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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

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$_{thlA}$], [pJIR750_act$_{thlA}$], [pJIR750_ac2t1]) were used in this work.

The two newly constructed acetone production plasmids ([pJIR750_ac1t1] and [pJIR750_ac2t1]) are based on pJIR750_act$_{thlA}$.[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*Δ*M15*, Δ(*lacZYA-argF*), U169, *rec*A1, *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, *rec*A1, *gyr*A96, relA1, *lac*, [F′ *proAB lacI*^{q} ZΔ*M15* 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* as well 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.

2.6 | Bioreactor studies

2.6.1 | 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 HColumn (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$_{thlA}$ 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

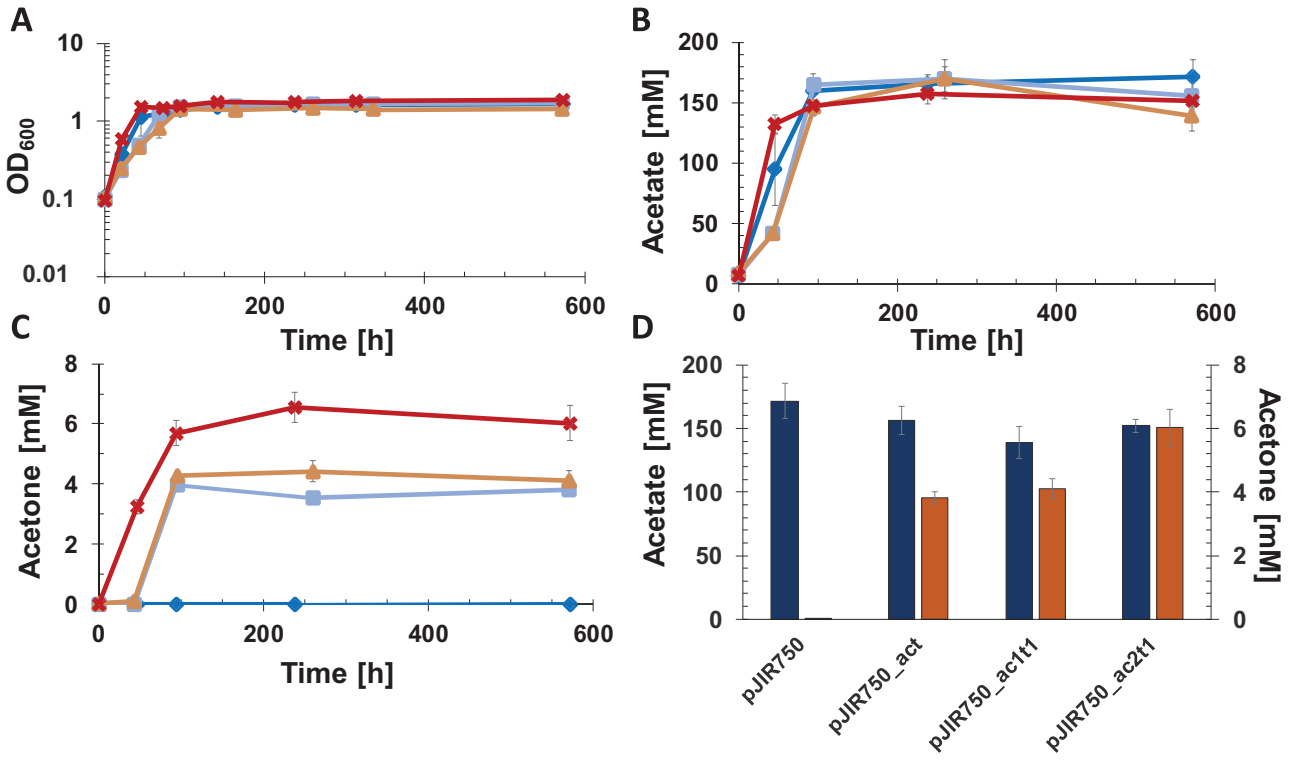

FIGURE 1 Autotrophic batch fermentations of recombinant *A. woodii* strains. *A. woodii* [pJIR750], *A. woodii* [pJIR750_act$_{thlA}$], *A. woodii* [pJIR750_ac1t1], and *A. woodii* [pJIR750_ac2t1] (◆, ■, ▲, and ×) in serum bottles. All data represent the mean with SDs of three biological replicates. (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

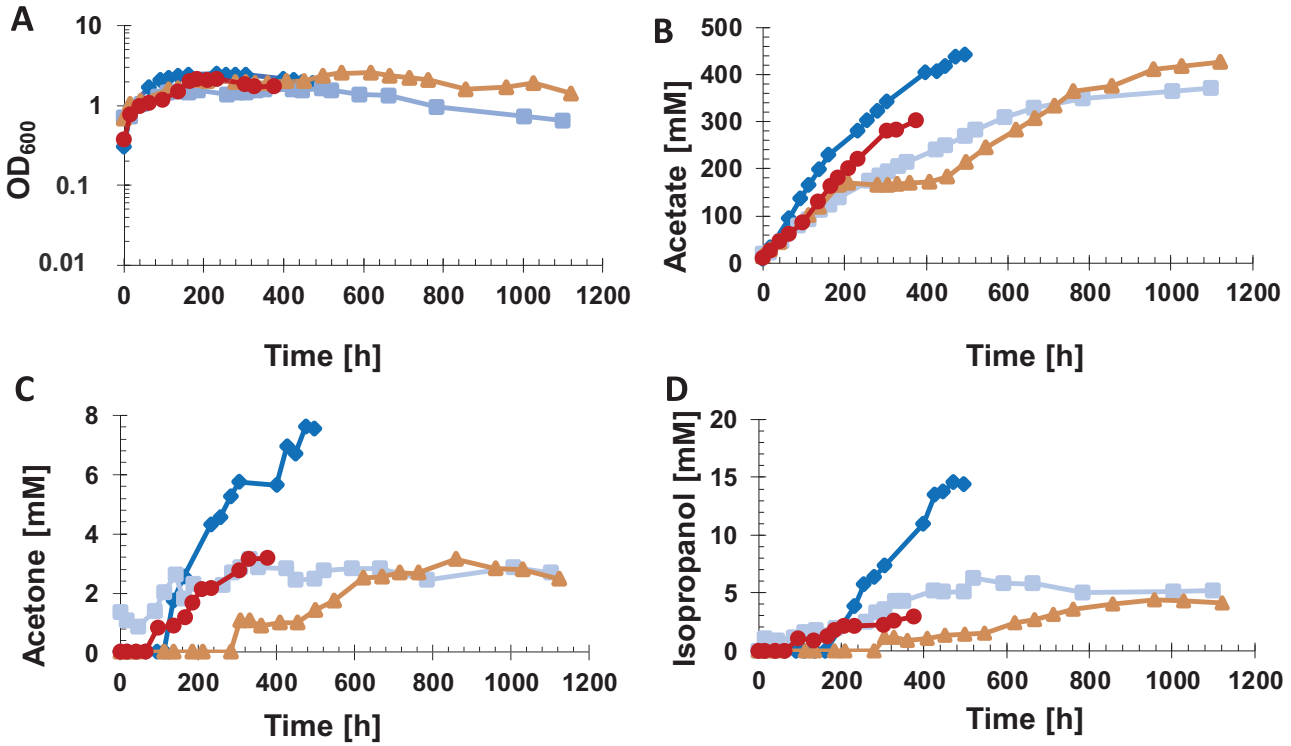

FIGURE 2 Autotrophic batch fermentations of recombinant *A. woodii* strains using stirred tank bioreactors with continuous gas supply. *A. woodii* [pJIR750_ac1t1], *A. woodii* [pJIR750_ac2t1], *A. woodii* [pJIR750_act$_{thlA}$], and *A. woodii* [pMTL84151_act$_{thlA}$] (◆, ■, ▲, and●). (A) Optical density 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 produced in only 230 h after inoculation with *A. woodii* [pJIR750_ac1t1]. In batch fermentation of *A. woodii* [pMTL84151_act_{thlA}], three times

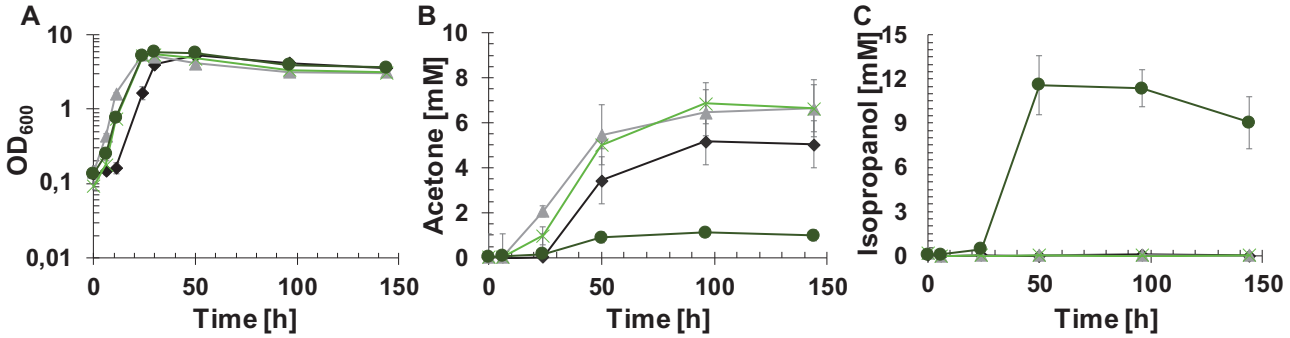

FIGURE 3 Heterotrophic batch fermentations of *C. acetobutylicum* wild type and recombinants.◆, *C. acetobutylicum* wild type; ▲, *C. acetobutylicum* [pIMP1_Padc_01070];×, *C. acetobutylicum* [pIMP1_Padc_17360]; ●, *C. acetobutylicum* [pIMP1_Padc_SADH]. Experiments were performed in serum bottles. All 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_{thlA}] 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 produced 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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