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Microbial CO_2 conversion to value-added products

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Declaration

I hereby declare that the contents and organization of this dissertation constitute my own original work and does not compromise in any way the rights of third parties, including those relating to the security of personal data.

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Abstract

Global warming due to the increase of atmospheric CO_2 concentration is the driving force for the development of strategies that aim to exploit CO_2 as raw material to produce interesting compounds for industry. According to the Carbon Capture and Utilization (CCU) concept, the development of new microbial factories, that exploit the CO_2 derived from industrial anaerobic digestion platforms to produce value-added chemicals, can contribute to mitigate climate change.

Acetone is a raw material used in the chemical industry both as a solvent and as a precursor for the synthesis of deeply used polymers and its worldwide demand is increasing. Acetogenic bacteria can produce acetate using CO_2 and/or CO as carbon source with H_2 as energy source. Among them, *Acetobaterium woodii* can grow both heterotrophically on organic compounds and autotrophically on CO_2 and H_2 to naturally produce acetate. By a metabolic engineering approach, *A.woodii* was modified with the genes coding for the enzymes of the acetone pathway derived from *Clostridium acetobulylicum*, to make it able to produce acetone.

In the autotrophic condition, microbial fixation depends on the mass transfer of the gaseous substrates – which is particularly low for H_2 - to the cells suspended in the liquid phase. By increasing substrates availability, performing fermentation processes at high pressure, increased compounds' productivities can be achieved.

The aim of this work is to investigate, at a laboratory scale, different autotrophic process conditions in pressurized bioreactors for acetone production, by employing an *A.woodii* modified strain.

In order to perform fermentations at high pressure, modified *A.woodii* resistance up to 11 bar was first assessed. The bacterium was grown in heterotrophic condition and different pressurization-depressurization protocols were tested. Results obtained confirmed the ability of *A.woodii* to survive and grow up to 11 bars.

Important operative parameters, such as inoculum strategy, optimal pH range, and definition of the feeding gas mix were tackled to optimize the pivotal pre-seeding step for autotrophic fermentations at high pressure. A gas mix composed

of 70% H_2 and 30% CO_2, pH control at 7, and inoculum in the reactor at OD_{600} > 0.2 using heterotrophic cultures of the bacterium in exponential phase were the preliminary conditions chosen.

A.woodii was then grown in an autotrophic gas and liquid batch fermentation at 10 bar. 1 L of culture was grown in a 2 L stirred reactor. The pressure was increased by providing the H_2:CO_2 mix. Results showed that the biocatalyst grew and carried on its main autotrophic metabolism at high pressure in this operating condition.

Next, tests in liquid batch and continuous gas supply were set up. First, a reference experiment at atmospheric pressure and then, fermentations at 10 bar, using different combinations of biomass and in-flow gas rates, were performed. Results showed the ability of *A.woodii* to survive and fix carbon. Nevertheless, in this operating condition, besides acetate and acetone, the carbon flow was redirected towards the production of formic acid while biomass production is avoided.

Interestingly, if, before inoculation, the fresh medium is pressurized with the gas feeding mix, biocatalyst behavior is similar to what was recorded when it was grown at high pressure. According to this result, it was suggested that this phenomenon could depend on an increased concentration of HCO_3^- in the medium. Tests were performed to verify this hypothesis, both at atmospheric and at high pressure, by testing different carbon dioxide partial pressures and maintaining constant the partial pressure of the hydrogen and the total pressure of the process. Results demonstrated that formic acid synthesis is fostered by higher CO_2 partial pressure.

Moreover, independently of the operating condition applied, experiments in the reactor and in serum bottles suggested an influence of acetate concentration in the medium on acetone production by modified *A.woodii* strain. At a concentration of acetate higher than 100-120 mM, a higher part of the carbon flow is directed towards acetone production.

An experiment in gas and liquid continuous allowed investigating the behavior of the biocatalyst in a range of pressure between 1.7 bar and 10 bar. At low pressures, two different in-flow gas rates were screened. Results confirmed that, at the highest pressure tested, bacteria growth was impaired and formic acid production was fostered, while the highest acetate and acetone productivities were recorded at 2.25 bar applying 20 ml/min of in-flow gas mix.

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Chapter 1

Introduction

1.1 CO_2-based chemical production

The natural carbon cycle is getting unbalanced by anthropogenic activities, which dig hydrocarbons to obtain energy and dump the carbon into the air in the form of greenhouse gases. Most of the carbon occurs in the form of carbon dioxide (CO_2). The five-year period 2015-2019 had an accelerating increase of carbon dioxide in the atmosphere, with a growth rate 18% higher than the former five-year period (2011-2015) (World Meteorological Organization, 2019). The concept of Carbon Capture and Storage (CCS) has a significant role in reducing the CO_2 concentration in the atmosphere. Currently, chemical and fuel production is based on a small number of large-scale facilities that usually rely on fossil raw material or natural gas (Clomburg et al., 2017). This model of production required high-volume feedstocks, or suitable infrastructure for their transport, and contributes to a considerable extent to anthropogenic CO_2 emissions. According to this model, to face climate change, the CCS approach proposes separating the CO_2 from the exhaust gas of the plans and storing it underground (Bruhn et al., 2016).

However, recently the concept of Carbon Capture and Utilization (CCU) attracts growing attention. This concept is based on recycling the CO_2 released in the air and re-using it in place of hydrocarbons in industrial processes. An innovative model of industrial production is related to this concept. The CO_2 used as feedstock can be provided by many plants operating at a small scale rather than to a smaller number of large-scale plants. The shift to smaller-scale facilities has

several advantages such as low capital infrastructure expenditure, more stakeholders involved, less dependence on high-volume resources, less dependence on the global market condition, and utilization of distributed and sustainable resources (Clomburg et al., 2017). Differently from CCS, most CCU technologies do not allow long-term CO_2 storage. Nevertheless, it can contribute to mitigating climate change. Depending on the final product in which carbon dioxide is embedded, storing time can range from the day (fuel) to centuries (cement). Moreover, it has to be noted that CCU also contributes to avoiding side effects (eutrophication, particulate matter formation, ozone depletion) and environmental risks related to the intensive exploitation and processing of fossil resources (Von Der Assen & Bardow, 2014). To reach a distributed manufacturing model to upgrade CO_2, bringing down the costs thanks to the cumulative expertise in processes and production methods (Dahlgren et al., 2013), integration to the plants already producing CO_2, as well as the exploitation of waste heat or renewable energy sources, are mandatory.

Knowledge about microbial CO_2 fixation and synthetic pathways carried on in CO_2-fixing microorganisms is increasing. Besides metabolic pathways in wild-type strains, advances in genetic tools allow developing strains able to overexpress CO_2-fixation enzymes or express heterologous enzymes for new anabolic pathways. Wild-type or genetically modified strains can be used as microbial factories: entities to be employed to produce natural products or new products of industrial interest. The utilization of such biological catalysts for CO_2 conversion can avoid the use of not renewable and more expensive inorganic catalysts and allow the production of compounds through processes working at milder operating conditions, saving energy consumption (Salehizadeh et al., 2020).

1.2 The ENGICOIN project

The ENGICOIN project is a project funded by the European Union under the Horizon 2020 Research and Innovation Program (grant Agreement 7606994) that aims to apply the approach of the small-scale facility. Different microbial factories are integrated into an industrial anaerobic digestion plant. The CO_2 present in the biogas, derived from the treatment of the organic fraction of the municipal waste, is converted to added-value products by three microorganisms. Light or hydrogen from renewable sources are used as the reducing agents, according to MF physiology (https://engicoin.eu/, 2018).

The three microbial factories are (Fig.1):

MF1) The cyanobacterium *Synechocystis* for the conversion of the CO_2 stream in lactic acid, using light as energy source.

MF2) The aerobic bacterium *Ralstonia eutropha* for the conversion of the CO_2 stream in polyhydroxyalkanoates (PHA), using H_2 as reducing agent.

MF3) The anaerobic bacterium *Acetobacterium woodii* for the conversion of the CO_2 stream in acetone, using H_2 as reducing agent.

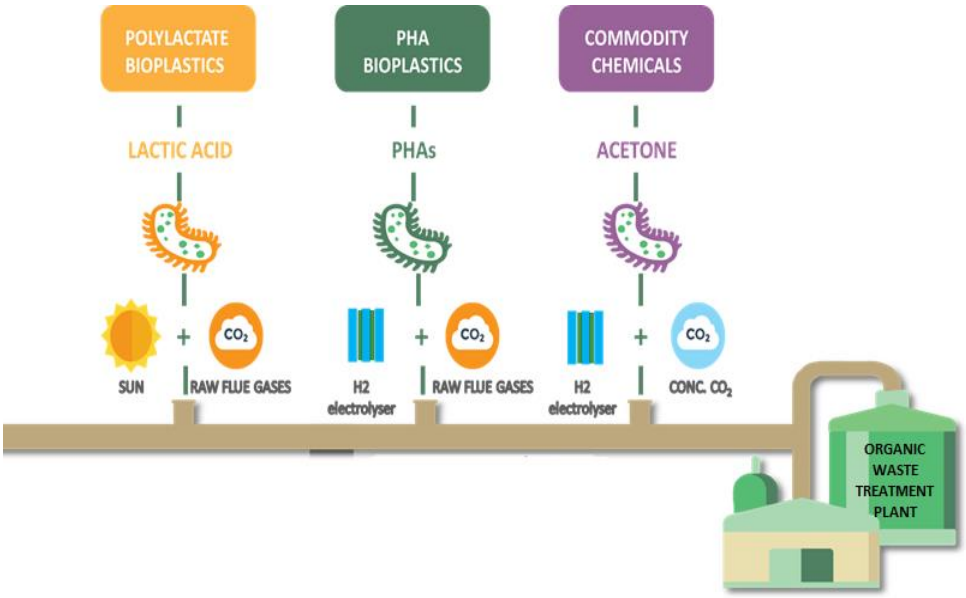


Figure 1. Schematic representation of the ENGICOIN Project (modified from https://engicoin.eu)

Thanks to the equipment and the expertise developed, the Istituto Italiano di Tecnologia (IIT) and the Politecnico di Torino are experimentally involved in the ENGIOCOIN project working on the MF3. Thus, this thesis is focused on the production of acetone from CO_2 and H_2 through *A.woodii* strains.

1.3 Synthesis of acetone

Acetone is the simplest ketone. It is colorless, liquid at ambient temperature, with a pungent smell. A 20 °C it is miscible with water and soluble in organic solvents.

The world demand for this compound is increasing. In 2020 the global market of acetone was 6.9 Million Tons and has been estimated that it will reach 7.3 Million

Tons by 2027 (https://www.researchandmarkets.com/reports, 2022). It is widely used as a solvent for resins, paints, and inks. Moreover, it is an intermediate for the synthesis of methacrylate and bisphenol-A, precursors of polymethyl methacrylate (PMMA) and polycarbonate plastics, respectively.

Nowadays, 95% of the acetone is produced by the cumene route and is based on fossil resources (Weber et al., 2014). This process is divided into two steps (Fig. 2). In the first step, cumene is synthesized from benzene and propene in an alkylation reaction. In the second step, cumene is oxidized to cumene hydroperoxide (CHP) which is then cleaved to phenol and acetone using acids (Schmidt, 2005; Zakoshansky, 2007).

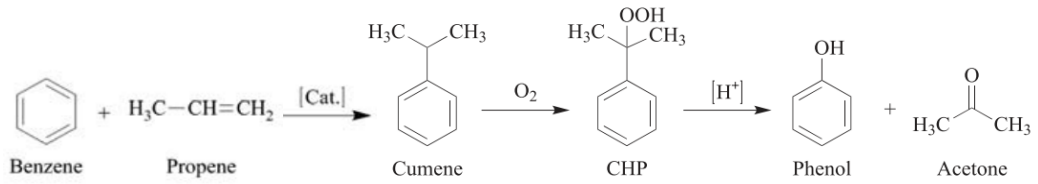


Figure 2. Synthesis of acetone via cumene route (modified from Weber et al., 2014).

Other ways for acetone production using inorganic catalysts are based on dehydrogenation of 2-propanol (Neamah, 2017; Said et al., 2016), and ethanol (Rodrigues et al., 2017).

Acetone synthesis utilizing biological catalysts is based on solventogenic bacteria belonging to the *Clostridium* genus (Jones & Woods, 1986). Among them, *C. acetobutylicum* is the model strain for studying acetone-butanol-ethanol (ABE) fermentation. Genes and enzymes involved in the pathway for acetone synthesis are identified and characterized (Schiel-Bengelsdorf et al., 2013). ABE fermentation can start from both organic substrates (sugars) and gaseous substrates (H_2, CO_2, and CO). Acetone, butanol and ethanol are synthesized in a 3:6:1 molar ratio. The fermentation process is divided into two phases. First, the acetogenic phase takes place. Exponentially growing cells produce acetate and butyrate. Then, in the following solventogenic phase, stationary cells take up acids and convert them into more reduced compounds (acetone, butanol, ethanol) (Li et al., 2020).

A schematic representation of the enzymes and intermediates involved in acetone synthesis is reported in Figure 3. Catabolism of sugars and C1-reduction pathway

converge in the Acetyl-CoA synthesis. The Thiolase (ThlA) catalyzes the condensation of two molecules of Acetyl-CoA in Acetoacetyl-CoA (AA-CoA) (Wiesenborn et al., 1988). The CoA transferase (CtfA/CtfB) transfers the CoA moiety from AA-CoA to acetate (Wiesenborn et al., 1989). The resulting acetoacetate is then decarboxylated to acetone by the Acetoacetate Decarboxylase (Adc) (Gerischer & Durre, 1990).

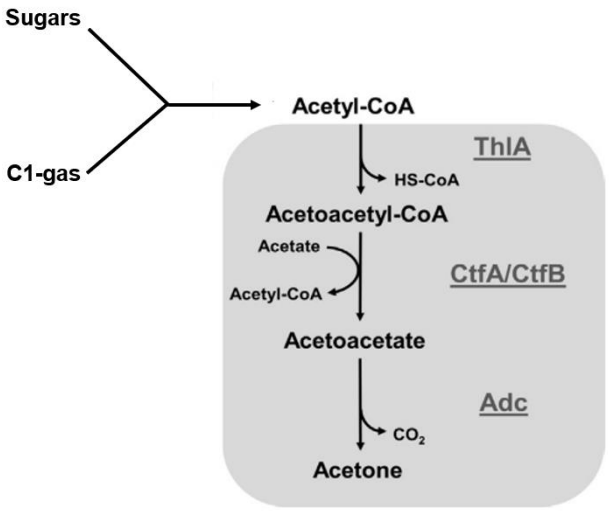


Figure 3. The metabolic pathway for acetone synthesis in *C. acetobutylicum* (modified from Hoffmeister et al., 2016)

The production of acetone and butanol using solventogenic bacteria was one of the first industrial fermentation processes developed, second only to ethanol in importance (Jones & Woods, 1986). Nevertheless, inhibition of biocatalyst growth due to solvents production and low yields make necessary continuous removal of products from bioreactors and complex downstream systems to separate and recover them from the fermentation broth (Karimi et al., 2015; Kujawska et al., 2015).

1.4 CO_2 conversion through *Acetobacterium woodii*

Acetogens are a group of bacteria belonging to different genera and widespread in nature that can reduce CO or CO_2 to Acetyl-CoA through the Wood-Ljungdalh pathway (WLP) (Drake et al., 2008; Schiel-Bengelsdorf & Dürre, 2012). They can be used to convert CO_2-rich waste gas streams into C1- C4 fuels or chemicals, like formate, acetate, ethanol, acetone, butanol (Bengelsdorf et al., 2018; Dürre, 2017; F. M. Liew et al., 2016; Müller, 2019; Takors et al., 2018). Compared to

other catalytic processes for CO_2 valorization, acetogens present several advantages due to their high metabolic efficiency, ability to handle variable gas compositions, high product specificity, and low susceptibility to poisoning. The main disadvantage consists of potential energy starvation in autotrophic conditions (Bertsch & Müller, 2015; Katsyv & Müller, 2020). In the case of chemolithoautotrophy, CO_2 fixation requires a reducing inorganic agent and acetogens use H_2 as electron donor (Heffernan et al., 2020). The attractiveness of CO_2/H_2 fermentation goes hand in hand with the competitiveness of the H_2 production technologies based on renewable energy sources. Even if complex technical hurdles have to be overcome before the hydrogen industry is truly sustainable, both environmentally and economically, the transition to low-CO_2 hydrogen production continues to accelerate by several means including different renewable production techniques (Ishaq & Dincer, 2020).

Among acetogens, *Acetobacterium woodii* (*A. woodii*) grows very robust and performs well compared to other acetogens on H_2/CO_2 (Groher & Weuster-Botz, 2016; Heffernan et al., 2020). Thanks to the cumulative knowledge about its metabolic and energetic pathways, it is a model organism to clarify the metabolism of this heterogeneous group of bacteria (Bengelsdorf et al., 2018). Moreover, techniques for its genetic manipulation are available (Hoffmeister et al., 2016; Straub et al., 2014). The suitability for the fermentation feedstocks, the information about its physiology with respect to other acetogens, and the molecular biology tools available for its modification made *A.woodii* the selected strain for the MF3 development.

A. woodii is a Gram-positive, strictly anaerobic bacterium, first described by Balch and coworkers in 1997 (Balch et al., 1977). It is a non-spore-forming, oval-rod shape bacterium, that often occurs in pairs, motile thanks to the presence of one or two flagella (Fig. 4). It grows heterotrophically on organic substrates (glucose, fructose, lactate, formate, glycerate, ethanol) and autotrophically, using CO_2 as carbon source and H_2 as reducing agent (Balch et al., 1977; Braun & Gottschalk, 1981; Mayer et al., 1977; Schwarz et al., 2020; Westphal et al., 2018).

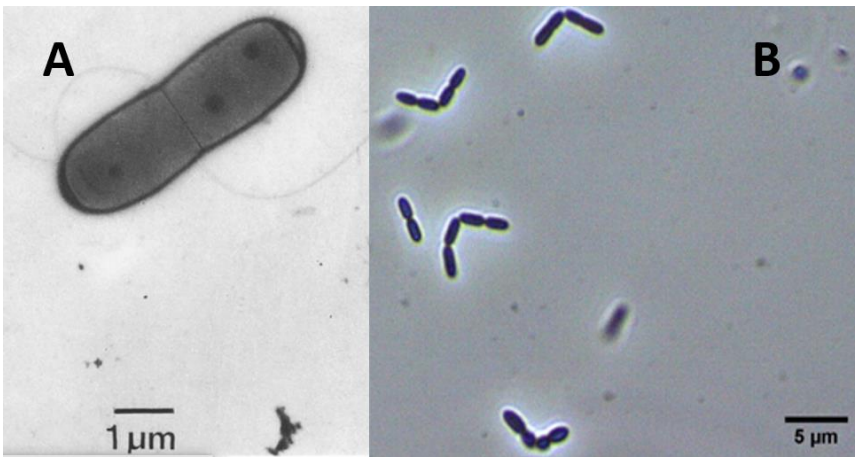


Figure 4. Cells of *Acetobacterium woodii*. (A) Electron microscopy (from Mayer et al., 1977); (B) Contrast phase, 100 X magnitude.

As mentioned before, this bacterium is one of the most studied acetogen, both at the genetical and physiological levels (Poehlein et al., 2012). It was a model to explore the WLP, which is the main metabolic pathway carried on in the autotrophic condition. The pathway consists of a methyl and a carbonyl branches, that converge in the synthesis of Acetyl-CoA, which is the pivotal intermediate for the anabolic pathways (Fig. 5).

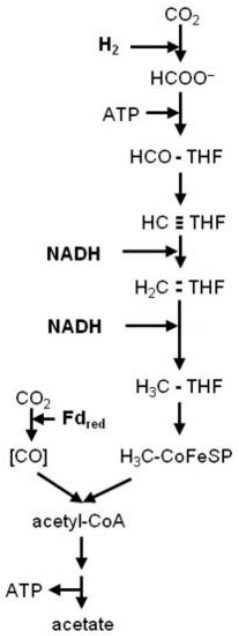


Figure 5. Wood-Ljungdahl pathway in *A.woodii* (modified from Katsyv & Müller, 2020).

In the methyl branch, one molecule of CO_2 is reduced to formate by a hydrogen-dependent CO_2 reductase (HDCR) (Schuchmann & Müller, 2013). HDCR has a formate dehydrogenase domain and a hydrogenase domain, connected by two smaller electron transfer proteins, containing Fe-S centers (Fig. 6). This enzyme directly reduces CO_2 using H_2 as electron donor, without any other cofactor involved (Müller, 2019). Formate is then bound to the cofactor tetrahydrofolic acid (THF), at the expense of ATP hydrolysis, and reduced to methenyl-THF. The following steps are catalyzed by two dehydrogenases NAD^+-dependent and lead to the reduction of the methenyl moiety bound to the THF to methyl-THF.

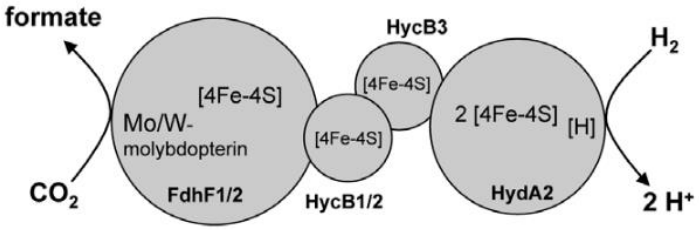


Figure 6. Hydrogen-dependent CO_2-reductase (HDCR) of *A.woodii* (from Poehlein et al., 2012)

The key enzyme of the carbonyl branch is the carbon monoxide dehydrogenase/acetyl-CoA synthase complex (CODH/ACS). It catalyzes the reduction of the CO_2 to CO, at the expense of reduced ferredoxin (Fd^{2-}), and then condenses together the methyl group, the carbonyl group, and free CoA to form acetyl-CoA (Ragsdale and Pierce, 2008). Acetyl-CoA is then turned into acetate via a phosphotransacetylase and an acetate kinase (Schiel-Bengelsdorf & Dürre, 2012; Schuchmann & Müller, 2014). In this last reaction, ADP is phosphorylated to ATP, so the pathway is energetically re-balanced. Nevertheless, it is to be noted that, even when the WLP is completed, no net ATP is gained (Katsyv & Müller, 2020). So, to support life, an additional source of ATP, other than substrate-level phosphorylation, must be present.

A.woodii has a fascinating, ancient energetic mechanism that allows generating an electrochemical gradient, based on Na^+ ions, across the cellular membrane that is responsible for ATP synthesis by oxidative phosphorylation (Biegel et al., 2009; Biegel & Müller, 2010; Müller et al., 2008; Reidlinger et al., 1994; Schiel-Bengelsdorf & Dürre, 2012; S. Schmidt et al., 2009). Energy conservation under the autotrophic condition in this bacterium, indeed, pointed out the role of two enzyme complexes located across the cytoplasmic membrane: the Rodobacter nitrogen fixation (Rnf) complex and the ATP synthase complex.

The RnF complex is composed of different subunits containing flavins and iron-sulfur centers: in the energetic metabolism, it couples the exergonic transfer of electrons from Fd^{2-} to NAD^+ to the vectorial translocation of Na^+ ions across the membrane, from the cytoplasm to the periplasm. Thus, thanks to the Rnf activity, an electrochemical gradient is built. This sodium ion potential across the membrane drives the ATP synthesis thanks to a Na^+-dependent membrane-integrated F_0F_1 ATPase (Fig. 7). 1 mole of ATP is synthesized from 3.3 moles of Na^+ translocated (Müller et al., 2001).

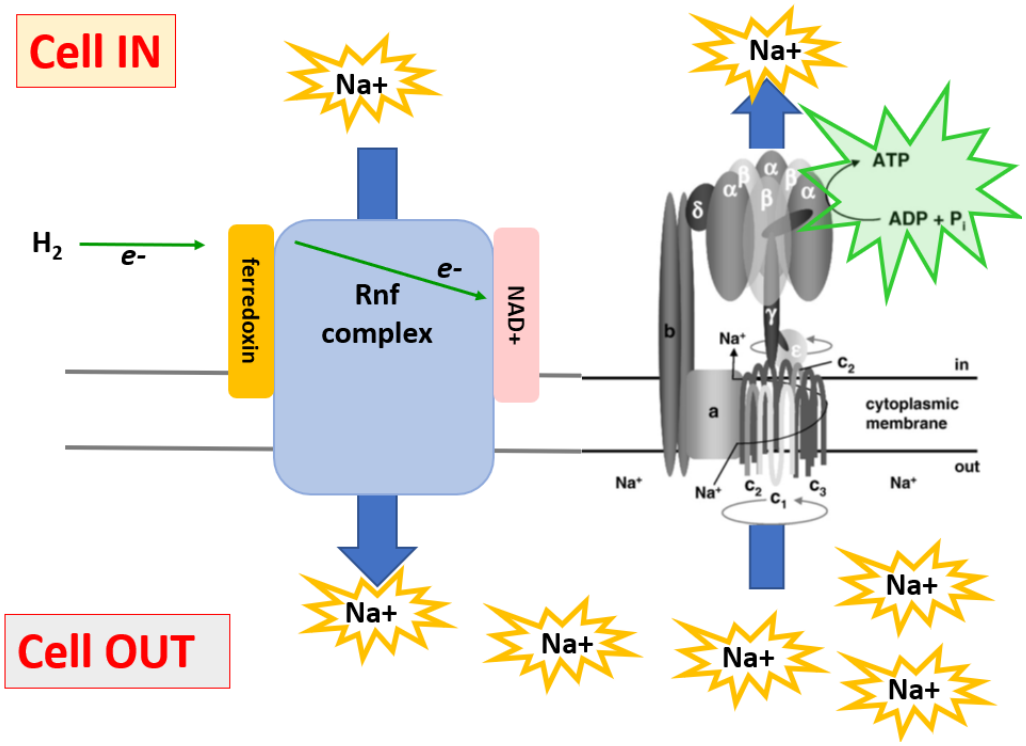


Figure 7. Oxidative phosphorylation in *A.woodii*. e^-: electrons. Green arrows indicate electron flow. ATP synthase picture from S. Schmidt et al., 2009.

The driving force for the generation of the electrochemical gradient across the membrane is the reduced ferredoxin. Moreover, this electron donor is also pivotal for the reduction of CO_2 in the carbonyl branch of the WLP. The oxidation of hydrogen is catalyzed by the soluble, flavin-based electron-bifurcating hydrogenase HydABCD (Schuchmann & Müller, 2012). This tetrameric complex couples the exergonic transfer of electrons from H_2 to NAD^+ to the endergonic reduction of Fd. The HydA subunit is the site for hydrogen oxidation, so the entry point for electrons. HydB is the binding site for flavins and is responsible for the bifurcating mechanism of electron flow to ferredoxin and NAD^+. HydC and HydD

are involved in electron transfer and complex stability, respectively (Fig. 8). It has to be noted that the activity of the electron-bifurcating hydrogenase, besides Fd^{2-}, provides also NADH, a pivotal cofactor for the dehydrogenases involved in the methyl branch of the WLP.

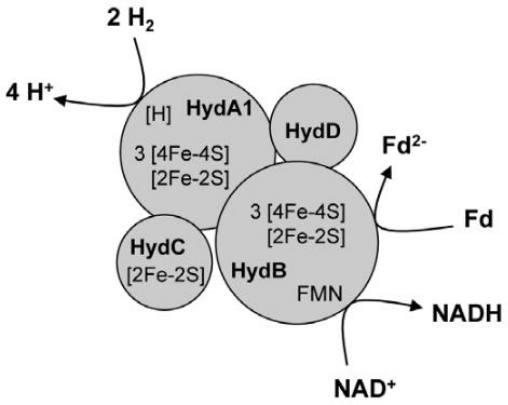


Figure 8. Electron bifurcating Hydrogenase of *A.woodii* (from Poehlein et al., 2012).

Acetate is usually the sole metabolic end product. Recombinant *A. woodii* strains were devised to enhance acetate production (Straub et al., 2014). The options evaluated involved increasing the carbon flow through the Wood-Ljungdahl pathway by the overexpression of the THF-dependent enzymes within this pathway or by overexpressing the phosphotransacetylase and acetate kinase. The genetically engineered *A. woodii* strains were used in stirred-tank reactors with continuous supply of gas consisting of CO_2 and H_2 and resulted in enhanced acetate productivity and final concentration (Straub et al., 2014).

Furthermore, the metabolism of *A. woodii* was engineered to produce acetone (Hoffmeister et al., 2016). In particular, the enzymes responsible for acetone synthesis (*thlA*- *cftA*/*cftB*- *adc* genes) from *C. acetobutylicum* were successfully cloned in plasmid constructions and inserted in *A.woodii*. The heterologous pathway implemented starts from Acetyl-CoA so it is an added branching point that competes with the natural main carbon flow directed toward acetate synthesis (Fig. 9).

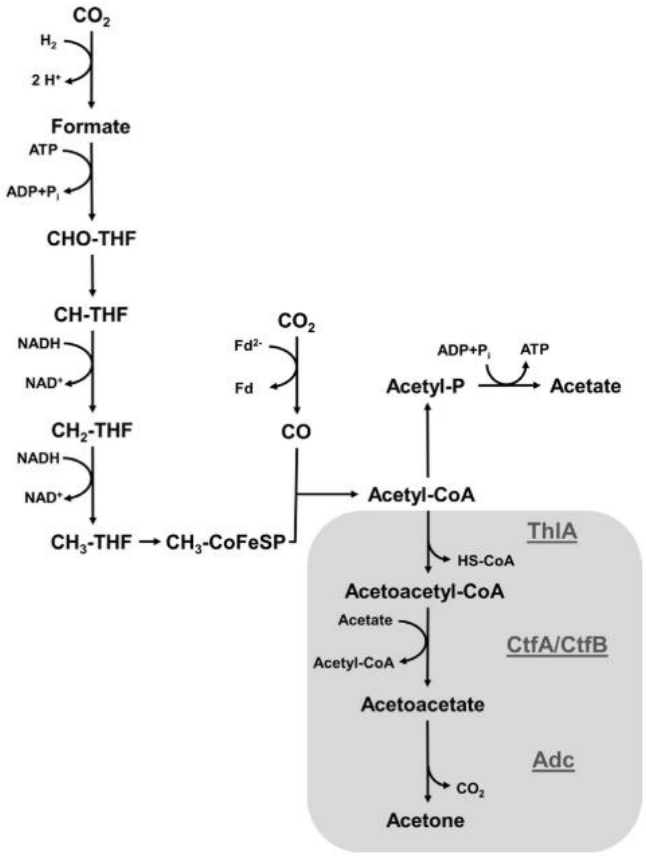


Figure 9. WLP coupled with the metabolic pathway for the synthesis of acetone in *A.woodii* (from Hoffmeister et al., 2016).

Recombinant strains obtained were able to synthesize acetone when fed with H_2+CO_2 gas mix, revealing that this exogenous pathway is feasible employing *A.woodii* as host.

1.5 High-pressure fermentations

In the synthesis of chemicals based on gas-fermentation, gaseous substrates are converted into added-value products. Due to the nature of the catalyst, often the reactions required for the conversion of gas happen in the liquid phase. This is the case of microbial factories grown in culture media that provide micro and macronutrients for biocatalyst growth and activity. Because the feedstock to be converted is gaseous, the maximum concentration of component *i,* provided as gas, in the liquid phase at the equilibrium is described by Henry's law:

$$C_i^* = pP_i \cdot H_i \qquad (1)$$

where $C_i^*$is the concentration (mol/L) of the compound *i* in liquid at the equilibrium, pP_i is the partial pressure of *i* in the gas phase and H_i is Henry's constant for *i* at a stated temperature.

When the system is not in the equilibrium state, the bioavailability of the substrate in the liquid phase is governed by the gas-liquid mass transfer rate, which can be described as follow:

$$dC_{i,L}/dt = K_L a \cdot (C_i^* - C_{i,L}) \qquad (2)$$

where $K_L a$ is the volumetric mass transfer coefficient, C_i^* is the concentration of compound *i* in the liquid medium at the equilibrium, and $C_{i,L}$ is the actual concentration of *i* in the liquid.

Combining equation (1) and (2), equation (3) can be obtained:

$$dC_{i,L}/dt = K_L a \cdot (pP_i \cdot H_i - C_{i,L}) \qquad (3)$$

According to equation (2), the dissolution of the gas in the medium is therefore dependent on two main parameters: the $K_L a$ and the difference between the actual concentration of the dissolved gas and its reachable concentration at the equilibrium. The $(C_i^* - C_{i,L})$ member is the driving force for gas dissolution in liquid. Even in a system not in the equilibrium state, equation (3) stresses the dependence of the maximum concentration achievable in liquid as function of the partial pressure of component *i* in the gas phase.

Moreover, pP_i can be written as function of the total pressure applied to the reactor as follow:

$$dC_{i,L}/dt = K_L a \cdot \left(y_i \cdot P_{tot} \cdot H_i - C_{i,L}\right) \qquad (4)$$

where y_i is the mole fraction of component *i* in the gas phase and P_{tot} is the reactor pressure.

In an ideal process, the mass transfer rate of the gaseous substrate should match with the reaction rate of conversion by the biocatalysts. In an actual process involving microbial factories, this ideal state of the system is difficult to achieve due to increasing biomass concentration, nutrient depletion from the medium, potential substrate limitation or inhibition for metabolic products concentration. Some of these issues can be solved by working in an optimized continuous liquid

operating condition. However, substrate limitation depends on the gas-liquid mass transfer. Common approaches to enhance this parameter are increasing the stirring speed (that increases the surface of contact between the gas and the liquid phase) or increasing the volumetric gas feed rate. Both these approaches act on the $K_L a$. Other approaches work on the driving force member of equation (4) by increasing the molar fraction of component *i* in the gas mixture of feeding or by increasing the fermentation pressure (Oswald et al., 2018; Van Hecke et al., 2019).

In the case of gas-fermentation for acetone production tackled in this thesis, gaseous substrates are H_2 and CO_2. At 30°C, H_i constants are $7.5*10^{-4}$ $mol*L^{-1}*bar^{-1}$ and $2.9*10^{-2}$ $mol*L^{-1}*bar^{-1}$ for H_2 and CO_2 respectively (Wilhelm et al., 1977). Due to the low value of H_i of the feeding gases, especially that of H_2, it is suggested that the productivities of microbial factories based on this gas mix are penalized by the maximum reachable bioavailability of feedstock in the liquid phase. Thus, increasing the partial pressures of H_2 and CO_2 or the pressure of the overall system are approaches proposed to overcome feed limitation and enhance productivities. These are the methods chosen for the ENGICON project and applied in this thesis.

1.5.1 High-pressure fermentation using acetogens

Increasing the pressure fermentation has been previously undertaken in gas fermentation processes using the acetogen *Clostridium ljungdhalii* grown on syngas. At a maximum pressure of 1.8 bar, no inhibition of the biocatalyst was reported and the final ethanol concentration was enhanced (Najafpour & Habibollah, 2006). Another work, conducted at 10.34 bar, describes acetate and ethanol as main fermentation products and CO uptake rates higher at 10.34 bar than at lower pressures (Department of Chemical Engineering. University of Arkansas, 1993). On a gas mix composed of CO_2 and H_2, *C. ljungdhalii* showed growth inhibition and a different distribution of metabolic products between 1 bar and 7 bar of total pressure, nevertheless, the overall yield was not affected (Oswald et al., 2018; Stoll et al., 2018). Experiments adopting this approach up to 3.5 bar were conducted also with *A. woodii* cultivations on CO_2 and H_2 (Demler & Weuster-Botz, 2011; Kantzow & Weuster-Botz, 2016). These works will be deeply discussed in the following chapters.

1.6 Aim of the work

The aim of this thesis is the study of recombinant strains of *A.woodii*, genetically modified for acetone synthesis, in autotrophic fermentation using H_2 and CO_2 as feeding gases. Increasing the pressure of the process up to 10 bar is the chosen approach to enhance substrate availability in the medium of growth to increase biocatalyst productivities. Thus, autotrophic fermentations in bioreactor were performed at different pressures to investigate the effects of the exposure to increasing concentrations of dissolved hydrogen and carbon dioxide on the recombinant *A. woodii* growth and products' spectrum. Biocatalyst behavior was screened in a range between 1.1 and 10 bar. Because no previous works are known in which resistance of *A.woodii* to the stated maximum pressure was assayed, this was the first topic tackled. Then, once verified survival of the bacterium in high-pressure condition, issues addressed were the gas mix to be provided in autotrophic fermentations and the strategy for bioreactor inoculation. After that the preliminary steps were defined, autotrophic fermentations at high pressure were set up. Gas and liquid batch, liquid batch and continuous gas supply, and gas and liquid continuous were the operating conditions investigated.

Chapter 2

Material and Methods

2.1 Bacterial strains

In this work, two genetically modified *Acetobacterium woodii* strains are used. Metabolic engineering of these strains was performed by the Clostridia Research Group led by Professor Nigel P. Minton at the University of Nottingham (UNOTT), in the United Kingdom. This group is responsible for the genetic modification of *A.woodii* for acetone production in the ENGICOIN project. Here, the main modifications are summarized to allow understanding the pivotal characteristics of the bacterial strains we worked with.

The genes coding for the metabolic pathway from Acetyl-CoA to acetone of *C. acetobutylicum* (genes *thlA*, *ctfA*, *ctfB*, *adc*) were first cloned in pMTL38151 plasmid under the control of the constitutively and strong promoter PtcdB. The construct was inserted in an *A.woodii* DSM 1030 strain previously modified with a genetic sequence that facilitates the expression of genes under the control of the PtcdB promoter. The *A.woodii* strain harboring the plasmid for acetone synthesis has been named *A.woodii* plAcC=O. To select and maintain the construction inside of the modified cells, plasmid pMTL83151 codes for a gene responsible for thiamphenicol resistance.

Nevertheless, antibiotic resistance is an unwanted feature for industrial plants. Thus, also another *A.woodii* strain with the genes coding for acetone synthesis integrated into the genome has been developed. The genetic features of the *A.woodii* background strain used were the same described above. The strain

harbored the sequences for acetone synthesis integrated into the genome has been named *A.woodii* intAcC=O and doesn't require thiamphenicol selection to be genetically stable.

In this thesis, both strains were used. Indeed, they were developed in two following steps. First, heterologous genes were cloned into the plasmid, and the expression and catalytic activity of *A.woodii* plAcC=O was tested in UNOTT. Once proven, it was sent to our laboratory in Politecnico di Torino for further testing. In parallel, colleagues in UNOTT completed the development of the second strain: *A.woodii* intAcC=O. For this reason, the largest number of experiments in thesis work was performed using the bacterium harboring the plasmid which was available before. The last experiment was performed using the final strain which is suitable for the pilot plant.

1.2 Culture media

Medium used for autotrophic cultivation of bacteria was as follow: KH_2PO_4 1.76 g/L; K_2HPO_4 8.44 g/L; NH_4Cl 1.0 g/L; NaCl 2.9 g/L; yeast extract 2 g/L; cysteine hydrochloride 0.5 g/L; $MgSO_4$ 0.180 g/L; resazurin 1 mg/L. Moreover, for each liter of the medium, 1.0 mL of trace element solution SL9, 1 mL of selenite-tungstate solution, and 2 mL of vitamin solution DMSZ 141 were added. The recipe of each preparation is reported below.

- Trace element solution SL9: nitrilotriacetic acid 12.8 g/L; $FeCl_2$*4 H_2O 2.0 g/L; $ZnCl_2$ 0.070 g/L; $MnCl_2$*4 H_2O 0.1 g/L; H_3BO_3 0.006 g/L; $CoCl_2$*6 H2O 0.19 g/L; $CuCl_2$ *2 H_2O 0.002 g/L; $NiCl_2$*6 H_2O 0.024 g/L; Na_2MoO_4 *2 H_2O 0.036 g/L.
- Selenite-tungstate solution: 0.5 g NaOH; 3 mg Na_2SeO_3*5 H_2O, 4 mg Na_2WO_4 *2 H_2O.
- Vitamin solution: Biotin 0.002 g/L; folic acid 0.002 g/L; pyridoxine-HCl 0.010 g/L, thiamine-HCl*2 H_2O 0.005 g/L; riboflavin 0.005 g/L; nicotinic acid 0.005 g/L; D-Ca-pantothenate 0.005 g/L; vitamin B12 0.0001 g/L; p-Aminobenzoic acid 0.005 g/L; lipoic acid 0.005 g/L.

If not otherwise stated, for heterotrophic fermentation, the medium was the autotrophic one plus the addiction of 6 g/L $KHCO_3$ and 20 mM fructose.

Every component was sterilized before use. Phosphate salts solution, $MgSO_4$, cysteine, and a mix composed of NH_4Cl + NaCl + yeast extract were prepared as separate solutions. For NH_4Cl + NaCl + yeast extract, pH was adjusted to 7.2 using NaOH. Sterilization was effected by autoclaving. Resazurin, trace elements, selenite-tungstate, and vitamin solution were prepared as 100X stock

and sterilized by filtration. Fructose was prepared as 10X stock and sterilized by filtration. In all cases, 0.22 μm sterile filters made of PES were used.
After that each component was prepared and sterilized, they were mixed to have the complete medium. It was stored in sterilized glass bottles in air until utilization.

2.3 High-pressure bioreactor – volume 2 L

The bigger high-pressure reactor used in this work is a custom-adapted bioreactor manufactured by the H.E.L group (UK) (Fig. 10). The core system consists of a stirred tank reactor with a total volume of 2 L. The vessel is oil-jacketed. An oil bath (Julabo, DE) connected to the jacket of the reactor enables sterilization operations (autoclaving of the vessel) and maintains the operating temperature throughout experiments.

5 piston pumps (Eldex, USA) allow liquid injection in the tank. Pumps were calibrated for each solution pumped (culture broth, base, acid, trace elements, and anti-foam) to ensure the correct in-flow rate.

6 Mass Flow Controller (MFC), listed below, are installed to supply four different gases (H_2, CO_2, N_2, CO). In particular, for H_2 and CO_2 two MCFs with different maximum and minimum thresholds are present, to being able to manage a broad range of in-flow gas rates.

- MFC for H_2 – high in-flow gas rate: maximum rate 5 L/min (Vögtlin Instruments, CH)
- MFC for CO_2 – high in-flow gas rate: maximum rate 2.5 L/min (Vögtlin Instruments, CH)
- MFC for N_2 – high in-flow gas rate: maximum rate 0.5 L/min (Vögtlin Instruments, CH)
- MFC for CO – high in-flow gas rate: maximum rate 1 L/min (Vögtlin Instruments, CH)
- MFC for H_2 – low in-flow gas rate: maximum rate 0.05 L/min (Bronkhorst High-Tech BV, NL)
- MFC for CO_2 – low in-flow gas rate: maximum rate 0.05 L/min (Bronkhorst High-Tech BV, NL)

Sparging of the gas is applied from the bottom of the vessel via a micrometric sparger. Stirring of the medium is mechanically maintained by one level of Ruston blades connected to an impeller driven by a motor (Heidolph Instruments

GmbH & Co, DE) and baffles. A proportional Back Pressure Regulation (BPR) valve (Norgren, USA) allows the control of the pressure at defined setpoints. A Coriolis Mass Flow Meter (MFM) (Bronkhorst High-Tech BV, NL), placed downstream of the BPR, measures mass flow rates of the gas mix coming from the gas outlet of the vessel. Gas samples out from the Coriolis MFM can be collected in a gas bag for offline analysis of its composition through gas chromatography.

The head plate of the reactor vessel is fitted with liquid level, pressure, temperature, pH, and redox (Sentek, UK) probes.

When sampling of liquid in the reactor, samples were withdrawn from a tube in the vessel head plate. The end of the tube rests below the level of the culture medium, and the internal pressure of the vessel drives liquid out. Out-flow from the sampling tube can be directed toward a manual sampling port or, in case of liquid continuous, to a valve (Shako, UK) for the removal of the culture medium at a stated rate. Before the sample collection, 3 ml of medium were withdrawn to the manual sampling port to purge the sampling tube. After purging, samples were collected and used for off-line measurements of biomass growth and fermentation products in liquid.

For the inoculum of the starter culture, in the head plate is placed an inoculum port, plugged by a rubber septum, and covered by a metallic screw cap. Inoculum is performed by piercing with a needle of a syringe the rubber septum. After injecting the starter culture, the rubber septum was sealed with the metallic cap.

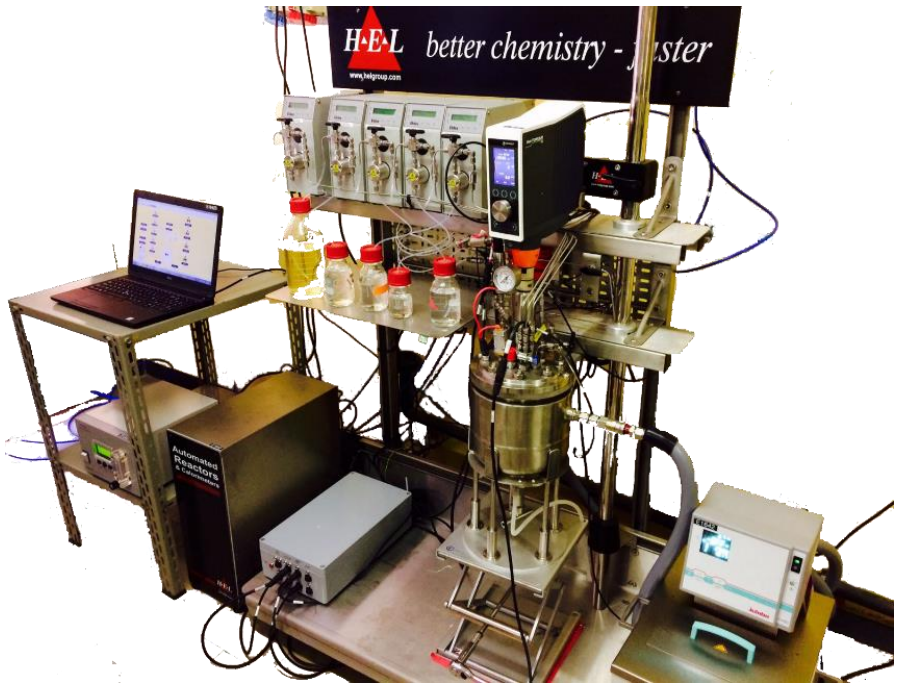


Figure 10. High-pressure bioreactor with 2 L vessel

2.4 High-pressure bioreactor – volume 0.5 L

The smaller high-pressure reactors used are a set of three parallel custom-made bioreactors manufactured by the H.E.L group (UK). Each reactor has a tank of 0.5 L total volume. The three vessels are allocated into a polyBLOCK responsible for heating, cooling and stirring. Each reactor has its own heating zone which is heated electrically. The polyBLOCK also houses a powerful magnetic drive below each reactor zone which couples with the PTFE-coated magnetic stirrer within each reactor. This PTFE disc is connected to a shaft suspended from the lid where a Rushton impeller is clamped. Each reactor can be individually stirred at stirring speeds ranging from: 30 to 200 rpm.

Three shared piston pumps (Eldex, USA) allow liquid injection in the vessel of each reactor. The liquid flow is split towards a specific reactor thanks to a Valco valve (Valco Instruments Co. Inc., Houston, USA).
Sparging of the gas in the medium is applied through a micrometric sparger placed in the bottom of the vessel. 4 MFCs for each reactor (reported in detail below) allow suppling of H_2, CO_2, N_2, CO.

- MFC for H_2 – maximum rate 100 mL/min (Vögtlin Instruments, CH)

- MFC for CO_2 – maximum rate 100 mL/min (Vögtlin Instruments, CH)
- MFC for N_2 – maximum rate 100 mL/min (Vögtlin Instruments, CH)
- MFC for CO – maximum rate 100 mL/min (Vögtlin Instruments, CH)

A proportional BPR valve (Norgren, USA) allows controlling the pressure inside each vessel at defined setpoints.

The head plate of each tank is fitted with pressure, temperature (a PT100 transducer), pH, and redox (Hamilton, Bonaduz, CH) probes. Hardware settings for gas and liquid samples withdrawn and reactor inoculation are the same described before (paragraph 2.3).

2.5 Heterotrophic fermentation of *A.woodii* strains

2.5.1. Heterotrophic fermentation in serum bottles

For fermentation in serum bottles in heterotrophic conditions, glass bottles with a volume of 160 mL plugged with rubber caps impermeable to gases were used (Fig. 11). Each bottle was filled with an aliquot of 30 mL of heterotrophic medium from sterile stock (paragraph 2.2). To make the anaerobiosis condition, serum bottles were gassed through bubbling of sterile N_2 in the medium for 40 minutes. Because this step led to the basification of the medium, 100 µL of H_2SO_4 1.5 M were added to bring back the pH to 7.2 - 7.3. In the case of fermentation using *A.woodii* plAcC=O, thiamphenicol 15 mg/L was added. Finally, cysteine was injected, and anaerobic serum bottles were stored for around 24 hours at room temperature in a dark area. The complete removal of oxygen was indicated by the color changing of the resazurin in the medium. Indeed, serum bottles turned from pink to colorless (Fig. 11).



Figure 11. Serum bottles filled with the heterotrophic medium. Left: completely reduced medium (colorless). Right: medium with oxygen (pink).

For the inoculum, a volume of bacteria preculture in exponential phase was inoculated in the colorless medium such that the final OD_{600nm} in the serum bottle was 0.1. If the starter culture was a frozen stock, 3 mL of frozen culture were used as inoculum. Serum bottles were incubated at 30°C and 200 rpm in an orbital shaker (Biosan, LV), in an upright position. In the case of experimental tests, if not otherwise stated, triplicates were performed.

2.5.1.1 A.woodii strain preservation

Empty serum bottles of 12 mL total volume were anaerobized using N_2 and then autoclaved. DMSO 100% was sterilized by filtering and then 5 mL were injected in a sterile serum bottle. Oxygen was removed by bubbling sterile N_2 for 15 minutes. 2.7 mL of heterotrophic culture of *A.woodii* strains in exponential phase were transferred in a sterile and anaerobic 12 mL-serum bottle and 0.3 mL of DMSO were added as cryoprotectant. Storing was performed at -20°C and culture was revitalized and replaced every four months.

2.5.2. Heterotrophic fermentation in bioreactor

2.5.2.1 Sterilization of the bioreactor and medium loading

Heterotrophic fermentations were conducted in the bioreactor with a total volume of 2 L. To perform fermentation in the bioreactor, sterilization of each equipment's components that meets the culture is needed.

Pumps were sterilized by pumping a solution of 70% ethanol at 1 mL/min for 1 hour and then switching off the pumps for one hour, leaving the sterilization

solution inside the tubes. Ethanol was then pulled pumping sterile deionized water. The inlet of the tubes was then plugged with a metal cap (Swagelok, USA) previously sterilized by autoclaving.

In case of liquid continuous, 1 L of 70 % ethanol was put in the vessel and the valve for liquid removal was activated at a rate of 15 mL/min. After 50 minutes, the valve was switched off and the sterilization solution was left inside the tube. Ethanol 70% in surplus was removed from the vessel by the outlet installed in the bottom.

Sterilization of the pipeline of in-flow and out-flow gas is performed by heating heaters plates fixed on the outside of the tubes.

The vessel is sterilized by *in situ* autoclaving. Temperature probe, liquid level probe, stirring components, baffles, and the sparger are embedded in the vessel, so they are sterilized with the same procedure. For autoclaving, 1 L of deionized water was put in the vessel and temperature was increased up to 121°C by heating the oil in the jacket. The system was maintained in the range of 121-124°C for 30 minutes and then was cooled by turning off the oil bath and leaving the oil coming back to room temperature. Usually, water after autoclaving was removed from the outlet placed in the bottom of the vessel. Nevertheless, in case of liquid continuous, it was removed by turning on the valve for liquid removal: in doing so, ethanol 70% left in the tube after sterilization process was rinsed using sterile water. When the vessel was empty, oxygen was removed by supplying 500 mL/min of N_2 at 1.5 bar constant pressure for 40 minutes.

0.9 L of the heterotrophic medium was prepared as described in paragraph 2.2 and then was transferred in a sterile bottle closed with a cap with two tubing ports (Duran, DE). This setting allows connecting the tube that gets through the first port to the pipeline for gassing medium with N_2 to remove oxygen. The second port is connected to an external tube and is a vent that avoids overpressure in the bottle. In-flow and out-flow of gases can be controlled by clamps placed on the tubes, close to the ports.

The medium was gassed with N_2 for 3 hours. Then, the bottle was disconnected from the pipeline and connected to the inlet of the sterilized culture medium pump of the reactor. 0.8 L of the medium was transferred into the vessel by activating the pump. After loading, cysteine was injected into the vessel from the inoculum

port. Stirring at 400 rpm and N_2 in-flow rate of 100 mL/min was supplied for 1 hour to remove oxygen eventually infiltrated during the loading procedure.

After waiting about 24 hours, a sample of the medium inside the vessel was withdrawn. If it is colorless, the medium was ready for the inoculum of bacteria.

2.5.2.2 *High-pressure fermentation in bioreactor*

To evaluate the resistance of *A.woodii* cells up to 11 bar pressure, a heterotrophic preculture of *A.woodii* plAcC=O, grown in serum bottles, in exponential phase (OD_{600nm} around 1), was used to inoculate 0.8 L of medium containing 20 mM fructose. The volume of preculture moved for the inoculum was calculated such that the OD_{600nm} in the reactor was around 0.1. The culture was then grown in the reactor at atmospheric pressure, in a nitrogen atmosphere, until it reached the exponential phase. Then the pressure inside the vessel was increased to 11 bar by sparging nitrogen with an in-flow rate of 500 mL/min.

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

i) Fast depressurization: opening the valve of the sampling port toward the manual sampling when the reactor was at 11 bar.

ii) Mild depressurization: pressure in the reactor was decreased by 1 bar every 30 minutes. The descent from 11 bar to 1 bar took 5 hours. Manual sampling was done when the pressure was 1.1 bar.

In both cases, a sample of cell culture was collected in a syringe directly connected to the tube for liquid sampling, by exploiting the overpressure present in the reactor, and taking care that no oxygen infiltered in the syringe during the procedure.

Bacterial vitality after the pressurization-depressurization process was assessed by re-inoculating 2.5 mL of pressurized and depressurized cultures into 50 mL of heterotrophic medium, in serum bottles, in a nitrogen atmosphere at 30°C, 200 rpm, in an upright position.

Cell-wall integrity was assessed by Gram staining. Cells were fixed on a glass slide by heating and Gram staining was effected. Reagents were purchased from Sigma-Aldrich (USA) and the test was performed following the instructions provided by the manufacturer. Slides were visualized by a light microscope (Nexcope - Ningbo Yongxin Optics Co., Ltd., CHN).

2.6. Autotrophic fermentation of *A.woodii* strains

2.6.1 Autotrophic fermentation in serum bottles

The procedure followed for the set-up of fermentation in serum bottles in autotrophy has the first steps analogous to what is described in paragraph 2.5.1 for fermentation in serum bottles in heterotrophic conditions. Briefly, 30 mL of autotrophic medium was injected in sterile serum bottles of 160 mL plugged with rubber caps. To make the anaerobiosis condition, serum bottles were gassed through bubbling of sterile N_2 in the medium for 40 minutes. As previously described for heterotrophic medium, gassing with N_2 makes the medium basic (pH 7.8 - 8). Then, nitrogen was replaced by feeding gases. If not otherwise stated, each serum bottle was gassed with 245 mL/min of a mix composed of 70% H_2 and 30% CO_2 (2.3:1 H_2:CO_2), plunging the needle for gas supply directly in the liquid. Dissolution of CO_2 in the medium acidifies the pH. After 7 minutes at the above-stated in-flow gas rate of the feeding mix, the pH didn't decrease anymore, remaining stable at 7.2, and gas change was considered complete. Finally, cysteine was injected, and anaerobic serum bottles were stored for around 24 hours at room temperature in a dark area, waiting for the medium became colorless. In the case of fermentation using *A.woodii* plAcC=O, thiamphenicol 15 mg/L was added.

For the inoculum, a heterotrophic culture was used. Bacteria grown on fructose in exponential phase were inoculated in a colorless autotrophic medium such that the final OD_{600nm} in the serum bottle was around 0.2. Serum bottles were incubated at 30°C and 200 rpm in an orbital shaker, in a vertical position.

2.6.1.2 Fermentation in serum bottles in fed-batch with pH control

Often, in closed systems like serum bottles, autotrophic tests are conducted in fed-batch to avoid the stop of bacteria growing due to feedstock depletion. In this operating condition, the exhausted gas in the headspace is replaced with the fresh gas-feeding mix. This action was done by entering two needles in the rubber cap: one was connected to the pipeline for gas supply, and the second acted as a vent to avoid overpressure in the bottle during gassing. If not otherwise stated, gas mix, composed of 70% H_2 and 30% CO_2, was provided at 245 mL/min for 2.5 minutes. Then, the needle for the gas outlet was removed, and a mild overpressure (0.4 barg) was created in the serum bottles before removing the needle for the gas inlet. Overpressure in the serum bottle was measured with a digital manometer

(Keller, CH) and the culture was incubated at 30°C and 200 rpm, in a vertical position.

A.woodii produces acids as the main products of its metabolism. For this reason, during long fermentations, even in serum bottles, basification of the medium is necessary. Unless otherwise specified, a sterile solution of NaOH 1M gassed with N_2 was utilized. A sample of 1.5 mL of the culture was drawn from the bottle and pH was measured using a pHmeter (XS Instruments, IT). According to the measured value, a variable volume of NaOH 1M was added in the serum bottle such that the pH rose back to 7.2 – 7.3. To confirm that the proper volume of the base was added, another sample of 1.5 mL was withdrawn, and pH was measured.

In autotrophic fermentations in serum bottles, both gas replacing and pH adjustment were usually accomplished daily and in the same operational time, to reduce as much as possible handling of the culture and time out from the shaking incubator. Experiments were performed in triplicates.

2.6.1.3 Serum bottle fermentation with $KHCO_3$ and KCl

For the fermentation in serum bottles with an increasing amount of $KHCO_3$ and KCl in the autotrophic medium, *A.woodii* plAcC=O was the strain used. The medium was prepared as described in paragraph 2.2 and $KHCO_3$ or KCl was put in addition to the components of the standard recipe. Briefly, a stock solution of autotrophic medium with 2.45 M of $KHCO_3$ or KCl was prepared, degassed first with N_2 and then with the 2.3:1 H_2:CO_2 gas mix. In parallel, sterile serum bottles were filled with different volumes of standard medium and gassed as described before (paragraph 2.6.1). The volume of the standard medium inside each serum bottle was calculated in a way that, by adding the 2.45 M stock of $KHCO_3$ or KCl to have the chosen concentration of salts, the volume inside the serum bottle reached 30 mL. Culture added with 150 mM KCl, 150 mM $KHCO_3$, 300 mM KCl, or 300 mM $KHCO_3$ were prepared. A control culture without added salts was prepared too. For each condition, a triplicate was performed. A fed-batch strategy was applied, and pH was controlled at 7.6 using sterile KOH 1M gassed with N_2.

Inoculum procedure and growing condition were the same described in paragraph 6.2.1.

2.6.2 Autotrophic fermentation in bioreactor

If not otherwise stated, autotrophic fermentations were conducted in the bioreactor with a total volume of 2 L. Reactor sterilization and anaerobiosis condition in the vessel were performed as described in the 2.5.2.1 paragraph.

0.9 L of the autotrophic medium was prepared as described in paragraph 2.2 and gassed with N_2 for 3 hours following the same procedure described above for the preparation of the heterotrophic medium. In the same manner, 0.8 L was loaded in the vessel and reduced with cysteine. Then, the medium was left in the reactor without shaking and temperature control for about 24 hours, waiting that it to become colorless.

Unless otherwise specified, before inoculation nitrogen dissolved in the liquid was changed with the feeding gas. To do so, a mix of 70% H_2 and 30% CO_2 was supply into the reactor at 75 mL/min for 3 hours at a constant pressure of 1.5 bar. In an analogous way to what was recorded for autotrophic medium in serum bottles, sparging with the feeding mix brough the pH at the suited value of 7.2-7.3. After the procedure of change of gas, a sample was withdrawn from the vessel to check that the medium was still colorless.

A heterotrophic culture in exponential phase (OD_{600nm} around 1) grown in serum bottles was used as the starter. The volume of preculture moved for the inoculum was calculated such that the OD_{600nm} in the reactor was around 0.2. If not otherwise stated, regardless of the operating condition tested in each specific fermentation, a first step of gas and liquid batch at low pressure (1.5 bar) was performed to allow cells to shift from the heterotrophic to the autotrophic metabolism before further stress. The gas mix supply was 2.3:1 H_2:CO_2, temperature 30°C and stirring 400 rpm. When pressure start to decrease, a sample was drawn from the reactor to check the increase of OD_{600}. If this parameter was confirmed the specific experimental conditions chosen for each test were applied.

pH during fermentations was kept around 7. An anaerobic bottle containing a sterile solution of NaOH 4.5 M gassed with nitrogen, with a port connected to a reactor pump, was used. pH control could be done by turning on the base pump manually until the pH probe recorded the setpoint value. Otherwise, it could be done by activating the continuous control from the software of the reactor. In this way, the pH pump autonomously switched on when the pH value measured by the probe was below 6.8.

In cases in which pressure was increased up to 10 bar using a mix containing CO_2, acidification of the medium is expected. Thus, during pressure increasing, pH control was turned off until the stated pressure value was reached. Once pressure stabilized, pH was adjusted to 7.

Except where otherwise specified, reactor and medium preparation, medium loading, gas change, and inoculum procedure were as described in this paragraph. Specific conditions probed for each experiment (operational condition, pressure, gas mix supplied, in-flow gas rate, dilution rate) are specified from time to time and described in the result and discussion chapter.

2.6.2.1 Fresh medium pressurization

For fresh medium pressurization, parallel reactors with a total volume of 0.5 L were used. After reactors sterilization, vessels were filled with 200 mL of autotrophic medium gassed first with N_2 to remove oxygen. The gas change in liquid from nitrogen to feeding gases was performed following two different protocols. In the first one, the bioreactor was sparged with 70% H_2 and 30% CO_2 mix at 1.1 bar for 3 hours at 80 mL/min in flow-gas rate. In the second protocol, instead, N_2 was removed using 100% H_2 at an in-flow rate of 80 mL/min for 2.25 hours. Subsequently, the vessel was pressurized up to 8 bar again, maintaining the same gas and the same in-flow rate. Then, the gas supplied was replaced by the 70% H_2 and 30% CO_2 mix, provided at 80 mL/min for 15 minutes. In this phase, pH dropped from 7.4 to 6.7. The pressure was then decreased and gas changing continued at 1.1 bar using the H_2:CO_2 gas mix at 80 mL/min for 3 hours. pH didn't change anymore, remaining around 6.7.

Reactors when then inoculated following the procedure described above for inoculum of bioreactors with autotrophic medium (paragraph 2.6.1)

2.7 Analytics

2.7.1 Optical density and biomass dry cell weight (DCW)

Optical density during experiments was measured at λ= 600 nm using a DH-5000 Spectrophotometer (HACH, USA) or a V-730 Spectrophotometer (Jasco, JP). Distilled water was used as the blank to set the reference for the measurements.

The specific growth rate (μ) was calculated from optical density measurements recorded during experiments (Maier, 2009). Cell growth during the exponential phase can be described as follow:

$$dX/dt = \mu X \qquad (5)$$

Where X is the optical density, t is the time and μ is the specific growth rate (1/time). By plotting the natural logarithm of the growth versus the time, it is possible to define a linear portion of the growth curve. In this portion, μ is constant, and by rearranging and integrating eq. (5) it is possible to obtain eq. (6):

$$lnX = \mu t + lnX_0 \qquad (6)$$

During fermentation, samples of 20 mL of 40 mL of growing culture were drawn and biomass was collected by centrifugation at 3005 x g, 20 minutes at 4 °C. Pellet was then washed three times with deionized water and dried at 80°C for 48 h in an oven (Memmert GmbH + Co. KG, DE). The dry pellet was weighted using an analytical balance (XS Instruments, IT). Measuring pellets of *A.woodii* plAcC=O grown autotrophically at atmospheric pressure, an experimental correlation factor of 0.38 was determined between DCW (g/L) and OD_{600}.

2.7.2 High-performance liquid chromatography (HPLC)

Quantification of fructose and liquid metabolic products was performed through high-performance liquid chromatography (HPLC). 2.5 mL of bacterial culture withdrawn from serum bottles or reactors were filtered using a 0.22 μm PES syringe filter and injected in vials for HPLC analysis. Measures were done by a Prominence HPLC System (Shimadzu, JP) equipped with a Refractive Index Detector and a Diode Array Detector. Compounds in 10 μL of filtered culture were separated through a Resez ROA-Organic Acid column (Phenomenex, USA), kept at 50°C using. H_2SO_4 0.005 M was the mobile phase and a constant flow rate of 0.7 ml/min was set.

2.7.3 Gas chromatography (GC)

Gas composition for in-flowing and out-flowing gas was measured using an Agilent 490 Micro GC (Agilent, CA, USA) or a Micro GC Fusion (Inficon, CH). Both instruments are equipped with the analytical columns Molsieve 5Å, using Argon as the carrier, for H_2, O_2, and N_2 analysis. Instead, CO_2 analysis was done using Helium as carrier and a PoraPLOT U column for Agilent 490 Micro GC or a Rt-U-Bond column for Micro GC Fusion respectively.

2.8 Carbon balance

Carbon balance (C-balance) was calculated for autotrophic fermentations considering the vessel of the bioreactor as the system.

Source of carbon (C-IN) were:

- CO_2 provided with the in-flow gas rate.
- Fructose, transferred into the autotrophic vessel by the inoculum grown in the heterotrophic media contains mole of this compound contains 6 moles of carbon.
- Formic acid, if this compound was consumed by bacteria during growth.

Considering the vessel as the system, carbon out (C-OUT) is represented by the CO_2 lost from the BPR valve. The weight of the out-flow gas mix was measured by the Coriolis MFM placed downstream of the BPR. The moles of CO_2 lost from the vessel were calculated by combining data from MFM with the volumetric fraction estimated by GC analysis.

The moles of CO_2 in the system are the difference between C-IN and C-OUT and should consist of metabolic products (main metabolic products for this bacterium are acetate, acetone, formic acid, and biomass), CO_2 dissolved in liquid as CO_2 itself or as HCO_3^- and CO_2 still available in the headspace.

Nevertheless, not for all experiments the weight of the out-flow gas was available due to constraints of the equipment. In these cases, estimation of the C-OUT was performed. Indeed, the sum of C-OUT and all the components containing carbon in the reactor should be equal to the C-IN calculated. So, C-OUT can be estimated as the difference between the C-IN and the carbon present in the reactor, in the above-mentioned forms.

Carbon in the system was calculated as the sum of the following components:

- Moles of C in liquid products. The HPLC analysis provides a quantification of the main products of *A.woodii* metabolism in liquid. The moles of C embedded in each compound were estimated by multiplying the concentration of each product by the volume of the sampling and the number of carbon contained in its molecular formula. In the case of the biomass, the molecular formula $C_{60}H_{87}O_{23}N_{12}P_1$ was considered (Villadsen et al., 2016).

- CO_2 dissolved in liquid. The concentration of carbon dioxide dissolved in the liquid phase at equilibrium is described by Henry's law. The constant H_{CO2} used for calculus takes into account the temperature of the process (30°C) and was 29.39 $mmol*L^{-1}*bar^{-1}$ (Wilhelm et al., 1977). The partial pressure of the gas was considered that provided according to the in-flow gas rate composition.
- CO_2 dissolved in liquid as HCO_3^-. The formula for the estimated concentration of bicarbonate in liquid was deeply treated in the work of Stumm and Morgan (Stumm & Morgan, 1995). For the operating condition of gas and liquid batch, the laws for estimation of bicarbonate concentration in closed CO_2 systems were considered. For gas continuous supply and liquid batch, instead, calculous was performed according to the laws for the estimation of bicarbonate concentration in open CO_2 systems. In both cases, the estimated HCO_3^- concentration was multiplied for the sample volume to obtain the moles.
- CO_2 in the headspace. The moles of CO_2 were calculated according to the law of the ideal gas. The volume considered was the difference between the total one (2 L) and the liquid volume still present in the reactor at the end of the fermentation. The carbon dioxide partial pressure was calculated according to the volumetric composition of the out-flow gas mix measured by the GC analysis.

For fermentations in which direct measurement of in-flow and out-flow gas mix were available, C-balance was calculated according to both methods above described. Calculus was done using the direct measurements of in-flowing and out-flowing gases recorded by the instruments connected to the reactor and by estimating the amount of carbon in the vessel, to find out the C-OUT. Results obtained by applying the two methods were compared and a good consistency was found.

2.8.1 α parameter

Taking into account the vessel as the system, the CO_2 trapped in any form in it could be considered as CO_2 converted, because it is provided to the system and not lost from its outlet. Nevertheless, according to what has been explained above, not all the CO_2 inside the reactor is effectively converted into metabolic products.

Indeed, a variable part of it is still available in the reactor, dissolved in the liquid (as CO_2 and as HCO_3^-) or in the headspace, ready to be consumed by bacteria, but

still not consumed. Assuming that microorganisms don't consume immediately the CO_2 provided to the system, the fraction of this gas available for metabolism but not already converted increased by increasing the pressure.

For this reason, in the results and discussion chapter, besides yields for each product, the fraction of the CO_2 provided effectively converted in metabolic products (acetate, acetone, formate, and biomass) is reported.

Moreover, the fraction of the total CO_2 converted in metabolic products used for the synthesis of a specific metabolite *j* (indicated as α) was calculated. This parameter allows highlighting the metabolic pathway in which the CO_2 effectively converted is addressed in the operating condition tested. The formula for the calculus of α is reported below:

$$\alpha_j = \frac{mol\ of\ CO2\ \ in\ product\ j}{mol\ of\ CO2\ \ converted\ in\ metabolic\ products} \qquad (7)$$

It should be noted that in the formula (7) calculus is referred to CO_2. So, each compound *j* has to be entered as CO_2 equivalents, according to its molecular formula.

2.8.2 Productivities calculation

To have a common unit of calculation, the volumetric productivity (r_j) for each product *j* is expressed in C-mmol$_j$*L^{-1}*h^{-1}. For the same reason, specific productivity (q_j) is expressed as C-mmol$_j$*g$_{biomass}^{-1}$*h^{-1}. In this way, each compound is considered as the CO_2 moles embedded in it.

Chapter 3

Results and Discussion

In this work, a genetically modified *Acetobacterium woodii* will be used to anaerobically convert CO_2 streams to acetone and other valuable compounds useful in industry (acetate). The genes coding for the metabolic pathways for acetone synthesis of *Clostridium acetobutylicum* were cloned under the control of the strong and constitutive promoter PtcdB. Two different genetically modified *A.woodii* strains have been used. The first one developed harbors the genes for acetone pathways in a plasmid (*A.woodii* plAcC=O). To select and maintain the construction inside of the modified cells, this plasmid codes for a gene responsible for thiamphenicol resistance. Nevertheless, antibiotic resistance is an unwanted feature for industrial plants, so a second strain (*A.woodii* intAcC=O) was developed, in which the same operon for acetone synthesis was inserted in the genome of the bacterium.

In this thesis, both strains are used. Indeed, in the meanwhile *A.woodii* intAcC=O was developed and optimized, experiments were performed using the bacterium harboring the plasmid. Once the final integrated strain was ready, tests were performed using this final biocatalyst.

3.1 Growth *A.woodii* plAcC=O in heterotrophic condition.

Since *A.woodii* strains were never used before in Politecnico di Torino's laboratory, preliminary tests were performed in a heterotrophic medium to evaluate the growth curve in optimal condition and to save stocks to revitalize the bacterium for further experiments. *A.woodii* plAcC=O has was grown in serum bottles, in N_2 atmosphere. Optical density and pH were recorded (Fig. 12 A). The maximum OD reached was 1.72 ± 0.14. Concomitant to the bacterial growth, the pH fell from 7.0 ± 0.22 to 5.8 ± 0.13. Fructose was completely consumed. Metabolic products were, as expected, acetate and acetone. Their maximum concentration was achieved at the end of the growth curve and reached 25.06 ± 5.82 mM and 5.85 ± 2.09 mM for acetate and acetone respectively (Fig. 12 B).

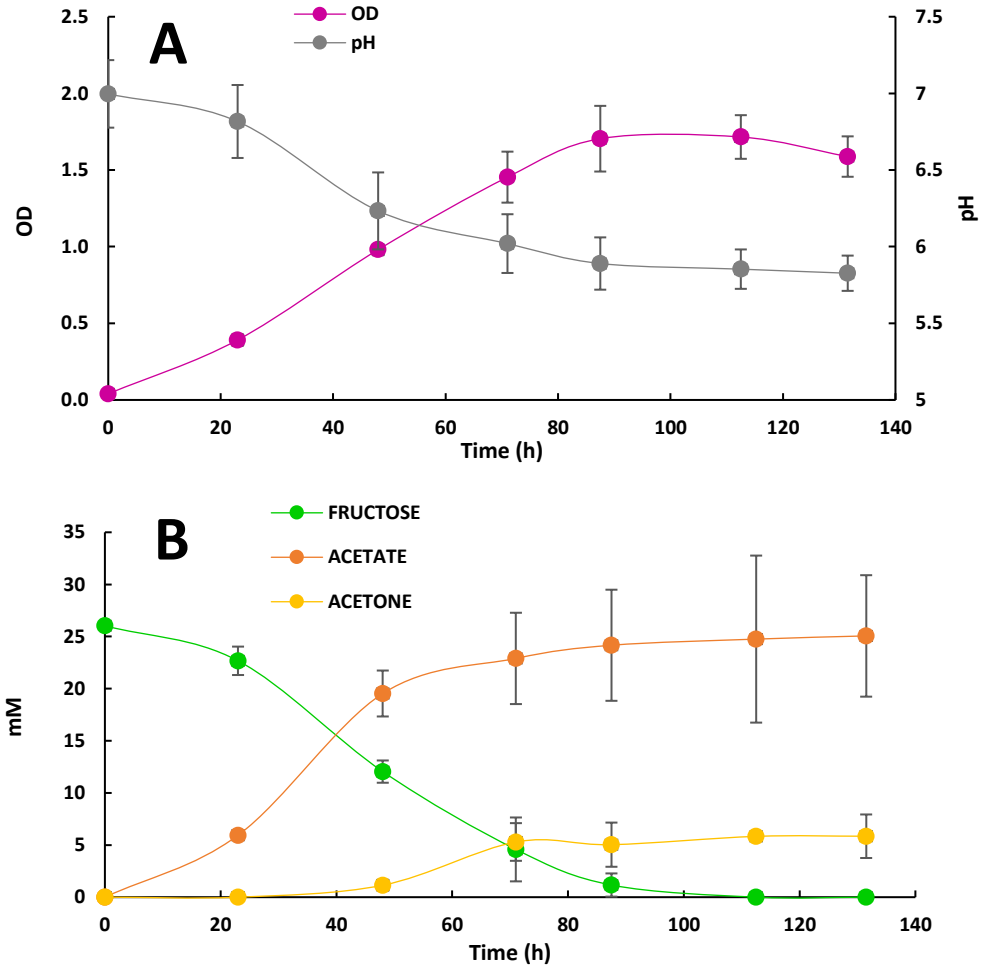


Figure 12. Growth of A.woodii plAcC=O in heterotrophic condition (20 mM fructose). (A) Optical density and pH trend; (B) Main metabolic products.

Tests using the wild-type strain in similar operating conditions (serum bottles providing 20 mM of fructose in the medium) reported values in the same order of magnitude with respect to what was recorded with our modified strain (Westphal et al., 2018). Wild-type cells grew up to OD_{600} 2.2. Concomitant to acetate synthesis (about 40 mM), pH fell from 7.3 to 6.3. It has to be noted that, nevertheless, using the wild-type strain a faster fermentation is reported: completely fructose depletion occurred in 45 hours while using *A.woodii* plAcC=O process was slower (≈ 90 hours). It could be due to the metabolic efforts related to the expression of the heterologous genes. This is also supported by another study in which *A.woodii* was modified with the same genes for acetone synthesis employed in this work, but cloned in a different plasmid and regulated by a different promoter (Hoffmeister et al., 2016). In autotrophic growth, insertion of the plasmid expressing the heterologous genes led to 47% less biomass with respect to the same strain transformed with the empty plasmid.

Obtained results demonstrate that the strategy for storing and revitalizing *A.woodii* plAcC=O was successful. Moreover, the conditions chosen to culture it heterotrophically were validated.

3.2 *A.woodii* plAcC=O resistance to pressure stress

In this work, conversion of CO_2 in added-value products by *A.woodii* has been thought to be at high pressure, to increase feedstock availability. In the ENGICOIN project, the maximum pressure stated for autotrophic growth is 10 bar. Nowadays in literature, no data are available on *A.woodii* grown at this pressure value.

To assess the ability of *A.woodii* plAcC=O to survive at high pressure and to discriminate between the effect on cell growth due to the total pressure of a general gas and the effects due to the partial pressure of a specific feeding gas, the first test in high pressure was performed culturing bacteria in the heterotrophic medium, using nitrogen to pressurize the reactor.

Even though the resistance of bacteria to 10 bar is needed, using a stream of gases to feed the reactor leads to a first step in the fermentation in which the pressure is not stable at the stated value, due to the response time of the equipment to the new condition set. To take into account a phase of fluctuating pressure close to the stated one before stabilization, *A.woodii* resistance to pressure in heterotrophy was assessed at 11 bar. This pressure value is also the maximum pressure level afforded by the reactor equipment.

A preculture of *A.woodii* plAcC=O in exponential phase was inoculated in the heterotrophic medium in the bioreactor. Experimental measurement of the OD_{600nm} in the vessel after inoculum was 0.14. The culture was then grown at atmospheric pressure until it reached the exponential phase (OD_{600} around 0.8). Then the reactor pressure was increased to 11 bar by sparging nitrogen with the maximum in-flow rate allowed by the MFC for supply N_2. This step took around 35 minutes to be completed. Cells were left for 1 hour at 11 bar.

After pressurization, *A.woodii* was stressed by depressurization. To test two operating conditions, cells were recovered following two different protocols:

i) a protocol in which cells reached the atmospheric pressure in a few seconds. Thanks to the 11 bar of overpressure, simply by opening the valve for liquid sampling, the medium with cells comes out from the reactor at the maximum rate allowed (D-fast).

ii) a protocol with a mild depressurization (D-slow). Pressure in the reactor was decreased from 11 bar to 1 bar in 5 hours and then sampling was performed.

Bacterial vitality after the pressurization-depressurization stress was evaluated by re-inoculating the collected culture in the heterotrophic medium, in serum bottles, in a nitrogen atmosphere, and following the growth curve. These results were compared to the growth curve obtained by re-inoculating 2.5 mL of *A.woodii* plAcC=O culture never stressed by pressure (control curve). Growth curves given by bacteria stressed by different protocols were similar (Fig. 13). Moreover, bacteria grown in the control condition showed a specific growth rate of 0.048 h^{-1}. In condition D-fast, the bacterium re-inoculated had a specific growth rate of 0.059 h^{-1}, whereas in D-slow condition it was 0.048 h^{-1}.

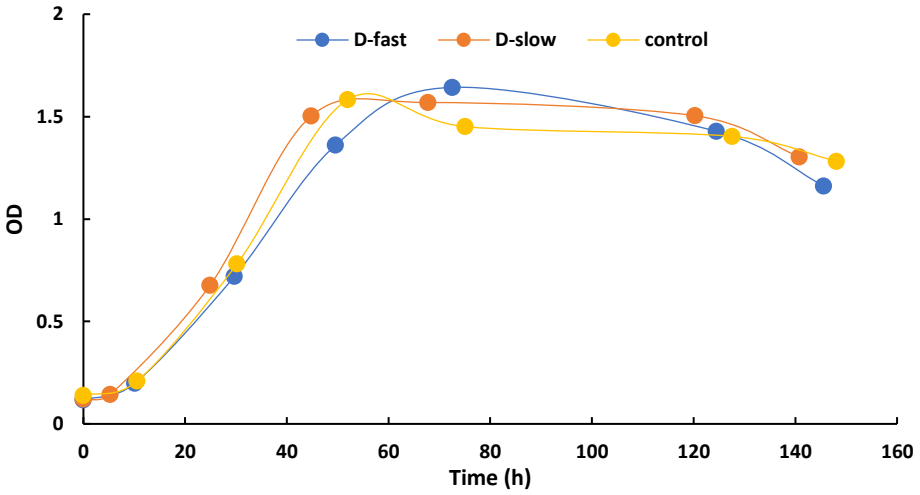


Figure 13. Growth of *A.woodii* plAcC=O in heterotrophic condition (20 mM fructose) after different pressurization and de-pressurization protocols.

Also, the growth curve of the culture left in the reactor was followed. As can be seen in figure 14, it had the typical profile of the growth curve of *A.woodii* grown in heterotrophic condition and the pressurization followed by mild-pressurization protocol didn't perturb it. The specific growth rate calculated was 0.042 h^{-1}, which was similar to the one found for *A.woodii* plAcC=O grown in heterotrophic condition in serum bottle without pressure stress.

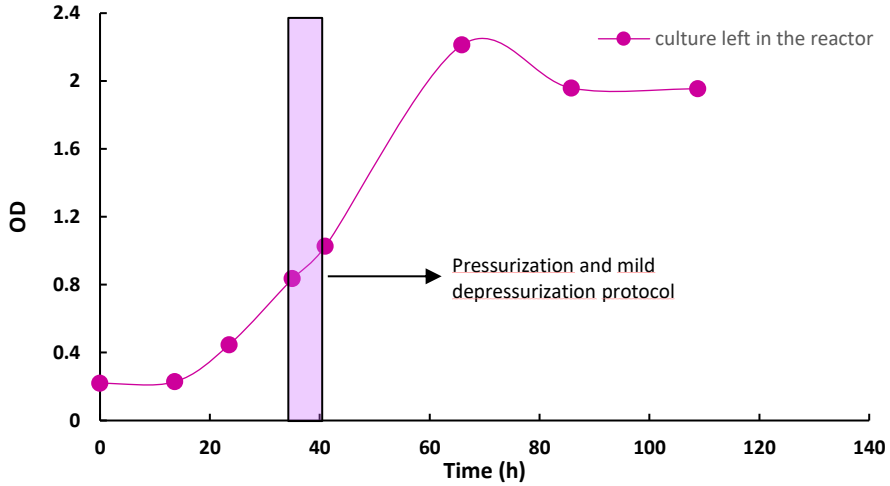


Figure 14. Growth curves of *A.woodii* plAcC=O grown in the reactor in heterotrophic condition. The pink rectangle represents the period in which the protocol of pressurization up to 11 bar and depressurization back to 1 bar was applied.

Moreover, assessment of morphological integrity of cells immediately after the sampling of the culture from the reactor was performed through Gram staining. *A.woodii* is a Gram-positive bacterium so damage to the cells could be detected by the presence of cellular debris or cells with Gram-negative staining, due to the injury to the cell wall. In both the D-fast (Fig. 15 A) and the D-slow case (Fig. 15 B), the morphological cell analysis showed pure cultures of intact Gram-positive cells with rod shape (frequently occurring in pairs) or chain of smaller cells. This result is consistent with the cell morphology described for wild-type *A.woodii* (Balch et al., 1977; Mayer et al., 1977).

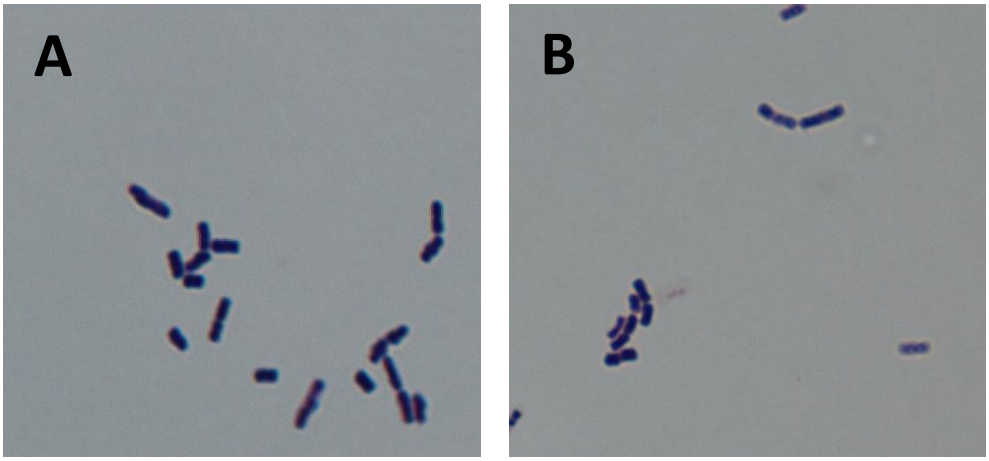


Figure 15. Gram staining of *A.woodii* plAcC=O after pressurization and depressurization protocol. (A) Cells after fast depressurization protocol (D-fast); (B) Cells after mild depressurization protocol (D-slow).

Results of pressurization with nitrogen in heterotrophy showed that *A.woodii* plAcC=O cells can bear a total pressure of 11 bar. Moreover, they are not damaged even by the fastest depressurization rate applicable. This data led to go on with experimental tests of fermentation at high pressure in autotrophy.

3.3. Growth of *A.woodii* plAcC=O in autotrophic condition in serum bottles.

The aim of this thesis is the development of a gas fermentation process based on engineered *A. woodii* strains exploiting CO_2 and H_2 gaseous feedstock under pressurized conditions up to 10 bar in a 2 L fermenter unit. Nevertheless, before testing performances in high pressure, some preliminary studies in autotrophy at atmospheric pressure were carried out, focused on some pivotal parameters such

as the optimal gas mix and the definition of pre-seeding procedure for the bioreactor.

Theoretically, several CO_2 - H_2 mixes can be used for fermentations. According to the method of production, hydrogen can be "labelled" into different colors: gray, blue and green (Ajanovic et al., 2022). Gray and blue hydrogen rely on fossil resources, while green hydrogen relies on renewable sources (wind, solar and hydro-based energy). Gray and blue H_2 are currently more cost-competitive than green H_2. Indeed, according to the National Renewable Energy Laboratory (NREL, USA) and the Klynveld Peat Marwick Goerdeler (KPMG) estimations, the price for H_2 that uses natural gas as the energy source is around 2 - 2.5 USD/Kg H_2, while green hydrogen worth is around 5.25 - 6 USD/Kg H_2. KPMG describes how costs for renewable energy are the bottleneck for green hydrogen production. Technology innovations and supply chain optimization besides investments in sources of renewable energy could determine a cost breakdown to the current price of the gray hydrogen within 2050 (https://home.kpmg/xx/en/home.html, 2020; James et al., 2016). As above stated, the ENGICOIN project envisages the use of green hydrogen to reduce CO_2. Considering the autotrophic feeding mix, the CO_2 is available as a by-product of the AD plant and would not have further usage. According to the European Emissions Trading System (ETS certificates) this feedstock is worth 0.008 USD/Kg CO_2 (Takors et al., 2018). Thus, hydrogen from renewable sources is the gas feedstock with a higher value to be provided to the reactor. For this reason, optimizing the gas ratio by lowering its amount in the mix is attractive for industrial applications.

A screening of two different H_2:CO_2 gas ratios at atmospheric pressure was performed:

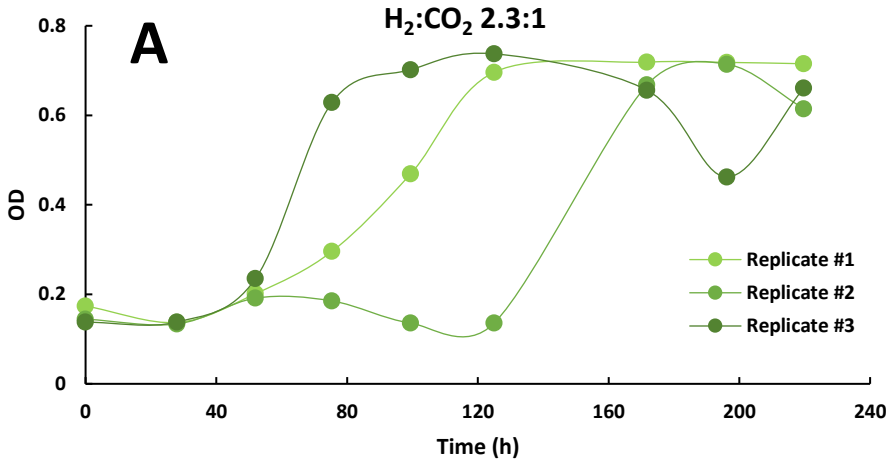
- H_2:CO_2 gas ratio 2.3:1 (in percentage 70% H_2 and 30% CO_2). This mix was chosen based on the literature. Straub and coworkers (2014) indicate this ratio in a work on the enhancement of autotrophic acetate production in *A.woodii*. Moreover, this is the same mix used by Kantzow and coworkers (2016) in a study regarding the effects of H_2 partial pressure on *A.woodii*-mediated fermentation.
- H_2: CO_2 gas ratio 3:1 (in percentage 75% H_2 and 25% CO_2). This feeding mix was suggested by the partners in UNOTT, based on their preliminary studies.

Experiments were performed at atmospheric pressure in serum bottles. Before the inoculum addiction, the autotrophic medium was gassed with the chosen H_2:CO_2 ratio (2.3:1 or 3:1). *A.woodii* plAcC=O for inoculum was grown in the

heterotrophic medium with 20 mM of fructose. As done before for the growth in heterotrophic condition, a volume of preculture was inoculated such that the OD_{600} in the serum bottles was around 0.1. Every 24 hours, the gas was refilled to avoid the block of the growth of bacteria due to feedstock depletion. Experiments were done in triplicate for each condition. OD_{600} and pH values were recorded. Samples for HPLC analysis were collected.

As shown by the growth curves in figure 16, the replicates in the same condition had a different lag phase, ranging from 2 to 5 days, and reached the same maximum OD_{600} (0.716 ± 0.0197) in both gas ratios conditions. Hoffmeister and coworkers (2016) studying different metabolically engineered *A.woodii* strains for acetone synthesis, fed with 70% H_2 and 30% CO_2 gas mix, reported maximum OD_{600} values consistent with the ones found out in this test.

Because delays in growth curves happened in both gas ratios, independently of which one was used, they couldn't be due to the different gas mixtures provided. However, a common behavior can be observed: regardless of the lag phase duration, the exponential phase of the growth curve started when the OD_{600} value was higher than 0.2.



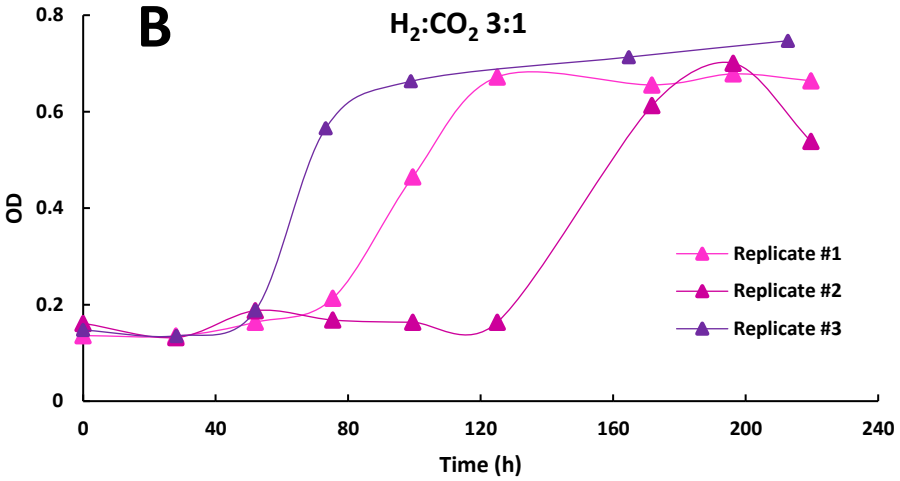


Figure 16. Growth curves in serum bottles of autotrophic culture of *A.woodii* plAcC=O fed with different gas ratios. (A) Autotrophic growth with H_2:CO_2 2.3:1 gas mix; (B) Autotrophic growth with H_2:CO_2 3:1 gas mix.

HPLC analysis of samples collected was performed. Acetate was the main metabolic product, as seen for the heterotrophic cultures, and, as expected, also acetone was synthesized. Nevertheless, formic acid was detected, which was not present in the heterotrophic condition. In particular, it was detected in the lag phase, then fell to 0 in the exponential phase and rose again in the stationary phase of all the growth curves in both 2.3:1 and 3:1 H_2:CO_2 gas mix, but in different amounts. For example, figure 17 shows the growth curve and the formic acid production of the culture grown with the shorter lag phase (replicate #1 of H_2: CO_2 2.3:1 gas ratio). Figure 18, instead, shows the growth curves and the formic acid production of *A.woodii* plAcC=O grown with the longer lag phase (replicate #3 for H_2:CO_2 2.3:1 gas ratio and replicate #2 for H_2:CO_2 3:1 gas ratio). In this latter case, a similar amount of formic acid (≈ 8 mM) in both gas ratios was synthesized during the lag phase. This value decreased to ≈ 0 mM during the exponential phase and increased again when the culture reached the stationary phase.

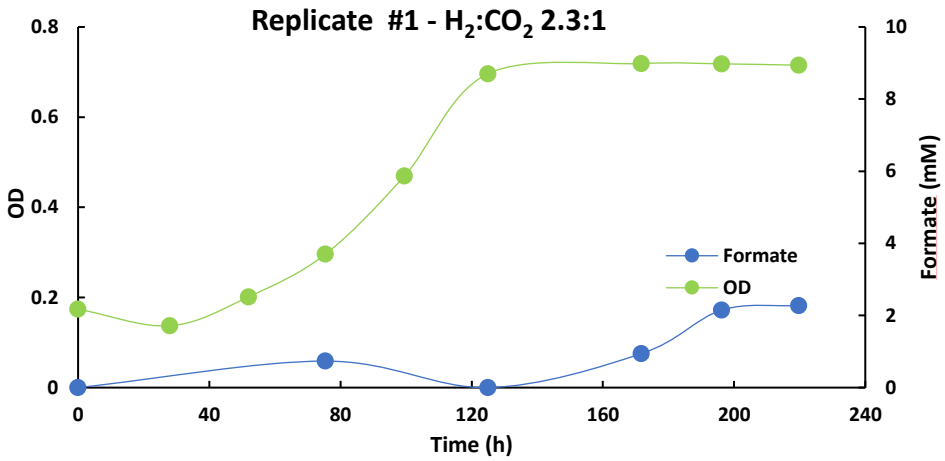


Figure 17. Growth curve and formic acid production in a serum bottle of the autotrophic culture of *A.woodii* plAcC=O with the shorter lag phase (replicate #1 grown with H_2:CO_2 2.3:1 gas mix).

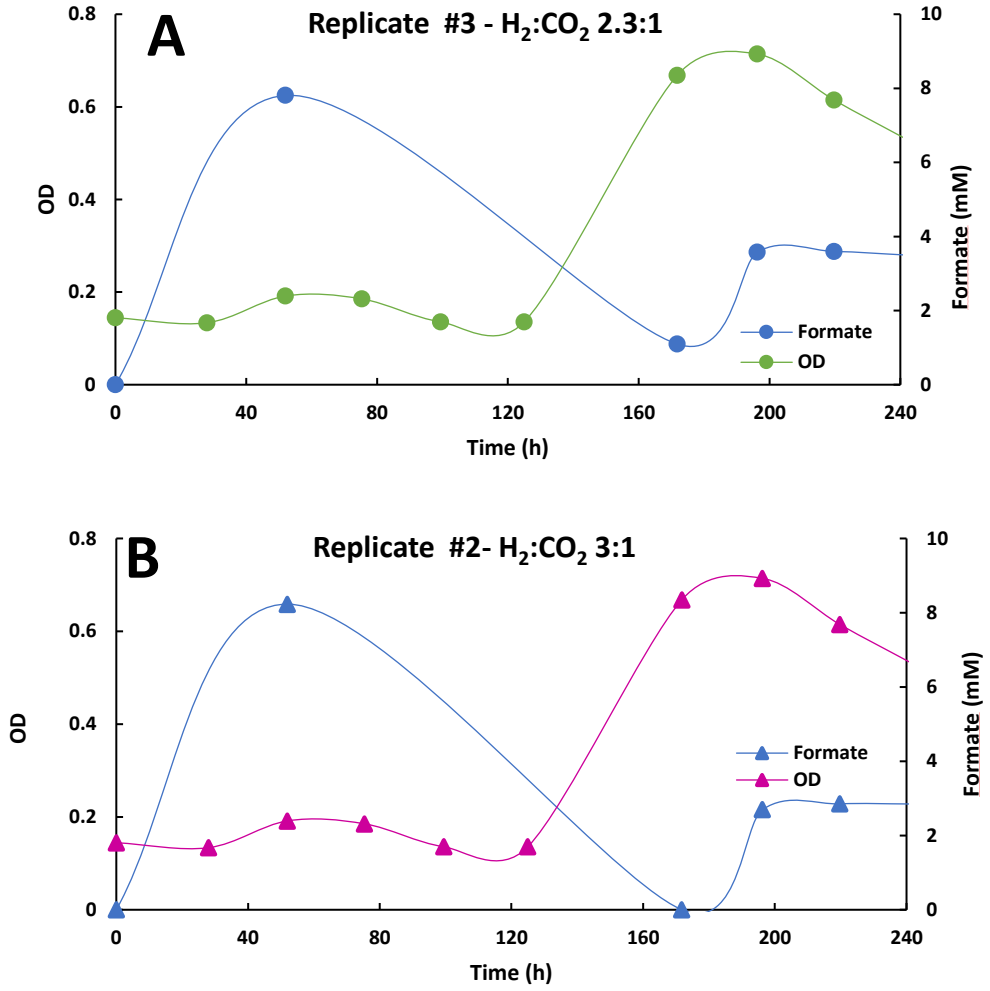


Figure 18. Growth curve and formic acid production in serum bottle of autotrophic culture of *A.woodii* plAcC=O with the longer lag phase. (A) Replicate #3 grown with H_2:CO_2 2.3:1 gas mix; (B) Replicate #2 grown with H_2:CO_2 3:1.

According to the data collected, another experiment with the same settings previously described but with the initial OD_{600} = 0.23 was set up, to test if this condition allows avoiding the lag phase of the growth curve and saving operational time. Again, the two above-mentioned gas ratios were tested. This time, replicates grew with the same behavior. As can be seen in figure 19, the choice of this initial OD_{600} avoided the lag phase, using both gas ratios. Moreover, growth curves of bacteria grown with H_2:CO_2 2.3:1 or H_2:CO_2 3:1 mix were similar.

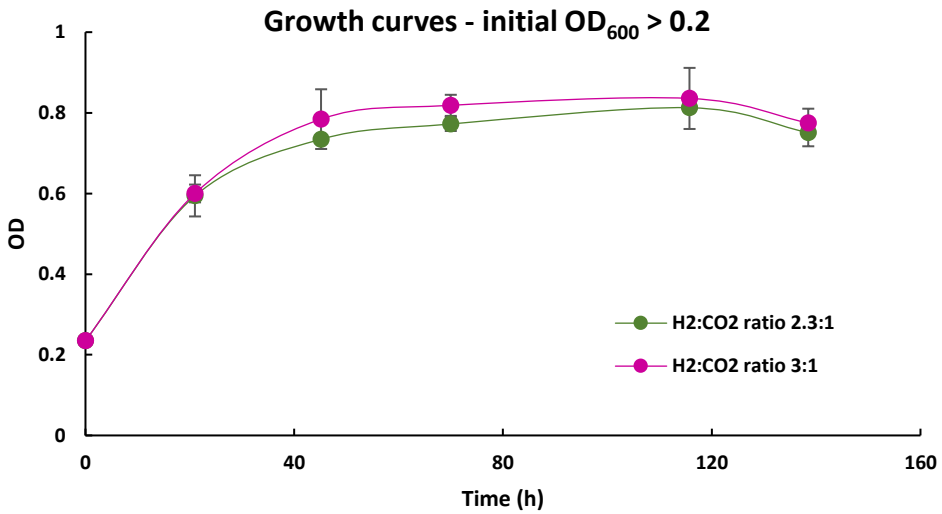


Figure 19. Growth curves in serum bottles of autotrophic cultures of *A.woodii* plAcC=O fed with different H_2:CO_2 gas ratios and initial inoculum OD_{600} > 0.2.

HPLC analysis showed that the acetate and acetone production was comparable between the gas mixtures: *A.woodii* grown with H_2:CO_2 2.3:1 gas mix was able to produce up to 63.1 ± 9.7 mM acetate and *A.woodii* grown with H_2:CO_2 3:1 gas mix was able to produce up to 64.7 ± 11.1 mM of the same product. Acetone was produced up to 2.1 ± 0.9 mM and 2.3 ± 1.4 mM using H_2:CO_2 2.3:1 and H_2:CO_2 3:1 respectively. Formate production was detected in a small amount at the beginning of the growth curves while was not present in the middle of the exponential phase. Finally, it was detected again in the stationary phase, reaching its maximum concentration: 1.56 ± 0.38 mM and 2.00 ± 0.32 mM for H_2:CO_2 2.3:1 and H_2:CO_2 3:1 gas mix, respectively.

Literature indicates that formic acid accumulation can be due to an impairment in the Wood-Ljungdahl pathway. The activity of the enzyme that mediates the second step of this pathway depends on ATP availability in the cell, so ATP scarcity could lead to formate accumulation. Many studies describe the Rnf and ATPase complexes as pivotal for the energetic metabolism of *A.woodii* grown in autotrophic condition. These proteins are responsible for ATP synthesis dependent on the Na^+ electrochemical gradient (see paragraph 1.4). Considering the Na^+ concentration in the medium used in this study, the ATPase complex has a maximum activity at pH ranging from 7 to 9 (Müller et al., 2001).

According to these data from the literature, a test was performed in which the pH of the culture was manually adjusted in a range between 7-7.3 every 24 h with

NaOH. To avoid the lag phase, serum bottles were inoculated with a preculture grown in heterotrophic condition at an initial OD_{600} > 0.2. Because no notable differences were detected in products synthesis using H_2:CO_2 2.3:1 or H_2:CO_2 3:1 mixes, experiments continued with H_2:CO_2 2.3:1 gas ratio as feed, to save hydrogen. For reference, the data collected were compared to a culture grown in the same condition without the pH control. In this latter case pH in the culture fell from 7 to 5.7 (Fig. 20).

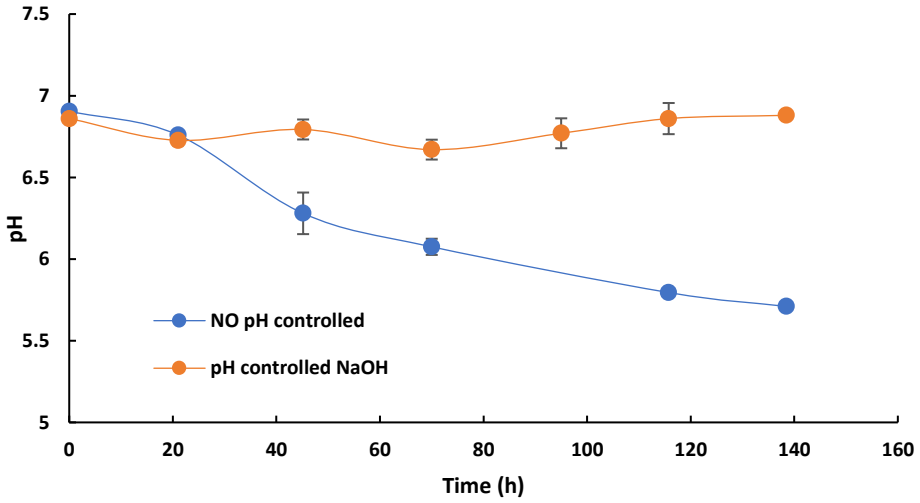


Figure 20. Comparison of pH trend between autotrophic cultures of *A.woodii* plAcC=O grown in autotrophic condition in serum bottles without or with pH control every 24h.

In figure 21, the cumulative amount of gas consumed, and the final biomass are shown respectively. Controlling the pH resulted in 120 % higher consumed gas and biomass than the ones reached without pH control during the culture growth.

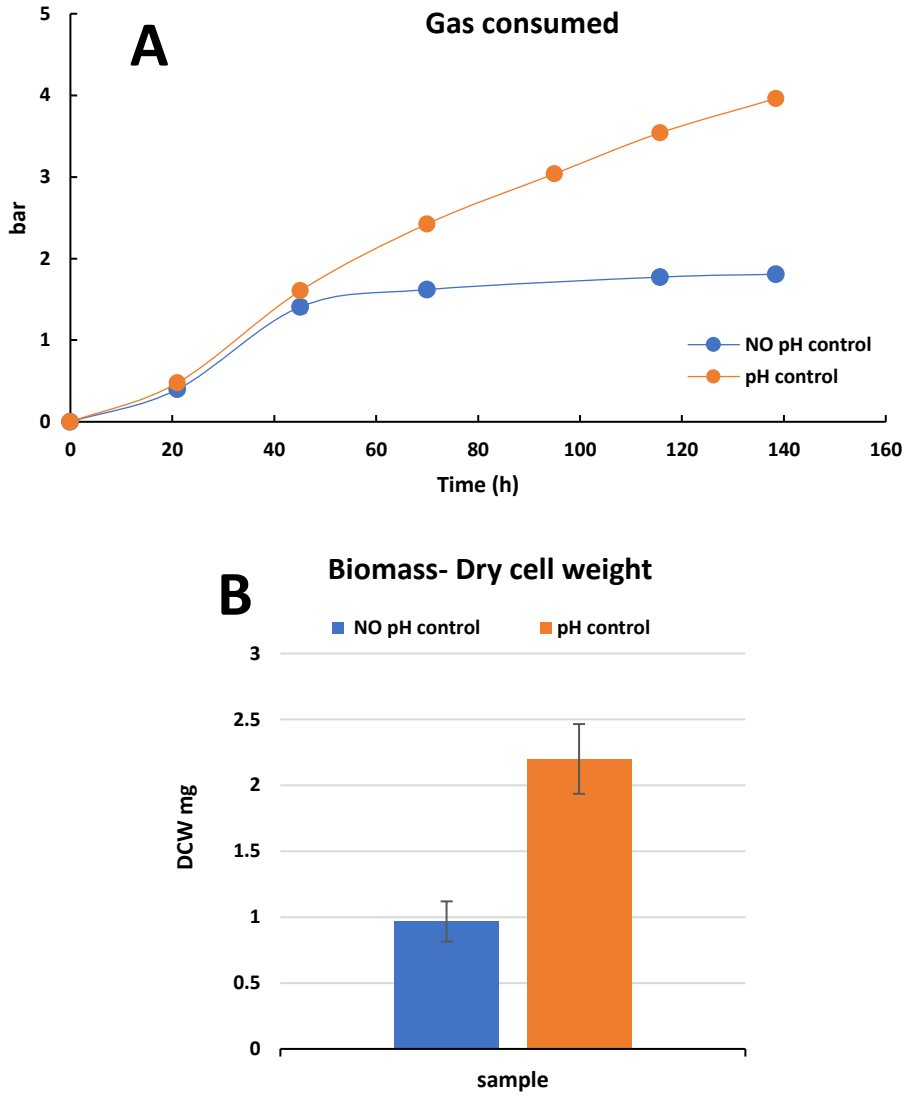
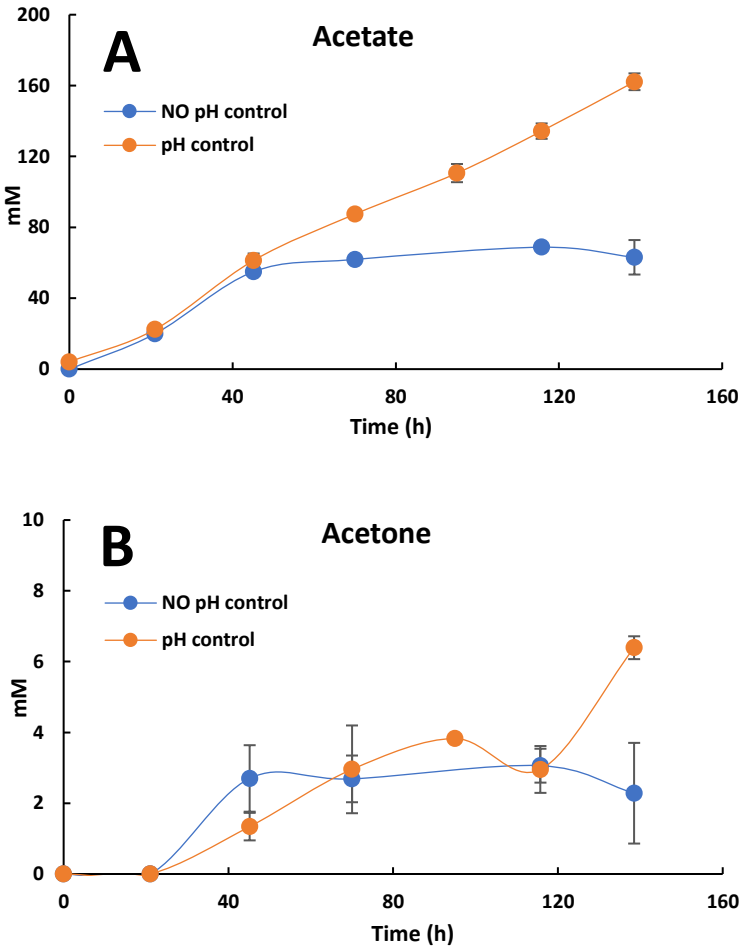


Figure 21. Comparison of gas consumed (A), and final biomass (B) between autotrophic cultures of *A.woodii* plAcC=O grown in autotrophic condition in serum bottles with or without pH control.

Consistently, HPLC analysis showed that controlling pH resulted in 150% higher acetate and acetone production than without pH control. *A.woodii* grown with pH control was able to produce up to 167.2 ± 4.7 mM acetate, while *A.woodii* grown without pH control was able to produce 63.1 ± 9.7 mM of acetate. Moreover, the strain synthesized up to 6.3 ± 0.3 mM and 2.3 ± 1.4 mM of acetone with or without pH control respectively. Regarding the formic acid, as seen for acetate and acetone, the amount increased controlling the pH. Nevertheless, differently

from the other two main fermentation products, this data is affected by error values ranging from 1.7 to 7.2 mM among the replicates (Fig. 22).

The concentration recorded for main metabolic products was in agreement with data described previously in the literature using modified *A.woodii* for acetone production harboring the same plasmid, but with the acetone operon controlled by a different promoter (Hoffmeister et al., 2016). These authors found out acetone synthesis up to 6 ±0.7 mM, while acetate production was lower than the one here measured (about 125 mM). pH control and initial OD_{600} were not mentioned as well as formic acid production. Nevertheless, it has to be noted that in the literature study reported, the fermentation was carried out for 1000 hours. In this study, instead, the stated concentrations were reached in 140 hours.



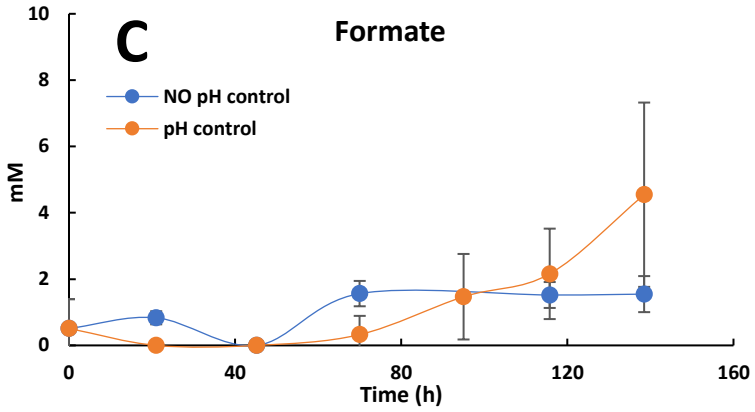


Figure 22. Comparison of production of acetate (A), acetone (B), and formate (C) between autotrophic cultures at atmospheric pressure of *A.woodii* plAcC=O grown with or without pH control.

According to the data collected, inoculation of the reactor was conducted with bacteria in exponential phase, grown in heterotrophy. Initial OD_{600} in the reactor should be > 0.2. The feeding gas ratio chosen was H_2:CO_2 2.3:1, and the pH was controlled at 7 with NaOH.

3.4 Growth of *A.woodii* plAcC=O in autotrophic condition, in gas and liquid batch, at high pressure.

To assess if *A.woodii* plAcC=O can survive at 10 bar in an atmosphere completely composed of feeding gases, an experiment in gas and liquid batch in the reactor was performed. Briefly, cells were inoculated in autotrophic medium and, after a period of adaptation, the pressure was increased up to 10 bar with an atmosphere of 70% H_2 and 30% CO_2. Pressure trend was logged for a short period (overnight) to evaluate if it decreased and its impact of it on biomass growth and metabolic profile.

The experiment was performed in the fermenter with a total volume of 2 L. The volume of the culture at the beginning was 1 L. The stirring speed was set to 400 rpm and the temperature was 30°C. The procedure for inoculum, the pH control, and the feeding gas mix were based on the preliminary experiments done in serum bottles. Therefore, the inoculum was a preculture of *A.woodii* plAcC=O grown in the heterotrophic medium. When moving into the reactor, bacteria were in exponential phase of the growth curve and a volume of preculture was transferred

such that OD_{600} was > 0.2 in the reactor. Before inoculation, the medium without cells was sparged with the gas mix used, as described in paragraph 2.6.2. By doing so, the medium was saturated with the gas chosen for feedstock before inoculation. After the inoculum, the pressure was increased to 1.5 bar to allow cells to shift from heterotrophic metabolism to autotrophic metabolism before increasing the pressure. After 15 h, the pressure was increased up to 10 bar by sparging feeding gasses inside the reactor with the in-flow rate of 150 mL/min utilizing the mix 2.3:1 H_2:CO_2. Using this in-flow gas rate the procedure for increasing pressure took 3.5 hours. By dissolving CO_2, pH fell to 6.2. So, it was risen to 7 by adding NaOH 4.5 M.

The batch fermentation was carried on for 15 hours. In this time, the pressure dropped from 10 bar to 5.1 bar. Pressure trend, OD_{600} growing, and residual fructose consumption are reported in figure 23.

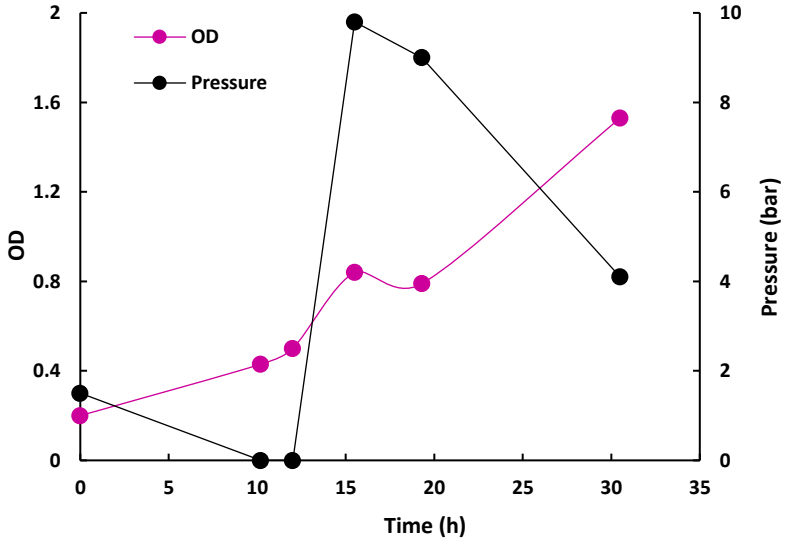


Figure 23. Pressure trend and growth curve of *A.woodii* plAcC=O in liquid and gas batch. The reactor was fed with 2.3:1 H_2:CO_2 gas mix.

As can be seen from figure 23, in 15 hours of batch at low pressure (1.5 bar). OD_{600} doubled its value. During the procedure of pressure increasing to achieve 10 bar, it rose from 0.5 to 0.84 (growth rate 0.148 h^{-1}). Nevertheless, in this step fructose transferred in the autotrophic vessel by inoculating the heterotrophic pre-colure was still present and fast consumed. Finally, OD_{600} increased to 1.53 in the

following 15 hours of batch at high pressure (growth rate 0.04 h^{-1}). HPLC analysis of the samples collected during the experiment (Fig. 24) allowed revealing that acetic acid production started immediately after the inoculum and that had a boost in the phase at high pressure. It could be due to both the shift from heterotrophic to autotrophic metabolism, after the complete consumption of fructose, and the enhanced availability of substrate for the WLP. The final concentration reached was 101.1 mM. The volumetric productivity recorded was 11.49 C-mmol*L^{-1}*h^{-1} while the specific productivity was 25.53 C-mmol*$g_{biomass}^{-1}$*h^{-1}. Acetone synthesis started after 20 hours from the inoculum. This result is consistent with what was seen before in autotrophic serum bottles. The final concentration achieved was 1.84 mM. Volumetric and specific productivities were 0.37 C-mmol*L^{-1}*h^{-1} and 0.82 C-mmol*$g_{biomass}^{-1}$*h^{-1} respectively. Formic acid production was detected during the step of increasing pressure, in which it reached the maximum recorded concentration of 6.2 mM. Nevertheless, at the end of the fermentation, it was not detected anymore, so probably it was reabsorbed and metabolized again through the WLP. Throughout the 15 hours of high-pressure fermentation, 4.9 bar of gas were consumed. According to the C-balance, 44.4% of the CO_2 provided was converted into biomass, acetate, and acetone while 47% was still available in the reactor, dissolved in the medium or in the headspace. Therefore 8.6% should likely be converted in other compounds synthezised in very small amounts and, for this reason, not detected and identified by the HPLC analysis. The greater part of the converted CO_2 was addressed to acetate synthesis: indeed, $\alpha_{acetate}$ was 93.8% while $\alpha_{acetone}$ was 3.1% (for the definition of α_i parameters see paragraph 2.8.1). Yields were 41.7% and 1.4% for acetate and acetone, respectively.

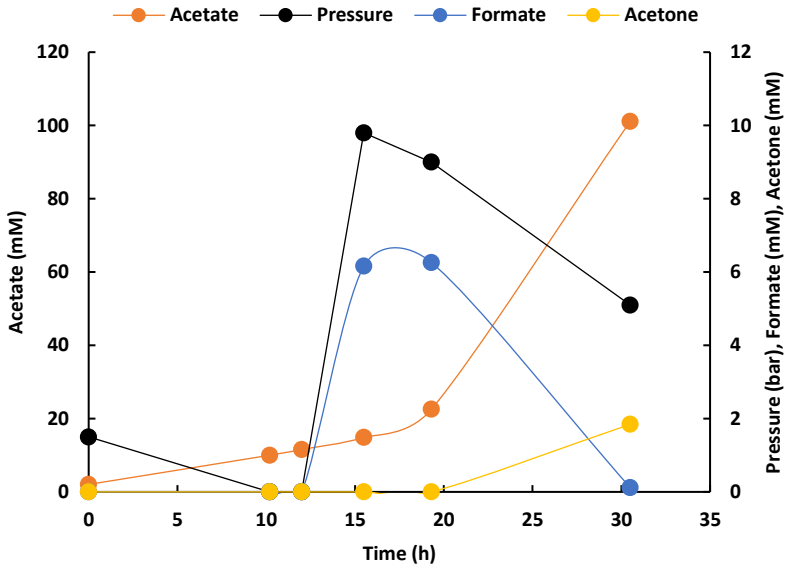


Figure 24. Metabolic profile of *A.woodii* plAcC=O in liquid and gas batch. The reactor was fed with 2.3:1 H_2:CO_2 gas mix.

The highest pressure reported in the literature for wild-type *A.woodii* grown in gas and liquid batch is 5.5 bar (Al Rowaihi et al., 2018). The gas mix used in that study was 85% H_2 and 15% CO_2, provided in such a way that, when the pressure dropped due to the uptake of gas by microorganisms, the drop was recorded by the equipment and the reactor was fed with the gas mix to build the pressure again. By applying this approach the maximum acetate concentration reached was 93.3 mM, so close to the maximum concentration reached in this work (101.1 mM). Nevertheless, in the study at 5.5 bar, the fermentation was carried out for a much longer period (200 hours) and both maximum volumetric and specific productivities were pretty lower (1.33 C-mmol*L^{-1}*h^{-1} and 16.66 C-mmol*$g_{biomass}^{-1}$*h^{-1}, respectively) than the ones achieved here at 10 bar.

The experiment in both gas and liquid batch, up to 10 bar, using feeding gases allowed understanding that *A.woodii* plAcC=O can survive, grow and carry on its main autotrophic metabolic pathway in this operating condition.

3.5 Growth of *A.woodii* plAcC=O in autotrophic condition, at atmospheric pressure, in gas continuous and liquid batch.

By using a strategy of both liquid and gas batch, when the feeding gases are consumed and converted into products dissolved in liquid, the pressure inside the reactor decreases. To maintain the pressure constant at a stated value, the following experiments were performed in continuous gas and liquid batch. In this way, if the gas provided by the in-flow gas rate is higher than the amount consumed by bacteria, the pressure doesn't decrease. Gases were sparged into the medium from a sparger installed in the bottom of the vessel. Moreover, the BPR valve of the reactor allowed maintaining a constant pressure value by its opening when the pressure inside the reactor exceeded the desired setpoint.

To assess the performance of *A.woodii* plAcC=O in the condition of continuous gassing and liquid batch without pressure stress, a first reference experiment was performed in which it was grown at atmospheric pressure. Inoculum strategy, gas feeding ratio, stirring, temperature, and pH control parameters were those previously described for growing in gas and liquid batch fermentation (paragraph 3.4). Immediately after the inoculum, culture was grown for around 24 hours in liquid and gas batch to allow the bacteria to shift between the heterotrophic and autotrophic metabolism before further stress. When continuous gassing started, the in-flow gas rate provided was 63.3 mL/min of H_2 and 27.5 mL/min of CO_2 (70 % H_2 and 30% CO_2 gas mix). To be sure to avoid the access of oxygen, coming from the air, into the reactor a very low overpressure (0.1 barg) was maintained inside the vessel compared to the actual atmospheric pressure, so the fermentation was carried on at a total pressure of 1.1 bar.

Figure 25 shows pressure and OD_{600} recorded during the experiment. As can be seen, when the continuous gassing started, the OD_{600} continued to grow, until reaching the maximum value of 0.9 after 77.25 hours. The specific growth rate calculated was 0.014 h^{-1}.

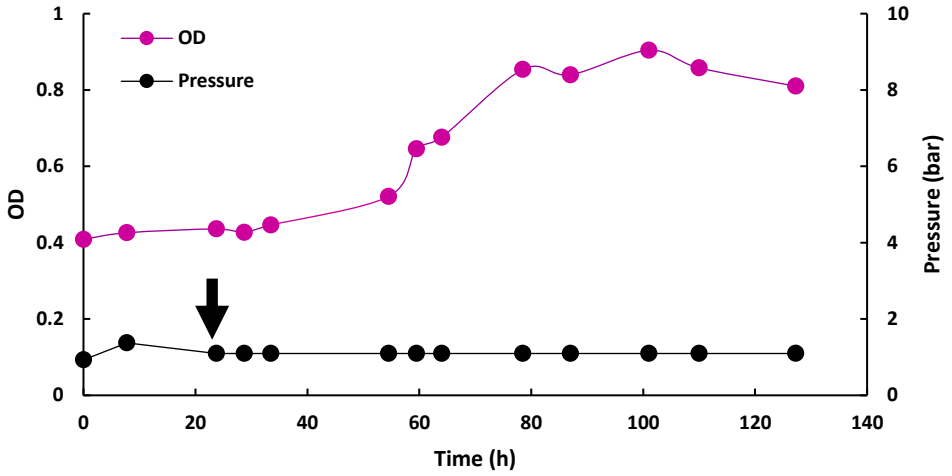


Figure 25. Growth curve of *A.woodii* plAcC=O in autotrophy at 1.1 bar, in liquid batch and continuous gassing. The arrow indicates the start of gas continuous.

Figure 26 illustrates the metabolic profile of *A.woodii* plAcC=O during the test. Bacteria synthesized acetate during all the time of the experiment, reaching a final concentration of 170 mM in the stationary phase. Acetone production had a similar trend and reached its maximum concentration of 8.6 mM at the beginning of the stationary phase. Nevertheless, after this maximum, it slightly diminished to 6.9 mM. Formic acid was produced up to 6.9 mM at the beginning of the continuous providing of gases, then it was reabsorbed in the exponential phase of the growth curve and again detected at the end of the stationary phase, where it reached a maximum value of 14.8 mM.

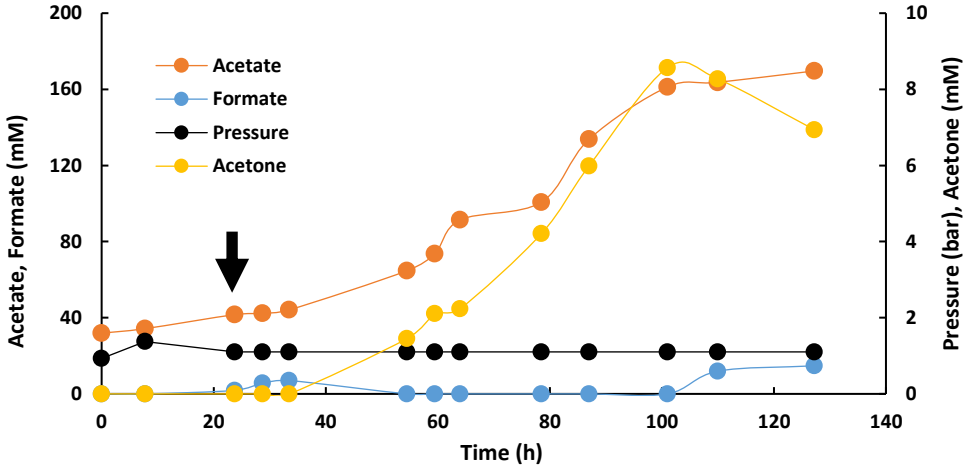


Figure 26. Metabolic profile of *A.woodii* plAcC=O in autotrophy at 1.1 bar, in liquid batch and continuous gassing. The arrow indicates the start of gas.

The conversion in liquid products of the CO_2 provided with the in-flow gas rate was 4.1%. According to the C-balance, most of the carbon dioxide provided was not converted and lost from the outlet of the reactor. Yields at the end of the fermentation were indeed 3.6% of acetic acid, 0.2% acetone, 0.15% formic acid, and 0.1% of biomass. The metabolism of bacteria was directed primarily towards acetic acid production: values of α were 87.3% for acetate, 5.2% for acetone and 3.7% for formic acid. The maximum volumetric productivity for acetate was 7.88 C-mmol*L^{-1}*h^{-1} in the exponential phase, while it was 0.63 C-mmol*L^{-1}*h^{-1} for acetone in the early stationary phase. The maximum volumetric productivity for formic acid was 1.32 C-mmol*L^{-1}*h^{-1} and was recorded at the end of the fermentation. The maximum specific productivities were 31.37 C-mmol*$g_{biomass}^{-1}$*h^{-1}, 1.95 C-mmol*$g_{biomass}^{-1}$*h^{-1}, and 5.88 C-mmol*$g_{biomass}^{-1}$*h^{-1} for acetate, acetone, and formic acid respectively. For each compound, the value of specific productivity indicated was observed in the same phase of the growth curve described above for the volumetric productivities.

The greater part of experiments described in the literature using *A.woodii* as biocatalyst is conducted a stirred tank bioreactor in the condition of liquid batch and continuous gassing, at atmospheric pressure. Table 1 summarizes the results of the studies in the last decade. Almost in all of them, data are on acetate because the wild-type strain was employed. Nevertheless, in the work of Hoffmeister and coworkers (2016), a modified *A.woodii* for acetone production was used. In that work, detailed information about acetate synthesis is missing, but information on acetone production is reported. For this reason, that work is summarized as well in the table to have a comparison with results on acetone obtained in the above-described experiment. It has to be noted that the genetic construct for acetone synthesis harbored by bacteria of Hoffmeister and coworkers was different from the one used in this study.

Table 1. Comparison between parameters and productivities form studies employing *A.woodii* as biocatalyst in stirred tank bioreactors in gas continuous and liquid batch, at atmospheric pressure. r = volumetric productivity; q = specific productivity.

Study	Stirring speed (rpm)	pH	In-flow gas rate (mL/min)	Gas mix H_2:CO_2:N_2	Initial OD	Max OD	μ_{max} (h^{-1})	Max conc. acetate (mM)	Max $r_{acetate}$ (Cmmol* L^{-1}*h^{-1})	Max $q_{acetate}$ (Cmmol* g^{-1}*h^{-1})	Max $r_{acetone}$ (Cmmol* L^{-1}*h^{-1})	Duration (h)
This study	400	7	90.8	70:30:0	0.4	0.9	0.04	170	7.88	31.37	0.63	127
Demler & Weuster-Botz, 2011	400	7	250	90:10:0	1.6	2.4	-	732	10.29	9.57	-	264
Straub et al., 2014	1200	7	500	40:17:43	1.1	4	0.05	744	35.12	28.50	-	89
Kantzow et al., 2015	1200	7	500	40:17:43	5	6.4	0.05	986	-	23.18	-	79
Groher & Weuster-Botz, 2016	600	7	83.3	80:20:0	0.4	2.3	0.08	623	28.24	34.00	-	144
Steger et al., 2017	400	6.8	70	80:20:0	0.1	-	-	361	2.68	-	-	312
Steger et al., 2017	200 + 400	6.8	70	40:10:50	0.1	-	-	492	2.23	-	-	840
Hoffmeister et al., 2016	800	7	500	80:20:0	-	2.1	0.05	1330	-	-	0.20	357

As can be seen from table 1, batch fermentations with continuous gas supply at atmospheric pressure allow dealing with limited gas-liquid mass transfer due to the low solubility of feeding gases increasing stirring, in-flow gas rate, and feedstock partial pressure. A huge range of stirring speed was attempted (200-1200 rpm), as also as the gas stream to feed the reactor (70-500 mL/min). Experiments had different duration and different amounts of biomass in the reactors. This leads to a big difference between the maximum concentration of acetate achieved and also to different volumetric productivities. According to the volumetric productivities of acetate, this study ranks between the lower value recorded, nevertheless, it has also the lowest final OD_{600}. Volumetric productivity is influenced by the number of biocatalyst cells in the volume considered, so the low final OD_{600} reached can explain this parameter. Considering the specific productivity of acetate, indeed, the value recorded in this study is among the higher recorded. Regarding acetone, Hoffmeister and coworkers reported a maximum concentration of 16.5 mM for this compound, while in this study a maximum concentration of 8.6 mM is achieved (data not reported in table 1). Nevertheless, the duration of the fermentation in the two works is different (357 versus 127 hours, respectively). The volumetric productivity measured was higher in this study even if the number of cells in the reactor is lower.

Almost all works listed before don't mention formic acid production during the experiment. Only Groher & Weuster-Botz (2016) referred to the synthesis of this compound. In particular, they describe that it was produced during the early phase of the experiment, then assimilated during the exponential phase, and finally synthesized again to a maximum concentration of 23.9 mM, after 144 hours of fermentation. The synthesis profile of this compound and its final concentration are consistent with the results recorded in the experiment above described.

3.6 Growth of *A.woodii* plAcC=O in autotrophic condition, in gas continuous and liquid batch, at high pressure.

Once evaluated the tolerance of the modified *A. woodii* strain to pressure stress and its performance in autotrophy in gas continuous supply and liquid batch at atmospheric pressure, fermentations in the latter operating condition at 10 bar were performed.

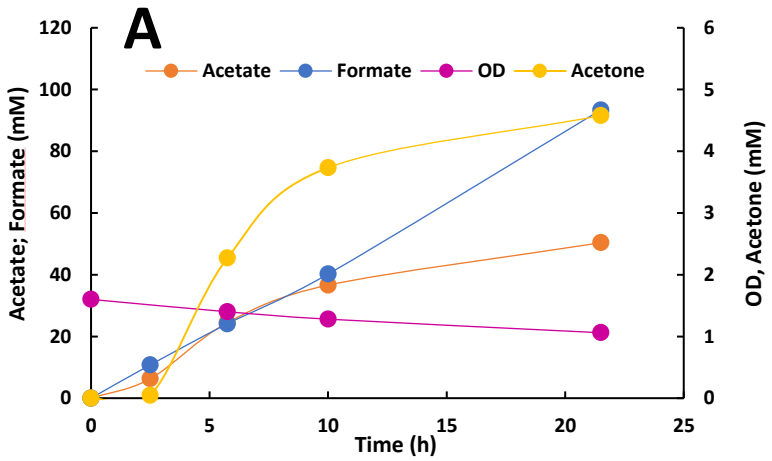
A.woodii plAcC=O was grown using the 2.3:1 H_2:CO_2 gas mix. Because in some cases gaseous substrates are found to be inhibitory (Van Hecke et al., 2019), a

first-round of tests was carried out in which the total pressure of 10 bar was applied with different combinations of in-flow gas rates and cells concentration in the bioreactor. In particular, the following combinations were tested:

a) high cell concentration (OD_{600}= 1.5) and high in-flow gas rate (82.5 mL/min), (Figure 27 A);
b) high cell concentration (OD_{600}= 1.2) and low in-flow gas rate (10 mL/min), (Figure 27 B);
c) low cell concentration (OD_{600}= 0.6) and low-flow gas rate (10 mL/min), (Figure 27 C).

For each experiment, reactor preparation and inoculum were performed as previously described (paragraph 2.6.2). In experiments a) and b) pressure was increased by sparging feeding gasses inside the vessel with the minimum in-flow gas rate allowed by the standard MCFs installed in the hardware of the reactor (82,5 mL/min of the mix 2.3:1 H_2:CO_2). Using this in-flow rate the procedure of increasing pressure took 5 hours. In experiment c), a new pair of MFCs for CO_2 and H_2 was available so it was possible to increase the pressure more slowly (20 mL/min). This procedure took around 20 hours to reach 10 bar.

Figures 27 A, 27 B, and 27 C report growth and metabolic products synthesis from the starting point of the continuous gassing at 10 bar during a), b) and c) experiments respectively. Thus, the 0 point on the abscissa axis corresponds to the condition of a total pressure of 10 bar maintained stable by the in-flow gas rates referred above. Similarly, the same point is the reference point to measure the production of acetate, acetone, and formate. This means that the concentration of these products at that point was considered the 0 mM concentration.



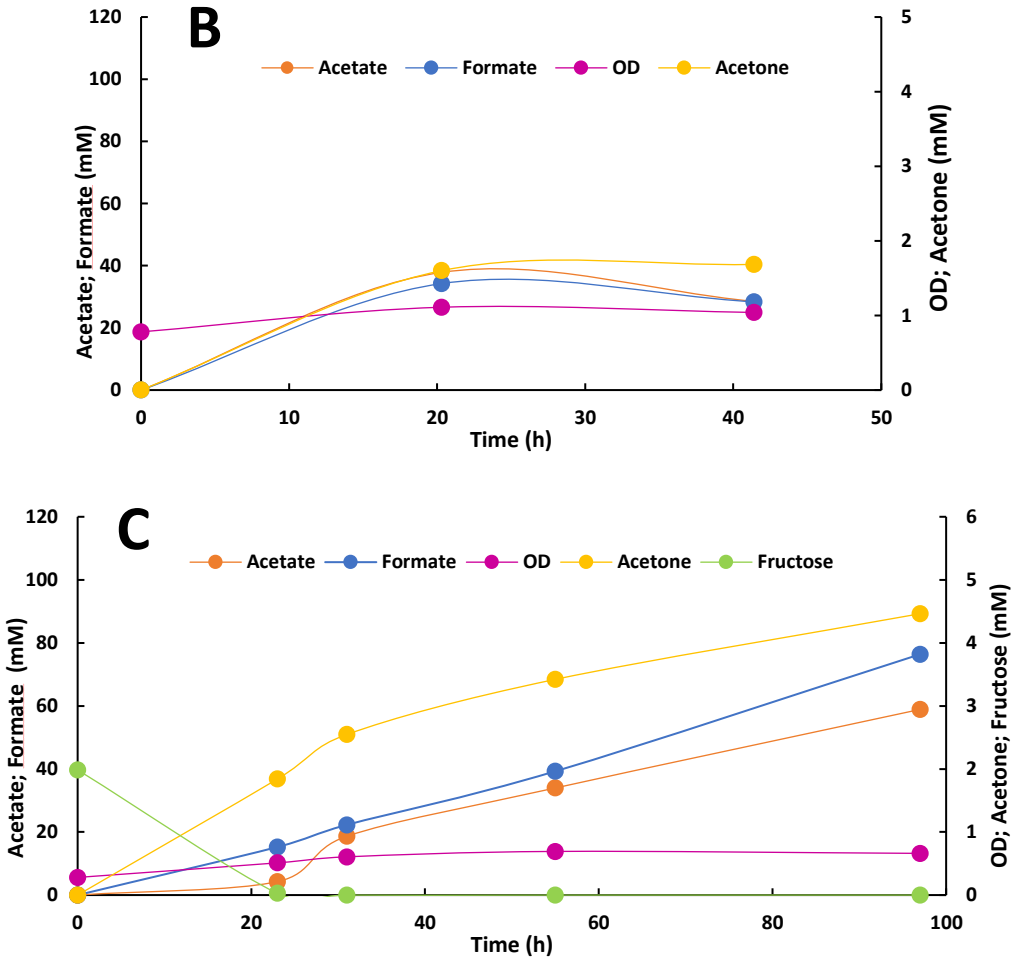


Figure 27. OD_{600} and metabolic profile of *A.woodii* plAcC=O in autotrophy at 10 bar in different conditions. (A) High OD_{600} and continuous high in-flow gas rate (82.5 mL/min) supply; (B) High OD_{600} and continuous low in-flow gas rate (10 mL/min) supply; (C) Low OD_{600} and continuous low in-flow gas rate (10 mL/min) supply.

Regarding the OD_{600} in condition a) it decreased constantly after the increasing of the pressure to 10 bar. In condition b) cell growth stopped quickly after reaching the same pressure, and then had a mild decline. In condition c) cells grew further in high pressure (from OD_{600}= 0.2 to OD_{600} = 0.6) and then stopped. Nevertheless, in this latter condition, fructose (1.9 mM), transferred into the reactor from pre-inoculum, was still present at the beginning of the high-pressure fermentation. When it was completely consumed also in this condition cells stopped growing. Data showed that, despite the in-flow gas rate difference between the conditions

and the different number of cells inside the reactor, increasing and maintaining the pressure at 10 bar using feeding gases inhibits the cell growth.

By increasing the pressure, more substrates for bioconversion are available. In fermentation carried on in the liquid batch operating condition, it could be possible that the accumulation of metabolic products in the medium inhibits the growth of cells. At higher pressure, a high amount of metabolites should be synthesized. As explained above, data reported in figure 27 considers the pressure increase to 10 bar as the starting point. Nevertheless, a small amount of acetate could be produced and gathered in the reactor before that step. Indeed, it was moved in the reactor from the previous inoculation step or synthesized while the pressure was increased. For this reason, the actual maximum concentration of metabolic products in the reactor medium could be slightly higher than the one reported in figure 27 (see also table 3). Thus, table 2 shows the maximum concentration of each metabolic product throughout each complete experiment at the high pressure described above, from the inoculum at atmospheric pressure to the final sampling at high pressure.

Table 2. The maximum concentration of metabolic products throughout complete experiments at 10 bar, in liquid batch and continuous gas supply.

	a) High biomass- High in-flow gas rate	**b)** High biomass- Low in-flow gas rate	**c)** Low biomass- Low in-flow gas rate
Max Acetate Concentration (mM)	168.94	175.98	167.45
Max Acetone Concentration (mM)	8.42	6.64	4.46
Max Formate Concentration (mM)	127.02	50.91	114.83

Different studies are reported in the literature that describe the maximum concentration of products endured by *A.woodii*. Kantzow and coworkers (Kantzow et al., 2015; Kantzow & Weuster-Botz, 2016), working with wild-type and modified strains, report the growth of the bacterium up to a concentration of 600 mM acetate. Kottenhahn and Muller (Kottenhahn et al., 2018), in a study on hydrogen production exploiting formate as the feedstock, describe that wild type *A.woodii* is not inhibited by formate concentration up to 600 mM. This is the maximum tested in the study, so it is possible that the bacterium could tolerate even a higher concentration of this metabolite. Moreover, ENGICOIN partners in UNOTT tested the ability of *A.woodii* plAcC=O to successfully survive at a concentration of acetone up to 500 mM. The maximum concentration of each

metabolic product reached during the fermentations at high pressure described above is far away from the tolerated concentrations reported in the literature. These data suggest that the growth inhibition of *A.woodii* in these experiments was not due to the buildup of products in the liquid medium.

Regarding liquid products, the metabolic profile had the same trend in all conditions. In table 3 are listed CO_2 conversion, yields of metabolic products, and productivities calculated for each test, considering the constant pressure of 10 bar as the starting point. Acetate, acetone, and formate were all synthesized in high-pressure fermentation. Nevertheless, both acetate and formate were the predominant products, while at atmospheric pressure acetate was the only main product. Moreover, in conditions a) and c) acetate and formate yields were similar, while in condition b) the yield of formate is lower with respect to the one of acetate. The highest volumetric productivity and specific productivity for acetate, acetone and formic acid were in the condition of high biomass and high in-flow gas rate fermentation (Table 3).

Table 3. CO_2 conversion, yields, and productivities of *A.woodii* plAcC=O grown in autotrophy at 10 bar with different in-flow gas rate and biomass concentrations.

	a) High biomass- High in-flow gas rate	**b)** High biomass- Low in-flow gas rate	**c)** Low biomass- Low in-flow gas rate
CO_2 conversion in liquid products (%)	13.21	20.00	19.54
Acetate Yield (%)	6.08	12.70	9.30
Acetone Yield (%)	0.97	1.25	0.66
Formate Yield (%)	6.17	4.64	9.03
Biomass Yield (%)	0	1.41	0.55
$\alpha_{acetate}$ (%)	46.0	63.5	47.6
$\alpha_{acetone}$ (%)	7.4	3.4	3.4
$\alpha_{formate}$ (%)	46.7	23.1	46.2
Max Acetate Concentration (mM)	67.83	41.56	61.40
Max Acetone Concentration (mM)	8.42	1.36	4.46
Max Formate Concentration (mM)	127.01	38.37	106.77
Max Volumetric Productivity Acetate (C-mmol*L^{-1}*h^{-1})	11.16	3.73	3.59
Max Volumetric Productivity Acetone (C-mmol*L^{-1}*h^{-1})	2.05	0.24	0.27
Max Volumetric Productivity Formate (C-mmol*L^{-1}*h^{-1})	4.62	1.69	0.88
Max Specific Productivity Acetate (C-mmol*$g_{biomass}^{-1}$*h^{-1})	22.52	10.38	16.94
Max Specific Productivity Acetone (C-mmol*$g_{biomass}^{-1}$*h^{-1})	4.15	0.66	1.60
Max Specific Productivity Formate (C-mmol*$g_{biomass}^{-1}$*h^{-1})	10.40	4.70	4.42

According to the C-balance, the maximum percentage of CO_2 converted in metabolic products is 20% (in condition c). Considering the α parameters, in conditions a) and c) the CO_2 converted was addressed in the same extent towards

acetate and formic acid production. Instead, in condition b) synthesis of acetate is favored with respect to formic acid, even though a substantial part of the carbon dioxide is anyway directed towards the synthesis of the latter compound. It should be noted that in fermentation at 10 bar a huge part of the CO_2 provided was not lost from the outlet of the reactor but stayed in the headspace of the vessel or dissolved in the liquid phase. Because the gas feeding ratio was not changed between the experiment at atmospheric pressure (paragraph 3.5) and the experiments at high pressure, this storage of CO_2 is due to its higher partial pressure at 10 bar with respect to 1.1 bar. In the condition with the higher in-flow gas rate (a), at the end of the experiment, 30.4% of the provided CO_2 remained in the reactor, still not converted by bacteria. In conditions of low in-flow gas rate (b and c), this percentage rose to around 50% of the total CO_2 provided. The storage of CO_2 was negligible in the previous experiment at atmospheric pressure, in which its value was 0.6%. Indeed, in that case, a large part of the CO_2 provided was lost from the outlet of the reactor.

Kantzow and Weuster-Botz (2016) studied *A.woodii* grown in liquid batch and continuous gassing at a total pressure of 3.5 bar. This is the highest pressure described for this operating condition using *A.woodii*. These researchers worked with two different gas mixes. The first one supplied was 40% H_2, 17% CO_2, 43% N_2, so hydrogen partial pressure (pP_{H2}) was 1.4 bar, while carbon dioxide partial pressure (pP_{CO2}) was 0.6 bar. The H_2:CO_2 ratio was 2.3:1. Feeding the reactor with this gas mix and using the wild-type strain as biocatalyst, they recorded growth and formic acid synthesis profiles similar to those observed in test b) above described: first a slight increase of biomass that then declined and a significant formate production beside acetate production. Supplying the second gas mix, 60% H_2, 26% CO_2, 14% N_2 (pP_{H2} =2.1 bar; pP_{CO2} = 0.91 bar - H_2:CO_2 ratio = 2.3:1), led to a behavior analogous to that described above in test a): complete stop of the growth and an even more enhanced formic acid production.

A previous study, conducted in anaerobic flasks, demonstrates a linear relationship between the initial pP_{H2} and the maximum formate concentration synthesized by wild-type *A.woodii* (Peters et al., 1999). Moreover, another study found out that the heterotrophic growth of this bacterium was impaired in an atmosphere consisting only of H_2. The growth of bacterium was immediately restored after replacing the H_2-atmosphere with the N_2-atmosphere (Braun & Gottschalk, 1981). Kantzow and Weuster-Botz (2016) suggest a metabolic overflow due to the inhibition of the enzymes of the central carbon and energy

metabolism by increasing hydrogen partial pressure. The first step of the WLP is the reduction of CO_2 to formate. This step doesn't require co-substrates or elector transfer systems. Further conversion of formate, instead, requires both ATP and tetrahydrofolate (THF). So, the lack of one of these compounds could be a bottleneck to move forward along the metabolic pathway to acetate. To test this hypothesis, in the same study, they grew a modified strain of *A.woodii* overexpressing enzymes of WLP for ATP synthesis with the 40% H_2, 17% CO_2, 43% N_2 gas mix. This overexpression partially restored cell growth and reduced formate production. According to these data from the literature and the data experimentally collected, the lack of ATP could explain a metabolic overflow. The condition of high biomass and low in-flow gas rate (condition b) could manage better this phenomenon.

3.7 Fresh medium pressurization

Some experiments were performed on a fresh autotrophic medium without cells to measure some reference parameters, like changing of redox potential and pH, between atmospheric pressure and high pressure (8 bar) using H_2:CO_2 2.3:1 gas mix.

For these experiments, a set of parallel pressurized bioreactors was used (paragraph 2.4). Their total volume was 0.5 L and the maximum threshold of pressure using hydrogen was 8 bar. The same procedure of the preparation of the reactor for fermentation with bacteria was performed. Briefly, bioreactors of 500 mL total volume, with 200 mL of autotrophic medium in the vessel, were first gassed with N_2 to remove oxygen. The following step of gassing with H_2:CO_2 was performed, instead, following two different protocols. In the first one, the standard procedure was applied: the bioreactor was sparged with 70% H_2 and 30% CO_2 mix at 1.1 bar. In the second protocol, instead, N_2 was removed using 100% H_2. Subsequently, the vessel was pressurized up to 8 bar again by using only 100% H_2, and then the gas mix was changed to 70% H_2 and 30% CO_2. In this phase, pH dropped from 7.3 to 6.7. The pressure was then decreased to 1.1 bar and gas change was continued at atmospheric pressure using the same H_2:CO_2 gas mix. pH didn't change anymore, remaining around 6.7.

After this procedure, bacteria in exponential phase from a heterotrophic preculture were inoculated in bioreactors, and pH was adjusted to 7 by NaOH. Cultures were grown with continuous H_2:CO_2 2.3:1 gassing at a rate of 10 mL/min and monitored for 24 hours at 1.1 bar.

Surprisingly, *A.woodii* plAcC=O exhibited a different behavior depending on the procedure used to remove N_2 and supply the H_2:CO_2 mix to the reactor. (Fig. 28).

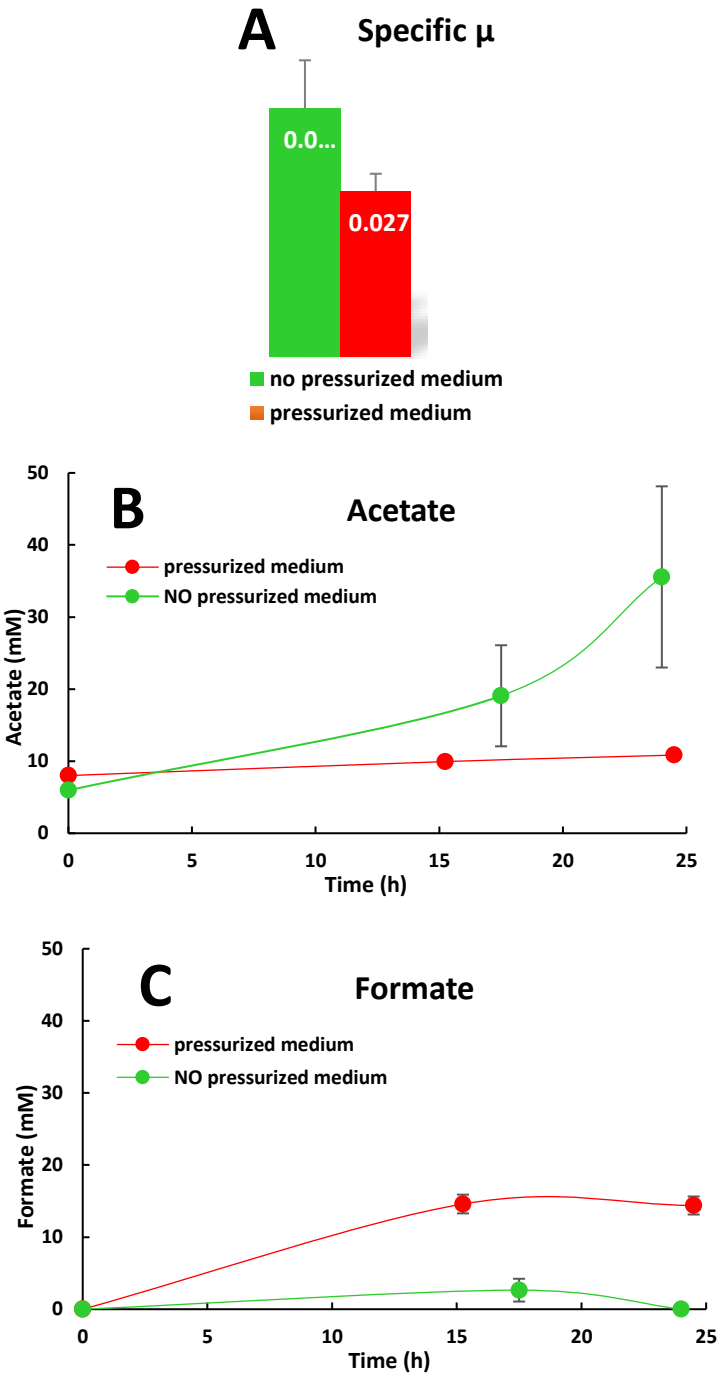


Figure 28. Growth and metabolic production of *A.woodii* plAcC=O inoculated in the reactor after different pressurization protocols of the fresh medium. (A) Specific growth rate; (B) Acetic acid production; (C) Formic acid production.

A.woodii plAcC=O grown in the medium not pressurized before inoculation reached a maximum OD_{600} = 0.79 ± 0.16 in 24 hours (specific growth rate = 0.041 ± 0.08 h^{-1}), like the specific growth rate previously recorded in serum bottles and in the bioreactor at atmospheric pressure. The metabolic profile showed only acetic acid production, up to a concentration of 35.56 ± 12.57 mM. No acetone nor formic acid were detected in 24 hours. Bacteria grown in the pre-pressurized medium, instead, reached a maximum OD_{600} = 0.33 ± 0.02 (specific growth rate = 0.027 ± 0.003 h^{-1}). Moreover, the metabolic profile showed production of both acetate and formic acid, up to 10.84 ± 0.27 and 14.60 ± 1.30 mM respectively. No acetone was detected after 24 hours. The metabolic profile in which both acetate and formate are predominant products is like the behavior observed in experiments in which bacteria were inoculated in the reactor and then grown at high pressure (paragraph 3.6).

In both cases, acetone production was not detected. Nevertheless, as can be observed from the previous tests in serum bottles, in liquid and gas batch at high pressure and in liquid batch and continuous gassing at atmospheric pressure, this compound wasn't produced immediately after the inoculum, like instead production of acetate was. These experiments were monitored for 24 hours after inoculum and probably acetone synthesis was not already started.

These experiments found out that the pressurization of the fresh medium, without bacteria inside it, anyway, leads them to a performance like that observed during growth in high-pressure. This suggests that not the pressure itself in autotrophic condition could be the reason for which cells shifted the metabolic production towards formic acid rather than acetate. It could be something else that happens in the medium at elevated pressure and that is not removable just bringing the pressure back to the atmospheric value. Feeding gas was composed of H_2 and CO_2. Nevertheless, hydrogen has low solubility in water and doesn't react with it. CO_2 instead has higher solubility with respect to H_2 in water and, by reacting with it, can be present in different compounds such as CO_2, H_2CO_3, HCO_3^- and CO_3^{2-}. The distribution between these forms is function of the pH. At pH 7, HCO_3^- is the predominant form (Sato & Amao, 2020; Stumm & Morgan, 1995). At high pressure, a bigger amount of CO_2 dissolves in liquid with respect to atmospheric pressure. This leads to a decrease in the pH that, in these experiments, was risen again to 7 by adding NaOH. It could be possible that, due to the pH effect, CO_2 equilibrium moves towards HCO_3^- form, which becomes the predominant one in liquid medium (Fig. 29).

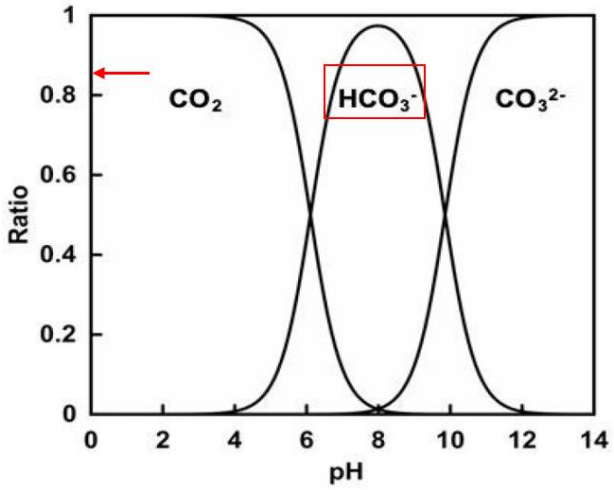


Figure 29. The molar fraction of carbon dioxide, bicarbonate, and carbonate under various pH (modified from Sato & Amao, 2020). The red arrow indicates the ratio of HCO_3^- at pH 7.

3.8 Growth of *A.woodii* plAcC=O in autotrophic condition, in serum bottles with different concentrations of $KHCO_3$

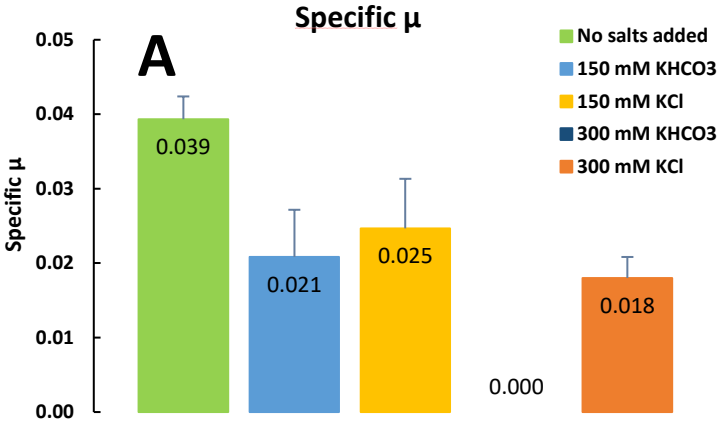
Recently, Schwarz and Müller (Schwarz & Müller, 2020) have reported increased formic acid production by resting cells of the acetogen *Termoanaerobacterium kivui* grown at atmospheric pressure with 300 mM of bicarbonate in the analytical buffer. This research group describes an impairing of ATP synthesis at a high concentration of bicarbonate that could slow down the WLP at the reaction catalyzed immediately after the FDH enzyme, leading to an accumulation of formate.

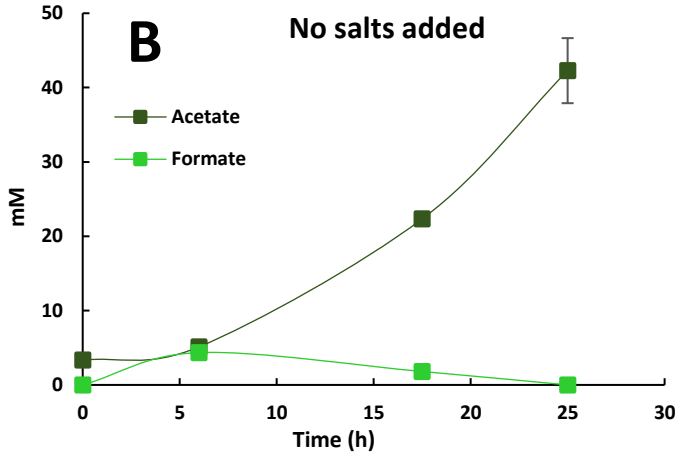
To assess if also *A.woodii* has the same behavior, an experiment in which *A.woodii* plAcC=O cultured in serum bottles with an increasing amount of $KHCO_3$ in the autotrophic medium was performed. Autotrophic serum bottles without $KHCO_3$, with 150 mM and 300 mM $KHCO_3$ were set up. Heterotrophic cultures in exponential phase were used as inoculum. The fed-batch strategy was applied, and pH was controlled at 7.6. KOH was chosen as base to maintain constant the pH in this experiment. Indeed, the generation of ATP in *A.woodii* is based on the Rnf-ATPase complex that is dependent on the Na^+ electrochemical gradient (Müller et al., 2001). Because it could be possible that for different

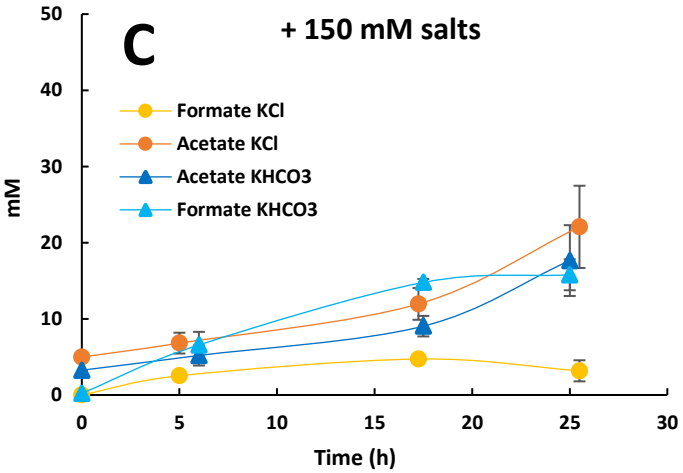
culture conditions different amounts of the base should be added, to avoid the difference of Na^+ concentration in serum bottles, KOH was used. Specific growth rate and metabolic profiles for each condition were analysed (Fig. 30). Results showed that the specific growth rate was strongly impaired with 300 mM $KHCO_3$ in the medium. HPLC analysis showed that acetic acid was the only product in serum bottles without $KHCO_3$, reaching a concentration of 42.28 ± 4.38 mM. In serum bottles with 150 mM and 300 mM $KHCO_3$, acetic acid production was placed side by side by formic acid production. Concentrations recorded after 25 hours with 150 mM bicarbonate in the medium were 17.64 ± 4.65 mM and 15.78 ± 2.03 mM for acetate and formate respectively. After 42 hours of fermentation, in medium supplemented with 300 mM $KHCO_3$, acetic acid concentration reached 16.84 ± 5.55 mM and formic acid concentration 27.36 ± 3.35 mM. No acetone was detected.

To assess if the decreasing of the specific growth rate and the production of formic acid are signals of metabolic stress related to bicarbonate or generally to the osmotic condition, an experiment analogous to the previous one in which $KHCO_3$ was replaced by KCl was set up. More in detail, serum bottles containing autotrophic medium added with 150 mM and 300 mM KCl were prepared. Serum bottles preparation, inoculum strategy, pH control, gas feeding, and sampling points were as previously described.

The specific growth rate in each condition and the metabolic profiles were analysed (Fig. 19r). Results showed that specific growth rate was impaired to a similar extent with 150 mM KCl or $KHCO_3$. By increasing the amount of added salts up to 300 mM, the specific growth rate of bacteria grown in medium added with 300 mM KCl was 0.018 ± 0.003 h^{-1} while in medium added with 300 mM $KHCO_3$ bacteria didn't grow. Acetic acid was the main product for both cultures with KCl added. In cultures with 150 mM of KCl in the medium, the concentration of this compound achieved 22.07 ± 5.40 mM in 25 hours of fermentation. A small amount of formic acid was also produced at the beginning of the curve and then was reabsorbed, even though at the end of the monitored time it was still present at a low concentration (3.17 ± 1.38 mM). In serum bottles with 300 mM of KCl, acetic acid concentration achieved 35.71 ± 5.91 mM after 42 hours of fermentation. Regarding the formic acid, after a mild production at the beginning of the curve, it was completely reabsorbed.


A
Specific µ
Specific µ
0.05
0.04
0.03
0.02
0.01
0.00
0.039
0.021
0.025
0.000
0.018
No salts added
150 mM KHCO3
150 mM KCl
300 mM KHCO3
300 mM KCl


B
No salts added
Acetate
Formate
mM
50
40
30
20
10
0
0
5
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15
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Time (h)


C
+ 150 mM salts
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Acetate KCl
Acetate KHCO3
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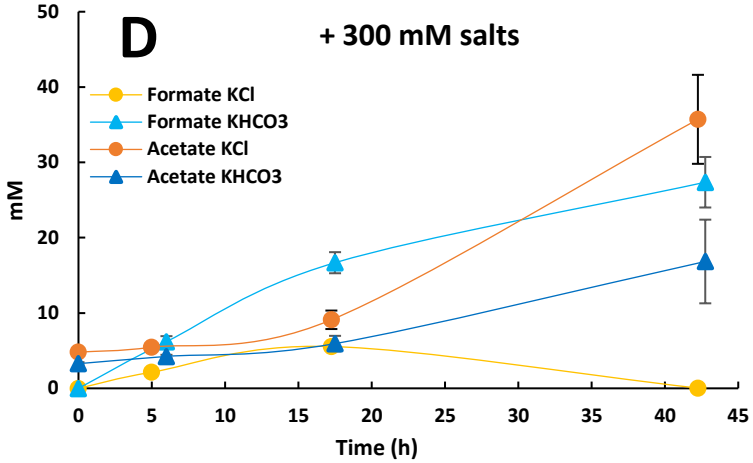


Figure 30. Growth and metabolic production of *A.woodii* plAcC=O in serum bottles with different concentrations of KCl or $KHCO_3$ added to the medium. (A) Specific growth rate; (B) metabolic profile without salts supplement; (C) metabolic profile with 150 mM salts added; (D) metabolic profile with 300 mM salts added.

According to the data collected from experiments in serum bottles with addiction of $KHCO_3^-$ or KCl, *A.woodii* growth and metabolism is influenced by salts concentration in the medium. $KHCO_3^-$ prompts formic acid production and inhibits cell growth. A recent work on resting cells of wild-type *A.woodii* (Schwarz et al., 2020) confirms that bicarbonate addiction to the test buffer during the experiment dramatically stimulated formate formation. Inhibitory effects of bicarbonate on F_1F_0 ATPase are already reported in the literature (Das & Ljungdahl, 2003; Lodeyro et al., 2001). Lodeyro and co-workers suggest that ATP hydrolysis and inhibition of ATP synthesis is affected by HCO_3^- because this ion prevents the binding of ADP to a low-affinity binding site on the F_1 subunit of the ATPase. This interaction modulates the relative affinities of the catalytic site for ATP and ADP.

3.9 Growth of *A.woodii* plAcC=O in autotrophic condition, in gas continuous and liquid batch at high total pressure and low CO_2 partial pressure.

Experiments in the reactor at atmospheric pressure demonstrated that *A.woodii* plAcC=O in liquid batch can grow and carry on WLP if the reactor is continuously fed with 2.3:1 H_2:CO_2 gas mix. Experiments at 10 bar pressure, in the same condition of liquid batch and continuous gassing, showed a stop in

bacteria growth and formic acid production in parallel to acetic acid production. Then, tests on fresh medium pressurization and tests of bacteria grown in serum bottles with an augmented concentration of $KHCO_3$ in the medium revealed that HCO_3^- dissolved in the liquid phase at 10 bar could be responsible for formic acid synthesis. The same experiments in serum bottles with increased KCl concentration revealed instead that salts concentration could be responsible for growth inhibition. Nevertheless, because no exogenous salts were provided at high pressure during fermentation, it stands to reason that providing CO_2 in the reactor at high pressure and pH control at 7 leads to HCO_3^- accumulation. This salt could be, therefore, responsible for the inhibition of bacteria growth too.

According to these data, a new set of experiments were performed, in which the partial pressure of the CO_2 was turned down. The total pressure in the vessel was maintained constant at 10 bar. To keep equal the partial pressure of the hydrogen to the value set in the former experiments, nitrogen replaced the reduction of CO_2 in the gas mix.

Accordingly, a first experiment was performed in which *A.woodii* plAcC=O was grown again at 10 bar, but pP_{CO2} in the feeding mix was reduced to the equivalent value that it should have using the 70% H_2 and 30% CO_2 mix at atmospheric pressure (paragraph 3.5). In particular the gas mix utilized was 2.3:0.1:0.9 H_2:CO_2:N_2 (in percentage 70% H_2, 3% CO_2, 27% N_2). Thus, compared to the gas mix previously provided to feed the reactor at high pressure, CO_2 was decreased to 10%.

Inoculum strategy, gas feeding ratio, stirring, temperature, and pH control parameters were those previously described for growing bacteria in continuous gassing and liquid batch. After the inoculum, culture was grown for around 24 hours in liquid and gas batch to allow the bacteria to shift between the heterotrophic and autotrophic metabolism. Then continuous gassing started, and pressure was increased. The total in-flow gas rate was 90.8 mL/min distributed as follows: 63.3 mL/min of H_2, 3 mL/min of CO_2 and 24.5 mL/min of N_2. In-flow rates of H_2 were equal to the test performed at atmospheric pressure (paragraph 5.3) but higher than the tests performed at high pressure (7 mL/min or 57.5 mL/min), (paragraph 3.6). Nevertheless, this value could not be further reduced due to equipment constraints. 63.3 mL/min of H_2, 3 mL/min of CO_2, and 24.5 mL/min of N_2 in-flow rates, indeed, were the values that satisfy both the

requirement for 2.3:0.1:0.9 H_2:CO_2:N_2 gas ratio and the stability of the in-flow gas rate of CO_2 according to the lower threshold of MFCs.

The experiment was divided into two sections: in the first one, the total pressure was 10 bar. In the second one, it was 1.7 bar. This is the lower pressure that could be reached when the Coriolis MFM is connected downstream to the BPR valve. This measuring instrument, indeed, is a bottleneck that gives an overpressure upstream. When the total in-flow rate was set to 90.8 mL/min, 1.7 bar is the lowest pressure reachable inside the reactor even with the total opening of the BPR valve. For this reason, in this set of experiments, the lowest pressure applied was 1.7 bar.

Figure 31 shows the pressure trend and the OD_{600} recorded during the experiment. As can be seen, when the continuous gassing started, at 10 bar pressure, the OD_{600} continue to grow but had two different slopes: until hour 43 specific growth rate was 0.027 h^{-1}, while from hour 47 cell growth slowed down, having a specific growth rate 0.005 h^{-1}. This phenomenon was different from what was expected. Indeed, according to what was described above, by decreasing the CO_2 provided it was expected to remove the inhibition due to salts dissolved in liquid, restoring the growth of biomass. The specific growth rate in the first part of the section at 10 bar could be due to the consumption of fructose transferred into the reactor with the inoculum, as previously seen for the experiment at 10 bar with a low in-flow gas rate and low biomass inside the reactor (Fig. 27 C). In the second part of the session, it should be like the specific growth rate reported with H_2:CO_2 2.3:1 gas mix at 1.1 bar. Nevertheless, in that case, the specific growth rate was higher: 0.014 h^{-1} (paragraph 3.5). When the pressure was decreased to 1.7 bar, the specific growth rate was 0.004 h^{-1}. However, even though it was slow, it was constant. It could be possible that faster cell growth was not supported by a too low amount of feeding gases dissolved in the liquid medium. Indeed, due to the presence of nitrogen in the mix, the CO_2 partial pressure was merely 0.051 bar, with respect to 0.33 bar in the experiment at 1.1 bar (paragraph 3.5). At the end of the experiment, OD_{600} was 1.3.

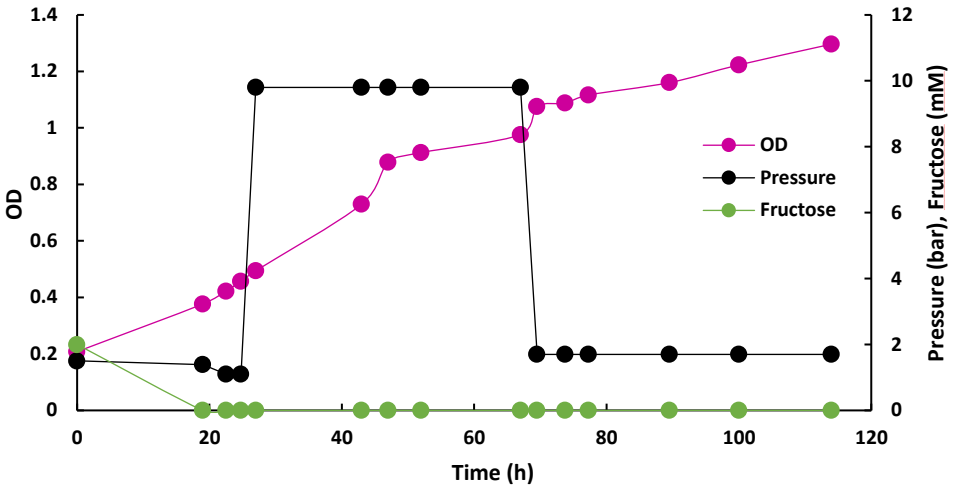


Figure 31. Pressure trend and growth curve of *A.woodii* plAcC=O in liquid batch and gas continuous with 2.3:0.1:0.9 H_2:CO_2:N_2 gas mix.

Figure 32 illustrates the metabolic profile of *A.woodii* plAcC=O during the experiment. Bacteria synthesized acetate during all the time of the experiment. The concentration of this compound had an always growing trend, reaching a final concentration of 199.48 mM at the end of the fermentation. Synthesis of acetone, as previously seen (paragraphs 3.3 and 3.5), started after 24 h from the inoculum and then had a specular trend to acetate, reaching its maximum concentration of 15.09 mM. Formic acid was produced at the beginning of the growth curve (up to 21.39 mM) in the phase of both liquid and gas batch. It was then reabsorbed and remained constant (about 1.8 mM) during the first part of the section at 10 bar. In the second part, it was synthesized again and reached a maximum concentration of 19.43 mM. Finally, in the section at 1.7 bar, it was completely reabsorbed. As expected, by lowering the partial pressure of the CO_2 in the feeding gas mix, formic acid production was strongly reduced and didn't alongside acetate production as previously recorded for experiments at high pressure using the H_2:CO_2 2.3:1 mix (paragraph 3.6).

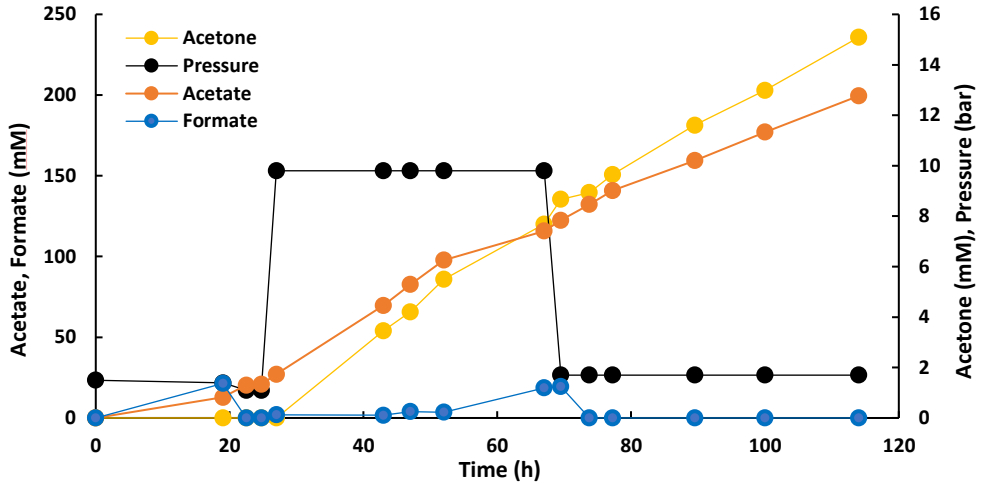


Figure 32. Metabolic profile of *A.woodii* plAcC=O grown autotrophically, in liquid batch and continuous gassing with 2.3:0.1:0.9 H_2:CO_2:N_2 gas mix.

According to the C-balance, 63.4% of the carbon dioxide provided during the 10 bar section was converted into liquid products. The metabolism of bacteria was directed primarily towards acetic acid production (Table 3). Yields at the end of the fermentation at high pressure were 49.6% for acetic acid, 5.4% for acetone, 4.3% for formic acid, and 4.0 % for biomass. The maximum volumetric productivity for acetate was 6.46 C-mmol*L^{-1}*h^{-1}, measured at the end of the part with the higher specific growth rate, while it was 0.77 C-mmol*L^{-1}*h^{-1} for acetone in the part with the lower specific growth rate. The maximum volumetric productivity for formic acid was 1.01 C-mmol*L^{-1}*h^{-1} and was recorded at the end of this section of fermentation at 10 bar. The maximum specific productivities were 22.94 C-mmol*$g_{biomass}^{-1}$*h^{-1}, 2.79 C-mmol*$g_{biomass}^{-1}$*h^{-1}, and 2.8 C-mmol*$g_{biomass}^{-1}$*h^{-1} for acetate, acetone, and formic acid respectively. Higher values of specific productivities of acetate and acetone were observed both at the beginning of the growth at 10 bar. The maximum specific productivity for the formate, instead, was observed at the end of the section.

Regarding the session at 1.7 bar, only 40.6% of the CO_2 provided with the in-flow gas rate was converted into metabolic products, while the rest was lost from the outlet of the reactor during the low-pressure fermentation. Yields were 35.1%, 4.6%, and 0.8% for acetate, acetone, and biomass, respectively. No formic acid was detected at the end of the fermentation. Regarding acetate, the maximum volumetric productivity was 4.91 C-mmol*L^{-1}*h^{-1} while the maximum specific productivity was 11.72 C-mmol*$g_{biomass}^{-1}$*h^{-}1. For acetone, the maximum volumetric productivity was 0.61 C-mmol*L^{-1}*h^{-1} and the maximum specific

productivity was 2.73 C-mmol*$g_{biomass}^{-1}$*h^{-1}. In all cases, these values were recorded at the beginning of the fermentation at low pressure. This could be due to the higher availability of CO_2 in the liquid phase still dissolved from the former section at 10 bar compared to the rest of the fermentation at low pressure. A small amount of CO_2, indeed, could be still present in the liquid phase as CO_2 itself or as HCO_3^- and could be an available storage for consumption by bacteria. As expected, yields, volumetric and specific productivities were lower at 1.7 bar than 10 bar using the 2.3:0.1:0.9 H_2:CO_2:N_2 gas mix. As above described, this could be due to the lower availability of the CO_2 in the liquid phase.

A second experiment was done to confirm the above-described results with a replicate. In doing so, the same strategy of reactor preparation and parameters (stirring, temperature, inoculum, gas feeding mix and rates, pH control) were applied. Also, the division of the experiment into two sections with two different pressures was maintained: the first section at 10 bar, the second at 1.7 bar.

In a similar manner to what was described in figure 31 for the first experiment, figure 33 describes the trend of the pressure and the OD_{600} recorded during the replicate.

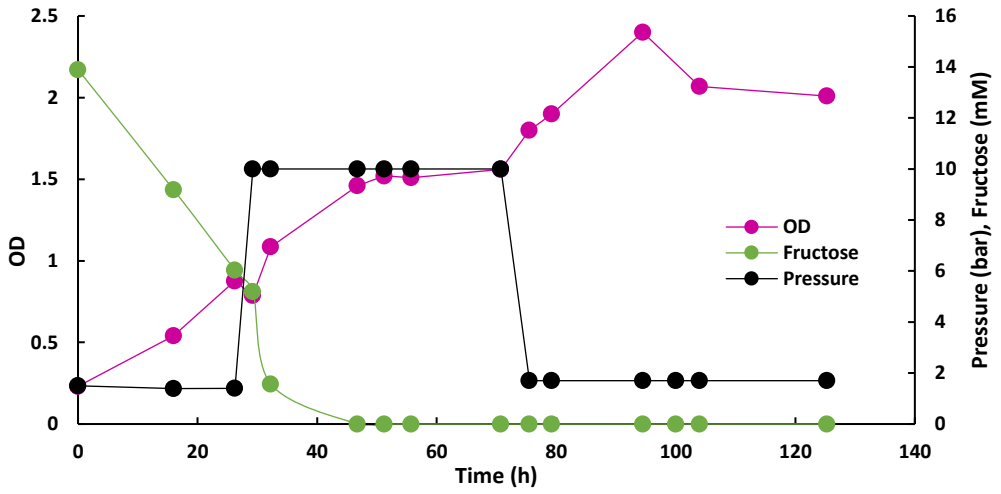


Figure 33. Pressure trend and growth curve of *A.woodii* plAcC=O in liquid batch and continuous gassing with 2.3:0.1:0.9 H_2:CO_2:N_2 gas mix.

As can be seen, also in this experiment at 10 bar pressure, the OD_{600} grew, but with two different slopes: until hour 32 specific growth rate was 0.049 h^{-1}, while in the second part of the section OD_{600} rose very slowly, with a specific growth

rate of 0.002 h^{-1}. This behavior is like what was seen in the previous experiment in the same condition, but with a higher specific growth rate in the first part of the section. Nevertheless, as can be seen in figure 33, a higher concentration of fructose was moved into the reactor with the heterotrophic inoculum. This is probably due to a mistake in the procedure of inoculum preparation. Indeed, as HPLC analysis clearly showed, at the beginning of this test, 13 mM of fructose were present in the vessel, instead of 1 or 2 mM as usually happened when the correct procedure was performed.

The specific growth rate in the second part of the section at 10 bar, instead, showed a comparable value with the one recorded in the first experiment in the same condition (0.002 h^{-1} and 0.005 h^{-1}, respectively).

The specific growth rate recorded during the fermentation at 1.7 bar was 0.017 h^{-1}. This value is lower compared to the specific growth rate observed in the first part of the former section at 10 bar. This trend is like what was observed in the previous experiment.

The metabolic profile of *A.woodii* plAcC=O is shown in figure 34**.** Acetate was produced throughout the experiment, and it had an always growing trend, reaching a final concentration of 220.96 mM at the end of the fermentation. Synthesis of acetone started later than in the previous experiment (70 hours from the inoculum), but it reached a higher final concentration (23.21 mM). This value is the highest concentration of acetone reported in this operating condition for modified *A.woodii*. Formic acid was produced up to 16.72 mM at the beginning of the growth curve, during the growth in both liquid and gas batch and in the earlier part of the fermentation at 10 bar, but it was completely reabsorbed, already in the section of the test at high pressure and wasn't synthesize anymore during the experiment.

Data from this replicate confirmed what was seen in the first experiment, in which the reactor was fed with the 2.3:0.1:0.9 H_2:CO_2:N_2 gas ratio. With respect to the experiments at 10 bar where the 2.3:1 H_2:CO_2 gas mix was provided (paragraph 3.6), at the same total pressure and hydrogen partial pressure (7 bar), by lowering the partial pressure of the CO_2 (0.3 bar) in the feeding gas mix, acetic acid was the main product and formic acid synthesis was strongly reduced.

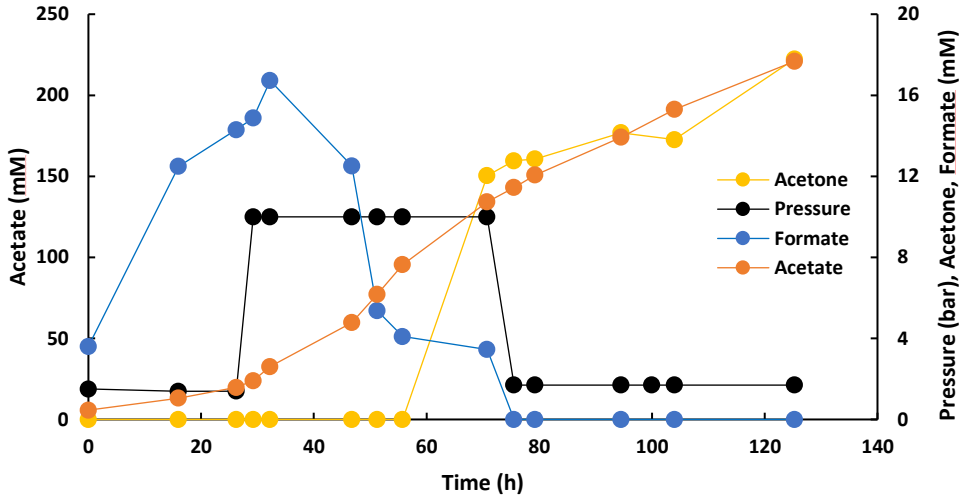


Figure 34. Metabolic profile of *A.woodii* plAcC=O grown autotrophically in liquid batch and continuous gassing using the 2.3:0.1:0.9 H_2:CO_2:N_2 gas mix.

Regarding the section of the replicate performed at 10 bar, carbon balance describes that 66.2 % of the carbon dioxide provided was converted. These values are consistent with those found out in the previous experiment (63.4 %). The same observation can be done about the yields. Indeed, yields at the end of the fermentation at high pressure were 53.13% for acetic acid, 7.0% for acetone, 0.8% for formate, and 5.3% for biomass. The maximum volumetric productivity for acetate was 8.11 C-mmol*L^{-1}*h^{-1} and it was 2.41 C-mmol*L^{-1}*h^{-1} for acetone. Both these values were recorded at the end of the section at high pressure. The maximum volumetric productivity for formic acid was 0.62 C-mmol*L^{-1}*h^{-1} and, as previously described, it was recorded in the earlier part of fermentation at 10 bar. The maximum specific productivities were 16.23 C-mmol*$g_{biomass}^{-1}$*h^{-1}, 4.13 C-mmol*$g_{biomass}^{-1}$*h^{-1}, and 1.73 C-mmol*$g_{biomass}^{-1}$*h^{-1} for acetate, acetone, and formic acid respectively. Higher values of specific productivities of acetate and formate were observed at the beginning of the growth at 10 bar. The maximum specific productivity for acetone, instead, was observed at the end of the same section.

Concerning the section at 1.7 bar, 39.4 % of the CO_2 provided with the in-flow gas rate was converted. Again, this value was consistent with what was measured in the previous test (40.6%). Yields were 34.4%, 3.4%, and 1.5 % for acetate, acetone, and biomass, respectively. No formic acid was detected at the end of the

fermentation. Regarding acetate, the maximum volumetric productivity was 4.08 C-mmol*L^{-1}*h^{-1} while the maximum specific productivity was 8.83 C-mmol*$g_{biomass}^{-1}$*h^{-1}. For acetone, the maximum volumetric productivity was 0.31 C-mmol*L^{-1}*h^{-1} while the maximum specific productivity was 1.6 C-mmol*$g_{biomass}^{-1}$*h^{-1}.

3.10 Growth of *A.woodii* plAcC=O in autotrophic condition, in gas continuous and liquid batch at high total pressure and high CO_2 partial pressure.

A last experiment was performed to close the set of tests in which CO_2 partial pressure was modified to understand how this parameter can influence the metabolism of *A.woodii* plAcC=O. In this test, 70% H_2 and 30% CO_2 gas mix was used and the in-flow gas rate was set to 90.8 mL/min, to have a condition analogous to the experiment at 1.1 bar (paragraph 3.5) and to the two replicates in which the CO_2 partial pressure was decreased to 0.3 bar (paragraph 3.9). As previously done, the first section of the experiment was performed at 10 bar (CO_2 partial pressure was 3 bar) and the second one was performed at 1.7 bar (CO_2 partial pressure was 0.51 bar).

To have a comparable test, all the other parameters (reactor preparation, strategy of inoculum in reactor, temperature, stirring, and pH control) were maintained identical to those applied in the above-mentioned experiments.

Figure 35 shows the pressure trend and the OD_{600} recorded during the experiment. Also this time, when the continuous gassing started at 10 bar pressure, the OD_{600} showed two different slopes: until hour 53 specific growth rate was 0.011 h^{-1}. Nevertheless, differently from what was seen in experiments with low pP_{CO2}, in the second part of the section, bacteria completely stopped growing.

Also, decreasing the pressure to 1.7 bar didn't restore bacteria growth. Indeed, after a little increase of OD_{600} immediately after the shift of the pressure, it continued to decline, throughout all the section at 1.7 bar. Considering only the section at 1.7 bar, the amount of dissolved H_2 and CO_2 in the liquid phase should be similar to the amount present in the experiment at 1.1 bar with the same gas mix (even higher due to the 0.6 barg difference between 1.1 and 1.7 bar). Thus, similar results in bacteria growth could be expected. Nevertheless, they were not similar. The stop of the growing, rather, resembled the behavior observed in experiments where 10 bar of the H_2:CO_2 2.3:1 mix was applied (paragraph 3.6).

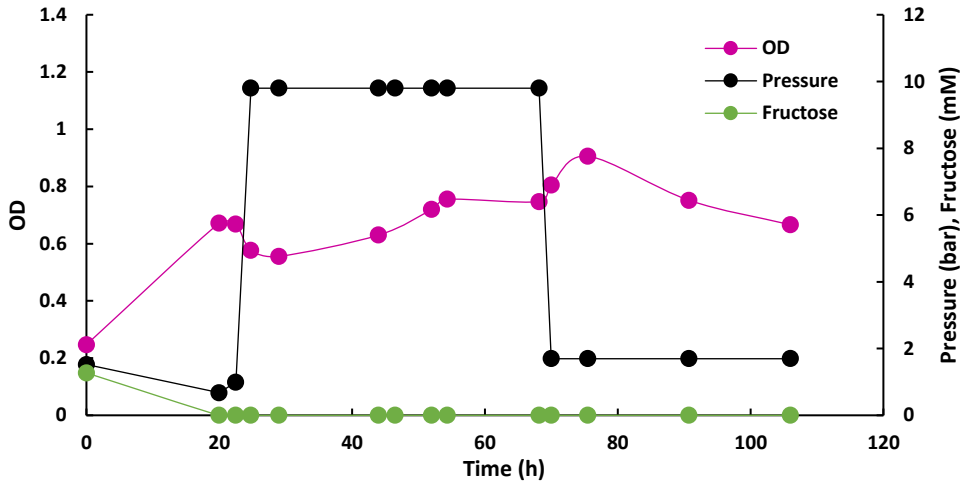


Figure 35. Pressure trend and growth curve of *A.woodii* plAcC=O in the reactor in liquid batch and continuous gassing using 2.3:1 H_2:CO_2 gas mix.

The analysis of the metabolic profile (Fig. 36) revealed that acetate and acetone synthesis had the same trend as what was seen in the previous experiment with the H_2:CO_2:N_2 gas mix. Acetate was produced throughout the experiment, reaching a final concentration of 131.9 mM. Synthesis of acetone started about 40 hours after the inoculum and reached a final concentration of 1.7 mM. Both these concentrations, however, were lower than those recorded in the experiments in which the pP_{CO2} was kept at 0.3 bar. Formic acid increased its concentration throughout the section at 10 bar, achieving a maximum value of 74.89 mM at the end of this step. In the following section at 1.7 bar, a low amount of this compound was reabsorbed but it finally stabilized its value at 64 mM ± 2.11 mM.

Data from this test were consistent with what was seen in fermentations carried out at 10 bar utilizing continuous gassing with 70% H_2 and 30% CO_2 gas mix. At 10 bar, bacteria growth was inhibited, and metabolic flow shifted towards formic acid production. Nevertheless, also lowering the pressure to 1.7 bar didn't restore bacteria growth nor allow complete formic acid reabsorption. A possible explanation of these phenomena at 1.7 bar could be the former step at 10 bar. As described when the fresh medium was pressurized, indeed, it brought to a high concentration of CO_2 in the liquid phase, that according to the pH, was prevalently in form of bicarbonate in the culture. Probably this salt was not immediately removed just by decreasing the pressure, so bacteria growth and WLP were still impaired.

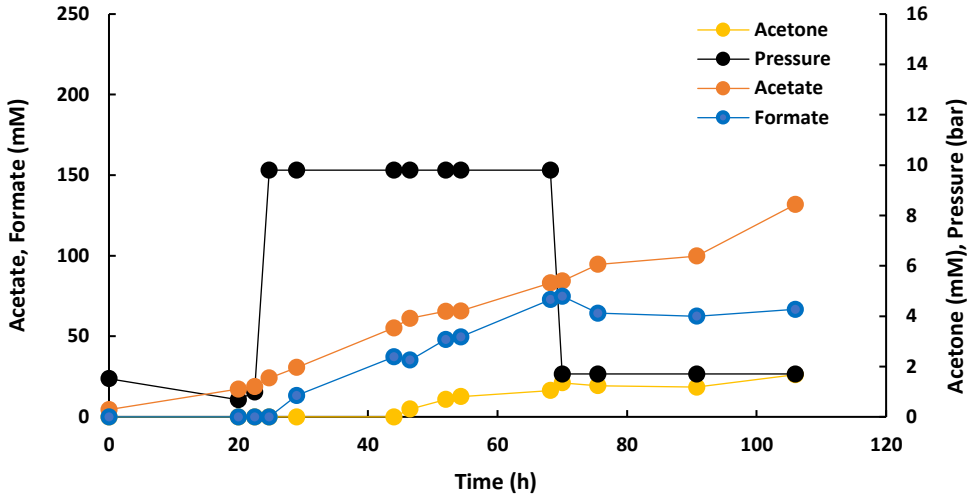


Figure 36. Metabolic profile of *A.woodii* plAcC=O grown autotrophically in the reactor, in liquid batch and continuous gassing using 2.3:1 H_2:CO_2 gas mix.

According to the C-balance, 7.0 % of the CO_2 provided was converted into liquid products. Metabolism was directed towards both acetic acid and formic acid production (Table 3). Yields at the end of the fermentation at high pressure were 4.6% for acetic acid, 0.1% for acetone, 2% for formic acid, and 0.4% for biomass. The maximum volumetric productivity for acetate was 4.81 C-mmol*L^{-1}*h^{-1}, for acetone was 0.37 C-mmol*L^{-1}*h^{-1} and for formic acid was 3.12 C-mmol*L^{-1}*h^{-1}. These values were recorded at the beginning of the section at 10 bar. The maximum specific productivities were 19.38 C-mmol*$g_{biomass}^{-1}$*h^{-1}, 1.49 C-mmol*$g_{biomass}^{-1}$*h^{-1}, and 14.54 C-mmol*$g_{biomass}^{-1}$*h^{-1} for acetate, acetone, and formic acid respectively.

Regarding the session at 1.7 bar, 3.2 % of the CO_2 provided with the in-flow gas rate was converted. Yields at the end of the step were 3.1%, 0.02%, and 0.02% for acetate, acetone, and biomass, respectively. As previously described, formic acid increased at the beginning of the step, but then a small amount was reabsorbed and finally its concentration stabilized. For this reason, considering the boundary points of the section, the overall yield for formic acid was 0%. Considering acetate, the maximum volumetric productivity was 4.91 C-mmol*L^{-1}*h^{-1} while the maximum specific productivity was 15.63 C-mmol*$g_{biomass}^{-1}$*h^{-}1. For acetone, the maximum volumetric productivity was 0.1 C-mmol*L^{-1}*h^{-1} while the maximum specific productivity was 0.36 C-mmol*$g_{biomass}^{-1}$*h^{-}1. In all cases, these values were recorded at the end of the fermentation at low pressure.

Tables 3 and 4 summarize CO_2 conversion, yields, and productivities recorded for all the experiments in which the partial pressure of the CO_2 was modified maintaining constant the pP_{H2} and the total pressure (paragraphs 3.9 and 3.10). Also, parameters recorded for the reference experiment at 1.1 bar are reported (paragraph 3.5).

Table 3. CO_2 conversion, yields, and maximum concentrations of metabolic products obtained growing *A.woodii* plAcC=O in autotrophy with different CO_2 partial pressures.

	1.1 bar	**10 bar replicate 1**	**10 bar replicate 2**	**1.7 bar replicate 1**	**1.7 bar replicate2**	**10 bar**	**1.7 bar**
Gas mix provided	H_2:CO_2 2.3:1	H_2:CO_2:N_2 2.3:0.1:0.9	H_2:CO_2:N_2 2.3:0.1:0.9	H_2:CO_2:N_2 2.3:0.1:0.9	H_2:CO_2:N_2 2.3:0.1:0.9	H_2:CO_2 2.3:1	H_2:CO_2 2.3:1
Tot in-flow rate	90.8	90.8	90.8	90.8	90.8	90.8	90.8
CO_2 conversion in liquid products (%)	4.1	63.4	66.2	40.6	39.4	7.0	3.2
Acetate Yield (%)	3.6	49.6	53.1	35.1	34.4	4.6	3.1
Acetone Yield (%)	0.2	5.4	7.0	4.6	3.4	0.1	0.02
Formate Yield (%)	0.15	4.3	0.8	0.0	0.0	2.0	0.0
Biomass Yield (%)	0.1	4.0	5.3	0.8	1.5	0.4	0.02
$\alpha_{acetate}$	87.3	78.3	80.2	86.5	87.3	65.5	98.7
$\alpha_{acetone}$	5.2	8.6	10.6	11.5	8.6	1.2	0.6
$\alpha_{formate}$	3.7	6.9	1.2	0.0	0.0	28.2	0.0
Max Acetate Conc (mM)	170.0	88.8	101.6	77.0	77.8	59.3	47.6
Max Acetone Conc (mM)	8.6	7.7	12.0	6.4	5.0	1.1	0.33
Max Formate Conc (mM)	14.8	16.7	0.0	0.0	0.0	59.5	0.0

3.10 Growth of A.woodii plAcC=O in autotrophic condition, in gas continuous and liquid batch at high total pressure and high CO2 partial pressure. 81

Table 4. Volumetric and specific productivities obtained growing *A.woodii* plAcC=O in autotrophy with different CO_2 partial pressures.

	1.1 bar	**10 bar replicate 1**	**10 bar replicate 2**	**1.7 bar replicate 1**	**1.7 bar replicate2**	**10 bar**	**1.7 bar**
Gas mix provided	H_2:CO_2 2.3:1	H_2:CO_2:N_2 2.3:0.1:0.9	H_2:CO_2:N_2 2.3:0.1:0.9	H_2:CO_2:N_2 2.3:0.1:0.9	H_2:CO_2:N_2 2.3:0.1:0.9	H_2:CO_2 2.3:1	H_2:CO_2 2.3:1
Max Volumetric Productivity Acetate (C-mmol*L^{-1}*h^{-1})	7.88	6.46	8.11	4.91	4.08	4.81	4.91
Max Volumetric Productivity Acetone (C-mmol*L^{-1}*h^{-1})	0.63	0.77	2.41	0.61	0.31	0.37	0.10
Max Volumetric Productivity Formate (C-mmol*L^{-1}*h^{-1})	1.32	1.01	0.62	0.0	0.0	3.12	0.0
Max Specific Productivity Acetate (C-mmol*$g_{biomass}^{-1}$*h^{-1})	31.37	22.94	16.23	11.72	8.83	19.38	15.63
Max Specific Productivity Acetone (C-mmol*$g_{biomass}^{-1}$*h^{-1})	1.95	2.79	4.13	2.73	1.6	1.49	0.36
Max Specific Productivity Formate (C-mmol*$g_{biomass}^{-1}$*h^{-1})	5.88	2.80	1.73	0.0	0.0	14.54	0.0

Lowering the pP_{CO2} led to reducing the synthesis of formic acid during the fermentation using *A.woodii* plAcC=O. In paragraph 3.6 was already described that Kantzow and coworkers, in experiments in liquid batch and continuous gassing at 3.5 bar using different pP_{H2}, recorded the stop of bacteria growth and formic acid production. These authors suggest that pP_{H2} is responsible for both behaviors. Nevertheless, from experiments conducted in this thesis, these two phenomena could be related to the pP_{CO2}, because in the above-described experiments pP_{H2} in the reactor was maintained constant. Kantzow and colleagues, in contrast, in their experiments kept constant the ratio between H_2 and CO_2 in the feeding mix. In this way, increasing the hydrogen partial pressure also the carbon dioxide partial pressure increases. Estimating the concentration of the HCO_3^- in the liquid medium according to the formula for open CO_2 systems (paragraph 2.8), the concentration of this ion in the experiments performed by Kantzow and coworkers using the 60% H_2, 26% CO_2, 14% N_2 gas mix at 3.5 bar (pP_{H2} = 2.1 bar) should be ≈ 120 mM. In this thesis, using the 70% H_2, 3% CO_2, 27% N_2 gas mix at 10 bar (pP_{H2} = 7 bar), the maximum HCO_3^- concentration should reach ≈ 28 mM. In this condition, formic acid production was not enhanced. Using the 70% H_2, 30% CO_2 gas mix at 10 bar (pP_{H2} = 7 bar), instead, the estimated maximum bicarbonate concentration in the liquid is ≈ 275 mM. This value is even higher than the one applied in the work of Kantzow and colleagues and the growth profile and formic acid synthesis were comparable.

It has to be noted that, even applying low pP_{CO2}, after about 48 hours of fermentation at 10 bar bacteria stopped growing. Interestingly, immediately after lowering the pressure from 10 bar to 1.7 bar, a rapid increase in biomass was recorded. This happened also in the fermentation conducted using 70% H_2, 30% CO_2 gas feeding: immediately after lowering the pressure at 1.7 bar, OD_{600} rose quickly, and then growth stopped again. This phenomenon could be compared to what was described by Braun and coworkers (1981) (Braun & Gottschalk, 1981). These authors grew wild-type *A.woodii* in the heterotrophic medium in H_2-atmosphere and recorded growth arrest. The growth of the bacterium was immediately restored after replacing the H_2-atmosphere with the N_2-atmosphere. It is possible, thus, that *A.woodii* growth is affected by both pP_{CO2} and pP_{H2}.

3.11 Influence of acetate on acetone production in *A.woodii* plAcC=O.

Performing experiments both in serum bottles and reactor, a particular behavior of *A.woodii* plAcC=O was noted: synthesis of acetone is influenced by the concentration of acetate.

Tests performed in serum bottles in which the pH was controlled (paragraph 3.3) achieved a higher concentration in acetate and acetone than the same condition without pH control. Similarly, in all experiments in the reactor in which acetate production was stressed, acetone reached a higher concentration, independently from the condition in which these tests were carried on. If concentration of acetate overcame the threshold of 100-120 mM, acetone synthesis was fostered. Data collected highlighted that, after a first step of production, often acetone concentration stabilized at a certain value which is different for different fermentations. Nevertheless, a common characteristic was that, if the concentration of acetate overcame the above-stated threshold, synthesis of acetone started again. Figures 37- 41 show some of the experiments in which this phenomenon was recorded. Each graph represents a different operating condition. The red circle indicates the stalemate of acetone synthesis, the green circle indicates acetate concentration ≈ 100-120 mM.

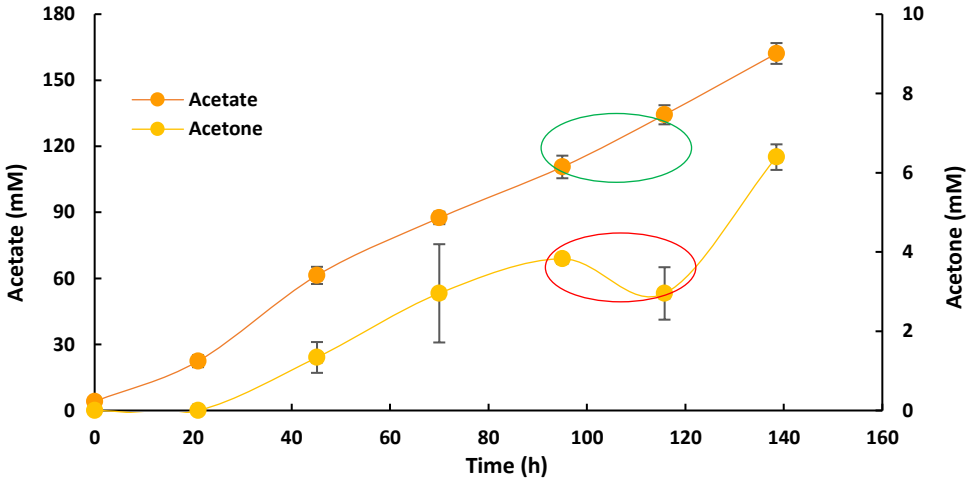


Figure 37. Acetate and acetone trends in the fermentation performed using *A.woodii* plAcC=O grown in serum bottles with pH control, fed with 2.3:1 H_2:CO_2 gas mix.

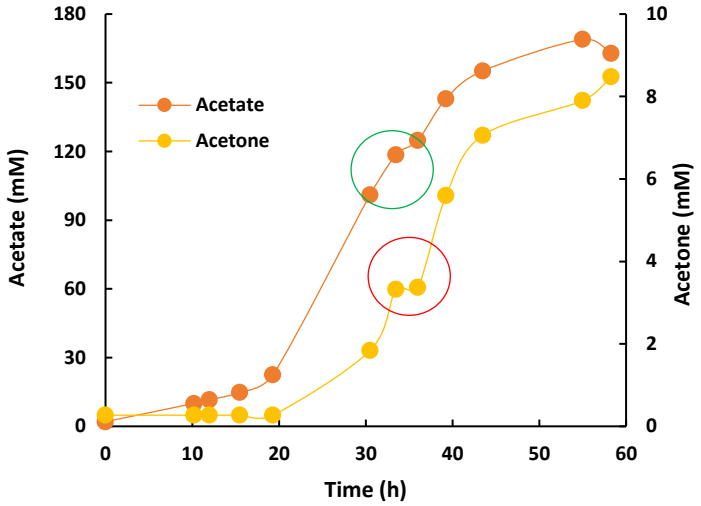


Figure 38. Acetate and acetone trends in the fermentation performed using *A.woodii* plAcC=O grown at high pressure in gas and liquid batch, fed with the 2.3:1 H_2:CO_2 gas mix.

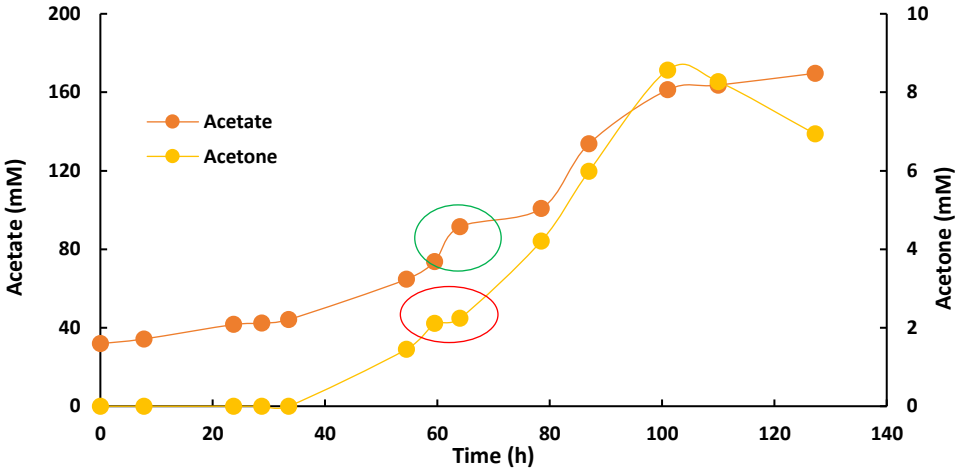


Figure 39. Acetate and acetone trends in the fermentation performed using *A.woodii* plAcC=O grown at atmospheric pressure in gas continuous and liquid batch, fed with 2.3:1 H_2:CO_2 gas mix.

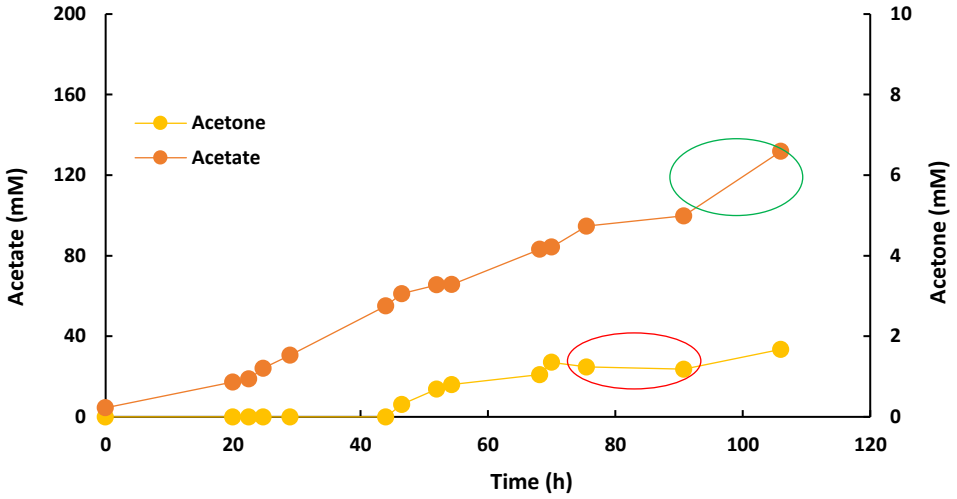


Figure 40. Acetate and acetone trends in the fermentation performed using *A.woodii* plAcC=O grown at high pressure in gas continuous and liquid batch at 10 bar, fed with 2.3:1 H_2:CO_2 gas mix.

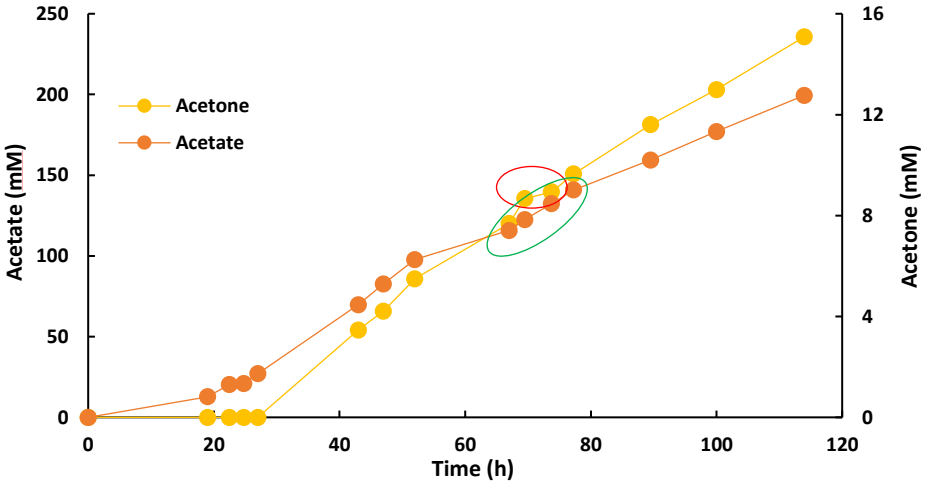


Figure 41. Acetate and acetone trends in the fermentation performed using *A.woodii* plAcC=O grown at high pressure in gas continuous and liquid batch at 10 bar, fed with 2.3:0.1:0.9 H_2:CO_2:N_2 gas mix.

To confirm this observation, an experiment in serum bottle was set up, in which 100 mM of exogenous acetate was added to the autotrophic medium immediately before the inoculum or when cells were in exponential phase. Addiction of acetic acid would lead to an acidification of the medium, because this compound was not neutral. To avoid a difference in cell growth due to the difference in pH in the medium, after the adding of exogenous acetate, pH was risen again to 7 using

NaOH. Moreover, as previuosly describes for the growth of bacteria in serum bottles, pH was checked and adjust to 7 daily, throughout the experiment. Serum bottles were inoculated from an heterotrophic culture of *A.woodii* plAcC=O. The gas mix used was 70% H_2 and 30% CO_2. Every 24 hours, the gas was refilled to avoid the block of the growth of bacteria due to feedstock depletion.

Figure 37 represents the condition of fed-batch serum bottles, in which exogenous acetate was not added to the culture medium. As can be seen, the test was carried on for 138.5 hours. The final acetate concentration was 162.2 mM ± 4.7 mM. Acetone synthesis started about 20 hours after the inoculum, reaching a concentration of 3.8 mM ± 0.12 mM and then remained stable around this value. When acetate concentration overcame the 100 -120 mM threshold, its synthesis resumed, reaching a final concentration of 6.39 mM ± 0.32 mM.

An experiment in fed-batch serum bottles, in which exogenous acetate was added before inoculation, was performed and carried on for 183 hours (Fig. 42 A). Thus, acetic acid was present in the culture broth from the beginning of the growth curve. The final concentrations for acetate and acetone were 222.9 mM ± 4.3 mM and 9.8 mM ± 0.89 mM, respectively. At 140 h the experimental point is missing. However, to have comparable values of acetate and acetone concentrations with the previous experiment, they were estimated by assuming a linear trend in compound concentrations between the two closer experimental points. By doing so, the estimated acetate concentration at 140h was 185 mM, and acetone concentration 6.75 mM. Both values are closer to the concentrations found when exogenous acetate was not added to the culture. As observed previously, and not expected this time, there was still a stalemate of acetone production between acetate concentrations ranging from 125 to 150 mM. Nevertheless, an interesting difference could be noted at the beginning of the growth curve: synthesis of acetone started immediately after the inoculum.

Finally, acetate and acetone trends of the culture in which exogenous acetate was added during the exponential phase of the growth curve are shown in figure 42 B**.** Also this test was 180 hours long. The final acetate concentration was 256.0 mM ± 35.1 mM and the final acetone concentration was 18.9 mM ± 5.6 mM. The sampling at 140h is missing and, as explained above, estimated concentrations of acetate and acetone at this time were calculated. They were 212.5 mM and 12.2 mM for acetate and acetone, respectively. By applying this strategy, thus, acetone concentration was two times fold the values recorded in the cases before. Interestingly, its synthesis didn't start immediately after the inoculum again. After

the addition of acetate, which corresponded also to the exponential phase of the growth curve, acetone kept an always increasing trend throughout the experiment.

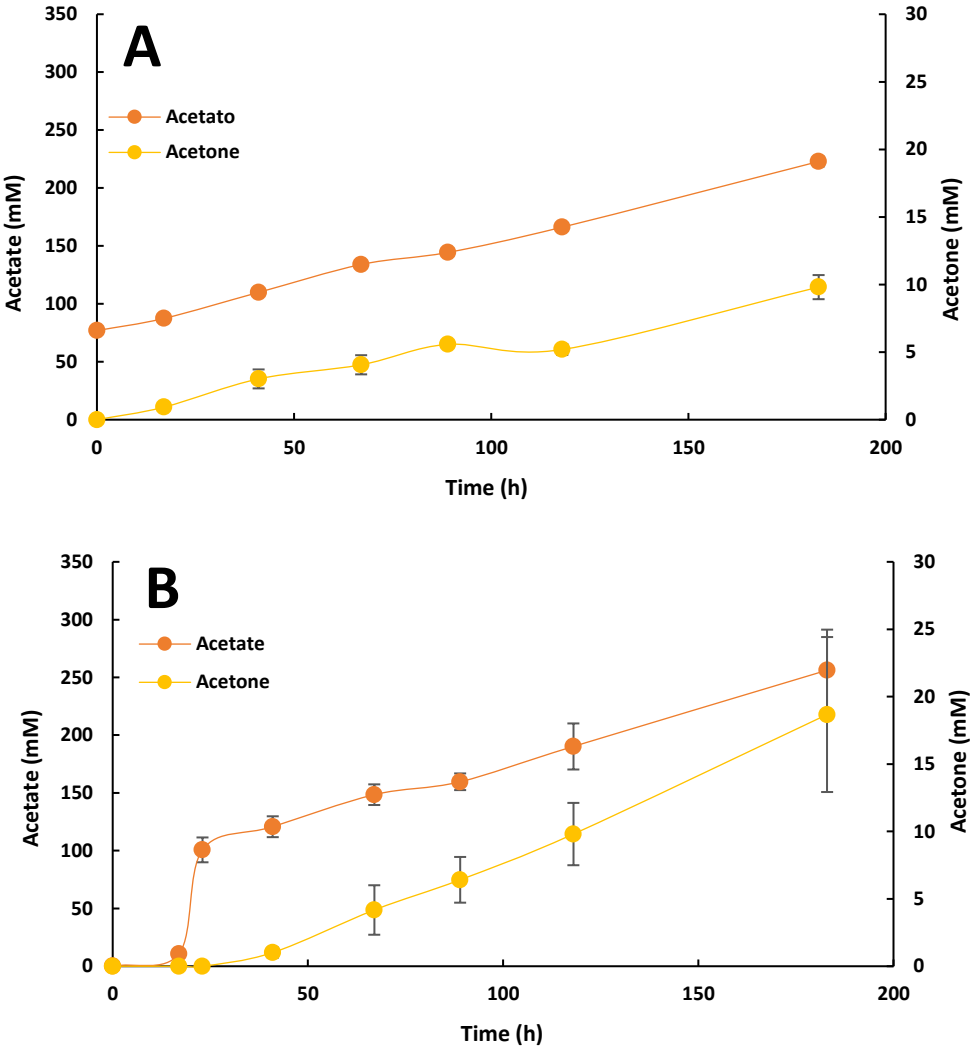


Figure 42. Acetate and acetone production by *A.woodii* plAcC=O in serum bottles with exogenous acetate added. (A) Addiction of acetate before the inoculum; (B) Addiction of acetate in exponential phase.

Hoffmeister and coworkers (2016), working with a modified *A.woodii* strain for acetone production, showed a similar influence of acetate concentration on acetone production. Nevertheless, in that case, the concentration of acetate in the medium that promoted the synthesis of acetone was 11-fold higher (1330 mM). The final concentration of acetone reached was 16.5 mM after 357 hours of fermentation.

Phosphate limitation was found to trigger solvent production in *C. acetobutylicum*. It was suggested that in this condition, WLP is limited in the final step by the shortage of Acetyl-P, and thus the Acetyl-CoA pool inside the cell increases (Bahl et al., 1982; Gottwald & Gottschalk, 1985). Other authors describe a similar behavior in wild-type *A.woodii* grown on glucose in a medium with phosphate limitation. They recorded ethanol production as scavenger mechanisms to manage the increasing pool of intracellular Acetyl-CoA (Buschhorn et al., 1989). This pivotal metabolite is the branching point between the WLP and the acetone pathway in the *A.woodii* plAcC=O strain. Phosphate depletion from the medium was not recorded in experiments in this thesis. Nevertheless, it could be possible that the increasing concentration of acetate in the medium leads to a metabolic rearrangement inside the cell, and so, once the acetate threshold was overcome, other metabolic pathways were enhanced.

3.12 Growth of *A.woodii* intAcC=O in autotrophic condition in gas and liquid continuous.

In the light of the data collected, an experiment was done to investigate the behavior of modified *A.woodii* in a screening of pressures between 1.7 and 10 bar. It was possible to perform this test, set up with both gas and liquid continuous, thanks to an upgrade of the bioreactor that enabled to continuously remove liquid at a stated liquid out-flow rate from the pressurized vessel. In literature, two studies describe continuous gas and liquid fermentation using *A.woodii* but in both cases, they were conducted at atmospheric pressure and using the cell retention to increase the amount of biomass inside the reactors (Hoffmeister et al., 2016; Kantzow et al., 2015). Nevertheless, to the best of my knowledge, no works are found about applying this operation condition scaling up the pressure to 10 bar.

Differently from all the experiments above, in this test *A.woodii* intAcC=O strain was the biocatalyst. Indeed, it was finalized by our partners in UNOTT, and, once ready, it was sent to our laboratory for testing. This strain has the genes coding for the synthesis of acetone, derived from *C. acetobutylicum*, integrated into the genome and was the final strain that will be used in the pilot plant because it doesn’t require antibiotics in the medium to maintain the modification.

According to findings of the influence of bicarbonate on the metabolic profile of *A.woodii* plAcC=O, in this test, the pH was set to 6.6 rather than 7. In this way,

the balance between the forms of the CO_2 in liquid is shifted towards aqueous CO_2 instead of HCO_3^-, with respect to pH 7. This should encourage the production of acetate and acetone rather than formic acid.

A working volume of 800 mL of autotrophic medium was introduced in the reactor. According to the protocol optimized in UNOTT on this new modified strain, 2 mM of fructose were added into the vessel to enhance bacteria growth before shifting to complete autotrophic metabolism. This medium was sparged for one hour at 1.1 bar with 71.7 mL/min of 2.3:1 H_2:CO_2 gas mix before inoculation. 200 mL of heterotrophic culture of *A.woodii* intAcC=O in exponential phase were inoculated, so the final working volume was 1 L of culture (measured OD_{600}= 0.171).

After the inoculum, fermentation proceeded for 24 hours in continuous gassing and liquid batch to allow the shift to autotrophic metabolism before starting the continuous of liquid too. Continuous gassing was provided at 10 mL/min using the 2.3:1 H_2:CO_2 gas mix at 1.7 bar.

Throughout the experiment in both liquid and gas continuous, 7 levels of pressure were screened: 1.7 bar, 2.25 bar, 2.7 bar, 3.3 bar, 5 bar, 7.6 bar, and 10 bar. Scaling up of the pressure was done after a complete change of the volume in the vessel. So, after that 1 L of fresh medium was introduced in the reactor, and in parallel 1 L of culture was removed, the pressure was increased to the following level. The duration of a step was dependent therefore on the dilution rate applied. The growth rate in the preliminary step of continuous gassing and liquid batch was 0.05 h^{-1} but it was influenced by the presence of fructose in the medium. The maximum specific growth rate recorded previously in autotrophy was 0.04 h^{-1}. Considering these values, the dilution rate applied to avoid washout of the cells was a maximum of 0.03 h^{-1}.

Figure 43 shows pressure trend, in-flow gas rate, $OD_{600,}$ and fructose consumption throughout the experiment while figure 44 shows pressure trend, in-flow gas rate, acetate, acetone, and formic acid production.

A detailed description of what was observed in each pressure level is provided in the bulleted list below.

Conversion of the CO_2 provided for each step and yields in main metabolic products (acetate, acetone, and formate), are reported in table 5. The average of the specific and volumetric productivities for each compound are summarized in table 6.

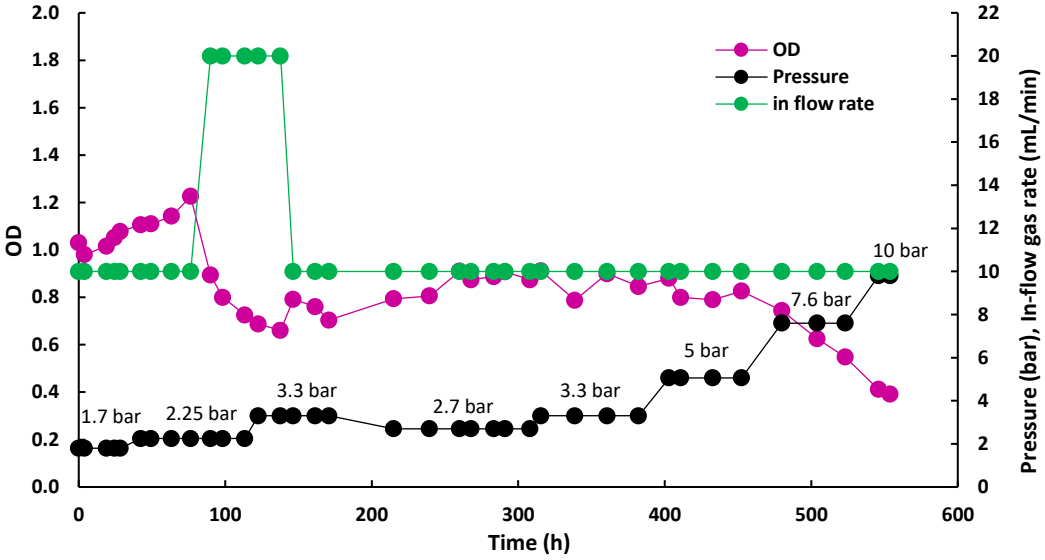


Figure 43. Pressure trend, OD_{600}, in-flow gas rate, and fructose concentration of *A.woodii* intAcC=O grown in gas and liquid continuous.

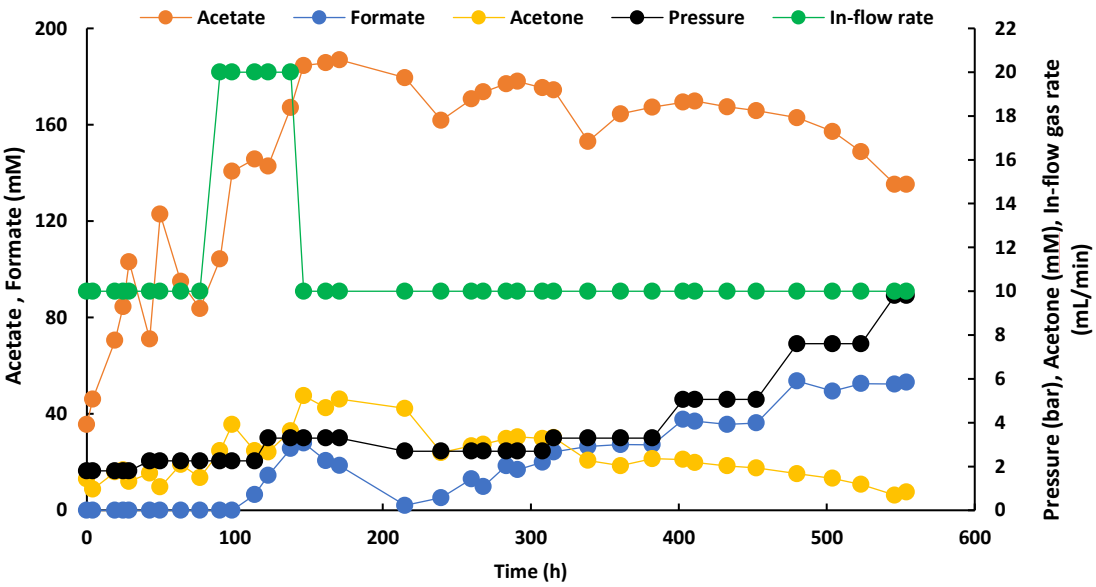


Figure 44. Pressure trend, in-flow gas rate, and concentration of the main metabolic products of *A.woodii* intAcC=O grown in gas and liquid continuous.

- Pressure 1.7 bar

In the first phase of the fermentation in gas and liquid continuous, OD_{600} maintained a value of 1.02 ± 0.04 by applying a dilution rate of 0.025 h^{-1}. Acetate was the main metabolic product. Its concentration was not only stable but increased inside the vessel of 67.6 mM. Acetate molar flow rate was 4.4 ± 1.8 mmol/h, while acetone molar flow rate was 0.006 ± 0.08 mmol/h. Formate was not detected.

- Pressure 2.25 bar

The dilution rate applied in this step was 0.03 h^{-1}. At the beginning of this stage, an in-flow gas rate of 10 mL/min was applied. Nevertheless, this in-flow rate was not sufficient to support a stable pressure. Indeed, this parameter fell from 2.25 bar to a stable value of 1.7 bar in 10 hours, even though a continuous gassing was provided. This phenomenon could be due to more gas solubilization in the liquid phase thanks to the pressure increasing and to the uptake of feedstock by bacteria in the reactor. OD_{600} increased from 1.10 to 1.23 even with the removal of the culture from the vessel and replacing it with the fresh medium through the liquid continuous. Again, acetate and acetone were the main metabolic products. 3.3 ± 4.7 mmol/h of acetate and 0.1 ± 0.2 mmol/h of acetone were synthesized, but, as the big standard deviation indicates, their production was not stable.

To stabilize the pressure, the in-flow gas rate was increased to 20 mL/min. By doing so, effectively, the pressure was maintained to the stated level and both acetate and acetone molar flow rates stabilized, at 5.7 ± 2.1 mmol/h and 0.14 ± 0.11 mmol/h respectively. OD_{600}, instead, was not stable and decreased to 0.73. A small amount of formic acid was detected in the reactor at the end of the step, reaching a concentration of 0.4 mM.

- Pressure 3.3 bar

Because OD_{600} decreased already in the previous step, the dilution rate in this pressure level was reduced to 0.015 h^{-1}. In-flow gas rate was kept at 20 mL/min and OD_{600} declining at 0.67 ± 0.02 before stabilizing. Besides acetate and acetone production, boosted formic acid production was detected. Molar flow rates were 2.9 ± 0.4 mmol/h, 0.1 ± 0.05 mmol/h and 1.0 ± 0.1 mmol/h for acetate, acetone and formate respectively.

Trying to increase biomass growth and turn down formate synthesis, the in-flow gas rate was set back to 10 mL/min, keeping the same dilution rate as before.

Even though biomass growth was not restored, formic acid synthesis lowered to 0.07 ± 0.19 mmol/h. As can be noted from the high standard deviation, the production of this compound was not stable, indeed it decreased during the step.

- Pressure 2.7 bar

The previous experiment on fresh medium pressurization suggested that it was not suitable to increase the pressure and then come back to lower levels. For this reason, immediately a step back in pressure was applied after the results obtained at 3.3 bar, to investigate what happens at an intermediate pressure between 2.25 and 3.3 bar. Thus, this step was performed at 2.7 bar. In-flow gas rate and dilution rate were kept constant at 10 mL/min and 0.015 h^{-1} respectively.

OD_{600} increased and stabilized at 0.89 ± 0.02. Formate production didn't record a downturn, indeed it was 0.3 ± 0.4 mmol/h. The standard deviation is high, but this time this is due to an increasing trend of the compound. Acetate production was 2.8 ± 0.2 mmol/h, comparable to the one found at 3.3 bar and 10 mL/min of in-flow gas rate; acetone molar flow rate had a slight decrease (0.06 ± 0.10 mmol/h).

- Pressure 3.3 bar

The following step was performed again at 3.3 bar, using a dilution rate of 0.015 h^{-1} and 10 mL/min of in-flow gas rate. These parameters were already applied before, but they were used once again to have a gradual ascent of the pressure towards 10 bar. OD_{600} had a value of 0.862 ± 0.02. The value of the molar flow rate of acetate was comparable to what was recorded before while the production of formic acid increased to 0.55 ± 0.23 mmol/h and the one of acetone lowered to 0.03 ± 0.02 mmol/h. The trends of these latter compounds were like what was observed in the former step at 2.7 bar.

- Pressure 5 bar

Raising the pressure up to 5 bar with 10 mL/min in-flow gas rate and 0.015 h^{-1} dilution rate didn't affect OD_{600} measured, that was 0.842 ± 0.04, close to the stable value previously recorded. The value of acetate, acetone, and formate production were comparable to those of the former step at 3.3 bar (molar flow rates were 2.52 ± 0.23 mmol/h, 0.07 ± 0.02 mmol/h, and 0.63 ± 0.25 mmol/h respectively)

- Pressure 7.6 bar

This pressure level had the same dilution rate and in-flow rate described before. OD_{600} decreased and, because the dilution rate was not reduced, washout of the cells occurred. In this step, OD_{600} indeed declined from 0.744 to 0.548 and didn't stabilize. Also, acetate (4.25 ± 0.51 mmol/h) and acetone (0.04 ± 0.02 mmol/h) molar flow rates showed the same descendent trend. Instead, formic acid synthesis was enhanced (0.94 ± 0.35 mmol/h).

- Pressure 10 bar

By further increasing the pressure up to 10 bar, cells washout continued to OD_{600} 0.392. Formic acid synthesis was stable (0.84 ± 0.08 mmol/h). Production of both acetate and acetone was lower than before: acetate molar flow rate measured was 3.56 ± 0.68 mmol/h, while it was 0.01 ± 0.02 mmol/h for acetone. As highlighted by the standard deviation, the production of this latter compound was low and not stable.

Table 5. Conversion of the CO_2 provided, yields and α in main metabolic products for each pressure step of gas and liquid continuous fermentation.

Pressure (bar)	In-flow gas rate (mL/min)	CO_2 conversion in metabolic products (%)	Yields (%)				α (%)		
			Acetate	Acetone	Formate	Biomass	Acetate	Acetone	Formate
1.7	10	96.7	96.4	0.2	0	0.07	99.7	0.2	0.0
2.25	10	84.0	82.6	1.3	0	0.07	98.3	1.5	0.0
2.25	20	78.6	75.1	2.7	0.8	0.03	95.5	3.4	1.0
3.3	20	43.2	35.5	1.3	6.3	0	82.1	3.0	14.6
3.3	10	59.6	56.2	3.0	0.3	0.001	94.3	5.0	0.5
2.7	10	70.9	65.5	2.0	3.4	0.02	92.4	2.8	4.7
3.3	10	67.6	59.5	1.1	6.9	0.03	88.0	1.6	10.2
5	10	71.4	62.7	0.9	7.8	0.02	87.8	1.2	10.9
7.6	10	65.2	52.9	0.5	11.8	0.01	81.1	0.8	18.1
10	10	55.1	44.3	0.4	10.4	0.005	80.4	0.7	18.9

Table 5 underlines that, at 10 mL/min in-flow gas rate, higher CO_2 conversion were at lower pressures. Indeed, the worst value recorded was at 10 bar, while the best was at 1.7 bar. As previously discussed, higher pressures lead to a higher concentration of CO_2 dissolved in the liquid phase. This gas dissolved, according to the pH, converts in different forms. By setting the pH at 6.6 rather than at 7, as

done before, less CO_2 is stored as HCO_3^- in the medium. Nevertheless, as noted before for *A.woodii* plAcC=O, also for *A.woodii* intAcC=O increasing the pressure led to a shift in autotrophic metabolism towards formic acid, rather than towards acetic acid production. Moreover, at 7.6 and 10 bar, formic acid yields recorded were comparable.
The lowest value of CO_2 conversion, however, was at 3.3 bar with an in-flow gas rate of 20 mL/min. A small shift in the pressure applied (form 2.25 bar to 3.3 bar) led to a 45% less carbon dioxide conversion and to a pronounced shift towards formic acid production also at a low-pressure level.

Acetate concentration for most of this experiment was above 120 mM in the vessel of the reactor (Fig. 44). Higher yields for acetone were recorded at lower pressures (range 2.25-3.3 bar). Nevertheless, these data are not enough to understand if, also in the integrated strain, acetone synthesis is influenced by acetate concentration. Even if this phenomenon happens, probably it is less pronounced than in the strain modified with the plasmid, where the genes coding for the enzymes of the pathway for acetone synthesis are in more copies.

Table 6. Average of the specific and volumetric productivities of acetate, acetone, and formic acid at each pressure step of gas and liquid continuous fermentation.

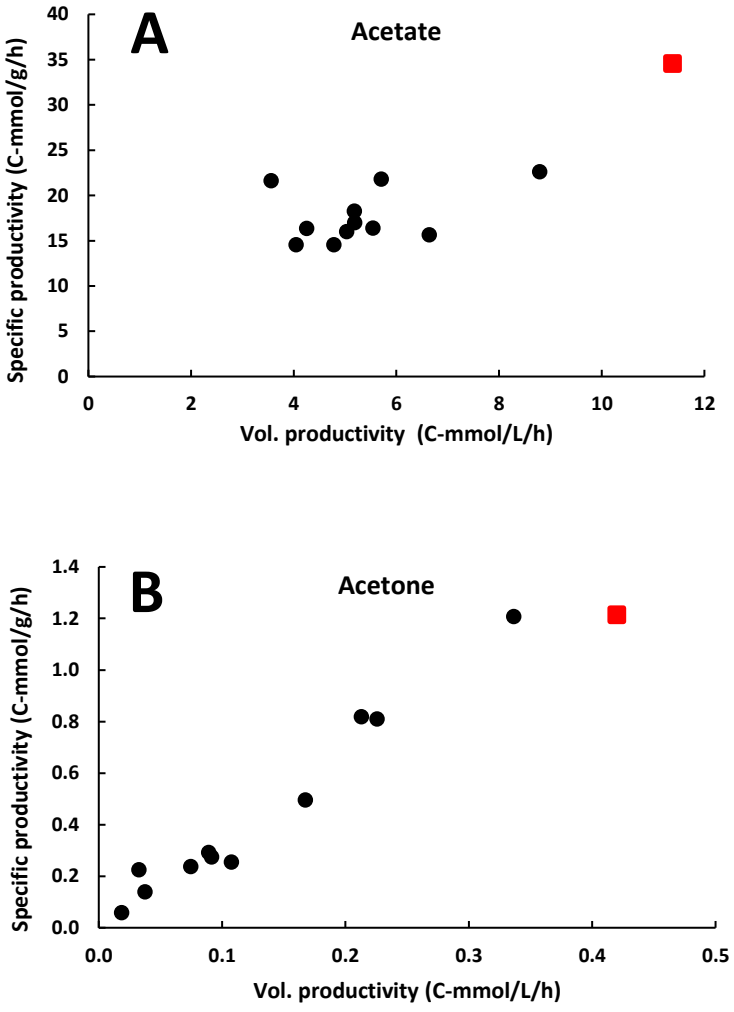
Pressure (bar)	In-flow gas rate (mL/min)	Volumetric productivities (C-mmol*L^{-1}*h^{-1})			Specific productivities (C-mmol*$g_{biomass}^{-1}$*h^{-1})		
		Acetate	Acetone	Formate	Acetate	Acetone	Formate
1.7	10	8.79	0.02	0	22.64	0.06	0
2.25	10	6.64	0.11	0	15.64	0.25	0
2.25	20	11.38	0.42	0.17	34.59	1.22	0.60
3.3	20	5.71	0.21	1.02	21.81	0.81	3.87
3.3	10	5.18	0.34	0.07	18.28	1.21	0.26
2.7	10	5.54	0.17	0.26	16.38	0.50	0.78
3.3	10	4.78	0.09	0.55	14.54	0.27	1.68
5	10	5.03	0.08	0.63	16.02	0.24	1.99
7.6	10	4.25	0.04	0.95	16.36	0.14	3.62
10	10	3.56	0.03	0.84	21.65	0.23	5.06

As shown in Table 6, volumetric productivities and specific productivities didn't go hand in hand. As an example, at 10 bar pressure, the highest specific productivity of formic acid was recorded, but it was not the higher volumetric productivity for this compound during the experiment. It happens essentially because the volumetric productivity is influenced by the number of biocatalyst cells inside the volume considered. The same observation could be done about

acetate in the same pressure condition: the specific productivity at 10 bar is comparable to the one recorded at 1.7 bar. However, at the highest pressure, washout of cells occurred and volumetric productivity declined and is only 40% of the one at 1.7 bar.

For this reason, it could be useful to find process parameters that allow combining the best specific productivity and the best volumetric productivity achievable.

Figure 45 shows specific productivities of the three main metabolic compounds as function of their volumetric productivities.



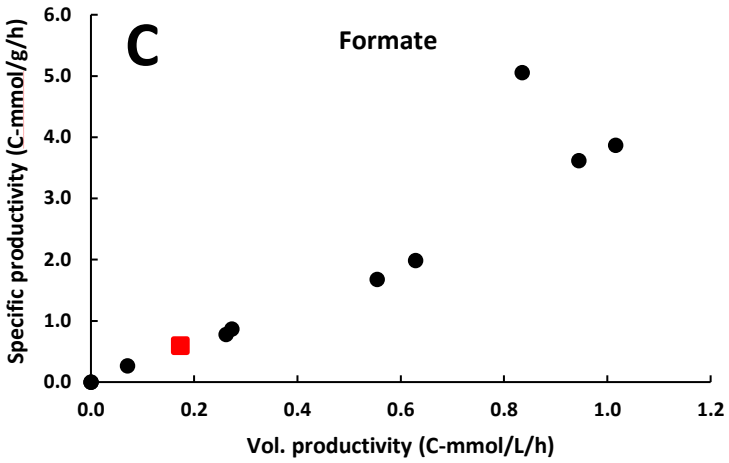


Figure 45. specific productivities as function of volumetric productivities for each main metabolic compound. (A) Acetate; (B) Acetone; (C) Formate.

As can be seen, for acetate and acetone there is a case in which the highest volumetric productivity corresponds also to the highest specific productivity. Both these cases belonged to the same process condition: reactor pressure set at 2.25 bar and in-flow gas rate set at 20 mL/min. In this condition, also formic acid was synthesized, but both its volumetric and specific productivities are among the lower recorded (0.17 C-mmol*L^{-1}*h^{-1} and 0.06 C-mmol*$g_{biomass}^{-1}$*h^{-1} respectively).

Chapter 4

Conclusions

The present work of thesis aims to exploit a CO_2 stream waste as a feedstock for the production of added-value chemicals. According to the CCU approach, it can contribute to mitigate climate change. Moreover, the shift from large-scale plants for chemicals production, often based on fossil raw materials, to small-scale plants exploiting renewable and distributed resources can contribute to a sustainable model of industrial production. This work was developed in the context of a project, named ENGICOIN, funded by the European Commission. It purposes to integrate three microbial factories placed downstream to an anaerobic digestion platform for organic waste treatment, to exploit the CO_2 from the biogas for the synthesis of compounds of industrial interest. In particular, this work was focused on the optimization of the fermentation process that allows converting the CO_2 stream in acetone by employing a modified *A.woodii*. This bacterium naturally produces acetic acid as the main fermentation product. It can grow heterotrophically on different organic compounds and autotrophically using CO_2 as carbon source and H_2 as reducing agent. H_2 and CO_2 are the substrates for the Wood-Ljungdahl Pathway (WLP) which leads to the synthesis of Acetyl-CoA. This metabolite is pivotal for the anabolic pathways and is the branching point between the acetate and the acetone pathways in acetogens. Wild-type *A.woodii* doesn't harbor the genes for the acetone biosynthetic pathways, so the strains employed in this thesis were genetically modified with the coding sequences for the enzymes responsible for the acetone synthesis of *C. acetobutylicum.* In particular, the first strain developed was a plasmidic strain and thiamphenicol is

required to maintain the genetic modification. The second strain developed was, instead, a strain in which the same genes are inserted in the genome of the bacterium. Because antibiotic selection is not required to keep stable the genetic modification in this latter strain, it is suitable for the process in the pilot plant.

For gas fermentation processes often low productivities are reported (Van Hecke et al., 2019). The low productivity can be due to the shortage of feedstocks dissolved in the growth medium that leads to low activity of the biocatalyst. Indeed, conversion processes often takes place in a liquid phase, while feedstocks are provided as gases. In an equilibrium state, the maximum concentration of a dissolved gas is described by Henry's law. This law underlines how the bioavailability of a gas in a liquid medium depends on the solubility constant, specific for each gas, particularly low for H_2, and the partial pressure of the gas. Therefore, to increase the concentration of substrates in the liquid medium, the approach proposed in this work was to accomplish gas fermentations at high-pressure. In particular, the highest pressure achievable by the bioreactor equipment is 10 bar. So, this was the maximum pressure tested in this work. The aim of the study was to investigate and define the best operating conditions for CO_2 conversion into added value compounds, and in particular acetone, by a modified *A.woodii* strain for acetone production, at laboratory scale. The results obtained will be the basis for the fermentation process carried on in the pilot plant.

The most part of the experiments performed were conducted in pressurized bioreactors that allow managing different operating process conditions: liquid and gas batch, liquid batch and continuous gas supply, and gas and liquid continuous. All these conditions were tested, and CO_2 conversion, yields and productivity parameters were evaluated.

No data in the literature are reported about the resistance of *A.woodii* at 10 bar. Therefore, the first test performed was a heterotrophic fermentation in which the modified bacterium was grown at 11 bar in a nitrogen atmosphere. Results obtained allow accessing that growth curve, viability, and cell integrity were not impaired by this pressure value.

The following steps were the definition of the best gas mix and inoculum strategy for the fermentation in the reactor in autotrophic condition. These preliminary data were collected in fed-batch experiments, in serum bottles, at atmospheric pressure. The gas mix chosen was 2.3:1 H_2:CO_2 (in percentage 70% H_2, 30% CO_2). pH control turned out to be pivotal for bacteria growth and was set to 7.

Inoculum strategy for autotrophic fermentations consisted of an heterotrophic culture of modified *A.woodii* in the exponential phase of the growth curve. Initial OD_{600} in the reactor should be greater than 0.2, to avoid the lag phase and save operational time in the reactor.

An experiment in gas and liquid batch, providing the 70 % H_2 and 30% CO_2 gas mix up to 10 bar, showed that the bacterium was able to manage this operating condition and carry on the WLP, synthesizing both acetate and acetone. The highest pressure previously reported in the literature in a gas and liquid batch fermentation, using wild-type *A.woodii,* was 5.5 bar. With respect to that work, the final concentration of acetate found here was similar, nevertheless it was reached in one-tenth of the time. Both specific and volumetric productivities for acetate were higher, confirming that increasing pressure could be a suitable approach to enhance compounds' productivities.

In liquid batch and continuous gas supply at 10 bar, tests were set up with different combinations of biomass and gas in-flow rates. A reference experiment at 1.1 bar (atmospheric pressure) was also performed. While bacterium grew and synthesized acetate as the main metabolic product at atmospheric pressure, at high pressure bacteria growth was inhibited and, besides acetate, an enhanced synthesis of formic acid was recorded. Experiments in which fresh medium, without cells, was pressurized with the feeding gas mix in the reactor were performed. The pressurization of the medium before bacteria inoculation led, however, to the inhibition of *A.woodii* growth and a to a shift of its metabolic profile toward formic acid. In this case, bacteria were never stressed by pressure. These results suggested that bicarbonate could be responsible for the modified behavior of the biocatalyst at higher pressure with respect to the atmospheric one. This ion, indeed, can occur in the medium due to the combination of pH control and increased dissolved CO_2, due to the increased process pressure. Tests in serum bottles with augmented concentration of exogenous salts ($KHCO_3$ or KCl) confirmed the effect of the bicarbonate on modified *A.woodii* physiology.

Tests in the reactor were performed in which the same total pressure (10 bar) and the same partial pressure of hydrogen (7 bar) were maintained as before but in which the partial pressure of the CO_2 ($pPCO_2$) was reduced to 10% (replaced by nitrogen). Results obtained showed that bacteria growth was not completely restored by decreasing the $pPCO_2$. However, formic acid synthesis was not enhanced anymore, and acetic acid was the main metabolic product. Moreover, the final concentration of acetone produced (23.21 mM) is the highest

concentration reported so far in this operating condition for an *A.woodii* strain modified to synthesize acetone. This result confirms that a moderate increase of the pressure of the feeding gases can promote higher productivities but also that within a range of 10 bar, using a 2.3:1 H_2:CO_2 gas mix, there is a threshold level of dissolved substrates that triggers a shift in metabolic products spectrum and an impairing of biocatalyst growth.

Independently from the operating condition applied, a common behavior was observed for the plasmidic modified strain of *A.woodii* for acetone synthesis: when the concentration of acetate in the medium overcame 100-120 mM, acetone synthesis was fostered. This phenomenon is probably due to an increased pool of Acetyl-CoA inside the cell and is an aspect to be taken into account to increase acetone productivity.

The gas and liquid continuous fermentation allow maintaining a viable culture inside the reactor for long time, avoiding nutrient depletion or inhibition due to the accumulation of the metabolic products. This operating condition was used to investigate the performance of the *A.woodii* strain, harboring the genes coding for acetone synthesis integrated into the genome, in a screening of pressures from 1.7 bar to 10 bar. Moreover, at low pressure levels (2.25 – 3.3 bar), two in-flow gas rates were tested. Results obtained highlighted that CO_2 conversion decreased by increasing the pressure from 1.7 to 10 bar, and that at the highest pressure (7.6 -10 bar) bacteria growth was inhibited, leading to the washout of cells form the reactor, and formic acid production was fostered. Nevertheless, a slight increase of pressure at values higher than the atmospheric one positively affected the productivities of acetate and acetone. Using the 70% H_2, 30% CO_2 gas mix, the best combination of pressure and in-flow gas rate found for the synthesis of these metabolic compounds were 2.25 bar and 20 mL/min, respectively.

Among acetogens, *A.woodii* is a reference strain. Its main metabolic and energetic pathways were disclosed both at genetic and enzymatic levels. Nevertheless, studies on the use of this catalysts in industrial applications are still limited. The good performance on H_2 and CO_2 streams and the availability of metabolic tools that allow modifications make this bacterium an attractive possibility to develop new microbial factories. This thesis highlighted that increasing fermentation pressure leads to a metabolic shift in the biocatalyst cell. Acetone synthesis and productivity were pretty low. According to results, acetate and acetone productions are linked. So, at a level of fermentation process development, implementing solutions that fostered acetate production leads to enhanced acetone

production. Nevertheless, to increase acetone productivity until industrial feasibility will depend deeply on the genetic progress to develop efficient strains for the synthesis of heterologous products.

It has to be noted that the metabolic shift at high pressure leads to the production of only one new metabolic product in this host: formic acid. This work demonstrated that both the partial pressure of each feeding gas and the operating condition applied can influence the metabolism of the bacterium. Acetate/acetone and formic acid synthesis can be tuned according to fermentation parameters applied. This can be the basis for a rational development of processes using *A.woodii* as biocatalyst. Like acetone, both acetate and formic acid, indeed, have a growing market. Acetic acid is a key raw material for many polymers, paints, adhesive and textile treatments (Cheung et al., 2012). Moreover, by coupling anaerobic acetic acid production and aerobic metabolisms, the synthesis of malic acid (Oswald et al., 2016), diesel fuels (Hu et al., 2016) and plastic polymers (Al Rowaihi et al., 2018) are shown to be feasible. Formic acid has multiple industrial applications, such as feed preservation, employment in textile and leather processing, component of cleaner formulation. Moreover, it is becoming an interesting compound for energy storage (Kottenhahn et al., 2018; Onishi et al., 2019; Roger et al., 2018; I. Schmidt et al., 2014; Schwarz & Müller, 2020). Indeed, interest towards hydrogen as energy source is increasing. Formic acid is considered as a possible form for its safer storage. HDCR of *A.woodii* is among the enzymes with the highest rates of CO_2 hydrogenation (Müller, 2019).

This work highlights how, by modifying just few process parameters and operating conditions, it is possible to redirect the metabolism of a modified strain of *A.woodii* for the synthesis of specific molecules. The possibility of using the bulk of the plant (gas production and clean-up, bioreactors, water treatment) to produce different chemicals is an attractive property in the industrial field (Köpke & Simpson, 2020). Experiments reported above highlight that *A.woodii* plAcC=O and *A.woodii* intAcC=O metabolisms can be directed towards the synthesis of a few metabolites, namely acetate, acetone and formic acid. It is likely that performing similar tests with wild-type *A.woodii* as the catalyst would lead to acetate and formic acid as main metabolic products. More studies are needed, both on carbon dioxide and hydrogen partial pressures, to precisely control the spectrum of synthesis. Experiments performed in this work focused on the influence of the pP_{CO2} on *A.woodii* physiology. Results suggest that this gas feedstock is responsible of the metabolic switch towards formic acid. Nevertheless, results also underline that hydrogen impacts at least on bacteria

growth. Indeed, lowering the pP_{CO2} and maintaining constant the pP_{H2} at 7 bar avoided formic acid production, but bacteria growth was still inhibited. Future experiments will be performed in order to investigate the influence of pP_{H2} on *A.woodii* physiology and products spectrum. H_2 threshold that allows bacteria growth will be assessed. pP_{H2} will be increased, while keeping constant the pP_{CO2}, and the process pressure at 10 bar. Kinetic of growth and production of metabolites will be monitored.

This work proposes to increase the pressure of gaseous feedstock to increase their dissolved amount in the liquid phase. Nevertheless, according to the pH, dissolved CO_2 in water can be stored as bicarbonate. *A.woodii* expresses a carbonic anhydrase enzyme that allows intracellular conversion of HCO_3^- into CO_2 (Braus-Stromeyer et al., 1997). Not only gaseous CO_2, but also HCO_3^- is a feedstock that can be directed towards the LWP. Thus, another approach to increase the amount of CO_2 available for bioconversion in the liquid phase could be providing CO_2 continuously in the medium at low partial pressure and setting the pH above 7. In this way, the gaseous feedstock will be converted into a bicarbonate pool and the carbon supply can be guaranteed even at low pP_{CO2}.

Because many parameters influence the growth and the metabolic spectrum of *A.woodii*, it could be useful to define a Design of Experiment (DoE) to investigate more than one parameter at a time. The three factors considered could be H_2-CO_2 ratio, pH and hydrogen partial pressure. The interaction between these factors could influence both bacteria growth and metabolism. In this way, it will be possible to assess the best combination that allows maximizing the productivity of the desired specific compound (acetate/acetone or formate).

To the best of my knowledge, ethanol is the only commodity compound currently produced at industrial scale through gas fermentation. This important milestone was achieved by LanzaTech Inc. company (USA). The catalyst employed is a naturally selected, non-engineered strain of *Clostridium autoethanogenum*, that synthetizes ethanol as a native product. Waste gas from steel mill plants is the feedstock of two demonstration facilities placed in China. The demonstration unit located at one of the BaoSteel's mills near Shanghai is operative since 2012, while the other, located at a Shougang steel mill near Beijing is operative since 2013 (Heijstra et al., 2017). The latter plant is currently a commercial scale gas fermentation plant (Köpke & Simpson, 2020). The syngas obtained from still mills is rich in CO. This composition makes Clostridium species particularly suitable for fermentation processes. It has to be noted that the fermentation

facilities are running directly on the off-gas produced by the still mill plants. The process is robust to gas composition fluctuation, presence of contaminants, intermittent gas supply, and equipment failure, replaced during the fermentation (Heijstra et al., 2017).

Recently, LanzaTech Inc. company describes a successful attempt to produce acetone and isopropanol (IPA) by gas fermentation at industrial pilot scale (F. E. Liew et al., 2022). The work is an interesting example of process optimization at three different levels. Each one is pivotal for the future development of microbial platforms for the gas fermentation at industrial level. A modified strain of *C.autoethanogenum* was used as bio-catalyst. This bacterium doesn't synthetize acetone or IPA as native products, so it was necessary to insert into the wild-type strain the genetic sequences coding for the pathway of acetone synthesis (or IPA), similarly to what described for *A.woodii* in this thesis work.

LanzaTech explored a collection of 272 ABE strains. This collection comprises also optimized adapted strains from the last industrial ABE operative plans in South Africa and Taiwan, which were operative until the 1980s. Some modified *C.autoethanogenum* strains were able to produce acetone to a titer of 100 mM and were chosen for further modification. Some gene knock-outs were made to eliminate by-products and to direct the carbon flow towards acetone production. Moreover, usually, the thiolase step is speculated to be the bottleneck of the acetone biosynthetic pathway, because it catalyzes an endergonic reaction. Through proteomic analysis, authors identify, instead, the low expression of the CftAB enzymes as the limiting step. Thus, the selected strain was further modified by increasing the expression of these selected proteins, resulting in an increased acetone selectivity. An optimized *C.autoethanogenum* strain with genomic integrated modifications was tested in a gas fermentation process kept in steady-state for ≈ 3 weeks. Pilot runs were performed in a 120 L fermenter, fed with a 50% CO, 10% H_2, 20% CO_2, 20% N_2 gas mix provided from 11.5 to 23 L/min, at 1.4 bar. The strain showed culture stability. Acetone volumetric productivity recorded was about 40 mmol*L^{-1}*h^{-1} with gas utilization up to 80%.

The reported work from LanzaTech suggests that gas fermentation processes for the synthesis of chemical commodities should join together three levels of optimization: pathway optimization, strain optimization and process optimization. Acetone production by gas fermentation using a modified strain of *A.woodii* allows exploiting different gas mixes respect to modified *C.autoethanogenum.* These bacteria share the LWP as the main autotrophic pathway. Thus, applying

the latest knowledge about the enzymes involved in the pathway, and a similar strain optimization approach, could improve acetone production in the modified *A.woodii* strain.

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