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Screening of conditions for the acetic acid production from H_2 and CO_2 by *Thermoanaerobacter kivui* in a pressurized stirred tank bioreactor

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ABSTRACT

Growing concerns regarding climate change have heightened interest in utilizing carbon dioxide as a valuable carbon source for chemical production. The acetogen *Thermoanaerobacter kivui* is a thermophilic bacterium that converts CO_2 and H_2 streams into acetic acid. Nevertheless, the limited solubility of the gaseous substrates in the liquid medium has often led to low productivity by the biocatalyst employed. This study aims to enhance the cell-specific acetic acid productivity of *T. kivui* by combining pressure, the composition of the inlet gas mix, and in-flow gas rate. Firstly, the combined effect of pressure and gas composition was assessed through a Design of Experiments approach. Tests were performed in a pressurized bioreactor and indicated that acetic acid cell-specific productivity was achieved at 10 bar, providing a 3:1 H_2:CO_2 blend. Subsequently, supplying this blend at high pressure into the vessel of the reactor, an in-flow gas rate screening was performed to identify completely the parameters that allowed the maximum acetic acid productivity. The optimal flow rate was 60 mL.min^{-1}, and the acetic acid cell-specific productivity reached 2.90 g.g^{-1}.h^{-1}. Additionally to the experiments in the bioreactor, tests in serum bottles were performed to investigate the influence of the osmotic condition due to different salts and acetic acid inhibition on *T. kivui*. Results indicated that salts and acetic acid concentration impaired bacteria growth and affected the production of further acetic acid. Nevertheless, a metabolic shift toward the production of formic acid was observed specifically by adding the HCO_3^- ion.

1. Introduction

Carbon dioxide is one of the most emitted greenhouse gases. Its growing concentration in the atmosphere is leading to a climate crisis due to a rise in the earth's temperature. Implementing a CO_2-based bioeconomy is one way to address this worldwide threat. Many bulk chemicals, currently obtained from fossil sources, can be produced using sustainable biological methods. Lithotrophic organisms employing CO_2 as a feedstock can be used for this purpose [1]. These organisms can derive the energy needed to fix the carbon dioxide from solar energy (photolithoautotrophs) or the oxidation of an inorganic electron donor such as H_2 (chemolithoautotrophs) [2]. Carbon dioxide can be taken from the air, industrial exhaust gases, or obtained from the gasification of biomass and waste streams. Among the chemolithoautotrophic organisms, the acetogenic bacteria produce a variety of chemicals, including butyrate, formate, acetate, and ethanol [3].

Acetogenic bacteria can run a reductive pathway in which the carbon dioxide is reduced to acetic acid [4]. The pathway is called the Wood-Ljungdahl Pathway (WLP) and a schematic representation is shown in Fig. 1. In the WLP, two molecules of CO_2 are reduced through the methyl and carbonyl branches and combined to obtain the acetyl-CoA. This pivotal intermediate is then further reduced to acetate (Fig. 1).

Acetic acid finds employment as a food preservative, solvent, or intermediate for many commercial-grade chemicals. It is primarily employed in the oxidative synthesis of vinyl acetate monomer, a crucial component of emulsion polymers, resins, and intermediates used in coatings, textiles, wires, and products made from acrylic fiber [5].

The hydrogenation of carbon dioxide is a demanding reaction due to its thermodynamic stability. The majority of chemical catalysts for CO_2 hydrogenation have low turnover frequencies, need high pressures and temperatures, or extremely costly additives, which renders them impractical and unprofitable [6]. Compared to the traditional catalytic Fischer-Tropsch synthesis, the use of biological catalysts is thought to have several benefits [1]. Energy expenses are decreased by the biocatalytic conversion occurring at low temperatures and pressure. Moreover, the biological catalysts are characterized by lower sensitivity to impurities [1], which makes upstream processes less articulated, and

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Nomenclature

Acronyms and abbreviations

A	Acetic acid
BPR	Back Pressure Regulation valve
CDW	Cell Dry Weight
DoE	Design of Experiments
F	Formic acid
HDCR	Hydrogen-Dependent CO_2 Reductase enzyme
HPLC	High-Performance Liquid Chromatography
MES	4-Morpholineethanesulfonic acid
MFC	Mass Flow Controller
MFM	Coriolis Mass Flow Meter
MLR	Multiple Linear Regression
OD	Optical Density
P	Pressure
PES	Polyethersulfone
q_A	Acetic acid cell-specific productivity
q_F	Formic acid cell-specific productivity
Q_g	Reactor inlet gas flow rate
r_A	Acetic acid volumetric productivity
r_F	Formic acid volumetric productivity
T	Temperature
WLP	Wood-Ljungdahl Pathway
$\eta_{CO2}A$	CO_2 conversion into acetic acid
$\eta_{H2}A$	H_2 conversion into acetic acid

Parameters

μ	Specific growth rate
b_i	Regression coefficients for each factor of DoE model equation
K_La	Volumetric mass transfer coefficient
q_p	Specific rate of formation of a product
r_P	Formation rate of a product
t	Time
V	Working volume
X	Cell concentration
X_0	Initial cell concentration
x_1	Factor of the DoE model corresponding to the pressure
x_2	Factor of the DoE model corresponding to the H_2:CO_2 inlet gas mixture ratio
X_{av}	Average biomass concentration
y	Predicted response of DoE model equation
ΔC_P	Change in product concentration
Δt	Time interval between two fermentation sampling points (h)
$\eta_i A$	Acetic acid yield
$C_{i,l}$	Current concentration of i in the liquid
C_i*	Concentration of compound i in the liquid phase at equilibrium
H_i	Henry's constant specific to compound i
P_{tot}	Total reactor pressure
pP_i	Partial pressure of compound i in the gas phase
y_i	Mole fraction of component i in the gas phase

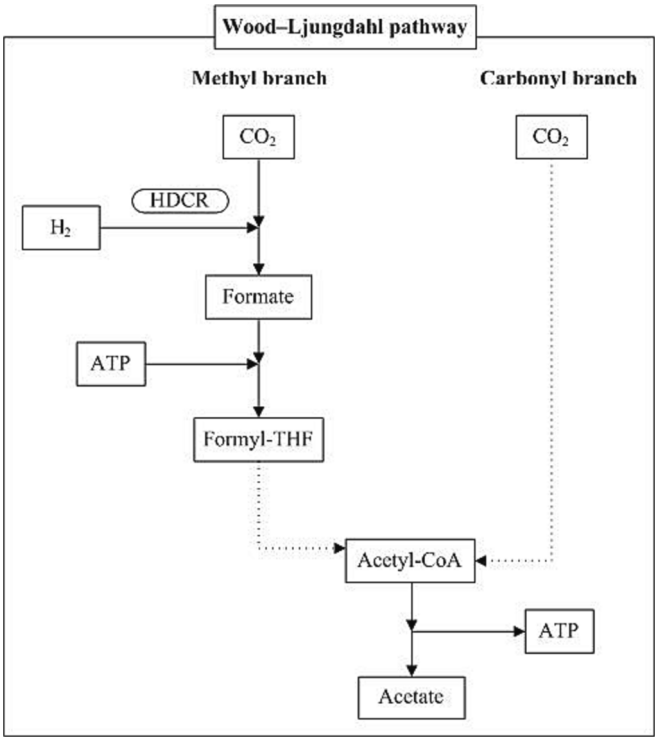

Fig. 1. Wood-Ljungdahl pathway. The dotted lines indicate that some steps in the metabolic pathways are omitted. (HDCR): Hydrogen-dependent CO_2 reductase enzyme.

higher product specificity. Nevertheless, low yields are often reported, so complex downstream systems could be necessary [7].

Among acetogens, *Thermoanaerobacter kivui* was chosen for the current study. This acetogen is a Gram-negative, strictly anaerobic, thermophilic bacterium that grows optimally at 66 °C and pH 6.5 [8]. It can utilize substrates like fructose, mannose, pyruvate, or glucose. Additionally, it handles variable gas compositions growing very robust on H_2 and CO_2 but also on CO [9–11]. *T. kivui* can be cultured on a mineral medium with the addition of feeding substrates. Expansive reagents such as yeast extract and vitamins are neither necessary nor stimulating, lowering fermentation costs [8].

Conducting fermentations at high temperatures (66 °C) decreases the risk of contamination with respect to processes in mesophilic conditions, reducing costs associated with sterilization and cooling. Moreover, the process heat can be exploited, offering opportunities for energy efficiency over mesophiles [12,13]. All organisms produce heat during metabolic processes. On a large scale, fermenters need cooling because the heat generated surpasses the dissipated heat through the fermentor wall. When employing thermophilic bacteria, the thermal driving force between the fermentation temperature and the ambient environment is higher than for mesophilic bacteria. Thus, removing excess heat is facilitated and potentially results in cost savings. An example is the Biological Methanation Demonstration Plant in Avedore, Denmark, which operates at 65 °C [14].

In the synthesis of chemicals through gas-fermentation, gaseous substrates are converted into high-value products. Given the nature of the biocatalyst, the reactions for gas conversion occur in the liquid phase. Therefore, the primary challenge in employing acetogens for CO_2 valorization is the risk of feedstock deficiency due to the low solubility of the gas provided in the liquid medium. At equilibrium, the maximum concentration of each component *i*, supplied as a gas in the liquid phase follows Henry's law (Eq. (1)):

$$C_i^* = pP_iH_i = y_iP_{tot}H_i \quad (1)$$

In Eq. (1), C_i* represents the concentration of compound *i* in the

liquid phase at equilibrium, *pPi* is the partial pressure of *i* in the gas phase, H_i stands for Henry's constant specific to *i* at the given temperature, y_i denotes the mole fraction of component *i* in the gas phase, and P_{tot} represents the total reactor pressure. In a non-equilibrium state, the availability of the substrate in the liquid phase depends on the mass transfer rate between the gas and liquid phases, as shown in Eq. (2) [15].

$$overall\ transfer\ rate = k_L a V\left(C_i^* - C_{i,L}\right) \quad (2)$$

In Eq. (2), k_La represents the volumetric mass transfer coefficient, *V* is the working volume, and C_i, the current concentration of *i* in the liquid. The mass transfer coefficient can be manipulated to enhance the overall transfer rate by increasing the stirring speed or the volumetric gas feed rate. Alternatively, elevating the molar fraction of component *i* in the gas mixture supplied or raising the fermentation pressure boosts the driving force component in Eq. (2) [16,17].

Previous research on CO_2 and H_2 fermentations involving acetogens investigated the impact of raising the total pressure. Growth inhibition and changes in metabolic product distribution were observed at pressures ranging from 1 to 7 bar for *Clostridium ljungdahlii* [16,18]. The same outcomes were described for *Acetobacterium woodii* from pressure between 1 and 10 bar, varying the inlet gas mix [19].

In this study, *T. kivui* was chosen as a biocatalyst to produce acetate through the H_2-CO_2 fermentation. The microorganism was cultured in a pressurized Continuous Stirred Tank Reactor (CSTR) in liquid batch and continuous gas supply. The work aimed to optimize the process parameters to allow the achievement of the highest cell-specific acetic acid productivity. Firstly, a Design of Experiment (DoE) approach was applied to investigate the bacterium performance in different combinations of pressure and inlet gas mix composition. DoE involves designing experimental conditions by simultaneously modifying two or more factors. This makes it possible to conduct a restricted number of experiments and to investigate the dependency between the factors. The data obtained from this approach can be used to build a multiple regression model. To the authors' knowledge, this is the first study to investigate the conditions for acetic acid production from H_2 and CO_2 by a biocatalyst employing DoE principles. The results of the DoE study were the basis for a subsequent flow rate screening to identify the optimal gas flow rate for reactor feeding. Moreover, some aspects of the behaviour of the biocatalyst were addressed in batch cultures in serum bottles. Tests were performed to evaluate the influence of the osmotic condition and acetic acid concentration on bacterial growth and metabolic profile in autotrophic cultivation.

2. Materials and methods

2.1. Microbial strain

In this work, a wild-type *Thermoanaerobacter kivui* strain was used. *T. kivui* (DSM 2030) was acquired by the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH.

2.2. Cultivation media

T. kivui was grown in DSM 171 medium. The medium for the autotrophic growth contained the following: 0.22 $g.L^{-1}$ K_2HPO_4, 0.22 $g.L^{-1}$ KH_2PO_4, 4.5 $g.L^{-1}$ $NaH_2PO_4x\ H_2O$, 6.1 $g.L^{-1}$ Na_2HPO_4 x 12 H_2O, 0.31 $g.L^{-1}$ NH_4Cl, 0.22 $g.L^{-1}$ $(NH_4)_2SO_4$, 0.45 $g.L^{-1}$ NaCl, 0.09 $g.L^{-1}$ $MgSO_4$ x 7 H_2O, 6 $mL.L^{-1}$ $CaCl_2$ x 2 H_2O (0.1 % w/v), 2 $mL.L^{-1}$ $FeSO_4$ x 7 H_2O (0.1 % w/v in 0.1 N H_2SO_4), 10 $mL.L^{-1}$ trace element solution (DSM 141), 0.5 $mL.L^{-1}$ Na-resazurin solution (0.1 % w/v), 0.5 $g.L^{-1}$ L-Cysteine-HCl x H_2O, 0.5 $g.L^{-1}$ Na_2S x 9 H_2O. 10 $g.L^{-1}$ of 4-Morpholineethanesulfonic acid (MES) was added to the medium when necessary. The trace element solution consists of 1.5 $g.L^{-1}$ nitrilotriacetic acid, 3 $g.L^{-1}$ $MgSO_4$ x 7 H_2O, 0.5 $g.L^{-1}$ $MnSO_4$ x H_2O, 1 $g.L^{-1}$NaCl, 0.1 $g.L^{-1}$ $FeSO_4$ x 7 H_2O, 0.18 $g.L^{-1}$ $CoSO_4$ x 7 H_2O, 0.1 $g.L^{-1}$ $CaCl_2$ x 2 H_2O, 0.18 $g.L^{-1}$ $ZnSO_4$ x 7 H_2O, 0.01 $g.L^{-1}$ $CuSO_4$ x 5 H_2O, 0.02 $g.L^{-1}$ $KAI(SO_4)_2$ x 12 H_2O, 0.01 $g.L^{-1}$ H_3BO_3, 0.01 $g.L^{-1}$ Na_2MoO_4 x 2 H_2O, 0.03 $g.L^{-1}$ $NiCl_2$ x 6 H_2O, 0.30 $mg.L^{-1}$ Na_2SeO_3 x 5 H_2O, 0.40 $mg.L^{-1}$ Na_2WO_4 x 2 H_2O. The autotrophic medium added with 5.04 $g.L^{-1}$ glucose was used for heterotrophic fermentations.

According to the experiment, a suitable volume of the autotrophic medium, lacking $FeSO_4$ x 7 H_2O, L-Cysteine-HCl and Na_2S, was aliquoted into serum bottles, gassed with nitrogen and heat-sterilized by autoclaving. $FeSO_4$ x 7 H_2O, L-Cysteine-HCl x H_2O, Na_2S x 9 H_2O, and when needed, glucose solutions were separately sterilized by filtration using a 0.22 μm polyethersulfone (PES) sterile filters and gassed with N_2 to purge oxygen. After cooling, a suitable amount of each filtered component was added to the autoclaved medium to obtain the complete medium.

2.3. T. Kivui strain preservation

T. kivui cells were stored using glycerol as a cryoprotectant. Glycerol was sterilized by autoclaving and gassed with N_2 to remove oxygen. Bacteria grew in the heterotrophic medium until the exponential phase. 3 mL of culture were transferred in a sterile serum bottle (12 mL total volume) previously gassed with N_2. Cell suspension added with 20 % glycerol was mixed gently to have a homogeneous suspension and stored at −80 °C.

2.4. Heterotrophic and autotrophic fermentations in serum bottles

A detailed description of the experimental procedures for the heterotrophic and autotrophic fermentations in serum bottles is provided in S1 and S2 sections of Supplementary Materials, respectively.

2.4.1. Serum bottle fermentation in fed-batch with $KHCO_3$ and KCl

For fermentations with an augmented concentration of salts in the culture, serum bottles filled with the autotrophic medium were prepared as described in Supplementary Materials – section S2, and 300 mM $KHCO_3$ or 300 mM KCl were added. Briefly, a salt stock solution of 2 M $KHCO_3$ or KCl was prepared in the autotrophic broth and sterilized using a 0.22 μm PES sterile filter. Each concentrated solution was then degassed for 2 h using 260 $mL.min^{-1}$ of an 80 % N_2 and 20 % CO_2 gas mixture. 4.5 mL of salt stock solution was added to 25.5 mL of standard autotrophic medium. Glucose was added to a final concentration of 0.36 $g.L^{-1}$ to shorten the initial lag phase. The inoculum and the gas-fed-batch strategy were performed as described in S2 of Supplementary Materials. In parallel, a control culture without additional salts in the autotrophic medium was set up. Experiments were performed in triplicates.

2.4.2. Serum bottle fermentation in fed-batch with increased acetic acid concentration

For fermentations with an increased acetic acid concentration in the culture, serum bottles filled with the autotrophic medium were prepared as described in in Supplementary Materials – section S2, and supplemented with 14.5 $g.L^{-1}$ or 28.3 $g.L^{-1}$ acetic acid. Briefly, a 17.5 M acetic acid stock solution was prepared, sterilized using a 0.22 μm PES filter, and gassed for 2 h using 260 $mL.min^{-1}$ of an 80 % N_2 and 20 % CO_2 gas mixture. A volume of acetic acid stock solution was added to each serum bottle to achieve the target concentration in the medium. Then, 6 M NaOH was used to adjust the pH back to 6.5. Glucose was added to a final concentration of 0.45 $g.L^{-1}$ to shorten the initial lag phase. The inoculum and the gas-fed-batch strategy were performed as described in S2 of Supplementary Materials. In parallel, a culture without additional acetic acid into the autotrophic medium was set up as a control. Experiments were performed in triplicates.

2.5. Autotrophic fermentation with continuous gas supply at high-pressure in the bioreactor

Autotrophic experiments in liquid batch and continuous gas supply were performed using a custom-adapted bioreactor fabricated by the H. E.L group (UK). A comprehensive description of the reactor's hardware can be found elsewhere [20]. In summary, the system's central component was a 2 L oil-jacketed stirred tank reactor. Key parameters such as pressure, temperature, pH, and redox potential were monitored using probes. Pressure control was achieved through a proportional Back Pressure Regulation (BPR) valve. Gas supply into the medium was accomplished using a micro sparger located at the vessel's base, with gas flow rates regulated by Mass Flow Controllers. To initiate the autotrophic growth experiments, a volume of 0.82 L of the autoclaved autotrophic medium was introduced into the vessel through a pump. The medium was supplemented with $FeSO_4$ x 7 H_2O. Subsequently, it underwent a purging process with sterile N_2 gas at a constant pressure of 1.1 bar, with a flow rate of 100 mL.min^{-1}, for 3 h. Then, Cysteine-HCl x H_2O and Na_2S x 9 H_2O were added into the medium. Before inoculation, an 80 % H_2 and 20 % CO_2 gas mixture was supplied at a flow rate of 72 mL.min^{-1} for 1 h at 1.1 bar. A heterotrophic preculture of *T. kivui* was injected into the vessel to attain an initial OD_{600nm} of approximately 0.3.

Independent of the operational mode investigated in each distinct fermentation, the initial step involved continuous gassing with an 80 % H_2 and 20 % CO_2 mixture at an in-flow gas rate of 20 mL.min^{-1}, constant pressure of 1.5 bar, and temperature of 66 °C. The impeller was set at 400 rpm. pH control was maintained at 6.5 using 3 M NaOH, which was previously autoclaved and purged with N_2. Once the OD_{600nm} inside the reactor reached approximately 0.5, the in-flow gas composition was switched to 50 % H_2 and 50 % CO_2 while keeping the gas rate constant at 20 mL.min^{-1}. This modification in the inlet gas mixture aimed to enhance the availability of CO_2 in the liquid, thereby preventing carbon source limitations for the bacteria. Parameters specific to each test were applied when the OD_{600nm} inside the reactor reached ≈ 0.6.

2.6. Analytical methods

2.6.1. Biomass and liquid products quantification

Bacteria growth was monitored through optical density measurements. Optical density was measured at 600 nm (OD_{600nm}) using a DH-5000 Spectrophotometer (HACH, USA). Distilled water was utilized as the blank to establish the baseline.

During fermentation, 20 mL samples of growing culture were drawn, and biomass was collected by centrifugation at 5000 rpm, for 15 min at 15 °C. The pellet was then washed three times with deionized water and dried at 80 °C for 24 h in an oven (Memmert GmbH + Co. KG, DE). The dry pellet was weighted using an analytical balance (XS Instruments, IT). An experimental cell dry weight (CDW) - optical density correlation factor of 0.394 g.L^{-1} was found (data not shown).

Metabolic products and glucose in the liquid phase were analyzed by High-performance liquid chromatography (HPLC) as described in [19].

2.6.2. pH measurement

Acids are the main products of *T. kivui's* metabolism. For this reason, monitoring pH and basifying the medium may be required during prolonged fermentations. In serum bottle fermentations, 1.5 mL of the culture was taken at each sampling point, and the pH was checked using a pHmeter (XS Instruments, IT). The pH was adjusted to 6.5 by adding a variable volume of a sterile solution of NaOH 1 M gassed with N_2. After the base addiction, 1.5 mL of culture was collected to assess the actual pH value in each serum bottle. In the bioreactor, the pH was continuously monitored. When necessary, it was automatically risen to 6.5 by injecting sterile NaOH 3 M, previously gassed with N_2, through a piston pump.

2.6.3. Gas analysis

The mass flow rates of the gas mixture exiting the vessel's gas outlet were measured using a Coriolis Mass Flow Meter (MFM) (Bronkhorst High-Tech BV, NL). The incoming and outgoing gas composition was analyzed using a Fusion Micro GC (Inficon, CH). The instrument was equipped with Molsieve 5 Å analytical columns, utilizing Argon as the carrier gas and an Rt-U-Bond column with Helium as the carrier gas. The Micro GC was not directly connected downstream of the MFM; therefore, gas composition analysis was performed offline. A sample of gas was first collected in a gas bag connected to the MFM outlet. Then, the gas bag was moved to the Micro GC and connected to allow gas injection for the composition analysis.

2.7. Calculations

2.7.1. Experimental design and data modeling

A Design of Experiments (DoE) approach [21] was employed to investigate the influence of pressure (P) and H_2:CO_2 ratio in the inlet gas mixture on the max acetic acid cell-specific productivity (q_A) while minimizing the experimental workload. Experiments were conducted in the above-described high-pressure bioreactor under autotrophic conditions with continuous gas supply. The DoE utilized a two-factor with three-level design, comprising 9 experiments, with a total of 11 experimental runs that encompassed three replicates at the most central point. The design factors were P values of 2, 6, and 10 bar and H_2:CO_2 inlet gas mixture ratios of 1:1, 2:1, and 4:1. The response variable of interest was acetic acid cell-specific productivity (g.g^{-1}.h^{-1}, see section 2.7.2). The gas inlet flow rate was kept constant at 20 mL.min^{-1}. Table 1 lists the experimental runs, with the real values of each factor and their coded versions. The R-based CAT (Chemometric Agile Tool) [22] was used to analyze data. The software employs a Design of Experiments (DoE) modeling engine that relies on the multiple linear regression (MLR) method outlined by Sergent *et al.* [23]. MLR is a regression technique that permits the modeling and examination of individual factors independently and in conjunction with one or more other factors. This approach offers flexibility for efficiently modeling the response being studied. Simultaneously, it allows for the inclusion or exclusion of non-significant terms in the model equation. This can be represented as in Eq. (3).

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 \quad (3)$$

In Eq. (3), y is the predicted response, and b_i corresponds to the regression coefficients for each factor (where x_1 = P, and x_2 = H_2:CO_2 ratio in the inlet gas mix). Eq. (3) addresses both the interaction between the factors (x_1x_2) and their quadratic terms (x_1^2 and x_2^2). The equation can be used to generate an isoresponse plot showing the response surface.

Following the analysis of the DoE results, the pressure and inlet gas mix conditions corresponding to the maximum specific productivity of acetic acid were selected. Subsequently, an inlet flow rate (Q_g) screening

Table 1
DoE plan to investigate the max acetic acid cell-specific productivity. (Expt): experiment name; (P): pressure; (original): factor real value; (coded): coded value of the real factor.

Expt.	P (bar) (original)	H_2:CO_2 mix (original)	P (bar) (coded)	H_2:CO_2 mix (coded)
R1	2	1: 1	−1	−1
R2	2	2: 1	−1	−0.33
R3	2	4: 1	−1	1
R4	6	1: 1	0	−1
R5.1	6	2: 1	0	−0.33
R5.2	6	2: 1	0	−0.33
R5.3	6	2: 1	0	−0.33
R6	6	4: 1	0	1
R7	10	1: 1	1	−1
R8	10	2: 1	1	−0.33
R9	10	4: 1	1	1

was performed by altering the in-flow gas rate (30, 37.5, 60, 80, 100, and 120 mL.min^{-1}) under the selected best pressure and gas mix condition.

2.7.2. Productivities and yield calculation

Productivities were calculated as described in [19].

Briefly, the volumetric production rate (r) was calculated as presented in Eq. (4).

$$r_P = \Delta C_P / \Delta t \quad (4)$$

In Eq. (4), r_P represents the formation rate of a product ($g_{product}.L^{-1}.h^{-1}$), Δt is the time interval between two fermentation sampling points (h) and ΔC_P is the change in the product concentration ($g.L^{-1}$) observed during the specified period.

The cell-specific production rate (q) was determined using the formula reported in Eq. (5).

$$q_P = \Delta C_P / (X_{av} \Delta t) \quad (5)$$

In Eq. (5), q_p represents the synthesis rate of a specific product ($g_{product}.g_{biomass}^{-1}.h^{-1}$), Δt is the time interval between two sampling points during the fermentation process (h), X_{av} is the average biomass concentration ($g.L^{-1}$) between the two selected sampling points, and ΔC_P is the increase in product concentration ($g.L^{-1}$) observed between those same sampling points.

The acetic acid yield ($\eta_i A$) was calculated as the conversion of CO_2 or H_2 into acetic acid using the formula provided in Eq. (6) [19].

$$\eta_i A = mol\ i\ converted\ into\ acetic\ acid / mol\ i\ in\ inlet \quad (6)$$

In Eq. (6), 'i' represents either the CO_2 or H_2 species. The numerator term represents the moles of 'i' converted into acetic acid, while the denominator term represents the moles of 'i' introduced into the reactor since the final conditions of the inlet gas mix and pressure were established.

The carbon and reducing equivalents balances were calculated. A detailed description is provided in section S3 of the Supplementary Materials.

3. Results and discussion

3.1. Heterotrophic and autotrophic fermentation in serum bottles

The results of the heterotrophic and autotrophic fermentations in serum bottles are presented and discussed in Sections S4 and S5 of the Supplementary Material, respectively.

3.2. Autotrophic fermentation with continuous gas supply at high-pressure in the bioreactor

3.2.1. Analysis of the experimental outcomes of the DoE study

The current study investigated process conditions for enhancing acetic acid cell-specific productivity by applying a Design of Experiments (DoE) approach to assess the influence of pressure and gas mix while minimizing the experimental workload (see section 2.7.1). Table 2 details the experimental conditions applied and the outcomes of the DoE study. Briefly, the outcomes of the experiments allowed their partition into three groups, according to the biocatalyst's performance in growth and metabolic profile. Below a description of one experiment of each group. Fig. 2 refers to the fermentation in which an H_2:CO_2 2:1 mix was supplied at 6 bar (experiment R5.1 in Table 2). It provides a graphical example of the trends in pressure, OD, acetate, and formate concentration over time. The OD increased up to 1.23. The period from the pressure increase at 6 bar (t = 50 h) until the culture reached the maximum OD (t = 62.5 h) corresponded to the highest specific productivity of acetic acid (1.44 g.g^{-1}.h^{-1}); then, its concentration plateauing shortly after the maximum OD was reached (31.00 g.L^{-1}). The subsequent formic acid production (5.95 g.L^{-1}) and a decrease in OD values coincided with the plateau in acetic acid production. A peak in formic acid can be noted at $t \approx 50$ in Fig. 2. It can be attributed to a sudden availability of gas in the medium due to the pressure-raising procedure with additional gas inflow (75 mL.min^{-1}). Once the pressure-increasing phase was concluded, the in-flow gas rate was lowered (20 mL.min^{-1}), less gas was provided to the system and formic acid was metabolized [24]. The growth trend and the metabolic spectrum described for the experiment conducted at 6 bar with 2:1 H_2:CO_2 (R5.1 in Table 2) were consistent across experiments conducted at 10 bar with an H_2:CO_2 ratio in the inlet gas mix of 2:1 (R8 in Table 2), 3:1 (R10.1 and R10.2 in Table 2), and 4:1 (R9 in Table 2), those at 6 bar with ratios of 2:1 (R5.2 and R5.3 in Table 2) and 4:1 (R6 in Table 2), and those at 2 bars with a 2:1 ratio (R2 in Table 2). In all the indicated experiments, OD ceased to rise just above 1, and the concentration of acetic acid stabilized between 24 and 30 g.L^{-1}. As OD started declining, formic acid synthesis initiated. Outcomes suggested that *T. kivui* growth trend and metabolic spectrum were independent of pressure conditions and the gas supplied, suggesting a nutrient deficiency in the medium or inhibition due to acetic acid concentration attained. In a study on *T. kivui* grown on glucose in continuous culture [25], the authors modeled the behaviour of the strain on this organic substrate and verified the model with experimental data. They found that the bacteria grew up to an acetate concentration of 34.5 g.L^{-1}. The outcomes of the current study on growing *T. kivui* in gas fermentation are congruent, suggesting that acetate concentration could affect bacteria growth. In all the

Table 2
Experimental conditions and results of the DoE study. (Expt): experiment name; (P): pressure; ([A]): acetic acid concentration; ([F]): formic acid concentration; (η_{CO2}A): CO_2 conversion into acetic acid; (η_{H2}A): H_2 conversion into acetic acid; (r_A): acetate volumetric productivity; (r_F): formate volumetric productivity; (q_A): acetate cell-specific productivity; (q_F): formate cell-specific productivity.

Expt	P (bar)	H_2:CO_2 inlet gas mix	μ_{max} (h^{-1})	Max [A] ($g.L^{-1}$)	Max [F] ($g.L^{-1}$)	η_{CO2}A (%)	η_{H2}A (%)	P upkeep	Max r_A ($g.L^{-1}.h^{-1}$)	Max r_F ($g.L^{-1}.h^{-1}$)	Max q_A ($g.g^{-1}.h^{-1}$)	Max q_F ($g.g^{-1}.h^{-1}$)
R1	2	1: 1	0.011	15.73	0.02	14 %	14 %	yes	0.15	0.00	0.58	0.01
R2	2	2: 1	0.037	30.81	0.64	46 %	23 %	no	0.41	0.02	0.85	0.05
R3	2	4: 1	0.000	13.53	0.06	11 %	3 %	yes	0.15	0.00	0.75	0.01
R4	6	1: 1	0.000	10.39	0.64	4 %	4 %	yes	0.06	0.02	0.34	0.12
R5.1	6	2: 1	0.051	31.01	5.95	38 %	19 %	no	0.53	0.24	1.44	0.64
R5.2	6	2: 1	0.043	30.54	4.79	38 %	19 %	no	0.49	0.23	1.27	0.74
R5.3	6	2: 1	0.046	29.52	4.32	37 %	19 %	no	0.51	0.16	1.46	0.55
R6	6	4: 1	0.053	26.06	2.83	53 %	13 %	no	0.59	0.18	1.76	0.53
R7	10	1: 1	0.008	13.40	1.87	6 %	6 %	no	0.13	0.04	0.62	0.21
R8	10	2: 1	0.064	27.67	9.37	31 %	15 %	no	0.80	0.27	2.74	0.65
R9	10	4: 1	0.032	25.53	4.79	45 %	11 %	no	0.53	0.12	2.08	0.37
R10.1	10	3: 1	0.046	24.13	4.54	33 %	11 %	no	0.64	0.13	2.30	0.45
R10.2	10	3: 1	0.044	24.54	3.86	30 %	10 %	no	0.67	0.15	1.91	0.33

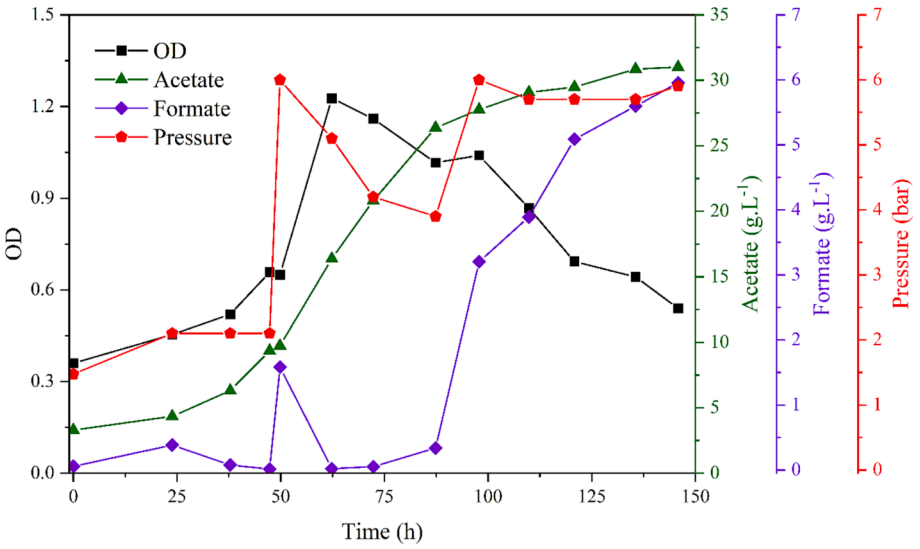

Fig. 2. Growth of T. kivui in autotrophic condition (2:1 H_2:CO_2) in bioreactor at 6 bar (experiment R5.1 in Table 2). At t = 38 h, the incoming gas composition was changed from 4:1 H_2:CO_2 to 1:1. At t = 47 h, the composition was changed to 2:1. The target OD (0.6) and the target pressure (6 bar) were achieved at t = 50 h, marking the official start time of the experiment (see also section 2.5).

experiments mentioned above, the pressure was not maintained at the initial value, resulting in greater gas consumption than supplied. Nonetheless, with increasing pressure (from 2 to 10 bar), the productivity of acetic acid increased.

Fig. 3 depicts the trend of pressure, OD, acetate, and formate concentration over time in the case of fermentation with an H_2:CO_2 1:1 mix and at a pressure of 6 bars (R4 in Table 2). Differently from the experiments described above, upon achieving the target experiment pressure, the OD immediately began to decrease, the concentration of acetic acid reached a plateau, and formic acid was synthetized. The growth curve and the metabolic behavior of *T. kivui* recorded at 6 bar with an H_2:CO_2 ratio of 1:1 were similar in the test conducted at 10 bar supplying the same gas mix (R7 in Table 2).

In the experiment conducted with an H_2:CO_2 inlet gas mix ratio of 4:1 at a pressure of 2 bar (R3 in Table 2), the metabolic behavior of *T. kivui* differed from the previous cases. Fig. 4 illustrates the trends in pressure, OD, acetate, and formate concentration over time under these pressure and H_2:CO_2 inlet gas mix conditions.

Similar to the experiments conducted with an H_2:CO_2 inlet gas mix ratio of 1:1 at pressures of 6 and 10 bar (R4 and R7 in Table 2, respectively), OD began to decrease when the pressure rose to 2 bar, and the concentration of acetic acid reached a plateau. However, there was no formic acid production. The different metabolic spectrum in this latter case can be attributed to the insufficient availability of CO_2 to support the metabolic activity of the biomass in the reactor, leading to cellular death and the almost absence of metabolic product synthesis.

Table 2 summarises the experimental conditions and the outcomes of the DoE study. The data indicates that there is no significant increase in the CO_2 and H_2 conversion into acetic acid with rising pressure. However, a noticeable improvement in acetate-specific productivity is observed. This means that the same acetate concentration could be achieved with fewer cells into bioreactor or with shorter process duration with respect to the atmospheric pressure. The highest acetate specific productivities were achieved in experiments conducted at high pressure (10 bar). The productivity in formic acid was influenced by the amount of dissolved CO_2 and its ratio with H_2. When the fraction of CO_2

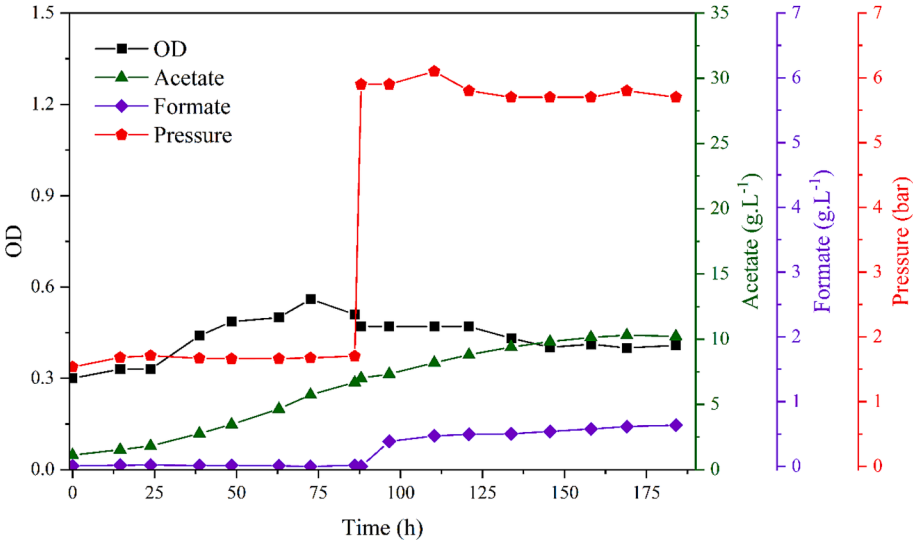

Fig. 3. Growth of T. kivui in autotrophic condition (1:1 H_2:CO_2) in bioreactor at 6 bar (experiment R4 in Table 2). At t = 63 h, the incoming gas composition was changed from 4:1 H_2:CO_2 to 1:1. The target OD (0.6) and the target pressure (6 bar) were achieved at t = 83 h, marking the official start time of the experiment.

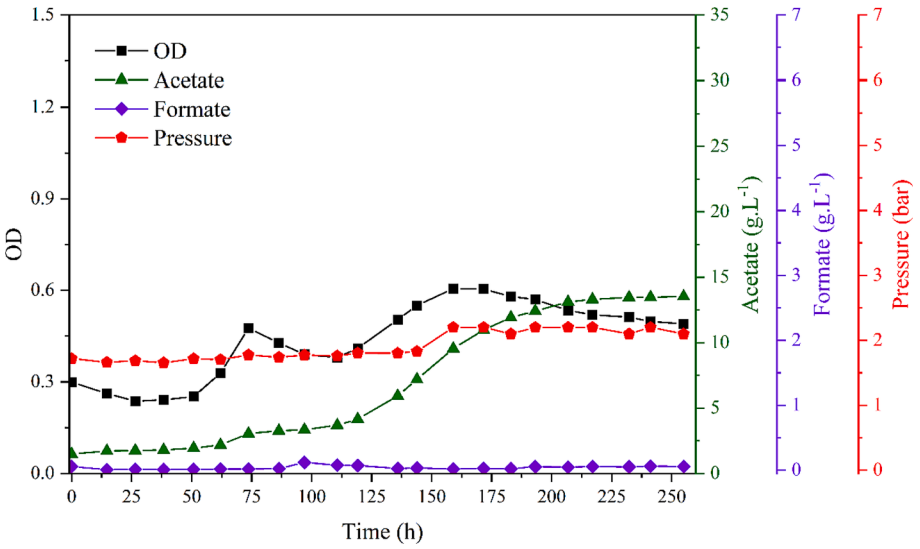

Fig. 4. Growth of T. kivui in autotrophic condition (4:1 H_2:CO_2) in bioreactor at 2 bar (experiment R3 in Table 2). At t = 86 h, the incoming gas composition was changed from 4:1 H_2:CO_2 to 1:1, and at t = 144 h, this composition was reverted back to 4:1. The OD = 0.6 and the 2 bar pressure were achieved at t = 159 h, marking the official start time of the experiment.

in the inlet gas mixture and the pressure increased, there was a corresponding rise in formic acid synthesis. This metabolic shift could be due to the dissolved CO_2 concentration in the liquid.

Formic acid is the first intermediate of the WLP pathway (Fig. 1). In a previous investigation [24], Schwarz and Muller proved that *T. kivui* can be a highly efficient whole-cell biocatalyst for the direct hydrogenation of CO_2 to formic acid. In the mentioned study, the addition of 300 mM of potassium bicarbonate into the culture medium switched the cells to the production of formic acid instead of acetic acid. According to other literature studies [23,24], the authors suggested that the change in metabolite production was caused by bicarbonate's inhibition of ATP synthases: lowering the cell's energy level prevents the formate from continuing along the WLP pathway [24].

In a bioreactor, operating at elevated $pP_{CO2,}$ the CO_2 concentration within the medium rises [17]. Additionally, maintaining a pH of 6.5 enhanced the conversion of CO_2 into HCO_3^- according to the CO_2 equilibrium in water [26]. Consequently, the combination of elevated pP_{CO2} and pH control could facilitate the accumulation of HCO_3^- in the culture medium. The HCO_3^- concentration in the liquid can be estimated using the formulas reported in the work of Stumm and Morgan [27]. Due to the continuous gas supply and liquid batch experimental set up, calculations were conducted following the laws for estimating bicarbonate concentration in open CO_2 systems. At 10 bar, the concentration of HCO_3^- would be 37.62 mM considering the gas mix H_2:CO_2 4:1; 47.02 mM considering the gas mix H_2:CO_2 3:1; 62.70 mM considering the gas mix H_2:CO_2 2:1; and 94.05 mM considering the gas mix H_2:CO_2. Tarraran *et al.* investigated the influence of these two process parameters on the metabolism of the mesophilic acetogen *A.woodii* [19]. In particular, a modified *A.woodii* strain for acetone production was used. The authors found that the bacterium exhibited the same metabolic performance when it grew at atmospheric pressure in a medium that was pressurized with an H_2-CO_2 mixture before the inoculation and when it was cultured at high pressure, providing the same gas mixture. Moreover, biomass growth arrest and fostered formic acid synthesis was reported by cultivating the modified *A. woodii* strain in serum bottles with 300 mM $KHCO_3$ in the medium [19]. Thus, literature data suggested that changing process parameters that vary the amount of CO_2 and HCO_3^- available in the medium can lead to a metabolic shift for acetogens growing on H_2-CO_2 blends.

3.2.2. Comparison of the experimental outcomes from the DoE study with the literature

Table 3 summarizes the cell-specific acetic acid productivities achieved in this study compared to data described in the literature over the last decade. Most of the literature regarding acetogens grown on CO_2-H_2 concerns mesophilic bacteria, thus the comparison also involved these microorganisms. The listed studies reported fermentations carried out in batch stirred tank bioreactors. Table 3 shows the extensive variation in stirring speeds (ranging from 400 to 1200 rpm) and gas flow rates supplied into reactors (from 20 to 500 $mL.min^{-1}$). Additionally, durations and biomass within the vessel varied, resulting in different maximum concentrations of acetate and formate. To the best of the authors' knowledge, the study by Kim and coworkers [11] is the only literature work on *T. kivui* reporting acetic acid cell-specific productivities. The values align with those of the present study at similar reactor pressures. The other experiments listed in Table 3 were conducted with mesophilic acetogenic bacteria. The experiment of the current study conducted at 2 bar with a 2:1 H_2:CO_2 blend (R2), was performed under pressure and inlet gas mix conditions similar to those in studies conducted by Kantzow and coworkers [28], Tarraran and coworkers [19], and Straub and coworkers [29]. In [19,28], and [29] the mesophilic *A.woodii* was the catalyst for the gas fermentation. Despite the considerable variability in the gas flow rate, the specific growth rate and maximum cell-specific acetic acid productivities are quite similar. The experiment of this study conducted at 2 bar with a 4:1 H_2:CO_2 mix (R3) was performed under the same pressure and inlet gas mix conditions as those in the work of Groher & Weuster-Botz [30]. Nevertheless, in Groher & Weuster-Botz's study, the gas flow rate and the biomass in the medium were higher. Moreover, the experiment's duration was longer. Thus, [30] reached a higher of acetic acid and formic acid concentration at the end of fermentation. In the current study, in the conditions applied in R3, probably the CO_2 dissolved in the liquid medium was not enough to sustain the biomass in the reactor, nor its growth. In the [30], the higher gas in-flow rate may have overcome this issue. Finally, the experiment of the present investigation conducted at 10 bar providing a 2:1 H_2:CO_2 inlet gas mix (R8) was performed under the same pressure and similar gas composition as the study conducted by Tarraran and coworkers at the highest pressure [19]. While, in Tarraran and colleagues' study, *A. woodii* ceased growth and had a lower specific productivity in acetic acid at 10 bar compared to atmospheric pressure, the growth of *T. kivui* was not influenced at 10 bar with the 2:1 H_2:CO_2

Table 3
Comparison of parameters and cell-specific acetic acid productivities among studies utilizing acetogenic microorganisms in batch liquid stirred tank bioreactors. (V): volume; (T): temperature; (P): pressure; (t): time; (Q_g): gas inlet flowrate; ([A]): acetic acid concentration; ([F]): formic acid concentration; (q_A): acetate cell-specific productivity; (q_F): formate cell-specific productivity; (*): strain genetically modified.

Study	Bacteria	V (L)	Stirringspeed (rpm)	Gas supply mode	T (°C)	P (bar)	t (h)	Q_g (mL.min^{-1})	H_2:CO_2:CO:N_2 inlet gas mix	Max OD	μ_{max} (h^{-1})	Max [A] (g.L^{-1})	Max [F] (g.L^{-1})	Max q_A (g.g^{-1}.h^{-1})	Max q_F (g.g^{-1}.h^{-1})
This study (R2)	*T. kivui*	1	400	Cont.	66	2	96	20	67:34:0:0	1.55	0.04	30.81	0.64	0.85	0.05
This study (R3)	*T. kivui*	1	400	Cont.	66	2	96	20	80:20:0:0	0.61	0	13.53	0.06	0.75	0.01
This study (R8)	*T. kivui*	1	400	Cont.	66	10	96	20	67:34:0:0	1.09	0.06	27.67	9.37	2.74	0.65
Kim *et al.*, 2016 [11]	*T. kivui*	1.2	500	Batch	65	1.5	72	–	33:19:11:37	0.14	–	0.53	–	0.80	–
Demler & Weuster-Botz, 2010 [31]	*A. woodii*	1	400	Cont.	30	1.9	264	250	89:11:0:0	2.40	–	44.00	–	0.29	–
Kantzow *et al.*, 2015 [28]	*A. woodii*	1	1200	Cont.	30	1	77	500	40:17:0:43	6.40	0.05	59.20	–	0.70	–
Groher & Weuster-Botz, 2016 [30]	*A. woodii*	1	600	Cont.	30	1	144	83.33	80:20:0:0	2.30	0.08	37.40	1.12	1.02	0.04
Groher & Weuster-Botz, 2016 [30]	*S. ovata*	1	600	Cont.	30	1	144	83.33	80:20:0:0	2.40	0.12	32.20	1.73	0.88	0.02
Tarraran *et al.*, 2023 [19]	*A. woodii**	1	400	Cont.	30	1	127	83.33	70:30:0:0	0.90	0.04	10.20	0.68	0.92	0.27
Tarraran *et al.*, 2023 [19]	*A. woodii**	1	400	Cont.	30	10	28	83.33	70:30:0:0	1.50	0	4.07	5.85	0.68	0.48
Straub *et al.*, 2014 [29]	*A. woodii**	1	1200	Cont.	30	1	89	500	40:17:0:43	3.4	0.052	44.7	–	0.86	–

gas blend (R8). Moreover, the maximum cell-specific acetic acid productivity increased with increasing pressure. This study and [19] differ for in-flow gas rate, pH, and temperature. Tarraran *et al.* supplied gas at 83.3 mL.min^{-1}, maintaining the reactor at 30 °C and a constant pH of 7. This study was conducted by providing 20 mL.min^{-1} of gas mix at 66 °C and a pH of 6.5. Under the operating conditions applied in [19], the dissolved CO_2 in the medium is higher, and the equilibrium is toward the HCO_3^- form due to the pH. It's worth noting that in both tests, a pressure increase corresponds to a rise in the concentration of formic acid produced and the maximum cell-specific formic acid productivity, which is comparable in both studies. To the best of our knowledge, the value of acetic acid cell-specific productivity obtained in the current study at 10 bar supplying an H_2:CO_2 2:1 mix of (R4) exceeds the highest value of acetic acid cell-specific productivity reported in the literature by more than 2.5 times for CO_2 fermentations conducted in batch stirred tank bioreactors with acetogenic microorganisms.

3.2.3. Acetic acid cell-specific productivity model

After screening the catalyst's performance according to the DoE study, a complete MLR regression model was constructed to develop the acetic acid cell-specific productivity curve response. Subsequently, the model was simplified by removing insignificant factors through a preliminary analysis, specifically the interaction and quadratic terms. The resulting equation retained only the statistically significant variables (Eq. (7)).

$$y = b_0 + b_1x_1 + b_2x_2 + b_{22}x_2^2 \quad (7)$$

Table 4 displays the significant terms of Eq. (7). Positive coefficients indicate a positive impact on the response variable (max q_A). The surface offers a three-dimensional representation of the interplay between the factors (x and y axes) and the response (z axis). The response surface took the shape of a paraboloid. The acetic acid cell-specific productivity increased at higher pressure, with a maximum at 10 bar. Concerning the gas mixture, the highest values were obtained with the H_2:CO_2 3:1 blend (Fig. 5). Likely, at a ratio of 4:1, the provided CO_2 was not sufficient. In contrast, at a ratio of 1:1, the CO_2 supplied to the culture medium was such that it inhibited the production of acetic acid. Thus, according to the model, the highest achievable cell-specific productivity of acetic acid occurred at 10 bar providing the H_2:CO_2 3:1 blend. To assess the predictive capability of the MLR model, two replicates were conducted under the predicted best condition of pressure and H_2:CO_2 ratio (namely R10.1 and R10.2 in Table 2 and Table 5). Table 5 presents the experimental results from these two experiments, their predicted values, and the upper and lower bounds. Experimental outcomes were in the range specified by the model. Thus, R10.1 and R10.2 confirmed the model's predictive capacity.

Table 4
MLR model coefficients and the explained data variance. Coefficients' significance is indicated by *, **, and *** (p-Values < 0.05, 0.01 and 0.001, respectively).

Coefficients	Value of the coefficients	Significance of the coefficients	Explained variance %
b_0	1.7934	0.0001***	69.08
b_1	0.5727	0.0099**	
b_2	0.4826	0.0213*	
b_{22}	−0.7449	0.0319*	

Table 2 shows that the experimental conditions in trials R10.1 and R10.2 (H_2:CO_2 3:1 at 10 bar) yielded a maximum acetic acid cell-specific productivity lower than the one experimentally observed in trial R8 (H_2:CO_2 2:1 at 10 bar). Nevertheless, the model applied in the current study determined the optimal condition not solely on the direct analysis of experimental values. A function based on the experimental values was constructed using the MLR method to give the maximum q_A across the entire tested domain as the response. Moreover, thanks to the

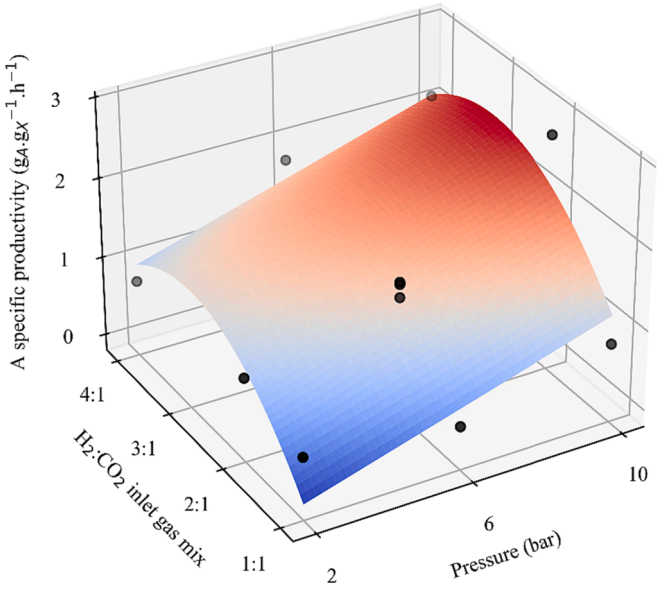

Fig. 5. Response surface for acetic acid (A) cell-specific productivity ($g.g^{-1}.h^{-1}$) as function of pressure and H_2:CO_2 inlet gas mix. (Black points •): experimental values.

Table 5
Experimental and predictive results of experiments R10.1 and R10.2.

Expt.	Experimental value	Lower	Predicted value	Upper
R10.1	2.30	1.82	2.44	3.07
R10.2	1.91	1.82	2.44	3.07

experimental triplicate conducted under one of the tested conditions (inlet gas mix H_2:CO_2 2:1 and 6 bar pressure - R5.1, R5.2, R5.3), the model also provides uncertainty limits for the predicted max q_A. The experimentally determined max q_A of each experiment fell within the uncertainty limits of the model's prediction for the specific condition tested. Accordingly, as shown in Table 5, the real max q_A from trials R10.1 and R10.2 (inlet gas 3:1 H_2:CO_2 and 10 bar pressure) falls within the uncertainty limits of the model's prediction. Therefore, considering the single experimental measurement and the calculated uncertainty limits, the condition selected as the best (3:1 H_2:CO_2 and 10 bar pressure) and the maximum q_A experimental value recorded in R8 are not in conflict.

3.3. Flow rate screening for autotrophic fermentation in a pressurized bioreactor with continuous gas supply

The outcomes of the DoE study found that the pressure was not maintained within the reactor throughout the experiment in many tested conditions, indicating that the gas flow rate provided to the reactor needed to be increased. Therefore, keeping the optimal conditions of the inlet gas mix (3:1 H_2:CO_2) and pressure constant, a flow rate screening was conducted from 30 to 120 $mL.min^{-1}$ in-flow gas rate (from R11 to R16 in Table 6). Nevertheless, even though the model pointed out an optimal pressure of 10 bar, in-flow rate screening tests were conducted at 8 bar. This choice was related to equipment safety constraints. When gas is provided into the vessel, fluctuations can happen around the target pressure until the system stabilizes. Supplying gas at high in-flow rates (up to 120 $mL.min^{-1}$) could cause the fluctuation to be quick. Thus, a lower maximum operating pressure (8 bar) was set to maintain a safety margin relative to the instrument's limit (10 bar). According to the predictions of the MLR model, at 8 bar the maximum cell-specific productivity should still be 89 % of the optimal value. Table 6 lists the experimental conditions and results of the flow rate screening study. For all the flow rates tested (30, 37.5, 60, 80, 100, and 120 $mL.min^{-1}$), an OD of approximately 1 and an acetic acid concentration of around 15 $g.L^{-1}$ were achieved in 24 h of high-pressure fermentation. The metabolic behavior of *T. kivui* was similar to that observed in the DoE experiments conducted at 10 bars with an H_2:CO_2 ratio in the inlet gas mix of 2:1 (R8), 3:1 (R10.1 and R10.2), and 4:1 (R9), those at 6 bars with ratios of 2:1 (R5.1, R5.2, R5.3) and 4:1 (R6), and those at 2 bars with a 2:1 ratio (R2) (see section 3.2.1). The screening found that the amount of gas supplied to the reactor was enough to maintain the pressure at the set value throughout the experiment at flow rates equal to or greater than 60 $mL.min^{-1}$. As the quantity of gas introduced into the reactor increased, the conversion of CO_2 and H_2 into acetic acid decreased (Table 6). CO_2 and H_2 balances indicated that from rates of 60 $mL.min^{-1}$ up to 120 $mL.min^{-1}$ (R13, R14, R15, R16) a great part of the gaseous substrate exited from the vent of the reactor vessel, probably because a larger gas quantity was provided than the bacteria could metabolize. According to the calculation reported in Supplementary Materials (section S3), in all flow rate screening tests the carbon balance was nearly closed (98 % and 99 %). The balance of the reducing equivalents ([H] = e- + H +) was closed between 87 % and 95 % in all cases. The latter balance was less accurate than the CO_2 balance probably due to the procedure of gas sampling and analysis downstream of the reactor vessel. As described in section 2.6.3, the gas composition measurement was performed offline and H_2 is a small molecule that can easily escape doing the connection steps between the analytical instruments.

Fig. 7 displays the maximum acetate and formate cell-specific productivity at each tested in-flow gas rate. Data indicates that a rise in the inlet gas rate to the reactor led to an increased cell-specific productivity of formic acid and a decreased cell-specific productivity of acetic acid. As described above (section 3.2.1), the shift in the metabolic production could be attributed to the greater amount of CO_2 available in the liquid medium supplying high in-flow gas rates (100–120 mL min^{-1}). As the gas flow rate into the reactor increases, the gas–liquid mass transfer also increases. When the pH is controlled at 6.5, the equilibrium of the CO_2 is shifted towards the HCO_3^- form that in *T. kivui* led to fostered formic acid

Table 6
Experimental conditions and results of the flow rate screening. (Expt): experiment name; (P): pressure; ([A]): acetic acid concentration; ([F]): formic acid concentration; ($\eta_{CO2}A$): CO_2 conversion into acetic acid; ($\eta_{H2}A$): H_2 conversion into acetic acid; (r_A): acetate volumetric productivity; (r_F): formate volumetric productivity; (q_A): acetate cell-specific productivity; (q_F): formate cell-specific productivity.

Expt	Q_g (mL. min^{-1})	μ_{max} (h^{-1})	Max [A] (g. L^{-1})	Max [F] (g. L^{-1})	$\eta_{CO2}A$ (%)	$\eta_{H2}A$ (%)	Max H_2 Rate ofconsumption (mg.h^{-1})	Max CO_2Rate of consumption (mg.h^{-1})	P upkeep	Max r_A (g.L^{-1}. h^{-1})	Max r_F (g.L^{-1}. h^{-1})	Max q_A (g.g^{-1}. h^{-1})	Max q_F (g.g^{-1}. h^{-1})	Max q_A / Max q_F
R11	30	0.042	16.08	0.98	85 %	28 %	121	958	no	0.90	0.20	3.03	0.63	4.84
R12	37.5	0.030	16.03	2.59	52 %	17 %	151	1175	no	0.94	0.19	2.90	0.57	5.09
R13	60	0.032	15.80	3.16	33 %	11 %	181	1402	yes	1.06	0.13	2.90	0.33	8.66
R14	80	0.031	15.40	4.42	25 %	8 %	128	1479	yes	0.88	0.21	2.65	0.62	4.25
R15	100	0.015	14.62	3.69	24 %	8 %	198	1772	yes	0.83	0.80	2.66	2.59	1.03
R16	120	0.049	12.56	2.68	19 %	6 %	242	1722	yes	0.62	0.64	1.92	2.78	0.69

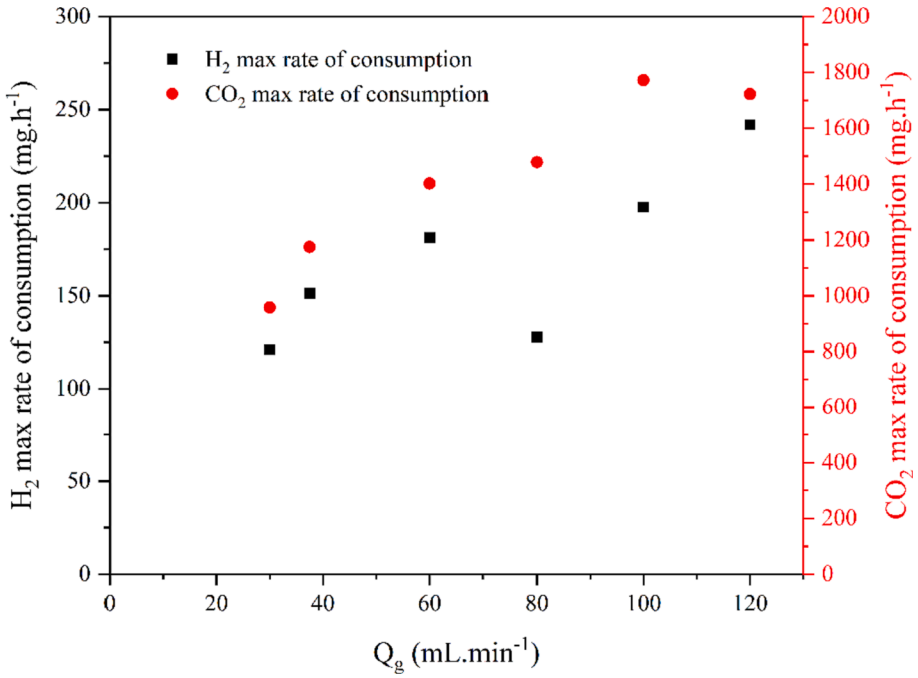

Fig. 6. Trend of maximum H_2 consumption rate and maximum CO_2 consumption rate with varying inlet gas flow rate (Q_g). Data obtained from experiments from R11 to R16 in Table 6.

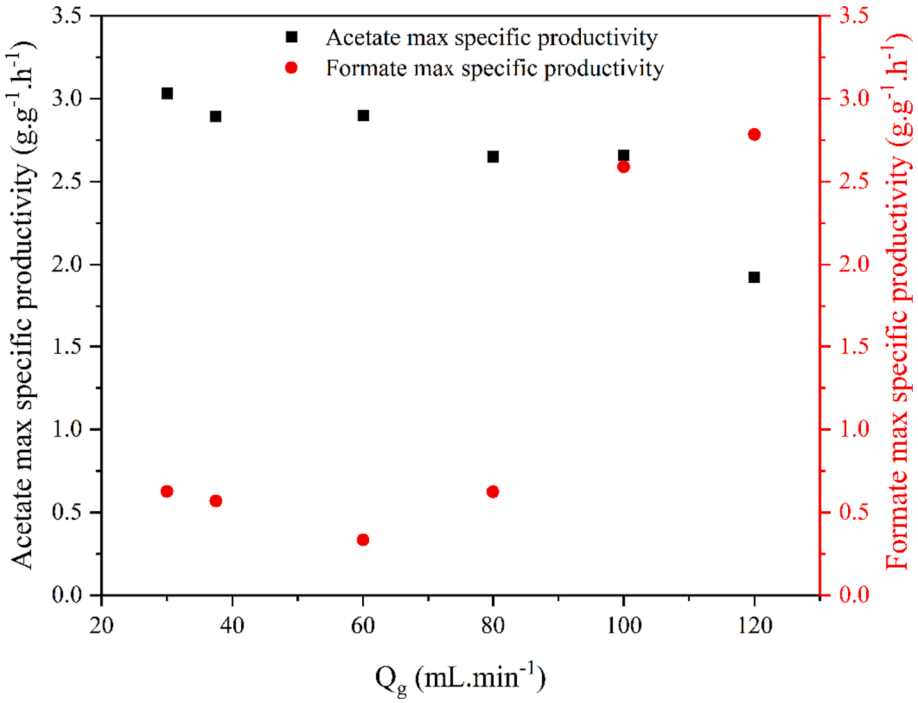

Fig. 7. Trend of maximum acetate cell-specific productivity and formate maximum cell-specific productivity with varying inlet gas flow rate (Q_g). Data obtained from experiments from R11 to R16 in Table 6.

production.

Fig. 6. Shows the trends of the maximum consumption rate for H_2 and CO_2 measured at each tested in-flow gas rate (see also Table 6 for the precise value of the x-axis). As the inlet gas flow rate provided to the reactor rose, the rate of H_2 consumption also increased. Nevertheless, at 80 mL.min^{-1}, there was a deviation from the trend of increasing H_2 consumption with higher input quantities. This outcome could be related to the metabolic product obtained: more formic acid was produced compared to the other tested configurations. Less H_2 is required to produce this metabolite than acetate. The maximum CO_2 consumption rate increased with the increasing gas flow rate until it reached a plateau at 100 mL.min^{-1}. It could be due to one or more enzymes involved in the metabolism of this compound in the cell that reached its/their catalytic activity limit.

This study aimed to maximize the cell-specific productivity of acetic acid, so formic acid production should be limited. Furthermore, higher

formic acid concentrations in the fermentation medium lead to increased acetate purification costs. Table 6 shows that considering the ratio between the maximum cell-specific productivities of acetic and formic acids (Max q_A/Max q_F), acetic acid production predominates over formic acid production for inlet gas flow rates below 100 mL.min^{-1}. Data concerning pressure maintenance pointed out that a minimum gas inlet flow rate of 60 mL.min^{-1} was required to sustain reactor pressure. Additionally, based on the maximum CO_2 consumption rate and productivity data, limiting the gas inlet flow rate to values below 100 mL.min^{-1} is advisable. In Fig. 8, the maximum volumetric productivity is plotted as a function of the maximum cell-specific productivity for acetic and formic acid for each tested in-flow gas rate. This graph helps identify the gas inlet flow rate condition that allows for maximizing acetate-specific and volumetric productivities and minimizing formate-specific and volumetric productivities. For acetic acid, they were maximized at a gas inlet flow rate of 60 mL.min^{-1} (R13). In the same condition, both specific and volumetric productivities of formic acid were minimized. Therefore, under the given gas mixture and pressure conditions (H_2:CO_2 3:1, 8 bar), the optimal gas flow rate for acetic acid production was 60 mL.min^{-1}.

3.4. Autotrophic fermentation in serum bottles with $KHCO_3$ and KCl

The DoE study and the flow rate screening suggested that dissolving more CO_2 into the liquid medium (increasing the CO_2 partial pressure or increasing the inflow gas rate into the reactor) led to fostered formic acid production besides acetic acid production. In experiment conditions applying high CO_2 partial pressure into the reactor (up to 3 bar), Tarraran and colleagues [19] reported a behavior of a modified *A.woodii* strain for acetone production similar to the one exhibited by *T. kivui*'s in the current study at high CO_2 partial pressures. As described above, controlling the pH at 6.5 shifts the equilibrium of the CO_2 provided in the liquid medium towards the HCO_3^- form. A previous study in the literature reported that the addition of 300 mM of bicarbonate to growing cultures of *T. kivui* led to fostered formic acid production [24]. Nevertheless, acids are the main metabolic products of *T. kivui*. Therefore, a higher base addition is required to maintain the pH constant when product concentration increases. The literature described that salt addiction to a culture medium can influence the growth of a microorganism and its intracellular metabolism [32–34]. Thus in the present work, an experiment in serum bottles was set up to investigate whether the growth of *T. kivui* and the production of formic acid were affected by bicarbonate or generally by the osmotic condition. Bacteria were inoculated in autotrophy in serum bottles with 300 mM of potassium bicarbonate or 300 mM of potassium chloride as a generic salt that does not contain bicarbonate in its formula. As a reference case, bacteria were inoculated in a medium without adding salts.

Fig. 9 displays the results obtained from the test. The bacterium developed a standard growth curve only in the reference case while adding 300 mM $KHCO_3$ or 300 mM KCl into the autotrophic medium, it was inhibited (Fig. 9a). In the standard culture condition, acetate was produced reaching 5.958 ± 0.212 g.L^{-1}. The glucose supplied at the beginning of the test was completely consumed, contributing 1.341 ± 0.727 g.L^{-1} to the final acetate concentration achieved. Instead, acetate production was abolished both adding 300 mM KCl or 300 mM $KHCO_3$ to the culture. Moreover, the glucose provided at the beginning of the fermentation was not fully consumed (data not shown), suggesting that the general osmotic condition impaired bacteria metabolism (Fig. 9b). The formic acid production was negligible in the reference condition (Fig. 9c). With 300 mM KCl into the medium, a higher concentration than the reference experiment was measured (0.595 ± 0.035 g.L^{-1}). Nevertheless, it should be noticed that formate synthesis started at the beginning of the growth curve and its concentration remained constant during the experiment. Instead, by adding 300 mM $KHCO_3$ into the medium, a considerable production of formic acid was recorded. Its maximum concentration was 3.717 ± 0.119 g.L^{-1}, and it was measured 80 h after inoculation (Fig. 9c). Therefore, outcomes indicated that both salts led to growth inhibition while formic acid production was enhanced by the addiction of $KHCO_3$ but not KCl.

In the above cited study [24], Schwarz and Muller added 300 mM of bicarbonate into a *T. kivui* growth culture at OD_{600nm} = 0.3 in the heterotrophic condition (5.04 g.L^{-1} glucose). They found an immediate growth arrest, stop of acetic acid production and formic acid synthesis, up to an amount congruent with that achieved in the current study. *T. kivui* cells showed a different behavior of *A. woodii* cells in the same condition (autotrophic medium + 300 mM salts). When 300 KCl was added to the medium, *A. woodii* growth was inhibited but not completely blocked and acetic acid synthesis was not affected [19]. *T. kivui* culture, instead, stopped its growth and acetic acid was not synthesized.

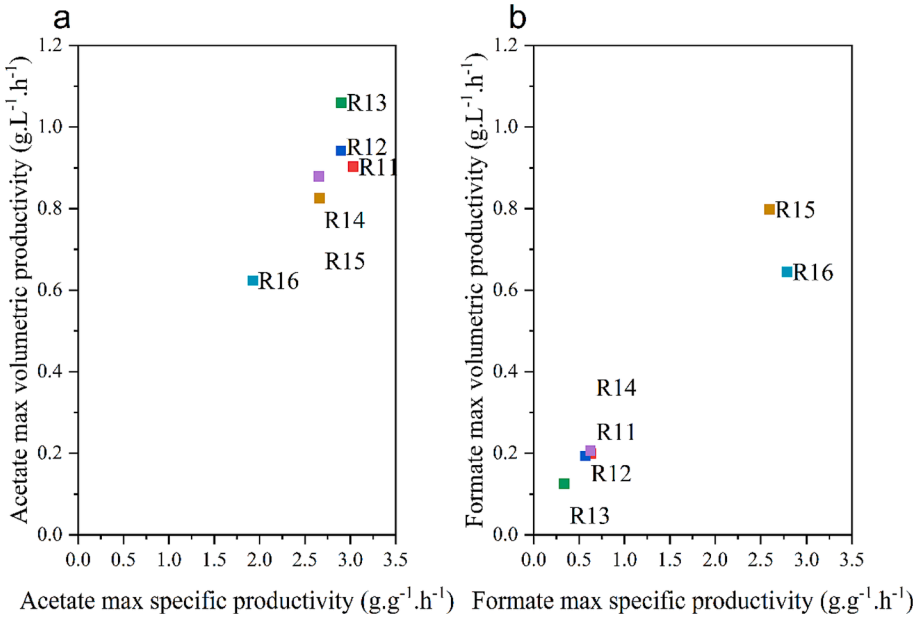

Fig. 8. Product maximum volumetric productivity in relation to the maximum cell-specific productivity. (a): acetic acid; (b): formic acid. (R…): experiment name according to Table 6. Data obtained from experiments R11 to R16 in Table 6.

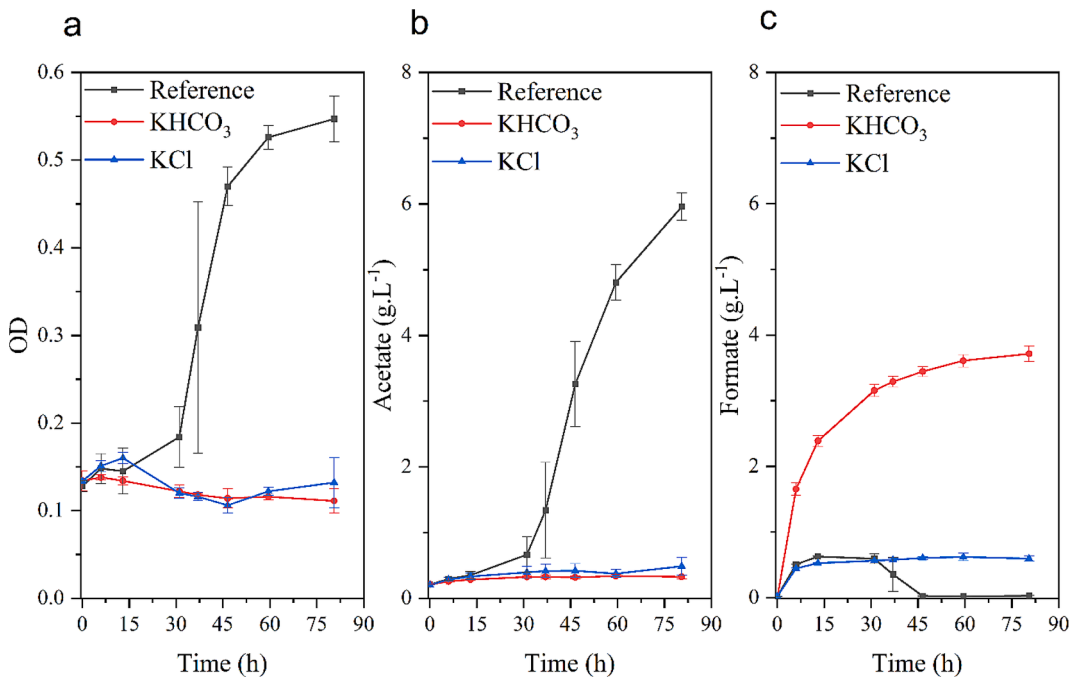

Fig. 9. Influence of the addition of 300 mM $KHCO_3$ or 300 mM KCl in the autotrophic medium. (**a**) *T. kivui* growth; (**b**) *T. kivui* acetate production; (**c**)*T. kivui* formic acid production. (Reference): bacteria growth in autotrophic medium without added salts.

3.5. Serum bottle fermentation in fed-batch with increased acetic acid concentration

Many experiments of the DoE reached an acetic acid concentration $\geq$ 24 $g.L^{-1}$ (R2, R5.1, R5.2, R5.3, R6, R8, R9, R10.1, R10.2 in Table 2); nevertheless, a slowdown in biomass growth was observed when the acetic acid concentration in the medium reached $\approx$ 15 $g.L^{-1}$. To assess the inhibition of bacteria growth due to the product's concentration, *T. kivui* was inoculated under autotrophic conditions with the addition of 14.5 or 28.3 $g.L^{-1}$ of exogenous acetic acid. The test was conducted in serum bottles. Exogenous acetic acid was added to the medium before the inoculum, and pH was adjusted to 6.5 with NaOH. In parallel, reference cultures in the autotrophic medium without added acetic acid were set up. Outcomes of the test indicated that *T. kivui* exhibited a typical growth curve and synthesized acetic acid only in the reference condition (acetate final concentration 6.254 $\pm$ 0.140 $g.L^{-1}$). The glucose provided at the beginning of the test was completely consumed, contributing 0.700 $\pm$ 0.022 $g.L^{-1}$ to the final acetate concentration achieved. Formic acid production was negligible. When 14.5 $g.L^{-1}$ or 28.3 $g.L^{-1}$ of acetic acid was introduced into the culture medium, bacteria did not grow or produce more acetic acid. Similarly to what was described in Section 3.4 for the KCl addiction to the culture medium, initial glucose was not completely consumed. A low amount of formate was synthetized, reaching a plateau at 0.160 $\pm$ 0.002 $g.L^{-1}$ and 0.180 $\pm$ 0.003 $g.L^{-1}$ with 14.5 $g.L^{-1}$ or 28.3 $g.L^{-1}$ exogenous acetic acid, respectively. A figure showing the detailed test results is reported in the Supplementary Materials (Fig S3).

According to the experiments outcomes in serum bottles, 14.5 $g.L^{-1}$ of acetic acid inhibited *T. kivui*'s metabolism. Nevertheless, it should be noticed that, before starting the experiment, NaOH was added to the culture medium to raise the pH to 6.5 after the addition of the exogenous acetate. For the bottles with 14.5 $g.L^{-1}$ acetic acid, 250 mM NaOH was added, while for the bottles with 28.3 $g.L^{-1}$ acetic acid, 450 mM NaOH was used. In Section 3.4, it was demonstrated that a salt concentration of 300 mM significantly impacted *T. kivui* 's metabolism. Growth, acetic acid, and formic acid production shown by the bacterium adding exogenous acetate were like when it was cultured with 300 mM KCl in the medium. Therefore, it was not clear whether *T. kivui* 's growth was inhibited by acetic acid concentration or by the amount of base required to maintain the pH at 6.5 at those acetic acid concentrations because the two factors are closely intertwined. Setting up a continuous liquid fermentation could be a strategy to overcome the issue. By removing the culture medium rich in acetate and base and replacing it with fresh medium, both the agents that could inhibit the bacterium will be soothed.

4. Conclusions

The carbon dioxide concentration in the atmosphere is leading to climate change. The synthesis of bulk compounds exploiting this molecule as a carbon source can contribute to developing a more sustainable economy. Among chemicals, acetic acid is widely employed and can be produced through gas fermentation. *T. kivui* is a thermophilic acetogen that exploits H_2 to reduce CO_2 into acetic acid. Low process productivities are often reported due to low substrate concentrations in the growth medium, resulting in diminished biocatalyst activity. The current work aimed to identify optimal conditions for achieving the highest acetic acid cell-specific productivity by *T. kivui*, combining pressure, H_2:CO_2 ratio in the inlet gas mix, and in-gas flow rate.

The core of the current study is a screening of pressure, H_2:CO_2 ratio in the inlet gas mix, and in-gas flow rate to optimize acetic acid production. First, an experimental campaign based on the Design of Experiments (DoE) approach was conducted in a pressurized bioreactor to assess the impact of combined pressure and H_2:CO_2 ratio in the inlet gas blend on the maximum acetic acid cell-specific productivity (q_A), keeping constant the inflow gas rate at 20 $mL.min^{-1}$. The experimental data of the DoE were the base for constructing a model for acetic acid cell-specific productivity. The model pointed out that working at 10 bar pressure, providing the H_2:CO_2 3:1 gas mix, allowed reaching the highest q_A. Besides acetic acid productivity, DoE tests suggested that dissolved CO_2 and its ratio with H_2 influenced formic acid productivity. When the concentration of dissolved CO_2 in the liquid phase increased, there was a corresponding increase in formic acid production. The gas in-flow rate provided in the DoE experiments (20 $mL.min^{-1}$) was insufficient to maintain the constant pressure, as the biomass inside the reactor grew, under many tested conditions. Consequently, a flow rate

screening was performed from 30 to 120 mL.min^{-1}. According to the model, the highest acetic acid cell-specific productivity could be reached at 10 bar and 3:1 H_2:CO_2 mix. Nevertheless, due to reactor hardware limit and safety constraints at rates above 80 mL.min^{-1}, in-flow rate tests were conducted at 8 bar, providing the 3:1 H_2:CO_2 blend. According to the model, the maximum cell-specific productivity should still be 89 % of the optimal value working at 8 bar rather than 10 bar. Gas-in rate screening results indicated that a minimum gas inlet flow rate of 60 mL.min^{-1} was needed to sustain the reactor pressure. The maximum rate of CO_2 consumption increased as the gas flow rate rose but leveled off at an inlet rate of 100 mL.min^{-1}. Moreover, acetic acid production significantly outweighed formic acid production for flow rates below 100 mL.min^{-1}. The highest acetic acid specific and volumetric productivities were achieved at 60 mL.min^{-1}. Therefore, at 8 bar pressure and 3:1 H_2:CO_2 gas blend, the optimal in-gas flow rate for acetic acid production was 60 mL.min^{-1}.

Finally, the current study investigated the influence of the osmotic condition and acetic acid inhibition on *T. kivui*. 300 mM $KHCO_3$ or 300 mM KCl were added to the autotrophic medium in serum bottles to test the effect of the osmotic conditions and different salts on bacteria growth and metabolic spectrum. Results suggested that the osmotic condition could affect *T. kivui*'s growth and impair acetate production. The main gas fermentation products in *T. kivui* are acids - acetic or formic. Thus, during the growth, the pH in the culture medium fell. To avoid growth arrest due to low pH is necessary to add base throughout the fermentation. Acetic acid inhibition on *T. kivui's* growth was tested by adding 14.5 or 28.3 g.L^{-1} of exogenous acetic acid to the autotrophic medium. Outcomes indicate that with 14.5 g.L^{-1} of exogenous acetic acid, growth, acetic acid, and formic acid production were like when the bacterium was cultured with 300 mM KCl into the serum bottle. Nevertheless, it was not possible to determine whether *T. kivui* 's growth was inhibited by the concentration of acetic acid or by the amount of base (NaOH) required to maintain the pH at 6.5 at those acetic acid concentrations. In fact, the two factors are closely intertwined, and more tests should be performed to address this issue.

The current work indicated that *Thermoanaerobacter kivui* could be a candidate for future large-scale fermentations exploiting CO_2. The fermentation design should consider several aspects to obtain acid acetic as the target molecule. The study indicated a combination of pressure, gas ratio, and inflow gas rate optimized to produce this latter compound. Furthermore, it highlighted some aspects that should be further investigated to optimize the scale-up of the process, such as the inhibition of salts and acetic acid.

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Author contributions

Francesco Regis: Conceptualization, Data curation, Investigation, Methodology, Writing - Original Draft, Writing - Review & Editing. **Loredana Tarraran**: Conceptualization, Data curation, Methodology, Writing – Review & Editing, Supervision. **Alessandro Monteverde**: Review, Resources, Supervision. **Debora Fino**: Resources, Supervision.

CRediT authorship contribution statement

Francesco Regis: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Loredana Tarraran:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Alessandro Monteverde:** Supervision, Resources. **Debora Fino:** Supervision, Resources, 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2024.149685.

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