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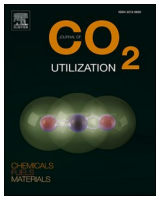

High-pressure fermentation of CO_2 and H_2 by a modified *Acetobacterium woodii*

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ABSTRACT

Global warming due to the increased atmospheric carbon dioxide concentration is the driving force for developing strategies that exploit CO_2 as raw material to produce interesting compounds for industry. According to this approach, *Acetobacterium woodii* was modified to convert CO_2 and H_2 into acetone. Gas fermentation was performed at high pressure to debottleneck the issue of the low availability of gaseous substrates in the liquid medium. This work aimed to investigate the catalytic performance of a modified *A. woodii* strain for acetone synthesis at 10 bar providing an H_2-CO_2 blend. First, tests were performed to assess the ability of the biocatalyst to survive heterotrophically at high pressure. Moreover, a reference test was set up in autotrophy at atmospheric pressure to confirm that it produced both acetate and acetone. Feeding the strain at 10 bar with the H_2-CO_2 mix resulted in growth inhibition and formic acid production. This outcome suggested a metabolism impairment due to bicarbonate build-up in the reactor at high CO_2 partial pressure. Thus, bacteria were grown at atmospheric pressure in a medium with an augmented exogenous salt concentration. Results confirmed that formic acid production and growth inhibition could be due to HCO_3^-. Furthermore, the modified *A. woodii* grown at atmospheric pressure in a sterile medium pressurized before inoculation showed the same outcomes. Finally, tests at 10 bar lowering the CO_2 partial pressure indicated that this gas was responsible for formic acid production but was not the only inhibitory factor for autotrophic cell growth at high pressure.

1. Introduction

The natural carbon cycle is being unbalanced by anthropogenic activities, which extract hydrocarbons to obtain energy and dump the carbon into the air in the form of greenhouse gases. Most of the carbon occurs in the form of carbon dioxide (CO_2). A significant step toward closing the carbon loop involves recycling the CO_2 released in the air and using it in place of hydrocarbons in energy and industrial processes.

Acetone is the simplest ketone and the world demand for this compound is increasing. In 2020 the global acetone market was 6.9 million Tons, estimated to reach 7.3 million Tons by 2027 [1]. It is widely used as a solvent and an intermediate for synthesizing materials such as polymethyl methacrylate (PMMA) and polycarbonate polymers. Nowadays, it is mainly produced from fossil fuels by the cumene route [2].

Acetogens are a group of bacteria belonging to different genera and widespread in nature that can reduce CO or CO_2 to Acetyl-CoA through the Wood-Ljungdahl Pathway (WLP) [3,4]. In literature, studies describe the underlying biochemical details and enzymes involved in the pathway in different acetogenic bacteria [5] and the energy conservation into the cells [6]. Acetogenic bacteria can be used to convert CO_2 and CO-rich waste gas streams into C1-C4 fuels and chemicals [5,7–9]. Compared to other catalytic processes for CO_2 valorization, acetogens present several advantages due to their high metabolic efficiency, ability

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to handle variable gas compositions, high product specificity and low susceptibility to poisoning. However, the main disadvantage is the potential energy starvation in autotrophic conditions [6,10].

Acetobacterium woodii is a model organism which has been studied to clarify common metabolic features of acetogens [11,12]. The cumulative knowledge about its metabolic [3,12] and energetic pathways [13–16] made it a reference organism among this heterogeneous group of bacteria. *A. woodii* grows heterotrophically on organic substrates such as glucose [17,20], fructose [18,19], lactate, glycerate, formate [19], and ethanol [20]. Moreover, it is capable of robust autotrophic growth, using CO_2 as a carbon source and H_2 as a reducing agent [12,21]. Acetate is usually the sole metabolic product. Nevertheless, techniques for its genetic manipulation are available [22,23] (Fig. 1).

Microbial acetone production has been studied for years via *Clostridium*-based acetone-butanol-ethanol (ABE) fermentation from organic substrates (e.g. sucrose, starch-based carbohydrates, lignocellulosic biomass). *Clostridium acetobutylicum* is the model strain for ABE fermentation. Genes and enzymes involved in the pathway for acetone synthesis are identified and characterized [24–26]. These genes can be exploited in metabolic engineering strategies to obtain modified bacteria strains (Fig. 1).

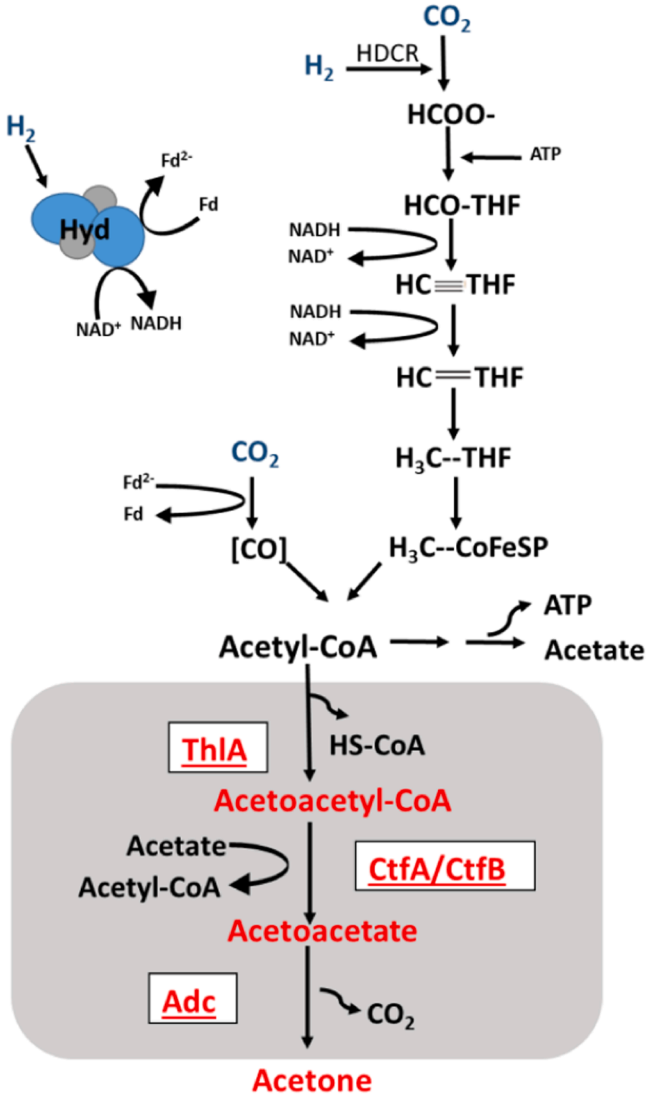

Fig. 1. Schematic representation of the Wood-Ljungdahl Pathway in *A. woodii* coupled with the acetone synthesis pathway of *Clostridium acetobutylicum* (red compounds in the gray square), based on [7,24]. Hyd: electron-bifurcating hydrogenase; HDCR: hydrogen-dependent CO_2 reductase; ThlA: thiolase A; CtfA/CtfB: acetoacetyl-CoA:acetate/butyrate CoA transferase; Adc: acetoacetate decarboxylase; THF: tetrahydrofolate, COFeSP: Corrinoid iron-sulfur protein.

A. woodii was previously engineered to produce acetone by implementing the synthetic pathway from *C. acetobutylicum* [24] or a mixed synthetic pathway from *C. acetobutylicum* and *Clostridium aceticum* [27]. The maximal acetone concentration reached in a stirred-tank bioreactor in a liquid batch with continuous gas supply fermentation was 0.96 g/L and the maximum productivity was 0.09 $g*L^{-1}*d^{-1}$ [24].

In gas-fermentation processes, microbial growth rate and productivity can be impacted by gas-liquid mass transfer limitations. In fact, low gas solubilities – especially for H_2 - could restrict biocatalytic activities due to low feedstock concentration in the liquid medium. Increasing the gas-liquid mass transfer can help to overcome this issue. Among the available methods [27,28], this work chose to increase the partial pressures of H_2 (pP_{H2}) and CO_2 (pP_{CO2}).

High-pressure gas fermentations have been previously undertaken using *A. woodii* cultivations on CO_2 and H_2 using different operative modes [29–31]. The highest pressure reported in autotrophic fermentation for wild-type *A. woodii* was 5.5 bar. Gaseous feedstock was provided in a fed-batch mode [32]. Studies on high-pressure fermentations in liquid batch and continuous gas supply are also present in the literature. In a first study [31], *A. woodii* was grown at different pressures with incremental pP_{H2} and constant pP_{CO2}, reaching a maximum pressure of 1.9 bar (pP_{H2} = 1.7 bar). Acetic acid productivity rose by increasing the pP_{H2} and it was the only product. The authors suggested that hydrogen was the limiting substrate for acetate synthesis. Nevertheless, the highest pressure applied in continuous gassing mode was 3.5 bar [30]. In this case, authors reported a significant formate production at increased pressure and suggested a relation between increasing pP_{H2} (up to 2.1 bar) and formate production.

In the present study, an *A. woodii* strain genetically modified to produce acetone was grown autotrophically from ambient pressure (≈ 1 bar) to 10 bar. Different pressure levels and gas blends were applied to investigate the effects on the recombinant *A. woodii* growth and products' spectrum.

2. Materials and methods

2.1. Microbial strain

A genetically modified *A. woodii* strain for acetone synthesis was used. *A. woodii* DSM 1030 was obtained from the DSMZ (Germany). First, the strain was modified to allow the expression of heterologous genes controlled by the TcdR sigma factor. 300 bp at the 3′ end of *pyrE* was deleted using a method based on allelic exchange previously described [33]. This deletion did not include the *pyrE* stop codon, and as a result an in-frame deletion was produced. This mutant was resistant to 5-Fluoroorotic acid (5-FOA) but required uracil supplementation to grow. Subsequently, the plasmid pMTL-JPB23 [33] was used to repair *pyrE* with the concomitant insertion of *tcdR* gene at the 3′ of the restored *pyrE*. Expression of the sigma factor TcdR enables the expression of heterologous genes via the P_{tcdB} promoter.

2.1.1. Plasmid pMTL24-1

To allow acetone synthesis, the *A.woodii* strain described in Section 2.1 was transformed with plasmid pMTL24–1. It is a 9670-bp plasmid that contains the acetone production operon of *C. acetobutylicum*, based on the pMTL80000-series plasmid pMTL84151 [34]. The operon is driven by the promoter P_{tcdB}, which is constitutively active when the plasmid is in a TcdR host. The plasmid backbone contains the *catP* thiamphenicol resistance gene from *Clostridium perfringens*. The plasmid also contains a Gram-negative replicon (ColE1) and a Gram-positive replicon (pCD6, derived from *Clostridioides difficile*). The genes for acetone synthesis were arranged in their original order (Fig. 2). These genes are *ctfA, ctfB, adc,* and *thl*. The plasmid was transformed into *A. woodii* using the electroporation method previously described [33]

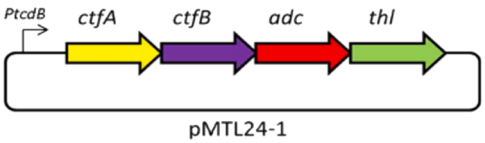

Fig. 2. Schematic plasmid map of the acetone production plasmid pMTL24–1. PtcdB: TcdR-dependent promoter driving the expression of the acetone production operon. The genes of the operon are: (*cftA*, *cftB*) CoA-transferase; (*adc*) acetoacetate decarboxylase; (*thl*) thiolase.

and maintained using thiamphenicol as a selective agent. The recombinant strain obtained was named *A. woodii* $p_{acetone}$.

2.2. Cultivation media

The medium used for the autotrophic cultivation of bacteria was as follows (per liter of distilled water): 1.76 g KH_2PO_4; 8.44 g K_2HPO_4; 1.0 g NH_4Cl; 2.9 g NaCl; 2 g yeast extract; 0.5 g cysteine hydrochloride; 0.180 g $MgSO_4$; 1 mg resazurin; 1 mL trace element solution SL9; 1 mL selenite-tungstate solution; 2 mL vitamin solution DMSZ 141.

The composition of the trace element was (per liter of distilled water): 12.8 g nitrilotriacetic acid; 2.0 g $FeCl_2$*$4H_2O$; 0.070 g $ZnCl_2$; 0.1 g $MnCl_2$*$4H_2O$; 0.006 g H_3BO_3; 0.19 g $CoCl_2$*$6H_2O$; 0.002 g $CuCl_2$*$2H_2O$; 0.024 g $NiCl_2$*$6H_2O$; 0.036 g Na_2MoO_4*$2H_2O$.

The composition of the selenite - tungstate solution was (per liter of distilled water): 0.5 g NaOH; 3 mg Na_2SeO_3*$5H_2O$, 4 mg Na_2WO_4*$2H_2O$.

The composition of the vitamins solution was (per liter of distilled water): 0.002 g biotin; 0.002 g folic acid; 0.010 g pyridoxine-HCl, 0.005 g thiamine-HCl*$2H_2O$; 0.005 g riboflavin; 0.005 g nicotinic acid; 0.005 g D-Ca-pantothenate; 0.0001 g vitamin B12; 0.005 g p-Aminobenzoic acid; 0.005 g lipoic acid.

The heterotrophic medium had the same recipe as the autotrophic medium with the addition of 6 g/L $KHCO_3$ and 3.6 g/L fructose. Thiamphenicol was used as a selective agent at a concentration of 15 mg/L.

2.3. Preparation of inocula for fermentation

Glass bottles with a total volume of 160 mL were filled with 30 mL of sterile heterotrophic medium. They were plugged with rubber caps and gassed with N_2. 100 µL of H_2SO_4 1.5 M were used to adjust the pH to 7.2–7.3. Thiamphenicol and cysteine were added, and anaerobic serum bottles were stored for around 24 h at room temperature. 3 mL of frozen culture were used as inoculum. Inoculated serum bottles were incubated at 30 °C in an orbital shaker (Biosan, LV) until the culture reached an optical density at 600 nm ($OD_{600\ nm}$) ≈ 1.

2.4. Batch fermentation in autotrophic serum bottles

Glass serum bottles (160 mL) were filled with 30 mL sterile autotrophic medium. To make the anaerobic condition, serum bottles were gassed with N_2. Then the feeding gas mix was provided by bubbling 14.7 L/h of a 70% H_2 and 30% CO_2 blend. The pH self-stabilized at 7.2. Thiamphenicol and cysteine were added, and anaerobic serum bottles were stored at room temperature until use. The inoculum was a heterotrophic culture in exponential phase. A culture volume was inoculated such that the final $OD_{600\ nm}$ in the bottle was around 0.2. Tests were conducted in a gas fed-batch mode to prevent the arrest of bacteria growth due to feedstock depletion. When refilled, the headspace of the serum bottle was first washed with fresh 70% H_2 and 30% CO_2 gas mix. The gas blend was supplied at 14.7 L/h through a needle placed into the septum of the serum bottle. Another needle was simultaneously inserted into the septum as a vent to avoid pressure increase inside the glass bottle. After washing with the fresh mix, an overpressure of ≈ 1.4 bar in the headspace was created by supplying the gas mix through the inlet needle and removing the outlet needle. After the inoculum, bottles were incubated upright at 30 °C in an orbital shaker.

2.4.1. Fermentation in serum bottles with $KHCO_3$ and KCl

Fermentations at high concentrations of salts were set up in serum bottles filled with 30 mL autotrophic medium. 150 mM KCl, 150 mM $KHCO_3$, 300 mM KCl, or 300 mM $KHCO_3$ were added to recipe's components in Section 2.2. In parallel, bacteria were grown in the standard autotrophic medium as a control. pH was adjusted at 7.6 using sterile KOH 1 M. Control test and tests with 150 mM of added salts were conducted for 25 h. Tests with 300 mM added salts were carried on for 42 h. For each condition, triplicates were performed.

2.5. Fermentations in bioreactors

2.5.1. Batch heterotrophic fermentation at high-pressure

High-pressure heterotrophic fermentations in batch mode were conducted in a custom-adapted bioreactor manufactured by the H.E.L group (UK). The details of the reactor hardware are described elsewhere [35]. Briefly, the core system was a 2 L oil-jacketed, stirred tank reactor. The head plate of the vessel was fitted with pressure, temperature, pH, and redox probes (Sentek, UK). Pression control was accomplished through a proportional Back Pressure Regulation (BPR) valve (Norgren, USA). Gases were sparged in the medium via a micrometric sparger placed at the vessel's bottom and in-flow gas rates were controlled by Mass Flow Controllers (MFC) (Vögtlin Instruments, CH; Bronkhorst High-Tech BV, NL).

The vessel was filled with 0.9 L heterotrophic medium and gassed with N_2. Cysteine was added and pH was adjusted to 7.2 with H_2SO_4 3 M.

To evaluate the resistance of *A. woodii* cells up to 11 bar pressure, a heterotrophic preculture of *A. woodii* $p_{acetone}$ was inoculated such that the starting $OD_{600\ nm}$ in the reactor was 0.14. The culture was grown at atmospheric pressure (≈ 1 bar), in a nitrogen atmosphere, at 30 °C, 400 rpm until $OD_{600\ nm}$ = 0.5. Then the pressure was increased to 11 bar by sparging nitrogen with an in-flow rate of 30 L/h.

Cells were left for 1 h in the reactor at 11 bar and then were recovered following two different protocols:

i) Fast depressurization (D-fast): the culture was collected by sampling from the vessel when the pressure inside the reactor was 11 bar. After the sampling, the pressure inside the reactor slightly decreased (≈ 10.9 bar). Nevertheless, N_2 was quickly provided to restore the pressure up to 11 bar.
ii) Slow depressurization (D-slow): pressure in the reactor was decreased by 1 bar every 30 min. The descent from 11 bar to 1 bar took 5 h. The sampling was done when the pressure was 1.1 bar.

Bacterial viability after the pressurization-depressurization protocol was assessed by re-inoculating 2.5 mL of pressurized/depressurized cultures into 30 mL of heterotrophic medium in serum bottles (Supplementary material 1).

2.5.2. Autotrophic fermentation with continuous gas supply

Autotropic experiments in liquid batch and continuous gas supply were performed at atmospheric pressure, 1.7 bar and at 10 bar. Fermentations were conducted in the 2 L bioreactor described in Section 2.5.1. 0.8 L of the autotrophic medium was loaded into the vessel, gassed with nitrogen, and reduced with cysteine. Before inoculation, a 70% H_2 and 30% CO_2 mix was supplied into the reactor at 4.5 L/h for 3 h at a constant pressure of 1.5 bar [35]. The pH stabilized at 7.2–7.3. A heterotrophic preculture of *A. woodii* $p_{acetone}$ was inoculated such that the starting $OD_{600\ nm}$ in the reactor was ≈ 0.2. Regardless of the operative mode investigated in each specific fermentation, a first step of gas and liquid batch at 1.5 bar in a 70% H_2 and 30% CO_2 atmosphere was

performed. When the $OD_{600\,nm}$ inside the reactor rose to ≈ 0.5, parameters chosen for each test were applied. The result section of each specific experiment describes the constant in-flow gas rate, the gas mix composition and the pressure setpoint applied. Fermentations were carried out at 30 °C and 400 rpm. pH was controlled at 7 using NaOH 3.5 M.

2.5.3. Medium pressurization

Fresh medium pressurization experiments were carried out in a 0.5 L custom-made bioreactor manufactured by the H.E.L group (UK). The vessels were allocated into a polyBLOCK that allowed heating and stirring. A magnetic drive placed below each vessel couples with the PTFE-coated magnetic stirrer within the reactor. This PTFE disc was connected to a shaft suspended from the lid where a Rushton impeller was clamped. Probes, MFCs for gas supply and BPR for pressure control were analogous to those reported above (Section 2.5.1). The vessel was filled with 0.2 L of autotrophic medium gassed with N_2 to remove oxygen. The temperature was set to 30 °C and stirred at 100 rpm. Subsequently, a 100% H_2 stream with an in-flow rate of 4.8 L/h was provided for 2.25 h. Then, the vessel was pressurized up to 8 bar. When the stated pressure was reached, the gas supplied was replaced by the 70% H_2 and 30% CO_2 mix, provided at 4.8 L/h for 0.25 h. The pressure was then decreased, and the continuous gassing of the medium continued for 3 h at 1.1 bar using 4.8 L/h of 70% H_2 and 30% CO_2 blend. As a control, an experiment was performed in which the pressure during the gas change phase from 100% H_2 to 70% H_2 and 30% CO_2, was constantly maintained at 1.1 bar. After gassing, the sterile medium in the vessel was inoculated following the procedure described above (Section 2.5.2). During fermentation, 0.6 L/h of the 70% H_2 and 30% CO_2 mixture was continuously supplied. pH was controlled at 7 using NaOH 3.5 M. Cultures were monitored for 24 h and experiments were performed in duplicates.

2.6. Analytical methods and calculation

Optical density during experiments was measured at λ = 600 nm ($OD_{600\,nm}$) using a DH-5000 Spectrophotometer (HACH, USA) or a V-730 Spectrophotometer (Jasco, JP). Distilled water was used as the blank to set the reference for the measurements. An experimentally determined correlation factor of 0.38 (data not shown) was used to calculate the dry cell weight in autotrophic fermentation.

Quantification of fructose and liquid metabolic products was performed through high-performance liquid chromatography (HPLC). 2.5 mL of bacterial culture withdrawn from serum bottles or reactors were filtered using a 0.22 μm PES syringe filter in vials for HPLC analysis. Measurements were done by a Prominence HPLC System (Shimadzu, JP) equipped with a Refractive Index Detector and a Diode Array Detector. Compounds were separated through a Resez ROA-Organic Acid column (Phenomenex, USA), kept at 50 °C. The mobile phase was H_2SO_4 0.005 M, flowing at 0.7 mL/min.

Gas composition for in-flowing and out-flowing gas was measured using an Agilent 490 Micro GC (Agilent, CA, USA) or a Fusion Micro GC (Inficon, CH). Both instruments were equipped with the analytical column Molsieve 5 Å, using Argon as the carrier. CO_2 analysis was done with a PoraPLOT U column (Agilent 490 Micro GC) or a Rt-U-Bond column (Fusion Micro GC) using Helium as the carrier.

Cell-specific production rate (q) and volumetric production rate (r) were calculated as follows [32]:

$$q_p = (1/c_{xav})*(dc_p)/dt \quad (1)$$

where q_p is the formation rate of a specific product (g product * g biomass^{-1} * d $^{-1}$); t is the time between two sampling points of the fermentation (d); c_{xav} is the average of biomass concentration (g*L^{-1}) between the two selected sampling points; c_p is the increase of product concentration (g*L^{-1}) between the two selected sampling points.

$$r_p = dc_p/dt \quad (2)$$

where r_p is the formation rate of a specific product (g product * L^{-1} * d $^{-1}$); t is the time between two sampling points of the fermentation (d); c_p is the increase of product concentration (g*L^{-1}) during the period considered.

CO_2 conversion in metabolic products was calculated as follows:

$$(\sum \text{mol } CO_{2\ \text{into acetate, formate, acetone, biomass}} / \text{mol } CO_{2\ \text{inlet}}) *100 \quad (3)$$

where $\sum mol\ CO_{2\ into\ acetate,\ formate,\ acetone,\ biomass}$ is the sum of the moles of CO_2 converted in each main metabolic product; $mol\ CO_{2\ inlet}$ are the total moles of CO_2 provided to the reactor.

3. Results and discussion

3.1. No effect of high pressure on A. woodii $p_{acetone}$ vitality in heterotrophic growth

Literature data concerning *A. woodii* survival at high pressure up to 10 bar are currently unavailable to the best of our knowledge. Thus, the first test of the present study consisted of assessing the ability of *A. woodii* $p_{acetone}$ to survive at 10 bar. To discriminate between the impact on cell growth of the total pressure of a general gas/mixture and the effect of the partial pressure of a specific feeding gas (CO_2 and H_2), the strain was cultivated in the heterotrophic medium, using nitrogen to pressurize the reactor. Feeding the reactor with a gas stream may cause a pressure fluctuation close to the set value at the beginning of the fermentation. *A. woodii* resistance in heterotrophy was assessed at 11 bar to take into account a phase of fluctuating pressure close to 10 bar.

An *A. woodii* $p_{acetone}$ culture in exponential phase was stressed by pressurization up to 11 bar and subsequent depressurization. Cells were recovered with a fast depressurization (D-fast) or a slow depressurization (D-slow) protocol, which required a few seconds or 5 h to return the cells to atmospheric pressure, respectively (Supplementary material 1).

Bacterial vitality after the pressure stress was evaluated by re-inoculating the collected culture in serum bottles with the heterotrophic medium and monitoring the growth curve. The results were compared to the curve of *A. woodii* $p_{acetone}$ unstressed by pressure challenge (control curve). Bacteria stressed by different pressurization/depressurization protocols and control showed similar growth curves (Fig. 3). The specific growth rates of the cultures were the following: control condition 0.048 h^{-1}; D-fast protocol 0.059 h^{-1} and D-slow protocol 0.048 h^{-1}. Moreover, the growth profile of the culture in the reactor was monitored: the pressurization and depressurization steps did not perturb the growth curve (Fig. 4). Culture had a specific growth rate of 0.042 h^{-1}.

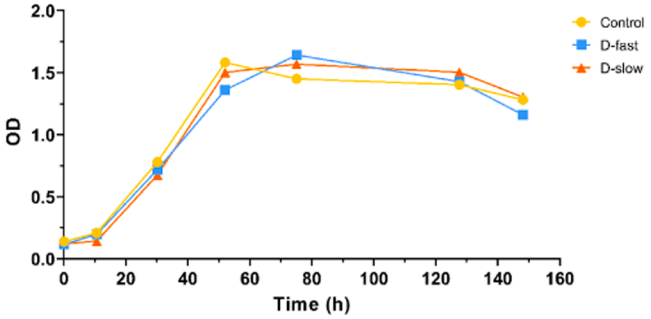

Fig. 3. Growth curves of *A. woodii* $p_{acetone}$ re-inoculated in heterotrophic serum bottles after different pressurization and depressurization protocols.

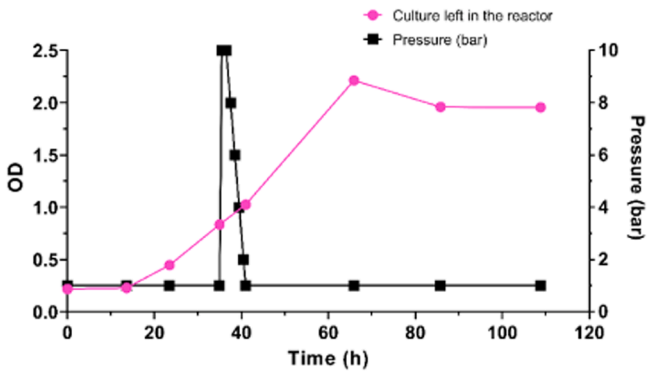

Fig. 4. Growth curve of *A. woodii* $p_{acetone}$ in the heterotrophic medium in the reactor stressed by pressure increase followed by the slow depressurization protocol.

3.2. Effects of high pressure on A. woodii $p_{acetone}$ in autotrophic growth

3.2.1. Reference CO_2/H_2-based fermentation of A. woodii $p_{acetone}$ strain at atmospheric pressure

Before high-pressure tests, a reference experiment was performed without pressure stress to evaluate the performance of *A. woodii* $p_{acetone}$ in a CO_2/H_2-based continuously-gassed and liquid batch fermentation (total pressure 1.1 bar). The continuous gassing with a 70% H_2 and 30% CO_2 blend was supplied with an in-flow gas rate of 5.3 L/h. The culture in the reactor reached a maximum $OD_{600\ nm}$ of 0.9 after 77.25 h. Bacteria produced acetate during the experiment, reaching a final concentration of 10.2 g/L in the stationary phase. Acetone achieved the maximum concentration (0.5 g/L) at the beginning of the stationary phase. However, from this maximum concentration it reduced slightly to 0.4 g/L. Formic acid was synthesized at the beginning of the continuous gassing, reaching a maximum of 0.3 g/L. Subsequently, this formate was consumed by the cells and again detected at the end of the stationary phase (0.7 g/L) (Fig. 5).

The maximum growth rate and productivities for each compound are reported in Table 1. The results obtained in the present study are compared to data described in literature in the last decade – summarized in Table 1- in which fermentations were carried in a batch stirred tank bioreactors with a continuous gas supply at atmospheric pressure. In most of the studies, wild-type *A. woodii* was the biocatalyst, so data are related to acetate production. However, Hoffmeister and coworkers [24] and Arslan and coworkers [27] used a modified *A. woodii* strain for acetone production. Thus, in these cases, it was possible to make a comparison on acetate and acetone productions.

The continuous gas supply allows for dealing with limited gas-liquid mass transfer increasing in-flow gas rate and stirring. As can be seen from Table 1, a wide range of stirring speeds was attempted (200–1200 rpm), as was the gas stream to feed the reactor (0.6–30 L/h). Moreover, experiments had different durations and amounts of biomass in the vessel, leading to different acetate maximum concentrations and volumetric productivities. According to the volumetric productivities of acetate, this study ranked among the lower values. Nevertheless, it also had the lowest OD. The number of cells in the liquid influences the volumetric production rate, so the low final OD in the present work can explain the result. Considering the acetate cell-specific productivity, the value reached in this study is among the highest recorded.

Dürre and coworkers [24] reported a maximum acetone concentration of 0.96 g/L. In the present experiment, acetone concentration achieved a lower value (0.5 g/L), consistent with the best-performing *A. woodii* modified strain in the study of Arslan et al. [27]. Nevertheless, the duration of the fermentation was different in the three works. The specific productivity measured in this study is the highest, even if the number of cells in the reactor is the lowest. However, the maximum acetone concentration reached in this study and previous studies [24, 27] has the same order of magnitude, which is quite low. Hoffmeister et al. [24] report that acetate concentration influenced acetone productivity. In their study, 1330 mM acetate in the medium promoted the synthesis of acetone. The authors suggested this effect could be due to the affinity of the CoA transferase of *C. acetobutylicum* (K_m value ≈ 1200 mM for acetate). In the work of Arslan et al. [27], CtfA/CtfB of C. *acetobutylicum* was replaced by the CoA transferase of *C. aceticum*, which has a lower k_m value for acetate (≈ 40 mM). The strain modified with the genes of *C. aceticum* reached a higher acetone concentration than the one modified with *ctfA/ctfB* of C. *acetobutylicum*.

Only Groher & Weuster-Botz [18] referred to the synthesis of formic acid. Bacteria produced this compound when the stirring was increased in the early fermentation phase. Then, it was subsequentely assimilated during the exponential phase. Finally, formate was synthesized again, reaching the maximum concentration of 1.1 g/L. The synthesis profile reported by these authors is consistent with the results obtained in the present study.

3.2.2. CO_2/H_2-based fermentations of A. woodii $p_{acetone}$ at 10 bar

The previous experiment indicated that *A. woodii* $p_{acetone}$ grew and synthesized acetone in continuous gas supply at atmospheric pressure. However, a deviation of the physiology of wild-type *A. woodii* was recorded during a CO_2/H_2-based continuously-gassed fermentation at a pressure higher than the atmospheric one (N_2:H_2:CO_2, gas blends up to 3.5 bar) [30]. Specifically, the metabolic profile of *A. woodii* shifted towards formic acid production and bacteria stopped growing. Nevertheless, in another experiment carried out at 1.9 bar (H_2:CO_2, 90:10) [31], wild-type *A. woodii* did not show any deviation in the metabolism or growth inhibition. The same results on metabolic profile and growth were described at 5.5 bar (85:15 H_2:CO_2) using a fed-batch gas feeding

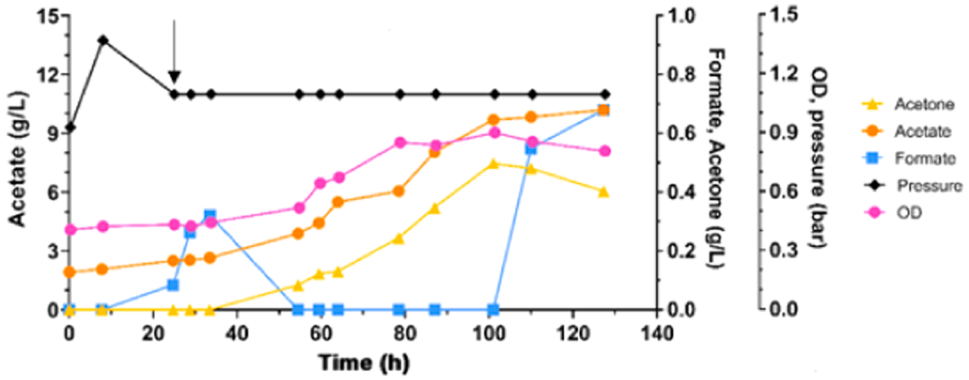

Fig. 5. Growth curve and main metabolic products of *A. woodii* $p_{acetone}$ in autotrophy at 1.1 bar, in liquid batch and continuous gassing (70:30 H_2:CO_2). The black arrow indicates the starting of the continuous gassing.

Table 1
Comparison of parameters and productivities between studies employing *A. woodii* as biocatalyst in stirred tank bioreactors in continuous gas supply, at atmospheric pressure. r = volumetric productivity; q = cell-specific productivity; μ_{max} = maximum growth rate; Mod = modification.

Study	*A. woodii* strain	In-flow gas rate (L/h)	Stirring speed (rpm)	Gas mix H_2:CO_2:N_2	Initial OD	Max OD	Working volume (L)	μ_{max} (h^{-1})	Max acetate (g/L)	Max $r_{acetate}$ ($g*L^{-1}*d^{-1}$)	Max $q_{acetate}$ ($g*g^{-1}*d^{-1}$)	Max acetone (g/L)	Max $r_{acetone}$ ($g*L^{-1}*d^{-1}$)	Duration (h)
This study	Mod: Acetone production	5.3	400	70:30:0	0.4	0.9	1	0.04	10.2	5.68	22.06	0.5	0.29	127
Straub et al., 2014 [23]	Mod: empty pJIR750	30	1200	40:16.7:43.3	1.1	3.4	1	0.052	44.7	25.31	20.54	-	-	89
Kantzow et al., 2015 [36]	Wild type	30	1200	40:17:43	4.8	6.4	1	0.05	59.2	-	16.7	-	-	77
Groher and Weuster-Botz, 2016 [18]	Wild type	5	200+ 600	80:20:0	0.4	2.3	1	0.08	37.4	20.37	24.25	-	-	144
Steger et al., 2017 [37]	Wild type	4.2	400	80:20:0	0.1	-	0.6–0.7	-	21.7	1.93	-	-	-	312
Steger et al., 2017 [37]	Wild type	4.2	200+ 400	40:10:50	0.1	-	0.6–0.7	-	29.57	1.61	-	-	-	840
Hoffmeister et al., 2016 [24]	Mod: Acetone production	30	800	20:80:0	-	2.2	1	-	79.87	-	-	0.96	0.09	357
Arslan et. al. 2022 [27]	Mod: Acetone production	0.6	250	35:25:40	-	2.5	1.3	-	26.3	-	-	0.44	-	500

strategy [32]. Therefore, following experiments assessed the effect on the metabolic spectrum and growth of *A. woodii* $p_{acetone}$ applying the CO_2/H_2 continuous-gassing mode at 10 bar. Since in some cases gaseous substrates were found to be inhibitory for biocatalysts [29], tests with different combinations of gas in-flow rates and cell concentration in the bioreactor were performed. The following combinations were tested: i) high cell concentration ($OD_{600\ nm}$ =1.5) and high in-flow gas rate (5.3 L/h); ii) high cell concentration ($OD_{600\ nm}$ = 1.2) and low in-flow gas rate (0.6 L/h); iii) low cell concentration ($OD_{600\ nm}$ = 0.6) and low in-flow gas rate (0.6 L/h). In all experiments, the gas mix provided was 70% H_2 and 30% CO_2. Results show that, in all conditions tested, cell growth stopped quickly after increasing pressure to 10 bar and then the $OD_{600\ nm}$ had a mild decline. Table 2 reports the calculated parameters for main metabolic products. For a comparison, parameters obtained from the reference experiment at atmospheric pressure (3.2.1 section) are also listed.

CO_2 conversion into metabolic products was enhanced by increasing the fermentation pressure (Table 2). Nevertheless, data showed that, regardless of the different in-flow gas rates and cell concentrations inside the reactor, maintaining 10 bar pressure with CO_2/H_2-based gas mix inhibited cell growth. Acetate, acetone, and formate were

Table 2
CO_2 conversion, yields, and productivities of *A. woodii* $p_{acetone}$ grown in autotrophy at 10 bar with different in-flow gas rates and biomass concentrations.

	Reference) Atmospheric pressure High in-flow gas rate	i) 10 bar High biomass- High in-flow gas rate	ii) 10 bar High biomass- Low in-flow gas rate	iii) 10 bar Low biomass- Low in-flow gas rate
Gas blend	H_2:CO_2, 70:30	H_2:CO_2, 70:30	H_2:CO_2, 70:30	H_2:CO_2, 70:30
In-flow gas rate (L/h)	5.3	5.3	0.6	0.6
CO_2 conversion in metabolic products (%)	4.1	13.21	35.86	19.54
Acetate yield (%)	3.6	6.08	25.99	9.30
Acetone yield (%)	0.2	0.97	1.52	0.66
Formate yield (%)	0.15	6.17	6.50	9.03
Biomass growth	yes	no	no	no
Max acetate concentration (g/L)	10.2	4.07	2.27	3.69
Max acetone concentration (g/L)	0.5	0.49	0.10	0.26
Max formate concentration (g/L)	0.68	5.85	1.58	4.91
Max volumetric productivity Acetate ($g*L^{-1}*d^{-1}$)	5.68	8.04	2.69	2.59
Max volumetric productivity Acetone ($g*L^{-1}*d^{-1}$)	0.29	0.95	0.11	0.12
Max volumetric productivity Formate ($g*L^{-1}*d^{-1}$)	1.46	5.12	1.86	0.98
Max cell-specific productivity Acetate ($g*g_{biomass}^{-1}*d^{-1}$)	22.06	16.27	7.48	12.21
Max cell-specific productivity Acetone ($g*g_{biomass}^{-1}*d^{-1}$)	0.91	1.93	0.31	0.74
Max cell-specific productivity Formate ($g*g_{biomass}^{-1}*d^{-1}$)	6.49	11.49	5.19	4.88
Duration (h)	127	28	21	121

synthesized in high-pressure fermentations. While acetate was the main product at atmospheric pressure, acetate and formate were the predominant products at high-pressure. Acetate and formate yields were similar in combinations i) and iii), while in combination ii) the yield of formate is lower than acetate. Among high-pressure fermentations, combination i) showed the highest specific productivities for all compounds. The highest ratio between acetate cell-specific productivity and formate cell-specific productivity was recorded by applying a 5.3 L/h gas rate at atmospheric pressure. *A. woodii* $p_{acetone}$ grown at 10 bar in CO_2/H_2 - continuous gassing showed the same metabolic profile and growth inhibition described in the literature for wild-type *A. woodii* at 3.5 bar in the same operative mode [30].

3.3. Bicarbonate influence on growth and metabolic profile of *A. woodii* $p_{acetone}$

A. woodii $p_{acetone}$ in autotrophic fermentation at 10 bar ceased growing and redirected the metabolic flow towards formic acid. At the beginning of the WLP, one molecule of CO_2 is reduced to formate by a hydrogen-dependent CO_2 reductase (HDCR, Fig. 1) [5]. This enzyme directly reduces CO_2 to formate using H_2 as an electron donor, without any other cofactor involved [6]. Further conversion of formate depends on the ATP availability in the cell. Thus, a shortage of ATP can lead to formate accumulation [30]. Literature studies [29,37] suggested that the pP_{H2} could cause the metabolic shift towards formic acid in *A. woodii*. Nevertheless, a more recent study describes an increased formic acid production by the acetogen *Thermoanaerobacter kivui* grown at atmospheric pressure with 300 mM of bicarbonate in the medium [21].

In the present work, *A. woodii* $p_{acetone}$ was fed with high pP_{H2} and pP_{CO2}. CO_2 solubility is higher than H_2 solubility in water. Moreover, CO_2 in aqueous solutions can be present in different forms such as CO_2, H_2CO_3, HCO_3^- and CO_3^{2-}. The distribution between the forms depends on the pH [38,39]. When the pH is 7, HCO_3^- should be the predominant form. To assess if bicarbonate had the same effects previously described on *T. kivui* [21], *A. woodii* $p_{acetone}$ was cultured autotrophically in serum bottles with an increased amount of $KHCO_3$ in the medium.

3.3.1. Fermentation with different salt types and concentrations in serum bottles

Autotrophic serum bottles without $KHCO_3$, with 150 mM and 300 mM $KHCO_3$ were set up. Serum bottles with 150 mM and 300 mM KCl in the autotrophic medium were also prepared to discriminate between effects due to bicarbonate or to the osmotic condition. Specific growth rate and metabolic profiles for each condition were analysed. Results are reported in Fig. 6.

Adding salts to the medium impaired bacteria growth. Nevertheless, 300 mM $KHCO_3$ completely stopped it. In serum bottles with 150 mM $KHCO_3$, final acetic acid and formic acid concentrations were similar, while in serum bottles with 300 mM $KHCO_3$, formic acid production overcame acetic acid production. The control culture and the cultures with KCl displayed the same metabolic profile: acetic acid was the main product.

Acetone was not detected during any of these growth tests. Nevertheless, they were carried on for a maximum of 42 h and it is possible that acetone synthesis had yet to begin. In fact, acetone was not produced immediately after the inoculum, as observed also in the autotrophic fermentation at atmospheric pressure (Section 3.2.1) and reported in other studies [24,27]. The delayed production could be due to a threshold of acetate concentration needed to boost the acetone pathway enzymes' activity. To test this hypothesis, specific studies on this topic are now ongoing. Preliminary results suggest that increasing acetate concentration at the fermentation's beginning can shorten the acetone synthesis delay.

This test showed that bicarbonate addiction to the medium stimulated formate production by *A. woodii* $p_{acetone}$, revealing a similar

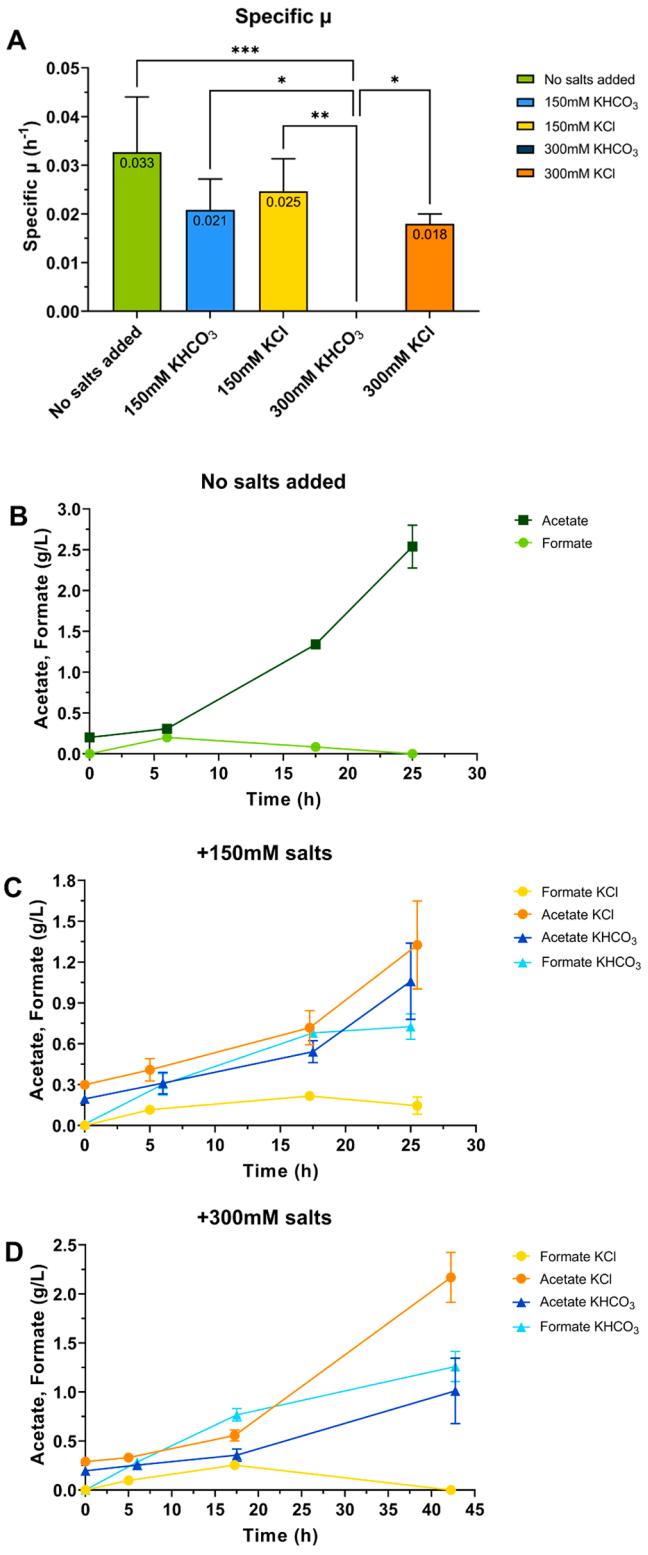

Fig. 6. Growth and metabolic production of *A. woodii* $p_{acetone}$ in serum bottles with different concentrations of KCl or $KHCO_3$ into the medium. (A) Specific growth rate, statistic: ordinary one-way ANOVA *P < 0.0332; **P < 0.0021; ***P < 0.0002; (B) metabolic profile without salts supplement; (C) metabolic profile with 150 mM of added salts; (D) metabolic profile with 300 mM of added salts.

behavior to that previously described for *T. kivui* [21]. In *T. kivui*, the authors measured an impairing of ATP synthesis at a high bicarbonate concentration in the medium. They suggested that the shortage of ATP could slow down the WLP after the first reaction of the pathway, leading to an accumulation of formate. *A. woodii* expresses an F_1F_0-ATPase localized in the cell membrane [41]. An inhibitory effect of bicarbonate on F_1F_0-ATPase has been reported in the literature [41,42], suggesting that HCO_3^- affects ATP synthesis and hydrolysis by modulating the affinities of the catalytic site of the ATPase for the substrates [43].

3.3.2. Medium pressurization

Tests with bacteria grown in serum bottles with an augmented concentration of $KHCO_3$ in the medium indicated that HCO_3^- dissolved in the liquid phase led to formic acid synthesis and growth inhibition. Working in the reactor at elevated pP_{CO2} increases the CO_2 concentration in the medium [29]. Moreover, according to the CO_2 equilibrium in water, controlling the pH at 7 promotes the conversion of CO_2 into HCO_3^- [40]. Thus, combining the elevated pP_{CO2} and the pH control could foster the HCO_3^-build in the reactor and affect bacteria metabolism. To assess this hypothesis, sterile autotrophic medium was first pressurized up to 8 bar and then *A. woodii* $p_{acetone}$ was inoculated and grown at atmospheric pressure, without pressure stress. The growth curve and the metabolic profile of the culture were monitored. As a reference, an experiment with the sterile medium continuously sparged with 70% H_2 and 30% CO_2 mix at 1.1 bar was performed.

A. woodii $p_{acetone}$ grown in pressurized medium exhibited a different behavior from the same strain grown in the un-pressurized medium (Table 3). In the reference experiment, only acetic acid was synthesized, and the specific growth rate of the strain was consistent with that recorded in a similar experiment (Section 3.2.1). Instead, *A. woodii* $p_{acetone}$ grown in the pressurized medium produced acetate and formic acid. In addition, the specific growth rate was lower in the pressurized medium than in the reference experiment. In both cases, no acetone was detected. Nevertheless, as observed above (Section 3.2.1), experiments were monitored for 24 h and it is possible that acetone synthesis had not started. These tests found that *A. woodii* $p_{acetone}$ grown in the sterile medium pressurized before the inoculation performed as *A. woodii* $p_{acetone}$ grown in high-pressure with H_2/CO_2 or in serum bottles with 300 mM $KHCO_3$.

3.3.3. High-pressure fermentation at low CO_2 partial pressure

Providing a 30% CO_2 and 70% H_2 blend to the reactor, the pP_{CO2} in fermentations could trigger the build-up of bicarbonate in experiments at 10 bar. Tests were therefore conducted with a lower amount of CO_2 in the feeding gas: a blend composed of 3% CO_2, 70% H_2, and 27% N_2 was tested. A replicate for this condition was performed. Moreover, as a control, an analogous experiment was set up in which the reactor was fed with the 30% CO_2 and 70% H_2 blend.

The experiments were divided into two sections: the first section reached a total pressure of 10 bar and was followed by a second section with a total pressure of 1.7 bar. 5.3 L/h of the gas mixture was continuously provided to the reactor.

Fig. 7 shows results from bacteria fed with the H_2:CO_2:N_2 70:3:27 blend. Detailed results obtained providing the H_2:CO_2 70:30 mix can be seen in Supplementary material 2 (Fig. SM2).

Table 3
Growth and metabolic profile of *A. woodii* $p_{acetone}$ grown in the pressurized sterile medium.

	μ_{max} (h^{-1})	Max $OD_{600\,nm}$	Max acetate (g/L)	Max formate (g/L)	Duration (h)
Not pressurized medium	0.041 ± 0.08	0.79 ± 0.16	2.14 ± 0.75	-	24
Pressurized medium	0.027 ± 0.003	0.33 ± 0.02	0.65 ± 0.02	0.67 ± 0.06	24

For the fermentation with H_2:CO_2:N_2 70:3:27, during the first 20 h at 10 bar (pP_{CO2} 0.3 bar), the specific growth rate was 0.027 h^{-1}. In the following 20 h, it decreased to 0.005 h^{-1}. The specific growth rate in the section at 1.7 bar (pP_{CO2} 0.05 bar) was 0.004 h^{-1}. Acetate synthesis started immediately after the inoculation. Synthesis of acetone started after 24 h. Formate was synthetized at the beginning of the fermentation and, at 10 bar, it was consumed. Cells synthesized it again only when the specific growth rate at 10 bar decreased. At 1.7 bar, formic acid was completely re-metabolized. The replicate of this experiment confirmed the growth and metabolic profile trends obtained in this test (Supplementary material 3 – Fig. SM3). In the control experiment (Fig. SM2), bacteria again showed two different growth rates at 10 bar (pP_{CO2} 3 bar). The specific growth rate was 0.011 h^{-1} until 27 h. Then bacteria ceased growing. The depressurization to 1.7 bar (pP_{CO2} = 0.05 bar) did not restore bacteria growth. Formic acid was synthesized since the beginning of the high-pressure fermentation, and its concentration increased during the 10-bar section. At 1.7 bar, a low amount was consumed, but it eventually stabilized its concentration at 2.95 ± 0.10 g/L.

Table 4 shows that carbon dioxide conversion into metabolic products in experiments with H_2:CO_2:N_2 70:3:27 is approximately ten-fold higher than in the experiment with H_2:CO_2 70:30. However, it should be noted that the amount of CO_2 provided in the latter experiment was ten-fold higher. It suggested that the total conversion was aligned using the different gas blends. Nevertheless, yields, specific productivities and final concentration of products indicate that in the fermentation with H_2:CO_2 70:30 at 10 bar, formic acid synthesis was boosted, while it was not when the blend with less CO_2 was used. Thus, results suggested that the pP_{CO2} affects *A. woodii* $p_{acetone}$ metabolic profile.

Even though the provided carbon dioxide amount affected *A. woodii* $p_{acetone}$ metabolism, it was not the only challenge for bacteria growing at 10 bar in autotrophy. Lowering the pP_{CO2} (H_2:CO_2:N_2 70:3:27 gas mix) did not allow bacteria growth. In both the gas blends tested (H_2:CO_2:N_2 70:3:27 and H_2:CO_2 70:30 gas mix), the pP_{H2} in the feeding mix was maintained at a constant 7 bar. The literature reported heterotrophic-growth impairment of wild-type *A. woodii* in an atmosphere consisting entirely of H_2 [44]. The growth of the bacterium was immediately restored by replacing the H_2-atmosphere with the N_2-atmosphere. Therefore, it is possible that pP_{H2} = 7 bar applied in the present study was at a level high enough to inhibit bacteria growth.

4. Conclusions

The present work focused on optimizing a gas fermentation process to convert a CO_2-H_2 stream into acetone by employing a modified *A. woodii* for acetone synthesis. Gas fermentation usually occurs in a liquid phase where feedstock is provided as a gaseous substrate and often low process productivities were reported. These low productivities could be due to the shortage of feedstock dissolved in the growth medium which would lead to low biocatalyst activity. Therefore, to increase the concentration of available substrates in the liquid medium, this work proposed to accomplish gas fermentations at high-pressure (max 10 bar).

The literature does not report data about *A. woodii* resistance at 10 bar. Thus, the first test performed was a heterotrophic fermentation in which the modified bacterium was grown at 11 bar in a nitrogen atmosphere. Results obtained led to the conclusion that this pressure value did not impair growth and cell viability.

Providing a 70% H_2 and 30% CO_2 gas blend in continuous gassing at atmospheric pressure, the modified strain produced acetic acid and acetone as main products. Nevertheless, at 10 bar, results showed growth inhibition and enhanced formic acid synthesis. Moreover, the metabolic shift was recorded also combining different biomass concentrations and in-flow gas rates into the reactor. Enhanced formic acid synthesis at high $KHCO_3$ concentration for the acetogen *T. kivui* was

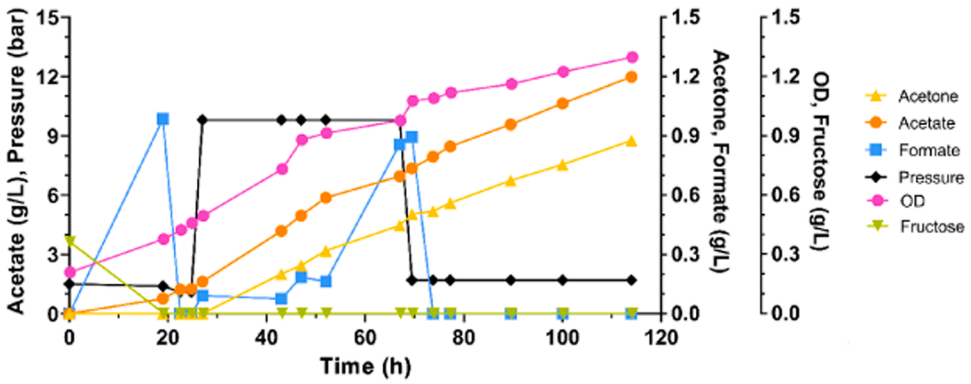

Fig. 7. Pressure trend, growth curve and metabolic profile of *A. woodii* $p_{acetone}$ grown autotrophically, in liquid batch and continuous gassing with a H_2:CO_2:N_2 70:3:27 gas mix.

Table 4
Yields, product concentrations, and productivities of fermentations at low and high pP_{CO2}. For the fermentation using H_2:CO_2:N_2 70:3:27 the average value of the two replicates is reported.

	H_2:CO_2:N_2 70:3:27		H_2:CO_2 70:30	
Total pressure (bar)	10	1.7	10	1.7
pP_{CO2}	0.3	0.051	3	0.51
pPH2	7	1.19	7	1.19
Total in-flow gas rate (L/h)	5.4	5.4	5.4	5.4
CO_2 conversion into metabolic products (%)	64.80 ± 1.98	40.00 ± 0.85	7.00	3.20
Acetate Yield (%)	51.35 ± 2.47	34.75 ± 0.49	4.60	3.10
Acetone Yield (%)	6.20 ± 1.13	4.00 ± 0.85	0.10	0.02
Formate Yield (%)	2.55 ± 2.47	0.00 ± 0.00	2.00	0.00
Max Acetate Conc (g/L)	5.72 ± 0.54	4.65 ± 0.03	3.56	2.86
Max Acetone Conc (g/L)	0.57 ± 0.18	0.33 ± 0.06	0.06	0.02
Max Formate Conc (g/L)	0.38 ± 0.24	0.00 ± 0.00	2.74	0.00
Max volumetric productivity Acetate ($g*L^{-1}*d^1$)	5.28 ± 0.72	3.12 ± 0.48	3.36	3.6
Max volumetric productivity Acetone ($g*L^{-1}*d^{-1}$)	0.72 ± 0.48	0.24 ± 0.00	0.24	0.00
Max volumetric productivity Formate ($g*L^{-1}*d^{-1}$)	0.96 ± 0.24	0.00 ± 0.00	3.36	0.00
Max cell-specific productivity Acetate ($g*g_{biomass}^{-1}*d^{-1}$)	14.16 ± 3.36	7.44 ± 1.44	13.92	11.28
Max cell-specific productivity Acetone ($g*g_{biomass}^{-1}*d^{-1}$)	1.68 ± 0.48	0.96 ± 0.24	0.72	0.24
Max cell-specific productivity Formate ($g*g_{biomass}^{-1}*d^{-1}$)	2.4 ± 0.72	0.00 ± 0.00	16.08	0.00

reported [45]. In the present study, tests in serum bottles with augmented concentration $KHCO_3$ or KCl in the medium showed that bicarbonate affected *A. woodii* physiology, leading to formic acid synthesis and inhibiting bacteria growth. Moreover, these results were confirmed by experiments in which the sterile fresh medium was pressurized with the feeding gas mix before inoculation.

Finally, by reducing the pP_{CO2} (70% H_2: 3% CO_2: 27% N_2 gas blend) at 10 bar, tests showed that formic acid synthesis was not increased anymore, confirming that this factor affected the metabolic profile of the modified *A. woodii*. Acetic acid and acetone were the main metabolic products also at high pressure. Nevertheless, bacteria growth was not completely restored. This result suggested that the pP_{H2} used in the tests (7 bar) was too high, resulting in the observed growth inhibition. Further experiments maintaining the pP_{CO2} and varying the pP_{H2} will be performed to investigate this observation further.

The H_2-CO_2 composition in the gas stream could be variable according to the gas source. For instance, in a biogas, the amount of CO_2 is around 30–40% and no H_2 is present [46]. In the biogas upgrading process, CO_2 is separated from methane and could be used as a carbon source for gas fermentation. Instead, Novak and coworkers [8] report that in a furnace gas from an industrial steel plant the amount of CO_2 is ≈ 20% and the H_2 ≈ 3.5%. Usually, the H_2 is the limiting component. Nevertheless, it is possible to add exogenous H_2 to the blend. As an example, H_2 can be produced via water electrolysis, exploiting the energy from renewable sources. Thanks to the availability of the equipment to control the gas in-flow (e.g., mass flow controllers), the end user can choose the gas mix to feed the reactor in order to obtain the best performance according to the biocatalyst.

Results of this work suggested that the physiology of modified *A. woodii* for acetone synthesis could be influenced by the amount of HCO_3^- dissolved in the liquid medium and that the metabolism of the strain could be controlled by modifying the pP_{CO2}. More studies should be performed to understand how to properly exploit modified and wild-type *A. woodii* as a biocatalyst in high-pressure gas fermentation. Studies should investigate optimal carbon dioxide and hydrogen partial pressures, the combination of pP_{CO2} and pH, stirring and in-flow gas rate.

The acetone concentration reached by this modified bacterium and other bacteria in the previous works [24,27] suggests that more efforts should be made to increase acetone production in *A.woodii*. Working on the biocatalyst development, a screening of acetone metabolic pathways from other organisms than *C. acetobutylicum* can be performed. In a study on a modified *C. autoethanogenum* for producing acetone and isopropanol, Liew et al. [47] explored a collection of 272 ABE strains and tested different combinations of promoters and enzymes, reaching an acetone concentration up to 100 mM. The same approach could be applied to develop modified *A.woodii* strains. Working on the process, improving medium composition to increase biomass concentration can lead to higher acetone volumetric productivity. For example, other authors used a higher concentration of trace elements for *A.woodii* cultivation in the bioreactor [8] than the one used in this study. Moreover, as mentioned above, further tests are ongoing to understand how to exploit acetate concentration to boost acetone synthesis in the modified *A. woodii* $p_{acetone}$ strain used in this work.

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Loredana Tarraran: Conceptualization, Methodology,

Investigation, Resourses, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Valeria Agostino**: Conceptualization, Methodology, Writing – review & editing. **Nicolò S. Vasile:** Software, Data curation. **Annalisa Abdel Azim**: Software, Resourses. **Giacomo Antonicelli:** Data curation, Visualization. **Jonathan Baker:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **James Millard:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Angela Re:** Writing – original draft. **Barbara Menin:** Writing – review & editing. **Tonia Tommasi:** Writing – review & editing. **Nigel P. Minton:** Supervision, Funding acquisition. **Candido F. Pirri:** Supervision, Funding acquisition. **Debora Fino:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jcou.2023.102583.

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