Microbial valorization of C1 waste gases for 2,3-butanediol production: a single and a double stage approach

Summary of the Ph.D. thesis – Luca Ricci

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Since the beginning of the first Industrial Revolution in the 19th century, the massive use of fossil-based resources has significantly increased the emission of Greenhouse Gases (GHG) and has led to the well-known problem of climate change (Royal Society, 2020). Among the harmful gases emitted by the main industrial players (iron, steel and cement industries), carbon dioxide was recognized as the primary contributor to climate change (Zhang et al., 2015).

Therefore, alternative carbon sources and new technologies must be identified and developed to reduce our dependency on fossil fuels and move toward sustainable production processes with a reduced carbon footprint. The utilization of C1 waste gases for the production of fuels and platform chemicals is considered one of the most promising approaches to satisfy these needs. Indeed, C1 waste gases are considered the primary enabler of a carbon-negative economy (Bae et al., 2022). Gas fermentation is one of the latest but more prominent technology for the utilization of C1 waste gases and their conversion into valuable products (Fackler et al., 2021). Indeed, gas fermentation via acetogenic bacteria of the genus *Clostridium* is an attractive technology that enables C1 industrial waste gases capture and conversion into valuable products (short-chain organic acids, alcohols and diols) while contributing to mitigate the current carbon cycle alterations (Fackler et al., 2021). Unlike the sugar fermentation process, gas fermentation does not compete with the food industry and has several technical and economic advantages compared to chemical processes, such as the Fisher-Tropsch Process (De Klerk et al., 2013). However, despite the commercialization of the ethanol production process by LanzaTech, many process parameters and conditions still need to be investigated and optimized to overcome the main limitations of the gas fermentation technology (Sun et al., 2019). Such limitations are mainly represented by the low gas to liquid mass transfer, slow bacterial growth rate, medium cost, low product yield and product recovery (Liew et al., 2013). Consequently, ongoing research to overcome these limitations mainly focuses on optimizing the cultivation techniques, gaseous substrate and growth medium compositions, process parameters and bioreactor configurations (Bae et al., 2022).

Gas fermentation is generally performed via acetogenic bacteria (Fackler et al., 2021), which are a group of phylogenetically and metabolically diverse anaerobic prokaryotes able to

use the Wood-Ljungdahl pathway (WLP) for the synthesis of acetyl-CoA and acetyl-CoA-derived products, from CO_2 and CO (Ragsdale and Pierce, 2008). For their ability to produce organic acids (acetate, lactate, butyrate, caproate), alcohols (ethanol, butanol, hexanol) and diols (acetoin, 2,3-BDO) from C1 gaseous substrates, acetogens have come to the forefront in the development of technological platforms exploitable in a carbon-negative economy (Bengelsdorf et al., 2018).

Among the several acetogenic bacteria, *C. ljungdahlii* and *C. autoethanogenum* are particularly interesting from an industrial point of view for their well-characterized ability to produce value-added products, such as acetate, ethanol, lactate, 2,3-butanediol (2,3-BDO) and acetoin, using C1 waste gaseous substrates (Köpke et al., 2011). Particularly, 2,3-BDO and acetoin are emerging platform chemicals with a wide range of industrially-relevant applications (Maina et al., 2022). Indeed, 2,3-BDO and acetoin are considered platform chemicals as they can be used in the manufacturing of foods, pharmaceuticals, cosmetics, anti-freezing agents, fuel additives, synthetic rubbers, and printing inks (Maina et al., 2022).

Therefore, in this study, gas fermentation technology was investigated mainly for the production of 2,3-butanediol. In particular, the influence of the main parameters known to significantly alter the performance of gas fermentation, such as the cultivation technique, gaseous substrate and growth medium compositions, biocatalyst, bioreactor configuration and process parameters (pH, temperature and pressure), was investigated.

Furthermore, as acetate is the predominant product generated using acetogens-mediated gas fermentation and as many technologies were recently developed to upgrade the "by-product" acetate into more industrially-relevant biochemicals (Ricci et al., 2021; Ricci et al., 2022), an alternative process scheme for further conversion of acetate into the target products, 2,3-butanediol and acetoin, was investigated. One of the main approaches to upgrading the "by-product" acetate, produced from the acetogens-mediated gas fermentation, into more valuable biochemicals is the realization of two-stage integrated bioprocesses (Ricci et al., 2022). Two-stage integrated bioprocesses consist of distinct and interlinked biotechnological processes where the products derived from one process stage are the substrates for the process that takes place in the subsequent stage. This process configuration leads to significant enhancement of the achievable titers, yields and carbon conversion to the target products compared to the gas fermentation process alone (Ricci et al., 2022).

For all the considerations stated above, this study wanted to meet two main aims and their relative sub-aims:

1. To produce 2,3-BDO from the microbial valorization of C1 waste gases using acetogens-mediated gas fermentation technology, by:

1.1) optimizing the cultivation technique
1.2) identifying the most appropriate gaseous substrate
1.3) identifying the most suitable biocatalyst
1.4) optimizing the medium composition
1.5) optimizing the process parameters
1.6) scaling up the process using bench-scale bioreactors by screening different gas flow rates, pH set values and bioreactor configurations

2. To develop a two-stage integrated bioprocess for the upgrading of the "by-product" acetate, generated through gas fermentation, into diols (2,3-butanediol and acetoin), by:

2.1) evaluating acetate toxicity in the model microorganism *Escherichia coli*
2.2) developing diols-producing *E. coli* strains
2.3) optimizing the acetate uptake and utilization pathways of the diols-producing *E. coli* strains through either an upregulation or a downregulation approach
2.4) scaling up the process using bench-scale bioreactors

Since this study wanted to reach two main aims, the Ph.D. dissertation was divided into two main sections of Results and Discussion: the C1 gases to 2,3-BDO, ethanol and acetate (Chapter 3.1) and the acetate to diols sections (Chapter 3.2), as illustrated in Figure 1 of this summary.

Particularly, in the C1 to 2,3-BDO, ethanol and acetate section, acetogens-mediated gas fermentation was investigated as a valuable technology to meet aim 1 and relative sub-aims. On the contrary, an additional aerobic process was investigated in the acetate to diols section to meet aim 2 and relative sub-aims.

Finally, a third section of Results and Discussion (Chapter 3.3), named "Two-stage integrated bioprocess", was created to perform the calculation of the main fermentation parameters of the theoretically integrated two-stage bioprocess and to verify the hypothesis that the proposed integrated bioprocess enhances the achievable product titers, yields and the carbon conversion to the target products, compared to gas fermentation process alone.

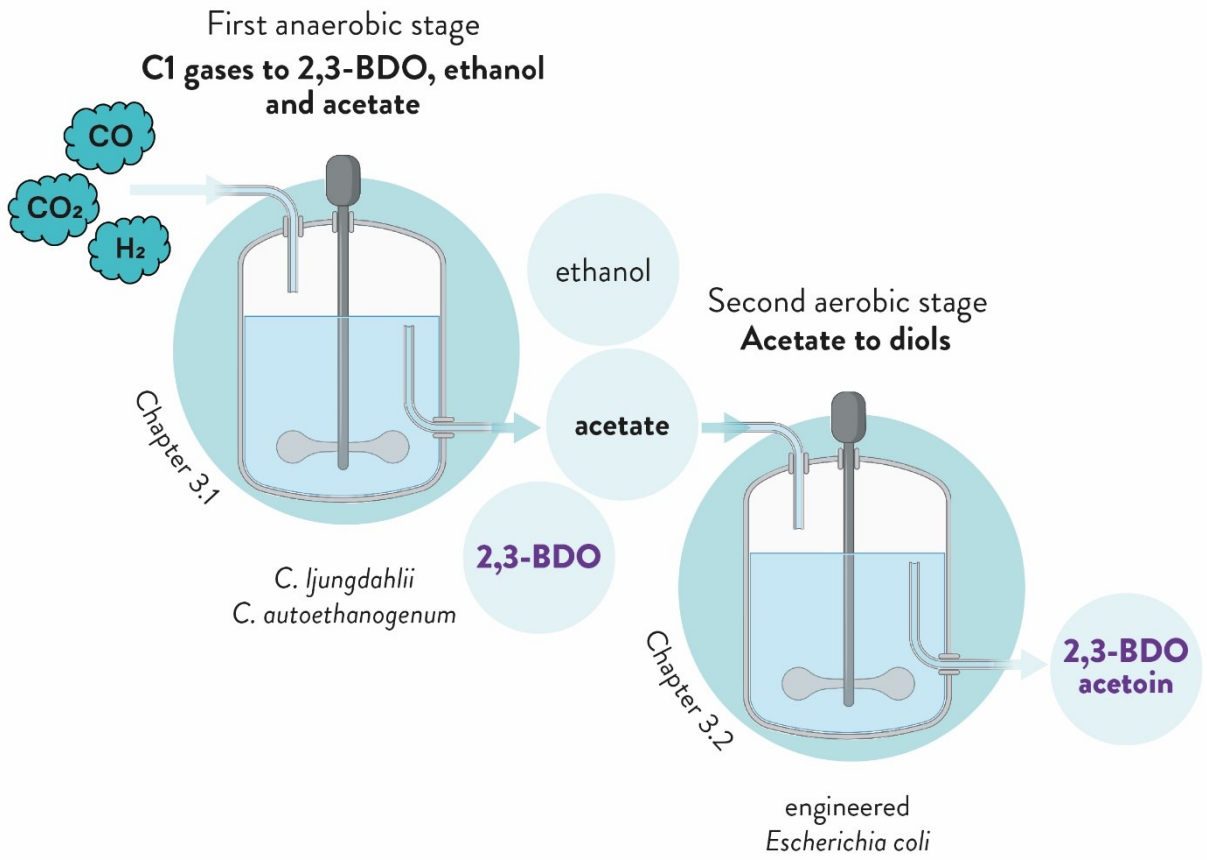

Figure 1: Visual representation of the two-stage integrated bioprocess proposed and of the structure of this study.

To these aims, the gas fermentation technology was deeply investigated to optimize the 2,3-BDO autotrophic production process mediated by acetogenic bacteria. Remarkably, the most appropriate cultivation technique, biocatalyst, gaseous substrate, gas ratio, process parameters and bioreactor configuration were individuated for 2,3-BDO and ethanol production using C1 waste gases as the sole carbon sources. Indeed, a multifaceted serum bottle-based screening campaign involving three different cultivation techniques, six gaseous substrates, eight medium formulations and two strains of acetogenic bacteria was performed to individuate the optimal conditions for the scale-up of the gas fermentation process using bench-scale bioreactors. Furthermore, a second aerobic process stage able to upgrade the "by-product" of the gas fermentation process, acetate, was developed to further valorize acetate into diols. In particular, advanced genetic engineering techniques were used to develop *E. coli* strains able to produce diols using acetate as the carbon source and to increase the activity of *E. coli* acetate uptake and utilization pathways. In addition, the acetate to diols aerobic second process stage was scaled up using bench-scale bioreactors. Finally, a two-stage integrated bioprocess, where C1 waste gases are firstly valorized into 2,3-BDO, ethanol and acetate in a first anaerobic

process stage, and secondly, the produced acetate is further upgraded to diols in a second aerobic process stage, was conceived. The main fermentation parameters of the theoretically integrated two-stage bioprocess were calculated in order to verify the hypothesis that the developed two-stage integrated bioprocess leads to an increase in product titer, yield and carbon conversion efficiency compared to the gas fermentation process alone.

C1 gases to 2,3-BDO, ethanol and acetate

The main aim of the C1 gases to 2,3-BDO, ethanol and acetate section of this study was to produce 2,3-BDO from the microbial valorization of C1 waste gases using acetogens-mediated gas fermentation technology by optimizing the main gas fermentation parameters.

To this aim, the cultivation techniques for the autotrophic growth and 2,3-BDO production of a model acetogenic bacteria were firstly optimized in serum bottle-based gas batch experiments. As a result, adopting a gas to liquid ratio of 9:1 and horizontal orientation of the serum bottles afforded a 2,3-BDO titer of 1.252 ± 0.041 g/L, which is 1.26-fold and 5-fold higher than the titers obtained when *C. ljungdahlii* was cultivated with gas to liquid ratio of 9:1 and vertically incubated serum bottles or with a gas to liquid ratio of 4:1 and vertically incubated serum bottles, respectively. It is plausible that the increased turbulence created by the agitation in horizontal mode and the higher amount of gaseous substrate provided using a gas to liquid ratio of 9:1, compared to a gas to liquid ratio of 4:1, favored 2,3-BDO production since increasingly reduced products, such as 2,3-BDO, particularly benefit from an excess supply of energy (Norman et al., 2019). Therefore, gas to liquid ratio of 9:1 and the horizontal orientation of serum bottles during incubation were selected as the most performing cultivation techniques in terms of 2,3-BDO production and used in the following serum bottle-based gas batch fermentation experiments.

Furthermore, this study generated comparable data on the growth and 2,3-BDO production of several gas batch fermentations using *C. ljungdahlii* and *C. autoethanogenum* grown on different gaseous substrates of primary applicative interest, such as $CO_2 \cdot H_2$, $CO \cdot CO_2$ and syngas (CO, CO_2, H_2 and N_2), and in different media featuring different compositions as regards trace metals, mineral elements, vitamins and amino acids. As a result of the screening of different gaseous substrates and biocatalysts, $CO \cdot CO_2$ and $CO_2 \cdot H_2$ were the most appropriate gas mixtures for 2,3-BDO and ethanol production, respectively, while *C. ljungdahlii* and *C. autoethanogenum* were identified as the most suitable biocatalysts for 2,3-BDO and ethanol production, respectively. These results were confirmed using either the "Tanner_mod" or the "Valgepea_mod" media. In particular, using *C. ljungdahlii* grown on $CO \cdot CO_2$ and "Tanner_mod" medium, 1.267 ± 0.024 g/L of 2,3-BDO were produced, which is 2.8-fold higher than the titer attained when *C. ljungdahlii* was grown on syngas. In contrast, only trace amounts

of 2,3-BDO were detected when $CO_2 \cdot H_2$ was employed. The favorable 2,3-BDO titer obtained by growing *C. ljungdahlii* on $CO \cdot CO_2$ depends on the energetically favorable reduction power of CO, which enhances the ATP yield of 2,3-BDO production, compared to H_2 (Katsyv and Müller, 2020). Moreover, using the gas mixture, consisting of both CO and CO_2, can favor 2,3-BDO production by increasing the levels of both CO_2 and reduced ferredoxin, the reactants of the first reaction for 2,3-BDO production, catalyzed by the PFOR enzyme. As a side result, using *C. autoethanogenum* grown on $CO_2 \cdot H_2$ and "Valgepea_mod" medium, 3.052 ± 0.201 g/L of ethanol were produced, which is 2.4- and 4.9-fold higher than the titers attained when *C. autoethanogenum* was grown on $CO \cdot CO_2$ and syngas, respectively. In addition, by a fair comparison of the biocatalysts, *C. ljungdahlii* produced a 2,3-BDO titer 3.8-fold higher than *C. autoethanogenum* from $CO \cdot CO_2$ while *C. autoethanogenum* produced an ethanol titer 5.8-fold higher than *C. ljungdahlii* from $CO_2 \cdot H_2$.

The main fermentation parameters and different medium compositions were investigated for the $CO \cdot CO_2$- and *C. ljungdahlii*-based 2,3-BDO production process using the key results from the initial experiments. Indeed, to optimize medium composition for enhanced 2,3-BDO production, the influence of six media featuring different concentrations of mineral elements, trace metals, amino acids and vitamins was characterized using $CO \cdot CO_2$ and *C. ljungdahlii*. The screening of different medium compositions reveals that the combination of mineral elements, Zinc and Iron increased concentrations exert a significant positive influence on 2,3-BDO titer and productivity of *C. ljungdahlii* grown on $CO \cdot CO_2$. Indeed, 2.028 ± 0.048 g/L of 2,3-BDO were produced by *C. ljungdahlii* cultivated in a medium where mineral elements, Iron and Zinc concentrations were increased, the "Tan_Val + Fe" medium. Notably, the titer obtained using the "Tan_Val + Fe" medium is 1.60-fold higher than the one obtained by *C. ljungdahlii* grown on $CO \cdot CO_2$ 4:1 and using the initial medium formulation of the "Tanner_mod" medium. This result can be explained by the fact that the butanediol dehydrogenase, which is the enzyme responsible for the conversion of acetoin into 2,3-BDO, is a Zinc-dependent enzyme (Köpke et al., 2011a). Zinc was one of the trace metals whose concentration mostly changed between the "Tanner_mod" and "Tan_Val + Fe" media with a 200-fold increase. Moreover, Iron, which concentration was 3.62-fold higher in the "Tan_Val + Fe" than in the "Tan_Val" media, is an essential cofactor of the Iron-Sulphur proteins, which are related to the Wood-Ljungdahl pathway (Schuchmann and Muller, 2014), and alcohol dehydrogenase enzymes (Scopes, 1983). Moreover, no significant differences were found in the 2,3-BDO titers obtained using media with increased vitamin or specific amino acid, such as methionine, concentrations, indicating that these elements play a minor influence on 2,3-BDO production by *C. ljungdahlii*.

Subsequently, the inhibition of a parallel metabolic pathway of the 2,3-BDO production pathway, which utilizes a 2,3-BDO precursor, acetolactate, for the synthesis of branched-chain

amino acids (Smart et al., 2017b), was tested through 2-HIBA addition using both *C. ljungdahlii* and *C. autoethanogenum*, cultivated in the "Tan_Val" medium and on CO · CO_2 as gaseous substrate. Interestingly, 2-HIBA addition was successfully proven to increase 2,3-BDO titers by 1.16- and 2.25-fold in *C. ljungdahlii* and *C. autoethanogenum*, respectively. 2.064 ± 0.041 g/L of 2,3-BDO were produced by *C. ljungdahlii*, which is the highest 2,3-BDO titer obtained in gas batch fermentation experiments and reported in the literature. The 2,3-BDO titer obtained corresponded to a yield of 0.158 ± 0.003 grams of 2,3-BDO per gram of C1 gaseous substrate. Notably, using the "Tan_Val + HIBA" medium, *C. ljungdahlii* fixed 71.875 ± 3.280 % of the input carbon into products and converted 22.646 ± 0.464 % of the input carbon into 2,3-BDO. Despite higher 2,3-BDO titers and yields were obtained using the "Tan_Val + Fe" and "Tan_Val + HIBA" media, the "Tan_Val" medium was used for the following experiments mainly because 2,3-BDO productivity was higher in the latter medium compared to the other two media tested and also because, nutrient addition, such as Iron and 2-HIBA, reduced specific growth rates and is known to influence the medium formulation costs significantly.

Therefore, the effects of different CO to CO_2 gas ratios were characterized for 2,3-BDO production by *C. ljungdahlii* using the "Tan_Val" medium. In particular, four different CO-based gas mixtures, differing in the CO to CO_2 gas ratios: pure CO, CO · CO_2 4:1, CO · CO_2 1:1 and CO · CO_2 1:4, were tested. The results reveal that CO · CO_2 4:1 is the most performing gaseous substrate for 2,3-BDO production using *C. ljungdahlii* grown in the "Tan_Val" medium and underline the positive influence on 2,3-BDO production of fine-tuned CO_2 addition in CO-based fermentation. Indeed, *C. ljungdahlii* grown on CO · CO_2 4:1 produced 1.781 ± 0.023 g/L of 2,3-BDO, which is 8.05-, 2.22- and 11.5-fold higher than the titers obtained using pure CO, CO · CO_2 1:1 and CO · CO_2 1:4, respectively. These results underline the positive influence of a 20 % CO_2 addition in CO-based fermentation and that further increasing the amount of CO_2 with respect to CO is counterproductive.

Subsequently, relevant process parameters, such as process pressure and temperature, were investigated using the most performing conditions for 2,3-BDO production, *C. ljungdahlii* cultivated in the "Tan_Val" medium and CO · CO_2 4:1 gaseous substrate. An increased process pressure did not lead to any effective advantage. On the contrary, the reduction of process temperature, either from 37° C to 30° C or from 37° C to 25° C, resulted in 2,3-BDO titers similar to the control condition (37° C), indicating the possibility of significantly reducing the process temperature and thus the maintenance costs related to the 2,3-BDO production process investigated in this study.

Parallelly to the CO · CO_2- and *C. ljungdahlii*-based 2,3-BDO production process, the main fermentation parameters and different medium compositions were also investigated for the CO_2 · H_2- and *C. autoethanogenum*-based ethanol production process. By testing four different

media featuring a different concentration of mineral elements, trace metals and vitamins, the crucial impact of Iron in ethanol production was highlighted since an ethanol titer 1.27-fold higher was produced by *C. autoethanogenum* from $CO_2 \cdot H_2$ in a medium with increased Iron concentration ("Valgepea_mod" medium), compared to a medium with reduced Iron concentration ("Valgepea_mod – w/o Fe" medium). Notably, *C. autoethanogenum*, cultivated in the "Valgepea_mod" medium and $CO_2 \cdot H_2$ gaseous substrate, fixed 96.399 ± 7.089 % of the input carbon into products and converted 69.157 ± 4.559 % of the input carbon into ethanol, resulting in a yield of 0.362 ± 0.024 grams of ethanol per gram of CO_2. Furthermore, the evaluation of a reduced process temperature, also for the *C. autoethanogenum*- and $CO_2 \cdot H_2$-based ethanol production process, reveals that comparable ethanol titers could be obtained by reducing the incubation temperature from 37° C to 25° C, resulting in the possibility of lowering the process maintenance costs.

Using the results mentioned above, obtained during serum bottle-based batch experiments, the gas fermentation process for 2,3-BDO production using *C. ljungdahlii* grown on $CO \cdot CO_2$ 4:1 and "Tan_Val" medium was scaled up using bench-scale bioreactors. Interestingly, a continuous gas flow rate of 15 mL/min and a constant pH control of 5.8 led to the production of 2.579 g/L of 2,3-BDO, a titer 2.18-fold higher than the one obtained without pH control and using the same gas flow rate. Moreover, by constantly controlling the process pH, a 5.58-fold higher acetate titer and a 7.13-fold higher ethanol titer were obtained, compared to the titers obtained using the same conditions but without controlling the pH. Hence, three different pH set values were tested in the following bioreactor-based experiment to individuate the optimal one to be kept for enhanced 2,3-BDO production. As a result, a pH set value of 6.0 was found to be optimal for 2,3-BDO and acetate production (2.874 g/L and 18.398 g/L, respectively), while a pH set value of 5.4 was revealed to be the preferable one for ethanol production (15.926 g/L). Subsequently, two other bioreactor configurations, different from the gas continuous and liquid batch configurations, were tested: the gas fed-batch - liquid batch and the gas - liquid continuous configurations. The gas fed-batch - liquid batch configuration was inappropriate for 2,3-BDO autotrophic production as cell growth and product titers were low. On the contrary, the application of a dilution rate of 0.02 h^{-1} in the gas - liquid continuous configuration led to the production of 3.105 g/L of 2,3-BDO, 15.914 g/L of acetate and 6.578 g/L of ethanol from $CO \cdot CO_2$ 4:1. Notably, by continuously adding gaseous substrate, fresh medium and by constantly removing the exhausted medium from the bioreactor's vessel, 2,3-BDO, acetate and ethanol productivities were maintained constants for nine days. These results indicate the importance of developing a continuous culture process for efficiently converting C1 gases into value-added products, such as 2,3-BDO, ethanol and acetate.

Therefore, in the C1 gases to 2,3-BDO, ethanol and acetate section of this study, the most performing cultivation technique, medium formulation, gaseous substrate, biocatalyst and

process parameters for the autotrophic 2,3-BDO production using C1 waste gases as substrate, were successfully individuated. The individuation of the optimal conditions for enhanced 2,3-BDO production translated into a series of attempts to scale up the gas fermentation process using bench-scale bioreactors that ultimately led to the development of a gas and liquid continuous process able to produce 3.105 g/L of 2,3-BDO and more importantly to constantly maintain 2,3-BDO, ethanol and acetate productivities for at least nine days. Hence, this study provides relevant insights for the further optimization and development of an efficient gas fermentation process for the microbial valorization of C1 waste gases, mainly CO and CO_2, into an industrially-relevant product with a wide range of applications, 2,3-butanediol. In particular, this study definitively states that $CO \cdot CO_2$ and *C. ljungdahlii* are the more suitable gaseous substrate and biocatalyst, respectively, for 2,3-BDO production. The results obtained through the testing of different medium compositions highlight the importance of undertaking comprehensive studies on medium composition influence on 2,3-BDO production, as those already performed by Saxena and Tanner (2011) and by Gao and co-workers (2013) for ethanol production using *C. ragsdalei*. The bioreactor-based experiments underline the relevant influence that pH control and bioreactor configuration play on the performance of gas fermentation. Furthermore, some relevant insights regarding the more suitable gaseous substrate, biocatalyst, medium composition and process parameters were also provided for ethanol production using $CO_2 \cdot H_2$-based acetogens-mediated gas fermentation.

Acetate to diols

The main aim of the acetate to diols section of this study was to investigate and develop an additional process stage to take advantage of the acetate produced during gas fermentation experiments and to further upgrade it into valuable products, such as diols (2,3-BDO and acetoin).

To this aim, *Escherichia coli* was selected as the most appropriate biocatalyst to be genetically engineered to upgrade the "by-product" acetate into diols. Acetate toxicity on *E. coli* growth was initially evaluated, and an initial acetate concentration in the range of 4 g/L and 8 g/L was identified as the most suitable for obtaining high biomass concentration, specific growth rate and acetate specific consumption rate without observing any toxic effect.

Subsequently, since *E. coli* is not naturally able to produce diols, four diols-producing *E. coli* strains were initially developed to test two 2,3-BDO-producing gene orders (*budC-budA-budB* and *budB-budA-budC*), two strengths of the plasmid vector (low-copy and high-copy numbers) and two *E. coli* strains (BL21 and W). The four *E. coli* strains initially developed were tested either in glucose (as a positive control to verify correct strains development) or acetate (as the target substrate) during shake flask-based experiments. Notably, *E. coli* W

carrying the high-copy number plasmid and *budB*, *budA*, *budC* genes order (*E. coli* W_pET_*budB-budA-budC*) was the sole strain able to produce a relevant diols titer (0.778 ± 0.012 g/L) using acetate as the sole carbon source. Therefore, this strain, re-named W-BDO, was used to investigate six different cultivation conditions. The investigation of alternative cultivation conditions confirmed that an initial acetate concentration of 5 g/L, 200 rpm, 37° C and an inducer (IPTG) concentration of 0.1 mM are the most suitable cultivation conditions for acetate fermentation using engineered *E. coli*. Notably, by testing an acetate fed-batch configuration in shake flask-based experiments, interesting results indicating that this configuration is suitable to increase diols titer were obtained.

Furthermore, the acetate uptake and utilization pathways of the diols-producing *E. coli* strain were engineered either by an upregulation or a downregulation approach. Consequently, thirteen new *E. coli* strains were developed. Seven strains were developed through the single or combined overexpression of the key genes involved in acetate uptake and utilization, such as *acs*, *ackA-pta*, *aceA*, *glcB*, *maeA* and *maeB*. Moreover, six strains were developed by deleting the genes related to the two repressors of the acetate uptake and utilization pathways, such as *pka* (that downregulates the Acs enzyme) and *iclR* (that downregulates the genes of the glyoxylate shunt). Notably, increased acetate specific consumption rates were obtained for *E. coli* W-BDO-AC (*E. coli* W_pET_*budB-budA-budC* – pCDF_*acs-aceA-glcB-maeA*) cultivated either in acetate batch or fed-batch configurations during shake flask-based experiments, compared to its relative control, *E. coli* W-BDO (*E. coli* W_pET_*budB-budA-budC*). In addition, *E. coli* W-BDO-AC, when cultivated in a fed-batch configuration, produced a higher diols titer than its relative control. Indeed, the optimized *E. coli* W-BDO-AC strain, cultivated in an acetate fed-batch configuration, afforded a diols titer of 1.160 ± 0.008 g/L, 1.36-fold higher than the one obtained using *E. coli* W-BDO cultivated in the same conditions. These results suggest that the overexpression of the genes related to the acetate uptake and utilization pathways, such as *acs*, *aceA*, *glcB* and *maeA,* is a valuable strategy to increase acetate consumption rate but also acetate-derived product titers in engineered *E. coli*. Subsequently, either the *acs* or *ackA-pta* acetate uptake pathways were overexpressed singularly or in combination with either *maeA* or *maeB* enzymes to verify which acetate uptake pathway (*acs* or *ackA-pta*) is the most suitable to be overexpressed to enhance substrate consumption rate and product titers, but also to verify which isoform of the malate to pyruvate-converting enzyme (*maeA* or *maeB*) is more active toward malate conversion into pyruvate, which is the first precursor for diols production. As a result, the overexpression of the *ackA-pta* and *maeA* genes of the acetate uptake and utilization pathways led to a strain, *E. coli* W-BDO-AC4 (*E. coli* W_pET_*budB-budA-budC* – pCDF_*ackA-pta-maeA*) able to produce the highest diols titer obtained in this study during shake flask-based acetate batch experiments (1.048 ± 0.007 g/L of diols). Particularly, the diols titer attained by *E. coli* W-BDO-AC4 is 1.60- and 10.17-fold higher than the ones obtained by its relative controls, *E. coli* W-BDO-AC3 (*E. coli*

W_pET_*budB-budA-budC* – pCDF_*acs-maeA,* 0.656 ± 0.005 g/L of diols) and *E. coli* W-BDO-AC6 (*E. coli* W_pET_*budB-budA-budC* – pCDF_*ackA-pta-maeB*, 0.103 ± 0.012 g/L of diols), respectively. Interestingly, the diols titer afforded by *E. coli* W-BDO-AC4 corresponded to a yield of 0.183 ± 0.002 grams of diols per gram of acetate. Moreover, *E. coli* W-BDO-AC4 was able to convert 33.670 ± 0.207 % of the input carbon into diols. Furthermore, the overexpression of the *ackA-pta* acetate uptake pathway in *E. coli* W-BDO-AC2 (*E. coli* W_pET_*budB-budA-budC* – pCDF_*ackA-pta*) resulted in 1.38-fold higher acetate specific consumption rate (0.420 ± 0.040 g/g*h, grams of acetate consumed per gram of biomass in one hour of fermentation) than the one obtained by overexpressing the *acs* acetate uptake pathway in *E. coli* W-BDO-AC1 (0.304 ± 0.013 g/g*h). Previous studies provided controversial results regarding which of the two acetate consumption pathways (*acs* or *ackA-pta*) is preferable to be overexpressed to increase the acetate consumption rate and product titers of *E. coli* (Xu et al., 2018; Leone et al., 2015; Niu et al., 2018; Jo et al., 2019; Yang et al., 2019; Li et al., 2019; Yang et al., 2020; Huang et al., 2018). On the contrary, the results obtained in this study clearly indicate that the overexpression of *ackA*, *pta* and *maeA* genes is more effective in enhancing both the acetate consumption rate and the diols production from acetate compared to the overexpression of *acs* and *maeB* genes. In addition, compared to their relative controls, increased acetate specific consumption rates were obtained in shake flask-based acetate batch experiments by the double deletion of *iclR* and *pka* in both *E. coli* W-BDO and *E. coli* W-BDO-AC. Particularly, *E. coli* W-BDO-AC_Δ*iclR*+Δ*pka* afforded a higher acetate specific consumption rate (0.398 ± 0.009 g/g*h) than the ones obtained by testing the other strains developed through the downregulation approach. Diols production was also slightly increased by the double deletion of *iclR* and *pka* in *E. coli* W-BDO-AC, compared to its relative control. In contrast, no significant improvements in the diols titer or acetate consumption rate were found for all the other strains developed through the downregulation approach. Although no significant improvements were observed in diols production, these results indicate that the double deletion of the two repressors of the acetate uptake and utilization pathways could be a valuable strategy, which needs further investigation, to enhance the acetate consumption rate.

Subsequently to shake flask acetate fermentation testing, the *E. coli* strains with increased abilities to produce diols and consume acetate, such as *E. coli* W-BDO (*E. coli* W_pET_*budB-budA-budC*), *E. coli* W-BDO-AC (*E. coli* W_pET_*budB-budA-budC* – pCDF_*acs-aceA-glcB-maeA*), and *E. coli* W-BDO-AC_Δ*iclR*+Δ*pka* (*E. coli* W_pET_*budB-budA-budC* – pCDF_*acs-aceA-glcB-maeA* – Δ*iclR* + Δ*pka*), were selected and used in the aerobic acetate fed-batch fermentation scale-up using bench-scale bioreactors. Interestingly, during acetate fed-batch bioreactor-based experiments, *E. coli* W-BDO-AC produced 1.074 g/L of diols, a titer 1.44-fold higher than its relative control, *E. coli* W-BDO, which produced 0.748 g/L of diols when cultivated in the same conditions. More relevantly, the optimized *E. coli* W-BDO-AC strain consumed acetate at a 1.92-fold faster specific rate (0.811 g/g*h) than *E. coli* W-BDO (0.422

g/g*h). These results confirmed that also using a higher system scale, such as bioreactors, the overexpression of the genes of the acetate and utilization pathways, such as *acs*, *aceA*, *glcB* and *maeA* is a valuable strategy to increase acetate consumption rate and acetate-derived product titers in acetate-grown engineered *E. coli*. A second bioreactor-based experiment performed using *E. coli* W-BDO-AC and *E. coli* W-BDO-AC_Δ*iclR*+Δ*pka* partially confirmed the results obtained using the same strains during shake flask-based experiments. Indeed, when tested in acetate fed-batch bioreactor configuration, *E. coli* W-BDO-AC_Δ*iclR*+Δ*pka* consumed acetate at a specific consumption rate 1.57-fold higher than *E. coli* W-BDO-AC. Nevertheless, *E. coli* W-BDO-AC produced 1.522 g/L of diols, while *E. coli* W-BDO-AC_Δ*iclR*+Δ*pka* produced only 0.662 g/L of diols. The highest diols titer gathered in the acetate to diols section of this study, 1.552 g/L, obtained by cultivating *E. coli* W-BDO-AC in acetate fed-batch configuration during bioreactor-based experiments, is the highest diols titer obtained using acetate-grown engineered *E. coli*, if compared to literature records.

In the acetate to diols section of this study, the feasibility of upgrading acetate, considered a "by-product" of acetogens-mediated gas fermentation, into diols, was proven. Innovative genetic engineering approaches were used to develop seventeen *E. coli* strains able to produce 2,3-BDO and acetoin from acetate and to increase the activity of the acetate uptake and utilization pathways of the diols-producing strains. The different genetic engineering approaches used resulted in a strain with a significantly improved ability to consume acetate and able to produce 1.552 g/L of diols, using acetate as the sole carbon source. Therefore, the results confirm that the realization of a second aerobic process stage placed downstream of the first anaerobic gas fermentation process is a valuable strategy deserving further investigation to develop efficient bioprocesses for the complete valorization of C1 waste gases and their conversion into industrially-relevant products. Moreover, since a previously optimized chemically defined medium (Novak et al., 2020; Noh et al., 2018) was utilized in this study, the results obtained underline the effectiveness of genetic engineering approaches to efficiently increase the consumption rate of toxic substrates, such as acetate, and consequently the generation of the target products. In particular, the results obtained clearly indicate for the first time that the overexpression of *ackA*, *pta* and *maeA* genes in *E. coli* is more effective in enhancing both the acetate consumption rate and diols production from acetate compared to the overexpression of *acs* and *maeB* genes.

Two-stage integrated bioprocess

Using the results from the two main sections of this study, we conceived an integrated two-stage bioprocess, where C1 waste gases are firstly valorized into 2,3-BDO, ethanol and acetate in a first anaerobic stage, and secondly, the produced acetate is further upgraded to 2,3-BDO and acetoin in a second aerobic stage (Figure 1).

As already mentioned, the integrated two-stage process was not tested experimentally in this study, but the two process stages were investigated separately. The integration of the two processes needs the development of an appropriate bioreactor system, as previously proposed by Hu and co-workers (2016). Moreover, in previous works where two-stage integrated bioprocesses were investigated (Hu et al., 2016; Rowaihi et al., 2018; Cestellos-Blanco et al., 2018), there was no need to separate the acetate produced in the first process stage, before being fed into the second process stage. Considering that *E. coli* needs specific amino acids for growing in an acetate-based medium, we can speculate that in the two-stage bioprocess conceived in this study, the acetate-containing fermentation broth, coming from the *C. ljungdahlii*–mediated C1 gas fermentation, could directly be used as the aqueous phase for the formulation of the medium needed by *E. coli* for growth and diols production. Specifically, the exhausted medium from the gas fermentation stage would need only the addition of essential amino acids, such as asparagine and methionine, and a pH adjustment from 4.8 to 7.0 before being employed as the acetate-based medium for the second aerobic stage.

Therefore, the two most performing batch processes, either for the gas fermentation or the aerobic acetate fermentation stages, were used as model examples of the two separated process stages and theoretically integrated into a two-stage bioprocess. In particular, the data from the processes that led to the highest diols yield were used to calculate the fermentation parameters of the theoretically integrated two-stage bioprocess. CO and CO_2 fermentation by *C. ljungdahlii* cultivated in the "Tan_Val + HIBA" (Chapter 3.1.5) was the most performing gas batch fermentation, affording yields of 0.158 grams of 2,3-BDO and 0.383 grams of acetate per gram of C1 gases. At the same time, the one carried out by *E. coli* W-BDO-AC4 was the most performing acetate batch fermentation (Chapter 3.2.4.2), resulting in a yield of 0.183 grams of diols per gram of acetate.

Considering the product yields of the two separated processes, the theoretically integrated bioprocess resulted in a yield of 0.228 grams of diols per gram of C1, which is 1.44-fold higher than the diols yield obtained during the first anaerobic stage alone. Although the total carbon conversion of the two-stage theoretically integrated bioprocess was lower than the one of the first anaerobic stage alone, due to carbon loss in the form of CO_2, a 1.61-fold higher carbon conversion to diols was obtained in the theoretically integrated two-stage bioprocess (36.474 %), compared to the first anaerobic stage alone (22.646 %). Furthermore, a 1.5-fold higher diols titer could be obtained by integrating the two process stages into a two-stage integrated bioprocess (Table 41). Therefore, through this calculation, we confirm that the realization of the two-stage integrated bioprocess would substantially increase product titer, yield and carbon conversion efficiency compared to the one-stage process alone.

This study clearly shows the feasibility of C1 waste gases fixation and diols production both via acetogens-mediated gas fermentation and via acetate in a two-stage integrated bioprocess. In particular, the knowledge acquired in this study could be used as a platform for C1 waste gases fixation and conversion into different industrially-relevant products. In the gas fermentation stage, other gaseous substrates and gas-fermenting microorganisms could also be investigated. Similarly, the generation of target products, different from diols, could be explored in the second aerobic acetate fermentation stage. In conclusion, biological production schemes are not yet wholly competitive with the traditional chemical production schemes that use fossil resources. Still, we believe that the industrial feasibility of the gas fermentation technology and two-stage integrated bioprocesses investigated in this study could be widely reached in the following decades.