Organic acids bioproduction from corn fiber using *Actinobacillus succinogenes*

by

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Abstract

Succinic acid (SA) and lactic acid (LA) are organic acids which are used to synthesize high value industry chemicals, which have applications in the food, agricultural, chemical, and pharmaceutical industries. Currently, these acids are produced primarily from fossil-fuel feedstocks, which are nonrenewable. This chemical process is expensive and ecologically questionable as it increases carbon footprints. Since SA and LA are intermediates in many living organisms, they can be produced biologically using a variety of microorganisms. One of the most promising microorganisms for SA and LA production is *Actinobacillus succinogenes* due to its resistance to these acids and other inhibitors, and its ability to utilize a variety of sugars. The aims of this study included SA bioproduction, investigation of the effect of N_2 and CO_2 bubbling on LA production, and LA bioproduction from corn fiber (CF) hydrolysates using *A. succinogenes*. CF is a byproduct of the dry grind corn ethanol process and has minimal lignin and high polysaccharide content. To produce SA and LA, CF was pretreated and hydrolyzed using enzyme. The hydrolysates were later fermented using *A. succinogenes*. When a control mimicking the sugars in hydrolysates was fermented, a maximum SA concentration of 25.3 g/L with a yield of 0.44 g/g sugars was obtained. After 3 months of *A. succinogenes* storage in 5% DMSO, the cells lost their ability to make SA and started making more LA. Cells in CO_2 bubbled media produced the highest LA concentration of 36.1 g/L LA with a 0.72 g/g glucose. Cells in CO_2 bubbled detoxified hydrolysate produced the highest LA concentration of all hydrolysates (22.5 g/L LA with a 0.57 g/g glucose yield) while cells in CO_2 bubbled control media mimicking the sugars in detoxified hydrolysate produced 28.5 g/L LA with a 0.73 g/g glucose yield. For future studies, better cryopreservation techniques of *A. succinogenes* will sustain the cells fermentation ability over time.

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1 Literature Review

Introduction

Recently, there has been a growing concern regarding the contribution of fossil-fuel based processes to global warming. This concern and the strong demand for environmentally friendly energy sources has sparked interest in developing more sustainable processes. The purpose is to use renewable resources and locally available raw materials to produce the same products as fossil fuel-based methods using less energy, decreased cost, and reduced carbon footprints **(Clark, 2014).**

The U.S Department of Energy has identified 12 building block chemicals that can be produced biologically from biobased feedstocks and subsequently be converted into high-value bio-based chemicals and products. Succinic acid ($C_4H_6O_4$) (SA), also known as 1,4 butanedioic acid or amber acid, is one of the top value-added chemicals among these 12 chemicals **(Werpy and Petersen, 2014).**

SA is a natural organic acid found in human bodies, plants, animals, and microorganisms. It is both an intermediate compound in the tricarboxylic acid cycle and an end product in various anaerobic metabolism pathways. Therefore, its synthesis can be achieved using microbial fermentation. SA is widely used as a precursor in synthesizing different high value industrial chemicals such as adipic acid, 1,4-butanediol, tetrahydrofuran, and 2-pyrrolidone, which have applications in the food, agricultural, chemical, and pharmaceutical industries. It can also be used to manufacture bio-based and biodegradable polymers such as polybutylene succinate and polyamides **(Ahn et al., 2016; Cok et al., 2014**; **Jiang et al., 2017)**. Additionally, SA has been used in the production of food additives, detergents, cosmetics, pigments, surfactants, dyes, resins, and coatings as well as an acidulant, sweetener, and flavoring in the food industry

(Nghiem et al., 2017; Kamzolova et al., 2009; Potera, 2005; Pateraki et al., 2016; Song and Lee, **2006**). In 2021, its global market size was valued at USD 222.9 million and expected to grow at annual rate of 9.7% from 2022 to 2030 **(Grandviewresearch, 2022)**

Conventional production of SA has been performed using petroleum feedstocks such as liquefied petroleum gas. The increase in crude oil prices directly impact the cost of these petroleum products, thereby increasing SA cost. The petrochemical process of producing SA involves partial oxidation of butane, followed by catalytic hydrogenation of maleic anhydride. In addition to being costly, it is also environmentally concerning as it increases carbon footprints by using heavy metal catalysts, organic solvents, high temperatures, and pressures **(Clark, 2014; Technavio, 2021)**.

These concerns led to an interest in discovering biological ways to produce SA using renewable feedstocks to replace fossil fuels. It is possible to produce SA biologically because it is part of every organism's central metabolism**.** Different microorganisms produce SA as an intermediate in their biochemical pathways. For instance, it is one of the co-products during ethanol fermentation by *Saccharomyces cerevisiae*, alongside glycerol, acetic acid, and lactic acid **(Nghiem et al., 2017).** As SA is a metabolite in the tricarboxylic acid (TCA) cycle, it is theoretically possible to produce two molecules of SA from one molecule of 6-carbon sugar and two molecules of carbon dioxide. This offers a theoretical SA yield of 1.12 g/g sugar **(McKinlay et al., 2010).**

Bio-based production of SA can be achieved by fermentation using a variety of microorganisms such as wild-type bacteria, engineered bacteria, and engineered yeast. Several studies have investigated SA production using microorganisms namely *Actinobacillus succinogenes, Basfia succiniciproducens, Corynebacterium glutamicum, Escherichia coli,*

Mannheimia succiniciproducens, Saccharomyces cerevisiae, and Yarrowia lipolytica (**Jiang et al., 2017; Pateraki et al., 2016**; **Vallecilla-Yepez et al., 2021)**. Among these, *A. succinogenes* has proved to be a better wild-type bacteria strain for SA production compared to other reported SA producing microorganisms due to its resistance to SA and other inhibitors (**Ferone et al., 2018; Guettler et al., 1996).** In addition to this, this strain can also utilize different sugar varieties ranging from pentoses, hexoses, and disaccharides such as glucose, xylose, arabinose, fructose, cellobiose, maltose, and lactose **(Bechthold et al., 2008; Jiang et al., 2017; Pateraki et al., 2016)**.

Several renewable second-generation biomasses including corn fiber (CF), sugarcane bagasse, corn stover, industrial hemp, and rapeseed straw have been investigated to produce SA using *A. succinogenes* **(Borges and Pereira, 2011; Chen et al., 2010, 2011; Kuglarz et al., 2016, 2018; Zheng et al., 2010; Vallecilla-Yepez et al., 2021)**. Among these biomasses, CF presents a unique potential as one of the most promising, renewable, and inexpensive biomasses for SA production. CF is a byproduct of the dry grind corn ethanol process. CF consists of the outermost layer of the corn kernel and is removed prior to fermentation to boost fermenter capacity. It is mainly composed of polysaccharides, and a small content of protein and oil **(Vallecilla-Yepez et al., 2021).** Currently, it is primarily used to feed cattle. This is not its most valuable exploitation as it can be converted into monomeric sugars, which could then be fermented into more valuable bioproducts and biofuels such as SA and LA.

The first step to convert CF into valuable bioproducts is pretreatment to disrupt the cell walls. The uniqueness of CF lies in its low lignin content compared to other cellulosic biomasses. This allows for a mild pretreatment method such as liquid hot water (LHW) to be used before saccharification. Enzymatic hydrolysis using low temperature can follow the LHW

pretreatment. This results in reduced inhibitory compounds compared to acid hydrolysis (**Yoo and Pan, 2017).** Enzymatic hydrolysis is also advantageous compared to acid hydrolysis because it is more environmentally friendly as it doesn't use heavy chemicals and solvents.

Several studies have investigated the production of SA from CF using *A. succinogenes*. For instance**, Chen et al. (2010)** obtained a SA yield of 72.0% from detoxified CF hydrolysate as a carbon source. Another study by **Chen et al. (2011)** reported a 67.7% SA yield from CF hydrolysate as the carbon source. In addition to that, **Guettler et al. (1996)** found a SA yield of 94% from CF hydrolysate using a variant of *A. succinogenes* 130Z. To obtain hydrolysate, these studies utilized dilute sulfuric acid for hydrolysis. On the other hand, **Vallecilla-Yepez et al. (2021)** utilized enzymatic hydrolysis to obtain hydrolysate from CF. The author reported a SA yield of 61% using *A. succinogenes* as a fermenting organism.

Lactic acid ($C_3H_6O_3$) (LA), also known as 2-hydroxypropanoic acid, is another crucial organic acid which is the most widely occurring hydroxycarboxylic acid. It was discovered by Swedish scientist C.W. Scheele in 1780 from sour milk **(Datta et al., 1995).** LA is naturally occurring in fermented foods such as sauerkraut, yoghurt, buttermilk, and sourdough breads. It is also an intermediate in many metabolic pathways in most living organisms, from anaerobes to human beings **(Datta et al., 1995; Reddy et al., 2008).** It can be manufactured by either chemical synthesis or microbial fermentation. Biological production is advantageous because it can utilize cheap raw materials including whey, molasses, starch waste, beet, cane sugar, and other affordable carbohydrate rich materials **(Anuradha et al., 1999; Richter & Berthold, 1998; Tsao et al., 1999; Vishnu et al., 2000).**

LA is a non-volatile, odorless organic acid which is classified as GRAS (Generally Recognized As Safe) to be used as a food additive by FDA **(Datta et al., 1995).** It has

applications across various industries such as food and beverage, pharmaceuticals, cosmetics, and biodegradable polymers. In the food industry, it serves as emulsifying agent in bakery products, an acidulant, flavoring, pH buffering agent, and preservative by reducing microbial spoilage **(Litchfield, 1996)**. Its water-retaining capacity allows it to be used as a moisturizer in cosmetics. It has also been used in pharmaceutical products such as ointments, lotions, and parenteral solutions. Moreover, it has been used to prepare biodegradable polymers used in the medical field, such as surgical sutures, prostheses, and drug delivery products **(Wee et al., 2006).** It also has the potential to be converted into useful chemicals including propionic acid, acetic acid, and acrylic acid since it possesses two reactive functional groups **(Demirci et al., 1993).** LA has gained considerable attention due to its potential to be used as a raw material for synthesis of fully biodegradable plastics. The most known is polylactic acid (PLA), a polymer of LA which is an environmentally friendly alternative to petrochemical plastics because it is biocompatible, biodegradable, and biobased. Replacing synthetic polymers with biodegradable bio-based polymers will decrease waste disposal problems, environmental contamination, and toxicity to the ecosystem and human health **(Balla et al., 2021).** LA's market is estimated over 150,000 tons per year, 90% of which is produced by lactic acid bacteria (LAB), a group pf related bacteria which produce LA as a major product through fermentation **(Sauer et al., 2008)**. *Lactobacillus* is the biggest genus of LAB. Different species in this genus have been used to make LA from raw starch materials **(Cheng et al., 1991; Vishnu et al., 2002; Naveena et al., 2005)**.

A few studies have also investigated the production of LA using *Actinobacillus succinogenes*, one of the major SA producing bacteria. **Rahim et al. (2023)** obtained 31.64 g/L LA and 0.88 g/L/h productivity from palm kernel cake hydrolysate fermentation. **Li et al. (2010)**

dual phase fermentation of glucose resulted in 135.6 g/L LA with 0.96 g LA/g glucose yield and 2.94 g/L/h productivity. **Wang et al. (2014)** obtained 183.4 g/L LA with 0.97 g LA/g glucose yield and 1.53 g/L/h productivity when microfiltration membrane was integrated in fermentation for separation.

To the author's knowledge, no study has investigated LA production from CF using *A. succinogenes*. Bio-production of SA and LA from CF has several advantages such as more profitability for corn ethanol plants originating from the new use for their CF, and environmental protection due to dangers associated with the current petroleum-based production of these acids. The following section discusses the different compositions of various lignocellulosic biomass that have been investigated in the past to produce various bioproducts and biofuels.

Chemical composition of biomass

To produce biofuels and bioproducts, complete characterization of biomass is important since the biomass will undergo further conversion processes **(Capareda, 2022).** Biomass composition will then dictate a suitable biofuel or product to produce. In addition to that, biomass composition also influences which pretreatment and hydrolysis methods to be used **(Varriale et al., 2022).** Lignocellulosic biomasses are mainly composed of carbohydrates and lignin. Thus, a comprehensive biomass analysis must measure these components **(Sluiter, 2008a).** It is also critical to remove all non-structural materials (extractives) from biomass before doing analysis to prevent any possible interference with further analytical steps. These can either be water or ethanol soluble materials. Water soluble materials include inorganic material, non-structural sugars, as well as nitrogenous material. Ethanol soluble materials include chlorophyll, waxes, and other minor components **(Sluiter, 2008b)**. The main structural components for lignocellulosic biomasses are cellulose, hemicellulose, and lignin **(Zhang et al., 2019).** Cellulose

is a linear homopolysaccharide made of D-glucopyranosyl units, while hemicellulose is a branched heteropolysaccharide made of hexose and pentose sugars. Lignin is made of phenylpropane units linked together mainly by ether bonds **(Jönsson et al., 2013)**. **Zhao et al. (2020a)** reviewed the chemical compositions of different lignocellulosic biomasses including hemp biomass, CF, corn stover, and sorghum bagasse. For hemp biomass, the author reported 36.5-75.6% cellulose, 10.1-32.8% hemicellulose, and 8.0-22.9% lignin content ranges. For CF, cellulose range was 13.0-18.0%, hemicellulose range was 35.0-45.3%, and lignin range was 1.3-18.0 %. For corn stover, cellulose range was 31.0-41.2%, hemicellulose range was 16.5-22.8, and lignin range was 12.6-25.4%. Finally, sorghum bagasse had 35.5-41.1% cellulose range, 18.4-25.9% hemicellulose range, and 15.4-24.5% lignin range. The review results with some additions are summarized in **Table 1** below.

Since CF has low lignin content compared to other lignocellulosic biomasses, this favors the bioconversion process as the structure is not very compact **(Zhao et al., 2020a; Zhao et al., 2020b).** This allows for a less severe pretreatment method, such as hydrothermolysis, also called liquid hot water, to be used. There are chemical bonds crosslinking cellulose, hemicellulose, and lignin together in lignocellulosic biomasses. These are resistant to enzymatic attack and microbial fermentation. The purpose of a pretreatment method is to disrupt this structure by breaking down these bonds, thereby solubilizing hemicellulose and lignin. This makes cellulose readily available to enzymes and fermenting organisms. A good pretreatment method should disrupt the physical and chemical connection between cellulose, hemicellulose, and lignin. It should also increase cellulose accessibility while minimizing generation of inhibitory compounds **(Zhou et al., 2023).** Hydrothermolysis was chosen for this study because it meets most of these criteria.

Table 1 Chemical composition for different biomass feedstocks (% dry basis)

Biomass type	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
CF	14.0	39.0	5.7	**(Van Eylen et al., 2011)**
	13.0	38.8	7.5	**(Noureddini & Byun, 2010)**
	16.4	45.3	1.3	**(Rasmussen et al., 2010)**
	18.0	35.0	18.0	**(Shrestha et al., 2008)**
	21	46.4	0.8	**Vallecilla-Yepez et al., 2021**
Hemp biomass	42.0	15.7	13.2	**(Kuglarz et al., 2014)**
	46.4	20.1	15.0	**(Kuglarz et al., 2018)**
	42.3	18.2	22.9	**(Gunnarsson et al., 2015)**
	40.1	16.6	17.8	**(Zhao et al., 2020a)**
	53.7	21.8	22.2	**(Emma et al., 2008)**
Corn stover	31.7	17.1	12.6	**(Liu et al., 2014)**
	34.4	22.8	18.0	**(Kumar et al., 2009)**
	31.0	20.1	25.4	**(Xu et al., 2016)**
	31.3	16.5	16.6	**(Wang et al., 2019)**
	33.1	17.6	17.3	**(Gong et al., 2015)**

Sorghum bagasse	40.4	20.0	19.8	**(Barcelos et al., 2016)**
	41.1	25.9	21.4	**(Sun et al., 2015)**
	37.8	21.2	16.7	**(Wu et al., 2011)**
	35.5	20.0	24.5	**(Jafari et al., 2017)**
	35.6	18.4	18.2	**(Umagiliyage et al., 2015)**

Actinobacillus succinogenes

A. succinogenes is a wildtype bacterial strain that was isolated from the bovine rumen. It is a facultative anaerobe, gram-negative rod, and exhibits pleomorphism (can alter its morphology, biological functions or reproductive modes depending on environmental conditions) **(Guettler et al., 1999)**. It can utilize a broad range of sugars (carbon sources) including arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sorbitol, sucrose, xylose, or salicin under anaerobic conditions. It can accumulate high SA concentrations (more than 70 g/L). This makes it one of the most efficient natural producers of SA. Biochemically, it is positive for tests such as catalase, oxidase, alkaline phosphatase, and b-galactosidase. However, it is negative for indole and urease tests **(Guettler et al., 1999; Pateraki et al., 2016).**

A. succinogenes has been used to produce SA using a variety of carbon sources. The **Table 2** below summarizes the results from the various studies.

Table 2 Different SA fermentation results for various carbon sources using *A succinogenes*

Biomass	Pretreatment	Yield	Productivity (g/L/h)	Reference
Sugarcane bagasse	Dilute acid	55.4%	1.014	Borges and Pereira (2011)
Sugarcane bagasse	Ultrasonic after dilute acid hydrolysis	79%	0.99	Xi et al. (2013)
Glycerol	N/A	0.87g SA/g glycerol	2.31	Carvalho et al. (2014)
Sake lees	Sulfuric acid	0.59 g/g sugars	1.21	Chen et al. (2012)
Spent yeast cells and corn fiber	Dilute sulfuric acid	67.7%	0.63	Chen et al. (2011)

As shown in **Table 2**, **Borges and Pereira (2011)** utilized sugarcane bagasse hemicellulose hydrolysate to produce SA using *A. succinogenes*. In the study, the authors used sugarcane bagasse containing 50% cellulose, 25% hemicellulose, and 25% lignin. After dilute acid pretreatment, the hemicellulose hydrolysate (liquid phase) obtained was composed mainly of xylose, which was used as substrate for fermentation in a bioreactor. SA concentration, productivity, and efficiency of 22.5 g/L, 1.014 g/L/h, and 55.4%, respectively, were obtained. Another study by **Xi et al. (2013)** used sugarcane bagasse that was pretreated ultrasonically after dilute acid hydrolysis. The sugar cane bagasse hemicellulose hydrolysate obtained was used as carbon source and fermented using *A. succinogenes*. SA yield of 79% and productivity of 0.99 g/L/h were obtained. **Carvalho et al. (2014)** utilized glycerol, a cheap byproduct of the biodiesel industry, as a carbon source for SA bioproduction by *A. succinogenes*. In the study, dimethyl sulfoxide acted as an external electron acceptor, promoting glycerol consumption and SA production. SA concentration of 49.62 g/L with a yield of 0.87g SA/g glycerol and a production rate of 2.31 g SA/L/h were reported. **Chen et al. (2012)** used sake lees, a byproduct of Japanese rice wine, to produce SA using *A. succinogenes*. In the study, sake lees were pretreated using

sulfuric acid, and the hydrolysate was supplemented with biotin. Fermentation by *A. succinogenes* resulted in SA yield of 0.59 g/g sugars with a productivity of 1.21 g/ L/h. Additionally, **Chen et al. (2011)** used biotin-supplemented enzymatic hydrolysate of spent yeast cells as a nitrogen source and corn fiber hydrolysate, obtained using dilute sulfuric acid hydrolysis, as carbon source. The mixture was fermented using *A. succinogenes*, yielding 67.7% SA with 0.63 g/L/h productivity.

Effect of different detoxification techniques

There are different lignocellulosic biomass pretreatment methods, including biological, physical, and thermochemical methods. Fungal pretreatment is an example of biological pretreatment. Physical pretreatment includes size reduction using mills, while thermochemical pretreatments include dilute acid, hot water, and alkaline pretreatments. All these types of pretreatments introduce byproducts which are toxic to fermenting microorganisms. These byproducts must be removed prior to fermentation to enhance the fermentation yield. They originate either from lignocellulosic extractives or degradation of hemicellulose and lignin **(Brethauer & Studer, 2015).** These toxic compounds include furan aldehydes [furfural, 5-hydroxymethyl-furfural(HMF)], aromatic carboxylic acids, aliphatic acids (acetic acid, formic acid, levulinic acid), macromolecules, phenolic compounds, and other unidentified degradation products. Furan aldehydes and aliphatic acids are carbohydrate degradation products, while phenolic compounds come from lignin and extractives **(Palmqvist & Hahn-Hägerdal, 2000; Jönsson et al., 2013)**. **Fig 1** below illustrates the formation of different inhibitory compounds.

The degree of toxicity of the compounds varies for different microorganisms. Thus, it is important to detoxify the hydrolysates to enable fermentative microorganisms to grow and ferment the substrate **(Buhner & Agblevor, 2004).** There are different detoxification methods

that have proved to reduce significantly or eliminate inhibitory compounds prior to fermentation, thereby increasing the product yields during fermentation. They include biological, physical, and chemical methods. Biological methods include the use of enzymes and fungi while physical methods include vacuum evaporation. Chemical methods include alkali treatments, use of solvents such as diethyl ether, and use of activated carbon. **Jönsson et al. (1998)** treated willow hemicellulose hydrolysate biologically with enzymes peroxidase and laccase, increasing ethanol productivity two to three times. This was attributed to the complete removal of phenolic monomers and acids. It was suggested that the detoxification mechanism involve oxidative polymerization of low molecular weight phenolic compounds.

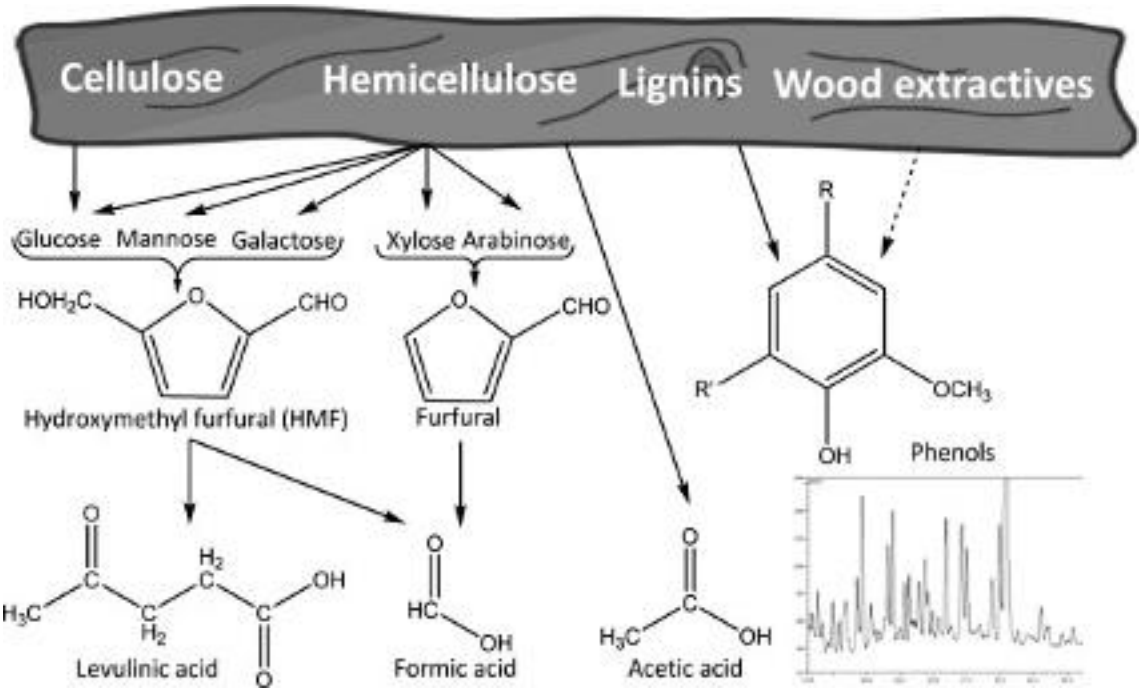

Figure 1 Main routes of formation of inhibitors [reproduced from Jönsson et al. (2013), published by BioMed Central Ltd.].

Palmqvist et al. (1997) used a biological method involving filamentous soft-rot fungus *Trichoderma reesi* to degrade inhibitors in willow hemicellulose hydrolysate, which increased ethanol productivity and yield by three and four times respectively. This was due to the removal

of acetic acid, furfural, and benzoic acid derivatives. Different continuous overnight extractions using solvents such as diethyl ether and ethyl acetate to detoxify hydrolysates chemically have been reported to increase product yields due to the removal of inhibitors such as acetic acid, furfural, vanillin, and 4-hydroxybenzoic acid **(Wilson et al., 1989).** Lignocellulosic hydrolysates have also been previously detoxified chemically by alkali treatments by increasing the pH to 9 - 10 using either $Ca(OH)_2$ (overliming) or NaOH, followed by readjustment to 5.5 by sulfuric acid. This has been reported to increase ethanol productivity and yield. $Ca(OH)_2$ has shown better hydrolysate fermentability than NaOH **(Zyl et al., 1988; Palmqvist et al., 1998).** The mechanism behind increased fermentation yield and productivity from overliming has been attributed to the precipitation of toxic compounds as well as the instability of some inhibitors at high pH.

Guo et al. (2013) studied the effect of spruce hydrolysate detoxification on inhibitory compounds and sugars during bacterial cellulose production. The methods investigated in the study included chemical methods using activated charcoal, alkali [sodium hydroxide, calcium hydroxide (overliming), and ammonium hydroxide], anion and cation exchange resins, and reducing agents (sodium sulfite and sodium dithionite). The results showed that activated charcoal treatment removed most of the furan aldehydes (over 94%) and phenols (over 88%) without affecting sugar concentration very much (less than 5% loss in glucose and xylose). It also removed over 30% and 40% acetic acid and formic acid respectively. The authors concluded that among all the detoxification techniques investigated, the activated charcoal treatment was the most efficient in removing furans, acetic acid, formic acid, and total phenolics.

Chen et al. (2010) investigated the effect of two detoxification techniques on furfural compounds and sugars during the production of SA by *A. succinogenes*. The methods studied

were sodium hydroxide neutralization as well as a combination of calcium carbonate neutralization with activated carbon adsorption. The results showed that calcium carbonate neutralization coupled with activated carbon adsorption was more effective. It reduced furfural compounds by 91.9% with only 5.2% loss in total sugars concentration. The NaOH neutralization was not effective at removing considerable furfural inhibitors. The study also investigated the effect of different carbon sources on SA yield. The carbon sources included the hydrolysate detoxified using sodium hydroxide, hydrolysate detoxified with calcium carbonate combined with activated carbon, and pure glucose. The results indicated that when pure glucose was used as a carbon source, the SA yield was the highest (more than 80%). This was followed by hydrolysate treated with calcium carbonate and activated carbon (more than 72% SA yield). Lastly, hydrolysate treated with sodium hydroxide had a SA yield of around 50%. This proved that hydrolysate detoxified with activated carbon and calcium carbonate was very efficient at reducing inhibitors, which yielded more SA compared to sodium hydroxide detoxified hydrolysate.

Lactic acid production using *A. succinogenes*

A few studies have investigated the microbial production of LA from different carbon sources using *Actinobacillus succinogenes*. Among them, **Rahim et al. (2023)** investigated the production of LA from pretreated palm kernel cake (PKC) hydrolysate, a plentiful, renewable, and inexpensive biomass feedstock. The hydrolysate was supplemented with 30 g/L of $MgCO_3$, and coconut-shell activated carbon. In the study, *A. succinogenes* 130Z dual phase (aerobic cultivation occurred in oxygen aerated fermentation media, followed by cultivation in anaerobic media sparged with carbon dioxide) yielded 31.64 g/L LA concentration and 0.88 g/L/h productivity. Another study by **Li et al. (2010)** utilized *A. succinogenes* 130Z in dual phase

fermentation, resulting in 135.6 g/L lactic acid with an overall yield of 0.96 g/g glucose and 2.94 g/L/h productivity. The study revealed lactate dehydrogenase activity, the enzyme responsible for LA production, was 18-fold higher in dual phase than monophase process (anaerobic). At the end of fermentation, the following organic acids were produced per mole of glucose: 1.93 mol LA, 0.37 mol SA, 0.69 mol acetic acid, and 0.21 mol formic acid. **Wang et al. (2014)** developed a novel method for LA production using *Actinobacillus succinogenes.* In the study, a microfiltration membrane was integrated in fermentation for separation, and LA was recovered using resin adsorption. This allowed for residual sugar and nutrients to be recycled back into fermenter, thereby overcoming product inhibition problem. LA production was increased from 141.2 g/L to 183.4 g/L, with a yield of 0.97 g/g glucose and 1.53 g/L/h productivity.

The Loss of SA production ability by *A. succinogenes*

A. succinogenes has been reported to lose its ability to make SA over time. A study by **Long et al. (2021)** observed complete loss of SA production and increase in LA production after approximately 10 months of *A. succinogenes* 130Z storage in the lab using 20% glycerol as cryoprotectant. Contamination and mutation of the SA producing strain over time were ruled out as causes for this loss using sequence comparisons between SA producing and non-producing strains. Another factor that was tested was storage stress. New freeze-dried *A. succinogenes* cells that produced SA initially, were tested after storing them in different cryoprotectants including 20% glycerol, 5% DMSO, and sucrose (0.15 M). Then, aliquots were subjected to 10 freeze-thaw cycles to mimic those used in the lab. The fermentation results showed a difference in cryoprotectant used, with DMSO and sucrose providing better protection than glycerol. The cells stored in glycerol showed a decreased SA production and an increase in LA production (as was observed with the cells stored for 10 months) compared with cells stored in DMSO and sucrose.

However, there was not a complete loss in SA production for the cells stored in glycerol, so this was not sufficient to determine definitively the cause of the SA loss observed after 10 months storage.

Vallecilla- Yepez (2022) reported a similar finding whereby *A. succinogenes* cells showed a much lower SA production and an increase in LA after 24 months of storage in 20% glycerol. The author did an investigation to determine the effectiveness between 20% glycerol and 5% DMSO as cryoprotectants. A fresh *A. succinogenes* strain was purchased and aliquots of cells with each cryoprotectant were stored at -80 °C for 7 days prior to fermentation. Fermentation results after 24 h showed that the strain stored in 20% glycerol produced 0.62 g/L SA and 15.4 g/L LA, while the strain stored in 5% DMSO produced 12.8 g/L SA with no LA. Applying moderate stresses such as cold, heat, starvation, and acid shocks before freezing have been shown to improve bacterial resistance to freezing by preparing the cells to react better to unfavorable conditions **(Reddy et al., 2009).** Other storage strategies that haven't been tested include freeze drying and cryopreservation in the vapor phase of liquid nitrogen at -130 °C. These strategies might be applied to *A. succinogenes* and potentially preserve the cells better over time. Also, future studies should be done to investigate the effects of storage conditions contributing to the loss of SA production by *A. succinogenes* over time.

The Effect of pH on SA and LA production

Several studies have investigated how pH and CO_2 levels influence either SA or LA production. **Samuelov et al. (1991)** investigated the role of CO_2 levels and pH on SA production from glucose by *Anaerobiospirillum succiniciproducens*. At pH 7.2, there was a long lag phase (20-25 h) for growth and LA was produced as a major product. When pH was adjusted to 6.2 with excess CO_2, phosphoenolpyruvate carboxykinase (enzyme responsible for SA production)

levels increased while lactate dehydrogenase and alcohol dehydrogenase levels (enzymes responsible for LA production) decreased. As a result, SA was the major product while trace amounts of LA were detected. Under both pH conditions, acetate and small amounts of ethanol were detected.

Another study by **Liu et al. (2008)** investigated the influence of pH on SA production from glucose using *A. succinogenes*. The authors reported that the organism did not grow and did not produce any SA at pH below 5.5. The optimal pH for SA production was 6.0-7.2, with 27.5 g/L maximum SA concentration when pH was maintained at 6.7. At pH below 6.0, the cells did not grow well, and SA production was lower (17.5g/L). Between pH 6.0-7.2, acetic acid was also detected (less than 5 g/L). The lower SA yields at low pH values were attributed to possible higher maintenance requirements.

Conclusion

SA and LA have gained a lot of attention because of their wide range of applications across various industries. This review comprehensively summarized the different uses of these acids and the downsides to the conventional petroleum-based production of these acids. The review informed on the composition of different lignocellulosic biomasses that have been used to produce different biofuels and bioproducts, as well as the potential of *A. succinogenes* for SA and LA production. The review also reported on the different detoxification techniques to reduce inhibitors and increase fermentation yields. Additionally, the review reported on the loss of SA production ability by *A. succinogenes* overtime and some recommendations for preserving the cells better. The review also reported on the effect of pH on SA and LA production. The information contained in this review can help to ensure a successful and sustainable SA and LA production.

2 Materials and Methods

Corn fiber preparation

Ground corn fiber (CF) was obtained from E Energy Adams, LLC (Adams, NE, USA). CF was evenly spread out in a thin layer and dried in aluminum trays in an oven at 40 °C until the moisture content was below 10%. CF was passed through a 20-mesh (0.85 mm) sieve stacked on top of an 80-mesh (0.18 mm) sieve stacked together in a Ro-Tap sieve shaker (W.S. Tyler, Mentor, Ohio). CF retained on the 80-mesh sieve was kept in sealed bags at room temperature for subsequent use. The fines which passed through the 80-mesh sieve were collected and used for ash analysis.

Composition analysis of corn fiber

Total extractives, oil, ash, moisture, and protein content were analyzed on unextracted CF samples. After extracting these components from CF, polysaccharides and lignin were also measured. CF moisture content (%) was measured using a Mettler Toledo HE73 moisture analyzer. Ash content was determined according to the NREL/TP-510-42622 method **(Hames et al., 2008).** Starch content was performed using a Megazyme total analysis kit (K-TSHK, Megazyme Ltd, Bray, Ireland). Extractives, including oil, were determined following the NREL/TP-510-42619 method with some modifications **(Sluiter, 2008)**. Sequential extraction was carried out in a Soxhlet apparatus (Electrothermal Ltd, UK). Hexane was used first to extract oil. This was followed by extraction using HPLC grade water and 190 proof, USP grade ethanol to remove water and ethanol soluble extractives, respectively. Collected solutions were loaded into a Rotavapor R-100 apparatus (BUCHI, Switzerland). The solvents were evaporated, and respective component of interest was retained and quantitatively measured. Protein extraction was performed following a method proposed by **Hojilla-Evangelista (1990)** with some

modifications. Here, ethanol soluble materials were first extracted using 4 ml 95% (v/v) ethanol/g corn fiber, and the mixture was heated at 75 °C for 1 h while stirring. After this, solids were removed from the mixture by vacuum filtration, placed on an aluminum tray, and dried overnight. Then, alkaline soluble materials were extracted from the dry solids using 15ml of 45% ethanol/55% 0.1M NaOH/g corn fiber (ethanol-extracted). The mixture was heated at 50 °C for 2 h while stirring. After this, solids were washed with HPLC grade water for 20 min until pH was 7. Finally, the solids were removed from the mixture by vacuum filtration and dried in an oven at 40 °C for 3 days. Dried solids were used for polysaccharides and lignin content measurement according to the NREL/TP-510-42618 method **(Sluiter et al., 2004).** CF samples before and after extraction were sent to Kansas State University Soil Testing Lab (Manhattan, KS, USA) for protein measurement using dry combustion method.

Corn fiber pretreatment

In this study, hot water pretreatment was used. A 15% solids mixture was produced by placing 75 g corn fiber (dry basis) and 425 g water into a 1L bench top pressure reactor (Parr Reactor Model 4848, Parr Instrument Co., Moline, IL, USA). The mixture was agitated at 250 rpm, heated to 180 °C, and held at temperature for 10 min. These pretreatment conditions were chosen according to the results from a study by **Vallecilla-Yepez et al. (2021)**.After that, the reactor was cooled to room temperature using cold water and a cooling coil and solids were separated from the liquid fraction of the mixture (known as prehydrolysate) under vacuum filtration using a coffee filter. The weight of pretreated solids and that of prehydrolysate obtained were recorded and their mass ratio was used later for enzymatic hydrolysis. Pretreated solids and prehydrolysate were stored at 4 °C for subsequent steps. To sterilize the solids, wet pretreated biomass was autoclaved at 121 °C for 20 min prior to enzymatic hydrolysis.

Prehydrolysate detoxification

After pretreatment, half of obtained prehydrolysate was detoxified using activated charcoal (SA 2, decolorizing, NORIT®, Marshall, TX). For the detoxification, 50g charcoal/L prehydrolysate was added. The mixture was stirred for 1 h. After, the mixture was separated under vacuum filtration using coffee filter paper. Both detoxified and undetoxified prehydrolysate were sterilized using 0.22 µm filtration units (Corning® Rapid-Flow Sterile Disposable Filter Units with PES Membrane, Oneonta, NY). Compositions of pretreated solids, undetoxified and detoxified prehydrolysate were determined. Sugar, hydroxymethylfurfural (HMF) and furfural concentrations before and after detoxification were compared.

Enzymatic hydrolysis of pretreated corn fiber

Water, undetoxified or detoxified prehydrolysate, acetate buffer (pH 5.0 and 50 mM), cellulase (Ctec2, Novozymes, Franklinton, NC, USA) in the ratio of 20 filter paper units (FPU)/g glucan, and pretreated corn fiber solids were added to 250 ml flasks. The enzyme FPU was determined following the procedure by **Adney & Baker (2008)**. The mixture was then incubated in a shaker at 50 °C and 200 rpm for 72 h. The same mass ratio between pretreated corn fiber solids and prehydrolysate obtained right after pretreatment was used when adding pretreated solids and prehydrolysate (detoxified or undetoxified) or water during enzymatic hydrolysis to mimic hydrolyzing a slurry of pretreated solids and prehydrolysate in a biorefinery. Hydrolysates obtained using water were labeled water hydrolysate (WH). Hydrolysates obtained using undetoxified or detoxified prehydrolysate were labeled undetoxified or detoxified hydrolysate (UH or DH), respectively.

Corn fiber hydrolysate preparation

After enzymatic hydrolysis, the slurry obtained was filtered under vacuum using Whatman #1 filter paper (ThermoFisher Scientific, Hanover Park, IL) to obtain WH, UH or DH. The filtrates were sterilized by pumping them through a 0.22 µm asymmetric polyethersulfone (aPES) membrane filtration unit. The sugar solutions were kept at 4 °C for future use as fermentation carbon sources.

Microorganism and media

A. succinogenes 130Z (NREL B-59377), donated by the USDA Agricultural Research Service (ARS) Culture Collection (Peoria, IL), and *A. succinogenes* 130Z, purchased from DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), were used to produce organic acids from corn fiber hydrolysates. Freeze-dried pellets were revitalized and subcultured in tryptic soy broth (TSB) media (Remel™, Lenexa, KS) with 10 g/L glucose (Fisher Chemical™, Pittsburgh, PA) **(Maharaj et al., 2014)** before being preserved with 5% dimethyl sulfoxide (DMSO) in 1ml culture tubes at -80 °C for inoculum preparation. Prior to fermentation, *A. succinogenes* culture was inoculated in seed medium (30.0 g TSB/L) in anaerobic culture tubes and incubated in a shaker at 37 ºC, 250 rpm for 14 -16 h. After incubation, the culture was washed with sterile 0.89% sodium chloride solution and resuspended with fermentation media. The fermentation medium for the flasks were composed of the following per L, based on **Maharaj et al. (2014)** with some modifications: 16.0 g yeast extract, 1.0 g NaCl, 1.36 g $NaC_2H_3O_2$, 0.20 g $MgCl_2.6H_2O$, and 0.20 g $CaCl_2.2H_2O$. The seed and fermentation media were sterilized using a 0.22 µm aPES membrane filtration unit.

Carbon sources, inoculum, and organic acids production

Carbon sources included WH, UH, DH, and a control that mimicked the sugars in hydrolysates (Control). Organic acids production was carried out in sealed anaerobic 250 ml flasks with a working volume of 100 ml. Before fermentation, the media was either bubbled with carbon dioxide or nitrogen for 5 min, or unbubbled, and $MgCO_3$ at 100% of initial glucose concentration was added to regulate pH and supply CO_2 simultaneously. Inoculum (2.5% v/v) was added to each experimental flask, and fermentation was performed in a shaker at 37 °C and 170 rpm for 48 h. Samples were taken every 24 h for measuring concentrations of sugars, succinic acid, lactic acid, formic acid, acetic acid, and ethanol.

Sample analysis methods

Concentrations of glucose, xylose, arabinose, succinic acid, lactic acid, formic acid, acetic acid, ethanol, HMF, and furfural were measured by high-performance liquid chromatography (HPLC) equipment (Agilent Technologies 1200 Series and 1260 infinity, Santa Clara, CA). In this study, glucose, xylose, and arabinose were eluted with deionized water as a mobile phase at a flow rate of 0.6 ml/min, separated using a carbohydrate ion exchange column (Aminex® HPX-86P, 300 x 7.8 mm, Bio-Rad, Hercules, CA) at 80 °C, and detected by a refractive index detector (Agilent Technologies 1260 Infinity, Santa Clara, CA) at 50 °C. Succinic acid, lactic acid, formic acid, acetic acid, ethanol, HMF, and furfural were eluted with 5mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min, separated using a carbohydrate exchange column (Aminex® HPX-87H, 300 x 7.8 mm, Bio-Rad, Hercules, CA) at 60 °C, and detected by a refractive index detector (Agilent Technologies 1260 Infinity, Santa Clara, CA) at 35 °C.

Calculations

Yields of sugars obtained in hydrolysate after enzymatic hydrolysis were calculated using the following equations:

Glucose yield (%) =

$$\frac{glucose\ concentration\ in\ hydroysate\ \left(\frac{g}{L}\right)+cellobiose*\frac{360}{342}\left(\frac{g}{L}\right)}{(mass\ \%\ glucan\ in\ pretreated\ solids*1.11*solids\ concentration\ in\ hydrolysis\ \left(\frac{g}{L}\right))+(mass\ \%glucan\ in\ prehydrolysate*1.11*solids\ concentration\ in\ hydrolysis\ \left(\frac{g}{L}\right))}$$

Xylose yield (%) =

$$\frac{xylose\ concentration\ in\ hydrolysate\ \left(\frac{g}{L}\right)}{\left(mass\ \%\ xylan\ in\ pretreated\ solids*\frac{150}{132}*solids\ concentration\ in\ hydrolysis\left(\frac{g}{L}\right)\right)+(mass\ \%\ of\ xylan\ in\ prehydrolysate*\frac{150}{132}*solids\ concentration\ in\ hydrolysis\ \left(\frac{g}{L}\right)}$$

For water hydrolysate, mass % glucan/xylan in prehydrolysate isn't relevant since there was no addition of prehydrolysate during hydrolysis.

Statistical analysis

All results are presented as mean values ± one standard deviation. A one-way analysis of Variance (ANOVA), followed by Tukey test were performed using SAS (Version 9.4 TS Level 1M6, SAS Inst. Inc, Cary, NC, USA). 95% confidence interval was used to determine the statistical differences among different groups.

3 Results and Discussion

CF composition

Raw biomass composition is shown in **Table 3**. Oil was extracted first from CF using hexane, followed by the removal of water and ethanol soluble extractives **(Sluiter, 2008).** Then, CF protein extraction was performed prior to sugar and lignin analysis as described in section 2.2. During the determination of structural carbohydrates and lignin, some protein is solubilized in the hydrolysis liquid during the acid hydrolysis step (**Sluiter et al., 2004).**The solubilized protein interferes with the accurate measurement of soluble lignin. Raw CF was analyzed for polysaccharides (glucan from cellulose and starch, xylan, galactan, arabinan, and mannan), total lignin, extractives (water and ethanol soluble extractives), oil, ash, and protein. Starch accounts for the noncellulosic glucan content.

Table 3 Raw corn fiber biomass composition. Data are average values of triplicate experiments ± one standard deviation.

Biomass constituents	**Composition (%, w/w)**
Glucan (cellulose)	14.8 ± 0.4
Glucan (starch)	4.9 ± 0.3
Xylan	16.0 ± 0.6
Galactan	1.7 ± 0.2
Arabinan	8.6 ± 0.5
Mannan	0.2 ± 0.01
Lignin	3.5 ± 0.2
Extractives	14.3 ± 0.4
Oil	10.7 ± 0.2
Ash	2 ± 0.1
Protein	20.1 ± 0.2

CF prehydrolysate detoxification

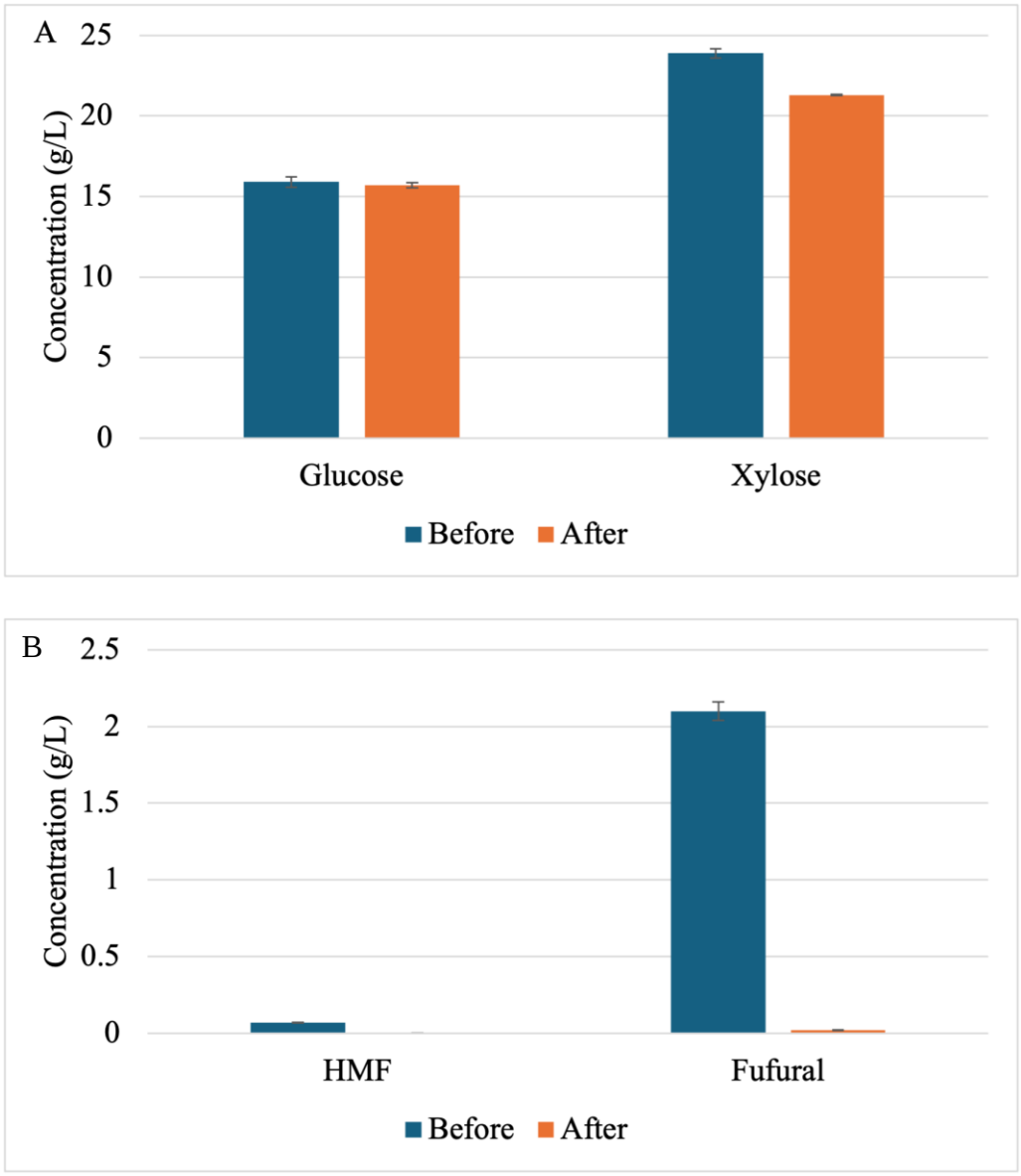

Figure 2 Effect of detoxification using activated charcoal on prehydrolysate sugars (A) and furan aldehydes (B). Data are average values for duplicate experiments, and error bars represent ± one standard deviation.

Because of CF's complex cell wall structure, hydrothermolysis was used to disrupt the cellulose, hemicellulose, and lignin chemical bonds. Any pretreatment method introduces byproducts that are toxic to fermenting organisms from either carbohydrates or lignin degradation **(Palmqvist & Hahn-Hägerdal, 2000; Jönsson et al., 2013).** Therefore, a detoxification method is often necessary to reduce or eliminate these inhibitory compounds after pretreatment. The most common inhibitors are furan aldehydes (furfural and HMF). Among different detoxification techniques that have been investigated in the past, activated charcoal has been the most efficient at removing furfural compounds with an insignificant effect on sugars.

For this study, prehydrolysate was analyzed in duplicate for sugars and furfural compounds before and after detoxification. Before detoxification, prehydrolysate had 15.9 g/L glucose, 23.9 g/L xylose, 0.07 g/L HMF, and 2.1 g/L furfural. After detoxification, it had 15.7 g/L glucose, and 21.3 g/L xylose, HMF was not detected, and 0.02 g/L furfural (Fig. 3.1). As shown in Fig 3.1, prehydrolysate detoxification using activated charcoal had a significant reduction on furan aldehydes with little effect on sugars. Furfural was reduced by more than 95% and HMF was removed completely while 1% glucose and around 11% xylose were lost during detoxification.

Previous studies have observed a similar trend using activated carbon detoxification. A study by **Guo et al. (2013)** investigated the effect of spruce hydrolysate detoxification using activated charcoal on inhibitory compounds and sugars during bacterial cellulose production. Activated charcoal treatment removed over 94% furan aldehydes with less than 5% loss in glucose and xylose. Another study by **Chen et al. (2010)** utilized calcium carbonate neutralization combined with activated carbon during detoxification of hydrolysate for SA production. The method reduced furfural compounds by approximately 92% with around 5%

loss in total sugars. The mechanism behind activated charcoal reducing inhibitors has been attributed to the fact that activated charcoal offers a large surface area and pores which facilitate the binding of organic impurities **(Ahuja et al., 2022).**

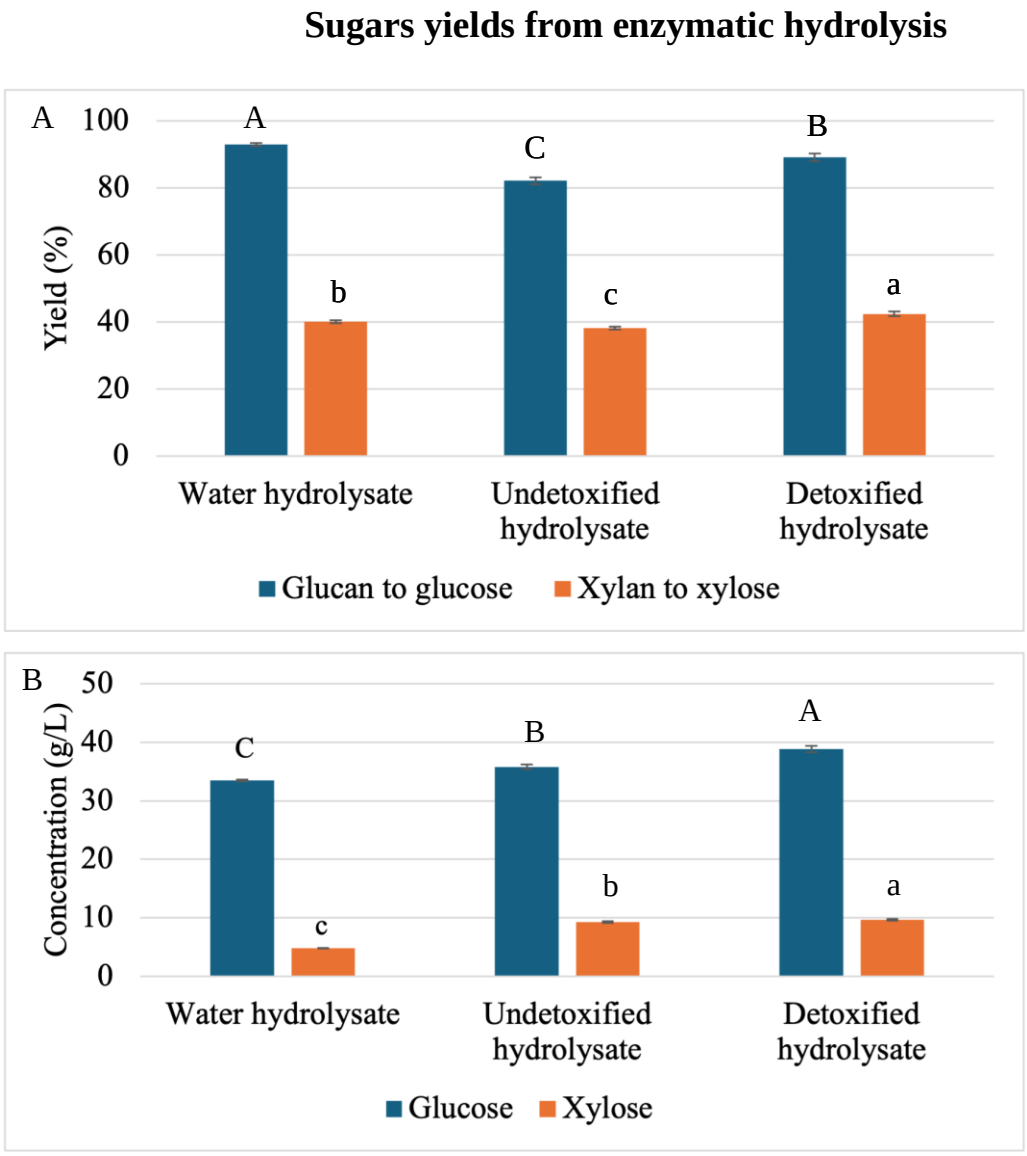

Figure 3 Glucose and xylose yields (A) and concentrations (B) after enzymatic hydrolysis for hydrolysates obtained using water, undetoxified, or detoxified prehydrolysate at 10.5% solids loading. Corn fiber was pretreated at 180 °C for 10 min with 15% solids. Data are average values for triplicate experiments, and error bars represent ± one standard deviation. Different capital letters represent significant differences of glucose among hydrolysate groups ($p < 0.05$), and different lower-case letters represent significant differences of xylose among hydrolysate groups ($p < 0.05$).

As shown in **Figure 3** A, glucose yield was the highest for water hydrolysate (92.9%), followed by detoxified hydrolysate (89.1%), and undetoxified hydrolysate (82.1%). Glucose yields for different hydrolysates were statistically different from each other. On the other hand, xylose yield was the highest for detoxified hydrolysate (42.4%), followed by water hydrolysate (40%), and finally undetoxified hydrolysate (38.2%). The xylose yields were also statistically different for different types of hydrolysates.

In terms of concentrations, glucose was the highest for detoxified hydrolysate (38.8 g/L). This was statistically different from undetoxified hydrolysate (35.8 g/L) and water hydrolysate (33.5 g/L). Xylose concentration was also the highest for detoxified hydrolysate (9.7 g/L), which was significantly different from undetoxified hydrolysate (9.3 g/L) and water hydrolysate (4.8) (**Figure 3** B). These trends observed for concentrations are different from yield trends because concentration only accounts for the total sugars (glucose or xylose) obtained after hydrolysis while yield accounts for the percentage of polymeric sugars (glucan or xylan) that were converted into monomers (glucose or xylose). Detoxified and undetoxified hydrolysates had higher sugar concentrations than water hydrolysate because the prehydrolysates that were added during hydrolysis to make these hydrolysates contained sugars. Undetoxified prehydrolysate contained 15.9 g/L glucose and 23.9 xylose while detoxified prehydrolysate contained 15.7 g/L glucose and 21.3 g/L xylose.

Enzymatic hydrolysis, which was used in this study, has been shown to result in fewer inhibitory compounds compared to acid hydrolysis (**Yoo and Pan, 2017).** A study that was done by **Vallecilla-Yepez et al. (2021)** on CF reported glucan to glucose yield of 90.2%, and xylan to xylose yield of 36.9% for CF enzymatic hydrolysis at 10% solids loading using water. These

results are comparable to the results obtained in this study, which used approximately 10.5% solids loading for enzymatic hydrolysis.

Several studies have investigated the effect of inhibitors such as furan derivatives (furfural and HMF), aliphatic acids (acetic acid, formic acid, and levulinic acid), and phenols on cellulose hydrolysis using cellulase. **Tejirian & Xu (2011)** reported that phenolics can inactivate cellulases by reversibly complexing them. It was also suggested that phenolics can inhibit enzymatic cellulolysis by adsorbing onto cellulose. **Jing et al. (2009)** investigated the inhibition performance by lignocellulose degradation products including organic acids, furan derivatives, and ethanol on cellulase enzyme Spezyme CP (Genencor, Palo Alto, CA, USA) for cellulose hydrolysis. The order of inhibition at the same concentration was as follows: lignin derivatives > furan derivatives > organic acids > ethanol. According to the author, these degradation products inhibit enzymatic hydrolysis by reducing enzyme activities and inactivating the enzymes, reversibly or irreversibly, thereby requiring more enzyme to obtain efficient cellulose conversion. In this study, the glucan to glucose and xylan to xylose yields, as well glucose and xylose concentrations after enzymatic hydrolysis for detoxified hydrolysate were significantly higher than those for undetoxified hydrolysate (**Figure 3** A and B). This difference can be attributed to a possible cellulase inhibition by inhibitory compounds which were more prevalent in undetoxified prehydrolysate compared to detoxified prehydrolysate.

SA production using *A. succinogenes*

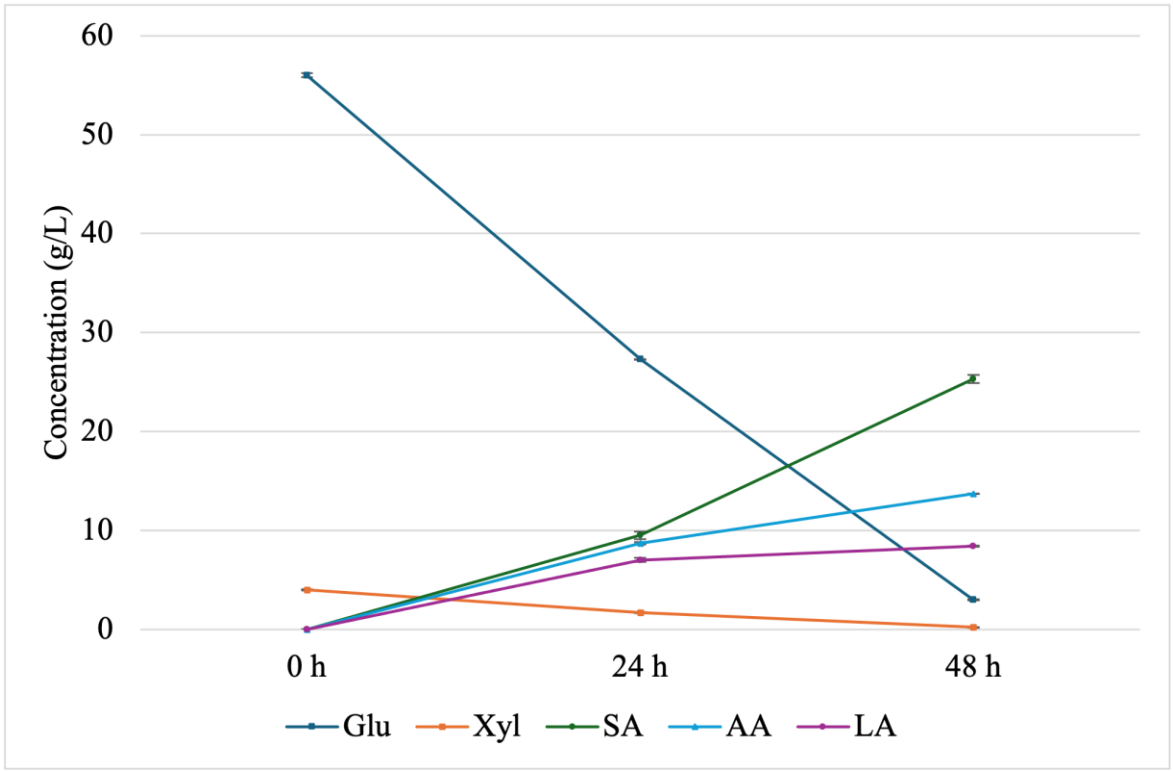

Figure 4 Time course of SA fermentation from control (initial glucose and xylose concentrations of 56 g/L and 4 g/L, respectively). Data are average values of duplicate experiments, and error bars represent ± one standard deviation. Variables shown are Glu: glucose, Xyl: xylose, SA: succinic acid, AA: acetic acid, and LA: lactic acid.

One of the major products of *A. succinogenes* fermentation is SA. For this study, glucose and xylose (the main sugars in CF) were simultaneously consumed to produce SA as a major product during *A. succinogenes* fermentation. In addition to SA, acetic acid (AA) and LA were also produced. As shown in **Figure 4**, after 24 h of fermentation, 9.5 g/L SA, 7 g/L LA, and 8.7 g/L AA were produced, while 48.8% of initial glucose and 42.5% of initial xylose remained. After 48 h, 25.3 g/L SA, 8.4 g/L LA, and 13.7 g/L AA were produced. Only 5.4% of initial glucose and 5% of initial xylose remained in solution. The yield of SA was 0.31 g SA/g sugars and 0.44 g SA/g sugars after 24 and 48 h of fermentation, respectively.

Different studies have reported different SA yields for various carbon sources using *A. succinogenes* fermentation. **Chen et al. (2010)** reported a 0.72g SA/g sugars yield from detoxified CF hydrolysate while **Chen et al. (2011)** obtained a 0.68g SA/g sugars yield from CF hydrolysate. These studies used dilute sulfuric acid for hydrolysis to obtain the hydrolysates. **Vallecilla-Yepez et al. (2021)** utilized enzymatic hydrolysis to obtain hydrolysate and reported a 0.61g SA/g sugars yield using *A. succinogenes*. The reason for the different SA yields for different studies can be attributed to different carbon sources that were used, different pH conditions, or the fact that the *A. succinogenes* organisms that were used in the studies were obtained from different suppliers. The results reported in this study (**Fig 4**) were for fermentation using *A. succinogenes* 130Z purchased from DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The initial pH for the fermentation media was 7.8, which declined to 6.1 after fermentation.

Different SA results were obtained when the same strain (*A. succinogenes* 130Z) was obtained from a different supplier - USDA Agricultural Research Service Culture Collection (Peoria, IL). **Table 4** shows the differences between the two organisms in terms of glucose, xylose, SA, LA, and AA concentrations at the start of fermentation (0 h), after 24 h, and after 48 h of fermentation.

Table 4 Differences in fermentation results between *A. succinogenes* 130Z strains ordered from DSMZ and USDA

	A. succinogenes 130Z from DSMZ			*A. succinogenes* 130Z from USDA		
	0 h	24 h	48 h	0 h	24 h	48 h
Glucose (g/L)	56	27.3	3	48	16.9	0.6
Xylose (g/L)	4	1.7	0.2	7.5	6.5	0.2
SA (g/L)	0	9.5	25.3	0	8.6	16.5
LA (g/L)	0	7	8.4	0	13.9	24.8
AA (g/L)	0	8.7	13.7	0	4.9	7.5

When the strain from USDA was used, the initial glucose and xylose concentrations were 48.0 g/L and 7.5 g/L, respectively. After 24 h of fermentation, 8.6 g/L SA, 13.9 g/L LA, and 4.9 g/L AA were obtained, with 35% of initial glucose and 86.6% of initial xylose remaining in solution. After 48 h of fermentation, 16.5 g/L SA, 24.8 g/L LA, and 7.5 g/L AA were obtained. Only 1.25% and 2.6% of initial glucose and xylose remained. The SA yield was 0.27 g SA/g sugars and 0.3 g SA/g sugars after 24 and 48 h of fermentation, respectively. In comparison, after 24 h of fermentation, the DSMZ strain produced a 0.3 g SA/g sugars yield, a 0.23 g LA/g sugars yield, and a 0.28 g AA/g sugars yield, while USDA strain produced a 0.27 g SA/g sugars yield, a 0.43 g LA/g sugars yield, and a 0.15 g AA/g sugars yield. After 48 h, DSMZ strain produced a 0.44 g SA/g sugars yield, a 0.15 g LA/g sugars yield, and a 0.24 g AA/g sugars yield, while USDA strain produced a 0.3 g SA/g sugars yield, a 0.45 g LA/g sugars yield, and a 0.14 g AA/g sugars yield. This shows that DSMZ strain had a higher SA yield and a lower LA yield than USDA strain. The initial media pH was 7.8, which declined to 6.0 after fermentation. For both fermentations, all flasks contained $MgCO_3$ at a concentration equal to the initial glucose concentration to regulate pH and supply CO_2 simultaneously. Also, acetate buffer (pH 5.0 and 50 mM) was added to all the flasks in both fermentations for pH regulation. Studies have shown that addition of $MgCO_3$ and acetate buffer increases SA production. **Vallecilla- Yepez et al. (2021)** reported a SA concentration of 28.7 g/L and a yield of 0.67 g SA/g sugars when acetate buffer was used during fermentation of sugar control mimicking CF hydrolysate using *A. succinogenes*, compared to 26.1 g/L SA concentration and 0.52 g SA/g sugars yield when no acetate buffer was used. SA yield when acetate buffer was used was significantly higher than SA when no acetate buffer was used. The authors did a study about the effect of $MgCO_3$ to glucose ratio on SA

production by CF hydrolysate fermentation and found that when $MgCO_3$:Glu ratio was 1:1, SA concentration reached 35.5 g/L compared to 30.5 g/L when the ratio was 0.8:1.

McKinlay et al. (2010) stated that *A. succinogenes* does not produce LA. The authors came to this conclusion because they believed that lactate dehydrogenase, an enzyme encoded in *A. succinogenes* genome, is only involved in lactate dehydrogenation to amino acids instead of LA production. Other studies that were done by **McKinlay et al. (2005)** and **McKinlay et al. (2007)** investigated the fermentative metabolic pathways of *A. succinogenes* in a chemically defined growth medium and by NMR and GC-MS analyses, respectively. The studies reported that *A. succinogenes* has a C3 pathway whose main products include formate, acetate, and ethanol, and a C4 pathway which produces succinate in its TCA cycle. The C4 pathway involves the reaction between phosphoenolpyruvate (PEP) and CO_2 to produce oxaloacetate (OAA), which starts the TCA cycle. Several enzymes play a role in the production of these products. For instance, pyruvate formate lyase catalyzes the production of formate, while alcohol dehydrogenase and acetate kinase are responsible for producing ethanol and acetate, respectively. On the other hand, PEP carboxykinase is responsible for the TCA cycle (C4 pathway), which produces succinate.

The results in this study do not agree with the conclusion that LA is not a byproduct of *A. succinogenes* fermentation because LA was detected in all fermentation experiments. In fact, several studies have reported LA production from *A. succinogenes* fermentation. For instance, **Li et al. (2010)** utilized *A. succinogenes* 130Z in dual phase fermentation (aerobic cultivation using oxygen, followed by anaerobic cultivation using carbon dioxide) resulting in 135.6 g/L lactic acid with an overall yield of 0.96 g/g glucose and 2.94 g/L/h productivity. At the end of fermentation, the following organic acids were produced per mole of glucose: 1.93 mol LA, 0.37

mol SA, 0.69 mol acetic acid, and 0.21 mol formic acid. Similarly, **Wang et al. (2014)** developed a novel method for LA production using *A. succinogenes.* In the study, a microfiltration membrane was integrated in fermentation for separation, and LA was recovered using resin adsorption. LA production was thereby increased from 141.2 g/L to 183.4g/L, with a yield of 0.97g/g glucose and 1.53g/L/h productivity.

Several studies have investigated how pH and CO_2 levels affect SA or LA production. For instance, **Samuelov et al. (1991)** investigated the role of CO_2 levels and pH on SA production from glucose by *Anaerobiospirillum succiniciproducens*. This organism follows the same pathway as *A. succinogenes* during SA production, which involves metabolizing glucose to PEP, then to OAA, and finally to SA. Thus, it can be speculated that the behaviors in both organisms would be comparable. In the study, at pH 7.2, LA was produced as a major product. When pH was adjusted to 6.2 with excess CO_2, PEP carboxykinase (enzyme responsible for SA production) levels increased while lactate dehydrogenase and alcohol dehydrogenase levels (enzymes responsible for LA production and ethanol, respectively) decreased. As a result, SA was the major product while trace amounts of LA were detected. Under both pH conditions, acetate and small amounts of ethanol were detected. The production of LA in this study shows that lactate dehydrogenase can in fact catalyze the production of LA depending on certain fermentation conditions.

Another study by **Liu et al. (2008)** investigated the influence of pH on SA production from glucose using *A. succinogenes*. The authors reported that the organism did not grow and did not produce any SA at pH below 5.5. The optimal pH for SA production was 6.0-7.2, with maximum SA concentration (27.5 g/L) when pH was maintained at 6.7. At pH below 6.0, the cells did not grow well, and SA production was lower (17.5 g/L). AA was also detected (less

than 5 g/L). The lower SA yields at low pH values were attributed to possible higher maintenance requirements. This shows that lactate dehydrogenase can in fact produce LA if the conditions for growth are favorable. This shows that a difference in pH can also play a role in SA production yields.

Since pH, organism strain, and other growth conditions were the same for both studies, the difference in SA yields and other byproducts between the two fermentations can be attributed to a difference in sugar concentrations of the carbon sources or the fact that organisms that were used were obtained from different suppliers.

The Loss of SA production ability by *A. succinogenes*

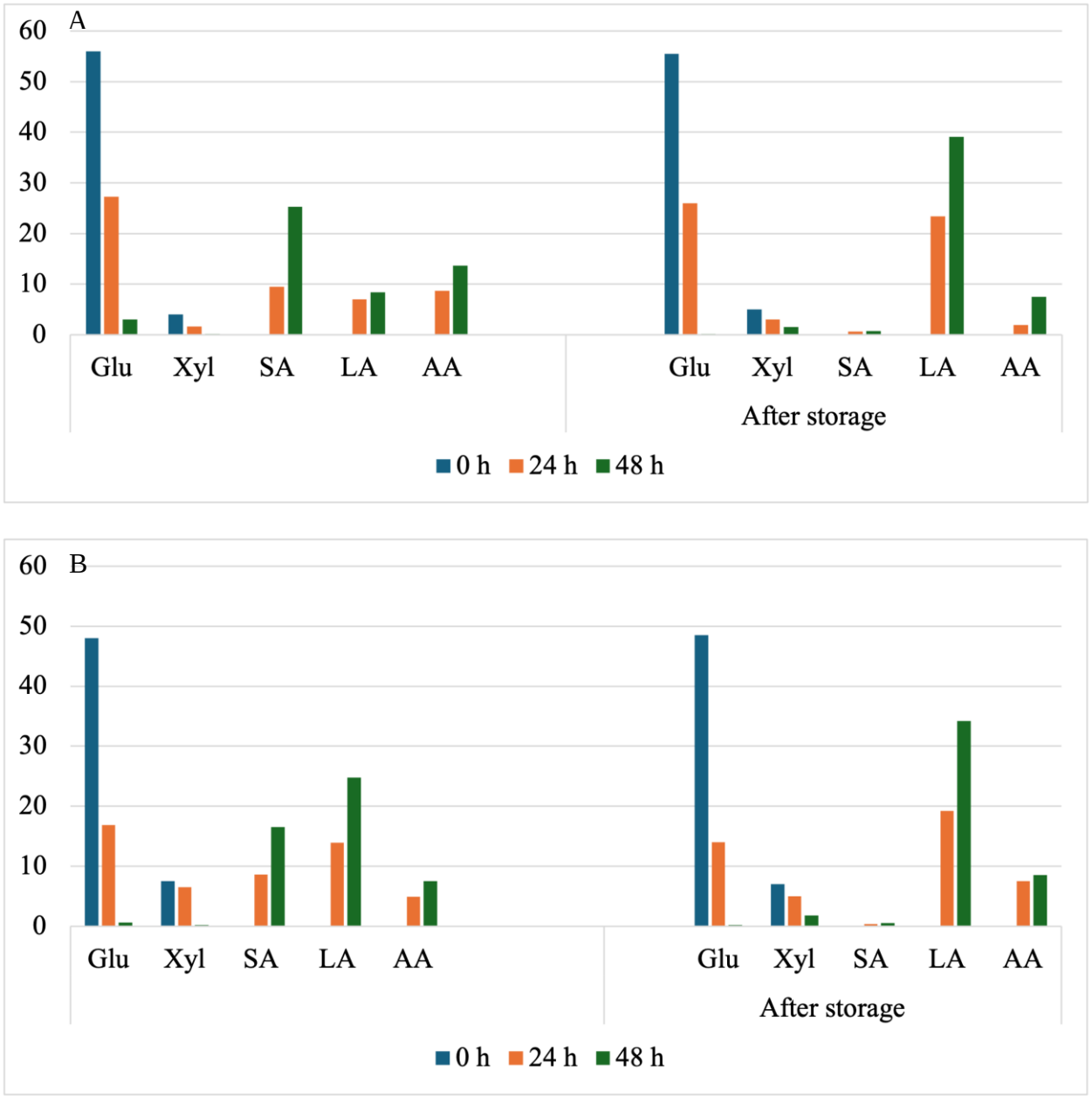

Figure 5 Organic acids production by DSMZ strain (A) and USDA strain (B) before storage (left) and after 3 months of storage (right) in 5% DMSO. Data are average values of duplicate experiments. Variables shown are Glu: glucose, Xyl: xylose, SA: succinic acid, LA: lactic acid, and AA: acetic acid. Data are average values of duplicate experiments

In this study, it was observed that *A. succinogenes* 130Z obtained from both DSMZ and the USDA lost the ability to produce SA after 3 months of storage at -80 °C using 5% DMSO as a cryoprotectant. From here on, "fresh strain" will refer to the organism before losing the ability to make SA, while "stored strain" will refer to the organism after losing the ability to make SA. As shown in **Fig 5** A, after 24 h of fermentation, 48.8% of initial glucose and 42.5% of initial xylose remained with the DSMZ fresh strain, while 46.8% of initial glucose and 60% of initial xylose remained with the DSMZ stored strain. Also, 9.5 g/L SA, 7 g/L LA, and 8.7 g/L AA were produced by the DSMZ fresh strain, while 0.7 g/L SA, 23.4 g/L LA, and 2 g/L AA were produced by the DSMZ stored strain. After 48 h, 5.4% of initial glucose and 5% of initial xylose remained in solution with the DSMZ fresh strain, while less than 1% of initial glucose and 32% of initial xylose remained in solution with the DSMZ stored strain. 25.3 g/L SA, 8.4 g/L LA, and 13.7 g/L AA were produced by the DSMZ fresh strain, while 0.8 g/L SA, 39.1 g/L LA, and 7.5 g/L AA were produced by the DSMZ stored strain. The yield of SA was 0.31 g SA/g sugars and 0.44 g SA/g sugars after 24 and 48 h of fermentation, respectively for the DSMZ fresh strain.

As shown in **Fig 5** B, after 24 h of fermentation, 35.2 % of initial glucose and 86.7% of initial xylose remained with the USDA fresh strain, while 28.9% of initial glucose and 71.4% of initial xylose remained with the USDA stored strain. In terms of organic acids production, the USDA fresh strain produced 8.6 g/L SA, 13.9 g/L LA, and 4.9 g/L AA, and the USDA stored strain produced a negligible amount of SA (0.4 g/L), 19.2 g/L LA, and 7.5 g/L AA. After 48 h of fermentation, only 1.25% of initial glucose and 2.7% of initial xylose remained with the USDA fresh strain, while 0.4% of initial glucose and 25.7% of initial xylose remained with the USDA stored strain. Additionally, 16.5 g/L of SA, 24.8 g/L LA, and 7.5 g/L AA were produced by the

USDA fresh strain, while a negligible amount of SA (0.5 g/L), 34.2 g/L LA, and 8.5 g/L AA were produced by the USDA stored strain.

After 48 h of fermentation, the USDA fresh strain had a 0.3 g SA/g sugars yield compared to the USDA stored strain, which barely made any SA. Also, the USDA fresh strain had a 0.45 g LA/g sugars yield and a 0.14 g AA/g sugars yield compared to a 0.64 g LA/g sugars yield and a 0.16 g AA/g sugars yield for the USDA stored strain. Similarly, after 48 h of fermentation, the DSMZ fresh strain had a 0.44 g SA/g sugars yield compared to the DSMZ stored strain, which barely made any SA. The DSMZ fresh strain had a 0.15 g LA/g sugars yield and a 0.24 g AA/g sugars yield compared to a 0.67 g LA/g sugars yield and a 0.13 g AA/g sugars yield for the DSMZ stored strain.

From the results above, after 3 months of storage, the fresh strains from both USDA and DSMZ lost their ability to produce SA and started making more LA instead. The same observations were reported by **Long et al. (2021)** who reported a complete loss of SA production and increase in LA production after approximately 10 months of *A. succinogenes* 130Z storage in the lab using 20% glycerol as cryoprotectant. In their study, the authors ruled out contamination and mutation as the causes for the loss after bacterial sequencing comparisons between the SA producing and the SA non-producing strain. They also investigated storage stress as another factor by comparing the effectiveness between different cryoprotectants including 20% glycerol, 5% DMSO, and sucrose (0.15M). DMSO and sucrose showed better protection than glycerol. However, there was not a complete loss in SA production for the cells stored in glycerol, so this was not sufficient to determine definitively the cause of the SA loss observed after 10 months storage.

Vallecilla- Yepez (2022) reported results similar to **Long et al. (2021)** whereby *A. succinogenes* cells showed a much lower SA production and an increase in LA after 24 months of storage in 20% glycerol. The author did an investigation to determine the effectiveness between 20% glycerol and 5% DMSO as cryoprotectants. A fresh *A. succinogenes* strain was purchased and aliquots of cells with each cryoprotectant were stored at -80 °C for 7 days prior to fermentation. Fermentation results after 24 h showed that the strain stored in 20% glycerol produced 0.62 g/L SA and 15.4 g/L LA, while the strain stored in 5% DMSO produced 12.8 g/L SA with no LA. These studies show that 5% DMSO was successful as a cryoprotectant to sustain *A. succinogenes* ability to make SA over time after storage. It is not clear why cells in the present study lost their ability to produce SA after 3 months of storage, even though 5% DMSO was used as a cryoprotectant. **Reddy at el. (2009)** showed that strategies like applying moderate stresses such as cold, heat, starvation, and acid shocks before freezing can improve bacterial resistance to freezing by preparing the cells to react better to unfavorable conditions. Other storage strategies that haven't been tested include freeze drying and cryopreservation in the vapor phase of liquid nitrogen at -130 °C. These strategies might be applied to *A. succinogenes* and potentially preserve the cells better over time. Also, future studies should be done to investigate the effects of storage conditions contributing to the loss of SA production by *A. succinogenes* over time.

A new *A. succinogenes* 130Z sample was obtained from USDA Agricultural Research Service Culture Collection (Peoria, IL) after the results above were observed; however, cell from this sample did not produce any SA, even though the cells were not subjected to cryostorage. The new bacterial cells did not produce any SA. However, they produced 32 g/L LA with a 0.62 g LA/g sugars and 7.4 g/L AA. These concentrations were comparable to those produced by cells

that were stored previously in DMSO for 3 months. This shows that *A. succinogenes* 130Z is not a good bacterial strain for large scale industrial SA production since it is very unstable and unpredictable.

Effect of gas bubbling on LA production by *A. succinogenes*

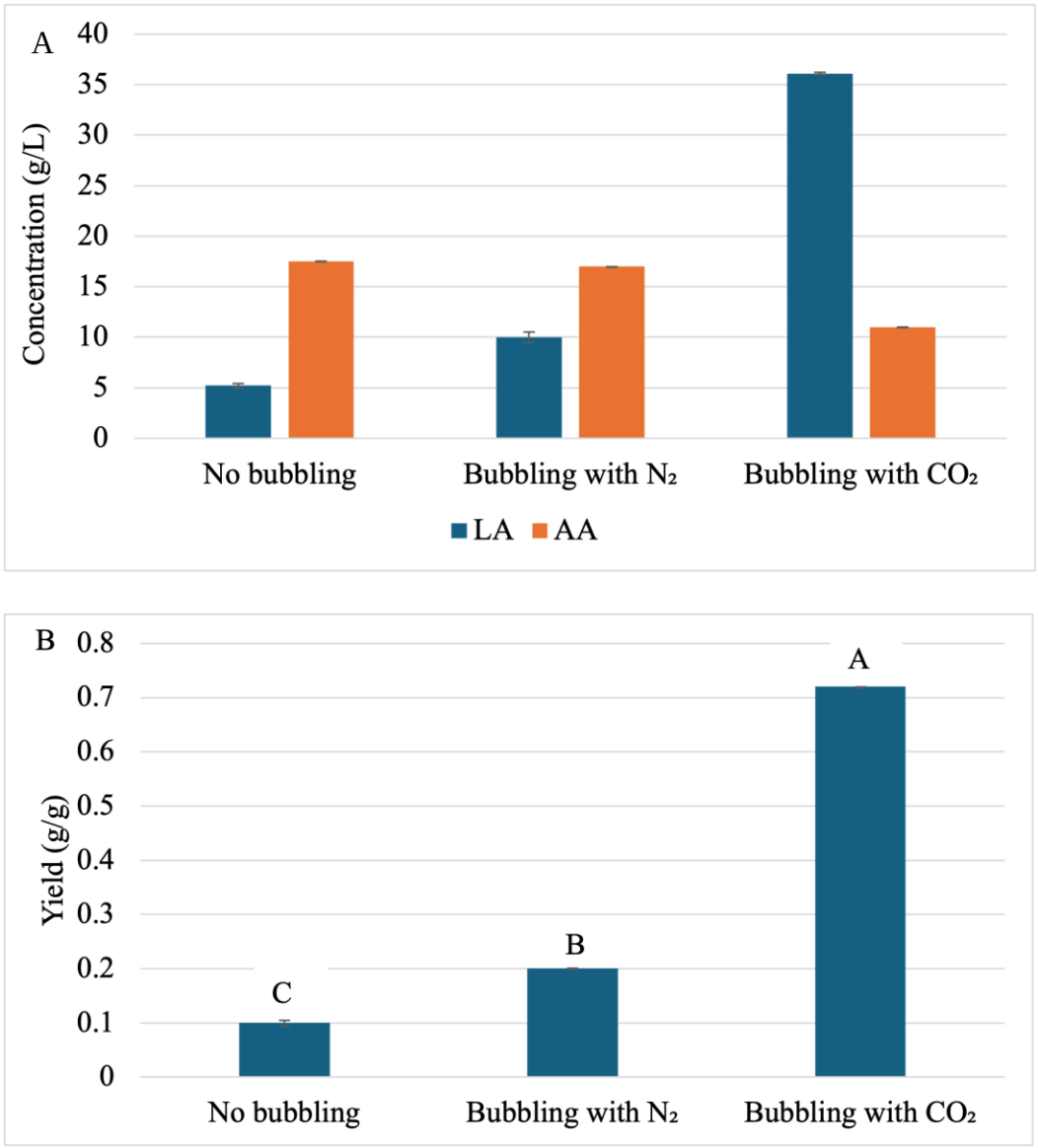

Figure 6 LA and AA concentrations (A) and LA yields (B) after 48 hours of fermentation for media that was either left unbubbled, bubbled with N_2, or bubbled with CO_2. Data are average values for triplicate experiments, and error bars represent ± one standard deviation. Different capital letters represent significant differences of LA among fermentation treatment groups ($p < 0.05$).

Because both the DSMZ and the USDA strains lost their ability to make SA and started producing more LA after 3 months of storage, the focus shifted towards LA production. The new USDA strain which did not produce any SA, even though the cells were not subjected to cryostorage, was used for subsequent studies. This study investigated the effect of bubbling fermentation media with either N_2 or CO_2 on LA production. The purpose of bubbling is to remove excess air from fermentation media. For all fermentation media, the initial glucose and xylose concentrations were 50 g/L and 2.5 g/L, respectively. After 24 h of fermentation, 84.2% of initial glucose remained for unbubbled media, 97.4% of initial glucose remained for N_2 bubbled media, while 52.3% of initial glucose remained for CO_2 bubbled media. After 48 h of fermentation, 20.4% of initial glucose remained for unbubbled media, 14.9% of initial glucose remained for N_2 bubbled media, while only 4% of initial glucose remained for CO_2 bubbled media. Most of the xylose was remaining after 48 h of fermentation for all media types (80%).

There was no SA production for all media since the organism had already lost its capacity to make SA. After 24 h of fermentation, there was no LA production for unbubbled media and N_2 bubbled media. On the other hand, cells produced 16.2 g/L LA with a 0.32g LA/g glucose yield from initial glucose in CO_2 bubbled media. Cells produced 5.6 g/L AA in unbubbled media, 1 g/L AA in N_2 bubbled media, and 6 g/L AA in CO_2 bubbled media. After 48 h of fermentation, cells in unbubbled media produced 5.2 g/L LA, 17.5 g/L AA, and 9.5 g/L FA. Cells in N_2 bubbled media produced 10 g/L LA, 17 g/L AA, and 10.3 g/L FA. On the other hand, cells in CO_2 bubbled media produced 36.1 g/L LA and 10.9 g/L AA. It is worth mentioning that after 48 h of fermentation, 6.8 g/L and 6.3 g/L of ethanol were detected in unbubbled and N_2 bubbled media, respectively. On the contrary, no ethanol or FA were detected in CO_2 bubbled media after 48 h of fermentation. As shown in **Fig 6** B, the LA yields were 0.1g LA/g glucose for

unbubbled media, 0.2g LA/g glucose for N_2 bubbled media, and 0.72g LA/g glucose for CO_2 bubbled media after 48 h of fermentation. These yields were calculated as the ratio between outputs (LA) to inputs (sugars). Only glucose was considered since xylose was not consumed significantly. This was considered because a biorefinery would be interested in knowing how much LA could be obtained from a certain initial amount of sugars. From these results, bubbling with CO_2 was chosen as the best LA producing treatment because it was able to produce the highest LA concentrations and yields.

Several studies have investigated the relationship between CO_2 availability and SA as well as LA production. **Samuelov et al. (1991)** did a study on the influence of CO_2 levels and pH on SA production by *Anaerobiospirillum succiniciproducens*. During the study, CO_2 was supplied as either CO_2 gas, Na_2CO_3, $MgCO_3$, $CaCO_3$, or a combination of these. The author reported that under excess CO_2 and pH 6.2, PEP carboxykinase increased and SA production was enhanced. There was no detection of lactate or alcohol dehydrogenase. On the other hand, at pH 7.2 and low CO_2, lactate and alcohol dehydrogenase activities increased, and LA was the main product. The authors reported that there is a threshold level of CO_2 for enhanced SA production. When the molar ratio of CO_2 to glucose was between 1.0 to 0.5, 90% of glucose was consumed and 65% of the carbon was converted to SA. LA and ethanol were not detected. However, when the molar ratio of CO_2 to glucose was reduced to 0.065, 45 to 60% of glucose was consumed, 50% of the carbon was converted into LA, 30% was converted into SA, and there was a balance between ethanol, FA, and AA. The authors reported that their fermentation results indicated that LA production in *Anaerobiospirillum succiniciproducens* is controlled by high pH while SA production is controlled by CO_2 availability. **Vallecilla- Yepez et al. (2021)** investigated the effect of $MgCO_3$:glucose (Mg:Glu) ratio on SA and byproducts production by *A. succinogenes*.

The authors reported that when Mg:Glu ratio was 0.8:1, SA concentration of 30.5 g/L was obtained after 36 h of fermentation. When the ratio was increased to 1:1, the SA concentration was 35.5 g/L. At 0.8:1 ratio, glucose was still present at the end of 36 h of fermentation. Also, LA and ethanol were detected. On the other hand, when the ratio was 1:1, no glucose was present at the end of 36 h of fermentation, and there was no detection of either LA or ethanol. It should be mentioned that AA and FA were detected for both Mg:Glu ratios. The results from these studies show that CO_2 availability enhances carbon flux to the SA route (C4 pathway), while reducing the byproducts formation including LA, AA, FA, and ethanol (C3 pathway).

To the author's knowledge, this is the first study to investigate the impact of bubbling gases (CO_2 and N_2) to organic acids production. In this study, $MgCO_3$ was used to supply CO_2 and regulate pH simultaneously. In all fermentation media, the Mg:Glu ratio was 1:1 before any bubbling. It is worth mentioning that a small experiment was performed when the fresh USDA strain (with SA production ability) was obtained. In the experiment, two flasks containing media were bubbled with CO_2 and two other flasks containing media were bubbled with N_2. The fresh USDA strain was used for fermentation in all flasks, and it was found that cells in N_2 bubbled media produced 17 g/L SA with a 0.4 g SA/g sugars and 10 g/L LA. On the other hand, cells in CO_2 bubbled media did not produce any SA and produced 31 g/L LA with a yield of 0.66 g LA/g sugars. The results from this experiment show that bubbling with N_2 favored SA production while bubbling with CO_2 favored LA production.

The results from **Figure 6** A and B show that bubbling with CO_2 had the highest LA concentrations and yields. This also indicates that higher CO_2 availability enhanced LA production instead of SA production. This is different from what the other studies mentioned above reported. The reason for this is because pyruvate can be directly converted to either LA or

acetyl-coA. Acetyl-coA can then be converted to formic acid and CO_2 plus either 1) ethanol or 2) acetate + ATP. As CO_2 is a product from pyruvate being converted into acetate and/or ethanol, bubbling with CO_2 caused CO_2 product inhibition, which caused pyruvate to be converted directly into LA as the main product.

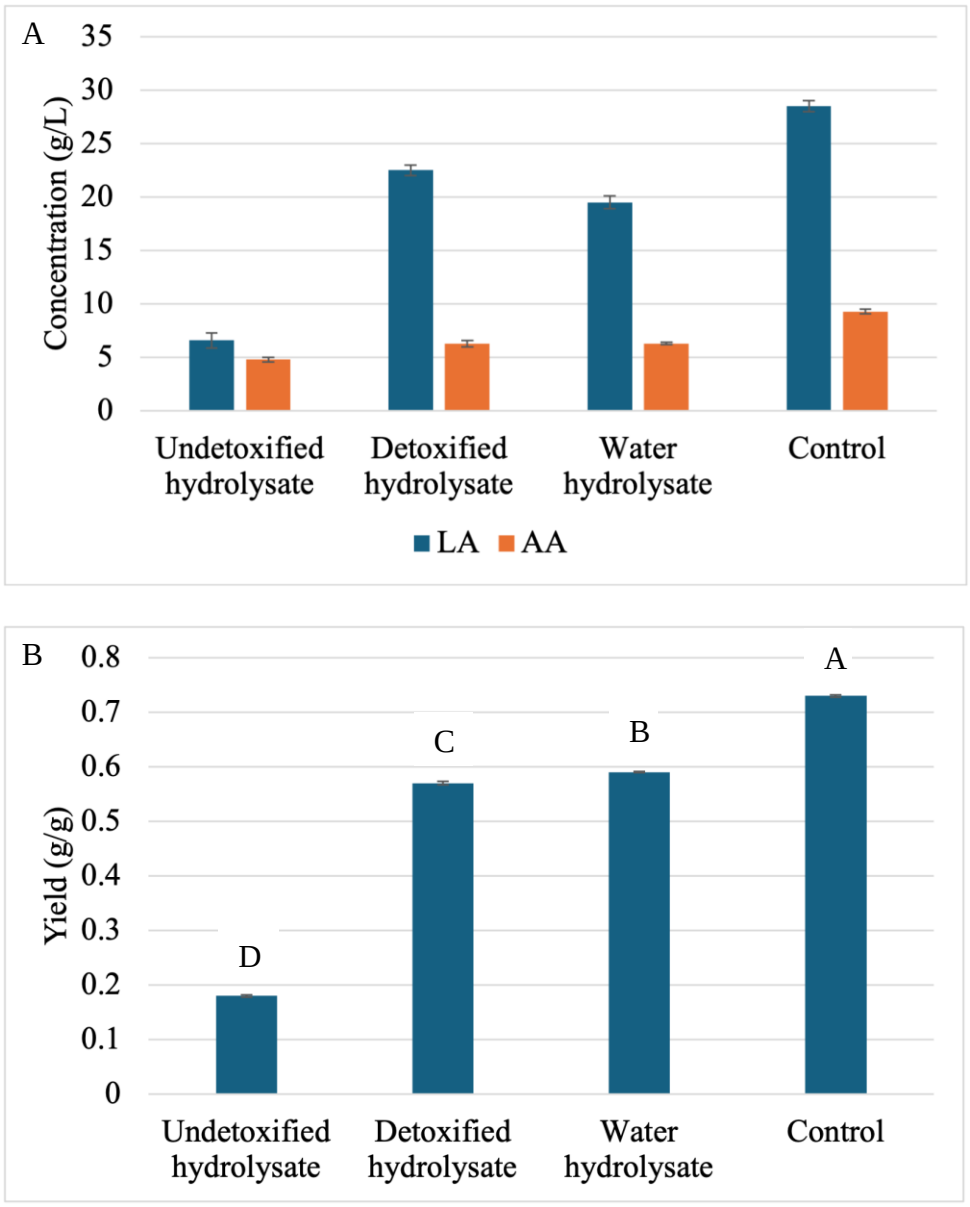

Figure 7 LA and AA concentrations (A) and LA yields (B) after 48 hours of fermentation of hydrolysates obtained using undetoxified or detoxified prehydrolysate, or water at 10.5 % solids loading. The control was mimicking detoxified hydrolysate with the highest sugar concentrations of all hydrolysates (39.5 g/L and 9.5 g/L glucose and xylose, respectively). Data are average values for triplicate experiments, and error bars represent ± one standard deviation. Different capital letters represent significant differences of LA among fermentation treatment groups ($p < 0.05$).

For this study, the feasibility of using CF hydrolysates for LA production were investigated. To achieve this, *A. succinogenes* fermentation of hydrolysates (undetoxified,

detoxified, and water) and a control mimicking the sugars in detoxified hydrolysate was carried out. Undetoxified hydrolysate had initial glucose and xylose concentrations of 37.4 g/L and 9 g/L, respectively. Detoxified hydrolysate had initial glucose and xylose concentrations of 39.5 g/L and 9.5 g/L, respectively. Water hydrolysate had initial glucose and xylose concentrations of 33.1 g/L and 5 g/L, respectively. Control had initial glucose and xylose concentrations of 39.5 g/L and 9.5 g/L, respectively. After 24 h of fermentation, 86.9 % of initial glucose remained for undetoxified hydrolysate, 88.7% of initial glucose remained for detoxified hydrolysate, 65% of initial glucose remained for water hydrolysate, and 42% of initial glucose remained for the control. After 48 h, 59% of initial glucose remained for undetoxified hydrolysate, 15.6% of initial glucose remained for detoxified hydrolysate, 18.7% of initial glucose remained for water hydrolysate, and 2.5% of initial glucose remained for control. Xylose was barely consumed for all hydrolysates and control (more than 98% remained) after 48 h.

In terms of organic acids production, there was no SA production for all hydrolysates and control since the bacteria had lost the ability to make SA. After 24 h of fermentation, no LA was detected for undetoxified hydrolysate, while a very small amount of LA (0.6 g/L) was detected for detoxified hydrolysate. Water hydrolysate had 7 g/L LA with a yield of 0.21g LA/g glucose, while control had 14.5 g/L LA with a yield of 0.37g LA/g glucose. The delay in LA production for both undetoxified and detoxified hydrolysate might have been caused by the inhibition from higher initial AA concentration in solution. The initial AA concentrations were 12 g/L, 12.1 g/L, 10 g/L, and 3 g/L for undetoxified hydrolysate, detoxified hydrolysate, water hydrolysate, and control, respectively. Hydrolysates had higher AA concentrations because of the acetate buffer that was added during hydrolysis. AA concentration in control originated from sodium acetate trihydrate which was used in preparation of fermentation media. **Larsson et al. (1999)** reported

that the reason for longer lag phase in cell growth caused by inhibitors might be because of the lowering in membrane permeability of the cell, which decreases sugars assimilation. Additionally, 4.1 g/L AA and 1.1 g/L FA were produced for undetoxified hydrolysate, 4.3 g/L AA and 1.2 g/L FA were produced for detoxified hydrolysate, 4.2 g/L AA and 0.7 g/L FA were produced for water hydrolysate, while 4.9 g/L AA and no FA were detected for control.

As shown in **Figure 7** A and B, after 48 h, 6.6 g/L LA with a yield of 0.18g LA/g glucose and 4.8 g/L AA were produced by undetoxified hydrolysate, 22.5 g/L LA with a yield of 0.57 g LA/g glucose and 6.3 g/L AA were produced by detoxified hydrolysate, 19.5 g/L LA with a yield of 0.59g LA/g glucose and 6.3 g/L AA were produced by water hydrolysate, while 28.5 g/L LA with a yield of 0.73g LA/g glucose and 9.3 g/L AA were produced by control. It is worth mentioning that after 48 h, 0.5g/L ethanol and 1.2 g/L ethanol were detected for undetoxified hydrolysate and detoxified hydrolysate, respectively. On the contrary, no ethanol was detected for both water hydrolysate and control.

A few studies have also investigated the production of LA using *A. succinogenes*. **Rahim et al. (2023)** obtained 31.64 g/L LA and 0.88 g/L/h productivity from palm kernel cake hydrolysate fermentation. **Li et al. (2010)** used dual phase fermentation (aerobic cultivation occurred in oxygen aerated fermentation media, followed by cultivation in anaerobic media sparged with carbon dioxide) of glucose resulted in 135.6 g/L LA with 0.96 g LA/g glucose yield and 2.94 g/L/h productivity. **Wang et al. (2014)** obtained 183.4 g/L LA with 0.97 g LA/g glucose yield and 1.53 g/L/h productivity when microfiltration membrane was integrated in fermentation for separation. The results in the present study show that the use of CF hydrolysates presents an alternative way of LA production using low cost and renewable carbon sources. As shown in **Figure 7** B, detoxified hydrolysate and water hydrolysate have comparable LA yields.

The use of detoxified hydrolysate is beneficial for reducing water use, especially for large scale industrial LA production in biorefineries, because instead of using water in hydrolysis, detoxified prehydrolysate can be used instead to make hydrolysate. The results in this study can be utilized by biorefineries whereby after pretreatment, all prehydrolysate can be detoxified and re-used in hydrolysis to make sugar hydrolysates for fermentation.

4 Summary, Conclusions, and Future Work

Summary and Conclusions

This thesis reported that corn fiber (CF), a byproduct of the dry grind corn ethanol process, has potential as an economical and renewable feedstock for succinic acid (SA) and lactic acid (LA) bioproduction. These organic acids have applications in food, agricultural, chemical, and pharmaceutical industries. Currently, they are produced primarily from fossil-fuel feedstocks using an expensive and ecologically questionable process. *Actinobacillus succinogenes* has the potential to be used for bioproduction of these acids.

It was shown that prehydrolysate detoxification using activated charcoal resulted in a significant reduction of furan aldehydes in prehydrolysate with little effect on sugar concentration. Furfural was reduced by more than 95% and HMF was removed completely while 1% glucose and 11% xylose were lost during detoxification. After detoxification, water, undetoxified or detoxified prehydrolysate, acetate buffer, cellulase, and pretreated solids were mixed during enzymatic hydrolysis, resulting in water, undetoxified, or detoxified hydrolysates. Glucan to glucose yield was the highest for water hydrolysate (92.9%) while detoxified hydrolysate had the highest glucose concentration (38.8 g/L).

When a control mimicking the sugars in hydrolysates in both sugar types and concentrations was fermented, a maximum SA concentration of 25.3 g/L with a yield of 0.44 g SA/g sugars was obtained. Unfortunately, after 3 months of storage in 5% DMSO, *A. succinogenes* cells lost their ability to make SA and started making more LA. Thus, focus shifted towards LA production. An experiment was done to compare the effect of N_2 and CO_2 bubbling, as well as no bubbling, on LA yields. Gas bubbling was done to remove O_2 from the media. It was found that cells in CO_2 bubbled media produced the highest LA concentration of 36.1 g/L

LA with a 0.72 g LA/g glucose yield. When detoxified, undetoxified, and water hydrolysates were bubbled with CO_2 and fermented, the cells in detoxified hydrolysate produced the highest LA concentration of all hydrolysates (22.5 g/L LA with a 0.57 g LA/g glucose yield). This thesis investigations show that the use of CF hydrolysates presents an alternative way of organic acids bioproduction using low cost and renewable carbon sources. Also, the use of detoxified hydrolysates for organic acids production will reduce water loss, especially for large scale industrial biorefineries, because instead of using water in hydrolysis, detoxified prehydrolysate can be used instead to make hydrolysate. This can be utilized by biorefineries whereby after pretreatment, all prehydrolysate can be detoxified and re-used in hydrolysis to make sugar hydrolysates for fermentation. However, as shown by the results in this thesis, *A. succinogenes* is not a good strain for industrial SA production as it loses its ability to make SA over time.

Future Work

To establish a successful and sustainable SA and LA production bioprocess, a high yield and resistant production strain, and suitable growth parameters are necessary. The author suggests metabolic engineering of *A. succinogenes* to enhance its production yields and to improve its resistance to cold temperatures to help preserve it better over time. Also, freeze drying and cryopreservation in the vapor phase of liquid nitrogen at -130 °C might be helpful tools to better preserve the cells over time. In addition to that, applying moderate stresses such as cold, heat, starvation, and acid shocks before freezing can help to improve bacterial resistance to freezing by preparing the cells to react better to unfavorable conditions (**Reddy at el., 2009)**. In the future, the author suggests using a bench scale bioreactor during fermentations to control pH

and other growth parameters better over the course of fermentation. This will enhance the output yields by avoiding fluctuations during fermentation and maintaining the growth conditions consistent over the course of fermentation.

5 References

Adney, B., & Baker, J. (2008). Measurement of Cellulase Activities: Laboratory Analytical Procedure (LAP); Issue Date: 08/12/1996. *Technical Report*

Ahn, J. H., Jang, Y.-S., & Lee, S. Y. (2016). Production of succinic acid by metabolically engineered microorganisms. *Current Opinion in Biotechnology*, *42*, 54–66. https://doi.org/10.1016/j.copbio.2016.02.034

Ahuja, V., Kshirsagar, S., Ghosh, P., Sarkar, B., Sutar, A., More, S., & Dasgupta, D. (2022). Process development for detoxification of corncob hydrolysate using activated charcoal for xylitol production. *Journal of Environmental Chemical Engineering*, *10*(1), 107097. https://doi.org/10.1016/j.jece.2021.107097

Anuradha, R., Suresh, A. K., & Venkatesh, K. V. (1999). Simultaneous saccharification and fermentation of starch to lactic acid. *Process Biochemistry*, *35*(3), 367–375. https://doi.org/10.1016/S0032-9592(99)00080-1

Balla, E., Daniilidis, V., Karlioti, G., Kalamas, T., Stefanidou, M., Bikiaris, N. D., Vlachopoulos, A., Koumentakou, I., & Bikiaris, D. N. (2021). Poly(lactic Acid): a versatile biobased polymer for the future with multifunctional properties—from monomer synthesis, polymerization techniques and molecular weight increase to PLA applications. *Polymers*, *13*(11), 1822. https://doi.org/10.3390/polym13111822

Barcelos, C. A., Maeda, R. N., Santa Anna, L. M. M., & Pereira, N. (2016). Sweet sorghum as a whole-crop feedstock for ethanol production. *Biomass and Bioenergy*, *94*, 46–56. https://doi.org/10.1016/j.biombioe.2016.08.012

Bechthold, I., Bretz, K., Kabasci, S., Kopitzky, R., & Springer, A. (2008). Succinic acid: a new platform chemical for biobased polymers from renewable resources. *Chemical Engineering & Technology*, *31*(5), 647–654. https://doi.org/10.1002/ceat.200800063

Borges, E. R., & Pereira, N. (2011). Succinic acid production from sugarcane bagasse hemicellulose hydrolysate by *Actinobacillus succinogenes*. *Journal of Industrial Microbiology & Biotechnology*, *38*(8), 1001–1011. https://doi.org/10.1007/s10295-010-0874-7

Brethauer, S., & Studer, M. H. (2015). Biochemical conversion processes of lignocellulosic biomass to fuels and chemicals – *A Review. CHIMIA, 69(10), 572*. https://doi.org/10.2533/chimia.2015.572

Buhner, J., & Agblevor, F. A. (2004). Effect of detoxification of dilute-acid corn fiber hydrolysate on xylitol production. *Applied Biochemistry and Biotechnology*, *119*(1), 13–30. https://doi.org/10.1385/abab:119:1:13

Capareda, S. C. (2022). 1—Comprehensive biomass characterization in preparation for conversion. In D. Mohan, C. U. Pittman, & T. E. Mlsna (Eds.), *Sustainable Biochar for Water*

and Wastewater Treatment (pp. 1–37). Elsevier. https://doi.org/10.1016/B978-0-12-822225-6.00015-4

Carvalho, M., Matos, M., Roca, C., & Reis, M. A. M. (2014). Succinic acid production from glycerol by *Actinobacillus succinogenes* using dimethylsulfoxide as electron acceptor. *New Biotechnology*, *31*(1), 133–139. https://doi.org/10.1016/j.nbt.2013.06.006

Chen, K., Jiang, M., Wei, P., Yao, J., & Wu, H. (2010). Succinic acid production from acid hydrolysate of corn fiber by *Actinobacillus succinogenes*. *Applied Biochemistry and Biotechnology*, *160*(2), 477–485. https://doi.org/10.1007/s12010-008-8367-0

Chen, K.-Q., Li, J., Ma, J.-F., Jiang, M., Wei, P., Liu, Z.-M., & Ying, H.-J. (2011). Succinic acid production by *Actinobacillus succinogenes* using hydrolysates of spent yeast cells and corn fiber. *Bioresource Technology*, *102*(2), 1704–1708. https://doi.org/10.1016/j.biortech.2010.08.011

Chen, K.-Q., Zhang, H., Miao, Y.-L., Jiang, M., & Chen, J.-Y. (2012). Enhanced succinic acid production from sake lees hydrolysate by dilute sulfuric acid pretreatment and biotin supplementation. *Journal of Sustainable Bioenergy Systems*, *2*(2), Article 2. https://doi.org/10.4236/jsbs.2012.22003

Cheng, P., Mueller, R. E., Jaeger, S., Bajpai, R., & Iannotti, E. L. (1991). Lactic acid production from enzyme-thinned corn starch using *Lactobacillus amylovorus*. *Journal of Industrial Microbiology*, *7*(1), 27–34. https://doi.org/10.1007/BF01575599

Clark, S., 2014. Global bio succinic acid market (applications and geography)—size, share, trends, analysis, research, future demand, scope and forecast, 2013–2020. *Allied Mark. Res*

Cok, B., Tsiropoulos, I., Roes, A. L., & Patel, M. K. (2014). Succinic acid production derived from carbohydrates: an energy and greenhouse gas assessment of a platform chemical toward a bio-based economy. *Biofuels, Bioproducts and Biorefining*, *8*(1), 16–29. https://doi.org/10.1002/bbb.1427

Datta, R., Tsai, S.-P., Bonsignore, P., Moon, S.-H., & Frank, J. R. (1995). Technological and economic potential of poly(lactic acid) and lactic acid derivatives. *FEMS Microbiology Reviews*, *16*(2–3), 221–231. https://doi.org/10.1111/j.1574-6976.1995.tb00168.x

Demirci, A., Pometto, A. L., & Johnson, K. E. (1993). Lactic acid production in a mixed-culture biofilm reactor. *Applied and Environmental Microbiology*, *59*(1), 203–207. https://doi.org/10.1128/aem.59.1.203-207.1993

Ferone, M., Raganati, F., Ercole, A., Olivieri, G., Salatino, P., & Marzocchella, A. (2018). Continuous succinic acid fermentation by *Actinobacillus succinogenes* in a packed-bed biofilm reactor. *Biotechnology for Biofuels*, *11*(1), 138. https://doi.org/10.1186/s13068-018-1143-7

Gong, W., Liu, C., Mu, X., Du, H., Lv, D., Li, B., & Han, S. (2015). Hydrogen peroxide-assisted sodium carbonate pretreatment for the enhancement of enzymatic saccharification of corn stover.

ACS Sustainable Chemistry & Engineering, *3*(12), 3477–3485. https://doi.org/10.1021/acssuschemeng.5b01278

Guettler, M.V., Jain, M.K., Rumler, D., 1996. Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. *US Pat. No. 5,573,931*.

Guettler, M. V., Rumler, D., & Jain, M. K. (1999). *Actinobacillus succinogenes* sp. Nov., a novel succinic-acid-producing strain from the bovine rumen. *International Journal of Systematic and Evolutionary Microbiology*, *49*(1), 207–216. https://doi.org/10.1099/00207713-49-1-207

Gunnarsson, I. B., Kuglarz, M., Karakashev, D., & Angelidaki, I. (2015). Thermochemical pretreatments for enhancing succinic acid production from industrial hemp (*Cannabis sativa* L.). *Bioresource Technology*, *182*, 58–66. https://doi.org/10.1016/j.biortech.2015.01.126

Guo, X., Cavka, A., Jönsson, L. J., & Hong, F. (2013). Comparison of methods for detoxification of spruce hydrolysate for bacterial cellulose production. *Microbial Cell Factories*, *12*(1), 93.

Hames, B., Ruiz, R., Scarlata, C., Sluiter, A., Sluiter, J., & Templeton, D. (2008). Preparation of Samples for Compositional Analysis: Laboratory Analytical Procedure (LAP); Issue Date 08/08/2008. *Technical Report*.

Hojilla-Evangelista, M.P. 1990. Sequential extraction processing: alternate technology for corn wet milling, Iowa State University.

https://www.grandviewresearch.com/industry-analysis/succinic-acid-market, accessed 07/02/2024

https://www.technavio.com/report/bio-succinic-acid-market-industry-analysis, accessed 07/08/2024

Jafari, Y., Karimi, K., & Amiri, H. (2017). Efficient bioconversion of whole sweet sorghum plant to acetone, butanol, and ethanol improved by acetone delignification. *Journal of Cleaner Production*, *166*, 1428–1437. https://doi.org/10.1016/j.jclepro.2017.08.132

Jiang, M., Ma, J., Wu, M., Liu, R., Liang, L., Xin, F., Zhang, W., Jia, H., & Dong, W. (2017). Progress of succinic acid production from renewable resources: metabolic and fermentative strategies. *Bioresource Technology*, *245*(Pt B), 1710–1717. https://doi.org/10.1016/j.biortech.2017.05.209

Jing, X., Zhang, X., & Bao, J. (2009). Inhibition performance of lignocellulose degradation products on industrial cellulase enzymes during cellulose hydrolysis. *Applied Biochemistry and Biotechnology*, *159*(3), 696–707. https://doi.org/10.1007/s12010-009-8525-z

Jönsson, L. J., Alriksson, B., & Nilvebrant, N.-O. (2013). Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnology for Biofuels*, *6*(1), 16. https://doi.org/10.1186/1754-6834-6-16

Jönsson, L. J., Palmqvist, E., Nilvebrant, N.-O., & Hahn-Hägerdal, B. (1998). Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. *Applied Microbiology and Biotechnology*, *49*(6), 691–697. https://doi.org/10.1007/s002530051233

Kamzolova, S. V., Yusupova, A. I., Vinokurova, N. G., Fedotcheva, N. I., Kondrashova, M. N., Finogenova, T. V., & Morgunov, I. G. (2009). Chemically assisted microbial production of succinic acid by the yeast *Yarrowia lipolytica* grown on ethanol. *Applied Microbiology and Biotechnology*, *83*(6), 1027–1034. https://doi.org/10.1007/s00253-009-1948-1

Kreuger, E., Escobar, F., Svensson, S. E., & Björnsson, L. (2008). Biogas production from hemp – evaluation of the effect of harvest time on methane yield. 5th International Symposium on Anaerobic Digestion of Solid Wastes and Energy Crops. https://res.slu.se/id/publ/22864

Kuglarz, M., Alvarado-Morales, M., Dąbkowska, K., & Angelidaki, I. (2018). Integrated production of cellulosic bioethanol and succinic acid from rapeseed straw after dilute-acid pretreatment. *Bioresource Technology*, *265*, 191–199. https://doi.org/10.1016/j.biortech.2018.05.099

Kuglarz, M., Alvarado-Morales, M., Karakashev, D., & Angelidaki, I. (2016b). Integrated production of cellulosic bioethanol and succinic acid from industrial hemp in a biorefinery concept. *Bioresource Technology*, *200*, 639–647. https://doi.org/10.1016/j.biortech.2015.10.081

Kuglarz, M., Gunnarsson, I. B., Svensson, S.-E., Prade, T., Johansson, E., & Angelidaki, I. (2014). Ethanol production from industrial hemp: effect of combined dilute acid/steam pretreatment and economic aspects. *Bioresource Technology*, *163*, 236–243. https://doi.org/10.1016/j.biortech.2014.04.049

Kumar, R., Mago, G., Balan, V., & Wyman, C. E. (2009). Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresource Technology*, *100*(17), 3948–3962. https://doi.org/10.1016/j.biortech.2009.01.075

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., & Nilvebrant, N.-O. (1999). The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme and Microbial Technology*, *24*(3), 151–159. https://doi.org/10.1016/S0141-0229(98)00101-X

Li, Q., Wang, D., Song, Z., Zhou, W., Wu, Y., Xing, J., & Su, Z. (2010). Dual-phase fermentation enables *Actinobacillus succinogenes* 130ZT to be a potential role for high-level lactate production from the bioresource. *Bioresource Technology*, *101*(19), 7665–7667. https://doi.org/10.1016/j.biortech.2010.04.058

Litchfield, J. H. (1996). Microbiological production of lactic acid. In S. L. Neidleman & A. I. Laskin (Eds.), *Advances in Applied Microbiology* (Vol. 42, pp. 45–95). Academic Press. https://doi.org/10.1016/S0065-2164(08)70372-1

Liu, Y.-P., Zheng, P., Sun, Z.-H., Ni, Y., Dong, J.-J., & Wei, P. (2008). Strategies of pH control and glucose-fed batch fermentation for production of succinic acid by *Actinobacillus succinogenes* CGMCC1593. *Journal of Chemical Technology & Biotechnology*, *83*(5), 722–729. https://doi.org/10.1002/jctb.1862

Liu, Z.-H., Qin, L., Zhu, J.-Q., Li, B.-Z., & Yuan, Y.-J. (2014). Simultaneous saccharification and fermentation of steam-exploded corn stover at high glucan loading and high temperature. *Biotechnology for Biofuels*, *7*(1), 167. https://doi.org/10.1186/s13068-014-0167-x

Long, D. S., Immethun, C. M., Vallecilla-Yepez, L., Wilkins, M. R., & Saha, R. (2021). One step forward, two steps back: transcriptional advancements and fermentation phenomena in *Actinobacillus succinogenes* 130Z. *Plos One*, *16*(5), e0245407. https://doi.org/10.1371/journal.pone.0245407

Maharaj, K., Bradfield, M. F. A., & Nicol, W. (2014). Succinic acid-producing biofilms of Actinobacillus succinogenes: Reproducibility, stability and productivity. *Applied Microbiology and Biotechnology*, *98*(17), 7379–7386. https://doi.org/10.1007/s00253-014-5779-3

McKinlay, J. B., Laivenieks, M., Schindler, B. D., McKinlay, A. A., Siddaramappa, S., Challacombe, J. F., Lowry, S. R., Clum, A., Lapidus, A. L., Burkhart, K. B., Harkins, V., & Vieille, C. (2010). A genomic perspective on the potential of *Actinobacillus succinogenes* for industrial succinate production. *BMC Genomics*, *11*(1), 680. https://doi.org/10.1186/1471-2164-11-680

McKinlay, J. B., Shachar-Hill, Y., Zeikus, J. G., & Vieille, C. (2007). Determining *Actinobacillus succinogenes* metabolic pathways and fluxes by NMR and GC-MS analyses of 13C-labeled metabolic product isotopomers. *Metabolic Engineering*, *9*(2), 177–192. https://doi.org/10.1016/j.ymben.2006.10.006

McKinlay, J. B., Zeikus, J. G., & Vieille, C. (2005). Insights into *Actinobacillus succinogenes* fermentative metabolism in a chemically defined growth medium. *Applied and Environmental Microbiology*, *71*(11), 6651–6656. https://doi.org/10.1128/AEM.71.11.6651-6656.2005

Naveena, B. J., Altaf, Md., Bhadriah, K., & Reddy, G. (2005). Selection of medium components by Plackett–Burman design for production of l(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using wheat bran. *Bioresource Technology*, *96*(4), 485–490. https://doi.org/10.1016/j.biortech.2004.05.020

Nghiem, N. P., Kleff, S., & Schwegmann, S. (2017). Succinic acid: technology development and commercialization. *Fermentation*, *3*(2), Article 2. https://doi.org/10.3390/fermentation3020026

Noureddini, H., & Byun, J. (2010). Dilute-acid pretreatment of distillers' grains and corn fiber. *Bioresource Technology*, *101*(3), 1060–1067. https://doi.org/10.1016/j.biortech.2009.08.094

Palmqvist, E., & Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates: inhibition and detoxification. *Bioresource Technology*, *74*, 17–24. https://doi.org/10.1016/S0960-8524(99)00160-1

Palmqvist, E., Galbe, M., & Hahn-Hägerdal, B. (1998). Evaluation of cell recycling in continuous fermentation of enzymatic hydrolysates of spruce with *Saccharomyces cerevisiae* and on-line monitoring of glucose and ethanol. *Applied Microbiology and Biotechnology*, *50*(5), 545–551. https://doi.org/10.1007/s002530051332

Palmqvist, E., Hahn-Hägerdal, B., Szengyel, Z., Zacchi, G., & Rèczey, K. (1997). Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment. *Enzyme and Microbial Technology*, *20*, 286–293. https://doi.org/10.1016/S0141-0229(96)00130-5

Pateraki, C., Patsalou, M., Vlysidis, A., Kopsahelis, N., Webb, C., Koutinas, A. A., & Koutinas, M. (2016). *Actinobacillus succinogenes*: advances on succinic acid production and prospects for development of integrated biorefineries. *Biochemical Engineering Journal*, *112*, 285–303. https://doi.org/10.1016/j.bej.2016.04.005

Potera, C. (2005). Making succinate more successful. *Environmental Health Perspectives*, *113*(12), A832–A835. https://doi.org/10.1289/ehp.113-a832

Rahim, N. A., Luthfi, A. A. I., Bukhari, N. A., Tan, J. P., Abdul, P. M., & Manaf, S. F. A. (2023). Biotechnological enhancement of lactic acid conversion from pretreated palm kernel cake hydrolysate by *Actinobacillus succinogenes* 130Z. *Scientific Reports*, *13*(1), 5787. https://doi.org/10.1038/s41598-023-32964-z

Rasmussen, M. L., Shrestha, P., Khanal, S. K., Pometto, A. L., & (Hans) van Leeuwen, J. (2010). Sequential saccharification of corn fiber and ethanol production by the brown rot fungus *Gloeophyllum trabeum*. *Bioresource Technology*, *101*(10), 3526–3533. https://doi.org/10.1016/j.biortech.2009.12.115

Reddy, G., Altaf, Md., Naveena, B. J., Venkateshwar, M., & Kumar, E. V. (2008). Amylolytic bacterial lactic acid fermentation—A review. *Biotechnology Advances*, *26*(1), 22–34. https://doi.org/10.1016/j.biotechadv.2007.07.004

Reddy, K., Awasthi, S., Madhu, A., & Siddalingaiya, P. (2009). Role of cryoprotectants on the viability and functional properties of probiotic lactic acid bacteria during freeze drying. *Food Biotechnology*, *23*, 243–265. https://doi.org/10.1080/08905430903106811

Richter, K., & Berthold, C. (1998). Biotechnological conversion of sugar and starchy crops into lactic acid. *Journal of Agricultural Engineering Research*, *71*(2), 181–191. https://doi.org/10.1006/jaer.1998.0314

Samuelov, N. S., Lamed, R., Lowe, S., & Zeikus, J. G. (1991). Influence of CO_2-HCO_3^- levels and pH on growth, succinate production, and enzyme activities of *Anaerobiospirillum succiniciproducens*. *Applied and Environmental Microbiology*, *57*(10), 3013–3019. https://doi.org/10.1128/aem.57.10.3013-3019.1991

Sauer, M., Porro, D., Mattanovich, D., & Branduardi, P. (2008). Microbial production of organic acids: expanding the markets. *Trends in Biotechnology*, *26*(2), 100–108. https://doi.org/10.1016/j.tibtech.2007.11.006

Shrestha, P., Rasmussen, M., Khanal, S. K., Pometto, A. L. I., & van Leeuwen, J. (Hans). (2008). Solid-substrate fermentation of corn fiber by *Phanerochaete chrysosporium* and subsequent fermentation of hydrolysate into ethanol. *Journal of Agricultural and Food Chemistry*, *56*(11), 3918–3924. https://doi.org/10.1021/jf0728404

Sluiter, A. (2008). Determination of extractives in biomass: laboratory analytical procedure (LAP); Issue Date 7/17/2005. *Technical Report*.

Sluiter, A. (2008). Determination of structural carbohydrates and lignin in biomass: laboratory analytical procedure (LAP); issue date: April 2008; revision date: July 2011 (version 07-08-2011). *Technical Report*.

Sluiter, A., Hames, B., Ruiz, R. O., Scarlata, C., Sluiter, J., & Templeton, D. (2004). Determination of structural carbohydrates and lignin in biomass. *Biomass Anal Technol Team Lab Anal Proced*, *2011*, 1–14.

Song, H., & Lee, S. Y. (2006). Production of succinic acid by bacterial fermentation. *Enzyme and Microbial Technology*, *39*(3), 352–361. https://doi.org/10.1016/j.enzmictec.2005.11.043

Sun, S.-L., Sun, S.-N., Wen, J.-L., Zhang, X.-M., Peng, F., & Sun, R.-C. (2015). Assessment of integrated process based on hydrothermal and alkaline treatments for enzymatic saccharification of sweet sorghum stems. *Bioresource Technology*, *175*, 473–479. https://doi.org/10.1016/j.biortech.2014.10.111

Tejirian, A., & Xu, F. (2011). Inhibition of enzymatic cellulolysis by phenolic compounds. *Enzyme and Microbial Technology*, *48*(3), 239–247. https://doi.org/10.1016/j.enzmictec.2010.11.004

Tsao, G. T., Cao, N. J., Du, J., & Gong, C. S. (1999). Production of multifunctional organic acids from renewable resources. *Recent Progress in Bioconversion of Lignocellulosics* (pp. 243–280). Springer. https://doi.org/10.1007/3-540-49194-5_10

Umagiliyage, A. L., Choudhary, R., Liang, Y., Haddock, J., & Watson, D. G. (2015). Laboratory scale optimization of alkali pretreatment for improving enzymatic hydrolysis of sweet sorghum bagasse. *Industrial Crops and Products*, *74*, 977–986. https://doi.org/10.1016/j.indcrop.2015.05.044

Vallecilla Yepez, L. (2022). Bioproduction of succinic acid from corn fiber by *Actinobacillus succinogenes*. The University of Nebraska-Lincoln. PhD Dissertation.

Vallecilla-Yepez, L., Ramchandran, D., Long, D., Saha, R., & Wilkins, M. R. (2021). Corn fiber as a biomass feedstock for production of succinic acid. *Bioresource Technology Reports*, *16*, 100868. https://doi.org/10.1016/j.biteb.2021.100868

Van Eylen, D., van Dongen, F., Kabel, M., & de Bont, J. (2011). Corn fiber, cobs and stover: enzyme-aided saccharification and co-fermentation after dilute acid pretreatment. *Bioresource Technology*, *102*(10), 5995–6004. https://doi.org/10.1016/j.biortech.2011.02.049

Varriale, L., Volkmar, M., Weiermüller, J., & Ulber, R. (2022). Effects of pretreatment on the biocatalysis of renewable resources. *Chemie Ingenieur Technik*, *94*(11), 1818–1826. https://doi.org/10.1002/cite.202200137

Vishnu, C., Seenayya, G., & Reddy, G. (2000). Direct conversion of starch to L(+) lactic acid by amylase producing *Lactobacillus amylophilus* GV6. *Bioprocess Engineering*, *23*(2), 155–158. https://doi.org/10.1007/PL00009119

Vishnu, C., Seenayya, G., & Reddy, G. (2002). Direct fermentation of various pure and crude starchy substrates to L(+) lactic acid using *Lactobacillus amylophilus* GV6. *World Journal of Microbiology and Biotechnology*, *18*(5), 429–433. https://doi.org/10.1023/A:1015526221744

Wang, C., Li, Q., Wang, D., & Xing, J. (2014). Improving the lactic acid production of *Actinobacillus succinogenes* by using a novel fermentation and separation integration system. *Process Biochemistry*, *49*(8), 1245–1250. https://doi.org/10.1016/j.procbio.2014.04.009

Wang, Z., Dien, B. S., Rausch, K. D., Tumbleson, M. E., & Singh, V. (2019). Improving ethanol yields with deacetylated and two-stage pretreated corn stover and sugarcane bagasse by blending commercial xylose-fermenting and wild type *Saccharomyces yeast*. *Bioresource Technology*, *282*, 103–109. https://doi.org/10.1016/j.biortech.2019.02.123

Wee, Y.-J., Kim, J.-N., & Ryu, H.-W. (2006). Biotechnological production of lactic acid and its recent applications. *Food Technology and Biotechnology*, *44*(2), 163–172. https://hrcak.srce.hr/clanak/161872

Werpy, T., Petersen, G. 2014. US Department of Energy: 2004. *Google Scholar*, *1-76*.

Wilson, J. J., Deschatelets, L., & Nishikawa, N. K. (1989). Comparative fermentability of enzymatic and acid hydrolysates of steam-pretreated aspenwood hemicellulose by *Pichia stipitis* CBS 5776. *Applied Microbiology and Biotechnology*, *31*(5), 592–596. https://doi.org/10.1007/BF00270801

Wu, L., Arakane, M., Ike, M., Wada, M., Takai, T., Gau, M., & Tokuyasu, K. (2011). Low temperature alkali pretreatment for improving enzymatic digestibility of sweet sorghum bagasse

for ethanol production. *Bioresource Technology*, *102*(7), 4793–4799. https://doi.org/10.1016/j.biortech.2011.01.023

Xi, Y., Dai, W., Xu, R., Zhang, J., Chen, K., Jiang, M., Wei, P., & Ouyang, P. (2013). Ultrasonic pretreatment and acid hydrolysis of sugarcane bagasse for succinic acid production using *Actinobacillus succinogenes*. *Bioprocess and Biosystems Engineering*, *36*(11), 1779–1785. https://doi.org/10.1007/s00449-013-0953-z

Xu, G.-C., Ding, J.-C., Han, R.-Z., Dong, J.-J., & Ni, Y. (2016). Enhancing cellulose accessibility of corn stover by deep eutectic solvent pretreatment for butanol fermentation. *Bioresource Technology*, *203*, 364–369. https://doi.org/10.1016/j.biortech.2015.11.002

Yoo, C.G., Pan, X., 2017. Pretreatment of lignocellulosic feedstocks. In: Li, Y., Khanal, S. K. (Eds.), *Bioenergy: Principles and Applications*. John Wiley & Sons, Hoboken, pp. 201–221

Zhang, Y., Huang, M., Su, J., Hu, H., Yang, M., Huang, Z., Chen, D., Wu, J., & Feng, Z. (2019). Overcoming biomass recalcitrance by synergistic pretreatment of mechanical activation and metal salt for enhancing enzymatic conversion of lignocellulose. *Biotechnology for Biofuels*, *12*(1), 12. https://doi.org/10.1186/s13068-019-1354-6

Zhao, J., Xu, Y., Wang, W., Griffin, J., Roozeboom, K., & Wang, D. (2020). Bioconversion of industrial hemp biomass for bioethanol production: A review. *Fuel*, *281*, 118725. https://doi.org/10.1016/j.fuel.2020.118725

Zhao, J., Xu, Y., Zhang, M., & Wang, D. (2020). Integrating bran starch hydrolysates with alkaline pretreated soft wheat bran to boost sugar concentration. *Bioresource Technology*, *302*, 122826. https://doi.org/10.1016/j.biortech.2020.122826

Zheng, P., Fang, L., Xu, Y., Dong, J.-J., Ni, Y., & Sun, Z.-H. (2010). Succinic acid production from corn stover by simultaneous saccharification and fermentation using *Actinobacillus succinogenes*. *Bioresource Technology*, *101*(20), 7889–7894. https://doi.org/10.1016/j.biortech.2010.05.016

Zhou, S., Zhang, M., Zhu, L., Zhao, X., Chen, J., Chen, W., & Chang, C. (2023). Hydrolysis of lignocellulose to succinic acid: a review of treatment methods and succinic acid applications. *Biotechnology for Biofuels and Bioproducts*, *16*(1), 1. https://doi.org/10.1186/s13068-022-02244-5

Zyl, C., Prior, B., Du-Preez, J., Zyl, C., Prior, B., & Du-Preez, J. (1988). Production of ethanol from sugar cane bagasse hemicellulose hydrolyzate by *Pichia stipitis*. *Applied Biochemistry and Biotechnology*, *17*(17), Article 17. https://eurekamag.com/research/016/773/016773236.php

1.1.1.1.1.1 Statistical data analysis appendix

1.1.1.1.1.1.1 SAS codes for Figure 3 A and B – Glucose

```
data hydrolysis;
  input hydrolysate $ Glucose yield;
  datalines;
water 33.3 92.5
water 33.5 92.8
water 33.6 93.3
undetox 36.1 83
undetox 35.9 82.4
undetox 35.4 81
detox 38.3 88
detox 38.8 89.1
detox 39.4 90.3
;
run;

proc glm data=hydrolysis;
  class hydrolysate;
  model Glucose yield = hydrolysate;
  means hydrolysate / tukey;
run;
```

1.1.1.1.1.1.2 Generated tables for Figure 3 A and B – Glucose

The GLM Procedure

Dependent Variable: Glucose

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	43.44666667	21.72333333	142.71	<.0001
Error	6	0.91333333	0.15222222		
Corrected Total	8	44.36000000			

The GLM Procedure

Dependent Variable: yield

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	178.1422222	89.0711111	105.20	<.0001
Error	6	5.0800000	0.8466667		
Corrected Total	8	183.2222222			

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Glucose

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.152222
Critical Value of Studentized Range	4.33917
Minimum Significant Difference	0.9774

The GLM Procedure

Tukey's Studentized Range (HSD) Test for yield

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.846667
Critical Value of Studentized Range	4.33917
Minimum Significant Difference	2.3052

1.1.1.1.1.1.3 SAS codes for Figure 3 A and B – Xylose

```
data hydrolysis;
  input hydrolysate $ Xylose yield;
  datalines;
water 4.7 39.5
water 4.8 40.3
water 4.8 40.3
undetox 9.4 38.7
undetox 9.2 37.9
undetox 9.2 37.9
```

```
detox 9.5 41.5
detox 9.8 42.8
detox 9.8 42.8
;
run;

proc glm data=hydrolysis;
  class hydrolysate;
  model Xylose yield = hydrolysate;
  means hydrolysate / tukey;
run;
```

1.1.1.1.1.1.4 Generated tables for Figure 3 A and B – Xylose

The GLM Procedure

Dependent Variable: Xylose

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	44.77555556	22.38777778	1439.21	<.0001
Error	6	0.09333333	0.01555556		
Corrected Total	8	44.86888889			

The GLM Procedure

Dependent Variable: yield

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	26.56888889	13.28444444	40.26	0.0003
Error	6	1.98000000	0.33000000		
Corrected Total	8	28.54888889			

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Xylose

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.015556
Critical Value of Studentized Range	4.33917
Minimum Significant Difference	0.3125

The GLM Procedure

Tukey's Studentized Range (HSD) Test for yield

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.33
Critical Value of Studentized Range	4.33917
Minimum Significant Difference	1.4391

1.1.1.1.1.1.5 SAS code for Figure 6 B

```
data fermentation;
  input bubbling $ LA yield;
  datalines;
unbubbled 5 0.1
unbubbled 5.2 0.1
unbubbled 5.4 0.1
N2 bubbled 10.5 0.2
N2 bubbled 10 0.2
N2 bubbled 9.5 0.2
CO2 bubbled 36.1 0.72
CO2 bubbled 36 0.72
CO2 bubbled 36.2 0.72
;
run;
```

```
proc glm data=fermentation;
  class bubbling;
  model LA yield = bubbling;
  means bubbling / tukey;
run;
```

1.1.1.1.1.1.6 Generated tables for Figure 6 B

The GLM Procedure

Dependent Variable: LA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	0	0.00000000	.	.	.
Error	2	0.08000000	0.04000000		
Corrected Total	2	0.08000000			

The GLM Procedure

Dependent Variable: yield

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	2075.220000	1037.610000	11972.4	<.0001
Error	6	0.520000	0.086667		
Corrected Total	8	2075.740000			

The GLM Procedure

Tukey's Studentized Range (HSD) Test for yield

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.086667
Critical Value of Studentized Range	4.33917
Minimum Significant Difference	0.7375

1.1.1.1.1.1.7 SAS code for Figure 7 B

```
data fermentation;
  input hydrolysate $ LA yield;
  datalines;
undetox 6 0.18
undetox 6.5 0.18
undetox 7.3 0.18
detox 22.1 0.57
detox 22.4 0.57
detox 23 0.57
water 20.1 0.59
water 19.5 0.59
water 19 0.59
control 28 0.73
control 28.5 0.73
control 29 0.73
;
run;

proc glm data=fermentation;
  class hydrolysate;
  model LA yield = hydrolysate;
  means hydrolysate / tukey;
run;
```

1.1.1.1.1.1.8 Generated tables for Figure 7 B

The GLM Procedure

Dependent Variable: LA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	768.6700000	256.2233333	858.85	<.0001
Error	8	2.3866667	0.2983333		
Corrected Total	11	771.0566667			

The GLM Procedure

Dependent Variable: yield

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.50122500	0.16707500	Infty	<.0001
Error	8	0.00000000	0.00000000		
Corrected Total	11	0.50122500			

The GLM Procedure

Tukey's Studentized Range (HSD) Test for LA

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.298333
Critical Value of Studentized Range	4.52877
Minimum Significant Difference	1.4281

The GLM Procedure

Tukey's Studentized Range (HSD) Test for yield

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0
Critical Value of Studentized Range	4.52877
Minimum Significant Difference	0