SUCCINIC ACID PRODUCTION FROM LIGNOCELLULOSIC BIOMASS BY *Actinobacillus succinogenes*

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ABSTRACT

SUCCINIC ACID PRODUCTION FROM LIGNOCELLULOSIC BIOMASS BY *Actinobacillus succinogenes*

Succinic acid is an expensive and high industrial value organic acid that can be used in many industries such as food, cosmetics, chemistry. It can be produced by bacterial fermentation. When cellulose and xylan in the lignocellulosic biomass are hydrolyzed into glucose and xylose with pretreatment process, they can be used as a carbon source in fermentation.

This study was designed for the production of succinic acid from pretreated corncob by *Actinobacillus succinogenes* ATCC-55618. Corncob was pre-treated by organasolv. The cellulose and xylan were hydrolyzed into monomers using commercial enzymes. The optimal enzyme dosages were sought at 50°C and pH 5.2, under which conditions pretreated corncob was hydrolyzed for separate hydrolysis and fermentation (SHF). The same test was repeated at 37°C and pH 6.8 to find the required enzyme dosages under the simultaneous saccharification and fermentation (SSF). The cellulose and xylan recoveries were 69.2% and 68.8% for SHF, 31.8 % and 41.4 % for SSF.

The SHF was conducted using the enzymatic hydrolysate and succinic acid yield was 0.48 g succinic acid/g sugar. In the SSF, the pretreated corncob was used as the carbon source, the succinic acid yields were 0.75 g succinic acid/g sugar.

This study shows that corncob treated with organosolv had a potential as carbon source for succinic acid production by *A. succinogenes* either via SHF or SSF. Although the conditions in the fermentation step were not optimum for the enzyme activity, the SSF was more successful than SHF considering the succinic acid yield on the carbohydrates.

ÖZET

Actinobacillus succinogenes İLE LİGNOSELÜLOZİK BİYOKÜTLEDEN SÜKSİNİK ASİT ÜRETİMİ

Süksinik asit gıda, kozmetik, kimya gibi birçok endüstride kullanılabilen pahalı ve endüstriyel değeri yüksek bit organik asittir. Süksinik asit, bakteriyel fermentasyonla üretilebilir. Lignoselülozik biyokütledeki selüloz ve ksilan, bir ön işlemden sonra glikoz ve ksiloza hidrolize edildiğinde, fermentasyonda karbon kaynağı olarak kullanılabilirler.

Bu çalışma, *Actinobacillus succinogenes* ATCC-55618 tarafından ile ön işleme tabi tutulmuş mısır koçandan süksinik acid üretimi için tasarlanmıştır. Mısır koçanı organasolv ile ön işleme tabi tutulmuştur. Selüloz ve ksilan ticari enzimler kullanılarak hidrolize edilmiştir. 50°C ve pH 5.2'de optimum enzim dozajları aranmış ve belirlenen bu koşullar altında, ön işleme tabi tutulmuş mısır koçanı, ayrı hidroliz ve fermentasyon (SHF) için hidrolize edilmiştir. Aynı test daha sonra eş zamanlı sakarifikasyon ve fermentasyon (SSF) için gerekli enzim dozajlarını bulmak adına 37°C ve pH 6.8'de tekrarlanmıştır. Selüloz ve ksilan geri kazanımı SHF için %69.2 ve %68.8, SSF için %31.8 ve %41.4 olarak belirlenmiştir.

SHF, enzimatik hidrolizat kullanılarak gerçekleştirilmiştir. Süksinik asit verimi 0,58 g süksinik asit/g şeker olmuştur.. SSF'de karbon kaynağı olarak ön işleme tabi tutulmuş mısır koçanı kullanılmıştır. SSF için süksinik asit verimi, 0.75 g süksinik asit/g şeker olarak belirlenmiştir..

Bu çalışma, organosolv ile muamele edilmiş mısır koçanının, SHF veya SSF yoluyla *A. succinogenes* tarafından süksinik asit üretimi için karbon kaynağı olarak bir potansiyele sahip olduğunu göstermektedir. Fermentasyon aşamasında kullanılan koşullar, enzimlerin aktivitesi için optimum olmasa da, karbonhidratlar üzerindeki süksinik asit verimi göz önüne alındığında SSF, SHF'den daha başarılı bulunmuştur.

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LIST OF SYMBOLS AND ABBREVIATIONS

ml .....milliliter
SSF..... simultaneous saccharification and fermentation
SHF……..…..separate hydrolysis and fermentation
µl……..microliter
L……...liter
°C……centigrade degree
mm……...……...…millimeter
g……...……gram
mM…….....millimolar
rpm…….. revolutions per minute
v……...volume
HPLC……high-pressure liquid chromatography
eqn .....equation
% .....percent
K_a ..... dissociation constant
ΔH_c .....heat of combustion
C_P ..... specific heat
nm ..... nanometer

CHAPTER 1

INTRODUCTION

Organic acids are used in foods for many reasons such as acidity regulators, flavoring agents. It is preferred to produce food additives by biotechnological methods. For this reason, the industry turns in this direction. Among these, many studies are carried out on the production of succinic acid. Succinic acid can be used in other industries and is among the platform chemicals and is very valuable. Succinic acid can be produced chemically as well as microbially.

Lignocellulosic biomass has a large volume in the world. The use of lignocellulosic biomass is preferred for the microbial production of cost-effective and sustainable organic acids. Pretreatment is required to produce fermentable carbohydrates from lignocellulosic biomass. This pretreatment increases energy demand and costs and can create environmental problems with the use of chemicals. For this reason, appropriate preprocesses must be found. Cellulose is generally used during fermentation because most microorganisms can easily transform. On the other hand, the number of organisms that efficiently ferment high xylan in lignocellulosic biomass is limited. For lignocellulosic biomass-based production to become sustainable, xylan must also be converted into products.

A.succinogenes, one of the most efficient microorganisms for succinic acid production, provides high efficiency from carbohydrates and it is an advantage to use xylose effectively. In this way, it can also be used to evaluate the xylan. There are examples of succinic production from *A.succinogenes* and Lignocellulosic biomass. There are also examples using xylose. For this reason, it is possible to evaluate the xylan as well. Different pre-treatments can be used in microbial production. Organasolv can also be applied as a method that removes lignin and hemicellulose with the aid of organic solvents.

In this study, the usability of organasolv in the production of succinic acid was tested. Corncob was chosen as a model for this because it emerges as a common agricultuıral residue in the world and its content is rich to provide the carbohydrate

required for fermentation. The removal of lignin from the corn cob was chosen as the first target. Fermentative sugars were released by enzymatic hydrolysis of xylan and cellulose in the solid. Of these, the conversion of glucose and xylose to succinic acid was investigated. In addition, the SSF and SHF method was used by combining enzymatic hydrolysis and fermentation. It is aimed to develop a sustainable process based on lignocellulosic biomass.

CHAPTER 2

LITERATURE REVIEW

2.1. Succinic Acid

Succinic acid is an organic acid that can be used for a wide variety of industrial applications. In the food industry, it is used as flavor additive, bacteriostatic, neutralizing agent. In chemical industries, succinic acid can be used for producing surfactants, detergents, dyes, biodegradable plastics, coating, etc (Saxena et al. 2016) Succinic acid has been designated as one of 12 sugar-based building blocks as it can later transform into new and useful molecules and ranked among the highest value-added chemicals from biomass by the U.S. Department of Energy (Werpy and Petersen 2004).

Amber is the fossilized resin of *Pinus succinifera* trees and obtained from the Baltic Sea, it was called the star of the north in ancient times and the Romans called amber succinum, inspired by the Latin word succus, which means plant extract and the word succinic acid also comes from here (Beck 1986) (Figure 2.1.). Succinic acid was first purified from amber by Georgius Agricola by dry distillation of the amber and fractions of amber were identified: succinic acid, amber oil, and amber colophony in 1546 (Matuszewska 2016).

Figure 2.1. Typical amber grain appearance

(Source: Schmidt et al. 2012)

Succinic acid (called butanedioic acid, 1,2-ethanedicarboxylic acid, or amber acid), a four-carbon dicarboxylic acid that is an intermediate of the tricarboxylic acid cycle and also frequently encountered in atmospheric aerosol samples and its chemical formula is $C_4H_6O_4$ (Saxena et al. 2016)(Figure 2.2.). Succinic acid exists in animal cells, some plants, spring water, or the structure of meteorites and it is soluble in alcohol, diethyl ether, anhydrous glycerol, acetone, aqueous solutions of acetone and glycerol, and also when heating occurs to the melting point of succinic acid, dehydration takes place and sublimes into anhydride (Fumagalli 2006). Some properties of succinic acid are given in Table 2.1.

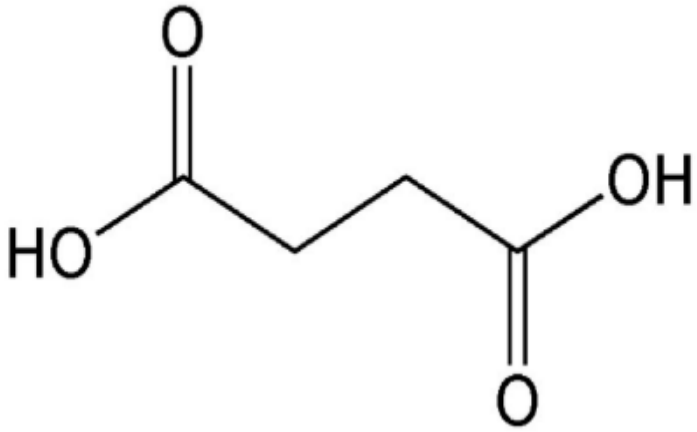

Figure 2.2. Chemical structure of succinic acid

(Source: Wang et al. 2020)

Table 2.1. Physical and chemical properties and some specifications of succinic acid (Saxena et al. 2016, Fumagalli 2006).

PROPERTIES and SPECIFICATIONS	
Molecular weight	118.09 g/mole
Melting point	185-187 $^{\circ}$C
Boiling point	235 $^{\circ}$C
Flash point	206 $^{\circ}$C (open cup)
Density	1.56 g/cm^3

Cont. on next page

Cont. of Table 2.1.

Physical state	Odorless white crystal
Specific gravity	1.552
Vapor density	3.04
Appearance in solution	Colorless, clear
Ash	0.1 %

2.2. Application Areas of Succinic Acid

Among the main applications of succinic acid, it is used as a surfactant in the detergent industry, as an acidity regulator and flavoring agent in the food industry, as an ion chelator in the electroplating sector as a corrosion inhibitor, and in the production of antibiotics, amino acids and vitamins in pharmacy and additionally, succinic acid is used in the production of succinic acid derivatives, which have a market share of 270000 tons per year such as adipic acid, 1,4 butanediol, tetrahydrofuran, 2-pyrrolidinone, succinic acid esters, succinate salts (Xu and Guo 2010). Succinic acid naturally processes the toxic acetaldehyde, which is formed as a metabolite of alcohol in the body, and therefore reduces the effects after alcohol intake and also dimethyl succinate obtained by esterification of succinic acid and also it is used as an environmentally friendly and innocuous solvent and used in water cooling systems of vehicles (Saxena et al. 2016). It has been stated that succinate can be used as an enhancer of growth and anthocyanin accumulation in plants based on the study carried out in carrot cells (Dougall and Weyrauch 1980) In addition, succinic acid and its derivatives are used in the leather industry to improve some of the leather properties such as water repellency(Saxena et al. 2016).

Succinate esters and salts have a wide range of uses, such as pharmaceutics additives or active ingredients, pH inhibitors, taste enhancers, antibacterial agents in the food industry, and fuel or deicer additives and the pyrrolidones are a class of succinic acid derivatives that are used as solvents, starting materials, or additives in the pharmaceutical, cosmetic, and food industries (Litsanov et al. 2014). It has been observed that succinate salts, which play a leading role in protein synthesis, increase the production of propionate in the rumen (Saxena et al. 2016).

2.3. Succinic Acid Production Technology

Succinic acid can be produced by different methods, and it is estimated that the succinic acid market will grow by 6.8% between 2018 and 2023 (Markets and Markets n.d.). Succinic acid was produced on a petroleum basis with chemical methods and caused high-cost production and its usage areas were limited. Today, succinic acid production has taken its place in the industry with methods based on biobased sugar fermentation (microbial methods).

With chemical technology, succinic acid is produced using different methods such as paraffin oxidation, catalytic hydrogenation, and electroreduction of maleic acid or maleic anhydride (Cheng et al. 2012). Paraffin oxidation technology uses a manganese or calcium catalyst to create a mixture of dicarboxylic acids and distillation, crystallization, and drying processes are also used to purify the succinic acid produced but using this process, succinic acid is produced in low yield and purity (Pateraki et al. 2016). The catalytic hydrogenation process is a maturation process using homogeneous or heterogeneous catalysts and with this process, succinic acid is produced with high yield and purity, but it is an expensive technology and may cause environmental problems (Pateraki et al. 2016). The production of succinic acid from fossil-derived maleic acid by hydrogenation has become undesirable due to the fact that fossil-derived products are a high-priced and non-renewable source (Ladakis et al. 2020). Microbial fermentation is a cleaner and more cost-effective method and for this reason, the production of succinic acid by microbial fermentation has been preferred (Xu and Guo 2010).

In microbial fermentation, many fungi, yeast, and gram-positive bacteria are used in the production of succinic acid. Microorganisms need carbon dioxide (CO_2) to produce succinic acid that an intermediate product of the TCA cycle and the end product of anaerobic metabolism. The reducing arm of the TCA cycle (fermentative pathway, anaerobically), the oxidative branch (aerobically), and finally the glyoxylate pathway (aerobically), are three different pathways of succinic acid production (Cheng et al. 2012). Since succinate does not accumulate in the cell and transforms into a different form during the oxidative pathway and the glyoxylate pathway, these methods are generally not used for succinic acid production, and the anaerobic route is preferred because of the absence of any enzyme that converts it into something else (Saxena et al. 2016). The primary production route of succinate is a derivation from phosphoenolpyruvate through several

intermediates of the TCA cycle, including oxaloacetate, malate, and fumarate. Oxaloacetate is transformed into malate, fumarate and then succinate by fermentative route (Cheng et al. 2012).

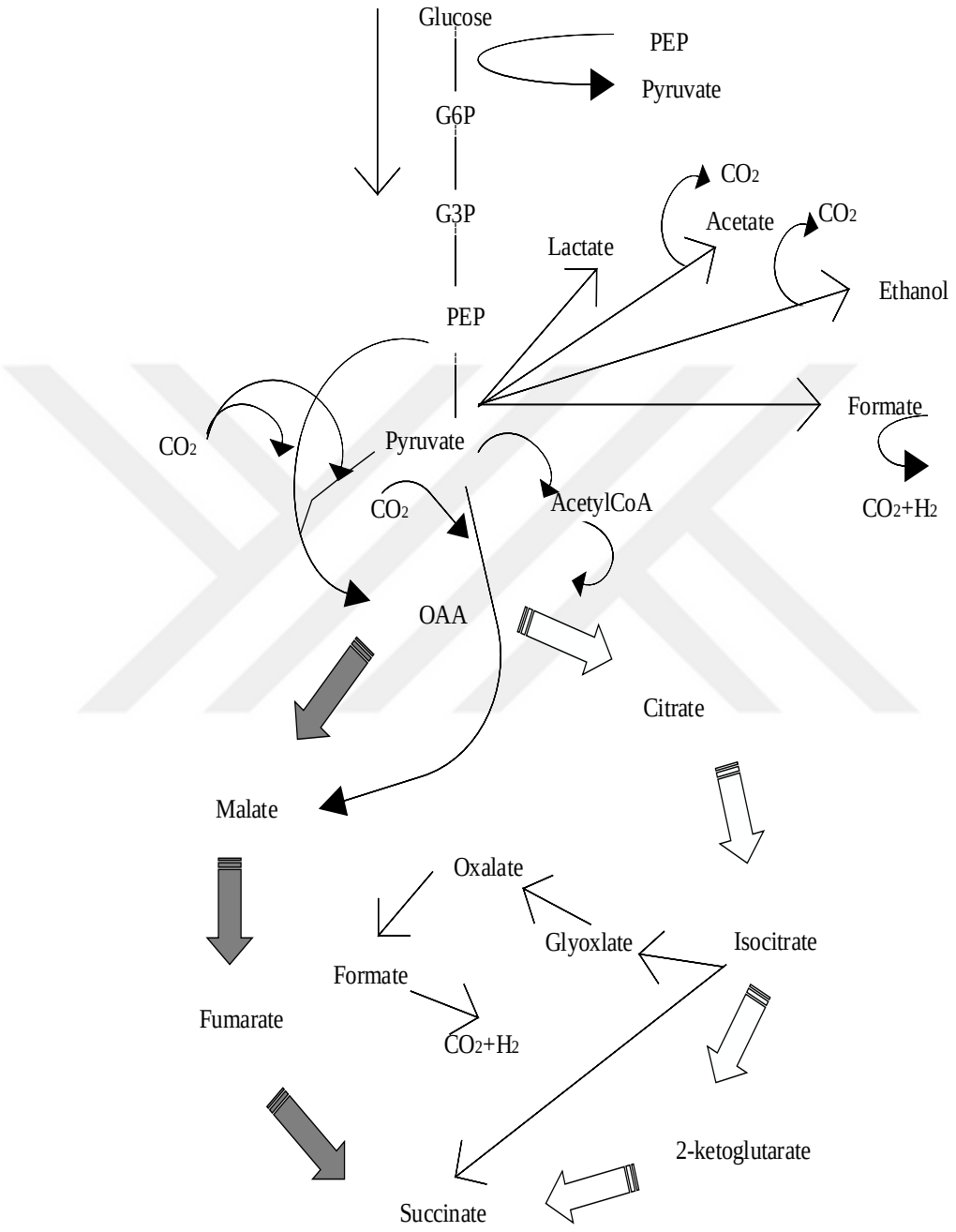

Figure 2.3. Metabolic pathway of succinic acid

(Source: Nghiem, Kleff, and Schwegmann 2017)

In Figure 2.3., the grey arrows showing the reductive (anaerobic) and empty white arrows indicating the oxidative (aerobic) tricarboxylic acid (TCA) cycle. Thin black thin arrows indicate contributing reactions and alternative pathways to succinic acid (OAA: Oxaloacetate, G6P: Glucose-6-Phosphate, G3P: Glyceraldehyde-3-Phosphate, PEP: Phosphoenolpyruvate.)

Microorganisms can produce two succinic molecules from each molecule of a 6-carbon sugar by the inclusion of two carbon dioxide molecules in the conversion of phosphoenolpyruvate (PEP) to oxaloacetate (OAA) with PEP carboxykinase(pck) during the use of the reductive pathway for succinic acid production (Nghiem, Kleff, and Schwegmann 2017). Then malate dehydrogenase (mdh), malic enzyme (sfc), fumarase (fum), and fumarate reductase (frd) are used and PEP carboxylation is regulated by the CO2 level, With the increase of CO2, the production of succinic acid increases, but also formic acid and ethanol production increases (Song and Lee 2006).

2.4. Microorganisms

Succinic acid, one of the final products of the Krebs cycle, can be produced by many microorganisms such as *Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, Mannheimia succiniciproducens,* and *Escherichia coli*.In Table 2.2. we can see some examples of succinic acid producer microorganisms.

Table 2.2. Succinic Acid Producer Microorganisms (Song and Lee 2006)

BACTERIA	FUNGI
Actinobacillus succinogenes	*Aspergillus niger*
Mannheimia succiniciproducens	*Aspergillus fumigatus*
Anaerobiospirillum succiniciproducens	*Byssochlamys nivea*
Recombinant *E. coli*	*Lentinus degener*
	Paecilomyces varioti
	Saccharomyces cerevisiae
	Penicillium viniferum

2.4.1. *Actinobacillus Succinogenes*

A.Succinogenes is a facultatively anaerobic, gram-negative, and ruminal microorganism that was first isolated from the bovine rumen and produced a higher amount of succinic acid in an environment containing high glucose compared to other strains and it can use C3, C5, C6 sugars, and disaccharides (Ahn, Jang, and Yup Lee 2016). *A.succinogenes* is the first choice for succinic acid production due to its production of the high amount of succinic acid, its ability to use cheap carbon sources, its performance in scalable biorefinery streams, its resistance to high concentrations of glucose, its high CO_2 availability, its non-pathogenicity and its tolerance to impurities such as HMF and furfural in hydrolysate (Dessie et al. 2018). Using the reducing arm of the TCA cycle, *Actinobacillus* is unique in this aspect. Glucose, fructose, xylose, arabinose, mannose, and sucrose are all effective substrates for *A. succinogenes* cultivation and thus could be used to produce succinate and *A. succinogenes* can efficiently accumulate succinate from a variety of complex carbon and nitrogen sources such as lignocellulosic biomass hydrolysates. The optimum pH value for this bacterium is 6.8. In the study where different pH values were tested, it was observed that the highest succinic acid production efficiency was at 6.8 when compared with lower and higher pH values(Wan et al. 2008).

In a study where cane molasses was used as raw material, bio-based succinic acid was produced with *A. succinogenes*. Cane molasses pre-treated with sulfuric acid was fermented with microorganisms in an anaerobic environment and succinic acid was produced at a concentration of 50.6 g/L with a yield of 79.5% (Liu et al. 2008). In another study in which Jerusalem artichoke tuber hydrolysate was used, succinic acid was produced from dilute acid pretreated hydrolysate with a yield of 74% and it was stated that artichoke tuber could be an alternative succinic acid production source(Gunnarsson, Karakashev, and Angelidaki 2014). *A. succinogenes* as can be seen in Figure 2.4. and 2.5.

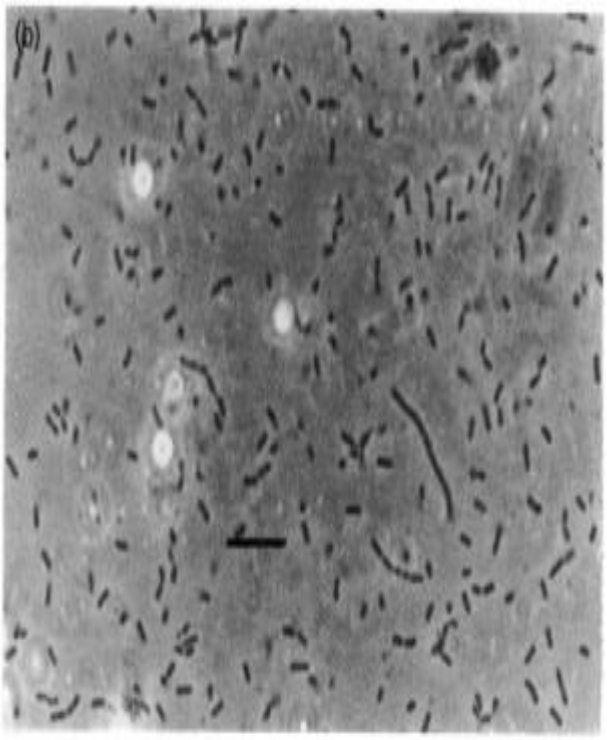

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Figure 2.4. *A.succinogenes* under the microscope

(Source: Guettler, Rumler, and Jain 1999)

Figure 2.5. *A.succinogenes* on TSB agar

2.5. Other Succinic Acid Producers

Anaerobiospirillum succiniciproducens is one of the bacteria that produces succinic acid anaerobically and it was isolated from dog (Beagle) faeces. It can effectively use many carbohydrates such as fructose, glucose, glycerol, lactose, starch for succinic acid (Ahn, Jang, and Lee 2016). In a study with *Anaerobiospirillum succiniciproducens*, wood hydrolysates (pretreated with steam explosion) were used (almost 27g/L glucose content) and 24 g/L succinic acid was produced by the addition of 10 g / L corn steep liquor as a nitrogen source (Lee, Lee, Hong, and Chang 2003). *Mannheimia succiniciproducens* is another type of bacteria obtained from Korean cows rumen and it can produce succinic acid using glucose, xylose, maltose fructose, arabitol (Ahn, Jang, and Lee 2016). In a study in which this bacterium was used, succinic acid was produced in two different media containing corn steep liquor and yeast extract using whey as carbon source and 71% yield was obtained with corn steep liquor and 72% with yeast extract (Lee, Lee, Hong, and Chang 2003). In addition, using metabolic engineering, the production of succinic acid was studied with the changes applied in many microorganisms. For example, a yeast, *Saccharomyces cerevisiae* whose metabolic engineering has been applied and some of its genes have been modified, produced 12.97 g/L succinic acid using glucose in the medium where $CaCO_3$ was added to urea and biotin, and it did this at pH 3.8 (Yan et al. 2014). The wild-type *E. Coli* is a bacterium that can produce a minimum amount of succinic acid in anaerobic environment, but studies have been carried out to increase the efficiency of producing succinic acid using recombinant gene technology. In a study related to this, the overexpression of the gene encoding malate dehydrogenase resulted in the production of 12.2 g succinic acid from 15.6 g glucose (Wang et al. 2009).

2.6. Raw Materials

Due to the need for water and soil to grow raw materials in bio-sourced productions, lignocellulosic biomass being sourced from agricultural waste, etc., provides recycling and provides an economically advantageous production for our world. Lignocellulosic wastes from industrial and agricultural sources are quite high worldwide. The estimated lignocellulosic biomass waste generated from grain crops worldwide is 2.9

× 103 million tons, waste from planting plants 5.4 × 102 million tons, and waste from oilseed crops is 1.4 × 10 million tons (Akhtar, Idris, and Aziz 2014). For example, it is estimated that more than 0.7 million tons of waste are generated each year in China (Zhao, Cheng, and Liu 2009).

A large number of lignocellulosic biomass have been used, tested, or proposed for the manufacture of succinic acid by fermentation. It is useful to compare feedstocks based on the following desirable qualities:

- low cost.
- low levels of contaminants.
- fast fermentation rate.
- high succinic acid yields.
- ability to be fermented with little or no pretreatment.
- year-round availability.

Lignocellulosic biomass is a complex organic structure that is abundant in nature and consists of cellulose, hemicellulose, and lignin. The main component of lignocellulosic biomass is cellulose which is a polymer of glucose linked by beta 1-4 glycoside bonds and it has a crystal and refractoriness structure (Khullar et al. 2013). They exist in different proportions in softwood and hardwood. Softwood biomasses include approximately 42% cellulose, 40%hemicellulose, 32% lignin and these ratios are 51% cellulose, 38% hemicellulose, 31% lignin in hardwood biomasses (Tarasov, Leitch, and Fatehi 2018).

Since most of the organic carbon in the biosphere is in the form of cellulose, the conversion of cellulose to other chemicals or fuels is of great importance. Hemicellulose is the second most abundant polymer found in lignocellulosic biomass and it forms a gel matrix around cellulose. Hemicelluloses differ in their composition. For example, hardwood hemicelluloses often contain xylan and softwood hemicelluloses often contain glucomannans. Heteropolymers of hemicelluloses consist of 5 or 6 carbon monosaccharides (pentoses: xylose, arabinose, hexoses: mannose, glucose, and galactose) and lignin, on the other hand, is a phenolic polymer and gives hardness to the

cell wall (Isikgor and Becer 2015). In succinic acid production, various lignocellulosic biomasses have been used such as corn stover (Zheng et al. 2010), cheese whey (Wan et al. 2008), rice husks (Bevilaqua et al. 2015), cane molasses (Liu et al. 2008), duckweed (Shen et al. 2018), and corncob (Yu et al. 2010). Lignocellulosic biomass is renewable, cost-effective, and abundant such as corn stover, corncob, rice husk, wheat stalk.

Table 2.3. Composition of some lignocellulosic biomasses (Saha 2003).

Biomass	Composition % (dry basis)		
	Cellulose	Hemicellulose	Lignin
Corn cob	45	35	15
Corn fiber	15	35	8
Corn stover	40	25	17
Wheat straw	30	50	20
Rice straw	35	25	12
Switchgrass	45	30	12
Coastal Bermuda grass	25	35	6
Sugarcane bagasse	40	24	25

2.7. Corn Cob

Under the name of lignocellulosic biomass, there are many agricultural products such as rice stalk, sunflower stalk, bamboo, cotton stalk, rice husk, rice straw, bagasse, and corncob have an important volume among them. Because corn is one of the most cultivated crops in the world. The corn cob, one of the important by-products of the corn plant, constitutes almost 20% of the corn plant, that is, 18-20 kg of corn cob comes out of 100 kg of the corn plant (Kapoor, Panwar, and Kaira 2016). Corn cob contains 35% hemicellulose, 45% cellulose, and 15% lignin on dry basis (Saha 2003). Xylan is the polysaccharide that makes up the majority of hemicellulose. Xylan can be obtained from corn cob, which can be used in different fields, can be used as a substrate for feed protein, or can be pretreated to obtain fermentable hydrolysates (Cao et al. 2004). The xylan in corn cob hemicelluloses contains approximately 35% arabinose, 6% galactose, 16%

glucuronic acid, and 53% xylose and xylose is one of the main structures in hemicellulose. Also, the production of xylose and multi-purpose xylitol from xylose is the more economical method with production from corncob than chemical production (Arumugam and Anandakumar 2016). In a study where corn cob was used as biomass, organosolv pretreatment (at 175 °C, for 2.5 h) was performed using ethanol (50 % w/w) and acetic acid (3 ml/L) as organic solvents and it was found that biogas production was more effective compared to alkali pretreatment and it was shown to be an alternative for biogas production (Sulbarán-Rangel et al. 2020).

2.8. Applications of Hemicellulose, Cellulose, and Lignin

Hemicellulose, cellulose, and lignin make up the lignocellulosic biomass and they are complex structures. These polymers are used in many different areas. Hemicelluloses can be used in the production of ethanol and xylitol (Tarasov, Leitch, and Fatehi 2018). Besides, hemicelluloses can be used in the paper production industry as hydrogels, thermoplastics, and additives, in cosmetics, and pharmacy as drug carriers (Farhat et al. 2017). Hemicellulose fibers can be used in wound dressing due to their moisture retention (Hu et al. 2020). It has an area of use in the food industry as a dietary fiber and fat substitute (Ebringerová 2005).

Cellulose can be used in the papermaking industry, veterinary foods, food industry, textile industry, cosmetics, and pharmaceutical industry, and especially an important part of the papermaking industry. Cellulose derivatives are often used in tablet or capsule formulations to modify the release of drugs and as tablet rheology control agents (Lavanya et al. 2015).

Lignin is used in carbon fiber production and dispersants production(Tarasov, Leitch, and Fatehi 2018). And it is used to improve the acceptance and high-temperature tolerance of batteries and also used to be an emulsion stabilizer, devolving on its molecular weight in some applications (Kienberger 2019).

2.9. Pretreatment of Lignocellulosic Biomass

The pretreatment of lignocellulosic biomass was first used to increase the digestibility of biomasses and to obtain affordable feeds with inexpensive lignocellulosic biomass (McMillan 1994). In short terms, the underlying reason for the pretreatment of lignocellulosic biomass is breaking the complex and stable structure of the lignocellulosic biomass and making it ready for enzymatic hydrolysis (Figure 2.6.). There are many different ways to the pretreatment of lignocellulosic biomass in the table (Table 2.4.).

Physical pretreatments mechanically break down the structure of the biomass and make it suitable for hydrolysis. With this process, the surface area for the reaction increases, and hence the hydrolysis efficiency increases (Aslanzadeh et al. 2014). The milling pretreatment, which is one of the physical pretreatments, is a pretreatment that can be done with the help of a mechanism that includes parts such as discs or balls, and in a study, sugarcane bagasse and straw are used as lignocellulosic biomass for ethanol production, the efficiency of disc and ball grinding mechanisms was tested and it was observed that both of them increased enzymatic digestibility as a result of fermentation performed by *Saccharomyces cerevisiae* (Silva et al. 2010).

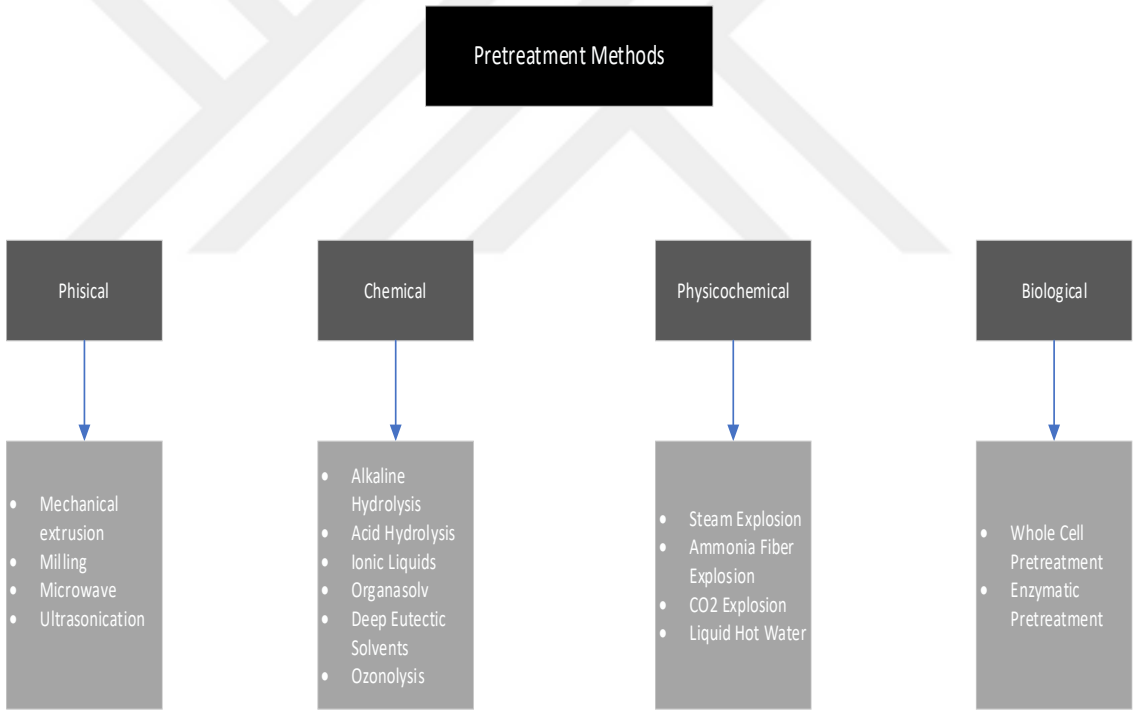
Chemical pretreatments also use chemical reactions to break the stubborn structure of the lignocellulosic raw material, and the most common of these pretreatments are alkali pretreatment, acid pretreatment, organosolv, ionic liquids (Jędrzejczyk et al. 2019). Dilute acid pretreatment by adding dilute acid(H_2SO_4, HCl, etc.) and heating, is one of these chemical methods and breaks the structure of lignocellulosic biomass and increases porosity, but it is a disadvantageous method in terms of recovery and corrosion(Badiei et al. 2014). In a study using corn fibers, bio-based succinic acid was produced using *A. succinogenes* for fermentation from fibers subjected to dilute acid hydrolysis with a yield of 72%, and it was stated that corn fibers could be used in the production of succinic acid as an alternative to glucose (Chen et al. 2011).

Physicochemical pretreatments, including steam explosion, ammonia fiber explosion, and liquid hot water, are methods that combine physical and chemical processes, and in these methods, in addition to high temperature and pressure, sometimes an inorganic compound is used to break down the lignocellulosic structure (Jędrzejczyk et al. 2019). For example, in a study using ammonia fiber explosion pretreatment, agave wastes were used as substrate and 85% sugar conversion was achieved by fermentation

by *Saccharomyces cerevisiae* after enzyme hydrolysis, and 40 g/L ethanol was produced and presented as an alternative method (Flores-Gómez et al. 2018).

Biological pretreatments include enzyme hydrolysis and whole-cell pretreatments. In these pretreatments, the hydrolytic and ligninolytic enzymes secreted by microorganisms provide the degradation of the structure and the preparation of the environment for the hydrolysis of the biopolymers, and for this, many microorganisms including fungi and bacteria can be used such as *Aspergillus niger, Fibrobacter succinoges, Aspergillus oryzae, Bacillus subtilis* (Sharma et al. 2019). In a biological pretreatment study using corn stalk as a substrate, white-rot fungus *Irpex lacteus* were used and the hydrolysis yield reached 82 % in 28 days (Du et al. 2011).

Table 2.4. Pretreatment methods (Baruah et al. 2018).



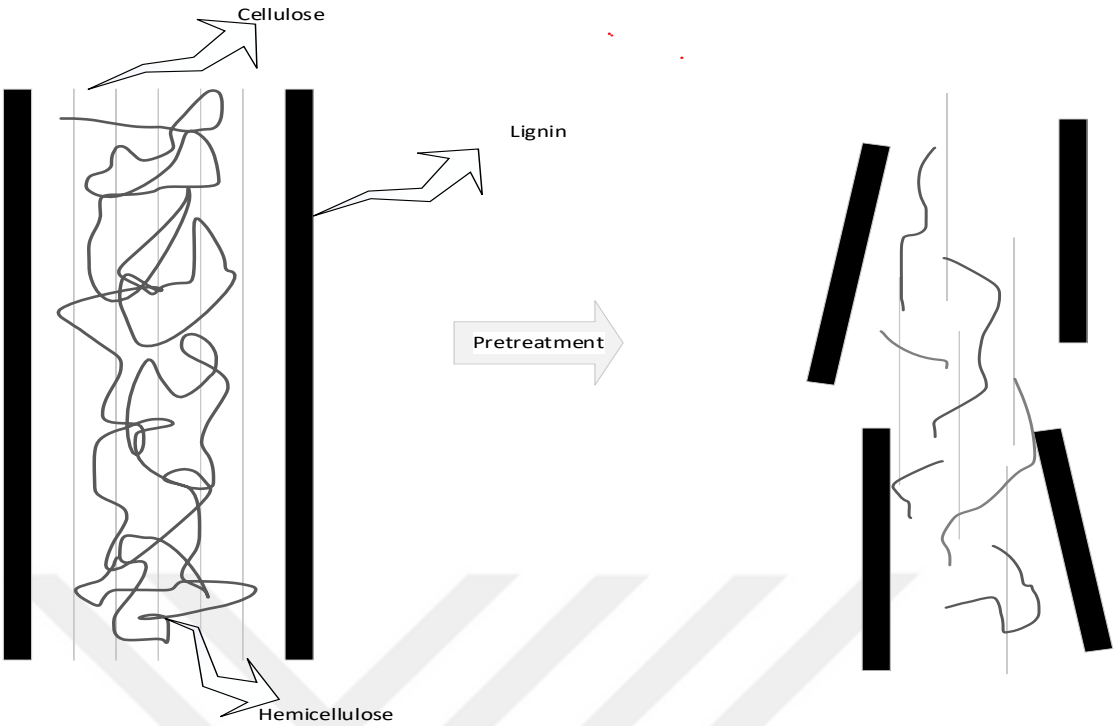


Figure 2.6. Effect of pretreatment on lignocellulosic biomass

(Source: Muley and Boldor 2017)

2.9.1. Organosolv Pretreatment

Organosolv pretreatment involves the hydrolysis of lignin bonds and lignin-carbohydrate bonds using organic solvents (such as methanol, ethanol, acetone, ethylene glycol), water, sometimes catalyst agent (acid or base), and a solid containing cellulose and hemicellulose. Organosolv pretreatment successfully removes lignin. Ethanol and methanol are preferred organic solvents compared to others in organosolv pretreatment because they are more affordable and they facilitate recycling due to their low boiling temperatures (Mesa et al. 2011). For comparison between methanol and ethanol, ethanol is more convenient to use because ethanol is less toxic than methanol (Zhao, Cheng, and Liu 2009).

It was determined that the biomasses treated with ethanol organosolv pretreatment have more enzymatic digestibility compared to other methods (Mesa et al. 2011). The advantages of organosolv pretreatment are as follows:

1) Organic solvents are easy to recover and can be reused for pretreatment.

2) The lignin isolated from the process can be used in some industries (Zhao, Cheng, and Liu 2009).

3) Compared to aqueous media, organic solvents have effects such as hydrogen transport, limiting diffusion effects, and improving the reaction by increasing catalytic activity (Zhang et al. 2016).

Besides these, organosolv pretreatment also has some disadvantages. These are that organic solvents are expensive, the energy consumed while recycling and the need for controlled work since organic solvents can be volatile and flammable (Baruah et al. 2018).

Organosolv pretreatment is used in a variety of biotechnological production from biomass such as sugarcane bagasse (Mesa et al. 2011), rice straw (Amiri, Karimi, and Zilouei 2014), wheat straw (Salapa et al. 2017). In a study, which olive tree pruning wastes were used, fermentable sugar was produced by organosolv pre-treatment from biomass using different concentrations of ethanol and different temperatures, and it was observed that the most efficient enzymatic hydrolysis (based on glucan) was achieved with a 15-minute pretreatment with 43% ethanol at 210 °C, and it was stated that organosolv pretreatment was more effective than other pretreatments (Díaz et al. 2011). In another study with bamboo, organosolv pretreatment with ethanol (75%) was catalyzed with dilute acid (2%), and cellulose was transformed into glucose yield of 83.4 % by enzymatic hydrolysis, and it was observed that fermentation inhibitors such as HMF and furfural were formed in less amount than the sulfuric acid-water treatment (Li et al. 2012).

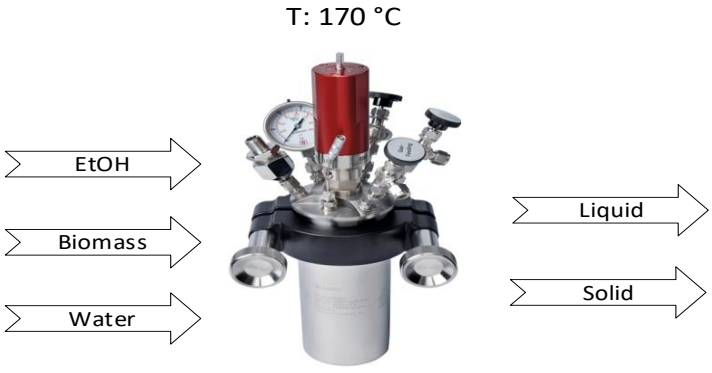


Figure 2.7. Organasolv pretreatment in the reactor

2.10. Enzymatic Hydrolysis

The most common method used for the production of fermentable sugars from pretreated biomass is enzyme hydrolysis and too many enzymes are used for this process (Ioelovich and Morag 2012). Lignin and hemicellulose bind to cellulose with too many cross-links and bonds, making it difficult for enzymes to form fermentable sugars. First of all, the complex structure is destroyed by various pretreatments, facilitating enzymes to act on cellulose and hemicellulose. Afterward, enzymes (such as cellulase, xylanase, exoglucanase) are added to the media, which will convert the cellulose in biomass and hemicellulose to fermentable monosaccharides and the medium contains buffer and pretreated biomass (Khullar et al., 2013).

In a study, pretreatment of yellow sorghum meal with concentrated phosphoric acid, enzyme hydrolysis was carried out together and, *A. Succinogenes* performed fermentation and 17.9 g / L succinic acid was produced from 29 g / L cellulosic glucose (Lo et al. 2020). A study has been done using alkaline pretreatment of cassava roots and pre-treatment with ozone and continued with enzyme hydrolysis, a maximum succinic acid concentration of 11.25 g / L was reached (Kanchanasuta et al. 2020).

2.10.1. Cellulases

Cellulases that are produced by fungi, bacteria, and protozoans and break down the structure of cellulose by breaking the beta -1,4- glycosidic bonds and release fermentable glucose (Sethi and Gupta 2014). This is done by three different types of enzymes, including exo beta-1,4-glucanase, endo beta-1,4-glucanase, and beta -1,4-glucosidase (Figure 2.8.). And the works they undertake are as follows:

- Exo beta-1,4-glucanase: It releases a cellobiose unit,
- Endo beta-1,4-glucanase: It hydrolyzes the beta -1,4- bonds in the cellulose randomly,
- Beta -1,4-glucosidase: It converts the cellobiose units secreted by the exo beta-1,4-glucanase to glucose (Li et al. 2009).

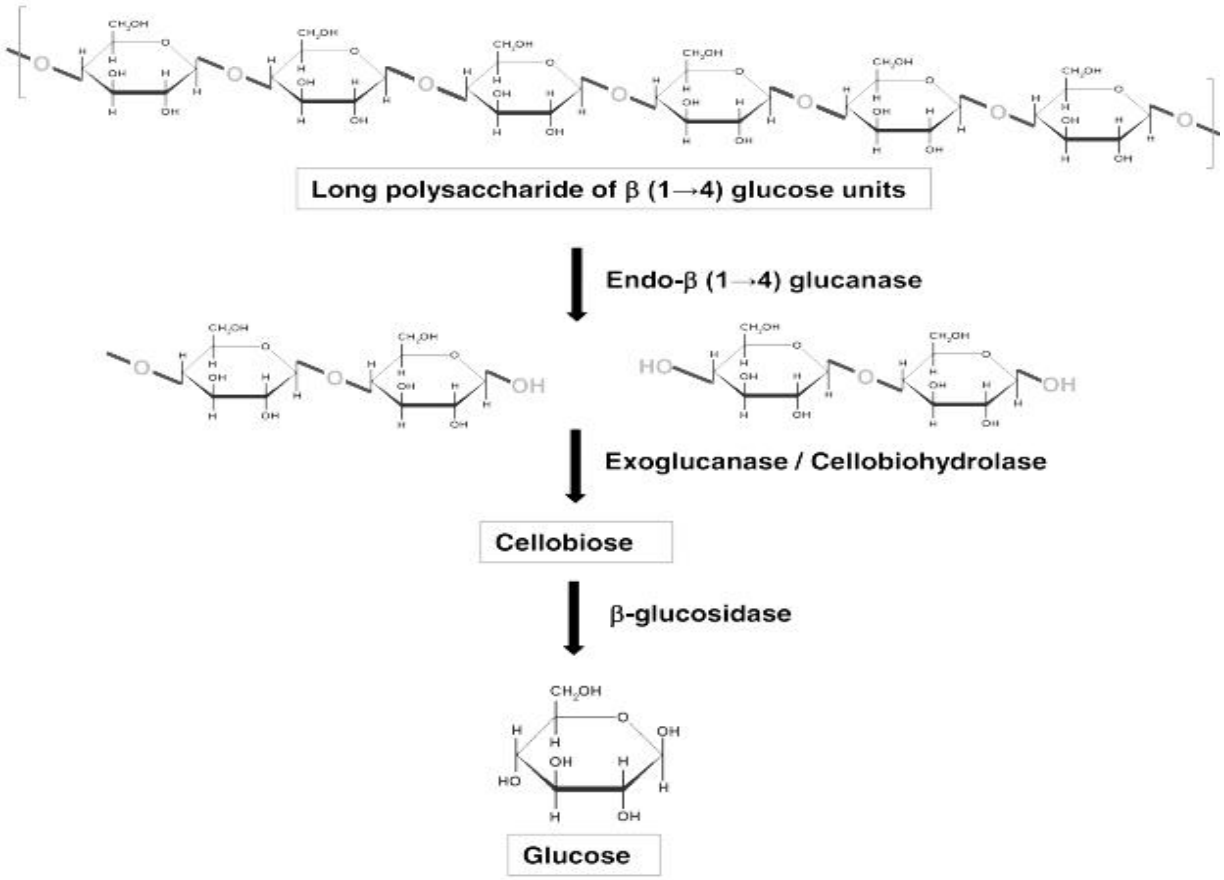


Figure 2.8. Enzymes involved in cellulose degradation

(Source: Lakhundi, Siddiqui, and Khan 2015)

Cellulases have a wide range of uses in the paper and pulp industry, such as dewatering, characterizing pulp fibers, improving fiber strength, bleaching kraft pulps, and they are also used in the production of biodegradable cardboard (Singh et al. 2016). In the food industry, cellulases are used to clarify fruit juices and olive oil, fruit nectar production, improve the malting of barley and increase the nutritional value and digestibility of feeds (Imran et al. 2016). Celluloses are used in the production of biobased ethanol with the conversion of cellulose to fermentable sugars (Kundu, Ghose, and Mukhopadhyay 1983). Cellulases provide an environmentally friendly alternative for the production of high-value chemicals such as lactic acid, succinic acid by producing sugars used in fermentation (Juturu and Wu 2014). In the textile industry, cellulases are used for the bio-stoning of denim, defibrillation of lyocell, improving the color brightness of fabrics and ensuring smoothness, and for the prevention of plant pathogens and diseases in agriculture (Bhat 2000).

2.10.2. Xylanases

Xylanase is produced by some organisms that are microorganisms, protozoans, and it can also be found in the rumen of some animals. In industrial terms, the most preferred microorganisms such as bacteria fungi are used in the production of xylanase. Xylan comprises D-xylose units linked by beta 1-4 glycosidic bonds and xylanase breaks these glycosidic bonds (Bhardwaj, Kumar, and Verma 2019).

A group of enzymes known as xylolytic enzymes carries out the hydrolysis of xylan. The main enzymes of this enzyme group are endo -1,4- beta xylanase, beta-xylosidase, alpha-L-arabinofuranosidase, alpha glucuronidase, acetylxylan esterase, and ferulic and p-coumaric acid esterase (Figure 2.9.). The tasks they undertake are as follows:

- Endo -1,4- beta xylanase: It breaks down the glycosidic bonds of the xylan structure and reduces the polymerization degree,
- Beta xylosidase: It hydrolyzes short xylooligosaccharides and xylobiose to release non-reducing end xyloses and is an exoglycosidase,
- Alpha-L-arabinofuranosidase: It hydrolyzes the bonds that bind arabinoses in xylan structure,
- Alpha glucuronidase: It hydrolyzes the bonds between xylose and glucuronic acid,
- Acetylxylan esterase: It removes the O-acetyl substituents,
- Ferulic and p-coumaric acid esterase: It breaks ester linkage between xylan and ferulic and p-coumaric acid esterase (Sunna and Antranikian 1997).

Xylanases are used in the production of cereal-based foods, in increasing the digestibility of animal feeds, in the pre-bleaching process in paper production (Pandya and Gupte 2012). In the food industry, they are used for clarification and extraction of fruit and vegetable juices and for increasing the quality of bakery products such as dough, biscuits, cakes (Bhat 2000). In addition, xylanases are used in the production of valuable organic acids and biofuels by releasing sugars that microorganisms will use in fermentation from lignocellulosic biomass (Pennacchio et al. 2018; Xin and He 2013).

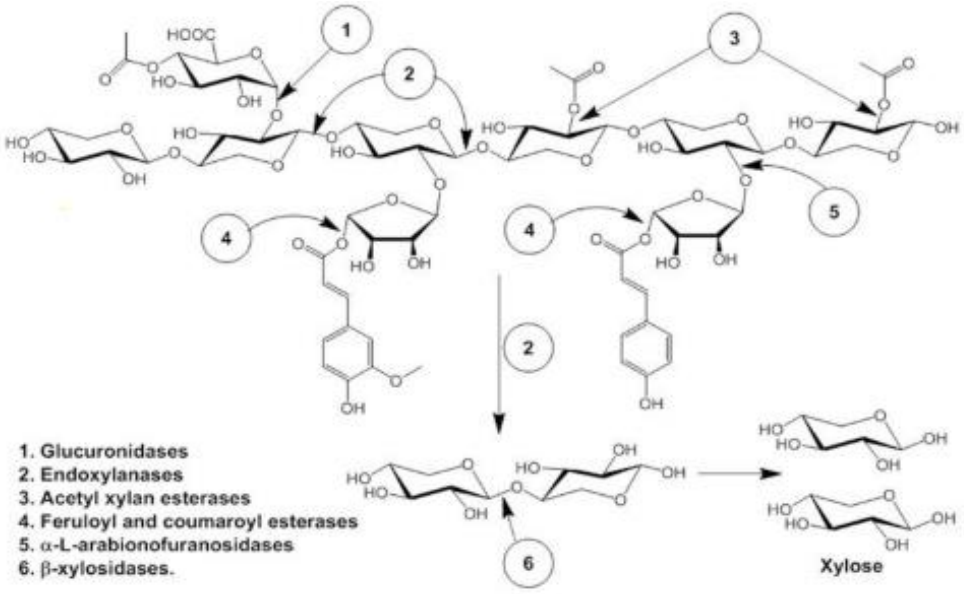


Figure 2.9. Enzymes involved in xylan degradation

(Source: Yadav et al. 2018)

2.11. Separate Hydrolysis and Fermentation & Simultaneous Saccharification and Fermentation

After the pretreatment, enzyme hydrolysis is applied to the lignocellulosic biomass, and if the sugar-containing hydrolysate is obtained from here and then the fermentation process is done separately, this process is called SHF, but if both processes, fermentation and enzyme hydrolysis, are carried out at the same time in the same medium, it is called SSF (Öhgren et al. 2007). In SHF, firstly, the enzymatic saccharification of the pretreated biomass is performed by providing the optimum temperature of the enzyme. Then microorganisms are added to the saccharified solution for fermentation. By processing the enzyme at the optimum temperature, both a higher sugar content in the hydrolysate and the use of fewer enzymes in the process, in general, can be achieved (Ishizaki and Hasumi 2013).

Comparing the two processes, SSF is a more advantageous application compared to SHF because of the integration of the process steps, simplification of the experimental process, and low cost can be achieved with SSF and the use of separate equipment is not required during the experiment. In addition, SSF provides a more efficient production by eliminating end-product inhibition, and the thing that provides this is that the

microorganism can use this sugar for growth and production immediately when sugar is produced (Saha et al. 2011). Many studies have shown that SSF is superior when compared to SHF. For example, in a study comparing SSF and SHF, wheat straw was used as a substrate, and ethanol production after steam explosion pretreatment was compared, as a result, while SSF lasted 30h, SHF lasted 96 h and productivity was 0.837 g / Lh for SSF and 0.313 g / Lh for SHF(Alfani et al. 2000).

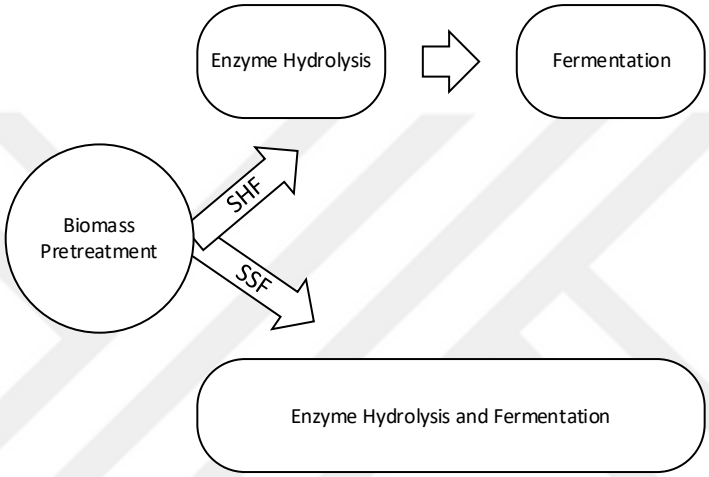


Figure 2.10. Difference between SHF and SSF.

CHAPTER 3

MATERIAL AND METHODS

3.1. Materials

Corn cob was selected as lignocellulosic biomass and it was obtained from the Ministry of Agriculture and Forestry, Aegean Agricultural Research Instıtue (Izmir, Turkey).*A. sucinogenes* NRRL B-59377(ATCC 55618) used throughout this study was kindly supplied by ARS (NRRL) Culture Collection. Celluclast and Accelarase XY were provided kindly by Novozymes (Denmark) and Genencor (Finland), respectively. And other materials used in the study are listed below:

- Tyriptic Soy Broth (Sigma Aldrich)
- KH_2PO_4 (Sigma Aldrich)
- K_2HPO_4 (Sigma Aldrich)
- $CaCl_2$ (Sigma Aldrich)
- $MgCl_2$ (Sigma Aldrich)
- Yeast Extract (Merck)
- NaCl (Sigma Aldrich)
- $MgCO_3$
- NaOH (Sigma Aldrich)
- Ethanol (Isolab)
- Soduim Citrate (Sigma Aldrich)
- Citric Acid (Sigma Aldrich)
- Sulphuric Acid (Sigma Aldrich)
- $CaCO_3$(Sigma Aldrich)
- Glucose Monohydrate, Xylose (Sigma Aldrich)
-Succinic Acid, Formic Acid (Sigma Aldrich)
- Xylan from Beechwood (Megazyme)
- 3,5 Dinitro Salicylic Acid (DNS) (Sigma Aldrich)
- Phenol

3.2. Methods

3.2.1. Organasolv Pretreatment of Lignocellulosic Biomass

The organosolv pretreatment of corn cob (grain size< 2mm, milled in a hammer mill) was performed with a high-pressure reactor (Berghof BR 600, Germany) and disrupted the lignocellulosic matrix of the corn cob [170°C, 70% EtOH, solid: liquid ratio, 1:10; 1h pretreatment] (Temelli 2020) 25 g milled corn cob (Figure 3.1. (a)), 175 ml EtOH and 75 ml deionized water were placed in the reactor with the steel tank. The tank was placed carefully, and the parameters were checked, and the heating process was started. The pedal mixer was set at 300 rpm to mix the contents. After reaching 170 °C, the reaction was continued for 1h and the reactor was cooled by circulation of tap water in the internal coils. Cooling was continued until the temperature was below 60 °C, and then the reactor was opened. The treated biomass was separated from the liquid part by filtering through cheesecloth and then was washed with deionized water and dried at 60 °C for overnight (Sabanci and Buyukkileci 2018). The solid reaching constant weight was ground by the grinder (Sinbo, Turkey). Prepared biomass was placed in sample bags and stored until use (Figure 3.1. (b)).

a)  b) 

Figure 3.1. Milled raw corncob (a) and pretreated corncob (b)

3.2.2. Enzymatic Hydrolysis of Lignocellulosic Biomass

Celluclast and Accelarase XY were used for the enzymatic hydrolysis. Enzymatic hydrolysis of lignocellulosic biomass was carried out in test tubes with a total reaction mixture volume of 7.5 ml containing 0.3 g biomass was used for each tube. The optimum enzyme hydrolysis conditions were determined by applying enzyme hydrolysis to biomass under different temperatures (37 and 50 °C), enzyme concentrations, solid: liquid ratios, and pH values (phosphate buffer-pH 6.8 and 50mM citrate buffer-pH 5.2). The hydrolysis process was carried out in a shaking incubator at 120 rpm. 48 h after the initiation of enzymatic hydrolysis, tubes were placed in a boiling water bath for 5 min to inactivate the enzymes. Then tubes were centrifuged and the liquid hydrolysate was separated from the solid part to use in SHF. The percent of xylan and cellulose conversion was calculated according to the HPLC results using equations 1 and 2:

Equation 1:

$$\text{Cellulose Conversion (\%)} = \frac{(Glucose\ released(g))xAnhidrous\ Factor\ (0.9)}{Cellulose\ in\ pretreated\ biomass(g)}x100$$

(Sabanci and Buyukkileci 2018)

Equation 2:

$$\text{Xylan Conversion (\%)} = \frac{(Xylose\ released(g))xAnhidrous\ Factor\ (0.88)}{Xylan\ in\ pretreated\ biomass(g)}x100$$

3.2.3. Fermentation Conditions

Inoculum prepared for use in fermentation. *A.succinogenes* was grown in tryptic soy broth (3ml) at 37 °C from the stock culture(30µl, at -80 °C in 40% glycerol) for 18h. Fermentation medium was prepared. The fermentation medium was composed of yeast extract (16g/L), KH2PO4 (3g/L), K2HPO4 (1.5 g / L), NaCl (1 g / L), MgCL2 (0.3g / L), CaCl2 (0.3g / L) (Zhu et al., 2012). As the carbon source glucose-xylose mixture(20g/L-20g/L) (XG medium), pretreated corn cob or enzymatic hydrolysate of the pretreated corncob was used. Fermentation was carried out with Hungate tubes and a bioreactor

(Biostat B). The bioreactor was used for SSF only. The pH of the fermentation medium was adjusted to 8.2 with 2 M NaOH and transferred to Hungate tubes. In the bioreactor pH of the fermentation medium was adjusted 6 M NaOH to 6.8. The CO_2 was pressed into it with a syringe and the rubber was slowly closed while the gas continued to be pressed, and the last syringe was removed from tubes. For the bioreactor, CO_2 was pressed continuously (0.45 l/m). $MgCO_3$ (up to 50% of the carbohydrates) was weighed separately for each tube and placed in eppendorfs. Eppendorfs containing $MgCO_3$ and Hungate tubes containing medium were sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the pH value was measured as 7.9. Next to the flame, sterile Hungate tubes were opened and $MgCO_3$ was added. At the same time, the inoculum was transferred to the fermentation medium (250µl inoculum for 7,5 ml medium) 18-20 h after inoculation and shaking at 120 rpm in the incubator and 150 rpm in the bioreactor (Figure 3.2. and Figure 3.3).

As a control, fermentation medium containing 20 g / L glucose and 20 g / L xylose was used for succinic acid production. To test the hypothesis of this study, the enzymatic hydrolysate was used as a sugar source in the fermentation medium at and at the same time, SSF process was performed under specified conditions with pretreated biomass at pH 6.8. Production of succinic acid was followed by HPLC.

In preliminary tests, it was observed that the concentrations of xylose and glucose in the culture medium decreased after autoclaving by about 35-40%. We speculated that the sugars may have absorbed by the solid MgCO3, which was added to the medium as a buffering agent. Said effect was tested by sterilizing the MgCO3 and the medium separately and mixing after the sterilization in the autoclave. The medium was prepared to have 20 g/L xylose and 20 g/L glucose, initially. MgCO3 required for each Hungate tube was sterilized separately. This test showed that separate sterilization could prevent the decrease in the carbohydrates concentration. Therefore, in the following cultivations, MgCO3 was sterilized separately and added afterward.

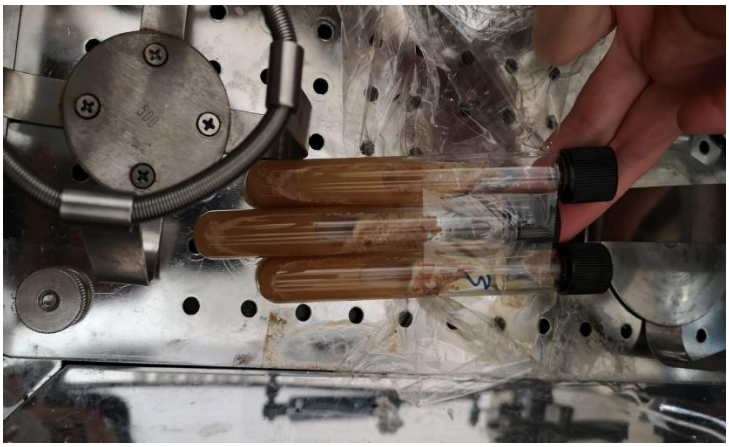


Figure 3.2. Hungate tubes in shaker incubator

Figure 3.3. Fermentation in the bioreactor

3.2.4. Simultaneous Saccharification and Fermentation

SSF was carried out in Hungate tubes (Figure 3.3.). For the SSF process, pretreated lignocellulosic biomass was used at a 1:15 solid: liquid ratio (0.5 g pretreated biomass and 7.5 ml culture medium). 30ul Accelarase XY and 100 ul Celluclast and the inoculum were added. Then, under CO_2 sparging $MgCO_3$ has added aseptically. Samples in Hungate tubes were placed in shaking incubator at 37 °C and shake at 120 rpm. Samples were taken at regular intervals for centrifuged and diluted for HPLC analysis.

In addition, to increase production, succinic acid production was monitored by adding enzyme and biomass at the end of 24 h, the same as the initial amount of enzyme and biomass.

3.2.5. Separate Hydrolysis and Fermentation

In this approach, enzymatic hydrolysates obtained as described above were used as the carbon source for succinic acid production. The hydrolysate was supplemented by medium ingredients (Figure 3.4.). The pH was adjusted to 8.2 with 4M NaOH. The medium was sterilized at 121°C for 15 min. *A. succinogenes* that have to reach the 18h were added into the medium, $MgCO_3$ was added aseptically under CO_2. The tubes were incubated as described for SSF. Samples were taken at regular intervals for 48 h, centrifuged, and then diluted for HPLC analysis.

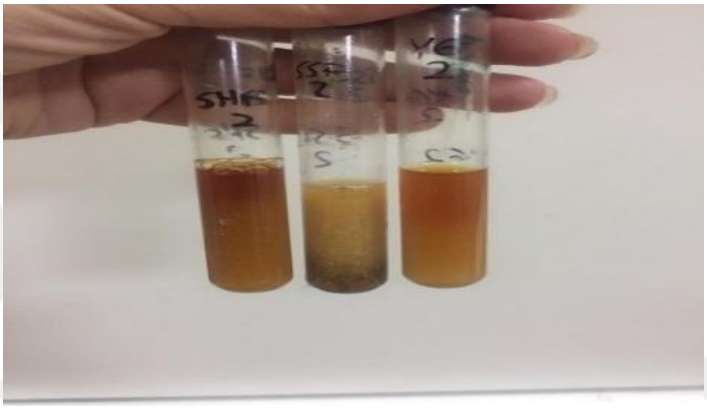


Figure 3.4. SHF, SSF, and XG medium before fermentation in Hungate tubes (From left to right)

3.3. Analytical methods

3.3.1. Acid Hydrolysis

Acid hydrolysis was performed to determine the polymeric content of the raw and pretreated lignocellulosic biomass and. For acid hydrolysis, 0,3 g dry biomass was treated with 3 ml 72% sulfuric acid in test tubes at room temperature for 1 h (Figure 3.5. (a)). The tubes were mixed by vortexing every 5 min. Then the content was diluted to 4% sulfuric acid by adding 84 ml of deionized water and kept at 121 °C for 1 h in the

autoclave (Figure 3.5. (b)). After the autoclaving process, 0,26 g $CaCO_3$ was added to 5 ml of the acid hydrolysate to increase the pH value to 5-6. It was then centrifuged to remove excess $CaCO_3$, diluted 5-fold and filtered by through membrane filters with a pore size of 0.45µm for the HPLC analysis. Xylose and glucose released as a result of the acid hydrolysis were measured in HPLC. The xylan and cellulose contents of the solid biomass were calculated using the xylose and glucose concentrations, respectively, using Equations 3 and 4.

Equation 3:

$$\text{Xylan (\%)} = \frac{\left(Concentration\ of\ xylose\ \left(\frac{g}{L}\right)\right) x AnhidroFactor\ (0.88) x 0.087}{Analyzed\ dry\ matter(g)} x100$$

Equation 4:

$$\text{Cellulose (\%)} = \frac{\left(Concentration\ of\ glucose\ \left(\frac{g}{L}\right)\right) x AnhidroFactor\ (0.9) x 0.087}{Analyzed\ dry\ matter(g)} x100$$

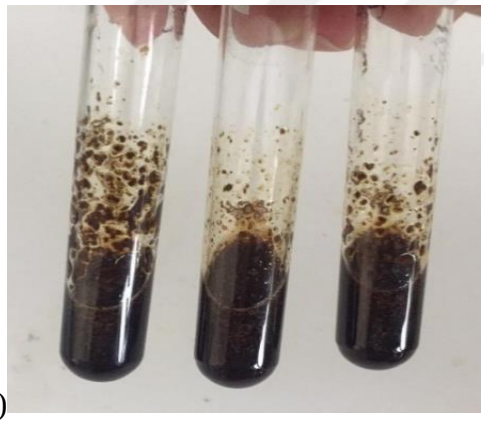
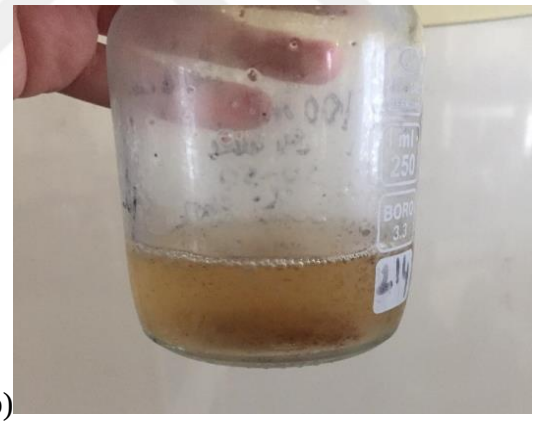
a)  b) 

Figure 3.5. Acid hydrolysis samples stored at room temperature in test tubes (a) after autoclave and the image of the diluted sample in a bottle (b)

3.3.2. Succinic Acid Analysis

Succinic acid concentrations in fermentation medium were analyzed by HPLC. The HPLC system was comprised of Perkin Elmer Series 200 pump., Series 200 refractive index detector (RID), Series 900 interface, and a standard computer. The system was controlled by Turbochrom Navigator software. The mobile phase was 5mM H2SO4 for the Aminex HPX-87H column for organic acid analyses. The mobile phase was filtered through 45 um cellulose acetate filter papers before use at HPLC. The column temperature was adjusted at 65 °C with MetaTherm column oven. The column was run at a flow rate of 0.6 ml/min for 15 min. The retention time of succinic acid was around 11.3 min. The quantification of succinic acid in the samples was done by comparing the peak areas with the standard curve. The standard curve was prepared by running succinic acid solutions with known concentrations (Appendix A).

One ml of fermentation sample was taken periodically from the SSF and SHF cultures and then centrifuged at 20000 RCF for 15 min. The supernatant was separated and was diluted 20 times with deionized water and filtered using 45 μm syringe filters (Sartorius). All standard solutions were prepared with deionized water and filtered.

. The properties of the column and the analysis conditions are given in Table 3.1 and the photo of the HPLC system is shown in Figure 3.6.

3.3.3. Glucose and Xylose Analysis

For the analysis of glucose and xylose, samples were taken periodically from SHF and XG cultures and the hydrolysis mixture. Glucose and xylose were detected in the same HPLC run used for succinic acid analysis. For the glucose, retention time is 8.2 minutes and for the xylose, it is 8.9 minutes. The calibration curves that about glucose and xylose were shown in Appendix A and the R^2 values of these sugars were greater than 0.99. Analysis conditions and retention times of succinic acid, xylose, and glucose are given in Table 3.1.

Table 3.1. The properties of the column and analysis conditions for HPLC analyses.

PROPERTY	SPECIFICATIONS
Type of Analysis	Succinic Acid Glucose and Xylose
Retention Time	Succinic acid: 11.4 min Glucose: 8.2 min Xylose:8.9 min
Column	Aminex HPX-87H ion exclusion Column (Biorad Laboratories)
Column Length	300 mm
Column Diameter	7.8 mm
Particle Size	9 μm
Guard Cartridge	Micro-Guard cation- H cartridge (30 x 4.6)
Mobile Phase	5 mM H_2SO_4
Flow rate	0.6 ml/min
Temperature	65° C
Detector	Refractive index
Elution Type	Isocratic Elution

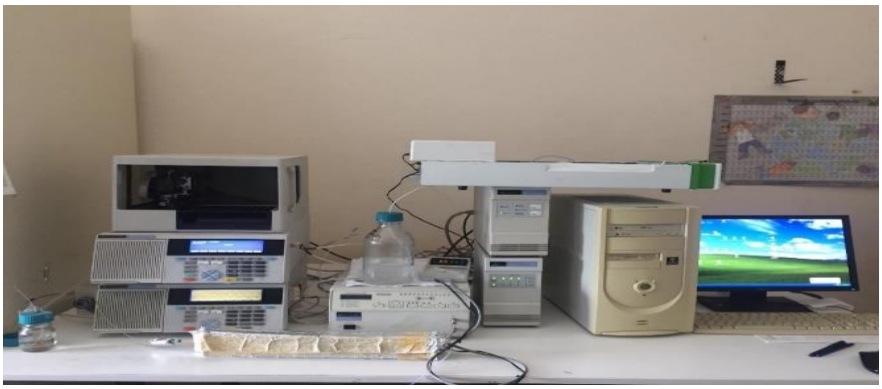


Figure 3.6. High-Pressure Liquid Chromatography(HPLC)

3.3.4. Enzyme Activity Assay

3.3.4.1. Xylanase Activity

The amount of reducing sugar released from beechwood xylan as a result of enzyme action was measured using the dinitrosalicylic acid reagent (DNS) method to assess xylanase activity (Bailey, Biely, and Poutanen 1992; Yegin 2017). 0.1 ml of appropriately diluted enzyme preparation was combined with 0.9 ml of substrate solution (0.5 %(w/v) beechwood xylan prepared in 0.05 M citrate buffer (pH 5.2)). The assay mixture was incubated for 5 min at 50°C in a water bath. Then, the reaction was stopped by adding 1.5 mL DNS to the mixture, which was then boiled for 5 min and cooled in ice-cold water in 1 min. For each sample, a control was run simultaneously that included all of the reagents but stopped the reaction before adding the enzyme. It was calculated how much reducing sugar was released by measuring the absorbance at 540nm. The calibration curve was prepared using xylose (Appendix B). The amount of enzyme needed to release 1 mol of xylose equivalent per minute under the assay conditions was described as one unit of xylanase activity. The activity of xylanase was determined using the formula below.

Equation 5:

Activity (U/ml) = $\frac{X}{150.13x5x0.1} xDF$

(X: µg xylose (from the standard graph), DF: Dilution factor, 150.13: Molecular weight of xylose, 5: Incubation time (min), 0.1: Enzyme amount (ml))

3.3.4.2. Cellulase Activity

The method used for cellulase activity measures the activity in 'filter paper units (FPU) per milliliter of the undiluted enzyme(Adney and Nrel 2008). In the activity determination, 1,0x6,0 cm Whatman No:1 filter paper is used as a substrate. The rolled filter papers were put into test tubes then 1 ml of citrate buffer (pH 4.8) and 0.5 ml of the enzyme were added and kept at 50 °C for 1 h. At the end of the period, the enzyme reaction was stopped by adding 3 ml of DNS. Glucose standards were prepared and the

same procedure was applied to generate the standard curve (Appendix B). All tubes were then boiled in a water bath for 5 min and then immediately cooled. To measure the color change, the samples were diluted and absorbance values read at 540 nanometers against blank. The activity of xylanase was determined using the formula below (Equation 6):

Equation 6:

$$\text{Filter Paper Activity (FPU)} = \frac{0.37}{[enzyme] releasing\ 2.0\ mg\ glucose} \text{x units/ml}$$

[enzyme]: The result when the enzyme concentration is determined which will release exactly 2.0 mg of glucose by plotting the glucose released against the enzyme concentration.

0.37 is calculated using the factor for converting the 2.0 mg of glucose-equivalents produced in the assay to mmoles of glucose (2.0/ 0.18016), as well as the volume of the enzyme being measured (0.5 mL)

0.37μmol/minute - mL = (2.0 mg glucose / 0.18016 mg glucose/μmol) / (0.5 mL enzyme dilution x 60 minu

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Cellulose and Xylan Content in Pretreated Corncob

Xylan and cellulose together with lignin form the three main compounds of lignocellulosic biomass. Determination of xylan and cellulose content of corn cob was necessary to determine the xylose and glucose yields obtained by enzymatic hydrolysis and succinic acid production yield obtained with these sugars in fermentation processes.

The cellulose and xylan contents of raw corn cob were determined as 31.6% and 29.7%, respectively. The previous studies stated that the corn cob consisted of 33.7% to 41.2% cellulose, 31.9% to 36% hemicellulose, and 6.1% to 15.9% lignin (Srivastava et al. 2021). Another source states that corncob consisted of 42% to 45% cellulose, 35% to 39% hemicellulose, and 14% to 15% lignin (Tayyab et al. 2018). Takada et. al (2018) found that the chaff part of the corncob contained 36.3% cellulose, 46.9% hemicellulose, and 18.8% lignin, while woody ring part contained 31.6%, 46.9%, and 15.7%, and pitch part contained 34.8%, 39.9%, and 17.3% cellulose, hemicellulose, and lignin, respectively. The values obtained in this study was consistent with the literature. The differences in the applied processes and the difference in the plant source was considered as possible reason for the differences (Takada et al. 2018). In the organosolv treated corncob, cellulose constituted a larger portion than the others, and it was found as 43% by acid hydrolysis. Xylan was the second main component, and its ratio was 38.7%. Lignin content was estimated as around 18%, by difference. In addition, acid hydrolysis was applied to the liquid obtained from the organosolv and it was calculated that 0.14 g cellulose and 2.6 g xylan passed into the liquid stream from 25g biomass. The total of xylan and cellulose mass in the pretreated solid and in the liquid is equal to their mass in the raw material. This showed that the pretreatment did not degrade the carbohydrates. Most of the xylose remained in the solid and most of the lignin was removed by organosolv pretreatment under the conditions used in this study. Unlike the other studies, only the pretreated solid was used in the following steps. Excluding the pretreatment

liquor prevented sugar degradation products and extractives from involving in the fermentation or hydrolysis medium. In pursuant of this ratio, the amount of xylan and xylose in 66 g/l biomass (0.5 g biomass in 7.5 ml buffer or medium) used for enzymatic hydrolysis 25.7 g and 31.7 g, while the amount of cellulose and glucose is 28.6 g and 32.4 g initially.

Table 4.1. Xylan and cellulose composition of the pretreated and raw corn cob.

Component	**Concentration (g/ 100 g dry matter) Raw corn cob**	**Concentration (g/100 g dry matter Pretreated corn cob**
Cellulose	31.6±0.51	43.3±0.65
Xylan	29.7±0.65	38.9±0.70

4.2. Effect of the Solid/liquid Ratio, Temperature, and Enzyme Concentration on the Enzyme Hydrolysis

The optimum enzymatic hydrolysis condition for the saccharification of organosolv treated corncob was determined by performing hydrolysis under different enzyme concentrations at pH 6.8 and 5.2, and at 37°C and 50°C using 1:15 solid:liquid ratio. Although the optimum temperature and pH recommended by the manufacturers of the xylanase and the cellulase were around 50°C and pH 5, we also tested 37°C and pH 6.8. Because *A. succinegenes* has been cultivated mostly at 37°C and near neutral pH. It would not grow efficiently under the optimal conditions of the enzymes used in this study. In the SSF, the optimum growth temperature and pH should be provided, and the performance of the enzymes under those conditions may be critical for succinic acid production.

All enzyme hydrolysis experiments were carried out for 48 h in triplicates. In the preliminary tests, it was observed that the amount of sugar produced by the enzyme did not change after 48 h. To find the optimal amounts of Celluclast and Accelarase XY,

enzymes were tested separately and together.

At 50°C Accelerase XY was tested at three concentrations, namely 10 µl (284 U/ml), 50 µl (1420 U/ml), and 100 µl (2840 U/ml) in citrate buffer at pH 5.2. The xylose yield increased slightly with enzyme dosage from 15.5 g/l to 20.2 g/l xylose (Fig 4.1). Under these conditions, the glucose released was less than 3.0 g. To keep the enzyme cost at a minimum, the lowest dosage (10 µl) was selected for further studies since it could yield a substantial amount of xylose. Thus, when combined with cellulase, this xylanase dosage was successful in saccharification of the treated corncob (Fig 4.2). By keeping xylanase dosage constant at 10 µl, cellulase was tested at four dosages, such as 25 µl (1.85 FPU/g), 50 µl (3.7 FPU/g), 100 µl (7.4 FPU/g), and 150 µl (11.1 FPU/g). The highest xylose (22.0 g/l) and glucose (20.1) were obtained with 100 µl cellulase. The xylan yield was 75.9% and cellulose yield was 63.9%, based on the xylan and cellulose contents of the pretreated biomass.

A similar test was conducted at 37°C in potassium phosphate buffer (at pH 6.8 to estimate the performance of the enzymes under the temperature and pH values of SSF. When xylanase was applied alone, the lower xylose concentrations were obtained compared to those obtained with the same enzyme dosages at 50°C (Figure 4.3). That was expected since the Accelerase XY could show a suboptimal activity at 37°C. The xylose concentration obtained with 10 µl xylanase was 6.2 g/L, 60% lower than the xylose obtained at 50°C. The effect of the xylanase dosage was not significant, so that, the two lowest dosages tested (10 µl (284 U/ml) and 30µl (852 U/ml)) were selected for future experiments. Afterward, both xylanase amounts were tested. An experiment was performed in which xylanase dosages were constant at 10 µl and 30 µl and the three dosages of cellulase (25, 50, and 100 µl) were tested (Figure 4.4.). The amounts of xylose and glucose were higher in the sets with 30 µl of xylanase than those with 10 µl of xylanase. The condition of 30 µl xylanase and 100 µl cellulase, which produced 10.6 g glucose and 10.7 g xylose, was considered suitable for saccharification at 37 °C. With 30 µl xylanase, increasing the cellullase to 150 µl did not increased the glucose and xylose formation (Figure 4.4). It was decided that 30 µl xylanase and 100 µl cellulase could be used for saccharification at 37°C. At the same cellulase dosage (100 µl) 10 µl xylanase yielded 8.3 g of glucose and 8.1 g of xylose, which were around 20% less than that obtained with 30 µl xylanase. Without xylanase in the hydrolysis mixture 100 µl of cellulase alone could release 5.2 g glucose and 3.5 g xylose (Figure 4.4). These concentrations were considerably less than those obtained with the joint action of

cellulase and xylanase. This could be ascribed to an association of the activities of the enzymes on the lignocellulosic structure. Organosolv treatment can change the structure of the network composed of cellulose, hemicellulose and lignin. Under the conditions of this study, this was achieved by partial removal of lignin (Temelli 2020). Lignin has been known to be a protective physical barrier against enzymatic hydrolysis (Öhgren et al. 2007). Thus, the pretreatment decreased the recalcitrance of the corncob to enzymatic hydrolysis. However, the cellulose, xylan ,and the lignin remaining in the pretreated corncob were probably in a network, which provided a partial resistance to the xylanase and cellulase activities. When xylanase and cellulase were used together, the partial hydrolysis of the xylan and cellulose, respectively, may have provided more sites for further activity of the other enzyme. This was more evident for cellulose hydrolysis. The hydrolysis of xylan by the xylanase may have allowed cellulase to act on the cellulose fibers in the pretreated corncob. Increasing the accessibility of the cellulose surface by cellulases plays a role in increasing the efficiency of cellulase hydrolysis (Qing and Wyman 2011). The use of enzymes such as xylanase targeting hemicelluloses together with cellulase in enzymatic hydrolysis increases the rate of enzymatic hydrolysis and increases the sugar concentration obtained (Marcos et al. 2013). Song et.al. (2016) conducted a cellulase and xylanase synergy experiment using corncob with a polymer content of 31.4 mg/ml. When 0.2 g/g cellulase and xylanase were used separately and 9.3 mg/ml glucose in hydrolysis with only cellulase, 1.7 mg/ml xylose in hydrolysis with only xylanase were obtained. When they were used together 10.1 mg/ml glucose and 2.6 mg/ml xylose were produced, which showed a slight synergistic effect (Song et al. 2016). To investigate the effect of organosolv pretreatment on enzyme hydrolysis, enzyme hydrolysis was performed with raw biomass under the same conditions as pretreated biomass. While the total sugar amount was 22 g/L at 37 °C in 6.8 pH phosphate buffer using 30 µl of xylanase and 100 µl cellulase in the pretreated biomass, the total sugar amount obtained with the raw biomass under the same conditions was only 2.1 g / L. This shows the positive effect of organosolv pretreatment on enzyme hydrolysis and hence in SSF. The recalcitrance of untreated corncob prevents the hydrolytic activity of cellulase and xylanase. Whereas organasolv treatment decomposed partially the lignocellulosic network thus more glucose and xylose could be released by the enzymes.

The effect of solid:liquid ratio on glucose and xylose formation in the enzymatic hydrolysis was tested. Solid: liquid ratio of 1:10 and 1:15 were tested at different enzyme concentrations and 50°C-pH 5.2 and 37°C-pH 6.8 (Figure 4.5.) It was observed that a

1:15 solid-liquid ratio was more suitable for hydrolysis. Because, the total amount of sugar produced in the same period is higher in the experiments done with 1:15 solid:liquid ratio. When the ratio was 1:10, the glucose concentration was 10.6 g/L, and xylose concentration was 7.3 g/L, using 100 µl Celluclast and 60 µl Accelarase XY at 37°C-pH 6.8. The glucose and xylose concentrations were 9.4 and 9.6 g/L with 1:15 solid:liquid ratio under the same conditions (Figure 4.5. (A)). At 50°C-pH 5.2, when the solid:liquid ratio was 1:10, the glucose concentration was 9.0 g/L and xylose concentration was 6.2 g/L with 100 µl cellulase and 60 µl xylanase. The concentrations were 15.0 and 12.5 g/L when solid:liquid ratio was decreased to 1:15 under the same conditions (Figure 4.5. (B)). It was noted that an increase in the amount of substrate in the hydrolysis mixture could cause substrate inhibition and problems with mixing and creates issues in mass transfer. (Taherzadeh and Karimi 2007). The data in this study also supported this finding.

This study showed that the concentrations of xylose and glucose obtained in the hydrolysis were less at 37°C-pH 6.8 than at 50°C-pH 5.2. The xylose obtained from the pretreated corncob, which contained 43.3% cellulose and 38.9% xylan, was 12.1 g/L and the glucose was 10.1 g/L, corresponding to 22.2 g/L total monosaccharides at 37°C-pH 6.8 using enzyme dosages determined for this temperature and pH values. Cellulose conversion yield was calculated as 31.8% and xylan conversion as 41.4%. The xylose obtained from the same pretreated biomass with enzyme amounts determined for 50°C-pH 5.2 was 20.1 and the glucose was 22 g/L. These corresponded to cellulose conversion yield of 69.2% and xylan conversion yield of 68.8%. The reason for this may be that the enzyme was not in the optimum temperature and pH conditions and its effectiveness was reduced. It has been stated that the most suitable conditions for cellulase activity are 50±5 °C and pH 4.5-5 (Taherzadeh and Karimi 2007). For xylanase activity, these conditions were 50-75° C and pH 4.5-7 (Marcos et al. 2013). The same authors also stated that temperature and enzyme/substrate ratio are more significant than the pH value. In contrast, Farinas et al. (2010)showed that pH profoundly affected the xylanase activity. The xylanase activities at pH 4.5 and 6.6 were measured as 10-13 IU/ml and 3.72 IU/ml, respectively, at 55 °C.

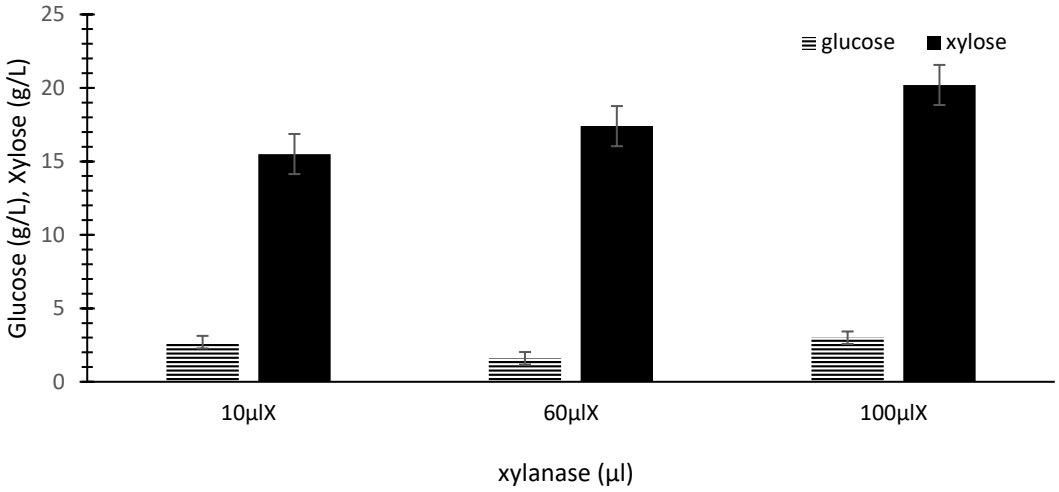


Figure 4.1. Effect of xylanase dosage on the hydrolysis of pretreated corncob.

X: xylanase. (0.5 g biomass in 7.5 ml citrate buffer at pH 5.2 and 50°C)

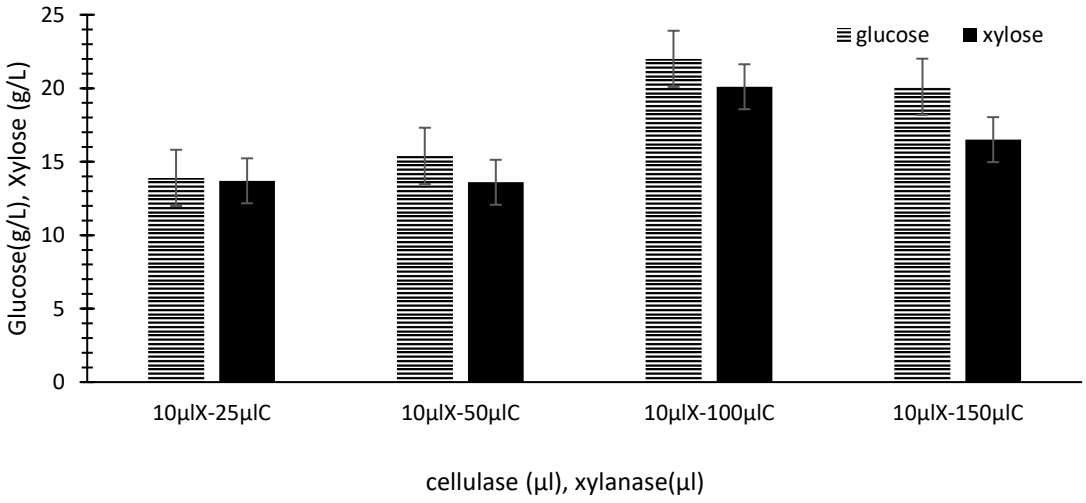


Figure 4.2. Effect of cellulase dosage on the hydrolysis of pretreated corncob under constant xylanase dosage.

X: xylanase; C: cellulase. (0.5 g biomass in 7.5 ml citrate buffer at pH 5.2 and 50°C)

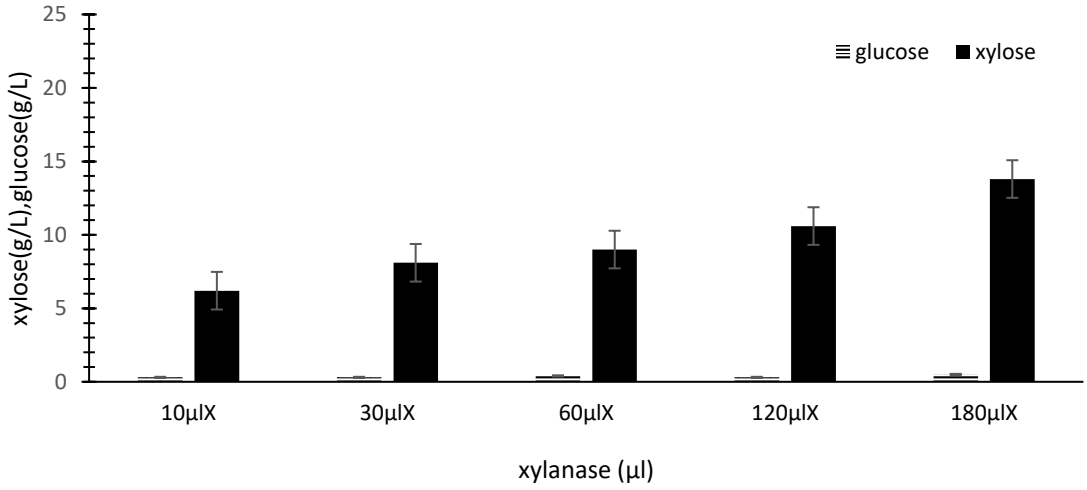


Figure 4.3. Effect of xylanase dosage on the hydrolysis of pretreated corncob.

X: xylanase. (0.5 g biomass in 7.5 ml phosphate buffer at pH 6.8 and 37°C)

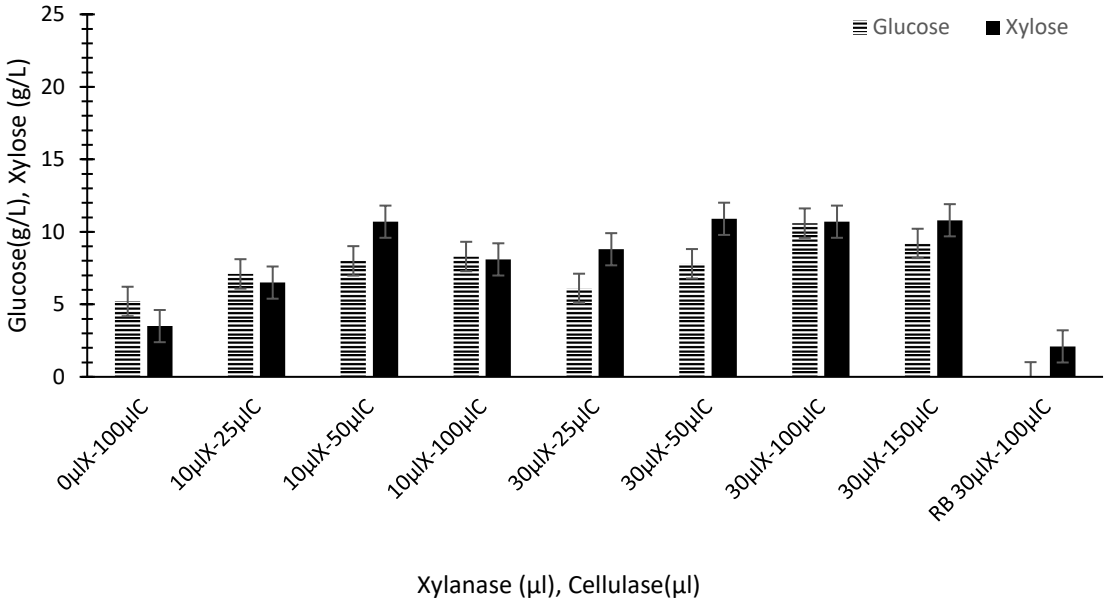
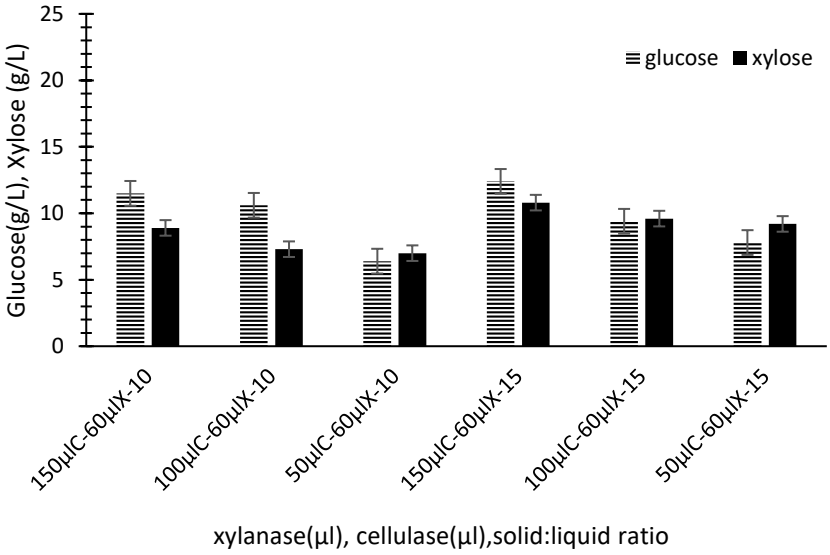
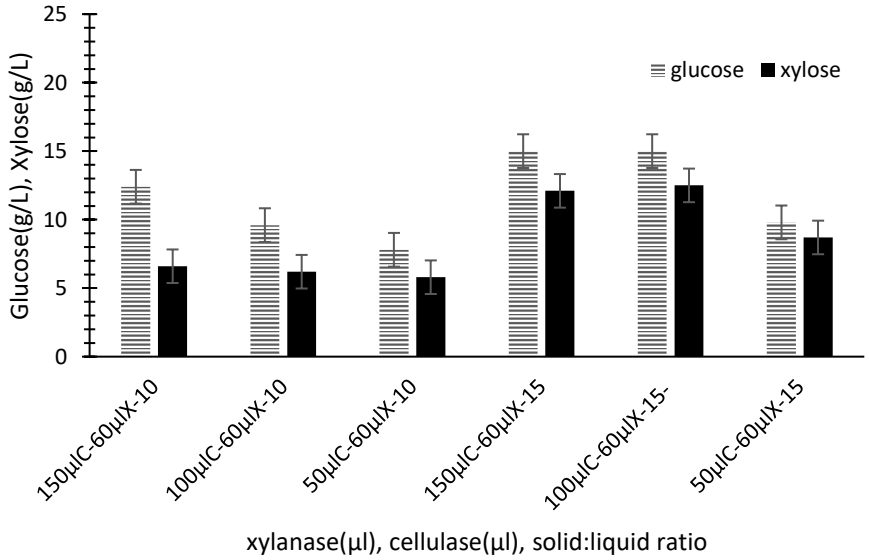


Figure 4.4. Effect of cellulase dosage on the hydrolysis of pretreated corncob under constant two xylanase dosage.

X: xylanase, C: cellulase, RB: Raw Biomass. (0.5 g biomass in 7.5 ml phosphate buffer at pH 6.8 and 37°C)



A)



B)

Figure 4.5. Effect of cellulase dosage on the hydrolysis of pretreated corncob under constant xylanase dosage for different solid:liquid ratio and different temperatures.

X: xylanase C: cellulase

A) At pH 6.8 and 37°C for 1:15 and 1:10 solid: liquid ratio

B) At pH 5.2 and 50°C for 1:15 and 1:10 solid: liquid ratio

4.3. Succinic Acid Production from pretreated corncob by *Actinobacillus succinogenes* by Simultaneous Saccharification and Fermentation Process

SSF is a process where enzymatic hydrolysis and fermentation steps can be performed in one place, and it is known to have some advantages over SHF. It has been mentioned that the inhibitory effect of hydrolytic products against enzymes can be eliminated with SSF, since the carbohydrates released are utilized by the microorganism (Lu et al. 2021). The immediate use of produced carbohydrates could also reduce the risk of contamination (Olsson and Hahn-Hägerdal 1996). In addition to that, having two steps in one place reduces equipment costs reduces the total processing time, which is also a significant advantage.

In this study, the whole SSF process was monitored for 44 h. In the preliminary tests, it was observed that the production of succinic acid stopped after this time. 16.0 g / L succinic acid and 3.1 g/L formic acid were produced in 44 h at 37 °C (Figure 4.6). It was seen that production started after 4 h. Throughout the cultivation the glucose and xylose concentrations were below 0.5 g/L, which showed that the carbohydrates released by the enzymes were utilized by the organism in a short time. There was almost linear production between 4 h36 h. The succinic acid concentration did not increase further after 36 h. Formic acid also increased in parallel with succinic acid (Figure 4.6). Formic acid is a known by-product in succinic acid fermentations (Yang et al. 2020). 29.7 g/L succinic acid and 5.5 g/L formic acid were produced in the study in which succinic production was carried out with *A. succinogenes* using a medium containing 45 g/L initial glucose (Du et al. 2007).

The pretreated corncob, used in the SSF as the carbon source, consisted of 43.3% cellulose and 38.9% xylan, as mentioned above. Since 0.5 g of corncob was suspended in 7.5 ml of culture medium, the xylan and cellulose concentrations in the medium were calculated as 28.9 and 25.9 g/L, respectively. These correspond to 54.8 g/L total carbohydrates in the polymeric form and 61.5 g/L monosaccharides. Therefore, with 16.0 g/L succinic acid production, the product yield was calculated as 0.26 g succinic acid per g of available carbohydrates.

SSF has been used in many ways to produce succinic acid, using different raw materials and pretreatments. When corn stover was used as lignocellulosic biomass, 35.3

g/L succinic acid was produced from 70 g/L initial substrate when SSF was applied with the addition of cellulose and cellobiose enzyme to the biomass pretreated with dilute alkaline pretreatment by *A.succinogenes* (Zheng et al. 2010). Similarly, sequential dilute acid microwave/alkali pretreated palm oil empty fruits were used as the carbon source and SSF was performed, 42.9 g/L succinic acid was produced by *A. succinogenes* and the conversion yield was stated as 0.61 g/g (Akhtar et al. 2020).

To increase the succinic acid concentration in the fermentation broth, further experiments were done. After 24 h more pretreated corncob and enzymes were added into the fermentation medium. The amounts of biomass and enzyme added was the same as the ones added in the beginning (0.5 g pretreated biomass, 30 µl Accelerase XY and 100µl Celluclast). After addition, CO_2 was sparged into the tubes. Samples were taken at 24 h intervals. 17.4 g/L succinic acid was produced in 72 h (Figure 4.7.). This did not have a positive effect compared to the SSF without secondary biomass and enzyme addition. It was speculated that the decrease in the pH in the tubes may have decreased the rate of growth and acid production, thus both with and without addition succinic acid production was poor. I has been known that the decrease of pH value for *A.succinogenes* has a negative effect on the growth of cells (Wan et al. 2008). The optimum pH range of *A.succinogenes* to grow is between 6 and 7.4 and pH affects the activity of the enzyme responsible for the production of succinic acid (Dessie et al. 2018). A similar test was repeated by adding only pretreated corncob but not the enzymes. In 72 h 16 g/L succinic acid was produced (Figure 4.8.), which was similar to the one obtained in the SSF experiments without corncob and enzyme addition. In these two tests, it was observed that sugar accumulated in the medium which can be considered as evidence for poor growth and carbohydrate utilization. In both addition experiments, the pH value at the end of fermentation is 5.8, which is slightly outside the optimum pH for *A. succinogenes*. The yield of succinic acid was 0.12 g succinic acid / g pretreated corncob when only biomass was added, while it was 0.13 g/g when biomass and enzyme were added. Expected yield increase was not observed. For this reason, the same analysis was repeated in the bioreactor, in which pH could be maintained at a constant value, (Figure 4.9.). The corncob and enzymes addition was done in 32 h and 24.7 g of succinic acid was produced from a total of 133 g/L biomass. The pH in the reactor was kept constant at 6.8. Higher succinic acid was produced at constant pH tests compared to under uncontrolled pH in the test tubes. In both phases, the succinic acid yield was around 0.18 and it was lower than the Hungate procedure.

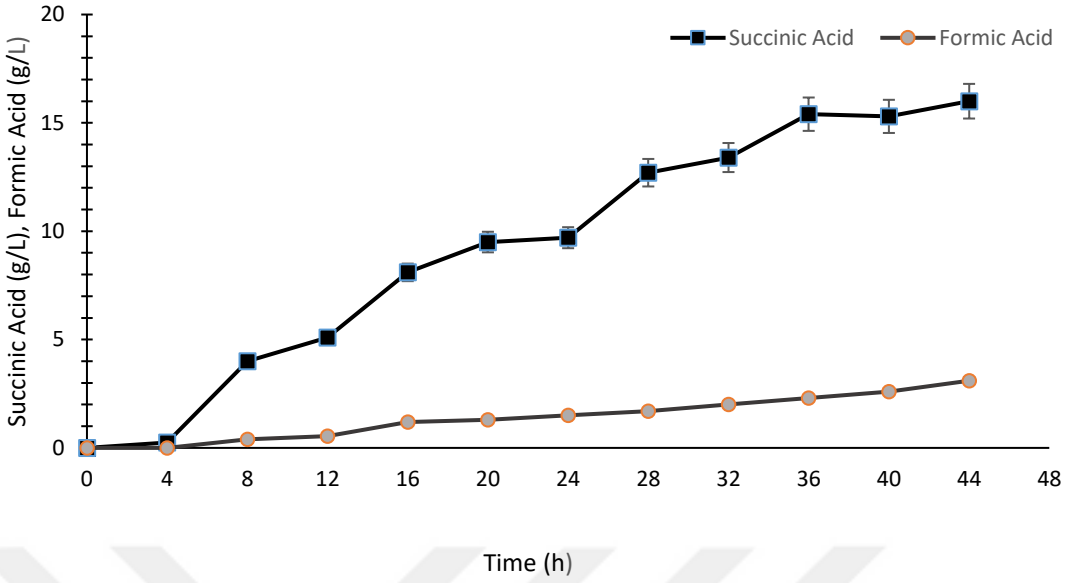


Figure 4.6. Kinetics of succinic acid and formic acid production in SSF.

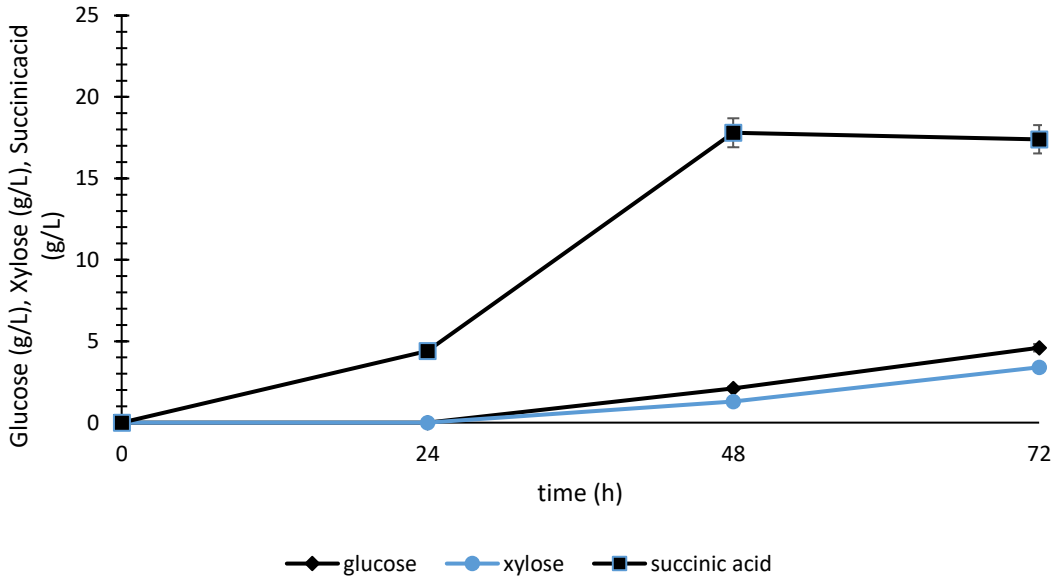


Figure 4.7. Effect of biomass and enzyme addition at 24h on SSF.

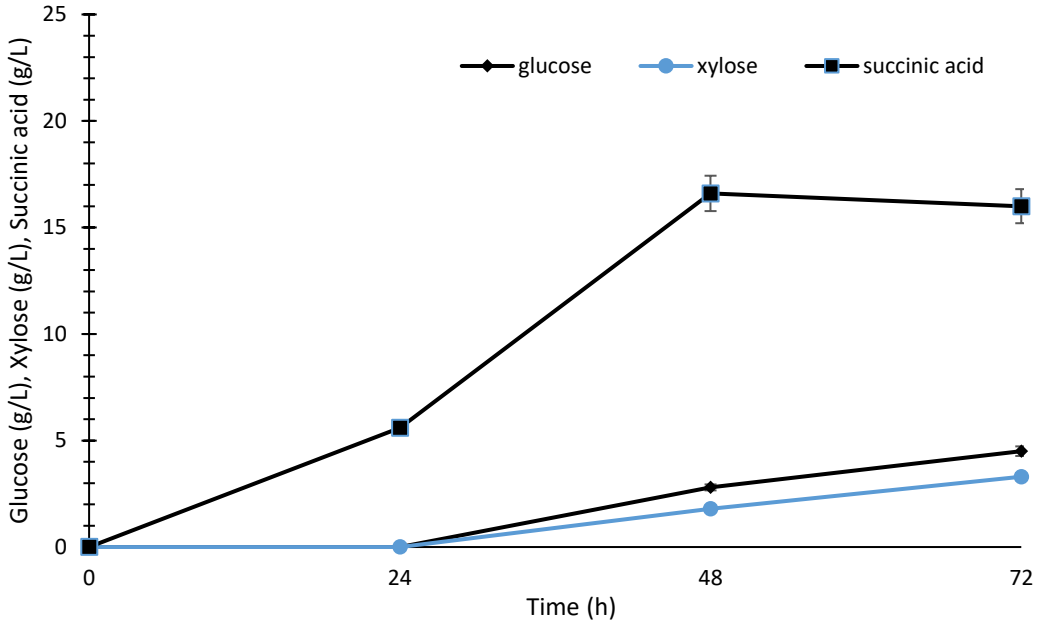


Figure 4.8. Effect of biomass addition at 24h on SSF.

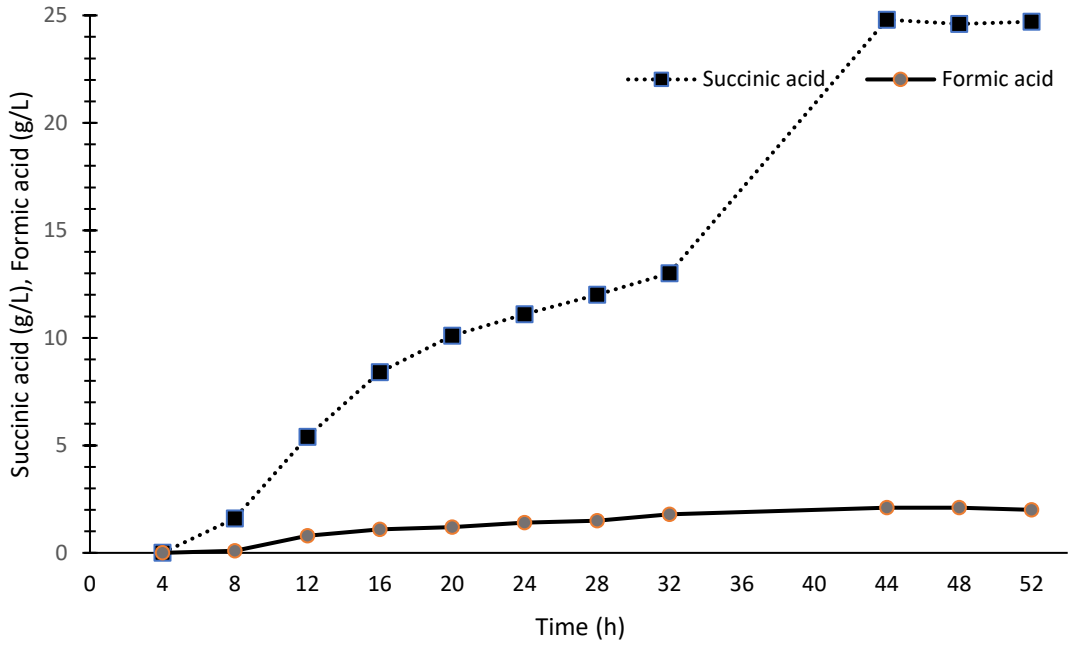


Figure 4.9. Effect of biomass and enzyme addition at 32h on SSF in pH 6.8 in the bioreactor.

4.4. Succinic Acid Production from pretreated corncob by *Actinobacillus succinogenes* by Separate Hydrolysis and Fermentation

SHF is a two-step production method in which enzymatic hydrolysis and fermentation take place consecutively. The most crucial advantage of SHF is that optimum conditions can be provided for both enzymatic hydrolysis and fermentation steps, unlike SSF. There are many studies in which SHF is preferred in the production of succinic acid. Zheng et. al. (2009)used an enzymatic hydrolysate from corn straw, which was pretreated with dilute alkali, and obtained 45.5 g/L succinic acid from a total xylose and glucose concentration of 58 g/L by *A. succinogenes* in 48 h. SHF method was used to produce succinic acid from stalks by *A.succinogenes* (Li et al. 2010). Using enzymatic hydrolysates from corn and cotton stalks, 17.8 g/L and 15.8 g/L succinic acid was produced, respectively, with product yields of 0.65-0.80 g/g reducing sugar. Succinic acid was produced from pretreated (dilute acid) corn stover hydrolysates with cellulase and 66.23 g/L succinic acid was produced with an initial glucose concentration of 100 g/L (Li et al. 2011). However, in the pretreatment with dilute acid hydrolysis, the liquid hydrolysate must first be neutralized. This causes dilution, which is a disadvantage when compared to organosolv in our study.

In this study, SHF was performed for 24 h, and during this process, sampling was done to monitor succinic acid and monosaccharide levels. With 26.4 g/L (13 g/L glucose and 13.4 g/L xylose) of initial total carbohydrate in the hydrolysate, 12.7 g/L succinic acid was produced in SHF (Figure 4.10; Figure 4.11). Formic acid (1.4 g/L) accompanied succinic acid production. The succinic acid yield was calculated as 0.48 g succinic acid/g sugar. The pH of the hydrolysate was 4.7 and to bring this pH to the optimum pH for the bacteria, more NaOH was added than SSF and XG medium, resulting in a lower initial sugar concentration than expected in the fermentation medium compared to the results of enzyme hydrolysis and this causes dilution in the initial sugar concentration. The succinic acid yield in the SHF was lower compared to SSF. Besides, the total process takes longer (48 h hydrolysis plus 24-h fermentation) compared to SSF process. Additional solid-liquid separation process (filtration or centrifugation) was applied to remove the biomass and obtain a clear hydrolysate after the hydrolysis in SHF as opposite the SSF. In the organic acid production (lactic, fumaric and succinic acid) where SHF and SSF were compared, different biomass was tested and it was stated that SSF was superior to SHF

in terms of organic acid production yield (Maslova et al. 2019). In our study, 12.7 g/L succinic acids were produced from 110.4 g/L biomass in SHF, while 16 g/L succinic acid were produced from 66 g/L biomass in SSF. Thus, the biomass consumed in fermentation is higher in SHF than in SSF. SSF appears to be more efficient than SHF in terms of succinic acid production. In SSF 0.24 g succinic acid/g of corn cob (pretreated), whereas in SHF 0.11 g succinic acid/g of corn cob was obtained. This can be ascribed to incomplete hydrolysis step. The accumulation of glucose and xylose may have inhibited the enzymes leaving fraction of the cellulase and xylan unhydrolyzed. In contrast, the rapid utilization of glucose and xylose in the SSF may have alleviated the inhibition, so that hydrolysis may have been more effective. Due to the increase in monosaccharide concentration during the hydrolysis step in SHF, enzymatic hydrolysis reactions are inhibited by hydrolytic products, which causes a decrease in the reaction rate and incomplete substrate hydrolysis (Margeot et al. 2009). From this point of view, monosaccharide accumulation is not observed in the SSF method and the yield increases with more effective hydrolysis.

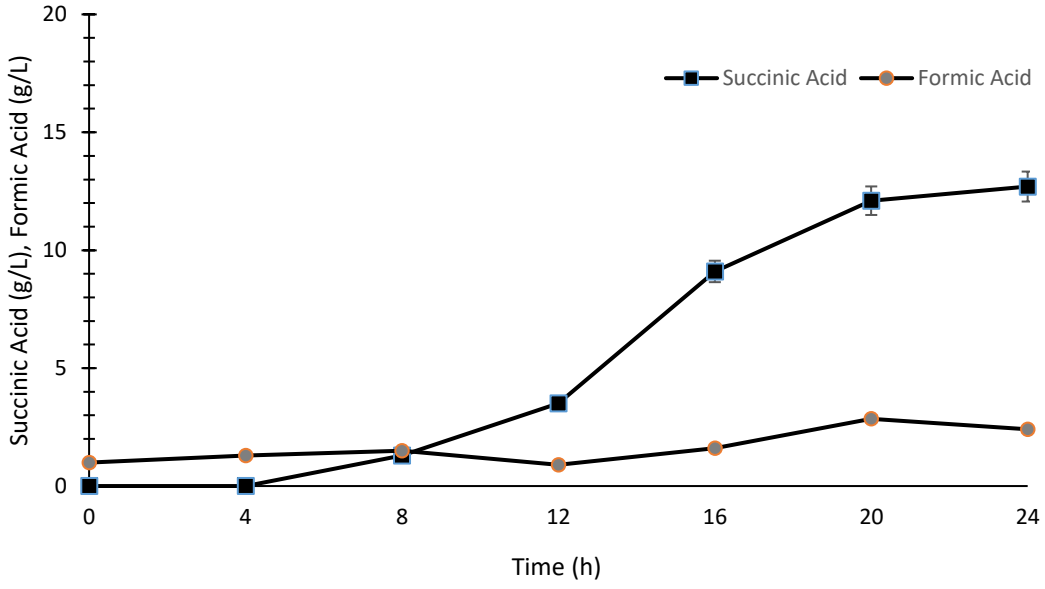


Figure 4.10. Kinetics of succinic acid and formic acid production in SHF.

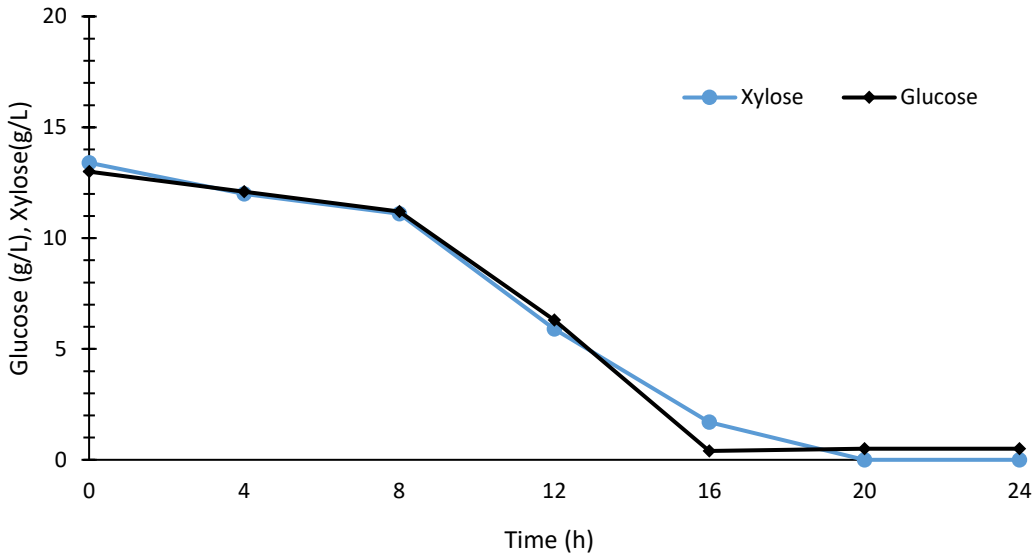


Figure 4.11. Kinetics of glucose and xylose consumption in SHF.

4.5. Succinic Acid Production by *Actinobacillus succinogenes* in Xylose- Glucose Medium

Succinic acid was produced by fermentation in a medium containing purified (analytical grade) xylose and glucose. This medium (XG) content aimed to have information about the use of xylose and glucose under the conditions as SSF and SHF processes. In the fermentation initiated with 20 g/L glucose and 20 g/L xylose, almost all of the carbohydrates were consumed and 20.4 g/L succinic acid and 3.6 g/L formic acid were produced in 20 h (Figure 4.12; Figure 4.13). Succinic acid production yield was calculated based on total carbohydrates was calculated as 0.51 g/g. In a previous study, xylose, glucose, arabinose, galactose and mannose were used as carbon sources (10 g of each) and all sugars were converted except galactose and 21.8-26.8 g/L succinic acid was produced by *A. succinogenes* (Almqvist et al. 2016). In the study in which continuous succinic acid production from xylose was performed, succinic acid was produced from 43-84 g/L xylose with a yield of 0.55-0.68 g/g by *A. succinogenes* and it was stated that the yield was lower than the same study with glucose (Bradfield and Nicol 2016). In the fermentation initiated with a glucose concentration of 100 g/L, 69.65 g/L succinic acid was produced by *A. succinogenes* (Li et al. 2011). It can be concluded that the yield

obtained in this study was in agreement with the previous studies.

Compared to SSF and SHF, the succinic acid production yield achieved with XG medium is notably higher than that achieved with SHF. Whereas, SSF was superior to SHF with its succinic acid production and product yield. It was observed that succinic acid and formic acid productions started after the 4 h of fermentation. Succinic acid production was seen after 4 h in both SSF and SHF. From this point of view, it was observed that in the XG medium the bacteria followed a parallel path to the other two media used to produce succinic acid. Glucose consumption started at a higher rate than xylose consumption (Figure 4.13). After this initial phase, the xylose consumption rate increased and the total time for the complete utilization of both monosaccharides were similar. As in SSF and SHF, it was observed that the medium became cloudy due to bacterial growth in the tubes at the end of fermentation in the XG medium, and $MgCO_3$ consumption caused a color change (Figure 4.14).

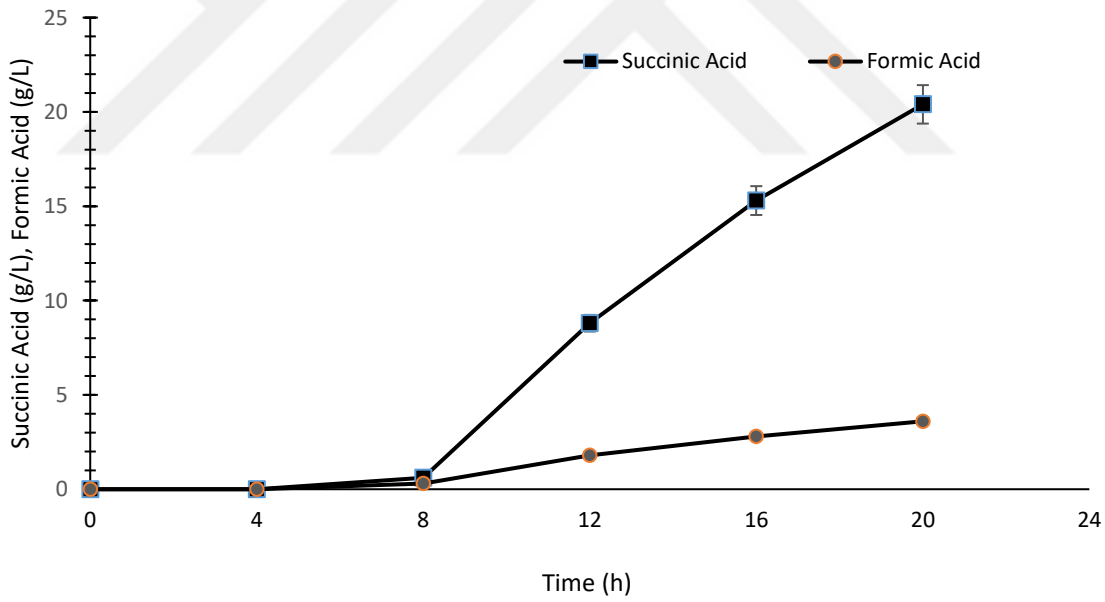


Figure 4.12. Kinetics of succinic acid and formic acid production in XG medium

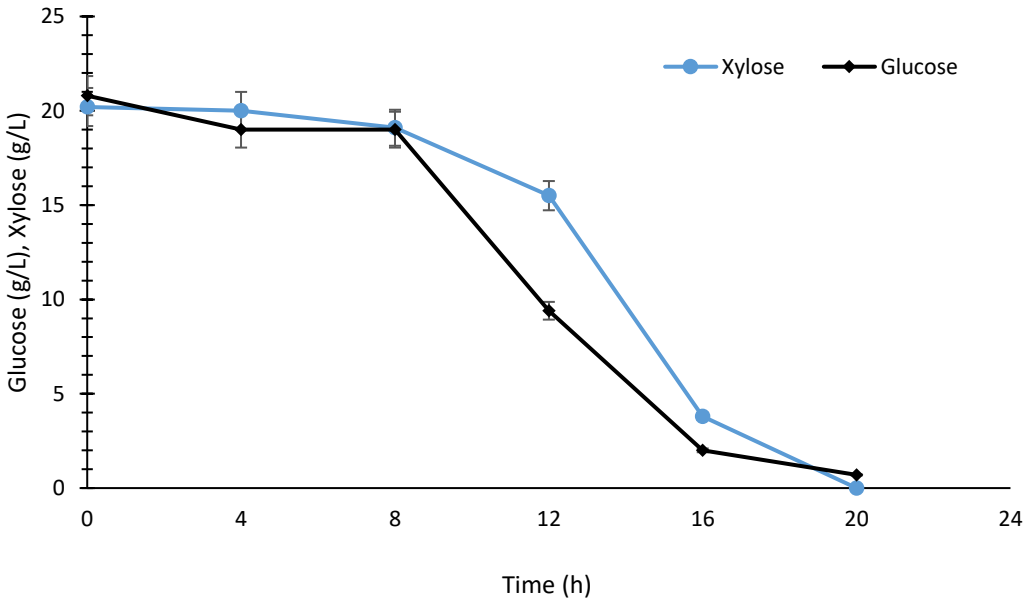


Figure 4.13. Kinetics of glucose and xylose consumption in XG medium.

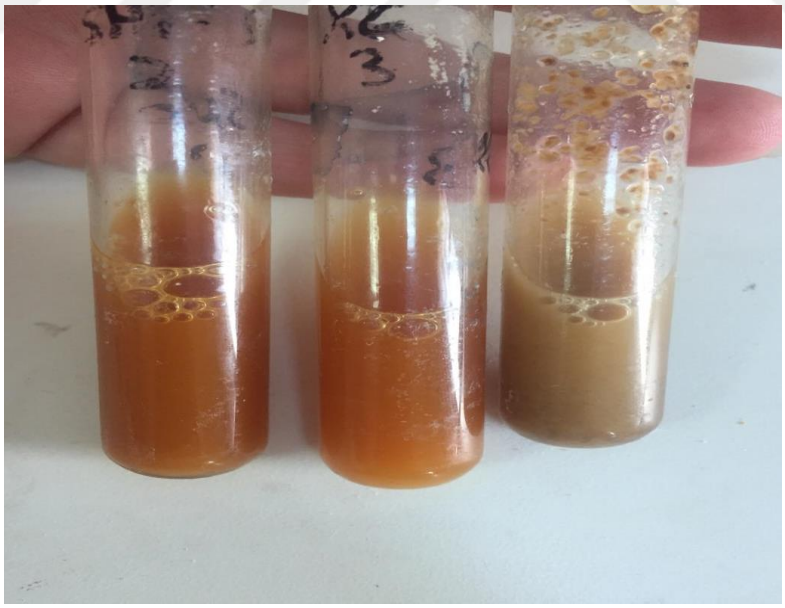


Figure 4.14. Hungate tubes containing samples discolored after fermentation.

(SHF, XG medium, and SSF samples from left to right, respectively)

CHAPTER 5

CONCLUSION

In this study, the potential of organosolv pretreated corncob as a feedstock for succinic acid by *A.succinogenes* was tested. Organosolv pretreatment was applied to the corncob to obtain fermentable sugars in the subsequent enzymatic hydrolysis. Two different approaches were used for the production of succinic acid. In the first method, succinic acid was produced in a SSF process, while in the second method, succinic acid fermentation was carried out using SHF. A set of experiments was carried out to determine the appropriate enzyme dosage and conditions for enzyme hydrolysis. Based on these experiments, it was concluded that the hydrolysate required for SHF can be obtained by applying 10 µl Accelarase XY and 100 µl Celluclast in citrate buffer at pH 5.2 at 50°C using 1:15 solid-liquid ratio. For SSF, the experiments were done with 1:15 solid-liquid ratio at 37°C using potassium phosphate buffer at pH 6.8. It was decided to use 30 µl Accelarase XY and 100 µl Celluclast enzyme addition.

With SSF, 16 g/L of succinic acid and 3.1 g/L of formic acid were produced and the succinic acid production yield was calculated as 0.24 g/g. On the other hand, succinic acid production yields were calculated as 0.48 g succinic acid/g sugar and 0.11 g succinic acid/g pretreated biomass by producing 12.7 g/L succinic acid and 2.4 g/L formic acids from 27.8 g/L total sugar with SHF.

Considering these results, it can be concluded that the SSF method is a more efficient method than the SHF method. Apart from this, when evaluated as succinic acid production per biomass used, SSF yielded more promising results than SHF. With the SSF method, all experiments are carried out in the same pot and it is easier to perform the process while using different equipment by carrying out two different processes for SHF is more challenging. While 48 h was sufficient for fermentation with the SSF method, 72 h was required in the enzyme hydrolysis for the SHF method. Considering the time spent to transfer the hydrolysates to the fermentation medium, it has been determined that the SSF method is more time-consuming. With both methods, xylan, which is the second basic structure in biomass, is converted to xylose and used in fermentation, thus, xylan was also used in succinic acid production.

This study showed that the corncob can be valorized in succinic acid production. Since *A. succinogenes* is capable of utilizing xylose as well as glucose, both cellulose and the xylan components of the corncob can be used as carbon source in succinic acid production. Organosolv does not use harsh or toxic chemicals. The modified organosolv used in this study does not form any degradation products. In addition to those, since the carbohydrates remained in the solid part, extractives from the lignocellulosic biomass were not involved in the hydrolysis and fermentation processes. Therefore a clean hydrolysate or a solid feedstock for SSF could be obtained. On the other hand, the hydrolysis yield of the organosolv pretreated corncob was not very high, and fractions of cellulose and xylan were not converted into fermentable sugars. To develop a sustainable succinic acid production process, hydrolysis, as well as fermentation yields should be maximized. Therefore more tests are required to increase the enzyme efficiencies and succinic acid yields for the available carbohydrates.

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APPENDICES

APPENDIX A

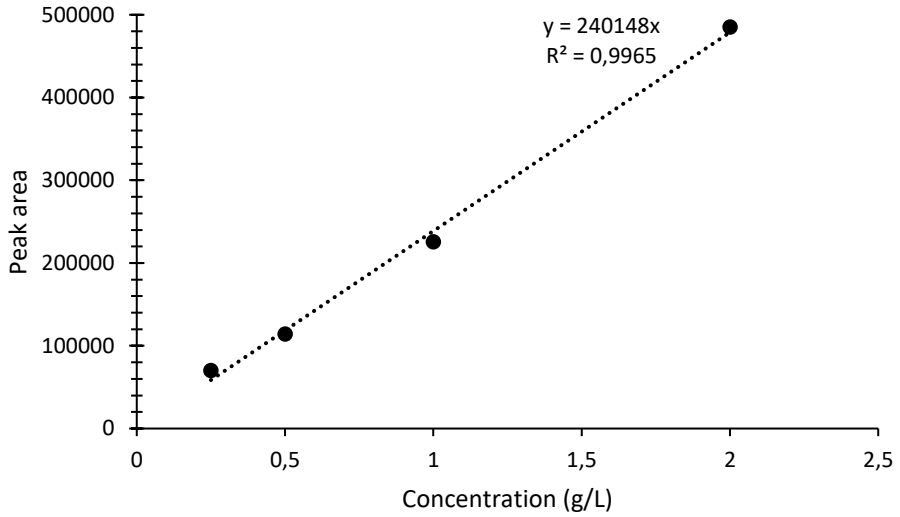

Figure A.1. Standard calibration curve of glucose in Aminex HPX-87H ion exclusion column for HPLC analysis

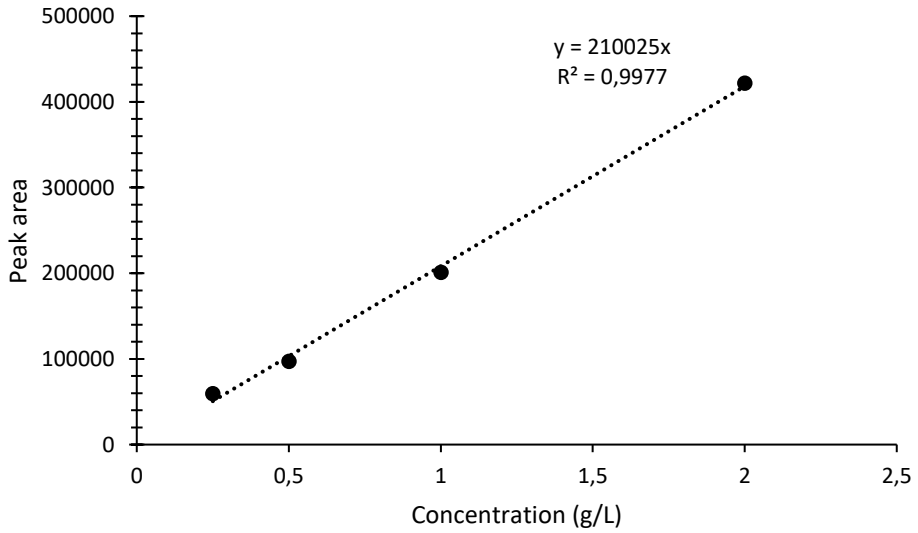

Figure A.2. Standard calibration curve of xylose in Aminex HPX-87H ion exclusion column for HPLC analysis.

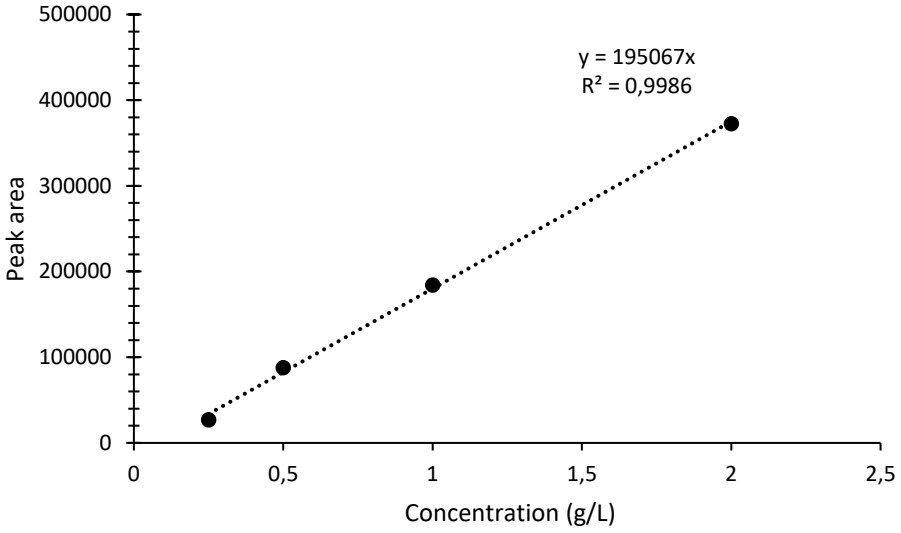

Figure A.3. Standard calibration curve of succinic acid in Aminex HPX-87H ion exclusion column for HPLC analysis.

APPENDIX B

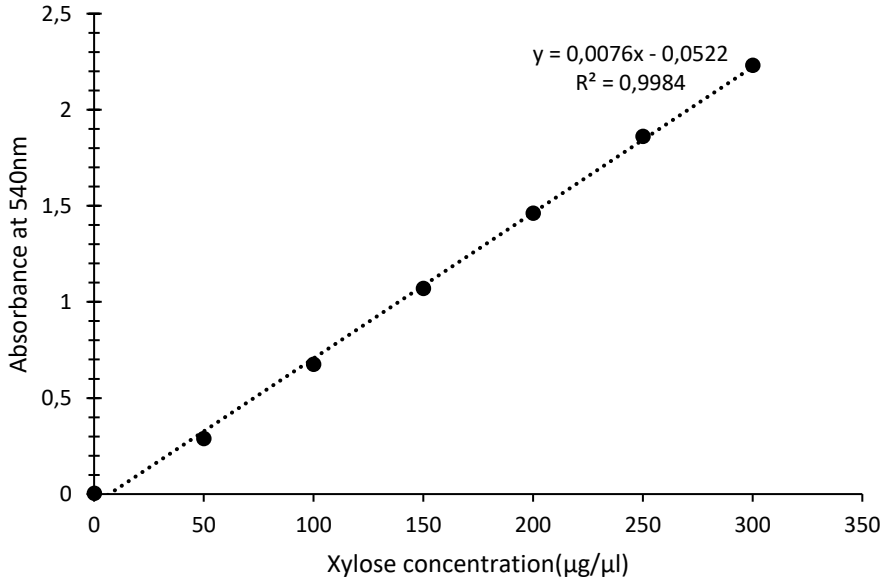

Figure A.4. Standard calibration curve of xylose at 540 nm for enzyme activity assay.

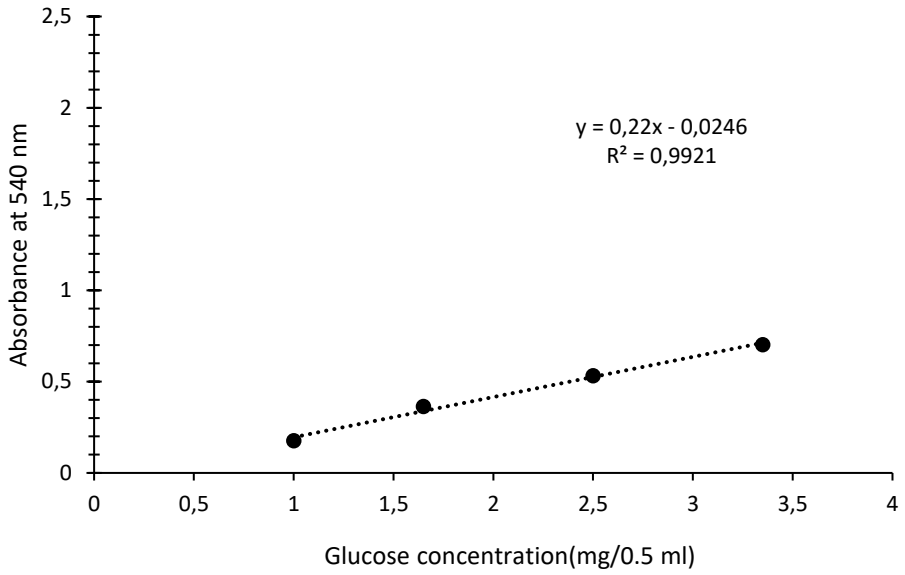

Figure A.5. Standard calibration curve of glucose at 540 nm for enzyme activity assay.