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Succinic acid production from organic waste

Ph.D. Dissertation

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Abstract

Succinic acid is a dicarboxylic acid widely used as a building block in several industrial sectors, including pharmaceuticals, cosmetics, and the chemical industry. It is a well-known precursor of polybutylene succinate, a biodegradable polymer employed in the production of bioplastics. Since the early 2000s, succinic acid has been recognized as a high-value platform chemical and was included in the Top Value-Added Chemicals list by the United States Department of Energy (DOE), owing to its potential for production from renewable biomass.

Currently, most of succinic acid is produced from non-renewable petrochemical sources, such as maleic acid or maleic anhydride, which are derived from fossil resources. This conventional production route is increasingly unsustainable due to several factors, including the volatility of fossil fuel prices and the significant environmental impact associated with their use, particularly greenhouse gas emissions, mainly CO_2. To address these limitations, alternative biological production routes have been extensively investigated.

Succinic acid can be produced as a metabolic intermediate in animal and plant tissues and by various microorganisms. Among these, bacteria and yeasts are of particular interest due to their ability to naturally produce high concentrations of succinic acid and their suitability for industrial fermentation processes. *Actinobacillus succinogenes* is a facultative anaerobic, capnophilic, non-pathogenic, Gram-negative, and non-motile bacterium classified as a biosafety level 1 (BSL-1) microorganism. Originally isolated from the bovine rumen and belonging to the Pasteurellaceae family, this strain exhibits remarkable metabolic versatility, being able to utilize a wide range of carbon sources, including glucose, xylose, arabinose, mannose, galactose, fructose, sucrose, lactose, cellobiose, mannitol, maltose, and glycerol. Moreover, *A. succinogenes* is capable of naturally producing succinic acid at high concentrations, making it a highly promising candidate for industrial-scale bio-succinic acid production.

This capability of the strain to metabolize a wide range sugars enables the use of lignocellulosic biomass as a renewable feedstock. Lignocellulosic biomass is abundantly available, as it largely consists of residues from agro-industrial processes, and its valorization aligns with circular economy principles. However, fermentable sugars such as

glucose and xylose are not readily available in lignocellulosic biomass, as they are embedded within a complex and recalcitrant matrix composed primarily of cellulose, hemicellulose, and lignin. Consequently, pretreatment processes are required to disrupt this structure and release fermentable sugars. These pretreatments often involve harsh conditions, including strong acidic or alkaline environments, high temperatures, and high pressures, which can lead to the formation of inhibitory compounds such as acetic acid and 5-hydroxymethylfurfural. These inhibitors negatively affect microbial growth and fermentation performance.

In addition, during the fermentation process for succinic acid production, several by-products are generated, with acetic, formic, and lactic acids being the most common. The presence of these compounds in the fermentation broth complicates downstream processing, making the separation and purification of succinic acid technically challenging and economically demanding.

This thesis investigates the feasibility of producing succinic acid via fermentation from lignocellulosic biomass, specifically wheat straw, as the primary feedstock. In the first phase of the study, different initial sugar concentrations and microbial strains were evaluated in a synthetic medium to identify the optimal balance between substrate availability and microbial performance.

Subsequently, the growth and fermentative capabilities of *Actinobacillus succinogenes* were assessed in a synthetic fermentation medium supplemented with inhibitory compounds to simulate the conditions typically found in hydrolysates obtained from the steam explosion of wheat straw. In the initial experiments, no additional carbon or nitrogen sources were supplied. Under these conditions, the strain exhibited strong inhibition, prompting the investigation of yeast extract and magnesium carbonate supplementation as strategies to enhance succinic acid production performance.

In the final fermentation trials, different dilutions of wheat straw hydrolysate were tested, and the concentrations of yeast extract and magnesium carbonate were optimized to avoid unnecessary supplementation while maintaining beneficial effects on fermentation efficiency.

Finally, an innovative approach for reducing by-product accumulation was explored. This strategy involved a second-stage fermentation process employing *Cupriavidus necator*, a bacterium capable of utilizing acetic and formic acids as carbon sources to produce polyhydroxybutyrate (PHB), another high-value bioproduct. This integrated bioprocess aims to decrease the concentration of inhibitory by-products, limit the reliance on costly downstream purification techniques, and improve the overall economic feasibility of bio-based succinic acid production through the co-production of an additional marketable compound.

1. Introduction

Starting from the 20th century, the use of non-renewable raw materials for energy production and for the synthesis of a wide variety of chemical compounds, such as plastics, has grown exponentially. However, the intensive exploitation of these resources entails a twofold challenge, related both to their progressive depletion and to the environmental impacts associated with their use. According to estimates by the International Energy Agency (IEA), by 2035 the global availability of fossil resources is expected to decrease by up to 75% (IEA, 2024). In parallel, the increase in greenhouse gas emissions resulting from conventional production processes has prompted public institutions and the industrial sector to move towards energy and chemical production systems based on renewable, biologically derived resources, in order to foster a more sustainable and resilient global economy.

In this context, biomass represents a widely available and potentially inexhaustible alternative to traditional petrochemical approaches, and it is increasingly recognized as a strategic resource to replace conventional energy and chemical production (Yana et al., 2022). In the early 2000s, based on scientific patents and academic data, the United States Department of Energy (US DOE), in collaboration with the National Renewable Energy Laboratory (NREL), developed a list of more than 300 molecules of industrial interest that can be obtained from renewable sources. Each of these molecules was accompanied by information regarding its chemical structure, source material, available production processes, and potential applications in consumer goods. The selection criteria included not only feedstock availability but also estimated process costs and market size. From the 300 identified compounds, an additional selection process led to the identification of 30 key molecules, defined as building blocks, whose biological production represents a functional equivalent to petrochemical synthesis. The introduction of bio-based products with comparable and compatible characteristics to their fossil-derived counterparts paves the way for alternatives with reduced environmental impact.

Subsequently, in 2004, the US DOE published the report Top Value-Added Chemicals from Biomass (Werpy et al., 2004), which marked a turning point in research orientation towards bio-based products. This document analyzed compounds that could be obtained through biomass utilization in industrial processes, producing a list of molecules with high strategic

potential. From an economic perspective, these molecules could ensure higher profit margins than those achieved through conventional production. Furthermore, the report examined analogies between base compounds obtained from fossil sources and those derivable from sugars, lignin, syngas, and triglycerides, although the term "base molecule" was not formally adopted. The main objective was to classify molecules that could be transformed into high-value-added chemicals and materials (Philp, 2014). From this analysis, a list of 12 building blocks derived from sugars was selected among the 30 most promising compounds initially identified.

Numerous studies have been carried out on these base molecules following the publication of the US DOE reports, even though the term has never been associated with a single, universally accepted definition. Indeed, in scientific literature, patents, technical reports, and official documents dealing with biomass-derived chemical building blocks, several terminologies have been used. Among the most recurrent expressions are: bio-platform molecules (Breeden et al., 2012), platform molecules (Martin Alonso et al., 2013), biomass-based platform chemicals (Lai et al., 2017), biomass-derived platform molecules (Serrano-Ruiz et al., 2011), base chemicals from biomass (Sanders et al., 2012), building-block chemicals from biomass (Eckert et al., 2007), and more generic terms such as platforms, referring for example to syngas or triglycerides.

Some scholars tend to define "platforms" as molecules containing multiple functional groups, since this characteristic enables them to be converted into a wide range of industrially relevant compound families (Wieschalka, 2012). When several building blocks combine or undergo transformation, they give rise to a broad spectrum of chemical products. During the biological, chemical, or thermal processes leading to the production of base molecules, certain intermediates are also generated which, due to their origin and application potential, can likewise be considered bio-derived platforms.

In recent years, both public and private entities have employed similar terminological variants, particularly within European and international projects, to refer to products obtained from biomass, also called bio-derived products. At present, the European Commission does not require that products be entirely bio-based to be defined as such, a position that contrasts with that of the United States Farm Bill, according to which a product

must be wholly or substantially composed of biological materials to be classified as bio-based.

According to the 2002 Farm Bill, bio-based products are defined as commercial or industrial goods not intended for food use, consisting largely or entirely of renewable biological resources, including agricultural, forestry, animal, or marine materials. The European definition, established in 2007, instead considers bio-based any product derived from biomass (excluding food and feed), defining biomass as materials originating from plants, crops, trees, algae, organic household waste, and animal or marine organisms (Biddy et al., 2007). These products are of considerable interest, particularly in the fields of cosmetics, food additives, pharmaceuticals, and many other high-value-added sectors.

The European Committee for Standardization (CEN) later updated the definition of biomass, specifying that although fossil fuels have a biological origin, they result from long-term geological transformations and therefore do not fall under the category of biologically derived materials. Consequently, fossil sources and materials derived from geological formations are excluded from the definition of biomass.

Given the wide variety of terms used to designate these compounds, an operational definition of a bio-derived base molecule can be proposed as follows:
"A chemical compound whose constituent elements are entirely obtained from biomass (i.e., materials of biological origin, excluding fossil sources), and which can serve as a building block in the synthesis of high-value-added chemicals." (Salma et al., 2021)

The complexity of reaction pathways can lead to ambiguity in identifying true base molecules, as multiple intermediates along the same transformation chain may be recognized as such.

As previously mentioned, among the more than 300 compounds evaluated, 12 building block or base chemicals were identified as the most promising candidates for sustainable production (Werpy & Petersen, 2004; Saxena et al., 2017). A strategic shift in their production, towards the use of renewable, low-impact raw materials, could represent a viable alternative to conventional processes, which are typically energy-intensive and fossil-based.

Among these compounds, succinic acid (SA) stands out for its high potential as a base molecule, being a key intermediate in the synthesis of numerous other compounds derived from renewable resources (Stichnothe et al., 2020; Louasté & Eloutassi, 2020). Owing to its versatility and industrial relevance, the global market for succinic acid has shown steady growth: its value increased from 157 million USD in 2015 to 234 million USD in 2022, an increase of 67%, and projections indicate it could surpass 500 million USD by 2030 (Ong et al., 2019; Grand View Research, 2022)

1.1. Succinic acid

Chemically, succinic acid (Figure 1.1), also known as 1,4-butanedioic acid, is a four-carbon dicarboxylic acid naturally occurring in various animal and plant tissues as well as in certain microorganisms. Historically, it was extracted from amber.

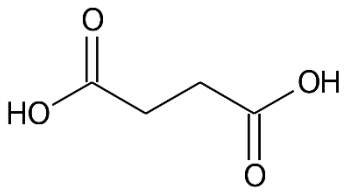

Figure 1.1 *Molecular structure of succinic acid ($C_4H_6O_4$), consisting of two terminal carboxyl groups connected by a saturated aliphatic chain.*

At present, over 50% of the industrial production of succinic acid originates from petrochemical feedstocks (Grand View Research, 2022). This production mainly occurs through three established processes: catalytic hydrogenation, electrolytic reduction, and paraffin oxidation, starting from maleic acid or maleic anhydride, both of which are obtained from fossil resources.

A typical example of a conventional two-step process involves the oxidation of butane or benzene to produce maleic anhydride (Phase I), followed by hydrogenation to succinic anhydride, which is subsequently hydrated to yield succinic acid (Phase II). This technology is well-established and ensures high production yields, meeting a global demand exceeding 30,000 tons per year (Lee et al., 2011; Saxena et al., 2017). However, such a process presents several environmental and economic drawbacks. Among the main issues associated with the use of fossil-based resources are the volatility of oil prices, the progressive depletion of these

sources, and the urgent need to mitigate environmental impact. These factors are driving the replacement of petrochemical compounds with bio-based alternatives produced from renewable feedstocks (Lee et al., 2011; Pascuta et al., 2022).

In this context, an increasing interest has emerged in recent years in the biotechnological production of succinic acid through microbial fermentation, which represents a more sustainable alternative to traditional chemical methods (Corona-González et al., 2008; Borges & Pereira, 2011). The applications of biologically derived succinic acid are numerous across various industrial sectors. In particular, it is employed as a food additive (E363 code), for example in beverages and processed meats, as well as in the formulation of surfactants, detergents, flavors, and fragrances, and in the production of biodegradable polymers, such as textile fibers (Nghiem et al., 2017; Saxena et al., 2017).

In the pharmaceutical sector, succinic acid serves as a key raw material for the synthesis of several high-value-added compounds, including adipic acid, 2-pyrrolidinone, various succinate salts, 1,4-butanediol, and maleic anhydride (Mitrea et al., 2017; Tan et al., 2017; Mitrea et al., 2022). Among the most recent and promising applications is the production of biodegradable plastics, particularly poly-butylene succinate (PBS), a polymer whose mechanical properties are comparable to those of polypropylene (Lu et al., 2021).

The solubility characteristics of succinic acid further enhance its application versatility: it is soluble in water, slightly soluble in ethanol, acetone, ether, and glycerin, while being insoluble in benzene, carbon disulfide, and petroleum ether (Saxena et al., 2017).

1.2. Biological synthesis of succinic acid

The biological synthesis of succinic acid occurs through the tricarboxylic acid cycle (TCA cycle or Krebs cycle) within the cell (Mitrea et al., 2022). Numerous studies have focused on optimizing succinate production via fermentation using microorganisms, primarily bacteria and yeasts.

From a biochemical perspective, succinate production can occur through metabolic or non-metabolic pathways, beginning in the mitochondria (anabolic and catabolic processes) and subsequently extending to the cytosol. Succinyl-CoA synthetase converts succinyl-CoA into

succinate, highlighting the central role of this enzyme within the Krebs cycle (Grimolizzi & Arranz, 2018).

Among the metabolic routes leading to succinic acid synthesis, the following pathways can be distinguished (Figure 1.2):

- Reductive pathway (reverse TCA cycle): This is the most common pathway among anaerobic microorganisms. It involves the consumption of two molecules of NADH and allows a theoretical yield of 1 mol of succinic acid per mol of glucose (1:1 ratio), which is, however, limited by NADH availability. The CO_2 fixation is primarily mediated by phosphoenolpyruvate carboxykinase (PCK). Phosphoenolpyruvate, adenosine diphosphate (ADP), and bicarbonate form oxaloacetate, accompanied by the generation of adenosine triphosphate (ATP) (Tsuji et al., 2013; Dessie et al., 2018). Oxaloacetate is converted to malate via malate dehydrogenase (cofactor: NADH), then to fumarate through fumarase, and finally to succinate via succinate dehydrogenase (Mitrea et al., 2017).
- Oxidative pathway: This route requires oxygen and generates an excess of NADH and other cofactors. Glycolysis converts glucose into pyruvate, which is then transformed into acetyl-CoA via pyruvate dehydrogenase. Acetyl-CoA reacts with oxaloacetate to form citrate, which, through decarboxylation steps, yields one molecule of succinic acid, two molecules of CO_2, and one molecule of NADH, with efficient ATP generation through oxidative phosphorylation (Beauprez et al., 2010; Carlson et al., 2016; Matei et al., 2020).
- Glyoxylate cycle: in the absence of oxygen, the glyoxylate shunt is activated, combining reductive and oxidative reactions to enhance succinate production. Oxaloacetate condenses with acetyl-CoA to form citrate and subsequently isocitrate. Isocitrate lyase then cleaves isocitrate to yield glyoxylate and succinic acid. The cycle is completed when glyoxylate condenses with an additional molecule of acetyl-CoA to produce malic acid and regenerate oxaloacetate (Beauprez et al., 2010; Zhu & Tang, 2017).
- 3-Hydroxypropionate (3HP) pathway: this pathway operates under aerobic conditions and is characteristic of cyanobacteria. It comprises 16 enzymatic steps, beginning with

acetyl-CoA, which is converted into malonyl-CoA, then to 3-hydroxypropionate, propionyl-CoA, and methylmalonyl-CoA (through epimerization and mutase reactions), leading to succinyl-CoA, and finally to succinic acid via succinyl-CoA synthetase. The theoretical yield is 1 mol of succinic acid per mol of acetyl-CoA, accompanied by the release of 2 mol of CO_2 (Liu et al., 2020; Vodnar et al., 2020; Liu et al., 2022).

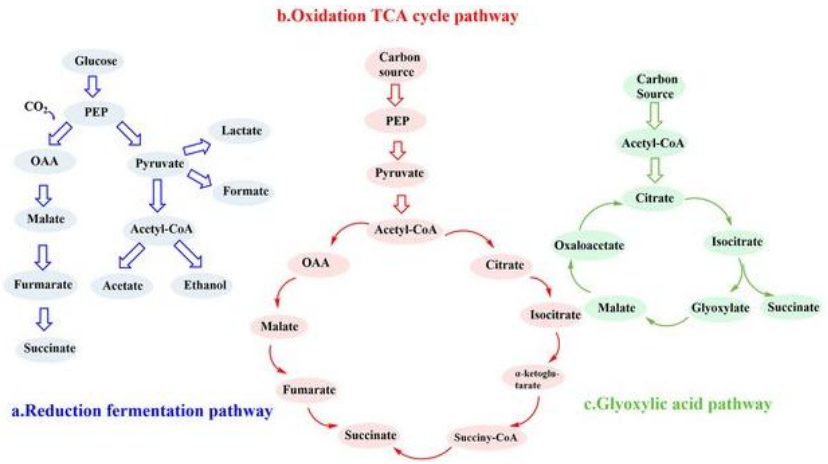

Figure 1.2 *Metabolic pathways for succinic acid biosynthesis. Schematic representation of the three principal metabolic routes leading to succinic acid production: (a) the reductive TCA (rTCA) cycle, a CO_2-fixing, anaerobic pathway that channels carbon flux from phosphoenolpyruvate (PEP) to oxaloacetate and subsequently to succinate; (b) the oxidative tricarboxylic acid (TCA) cycle, in which succinate is formed as an intermediate during aerobic respiration; and (c) the glyoxylate cycle, which bypasses the decarboxylation steps of the TCA cycle to generate succinate from acetyl-CoA under specific metabolic conditions (Zhou et al., 2023).*

1.3. Succinic acid-producing microorganisms

Recent studies have shown that several prokaryotes belonging to different genera, such as *Actinobacillus*, *Anaerobiospirillum*, *Basfia*, *Corynebacterium*, *Bacillus*, and *Enterococcus,* are the main natural producers of succinic acid. Many of these microorganisms, most of which have been isolated from the bovine rumen, accumulate succinic acid as the end product of fermentation. This compound serves as a key precursor in the biosynthesis of propionic acid, which accounts for approximately 20% of the total volatile fatty acids

(VFAs). Propionic acid is absorbed through the ruminal wall and subsequently oxidized to generate energy and milk (Hong et al., 2004; Narisetty et al., 2022).

Although succinic acid is an intermediate of the Krebs cycle, certain strains capable of producing it, such as *Actinobacillus succinogenes* and *Anaerobiospirillum succiniciproducens*, can accumulate it even under anaerobic conditions as the final product of fermentation. Several strains are able to naturally produce large quantities of succinic acid, including *A. succinogenes* and *B. succiniciproducens*. However, over the years, the genetic knowledge of model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* has been exploited to engineer these microorganisms and induce succinic acid production at concentrations suitable for industrial processes.

Table 1.1 summarizes the various microorganisms employed for the production of succinic acid using glucose as the carbon source.

Most microorganisms exploit anaerobic conditions and, consequently, the reductive branch of the TCA cycle, operating optimally at a near-neutral pH (Hong et al., 2004; Pateraki et al., 2016; Ahn et al., 2017). They also possess the ability to fix CO_2, and in several cases, they perform anaerobic respiration using fumarate as the terminal electron acceptor.

At the biochemical level, the electron carriers involved are ubiquinone and menaquinone, which are employed in aerobic and anaerobic respiration, respectively. Most native succinic acid producers, including *A. succinogenes* and *B. succiniciproducens*, possess metabolic pathways for the synthesis of menaquinone but not ubiquinone. The menaquinone-based electron transport system enables the use of fumarate as the final electron acceptor, leading to the formation of succinic acid through the reaction catalyzed by fumarate reductase (McKinlay et al., 2010; Ahn et al., 2020).

Phosphoenolpyruvate (PEP) plays a key role in the succinic acid metabolism of these microorganisms, serving as a branching point for the major metabolic fluxes. The key enzyme involved is phosphoenolpyruvate carboxykinase (PEPCK), encoded by the pckA gene, an efficient CO_2-fixing enzyme that catalyzes the conversion of PEP to oxaloacetate (OAA) with ATP formation ($PEP + CO_2 + ADP \rightarrow OAA + ATP$).

Table 1.1 *Microorganisms producing succinic acid from glucose. (AA = acetic acid; FA = formic acid; PA = pyruvic acid; LA = lactic acid; MA = malic acid; CA = citric acid; ICA = isocitric acid; PPA = propionic acid).*

Strain	Culture mode	Succinic acid concentration ($mg \cdot L^{-1}$)	Fermentation by-product	Reference
Actinobacillus succinogenes GXAS	Anaerobic; batch	54.6	AA, FA, PA, PPA	Shen et al. (2016)
Actinobacillus succinogenes 130Z	Anaerobic; batch	29-39	AA, FA, PA	Min et al. (2014)
Actinobacillus succinogenes DSM22257	Anaerobic; continuous	26.5	AA, FA, PA	Ferone et al. (2017)
Anaerobiospirillum succiniciproducens ATCC5348	Anaerobic; batch	34.4	AA	Yan et al. (2014)
Basfia succiniciproducens LPK7	Anaerobic; fed-batch	70.3	AA, LA, PA, MA	Ahn et al. (2017)
Escherichia coli HL27659K	Aerobic; continuous	7.0	AA, PA	Thakker et al. (2013)
Cornybacterium glutamicum NC_3	Micro-aerobic; fed-batch	81.0	AA, PA	Xu et al. (2016)
Yarrowia lipolytica	Fed-batch	30.1	CA, ICA	Cui et al. (2017)
Saccharomyces cerevisiae	Fed-batch	57.8	CA, ICA, PA	Wahl et al. (2017)

Succinic acid production through the reductive pathway is highly dependent on CO_2 availability, and the naturally CO_2-rich environment of the bovine rumen greatly enhances the biosynthesis of this acid (Hungate, 2013).

Among the various microorganisms tested for industrial-scale succinic acid production, *Actinobacillus succinogenes* has proven to be the most promising, owing to its favorable

physiological and metabolic characteristics (Pateraki et al., 2016; Nghiem et al., 2017; Dessie et al., 2018).

1.4. *Actinobacillus succinogenes*

Actinobacillus succinogenes is a facultative anaerobic, capnophilic, non-pathogenic, Gram-negative, and non-motile bacterium, classified as a biosafety level 1 (BSL-1) microorganism. This bacterium was first isolated from the bovine rumen and belongs to the *Pasteurellaceae* family. The optimal growth temperature is 37 °C, with a pH range between 6.5 and 7.5 (Yang et al., 2020).

This strain exhibits high metabolic versatility, being able to utilize a wide variety of carbon sources, including glucose, xylose, arabinose, mannose, galactose, fructose, sucrose, lactose, cellobiose, mannitol, maltose, and glycerol. Moreover, it can naturally produce succinic acid in high concentrations (Table 1.2).

Glucose generally represents the preferred carbon source for the synthesis of biologically derived chemical compounds. The bacterium *Actinobacillus succinogenes* is capable of efficiently converting glucose into succinic acid. Metabolically, glucose is catabolized to PEP/pyruvate through glycolysis and the oxidative pentose phosphate pathway. However, the TCA cycle in this microorganism is partially incomplete due to the absence of key enzymes such as citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase, which prevents the production of succinic acid via the oxidative route. The glyoxylate shunt is also absent. Therefore, the main pathway for succinic acid synthesis is the reductive branch of the Krebs cycle, in which the enzyme phosphoenolpyruvate carboxylase (PEPC) plays a crucial role in linking the three-carbon and four-carbon metabolic pathways (Yang et al., 2020).

In recent years, extensive studies have been carried out on the ability of this microorganism to utilize alternative carbon sources for succinic acid production. Xylose, one of the most abundant pentose sugars in lignocellulosic hydrolysates, cannot be metabolized by most microorganisms, which limits the economic sustainability of using such biomasses as

substrates. However, *A. succinogenes* possesses the capability to metabolize xylose and convert it into succinic acid (Salvachúa et al., 2016).

Table 1.2 *Succinic acid production by Actinobacillus succinogenes from different carbon sources.*

Substrate	Strain	Fermentation mode	Succinic acid concentration ($g \cdot L^{-1}$)	Reference
Glucose	130Z	Continuous	48.50	Bradfield & Nicol (2014)
		Batch	66.40	Guettler et al. (1999)
	ATCC55618	Batch	73.00	Thuy et al. (2017)
		Fed-bacth	100.54	
Xilose	130Z	Continuous	29.40	Bradfield & Nicol (2016)
		Batch	48.00	Salvachùa et al. (2016)
	CGMCC1593	Batch	32.60	Liu et al. (2008)
Sucrose	NJ113	Fed-batch	60.40	Jiang et al. (2014)
	CGMCC1593	Batch	40.10	Liu et al. (2008)
Maltose	BE-1	Batch	16.80	Li et al. (2010)
	CGMCC1593	Batch	38.80	Liu et al. (2008)
Fructose	BE-1	Batch	14.20	Li et al. (2010)
	CGMCC1593	Batch	38.40	Liu et al. (2008)
Glycerol	130Z	Fed-batch	49.60	Carvalho et al. (2014)
		Batch	29.30	Vlysidis et al. (2011)
	ATCC55618	Continuous	31.70	Schindler et al. (2014)

Moreover, the strain exhibits good tolerance to high initial sugar concentrations, such as glucose (up to 158 $g \cdot L^{-1}$) (Lin et al., 2008), and to fermentation by-products resulting from PEP/pyruvate metabolism, including acetate (10.8 $g \cdot L^{-1}$), ethanol (42 $g \cdot L^{-1}$), and formate (10 $g \cdot L^{-1}$) (Song & Lee, 2006; Carvalho et al., 2016).

These characteristics make *A. succinogenes* an extremely attractive candidate for use in industrial-scale bio-based succinic acid production.

1.5. Industrial fermentation

To date, succinic acid (SA) has been produced on an industrial scale primarily through a petrochemical process, starting from n-butane via maleic anhydride. Although this conventional, fossil-based route remains economically competitive, with an average cost of approximately 2.0 $USD \cdot kg^{-1}$, biotechnological alternatives based on microbial fermentation of agro-industrial residues and by-products have been proposed. These approaches not only offer significant environmental benefits but also represent a strategic opportunity for sustainable development, enabling a reduction of over 60% in greenhouse gas emissions compared to conventional petrochemical processes (Ferone et al., 2017; Musonda et al., 2020; Stegmann et al., 2020).

Since 2012, several companies have established industrial or pilot-scale plants for the production of succinic acid from renewable sources (Table 1.3).

For instance, Reverdia developed a direct acidic pH fermentation process using corn starch and *Saccharomyces cerevisiae*, achieving an annual production capacity of 10,000 tons. Myriant, in contrast, employed non-genetically modified *Escherichia coli* and sugars derived from sorghum and other commercial sources, reaching a production of 13,600 tons per year (Jansen & van Gulik, 2014). Succinity attained a production capacity of 10,000 tons per year using renewable substrates such as glycerol, glucose, and sucrose, through the application of the strain *Basfia succiniciproducens* (Sosa-Fernandez & Velizarov, 2018). BioAmber, in collaboration with Mitsui (Canada), utilized a genetically engineered yeast (*Candida kruseii*) licensed from Cargill, primarily using corn-derived glucose as a substrate to produce 30,000 tons per year, whereas BioAmber's French facility employed *E. coli* and wheat-derived

glucose to achieve a production of 3,000 tons annually (Li & Mupondwa, 2021; Li et al., 2021).

Table 1.3 *Companies with commercial-scale succinic acid production plants.*

Company	Plant localization	Microorganism	Capacity (T)	Activity	Reference
BioAmber	Sarnia (Canada)	*Candida kruseii*	30000	2015	Li & Mupondwa (2021)
	Pomacle (France)	*Escherichia coli*	3000	2010	Li et al. (2021)
Reverdia	Cassano Spinola (Italy)	*Saccharomyces cerevisiae*	10000	2012	Carlson et al. (2016)
Myriant	Lake Providence (USA)	*Escherichia coli*	13600	2013	Jansen & van Gulik (2014)
Succinity	Montmelò (Spain)	*Basfia succiniciproducens*	10000	2014	Sosa-Fernandez & Velizarov (2018)

Despite the industrial-scale implementation of bio-based succinic acid production by these companies, several technical and economic challenges persist. These challenges primarily concern the availability and cost of carbon sources, the selection of optimal microbial strains, the presence of inhibitory compounds that can reduce product yield, as well as difficulties associated with downstream processing and purification, and the overall scalability of the production process (Barbosa et al., 2024).

1.6. Lignocellulosic biomass

In recent decades, the use of lignocellulosic biomass for the production of biofuels and chemicals has attracted increasing attention. Such biomass mainly derives from residues and by-products of various agro-industrial production chains and is widely available on a global scale, with an estimated annual production of approximately 2×10^{11} tons (Kumar et al., 2020). The most abundant sources include rice, wheat, maize, and sugarcane, as they are extensively cultivated for human and animal consumption (Vivek et al., 2017).

Lignocellulosic biomass represents a complex matrix, whose the main components present in significant quantities are cellulose (40–60% of dry weight), hemicellulose (10–40% of dry weight), and lignin (15–30% of dry weight) (Lu et al., 2021) (Figure 1.3).

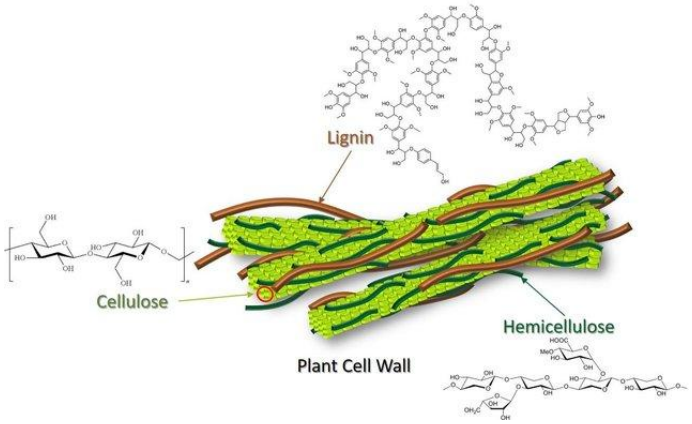

Figure 1.3 *Structure of lignocellulosic biomass (Jensen et al., 2017)*

Cellulose is a homopolymer composed of glucose units linked by β-1,4-glycosidic bonds. Its highly crystalline and compact structure requires specific treatment conditions to enable depolymerization into simple sugars, such as glucose (Deng et al., 2023; Cazier et al., 2024).

Hemicellulose is characterized by a higher heterogeneity compared to cellulose. It consists of a mixture of sugars, including xylose, arabinose, mannose, and glucose. Its less ordered and more amorphous structure makes it more easily degradable, although adequate pretreatment is still required. Some studies also suggest that hemicellulose plays a key role in maintaining the structural flexibility of plant cell walls (Li et al., 2024).

Lignin is one of the main structural components responsible for plant rigidity and mechanical resistance. It is a three-dimensional phenolic polymer, insoluble in water, which surrounds cellulose fibers and protects them from enzymatic and microbial attack (Woźniak et al., 2025).

The percentage composition of these three fractions can vary significantly depending on the type of biomass used, as summarized in Table 1.4.

Table 1.4 *Lignocellulosic biomass composition from different sources (% dry weight)*

Lignocellulosic biomass	Cellulose %	Hemicellulose %	Lignin %	Reference
Maize stalks	50	20	30	Christopher et al. (2017)
Maize stove	38	23	20	Wan & Li (2010)
Sugarcane basse	35	36	16	Sasaki et al. (2003)
Rice straw	36	21	24	Imman et al. (2015)
Wheat straw	40	21	23	Khan & Mubeen (2012)
Mischantus straw	42	25	23	Dąbkowska et al. (2019)

The composition of lignocellulosic biomass is closely related to its origin. For instance, maize stalks exhibit the highest cellulose content (50% of dry weight) and lignin content (30% of dry weight), while they show the lowest hemicellulose content among the analyzed biomasses (20% of dry weight) (Christopher et al., 2017). The highest hemicellulose content has been observed in sugarcane bagasse, accounting for 36% of dry weight (Sasaki et al., 2003). Straws (from wheat, rice, and Miscanthus), on the other hand, are the biomasses with the highest lignin content, exceeding 23% of dry weight.

Before fermentation, it is essential to subject the biomass to a series of pretreatment steps aimed at separating and making cellulose and hemicellulose accessible, as they are tightly bound within the lignocellulosic matrix. Subsequently, these polysaccharide fractions are hydrolyzed into their respective sugar monomers, primarily glucose and xylose, which can be utilized by microorganisms during the fermentation process.

1.7. Lignocellulosic biomass pre-treatments

Due to the structural complexity of the lignocellulosic matrix, pretreatment processes represent a crucial step to make fermentable sugars available to the microorganisms

employed in fermentation. Pretreatment alters the structure of lignocellulose, facilitates lignin removal, and increases cellulose accessibility to enzymatic hydrolysis, allowing the conversion of polysaccharides into simple sugars such as glucose and xylose.

Enzymatic hydrolysis is one of the most effective strategies for converting cellulose and hemicellulose into simple sugars. This process relies on the use of cellulolytic and hemicellulolytic enzymes, which catalyze the cleavage of bonds within the cellulose and hemicellulose structures, releasing glucose, xylose, arabinose, mannose, and sucrose units (Yang et al., 2011). These enzymes are commonly produced by microorganisms, particularly fungi of the genera *Trichoderma reesei* and *Aspergillus niger*, which are widely used in biotechnology for cellulase and hemicellulase production (Jia et al., 2021).

Enzymatic hydrolysis occurs in several stages. Initially, the biomass undergoes mechanical or thermo-chemical pretreatment aimed at increasing its accessibility to enzymatic action. Subsequently, enzymes are added to the treated substrate under optimal operational conditions, typically around 50 °C and pH 4.8. Under these conditions, the enzymes progressively degrade cellulose and hemicellulose, first generating oligosaccharides and then simple sugars, which can be used in fermentative processes for the production of biofuels and biochemicals.

Numerous pretreatment techniques have been developed, which can generally be classified into three main categories: physical, chemical, and physico-chemical (Table 1.5).

Physical methods include mechanical treatments such as milling and ultrasonication, whichrequire high energy input and are associated with significant operational costs. Another limitation of physical methods lies in their relatively low yields. Nevertheless, Putri et al. (2023) investigated the combined use of peracetic acid and alkaline peroxide with ultrasound on rice straw and bagasse, achieving an increase in cellulose content of 107% and 68%, respectively, compared to the untreated biomass.

Table 1.5 *Comprehensive comparison of lignocellulosic biomass pretreatment methods*

Type	Process	Advantages	Dsisadvantages
Physical	Milling	• Low processing costs	• Poor selectivity
	Ultrasuond	• High processing speed • Moderate selectivity	• Limited scalability • High equipment costs
	Hydrothermal	• Environmentally sustainable • No solvents required	• Not applicable to all feedstocks • High energy consumption
Chemical	Acid	• High sugar release efficiency	• Corrosive • Generation of toxic by-products • Requires neutralization
	Alkaline	• Efficient lignin removal	• Large waste generation • Requires neutralization
	DES (Deep eutectic solvents)	• Innovative • High selectivity	• High solvent cost • Limited scalability
Physico-chemical	Organosolv	• Effective lignin removal • Generation of high-value by-products	• High solvent cost • Process complexity
	Steam-explosion	• Enhances subsequent hydrolysis • Requires little solvent • Low cost	• Possible sugar degradation

Chemical pretreatments, on the other hand, mainly employ acids (such as hydrochloric, sulfuric, and phosphoric acids), bases (sodium hydroxide, calcium hydroxide, and ammonia), and environmentally friendly organic solvents (organosolv methods). Jampatesh et al. (2019) compared the acid pretreatment of rice straw using 1 M hydrochloric, phosphoric, and sulfuric acids, testing different hydrolysis times (15, 30, 45, and 60 minutes). The best sugar recovery (10 $g \cdot L^{-1}$ glucose and 18 $g \cdot L^{-1}$ xylose) was obtained with HCl after 60 minutes. Similarly, Lo et al. (2020) optimized the acid pretreatment of sweet sorghum bagasse at 50 °C for 1 hour, achieving a recovery of 29.2 $g \cdot L^{-1}$ of cellulose-derived

glucose after enzymatic hydrolysis. These methods are widely adopted due to their high conversion yields; however, the handling and disposal of acids and bases represent significant environmental and economic limitations.

Among physico-chemical pretreatments, steam explosion is the most extensively used. This technique involves exposing lignocellulosic biomass to high-temperature (160–260 °C) and high-pressure (5–50 atm) saturated steam for short periods (Lee et al., 2003). Kuglarz et al. (2018) applied this method to rapeseed straw using a 1% H_2SO_4 solution in a reactor at 180 °C for 10 minutes, varying the biomass concentration (10, 15, 20, and 25% w/v) and enzyme loading (cellulases). The highest glucan recovery (98.7%) was obtained with a biomass concentration of 20% w/v, corresponding to 23.3 $g \cdot L^{-1}$ glucose and 4.5 $g \cdot L^{-1}$ xylose after enzymatic hydrolysis (with 13% enzyme dosage).

Steam explosion is considered advantageous both economically and environmentally, especially when compared to the aforementioned techniques. However, the extreme operating conditions (high temperatures and pressures) can promote the formation of undesirable compounds such as organic acids, furans (e.g., furfural and HMF – hydroxymethylfurfural), and phenolic derivatives. These by-products are well-documented for their inhibitory effects on microbial growth, thereby reducing both the efficiency of enzymatic hydrolysis and the overall fermentation productivity for succinic acid synthesis.

1.8. Inhibitor compounds of fermentative processes

Pretreatment processes of lignocellulosic biomass, carried out under either acidic or alkaline conditions and often involving high temperatures (as in the case of steam explosion), have been shown to achieve high sugar conversion yields. However, such extreme operating conditions also lead to the formation of undesirable by-products, including organic acids (such as acetic acid, lactic acid, and formic acid), furans (such as 5-hydroxymethylfurfural and furfural), and phenolic compounds. These substances are recognized as microbial growth and productivity inhibitors in fermentative processes (Table 1.6).

Table 1.6 *Inhibitory compounds generated during lignocellulosic biomass pretreatments and their critical concentrations that inhibit the growth of Actinobacillus succinogenes.*

Compound class	Compound	Critical concentration ($g \cdot L^{-1}$)	References
Furans	Furfural	6.0	Dessie et al. (2019)
	5- Hydroxymethylfurfural	10.0	
Acids	Formic acid	18.0	Jonsson & Martin (2016)
	Lactic acid	33.7	
	Acetic acid	55.0	
Phenols	Cinnamic acid	1.47	Xu et al. (2022)
	Benzoic acid	1.47	
	p-Hydroxybenzaldehyde	2.20	

Furans, for example, can interfere with key enzymes involved in glycolysis and the tricarboxylic acid (TCA) cycle, thereby reducing both sugar uptake and final product yield (Chen et al., 2021). Moreover, furfural can damage mitochondrial structures by inducing the intracellular accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), leading to cytotoxicity (Tian et al., 2011). A study by Dessie et al. (2019) demonstrated that a combination of furfural and HMF at concentrations of 3 $g \cdot L^{-1}$ completely inhibited succinic acid production by *A. succinogenes*. It has also been observed that furfural can enhance the toxicity of other compounds, such as phenols and acetic acid, toward microorganisms. In a recent investigation, Xu et al. (2022) reported that certain phenolic compounds, particularly cinnamic acid and benzoic acid at concentrations of 1.47 $g \cdot L^{-1}$, exert a strong inhibitory effect on *A. succinogenes* by disrupting glucose uptake systems, heat shock proteins (HSPs), and chemotaxis mechanisms.

Furans originate from the dehydration of pentose and hexose sugars released during hemicellulose hydrolysis, while phenolic compounds mainly result from the degradation of lignin and other extractable substances. Among the most frequently detected phenols during

lignocellulosic biomass pretreatment are p-hydroxybenzoic acid, vanillin, vanillic acid, cinnamic acid, benzoic acid, and syringic acid (Dąbkowska et al., 2019).

Acetic acid is formed through the cleavage of acetyl groups present in degraded hemicellulose. Formic acid may arise from the decomposition of HMF under acidic conditions or from the degradation of polysaccharides under alkaline environments, both leading to formic acid formation (Kumar et al., 2020). Furthermore, acetic, formic, and lactic acids can also be produced as metabolic by-products during microbial succinic acid synthesis. It has been reported that formic acid inhibits the growth of *Actinobacillus succinogenes* at concentrations between 11 and 18 $g \cdot L^{-1}$, while acetic and lactic acids exhibit inhibitory effects at 33.7 $g \cdot L^{-1}$ and 55 $g \cdot L^{-1}$, respectively (Jönsson & Martin, 2016). Formate and acetate share a similar inhibitory mechanism, interfering with phosphate transport across the cell membrane, increasing ATP demand, and consequently hindering metabolic processes.

1.9. Production of succinic acid using lignocellulosic biomass

Table 1.7 reports recent studies concerning the use of different lignocellulosic biomasses as carbon sources for succinic acid production. In the study by Vallecilla-Yepez et al. (2021), the highest succinic acid concentration among the reviewed works was achieved, 27.8 $g \cdot L^{-1}$, using a hydrolysate derived from corn stover previously subjected to high-temperature water treatment followed by enzymatic hydrolysis.

Dąbkowska et al. (2019) achieved a succinic acid concentration of 23.5 $g \cdot L^{-1}$ by optimizing the ratio between Miscanthus straw hydrolysate and the culture medium, which was set at 75:25 (v/v). Sawisit et al. (2018) investigated the simultaneous saccharification and fermentation (SSF) process in both batch and fed-batch modes. After alkaline pretreatment of rice straw, the results showed that succinic acid production was higher in the fed-batch process compared to the batch process, using the engineered strain *Escherichia coli* KJ122.

Table 1.7 *Lignocellulosic biomass used for succinic acid production via fermentation. Reported are the pretreatments prior to fermentation, the microorganisms employed, the initial sugar concentration (Ci), and the final succinic acid (SA) concentration (Cf). NR = not reported.*

Biomass	Pretreatment	Microorganism	C_i sugars $g \cdot L^{-1}$	C_f SA $g \cdot L^{-1}$	Reference
Oil palm trunk	Carboxylic acids and sulfuric acid	*A. succinogenes*	33.6	2.5	Bukhari et al. (2020)
			44.8	10.6	
Sweet sorghum bagasse	Phosphoric acid	*A. succinogenes*	32.7	17.8	Lo et al. (2020)
Sugarcane bagasse	Peracetic acid and alkaline peroxide combined with ultrasound	*Proteus vulgaris*	20.0	5.1	Putri et al. (2023)
	Sodium hydroxide	*A. succinogenes*	130.0	41.0	Chen et al. (2021
Corn stover	High-temperature water	*A. succinogenes*	NR	27.8	Vallecilla-Yepez et al. (2021)
Rice straw	Peracetic acid and alkaline peroxide combined with ultrasound	*Proteus vulgaris*	20.0	3.6	Putri et al. (2023)
	Sodium hydroxide	*E. coli*	100.0	32.0	Sawisit et al. (2018)
	Acid followed by autoclaving	*E. coli*	100.0	14.0	Jampatesh et al. (2019)
Miscanthus straw	Organosolv	*A. succinogenes*	44.2	23.5	Dąbkowska et al. (2019)
Rapeseed straw	Steam explosion	*S. cerevisiae*	23.3	9.3	Kuglarz et al. (2018)
Wheat straw	NR	*Fibrobacter succinogenes*	NR	1.5	Li et al. (2010)

Chen et al. (2021) developed an innovative *in-situ* process called semi-simultaneous saccharification and co-fermentation (SSSCF), achieving a succinic acid concentration of 41 $g \cdot L^{-1}$. The process was conducted on non-dried, alkaline-pretreated sugarcane bagasse residues subjected to enzymatic hydrolysis, to which the growth medium and a 10% (v/v) inoculum of *Actinobacillus succinogenes* were subsequently added directly in the same fermenter.

In the work of Lo et al. (2020), after optimizing the acid pretreatment of sweet sorghum bagasse (at 50 °C for 1 hour), 32.7 g of fermentable sugars were obtained after enzymatic hydrolysis. This hydrolysate was then used in a 3-liter reactor, to which 2.5 M Na_2CO_3 and CO_2 were added, resulting in a final succinic acid concentration of 17.8 $g \cdot L^{-1}$.

Jampatesh et al. (2019) obtained a maximum succinic acid concentration of 14 $g \cdot L^{-1}$ using 70% of the hydrolysate derived from rice straw as the fermentation substrate. Bukhari et al. (2020) tested and compared several organic acids (oxalic, citric, and formic) for the pretreatment of wood obtained from oil palm trunks. The authors observed that, although oxalic acid allowed the highest glucose recovery (over 60%), the best results in terms of succinic acid production were obtained when the biomass was treated with citric acid (2.5 $g \cdot L^{-1}$ vs. 10.6 $g \cdot L^{-1}$).

The analysis of these studies highlights how the starting biomass and the type of pretreatment applied strongly influence the yield of fermentable sugars and, consequently, succinic acid production.

1.10. Recovery of the succinic acid from fermentation broth

Efficient industrial production of succinic acid requires substantial progress in three key areas:

- selection, preparation, and pretreatment of feedstocks;
- formulation of the fermentation medium, optimization of operating conditions, and engineering of microbial strains;
- recovery and purification of the final product.

While the first two aspects have been extensively investigated and are well documented in the literature, downstream stages of the process have received comparatively less attention. This gap is significant, as the downstream processing represents a critical issue in the fermentative production of chemicals, including succinic acid. In most cases, the fermentation broth constitutes a highly complex matrix in which succinate represents the predominant product, typically accounting for roughly 15% of the overall mixture. Recent analyses indicate that downstream process can account for 60–80% of the overall production cost (Mancini et al., 2020).

The downstream processing of bio-based succinic acid generally involves three principal stages (Dessie et al., 2021). The first stage is clarification, during which suspended solids, such as microbial cells, cell debris, and other coarse particles, are removed, typically through centrifugation or membrane-based separation.

The second stage consists of primary recovery, in which the bulk aqueous mixture is treated to separate impurities and by-products, yielding a crude stream enriched in succinate. At this point, the physicochemical characteristics of succinic acid and of the co-produced metabolites play a decisive role. For instance, because this acid has a high boiling point, compounds with substantially lower boiling points, such as water, acetic acid, or other volatile impurities, can be selectively removed via evaporation. Additional separation strategies, including solvent extraction, adsorption, distillation, electrodialysis, and precipitation, may further enhance the recovery efficiency.

In the final stage, the crude product is purified and concentrated to obtain high-purity succinic acid. This typically involves sequential operations such as solvent removal, evaporation, crystallization, and drying. Collectively, these three stages underscore that succinic acid purification cannot realistically be achieved through a single unit operation; instead, downstream process relies on an integrated multistep approach that combines complementary techniques.

Table 1.8 summarizes various studies that have examined downstream techniques for the separation and purification of succinic acid produced via fermentation. Across these works, reported succinic acid yields typically surpass 90% and product purities exceed 99%, aligning well with industrial targets (90–100% yield and ≥99.5% purity). However, high

yield and purity alone do not guarantee the viability of large-scale production of bio-based succinic acid. A comprehensive assessment must also consider economic performance and environmental sustainability.

Table 1.8 *Recent studies on the downstream processing of bio-based succinic acid with the highest yield and purity. NF = ultrafiltration; AC = activated carbon; RO = reverse osmosis.*

Starting broth	Microorganism	Downstream processing methods	Yield %	Purity %	Reference
Synthetic medium	*E. coli*	Two steps membrane process of NF followed by RO	>92.00	99.50	Khunnonkwao et al., 2018
		Integrated membrane-based fermentation and separation process	90.20	99.40	Wang et al., 2014
Cassava root hydrolysate	*A. succinogenes*	NF followed by cooling crystallization	91.86	99.35	Thuy et al., 2017
Cassava hydolysate	*E. coli*	Two-stage crystallization	95.00	99.00	Xiao et al., 2020
Corn stover	*A. succinogenes*	Cation exchange, AC and crystallization	91.00	99.93	Karp et al., 2018

In this context, an additional fermentation step aimed at lowering the concentration of inhibitory by-products, such as acetic and formic acids, may represent a promising strategy to reduce the reliance on downstream separation and purification operations, and consequently their associated costs. Certain microorganisms, including *Cupriavidus necator*, are able to metabolize these fermentation by-products and convert them into value-added compounds such as polyhydroxyalkanoates (PHAs) (Marudkla et al., 2018; Jawed et al., 2022; Lhamo & Mahanty, 2024). By exploiting this metabolic capability, the process can circumvent the use of energy-intensive separation methods or membrane systems that require frequent replacement. Moreover, the concurrent synthesis of a commercially valuable biopolymer, such as PHB, provides an additional revenue stream that can significantly improve the overall economic sustainability of the production process.

2. Thesis goals

The aim of this doctoral research is to develop and optimize an integrated bioprocess for the biological production of succinic acid from wheat straw, an abundant yet underutilized lignocellulosic feedstock. Wheat straw has significant potential as a renewable raw material; however, its complex structure and recalcitrance to fermentation present substantial challenges that must be addressed to unlock its industrial applicability.

Previous studies have shown that wheat straw often results in low yields of target products, such as succinic acid, with reported titres of approximately 1.5 $g \cdot L^{-1}$, likely due to its resistance to enzymatic degradation and the formation of inhibitory compounds during pretreatment (e.g., furfural, hydroxymethylfurfural, and acetic acid) that hinder microbial metabolism. These limitations underscore the need for a comprehensive optimization strategy that considers feedstock characteristics and fermentation performance.

Against this backdrop, the primary objective of this doctoral work is to establish a robust and scalable fermentation process using wheat straw as a feedstock for the production of succinic acid with performance levels of potential industrial relevance.

To achieve this overarching goal, the research is structured around the following specific objectives:

- Optimize fermentation operational parameters to enhance succinic acid production.
    - ✓ Determine optimal concentrations of fermentable sugars and inhibitory compounds that support efficient growth and metabolism of the selected microorganism, *Actinobacillus succinogenes*.
    - ✓ Fine-tune process conditions (carbon and nitrogen source) to maximize product concentration, yield, and productivity.
- Develop and evaluate a secondary biological treatment for by-product valorisation.
    - ✓ Investigate the use of *Cupriavidus necator* to reduce the concentration of by-products, specifically acetic and formic acids, formed during the first fermentation step.

- ✓ Explore the potential of this biological removal stage not only to reduce downstream processing costs and environmental impact but also to generate a second high value-added product, polyhydroxybutyrate (PHB), as part of an integrated bioprocess.

By addressing these objectives, this research seeks to contribute to the advancement of sustainable biorefinery concepts that transform lignocellulosic residues into valuable biochemical products, thereby improving process viability and supporting circular economy strategies in the bio-based industry.

3. Preliminary batch tests for succinic acid production and co-products through fermentation by *Actinobacillus succinogenes*

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3.1. Introduction

Sustainable bioprocesses and green chemicals will be necessary for the transition to bio-based production. One of the most interesting green chemicals is succinic acid (SA) for its great potential in bio-based economy as precursor of bioplastics/biopolymers. Although SA can be produced through bio-based processes it is still produced by a petrochemical route by using fossil fuel sources at a price of 2 $\$\cdot kg^{-1}$ while bio-based production resulted at uncompetitive price of 2.6 – 4.5 $\$\cdot kg^{-1}$ (Morales et al., 2016) with consequences on economy and on environmental problems (Mancini et al., 2022).

Bio-based succinic acid can be produced by many microorganisms as an intermediate and end-product of fermentative biochemical pathways, including the Krebs cycle. The bacteria *Actinobacillus succinogenes* was one of the most promising microorganisms to produce bio-based succinic acid; in fact, it could utilize a wide range of sugars, in particular glucose till to 158 $g\cdot L^{-1}$ (Lin et al., 2008), and this characteristic made it interesting for use on a commercial scale (Yang et al., 2020). The succinic acid production by the strain *Actinobacillus succinogenes* occurred mainly under anaerobic conditions at 37 °C by using principally glucose or fermentative sugars from biomass and agro-industrial residues. This strain is an anaerobic facultative so it can grow under oxidative conditions, but in those conditions unwanted by-products as lactic acid can be produced that decreased succinic acid concentration.

Some works were published on different fermentation strategies, batch tests on succinic acid production starting from single and mixed sugars (Molino et al., 2020), fed batch tests (Hoefel et al., 2012) that were the most common approaches; furthermore, continuous succinic acid production by immobilized cells was developed (Ercole et al., 2021). Bio-based succinic acid production has been also developed at industrial scale, but there are still critical issues such as the low productivity and yield, and the high costs for the recovery and purification to obtain high purity bio-succinic acid. One of the most critical issues on succinic acid production has been addressed when agro-industrial wastes were used, especially lignocellulosic biomass residues as feedstock. Fermentative processes can be influenced by several parameters (like pH, temperature, inoculum and starting sugars concentration), but in particular, the composition of hydrolysates obtained from

lignocellulosic biomasses can induce the presence of unwanted compounds that adversely affect the process.

Furthermore, the concentration between inoculum and sugars is also one of the important parameters in the fermentation process, as the microorganism's growth can be subject to substrate inhibition due to amounts of glucose that can be too high relative to the strain concentration.

In this study, batch tests on succinic acid production were performed at different concentrations of bacterial strain *Actinobacillus succinogenes* at a given glucose concentration to evaluate the effect of inoculum size on glucose consumption, succinic acid production and by-products. Multiple samples were collected at different times to assess microbial growth by means of a spectrophotometer at 600 nm for optical density measurements and organic acids concentration by uHPLC.

3.2. Materials and methods

3.2.1. Materials and chemicals

The strain *Actinobacillus succinogenes* 130Z was bought from the CCUG (Culture Collection University of Gothenburg) as active freeze-dried pellets. The Tryptic Soy Broth (TBS) was used as a growth medium. TBS was composed of casein peptone (8.5 $g \cdot L^{-1}$), soia peptone (1.5 $g \cdot l^{-1}$), NaCl (2.5 $g \cdot L^{-1}$), K_2HPO_4 (1.25 $g \cdot L^{-1}$) and glucose (1.25 $g \cdot L^{-1}$). The broth was sterilized at 121 °C for 10 min before use. For the carbohydrates and organic acids quantification, uHPLC/LC-MS grade reagents were used. Analytical standards (monosaccharides and organic acids kit) were used for analytical quantification.

3.2.2. Batch test on succinic acid production

A. succinogenes freeze-dried pellet was weighed and transferred into the TBS medium and gently shaken to allow uniform distribution. The experimentation was carried out on batch tests at initial glucose concentration of 1.25 $g \cdot L^{-1}$ and at different concentrations of bacterial strain *Actinobacillus succinogenes* (test 1: 0.38 $g \cdot L^{-1}$) and (test 2: 1.36 $g \cdot L^{-1}$) (Figure 3.1). Growth of the strain, glucose consumption and organic acids concentration were monitored.

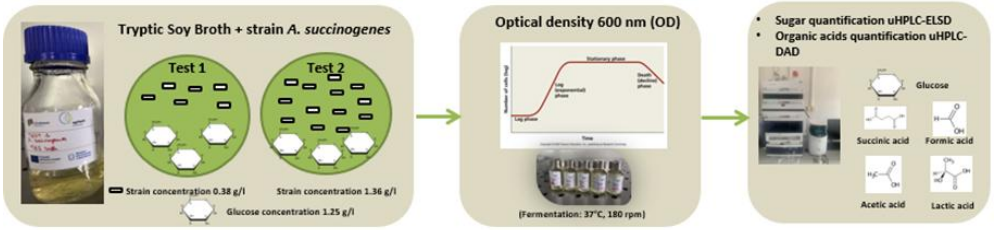

Figure 3.1 *Experimental set-up*

Each sample was transferred in a temperature-controlled shaker at 37 °C, stirring at 180 rpm, in the dark under not strictly anaerobic conditions for the growth of *A. succinogenes*. The growth of the strain was monitored by measuring the absorbance at 600 nm by a dual beam spectrophotometer. Strain concentration ($g \cdot L^{-1}$) was calculated by the following equation that was elaborated at OD value at 600 nm of specific quantity of the strain (dry weight):

$$A.\,succinogenes\ concentration\ (g \cdot L^{-1}) = 0{,}079x + 0{,}078$$

3.2.3. Quali-quantitative analysis of carbohydrates and organic acids

For sugar analysis, the uHPLC 1290 Infinity II instrument with ELSD (Evaporative Light Scattering, model 1260 Infinity II) detector was used following the method Agilent 5991-8984EN. The chromatographic column HILIC-Z (2.1 x 100 mm, 2.7 µm) was used at 80 °C to quantify glucose. Ammonium Acetate (0.1 M) and acetonitrile were used as mobile phase at a gradient of 95-80% in 12 minutes at a flow rate of 0.4 $mL \cdot min^{-1}$. The ELSD detector was used for the identification of sugars at the following parameters: evaporation 60 °C, nebulization 30 °C, 3.6 bar (1.5 SLM), 30 Hz (signal frequency).

For the analysis of carboxylic acids, the uHPLC 1290 Infinity II instrument was used following the method Agilent 5989-5672EN. Samples and standards were injected at a volume of 20 µL by using a Hi-Plex-H column (7.7 x 300 mm, 8 µm) at 50 °C. Isocratic gradient of 100 % H_2SO_4 0.01 M was used at a flow rate of 0.6 $mL \cdot min^{-1}$. Detection of carboxylic acids was done at the wavelength of 210 nm by a diode array detector (DAD).

3.2.4. Succinic acid production performance

Glucose consumption was calculated as:

$$Glucose\ consumption\ (\%) = \frac{C_0 - C_t}{C_0 * 100}$$

where C_0: Initial concentration of glucose, C_t was the concentration of glucose at a fixed time.

Succinic acid yield was calculated as:

$$Succinic\ acid\ yield\ (g \cdot g^{-1}) = \frac{SA\ concentration\ (g)}{Starting\ glucose\ concentration\ (g)}$$

Succinic acid selectivity was calculated as follows:

$$Selectivity\ (g \cdot g^{-1}) = \frac{conc_{SA}}{conc_{SA} + conc_{FA} + conc_{AA}}$$

3.3. Results and discussion

In this work, two different concentrations of *A. succinogenes* were tested under batch conditions to evaluate the effect of different initial strain concentration on succinic acid production performance. To this aim, a synthetic growth medium, the tryptic soy broth (TBS), was used at fixed glucose concentration (1.25 $g \cdot L^{-1}$). The choice of this culture medium was due to its composition which provided all essential nutrients for the growth of the strain and the fact that it was also used in previous studies in which *A. succinogenes* was tested to produce succinic acid (Lin et al., 2008; Van Heerden & Nicol, 2013). This preliminary step turned out to be fundamental to better understanding the optimal initial sugar-strain concentration ratio before to move towards the use of more complex starting feedstocks such as agro-industrial and/or lignocellulosic matrices.

Bacterial growth was monitored till to 48 hours, but the maximum concentration of the strain was obtained at 24 hours at the low strain concentration (test 1: 0.38 $g \cdot L^{-1}$) and at 8 hours at the high strain concentration (test 2: 1.36 $g \cdot L^{-1}$). In Table 3.1 the final concentration (48 h) and the maximum concentration of strain were reported.

Table 3.1 *A. succinogenes concentrations (mg·L^{-1}) Test No 1 and Test No 2*

	Test 1	**Test 2**
Initial concentration (C_0) strain (mg·L^{-1})	380	1360
Final concentration strain (mg·L^{-1})	1800 (48h)	2290 (48h)
Max. concentration growth strain (mg·L^{-1})	3000 (24h)	4710 (8h)

The strain of test 1 reached the highest concentration at 24 hours, equal to 3000 mg·L-1, while at the end of the growth, the concentration decreased to 1800 mg·L^{-1}. Test 2 reached the highest concentration 4710 mg·L^{-1} at 8 hours (Figure 3.2), and the concentration decreased at 24 h and 48 h by reaching 3650 mg·L-1 and 2290 mg·L-1, respectively. The results obtained showed that the greatest growth occurred in the first 8-24 hours, reaching a *plateau* or decrease in strain concentration thereafter for the two concentrations tested. The trend of the growth resulted in a slight difference between test 1 and test 2. Although the strain reached the highest concentration in test 2, a better growth rate was observed in test 1 in which the highest concentration at 24 hours was 7.8 times higher than the initial concentration while in test 2 the highest concentration at 8 hours was 3.5 times higher than the initial concentration. The different progress of biomass growth was mainly due to the relationship between inoculum concentration and glucose concentration. In test 1, the ratio between inoculum and glucose was 0.3, while in test 2, the ratio was 1.1. In test 2, the biomass concentration reached a high point at 8 hours and rapidly decreased until 48 hours. In contrast, this behavior was less evident in test 1 where the strain concentration decreased after 24 hours. This growth trend can be explained as the limiting effect of the glucose concentration in relation to the initial strain concentration as reported in the kinetics study of Corona-González et al., 2008. Corona-González et al., 2008 demonstrated that high glucose concentration (> 20.4 g·L^{-1}) affected biomass concentration that reached the maximum concentration (4.9 g·L^{-1}) at 20 hours but after that time of growth the A. succinogenes concentration rapidly decreased.

In Figure 3.2, glucose concentration and organic acids concentration were shown together with strain concentration.

The glucose concentration decreased to 986 mg·L^{-1} in test 1 and 1032 mg·L^{-1} in test 2, with a final consumption at 48 hours of 21.1 % in test 1 and 17.4 % in test 2. Glucose consumption was observed at around 19.7% after 24 hours in test1, while in test 2, the glucose consumption was 15.7% at 24 hours. On average, 20% glucose consumption was found with no significant differences between the two tests ($p>0.05$).

As shown in Figures 3.2 and 3.3, the peak of organic acid concentration was observed at 24 hours for all tests. The concentration of succinic acid reached 288.6 mg·L^{-1} and 428.3 mg·L^{-1} respectively, in tests 1 and 2. The production of by-products acetic and formic acids was higher than succinic acid. Acetic acid was the most abundant by-product, and its concentration was equal to 894.8 mg·L^{-1} at 24 hours (Test 1) and 879.1 mg·L^{-1} at 24 hours (test 2). Selectivity calculation demonstrated for both tests that the concentration of by-products was higher than succinic acid, but the ratio between succinic acid and by-products become better in test 2. Also, succinic acid yield was slightly increased in test 2 (0.24 g·g^{-1}) respect to test 1 (0.19 g·g^{-1}). The productivity of succinic acid resulted lower in test 1 (12 mg·L^{1}·h^{-1}) than the value in test 2 (18 mg·L^{-1}·h^{-1}).

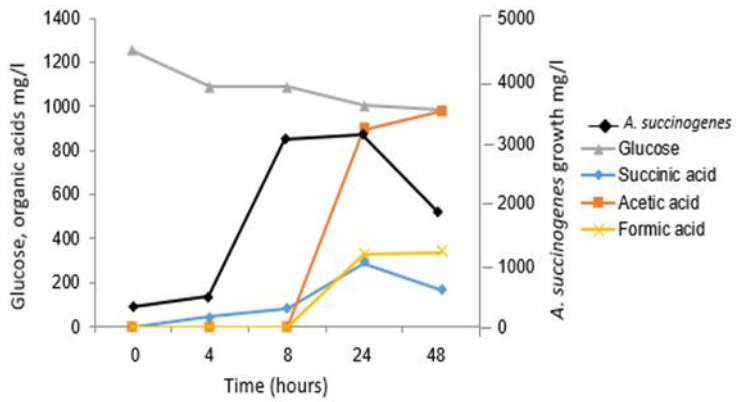

Figure 3.2 *TEST 1: 0.38 g·L^{-1} strain concentration: Actinobacillus succinogenes growth curves (line black), glucose concentration (grey line), succinic acid concentration (blue line), formic acid concentration (yellow line), acetic acid (orange line).*

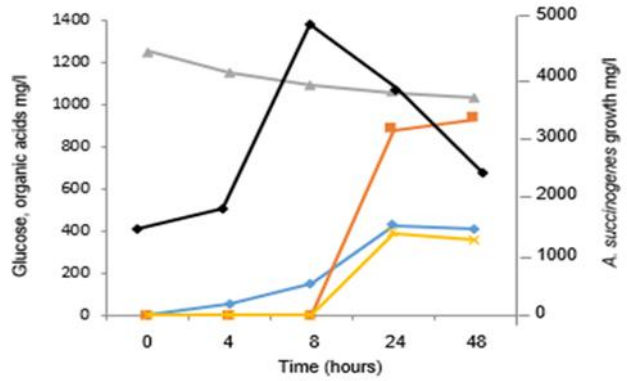

Figure 3.3 *TEST 2: 1.36 g·L^{-1}strain concentration: Actinobacillus succinogenes growth curves (line black), glucose concentration (grey line), succinic acid concentration (blue line), formic acid concentration (yellow line), acetic acid (orange line).*

The results on the low productivity of succinic acid were in line with those obtained by the authors Salma et al., 2021, that reached succinic acid productivity <12 mg·L^{-1}·h^{-1} in tests under micro-aeration conditions, highlighting how maintaining anaerobic conditions was an essential factor to improve the performance of the succinic acid production process.

The results obtained in this study were achieved under near optimal conditions, such as utilizing a synthetic broth with a standard composition suitable for bacterial growth. However, bio-based production of succinic acid presents some several issues that need to be fixed before to start industrial-scale production. Firstly, the choice of lignocellulosic biomasses as abundance, low cost and high carbohydrates content feedstock has been one of the most critical due to their complex structure and high mechanical strength. In lignocellulosic biomasses, sugars were not directly available for the microorganisms, and pre-treatments were needed to make them available to the fermentation process. But these preliminary pre-treatments processes could be expensive and could generate some inhibitory compounds for microorganisms, such as furans, phenolic compounds and acids (Xu et al., 2022). Furan derivatives appeared to have a strong toxic effect on *A. succinogenes*. In fact, Dessie et al. (2019) observed that, even if present in low concentrations (3 g·L^{-1}), hydroxymethylfurfural and furfural completely inhibited the growth of the strain. Among the acids, the most common was the acetic acid, that could also be produced by the strain as end-product during fermentation process. In this study, a high acetic acid concentration was produced (978.6 and 931.1 mg·L^{-1} in test 1 and test 2, respectively, at 48h than succinic acid

under the conditions tested, which did not differ from the initial inoculum concentration but was certainly due to the operating conditions in micro-aeration. According to the study of Li et al. (2010), the strain *A. succinogenes* at a fixed initial concentration (0.03 $mg \cdot L^{-1}$) could tolerate up to 10 $mg \cdot L^{-1}$ of acetic acid, with a slowdown in growth over 12 hours and an inhibition rate of 86% was observed than control without acetic acid. In fact, when the concentration of acetic might be critical, a toxic effect was revealed such as the block of the functions of many key enzymes. Therefore, using a more complex medium that better represents the composition of agro-industrial residues or lignocellulosic biomass could present various critical issues, such as observing an inhibitory effect on the growth of the strain and consequently low yield of succinic acid. Another critical aspect of the production of bio-succinic acid was the downstream process for the recovery and purification of the final product. Many techniques were tested, however, some issues still need to be addressed such as the use of large number of chemicals to fulfil the process, that was the biggest problem in reactive extraction. The precipitation, that was one of the most widely used methods to recovery succinic acid, resulted in the production of large quantities of solid waste, such as gypsum. Even more advanced techniques that used membranes, such as electrodialysis, was not yet applicable on an industrial scale for fouling troubles. The effects of these issues were low yields and purity of the final product, and high production costs, which limited industrial scale application of these methods. The techno-economic analysis performed by Li & Mupondwa (2021) confirmed that the choice of starting biomass and the downstream process were two critical aspects. In fact, the simulation of the industrial scale production of bio-succinic acid demonstrated that the cost of the raw material accounted for 31.5% of the operating costs of which only the initial feedstock (corn syrup) was equal to 31.2 % of the total operating costs. Instead, the downstream purification process made up 83% of utility cost.

Table 3.2 *TEST 1-2-Glucose consumption, SA, AA and FA concentration ($mg \cdot L^{-1}$), SA yield ($g \cdot g^{-1}$) and selectivity ($g \cdot g^{-1}$)*

	Test 1		Test 2	
	24h	48h	24h	48h
Glucose consumption 24 h (%)	19.7	21.1	15.7	17.4
SA Concentration ($mg \cdot L^{-1}$)	288.6	169.7	428.3	408.9
AA Concentration ($mg \cdot L^{-1}$)	894.8	978.6	879.1	931.1
FA Concentration ($mg \cdot L^{-1}$)	331.4	339.1	389.1	358.4
SA yield ($g \cdot g^{-1}$)	0.23	-	0.34	-
SA productivity ($mg \cdot L^{-1} \cdot h^{-1}$)	12	-	18	-
Selectivity SA/AA ($g \cdot g^{-1}$)*	0.19	0.11	0.25	0.24

3.4. Conclusions

The results obtained in this paper demonstrated that the bacterial and sugar concentrations tested did not affect the overall performance of the process because comparable glucose consumption was achieved among the different initial strain concentrations. However, after these preliminary results further tests will be necessary to better investigate other processes parameters before to study the potential use of more complex feedstock as lignocellulosic biomasses.

3.5. Acknowledgments

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4. Preliminary assessment on *Actinobacillus succinogenes* growth and succinic acid production for bioplastics

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4.1. Introduction

The petroleum-based plastics have been one of the most widely used materials for their low price and the durability. In 2016 from 9 to 23 million metric tons of plastics polluted lakes, rivers and oceans, and 13 to 25 million metric tons contaminated the terrestrial environment (Borrelle et al., 2020). The disposal of plastics has been a critical issue. since the plastics were designed to be durable and persist in the environment for a long time. Furthermore, the life cycle of plastic resulted to impact strongly on CO_2 emissions (1.7 Gt $CO_2 \cdot year^{-1}$) respect to other sector as global aviation industry (0.4 Gt $CO_2 \cdot year^{-1}$) (Jiao et al., 2024).

To reduce the environmental impact of fossil-based plastics, biodegradable and compostable polymers have recently been tested and developed as polyhydroxyalkanoate (PHAs), polylactic acid (PLA), polybutylene succinate (PBS). Economic interest in biopolymers as substitutes for fossil plastics was growing exponentially in fact global bioplastics production was set to increase significantly from around 2.18 million tons in 2023 to approximately 7.43 million tons in 2028 (European bioplastics, 2023).

Among biopolymers, PBS was considered one of the most interesting for his mechanical endurance, ductility, and toughness (Barletta et al., 2022). PBS was principally produced from condensation polymerization of succinic acid and butan-1,4-diol. Succinic acid (SA) was one of the principal chemical building blocks to produce PBS. To produce SA, biotechnological routes were developed by using microbial fermentation. The microorganisms can use most sugars (principally glucose) derived also from renewable sources as agro-industrial by-products. The anaerobic facultative strain *Actinobacillus succinogenes* was one of the most tested microorganisms for the capability of using different sugars sources (monosaccharides and disaccharides) and to produce large quantities of succinic acid (Yang et al., 2020).

In this context, the possibility of utilizing a lignocellulosic biomass as feedstock was interesting, because these raw materials were rich in glucose and xylose, and they were available in large quantities. However, the complex matrix of lignocellulosic biomass needed pre-treatments to convert polysaccharides into fermentable sugars. The non-specificity of the pre-treatment processes and the harsh operational conditions of some treatments as steam explosion (such as the high temperatures and pressure) leaded to the formation of unwanted

compounds, capable of inhibiting the growth and consequently the productivity of the microorganisms. These inhibitors can be divided into three main groups: organic acids (acetic acid, lactic acid, formic acid, levulinic acid), furan derivates (5-hydroxymethylfurfural and furfurals), and phenolic compounds (Basak et al., 2020).

The aim of this paper was to assess the growth of *A. succinogenes* for the production of succinic acid in the presence of two of the most representative inhibitors (acetic acid and furfural) that were formed under pre-treatment processes of lignocellulosic biomasses. The growth of *A. succinogenes* was evaluated on the inoculum without and with adaptation.

4.2. Materials and methods

The freeze-dried pellets of the strain *Actinobacillus succinogenes* 130Z (CCUG-43843) was supplied by Culture Collection University of Gothenburg (CCUG, Gothenburg, Sweden). The strain was grown in Tryptic Soy Broth (TSB) (22092-500G Sigma) at 37 °C and 180 rpm in a stirring incubator (New Brunswick Scientific Excella E24 Incubator Shaker Series) in the dark (Molino et al., 2020).

The effect of sugars and potential inhibitors were directly tested on *A. succinogenes* without pre-inoculum adaptation and in tests after inoculum adaptation. The growth of *A. succinogenes* without adaptation was tested at a concentration of glucose equal to 229 $mg \cdot L^{-1}$, glucose and xylose (229 $mg \cdot L^{-1}$ and 114 $mg \cdot L^{-1}$) and acetic acid and furfural equal to 14 $mg \cdot L^{-1}$ and 6 $mg \cdot L^{-1}$ (Table 4.1).

Table 4.1 *D-(+)-glucose, D-(+)-xylose, acetic acid and furfural concentrations at the start of fermentation experiment without pre-inoculum adaptation*

Compound	**Concentration ($mg \cdot L^{-1}$)**			
	CTL-G	CTL-G/X	IN-AA	IN-AA/F
A.Succinogenes	300	300	300	300
D-(+)-Glucose	229	229	229	229
D-(+)-Xylose	0	114	114	114
Acetic acid	0	0	14	14
Furfural	0	0	0	6

Inoculum adaptation was performed in two subcultures (SUB1 and SUB2) that were grown at the same conditions at 37°C, 180 rpm in the dark at two different glucose concentrations of 100 mg·L^{-1}and 1250 mg·L^{-1} respectively. In Table 4.2, the concentrations of strain, sugars and potential inhibitors after inoculum adaptation were reported. A control (CTL) was prepared to compare the growth of *A. succinogenes* under favorable conditions respect to the presence of potential inhibitors, acetic acid (AA) and AA with furfural at low (L) and high concentration (H).

Table 4.2 *D-(+)-glucose, D-(+)-xylose, acetic acid and furfural concentrations at the start of fermentation experiment.*

Compound	**Concentration (mg·L^{-1})**							
	CTL-G-L	CTL-G/X-L	IN-AA-L	IN-AA/F-L	CTL-G-H	CTL-G/X-H	IN-AA-H	IN-AA/F-H
A.succinogenes	370	370	370	370	1100	1100	1100	1100
D-(+)-Glucose	367	367	367	367	1130	1130	1130	1130
D-(+)-Xylose	0	171	171	171	0	428	428	428
Acetic acid	0	0	21	21	0	0	52.5	52.5
Furfural	0	0	0	6	0	0	0	15

The bacterial growth was monitored by measuring the optical density (OD) at a wavelength of 600 nm by mean of a dual beam spectrophotometer (Perkin-Elmer Lambda 365). A calibration curve was built to estimate the concentration of the strain (equation 1), by using OD values at known concentrations of the strain:

Actinobacillus succinogenes concentration (g·L^{-1}) = 12.54*OD 660 value – 0.9708 (1)

After the spectrophotometric analysis, the samples were centrifuged for 10 minutes at 4 °C and 13000 rpm to separate the bacteria form the broth. The broth was filtered by a syringe filter of nylon (0.20 µm of porosity) and then it was stored at -20 °C for the following analysis.

The quantification of SA was carried out by ultra-high performance liquid chromatography, u-HPLC 1290 Infinity II (Agilent Technologies Inc., Santa Clara, USA) using aa Diode Array Detector (DAD) at a wavelength of 210 nm. The Hi-Plex H Column, 7.7 x 300 mm, 8 µm (p/n 1170-6830 Agilent) was used to separate organic acids following the chromatographic conditions reported in the method Agilent 5991-8984EN. Diluted analytical standards of organic acids (47264 Supelco) were prepared for identification and quantification

All tests were carried out in triplicate, the Data Analysis ToolPack (Microsoft Excel 365, Microsoft Corp., USA) was used to calculate the standard deviation. IBM SPSS Statistics v26® was used for ANOVA (One way analysis of variance) statistical analysis.

4.3. Results and discussion

The tests were designed to evaluate the effects of two of the most common inhibitors derived from the pretreatments of the lignocellulosic biomass: acetic acid and furfural. The effect of inhibitors was evaluated firstly on *A, succinogenes* strain without a pre-adaptation (Figure 4.1). *A, succinogenes* without a pre-adaptation reached the highest concentration (3.0 ± 0.2 $g \cdot L^{-1}$) after 72 hour starting from the initial strain concentration of 0.3 $g \cdot L^{-1}$ without any difference between the test with glucose (229 $mg \cdot L^{-1}$), and glucose and xylose (229 $mg \cdot L^{-1}$ and 114 $mg \cdot L^{-1}$) ($p>0.05$) (Figure 4.1). The effect of inhibitors was more evidence after 48 hours for AA in IN-AA and the mixture AA and furfural (IN-AA/F) when the strain concentrations reached the value equal to 1.83 ± 0.1 $g \cdot L^{-1}$ and 2.24 ± 0.3 $g \cdot L^{-1}$ respectively.

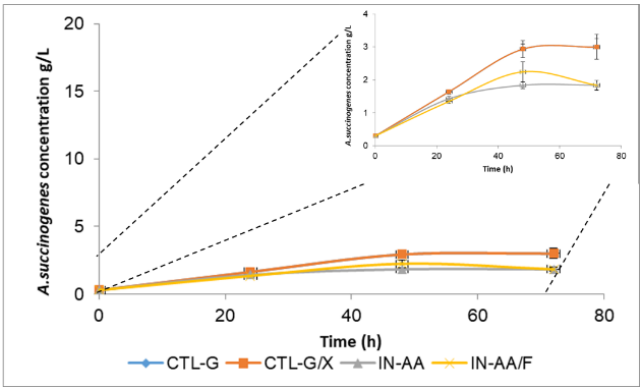

Figure 4.1 *Growth curve of A. succinogenes (mg·L^{-1}) in tests without pre-inoculum adaptation. Error bars indicate the standard deviation.*

For tests with inoculum pre-adaptation, the initial concentration of strain (0.17 g·L^{-1}) for pre-adaptation was tested at two glucose concentrations 0.10 g·L^{-1} (Sub1) and 1.25 g·L^{-1} (Sub 2) for 144 hours. At 24 hour the strain concentration was similar (1.35 ± 0.01 g·L^{-1}vs 1.31 ± 0.04 g·L^{-1} respectively). At the final growth state (144 h), *A. succinogenes* reached the highest concentration of 4.85 ±0.02 g·L^{-1} from the starting concentration of glucose of 1.25 g·L^{-1}glucose. In sub 1 the strain reached the concentration equal to 1.90 ± 0.03 g·L^{-1} at an initial glucose concentration of 0.10 g·L^{-1}.

After inoculum adaptation, the tests on the effect of inhibitors were started at an initial concentration of *A. succinogenes* equal to 0.37 g·L^{-1} (Test L) and 1.1 g·L^{-1} (Tests H) (Figure 2). Given the response of the strain without an adaptation phase, it was decided to increase the concentrations of all compounds including AA and furfural and to test them a low and a high concentration (Figure 4.2) on inoculum after per-adaptation. In all the analyzed samples, the best concentration value was recorded after 144 hours of fermentation.

In the tests at low concentrations of sugars (367 mg·L^{-1} glucose and 171 mg·L^{-1} xylose) (Figure 4.2A), the growth of the strain was similar in the tests with glucose and with the mixture of glucose-xylose: 10.5 ± 0.3 (CTL-G-L) and 10.7 ± 0.0 g·L^{-1} (CTL-G/X-L), respectively.

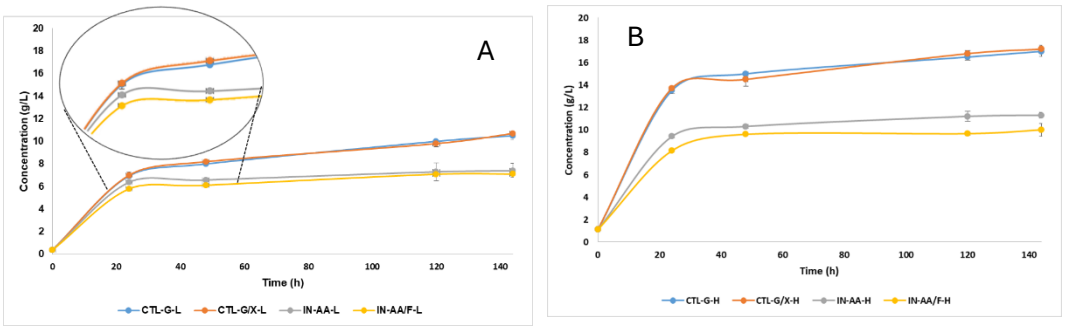

Figure 4.2 *Growth curve of A. succinogenes (g·L^{-1}) in tests with low (A) and high (B) concentrations of sugars and inhibitors. Error bars indicate the standard deviation.*

A similar growth trend was observed when acetic acid (AA) and the mixture of AA and furfural were used and the concentration of the strain decreased till to 7.4 ± 0.6 (CTL-AA-L) and 7.1 ± 0.1 $g \cdot L^{-1}$ (CTL-AA/F-L). The growth of *A. succinogenes* started to decrease after 24 under the effect of the AA and the mixture AA (21 $mg \cdot L^{-1}$) and furfural (6 $mg \cdot L^{-1}$) respect to the control (CTL-G-L) (Figure 4.2A). In the tests at high concentrations of sugars and inhibitors (Figure 4.2B), he highest concentrations of *A. succinogenes* were detected in CTL-G-H equal to 17.0 ± 0.4 $g \cdot L^{-1}$ when glucose concentration was 1130 $mg \cdot L^{-1}$, and in CTL-G/X-H equal to 17.2 ± 0.3 $g \cdot L^{-1}$ when glucose (1130 $mg \cdot L^{-1}$) and xylose (428 $mg \cdot L^{-1}$) were used. The effect of the mixture AA and furfural was stronger at 24 hours than the recorded effect of AA when strain concentration was equal to 11.3 ± 0.3 $g \cdot L^{-1}$. For a better evaluation of the effect of potential inhibitors on *A. succinogenes* strain, the inhibition rate was calculated for all tests (Table 3). Strain growth decreased compared to the control in the presence of acetic acid and the acetic acid and furfural mixture at all concentrations tested for both the pre-adapted strain and the strain without pre-adaptation. Although acetic acid was tested at lower concentrations (14 $mg \cdot L^{-1}$) for the strain without an adaptation phase (Table 4.3), acetic acid inhibition rates were higher up to 38.6% at 72 hours of growth. This inhibition rate recorded at 72h on inoculum without pre-adaptation under AA 14 $mg \cdot L^{-1}$ was also higher than the inhibition rate (33.5%) observed at 144 hours on bacterial growth when 52.5 $mg \cdot L^{-1}$ of acetic acid was used on inoculum after pre-adaptation.

Table 4.3 *Percentage of growth inhibition of A. succinogenes (%): test without inoculum adaptation (IN-AA and IN-AA/F) and test with inoculum adaptation (IN-AA-L, IN-AA/F-L, IN-AA-H and IN-AA/F-H). These values were calculated in comparison with the controls. ND = not detected*

Time (h)	**Inhibition growth (%)**					
	IN-AA	IN-AA/F	IN-AA-L	IN-AA/F-L	IN-AA-H	IN-AA/F-H
24	13.0	20.6	8.1	16.6	30.4	39.8
48	37.4	37.4	17.8	23.6	31.3	36.0
120	38.7	63.1	27	29.1	32.1	41.5
144	ND	ND	29.5	32.4	33.5	41.2

It was noted that the inhibition effect of acetic acid, alone was slightly lower than the mixture with furfural when the strain was tested without adaptation. The inhibition rate reached the

value equal to 63.1% in co-presence of acetic acid and furfural at the concentration of 14 $mg \cdot L^{-1}$ and 6 $mg \cdot L^{-1}$ on *A. succinogenes* without adaptation. After strain adaptation, the most evident difference between the effect of acetic acid and the mixture was observed at 24 hours in the test at low concentrations (8.1 % IN-AA-L vs. 16.6 % IN-AA/F-L). The highest effect of the mixture AA and furfural was observed at 120 hours (41.5 % inhibition rate) when the high concentration of the inhibitors was used on the strain after adaptation of the strain. The differences found between the strain concentration obtained for inoculation without adaptation and that with adaptation were in line with that observed by Escanciano et al., 2022. The authors (Escansiano et al., 2022) observed that the inoculum without adaptation grew 1.4 times less than the inoculum with adaptation in favorable conditions and in the absence of inhibitors, while in this work the concentration of the strain without adaptation was approximately 3.3 times lower than the inoculum with adaptation. The effect of furfural on *A. succinogenes* growth was also detected by Xu et al., 2015 at increasing concentration of this compound that reduced OD600 value of 50% respect to control at a furfural concentration of 2 $g \cdot L^{-1}$. The inhibitory effects of some compounds, such furans derivates and weak acids (acetic acid) was also investigated on the growth of *Bacillus subtilis* (van der Maas et al., 2021). Van der Maas et al. (2021) found that acetic acid caused inhibition at concentration of 2 g/L, with a reduction of 29% of strain concentration. Furthermore, in our study was observed a greater inhibitory effect of the mixture of furfural and acetic acid, at both low and high concentrations. This finding was similar to the data detected by Franden et al. (2013) that observed a greater growth inhibition (31%) on *Zymomonas mobilis* when acetic acid was in combination with furan than the reduction caused by acetic acid alone (23%).

Strain productivity was markedly lower in the case of the inoculum without an adaptation step reaching levels of 0.07 $g \cdot L^{-1} \cdot h^{-1}$ at 24 h in the presence of both glucose and glucose/xylose mix (Figure 4.3A) with productivity in the presence of the inhibitors decreasing to 0.06 $g \cdot L^{-1} \cdot h^{-1}$. In the tests with the adapted inoculum, the highest productivity was obtained in the CTL-G-H and CTL-G/X-H tests with a value of 0.6 $g \cdot L^{-1} \cdot h^{-1}$ (10 times higher than the value of the strain without adaptation step). At the high concentrations of the inhibitors, it can be observed that the productivity of the strain decreased to 0.4 $g \cdot L^{-1} \cdot h^{-1}$ (IN-AA-H) and 0.3 $g \cdot L^{-1} \cdot h^{-1}$ (IN-AA/F-H).

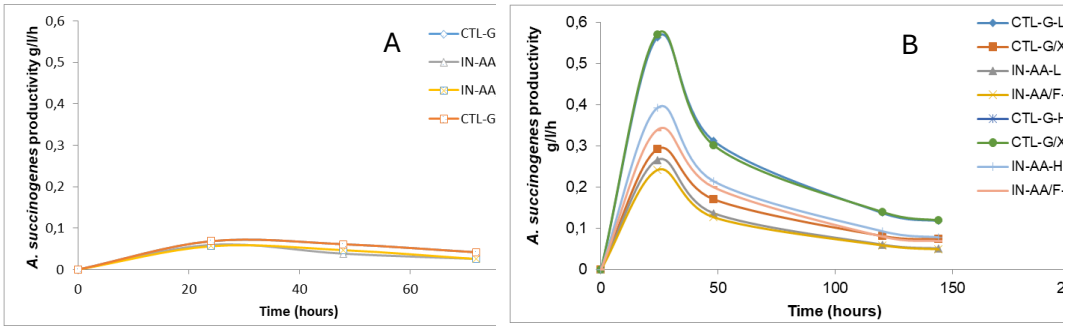

Figure 4.3 *A. succinogenes productivity (g/l/h): A) strain without adaptation; B) strain with adaptation*

In terms of productivity of SA the maximum succinic acid concentration was reached when the high concentration of sugars was used 390 ± 7 mg·L^{-1} (CTL-G/X-H) (Table 4.3). SA concentration dropped in the samples with inhibitory compounds, mostly under acetic acid and furfural effects (50 ± 3 mg·L^{-1} in IN-AA/F-L and 160 ± 9 mg·L^{-1} in IN-AA/F-H), thus highlighting a combined toxicity effect for the strain both at low and high concentrations of AA and furfural. In addition, despite inoculation without adaptation achieved low productivity values showing a slight response to the conditions tested, the strain was able to produce in 24 h in the absence of inhibitors up to 62 mg·L^{-1}which was reduced to 41 mg·L^{-1} in the presence of acetic acid and furfural.

Table 4.3 *SA concentration (mg/) after 24h in all tests (*without adaptation).*

Succinic acid concentration (mg·L^{-1})											
CTL-G	CTL-G/X	IN-AA	IN-AA/F	CTL-G-L	CTL-G/X-L	IN-AA-L	IN-AA/F-L	CTL-G-H	CTL-G/X-H	IN-AA-H	IN-AA/F-H
62	78	58	41	108	102	74	50	384	390	277	160

So, the effect of acetic acid was observed at each tested concentration on inoculum without adaptation and with adaptation. The synergistic inhibitory effect of acetic acid and furfural

was strongly observed in all tests but the strain demonstrated the capacity to tolerate them. The highest reply of the strain against the inhibitors was observed at the highest initial strain concentration (1.1 $g \cdot L^{-1}$) after a pre-inoculum adaptation. In line with these findings, Dessie et al. (2019) observed that hydroxymethylfurfural and furfural, when together in the fermentation broth, strongly inhibited (93.4%) the succinic acid production by *A. succinogenes*, even at the concentrations of both equal to 2 $g \cdot L^{-1}$.

4.4. Conclusions

These results highlighted that *Actinobacillus succinogenes* suffered without a pre-adaptation cultivation under the presence and the absence of inhibitors respect to pre-adapted inoculum. Unfavourable conditions were due to the presence of inhibitors compounds as acetic acid and the mixture of acetic acid and furfural that caused a decrease of *A. succinogenes* growth. The inhibition effects of these compounds were strongly evident on the inoculum without pre-adaptation. The strain after the pre-adaptation phase was better able to tolerate acetic acid despite the increased concentration tested. *A. succinogenes* suffered the most inhibitors effects when acetic acid and furfural were together both at low and high concentrations. Therefore, this preliminary study can provide the basis for further insights into the effects of fermentation inhibitory compounds on *A. succinogenes*. A better understanding of this critical issue can be fundamental to optimizing the fermentation process and therefore the production of succinic acid.

4.5. Acknowledgments

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5. Inhibitors derived from wheat straw hydrolysate can affect the production of succinic acid by *Actinobacillus succinogenes*

This chapter has been published as:

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Process Biochemistry, 147, 228-239.

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5.1. Introduction

The global strategy of bio-based economy evolved towards sustainable bio-based production that is considered a priority for a low-carbon based economy and for a better future (E4 Tech, 2017; Commission Expert Group on Bio-Based Products, 2017). In the last fifteen years, one of the most interesting bio-based products has been bio-succinic acid (Bio-SA). Succinic acid was classified in the first 10 high values chemicals as a precursor for many industrial compounds and for the synthesis of biodegradable polymers such as polybutyrate succinate (PBS), polyamides and various green solvents (Saxena et al., 2017; Werpy & Petersen, 2004). Furthermore, the global demand of succinic acid is increasing and is expected to have a market value of over 500 million USD by 2030 (G.V. Research Succinic Acid Market Size, 2021).

Up to the present day, SA has been industrially produced from n-butane through maleic anhydride by means of a petrochemical process. Although the production of succinic acid from fossil sources is still cost–effective with a price around 2.0 USD·kg^{-1}, biotechnological routes have been proposed through microbial fermentation from agro-industrial residues/wastes. These processes may in fact lead to noticeable environmental benefits and new opportunities of growth for the future with the reduction of more than 60% of greenhouse gases emission when compared to the carbon footprints of petrochemical-based process (Ferone et al., 2017; Musonda et al., 2020; Stegmann et al., 2020).

Since 2012, four companies have launched commercial/pilot plants to produce bio-based succinic acid. Reverdia developed a direct fermentation at low pH of corn starch to produce 10,000 T·year^{-1} of Bio-SA by using *Saccharomyces cerevisiae*. Myriant featured non-genetically modified *Escherichia coli* and sugars derived from sorghum and other commercial sugars to produce13,600 T·year^{-1} (Jansen & van Gulik, 2014). Succinity reached a capacity of 10,000 T·year^{-1} of Bio-SA by valorizing renewable resources as glycerol, glucose, and sucrose through *Basfia succiniciproducens* strain (Sosa-Fernandez & Velizarov, 2018). BioAmber-Mitsui (Canada) used the engineered yeast *Candida kruseii* (licensed from Cargill) and mainly cereal-based (corn glucose) feedstock with a production of 30,000 T, while BioAmber-France used *E. coli* and wheat glucose as feedstock for a production of 3000 T (Li et al., 2021a; Li et al., 2021b).

Despite the fact that some companies started the industrial production of Bio-SA, there are still many technical-economic difficulties to be faced that are principally related to carbon sources, strain selection and inhibitors compounds that affect productivity and succinic acid conversion yield, as well as the down streaming process for succinic acid recovery and overall process scalability (Barbosa et al., 2024).

Among the most promising carbon sources, lignocellulosic biomasses that amounted to around $2*10^{11}$ T·year^{-1} (Anu Kumar et al., 2020) are noticeable sources of sugars for the production of succinic acid (Li et al., 2021c). These renewable resources can be produced as post-harvest residues from intensive cultivation of rice, wheat, maize, and sugarcane (Vivek et al., 2017). These resources contain carbohydrates polymers distributed in these fractions: cellulose 40-60% on dry weight (dw), hemicellulose 10-40% dw and lignin 15-30% dw, extractives (1-2% dw); and ashes (2-3 % dw) (Lu et al., 2021; Khan & Mubeen, 2012; Blasi et al., 2023). For the utilization of the carbohydrates feedstock, pre-treatments are necessary to alter the cellulose-hemicellulose-lignin matrix, to remove lignin, to make xylans and glucans available for the enzymatic attack and subsequently to convert them into fermentable sugars for microorganisms (Beig et al., 2021; Zhang et al., 2022). The pretreatments can be physical (grinding, ultrasound assisted), chemical (acidic and alkaline hydrolysis), chemical-physical (steam explosion) and biological (enzymatic treatments) (Li et al., 2010). The most tested pretreatments on corn, rice, wheat straw and sugarcane bagasse were dilute alkaline hydrolysis (Li et al., 2009), which has also been tested in autoclave reactor (121 °C, 5 min) (Sawisit et al., 2018) and at different temperatures (H_3PO_4 5 - 99°C) (Lo et al., 2020), or acid hydrolysis with steam explosion (Zheng et al., 2010) and autoclave reactor (Kuglarz et al., 2018). While less aggressive methods were also tried with hot water on corn fiber (Jampatesh et al., 2019), peracetic and alkaline peroxide assisted ultrasound on rice straw and sugarcane bagasse (Vallecilla-Yepez et al., 2021), alternative solvents as glycerol:water mixture (Putri et al., 2023) (Table 1, Supplementary materials S1).

Hydrolysis pretreatments (acidic and alkaline), in particular acidic conditions, at high temperatures such as steam explosion allowed to reach high sugars conversion yield but strong operative conditions generated unwanted by-products, such as organic acids (Acetic acid (AA), lactic acid (LA), formic acid (FA)), furans (5-hydroxymethyl-furaldehyde (HMF)

and furfural), and phenolic compounds. Furans are produced from the dehydration of pentose and hexose sugars generated from the hydrolysis of hemicellulose, and phenolic compounds are generated from the degradation of lignin and extractives. The most common phenols generated during the pretreatment of lignocellulosic biomass were p-hydrozybenzoic acid, vanillin, vanillic acid, cinnamic acid, benzoic acid, and syringic acid (Dąbkowska et al., 2019). Acetic acid is produced from the hydrolysis of acetyl groups of degraded hemicellulose. Formic acid can be generated from the degradation of HMF in acidic condition or degradation of polysaccharides under alkaline conditions that also produced FA (Kumar et al., 2020). Acetic acid, formic acid and lactic acid can be also generated as by-products of metabolic pathways during the process of SA production. Formic acid can block the growth of A. *succinogenes* at concentrations between 11 and 18 $g \cdot L^{-1}$, acetic acid at the concentration of 33.7 $g \cdot L^{-1}$ while lactic acid at the concentration of 55 $g \cdot L^{-1}$ (Jonsson & Martin, 2016). Formate and acetate have a similar mechanism of growth inhibition of the strain since they interfered on phosphate transport across the cell membrane by chemically interfering on an over requirement for ATP.

These multiple by-products were identified as inhibitors of the growth of microorganism and consequently of the productivity of the fermentative processes. Furans were able to activate different key enzymes in the microbial pathways of glycolysis and tricarboxylic acid cycle metabolism, thus reducing the sugar intake rate and target product conversion rate (Chen et al., 2021). On the other hand, the furfurals could destroy the mitochondrial structure by inducing the accumulation of reactive oxygen (such as H_2O_2) in the cells and cytotoxicity (Tian et al., 2011). Dessie et al. found that the combination of furfural and HMF at a concentration of 3 $g \cdot L^{-1}$ completely stopped the production of succinic acid by *A. succinogenes* (Allen et al., 2010). Indeed, it was observed that furfurals can enhance the toxicity of other substances, such as phenols and acetic acid, to microorganisms (Dessie et al., 2019). In a recent study, Xu et al. (2022) highlighted that the phenolic compounds, cinnamic and benzoic acid at a concentration of 12 mM had strong inhibitory effect on *A. succinogenes* by altering glucose uptake system, heath shock protein (HSP), and chemotaxis (Luo et al., 2020).

A recent study demonstrated the techno-economic feasibility of a simulated process from a sugarcane bagasse hydrolysate for the production of SA integrated in a biorefinery for the production of ethanol and electricity, can resulted in a lowering of the Bio-SA price to 2.32 USD·kg^{-1}. Despite this, most of the studies on the production of SA from corn, rice and wheat straw, and sugarcane bagasse have been carried out on a laboratory scale (Zheng et al., 2010; Kuglarz et al., 2018; Jampatesh et al., 2019; Vallecilla-Yepez et al., 2021) (Supplementary materials S1). Zheng et al. (2010) investigated SA production from corn straw hydrolysate treated by alkaline hydrolysis and enzymatic treatment by obtaining 45 g·L^{-1} of succinic acid at a yield of 81% starting from 58 g·L^{-1} of sugars (Zheng et al., 2010). Through simultaneous saccharification and fermentation, SA was produced at a concentration of 47.4 g·L^{-1} and at a yield equal to 0.72 g·g^{-1} by using corn stover (Zheng et al., 2010). Sawisit et al. (2018) obtained 48 g·L^{-1} of SA at a yield of 0.84 g·g^{-1} pretreated rapeseed straw after steam explosion by using *A. succinogenes* through the fermentation of liquid hydrolysate and fermentation after ethanol production (Sawisit et al., 2018). Kuglarz et al. (2018) obtained from rapeseed straw treated by steam explosion and enzymatic hydrolysis 5.8 g·L-^{1} of SA at a yield of 48% without an investigation on inhibitors (Kuglarz et al., 2018).

Lo et al. (2020) performed batch fermentation of sweet sorghum bagasse by *A. succinogenes* to obtain 17.8 g·L^{-1} of SA and a yield of 0.61 $g_{SA}·g^{-1}{}_{glucose}$ and HMF was detected around 1-2 g·100 of feedstock when acid hydrolysis was carried out at 80 °C for 60 min (Lo et al., 2020). Jampatesh et al. (2019) produced 78 g·L^{-1} of SA at a yield of 0.86 g·g-1 by using the modified strain *E. coli* AS1600a from rice straw pretreated by acid hydrolysis and autoclaving that contained a total inhibitors concentration of 4.3 g·L^{-1} of AA, FA and HMF (Jampatesh et al., 2019). Chen et al. (2021) produced 23 g·L^{-1} of succinic acid 0.64 g·g-1 by using sugarcane bagasse hydrolyzed by sodium hydroxide (Chen et al., 2021). Total phenols concentration was 0.47 g·L^{-1} and succinic acid production decreased from 23 g·L^{-1} to 19 g·L^{-1} when phenolic compounds concentration increased.

Starting from the current state of knowledge, inhibitory compounds have often been contained simultaneously at different concentrations in the pretreated lignocellulosic biomass. Few works have highlighted the problematic presence of inhibitors in the

lignocellulosic hydrolysate and their effect on Bio-SA production (Lo et al, 2020; Kuglarz et al., 2018; Jonsson & Martin, 2016) also because the most recent studies tried to make the pretreatment process less impactful by using alternative methods. In this study starting from a very effective pre-treatment technology such as steam explosion for one of the less studied and more recalcitrant lignocellulosic biomasses such as wheat straw, the authors aimed to evaluate the production performance of succinic acid in the presence of multiple inhibitors by evaluating their effect on several parameters: different initial concentrations of inoculum, the mixture of sugars (glucose and xylose) at different concentrations, concentrations of inhibitors and how these can influence the resistance of the *A. succinogenes* strain to the presence of inhibitors in a rich growth medium and diluted wheat straw hydrolysate.

Succinic acid production was investigated by using standard growth medium, standard growth medium supplemented with inhibitors compounds (furfural and acetic acid), and diluted wheat straw hydrolysate. Two concentrations of inoculum, sugars (glucose and mixture glucose and xylose) and two concentrations of AA and furfural were tested under batch fermentation by using *A. succinogenes* strain.

5.2. Material and methods

5.2.1. Material and microorganism

Actinobacillus succinogenes strain 130Z (CCUG-43843) was supplied by Culture Collection University of Gothenburg (CCUG, Gothenburg, Sweden) as freeze-dried pellets. The growth medium was a Tryptic Soy Broth (TSB) (22092-500G Sigma) that contained (per liter) 17.0 g of peptone from casein, 3.0 g of peptone from soy, 5.0 g of NaCl, 2.5 g of K_2HPO_4 and 5.0 g of D-(+)-glucose. Before the use TSB was sterilized for 15 minutes at 121 °C

5.2.2. Wheat straw treatment

Wheat straw was provided and treated in ENEA Research Center of Trisaia (Rotondella (MT), Italy). The raw lignocellulosic biomass was preliminarily characterized after a fine milling phase to reduce particle size at 50 mesh. The composition of wheat straw (WS) was determined as reported in table 1 as percentage on dry weight (% $w \cdot w^{-1}$). Extractives

quantification was performed following the methods proposed by Sluiter et al. (2015). In particular, the biomass was extracted using water and ethanol in a Soxhlet apparatus for 6 hours per cycle. The oven dry weight (dw) was measured by drying the biomass or apparatus at 105 ± 5 °C until a constant weight was achieved (Sluiter et al., 2015). The characterization of lignocellulosic materials in terms of sugars, lignin, and ashes was carried out. Acid-insoluble lignin was determined gravimetrically via filtration on Whatman GFA filters following the NREL protocol proposed by Sluiter et al. in 2008 and ash content was determined using a muffle furnace at 575°C overnight (Sluiter et al., 2008). Sugars analyses were performed by using ion chromatography (HPIC) with DIONEX ion chromatograph model DX300 equipped with a column Nucleogel Ion 300 OA, refractive index ED50 as detector, and H_2SO_4 0.05 M (40 °C, 0.4 mL·min^{-1}) as mobile phase, and the analysis of furfural and HMF was performed using the HP 1100 system equipped with diode array UV detector (Liuzzi et al., 2019).

Table 5.1 *Composition of wheat straw (% dw.). Average values of three replicates*

	Composition (% dw)				
	Glucan	Xylan	Organic extractives	Insoluble lignin	Ashes
WS	38.4 ± 3.2	16.7 ± 1.1	4.3 ± 0.3	20.6 ± 1.1	6.2 ± 0.1

Wheat straw was treated following a catalyzed acid steam explosion treatment following the optimized operational conditions for the purpose of guaranteeing a high hemicellulose recovery and a high hydrolysis of cellulose (Caporusso et al., 2023). The biomass was minced in a blanking machine that reduced the size to measures between 1.7 and 5.5 mm. Then, 1 kg of raw biomass was immersed in 30 L of 0.5 M H_2SO_4 solution, for 10 minutes, and then transferred to the filter press to eliminate the liquid in excess. After the impregnation and the mechanical presmeasure dry matter of the wet biomass was determined around 31.2% (w·w^{-1}). After that, the steam explosion was carried out in a batch reactor (volume of 10 L) at 203 °C for 5 minutes (Caporusso et al., 2023).

After the steam explosion treatment, the hydrolysis tests were conducted by using the enzymatic blend Cellic Ctec2® (Danish company Novozymes A/S) adding 15FPU per gram of cellulose. The determination of enzyme activity (FPU) was carried out using the method

of Ghose (Ghose, 1992). The hydrolysis was conducted at a solid-liquid ratio of 5% $w \cdot v^{-1}$, temperature of 50°C and pH of 4.8 (obtained by adding a concentrated solution of NaOH to hemicellulose from acid pretreatment) and was stopped at 72 hours. Temperature control and agitation were performed by a shake plate incubator. The hydrolysate was filtered and sterilized. The final composition is reported in Table 5.2.

Table 5.2 *D-(+)-glucose, D-(+)-xylose, acetic acid and furfural concentration in wheat straw hydrolysed (WSH)*

	Concentration ($g \cdot L^{-1}$)			
	D-(+)-glucose	D-(+)-xylose	Acetic acid	Furfural
WSH	22.9 ± 1.3	11.4 ± 0.8	1.9 ± 0.2	0.9 ± 0.1

5.2.3. Pre-inoculum

Before starting the batch fermentation tests on succinic acid production, *A. succinogenes* 130Z cells were grown in 150 mL sealed anaerobic bottle containing 120 mL TSB growth medium, for 6 days in a stirring incubator (New Brunswick Scientific Excella E24 Incubator Shaker Series) in the dark, at 37 °C and 180 rpm. Two starters for the inoculum were prepared at a final glucose concentration of 100 $mg \cdot L^{-1}$ and 1250 $mg \cdot L^{-1}$ respectively. Before that starter was transferred in fermentation bottles, a sample was taken to measure microbial growth and further analysis on the concentrations of sugars and acids organics.

5.2.4. Experimental set-up

Fermentation tests were performed under batch condition in sealed anaerobic bottle at a working volume of 250 mL with an inoculum of *A. succinogenes* at the ratio of 25% (v/v) in all tests. The batch tests were performed at 37 °C and 180 rpm in the dark in a stirring incubator (New Brunswick Scientific Excella E24 Incubator Shaker Series).

The fermentation was carried out by using the standard growth medium (TSB) as control (C1) and the growth medium was prepared at two glucose concentrations (Table 3, Figure 1): 0.4 $g \cdot L^{-1}$ (C1- L) and 1.1 $g \cdot L^{-1}$ (C1-H).

To evaluate the effect of several substrates as carbon sources (glucose and xylose) fermentation was carried out by using standard growth medium (TSB) supplemented with xylose, named (Control 2 C2), at two concentrations 0.2 $g \cdot L^{-1}$ (C2-L) and 0.4 $g \cdot L^{-1}$ (C2-H). In order to evaluate the effect of the inhibitors, acetic acid was added to standard growth medium (Test – I1) at the two concentrations of 21 $mg \cdot L^{-1}$ (I1-L) and 52.5 $mg \cdot L^{-1}$ (I1-H). And in a second test acetic acid at the same concentration of 21 $mg \cdot L^{-1}$ and 52.5 $mg \cdot L^{-1}$, plus furfural at the two concentrations of 6 $mg \cdot L^{-1}$ (I2—L) and 15 $mg \cdot L^{-1}$ (I2-H) were added to the standard growth medium.

Finally, fermentation was carried out by using two dilutions of wheat straw hydrolysate (WS-tests) obtained from steam explosion and enzymatic hydrolysis, that contained glucose, xylose, acetic acid and furfural at the same two concentrations tested on standard growth medium (tests C1-C2 and I1-I2) (Figure 5.1). WS-L contained as carbon sources glucose 0.4 $g \cdot L^{-1}$ and xylose 0.2 $g \cdot L^{-1}$, and inhibitors acetic acid (AA) and furfural (F) at the concentration of 21 $mg \cdot L^{-1}$ and 6 $mg \cdot L^{-1}$. The composition of WS-H was glucose 1.1 $g \cdot L^{-1}$ and xylose 0.4 $g \cdot L^{-1}$), acetic acid (AA) and furfural (F) at the concentration of 52.5 $mg \cdot L^{-1}$ and 15 $mg \cdot L^{-1}$. For the fermentation by using diluted wheat straw, no additional carbon sources or nitrogen sources were used in order to evaluate the effect of a crude diluted hydrolyzed.

In addition, the effect of the different strain concentrations in the inoculum was also evaluated. For the test at low carbon sources and inhibitors the strain concentration of strain was 0.4 $g \cdot L^{-1}$ while the strain concentration was 1.1 $g \cdot L^{-1}$ for the test at high compounds concentrations.

The effects of the two different concentrations of carbon sources (glucose and xylose) and inhibitors, acetic acid, and together acetic acid and furfural between tests by using standard growth medium and diluted hydrolysate and the effect of two strain concentrations were evaluated on bacterial growth, sugars concentration, succinic acid production and by-products. In order to evaluate bacterial growth, monosaccharides and organic acids concentrations, samples were collected periodically (0, 24, 48, 120, and 144 hours) and prepared for further analysis and stored at -20 °C.

Table 5.3 *Concentrations of D-(+)-glucose, D-(+)-xylose, acetic acid (AA) and furfural (FU) for each test*

	Concentration (g·L^{-1})		**Concentration (mg·L^{-1})**	
	Glucose	Xylose	Acetic acid	Furfural
C1-L	0,4	0,0	0	0
C2-L	0,4	0,2	0	0
I1-L	0,4	0,2	21	0
I2-L	0,4	0,2	21	6
WS-L	0,4	0,2	21	6
C1-H	1,1	0,0	0	0
C2-H	1,1	0,4	0	0
I1-H	1,1	0,4	52,5	0
I2-H	1,1	0,4	52,5	15
WS-H	1,1	0,4	52,5	15

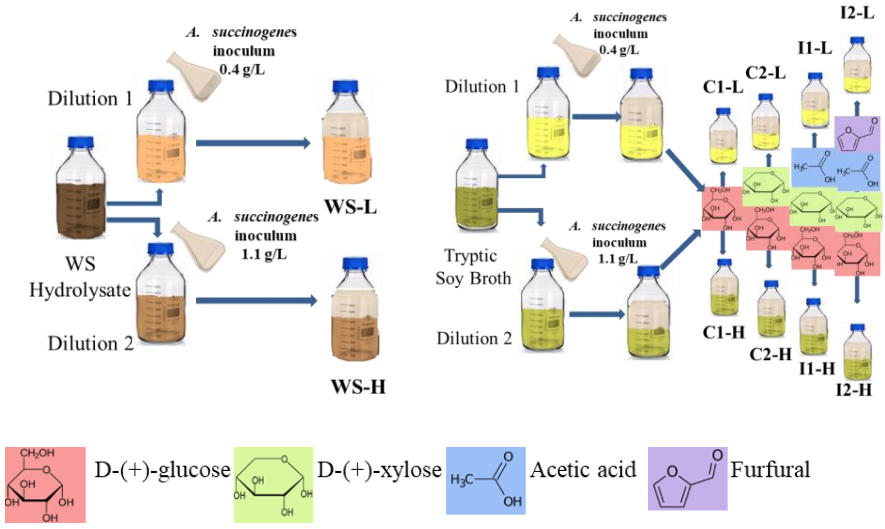

Fig. 5.1 *Experimental design*

5.2.5. Analytical methods

In all tests the bacterial growth was monitored by measuring the optical density (OD) at a wavelength of 600 nm (OD_{600}) by mean of a dual beam spectrophotometer (Perkin-Elmer Lambda 365). A calibration curve was obtained by interpolating OD values at 600 nm with known strain concentration value to calculate bacterial concentration ($g \cdot L^{-1}$). After OD_{600} measurement, the samples were centrifuged for 10 minutes at 4°C and 13000 rpm. Bacterial pellet was separated, and the supernatant was filtered by using a nylon syringe filter, pore size 0.20 µm and stored at -20 °C for the following analysis of sugars (glucose and xylose) and organic acids (succinic acid, lactic acid, formic acid, and acetic acid).

The quantification of monosaccharides was carried out by ultra-high performance liquid chromatography, u-HPLC 1290 Infinity II (Agilent Technologies Inc., Santa Clara, USA) by using the detector ELSD (Evaporative Light Scattering Detector). InfinityLabPoroshell 120 HILIC-Z column (2.1 x 100 mm, 2.7 µm) was used to detect glucose and xylose, at a temperature of 80 °C, by using a mobile phase of ammonium acetate (0.01 M, pH 7.0) and acetonitrile (LC/MS grade) at a rate of 0.4 $mL \cdot min^{-1}$, with a gradient of 95-80% for 12 minutes and 3 minutes of re-equilibration. The ELSD detector was set at a temperature of 60 °C, nitrogen flow rate was equal to 3.5 psi, signal frequency was 30 Hz (Agilent p/n 685775-924). Samples and analytical standards were prepared by using acetonitrile to reach acetonitrile and water 9:1 v/v ratio. Analytical standards were prepared at a different concentration in the range 12.5 $mg \cdot L^{-1}$-250 $mg \cdot L^{-1}$. Volume injection was fixed at 2.5 µl.

Carboxylic acids (SA, LA, FA, and AA) were quantified by using u-HPLC 1290 Infinity II. Diode Array Detector (DAD) was used to identify them at a wavelength of 210 nm. The Hi-Plex H Column, 7.7 x 300 mm, 8 µm was used at a temperature of 50 °C with 100% isocratic mobile phase 0.01 M H_2SO_4 at a flow rate of 0.6 mL/min (p/n 1170-6830 Agilent). Diluted fermented broth samples and analytical standards of succinic, lactic, formic, and acetic acid (47264 Supelco) were prepared diluted by using water (LC/MS grade) and injected at a volume of 20 µl.

5.2.6. Statistical analysis

The inhibition rate on bacteria growth (IR) expressed as percentage was calculated following the calculation 1.

$$IR = (B_{ctrl} - B_{test}) * 100/B_{ctrl} \quad (1)$$

Where B_{ctrl} is the average value of concentration of the strain ($g \cdot L^{-1}$) in control C1 and B_{test} is the bacterial concentration ($g \cdot L^{-1}$) in the other tests. The inhibition rate was calculated for control and test at the same time for each sampling time of 24, 48 and 144 hours.

Glucose consumption (Gcons) was calculated as percentage between the initial concentration (C_0) and the concentration (C) at the chosen sampling time for each test (2).

$$G_{cons} = \frac{(C_0 - C)}{C_0} * 100 \quad (2)$$

Succinic acid (SA) yield was calculated as reported in (3).

$$Yield_{SA} = \frac{\text{succinic acid produced } (g)}{\text{Initial glucose amount } (g)} \quad (3)$$

Succinic acid productivity was calculated as the ration between the succinic acid concentration at a fixed time and the time (hour) of the experimentation.

$$Productivity = \frac{SA\ concentration\ (mg \cdot L^{-1})}{time\ (h)} \quad (4)$$

The ratio SA/AA was calculated by dividing the SA concentration ($mg \cdot L^{-1}$) and AA concentration ($mg \cdot L^{-1}$) (5)

$$SA/AAratio = \frac{SA\ concentration\ (mg \cdot L^{-1})}{AA\ concentration\ (mg \cdot L^{-1})} \quad (5)$$

The selectivity was calculated as following (6):

$$Selectivity\ (g \cdot g^{-1}) = \frac{conc_{AS}}{conc_{AS} + conc_{AF} + conc_{AA}} \quad (6)$$

Where $Conc_{SA}$ is the concentration of succinic acid ($mg \cdot L^{-1}$), $Conc_{AA}$ is the concentration of acetic acid ($mg \cdot L^{-1}$), and $Conc_{FA}$ is the concentration of formic acid ($mg \cdot L^{-1}$)

All tests were carried out in triplicate, the average values, and the standard deviation have been calculated.

Past software was used to calculate Two Way ANOVA tests that were performed following Holm-Sidak test for the comparison between control and tests. The test was also performed comparing the same experimental time between control and each test.

5.3. Results and discussion

5.3.1. Effect of inhibitors on bacterial growth

Acetic acid and furfural were the most common by-products generated after chemical (acid and alkaline hydrolysis) and thermo-chemical treatments (steam explosion) of lignocellulosic biomasses. In wheat straw hydrolysate, furfural can typically be found in concentrations between 2-7.1 mM (Alvira et al., 2016). Other by-products, such as vanillin, ferulic and coumaric acids can be formed at lower concentrations than furfural and acetic acid (Alvira et al., 2016).

In this study the concentration of furfural was found (<9.4 mM) equivalent to 0.9 ± 0.1 $g \cdot L^{-1}$ and the concentration of AA was less than 1.9 ± 0.1 $g \cdot L^{-1}$ in wheat straw hydrolysate. For these reasons, acetic acid and furfural were chosen to compare their effect on strain growth and SA production and by-products both in hydrolysate and standard growth medium.

Two initial concentrations of *A. succinogenes* strain were considered that were 0.4 $g \cdot L^{-1}$ for the tests at low sugars and inhibitors concentrations and 1.1 $g \cdot L^{-1}$ for the tests at their high concentration.

The strain grew exponentially in the first 24 hours in all samples irrespective of the initial inoculum concentration and the presence of xylose, and inhibitors in the growth medium, whereas the growth trend was markedly lower when using wheat straw hydrolysate. Starting from a high inoculum concentration of 1.1 $g \cdot L^{-1}$ in the C1-H control, a significantly higher strain concentration was reached at 24 hours than in the C1-L control. Any difference was observed on bacterial growth trend between the control and the sample in which xylose was also contained. After 24 hours, the strain reached a clear plateau phase in the presence of the inhibitors, both acetic acid and the mixture AA and furfural in the growth medium at all tested concentrations. The maximum concentration of the strain that grew in the most diluted hydrolysate (WS-L) was 4.0 ± 0.7 $g \cdot L^{-1}$ after 144 hours of fermentation and an initial lag

phase, whereas when a more concentrated hydrolysate was used (WS-H), the strain reached a concentration of 7.0 ± 0.5g·L^{-1} (Figure 5.1, Supplementary materials, S1).

The percentage of growth inhibition was calculated between tests and the control C1 (C1-L and C1-H) (Table 5.4). Growth inhibition of the strain was observed in the presence of acetic acid at both concentrations of 21 mg·L^{-1} and 52.5 mg·L^{-1}, and together with furfural at the two concentrations tested (6 mg·L^{-1} and 15 mg·L^{-1}), both in the supplemented growth medium and in the wheat straw hydrolysate (WS-L and WS-H). Inhibition rates of the strain's growth were higher for the strain that grew in the diluted hydrolysates respect to the strain grown in the growth medium supplemented with inhibitors. This difference was basically due to the composition of the growth medium, which contained nitrogen sources such as casein and peptone, a source of energy for strain growth even in the presence of the inhibitors, that were absent in the hydrolysate.

Table 5.4 *A. succinogenes concentration (g·L^{-1}) and inhibition rate (IR)(%)*

	A. succinogenes concentration (g·L^{-1})		Inhibition rate (IR) (%)	
TEST	0h	24 h	48 h	144 h
C1-L	0.4	0	-	-
C2-L	0.4	0	-	-
I1-L	0.4	8	18	29
I2-L	0.4	17	24	32
WS-L	0.4	74	71	62
C1-H	1.1		-	-
C2-H	1.1		-	-
I1-H	1.1	30	31	33
I2-H	1.1	40	36	41
WS-H	1.1	66	60	59

In the growth medium supplemented with inhibitors, IR increased as the concentration of AA produced during fermentation in all the tests. Where acetic acid as unique inhibitor, had a starting concentration of 52.5 mg·L^{-1}, IR was equal to 30% at 24h and increased weakly

up to 33% at 144h despite the acetic acid produced being equal to 224 ± 3.5 $mg \cdot L^{-1}$ but the strain concentration managed to counteract this concentration better.

In the presence of AA and furfural in the growth medium, the rate of growth inhibition was higher at 24h than in tests with a single inhibitor, 17% at the initial contraction of AA 21 $mg \cdot L^{-1}$ and furfural 6 $mg \cdot L^{-1}$, and 40% at the initial concentrations of AA 52.5 $mg \cdot L^{-1}$ and furfural 15 $mg \cdot L^{-1}$. Inhibition tended to increase during the fermentation process for acetic acid production.

A. succinogenes growth was strongly decreased when the two diluted wheat straw hydrolysates were used. At 24h, the IR of the strain (at initial concentration of 0.4 $g \cdot L^{-1}$) was higher (77%) in the diluted hydrolysate that contained less inhibitors (WS-L), AA 21 $mg \cdot L^{-1}$ and furfural 6 $mg \cdot L^{-1}$, because the strain was undergoing a latency phase. And the inhibition effect appeared to reduce slightly from 48h to 144h because the microorganism started to grow.

A. succinogenes better tolerated the more concentrated wheat straw hydrolysate (WS-H) starting from bacterial concentration equal to 1.1 $g \cdot L^{-1}$, acetic acid at 52.5 $mg \cdot L^{-1}$ and furfural at 15 $mg \cdot L^{-1}$ with a IR% equal to 66% at 24h that decreased till to 144h. A decrease in the growth inhibition rate was observed despite the fact that acetic acid was produced by the strain and reached the concentration of 215 ± 2.7 $mg \cdot L^{-1}$.

Compared with previous studies in which higher concentrations of these inhibitors were tested, lower concentrations were tested to maintain a ratio of bacterial to glucose concentration around 1:1 as demonstrated in a previous work (Molino et al., 2020). Li et al. (2010) tested different concentrations of AA from 10 $g \cdot L^{-1}$ to 80 $g \cdot L^{-1}$ and an initial *A. succinogenes* concentration of 0.03 $g \cdot L^{-1}$ showing that the strain can tolerated up to 10 $g \cdot L^{-1}$ of acetic acid. The authors reached a bacterial concentration of 0.06 $g \cdot L^{-1}$ but the growth was slow over 12 hours and an inhibition rate of 86% was recorded. (Li et al., 2010). On the other hand, *A. succinogenes* can tolerate furfural up to the maximum concentration of 0.28 $g \cdot L^{-1}$ (Shen et al., 2022). Nevertheless, a different inhibition trend was observed in this work between the strain growth in the medium (simulated) and the hydrolysate due to the production of AA in the simulated that increased the inhibition rate. Among the organic acids, formic acid was also produced that can blocked the growth of the strain at the critical concentration of 18 $g \cdot L^{-1}$ (Du et al., 2007). Specifically, inhibition rates in samples in which

growth medium was supplemented with acetic and furfural acid at both low and high concentrations were similar from 48h to 144h for acetic and formic acid production trends. In the hydrolysate, on the other hand, inhibition rates were higher but decreased slightly in response to strain growth, and AA and FA concentrations were still lower than those produced in the simulated as shown in the following paragraphs (5.3.3 and 5.3.4).

5.3.2. Glucose and mixed sugars consumption

Glucose and xylose were the main fermentable sugars released after the pre-treatments of a lignocellulosic biomass that accounted for around 90% of the total sugars. Even if glucose was the monosaccharide principally investigated for the production of succinic acid, xylose also enabled its production as demonstrated by using a hydrolysate of corn stover (xylose concentration 57.0 $g \cdot L^{-1}$), with a conversion equal to 80% and SA production of 32.5 $g \cdot L^{-1}$ in a continuous fermentation process by a biofilm reactor (Bradfield et al., 2015). Even though, *A. succinogenes* was also able to use till to 60 $g \cdot L^{-1}$ pure xylose to produce around 6 $g \cdot L^{-1}$ of succinic acid in shake flask fermentation (Jokodola et al., 2022), SA production can be affected by the co-utilization of glucose and xylose since their ratio can affect the intracellular ATP production by interfering with metabolic pathways (Zhang et al., 2016). Zhang et al. (2016) tested several glucose-xylose ratios for a total sugar concentration of 60 $g \cdot L^{-1}$ and the results showed an increase in succinic acid production when glucose was higher in the ratio of the two sugars (Zhang et al., 2016). A similar result was observed on the production of SA from pure glucose and the mixture with sucrose, fructose, and xylose, where better performance of succinic acid production was observed in the presence of glucose as the unique sugar source compared to the glucose:xylose ratio 0.9:0.1 (Molino et al., 2020).

In this study, since the glucose:xylose ratio was found equal to 0.7:0.3 in the wheat straw hydrolysate, this ratio has been repeated in the control tests with TSB growth medium (C2) and in the simulated hydrolysate samples. A predominant consumption of glucose was observed in all tests irrespective of the concentration and the presence of xylose, which did not significantly influence glucose consumption trends. Xylose was poorly consumed (3.5% xylose consumption) in control from an initial concentration of 0.2 $g \cdot L^{-1}$ while, in the control (C2-H) at a high concentration of sugars (glucose 1.1 $g \cdot L^{-1}$ and xylose 0.4 $mg \cdot L^{-1}$), 6.1%

xylose was consumed. The consumption decreased from 6.1 % to 4.2% and to 5.4% where the growth medium (TSB) contained acetic acid and acetic acid and furfural respectively. When wheat straw hydrolysate was used 3.7 % xylose was consumed.

The Figure 5.2 showed the percentage of the consumed glucose during the fermentation. In the control tests, the strain consumed up to 32 ± 2% of glucose from an initial concentration of 0.4 $g \cdot L^{-1}$ (C1-L, Figure 5.2A) and 51 ± 2% glucose from 1.1 $g \cdot L^{-1}$ (C1-H, Figure 5.2B) after 144 hours. The results showed that the consumption of glucose decreased in the presence of inhibitory compounds as evidenced by the decreased growth of the strain as discussed in the previous paragraph. When AA was contained in the supplemented growth medium as a single inhibitor, at the two initial concentrations 21 $mg \cdot L^{-1}$ (I1-L) and 52.5 $mg \cdot L^{-1}$ (I1-H), glucose was consumed up to 24 ± 3 % and 25 ± 3% respectively, without significant differences ($p>0.05$). The highest concentration of acetic acid (52.5 $mg \cdot L^{-1}$ in I1-H) significantly affected glucose consumption, which decreased by almost 50% compared to glucose consumption in the control (C1-H).

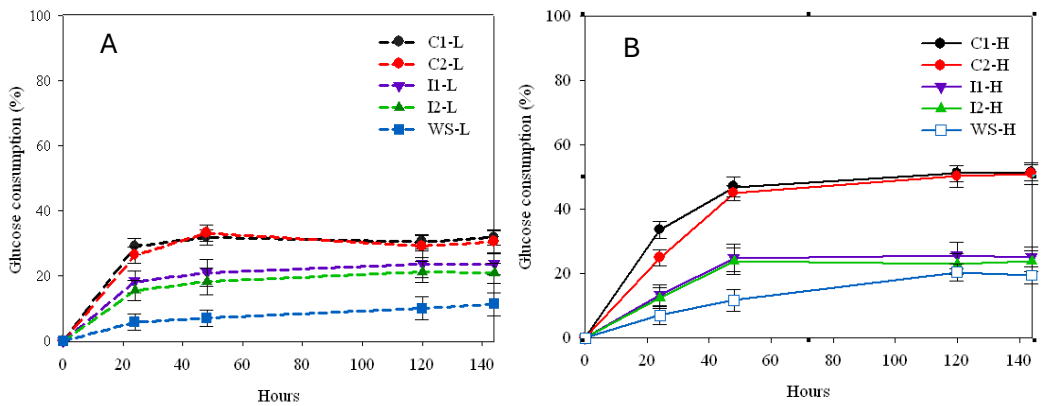

Fig. 5.2. *Glucose consumption (%) in test L (low concentration – dashed lines) and H (high concentration – continuous lines). Circles represent Control 1 and Control 2; triangles represent tests with inhibitors compounds; squares represent the wheat straw sample. Error bars indicated standard deviation.*

The greatest evidence of the reduction in glucose consumption in the presence of both inhibitors (AA and furfural) was observed at their highest concentration (I2-H) (AA 52.5 $mg \cdot L^{-1}$ of and furfural 15 $mg \cdot L^{-1}$) in the supplemented growth medium. In the hydrolysate,

the highest amount of glucose consumed was observed at 144h that was around 19% ± 3 when a more concentrated wheat straw hydrolysate was used (WS-H). The greatest difference in glucose consumption between the two wheat straw dilutions was observed at 144 hours. As the consumption rates were very similar for the dilutions at 24 hours (6% ± 1 and 7 % ± 1), the differences observed on the glucose were due to the growth of the strain that started higher in the more concentrated hydrolysate.

5.3.3. Effect of inhibitors on succinic acid production

The concentration of succinic acid produced by *A. succinogenes* is shown for the samples at low concentrations of strain, sugars and inhibitors (Figure 5.3b) and those at high concentrations (Figure 5.3b). The highest concentration of SA was recorded when inhibitors were absent and without significant differences in the presence of xylose, while SA decreased significantly in all tests in which unwanted compounds were present both at low and high concentrations than the control tests ($p<0.05$). At an initial glucose concentration of 0.4 $g \cdot L^{-1}$ the SA maximum concentration was 200 ± 10 $mg \cdot L^{-1}$. Respect to control, the highest decrease of SA concentration in supplemented growth medium was observed when AA and furfural was added at the initial concentration of 21 $mg \cdot L^{-1}$ and 6 $mg \cdot L^{-1}$ (Figure 5.3a). In this case, SA production amounted to 66 ± 3 $mg \cdot L^{-1}$ compared to 200 ± 10 $mg \cdot L^{-1}$ in the control, with a statistically significant difference ($p <0.017$). The presence of AA at a concentration of 21 $mg \cdot L^{-1}$, on the other hand, yielded up to 95 ± 5 $mg \cdot L^{-1}$ of succinic acid. The concentration of SA obtained by using the less concentrated wheat straw hydrolysate (WS-L) was recorded as less representative and amounted to 10 ± 7 $mg \cdot L^{-1}$ (Figure 5.3a).

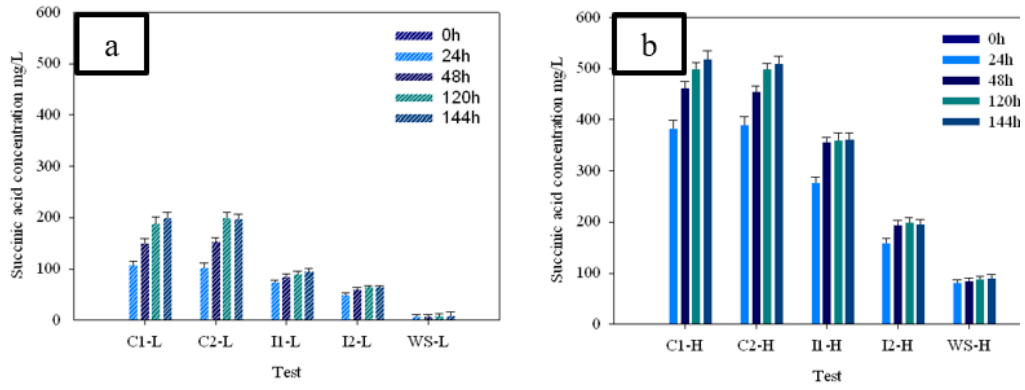

Fig. 5.3 *Succinic acid concentration (mg·L^{-1}) in a) test L (low concentration – dashed vertical bars) and b) test H (high concentration – full vertical bars). Control 1 (C1), control 2 (C2), test with acetic acid (I1), test with acetic acid and furfural (I2), wheat straw sample (WS). Error bars indicated standard deviation.*

The highest production of SA was equal to 520 ± 16 mg·L^{-1} and it was observed in the control at starting glucose concentration of 1.1 g·L^{-1} (Figure 5.3a). Any important difference was observed on SA concentration when xylose was used. The combined effect of acetic acid and furfural was also observed in this case and succinic acid concentration reached 196 ± 9 mg·L^{-1}. The concentration of succinic acid produced by using the more concentrated wheat straw hydrolysate was 90 ± 7 mg·L^{-1} (WS-H), which was significantly 9 times higher than the achieved by using the more diluted wheat straw hydrolysate (WS-L test: 10 ± 7 mg·L^{-1}). The SA concentration 90 ± 7 mg·L^{-1} (WS-H) was also approximately 2.2 times lower than the corresponding simulated in the growth medium with acetic acid and furfural (I2-H, AA 52.5 mg·L^{-1} and furfural 15 mg·L^{-1}) at the high concentration.

An overview of the final concentration and percentage reduction of succinic acid is reported in Table 5 for all tests compared to control (C1). The percentages of SA reduction in the tests of standard medium supplemented with AA were 53% at the low initial concentration of AA (21 mg·L^{-1}) and 30% at 52.5 mg·L^{-1}. The reduction rate 53% of SA was not due to acetic acid concentration but to the less growth of the strain compared with the corresponding test at higher AA concentration.

The inhibition effect of the acetic acid and furfural mixture seemed to be not dependent on their two concentrations with a reduction in succinic acid concentration of 67% and 62% at

their low and high concentrations respectively. The similarity between the two inhibition rates was also due to the effect of FA produced in greater amounts than acetic acid in the I2-L test.

In contrast, the inhibition effect of the compounds contained in the wheat straw hydrolysate was greatest at their lowest concentrations with a 95% reduction in the concentration of SA, while at the highest concentrated hydrolysate the reduction was fewer (83%).

Table 5.5 *Inhibitors effect on succinic acid concentration respect to controls at low and high concentration of unwanted compounds*

Succinic production mg·L^{-1} and reduction (%)				
Carbon source	NO inhibitors	AA 21 mg·L^{-1}	AA 21 mg·L^{-1} + Furfural 6 mg·L^{-1}	AA 21 mg·L^{-1} + Furfural 6 mg·L^{-1} in wheat straw
Glucose 367 mg·L^{-1}	200 ± 10 (100%)	95± 5* (53%)	66± 3* (67%)	10± 7* (95%)
Glucose 367 mg·L^{-1} + Xylose 171 mg·L^{-1}	198± 9 (100)			
A. succinogenes Final concentration g·L^{-1}	10.5/10.7	7.4	7.1	4.0
Carbon source	NO inhibitors	AA 52.5 mg·L^{-1}	AA 52.5 mg·L^{-1} + Furfural 15 mg·L^{-1}	AA 52.5 mg·L^{-1} + Furfural 15 mg·L^{-1} in wheat straw
Glucose 1130 mg·L^{-1}	520±16 (100%)	362±13* (30%)	196±9* (62%)	90±7* (83%)
Glucose 1130 mg·L^{-1} + Xylose 428 mg·L^{-1}	510±14 (100%)			
A. succinogenes Final concentration g·L^{-1}	17/17.2	11.3	10	7.02

The effects on the succinic acid production of the main inhibitory compounds generated by the pre-treatment of lignocellulosic biomass as furans, acids and phenolic compounds were already partially investigated as reported in Table 5.5. Dessie et al. (2019) demonstrated that at the same concentration of HMF and furfural, *A. succinogenes* tolerated more HMF (Dessie et al., 2019). In a batch fermentation process starting from 30 g·L^{-1} of glucose, if furfural concentration was 3 g·L^{-1} the SA production reduced of 84% while when both furfural and

HMF were present SA concentration decreased of 93.2% despite the total concentration of inhibitors was less than 3 $g \cdot L^{-1}$ (Dessie et al., 2019). This phenomenon was due to a synergistic effect of the inhibitors. In this study, only furfural was detected in hydrolysate, so the synergistic effect of the two furans was not observed.

Salvachúa et al. (2016), observed the synergistic effect of furans together with acetic acid by using a diluted corn stover hydrolysate in which the concentrations of AA were 5.8 $g \cdot L^{-1}$, HMF 1.4 $g \cdot L^{-1}$, and furfural 0.17 $g \cdot L^{-1}$ that decreased SA concentration to 95% (Salvachúa et al., 2016). In the same study, acetic acid, at the concentration of 5.8 $g \cdot L^{-1}$, but as the only inhibitor, affected SA concentration only for the 30% (Salvachúa et al., 2016). This synergetic behaviour of acetic acid and furans was also observed in this study, in addition at this effect authors highlighted the strong effect of formic acid respect to acetic acid on the reduction of SA equal to 67% (Tables 5.5 and 5.6). In addition to the synergistic effect with furans, it was also observed that acetic acid, when present as a single inhibitor, can cause a delaying effect on succinic acid production at 24 hours (Salvachúa et al., 2016).

The effect of the inhibitors was also tested on other microorganisms such as *Basfia succiniciproducens* using a hydrolysate of *Arunndo donax* at different dilutions (Cimini et al., 2016). The more concentrated hydrolysate (90% hydrolysate) (containing AA 8 $g \cdot L^{-1}$, HMF 63 $mg \cdot L^{-1}$ and furfural 45 $mg \cdot L^{-1}$) totally inhibited SA production up to 20 hours. After this inhibition, at 48 hours, succinic acid concentration reached the same value (5 $g \cdot L^{-1}$) obtained when a more diluted hydrolysate (20% hydrolysate) at the concentration of acetic acid equal to 2 $g \cdot L^{-1}$ was used.

Xu et al. (2015) tested other compounds, such as vanillin and syringaldehyde, in combination with furfural and HMF, at different concentrations, starting from a corn cob hydrolysate on an engineered *C. glutamicum* strain (Xu et al., 2015). Syringaldehyde inhibited more SA production than furfural and HMF. The corn cobs hydrolysate was tested with and without detoxification treatment, and the hydrolysate without detoxification reduced SA production by 50% compared to the detoxified hydrolysate (Xu et al., 2015).

Table 5.6 *The main results on the effect of inhibitors on succinic acid production.*

Inhibitors	Inhibitors concentration ($g \cdot L^{-1}$)	Strain	SA reduction (%) and concentration ($g \cdot L^{-1}$)	References
Furfural	3	*Actinobacillus succinogenes*	84% (3.5)	Dessie et al., 2019
HMF	3		62% (8.4)	
Furfural	3		83.3% (2.7)	
HMF	3		75% (4.0)	
Furfural + HMF	2 (F) + 2 (HMF)		93.2% (1.1)	
Acetic Acid	5.8	*Actinobacillus succinogenes*	30% (5)	Salvachùa et al., 2016
Furfural + HMF + Acetic acid	1.4 (F) + 0.17 (HMF) + 5.8 (AA)		95% (5)	
Furfural + HMF + Acetic acid	1.4 (F) + 0.17 (HMF) + 2.3 (AA)		25% (30)	
Acetic acid	2	*Basfia succiniciproducens*	75% (5)	Cimini et al., 2016
Acetic acid + HMF + furfural	8 (AA) + 0.063 (HMF) + 0.045 (FU)		0% 24 h 75% (5) 48 h	
Acetic acid	>10	*Corynebacterium glutamicum*	92%	Xu et al., 2015
Furfural	0-2		20% (1.5)	
5-HMF	0-2		30% (1.5)	
Vanillin	0-2		60% (1.5 & 2.0)	
Syringaldehyde	0-2		50% (1.5 and 2.0)	
Inhibitors mixtures	0-2		60%	
Acetic acid	0.021 (AA) 0.052.5 (AA)	*Actinobacillus succinogenes*	53% (0.095± 0.05) 30% (0.362±0.13)	In this study
Acetic acid + furfural	0.021 (AA) + 0.006 (FU)		67% (0.066± 0.03)	In this study
Acetic acid + furfural	0.052.5 (AA) + 0.015 (FU)		62% (0.196±0.9)	In this study

5.3.4. Effect of inhibitors on by-products

The decrease of by-products such as acetic, formic, and lactic acid is one of the most challenging topics to improve the production of succinic acid. In this study, the concentration of the acetic and formic acids produced during the fermentation in all the tests were evaluated and the effect of the inhibitors on their production was also observed (Figures 5.4 and 5.5). An interesting decrease of the acetic acid was recorded when xylose was used as feedstock with glucose, both at the concentrations of 0.2 $g \cdot L^{-1}$ (C2-L) and 0.4 $g \cdot L^{-1}$ and (C2-H) (Figure 5.4a and b). In particular, when xylose was 0.2 $g \cdot L^{-1}$, the concentration of AA was 2.6 times lower respect to control (C1-L) when only glucose was used.

When acetic acid was added at the initial concentration of 21 $mg \cdot L^{-1}$ (I1-L) in the standard growth medium alone and with furfural (I2-L) the AA concentration slightly increased without significant difference in the two tests ($p>0.05$). In the diluted hydrolysate, its concentration slightly increased till to 70 $mg \cdot L^{-1}$.

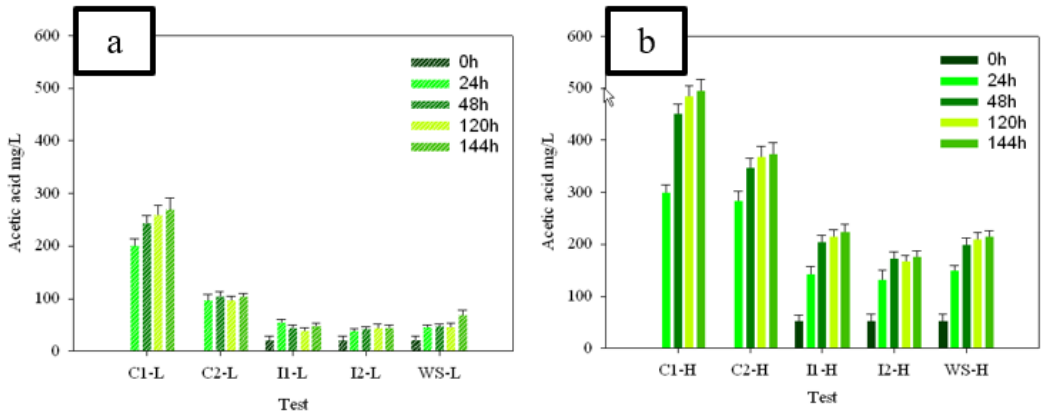

Fig. 5.4 *Acetic acid concentration (mg·L-1) in a) test L (low concentration – dashed vertical bars) and b) test H (high concentration – full vertical bars). Control 1 (C1), control 2 (C2), test with acetic acid (I1), test with acetic acid and furfural (I2), wheat straw sample (WS). Error bars indicated standard deviation.*

The effect of glucose-xylose mixture was also observed on AA at their high concentration (Glucose 1.1 $g \cdot L^{-1}$ and xylose 0.4 $g \cdot L^{-1}$) (C2-H). In this case, the final concentration of acetic acid was only 1.3 times lower than control (C1-H). Starting at acetic acid concentration of 52.5 $mg \cdot L^{-1}$, its concentration increased in supplemented growth medium and hydrolysate, but the production of AA was reduced respect to control (C1-H).

AA reached the concentration of 224 ± 3.5 mg·L^{-1} and 215 ± 2.7 mg·L^{-1} without statistically difference ($p>0.05$) in the supplement standard growth medium, respectively in I1-H and I2-H. A slight, but non-significant, decrease of AA production was observed in supplemented TSB with acetic acid and furfural (I2-H) ($p>0.05$).

In addition, the concentration of formic acid was also evaluated (Figures 5.5). An unexpected high production of formic acid was observed in the supplemented growth medium with acetic acid at 21 mg·L^{-1} and furfural at 6 mg·L^{-1} (I2-L) respect to the others group tests (Figure 5.5a).

Formic acid production did not differ significantly between control C1-H, supplemented growth medium with the mixture of sugars (glucose 1.1 g·L^{-1} and xylose 0.4 g·L^{-1}), and added AA 52.5 mg·L^{-1} (Figure 5.5b) ($p>0.05$). In the co-presence of AA and furfural (15 mg·L^{-1}), formic acid decreased considerably in both the TBS growth medium (I2-H) and in the hydrolysate (WS-H).

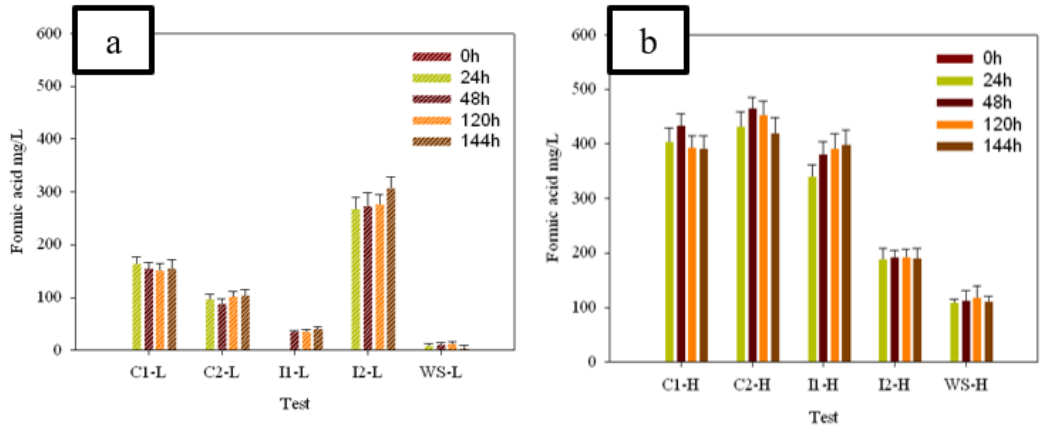

Fig. 5.5 *Formic acid concentration (mg·L-1) in a) test L (low concentration – dashed vertical bars) and b) test H (high concentration – full vertical bars). Control 1 (C1), control 2 (C2), test with acetic acid (I1), test with acetic acid and furfural (I2), wheat straw sample (WS). Error bars indicated standard deviation.*

5.3.5. Succinic acid production performance and strategies

The results indicated that the growth of the strain, glucose consumption and succinic acid production and the other by-products were strongly influenced by the presence of the inhibitors both under growth conditions in a rich medium, such as TSB, and under wheat

straw hydrolysate at the two tested dilutions. But the different initial concentration of inoculum equal to 0.4 $g \cdot L^{-1}$ and 1.1 $g \cdot L^{-1}$ demonstrated that the more concentrated inoculum was able to grow more, to consume more glucose, to produce more SA when using a rich growth medium. The more concentrated inoculum was able to also grow and counteract the effects of using dilute raw hydrolysed. The negative effect of acetic acid production was recorded but less pronounced than the synergistic effect of AA and furfural on succinic acid. Yield ($gr_{SA} \cdot gr^{-1}_{Glucose}$), the 24-hour productivity, and the ratio between SA and AA, and the selectivity were evaluated (Table 5.7) to better understand the performance of the succinic acid production.

In this study, under conditions of the absence of inhibitor stress *A. succinogenes* produced succinic acid at the concentration equal to 520 ± 16 $mg \cdot L^{-1}$ and at a yield of 47.3%. When less glucose and less inoculum were used succinic acid concentration was 200 ± 10 $mg \cdot L^{-1}$ at a yield of 55.6%.

Table 5.7 *Process performance of succinic acid production*.

TEST	Succinic acid concentration ($mg \cdot L^{-1}$)	Yield ($gr_{SA} \cdot gr^{-1}_{Glu}$)	Productivity ($mg \cdot L^{-1} \cdot h^{-1}$)-24	Ratio SA:AA	Selectivity ($g_{SA}/g_{SA}+g_{AA}+g_{FA}$)
C1-L	200 ±10	55.6%	4.5	0.7	0.3
C2-L	198 ±9	53.5%	4.3	1.9	0.5
I1-L	95 ±5	25.7%	3.1	2.0	0.5
I2-L	66 ±3	17.8%	2.1	1.5	0.2
WS-L	10 ±7	2.7%	0.3	0.1	0.1
C1-H	520 ±16	47.3%	16.0	1.05	0.4
C2-H	510 ±14	46.4%	16.3	1.4	0.4
I1-H	362 ±13	32.9%	11.5	1.6	0.4
I2-H	196 ±9	17.8%	6.7	1.1	0.3
WS-H	90 ±7	8.2%	3.4	0.4	0.2

To better understand the potentiality of diluted wheat straw hydrolysate as unique feedstock of carbon sources (glucose and xylose) for the production of SA, sugars concentrations and

organic acids were reported in Figure 5.6, where the data obtained from the use of the more concentrated hydrolysate were reported compared to control.

In absence of inhibitors (Figure 5.6a) *A. succinogenes* grew in a rich growth medium and quickly consumed about 50% (51.3 ± 2.4%) of glucose at 48 hours and produced at the end of the fermentation SA 520 ±16 $mg \cdot L^{-1}$ (144 h) at a yield of 47.3% respect to the initial amount of glucose. Respect to the others organic acids, the production of SA increased till the end of the fermentation without significant difference respect to the produced AA that was explained with a ratio SA:AA equal to 1.0. While FA concentration decreased from 48h (Figure 5.6a).

In Figure 5.6b, xylose concentration was not reported due to the low consumption that was around 6 % respect to the starting concentration of 0.4 $g \cdot L^{-1}$. Under the co-presence of two carbon sources, the strain preferred to use glucose to produce a similar concentration of SA around 510 ± 14 $mg \cdot L^{-1}$ at 144 h at a yield of 46.4%. In the presence of glucose alone or glucose and xylose, no change was found on the growth trend of the strain. Although *A. succinogenes* can utilize several monosaccharides and other disaccharides and reducing sugars (Yang et al., 2020), it was abundantly demonstrated that the strain preferred to use glucose that can tolerate up to the concentration of 158 $g \cdot L^{-1}$ (Lin et al., 2008). By using glucose as carbon source, *A. succinogenes* can produce relatively large quantities of SA till to 70 $g \cdot L^{-1}$ at a yield of 0.6 $g_{SA} \cdot g^{-1}_{glucose}$, and a productivity equal to 1.4 $g \cdot L^{-1} \cdot h^{-1}$ (Thuy et al., 2017).

Although xylose was partially consumed by the strain at the two concentrations, it was observed that the SA:AA ratio increased to 1.4 and 1.9 respectively when the initial xylose concentrations were 0.2 $g \cdot L^{-1}$ and 0.4 $g \cdot L^{-1}$. The increase in the value of SA:AA ratio was due to the decrease in AA production in both tests, but the concentration of formic acid was recorded higher. In addition, when glucose and xylose were at the initial concentrations equal to 0.4 $g \cdot L^{-1}$ and 0.2 $g \cdot L^{-1}$, and the concentration of SA was 198 ± 9 $mg \cdot L^{-1}$, the selectivity was also improved (0.5 $g_{SA}/g_{SA} + g_{AA} + g_{FA}$) (Table 5.7) as the concentration of both AA and FA decreased (Figures 5.4 and 5.5).

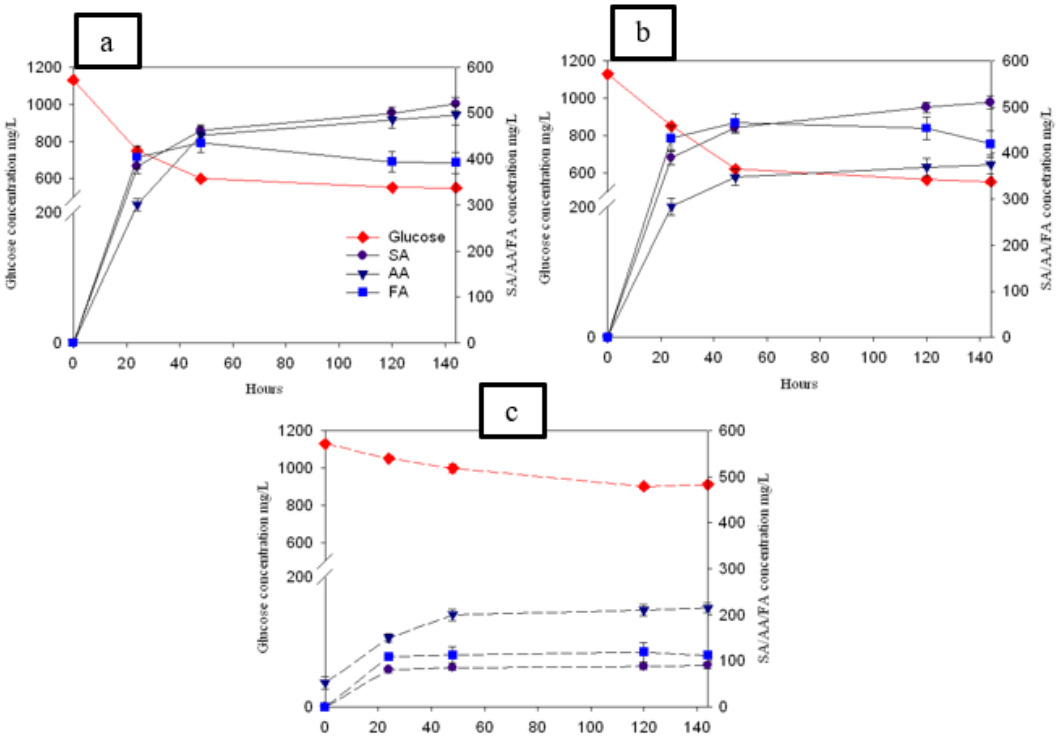

Fig. 5.6 *Glucose and organic acid concentration (Succinic acid SA, Acetic acid AA, Formic acid FA) in: a) standard growth medium supplemented with glucose 1.1 g·L-1, b) standard growth medium supplemented with glucose 1,1 g·L-1 and xylose 0.4 g·L-1, c) wheat straw hydrolysate (high concentration of sugars and inhibitors). Error bars indicated standard deviation.*

This selectivity value was also calculated in the presence of acetic acid at the initial concentration of 21 $mg \cdot L^{-1}$ given the higher production of succinic acid compared to the other byproducts (acetic and formic acid) (Table 5.7).When the hydrolysate was used (Figure 5.6C), the strain underwent a reduction in growth capacity with an observable initial latent phase (66% inhibition rate) demonstrated by the trend of glucose concentration reaching only at the end of the experiment a concentration of 910 $g \cdot L^{-1}$ when the growth inhibition rate dropped to 59%. At the end of fermentation the SA produced was 90 ± 0.7 $mg \cdot L^{-1}$ with a predominance of production of the other organic acids especially AA with a concentration of 215 ± 2.7 $mg \cdot L^{-1}$. In fact, the SA/AA ratio decreased to 0.42 and the selectivity reached 0.2 $g_{SA}/g_{SA}+g_{AA}+g_{FA}$. The best yield of SA was obtained at 24h with the 8.2% $g_{SA} \cdot g^{-1}{}_{glucose}$ respect to the initial glucose amount.

A slower initial phase of glucose consumption when using a dilute lignocellulosic biomass hydrolysate, and a reduction in succinic acid production were also observed by the authors

Salvachúa et al. (2016). In this study, a corn stover hydrolysate obtained by acid hydrolysis at 120 °C was tested. Glucose was firstly utilized by the *A. succinogenes* strain but whose consumption started as well as for xylose after 48 h when the strain grew in hydrolysates at furfural concentration 1.7 $g \cdot L^{-1}$, HMF 0.17 $g \cdot L^{-1}$ and acetic acid at 2.3 $g \cdot L^{-1}$ and 5.8 $g \cdot L^{-1}$. SA production also started with latent phase after 48h, and in the hydrolysate with higher acetic acid concentration, SA concentration was 4 times lower than in the control with productivity almost 10 times lower (0.7 $g \cdot L^{-1} \cdot h^{-1}$) at the time when productivity was highest in the control (6.36 $g \cdot L^{-1} \cdot h^{-1}$) (Salvachúa et al., 2016).

The results obtained in this work on SA production by using the two diluted wheat straw hydrolysates were certainly lower than the results already obtained using a wheat straw hydrolysate after alkali treatment obtaining SA production of 18.9 $g \cdot L^{-1}$ with a yield of 74% from 19.4 $g \cdot L^{-1}$ glucose with 57% sugar consumption (Zheng et al., 2009). It should be noted, however, that in the work of Zheng et al. (2009) the lowest succinic acid production performance was precisely observed when wheat straw was used compared to hydrolysate of other biomasses such as corn straw and core, and rice straw. The difficulty to be degraded by microorganisms was related to the complex, multi-layered structure of wheat straw composed of the crystalline cellulose with a density of 1.59 $g \cdot cm^{3-1}$ higher than the amorphous hemicelluloses and lignin, the hemicelluloses, lignin, and the complex formed between lignin and carbohydrates (Bradfield et al., 2015). For this reason, this challenging feedstock was chosen in this study for a preliminary assessment of succinic acid production by using wheat straw hydrolysate as unique source and to assess the effect of the inhibitors (acetic acid and furfural) produced after its pre-treatment by steam explosion and enzymatic treatment. Cimini et al., tested several dilutions of *Arundo donax* hydrolysate obtained from acid hydrolysis (acetate buffer), sterilization and enzymatic hydrolysis for SA production by the strain *Basfia succiniciproducens*. The authors observed as SA yield decreased as the concentration of the hydrolysate increased till to 90% that contained the highest inhibitors concentrations (AA 8 $g \cdot L^{-1}$, HMF 63 $mg \cdot L^{-1}$, furfural 45 $mg \cdot L^{-1}$). The increase of inhibitor concentrations caused a decrease in glucose and xylose consumption and an onset of succinic acid production after 24 h with a productivity of 0.09 $g \cdot L^{-1} \cdot h^{-1}$ (Cimini et al., 2016). The trends of decrease of SA production when using wheat straw hydrolysate were common problems highlighted in most of the papers where lignocellulosic biomass were used as

feedstock. It should also be pointed out that in works lignocellulosic biomass such as sweet sorghum bagasse or rapeseed straw was pre-treated by acid hydrolysis or steam explosion and used as a substrate for *A. succinogenes* strain. Therefore, SA yield was quite lower such as 61% or 48% (Sawisit et al., 2018; Lo et al., 2020) compared to studies in which modified strains (*E. coli* AS1600a) were used, which was able to produce SA at the yield of 86% in the presence of inhibitors at the total concentration of 4.3 $g \cdot L^{-1}$ (Kuglarz et al., 2018). Recently, in order to implement the utilisation of wheat straw for succinic acid production, the fungus *Aspergillus niger* was engineered by using the RNP-based CRISPR-Cas9 system to obtain an increase in succinic acid production up to 15 $g \cdot L^{-1}$ from 100 $g \cdot L^{-1}$ glucose at a yield of 15% $g_{SA} \cdot g^{-1}_{glucose}$. Conversely, the wild type did not show SA production under the same conditions (Yang et al., 2020). The same engineered strain was tested for succinic acid production by using sugarcane molasses and wheat straw hydrolysate filtered and supplied to micronutrients (biotin), calcium carbonate and ammonium nitrate, and other minerals salts. The authors (Yang et al., 2020) obtained higher succinic acid production by using molasses (20 $g \cdot L^{-1}$) from 110 $g \cdot L^{-1}$ sucrose, 55 $g \cdot L^{-1}$ glucose and fructose respectively at a yield of 20% $g_{SA} \cdot g^{-1}_{sugars}$. When wheat straw hydrolysate was tested the authors observed a delayed phase and the production of succinic acid started after 48 hours when was used. A less final concentration of SA equal to 9 $g \cdot L^{-1}$ was obtained after 144 hours at a yield of 9% $g_{SA} \cdot g^{-1}_{sugars}$ from an initial stock of sugars of 45 $g \cdot L^{-1}$ xylose and 65 $g \cdot L^{-1}$ glucose (Yang et al., 2020).

In addition, the obtained results on SA production by using a glucose rich medium were in line with the results obtained by other authors as Zhou et al. (2023), that performed batch tests without the use of any fermentation strategies, without resorting to direct and indirect sources of CO_2 or pH modifiers, that might be essential in improving SA production (Zhou et al., 2023).

The yield obtained in this study (8.2 $g_{SA} \cdot g^{-1}_{glucose}$) where *A. succinogenes* was used, and the achieved SA production by using a wheat straw hydrolysate (90 ± 0.7 $mg \cdot L^{-1}$) as the unique carbon source without the supplementation of nitrogen source and the lag phase observed in the 24 hours are in line with the results obtained by Yang et al. (2020) that tested the engineered fungus *A. niger* for the production of SA from wheat straw hydrolysate.

In this study, a concomitant cause of the presence of the inhibitors in wheat straw hydrolysate and resulting in low SA production was the deficiency of a nitrogen source such as yeast extract. Indeed, Putri et al. (2023) showed that as the concentration of yeast extract increased in the process for the production of succinic acid from rice straw, the yield and concentration of SA increased too (Putri et al., 2023).

The results presented in literature showed that the use of wheat straw is quite challenging for succinic acid production. The findings achieved in this study from these preliminary batch fermentation tests emphasized the ability of the strain *A. succinogenes* to utilize the glucose feedstock contained in the wheat straw hydrolysate, despite the synergistic action of the inhibitors and the absence of nitrogen sources, the starting more concentrated inoculum seemed a great strategy to increase resistance against inhibitors normally present in wheat straw hydrolysate.

5.4. Conclusions

The wheat straw hydrolysate was used as unique carbon source without nitrogen supplement as low-cost feedstock for succinic acid production. The wheat straw hydrolysate contained inhibitors as acetic acid and furfural generated from steam-explosion pre-treatment. This study evidenced as different inhibitory effects was observed in relation to the growth of the strain, and succinic acid production performance in supplemented rich growth medium and in the crude hydrolysate. The presence of more carbon sources (glucose and xylose) in the hydrolysate decreased the production of acetic acid respect to succinic acid. And the use of a more concentrated inoculum seemed to be a great strategy to overcome the inhibition effects of unwanted compounds in wheat straw hydrolysate. The results obtained can surely improve the knowledge towards a better valorisation of wheat straw residues for succinic acid production. Certainly, the use of wheat straw hydrolysate needs to be further investigated to develop targeted strategies for pre-treatment and fermentation to improve succinic acid production.

5.5. Acknowledgments

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5.6. Supplementary materials

5.6.1. Figures

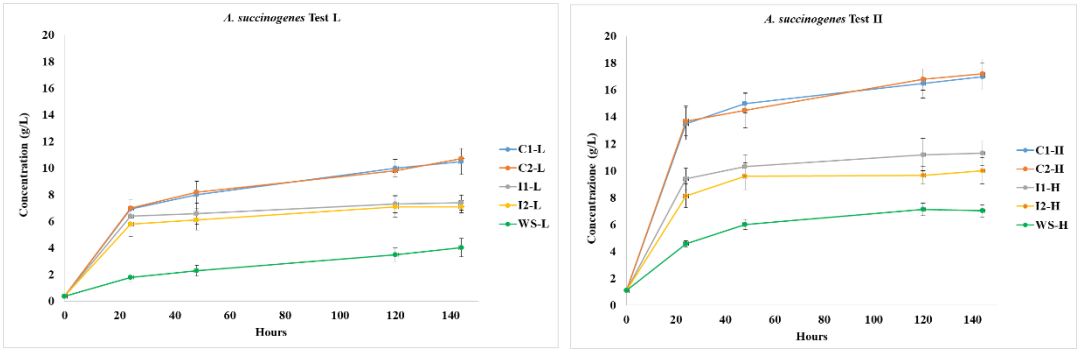

Figure S1. *A. succinogenes strain concentration in each tests at low concentration of compounds TEST –L and high concentration TEST-H*

6. Valorization of wheat straw hydrolysate into succinic acid by fermentation with *Actinobacillus succinogenes*

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Bioresource Technology

6.1. Introduction

Succinic acid (SA), a four-carbon dicarboxylic acid, is a well-established and high-value platform chemical with many applications in the pharmaceutical, food, and agricultural fields (Zeikus et al., 1999; McKinley et al., 2010). It also serves as a crucial precursor in the production of polybutylene succinate (PBS), a biodegradable polymer widely employed in the bioplastics industry (Saxena et al., 2017). Due to its versatility and industrial significance, the global market for SA has experienced steady growth. Its value increased from USD 157 million in 2015 to USD 234 million in 2022, a 67% rise, with projections indicating it could exceed USD 500 million by 2030 (Ong et al., 2019; Grand View Research, 2022). Currently, over 50% of the industrial SA supply is derived from petroleum-based feedstocks, such as n-butane (Grand View Research, 2022). This reliance poses environmental challenges, particularly related to greenhouse gas (GHG) emissions, as well as economic risks due to fluctuating fossil fuel prices (Xu et al., 2021). In response to these concerns, the European Union and other international stakeholders have introduced policies promoting sustainable production technologies as part of broader strategies aimed at climate neutrality (Lithourgidis et al., 2023). In this context, biotechnological production of SA, especially through microbial fermentation of agro-industrial residues and organic waste, has emerged as a promising alternative. These bioprocesses can reduce GHG emissions by over 60% compared to conventional petrochemical routes (Musonda et al., 2020).

Certain microorganisms are able to produce SA as a fermentation product. Notable strains include *Actinobacillus succinogenes*, *Basfia succiniciproducens*, and *Anaerobiospirillum succiniciproducens* (Dessie et al., 2021; Robertiello et al., 2023; Lee et al., 2000). Additionally, model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* have been genetically engineered to enhance succinate yields (Valle et al., 2022; Malubhoy et al., 2024). Among these, *A. succinogenes* is currently the most widely employed strain for industrial SA fermentation (Zacharopoulos et al., 2025), due to several favorable traits. These include the ability to utilize a broad spectrum of carbon sources, like pentoses (xylose, arabinose), hexoses (glucose, galactose, mannose, fructose), disaccharides (sucrose, lactose), reducing sugars (cellobiose, maltose), and sugar alcohols such as mannitol and sorbitol (Yang et al., 2020). Moreover, this strain can tolerate high initial glucose and

glycerol concentrations, up to 158 $g \cdot L^{-1}$ and 125 $g \cdot L^{-1}$ respectively (Lin et al., 2008; Zacharopoulos et al., 2023). High productivity of SA by *A. succinogenes* requires strict anaerobic conditions, as the acid is primarily produced via the reductive branch of the tricarboxylic acid (TCA) cycle (Escanciano et al., 2024). The process also depends on an external CO_2 supply, which can be provided as a gas or in salt form, such as magnesium carbonate ($MgCO_3$).

These metabolic characteristics position *A. succinogenes* as a promising organism for lignocellulosic biomass–based fermentation processes, representing a key step toward lowering the production cost of bio-based SA by up to 50% (Lu et al., 2021). Lignocellulosic feedstocks are abundantly available, estimated at around 2×10^{11} tons per year, and largely derived from agricultural residues of crops like rice, corn, and wheat (Kumar et al., 2020; Vivek et al., 2017). In Europe alone, wheat by-products generate approximately 144 million tons annually (Gillman, 2017), with wheat straw being one of the most prominent. This feedstock is rich in fermentable carbohydrates: cellulose (34–40% dry weight), hemicellulose (21–26%), and lignin (11–23%) (Liu et al., 2015). However, microorganisms cannot directly metabolize the polymeric carbohydrates in raw biomass. To release fermentable sugars (glucose, xylose, sucrose), the lignocellulosic matrix must undergo pretreatment to disrupt the cellulose–hemicellulose–lignin structure. This is followed by enzymatic hydrolysis of glucans and xylans into monomeric sugars. Pretreatment also serves to remove lignin, which can inhibit microbial activity (Beig et al., 2021).

Lignocellulose pretreatment methods often involve harsh conditions, such as high temperatures and pressure, and the employment of strong acids or bases, which influence sugar yields and lead to the formation of inhibitory compounds (Chen et al., 2017). Among the most common inhibitors are furans, such as 5-hydroxymethylfurfural (5-HMF) and furfural, generated from the dehydration of pentoses and hexoses during hemicellulose hydrolysis. These compounds are toxic because they induce reactive oxygen species (ROS) formation (like H_2O_2), disrupt mitochondrial function, and cause cytotoxicity (Batool et al., 2021). Notably, the combination of 5-HMF and furfural at 3 $g \cdot L^{-1}$ can completely inhibit the growth of *A. succinogenes* (Dessie et al., 2019).

Phenolic compounds, such as benzoic acid, syringic acid, and vanillin, arise from lignin degradation. Xu et al. (2019) reported that cinnamic acid and benzoic acid exhibit strong inhibitory effects on *A. succinogenes* even at low concentrations (1.77 $g \cdot L^{-1}$ and 1.46 $g \cdot L^{-1}$, respectively). These compounds interfere with glucose uptake, affect heat shock protein expression, and impair chemotaxis. Another common inhibitor released during pretreatment is acetic acid (AA), derived from the hydrolysis of acetyl groups in hemicellulose. AA is also a metabolic by-product of SA fermentation, along with other weak acids such as lactic acid and formic acid (FA). These organic acids disturb cellular metabolism by altering the redox balance and cofactor availability ($NADH/NAD^+$). Despite this, *A. succinogenes* is relatively tolerant to AA, remaining active at concentrations up to 33.7 $g \cdot L^{-1}$ (Lin et al., 2008; Zacharopoulos et al., 2024). However, its toxicity can increase synergistically when AA is combined with other inhibitors like furfural and phenolic compounds (Xu et al., 2019).

A major challenge in employing this lignocellulosic hydrolysate as a carbon source is the presence of potentially toxic inhibitory compounds, such as AA, furfural, and 5-HMF, which are generated during biomass pretreatment. According to Sulzenbacher et al. (2023), even when optimized steam explosion conditions were applied, such as milder residence times and temperatures that improve sugar yield the formation of these inhibitors remained unavoidable. In their study, inhibitors concentrations reached 3.0 – 3.2 $g \cdot L^{-1}$ for AA, 0.1 – 0.15 $g \cdot L^{-1}$ for furfural, and approximately 0.02 $g \cdot L^{-1}$ for 5-HMF. To mitigate the inhibitory effects, Casella et al. (2024) employed a diluted wheat straw hydrolysate without the addition of external nitrogen or carbon sources. Starting from an initial glucose concentration of 1.1 $g \cdot L^{-1}$ and xylose concentration of 0.4 $g \cdot L^{-1}$, they achieved a SA titer of 0.09 $g \cdot L^{-1}$, with a yield of 8.2% ($g_{SA}/g_{glucose}$).

Rich in amino acids, vitamins, nucleosides, polypeptides, and minerals, yeast extract serves as an excellent nutrient medium for microbial fermentation in both laboratory and industrial settings, particularly for auxotrophic strains (Huynh et al., 2020).

In SA production by *A. succinogenes*, a crucial factor is the availability of CO_2. Due to the limited solubility of gaseous CO_2 at atmospheric pressure (1 atm), various carbonate and bicarbonate salts have been used as indirect CO_2 sources to increase their concentration in the fermentation broth (Zou et al., 2011). Magnesium carbonate ($MgCO_3$) was considered a

favorable carbon source for several reasons. It played a key role in buffering the pH of the fermentation medium and supplied Mg^{2+} ions, which are essential for the activity of phosphoenolpyruvate carboxykinase, an important enzyme involved in SA biosynthesis (Lee et al., 1999).

In this study, we identified the conditions for optimal succinic acid (SA) production using *Actinobacillus succinogenes* and a hydrolyzed wheat straw feedstock obtained through steam-explosion pretreatment. A series of experiments, with different hydrolysate dilution levels, and initial concentrations of yeast extract and $MgCO_3$ were conducted to optimize the composition of the culture medium to obtain a higher SA production. The results of these experiments were used to fit two Gaussian Procession surrogate models, which then were utilized to identify the best conditions for maximizing SA production and yield by *A. succinogenes* using hydrolysate derived from lignocellulosic biomass, in this case, wheat straw.

6.2. Material and methods

6.2.1. Microorganism, pre-culture medium and inoculum

Actinobacillus succinogenes strain 130Z (CCUG-43843) was supplied by the Culture Collection University of Gothenburg (CCUG, Gothenburg, Sweden) as freeze-dried pellets and is kept cryopreserved in 20% glycerol solution at -80° C. To revive the strain, it is initially transferred from the cryopreservation vial to a 37 g·L-1 Brain Heart Infusion (BHI) solution and incubated for 24 hours at 37 °C. Afterwards, the revived *A. succinogenes* was transferred to TSB (Tryptic Soy Broth) growth medium, containing 5.0 $g \cdot L^{-1}$ of D-Glucose, 17.0 $g \cdot L^{-1}$ of peptone from casein, 3.0 $g \cdot L^{-1}$ of peptone from soybean, 5.0 $g \cdot L^{-1}$ of sodium chloride (NaCl), and 2.5 $g \cdot L^{-1}$ of dibasic potassium phosphate (K_2HPO_4). Furthermore, to improve the growth of the strain, D-(+)-Glucose at a concentration of 15 $g \cdot L^{-1}$ was added to the growth medium. The inoculum was left to incubate for 24 hours at 37°C to reach the exponential phase of growth. The preculture and the inoculum media were both sparged with nitrogen for 15 minutes to secure the anaerobic conditions, and they were sterilized in autoclave, for 15 minutes at 121 °C.

6.2.2. Hydrolyzed wheat straw

The hydrolyzed wheat straw was provided by the ENEA Research Centre of Trisaia (Rotondella, Matera, Italy). The raw material was treated as follows: the wheat straw was milled before to reduce the particle size at 50 mesh, then the biomass was treated following a catalyzed acid steam explosion following the optimized operational conditions to guarantee a high hemicellulose recovery and a high hydrolysis of cellulose. A blanking machine was used to reduce the raw material in particles of size between 1.7 and 5.5 millimeters. Then, 1 kg of biomass was immersed for 10 minutes in a solution of 30 L of 0.5 M sulfuric acid (H_2SO_4), and after, to eliminate the liquid in excess, it was transferred to the filter press. After these treatments, the dry matter of wet biomass was determined around 31.2% (w/w). Subsequently the steam explosion was carried out in a batch reactor with a volume of 10 L, at 203 °C for 5 minutes (Caporusso et al., 2023). The slurry derived from this process was subjected to hydrolysis, using an enzymatic blend Cellic Ctec2® (Novozymes A/S, Denmark) adding 15 FPU per gram of cellulose. The hydrolysis was conducted under these parameters: solid-liquid ratio of 5% (w/v), at 50 °C and controlled pH of 4.8 (obtained after the addition of NaOH to hemicellulose from acid pretreatment), for 72 hours. The temperature and agitation were maintained constant through a shake plate incubator. After this, the hydrolysate was filtered and sterilized. To prevent inhibition phenomena from compounds like furfural and AA, the hydrolysate was diluted 1/5 (v/v) with ultrapure sterile water. The final composition was reported in Table 6.1.

Table 6.1 *Diluted hydrolyzed wheat straw composition. AA=Acetic acid; 5-HMF=5-hydroxymethylfurfural.*

	Concentration ($g \cdot L^{-1}$)				
	D-Glucose	D-(+)-Xylose	AA	Furfural	5-HMF
WSH 1:5	23.07	4.05	0.65	0.11	0.035

6.2.3. Experimental set-up

Fermentation tests were performed in batch conditions in sealed anaerobic bottles at a working volume of 100 ml with an inoculum of *A. succinogenes* of 10% (v/v). The bottles

with fermentation media were sparged with CO_2 for 15 minutes and then were sterilized in autoclave for 15 minutes at 121 °C. The tests were conducted at 37 °C and 120 rpm in a stirring incubator. A standard growth medium (TSB) supplemented with glucose at a titer of 23 $g \cdot L^{-1}$ was used as control (C). In all tests K_2HPO_4, NaCl, $CaCl_2$ and $MgCl_2$ were added at fixed concentration (3 $g \cdot L^{-1}$, 1 $g \cdot L^{-1}$, 0.2 $g \cdot L^{-1}$ and 0.2 $g \cdot L^{-1}$ respectively).

To evaluate the effects of the addition of nitrogen and carbon source, full factorial experimental design (2^3) with a center point was utilized, assessing the effects of different combinations of diluted hydrolysate, yeast extract and magnesium carbonate, in the fermentation of SA (Masetto et al., 2001). Each condition was conducted in biological triplicate to ensure reproducibility. The experimental design was prepared using R-studio (R v. 4.5.1) as reported in Table 6.2. There were tested 2 different dilutions of hydrolyzed wheat straw (50 and 90% v/v), combined with low and high concentrations of yeast extract (10 and 25 $g \cdot L^{-1}$) and magnesium carbonate (30 and 60 $g \cdot L^{-1}$).

Table 6.2 *Concentration of D-glucose, D-(+)-Xylose, yeast extract (YE), carbonate magnesium ($MgCO_3$), AA (AA) and Furfural (Fur) in starting fermentation media. LH = low concentration hydrolysate (50% v/v); MH = medium concentration hydrolysate (70% v/v); HH = high concentration hydrolysate (90% v/v); T1 = low concentration of yeast extract and $MgCO_3$; T2 = low concentration of yeast extract and high concentration of $MgCO_3$; T3 = high concentration of yeast extract and low concentration of $MgCO_3$; T4 = high concentration of yeast extract and $MgCO_3$.*

Test	**Concentration ($g \cdot L^{-1}$)**					
	D-Glucose	D-(+)-Xylose	YE	$MgCO_3$	AA	Fur
CTRL	23	0	10	30	0	0
LH-T1	11.5	2	10	30	0.3	0.1
LH-T2	11.5	2	10	60	0.3	0.1
LH-T3	11.5	2	25	30	0.3	0.1
LH-T4	11.5	2	25	60	0.3	0.1
MH	16.1	2.8	15	45	0.5	0.1
HH-T1	20.8	3.6	10	30	0.6	0.1
HH-T2	20.8	3.6	10	60	0.6	0.1
HH-T3	20.8	3.6	25	30	0.6	0.1
HH-T4	20.8	3.6	25	60	0.6	0.1

6.2.4. Analytical methods

Bacterial growth was monitored in all the samples by measuring the optical density (OD) at a wavelength of 660 nm (OD_{660}) with the use of a spectrophotometer Shimadzu UV-1280 UV-Vis. Then the samples were centrifuged for 15 minutes at 12000 rpm, and the supernatant was collected, filtered by using a nylon syringe filter, pore size 0.20 μm and stored at -20 °C for the following analysis of sugars (glucose and xylose) and weak acids (SA, LA, FA and AA). To quantify the concentration of these compounds a High-Performance Liquid Chromatography (HPLC) was carried out using a Shimadzu Nexera XR HPLC equipped with a Bio-Rad Aminex HPX-87H Ion Exclusion Column. The eluent used for the analysis was a sulfuric acid solution (H_2SO_4, 12 mM), with a flow of 0.6 $mL \cdot min^{-1}$ and a temperature of 63 ˚C. The samples and the analytical standards of glucose, xylose, SA, LA, FA and AA were prepared diluted with ultrapure water and injected at volume of 20 μL.

6.2.5. Mathematical methods and statistical analysis

The μ_{max} was a specific growth rate of bacterial cells, and in the case of batch cultivation was calculated as the difference between the logarithmic optic density (660 nm) of initial (Log OD_{T0}) and peak points (Log OD_{T24}) of the exponential growth phase divided by the duration time of this phase (1).

$$\mu_{max} = \frac{(LOG\ OD_{T24} - LOG\ OD_{TO})}{24 - 8} \qquad (1)$$

The sugars consumption (S_{cons}) was calculated between the starting (S_0) and final (S_f), after 28 hours of fermentation, as percentage (2).

$$S_{cons} = \frac{(S_0 - S_F)}{S_0} * 100 \qquad (2)$$

The yield of SA (SA_y) was calculated as the ratio between the final concentration of SA (SA_f) and the initial concentration of sugars (S_0) (3).

$$SA_y = \frac{SA_f}{S_0} \qquad (3)$$

SA productivity (SA_{prod}) was calculated as reported (4).

$$SA_{prod} = \frac{SA_f}{T_{28h}} \qquad (4)$$

The selectivity (SA_{sel})was calculated as the ratio between the final succinic acid concentration and the final amount of succinic (SA_f), formic (FA_f) and acetic acids (AA_f) (5).

$$SA_{sel} = \frac{SA_f}{SA_f + FA_f + AA_f} \qquad (5)$$

All tests were carried out in triplicate. The media values and the standard deviation have been calculated. A One-Way ANOVA (Analysis of Variance) was performed using software IBM SPSS Statistic 20 (International Business Machine Corporation, New York, USA) for a comparison among the tests. A Tukey post-hoc test at the 0.05 level of significance was applied.

6.2.6. Hydrolysate concentration and additives optimization

As described in the previous paragraph, batch fermentations based on a factorial experimental design were conducted. For each fermentation run, the succinate yield (grams of succinate per gram of sugar consumed) and the fraction of SA in the total product mixture (SA purity) were measured and used as the response variables for the construction of surrogate models. These surrogate models of the experimental responses were constructed using Response Surface Methodology (RSM).

Response Surface Methodology (RSM) is a parametric statistical modeling technique used to simulate and optimize processes influenced by multiple input variables (Montgomery, 2017). In this study, RSM was applied to model and optimize fermentation conditions for maximizing succinate yield and SA selectivity over AA. RSM constructs second-order polynomial regression models to capture the relationships between process inputs and the desired outputs (Myers et al., 2016). Here we considered three input parameters: Hydrolysate dilution, magnesium carbonate and yeast extract concentration.

The RSM models for the two optimization parameters, were Implemented in Python (v. 3.15.5) using the scikit-learn library. The model inputs were the experimental data gathered

by the experimental design presented in Table 2. The RSM models were developed as second-order polynomial regression models. The general form of such a model is given in eq. 6.

$$Y = \beta_0 + \sum_{i=0}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j}^{k} \beta_{ij} X_j + \varepsilon \qquad (6)$$

where Y represents the predicted response, X_i are the coded input variables, β are the regression coefficients, and ε is the residual error term.

Polynomial feature expansion was performed using the *PolynomialFeatures* class from *sklearn.preprocessing*, which generated interaction and quadratic terms up to the second order. These transformed predictors were then fitted using ordinary least squares (OLS) regression via *LinearRegression* from *sklearn.linear_model*.

The model performance was assessed using the coefficient of determination R^2. Moreover, the calculated continuous response surfaces were plotted together with experimental validation points.

The two response surfaces were used as surrogate models for multi-objective optimization of the substrate. The optimization was carried out using the Non-dominated Sorting Genetic Algorithm II (NSGA-II). NSGA-II is a population-based multi-objective evolutionary algorithm that sorts candidate solutions into Pareto fronts (Deb et al., 2002). For the optimization in Python the *pymoo* package was used, with a population size of 200 and 200 generations. Finally, a selected set of Pareto-optimal conditions was validated experimentally.

6.3. Results and discussion

6.3.1. Strain growth

The main focus of this work was to optimize the initial concentration of two key compounds for the growth of *A. succinogenes*: the yeast extract and the magnesium carbonate ($MgCO_3$). In this study, various combinations of these compounds were tested at both low (10 $g \cdot L^{-1}$ yeast extract and 30 $g \cdot L^{-1}$ $MgCO_3$) and high concentrations (25 $g \cdot L^{-1}$ yeast extract and 60

$g \cdot L^{-1}$ $MgCO_3$) to evaluate their impact on strain growth and, consequently, on SA production.

Table 6.3 reports the growth data of *Actinobacillus succinogenes*. In terms of OD_{660}, the best condition in the 50% (v/v) diluted hydrolysate was achieved with the addition of 10 $g \cdot L^{-1}$ yeast extract and 30 $g \cdot L^{-1}$ magnesium carbonate (LH-T1), reaching an OD_{660} of 11.733 (± 0.012) after 24 hours of fermentation.

Similarly, in the 90% (v/v) diluted hydrolysate, the highest OD_{660} value (16.247 ± 0.025) was observed under the same formulation with lower concentrations of the two additives (HH-T1).

When magnesium carbonate was added at a concentration of 60 $g \cdot L^{-1}$, no improvements were observed. In fact, the lowest OD_{660} values were recorded when it was combined with 10 $g \cdot L^{-1}$ of yeast extract, in both the 50% and 90% (v/v) diluted hydrolysates (LH-T2 and HH-T2), reaching 4.033 ± 0.107 and 5.713 ± 0.550, respectively.

Table 6.3 *Growth data of Actinobacillus succinogenes reported in OD_{660} value, at 3 different sampling times (0, 8 and 24 hours), with standard deviation for each value. The μ_{max} (h^{-1}) was reported for the conditions tested. Different letters within each column indicate significant differences according to Tukey post-hoc test (p =0.05). LH = low concentration hydrolysate (50% v/v); MH = medium concentration hydrolysate (70% v/v); HH = high concentration hydrolysate (90% v/v); T1 = low concentration of yeast extract and $MgCO_3$; T2 = low concentration of yeast extract and high concentration of $MgCO_3$; T3 = high concentration of yeast extract and low concentration of $MgCO_3$; T4 = high concentration of yeast extract and $MgCO_3$.*

Test	OD 660 nm			μ_{max} (h^{-1})
	Time (h)			
	0	8	24	
CTRL	0.213 ± 0.013	0.133 ± 0.042	11.427 ± 0.030	1.11 b
LH-T1	0.373 ± 0.012	0.423 ± 0.042	11.733 ± 0.012	1.09 b
LH-T2	0.387 ± 0.035	0.637 ± 0.040	4.033 ± 0.107	0.62 f
LH-T3	0.397 ± 0.015	2.370 ± 0.200	5.150 ± 0.200	0.69 e
LH-T4	0.510 ± 0.036	2.337 ± 0.136	4.103 ± 0.136	0.59 f
MH	0.873 ± 0.189	1.423 ± 0.071	4.737 ± 0.379	0.67 e
HH-T1	0.633 ± 0.015	0.863 ± 0.032	16.247 ± 0.025	1.21 a

HH-T2	0.723 ± 0.237	1.323 ± 0.105	5.713 ± 0.550	0.75 d
HH-T3	0.847 ± 0.042	1.280 ± 0.030	7.447 ± 0.326	0.87 c
HH-T4	0.850 ± 0.050	1.720 ± 0.157	6.343 ± 0.151	0.79 d

The addition of yeast extract at a concentration of 25 $g \cdot L^{-1}$ did not lead to any improvement in the growth of the strain in either hydrolysate dilution. In both cases, whether combined with low or high concentrations of magnesium carbonate, a reduction in OD_{660} values was observed after 24 hours of fermentation, compared to the best-performing condition, with decreases ranging from approximately 55% to 60%.

To gain deeper insight into the strain's growth behavior, the maximum specific growth rate (μ_{max}) was also calculated. This is a key parameter in microbial growth kinetics, representing the highest possible growth rate under optimal conditions. It indicates how fast a microbial population can grow when no limiting factors are present.

The results obtained from the μ_{max} calculations reflect the trend previously observed in OD_{660} growth values, with the HH-T1 test condition showing a statistically significant improvement compared to all other conditions (1.21 h^{-1}). This outcome is influenced by the higher initial concentrations of glucose and xylose in the broth (20.8 and 3.6 $g \cdot L^{-1}$, respectively) compared to the 50% diluted hydrolysate (11.5 and 2 $g \cdot L^{-1}$ of glucose and xylose, respectively).

However, despite the lower initial sugar content, the LH-T2 condition ranked as the second-best statistically in terms of μ_{max}, reaching 1.09 h^{-1}. In tests with higher concentration of yeast extract and magnesium carbonate, both with hydrolysate diluted at 50% and 90% (v/v), there were not seen improvement in strain growth. These results could be explained by the negative effects caused by excessive concentrations of these compounds on the metabolism of microorganisms. In fact, Tachibana et al. (2019) observed that certain amino acids, such as methionine, can negatively affect microbial growth. Therefore, if the yeast extract contains an overabundance of amino acids with potential regulatory or inhibitory effects on gene expression or metabolism, this may alter the fermentation behavior. Similarly, high concentrations of magnesium carbonate could also inhibit microbial growth. Although

magnesium ions normally act as beneficial cofactors, their excess can induce osmotic stress, leading to growth inhibition (Qu et al., 2022).

6.3.2. Sugars consumption

In hydrolysates derived from lignocellulosic biomass, glucose and xylose are the most abundant sugars following pretreatment. *Actinobacillus succinogenes* can use both sugars to produce SA and is well known for its ability to metabolize a wide range of carbon sources.

In a study by Salma et al. (2021), fructose was used as a carbon source and was consumed by the strain at 99.7%, starting from an initial concentration of 9.01 $g \cdot L^{-1}$. Under the same conditions, a glucose-only medium was also tested, showing a 91.9% consumption of this sugar.

However, glucose appeared to be the preferred carbon source for *A. succinogenes*. Indeed, Casella et al. (2024) observed that, in a medium containing both glucose and xylose, xylose was poorly consumed (6.1%) compared to glucose (51%) when their initial concentrations were 0.4 $g \cdot L^{-1}$ and 1.1 $g \cdot L^{-1}$, respectively. Yet, Lee et al. (2022) achieved complete consumption of glucose (50 $g \cdot L^{-1}$) and xylose (8.92 $g \cdot L^{-1}$) using a hydrolysate obtained from Napier grass in batch fermentation.

In the present work, two dilutions of wheat straw-derived hydrolysate were used (50% and 90% v/v). The initial concentrations of glucose and xylose were 11.5 $g \cdot L^{-1}$ and 2 $g \cdot L^{-1}$ in the more diluted hydrolysate, and 23 $g \cdot L^{-1}$ and 4 $g \cdot L^{-1}$ in the more concentrated one.

After 28 hours of fermentation, both glucose and xylose were consumed by the strain, with no apparent preference for either sugar. In fact, the total sugar concentration at the end of the process was reduced to zero in tests LH-T2 and HH-T3, and to a maximum of 0.77 $g \cdot L^{-1}$ in test HH-T3. The overall sugar consumption ranged from 96.4% in the latter test up to 100% (Figure 6.1a). Therefore, under all tested conditions, *A. succinogenes* was able to completely utilize the carbon sources present in the wheat straw hydrolysate.

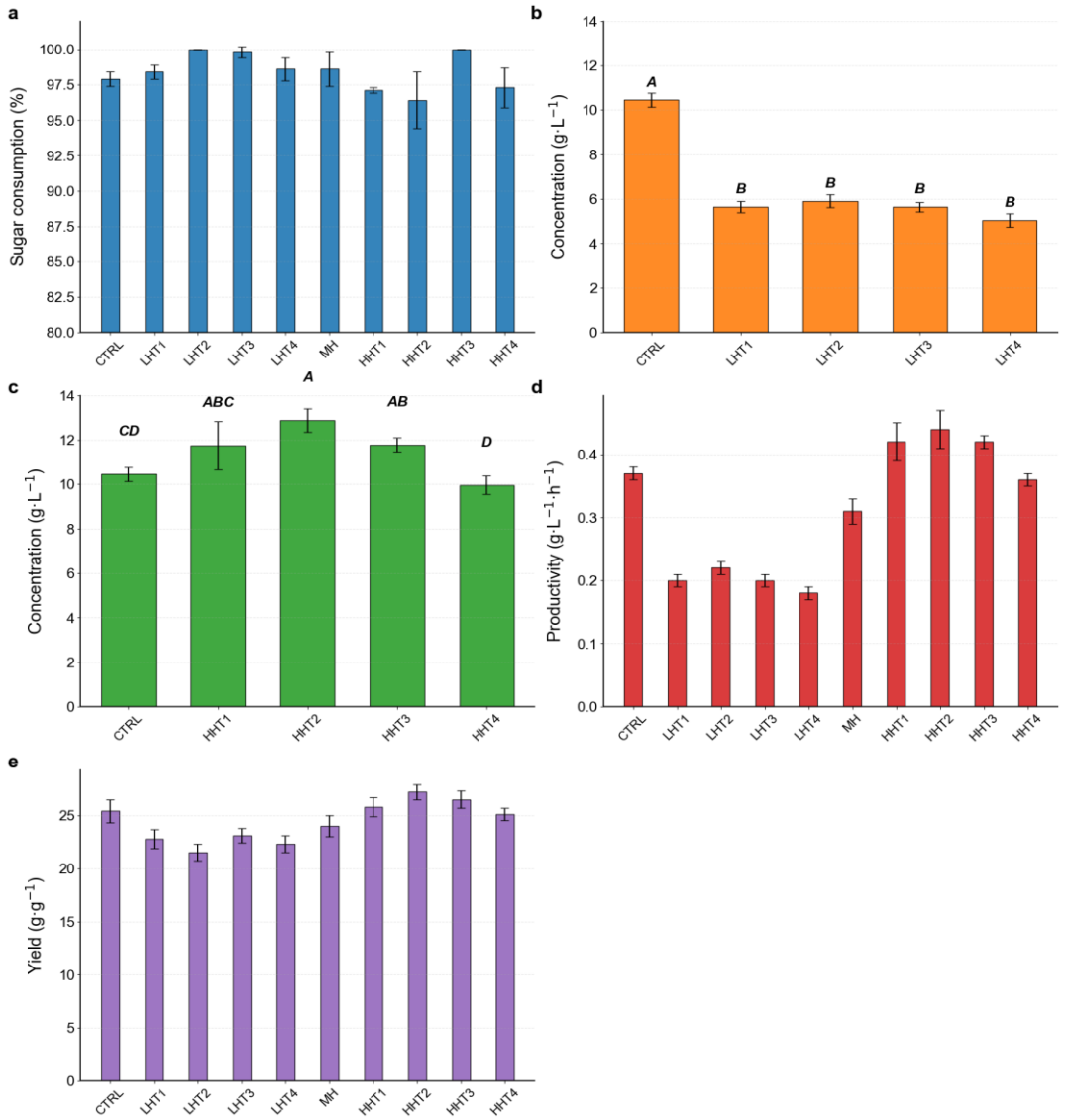

Figure 6.1 *(**a**) Sugars consumption (%) in all conditions tested after 28 hours of fermentation. (**b** and **c**) SA concentration after 28 hours of fermentation (g·L^{-1}). Different letters within each column indicate significant differences according to Tukey post-hoc test (p = 0.05). (**d**) Productivity of SA in the different tested conditions (g·L^{-1}·h^{-1}) (**e**) SA yield over the total sugars (%).*

6.3.3. Succinic acid production performance and by-products formation

The production of SA using hydrolysates derived from lignocellulosic biomass has been the focus of extensive research. This interest stems from the large amount of sugars potentially available for the microorganisms used in the fermentation process. One of the main

challenges lies in optimizing the initial parameters, as several issues arise. Indeed, the lignocellulosic biomass used to produce the hydrolysate and the pretreatment methods applied to it can vary greatly, and these differences are reflected in the type and initial concentrations of sugars.

In a study by Kuglarz et al. (2018), using a hydrolysate derived from rapeseed straw after an H_2SO_4-catalyzed pretreatment, the concentration of xylose (31.8 $g \cdot L^{-1}$) was found to be higher than that of glucose (0.5 $g \cdot L^{-1}$). However, it is more common to observe a higher glucose concentration compared to xylose, as reported by Sawisit et al. (2018), where glucose reached 75.9 $g \cdot L^{-1}$ and xylose 13.6 $g \cdot L^{-1}$ after a NaOH (1 M) pretreatment of rice straw. Similarly, in hydrolysates derived from wheat straw, glucose was predominant over xylose, as reported by Casella et al. (2024).

The main issue associated with the use of this raw material, apart from its variability, was the presence of inhibitory compounds derived from pretreatment processes, such as AA and furan derivatives. In fact, Casella et al. (2024) reported an inhibition of *A. succinogenes* growth of around 60%, even when the hydrolysate was highly diluted (2% v/v), due to the presence of these compounds. However, in that study, no nitrogen source (such as yeast extract) or magnesium was added to improve the growth conditions for the strain.

Building upon these findings, the present work aimed to enhance the growth of *A. succinogenes* and the subsequent production of SA by testing the addition of yeast extract and $MgCO_3$. Different combinations of these compounds were evaluated to optimize their initial concentrations and, consequently, the fermentation performance.

In Figures 6.1b and 6.1c were reported the concentrations of SA at the end of the fermentation process (28 hours). It was possible to note that when the 50% (v/v) diluted hydrolysate was used no statistical differences were produced by the different combinations of yeast extract and magnesium carbonate. The maximum titer of this organic acid with this diluted hydrolysate was 5.91 (± 0.29) $g \cdot L^{-1}$ obtained when the yeast extract was in concentration of 10 $g \cdot L^{-1}$ and $MgCO_3$ in 60 $g \cdot L^{-1}$. But the other tested conditions were not so different, and the reason behind this result was that the starting concentration of total sugars could be the limiting factor. When a more concentrated hydrolysate (90% v/v) with higher starting concentrations of total sugars (24.4 $g \cdot L^{-1}$ vs 13.5 $g \cdot L^{-1}$) was possible to see

that the maximum concentration of SA was reported in HH-T2 (12.87 ± 0.53 $g \cdot L^{-1}$. In this pool of tests, the worst condition was the one where the yeast extract and magnesium carbonate were added at maximum concentration, 25 $g \cdot L^{-1}$ and 60 $g \cdot L^{-1}$ respectively, with a final titer of SA equal at 9.95 ± 0.41 $g \cdot L^{-1}$.

The yield of SA in this test was calculated based on the total sugar content of the broths (Figure 6.1e). The best performance was obtained using the least diluted hydrolysate, supplemented with 10 $g \cdot L^{-1}$ of yeast extract and 60 $g \cdot L^{-1}$ of magnesium carbonate, achieving a yield of 62.08%. A similar result (61.77%) was observed under the same hydrolysate concentration when combined with 25 $g \cdot L^{-1}$ of yeast extract and 30 $g \cdot L^{-1}$ of $MgCO_3$. Overall, when analyzing this parameter, the tests conducted with the hydrolysate diluted to 50% (v/v) showed lower yields compared to those using the more concentrated hydrolysate across all tested conditions.

A trend similar to that observed for yield was also found for SA productivity, measured after 28 hours of fermentation (Figure 6.1d). The highest productivity values were obtained in the tests using the 90% (v/v) diluted hydrolysate. Among all conditions, the best result was achieved with HH-T2, reaching 0.44 $g \cdot L^{-1} \cdot h^{-1}$. In contrast, when the 50% (v/v) diluted hydrolysate was used, the productivity was lower than that observed with the 90% (v/v) dilution. The lowest productivity (0.18 $g \cdot L^{-1} \cdot h^{-1}$) was obtained under the condition combining 25 g/l of yeast extract and 60 g/l of magnesium carbonate.

In Table 6.4, several studies on the use of hydrolysates derived from different lignocellulosic biomasses are reported. As shown, a wide range of biomass types can be employed; however, the crucial factor is the sugar content potentially available for fermentation, the availability of nitrogen and carbon sources. The nature of the raw material and the pretreatment method used to convert carbohydrates into fermentable sugars, such as glucose and xylose, can strongly influence process performance and, consequently, SA production.

Table 6.4 *Hydrolysate derived from lignocellulosic biomass used in SA production. There were reported starting concentrations of total sugars, yeast extract (YE) and carbon sources. SA final concentration and yield were used as parameters to evaluate the performance of the fermentation. (NR = not reported)*

Raw material	Starting concentration ($g \cdot L^{-1}$)			SA final concentration ($g \cdot L^{-1}$)	SA yield (%)	Reference
	Total sugars	YE	Carbon source			
Wheat straw	24.4	10	60 ($MgCO_3$)	12.87	62.1	This work
	0.6	0	0	0.01	2.7	Casella et al. (2024)
	1.5	0	0	0.09	8.2	
	NR	0	0	2.00	NR	Li et al. (2010)
Oil palm trunks	22.6	15	40 ($MgCO_3$)	10.62	47.0	Bukhari et al. (2020)
Rice husks	22.0	8.4	1.4 (Na_2CO_3)	12.50	59.9	Bevilacqua et al. (2015)
Sweet sorghum bagasse	29.2	10	2.5 (Na_2CO_3)	17.80	61.0	Lo et al. (2020)
Corn fiber	46.2	NR	NR	26.30	53.0	Yoo et al. (2016)
Rice straw	NR	15	NR	3.64	18.0	Putri et al. (2023)
Sugarcane bagasse	NR	10	NR	5.10	25.0	

Indeed, although the initial sugar concentrations reported by Lo et al. (2020) and Yoo et al. (2016) were higher than those in the present work, their SA yields were lower (61.0% and 53.0% vs. 62.1%, respectively). Similarly, in the studies of Bukhari et al. (2020) and

Bevilacqua et al. (2015), where hydrolysates derived from oil palm trunks and rice husks were used, the initial total sugar concentrations were comparable to those of this work (22.6 and 22.0 vs. 24.4 $g \cdot L^{-1}$), but the SA concentrations and yields were lower (10.62 $g \cdot L^{-1}$ - 47.0% and 12.50 $g \cdot L^{-1}$ - 59.0%, respectively). These findings highlight that, for each biomass type, process optimization is required to achieve efficient fermentation.

A key step in this optimization involves the addition of yeast extract and magnesium carbonate at appropriate concentrations. These two compounds are essential for microbial activity, particularly for *Actinobacillus succinogenes*, as yeast extract provides a nitrogen source that supports bacterial growth and metabolism (Lee et al., 1999). The importance of this compound and the optimization of its concentration were further investigated by Putri et al. (2023), who tested three yeast extract levels (5, 10, and 15 $g \cdot L^{-1}$) using two different feedstocks: rice straw and sugarcane bagasse. They observed that yeast extract addition enhanced SA production, but the optimal concentration differed between the two hydrolysates. Specifically, for rice straw, the highest production and yield (3.64 $g \cdot L^{-1}$ and 18.0%) were achieved with 15 $g \cdot L^{-1}$ yeast extract, whereas for sugarcane bagasse, the best results were obtained at 10 $g \cdot L^{-1}$. Higher yeast extract concentrations did not further improve performance in the latter case (Putri et al., 2023). A similar trend was observed with magnesium carbonate ($MgCO_3$), a compound that plays a major role in controlling the pH of the fermentation broth and serves as a CO_2 donor. $MgCO_3$ is often preferred over other carbon sources, such as sodium carbonate (Na_2CO_3), as it provides magnesium ions necessary for phosphoenolpyruvate carboxykinase, a key enzyme in SA biosynthesis (Zhang et al., 2012). Zou et al. (2011) tested various $MgCO_3$ concentrations (30, 40, 50, and 60 $g \cdot L^{-1}$) in *A. succinogenes* cultures and found that, although SA production remained stable above 40 $g \cdot L^{-1}$, productivity decreased. To improve SA production from biomass, it is also necessary to optimize the concentrations of compounds such as yeast extract and magnesium carbonate in the initial broth. This optimization helps to avoid the use of excessive amounts, thereby reducing production costs. Yeast extract, in particular, is one of the most expensive supplements (Jiang et al., 2010); therefore, adding it without any corresponding improvement in SA yield is economically and environmentally undesirable.

In the present work, when hydrolyzed wheat straw diluted to 50% (v/v) was used, no statistically significant differences were observed among the tested conditions regarding SA production. This suggests that yeast extract and magnesium carbonate concentrations did not markedly influence fermentation performance, likely because the low initial sugar concentration acted as a limiting factor. Conversely, when using more concentrated hydrolysates, noticeable differences were observed. The best results in terms of SA concentration and yield were obtained when yeast extract and magnesium carbonate were added at 10 $g \cdot L^{-1}$ and 60 $g \cdot L^{-1}$, respectively. This finding expands on the results of Zou et al. (2011), showing that under the tested conditions, adding $MgCO_3$ above the previously reported 40 $g \cdot L^{-1}$ threshold improved SA production and overall fermentation performance.

On the other hand, it was seen that increasing yeast extract concentration beyond 10 $g \cdot L^{-1}$ did not further enhance the process when hydrolyzed wheat straw was used. Across all tested conditions, the proportion of SA in the final product pool ranged from 37.0% to 43.1%. This performance could be further improved when the process is conducted in a controlled fermenter, where parameters such as pH and CO_2 supply can be precisely regulated, unlike in batch systems. Indeed, carbon dioxide availability is a critical parameter in fermentations involving *A. succinogenes*, as an insufficient CO_2 supply can shift microbial metabolism toward by-product formation, such as acetic and formic acids (Herselman et al., 2017).

The results obtained in this work showed the importance of adding yeast extract and magnesium carbonate to the hydrolyzed wheat straw to improve the performance of fermentation. In fact, the concentration of SA detected was higher than what was seen in the paper of Li et al. (2010) and Casella et al. (2024) where the same raw material was used. Indeed, also if in these works a hydrolysate derived from wheat straw was used, yeast extract and magnesium carbonate were not added in the starting fermentation broth. Therefore, the addition of these two compounds can help the strain to overcome the problem derived from the presence of inhibitor compounds, like furan derivates and AA, even if in low concentrations, that were commonly found in hydrolysate derived from pretreated wheat straw, creating an optimal condition for the growth of the microorganism and consequently for SA production.

6.3.4. RSM model and substrate optimization

Following the methodology described in the materials and methods section a response surface model was created. The inputs of the model were the dilution of the hydrolysate and the concentrations of magnesium carbonate and yeast extract. The outputs of the model were the SA acid yield and selectivity. These outputs were selected as they can be later used for the optimization of the SA fermentation. Despite the small amount of experiments the fitting of the model was good, achieving values of $R^2 = 0.991$ for SA yield and $R^2 = 0.972$ for SA selectivity. The solutions of the models were then plotted in triangular plots, with a validation experimental point superimposed on top of them (Figure 6.2).

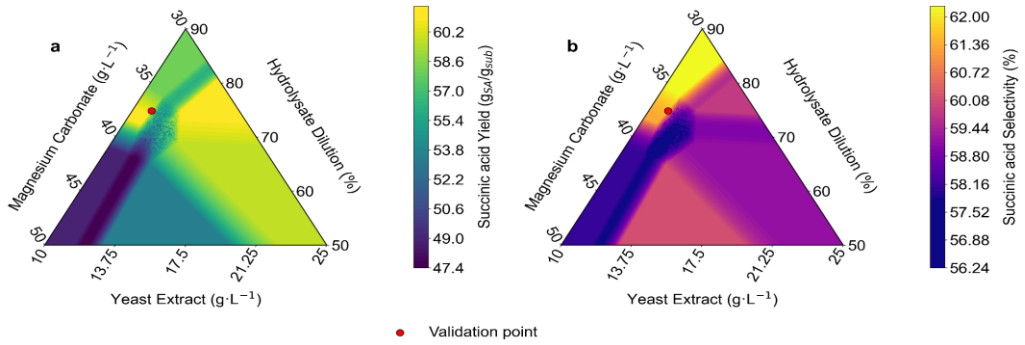

Figure 6.2 *Ternary plots visualizing the results of the RSM models a. SA yield (gL-1) b. SA selectivity (%). The red dot is the validation experiment.*

The validation point acted as the validation experiment for the optimization, as the values for hydrolysate dilution, magnesium carbonate and yeast extract concentrations, were calculated using the optimization algorithm and are presented in Table 6.6.

Table 6.6 *Concentration of D-glucose, D-(+)-Xylose, yeast extract (YE), carbonate magnesium ($MgCO_3$), AA (AA) and Furfural (Fur) and values for SA and selectivity in validation experiment.*

Test	Concentration (g·L^{-1})							
	D-glucose	D-(+)-Xylose	YE	$MgCO_3$	AA	Fur	Yield	Selectivity
HH-T2	20.8	3.6	10	45	0.6	0.1	59.39	61.51

The goal of the optimization is to obtain the substrate combination, which will maximize both the SA yield and selectivity at the same time. For this reason, the Pareto font was computed (Figure 6.3)

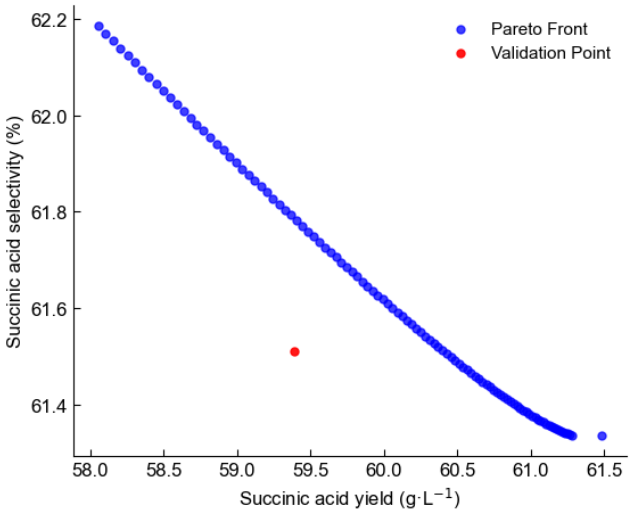

Figure 6.3 *Computed Pareto front (blue dots) and experimental validation results (red dots) for SA yield against SA selectivity.*

From the Pareto font, a clear compromise, with almost linear relationship, between the SA yield and selectivity can be observed. Maximizing one will result in a drastic reduction in the value of the other parameter. The calculated point lies below the calculated Pareto front, signifying that the model overestimates the system performance slightly (less than 2%). Since the experimental point lies in the middle of the yield and the selectivity values, the substrate optimization can be deemed successful as it can equally maximize both values.

The optimization of the fermentation broth was crucial to achieve higher SA concentrations. Indeed, numerous studies in the literature have pursued this objective using different starting substrates, such as xylose mother liquor, glycerol, or sugarcane bagasse (Kanchanasuta et al., 2021; Shen et al., 2022; Jokodola et al., 2022). However, the optimization of SA production from wheat straw hydrolysate has not been extensively explored, mainly due to

the challenging composition of this substrate. The hydrolysate derived from wheat straw contains several inhibitory compounds, including furan derivatives and AA.

In studies where wheat straw hydrolysate was used, the SA yield remained low. For instance, Li et al. (2010) reported a maximum concentration of only 2 $g \cdot L^{-1}$ at the end of fermentation. Similarly, in the recent work of Casella et al. (2024), the use of wheat straw hydrolysate did not result in a high SA concentration even after 48 hours of fermentation. Notably, neither of these studies included the addition of yeast extract or magnesium carbonate. These findings highlight the importance of supplementing the medium with these compounds to enhance fermentation performance.

In the present work, a maximum SA concentration of 12.87 $g \cdot L^{-1}$ was achieved, six times higher than that reported by Li et al. (2010). This result was obtained with 10 $g \cdot L^{-1}$ of yeast extract and 60 $g \cdot L^{-1}$ of magnesium carbonate when using 90% (v/v) hydrolysate. The optimization process was therefore essential to determine the appropriate levels of yeast extract and magnesium carbonate, avoiding excessive use that would unnecessarily increase production costs without improving yield.

6.4. Conclusions

In this study, the fermentation process involving *Actinobacillus succinogenes* using hydrolyzed wheat straw was feedstock, was optimized. The addition of magnesium carbonate and yeast extract to the fermentation medium enhanced both microbial growth and SA production. To determine the optimal initial concentrations of yeast extract and magnesium carbonate, a Response Surface Model (RSM) was developed to analyze the experimental data and validate the obtained results. The optimal conditions identified, corresponding to the addition of 10 $g \cdot L^{-1}$ yeast extract and 60 $g \cdot L^{-1}$ magnesium carbonate to a 90% (v/v) diluted hydrolysate, should be further evaluated under controlled fermentation conditions, such as in a bioreactor, to confirm and potentially enhance the performance of *A. succinogenes*.

7. Acetic acid removal from fermentation broth

7.1. Introduction

One of the most crucial challenges in the production of bio-based succinic acid is to effectively separate this acid (or its salt form, succinate) from a mixture of microbial cells and various compounds, such as water, residual sugars, byproducts (like ethanol, formic, lactic and acetic acids), macromolecules (especially proteins, nucleic acids and polysaccharides) and salts. The removal of all the impurities was fundamental to obtain succinic acid with high purity of approximately 98%, that could be used to produce biopolymers, such as that butylene succinate-based (Alexandri et al., 2019). Mancini et al. (2020) showed that downstream process to obtain marketable bio-succinic acid could cover up to 80% of the total production costs. The separation of succinic acid from a complex mixture, such as the fermentation broth, was challenging because there could be some components that had physiochemical properties similar to the target compound. In fact, to date, to obtain succinic acid with high purity characteristics, the downstream process comprises three major steps:

1) The broth has to be clarified, removing microbial cells, debris and large particles through centrifugation or membrane technologies.
2) A primary recovery process separates byproducts/impurities from the bulk aqueous solution to generate crude products mainly containing succinate. Physiochemical properties of SA and its fermentative impurities play a significant role in this step of downstream process.
3) A concentrated pure SA could be generated after removal of solvents and remaining impurities through successive processes including evaporation, crystallization, drying and so on (Dessie et al., 2023).

The use of expensive membranes or high-energy techniques, like distillation, could increase the production costs of succinic acid. In fact, the removal of some compounds is not easy for their physico-chemical characteristics similar to succinic acid. Among them, the presence of acetic acid, a known by-product of the fermentation of *A. succinogenes*, turns out to be a problem in the recovery and purification phase of succinic acid from the culture broth, as they were chemically similar, and separating them is a complex operation that increases costs. The acetic acid removal from fermentation broth can become a preliminary step for

purification of succinic acid and a part of fermentation processes to remove a product inhibitor (Lee & Hyun, 2010). This organic acid could be present in hydrolysate derived from lignocellulosic biomass and in broth after fermentation process. Indeed, acetic acid is generated from the hydrolysis of the acetyl group during degradation of hemicellulose of the lignocellulosic biomass (Kumar et al., 2020). Furthermore, *Actinobacillus succinogenes* can produce this acid during the fermentation through the pyruvate pathway (Salma et al., 2021)

Cupriavidus necator, also known as *Ralstonia eutropha* or *Alcaligenes eutrophus*, is a Gram-negative β-proteobacteria, capable of autotrophic growth, that can be used to remove acetic acid from fermentation broth. In fact, this microorganism can use volatile fatty acids, like acetic acid, as carbon source, to produce polyhydroxybutyrate (PHB) (Jawed et al., 2022). *C. necator* can intracellularly metabolize various carbon sources, such as glucose and acetic acid, into acetyl-CoA, which under balanced growth conditions is directed into the TCA cycle. Under unbalanced or stress conditions, however, acetyl-CoA is redirected toward the synthesis of polyhydroxyalkanoates (PHAs). Among volatile fatty acids, acetic acid can serve as a direct precursor for PHA formation, as it is readily converted into acetyl-CoA, the central metabolic intermediate that initiates PHA biosynthesis (Sun et al., 2020; Szacherska et al., 2021).

The aim of this work was to reduce the concentration of acetic acid in the fermentation broth through a biological approach, leveraging the ability of *C. necator* to use this acid as a carbon source for PHA production, a potential value-added product within the succinic acid production chain. In this way, multiple benefits could be achieved, including avoiding the use of costly separation methods and simultaneously generating a marketable chemical, such as PHA, thereby reducing the overall costs of biological succinic acid production.

7.2. Material and methods

7.2.1. Bacterial strain and culture media

Cupriavidus necator DSM 545 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). All media used in the experiments were autoclaved at 121 °C for 20 min. The strain was stored in cryovials containing a 20% glycerol

solution at −80 °C. To revive the culture, the strain was transferred from the cryovial to TSB (Tryptic Soy Broth) medium, containing 5.0 $g \cdot L^{-1}$ D-glucose, 17.0 $g \cdot L^{-1}$ casein peptone, 3.0 $g \cdot L^{-1}$ soybean peptone, 5.0 $g \cdot L^{-1}$ sodium chloride (NaCl), and 2.5 $g \cdot L^{-1}$ dibasic potassium phosphate (K_2HPO_4), and incubated for 24 h at 30 °C under aerobic conditions. Following this revitalization step, *C. necator* was inoculated into a sealed aerobic bottle containing the same growth medium and incubated under identical conditions.

7.2.2. Experimental set-up

Fermentation tests were conducted in batch mode using sealed aerobic bottles with a total working volume of 100 mL and a 3% (v/v) inoculum of *Cupriavidus necator*. The bottles containing the fermentation medium were sterilized by autoclaving at 121 °C for 15 minutes prior to inoculation. After the addition of the microorganism, the bottles were incubated at 30 °C and 100 rpm in a shaking incubator for 120 hours. Samples were collected at different defined times (after 0, 6, 24, 30 and 120 hours of fermentation). The media were prepared with fixed concentrations of yeast extract (20 $g \cdot L^{-1}$), salts (NaCl, $CaCl_2$, and $MgCl_2$ at 1.0, 0.2, and 0.2 $g \cdot L^{-1}$, respectively), and K_2HPO_4 (3 $g \cdot L^{-1}$).

To assess the reduction of the acetic acid concentration, the consumption of other acids, such as succinic and formic acid, and their potential inhibitory effects on strain growth, these compounds were added to the broth prior to *C. necator* inoculation at concentrations reported in Table 7.1. D-glucose was included in three tests to evaluate the effect of providing an additional carbon source beyond the organic acids. A control test was prepared with only glucose and without the addition of the acids.

Table 7.1. *Concentration of acetic, succinic and formic acids, and of D-glucose in the broth prior to C. necator inoculation. HA = high concentration acids; MA = mild concentration acids; LA = low concentration acids; NG = without glucose; G = with glucose.*

Samples	Acetic acid	Succinic acid	Formic acid	D-glucose
		$g \cdot L^{-1}$		
CTRL	0	0	0	20
HA-NG	10	13	8	0
HA-G	10	13	8	20
MA-NG	5	6.5	4	0
MA-G	5	6.5	4	20
LA-NG	2.5	3.3	2	0
LA-G	2.5	3.3	2	2

7.2.3. Analytical methods

The strain's growth was monitored in all samples by measuring the optical density (OD) at 600 nm using a Shimadzu UV-1280 UV-Vis spectrophotometer. After measurement, the cultures were centrifuged at 12,000 rpm for 15 minutes, and the resulting supernatant was collected, filtered through a 0.20 μm nylon syringe filter, and stored at -20 °C for subsequent analysis of sugars (glucose) and organic acids (succinic, formic, and acetic acid).

Quantification of these compounds was performed by High-Performance Liquid Chromatography (HPLC) using a Shimadzu Nexera XR system equipped with a Bio-Rad Aminex HPX-87H ion-exclusion column. The mobile phase consisted of a 12 mM sulfuric acid (H_2SO_4) solution, delivered at a flow rate of 0.6 $mL \cdot min^{-1}$ and a column temperature of 63 °C. Samples and analytical standards of glucose, succinic, formic, and acetic acids were diluted in ultrapure water and injected at a volume of 20 μL.

7.2.4. Mathematical methods and statistical analysis

The acids consumption was calculated between the starting (A_0) and final (A_f) concentrations, after 120 hours of fermentation, as percentage for all the three compounds investigated.

$$A_{cons} = \frac{(A_0 - A_F)}{A_0} * 100 \qquad (1)$$

The glucose reduction in the broths was calculated as follows.

$$G_{cons} = \frac{(G_0 - G_F)}{G_0} * 100 \qquad (2)$$

All tests were carried out in triplicate. The media values and the standard deviation have been calculated. A One-Way ANOVA (Analysis of Variance) was performed using software IBM SPSS Statistic 20 (International Business Machine Corporation, New York, USA) for a comparison among the tests. A Tukey post-hoc test at the 0.05 level of significance was applied.

7.3. Results and discussion

The profiles of the growth of *C. necator* with various acids concentration and glucose supplementation are shown in Figure 7.1.

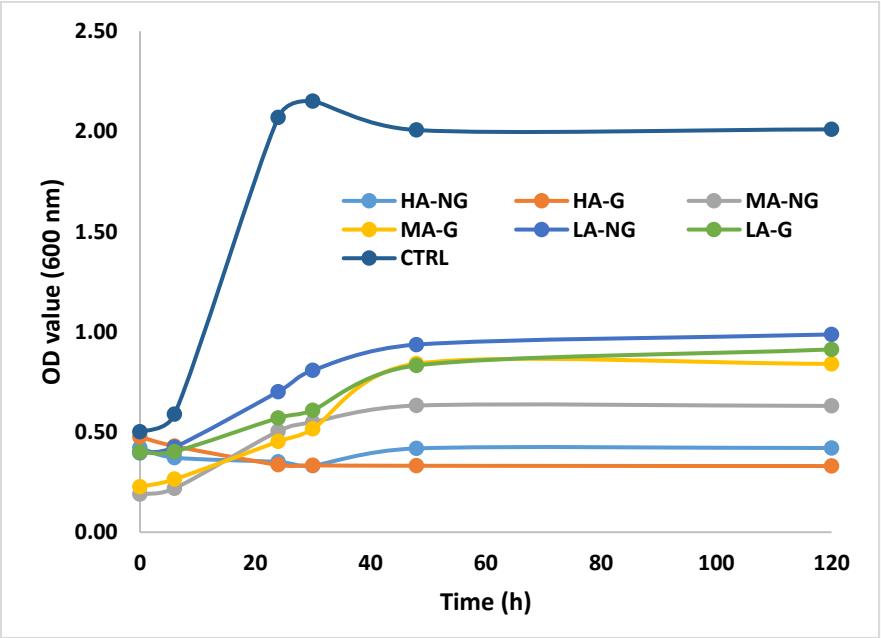

Figure 7.1 *Growth curves of Cupriavidus necator in all tested conditions (OD 600 nm). HA = high concentration acids; MA = mild concentration acids; LA = low concentration acids; NG = without glucose; G = with glucose.*

In the control condition, the strain showed the highest growth (2.15 ± 0.08 OD_{600}) after 30 hours of fermentation. In samples with low and moderate acid concentrations, also when glucose was supplemented, the growth phase was delayed until 48 hours, with the highest values reached at this later sampling time. Among the conditions in which acids were added to the growth medium, the test with low acid concentrations and no glucose supplementation (LA-NG) yielded the highest OD_{600} value (0.99 ± 0.04).

When succinic, formic, and acetic acids were supplied at the highest concentrations (13, 8, and 10 $g \cdot L^{-1}$, respectively), no growth of the strain was observed, even with glucose supplementation. Although the ability of *C. necator* to use volatile fatty acids, such as acetic acid, as carbon sources, is well documented in the literature, a complete growth inhibition has been reported when acetic acid exceeds 10 $g \cdot L^{-1}$ (Kedia et al., 2014). Consistent with this, our results showed that below this threshold, *C. necator* was still able to grow when succinic, formic, and acetic acids were added at concentrations of 6.5, 4, and 5 $g \cdot L^{-1}$, respectively.

Furthermore, under moderate acid concentrations, the glucose supplementation appeared to have a beneficial effect on the growth of the strain (0.84 ± 0.08 in MA-G vs. 0.63 ± 0.04 in MA-NG, OD_{600}). This finding is in according with Marudkla et al. (2018), who demonstrated that the glucose addition to the fermentation broth mitigated the negative effects of acetic acid on the strain growth and reduced the extended lag phase caused by this acid. In contrast, under low acid concentrations, the glucose supplementation did not improve the growth of *C. necator* (0.99 ± 0.03 in LA-NG vs. 0.91 ± 0.05 in LA-G, OD_{600}).

Figure 7.2 shows the glucose consumption in the tests where this compound was added to growth broth. In the test with a high concentration of acids (HA-G), no glucose was consumed, as expected because of the absence of growth in the strain. When the acids were in the broth at mild concentrations (MA-G), the lowest concentration of the sugar detected was after 120 hours-fermentation, reaching 17.5 ± 0.7 $g \cdot L^{-1}$ (-12.5%). Furthermore, total glucose consumption was observed in sample with low concentration of acids (LA-G). The ability of *C. necator* to utilize sugars such as glucose and fructose has been well documented by Bellini et al. (2024), who demonstrated that a sugar-rich syrup derived from cereal waste can be used for PHB production. Although the strain preferentially consumes volatile fatty

acids, such as acetic and butyric acids, as primary carbon sources for polymer accumulation, simple sugars like glucose and fructose can also enhance the biomass growth (Ertan et al., 2021).

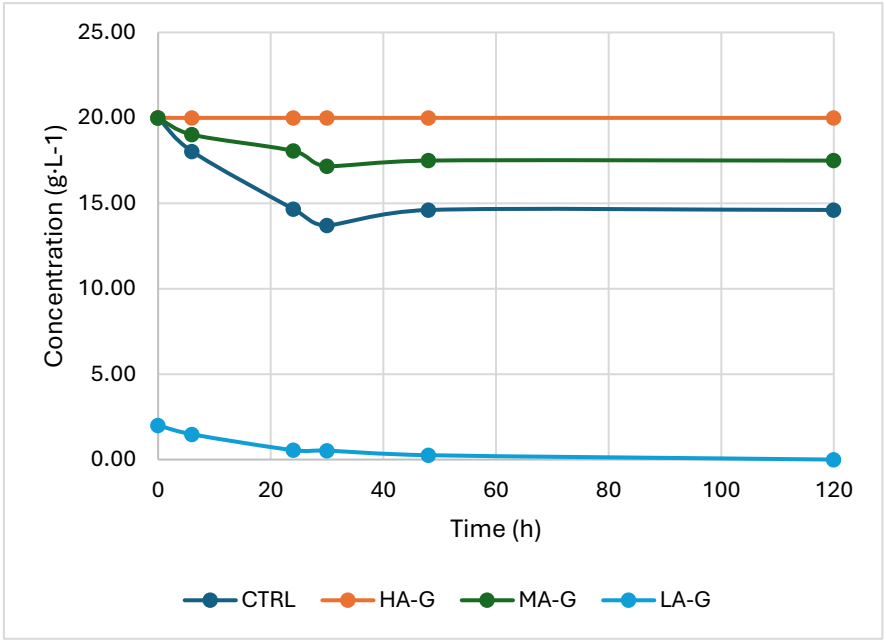

Figure 7.2 *The glucose consumption under the tested conditions where this sugar was added to the growth broth. Abbreviations: HA = high concentration acids; MA = mild concentration acids; LA = low concentration acids.*

The analysis of the acid reduction represented the core focus of this work. This was primarily due to the fact that, during *Actinobacillus succinogenes* fermentation for succinic acid production, several by-products, such as acetic and formic acids, were also formed. The accumulation of these compounds is undesirable because, once present in the fermentation broth, acetic and formic acids must be removed in order to obtain succinic acid with the purity required by the market.

Figure 7.3 reports the percentage reduction of these acids observed after 120 hours of incubation. Notably, the highest reduction of acetic acid was achieved in the broth with a low acid concentration and without glucose supplementation (LA-NG), reaching 19.60 ± 0.55%. An equally noteworthy result was obtained under mild acid concentrations with

glucose addition (MA-G), where a reduction of 14.56 ± 0.51% was recorded. Despite the lower percentage, this condition led to a comparable absolute amount of acetic acid removed (3.10 vs. 3.06 g·L^{-1}), even though the initial acid concentration in the broth was higher. From an operational standpoint, this is a relevant outcome, as it indicates that extensive dilution of the *A. succinogenes* fermentation broth may not be required. Consequently, this finding effectively increases the tolerable concentration threshold of acetic acid, reported as inhibitory for *C. necator* at 3 g·L^{-1} by Garcia-Gonzalez & De Wever (2018).

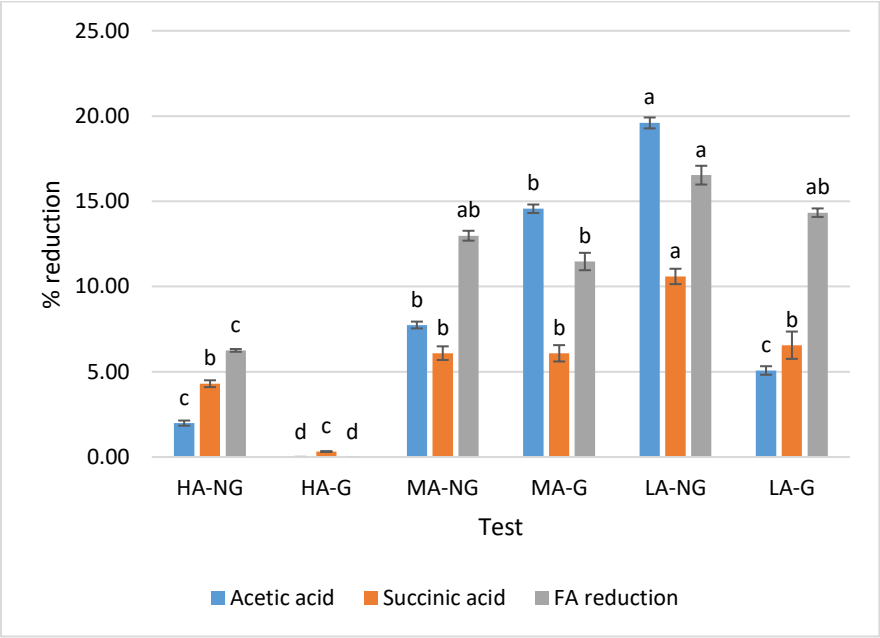

Figure 7.3 *The percentage reduction of acetic, succinic and formic acids after 120 hours-fermentation under tested conditions. Abbreviations: HA = high concentration acids; MA = mild concentration acids; LA = low concentration acids; NG = without glucose; G = with glucose. Error bars indicated standard deviation. Different letters on each bar indicate significant differences among different samples according to Tukey post-hoc test (p = 0.05).*

Moreover, this strain is capable of utilizing formic acid, a volatile fatty acid, Al Battashi et al. (2021). Interestingly, the strain was also able to metabolize acids outside this class of compounds, such as succinic acid itself. Indeed, succinic acid concentration decreased by up to 10.59 ± 0.32% in the broth with low acid concentration and without glucose addition (LA-

NG). This reduction is potentially a concern, as in a process producing succinic acid, any decrease in its final concentration results in a lower process efficiency and, consequently, economic losses.

7.4. Conclusions

The partial removal of acetic and formic acids through a secondary biological step using *Cupriavidus necator* could significantly improve the overall sustainability and cost-effectiveness of biologically produced succinic acid. This approach reduces the need for energy-intensive separation technologies or membrane systems that require frequent replacement. Nevertheless, careful attention must be paid to the unintended consumption of succinic acid, as minimizing its loss is essential to ensure that the reduction of acetic and formic acids does not compromise the overall process yield.

In addition, the simultaneous production of a value-added biopolymer such as PHB offers an opportunity to generate additional revenue, further enhancing the economic viability of the process. However, further studies are required, since current research on *C. necator* is largely focused on its use for PHB production rather than on its potential role in succinic acid purification.

Overall, the results of this work suggest that integrating this biological treatment step into a succinic acid production process could represent a promising downstream strategy, enabling acid reduction without the use of membranes or other environmentally burdensome technologies.

8. Conclusions and future perspectives of the work

The objective of this research work was to produce succinic acid through a fermentative process carried out by *Actinobacillus succinogenes*, using fermentable sugars obtained from the pretreatment of wheat straw. This lignocellulosic biomass is an agricultural by-product derived from different parts of the wheat plant, such as stems and leaves, and is rich in cellulose, hemicellulose, proteins, lignin, and ash. Its high carbohydrate content and broad availability, estimated at 170 million tons in Europe (Ćilerdžić et al., 2017), make wheat straw an attractive candidate for biotechnological applications, particularly for fermentation processes. Currently, as a residue of cereal production, wheat straw is often burned in the field despite the practice being illegal (Song et al., 2016), contributing significantly to greenhouse gas emissions such as CO_2.

In contrast, when this biomass is used for succinic acid production via fermentation with *A. succinogenes*, CO_2 becomes a required input to enhance product yields, since this microorganism grows optimally under anaerobic conditions. In this context, CO_2 not only supports these conditions but also serves as a carbon source for *A. succinogenes*. Therefore, redirecting wheat straw toward succinic acid bioproduction provides a dual benefit: it valorizes an agricultural residue by converting it into a high-value compound and simultaneously offers an environmental advantage by avoiding CO_2 emissions from straw burning while potentially contributing to this gas sequestration within the fermentation process.

The chemical composition of wheat straw, especially its high levels of cellulose (40–60% of dry weight) and hemicellulose (10–40% dw) (Lu et al., 2021; Khan & Mubeen, 2012; Blasi et al., 2023), further supports its suitability for fermentation-based production of value-added biochemicals. Through appropriate pretreatments and enzymatic hydrolysis, these polysaccharides can be converted into simple sugars such as glucose and xylose, which can subsequently serve as carbon sources for microbial fermentation.

To date, wheat straw has been predominantly employed in fermentation processes for ethanol production (Ingrao et al., 2021). Its use for succinic acid synthesis, however, has been far less explored (Zheng et al., 2009; Li et al., 2010), largely due to the challenges associated with its processing. Pretreatments such as steam explosion, required to break down the

lignocellulosic matrix, are conducted under harsh temperature and pressure conditions that generate inhibitory by-products. The thermal degradation of pentoses and hexoses yields compounds such as organic acids (like acetic acid) and furan derivatives (as hydroxymethylfurfural and furfural), which are well-known microbial growth inhibitors.

In Table 8.1, the works conducted to date in which wheat straw has been used for succinic acid production are summarized. As can be observed, prior to the start of this doctoral work in 2023, only two studies were found in the literature employing wheat straw as the initial biomass. In the study by Zheng et al. (2009), the best process performance was reported, achieving a succinic acid concentration of 18.9 $g \cdot L^{-1}$ after fermentation with *A. succinogenes*, corresponding to a yield of 74%. Conversely, in the work of Li et al. (2010), where *Fibrobacter succinogenes* was used, the final succinic acid concentration was significantly lower, 0.5 $g \cdot L^{-1}$ with a yield of 12%.

Table 8.1. *Succinic acid production starting from wheat straw. SA = succinic acid*

Pre-treatments	Microorganism	Fermentation process	SA concentration ($g \cdot L^{-1}$)	Yield (%)	Reference
Dilute-alkaline pretreatment and enzymatic hydrolysis	*A. succinogenes* CGMCC1593	Batch fermentation in anaerobic condition	18.9	74	Zheng et al. (2009)
Sun-dried and ground to a final diameter of approximately 2 mm by the blender	*Fibrobacter succinogenes*	Batch fermentation in anaerobic condition	0.5	12	Li et al. (2010)

In both of these studies, no information was provided regarding the presence of inhibitory compounds in the growth media used for succinic acid production. In the present work, following an initial optimization phase performed using synthetic media to determine the optimal starting conditions (as described in Chapter 2), a series of experiments was carried out in Chapter 3 using the optimized growth medium (TSB). To this medium, the inhibitory compounds acetic acid and furfural were added at concentrations corresponding to those

found in the wheat straw hydrolysate, which had been appropriately diluted to ensure inhibitor levels below the critical thresholds reported in the literature.

In Chapter 4, the production performance of the strain was evaluated using both synthetic media and different dilutions of wheat straw hydrolysate (2% and 5% v/v). The results showed that, under low initial concentrations of glucose and xylose (0.4 and 0.2 $g \cdot L^{-1}$, and 1.1 and 0.4 $g \cdot L^{-1}$, respectively), and without supplementation with additional carbon sources (such as CO_2 or $MgCO_3$) or nitrogen sources (such as yeast extract), succinic acid production and overall strain performance were strongly affected by the presence of inhibitory compounds. Specifically, succinic acid levels at the end of fermentation decreased by 95% and 83% compared with their respective controls.

For this reason, in the subsequent phase described in Chapter 5, a less diluted hydrolysate (50% and 90%) was tested to provide higher initial sugar concentrations. Furthermore, different amounts of $MgCO_3$ and yeast extract were added to the media in order to determine the most suitable combination of these two supplements for the strain. In terms of succinic acid concentration and yield, the best performance was obtained using a 90% (v/v) hydrolysate supplemented with 60 $g \cdot L^{-1}$ magnesium carbonate and 10 $g \cdot L^{-1}$ yeast extract, resulting in 12.87 $g \cdot L^{-1}$ of succinic acid and a yield of 62.1% ($g \cdot g_{glucose}^{-1}$).

Table 8.2 reports several data points from studies in which other lignocellulosic biomasses were used as carbon sources for fermentative microorganisms. As can be observed, wheat straw appears to be a challenging biomass in terms of utilization and process optimization. Despite the extensive optimization performed in the present work, its performance remains lower than that reported in studies using other biomasses, such as rice straw, miscanthus straw, or sugarcane and sweet sorghum bagasse. Although the results achieved here represent a clear improvement compared to previous studies employing wheat straw, such as Li et al. (2020), the succinic acid concentrations and yields remain below those obtained with alternative feedstocks.

Table 8.2 *Succinic acid production from different lignocellulosic biomass*

Raw material	SA final concentration ($g \cdot L^{-1}$)	SA yield (%)	Reference
Wheat straw	12.87	62.1	This work
	2.00	12.0	Li et al. (2010)
Sweet sorghum bagasse	17.10	61.0	Lo et al. (2020)
Rice straw	47.70	84.0	Sawisit et al. (2018)
	78.00	86.0	Jampatesh et al. (2019)
Miscanthus straw	18.00	75.0	Dąbkowska et al. (2019)
Sugarcane bagasse	23.00	64.0	Chen et al. (2021)

Furthermore, even under the best conditions identified in this study, the fermentation broth contained high concentrations of by-products such as acetic acid and formic acid (10 and 8 $g \cdot L^{-1}$, respectively). Acetic acid, in particular, presents a major challenge for downstream processing due to its chemical similarity to succinic acid, requiring the use of costly separation techniques. To address this issue and potentially reduce the final costs of the process, Chapter 6 describes a set of experiments using *Cupriavidus necator*, a bacterium known for its ability to utilize volatile fatty acids, including acetic and formic acid, as carbon sources to produce high-value compounds such as PHB. The results of this experiment are promising, showing that in a broth containing 5 and 4 $g \cdot L^{-1}$ of acetic and formic acid, respectively, the strain was able to remove 19.6% and 16.5% of these compounds.

Overall, although wheat straw proved to be a challenging starting biomass, the optimization of the fermentative process, including adjustments to the initial sugar concentration and the supplementation of growth-supporting compounds such as magnesium carbonate and yeast extract, led to satisfactory outcomes in terms of succinic acid concentration and yield, especially when compared to the literature available for this specific feedstock. Moreover, the use of *Cupriavidus necator* in a secondary fermentation step for the biological removal of by-products such as acetic and formic acid, and the simultaneous production of PHB, provides valuable insights into possible strategies for improving both the production and purification of succinic acid from lignocellulosic biomasses.

Despite the encouraging results obtained in this work, several aspects can be further investigated to improve the overall feasibility and competitiveness of succinic acid production from wheat straw. One of the main future perspectives concerns the optimization of the pretreatment and hydrolysis steps. Since the generation of inhibitory compounds represents a major bottleneck for microbial fermentation, the development or integration of milder pretreatment strategies, as well as detoxification approaches specifically tailored to wheat straw hydrolysates, could significantly enhance microbial performance and product yields. Techniques such as biological detoxification, adsorption-based inhibitor removal, or adaptive laboratory evolution of the production strain may contribute to increasing tolerance toward inhibitory compounds while maintaining high succinic acid productivity.

From a bioprocess perspective, further improvements could be achieved through advanced fermentation strategies. The transition from batch to fed-batch or continuous fermentation modes may allow better control of substrate availability and inhibitor concentrations, potentially leading to higher titers and yields. Additionally, the implementation of in situ CO_2 supply systems or alternative buffering strategies could reduce the dependence on high concentrations of magnesium carbonate, thereby lowering operating costs and simplifying downstream processing.

Another promising direction involves the metabolic and genetic optimization of *Actinobacillus succinogenes*. Targeted metabolic engineering aimed at redirecting carbon fluxes toward succinic acid while minimizing by-product formation, particularly acetic and formic acids, could substantially improve process efficiency. In parallel, the exploration of alternative succinic acid–producing microorganisms or microbial consortia specifically adapted to lignocellulosic hydrolysates may provide additional opportunities for process intensification.

The results obtained with *Cupriavidus necator* also open interesting perspectives for the development of integrated biorefinery concepts. The combination of succinic acid fermentation with a secondary biological step for by-product valorization represents a sustainable approach to reduce purification costs while generating additional high-value products such as polyhydroxyalkanoates. Future studies could focus on optimizing this sequential or co-culture strategy, as well as evaluating its scalability and economic viability.

Finally, comprehensive techno-economic and life cycle assessments will be essential to evaluate the real industrial potential of the proposed process. Such analyses would allow the identification of the most critical cost drivers and environmental hotspots, guiding future research toward the most impactful improvements. In this context, the integration of wheat straw–based succinic acid production within existing agricultural and industrial infrastructures could represent a key step toward the development of sustainable and circular bio-based production chains.

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