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Expanding the product portfolio of carbon dioxide and hydrogen-based gas fermentation with an evolved strain of *Clostridium carboxidivorans*

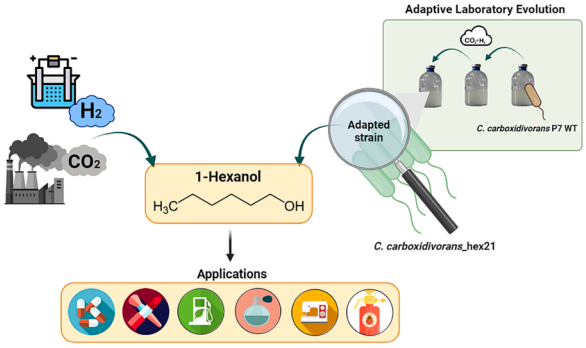
G. Antonicelli [a,b,1], L. Ricci [a], L. Tarraran [a,c], S. Fraterrigo Garofalo [c], A. Re [c], N.S. Vasile [a,b], F. Verga [b], C.F. Pirri [a,c], B. Menin [a,d], V. Agostino [a,1,*]

[a] Centre for Sustainable Future Technologies, Fondazione Istituto Italiano di Tecnologia, Via Livorno 60, 10144 Turin, Italy
[b] Department of Environment, Land and Infrastructure Engineering, Politecnico di Torino, Corso Duca degli Abruzzi 24, 10129 Turin, Italy
[c] Department of Applied Science and Technology, Politecnico di Torino, Corso Duca degli Abruzzi 24, 10129 Turin, Italy
[d] Istituto di Biologia e Biotecnologia Agraria, Consiglio Nazionale delle Ricerche, Via Alfonso Corti 12, 20133 Milan, Italy

HIGHLIGHTS

- Development of a new strain: *C. carboxidivorans* hex21.
- Direct hexanol production from CO_2:H_2-based gas fermentation.
- Highest maximum hexanol productivity reported so far.
- Enhanced hexanol selectivity: carbon partitioning > 40%.
- Odd-chain carboxylates and alcohols production.

GRAPHICAL ABSTRACT



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ABSTRACT

CO_2:H_2-based gas fermentation with acetogenic *Clostridium* species are at an early stage of development. This work exploited the Adaptive Laboratory Evolution technique to improve the growth of *C. carboxidivorans* P7 on CO_2 and H_2. An adapted strain with decreased growth lag phase and improved biomass production was obtained. Genomic analysis revealed a conserved frameshift mutation in the catalytic subunit of the hexameric hydrogenase gene. The resulted truncated protein variant, most likely lacking its functionality, suggests that other hydrogenases might be more efficient for H_2-based growth of this strain. Furthermore, the adapted strain generated hexanol as primary fermentation product. For the first time, hexanol was produced directly from CO_2:H_2 blend, achieving the highest maximum productivity reported so far via gas fermentation. Traces of valerate, pentanol, eptanol and octanol were observed in the fermentation broth. The adapted strain shows promising to enrich the product spectrum targetable by future gas fermentation processes.

* Corresponding author.
E-mail address: valeria.agostino@iit.it (V. Agostino).
[1] Antonicelli and Agostino co-first authors.

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1. Introduction

Gas fermentation technology with acetogens is one of the strategies to transit from a fossil to a sustainable bio-based economy. Acetogenic bacteria naturally convert C1-gases into organic acids, alcohols and diols (Sun et al., 2019). Both H_2 and CO can act as electron donors to sustain the autotrophic growth of acetogens. In recent decades, the research community has dedicated extensive efforts to understanding and developing CO-rich and syngas-based bioprocesses. So far, the use of acetogens has proven commercially viable only in fermentation based on CO-rich industrial off-gases (Köpke and Simpson, 2020). Despite the importance of these gas fermentation options, it is worth noting that the main fraction of carbon source in these waste streams is CO rather than CO_2. In addition, it is known that during the oxidation of CO as an electron donor, carbon is lost in the form of CO_2, as reported in several studies (Valgepea et al., 2018; Hermann et al., 2020). More research should be devoted in the valorization of CO_2-rich waste gases with acetogenic *Clostridium* species to upgrade waste streams where CO is absent. Indeed, sources of CO_2 emissions are abundant and highly diversified in purity, scale, and distribution. The aim of this work is to fill this gap. Differently from syngas and CO-based industrial feedstock, pure CO_2-based fermentations need to be supplemented with reducing power, such as green H_2, allowing complete microbial conversion of CO_2 into biomass and final products (carbon negative). Currently, CO_2:H_2-based bioprocesses are in an early stage of development, mainly resulting in the production of acetate (Kantzow et al., 2015; Molitor et al., 2019) and low titer ethanol (Heffernan et al., 2020). Besides ethanol, another alcohol of commercial interest is hexanol. Hexanol is a C6-alcohol used as a drop-in molecule for multiple market segments: solvents, flavors and fragrances, plasticizers, detergent, coatings and pharma/fine chemicals. The current production of hexanol is based on fossil fuels or kernel palm oil. A commercially sustainable bio-based process is not available. *Clostridium carboxidivorans* P7 naturally generates hexanol, beyond ethanol and butanol, using syngas and CO-based gaseous feedstock. *C. carboxidivorans* P7 is one of symbolic strains in "acetogens" studies, originally isolated from an agricultural settling lagoon in Oklahoma (Liou et al. 2005). However, its product yields and selectivity towards this C6-alcohol are lower compared to C2-C4-alcohols, except for the recent works of Oh and colleagues (Oh et al., 2022; Oh et al., 2023). To the best of the authors' knowledge, only Lakhssassi and co-workers (2020) studied the physiology of *C. carboxidivorans* P7 wild-type (WT) and of ethyl methanesulfonate-induced mutants grown on CO_2:H_2 substrates. The mutated strains produced ethanol and butanol, but not hexanol. Several approaches can be applied to improve strain growth and hexanol synthesis in desired fermentation conditions. When dealing specifically with *C. carboxidivorans* P7, metabolic engineering is still probit and random mutagenesis is limited by the occurrence of uncontrolled genomic mutations. A different approach consists in the adaptation of *C. carboxidivorans* P7 WT to the desired growth conditions via adaptive laboratory evolution (ALE). ALE was successfully exploited in acetogens, such as *Eubacterium limosum* for growth enhancement in the presence of CO (Kang et al., 2020), *Sporomusa ovata* to accelerate the autotrophic metabolism (Tremblay et al., 2015) and in *Desulfosporosinus orientis* to improve acetate production (Agostino et al., 2020). Following this strategy, the present work aims to leverage the ALE technique for developing an evolved strain of *C. carboxidivorans* P7 with improved growth on CO_2:H_2-based substrate. The successfully generated adapted strain not only showed higher growth performance than the WT, but was also able to produce hexanol as the primary fermentation product. This is, to the best of authors' knowledge, the first example of direct hexanol production via CO_2:H_2-based gas fermentation. A thorough phenotypic characterization of the evolved strain was carried out, evaluating key physiological parameters of gas fermentation process. Eventually, key genes that had undergone mutations and the stability of these variants were investigated, by a whole genome re-sequencing (WGrS) followed by insertions (Ins), deletions (Dels) and single nucleotide polymorphisms (SNPs) detection and annotation analysis.

2. Material and methods

2.1. Microorganism, medium and fermentation conditions

Clostridium carboxidivorans P7 (DSM 15243) was purchased from DSMZ. *C. carboxidivorans* was routinely maintained anaerobically in 1754 PETC-modified medium + D-glucose (5 g/L), composed as follows (per liter): Na-resazurin 0.001 g, yeast extract 0.5 g, MES buffer 5 g, NaCl 0.8 g, NH_4Cl 1.0 g, KCl 0.1 g, K_2HPO_4 0.1 g, $CaCl_2x2H_2O$ 0.04 g, $MgCl_2x6H_2O$ 0.2 g, 10 mL of 1754 PETC Trace Elements solution, 10 mL of 141 DSMZ Vitamins solution, L-cysteine-HCl 0.15 g, Na_2Sx9H_2O 0.15 g. Cryopreservation of the strains was performed using anaerobic glycerol stocks (25%) with a heterotrophic culture (104c DSMZ medium without Na_2CO_3) for the WT and an autotrophic culture for the adapted strain. Fermentations with WT and adapted strains were performed in triplicate in 250 mL serum bottles (OEA labs, UK) with 25 mL of 1754 PETC-modified medium. Batch heterotrophic cultivation was peformed at 37 °C with static incubation. Batch autotrophic cultivation was conducted with 1754 PETC-modified medium without D-glucose, using CO_2:H_2, CO or syngas (CO:H_2:CO_2:N_2) (56:20:9:15) mixture at 1.75 bar. A horizontal incubation at 100 rpm and temperature of 25° or 37 °C were used. Fed-batch gas experiments were carried out by re-pressurizing the bottles daily at 1.75 bar.

2.2. Adaptive laboratory evolution experiment

The ALE experiment was conducted under batch conditions at 25 °C, using CO_2:H_2– based gas mixture (CO_2:H_2) (20:80) as the selective pressure. Heterotrophic pre-culture (10% volume per volume), originating from glycerol stock, was used as inoculum for the ALE experiment. The test was performed in triplicate, and the following parameters were measured daily: optical density at 600 nm (OD_{600}), pH and pressure. 10 consecutive culture passages were done by transferring cells in the late exponential /early stationary phase into fresh medium. The production profiles of transfers 1, 3, 5, 8 and 10 were characterized.

2.3. Analytical methods

Quantification of C2-C6 organic acids and C2-C6 alcohols was performed by HPLC as described in Ricci et al. (2021), with the following modifications: flow rate 1 mL min^{-1} and an oven temperature of 60 °C. To characterize the entire metabolite profile of C. *carboxidivorans'* supernatant (gas fed-batch fermentation), the GS-MS analysis of diethyl ether extracted samples was perfomed using an Agilent 7890A GC System equipped with a Rxi-5 ms column (30 m × 0.25 mm ID × 25 μm; Restek) and a quadrupole mass detector. Helium was the carrier gas (0.4 mL/min), the injection volume was 1 μL using the split mode with a split ratio of 20:1 at 280 °C. The column was initially maintained at 40 °C for 5 min and then the temperature was then increased to 100 °C at 10 °C/min, and to 280 °C at 5 °C/min and kept constant for 10 min. The mass analyzer worked in electron ionization mode (70 eV) and in full scan mode (50–600 a.m.u). Off-line gas analysis (CO, CO_2, H_2) of the serum bottles headspace was conducted with a two channels MicroGC Agilent 490 (Agilent, USA), equipped with Thermal Conductivity Detector, MS 5A PLOT (0,25 mm d.i., 10 m) capillary column and Pora PLOT Q (0,25 mm d.i., 10 m) capillary column. The gas sampling was performed by using a 2.5 mL glass tight syringe (Hamilton, USA).

2.4. Calculation of main fermentation metrics

To estimate the biomass concentration, an experimentally determined correlation factor of 0.3 (data not shown) was used to calculate the cell dry weight (CDW) from OD_{600} data. The volumetric productivity

(q_p) and the specific productivity (r_p) were calculated for each metabolite every day of fermentation. Yield for each metabolite or biomass ($Y_{p/s}$) was calculated as the ratio between the moles of carbon (C-mols) converted into the specific product and the total C-mols of the substrate dissolved in the liquid phase (either CO_2 or CO). A biomass molecular weight of 24 g mol^{-1} (chemical formula: $CH_{1.8}O_{0.5}N_{0.2}$) was used for calculation (Villadsen et al. 2011). The C-mols of substrate dissolved in the liquid phase were calculated by taking into account the gas change in the headspace and the carbon fraction solubilized in the medium during the degassing procedure. The C-mols in the headspace were calculated by applying the Ideal Gas Law, while the solubilized fraction of gases during the medium degassing procedure was calculated by Henry Law.

2.5. Statistical analysis

All the presented data derive from triplicate experiments and are shown as mean values ± standard error of the mean (SEM). GraphPad Prism 5.1 (USA) software was used for data analysis and plots elaboration. Statistical analysis was performed as reported in Ricci et al. (2021).

2.6. Whole-genome resequencing and mutation screening

To perform WGrS, genomic DNA was extracted from pure cultures of either the adapted or wild-type strain using Qiagen DNeasy Powersoil Pro Kit (Qiagen) and following the manufacturer's instructions. Glycerol stocks were activated in heterotrophic conditions and incubated for 24 h at 37 °C. 12 mL of culture was centrifuged and the resulting pellet, after a washing in 10% NaCl, was used for DNA extraction. The extracted DNA quantity and quality (A260/A280 > 2.0 A260/230 > 2.0) were assessed using a NanoDrop (Thermo Scientific, USA). The WGrS was performed by Eurofins (Eurofins, Luxembourg) with the Genome Sequencer Illumina NovaSeq 6000 (Illumina, USA) with 2x150 bp-paired-end reads. InDels and SNPs of the new DNA sequences were mapped against the wild-type genome of *C. carboxidivorans* P7, deposited in NCBI (RefSeq NZ_CP011803.1). The annotation of the detected SNPs, Ins and Dels was performed using dbSNP (NCBI-NIH), while the allocation of effects at the protein level was performed using Ensembl (EnsemblGenomes.com).

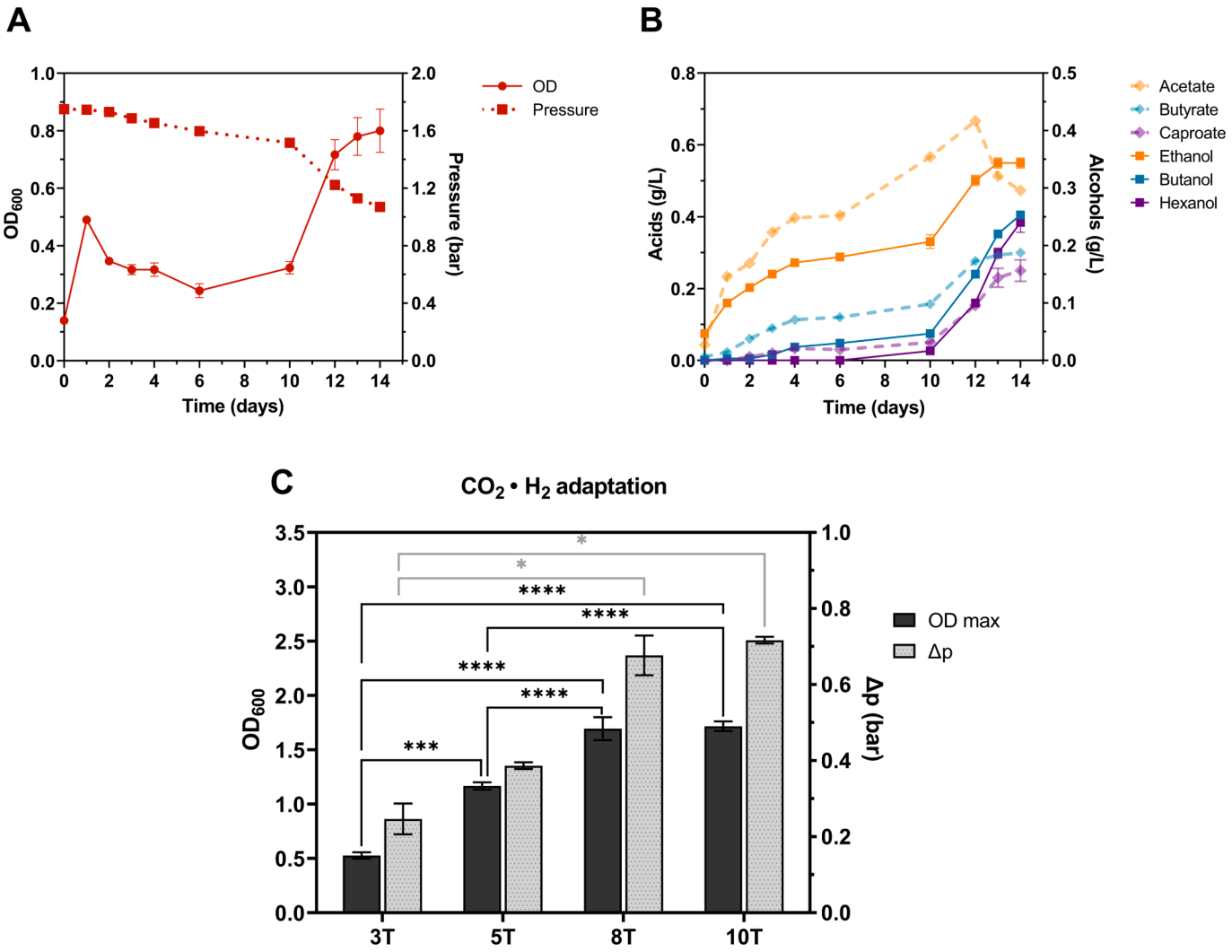


Fig. 1. Physiology of *C. carboxidivorans* P7 WT cultivated on CO_2:H_2 at 25 °C and adaptive laboratory evolution (ALE) experiment. Heterotrophic seed-culture was used as inoculum. (A) Optical density profiles (solid lines), pH profiles (dotted lines), (B) products profiles. Organic acids are represented with diamond symbols and dotted lines, while alcohols with square symbol and solid lines. (C) ALE experiment, OD_{600} and headspace gas pressure decrement (ΔP) after three days of fermentation. Data represent the average ± standard error of the mean (SEM) of triplicate experiments. The paired one-tailed *t*-test was carried out for statistic differences ($p < 0.0332$(*) $p < 0.0021$ (**) $p < 0.0002$ (***) $p < 0.0001$ (****)). T = culture transfer.

3. Results and discussion

3.1. *C. carboxidivorans P7 physiology on CO_2:H_2 and adaptive laboratory evolution*

Batch experiments using heterotrophic pre-culture were performed to evaluate the growth rate and biomass production of *C. carboxidivorans* P7 WT using a CO_2:H_2-mixture (1:4). Based on the results obtained by Lakhssassi and co-workers (2020), namely that the optimal growing temperature of 37 °C resulted in an OD_{600} of 0.2 while 25 °C in an OD_{600} of 0.7, a 25 °C cultivation temperature was chosen for experiments. Starting from two different glycerol stocks, replicate tests resulted in growth curves with a long lag phase of 10–12 days, reaching OD_{600} values of 0.8 at 14 days of fermentation (Fig. 1A). It is worth pointing out that, unlike the results obtained by Lakhssassi and co-workers (2020), in the present study the product spectrum of *C. carboxidivorans* P7 WT also included caproate and hexanol (Fig. 1B). Notably, to the best of authors' knowledge, this is the first time that hexanol production was demonstrated from a gas mixture composed of CO_2 and H_2. The main differences between the cultivation strategy of this study and the one used by Lakhssassi and co-workers (2020) are i) medium composition (Section 2.1); ii) gas to liquid volumetric ratio (9:1 vs. 3:1); iii) serum bottle orientation during fermentation (horizontal vs. vertical). As already reported in previous studies with other gaseous substrates, increasing the gas to liquid volumetric ratio and using horizontal incubation of the serum bottles can improve the gas availability for cells and the production of alcohols (Ribeiro et al., 2017, Lee et al., 2019; Ricci et al., 2021). To reduce the lag phase of the growth on CO_2:H_2-based substrate and increase biomass production, the ALE technique was adopted using CO_2:H_2 as a selective pressure. Fig. 1C compares the OD_{600} and the headspace pressure decrement (ΔP) obtained after three days of fermentation, among the different culture transfers of the ALE experiment. In agreement with results described above, the first autotrophic culture showed no growth and gas consumption. As the number

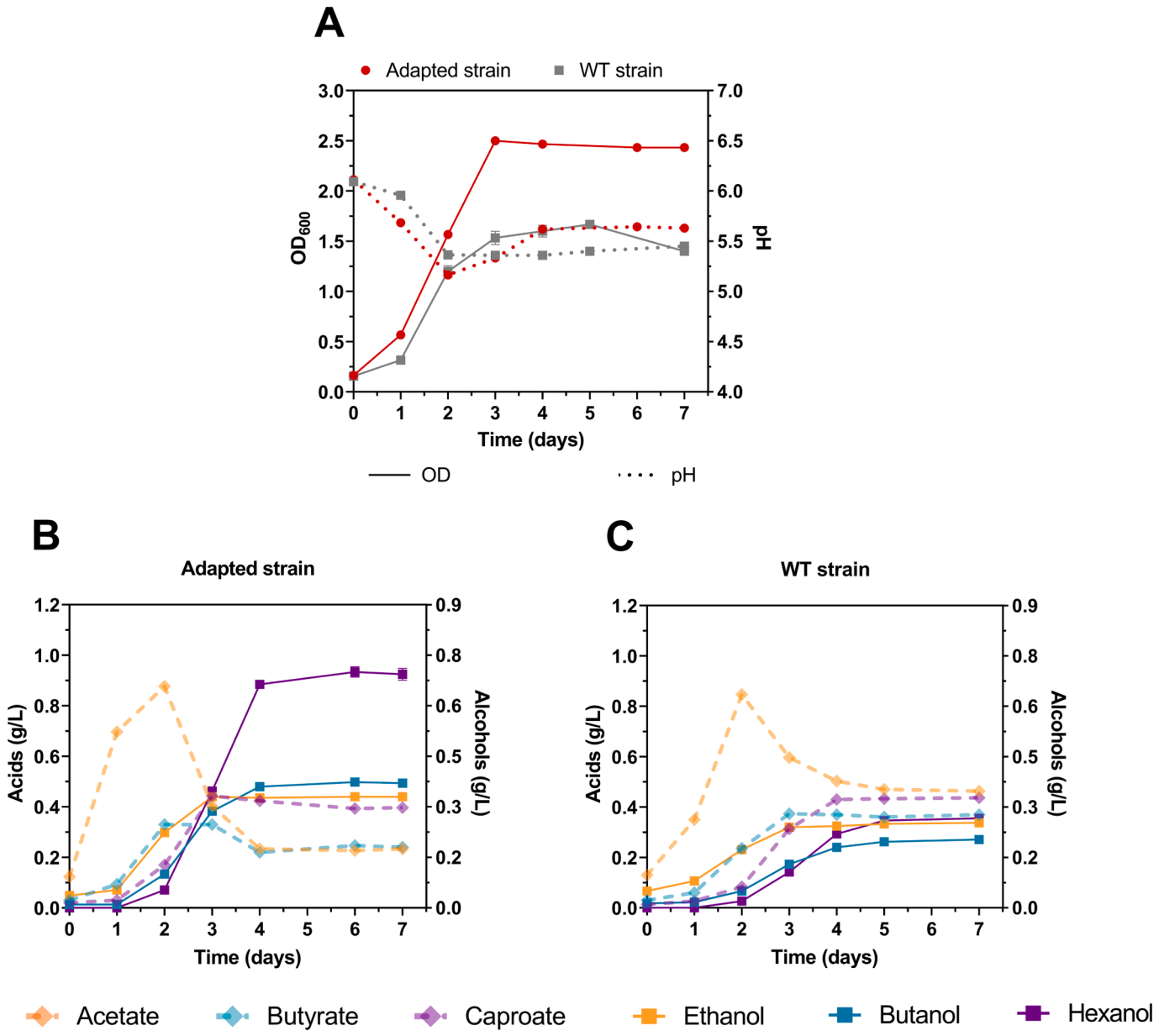


Fig. 2. Comparison of adapted strain vs WT strain, grown on CO_2:H_2 at 25 °C. (A) Optical density profiles (solid lines), pH profiles (dotted lines), (B) product profiles of adapted strain and (C) product profiles of WT strain. Organic acids are represented with diamond symbols and dotted lines, while alcohols with square symbol and solid lines. Data represent the average ± standard error of the mean (SEM) of triplicate experiments.

of cultural transfers increased, the growth and gas consumption improved, reaching a maximum OD_{600} of 1.7 and a ΔP of 0.7 bar at the 8th culture transfer (8T). No statistical difference in growth performances was observed with further transfers. Thus, the 8T of the ALE experiment was considered as the final step for obtaining a strain adapted to CO_2:H_2-mixture (1:4). Glycerol stocks of the 8T were stored at −80 °C and used to perform phenotypical characterization and WGrS. The comparison of growth rate and the product spectrum between the adapted strain (8T) and the WT strain was performed by using an autotrophic seed culture at mid-exponential phase. The time between the reactivation of the glycerol stock and the inoculation of fermentation experiment was about 20 days for WT and about 5 days for the adapted strain. This result confirms the slow adaptability of the WT strain and the improved growth ability of the adapted strain to the CO_2:H_2-based gas mixture. Both the WT and the adapted strain inoculated with a pre-adapted autotrophic seed culture showed 3–4 days of exponential growth phase with no lag phase on CO_2:H_2 (Fig. 2A). The adapted strain outperformed the WT in terms of maximum OD_{600} and specific growth rate (μ) by 1.5- and 1.14- fold, respectively (OD P value = 0.0008 (***); μ P value = 0.0451 (*)). In addition, the adapted strain showed faster and higher gas consumption and a different pH profile than the WT (Fig. 2A). The pH in acetogenic *Clostridium* species is strongly related to acetogenic and solventogenic phases of the fermentation and, thus, to the product spectrum. Both strains showed decreased pH values and acids generation during the first two days of fermentation (mainly acetate) (Fig. 2B-C). Thereafter, the two strains behaved differently. The pH of the WT strain stabilized and only a part of acetate was consumed, with consequent lower production of alcohols than organic acids (acetate, butyrate and caproate). The hexanol titer was 0.27 g/L with a maximum volumetric productivity (q_{hex}) of 0.005 $gL^{-1}h^{-1}$. Conversely, the adapted strain partially consumed all three acids, primarily acetate, with concomitant high production of alcohols, mainly butanol and hexanol. At the end of the fermentation, hexanol was the primary product generated by the adapted strain, reaching a final titer of 0.7 g/L and a q_{hex} of 0.013 $gL^{-1}h^{-1}$, which are 2.6-fold higher than the values of the WT (max $titer_{hex}$ P value = 0.0011 (**), q_{hex} P value = 0.0051 (**); T test). As in the case of *S. ovata*, *E. limosum* and *D. orientis* (Section 1), the ALE strategy also proved effective for *C. carboxidivorans*, with positive effects on CO_2:H_2-based growth and unexpected increased hexanol production.

It should be mentioned that besides the stress due to the continuous propagation of the strain using a not energetically favorable electron donor (H_2), in ALE serum bottle experiments the cells may also be subjected to gas–liquid mass transfer stress.

3.2. Physiological characterization of the adapted strain in batch fermentation

Process parameters, such as H_2 to CO_2 ratio, gas mixture composition and temperature, influence the growth, products spectrum and, yields of

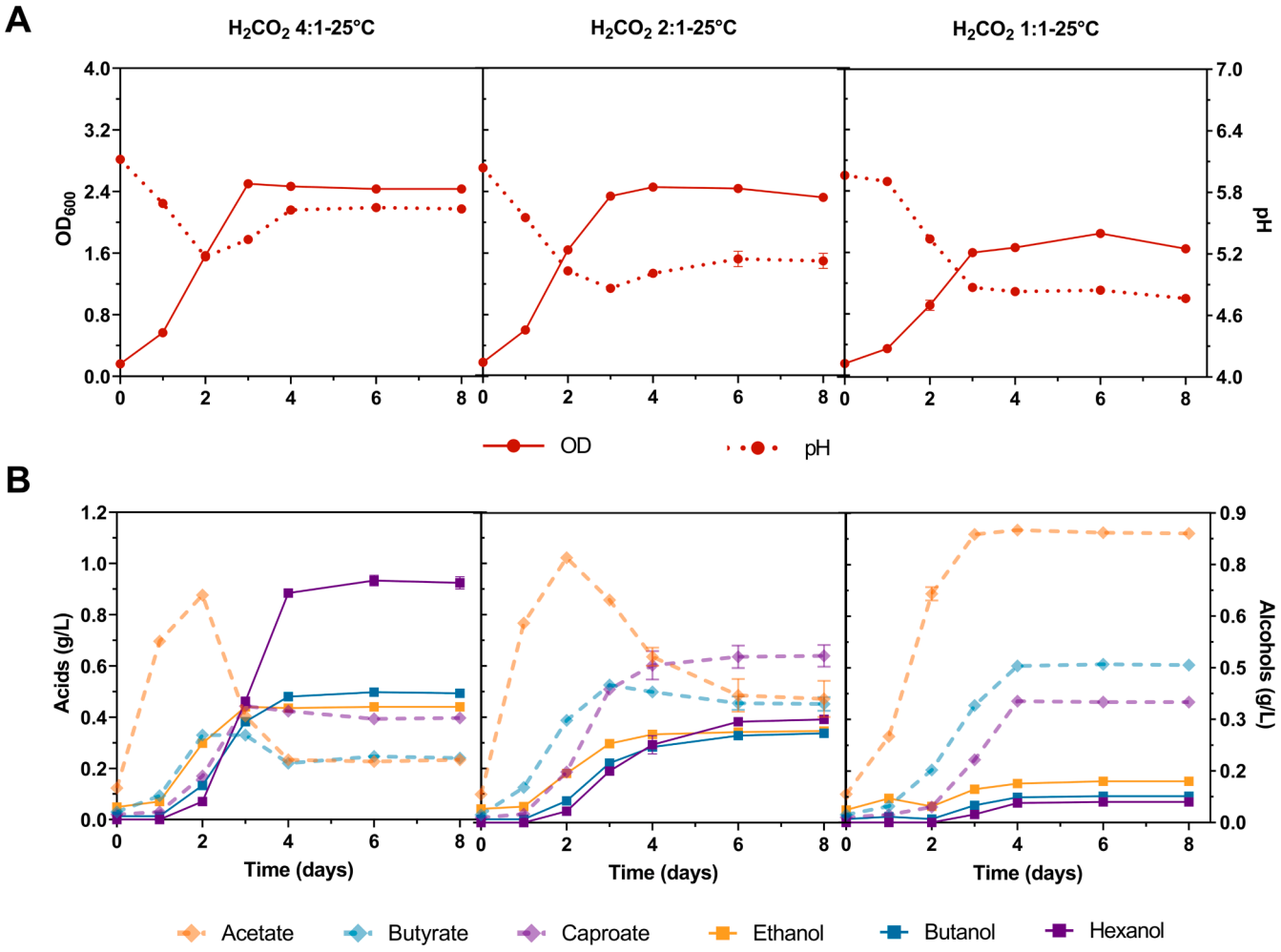


Fig. 3. Growth and product profiles of the adapted strain with different H_2 to CO_2 ratio. (A) Optical density profiles (solid lines), pH profiles (dotted lines) and (B) products profiles. Organic acids are represented with diamond symbols and dotted lines, while alcohols with square symbol and solid lines. Data represent the average ± standard error of the mean (SEM) of triplicate experiments.

acetogens (Zhu et al., 2020; Ricci et al., 2021; Oh et al., 2022). Accordingly, the effects of these parameters on the performance of the adapted strain were evaluated. The main metrics and performances using different cultivation strategies are summarized in supplementary material (see supplementary material-Excel file). To enable a fair comparison of the presented results with the ones of Lakhssassi and co-workers (2020), in the previous section (3.1) the adapted and WT strains were autotrophically cultivated with a H_2 to CO_2 ratio of 4:1 at 25 °C. This condition represents the reference cultivation strategy of next fermentation experiments (Figs. 3 and 4).

3.2.1. *Influence of H_2 to CO_2 ratio*

The theoretical stoichiometry of the catabolic reactions for producing C2-C6 acids and alcohols from CO_2:H_2 varies (Fernández-Naveira et al., 2017a). Alcohol production needs more H_2 than organic acid production. The theoretical ratio between H_2 and CO_2 for the production of C2-C6 alcohols is 3:1, while for the acids varies from 2.1:1 to 2.66:1. Indeed, in previously reported studies, H_2 to CO_2 ratios ranged from 2.35:1 to 4:1 for acetate and ethanol production (Heffernan et al., 2020). The physiology of the adapted strain using "not optimal" H_2 to CO_2 ratios of 2:1 and 1:1 was evaluated, in order to understand the shift in product profile based on this parameter (Fig. 3). The adapted strain, cultivated with a H_2 to CO_2 ratio of 2:1, resulted in a similar growth trend compared to the 4:1 reference cultivation strategy, achieving a similar μ of 0.038 h^{-1} (no statistical difference, μ P value = 0.0989, one-way ANOVA) (see supplementary material-Excel file). However, the product spectrum of the 2:1-condition differed from the reference one, showing higher production of organic acids (1.59 g/L) than alcohols (0.83 g/L) (see supplementary material for statistical analysis). At the end of the fermentation, the primary product was caproate (0.65 g/L), corresponding to 21% of the dissolved carbon (Fig. 5). Hexanol production performances decreased compared to the 4:1-reference condition, with low values of q_{hex}, $yield_{hex}$ and alcohol selectivity (see supplementary material-Excel file). The 1:1-condition behaved differently than the reference one (4:1) in terms of growth, pH and especially product profile (Fig. 3), achieving lower values of maximum biomass concentration and μ, 0.56 g/L and 0.032 h^{-1} respectively. Contrary to the reference cultivation strategy, the pH of 1:1-culture decreased to a value of 4.8 on the third day of fermentation and remained constant until the end. This result is consistent with the production profile: 2.20 g/L of acids and only 0.26 g/L of alcohols were generated (see supplementary material for statistical analysis). No acids re-assimilation and switch from acetogenesis to solventogenesis were observed in the 1:1-condition. Growth and metabolite generation stopped after 3–4 days of fermentation, most probably due to the "acid crash" phenomenon, caused by the fast accumulation of acids in the culture (Wang et al., 2011; Ramió-Pujol et al., 2015). The main product of the fermentation was acetate (1.12 g/L), with negligible amount of hexanol (Fig. 3B). Only 33% of the total supplied CO_2 was dissolved in the liquid phase, with 29% of the dissolved CO_2 used for acetate production, 22% for butyrate and 19% for caproate (Fig. 5). The main difference with the reference condition lies in the mmoles of H_2 dissolved into the liquid broth and available for the strain (see supplementary material- Excel file). The low H_2 availability of 1:1-condition is consistent with the product spectrum obtained. Interestingly, if the goal of fermentation is to produce C2-C6 acids from CO_2:H_2-based mixture, 1:1-condition could represent the best cultivation strategy using the adapted strain.

3.2.2. *Influence of gas mixture type used as substrate*

To figure out which is the best gas mixture for hexanol production

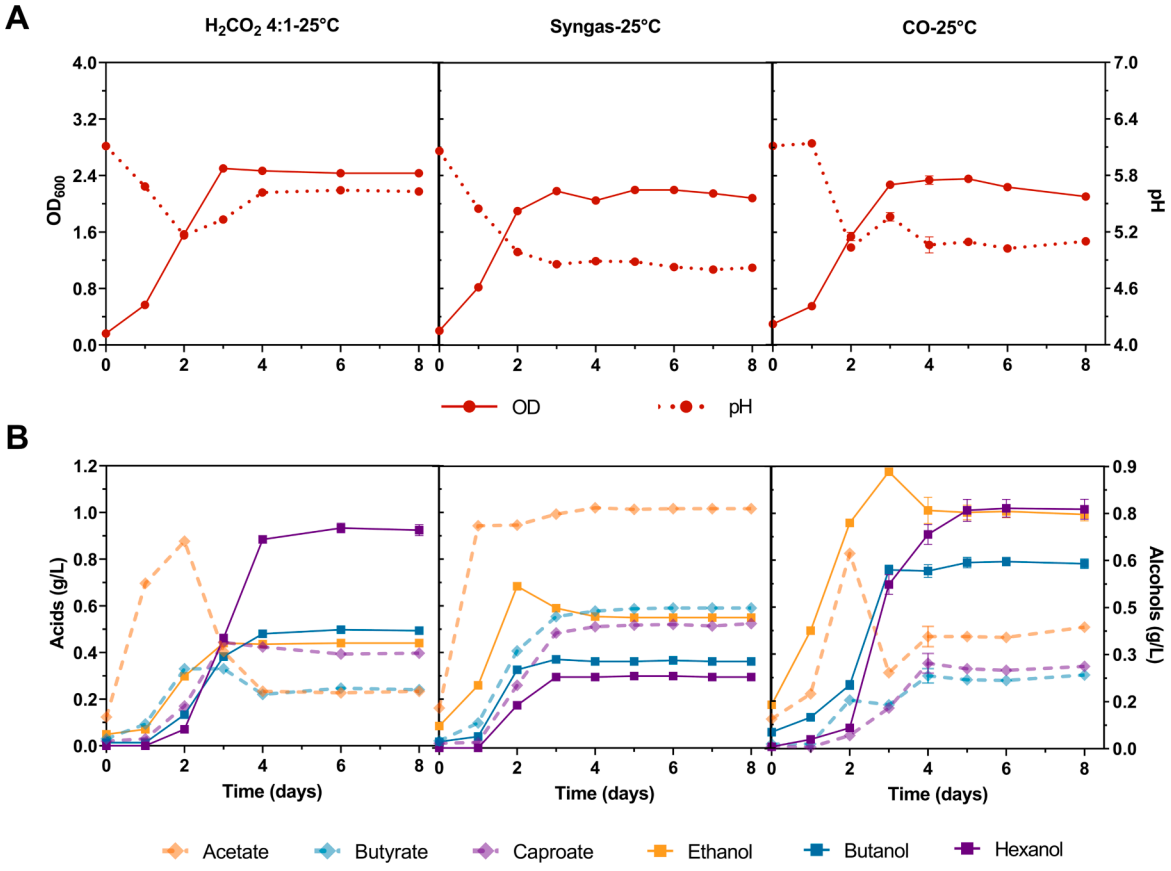
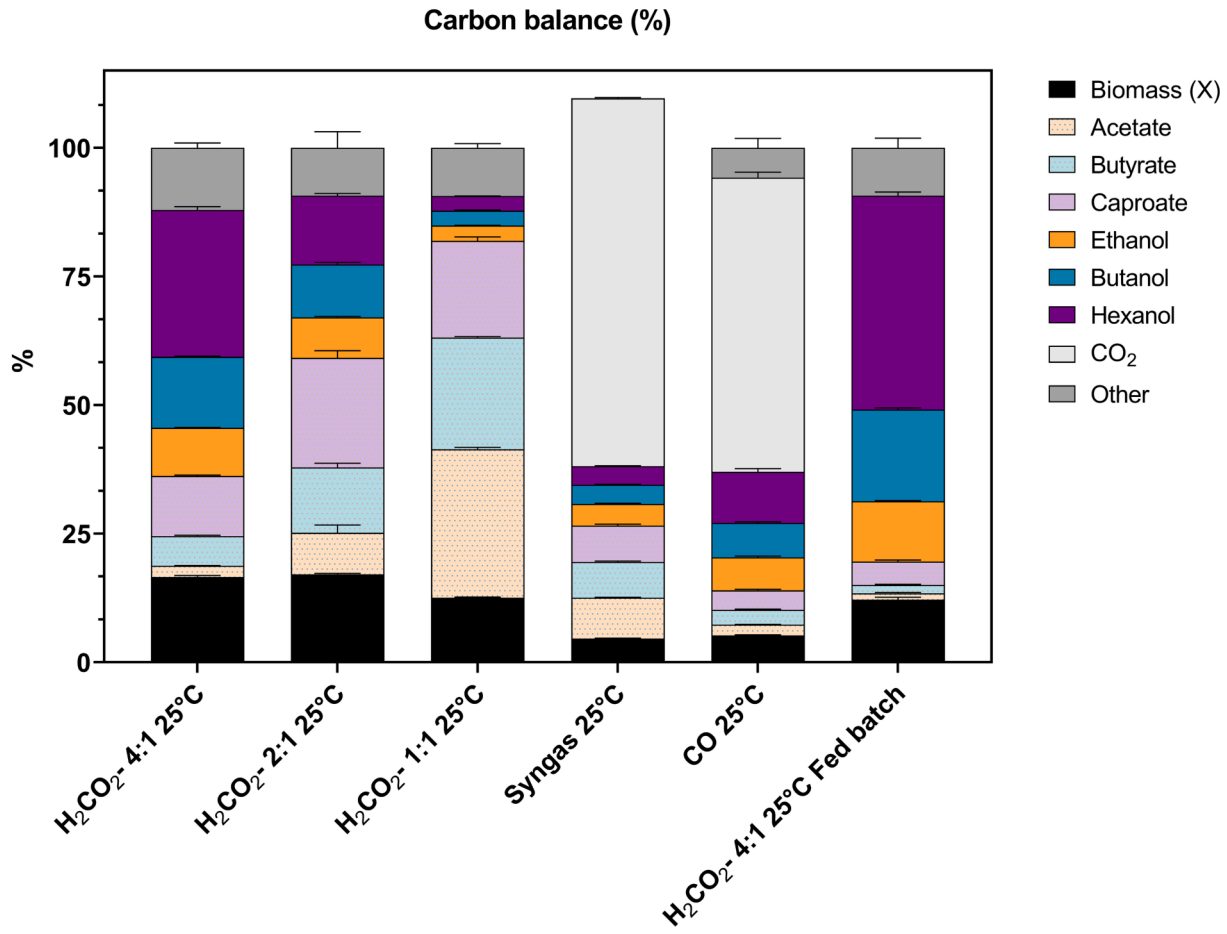


Fig. 4. Growth and product profiles of the adapted strain grown with different gas mixture at 25 °C. (A) Optical density profiles (solid lines), pH profiles (dotted lines) and (B) products profiles. Organic acids are represented with diamond symbols and dotted lines, while alcohols with square symbol and solid lines. Data represent the average ± standard error of the mean (SEM) of triplicate experiments.



	X	Acet	But	Capr	EtOH	ButOH	HexOH	CO_2	Other	Total
4:1 25°C	16	2	6	12	9	14	29	/	12	100
2:1 25°C	17	8	13	21	8	10	14	/	9	100
1:1 25°C	13	29	22	19	3	3	3	/	8	100
Syngas 25°C	5	8	7	7	4	4	4	71	/	110
CO 25°C	5	2	3	4	6	7	10	57	6	100
4:1 Fedbatch	12	1	2	4	12	18	42	/	9	100

Fig. 5. Carbon balance, based on the dissolved carbon, of the adapted strain cultivated in different conditions. Data represent the average of triplicate experiments. The unit of the values in the table is %.

with the adapted strain, its physiology at 25 °C using either syngas or pure CO was characterized. Slightly less biomass and a higher μ were obtained by cultivating the adapted strain on syngas or CO compared to CO_2:H_2 reference condition (Fig. 4A). Concerning hexanol, only the CO-culture showed a significant titer (0.76 g/L), similar to the CO_2:H_2 reference condition (see supplementary material for statistical analysis). The CO-condition achieved higher r_{hex} and q_{hex} (0.029 g_{hex} $g_x^{-1}h^{-1}$ and 0.019 $gL^{-1}h^{-1}$) than the CO_2:H_2 reference one (0.019 g_{hex} $g_x^{-1}h^{-1}$ and 0.013 $gL^{-1}h^{-1}$). Nevertheless, the CO_2:H_2-condition was more selective toward hexanol (hexOH: butOH: etOH = 1: 0.53: 0.47) than the CO-culture (hexOH: butOH: etOH = 1: 0.78:0.99), where a substantial amount of ethanol (0.75 g/L) was also produced. Furthermore, for what concerns the carbon balance, 29% of the dissolved carbon was converted into hexanol in the CO_2:H_2-based fermentation. In contrast, in the CO-based fermentation, only 10% of the dissolved carbon was converted into hexanol, while 57% was lost in the form of CO_2 (Fig. 5). The 68% of

the total CO input was dissolved into the liquid medium during the experiment. Syngas-grown culture generated only 0.23 g/L of hexanol, with low productivity values (see supplementary material-Excel file). 2.3-times more acids than alcohols were produced in this fermentation condition, with acetate being the main product (1 g/L). Acid re-assimilation and switching to the solventogenic phase were not observed. 75% of the total supplied carbon was dissolved into the fermentation broth. Only 3.6% of the dissolved carbon was turned into hexanol, while 71% was lost as CO_2 (Fig. 5). These findings clearly demonstrate that utilizing H_2 as the electron donor for CO_2 valorization is the optimal strategy for developing a carbon-negative gas fermentation process aimed at hexanol production.

3.2.3. *Influence of the cultivation temperature*

Literature studies on *C. carboxidivorans* WT strain, cultivated using syngas, showed an increased hexanol production of 66-times at 25 °C and of 2.6-times at 30 °C, compared to 37 °C (Shen et al., 2017; Oh et al., 2022). Additionally, another work highlighted an hexanol toxicity increment for *C. carboxidivorans* grown at 37 °C (hexanol half-maximal inhibitory concentration -IC50- of 1.2 g/L) compared to 30 °C (hexanol IC50 of 1.8 g/L) (Kottenhahn et al., 2021). Experiments with the adapted strain cultivated at 37 °C, using different gas mixtures, gave similar results showing low hexanol production (see supplementary material). CO_2:H_2-culture showed a distributed product profile, CO-culture generated mainly ethanol (1.7 g/L) and acetate (1.6 g/L), while syngas-culture produced mainly acetate (2.23 g/L). However, all the gas conditions used led to higher μ (μ-CO_2:H_2 = 0.078 h^{-1}, μ-CO = 0.13 h^{-1} and μ-syngas = 0.10 h^{-1}), compared to the 25 °C-conditions.

3.3. *Gas fed-batch fermentation with the adapted strain*

The screening campaign described in section 3.2 confirms that the reference cultivation condition (25 °C and H_2:CO_2 = 4:1) is the optimal fermentation strategy for hexanol production using the adapted strain in batch condition. Hence, the adapted strain performance during a gas fed-batch fermentation using the reference condition was evaluated. As shown in Fig. 6, the hexanol titer at the end of fermentation (8 days) was 2.4-times higher than the titer obtained in the batch control, 1.67 g/L and 0.7 g/L, respectively (P value = 0.0003 (***)). Furthermore, in the gas fed-batch condition, the hexanol selectivity increased compared to the other alcohols (see supplementary material -Excel file). The most critical fermentation metrics for bioprocess development and scale-up are: q_p, r_p and the substrate partitioning into the product. In the gas fed-batch condition, the adapted strain reached a q_{hex} of 0.031 $gL^{-1}h^{-1}$ (0.744 g/L day^{-1}) and converted >40% of the dissolved carbon into hexanol, the highest productivity and carbon partitioning reported so far in literature using *C. carboxidivorans* P7 (Fig. 5). 84% of the total carbon input was dissolved in the liquid during this cultivation condition. Furthermore, compared to the best hexanol production performances reported in literature (Shen et al., 2017; Oh et al., 2022; Oh et al., 2023), the adapted strain grown in CO_2:H_2-based fed-batch condition generated hexanol as the primary fermentation product without the production of CO_2. In the CO-based fed-batch fermentations, described by Oh and colleagues (2022, 2023), >65% of the dissolved carbon was converted into CO_2 and only 25% into hexanol (calculated from the reported paper data). However, they obtained the highest hexanol titer reported so far in literature of 5 g/L, after 23 days of CO-based fermentation with the

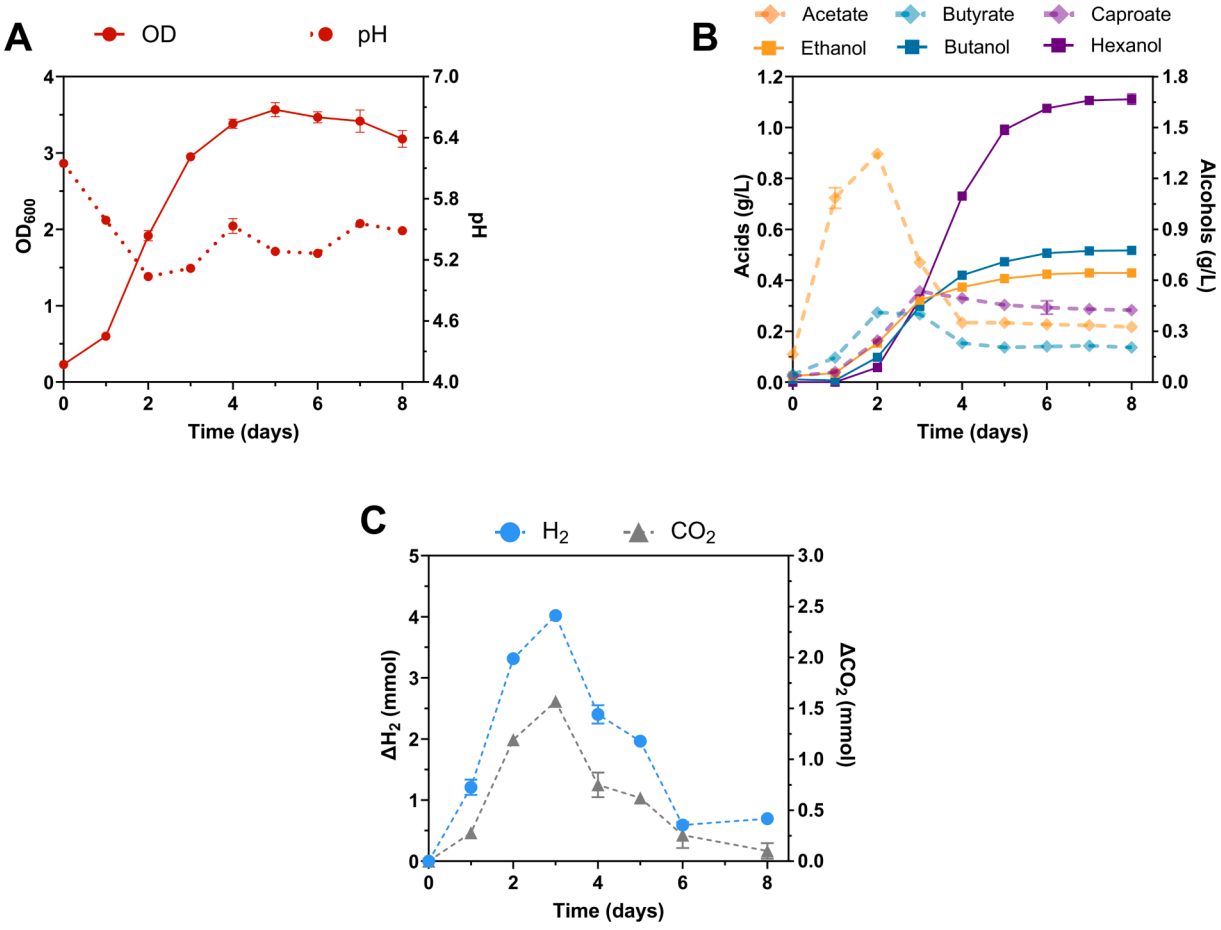


Fig. 6. CO_2:H_2-based fed-batch fermentation with the adapted strain (A) Optical density profiles (solid lines), pH profiles (dotted lines) and (B) products profiles. Organic acids are represented with diamond symbols and dotted lines, while alcohols with square symbol and solid lines. (C) H_2 and CO_2 mmoles dissolved in the liquid phase. Data represent the average ± standard error of the mean (SEM) of triplicate experiments.

use of in-situ hexanol extraction technique. Nevertheless, data on q_{hex} are not available in the mentioned study. Finally, GC–MS analysis of the fed-batch fermentation supernatant showed unexpected results. The product profile of the adapted strain also included traces of: valerate, pentanol, heptanol and octanol.

None of these compounds have been reported in literature as products naturally generated by pure culture acetogens. Octanol synthesis was only described by Richter and co-workers (2016), using a co-culture system based on the chain elongator strain *Clostridium kluyveri* and *C. ljungdahlii*. The truly outstanding finding is the adapted strain production of odd-chain carboxylates and alcohols. As well described in literature, odd-chain organic acids are generated through chain elongation of propionate and valerate with ethanol (Candry et al., 2020; Roghair et al., 2018). The corresponding C5 and C7 alcohols can then be produced through aldheyde ferrodoxin oxidoreductase (AOR)-pathway, as usually occurs for ethanol, butanol and hexanol in acetogens. Although propionate production was not observed in the adapted strain-cultures, Fernández-Naveira and co-workers (2017b) reported its production during *C. carboxidivorans* P7 WT glucose-based fermentation. These results indicate that the adapted strain has apparently the ability to perform not only even-chain elongation but also odd-chain elongation. Further studies will investigate deeply this unexpected finding.

3.4. Whole genome resequencing for genomic characterization of the adapted strain

WGrS was used to investigate the presence of mutations possibly associated with the observed phenotypical behavior of the adapted strain, such as growth rate enhancement on CO_2:H_2 and improved hexanol production performance. Variants identification was performed on 9 samples of *C. carboxidivorans*, as described in Table 1. All new glycerol stocks for adapted strain maintenance were prepared with the third autotrophic transfer culture derived from a previous glycerol stock. Sample A and B were the internal control genomes, since the same glycerol stock was used as inoculum for the ALE experiment. NCBI-published genome of *C. carboxidivorans* P7 WT (RefSeq NZ_CP011803.1) was used as reference for variant analysis. 63/64 mutations in the samples (A and B) of the parental strain (inoculum for the ALE experiment) were identified, probably due to some spontaneous mutations that usually occur during microbial strain maintenance in the laboratory. Moreover, in the adapted strain samples (C to F2) occurred new mutations, that were not present in the NCBI-published reference strain or in the parental strain. Special attention have been focused on the genomic mutations that were detected only in all the re-sequenced genomic samples of the adapted strain (C to F2). Mutations with a mutation frequency of 99–100% and a medium/high mutation impact were considered as "key mutations". As result, three key mutations were identified (Table 2).

Table 1
List and description of the re-sequenced genomic samples of parental and adapted strains.

Sample	Strain	Starting Glycerol stock	Growth condition for DNA extraction
A	parental *	wild-type	heterotrophic
B	parental *	wild-type	autotrophic
C	adapted	8th transfer of ALE experiment	heterotrophic
D1	adapted	3rd autotrophic transfer of sample C	heterotrophic
D2	adapted	3rd autotrophic transfer of sample C	autotrophic
E1	adapted	3rd autotrophic transfer of sample D2	heterotrophic
E2	adapted	3rd autotrophic transfer of sample D2	autotrophic
F1	adapted	3rd autotrophic transfer of sample E2	heterotrophic
F2	adapted	3rd autotrophic transfer of sample E2	autotrophic

* wild-type after one year of laboratory maintenance.

3.4.1. Mutations on hydrogenase genes

Hydrogenases are the main enzymes involved in H_2 oxidation reactions. In the majority of acetogenic *Clostridium* species, grown on CO_2 and H_2 (e.g. "*C. autoethanogenum*", *C. ljungdahlii* and *C. drakei*), the most expressed hydrogenase gene cluster encodes the electron bifurcating hexameric [FeFe]-hydrogenase ($HytABCDE_1E_2$) in functional complex with the formate dehydrogenase (Mock et al., 2015; Zhu et al., 2020; Song et al., 2020). The frameshift mutation 1 is an insertion of two adenine nucleotides in position 167 of the locus tag Ccar_RS16050, encoding for the catalytic subunit HytA of the hexameric hydrogenase of *C.carboxidivorans* P7 (Di Leonardo et al., 2022). This frameshift completely changes the sequence reading frame and generates a stop codon (TAG) at position 238. The translation generates a truncated protein of 80 amino acids (aa), which likely lacks functionality (see supplementary material) and thus could severely influence the functionality of the entire hexameric hydrogenase. This ALE-induced mutation is fascinating when compared with the work of Nagaraju and Köpke (2017), where each of the six hydrogenases of "*C. autoethanogenum*" were independently disrupted via genome editing. Surprisingly, under the CO_2:H_2-fermentation condition, the two mutants lacking two different subunits of $HytABCDE_1E_2$ (ΔCAETHG_2797-Clone1 and ΔCAETHG_2798-Clone1) achieved higher biomass formation and 20-times higher ethanol titer, compared to the WT. The adapted strain developed here achieved a similar performance, in terms of growth and alcohol production (hexanol). Both studies, thus, suggests that $HytABCDE_1E_2$ cannot be considered the unique functional hydrogenase of "*C. autoethanogenum*" and *C. carboxidivorans* during growth on CO_2:H_2. If the hexameric hydrogenase is not functional, other hydrogenases present in acetogenic *Clostridium* species might play a more favorable and/or efficient role during CO_2:H_2– based growth (Di Leonardo et al., 2022). Mutation 2 resides in the locus tag Ccar_RS18600 of *C. carboxidivorans* P7 WT, whose protein product has not been characterized yet. This gene is predicted to encode a putative monomeric group B [FeFe]-hydrogenase by HydDB (Søndergaard et al., 2016). Nevertheless, the recent study of Di Leonardo and co-workers (2022) did not includes this gene among the hydrogenases shortlisted for *C. carboxidivorans*, because it faithfully displays only two of three H cluster binding motifs deemed characteristic of [FeFe]-hydrogenases (Vignais and Billoud, 2007; Poudel et al., 2016). However, in transcriptomic profiles of *C. ljungdahlii* grown on CO_2 and H_2, a putative monomeric group B [FeFe]-hydrogenase (CLJU_c37220) resulted in being the second most highly expressed hydrogenase (Aklujkar et al., 2017) and the hydrogenase most highly expressed with this gas blend compared to CO (Zhu et al., 2020). The identified missense variation of the adapted strain resulted in a change in the aa sequence at position 100, where a proline is replaced by a serine. Proline contains a secondary distinctive amine group, which gives an exceptional conformational rigidity compared to the other aa. Therefore, it can be speculated that this substitution may affect the correct folding of the protein. To better understand the impact of mutation 2 on the protein function, it might be beneficial to express the mutated gene in a heterologous system and perform enzymatic function assays.

3.4.2. Mutation on Rex transcriptional factor

Mutation 3 is a missense point mutation on the *rex* gene with a single nucleotide change (Table 2). The redox-responsive transcriptional regulator Rex is a transcription factor capable of sensing the intracellular NADH/NAD^+ redox balance in bacteria. Physiological evidence, supporting the involvement of Rex in the regulation of fermentative pathways, was observed in different *Clostridium* species typically used in

Table 2
Key mutations present in the re-sequenced genomic samples of the adapted strain (C to F2). Mut. = mutation, AA = amino acid and mod. = moderate.

Mut.n°	Locus tag	NCBI Description	Mut. type	Mut. impact	Mut. freq.	Codon change	AA change
1	Ccar_RS16050	[FeFe] hydrogenase, group A	Frame-shift	high	99–100%*	166_167 dupAA	Gly57fs[#]
2	Ccar_RS18600	4Fe-4S dicluster domain	Missen-se	mod.	99–100%*	298C > T	Pro100Ser
3	Ccar_RS18645	redox-sensing transcriptional repressor Rex	Missen-se	mod.	99–100%*	512 T > C	Val171Ala

*Sample C resulted in a lower mutation frequency of 40–50%. [#]frame-shift.

acetone-butanol-ethanol fermentation (Wietzke and Bahl, 2012; Zhang et al., 2014, Schwarz et al. 2017). Recently, the influence of Rex on acetogenic metabolic pathways was investigated in *C. ljungdahlii* by Liu and co-workers (2022). The generated mutant strain, carrying a deletion in the *rex* gene (CLJU_c37250), showed an up-regulation of ethanol production pathways and a down-regulation of the acetate pathway, compared to the WT. However, the mutation 3 of the adapted strain is a SNP missense variation resulting in the exchange of a valine for an alanine at position 171. Both aa have an aliphatic side chain and are nonpolar. Therefore, it is not easy to predict whether and to what extent the functionality of the transcriptional regulator Rex could potentially be affected by this missense mutation.

3.4.3. Stability of the key mutations obtained via ALE

The presence of the above discussed key mutations is consistent with the different phenotypical behavior of the autotrophically grown adapted strain, compared with the parental strain (Fig. 2). The adapted strain conserved its improved phenotipycal behavior in gas fed-batch growth condition over time (see supplementary material). The re-sequencing of multiple genomic samples coming from sequentially and timely different glycerol stocks of the adapted strain (Table 1) allowed to prove also the conservation of the key mutations. WGrS of both heterotrophic and autotrophic cultures was performed and similar results were obtained (Table 2). Thus, the stability of the key mutations is independent of the carbon source used by the adapted strain. The adapted strain generated and characterized in this work was named as *C. carboxidivorans*_hex21. Metabolic engineering and synthetic biology are well applied in acetogenic *Clostridium* species such as *C. ljungdahlii* and "*C. autoethanogenum*" (Liew et al., 2022). On the contrary, no genome integration toolkit are available for *C. carboxidivorans*. Only Cheng and co-workers (2019) reported a plasmid-based metabolic engineering strategy that resulted in mutants with slight improvements in ethanol production from syngas, compared to *C. carboxidivorans* WT. In the current work, the ALE technique proved to be a successful strategy to improve *C. carboxidivorans* growth and production performances with the CO_2:H_2 blend, leading to conserved mutations on genes encoding critical proteins in hydrogen metabolism and a transcriptional factor. Future work will be devoted to an in-depth comparative study between *C. carboxidivorans*_hex21 and the WT, to investigate the effect of ALE on the whole trascriptome and proteome of the strains. Furthermore, this study highlighted possible new engineering targets. The deletion of the hexameric hydrogenase catalytic subunit in *C. carboxidivorans* WT and in other acetogenic *Clostridium* strains will validate the hypothesis that owning a nonfunctional hexameric hydrogenase leads to improved strain growth and alcohols production performance from CO_2 and H_2.

4. Conclusion

In this study, an adapted strain of *C. carboxidivorans* P7 with improved growth on the CO_2:H_2-based blend compared to the wild type was developed. The adapted strain produced hexanol as the primary fermentation product, with a q_{hex} of 0.744 $gL^{-1}day^{-1}$, which is -to the best of authors' knowledge- the highest value reported in literature. This work expanded the product portfolio of gas fermentation from CO_2 and H_2, since direct hexanol production from this gas mixture was never demonstrated before. The obtained results set a promising groundwork for developing a bio-based carbon negative alternative process for hexanol production.

CRediT authorship contribution statement

G. Antonicelli: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Writing – original draft. **L. Ricci:** Methodology, Formal analysis, Writing – review & editing. **L. Tarraran:** Methodology, Writing – review & editing. **S. Fraterrigo Garofalo:** Methodology, Writing – review & editing. **A. Re:** Formal analysis, Resources, Writing – review & editing. **N. Vasile:** Supervision, Writing – review & editing. **F. Verga:** Supervision, Writing – review & editing. **F. Pirri:** Funding acquisition, Writing – review & editing. **B. Menin:** Methodology, Supervision, Writing – review & editing. **V. Agostino:** Project administration, Conceptualization, Investigation, Methodology, Data curation, Resources, Supervision, Writing – original draft.

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.

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Appendix A. Supplementary data

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