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 CO₂ and hydrogen was tested. The acetone biosynthesis pathway was constructed by combining genes from *Clostridium acetobutylicum* and *Clostridium aceticium*. 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 CO₂ and H₂ 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.⁴¹ 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.⁴² 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.⁴³ 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.\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[9]} In that sense, acetogenic bacteria, able to grow on CO and/or CO\textsubscript{2}, represent a promising microbial platform that can combine GHG reduction with fuels and bulk chemicals production.\textsuperscript{[2]} Exhaust emissions from certain heavy industrial processes, such as steel making, may contain gases such as CO\textsubscript{2}, CO, and H\textsubscript{2} in different ratios,\textsuperscript{[10,11]} as well as some other minor compounds and impurities.\textsuperscript{[12]} CO, CO\textsubscript{2}, and H\textsubscript{2} gas mixtures (i.e., syngas) can also be generated from a variety of renewable or waste-derived feedstocks such as lignocellulosic biomass.\textsuperscript{[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\textsubscript{2} gases.\textsuperscript{[13,14]} In this pathway, C\textsubscript{1} 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.\textsuperscript{[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.\textsuperscript{[21,18–22]} 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.\textsuperscript{[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. coskataii, C. autoethanogenum and Acetobacterium woodii.\textsuperscript{[24–29]}

A. woodii is a model organism of acetogenic bacteria with its genome already sequenced and its energy conservation pathway elucidated.\textsuperscript{[14,30]} Acetone production in recombinant A. woodii strains using genes from C. acetobutylicum has already been shown.\textsuperscript{[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\textsuperscript{[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\textsubscript{2}+H\textsubscript{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 (DH5\textalpha{} and XL1-Blue MRF\textsuperscript{+}) were cultivated under aerobic conditions at 37°C in LB medium.\textsuperscript{[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\textsuperscript{[25]} under anaerobic conditions at 30°C. For cultivation of C. acetobutylicum DSM 792, CG medium was used,\textsuperscript{[33]} and C. beijerinckii DSM 6423 was grown in 2xYT medium.\textsuperscript{[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 Zippy 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.\textsuperscript{[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.\textsuperscript{[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−, Δ (araD159, proA2, proC, trpE), supE44, Δ (rB−), endA1, hsdR17 (rK−, mK−), phoA, uvrD, relA | Life Technologies, Carlsbad, CA, USA                                                  |
| E. coli XL1-Blue MRF'      | Δ (mcrA)183, Δ (mcrB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA, [F' proABlacZ15 ΔM15 Tn10 (TetR)] | Agilent Technologies, Santa Clara, CA, USA                                           |
| A. woodii DSM 1030         | wild type                                                                                | DSMZ GmbH, Brunswick, Germany                                                        |
| A. woodii DSM 792          | wild type                                                                                | DSMZ GmbH, Brunswick, Germany                                                        |
| C. acetobutylicum DSM 1496 | wild type                                                                                | DSMZ GmbH, Brunswick, Germany                                                        |
| C. beijerinckii DSM 6423   | wild type                                                                                | DSMZ GmbH, Brunswick, Germany                                                        |
| pDrive                     | ColE1 ori−, f 1 ori, ApB (bla), KmB (aph), lacPOZ'                                      | Qiagen GmbH, Hilden, Germany                                                         |
| pJIR750                    | CmR, pMB1ori−, lacZ, pIP404 ori+                                                         | Bannam and Rood (1993)                                                               |
| pMTL84151                  | CmR, ColE1ori−, lacZ, pCD6 ori+, traI                                                   | Heap et al. (2009)                                                                  |
| pANS1                      | p15A ori−, SpB (spcE), Φ3T I                                                            | Böhrlinger, 2002                                                                   |
| pIMP1_catP                 | ColE1 ori−, p15M13 ori−, ApB (bla), EmB (ermC), CmR (catP)                               | Linder, unpublished                                                                 |
| pMTL84151_act_plmha        | pMTL84151, Pama, ada (CA_P0165), ctfA/ctfB (CA_P0163/CA_P0164), thlA (CAC2873) from C. acetobutylicum | Hoffmeister et al. (2016)                                                            |
| pJIR750_act_plmha          | pJIR750, Pama, ada (CA_P0165), ctfA/ctfB (CA_P0163/CA_P0164), thlA (CAC2873) from C. acetobutylicum | Hoffmeister et al. (2016)                                                            |
| pJIR750_act11              | pJIR750, act11 including native ribosomal binding sites upstream of adc as well as ctfA | This study                                                                            |
| pJIR750_act21              | pJIR750, Pama, ctfA/ctfB (CACET_c04240/CACET_c04250) from C. acetobutylicum             | This study                                                                            |
| pIMP1_Padc                 | pIMP1_catP, Pasm from C. acetobutylicum                                                  | This study                                                                            |
| pIMP1_Padc_01070           | pIMP1_catP, Pasm and gene with locus tag Awo_c01070 from A. woodii                      | This study                                                                            |
| pIMP1_Padc_17360           | pIMP1_catP, Pasm and gene with locus tag Awo_c17360 from A. woodii                      | This study                                                                            |
| pIMP1_Padc_SADH            | pIMP1_catP, Pasm-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               | GGTTACCAGGAGGTGACCTTTATA   | Amplification of adc from C. acetobutylicum for pDrive+adcRBS                        |
| adc_EcoRI_rev              | GAATTCTTACTAAGATAACTATATAACT |                                                                                      |
| ctfA_BamHI_fwd             | GGTATCCGAGGAGTGAAATGAGAAT | Amplification of ctfA/ctfB from C. acetobutylicum for pDrive+ctfA/ctfB             |
| ctfB_KpnI_rev              | GGATCCCTTCTTTAACAAGCCATCGTTCTAG |                                                                                      |
| ctfA/B_Cacet_fwd           | CATAAAAGTACCCCTCTCTGACCTTTTATAATTCCATAACCTTTAATCTGACCGAAAAATGCTGAGAAAAAGGATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of ctfA/ctfB from C. acetobutylicum for pJIR750_ac2t1 |
| ctfA/B_CacetSacII_rev      | ATATATTTAAGCAAGGGTCGAGATGGAACGGTGATGTGAGTGAATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of Pasm from C. acetobutylicum for pIMP1_Padc                                  |
| Padc_fwd                   | TGCGATTACGCTGGTACGGAAGGATGAGGAGATGAGTGAATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of Pasm from C. acetobutylicum for pIMP1_Padc                                  |
| Padc_rev                   | ATGTACATTTAGGAAGGGTCGAGATGGAACGGTGATGTGAGTGAATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of Pasm from C. acetobutylicum for pIMP1_Padc                                  |
| Awo_c01070_fwd             | AGTGATACATTTAGGAAGGGTCGAGATGGAACGGTGATGTGAGTGAATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of Pasm from C. acetobutylicum for pIMP1_Padc                                  |
| Awo_c01070_rev             | ATGTACATTTAGGAAGGGTCGAGATGGAACGGTGATGTGAGTGAATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of Pasm from C. acetobutylicum for pIMP1_Padc                                  |
| Awo_c17360_fwd             | ATGTACATTTAGGAAGGGTCGAGATGGAACGGTGATGTGAGTGAATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of Pasm from C. acetobutylicum for pIMP1_Padc                                  |
| Awo_c17360_rev             | ATGTACATTTAGGAAGGGTCGAGATGGAACGGTGATGTGAGTGAATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of Pasm from C. acetobutylicum for pIMP1_Padc                                  |
| SADH_CLOBI_fwd             | ATATATTTAAGCAAGGGTCGAGATGGAACGGTGATGTGAGTGAATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of SADH-encoding gene from C. beijerinckii for pIMP1_Padc_SADH            |
| SADH_CLOBI_rev             | ATATATTTAAGCAAGGGTCGAGATGGAACGGTGATGTGAGTGAATCCGAGGAGTGGTAGGTAGTAGGACCC | Amplification of SADH-encoding gene from C. beijerinckii for pIMP1_Padc_SADH            |
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*. For a positive control, pIMP1_Padc_SADH was constructed using the corresponding genes from *C. acetobutylicum*. The genes were amplified from genomic DNA of *C. acetobutylicum* via PCR using CloneAmp HiFi PCR Premix (Takara Bio Inc., Kusatsu, Shiga Prefecture, Japan) and ligated into the linearized pJIR750_actth1A (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 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] and *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, Elchingen, Germany) 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, OD₆₀₀ 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 N₂ + CO₂ (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 H₂ + CO₂ (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, OD₆₀₀, 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): NH₄Cl, 0.20 g; yeast extract, 2 g; KH₂PO₄, 1.76 g; K₂HPO₄, 8.44 g; MgSO₄ × 7 H₂O, 0.33 g; NaHCO₃, 10 g; L-Cysteine-HCl, 0.30 g; Na₂S × 9 H₂O, 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; nicotinic acid, 50 μg; D-Ca-pantothenate, 50 μg; vitamin B₁₂, 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): nitriiloacetic acid, 12.8 g; MnCl₂ × 4 H₂O, 0.1 g; NaCl, 5 g; FeCl₂ × 4 H₂O, 2 g; CoCl₂ × 6 H₂O, 0.2 g; ZnCl₂, 70 mg; CuCl₂ × 2 H₂O, 2 mg; H₂BO₃, 6 mg; Na₂MoO₄ × 2 H₂O, 36 mg, and NiCl₂ × 6 H₂O, 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. MgSO₄, 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,N,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% N₂, 25% CO₂, and 35% H₂, was fed through a microsparger into the reactor at a flow rate 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-2000, Pacisa & Giralt, Madrid, Spain, or, Ultrospec 1100 Pro, Amersham Biosciences Europe GmbH, Freiburg, Germany, respectively) at a wavelength of 600 nm (OD₆₀₀). 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 H₂SO₄ 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 H₂SO₄ 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. H₂ 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 pJR750act_thlA was the insertion of the native ribosome binding site (RBS) of the acetoadetate decarboxylase (adc) (CA_P0165) and CoA transferase (ctfA) genes (CA_P0163), resulting in plasmid pJR750_actt1. 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 Kₘ value
FIGURE 1  Autotrophic batch fermentations of recombinant A. woodii strains. A. woodii [pJIR750], A. woodii [pJIR750_actthlA], 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). 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). 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). 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). 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_actthlA] and A. woodii [pJIR750] 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_actthlA], 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_actthlA] and A. woodii [pJIR750_ac1t1]. Acetone concentrations of culture supernatants of A. woodii [pJIR750_actthlA], 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_actthlA], 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
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_actthlA], strain *A. woodii* [pMTL84151_actthlA] 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_actthlA]. 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 OD600 
value of 2, at t = 185 h, the biomass concentration remained nearly con-
stant until reaching t = 450 h. Even though acetone and isopropanol 
first appeared in the HPLC analysis during this period, no consid-
erable changes in the concentrations of any metabolites were observed. 
Later on, the second growth phase started, the biomass OD600 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 accumula-
tion 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_actthlA], was somewhat 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 
OD600 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, simultane-
uous with the initial production of acetone and isopropanol, and a maxi-
mum biomass OD600 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 pro-
duction. Acetic acid accumulation was also slower than with the first 
recombinant strain and, after t = 375 h, acetic acid reached its maxi-
mum 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). Padc was chosen to ensure gene 
expression during acetone production in C. acetobutylicum. Both plas-
mids 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 iso-
propanol 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 per-
formed best.

High growth rates and high biomass concentrations are required 
to achieve high metabolite concentrations and process productiv-
ity. A. woodii [pJIR750_ac1t1] and A. woodii [pJIR750_actthlA] showed the 
highest biomass concentrations amongst all transformants. How-
ever, A. woodii [pJIR750_actthlA] reached its maximum biomass OD600 
value 550 h after the beginning of the process, whereas A. woodii 
[pJIR750_ac1t1] reached its maximum biomass OD600 value at t = 160 
h. The maximum biomass OD600 for A. woodii [pJIR750_ac1t1] was 
2.54, which is higher than that reported recently for the genet-
ically engineered acetone-producing A.woodii [pMTL84151_actthlA] 
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 concen-
trations were observed with A. woodii [pJIR750_ac1t1] and A.woodii 
[pJIR750_actthlA], 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_actthlA], 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_actthlA], three times
higher acetic acid concentration (1330 mM) was reported\cite{25} in a shorter process time (360 h) and with a lower biomass accumulation (OD\textsubscript{max} = 2.2), than what was observed with \textit{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.\cite{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\textsuperscript{-1}) compared to the previously reported one and the gas fed to the bioreactor in this case contained N\textsubscript{2} besides H\textsubscript{2} and CO\textsubscript{2}. Other gases than H\textsubscript{2}, CO\textsubscript{2}, or CO\textsubscript{2} for example, N\textsubscript{2}, can actually also be found in syngas and in many industrial emissions.

\textit{A. woodii} [pJIR750\_ac1t1] produced the highest concentration of acetone, reaching 442 mg L\textsuperscript{-1}. A total amount of 16.5 mM acetone production was reported before\cite{25} with the recombinant strain \textit{A. woodii} [pMTL84151\_act\textsubscript{fda}] 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 \textit{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 \textit{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.\cite{25} The analytical system for batch fermentations used in that study\cite{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 \textit{C. ljungdahlii} and \textit{A. woodii} was shown before.\cite{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. bei-jerinckii is one of the major isopropanol-producing bacteria using sugars or lignocellulosic hydrolysates 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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