii* [pJIR750_ac2t1], followed a similar biomass growth profile as the first one. However, a maximum biomass OD_{600} of only around 1.5 was reached and no further increase was then seen after that. Contrary to all other three strains, in this case, acetone and isopropanol accumulation occurred at the same time as acetic acid production and they were all detected immediately upon inoculation. In this strain, compared to acetic acid, there was thus no delay in the production of acetone and isopropanol, contrary to what was observed in all the other three strains. After about 500–600 h, slow and gradual biomass decay took place and, at the same time, accumulation of any metabolites did also slow down or even stop. The production of acetone did actually already reach steady state a few hours before the other metabolites, as it was the first one whose production stopped. After 1100 h, at the end of the experiment, basically no further acetic acid accumulation was observed, and its concentration remained nearly constant, while acetone and isopropanol production leveled off sooner. The highest acetone concentration was reached at t = 330 h, while isopropanol production continued to increase and reached its maximum concentration at t = 520 h. The maximum acetic acid, acetone, and isopropanol concentrations detected by HPLC were 373 mM, 3.2 mM, and 6.3 mM, respectively. In this case, the acetone:acetic acid ratio was 0.008 and the acetone:isopropanol ratio was 0.49.

Apparently, two distinctive growth phases and slower growth were observed in the biomass profile of the third recombinant strain, *A.woodii* [pJIR750_act$_{thlA}$]. Each of the two growth phases appeared to

be simultaneous to fast exponential productions of acetic acid, while a clear plateau of several days was observed in terms of growth as well as acetic acid production in between those two exponential phases. Acetone and isopropanol production both started near the beginning of that plateau and inactive growth phase. After reaching an OD_{600} value of 2, at t = 185 h, the biomass concentration remained nearly constant until reaching t = 450 h. Even though acetone and isopropanol first appeared in the HPLC analysis during this period, no considerable changes in the concentrations of any metabolites were observed. Later on, the second growth phase started, the biomass OD_{600} value reached 2.6 at t = 620 h, and an additional increase in the formation of all metabolites, that is, acetic acid, acetone, isopropanol, took place. After t = 1122 h, near the end of the experiment, the concentrations of metabolites had reached nearly constant values, and a maximum acetic acid concentration of 427.3 mM was measured. Maximum accumulation of acetone and isopropanol was also observed, corresponding to 3.2 mM and 4.4 mM, respectively. The acetone:acetic acid ratio was 0.007 and the acetone:isopropanol ratio was 0.73.

The biomass growth pattern of the fourth recombinant strain, *A. woodii* [pMTL84151_act$_{thlA}$], was somehow similar to the third one. Two clear distinct growth phases were observed and even though a fast growth was observed on the first day of the process, once the biomass OD_{600} value was close to 1, growth reached a kind of short plateau and a decrease in the growth rate was observed between t = 40 and 96 h. Later on, a second faster growth phase was observed, simultaneous with the initial production of acetone and isopropanol, and a maximum biomass OD_{600} value of 2.1 was reached, 166 h after inoculation. Here again, acetic acid accumulation was observed immediately after inoculation, while there was a delay in acetone and isopropanol production. Acetic acid accumulation was also slower than with the first recombinant strain and, after t = 375 h, acetic acid reached its maximum concentration of 302 mM in the fermentation medium. Acetone and isopropanol first appeared 100 h after inoculation, when the acetic acid concentration was 86 mM. Unlike the previous recombinant strain, here acetone and isopropanol were produced simultaneously and at very similar rates. At the end of the process their final concentrations were 3.2 mM and 3 mM, respectively. In terms of ratios, this means an acetone:acetic acid ratio of 0.011, which is lower than in the previous strain, and an acetone:isopropanol ratio of 1.07.

3.3 | Attempts to identify the SADH activity in *A. woodii*

In an attempt to identify the gene encoding the SADH responsible for isopropanol formation in *A. woodii*, two candidate genes (locus tags Awo_c01070 and Awo_c17360) were examined. The gene with the locus tag Awo_c01070 is annotated as a galactitol 1-phosphate 5-dehydrogenase. This gene was selected as a candidate because it shows 33% identity at the protein level to the SADH from *Clostridium ljungdahlii* (CLJU_c24860). The gene Awo_c17360 is annotated as an iron-containing alcohol dehydrogenase and was therefore selected as well. Both genes were subcloned under control of the promoter of the acetoacetate decarboxylase gene (*adc*) of *C. acetobutylicum* in the vector backbone pIMP, resulting in plasmids pIMP1_Padc_01070 and pIMP1_Padc_17360 (Table 1). P_{adc} was chosen to ensure gene expression during acetone production in *C. acetobutylicum*. Both plasmids were transformed into *C. acetobutylicum* and growth experiments were performed. As a positive control, the strain *C. acetobutylicum* [pIMP1_Padc_SADH] was used, harboring the known SADH-encoding gene from *C. beijerinckii* DSM 6423 (CLOBI_40010). Successful isopropanol production in recombinant *C. acetobutylicum* carrying the *C. beijerinckii* SADH-encoding gene has already been reported.[34] As a negative control, *C. acetobutylicum* wild type strain was used. As shown in Fig. 3, the strains *C. acetobutylicum* [pIMP1_Padc_SADH], *C. acetobutylicum* [pIMP1_Padc_01070], and *C. acetobutylicum* [pIMP1_Padc_17360] showed a similar growth behaviour and product pattern (Table 3). No isopropanol could be detected in supernatants of the strains *C. acetobutylicum* [pIMP1_Padc_01070], *C. acetobutylicum*[pIMP1_Padc_17360], and the wild type. The positive control *C. acetobutylicum* [pIMP1_Padc_SADH] produced 11.6 mM isopropanol in the early stationary growth phase. During further cultivation, isopropanol concentration decreased to a final value of 9 mM (Figure 3).

4 | DISCUSSION

The uncontrolled batch experiments indeed revealed a better acetone production in recombinant *A. woodii* when another CoA transferase was induced. However, in bioreactor fermentations with continuous gas supply the strain carrying a construct with improved RBS performed best.

High growth rates and high biomass concentrations are required to achieve high metabolite concentrations and process productivity. *A. woodii* [pJIR750_ac1t1] and *A. woodii* [pJIR750_act$_{thlA}$] showed the highest biomass concentrations amongst all transformants. However, *A. woodii* [pJIR750_act$_{thlA}$] reached its maximum biomass OD_{600} value 550 h after the beginning of the process, whereas *A. woodii* [pJIR750_ac1t1] reached its maximum biomass OD_{600} value at t = 160 h. The maximum biomass OD_{600} for *A. woodii* [pJIR750_ac1t1] was 2.54, which is higher than that reported recently for the genetically engineered acetone-producing *A.woodii* [pMTL84151_act$_{thlA}$] autotrophically grown in a batch operated stirred-tank bioreactor.[25]

A.woodii is one of the most studied acetogens and it is known to have a high autotrophic acetic acid formation performance.[39] In the present study, acetic acid was the main product of all four studied engineered strains as well. The highest acetic acid concentrations were observed with *A. woodii* [pJIR750_ac1t1] and *A.woodii* [pJIR750_act$_{thlA}$], reaching 438 mM and 427.3 mM, respectively. The most important difference between these transformants was again the process duration. About 60% of the total produced acetic acid accumulated during the last 670 h of the process with *A.woodii* [pJIR750_act$_{thlA}$], while the same amount acetic acid was already pro- duced in only 230 h after inoculation with *A. woodii* [pJIR750_ac1t1]. In batch fermentation of *A. woodii* [pMTL84151_act$_{thlA}$], three times

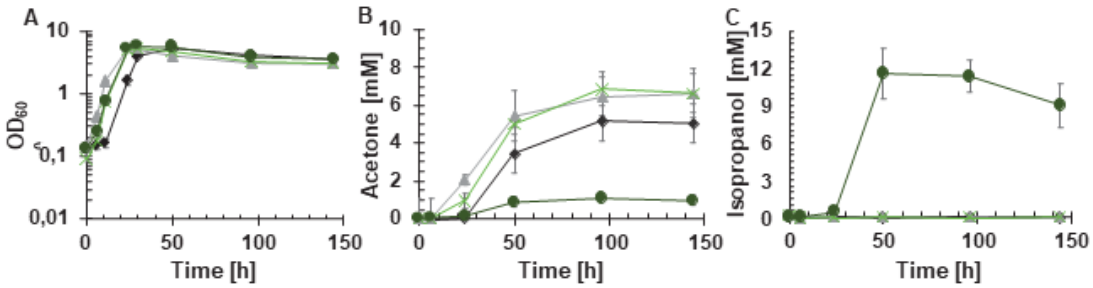

FIGURE 3 Heterotrophic batch fermentations of *C. acetobutylicum* wild type and recombinants. ♦, *C. acetobutylicum* wild type; ▲, *C. acetobutylicum* [pIMP1_Padc_01070]; ×, *C. acetobutylicum* [pIMP1_Padc_17360]; ●, *C. acetobutylicum* [pIMP1_Padc_SADH]. Experiments were performed in serum bottles. All data represent the mean with SDs of three biological replicates. (A) Optical density measured at 600 nm, (B) acetone production, (C) isopropanol production

TABLE 3 Growth and product formation of *C. acetobutylicum* strains with SADH- and putative SADH-encoding genes

		max. product concentrations [mM]	
Strains	max. OD_{600}	acetone	isopropanol
C. acetobutylicum wild-type	5.2 ± 0.1	5.2 ± 0.2	-
C. acetobutylicum pIMP1_Padc_01070	5.5 ± 0.3	6.6 ± 1.0	-
C. acetobutylicum pIMP1_Padc_17360	5.5 ± 0.3	6.9 ± 0.9	-
C. acetobutylicum pIMP1_Padc_SADH	5.9 ± 0.1	1.1 ± 0.1	11.6 ± 1.3

-, not detected.
All data represent the mean with SDs of three biological replicates.

higher acetic acid concentration (1330 mM) was reported,[25] in a shorter process time (360 h) and with a lower biomass accumulation (OD_{max} = 2.2), than what was observed with *A. woodii* [pJIR750_ac1t1] in the present study . A possible explanation for such difference between the productivities of the mutant strains could certainly be the operating process conditions. The poor aqueous solubility of the gas components is considered to be among the main reasons for low productivities of gas fermentation processes. In order to cope with this problem, increased agitation as well as higher gas flow rates and pressures were successfully applied in some studies and appeared to improve the gas fermentation process.[39,40] However, this does also significantly increase operating costs, which may then become prohibitive at large scale. Slower agitation and low gas feeding rates applied in the present study reduce gas transfer and substrate availability to the biomass, resulting in reduced metabolic activities and lower production of metabolites. The gas flow rate was as much as 50 times lower in the present study (10 vs. 500 ml min^{-1}) compared to the previously reported one and the gas fed to the bioreactor in this case contained N_2 besides H_2 and CO_2. Other gases than H_2, CO_2, or CO, for example, N_2, can actually also be found in syngas and in many industrial emissions.

A. woodii [pJIR750_ac1t1] produced the highest concentration of acetone, reaching 442 mg L^{-1}. A total amount of 16.5 mM acetone production was reported before[25] with the recombinant strain *A. woodii* [pMTL84151_act_{bhA}] in batch gas fermentation and 52 mM acetone, so far the highest acetone accumulation in an engineered acetogen, from continuous gas fermentation with cell retention. Despite lower metabolic activities and lower acetic acid and acetone production in *A. woodii* [pJIR750_ac1t1], due to the lower substrate supply and consumption, a significant improvement can still be seen, considering the acetone to acetic acid ratio, observed in these recombinant strains. To the best of our knowledge, the acetone:acetic acid ratio of 0.017 reached in *A. woodii* [pJIR750_ac1t1], is the highest ever reported ratio, which may therefore be considered to perform better in terms of acetone production.

Interestingly, isopropanol was found in all our recombinant strains while this was not the case in the experiments of Hoffmeister et al..[25] The analytical system for batch fermentations used in that study,[25] however, did not allow separation of acetone and isopropanol. Acetone and isopropanol peaks may easily overlap in chromatographic analyses, if not optimized, and could have been the case in that previous study. This might be the reason for the higher acetone concentration, due to additional undetected isopropanol formation, compared to the values reported here. Successful expression of the acetone production pathway genes in the acetogenic bacteria *C. ljungdahlii* and *A. woodii* was shown before.[25,41] Here, acetone was pro- duced autotrophically by the four recombinant strains under study. However, accumulation of isopropanol in the fermentation medium

was, surprisingly, also detected in the bioreactor experiments of all recombinants.

Isopropanol, also called isopropyl alcohol, is another important valuable organic molecule which is used in pharmaceutical, cosmetics, and painting. Isopropanol is also utilized as a gasoline and diesel additive and also disinfection is one of its most important applications.[42] The market demand of isopropanol reaches 2 million tons per year, with growing demand as a result of the recent pandemic.[43] Traditionally, isopropanol has been manufactured with two possible methods, that is, indirect hydration of propylene and direct hydration of propylene.[42] Isopropanol can be produced through fermentation as well and *C. beijerinckii* is one of the major isopropanol-producing bacteria using sugars or lignocellulosic hydrolysate materials as carbon source.[43,44] It is also known that some gas fermenting acetogens, such as *C. ragsdalei*, are able to convert exogenous acetone into isopropanol,[45–47] with the function of a single SADH. Even though *A. woodii* was reported not to have such primary-SADH in its genome,[25,46] in the present study acetone conversion to isopropanol was observed in all recombinants. The highest maximum isopropanol concentration of 14.5 mM was observed in *A. woodii* [pJIR750_ac1t1], two times more than the maximum acetone concentration (7.6 mM) observed in this transformant.

Studies describing the production of isopropanol in syngas-fermenting engineered acetogens are very scarce. Recently, isopropanol formation was confirmed in an engineered *C. ljungdahlii* [48] and to the best of our knowledge the highest isopropanol production of 648 mg L^{-1}, which is 75% of what was observed in the present study (875 mg L^{-1}), from an engineered strain, was reported in *C. autoethanogenum*.[49] After submission of this manuscript, a report by Jia et al. was published, describing the production of up to 13.4 g L^{-1} by a recombinant *C. ljungdahlii* .[50] Considering these results, it can be concluded that *A. woodii* might have an alternative acetone-isopropanol conversion pathway in its metabolism and it could be a better isopropanol producer. Unfortunately, the respective gene could not yet be identified. This should be a further target for optimization.

5 | CONCLUSIONS

Production of acetone and isopropanol from CO_2 is possible with engineered strains. According to the findings, *A. woodii* [pJIR750_ac1t1] was shown to be the most promising engineered strain for an efficient acetone production from a gas mixture of CO_2 and H_2 in terms of biomass growth and metabolite production. It also produced the highest amount of isopropanol from anaerobic gas mixtures reported so far. The metabolism of isopropanol formation in *A. woodii* could be a new target for future improvement.

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CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

PERMISSION STATEMENT

The manuscript does not contain experiments with animals or humans.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions

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2.2. Heterologous 1,3-propanediol production using different recombinant *Clostridium beijerinckii* DSM 6423 strains

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Heterologous 1,3-Propanediol Production Using Different Recombinant *Clostridium beijerinckii* DSM 6423 Strains

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Abstract: 1,3-propanediol (1,3-PDO) is a valuable basic chemical, especially in the polymer industry to produce polytrimethylene terephthalate. Unfortunately, the production of 1,3-PDO mainly depends on petroleum products as precursors. Furthermore, the chemical routes have significant disadvantages, such as environmental issues. An alternative is the biobased fermentation of 1,3-PDO from cheap glycerol. *Clostridium beijerinckii* DSM 6423 was originally reported to produce 1,3-PDO. However, this could not be confirmed, and a genome analysis revealed the loss of an essential gene. Thus, 1,3-PDO production was genetically reinstalled. Genes for 1,3-PDO production from *Clostridium pasteurianum* DSM 525 and *Clostridium beijerinckii* DSM 15410 (formerly *Clostridium diolis*) were introduced into *C. beijerinckii* DSM 6423 to enable 1,3-PDO production from glycerol. 1,3-PDO production by recombinant *C. beijerinckii* strains were investigated under different growth conditions. 1,3-PDO production was only observed for *C. beijerinckii* [pMTL83251_$P_{ptb\text{-}adc}$_1,3-PDO.diolis], which harbors the genes of *C. beijerinckii* DSM 15410. By buffering the growth medium, production could be increased by 74%. Furthermore, the effect of four different promoters was analyzed. The use of the constitutive *thlA* promoter from *Clostridium acetobutylicum* led to a 167% increase in 1,3-PDO production compared to the initial recombinant approach.

Keywords: *Clostridium beijerinckii*; *Clostridium pasteurianum*; *Clostridium diolis*; 1,3-propanediol production; glycerol fermentation; promoter effects

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1. Introduction

The demand for 1,3-propanediol (1,3-PDO) has been increasing in the last few years and will continue to rise in the coming years. With a "Compound Annual Growth Rate" of 14.2% as of 2018, an optimistic market expectation for 1,3-PDO is US$ 1,442.77 million in 2027 [1]. 1,3-PDO is an important basic chemical and is widely used as an organic solvent in the food, cosmetics, and pharmaceutical industries. However, the main application of 1,3-PDO is in the polymer industry as a raw material or intermediate, especially as a key monomer in the production of polytrimethylene terephthalate [2,3]. In the past, ethylene oxide hydroformylation and acrolein hydration-hydrogeneration were the two processes mainly used for the chemical synthesis of 1,3-PDO. Both processes depend on petroleum products as precursors for 1,3-PDO production [4–6]. However, those processes have many disadvantages, such as high investment, technical difficulties, substrate toxicity, and environmental issues [2,7]. Therefore, a biobased synthesis of 1,3-PDO is desirable.

A biosynthetic 1,3-PDO production approach was developed by DuPont and Genencor in the early 2000s using recombinant *Escherichia coli* cells and glucose as substrate [8]. The process was commercialized by DuPont Tate & Lyle in Loudon (USA) in 2006, with a production capacity of 63,500 tons per year [9–11]. Another possibility is the use of glycerol or crude glycerol as a substrate for the microorganisms [12,13]. About 10% crude glycerol is generated as the main by-product from the biodiesel production. With the expanding biodiesel market, the availability of crude glycerol has increased over the last few years. As

a result, the price of crude glycerol is dropping [14,15]. Cheap substrates are of particular importance since the cost of substrates accounts for 50–60% of the total production cost of 1,3-PDO [16]. Thus, the use of cheap glycerol from biodiesel as a substrate for the production of 1,3-PDO could be a good alternative to the original chemical production routes. Therefore, the establishment of a biological production of 1,3-PDO via fermentation of glycerol is desired for a sustainable 1,3-PDO production.

Several microorganisms are known to naturally reduce glycerol to 1,3-PDO including *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Enterobacter agglomerans* [17–19]. A big disadvantage of some of these microorganisms is their pathogenicity and the associated restrictions on industrial use [7,19,20]. However, a few clostridial species are also able to use glycerol for the production of 1,3-PDO such as *Clostridium pasteurianum*, *Clostridium butyricum*, *Clostridium diolis* (reclassified now as *Clostridium beijerinckii* [21]), and some *Clostridium beijerinckii* strains [22–26]. These different clostridial species harbor different glycerol reduction pathways. In the case of *C. pasteurianum*, glycerol is dehydrated via a coenzyme B_{12}-dependent glycerol dehydratase (DhaB, DhaC, DhaE) to 3-hydroxypropionaldehyde (3-HPA), followed by the conversion of 3-HPA via a NADH-linked 1,3-PDO dehydrogenase (DhaT) to 1,3-PDO [27]. The glycerol reductive pathway of *C. butyricum* and *C. beijerinckii* consists of a coenzyme B_{12}-independent glycerol dehydratase (DhaB1, DhaB2) for the dehydration of glycerol to 3-HPA, also followed by the conversion of 3-HPA to 1,3-PDO via a NADH-linked 1,3-PDO dehydrogenase (DhaT) [28,29]. However, glycerol is not only reduced to 1,3-PDO but also oxidized and used for biomass and by products e.g., acetate, butyrate, ethanol, and butanol [30]. An overview of the glycerol consumption pathway is given in Figure 1.

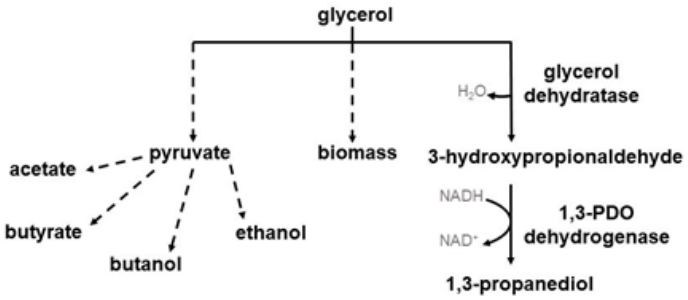

Figure 1. Overview of the glycerol consumption pathway in clostridial species. Glycerol can either be converted reductively, forming 3-HPA and finally 1,3-PDO, or used for biomass and the production of byproducts.

In the past, most publications focused on the conversion of glycerol to 1,3-PDO with wild-type clostridial strains. Clostridia are difficult to manipulate genetically, due to the limited availability of genetic tools as well as the occurrence of native restriction-modification systems, resulting in major obstacles in obtaining recombinant strains [31,32]. Therefore, development of new genetic tools and methods to overcome restriction-modification systems are essential for the construction of an optimized production strain for the waste valorization from glycerol to 1,3-PDO in the future. One of these new developments is the newly published transformation protocol for *C. beijerinckii* DSM 6423 [31], which was used in this study to create recombinant 1,3-PDO production strains.

The two different 1,3-PDO production pathways form *C. pasteurianum* DSM 525 and *C. beijerinckii* DSM 15410 were expressed in *C. beijerinckii* DSM 6423 in a plasmid-based manner. Furthermore, the effect of different promoters on 1,3-PDO production was examined in this study. The results presented here demonstrate the heterologous production of 1,3-PDO with genetically engineered *C. beijerinckii* DSM 6423 strains for the first time.

2. Material & Methods

1. Strains and Cultivation

Bacterial strains used in this study are listed in Table 1. *C. beijerinckii* strains were cultivated under anaerobic conditions at 37 °C. YTG medium was used as a complex medium, for solid medium 2YTG medium containing 1.5% (*w*/*v*) agar was prepared. 2YTG medium contained (per L): tryptone, 16 g; yeast extract, 10 g; NaCl, 5 g; glucose, 20 g [31]. Growth experiments were performed in minimal medium referred to as glycerol medium. Glycerol medium contained (per L): K_2HPO_4, 4 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; yeast extract, 1 g; cysteine-HCl · H_2O, 1.2 g; resazurin, 1 mg; trace element solution, 1 mL. Composition of trace element solution (per L): $CoCl_2 \cdot 2H_2O$, 0.2 g; $CuCl_2 \cdot 2H_2O$, 0.02 g; H_2BO_3, 0.06 g; HCl (37%), 0.9 mL; $MnCl_2 \cdot 4H_2O$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.035 g; $NiCl_2 \cdot 6H_2O$, 0.025 g; $ZnCl_2$, 0.07 g. In case of growth experiments with buffered medium, glycerol medium was prepared with 10.46 g/L 3-(N-morpholino)propanesulfonic acid (MOPS). For the construction and growth of recombinant *C. beijerinckii* strains, media were supplemented with the appropriate antibiotic. *E. coli* strains were cultivated aerobically under shaking conditions (180 rpm) at 37 °C in LB medium [33]. The solid medium contained 1.5% (*w*/*v*) agar. For cloning purposes, media were supplemented with the respective antibiotic.

Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Features	Reference
C. beijerinckii DSM 6423	wild-type	DSMZ * GmbH, Brunswick, Germany
E. coli XL1-Blue MRF'	Δ(*mrcA*)183, Δ(*mrcCB-hsdSMR-mrr*)173, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac*, [F' *proAB lacI*^{q} ZΔ*M15* Tn10 (Tet^R)]	Agilent Technologies, Santa Clara, CA, USA
E. coli SCS110	*rpsL*, (Str^R), *thr*, *leu*, *endA*, *thi-1*, *lacy*, *galK*, *galT*, *ara*, *tonA*, *tsx*, *dam*, *dcm*, *supE44D*, (*lac-proAB*), [F' *traD36 proAB lacI*^{q}ZΔ*M15*]	Agilent Technologies, Santa Clara, CA, USA
pMTL83251	clostridial shuttle vector; Em^R	[34]
pMTL83251_P*thlA*_FAST	pMTL83251 containing P*thlA* from *C. acetobutylicum* and *feg* (FAST-encoding gene)	[35]
pMTL83251_P*bgaL*_FAST	pMTL83251 containing P*bgaL* from *C. perfringens* and *feg*	[35]
pMTL83251_P*bld*_FAST	pMTL83251 containing P*bld* from *C. saccharoperbutylacetonicum* and *feg*	[36]
pMTL83251_P*pta-ack*_1,3-PDO.CLOBI	pMTL83251 containing 1,3-PDO gene cluster from *C. beijerinckii* DSM 6423 and *pta-ack* promoter from *C. ljungdahlii*	This study
pMTL83251_P*pta-ack*_1,3-PDO.diolis	pMTL83251 containing 1,3-PDO gene cluster from *C. beijerinckii* DSM 15410 and *pta-ack* promoter from *C. ljungdahlii*	This study
pMTL83251_P*thlA*_dhaBCET.Cpas	pMTL83251 containing *dhaBCE* and *dhaT* from *C. pasteurianum* DSM 525 and *thlA* promoter from *C. acetobutylicum*	This study
pMTL83251_P*bgaL*_1,3-PDO.diolis	pMTL83251 containing 1,3-PDO gene cluster from *C. beijerinckii* DSM 15410 and *bgaL* promoter from *C. perfringens*	This study
pMTL83251_P*thlA*_1,3-PDO.diolis	pMTL83251 containing 1,3-PDO gene cluster from *C. beijerinckii* DSM 15410 and *thlA* promoter from *C. acetobutylicum*	This study
pMTL83251_P*bld*_1,3-PDO.diolis	pMTL83251 containing 1,3-PDO gene cluster from *C. beijerinckii* DSM 15410 and *bld* promoter from *C. saccharoperbutylacetonicum*	This study

* German Collection of Microorganisms and Cell Cultures (DSMZ).

2.2. Construction of Recombinant C. beijerinckii Strains

Standard molecular cloning techniques were performed according to established protocols [33]. All primers used for the construction of the different 1,3-PDO production plasmids are listed in Table 2. Primers were designed using the "NEBuilder®" online tool and synthesized by Biomers.net GmbH (Ulm, Germany). Genomic DNA or plasmid DNA served as a template for amplification via PCR. Amplification was performed using "CloneAmp™ HiFi polymerase" (Takara Bio USA, Inc., Mountain View, CA, USA) or "Phusion™ Green High-Fidelity DNA Polymerase" (Thermo Fischer Scientific Inc., Waltham, MA, USA). Linearization of vectors and plasmids was achieved using "FastDigest™ restriction enzymes" (Thermo Fischer Scientific Inc., Waltham, MA, USA). Purification of DNA fragments from agarose gels or solutions after PCR as well as after plasmid linearization were performed using the "Zymoclean™ Gel DNA Recovery Kit" (ZYMO Research Corp., Irvine, CA, USA) or the "DNA Clean &Concentrator® Kit" (ZYMO Research Corp., Irvine, CA, USA), respectively. The procedure was carried out as described by the manufacturer. Assembly of purified DNA fragments was performed with the "NEBulder® HiFi DNA Assembly Master Mix" (New England Biolabs® Inc., Ipswich, MA, USA), according to the manufacturer's instructions. After assembly, 3.5-5 µL of cloning mixture were used to transform the chemically competent *E. coli* strain XL1-Blue MRF'. Plasmid preparation from *E. coli* cells was performed using the "Zyppy™ Plasmid Miniprep Kit" (ZYMO Research Corp., Irvine, CA, USA) following the manufacturer´s instructions. The accuracy of the desired plasmid was checked by an analytic digestion, again using "FastDigest™ restriction enzymes" (Thermo Fischer Scientific Inc., Waltham, MA, USA). Subsequently, plasmids that showed the expected DNA fragments were sent to Microsynth AG (Balgrach, Switzerland) for sequencing. An overview of the newly constructed plasmids and their relevant characteristics is shown in Table 1.

Table 2. Primers used in this study.

Primer	Sequence	Purpose
dhaB1/2CoT.diol_fwd dhaB1/2CoT.diol_rev	ttaaatttaaagggaggactctagaatgataagtaaaggatttagtacc gcaggcttcttatttttatgctagcttaataagcagctttaaatatatttacg	amplification of 1,3-PDO gene cluster from *C. beijerinckii* DSM 15410
PthlA.Cpas_fwd PthlA.Cpas_rev	cgaattcgagctcggtacccgggtcaagaagaggcacctcatc ccctcctggtcaccaaattttgatacggggtaacag	amplification of *thlA* promoter from *C. acetobutylicum*
dhaBCE_fwd dhaBCE_rev	aaatttggtgaccaggaggggatcccatgaagtcaaaacgatttcaag aattcctcctctagtcctctattctaacttatttc	amplification of *dhaBCE* from *C. pasteurianum* DSM 525
dhaT_fwd dhaT_rev	agaggactagaggaggaattataaaatgagaatg atggacgcgtgacgtcgactttaaaatgcttctctaaatattttaactatatc	amplification of *dhaT* gene from *C. pasteurianum* DSM 525
PbgaL_1,3-PDO_fwd PbgaL_1,3-PDO_rev	cgaattcgagctcggtacccgggtaatttagatattaattctaaattaagtgaaat taaatcctttacttatcattctcgagaccctcccaatacatttaaaataa	amplification of *bgaL* promoter from pMTL83251_P_{bgal}_FAST
PthlA_1,3-PDO_fwd PthlA_1,3-PDO_rev	cgaattcgagctcggtacccgggtcaagaagaggcacctcatc taaatcctttacttatcattctcgagacctctctaaattttgatacgg	amplification of *thlA* promoter from pMTL83251_P_{thlA}_FAST
Pbld_1,3-PDO_fwd Pbld_1,3-PDO_rev	cgaattcgagctcggtacccggggatatttcccccataagtaaag taaatcctttacttatcattctcgagtcctccttatgatttaaaaattaataac	amplification of *bld* promoter from pMTL83251_P_{bld}_FAST
16S-27F 1492r	ataagcttggatccagagtttgatcctggctcag actcgaggatatcggttaccttgttacgactt	amplification of 16S rRNA gene

To construct pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis, the plasmid pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.CLOBI was digested using the restriction enzymes *Xba*I, *Hind*III, and *Nhe*I to remove the 1,3-PDO gene cluster from *C. beijerinckii* DSM 6423. The PCR-amplified 1,3-PDO gene cluster from *C. beijerinckii* DSM 15410 (locus tag K684DRAFT_00976-00979; primers: dhaB1/2CoT.diol_fwd and dhaB1/2CoT.diol_rev) was cloned into the digested pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.CLOBI, still harboring the *pta-ack* promoter from *C. ljungdahlii*. For cloning of pMTL83251_P_{thlA}_dhaBCET.Cpas, plasmid pMTL83251 was linearized using restriction enzymes *Sal*I and *Sma*I. Digested plasmid was ligated with P_{thlA} (primers: PthlA.Cpas_fwd and PthlA.Cpas_rev) amplified from *C. acetobutylicum* as well as *dhaBCET*

(locus tags: Ga0078015_112319-112317; primers: dhaBCE_fwd and dhaBCE_rev) and *dhaT* (locus tag: Ga0078015_112312; primers: dhaT_fwd and dhaT_rev) DNA fragments amplified from genomic DNA from *C. pasteurianum* DSM 525. Plasmids for testing the effects of different promoter sequences were based on the plasmid pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis. The *pta-ack* promoter was removed using the restriction enzymes *Sma*I and *Xba*I. The P_{thlA} fragment from *C. acetobutylicum* was amplified from the plasmid pMTL83251_P_{thlA}_FAST (primers: PthlA_1,3-PDO_fwd and PthlA_1,3-PDO_rev) and ligated into the digested pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis, resulting in the plasmid pMTL83251_P_{thlA}_1,3-PDO.diolis. To construct the plasmid pMTL83251_P_{bld}_1,3-PDO.diolis, the PCR-amplified P_{bld} DNA fragment from *C. saccharoperbutylacetonicum* (amplified from pMTL83251_P_{bld}_FAST; primers: Pbld_1,3-PDO_fwd and Pbld_1,3-PDO_rev) was cloned into the digested pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis plasmid. P_{bgal} form *C. perfringens* was amplified from the plasmid pMTL83251_P_{bgaL}_FAST using primers PbgaL_1,3-PDO_fwd and PbgaL_1,3-PDO_rev, and ligated into the *Sma*I and *Xba*I digested pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis plasmid to assemble the plasmid pMTL83251_P_{bgaL}_1,3-PDO.diolis.

Prior to the transformation of *C. beijerinckii*, the newly constructed plasmids were transformed into electrocompetent *E. coli* SCS110 cells. Those *E. coli* cells are *dcm* and *dam* deficient, leading to unmethylated plasmid DNA after replication and isolation of plasmids. Transformation of *C. beijerinckii* was performed as described by Diallo et al., 2020 [31].

3. Strain Verification

Newly constructed *C. beijerinckii* strains and strains used for growth experiments were verified by 16S rRNA gene sequencing, and plasmids were retransformed in *E. coli* XL1-Blue MRF' for further analysis. Therefore, genomic DNA was isolated using the "MasterPure™ Gram Positive DNA Purification Kit" (Lucigen Corp., Middleton, WI, USA). Genomic DNA served as a template for amplification of the 16S rRNA gene (primers: 16S-27F and 1492r) using the "ReproFast proofreading polymerase" (Genaxxon Bioscience GmbH, Ulm, Germany). The amplified 16S rRNA gene was sequenced by Microsynth AG (Balgrach, Switzerland), and the sequence was blasted using NCBI blastn with the RNA/ITS database. For plasmid verification, *E. coli* XL1-Blue MRF′ was transformed with 3 µL of genomic DNA. After growth, plasmids were isolated and checked via analytic digestion.

4. Growth Conditions of Batch Experiments

For batch growth in bottles, the different *C. beijerinckii* strains were inoculated in a glycerol medium. The medium was prepared as described above. The pH of the glycerol medium was adjusted to 7.5 with KOH. After preparing the medium, 50 mL aliquots were filled in bottles and closed airtight. The gas phase was exchanged with N_2:CO_2 (80:20), and bottles were autoclaved. Before inoculation, the medium was supplemented with 40 mM xylose and 100 mM glycerol. For recombinant *C. beijerinckii* strains, clarithromycin (5 µg/mL) was added to the medium. To analyze the influence of vitamin B_{12} supplementation, 5 mg/L sterile vitamin B_{12} was added to the autoclaved glycerol medium of *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET. Cpas]. OD_{600} and pH were monitored during the growth experiments. 2-mL samples for the analysis of substrate consumption and product concentration were taken. The samples were withdrawn with syringes and frozen until analysis took place.

5. Analytical Methods

The 2-mL samples withdrawn during growth experiments were thawed and subsequently centrifuged (18,000× *g*; 30 min; 4 °C). Acetate, butyrate, butanol, ethanol, acetoin, and isopropanol concentrations were determined using a "Clarus 600 gas chromatograph" (Perkin Elmer Inc., Waltham, MA, USA) equipped with a flame ionization detector heated to 300 °C and a flowrate of synthetic air of 450 mL min^{-1}. H_2 was used as the carrier gas (45 mL min^{-1}). Prior to analysis, 480 µL supernatant was acidified with 20 µL 2 M HCl. 1 µL of acidified supernatant was injected onto an "Elite-FFAP" column (30 m × 0.32 mm; Perkin Elmer Inc., Waltham, MA, USA) with the injector heated to 225 °C. For analysis, the

following temperature profile was used: 40 °C for 3 min, 40 °C to 250 °C by 40 °C min^{-1}, 250 °C for 1 min. Xylose and 1,3-PDO concentrations were measured using an "Agilent 1260 Infinity Series HPLC" system (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector and a diode array detector. 20 µL of supernatant were injected into a "CS organic acid" precolumn (40 × 8 mm) followed by a "CS organic acid" column (300 × 8 mm; CS-Chromatographie Service GmbH, Langerwehe, Germany). The column was heated to 40 °C, and a mobile phase consisting of 5 mM H_2SO_4 with a flow rate of 0.6 mL min^{-1} was used. Glycerol concentration was determined using the "Glycerol Assay Kit MAK117" (Sigma-Aldrich®, St. Louis, MO, USA) following the manufacturer's manual. For glycerol standards, 1 mM, 0.8 mM, 0.6 mM, 0.4 mM, 0.2 mM, and 0.1 mM were used. Prior to analysis, the supernatant of the growth experiment samples was diluted (1:200 or 1:100) to fit the standard range used for the assay.

3. Results

1. *Construction of Recombinant 1,3-PDO Production C. beijerinckii Strains*

Two types of glycerol dehydratases are present in different clostridial strains. One is a coenzyme B_{12}-dependent glycerol dehydratase, and the other is a coenzyme B_{12}-independent glycerol dehydratase. The reported production of 1,3-PDO by *C. beijerinckii* DSM 6423 [37] could not be reproduced. Genome analysis showed that the essential gene encoding 1,3-PDO dehydrogenase was missing (Figure 2A). Therefore, we attempted to reconstitute the 1,3-PDO production ability. To examine whether *C. beijerinckii* DSM 6423 can use both glycerol dehydratases to produce 1,3-PDO, two different plasmids were constructed and transformed into *C. beijerinckii*. The strain *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] contains a plasmid harboring the *dhaB*, *dhaC*, and *dhaE* genes (locus tags: Ga0078015_112319-112317) encoding the coenzyme B_{12}-dependent glycerol dehydratase, as well as the *dhaT* gene (locus tag: Ga0078015_112312) encoding the 1,3-PDO dehydrogenase of *C. pasteurianum* DSM 525 [27,38]. The constitutively active *thlA* promoter of *C. acetobutylicum* was used to ensure expression. Figure 2B shows the 1,3-PDO gene cluster of *C. pasteurianum*. The 1,3-PDO gene cluster contains four more genes besides *dhaB*, *dhaC*, *dhaE*, and *dhaT*, which are not present on the plasmid. It is assumed that enzymes encoded by these genes are involved in the reactivation of coenzyme B_{12}, due to the high similarity to genes also present in the 1,3-PDO gene cluster of *C. freundii* [39]. In contrast, the second recombinant strain of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] carries the plasmid pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis harboring the whole 1,3-PDO gene cluster of *C. beijerinckii* DSM 15410 (formerly *C. diolis* DSM 15410). The 1,3-PDO gene cluster of *C. beijerinckii* DSM 15410 is shown in Figure 2C. The two genes *dhaB1* and *dhaB2* (locus tag: K684DRAFT_00976 and K684DRAFT_00977) encode a coenzyme B_{12}-independent glycerol dehydratase, and *dhaT* (locus tag: K684DRAFT_00979) the 1,3-PDO dehydrogenase [28]. Furthermore, the 1,3-PDO gene cluster also includes the *pduO* gene (locus tag: K684DRAFT_00978). This gene is annotated as an ATP:cob(I)alamin adenosyltransferase, necessary for the conversion of vitamin B_{12} to coenzyme B_{12} [40] and is also present on the 1,3-PDO production plasmid. The 1,3-PDO gene cluster was cloned under the control of the constitutively active *pta-ack* promoter of *C. ljungdahlii* [41]. To examine the effect of different promoters on 1,3-PDO production, three additional plasmids were constructed. The plasmid pMTL83251_P_{thlA}_1,3-PDO.diolis harbors the early growth phase-associated *thlA* promoter of *C. acetobutylicum* [42] instead of the *pta-ack* promoter, likewise pMTL83251_P_{thlA}_dhaBCET.Cpas. For the plasmid pMTL38251_P_{bld}_1,3-PDO.diolis, the exponential growth phase-associated *bld* promoter of *C. saccharoperbutylacetonicum* [43] was used. Plasmid pMTL83251_P_{bgaL}_1,3-PDO.diolis includes the lactose-inducible *bgaL* promoter of *C. perfringens* [44].

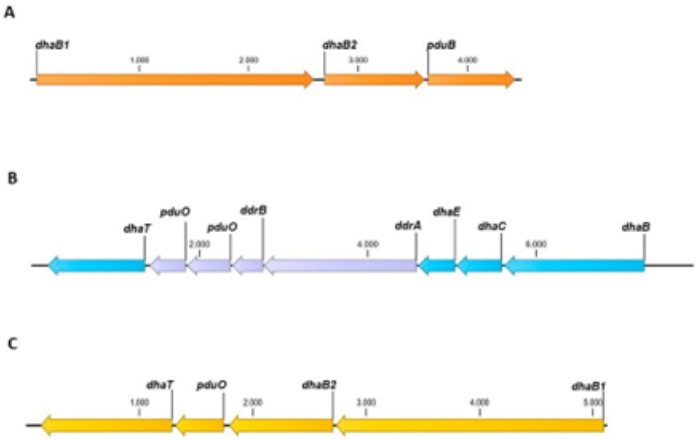

Figure 2. Overview of the 1,3-PDO gene clusters from *C. beijerinckii* DSM 6423 (**A**), *C. pasteurianum* DSM 525 (**B**) and *C. beijerinckii* DSM 15410 (**C**). Genes in blue and yellow were incorporated into the plasmids pMTL83251_P_{thlA}_dhaBECT.Cpas and pMTL83251_$P_{pta\text{-}adc}$_1,3-PDO.diolis, respectively. *dhaB1/B2*, genes for coenzyme B_{12}-independent glycerol dehydratase; *pduB*, gene for propanediol utilization microcompartment protein; *dhaBCE*, genes for coenzyme B_{12}-dependent glycerol dehydratase; *ddrA*, gene for glycerol reactivation factor large subunit; *ddrB*, gene for glycerol reactivation factor small subunit; *pduO*, gene for ATP:cob(I)alamin adenosyltransferase; *dhaT*, gene for 1,3-PDO dehydrogenase.

3.2. Recombinant 1,3-PDO Production

Different batch growth experiments were carried out to evaluate if the constructed recombinant *C. beijerinckii* strains produced 1,3-PDO and under what conditions the highest 1,3-PDO concentration could be achieved. Since *C. beijerinckii* DSM 6423 could not grow with glycerol as the sole carbon source, xylose was also added to the glycerol medium. The first batch growth experiment was performed with unbuffered glycerol medium for cultivation of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}adc}$_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251], and the wild-type *C. beijerinckii* (Figure 3). Compared to the control strains *C. beijerinckii* [pMTL83251] and the *C. beijerinckii* wild-type, the recombinant strain *C. beijerinckii* [pMTL83251_$P_{pta\text{-}adc}$_1,3-PDO.diolis] grew faster and reached the stationary phase after 24 h with an OD_{600} of 0.73. However, the three analyzed *C. beijerinckii* strains reached a final OD_{600} of about 1 (Figure 3A). The drastic pH drop during the first 24 h of cultivation was only detected in *C. beijerinckii* [pMTL83251_$P_{pta\text{-}adc}$_1,3-PDO.diolis] (Figure 3B). Unexpectedly, 1,3-PDO could only be detected in the growth medium of the recombinant strain *C. beijerinckii* [pMTL83251_$P_{pta\text{-}adc}$_1,3-PDO.diolis]. After almost 250 h of incubation, 18.9 mM 1,3-PDO was measured in case of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}adc}$_1,3-PDO.diolis] (Figure 3C). The glycerol concentration in the case of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}adc}$_1,3-PDO.diolis] decreased during the experiment by 33.3 mM. In contrast, the glycerol concentration of the wild-type strain and *C. beijerinckii* [pMTL83251] decreased by 7 mM and 13 mM, respectively (Figure 3D). Xylose was not consumed completely by any of the strains tested. The main product of the analyzed *C. beijerinckii* strains was butyrate (wild-type: 20.9 mM; *C. beijerinckii* [pMTL83251]: 21.3 mM; *C. beijerinckii* [pMTL83251_$P_{pta\text{-}adc}$_1,3-PDO.diolis]: 20.4 mM) (Figure 3E). Only traces of ethanol were detected for all tested *C. beijerinckii* strains (0.2 mM). The lowest acetate concentration (2.2 mM) was measured in case of the wild-type strain. However, this strain also produced the most butanol, with 4.5 mM during the growth experiment. *C. beijerinckii* [pMTL83251] produced 3.6 mM acetate and 2.9 mM butanol throughout the cultivation. The highest acetate concentration was measured in the growth medium of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}adc}$_1,3-PDO.diolis] (6.4 mM). However, this strain produced only traces of butanol (0.2 mM).

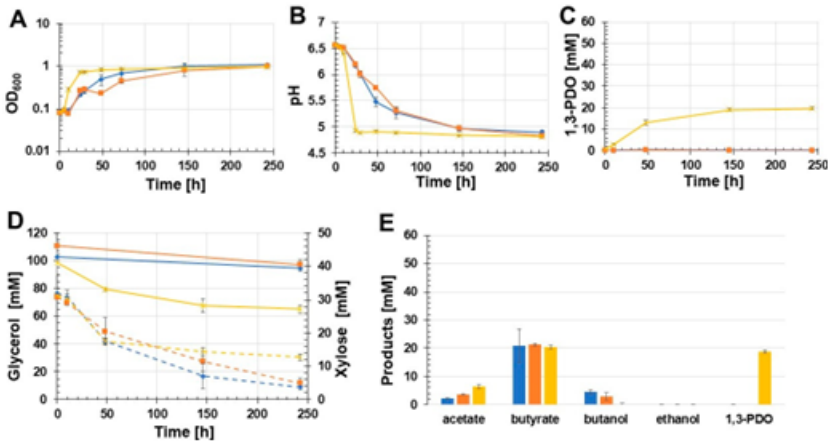

Figure 3. Results of the batch growth experiment with unbuffered glycerol medium performed with the recombinant strains *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251], and the wild-type *C. beijerinckii* strain. OD_{600} (**A**), pH (**B**), 1,3-PDO production (**C**), substrate consumption (**D**), and concentration of products (**E**). Wild-type: blue rhombus; *C. beijerinckii* [pMTL83251]: orange squares; *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis]: yellow crosses; glycerol consumption: solid lines; xylose consumption: dashed lines; error bars represent standard deviations, n = 3.

3.3. Influence of Buffered Glycerol Medium on 1,3-PDO Production

Due to the drastic drop in pH values in the first growth experiment (Figure 3B), the effect of MOPS-buffered medium on the production of 1,3-PDO was examined. The following batch growth experiment was executed with the two recombinant strains *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] and *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis]. As controls, the wild-type strain as well as *C. beijerinckii* [pMTL83251] were used. The results of this batch growth experiment are shown in Figure 4. Again, *C. beijerinckii* [pMTL 83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] reached the stationary phase after 24 h of incubation (OD_{600}: 0.85). In contrast, the OD_{600} of *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] as well as the wild-type strain and *C. beijerinckii* [pMTL83251] decreased after 24 h and 48 h, respectively. Afterwards, the OD_{600} of all three strains rose again. All tested strains reached a similar final OD_{600} after 243 h (Figure 4A). Compared to the first growth experiment, the pH dropped in the case of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] during the first 48 h of incubation only to a pH value of 5.47. Afterwards, the pH value decreased to a final value of 5.39 (Figure 4B). Again, production of 1,3-PDO was only detected in the case of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis]. The amount of 1,3-PDO was increased to 32.8 mM 1,3-PDO for the recombinant *C. beijerinckii* strain harbouring the 1,3-PDO production genes of *C. beijerinckii* DSM15410 (Figure 4C). Minor concentrations of 1,3-PDO production could be measured for the wild-type strain, *C. beijerinckii* [pMTL83251], and *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] (0.7, 0.4, and 0.2 mM, respectively). A distinct decrease of 40.1 mM glycerol was only observed in the case of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis]. The other three examined *C. beijerinckii* strains *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas], *C. beijerinckii* [pMTL83251], and the wild-type strain consumed only minor amounts of glycerol (3.5 mM, 14.1 mM, and 12.6 mM, respectively) (Figure 4D). The supplemented xylose was consumed completely by the strains *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas], *C. beijerinckii* [pMTL83251], and the wild-type strain during the growth experiment. In contrast, *C. beijerinckii* [pMTL 83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] only consumed 18.1 mM of the added xylose (Figure 4D). As in the first growth experiment, butyrate was the main product. The highest butyrate production was detected in the culture broth of *C. beijerinckii* [pMTL83251] with 40.1 mM, followed by

C. beijerinckii [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] and *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas], which produced 39.6 mM and 38.1 mM butyrate, respectively. The wild-type strain produced 33.3 mM butyrate. The highest acetate production was also detected in the culture broth of the strain *C. beijerinckii* [pMTL83251] at 3.1 mM. The strains *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas], as well as the wild-type strain, produced 2.2 mM, 3.0 mM, and 1,3 mM acetate. Similar to the first growth experiment, the highest butanol concentration was obtained by the wild-type strain (4.1 mM butanol). In the case of *C. beijerinckii* [pMTL83251] and *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], 1.5 mM and 0.5 mM butanol were produced. No butanol could be detected in the culture medium of *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas]. Only traces of ethanol were detected by all tested *C. beijerinckii* strains (0.4 mM). An overview of the synthesized products is shown in Figure 4E. The use of MOPS-buffered glycerol medium resulted in a 74% increase in 1,3-PDO production using *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis].

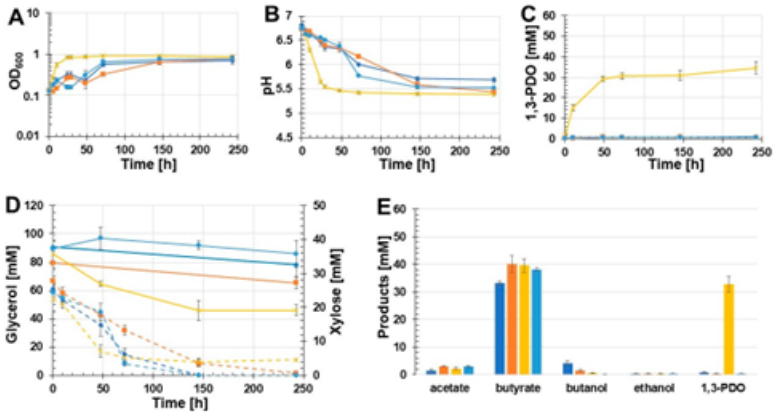

Figure 4. Results of the batch growth experiment using MOPS buffered glycerol medium performed with the recombinant strains *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas], *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251], and the wild-type *C. beijerinckii* strain. OD_{600} (**A**), pH (**B**), 1,3-PDO production (**C**), substrate consumption (**D**), and concentration of products (**E**). Wild-type: blue rhombus; *C. beijerinckii* [pMTL83251]: orange squares; *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis]: yellow crosses; *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas]: light blue circles; glycerol consumption: solid lines; xylose consumption: dashed lines; error bars represent standard deviations; $n = 3$.

3.4. Effect of Vitamin B_{12} Supplementation

Since the used glycerol dehydratase of *C. pasteurianum* is coenzyme B_{12}-dependent, the effect of supplementation of vitamin B_{12} in the MOPS-buffered growth medium of *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] was studied. Therefore, the glycerol medium of *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] was supplemented with vitamin B_{12}. The results are shown in Figure 5. During this growth experiment, the recombinant strain *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] grew slower than during previously described growth experiments. Cells reached the stationary phase after 48 h with an OD_{600} of 0.62. As reported before, a decrease of the OD_{600} after 24 h and 48 h was observed for *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] as well as the wild-type strain and *C. beijerinckii* [pMTL83251]. After 243 h of incubation, the OD_{600} values of *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas], *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], and the wild-type were similar; only the OD_{600} of *C. beijerinckii* [pMTL83251] was lower with a value of 0.69 (Figure 5A). In parallel to the slower growth, the pH of the culture broth of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] also dropped

slower than described for the previous experiments (Figure 5B). Throughout the growth experiment, *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] produced 43.1 mM 1,3-PDO. Again, the three other *C. beijerinckii* strains synthesized only traces of 1,3-PDO (wild-type and *C. beijerinckii* [pMTL83251]: 0.7 mM; *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas]: 0.6 mM) (Figure 5C). The 1,3-PDO-producing strain *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] consumed 66.9 mM glycerol during batch growth. *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] used 27.3 mM glycerol. The two control strains consumed only around 10 mM glycerol. In contrast, xylose was consumed completely by the wild-type strain, *C. beijerinckii* [pMTL83251], and *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] (Figure 5D). In the case of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], 1,3-PDO was the main product. Compared to the other three tested strains, *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] showed the lowest butyrate (19.9 mM) and acetate (1.1 mM) concentrations. The highest butyrate and acetate amounts were observed with the control strain *C. beijerinckii* [pMTL83251], i.e., 23.6 mM butyrate and 2.4 mM acetate. *C. beijerinckii* [pMTL83251_ P_{thlA}_dhaBCET.Cpas] produced 20.9 mM butyrate and 1.6 mM acetate during growth. The wild-type strain produced butyrate (22.7 mM), acetate (1.5 mM) and butanol (1.4 mM). Traces of butanol were also observed in cultures of *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] (0.4 mM). All examined *C. beijerinckii* strains synthesized traces of ethanol (0.3–0.2 mM). An overview of the products is provided in Figure 5E. Overall, a benefit of the vitamin B_{12} supplementation could not be observed.

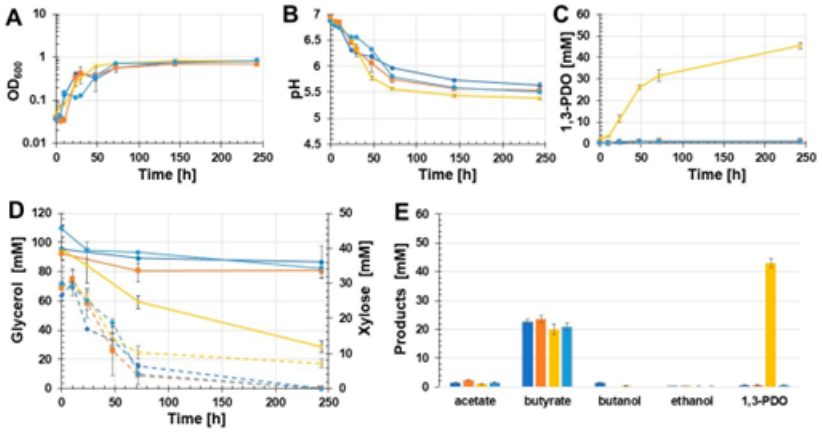

Figure 5. Results of the batch growth experiment using MOPS buffered glycerol medium performed with the recombinant strains *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas], *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251], and the wild-type *C. beijerinckii* strain. The medium of *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] was supplemented with vitamin B_{12}. OD_{600} (**A**), pH (**B**), 1,3-PDO production (**C**), substrate consumption (**D**), and concentration of products (**E**). Wild-type: blue rhombus; *C. beijerinckii* [pMTL83251]: orange squares; *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis]: yellow crosses; *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas]: light blue circles; glycerol consumption: solid lines; xylose consumption: dashed lines; error bars represent standard deviations; $n = 3$.

3.5. Effect of Different Promoters on Recombinant 1,3-PDO Production

To exclude that the *thlA* promoter is not functional in *C. beijerinckii* and therefore *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] could not produce 1,3-PDO in the previously performed batch growth experiments, and to examine if other promoters have a positive effect on the 1,3-PDO production, four different promoters were tested. The batch experiments were again performed with MOPS-buffered medium using the recombinant strains *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251_P_{bgaL}_1,3-PDO.

diolis], *C. beijerinckii* [pMTL83251_P*bld*_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251_ P*thlA*_1,3-PDO.diolis], as well as the wild-type *C. beijerinckii* strain (Figure 6). *C. beijerinckii* [pMTL83251_ P*thlA*_1,3-PDO.diolis] reached the stationary phase after 10 h, whereas *C. beijerinckii* [pMTL8325 1_P*pta-ack*_1,3-PDO.diolis] reached the stationary phase after 24 h. 1,3-PDO gene expression of *C. beijerinckii* [pMTL83251_P*bgaL*_1,3-PDO.diolis] was induced via supplementation of 20 mM lactose after 10 h of cultivation (OD_{600}: 0.19). Before induction, the *C. beijerinckii* [pMTL83251_P*bgaL*_1,3-PDO.diolis] cultures grew similarly. However, in contrast to the induced cultures, the OD_{600} of the uninduced strain did not increase but even decreased. After 48 h, the OD_{600} of the uninduced strain *C. beijerinckii* [pMTL83251_P*bgaL*_1,3-PDO.diolis] increased again. The strains containing the inducible *bgaL* promoter showed the lowest final OD_{600} (induced: 0.61; uninduced: 0.57) (Figure 6A). As observed in the growth experiments before, the pH of the 1,3-PDO production strains dropped faster than the pH values of the control cultures (Figure 6B). 1,3-PDO was produced by all recombinant production strains. Surprisingly, the 1,3-PDO production strain harbouring the *thlA* promoter and the 1,3-PDO genes of *C. beijerinckii* DSM 15410 produced at 50.4 mM the highest 1,3-PDO concentration after 240 h of incubation. Throughout the course of the experiment, *C. beijerinckii* [pMTL83251_P*bld*_1,3-PDO.diolis] produced 37.4 mM 1,3-PDO. 36.3 mM 1,3-PDO were detected in induced cultures of *C. beijerinckii* [pMTL83251_P*bgaL*_1,3-PDO.diolis]. In contrast, the uninduced strain *C. beijerinckii* [pMTL83251_P*bgaL*_1,3-PDO.diolis] only produced 2.1 mM 1,3-PDO. During this growth experiment, *C. beijerinckii* [pMTL83251_P*pta-ack*_1,3-PDO.diolis] produced only 27.3 mM 1,3-PDO. In the wild-type, traces of 1,3-PDO could be detected (0.3 mM). The 1,3-PDO values produced by the different tested strains are shown in Figure 6C. Throughout the growth experiment, *C. beijerinckii* [pMTL83251_P*thlA*_1,3-PDO.diolis] consumed 63.4 mM glycerol (Figure 6D). The induced *C. beijerinckii* [pMTL83251 _P*bgaL*_1,3-PDO.diolis] as well as *C. beijerinckii* [pMTL83251_P*bld*_1,3-PDO.diolis] showed similar glycerol consumption (53.2 mM and 52.1 mM, respectively). Surprisingly, the glycerol concentration in the culture broth of *C. beijerinckii* [pMTL83251_P*pta-ack*_1,3-PDO.diolis] decreased by only 37.6 mM, matching the low 1,3-PDO production during growth (Figure 6D). Again, the non-1,3-PDO-producing strains metabolized the added xylose completely. Interestingly, the strain *C. beijerinckii* [pMTL83251_P*thlA*_1,3-PDO.diolis] consumed only 11 mM xylose (Figure 6D). 1,3-PDO was the main product in case of the induced *C. beijerinckii* [pMTL83251_P*bgaL*_1,3-PDO.diolis] strain and *C. beijerinckii* [pMTL83251_P*thlA*_1,3-PDO.diolis] (Figure 6E). In contrast to the approaches before, the by-product concentrations varied. Matching the low xylose consumption, *C. beijerinckii* [pMTL83251_P*thlA*_1,3-PDO.diolis] produced a low amount of butyrate (30 mM). However, more acetate (10.9 mM) was obtained than by most of the other tested strains. The highest acetate concentration was observed for the induced *C. beijerinckii* [pMTL83251_P*bgaL*_1,3-PDO.diolis] strain at 18.5 mM. The lowest acetate production was observed for the wild-type strain at only 1.6 mM. The other tested strains formed between 3.9 mM and 2.6 mM acetate. In contrast to the low acetate production, the wild-type strain showed the highest butyrate concentration (54.5 mM). In the culture broth of the induced *C. beijerinckii* [pMTL83251_P*bgaL*_1,3-PDO.diolis] strain, only 28.5 mM butyrate was measured. In contrast, the uninduced strain produced 35.8 mM butyrate. *C. beijerinckii* [pMTL83251_P*pta-ack*_1,3-PDO.diolis] and *C. beijerinckii* [pMTL83251_P*bld*_1,3-PDO.diolis] produced similar amounts of butyrate (48.6 mM and 48.3 mM, respectively). Traces of ethanol were detected in all studied strains (0.4–0.2 mM). The wild-type strain, *C. beijerinckii* [pMTL83251_P*pta-ack*_1,3-PDO.diolis], and the uninduced *C. beijerinckii* [pMTL83251_P*bgaL*_1,3-PDO.diolis] strain synthesised traces of butanol (0.3–0.1 mM). 1,3-PDO production was increased with all tested promoters compared to the originally used *pta-ack* promoter.

Table 3 shows a summary of the performed batch growth experiments. A distinct increase in 1,3-PDO production could be obtained by using a buffered medium, resulting in an overall improvement of 82%. Furthermore, the use of buffered medium also resulted in a higher yield of 0.71 $mol_{1,3.PDO}/mol_{glycerol}$. For those calculations, the mean of all experiments performed in buffered glycerol medium was used. The second improvement could be ob-

served by exchanging the *pta-ack* promoter. The highest yield of 0.79 and 50.4 mM 1,3-PDO was obtained with the strain *C. beijerinckii* [pMTL83251_P_{thlA}_1,3-PDO.diolis]. Comparing the 1,3-PDO production utilizing unbuffered medium and *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], the 1,3-PDO production could be improved by 167% by using the strain *C. beijerinckii* [pMTL83251_P_{thlA}_1,3-PDO.diolis] in MOPS-buffered glycerol medium.

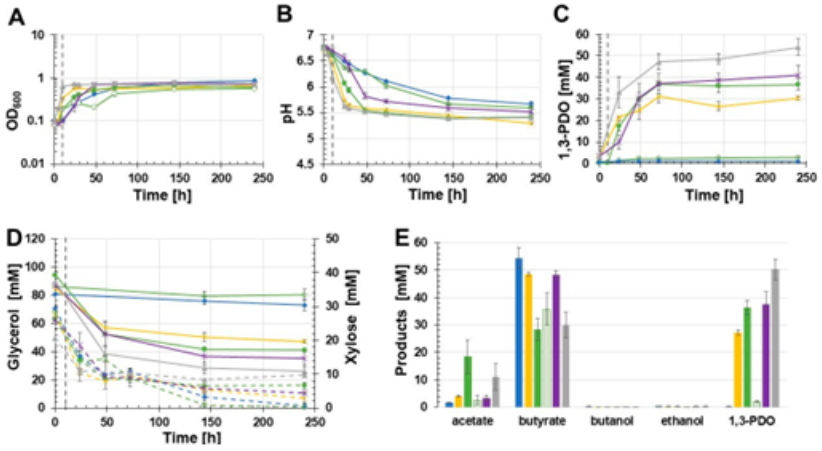

Figure 6. Results of the batch growth experiment using MOPS buffered glycerol medium performed with the recombinant strains *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251_P_{bgaL}_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251_P_{bld}_1,3-PDO.diolis], *C. beijerinckii* [pMTL83251_P_{thlA}_1,3-PDO.diolis], and the wild-type *C. beijerinckii* strain. OD_{600} (**A**), pH (**B**), 1,3-PDO production (**C**), substrate consumption (**D**), and concentration of products (**E**). Wild-type: blue rhombus; *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis]: yellow crosses; *C. beijerinckii* [pMTL83251_P_{bgaL}_1,3-PDO.diolis] induced: green circles; *C. beijerinckii* [pMTL83251_P_{bgaL}_1,3-PDO.diolis] uninduced: green empty circles; *C. beijerinckii* [pMTL83251_P_{bld}_1,3-PDO.diolis]: purple stars; *C. beijerinckii* [pMTL83251_P_{thlA}_1,3-PDO.diolis]: gray triangle; glycerol consumption: solid lines; xylose consumption: dashed lines; induction of gene expression using 20 mM lactose: gray vertical dash lines; error bars represent standard deviations, $n = 3$.

Table 3. Summary of all performed batch growth experiments.

1,3-PDO Producing *C. beijerinckii* Strain	Glycerol Consumption [mM]	1,3-PDO Production [mM]	Yield [$mol_{1,3\text{-}PDO}/mol_{glycerol}$]	Improvement [%] [1]
C. beijerinckii [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] (unbuffered glycerol medium)	33.3	18.9	0.57	—
C. beijerinckii [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] (MOPS buffered glycerol medium)	48.3 [2]	34.4 [2]	0.71 [2]	82 [2]
C. beijerinckii [pMTL83251_P_{bgaL}_1,3-PDO.diolis] induced	53.1	36.3	0.68	92
C. beijerinckii [pMTL83251_P_{bld}_1,3-PDO.diolis]	52.1	37.4	0.72	98
C. beijerinckii [pMTL83251_P_{thlA}_1,3-PDO.diolis]	63.4	50.4	0.79	167

[1] Compared to 1,3-PDO production in unbuffered glycerol medium. [2] Average values of all performed batch growth experiments with buffered glycerol medium.

4. Discussion

C. beijerinckii DSM 6423 (=*C. beijerinckii* NRRL B-593) is described as a natural 1,3-PDO producer [22,37,45]. However, the strain obtained from the DSMZ was unable to do so due to the loss of the 1,3-PDO dehydrogenase gene. In contrast to the published literature, the *C. beijerinckii* DSM 6423 wild-type strain only produced traces of 1,3-PDO. To reconstitute the 1,3-PDO production of the wild-type strain, a newly published transformation protocol of *C. beijerinckii* was employed to establish recombinant 1,3-PDO production in *C. beijerinckii* DSM 6423. Therefore, the heterologous gene expression of two different 1,3-PDO gene clusters, the effect of buffered growth media on the recombinant strains, and the use of different promoters for the gene expression were examined. The data presented clearly show that higher concentrations of 1,3-PDO could only be produced with recombinant *C. beijerinckii* DSM 6423 strains. In the published study of Gungormusler et al. the wild-type produced up to 131 mM 1,3-PDO (10 g/L) [37]. Genome analysis of *C. beijerinckii* DSM 6423 showed that the 1,3-PDO gene cluster is not complete. The *dhaB1* and *dhaB2* genes encoding a supposedly coenzyme B_{12}-independent glycerol dehydratase could be identified. However, a *dhaT* gene encoding a 1,3-PDO dehydrogenase could not be found in the genome of *C. beijerinckii* DSM 6423. It is possible that another gene with a similar function can also convert 3-HPA to 1,3-PDO, resulting in low 1,3-PDO production. For example, alcohol dehydrogenases could also take over the same function due to their broad substrate specificity [46].

Higher concentrations of 1,3-PDO were only achieved using recombinant *C. beijerinckii* strains carrying the 1,3-PDO gene cluster of *C. beijerinckii* DSM 15410. The plasmid-based expression of the 1,3-PDO genes (*dhaBCE* and *dhaT*) of *C. pasteurianum* did not lead to an increase in 1,3-PDO production compared to the wild-type strain and *C. beijerinckii* [pMTL83251]. As mentioned before, the glycerol dehydratase complex of *C. pasteurianum* is coenzyme B_{12}-dependent [27]. However, very little is known about the synthesis of coenzyme B_{12} in *C. beijerinckii* DSM 6423. In many cases, supplementation of vitamin B_{12} was necessary to produce 1,3-PDO when a coenzyme B_{12}-dependent glycerol dehydratase was used [47]. In this study, even with supplementation of vitamin B_{12} (2.5 mg/L), *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] was unable to produce 1,3-PDO. The reason could be that *C. beijerinckii* DSM 6423 does not harbour the necessary genes for the conversion of vitamin B_{12} to coenzyme B_{12}. Furthermore, Fokum et al. recently showed that the addition of vitamin B_{12} in high concentrations (7.5–10 mg/L) to the culture medium has a negative effect on the 1,3-PDO production of *C. beijerinckii* CCIC 22954 [48]. For industrial approaches, it is also more desirable to use a coenzyme B_{12}-independent production strain, in order to keep cultivation costs low due to expensive vitamin B_{12} supplementation.

A further increase in 1,3-PDO production was accomplished by buffering the glycerol medium and preventing a fast pH decrease. The activities of enzymes as well as cofactors within the cell depend strongly on the pH. Therefore, a more stable pH throughout the bacterial cultivation is desired [49]. A positive effect of a controlled pH throughout the fermentation of glycerol was already described before [37]. Experiments with mixed cultures showed that the highest 1,3-PDO production yields could be measured at pH 7 and 8 [50]. Furthermore, most studies on the production of 1,3-PDO from glycerol are performed in fermenters with a controlled pH. Thus, it would be interesting to examine our recombinant production strains in a fermentation experiment with a steady pH.

The biggest improvements were made by exchanging the *pta-ack* promoter. Unfortunately, the direct activity test as described before for *C. saccharoperbutylacetonicum* and *Eubacterium limosum* [35,36] could not be applied for *C. beijerinckii* DSM 6423. In preliminary tests, *C. beijerinckii* carrying pMTL83251_P_{bgaL}_FAST did not show differences in fluorescence compared to the wild-type strain. Thus, growth experiments were performed to examine the effects of different promoters. Although the lactose-inducible *bgaL* promoter of *C. perfringens* did not lead to the highest 1,3-PDO production, the inducible promoter system is almost tight in *C. beijerinckii* DSM6423 as indicated by the low 1,3-PDO concentration of uninduced cultures. Based on the presented results, the *bgaL* promoter can be

described as the second inducible promoter for gene expression in *C. beijerinckii* DSM 6423. Another inducible system is based on the *xylB* promoter from *Clostridium difficile* strain 630, in which gene expression is induced by the addition of xylose [31,51]. The *xlyB* promoter was not examined in this study, since xylose was used as substrate in the growth experiments. Due to the close relationship of *C. saccharoperbutylacetonicum* and *C. beijerinckii*, both strains belong to the second clade of solvent-forming clostridia [52], which is why the *bld* promoter was also tested. Furthermore, the *bld* promoter showed the highest activity in *C. saccharoperbutylacetonicum* compared to promoters *thlA*, *bgaL*, and *pta-ack* [36]. Thus, we assumed high activity of the *bld* promoter in *C. beijerinckii*. However, the highest 1,3-PDO concentration and yield were detected in the case of *C. beijerinckii* [pMTL83251_P_{thlA}_1,3-PDO.diolis] harboring the *thlA* promoter. This promoter also showed high activity in *C. saccharoperbutylacetonicum* [36].

The use of recombinant clostridial strains to produce 1,3-PDO was already reported before. In 2005, Gonzalez-Pajuelo et al. published an article using genetically modified *C. acetobutylicum* strains [53]. *C. acetobutylicum* was chosen as a host strain, as no genetic tools were available for *C. butyricum* at that time. The recombinant *C. acetobutylicum* strain was engineered for the heterologous production of 1,3-PDO using the genes from *C. butyricum*. Since the described fermentation approaches were fed-batch experiments, a comparison to this study is difficult. The modified *C. acetobutylicum* strain produced more 1,3-PDO compared to the tested strains of the present study due to the different cultivation method. However, all the recombinant *C. beijerinckii* strains presented here showed higher yields when grown in buffered glycerol medium. In comparison with other wild-type clostridial strains, the obtained concentrations are lower [7], but the yield is higher in the case of *C. beijerinckii* [pMTL83251_P_{thlA}_1,3-PDO.diolis]. As mentioned before, a comparison of batch growth cultures and fermentation experiments under controlled conditions (pH) are hardly possible. However, continuous culture experiments with the newly constructed strains might even show higher 1,3-PDO production, thus proving the way for a cost-competitive bioprocess, when compared to chemical synthesis routes.

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2.3. *Catenovulum adriaticum* sp. nov., isolated from algae in the harbor of Susak (Croatia)

Schoch, T., Reich, S. J., Flaiz, M., Stöferle, S., Riedel, C. U., & Dürre, P. *Catenovulum adriaticum* sp. nov., isolated from algae in the harbor of Susak (Croatia)

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Catenovulum adriaticum sp. nov., isolated from algae in the harbor of Susak (Croatia)

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Keywords

Catenovulum adriaticum, agarolytic, genome sequencing, polyphasic taxonomy.

Abbreviations

dDDH, digital DNA-DNA hybridization; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, aminolipid; GL, glycolipid; L, unidentified lipids; PL, phospholipid.

The GeneBank accession numbers are CP109965 (chromosome), CP109966 (pCadTS8_1), CP109967 (pCadTS8_2), and OP964652 (16S rRNA gene sequence).

Abstract

The use of algae as feedstock for industrial purpose such as bioethanol production is desired. During the search of new agarolytic marine bacteria a novel Gram-stain-negative, strictly aerobic, and agarolytic bacterium, designated as TS8^T, was isolated from algae in the harbor of the island Susak, Croatia. The cells are rod-shaped and motile. The G+C content of the sequenced genome is 38.6 %. Growth was observed at 11-37 °C, with 0.5-13 % (w/v) NaCl, and at pH 6.0-9.0. The main fatty acids are summed features 3 ($C_{16:1}$ $\omega6c$ and/or $C_{16:1}$ $\omega7c$), summed features 8 ($C_{18:1}$ $\omega7c$ and/or $C_{18:1}$ $\omega6c$), and $C_{16:0}$. The main respiratory quinone is ubiquinone-8. The major polar lipids are phosphatidylethanolamine and phosphatidylglycerol. Analysis of 16S rRNA gene sequences indicates that the newly isolated strain belongs to the genus *Catenovulum*. Based on the 16S rRNA gene sequence, strain TS8^T is closely related to *C. sediminis* D2^T (95.7 %), *C. agarivorans* YM01^T (95.0 %), and *C. maritimum* Q1^T (93.2 %). Digital DNA-DNA hybridization values of TS8^T and the other *Catenovulum* strains are below 25 %. Based on genotypic, phenotypic, and phylogenetic data, strain TS8^T represents a new species of the genus *Catenovulum*, for which the name *Catenovulum adriaticum* sp. nov. is proposed. The type strain is TS8^T (=DSM 114830^T = NCIMB 15451^T).

In 2011, the genus *Catenovulum* was proposed by Yan et al. [1]. Up to now, three type strains are described for this genus: *Catenovulum agarivorans* [1], *Catenovulum maritimum* [2], and *Catenovulum sediminis* [3]. Bacteria of the genus *Catenovulum* are rod-shaped, Gram-stain-negative, and agarolytic. All described *Catenovulum* strains were isolated form seawater, alga or sediment samples collected in the Yellow Sea, China [1-3]. Agarolytic bacteria are of industrial interest especially for the utilization of algae as feedstock for biofuel production such as ethanol [4]. Therefore, isolation of new agarolytic bacteria with high performing agarases

48 are desired. During the search for agarolytic marine bacteria, the strain TS8^T was isolated from
49 algae in the harbor of the island of Susak, Croatia (44°30'45.3"N 14°18'35.3"E). We propose
50 that this strain represents a novel species of the genus *Catenovulum*.

51 For the isolation, artificial seawater medium was used containing (per L): NaCl, 27 g; $MgSO_4$
52 7 H_2O, 6.6 g; $MgCl_2$ 6 H_2O, 5.6 g; $CaCl_2$ 2 H_2O, 1.5 g; KNO_3, 1 g; KH_2PO_4, 0.07 g; $NaHCO_3$,
53 0.04 g; Tris-HCl (1 M, pH 7.6) 20 mL; chelated iron solution 1 mL; trace metal solution 1 mL.
54 For solid medium, 1.5 % (w/v) agar was added to the medium. Thus, agar represents the sole
55 carbon source in this medium. Trace metal solution consisted of (per L): H_3BO_3, 0.6 g; $MnCl_2$
56 4 H_2O, 0.4 g; $(NH_4)_6Mo_7O_{24}$ 4 H_2O, 0.37 g; $CuCl_2$ 2 H_2O, 0.04 g; $ZnCl_2$, 0.04 g; $CoCl_2$ 6 H_2O,
57 0.015 g. The chelated iron solution was prepared by dissolving 14.6 g EDTA in 90 mL
58 demineralized water and pH was adjusted to 7.6. Afterwards, 0.24 g $FeCl_3$ 4 H_2O was added to
59 the solution and dissolved. The solution was filled up to the final volume of 100 mL with
60 demineralized water. In June 2017, during a field trip to Croatia new agarolytic bacteria should
61 be isolated. For the isolation, algae samples were pressed on artificial seawater agar plates. The
62 agar plates were incubated at 30 °C. Formation of holes, shallow craters, and dents in the
63 surface indicated degradation and utilization of agar. Cell material from these areas was
64 streaked on new artificial seawater agar plates. This process was repeated, until pure cultures
65 were obtained, one of these resulted in the strain TS8^T. Based on 16S rRNA gene sequences
66 similarity, the other isolated strains were identified as *Alteromonas gracilis*, *Agarivorans albus*,
67 *Agarivorans litoreus*, and *C. maritimum*. For all experiments, if not otherwise stated, TS8^T cells
68 were incubated in artificial seawater medium at 30 °C. Cells were stored at -80 °C in 50 % (v/v)
69 glycerol.

70 For genome sequencing and assembly, high molecular weight genomic DNA was prepared
71 using the MasterPure Complete DNA & RNA Purification Kit (Biozym, Hessisch Oldendorf,
72 Germany) according to the manufacturer's protocol. DNA concentrations were determined

using the Qubit dsDNA HS Assay kit as recommended by the manufacturer (Life Technologies GmbH, Darmstadt, Germany). Illumina sequencing was performed as described before [5]. Illumina read data was then filtered using fastp before further analysis [6]. For nanopore sequencing, a native barcoded library was prepared using the rapid barcoding kit (SQK-RBK004, Oxford Nanopore Technologies, Oxford, UK) and sequenced using a MinION device equipped with a R9.4.1 flowcell (both ONT). Base calling of raw data was done using the super-accurate base calling model of the guppy algorithm (v. 6.0.1, ONT) on a CUDA-capable RTX 3070 GPU (Nvidia). Reads were then filtered using filtlong v. 0.2.1 [7] with recommended settings. Both data sets were then used in a hybrid assembly approach using the Unicycler assembly pipeline (v. 0.5.0, [8]). Downstream short-read polishing for error correction was done with Polypolish v. 0.5.0 [9] and POLCA (from the MaSuRCA package v. 4.0.8 [10]). Annotation was done via the NCBI Prokaryotic Genome Annotation Pipeline. The annotated *Catenovulum adriaticum* TS8^T genome is accessible at GenBank under the accession numbers CP109965 (chromosome), CP109966 (pCadTS8_1), and CP109967 (pCadTS8_2). 16S rRNA gene sequence similarity was analyzed with the NCBI online tool Blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). As database, the rRNA/ITS database was chosen. For phylogenetic analysis, 16S rRNA genes were aligned using the ClustalW algorithm and neighbour-joining, maximum-likelihood and maximum-parsimony trees were constructed using Mega 11 [11]. Digital DNA-DNA hybridization (dDDH) was performed using the Type (Strain) Genome Server (TYGS) by the DSMZ (https://tygs.dsmz.de/) [12]. The recommended formula d_4 was used.

The genome sequencing of strain TS8^T resulted in three circular contigs. The first one represents the circular chromosome with a length of 3,198,344 bp, the other two contigs correspond to the megaplasmids pCadTS8_1 and pCadTS8_2 with a length of 672,341 bp and 646,854 bp, respectively. An overview of the main genome features of TS8^T as well as its close relatives

C. agarivorans, *C. maritimum* and *C. sediminis* is shown in Table 1. The four compared *Catenovulum* strains showed similar genome size (4.5 – 5.0 Mbp), G+C content (approx. 40 %), and number of protein coding genes (3645-4056 genes). In the genome of TS8^T, 7 identical 16S rRNA genes are present. The sequence of the 16S rRNA gene was used for 16S rRNA gene comparison. BLAST analysis showed that *C. sediminis* D2^T (95.7 %), *C. agarivorans* YM01^T (95.0 %), and *C. maritimum* Q1^T (93.2 %) shared the highest 16S rRNA gene sequence similarities to strain TS8^T. However, the 16S rRNA gene sequence identity threshold value for same species is above 97 % [13]. The close relationship of TS8^T and other *Catenovulum* strains is also shown in the topological structure of the neighbour-joining tree (Fig. 1), maximum-parsimony tree (Fig.2), and maximum-likelihood tree (Fig. 3). All three phylogenetic trees show that the strain TS8^T clusters with the other strains of the genus *Catenovulum*. To support the finding that the isolated strain TS8^T is indeed a new species, dDDH was performed. The dDDH analysis showed values between *C. agarivorans* YM01^T, *C. maritimum* Q1^T, and TS8^T of 21.5 %. Since the type strain *C. sediminis* D2^T is not uploaded to the TYGS database, the genome sequence of *C. sediminis* WS1-A (GenBank assembly accession: GCA_007289895.1) was used for dDDH. Analysis showed a dDDH value of 24.3 % between TS8^T and the tested *C. sediminis* strain. This result supports the assumption that the isolated strain TS8^T is a new species of the genus *Catenovulum* since organisms with a dDDH value below 70 % are considered as different species [14].

To determine the phenotypic features of strain TS8^T, cells were incubated on artificial seawater agar plates at 30 °C for two days. Motility of TS8^T was examined using the hanging drop method. The presence of flagella as well as the size and shape of the TS8^T cells were examined by scanning electron microscopy and light microscopy. Therefore, cells were grown in liquid artificial seawater supplemented with 50 mM galactose. The same growth conditions were used to prepare cells for Gram-staining, which was performed as described previously [5]. Oxidase

123 and catalase activity were tested with standard protocols. Catalase activity was identified by
124 bubble formation in 3 % (v/v) H_2O_2. Oxidase activity was tested with 1 % (w/v) N,N,N′,N′-
125 tetramethyl-p-phenylenediamine-dihydrochloride. Anaerobic growth of strain TS8^T was tested
126 by preparing artificial seawater agar plates as described before in an anaerobic chamber (gas
127 atmosphere: 95 % N_2 + 5 % H_2) with anaerobic water. Anaerobic water was obtained by
128 cooking demineralized water for 20 min and afterwards gassed with N_2 for 20 min. After
129 autoclaving the agar plates were poured in the anaerobic chamber. Agar plates were inoculated
130 and incubated at 30 °C for two weeks in the anaerobic chamber. To determine the temperature
131 range supporting growth of TS8^T, inoculated artificial seawater agar plates were incubated
132 aerobically at 4, 11, 15, 18, 20, 25, 30, 37, and 40 °C for two weeks in duplicates. Growth was
133 evaluated by formation of visible colonies. To examine different growth conditions such as
134 NaCl concentrations and pH values, liquid artificial seawater medium was supplemented with
135 50 mM galactose as substrate and tests were performed in triplicates. Several NaCl
136 concentrations were tested with 5 mL liquid medium containing 0.5, 1.5, 2.7, 5, 6.5, 8, 10, 13,
137 15 % (w/v) NaCl. The effect of different pH values (pH 5.5-9.0, at intervals of 0.5 unit) was
138 also tested in 5 mL liquid medium. The following buffer systems were used to adjust the pH of
139 the artificial seawater medium: Tris (pH 7.0-9.0), MOPS (pH 6.5-7.5), and MES (pH 5.5-7.0).
140 All phenotypic features were tested in triplicates. Additionally, API 20E, API 50CH, and API
141 ZYM test strips (bioMérieux) were used for characterization of further physiological properties.
142 The API 20E and API ZYM tests were performed in duplicates and according to the
143 manufacturer's instructions. Biolog GEN III MicroPlates were also performed as described by
144 the manufacturer and executed in duplicates. However, the results were not consistent and, thus,
145 cannot be used for characterization of TS8^T. For the API 50CH test, artificial seawater medium
146 was used without Tris-HCl and the pH was adjusted to 7.8. Afterwards, 0.18 g/L phenol red
147 was added and the inoculation medium was autoclaved.

148 Colonies of the newly isolated strain TS8^T were small, pale white, convex, smooth, and circular
149 (> 1 mm), when grown on artificial seawater agar plates at 30°C for two days. Cell growth leads
150 to formation of dents in the agar medium. As agar is the sole carbon source present in artificial
151 sweater agar, this suggests degradation and utilization of agar by TS8^T. In line, the genome of
152 TS8^T contains two genes for putative agarases on the chromosome and four genes for putative
153 agarases on pCadTS8_2 (Supplementary Tab.1). The cells of TS8^T were rod-shaped, 1.0-2.5
154 µm long and 0.4-0.8 µm wide, motile, and Gram-stain-negative (Supplementary Fig.1).
155 Scanning electron microscopy images indicated presence of flagella (Supplementary Fig.2) and
156 genes necessary for flagella formation are present in the genome. TS8^T was able to grow at a
157 temperature range of 11-37 °C with an optimal growth temperature of 30 °C. Growth could be
158 observed with 0.5-13.0 % (w/v) NaCl in the artificial seawater medium. Cells could grow within
159 a pH range of 6.0-9.0, however, TS8^T did not grow when MOPS was present in the medium.
160 Cells of TS8^T were oxidase- and catalase-positive.

161 The main characteristics of TS8^T and the other three *Catenovulum* type strains are shown in
162 Table 2. According to the biochemical characterization, TS8^T showed activity of alkaline
163 phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-
164 phosphohydrolase, β-galactosidase, and α-glucosidase. Also, weak acid phosphatase activity
165 was observed. Strain TS8^T showed no activity for lipase (C14), valine arylamidase, cysteine
166 arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, N-
167 acetyl-β-glucosaminidase, α-mannosidase, and α-frucosidase. TS8^T produced acids from the
168 following carbon sources: glycerol, D- and L-arabinose, D-ribose, D- and L-xylose, D-
169 galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, amygdalin, arbutin, salicin, D-
170 cellobiose, D-maltose, D-lactose, D-sucrose, D-raffinose, glycogen, D-turanose, D-lyxose, D-
171 tagatose, D-and L-frucose, and potassium 2-ketogluconate.

The analyses of polar lipides, respiratory quinones, and cellular fatty acids were carried out by the DSMZ as a commercial service (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The two *Catenovulum* strains *C. agarivorans* YM01^T (=DSM 23111^T) and *C. maritimum* Q1^T (=DSM 28813^T) were used as related type strains. For sample preparation, the three strains were grown in artificial seawater medium supplemented with 50 mM galactose for 24 hours at 30 °C under shaking conditions. Afterwards, the cells were harvested, washed twice with 200 mL 0.8 % (w/v) NaCl solution, and lyophilized. Therefore, cells were frozen in liquid nitrogen, placed into the lyophilisator (Alpha 1-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany), and dried at -54 °C at 0.01 bar for 24 h.

The major polar lipids of strain TS8^T were phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). Moderate and minor amounts of aminolipid (AL), glycolipid (GL), and four unidentified lipids (L) were detected (Supplementary Fig. 3). The major polar lipids of both strains *C. agarivorans* YM01^T and *C. maritimum* Q1^T were PE, AL, and one unidentified (Supplementary Fig.3). In contrast to the reference strains, no phospholipid (PL) was detected for the polar lipids of TS8^T. Furthermore, the amount of PE and PG is much higher for the strain TS8^T than for *C. agarivorans* YM01^T and *C. maritimum* Q1^T. The respiratory quinones of strain TS8^T were ubiquinone-8 (91.8 %), Q-7 (5.0 %), and menaquinone MK-7 (3.2 %). In comparison, ubiquinone-8 was also the major respiratory quinone of the reference strains *C. agarivorans* YM01^T and *C. maritimum* Q1^T (95.7 % and 95.6 %, respectively). The percentage of MK-7 differs from the other two strains, TS8^T showed the highest percentage of MK-7 compared to *C. agarivorans* YM01^T (0.1 %) and *C. maritimum* Q1^T (0.3 %). For the strain TS8^T main cellular fatty acids are summed features 3 ($C_{16:1}$ $\omega6c$ and/or $C_{16:1}$ $\omega7c$), summed features 8 ($C_{18:1}$ $\omega7c$ and/or $C_{18:1}$ $\omega6c$), and $C_{16:0}$. These match the major cellular fatty

196 acids of the reference strains *C. agarivorans* YM01^T and *C. maritimum* Q1^T. All detected
197 cellular fatty acids of the three examined strains are listed in Table 3.

198

199 Based on the phenotypic, physiological, and biochemical characteristics and chemotaxonomic
200 analysis, strain TS8^T is considered to represent a novel species of the genus *Catenovulum*. The
201 name *Catenovulum adriaticum* sp. nov. is proposed as name for strain TS8^T.

202

203

204 DESCRIPTION OF *CATENOVULUM ADRIATICUM* SP. NOV.

205 *Catenovulum adriaticum* (a.dri.a'ti.cum. L. neut. adj. *adriaticum*, belonging to the Adriatic
206 Sea).

207 Cells are Gram-stain-negative, rod shaped (0.4-0.8 µm x 1.0-2.5 µm), strictly aerobic and
208 motile. Colonies grown on artificial seawater agar plates are pale white, convex, smooth, and
209 circular (> 1 mm) after incubation for two days at 30 °C. Cell growth occurs with 0.5-13 %
210 (w/v) NaCl, at 11-37 °C, and at pH 6.0-9.0. Cells showed activity of alkaline phosphatase,
211 esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, β-
212 galactosidase, α-glucosidase, and weak activity of acid phosphatase. Activity of lipase (C14),
213 valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-
214 glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-frucosidase
215 is absent. Acids were produced from glycerol, D- and L-arabinose, D-ribose, D- and L-xylose,
216 D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, amygdalin, arbutin, salicin, D-
217 cellobiose, D-maltose, D-lactose, D-sucrose, D-raffinose, glycogen, D-turanose, D-lyxose, D-
218 tagatose, D-and L-frucose, and potassium 2-ketogluconate.

The polar lipids are PE, PG, AL, GL, and four unidentified lipids. Main cellular fatty acids are summed features 3 ($C_{16:1}$ $\omega 6c$ and/or $C_{16:1}$ $\omega 7c$), summed features 8 ($C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$), and $C_{16:0}$. The main respiratory quinone is uniquinone-8.

The type strain TS8^T (= DSM 114830^T = NCIMB 15451^T) was isolated from algae collected from the harbor wall in Susak, Croatia (44°30'45.3"N 14°18'35.3"E). The G+C content of the genomic DNA of the type strain is 38.6 %.

The sequences of the chromosome and the two megaplasmids have been deposited under the GeneBank accession numbers CP109965 (chromosome), CP109966 (pCadTS8_1), CP109967 (pCadTS8_2). The GeneBank accession number for the 16S rRNA gene sequence is OP964652.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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288

289 **Table 1. Genome features of strain TS8^T and sequenced related species.**

	C. adriaticum TS8^T			*C. agarivorans* YM01^T	*C. maritimum* Q1^T	*C. sediminis* WS1-A^2	
Feature	**Chromosome**	**pCadTS8_1**	**pCadTS8_2**	**Genome**[1]	**Genome**[1]	**Chromosome**	**Plasmid**
Length [bp]	3,198,344	672,341	646,854	~ 5,030,000	~ 4,550,000	~ 4,110,000	~ 760,000
G+C content [%]	38.5	39.2	38.1	40.0	37.9	40.8	40.5
Protein coding genes	2804	403	478	4056	3645	3398	531
rRNA genes (5S, 16S, 23S)	8, 7, 7	0, 0, 0	0, 0, 0	n.a.[1]	n.a.[1]	7, 6, 6	0, 0, 0
tRNA	66	0	0	53	60	61	0
ncRNA	3	0	0	4	4	4	0

290 ^{T} type strain; [1] not applicable, assembly on scaffold level; [2] genome sequence of type strain *C. sediminis* D2^T not available.

291

292 **Table 2. Characteristics of strain TS8^T compared with the three type strains of the genus**

293 ***Catenovulum.***

294 1, strain TS8^T (this study); 2, *C. agarivorans* YM01^T [1]; 3, *C. maritimum* Q1^T [2]; 4,

295 *C. sediminis* D2^T [3].

Feature	1	2	3	4
origin of type strain	Adriatic Sea, Croatia	Yellow Sea, China	Yellow Sea, China	Yellow Sea, China
Cell morphology				
Pigmentation	pale white	pale yellow	pale white	pale white
Cell shape	rod shaped	rod shaped, form long chains	slightly curved rod shaped	rod shaped
Cell size [μm]	0.4-0.8x1.0-2.5	0.4-0.6x0.8-1.5	0.4-0.9x1.2-3.0	0.7-1.2x1.5-3.0
Range for growth				
Temperature [°C]	11-37	10-42	10-37	10-42
NaCl (%, w/v)	0.5-13.0	0.5-3.0	0.5-5.5	0.5-8.0
pH	6.0-9.0	7.5-10.0	6.0-8.5	6.0-9.0
Enzyme activities				
α-galactosidase	-	+	-	-
β-galactosidase	+	+	ND	-
α-glucosidase	+	+	+	-
β-glucosidase	-	+	-	-
esterase(C4)	+	+	-	-
esterase lipase (C8)	+	+	-	-
Acid production from				
D-galactose	+	+	+	ND
glycerol	+	-	-	ND
D-glucose	+	+	+	ND
D-fructose	+	+	-	+
D-lactose	+	-	+	ND

296 ^{T} type strain; ND, no data available.

297

298 **Table 3. Cellular fatty acid composition of strain TS8^T, *C. agarivorans* YM01^T; 3,**

299 ***C. maritimum* Q1^T.**

	TS8^T	*C. agarivorans* YM01^T	*C. maritimum* Q1^T
$C_{10:0}$	0.3	---	0.2
$C_{12:0}$	1.0	1.7	1.0
$C_{14:0}$	0.5	1.5	1.3
$C_{15:0}$	0.2	---	0.8
$C_{16:0}$	**29.9**	**30.8**	**24.0**
$C_{17:0}$	0.3	1.7	2.0
$C_{18:0}$	2.0	4.4	2.5
10-methyl $C_{19:0}$	---	0.5	0.8
$C_{10:0}$ 3-OH	3.5	4.0	5.8
$C_{16:0}$ iso 3-OH	---	0.4	---
$C_{16:0}$ 3-OH	---	0.7	---
$C_{12:1}$ $\omega 7c$	---	1.5	4.3
$C_{20:1}$ $\omega 7c$	0.3	0.7	0.4
Summed Feature 2	1.2	0.5	0.6
Summed Feature 3	**40.5**	**23.4**	**26.7**
Summed Feature 8	**20.2**	**28.6**	**28.2**

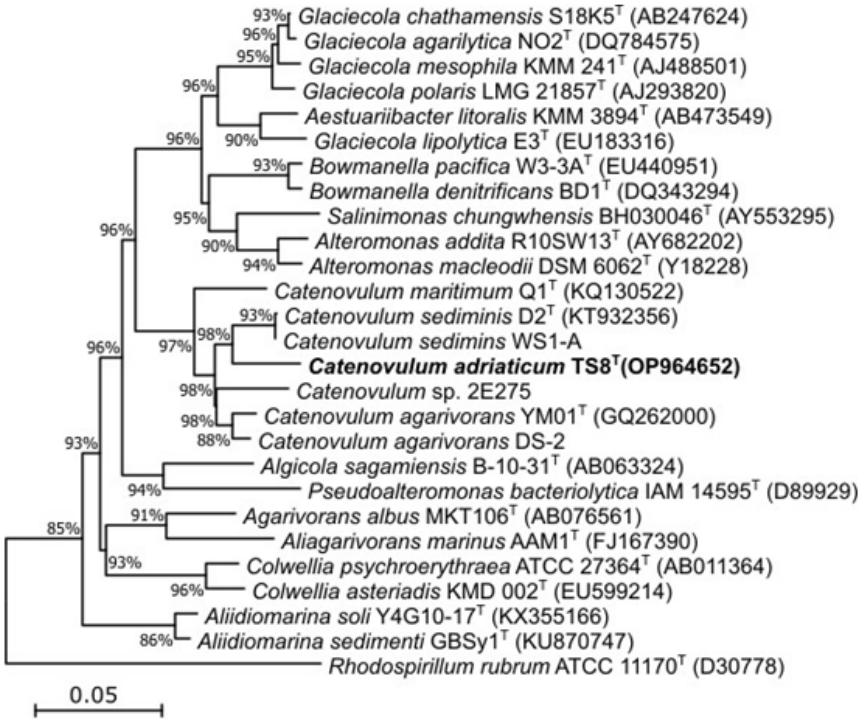
300 ^{T} type strain; ---, not detected.

301 Summed Feature 2, $C_{14:0}$ 3-OH/$C_{16:1}$ iso I

302 Summed Feature 3, $C_{16:1}$ $\omega 6c$/$C_{16:1}$ $\omega 7c$

303 Summed Feature 8, $C_{18:1}$ $\omega 7c$/$C_{18:1}$ $\omega 6c$

304

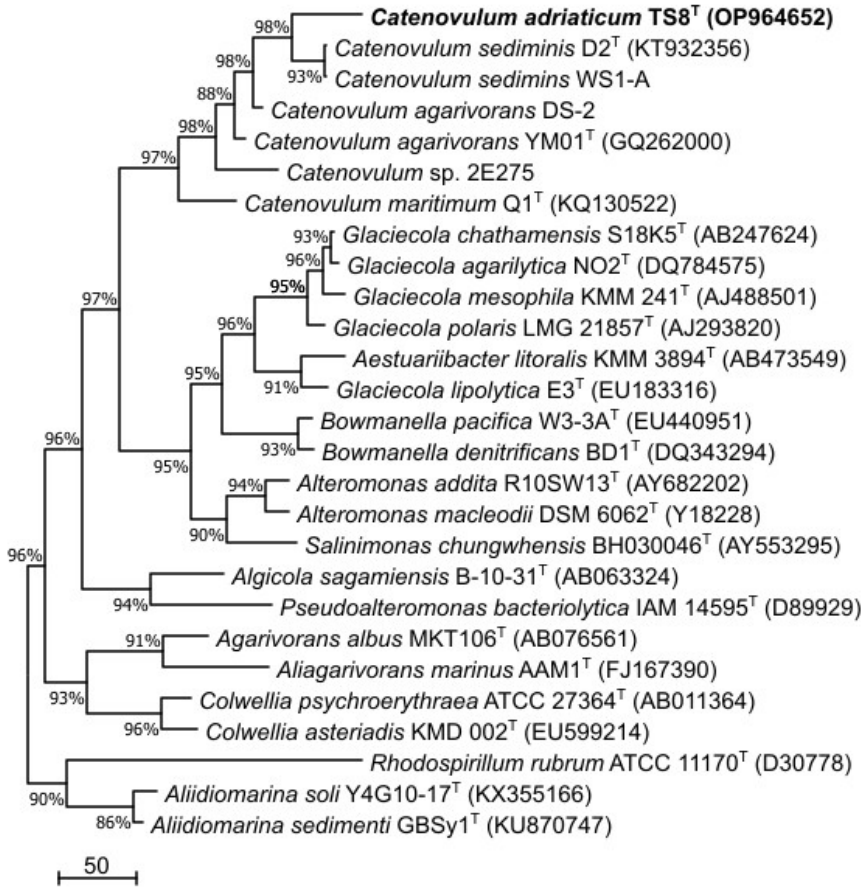


305

306 **Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain**

307 **TS8T, strains of the genus *Catenovulum* and other related taxa.**

308



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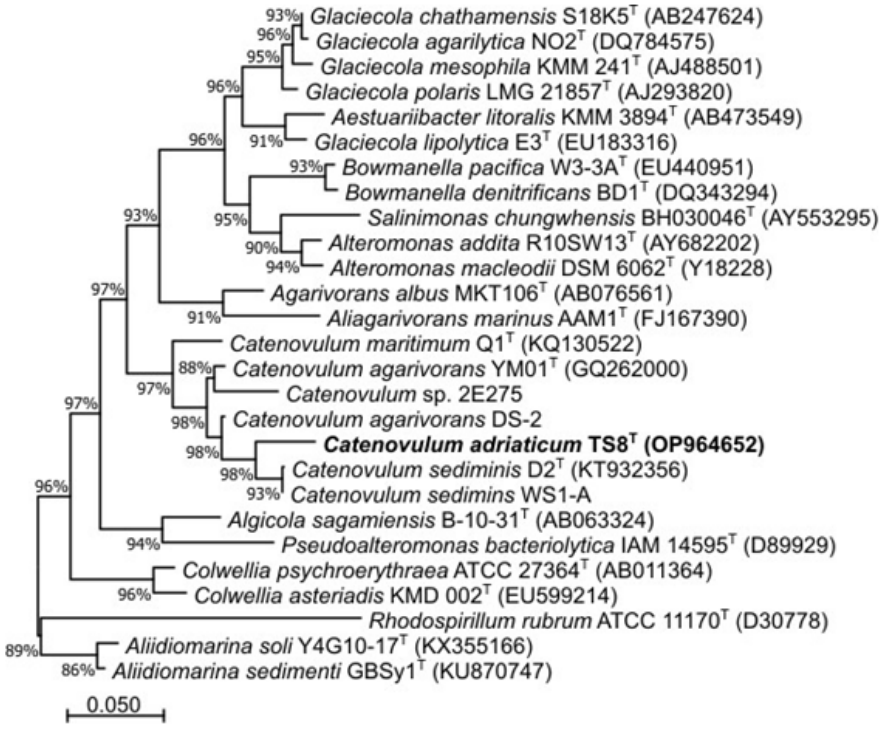
310 **Fig. 2 Maximum-parsimony tree based on 16S rRNA gene sequences of the strain TS8^T,**

311 **strains of the genus *Catenovulum* and other related taxa.**

312



313

314 Fig. 3 Maximum-likelihood tree based on 16S rRNA gene sequences of the strain TS8^T,

315 strains of the genus *Catenovulum* and other related taxa.

Supplementary Materials

Catenovulum adriaticum sp. nov., isolated from algae in the harbor of Susak (Croatia)

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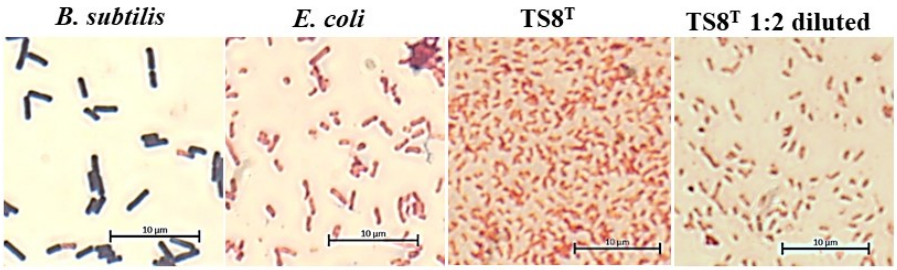
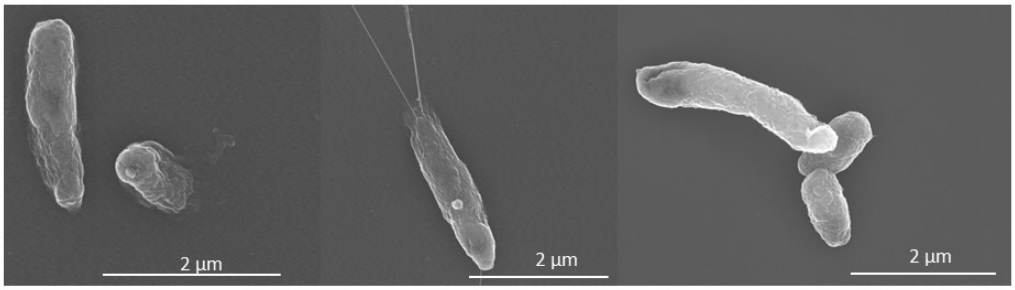
Supplementary Tab.1 Putative agarases of TS8[T].

Conserved domains were determined by the online tool "NCBI Conserved Domains Search"

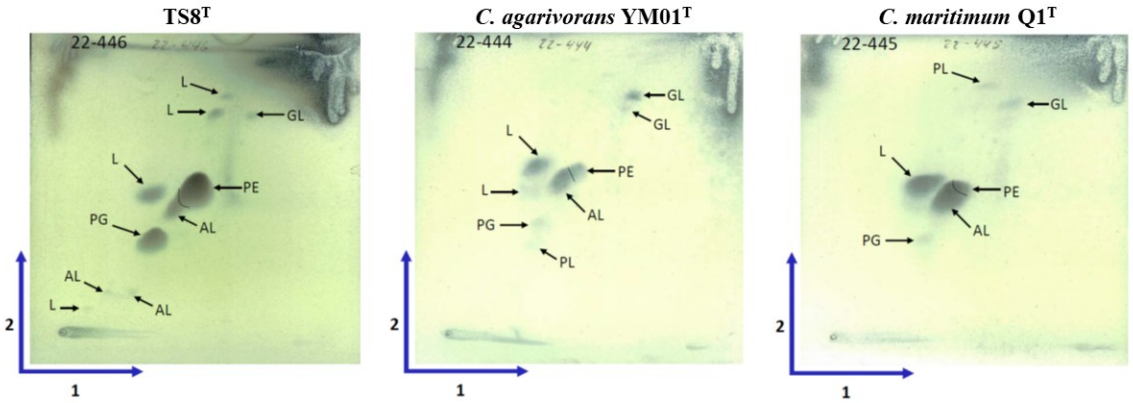
Contig	Locus tag	NCBI conserved domains database search	Length [AA]
CP9965	OLW01_06835	LamG superfamily, GH16_beta_agarase	334
CP9965	OLW01_08105	CBM6_agarase-like	937
CP9967	OLW01_16635	LamG superfamily, RICIN superfamily, GH16_beta_agarase	445
CP9967	OLW01_16995	LamG superfamily, BglS superfamily, GH16_beta_agarase	283
CP9967	OLW01_17045	LamG superfamily, BglS superfamily, GH16_beta_agarase	313
CP9967	OLW01_18205	DUF1565 superfamily, Beta_helix superfamily, Polysaccharide Lyase Family 6	754



Supplementary Fig. 1: Gram staining of TS8^T and the reference strains *Bacillus subtilis* DSM 402 as well as *Escherichia coli* DSM 498. Bars, 10 µm.



Supplementary Fig. 2: Scanning electron microscopy images of strain TS8^T. Bars, 2 µm.



Supplementary Fig. 3: Two-dimensional TLC plate image of lipids of TS8^T, *C. agarivorans* YM01^T, and *C. maritimum* Q1^T.

PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, aminolipid; GL, glycolipid; PL, phospholipid; L, unidentified lipid.

2.4. Reclassification of *Clostridium aurantibutyricum* Hellinger 1944 and *Clostridium roseum* (ex McCoy and McClung 1935) Cato et al. 1988

Schoch, T., Höfele, F., Odeh, H., Winter, L. M., Stöferle, S., Karl, M., Bengelsdorf, F., Poehlein, A., Daniel, R., & Dürre, P. (2022). Reclassification of *Clostridium aurantibutyricum* Hellinger 1944 and *Clostridium roseum* (ex McCoy and McClung 1935) Cato et al. 1988. *International Journal of Systematic and Evolutionary Microbiology*, *72*, 005589.

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MICROBIOLOGY SOCIETY

Reclassification of *Clostridium aurantibutyricum* Hellinger 1944 and *Clostridium roseum* (ex McCoy and McClung 1935) Cato *et al.* 1988

Teresa Schoch[1]*,†, Franziska Höfele[1]†, Hannah Odeh[1], Lisa-Maria Winter[1], Sophia Stöferle[1], Michael Karl[1], Frank Bengelsdorf[1], Anja Poehlein[2], Rolf Daniel[2] and Peter Dürre[1]

Abstract

Clostridium aurantibutyricum, *Clostridium felsineum* and *Clostridium roseum* share a very high similarity based on multi-locus sequence analysis. In this study, their correct taxonomic status was determined using genomic and phenotypic investigations. Average nucleotide identity based on MUMmer alignment of the genomes and *in silico* DNA–DNA hybridization resulted in values of 98.55–100 and 78.7–100%, respectively, strongly indicating that all strains are members of the same species. In addition, morphological investigations, fatty acid analyses and substrate utilization tests revealed no striking differences between the strains. Therefore, we propose the reclassification of *C aurantibutyricum* and *C roseum* as later heterotypic synonyms of *C. felsineum*. The type strain is lodged in several culture collections (ATCC 17788^T=DSM 794^T=NCIMB 10690^T).

Three species *Clostridium aurantibutyricum*, *Clostridium felsineum* and *Clostridium roseum* belong to the solventogenic clostridia and form a coherent cluster, which is phylogenetically related to the *Clostridium acetobutylicum* group [1]. Of the three, *C. felsineum* was the first to be isolated in 1917 [2], during investigations on warm water retting of flax and hemp in Bologna, Italy. Hence, the species was designated '*felsineum*', which was derived from the Etruscan name of Bologna, i.e. Felsina (original name of the microbe: *Bacillus felsineus*). A taxonomic description followed in 1939 [3]. In 1930, Sir Frederick Andrewes isolated a pink-pigmented anaerobic bacterium from German maize, which resembled *C. acetobutylicum*. The new strain was later studied in detail and designated *Clostridium roseum* [4–6]. Finally, the name *Clostridium aurantibutyricum* was given to a bacterium that was originally isolated by Chaim Weizmann in May 1939. Weizmann is the originator of acetone–butanol–ethanol (ABE) fermentation, the starting point of modern industrial biotechnology [7, 8]. The description of *C. aurantibutyricum* is usually linked to a citation of Esther Hellinger from 1944 (Commemorative Volume to Dr. Weizmann's 70th Birthday, Private print, November 1944. pp. 37–46), which, however, is not publicly available. The description was openly published in 1947 [9]. Hellinger already stated certain resemblance of *C. aurantibutyricum* to *C. felsineum* and *C. roseum*, but thought a new species to be justified because of mostly gradual metabolic differences.

A detailed study on solventogenic clostridia revealed average nucleotide identity based on MUMmer alignment (ANIm) values between 98 and 100% of the type strains of *C. aurantibutyricum*, *C. felsineum* and *C. roseum* plus an additional strain of *C. roseum*, indicating that all these strains belong to a single bacterial species [1]. The respective genome sequences have been published under the GenBank accession numbers CP096988–CP096990, CP096980–CP096982, CP096983–CP096985 and CP096986–CP096987, respectively. The aim of the present study was to provide additional evidence for the reclassification of these clostridia as a single species.

All strains (*C. aurantibutyricum* DSM 793^T, *C. felsineum* DSM 794^T, *C. roseum* DSM 7320^T, *C. roseum* DSM 6424) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Brunswick, Germany). They were routinely grown

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Keywords: acetone–butanol–ethanol (ABE) fermentation; heterotypic synonym; genome analysis.
Abbreviations: ANI, average nucleotide identity; ANIm, average nucleotide identity based on MUMmer alignment; dDDH, digital DNA–DNA hybridization; HWD, high molecular weight DNA; MLSA, multilocus sequence analysis.
†These authors contributed equally to this work
Four supplementary tables and three supplementary figures are available with the online version of this article.

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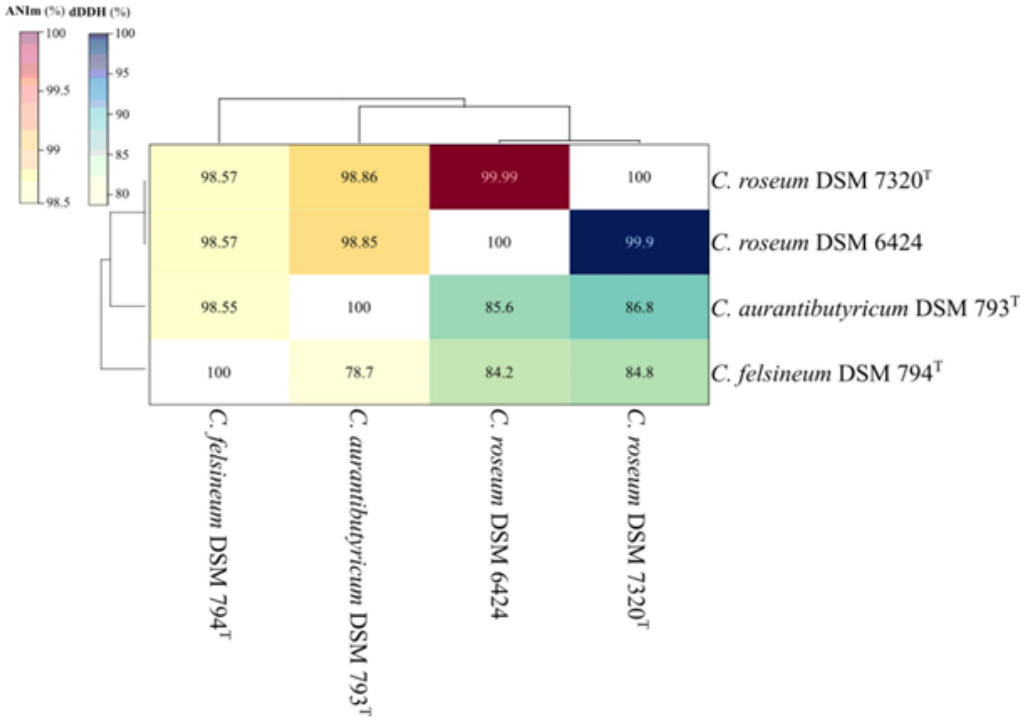

Fig. 1. Heat map of dDDH and ANIm pairwise genomes comparison. Pairwise genome comparison: the heat map shows dDDH (bottom right) and ANIm (top left) values of pairwise whole-genome comparisons along with the tree cladogram to show relationships.

under anaerobic conditions at 37 °C in DSM 104b PY+X medium containing (per litre): trypticase peptone, 5 g; peptone from meat, 5 g; yeast extract, 10 g; salt solution, 40 ml; Na–resazurin solution (0.1% w/v), 0.5 ml; cysteine–HCl·H_2O, 0.5 g; D-glucose, 5 g. Composition of salt solution (per litre): $CaCl_2 \cdot 2H_2O$, 0.25 g; $MgSO_4 \cdot 7\ H_2O$, 0.50 g; K_2HPO_4, 1 g; KH_2PO_4, 1 g; $NaHCO_3$, 10 g; NaCl, 2 g. For substrate testing, a basal minimal medium was used (per litre): K_2HPO_4, 4 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; yeast extract, 1 g; 3-(*N*-morpholino)propanesulfonic acid, 10.46 g; cysteine–HCl·H_2O, 1.2 g; resazurin, 1 mg; trace element solution, 1 ml. Composition of trace element solution (per litre): $CoCl_2 \cdot 2H_2O$, 0.2 g; $CuCl_2 \cdot 2H_2O$, 0.02 g; H_2BO_3, 0.06 g; HCl (37%), 0.9 ml; $MnCl_2 \cdot 4H_2O$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.035 g; $NiCl_2 \cdot 6H_2O$, 0.025 g; $ZnCl_2$, 0.07 g. The tested substrate was added to a final concentration of 20 mM. The growth experiments were performed under anaerobic conditions in 50 ml minimal medium at 37 °C. During the 136 h of incubation OD_{600} was measured to determine growth of the cells. High molecular weight DNA (HWD) was isolated with the MasterPure Complete DNA and RNA Purification Kit (Biozym) as recommended by the manufacturer. Quality of isolated DNA was initially checked by agarose gel electrophoresis and validated on an Agilent Bioanalyzer 2100 using an Agilent DNA 12000 kit as recommended by the manufacturer (Agilent Technologies). Concentration and purity of the isolated DNA was first checked with a Nanodrop ND-1000 (PeqLab Erlangen) and exact concentration was determined using the Qubit dsDNA HS assay kit as recommended by the manufacturer (Life Technologies). Illumina shotgun libraries were prepared using the Nextera XT DNA sample preparation kit. To assess quality and size of the libraries, samples were run on an Agilent Bioanalyzer 2100 using an Agilent High Sensitivity DNA kit as recommended by the manufacturer (Agilent Technologies). Concentration of the libraries were determined using the Qubit dsDNA HS Assay Kit as recommended by the manufacturer (Life Technologies). Sequencing was performed on a MiSeq system with the reagent kit version 3 with 600 cycles (Illumina) as recommended by the manufacturer. For Nanopore sequencing, 1.5 µg HWD was used for library preparation using the Ligation Sequencing Kit 1D (SQK-LSK109) and the Native Barcode Expansion Kit (EXP-NBD114) as recommended by the manufacturer. Sequencing was performed for 72 h using a MinION device Mk1B and a SpotON Flow Cell R9.4.1 as recommended by the manufacturer (Oxford Nanopore Technologies) using MinKNOW software version 21.10.4 for sequencing and Guppy version 6.0.1 in high-accuracy mode for basecalling and demultiplexing. Unicycler version 0.4.7 [10] was used with default settings to perform a hybrid assembly, resulting in a closed genome. Annotation was performed with Prokka version 1.13.3 [11] and default settings. For comparison of the 16S rRNA genes, the sequences were amplified and sequenced with the following primers (16 S-27F: ATAAGCTTGGAT

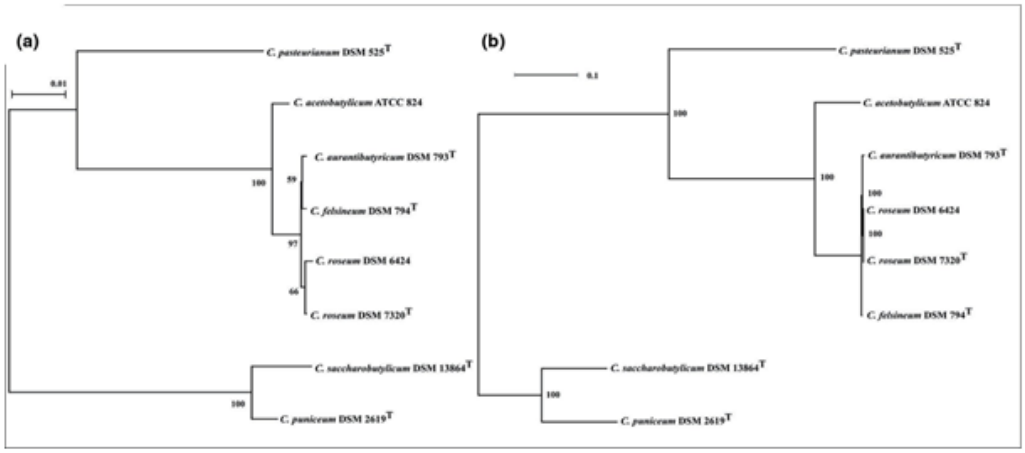

Fig. 2. Phylogenetic analysis of 16S rDNA sequences (a) and core orthologous groups (b). The clustering of the 16S rRNA gene sequences was tested by a bootstrap approach with 1000 repeats. The lengths of the tree branches were scaled according to the number of substitutions per site (see size bar). MLSA tree-based on 1081 orthologous groups with maximum likelihood method and 500 repetitions.

CCAGAGTTTGATCCTGGCTCAG; 1494 r: ACTCGAGGATATCGGTTACCTTGTTACGACTT [12]). For examination of the similarities, the NCBI BLAsTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used.

16S rRNA gene comparisons showed high sequence similarities (Table S1, available in the online version of this article). The genome sizes of *C. aurantibutyricum* and the two *C. roseum* strains differed by 74275, 33588 and 110204 bp and were 98.57% (both *C. roseum*) and 98.55% (*C. aurantibutyricum*) similar to the genome size of *C. felsineum*. A high similarity of 98% of the 16S rRNA gene sequences had also been reported previously for *C. felsineum* and *C. acetobutylicum* [13]. An overview of the genome features of the four strains is shown in Table S2. DNA–DNA hybridization was examined digitally (dDDH), using the Genome-to-Genome Distance Calculator 3.0 provided by the DSMZ server (https://ggdc.dsmz.de/ggdc.php) [14]. ANIm values had been determined previously [1]. The ANIm and dDDH values are depicted in Fig. 1, showing 98.55–100% for ANIm and 78.7–100% for dDDH. These numbers clearly exceed the 95 and 70% threshold, respectively, that has been proposed for species definition [15, 16]. Fig. 2 shows the phylogenetic analysis of *C. felsineum*, *C. roseum*, *C. aurantibutyricum* and related taxa. For Multilocus sequence analysis (MLSA)-based phylogeny, proteinortho version 6.031 [17] was used to generate clusters of ortholo- gous groups, inparalogs were removed, MUsCLE was used to align the remaining sequences, and poorly aligned positions were automatically filtered from the alignments using Gblocks [18, 19]. A maximum-likelihood tree from 1081 orthologs was inferred with 500 bootstraps with RAxML version 8.1.22 [20]. The PO_2_MLSA.py script is available at github [21]. The 16S rRNA-based dendrogram was prepared using MEGA11 and the maximum-likelihood method [22]. The clustering of the sequences was tested by a bootstrap approach with 1000 repeats. The trees were visualized using Dendroscope version 3.5.9 [23].

Light and scanning electron microscopy showed rods of approx. 4.4–7.1 µm length and 0.6–1 µm diameter for all four strains (Fig. S1). For scanning electron microscopy, the cells were adsorbed on silicon platelets and fixed for 1 h with a final concentration of 2.5% glutaraldehyde in 0.1 M phosphate buffer with 1% sucrose. Afterwards, samples were postfixed with 2% of osmium tetroxide in phosphate buffer for 20 min, dehydrated in a graded series of propanol and then critical point dried using carbon dioxide in a CPD BalTec030 critical point dryer. Finally, samples were mounted on specimen stubs and rotary coated by electron beam evaporation with about 2 nm of platinum. Samples were imaged in a Hitachi S-5200 scanning electron microscope at an accelerating voltage of 10 kV using the secondary electron signal. For transmission electron microscopy (TEM), cells were harvested by centrifugation and washed with fresh medium. After another centrifugation step (5 min, 604 g, room temperature), the cells were suspended in a 1:1 mixture of fresh medium and fixation solution containing 5% (v/v) glutaraldehyde, 58.4 mM sucrose, 0.2 M phosphate buffer (PBS, pH 7.3). Then, cells were washed 5 times with 0.01 M PBS buffer and postfixed in 2% (v/v) aqueous osmium tetroxide. Cells were dehydrated in graded series of 1-propanol, block-stained in 1% (w/v) of uranyl acetate and embedded in Epon (Sigma-Aldrich Chemie GmbH). Ultra-thin sections (80 nm) were contrasted with 0.3% (w/v) lead citrate for 1 min and imaged in a Jeol 1400 TEM device (JEoL) at an acceleration voltage of 120 kV. For Gram-staining, cells were transferred onto a slide, air-dried, and fixed with heat. Afterwards, cells were stained with crystal violet solution for 3 min. Lugol's iodine solution was used to wash off and coat the cells for 1 min. Then, the solution was washed off with 96% ethanol. Residual ethanol was removed by rinsing the slide with demineralized water. For counterstaining, cells were coated for 1 min with a 0.5%

safranin solution and afterwards rinsed off with demineralized water. TEM indicated a Gram-positive cell wall (Fig. S1), which fits well to the detected (Fig. S2) as well as reported Gram-positive staining of young cells of *C. roseum* and *C. aurantibutyricum* [4, 9]. However, aged cultures of *C. roseum* were reported to stain Gram-negative [4], which was found to be true for all four strains in this study (Fig. S2). For fatty acid analysis, cells were cultivated under anaerobic conditions in the described minimal media supplemented with 100 mM glucose at 37 °C. After harvesting at on OD_{600} of 1 and lyophilization of the cells, fatty acid analysis was performed by DSMZ as a commercial service. DSMZ uses the Sherlock Microbial Identification System and the MIS standard software. The main fatty acid of all four strains is $C_{16:0}$, ranging from 44.9 to 60.3% (Table S3). Several cyclo fatty acids were also detected in all strains. These compounds have been reported to be characteristic of *Clostridium* species and anaerobic bacteria, respectively [24–26].

Substrate utilization tests are summarized in Table S4. The results were remarkably similar for all four strains. Carbon sources were fermented to acetate, butyrate and lactate. A small amount of butanol was detected after growth of all four strains with sucrose as substrate. Acetone could not be detected. Furthermore, all strains produced minor amounts of ethanol using xylose or fructose as carbon sources. Glycerol, inulin and maltose could not be used as carbon sources by all four strains.

The eponymous pink colour of *C. roseum* is caused by clostyrylpyrones A–D, a novel polyketide family [27]. Respective genes for biosynthesis were identified as *cspA–E* in *C. roseum* DSM 6424, and no homologous gene clusters were identified from public databases [27]. The same chromosomal arrangement was found in *C. roseum* DSM 7320^T. However, the adjacent gene sequences in *C. felsineum* are very similar, indicating a gene cluster insertion in the two *C. roseum* strains. In case of the *C. roseum* strains, the insertion is approximately 25000 bp long and compared to the chromosome of *C. felsineum* the insertion is placed between the two genes CLFE_11180 and CLFE_11190 (Fig. S3). Comparison to the genome of *C. aurantibutyricum* showed that the upstream region is also present. However, a more complex gene rearrangement must have occurred in this strain, since the gene with the locus tag CLAUR_39130 (which was found in the other three strains app. 26000 bp downstream of the insertion site) was here found close to the insertion site (Fig. S3). On the other hand, *C. felsineum* and *C. aurantibutyricum* were also reported as pink-coloured organisms in the original descriptions [2, 9]. Thus, further pigments in these chromogenic strains still await elucidation.

In conclusion, the data presented show that there is no taxonomic evidence supporting the separate species designations of *C. felsineum*, *C. aurantibutyricum* and *C. roseum*. Thus, we propose *Clostridium aurantibutyricum* Hellinger 1944 and *Clostridium roseum* (ex McCoy and McClung 1935) Cato *et al.* 1988 as later heterotypic synonyms of *Clostridium felsineum* (Carbone and Tombolato 1917, Bergey, Breed, Murray and Hitchens 1939) (Approved Lists 1980).

EMENDED DESCRIPTION OF *CLOSTRIDIUM FELSINEUM*

The description of *Clostridium felsineum* is identical to that published before [2, 3] with the following modifications. Cells are 4.4–7.1 µm long and 0.6–1 µm in diameter. Young cells stain Gram-positive, whereas older cells stain Gram-negative. Organic acids are produced from arabinose, fructose, galacturonic acid, glucose, glycerol +glucose, lactose, mannose, pectin, sucrose and xylose. Major metabolic products are acetate, butyrate and lactate, and minor products are ethanol and butanol. The genome of *C. felsineum* has a size of app. 5 Mbp with a G+C content of 29.9–30.1 mol% and contains at least one plasmid. The type strain is *C. felsineum* DSM 794^T and is also lodged in two more culture collections (ATCC 17788^T=NCIMB 10690^T).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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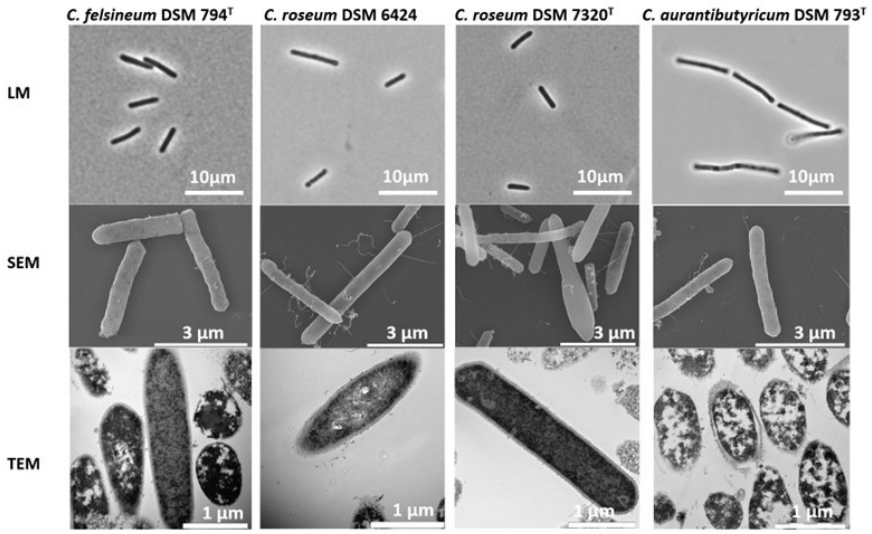
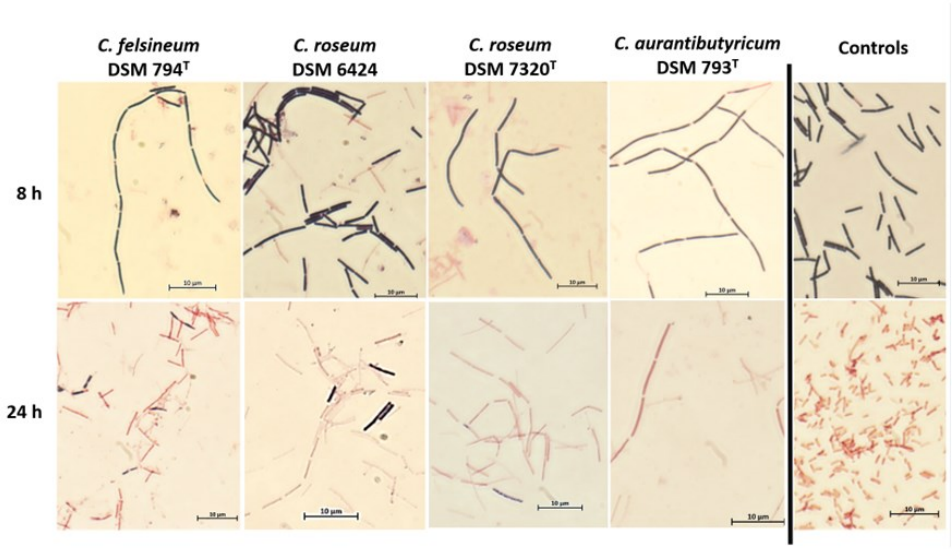
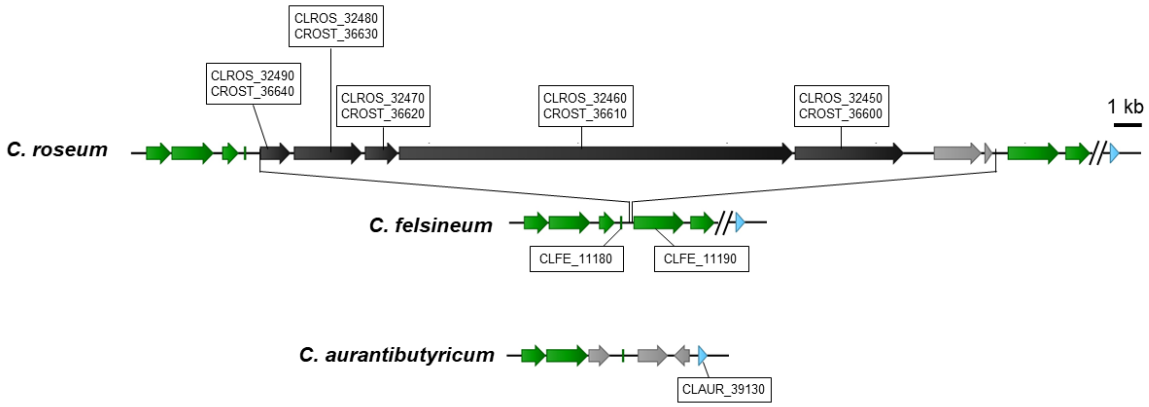
Supplementary Materials



Supplementary Fig. 1: Microscopy images of *C. felsineum* DSM794^T, *C. roseum* DSM 6424, *C. roseum* DSM 7320^T and *C. aurantibutyricum* DSM 793^T.
LM, light microscope images; SEM, scanning electron microscope images; TEM, transmission electron microscope images.



Supplementary Fig. 2: Gram staining of *C. felsineum* DSM 794^T, *C. roseum* DSM 6424, *C. roseum* DSM 7320^T, and *C. aurantibutyricum* DSM 793^T.
First row: cells were grown for 8 h; second row: cells were grown for 24 h; controls: first row *Bacillus subtilis* DSM 402; second row *Escherichia coli* DSM 498.



Supplementary Fig. 3: Schematic overview of the genome region of the *cspA-E* gene cluster integration.

Green and blue arrows symbolize homologous genes, black arrows mark the *csp* gene cluster of *C. roseum*, and grey arrows label unique genes of the strains.

3. Summary

The possibility of waste valorisation and the opportunities to use genetically engineered anaerobic bacteria as well as the importance of isolation and reclassification of bacteria was investigated in the presented publications.

The construction and improvement of recombinant autotrophic bacterial chassis strains for waste gas fermentation was investigated in the first part of the thesis. For an improved acetone production using CO_2 + H_2 as substrate, new recombinant *A. woodii* strains were constructed. Therefore, two new acetone production plasmids were constructed and transformed into *A. woodii*. The first plasmid pJIR750_ac1t1 harbours a ribosomal binding site upstream of the *adc* gene from *C. acetobutylicum*, which was missing in the original plasmid pJIR750_act$_{thlA}$ used by Hoffmeister et al. (2016). For the second plasmid pJIR750_ac2t1, the genes for the acetoacetyl-CoA:acetate/butyrate-CoA transferase (*ctfA/B*) from *C. acetobutylicum* were exchanged by the corresponding genes from *C. aceticum,* having a much lower K_m value for acetate. Autotrophic batch fermentation experiments in bottles showed that the addition of the ribosomal binding site did not lead to a much higher acetone production. The original acetone production strain *A. woodii* [pJIR750_act$_{thlA}$] produced 3.8 mM, whereas the newly constructed strain *A. woodii* [pJIR750_ac1t1] with the ribosomal binding site, produced 4.1 mM acetone. The exchange of the *ctfA/B* gene resulted in an increase of acetone production by the new recombinant strain *A. woodii* [pJIR750_ac2t1]. With this strain 6.0 mM acetone was produced throughout the autotrophic batch experiment in bottles. In contrast, *A. woodii* [pJIR750_ac1t1] produced the highest acetone concentration with 7.6 mM when tested in bioreactor fermentation experiments under anaerobic conditions. In comparison, *A. woodii* [pJIR750_ac2t1] only produced 3.2 mM acetone. Interestingly, all tested acetone production strains also produced isopropanol throughout the bioreactor fermentations. To identify the native secondary alcohol dehydrogenase of *A. woodii*, responsible for the conversion of acetone to isopropanol, growth experiments with the natural acetone producer *C. acetobutylicum* harbouring plasmids with possible candidate genes were performed. Unfortunately, only in case of the control strain *C. acetobutylicum*

[pIMP1_Padc_SADH] isopropanol production could be detected. These results showed that an improvement of acetone production by optimising the heterologous acetone production pathway is possible. Furthermore, the isopropanol formation by *A. woodii* could be a target for future industrial purposes and improvements.

The second part of the thesis focuses on the recombinant 1,3-PDO production from glycerol. Here, the waste valorisation of cheap glycerol to 1,3-PDO with genetically modified *C. beijerinckii* strains was investigated. As host organism, the natural producer *C. beijerinckii* DSM 6423 was chosen, however, preliminary experiments showed that this strain could not grow with glycerol as sole carbon source and only traces of 1,3-PDO were produced. To reinstall 1,3-PDO production in *C. beijerinckii* DSM 6423 a plasmid-based approach was tested. Thus, two plasmids were constructed. The plasmid pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis contained the 1,3-PDO gene cluster of *C. beijerinckii* DSM 15410 including a coenzyme B_{12}-independent glycerol dehydratase. For the second plasmid pMTL83251_P_{thlA}_dhaBCET.Cpas, the 1,3-PDO genes of *C. pasteurianum* DSM 525 were used, harbouring a coenzyme B_{12}-dependent glycerol dehydratase. 1,3-PDO production of the newly constructed recombinant *C. beijerinckii* strains was tested in batch experiments. Throughout the different growth experiments, 1,3-PDO production could only be detected in larger amounts when the 1,3-PDO gene cluster of *C. beijerinckii* DSM 15410 was expressed. Even with vitamin B_{12} supplementation, *C. beijerinckii* [pMTL83251_P_{thlA}_dhaBCET.Cpas] did not produce 1,3-PDO. Buffering the cultivation medium with MOPS resulted in increase in 1,3-PDO production by 82 %, which showed that a massive pH drop during the exponential phase resulted in less 1,3-PDO production. Furthermore, the exchange of the original *pta-ack* promoter sequence resulted in an improvement of 1,3-PDO production in case of all tested promoters. The highest 1,3-PDO production of 50.4 mM was achieved for *C. beijerinckii* [pMTL83251_P_{thlA}_1,3-PDO.diolis] in buffered medium. Compared to the results of the original production strain *C. beijerinckii* [pMTL83251_$P_{pta\text{-}ack}$_1,3-PDO.diolis] in unbuffered medium, the 1-3-PDO production could be improved by 167 %. Furthermore, with the use of the lactose-inducible *bgaL* promoter, this promoter was also tested for

recombinant 1,3-PDO production, a second inducible promoter system could be established in *C. beijerinckii* DSM 6425.

New feedstocks for industrial purposes are explored by many investigations. One interesting substrate is agar from algae, therefore, novel agarolytic bacteria strains are desired. In the third part of the thesis, the isolation, characterisation, and description of a novel agarolytic bacteria strain, designated as TS8^T, was presented. During an ecology field trip to Croatia in 2017, agarolytic bacteria strains were isolated from algae. For genome-based taxonomic approaches such as dDDH and 16S rRNA gene sequence analysis, the genome of the novel bacterium was isolated and sequenced. Genome sequence of TS8^T resulted in three circular contigs (chromosome: 3,198,344 bp; megaplasmid pCadTS8_1: 672,341 bp; megaplasmid pCadTS8_2: 646,854 bp). Analysis of the 16S rRNA gene sequence similarity indicated that the isolated strain belongs to the genus *Catenovulum*. Based on 16S rRNA gene sequence similarity strain TS8^T is closely related to *Catenovulum sediminis* D2^T (95.7 %), *Catenovulum agarivorans* YM01^T (95.0 %), and *Catenovulum maritimum* Q1^T (93.2 %). These results were supported by dDDH analysis with values below 25 %. The isolated strain is Gram-stain-negative, strictly aerobic, rod-shaped, and motile. Cell growth on seawater agar plates led to formation of dents in the medium indicating agarolytic activity of strain TS8^T. Furthermore, the genome of TS8^T harbours six genes for putative agarases. Main cellular fatty acids were summed features 3 ($C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c*), summed features 8 ($C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c*), and $C_{16:0}$. The main respiratory quinone was Q-8. Based on the phenotypic, physiological, and biochemical characteristics and chemotaxonomic analysis, strain TS8^T was considered to represent a novel species of the genus *Catenovulum*. *Catenovulum adriaticum* was proposed as name for strain TS8^T. Strain TS8^T was deposited at the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) as well as at the National Collection of Industrial Food and Marine Bacteria (NCIMB, Aberdeen, United Kingdom).

To declutter the bacterial taxonomy, many bacterial strains are reclassified based on novel genome-based methods. The fourth part of the thesis shows the efforts

that were made to reclassify the species *C. felsineum* DSM 794^T, *C. aurantibutyricum* DSM 793^T, and *C. roseum* (strains *C. roseum* DSM 7320^T and *C. roseum* DSM 6424) based on genomic and phenotypic investigations. Cells of all four strains were rod-shaped and similar in length (4.4 - 7.1 µm) and diameter (0.6 - 1.0 µm). Young cells of all four tested strains stained Gram-positive, whereas aged cells stained Gram-negative. Substrate and product spectrum of the four tested strains were also remarkably similar. The ANIm value (98-100 %) also supported the hypothesis that all examined strains belong to a single bacterial species. Furthermore, based on the genetic arrangement of the genes for the synthesis of clostyrylpyrones A-D, a possible gene reorganization could be reconstructed. Respective genes for the synthesis were identified as *cspA-E* in *C. roseum* DSM 6424. *C. roseum* DSM 7320^T contains the same chromosomal arrangement. In case of *C. felsineum*, the adjacent gene sequences were very similar, indicating a gene cluster insertion in the two *C. roseum* strains. In contrast, the upstream region of the insertion side was also present in the genome of *C. aurantibutyricum*. However, downstream a more complex gene rearrangement must have been occurred in this strain. Based on the presented data, the strains were reclassified and belong now to the species *C. felsineum*.

4. Literature

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5. Abbreviations

A

ABE: acetone-butanol-ethanol

Adc: acetoacetate decarboxylase

B

bp: base pairs

C

°C: degree Celsius

CO: carbon monoxide

CoA: coenzyme A

CO2: carbon dioxide

CtfA/B: acetoacetyl-CoA:acetate/butyrate-CoA transferase

D

dDDH: digital DNA-DNA-hybridization

DhaB: coenzyme B_{12}-dependent glycerol dehydratase, large subunit

DhaB1: coenzyme B_{12}-independent glycerol dehydratase, large subunit

DhaB2: coenzyme B_{12}-independent glycerol dehydratase, small subunit

DhaC: coenzyme B_{12}-dependent glycerol dehydratase, medium subunit

DhaE: coenzyme B_{12}-dependent glycerol dehydratase, small subunit

DhaT: NADH-linked 1,3-PDO dehydrogenase

DNA: desoxyribonucleic acid

E

e.g.:	lat. exempli gratia (for example)
et al.:	lat. et alii (and others)

G

GFP:	green fluorescent protein

H

H_2:	hydrogen
HPA:	hydroxypropionaldehyde

I

i.e.:	lat. id est (that is)

M

mM:	millimolar
Mt:	million tons

N

NADH:	reduced nicotinamide adenine dinucleotide

P

PDO:	propanediol

R

RNA: ribonucleic acid

rRNA: ribosomal RNA

S

syngas: synthesis gas

T

t: tons

ThlA: thiolase

U

UK: United Kingdom

US: United States

6. Publications

List of publications

1) Arslan, K., **Schoch, T.**, Höfele, F., Herrschaft, S., Oberlies, C., Bengelsdorf, F., Veiga, M. C., Dürre, P., & Kennes, C. (2022). Engineering *Acetobacterium woodii* for the production of isopropanol and acetone from carbon dioxide and hydrogen. *Biotechnology Journal*, *17*(5), 2100515. https://doi.org/10.1002/biot.202100515

2) **Schoch, T**., Höfele, F., Odeh, H., Winter, L. M., Stöferle, S., Karl, M., Bengelsdorf, F., Poehlein, A., Daniel, R., & Dürre, P. (2022). Reclassification of *Clostridium aurantibutyricum* Hellinger 1944 and *Clostridium roseum* (ex McCoy and McClung 1935) Cato et al. 1988. *International Journal of Systematic and Evolutionary Microbiology*, *72*(10), 005589. https://doi.org/10.1099/ijsem.0.005589

3) **Schoch, T.,** Baur, T., Kunz, J., Stöferle, S., & Dürre, P. (2023). Heterologous 1,3-propanediol production using different recombinant *Clostridium beijerinckii* DSM 6423 strains. *Microorganisms*, *11*(3), 784. https://doi.org/10.3390/microorganisms11030784

Declaration of own contributions

For the publication “Engineering *Acetobacterium woodii* for the production of isopropanol and acetone from carbon dioxide and hydrogen” all recombinant *A. woodii* strains (expect *A. woodii* [pJIR750_act]) were constructed by me. Experiments for identification of the secondary alcohol dehydrogenase in *A. woodii* were planned and supervised by me. Data of the bottle growth experiments as well as for the identification of the secondary alcohol dehydrogenase was analysed and visualized by me. The bioreactor experiments were performed by our project partners in Spain. Furthermore, I prepared parts of the original manuscript and revised parts of the manuscript during the revision process.

My contribution to “Heterologous 1,3-propanediol production using different recombinant *Clostridium beijerinckii* DSM 6423 strains” involved strain construction and performance of all presented experiments (except cloning of plasmids harbouring different promoters, construction of corresponding *C. beijerinckii* strains, and the related growth experiment). All recorded data were analysed, as well as all visualisations were done by me. Acting as the corresponding author, the manuscript was written and submitted by me.

For the prepared manuscript “*Catenovulum adriaticum* sp. nov., isolated from algae in the harbor of Susak (Croatia)” I isolated and performed all the presented experiments (except genome sequencing). Determination of growth characteristics, evaluation of the cellular fatty acid, major polar lipids and respiratory quinones composition was performed by me. Furthermore, I deposited *C. adriaticum* at the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) and the National Collection of Industrial Food and Marine Bacteria (NCIMB, Aberdeen, United Kingdom). All figures (except phylogenetic tree) and tables (except genome comparison) were created by me. In addition, the manuscript was written and submitted by me as the corresponding author.

For the publication “Reclassification of *Clostridium aurantibutyricum* Hellinger 1944 and *Clostridium roseum* (ex McCoy and McClung 1935) Cato et al. 1988” my own contribution included planning, supervision of all experiments performed in the institute for Microbiology and Biotechnology at University of Ulm. Furthermore, parts of the cultivation experiments were performed by me. Respective parts of the manuscript were written by me. I submitted and revised the manuscript during revision process as the corresponding author.

Contribution to conferences

Poster contribution

Schoch, T., Erz, C., Hoffmeister, S., Bengelsdorf, F. R., & Dürre, P. Production of acetone using recombinant *Acetobacterium woodii* strains. *2018*. Clostridium XV. Munich, Freising, Germany.

Schoch, T., Nick, S., Erz, C., Hoffmeister, S., Bengelsdorf, F. R., & Dürre, P. Production of acetone using recombinant *Acetobacterium woodii* strains. *2019*. C1-net. Nottingham, United Kingdom.

Schoch, T., Nick, S., Erz, C., Hoffmeister, S., Bengelsdorf, F. R., & Dürre, P. Production of acetone using recombinant *Acetobacterium woodii* strains. *2019*. VAAM. Mainz, Germany.

Schoch, T., Bengelsdorf, F. R., & Dürre, P. *Clostridium beijerinckii* DSM 6423: Transformation and plasmid-based overexpression of 1,3-propanediol production gene cluster. *2020*. VAAM. Leipzig, Germany.

Schoch, T., Stöferle, S., & Dürre, P. Recombinant 1,3-propanediol production using *Clostridium acetobutylicum*. *2022*. VAAM. virtual conference.

Danksagung

Aus datenschutzrechtlichen Gründen entfernt.

Lebenslauf

Aus datenschutzrechtlichen Gründen entfernt.

Eigenständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Alle aus der Literatur stammenden Quellen und alle sinngemäßen oder wörtlichen Zitate sind als solche kenntlich gemacht und im Literaturverzeichnis aufgeführt. Außerdem versichere ich, dass die vorliegende Arbeit weder vollständig noch in Auszügen an einer anderen Stelle zur Erlangung eines akademischen Abschlusses eingereicht wurde.

Ulm, den

Teresa Schoch