CONTINUOUS ENZYMATIC HYDROLYSIS OF BIOMASS

Lappeenranta–Lahti University of Technology LUT

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ABSTRACT

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Continuous enzymatic hydrolysis of biomass

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Enzymatic hydrolysis is well known biological process for converting complex substrates into smaller units. In enzymatic hydrolysis of biomass, the enzymes degrade polymeric chain of cellulose into monomeric sugar units – glucose. The process has advantages such as mild operating conditions, high selectivity, and low energy consumption. The process is mainly used in bioethanol processing at the moment, but monomeric sugars as multifunctional platform chemicals has gained increasing attention.

Conventionally the process is done in batch reactor, but the advantages of continuous processing have raised attention. By changing the batch process into a continuous process, the aim is to improve runnability, enhance the use of tank volume, and prevent contamination better. With continuous process the water consumption could be reduced since the tanks are full at all times. Cleaning and disinfection after each batch consumes more water compared to continuous process where cleaning sequences can be reduced. Due to the decreased water consumption the wastewater capacity can also be reduced. In addition, contamination microorganisms have less or no time to grow when the biomass is changing all the time.

The trials were conducted in a pilot scale one step continuous enzymatic hydrolysis with a simple continuous tank reactor in which the feed was fed to the bottom of the reactor and the outlet was on the surface. Due to pumpability difficulties the steady operation was challenging to reach. Promising results were obtained from the three successful trials. With 14,8 h, 25,9 h, and 105,5 h retention times 48 %, 70 %, and 95 % conversions were reached. The conversions were at the same level as in reference batch process. The continuous operating did not have an effect to the sugar purity compared to the batch process. Scaling up the process into a larger scale holds the potential to overcome pumpability challenges and be a feasible process for producing sustainable green sugars and lignin.

TIIVISTELMÄ

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Biomassan jatkuvatoiminen entsymaattinen hydrolyysi

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Entsymaattinen hydrolyysi on biologinen konversio prosessi, jossa kompleksiset substraatit hajotetaan pienempiin yksiköihin. Biomassan entsymaattisessa hydrolyysissä entsyymit hajottavat selluloosan polymeerisen ketjun monomeerisiksi sokeriyksiköiksi - glukoosiksi. Prosessilla on useita etuja kuten miedot operointiolosuhteet, korkea selektiivisyys ja matala energian kulutus. Tällä hetkellä prosessia käytetään enimmäkseen bioetanolin valmistukseen, mutta monomeeriset sokerit ovat kasvattaneet huomiota monikäyttöisinä jalostuskelpoisina kemikaaleina.

Perinteisesti entsymaattinen hydrolyysi on tehty panostoimisena prosessina, mutta jatkuvatoimisen prosessin edut ovat myös herättäneet kiinnostusta. Vaihdettaessa jatkuvatoimiseen prosessiin pyrkimys on parantaa prosessin ajettavuutta, saada käytettävissä oleva tankkitilavuus tehokkaampaan käyttöön ja parantaa kontaminaation hallintaa. Jatkuvatoimisessa prosessissa vedenkulutusta on mahdollista vähentää harvemmilla pesusekvensseillä. Vähentynyt vedenkulutus pienentää myös tarvittavaa jäteveden puhdistuskapasiteettia. Lisäksi kontaminaatiota aiheuttavilla organismeilla on vähemmän aikaa lisääntyä, kun biomassa on jatkuvassa liikkeessä.

Koeajot suoritettiin pilottimittakaavassa yksivaiheisena entsymaattisena hydrolyysinä. Kokeet tehtiin yksinkertaisessa säiliöreaktorissa, johon biomassa syötettiin pohjalle ja ulostulo virtasi pois pinnalta. Pumppausongelmat vaikeuttivat tasaista operointia. Kolmesta onnistuneesta koeajosta saatiin kuitenkin lupaavia tuloksia. Viipymäajoilla 14,8 h, 25,9 h ja 105,5 h päästiin 48 %, 70 % ja 95 % prosentin konversioihin. Konversiot olivat samaa luokkaa panostoimisen prosessin kanssa. Jatkuvatoimisuudella ei ollut vaikutusta sokerin laatuun verrattaessa panostoimiseen prosessiin. Prosessilla on selkeästi potentiaalia ja isommassa mittakaavassa pumppauksen ongelmat voivat ratketa isompien linjojen ja pumppujen avulla, jolloin prosessilla voidaan tuottaa kestävästi vihreitä sokereita ja ligniiniä.

SYMBOLS AND ABBREVIATIONS

Roman characters

A	Absorbance	-
a	Absorptivity coefficient	l/mg*cm or l/g*cm
A_{max}	Maximum cellulase adsorption	mg $\mu mol_{cellulase}$/g
B	Titrate consumption in reference	ml
B_0	Brix in the beginning	-
B_I	Brix increase	-
B_{max}	Maximum sugar brix in the determined dry matter content	-
B_T	Brix at moment T	-
c	Container	g
$c_{0,glucan}$	Proportion of glucan in the fresh feed	-
$C_{dry\ matter}$	Dry matter content in sample	-
$c_{glucose}$	Glucose content in the sample	mg/l
d	Dilution factor	-
E_a	Absorbed cellulase	mg or $\mu mol_{cellulase}$/L
E_f	Free cellulase	mg or $\mu mol_{cellulase}$/L
F	Coefficient for nitrogen to protein	-
K_P	Dissociation constant	-
M	Mass of dry insoluble lignin	g
m	Mass of the sample	mg
N	Normality of titrate acid	-
$N\%$	Nitrogen content	%

r_1	Reaction rate catalyzed by endoglucanase	-
r_2	Reaction rate catalyzed by β-glucosidase	-
r_3	Reaction rate catalyzed by cellobiohydrolase or glycohydrolase	-
S	Cellulose concentration	$g_{cellulose}/L$
s	Dry sample and container	g
t	Titrate consumption	ml
T	Wet sample	g
t_B	Batch time in the beginning of the process	h
t_R	Retention time of continuous process	h
W	Mass of total dry matter	g
W_{max}	Maximum cellulase adsorption	-
x_c	Changed proportion of slurry after the continuous operation has started	-
X_{CF}	Conversion factor to convert polysaccharide to monosaccharide including water uptake during hydrolysis	-

Greek characters

ρ	Density	kg/l or kg/m^3

Abbreviations

5-HMF	5-hydroxymethylfurfural
AFEX	Ammonia fibre explosion
BSA	Bovine serum albumin
CAH	Concentrated acid catalyzed hydrolysis

CBH	Cellobiohydrolase enzyme
CDH	Cellobiose dehydrogenase enzyme
CSTR	Continuously stirred tank reactor
DAH	Dilute acid catalyzed hydrolysis
DP	Degree of polymerization
EC	Enzyme Commission number
EG	Endoglucanase enzyme
FPU	Filter paper unit
HPLC	High performance liquid chromatograph
HTC	Hydrothermal carbon
LAB	Lactic acid bacteria
LCB	Lignocellulosic biomass
LCC	Lignin-carbohydrate complex
LHW	Liquid hot water
LPMO	Lytic polysaccharide mono-oxygenase enzyme
MBR	Membrane bioreactor
RI	Refractive index
SE	Steam explosion
SMP	Skimmed milk powder
SP	Soy protein
UF	Ultrafiltration
βG	β-Glucosidase enzym

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Appendix 1. Total process times, average retention times, conversions from liquid samples, lignin content in solid samples, and conversions from solid samples.

Appendix 2. Composition of solid sample compared to conversion.

1 Introduction

Climate change and environmental problems have brought increased attention to the potential of biobased materials as a sustainable option to fossil fuels and chemicals. The production of fossil-based fuels and chemicals is not sustainable and creates large amounts of greenhouse gases, while also creating a dependence on oil-rich areas. Biobased chemicals and fuels can reduce the climate impact and promote independence in chemical production. Renewable biobased chemicals can create a sustainable future.

As a raw material lignocellulosic biomass has been acknowledged to have potential for biofuels and biochemicals for at least the past 80 years (Himmel et al., 2007). Lignocellulosic biomass is multifunctional and highly renewable, making it an attractive option for producing biobased platform chemicals such as green sugars. Enzymatic hydrolysis is a promising process for producing these green sugars and high-quality lignin. Green sugars and lignin can then be refined further into various products, including biofuels, biochemicals, and biopolymers. Enzymatic hydrolysis has advantages over many chemical processes in terms of yields, selectivity, energy costs, and operating conditions (Yang et al., 2011). Although, enzymes are considered costly, the development of enzyme production has decreased the enzyme costs making enzymatic hydrolysis an intriguing option for biochemicals production.

While enzymatic hydrolysis is currently used mainly in the bioethanol process before or during fermentation, there is increasing interest in optimizing it as a process for producing monomeric sugars as such. Conventionally, enzymatic hydrolysis is done in batch processes, which can achieve high conversion rates with long retention times but also has limitations such as efficient use of tank volume. The increasing demand of bioproducts is driving the development into continuous processing in which more efficient operation time wise is achieved. Continuous operation of enzymatic hydrolysis is an intriguing option for more efficient and sustainable processing, although research in this area is limited. Continuous operation might have benefits such as decreased water consumption, decreased contamination risk, and enhanced use of tank volume.

To address the issue a study of continuous enzymatic hydrolysis using a simple continuously stirred reactor was conducted. The aim was to determine whether this process configuration

could achieve high enough conversion rates to be viable process option compared to the batch process.

2 Lignocellulosic Biomass

Lignocellulosic biomass (LCB) is a building material of plants and highly renewable raw material for biorefineries. As a raw material LCB can be such as wood, crops, grasses, forest residues, industrial residues, municipal solid wastes, and energy crops that can be utilized in biorefinery processes such as enzymatic hydrolysis. The primary source of lignocellulose in bioethanol production is still first-generation biomasses such as food crops. The second-generation biomass is nowadays preferred over the first generation since they do not compete with food production unlike first generation biomasses. Third-generation biomasses such as marine algae are also being researched, although the pretreatment of this type of biomass is currently relatively expensive. (Vasić et al., 2021) LCB primarily consists of cellulose, hemicellulose, and lignin but there are also minor compounds present in the structure. In this chapter these three main compounds are presented in more detailed and minor compounds are identified later in this chapter. In addition, structural differences between different LCB raw materials are studied.

2.1 Compounds of Lignocellulosic Biomass

Lignocellulosic biomass consists mainly of three compounds which are cellulose, hemicellulose, and lignin. Raw materials have different compositions of these three but approximately there is 30-50% of cellulose, 15-35% hemicellulose, and 10-20% lignin in every biomass. (Akhtar et al., 2016) Cellulose forms the base of the structure in which hemicellulose and lignin attach to. In addition of these three main compounds there are small amounts of extractives, pectin, proteins, starch, and inorganic compounds. (Sjöström, 1993) Cellulose and hemicellulose can be degraded into their building units, monomeric sugars, in the enzymatic hydrolysis. Lignin can also be degraded into monomeric building units, but in enzymatic hydrolysis lignin is usually recovered in solid polymeric structure.

2.1.1 Cellulose

Cellulose is the main compound in lignocellulosic biomass. It is found from the secondary cell wall in the cell structure of plant and in some algae (Betts et al., 1991; Sjöström, 1993). Cellulose consists of linear structure of glucose units which is called glucan. The linear glucose units are attached to each other with β-1,4-glycosidic bonds. (Bai et al., 2019; Betts et al., 1991) There can be from hundreds to thousands of glucose units in one cellulose chain (Jayasekara et al., 2019). From the chain structure can be found cellobiose units which consist of two glucose units. In cellobiose glucose units can be in different configuration in 180˚ along the axis of the cellulose chain. (Betts et al., 1991) The structure of glucose units and cellobiose units in cellulose chain is represented in Figure 1.

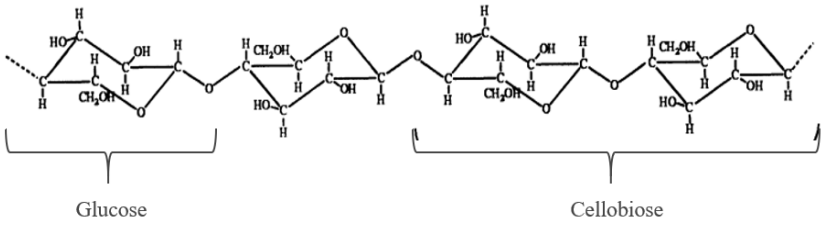

Figure 1. Structure of cellulose with glucose and cellobiose units (Betts et al., 1991).

Cellulose can have crystalline and amorphous regions in its structure. Crystalline regions have highly ordered structure unlike in amorphous regions. (Alen, 2000) Cellulose has high tendency of forming intra- and intermolecular hydrogen bonds which create the compact crystalline structure. The poor solubility of cellulose is also caused by the hydrogen bonds. (Jayasekara et al., 2019) Crystallinity and matrix structure gives cellulose its recalcitrance structure (Kallioinen, 2014).

2.1.2 Hemicellulose

Hemicellulose is group of heteropolysaccharides in the LCB structure. They consist of hexoses, pentoses, or deoxyhexoses which usually form a pyranose structure. Usually occurring hexoses in hemicelluloses are D-glucose, D-mannose, and D-galactose. Pentoses that usually occur in hemicellulose are D-xylose, L-arabinose, and D-arabinose. L-rhamnose, 6-deoxy-L-mannose, L-fucose, or 6-deoxyl-L-galactoses are deoxyhexoses that can occur also in the structure. (Alen, 2011) In addition, small amounts of uronic acids can be found. (Alen, 2000) Hemicellulose can be classified by the main polymeric chain's predominant monosaccharide. There are four classification groups which are xylans, mannans, β-(1,3;1,4)-glucans, and xyloglucans. (Broeker et al., 2018) Unlike cellulose, the structure of hemicellulose is usually highly branched. Hemicellulose structures are usually smaller than cellulose structures and it does not have crystalline regions. (Betts et al., 1991)

2.1.3 Lignin

Lignin is a natural polymer that occurs in the plant structure. It is amorphous and has three dimensional and irregular structure with aromatic rings. (Alen, 2011; Betts et al., 1991) Lignin has many functions in the plant structure. Mainly it acts as a bonding agent between cells. It also resists chemical and biological stress. (Betts et al., 1991) Lignin structure consists of three main building units which are coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol. These units are attached to each other with ether or carbon-carbon linkages. (Alen, 2011)

Lignin is structurally very stable due to the intermonomeric linkages. The main functional groups of lignin are phenolic hydroxyl, aliphatic hydroxyl, methoxyl, and carbonyl groups by which lignin reacts with other compounds. Lignin occurs in lignocellulosic structure as lignin-carbohydrate complex (LCC). Lignin is found to be linked at least with all the hemicellulose units and is believed to link with cellulose units too. Due to the complex structure lignin cannot be recovered from the LCB as a whole molecule. (Alen, 2000) Lignin composition also varies between different raw materials in molecular weight and linkages. Environmental factors also affect to the lignin structure. (Pollegioni et al., 2015) Due to these

factors, lignin structure is not perfectly known and probably cannot be determined. Nevertheless, there are assumptions of the structure. Schematic figures of softwood and hardwood lignin structures are presented the in Figure 2.

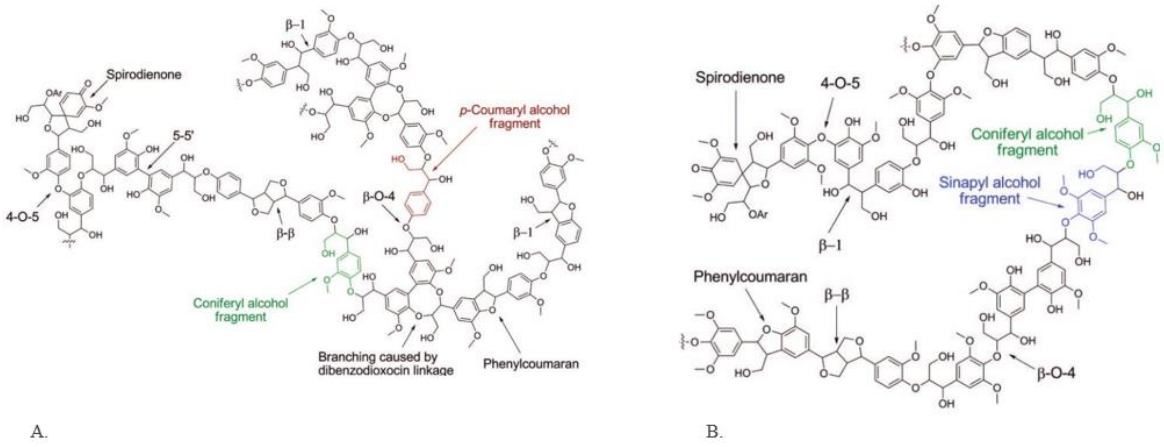

Figure 2. Schematic figures of A. Softwood lignin structure and B. Hardwood lignin structure (Zakzeski et al., 2010).

Lignin structure also changes during the process in which it is separated. Different processes create different lignin with different properties such as kraft, organosolv, soda, and enzymatic hydrolysis lignin. The lignins precipitated with different processes are called technical lignins which have different properties than natural lignin. These changes can occur also in pretreatment processes which needs to be considered in the process design. Compared to other lignin extraction methods the enzymatic hydrolysis lignin structure is considered to remain relatively unchanged (Sharma et al., 2020). Enzymatic hydrolysis lignin is also odourless and sulfur free which makes its further processing easier (Eraghi Kazzaz et al., 2020). Technical lignins have many value added applications for example in resins. (Harmsen et al., 2010)

2.1.4 Other minor compounds

In addition of the three main compounds there are minor polysaccharides such as pectin and starch in LCB. Also, some amounts of extractives and inorganic compounds can be found from the structure. The concentrations of these minor compounds vary between different raw materials. For example, growing conditions affect to the amount of inorganics present in the structure. (Rowell et al., 2005)

Pectin is a polysaccharide which consists of D-galactouronic acid units which are linked to each other with α-(1→4) linkages. Also, L-arabinofuranose and D-galactopyranose units are sometimes found in the pectin structure as minor parts. In the cell wall pectin is found in the membrane in the border pits between wood cells. (Rowell et al., 2005) In many biomasses pectin amounts are low. Pectin is not usually found from straw, woody, or grass biomasses. Except eucalyptus and sugarcane are known to have some amounts of pectin in their structure. In woody biomass highest amounts of pectin are found from inner structural parts of wood bark where it acts as a binder. Usually, fruit and vegetable peels have high amounts of pectin in their structure. For example, potato peel has 19-21 % of pectin. (Shrestha et al., 2021) Pectin can have an effect to enzymatic hydrolysis of cellulose and hemicellulose since it is known to mask those structures. Thus, it might hinder the accessibility of the enzymes. Pectin content needs to be considered in pretreatment if the biomass has high amounts of it in a way that it will not affect to the hydrolysis.

Starch is polysaccharide which consists of glucose units. It is the structure in which plants reserve sugars. It can be also found in small concentrations in the cell wall structure. Starch consists of D-glucapyranose units which are linked with α-(1→4) linkages. This structure creates amylose. There is also highly branched structure of these same units and linkages which is called amylopectin. (Rowell et al., 2005) Starch is stored in grains in the plastid stroma of living cells. It is also present in all tree organs such as leaves, stems, and roots. Usually, the starch content is highest in young trees and especially near the phloem. The starch content decreases close to the pitch. (Herrera-Ramírez et al., 2021) Most starch rich biomasses are first generation biomasses which decreases the willingness of using starch as raw material for enzymatic hydrolysis.

Extractives are components in biomasses that are not chemically compound to the components in the structure (Li et al., 2016). The extractives present in LCB structure mainly

consists of fats, fatty acids, fatty alcohols, phenols, terpenes, steroids, resin acids, rosin, waxes etc. Softwoods tend to have higher amounts of extractives in their structure than hardwoods. The highest concentrations of extractives are usually found from heartwood but bark also contains high concentrations of extractives. (Rowell et al., 2005) Herbaceous and agricultural fibres, grasses, and residues such as straws and corns strove straws have higher amount of extractives. Extractives are known to have an effect to the pretreatments in enzymatic hydrolysis. (Li et al., 2016)

Inorganics or ash is a group of minor inorganic compounds. Usually, the inorganics are carbonates such as calcium carbonate, magnesium carbonate, iron carbonate, complex silicates of aluminium, magnesium, calcium, iron, natrium, or potassium. There are small amounts of alkali metals and phosphates, halogen minerals such as sodium chloride, potassium chloride and some amounts of heavy metals such as zinc, copper, cobalt, nickel, chromium, lead, vanadium, and cadmium. The amounts of these inorganic elements vary between the raw materials, single plants, and the environments they are growing in. (Dzurenda et al., 2016)

2.2 Structures of different biomasses

There are various sources of LCB available for biorefineries, but wood is the most commonly utilized biomass due to several reasons. Unlike wood, the collection or harvesting of crops and grasses is typically only feasible in late summer or fall. This leads to limited availability of the biomass as a feedstock during other times of the year. Especially crops are prone to microorganism growth in storage which can affect to the quality of the raw material. Wood on the other hand is relatively easy to storage. Other problem with these kind of biomasses is that their bulk density is lower which causes bigger transportation costs and might require densification on the harvesting site. (Kaliyan et al., 2009) Also, non-wood biomasses contain higher amounts of inorganic compounds, silica, and proteins (Alen, 2011). In addition, agricultural residues have important role in the soil. If harvested the soil is more prone to erosion and the nutrients are collected away which increases the need of fertilizers. (Asikainen, 2011) The wood biomass is easy to handle, and the raw material is uniform which eases the pretreatment and process optimization. The chemical compositions of

cellulose, hemicellulose, and lignin in different lignocellulosic biomasses is presented in Table 1. The main focus in this study is on wood feedstock and its properties as a raw material in enzymatic hydrolysis

Table 1. Chemical compositions of different lignocellulosic raw materials.

Raw material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Pine	43	21	28	(Galbe et al., 2015)
Spruce	45	23	28	(Galbe et al., 2015)
Poplar	45	19	26	(Hamelinck et al., 2005)
Eucalyptus	50	14	28	(Hamelinck et al., 2005)
Corn stover	37	22	19	(Yang et al., 2016)
Switchgrass	32	25	18	(Hamelinck et al., 2005)
Wheat straw	35	22	16	(Østergaard Petersen et al., 2009)
Sugarcane bagasse	41	21	26	(Rabelo et al., 2011)
Hemp	64	15	4	(Thygesen et al., 2006)

The difference between grasses and higher growing plants such as woods is that lower pectin and glycoprotein content. (Betts et al., 1991) Marine biomass structures have different composition compared to the plant-based lignocelluloses. Generally, macroalgae contains 25-60% of carbohydrates, 5-20% proteins, 0,5-5% lipids, and 15-40% ash. (Vasić et al., 2021) Thus, marine biomasses need different preparations, pretreatments, and processing conditions.

Wood feedstock can be divided into softwood and hardwood. Cellulose contents in softwood and hardwood are usually very close to each other. (Alen, 2000) Softwoods and hardwoods have different total amount of hemicellulose but also the amount of glucomannan and xylan varies. Hardwoods usually have more xylose and acetylated hydroxyl groups and less mannose and galactose units than softwood. Softwoods primary components of hemicellulose are glucomannan (galactoglucomannan) and xylan (arabinoglucuronoxylan). From 100 % dry wood the amount of glucomannan is approximately 15-20 % and xylan is 5-10%. (Alen, 2011)

3 Enzymes degrading lignocellulosic biomass

This chapter focuses on the enzymes used for enzymatic hydrolysis of biomass. In enzymatic hydrolysis enzymes degrade the polymeric cellulose chain into monomeric glucose. Enzymes are considered the most expensive factor in the process, accounting for over 50% of the total costs. (Tolan et al., 2010) Therefore, optimizing the enzyme dosage is crucial for achieving the required conversion while ensuring the viability and profitability of the process.

Enzymes can be defined as proteins that catalyze chemical reactions by binding to and metabolizing compounds, which are referred to as substrates. There are two different metabolic reactions that can happen: anabolic or catabolic reactions. In anabolic reactions enzymes use energy to build more complex structures from simpler structures. In catabolic reactions enzymes break molecules to simpler structures and release energy. (Encyclopaedia Britannica, 2023) In enzymatic hydrolysis of biomass, the catabolic reactions hydrolyse the polymeric sugars structures into monomeric structures. Enzymes can be either specific or non-specific with the substrates. Specific enzymes only act on one specific substrate and non-specific enzymes can act on multiple different kind of substrates that have structural similarities. Enzymes work via their active site by which they attach to the substrate forming enzyme-substrate complex. As a result, the product or products are formed and released while enzyme remains unchanged and can then move on to catalysing other substrates. Enzymes can be classified by their catalysation reaction into six groups: oxidoreductases, transferases, hydrolases, lyases, ligases, and isomerases. From which the hydrolases are the most interesting group in enzymatic hydrolysis of biomass. (Blanco et al., 2017) Enzymes are also classified with Enzyme Commission number or EC which represents the enzymes and their genes. EC numbers also identify the chemical reaction that the enzyme catalyzes. (Hu et al., 2012)

The main purpose of the enzymes in enzymatic hydrolysis is to decay the cellulose and hemicellulose to free monomeric sugars (Álvarez et al., 2016). The enzymes are produced by bacteria or fungi from which the filamentous fungi is the main source of commercial enzymes (Álvarez et al., 2016; Kallioinen, 2014). Because of the complex structures of cellulose and hemicelluloses the degradation of these components need multiple different

enzymes. (Kallioinen, 2014) These different enzymes belong to the groups of cellulases and hemicellulases. (Álvarez et al., 2016) Cellulases and hemicellulases and their working principles in enzymatic hydrolysis are studied in this chapter.

3.1 Cellulases

Cellulases are enzymes that are produced by fungi, bacteria, actinomycetes, protozoans, plants, and animals. Cellulases belong to glycoside hydrolases that have been classified into 115 different families in the enzyme classification and cleave mainly β-1-4-glycosidic bonds. (Jayasekara et al., 2019; Kallioinen, 2014) Even thought, there are only one type of glycosidic bond in cellulose, different cellulase enzymes are needed to produce monomers (Rahikainen, 2013). The degradation of cellulose produces glucose, cellobiose, and oligomers as products. The cleaving of the bond is done by the enzyme using acid-based catalysis: a general acid and nucleophile (Jayasekara et al., 2019). There are two subsequent pathways which occur in enzymatic hydrolysis of cellulose to glucose: 1. dissolution of crystalline cellulose and 2. hydrolysis of disrupted cellulose bonds (Bhaumik et al., 2016). The enzymes act on the cellulose with inversion and retention mechanisms. Both of them are catalyzed by two carboxyl groups which are located in the active site of the enzyme (Wertz et al., 2010).

Cellulases can be classified into cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4), and β-glucosidases (EC 3.2.1.21) depending on their working mechanisms. In addition there are auxiliary enzymes, expansins, and swollenins affecting to the depolymerization process. (Kallioinen, 2014) Cellulases belong to a superfamily of hydrolases. Hydrolases are enzymes that use water to degrade larger molecules. (Rahman et al., 2018) One of the most used and studied fungi which produces cellulase enzymes is *Trichoderma reesei*. This fungi is very active producer of cellulase enzymes which makes it most commercially consumed enzyme producer. (Rahikainen, 2013).

3.1.1 EC 3.2.1.4 Endoglucanases

Endoglucanases (EGs) are enzymes that degrade the cellulose chain within the chain unlike CBHs (Rahikainen, 2013). The active site of the EG is open groove like structure in the three-dimensional structure of the enzyme. This allows the enzyme to attach in the middle of the chain. (Henriksson et al., 1996) EGs are classified to belong to glycoside hydrolase families (Rahman et al., 2018). Although endoglucanases are usually in this study referred to β-1-4-endoglucanases, there are other endoglucanases that can cleave for example β-1-6-glycosidic bonds (Rahman et al., 2018). The cleaving of glycosidic bonds in the middle of the chain creates more attachment sites for CBHs that depolymerize the chains only from ends (Rahikainen, 2013). Thus, it increases the possibilities to find a chain for the CBH. EGs mainly focus on degrading the amorphous areas of the cellulose (Kleman-Leyer et al., 1996). EG activity can be detected with change of color or viscosity in the solution or slurry (Rahman et al., 2018).

3.1.2 EC 3.2.1.91 Cellobiohydrolases

Cellobiohydrolases or CBHs are enzymes that depolymerize cellulose producing cellobiose units (Kallioinen, 2014). It is usually the bulk of the cellulolytic cocktail in enzymatic hydrolysis (Teter et al., 2014). CHBs decay the cellulose chain from both ends. CBH I (Cel7A) from the reducing-end and CBH II (Cel6A) from the non-reducing end. (Rahikainen, 2013; Teter et al., 2014) CBHs always start the cleaving form end of the cellulose chain and cannot affect to the bonds in the middle of the chain. (Teter et al., 2014) This is caused by a tunnel-shaped active site on the enzymes three dimensional structure (Igarashi et al., 2009). This progressive method of proceeding in the degradation helps the enzyme to stay bound after the catalytic cycle. CBHs can proceed from one end of the chain to the other decaying it on the way without releasing the chain. (Igarashi et al., 2009; Rahikainen, 2013) Unlike many other catalysts CBH can and mostly does decay crystalline cellulose (Shrotri et al., 2017). CBH II is found to be less processive than CBH I in the studies (Igarashi et al., 2009). The cellobiose product inhibits the CBH actions (Olsen et al., 2016).

3.1.3 EC 3.2.1.21 β-glucosidases

β-glucosidases (βGs) catalyze oligosaccharides and cellobiose into glucose. βGs can be classified into three groups: aryl-β-glucosidases, cellobiases, and broad specificity β-glucosidases. Aryl-β-glucosidases prefer to cleave aryl-β-glycoside bonds and cellobiases depolymerise only cello-oligosaccharides. Unlike the first two groups, broad specificity β-glucosidases act similarly to both substrate groups cleaving both. (Yeoman et al., 2010) The glycosylation of cellobiose unit by βG is presented in Figure 3.

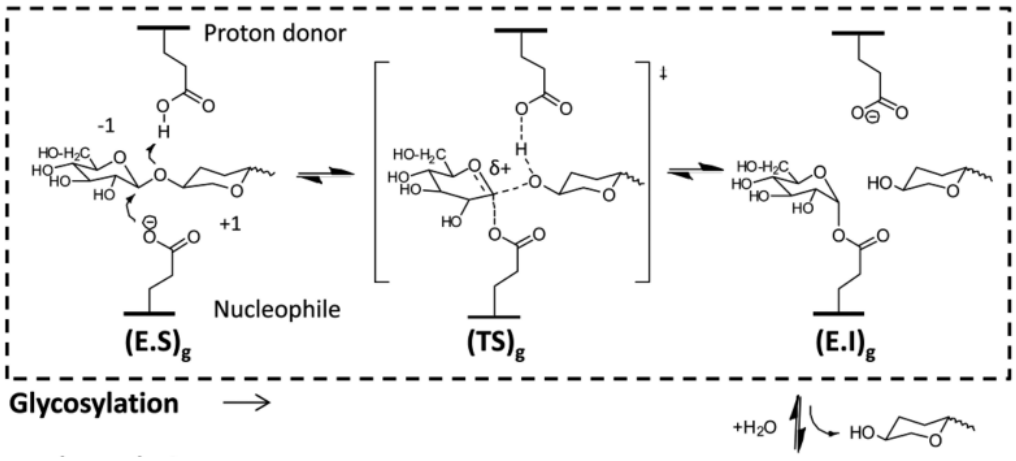

Figure 3. Glycosylation of cellobiose unit by β-glucosidase (Badieyan et al., 2012).

βGs act as rate limiting factor in the whole cellulolytic process. (Yeoman et al., 2010) Due to the ability of decaying the intermediate cellobiose in the process decreases the inhibition of CBH and also produces the wanted end product. Thus, βG is important part of the synergism of the enzymes in the enzymatic hydrolysis. (Gusakov et al., 2007) Glucose is known to inhibit many βGs but there are also many studies about glucose tolerant and glucose stimulated βGs. (Salgado et al., 2018)

3.1.4 Auxiliary enzymes for hydrolysis of cellulose

Auxiliary enzymes are a more recent found in the cellulose degrading enzymes. They cleave cellulose in addition of the well-known cellulases. Here are presented the most well-known auxiliary enzymes the lytic polysaccharide mono-oxygenases (LPMOs) and cellobiose dehydrogenases (CDHs). Also, swollenins and expansins which can promote the enzymatic hydrolysis are presented later in the chapter.

LPMOs (EC 1.14.99.54) cleave the glycosidic bonds using oxidative mechanisms and act synergistically with cellulase (Westereng et al., 2011). They are also known to be able to hydrolyse the crystalline regions of cellulose (Kallioinen, 2014). The LPMOs are a copper depended enzymes that use small redox-active cofactors such as ascorbate and galate (Quinlan et al., 2011). CDHs (EC 1.1.99.18) are redox enzymes which role in degradation of cellulose is yet unknown. It is known that CDHs affect to the decaying of cellulose, hemicellulose, and even lignin. CDHs are ping-pong type enzymes that act on the reducing end of cellobiose. (Henriksson et al., 2000) CDH has high specificity for cellobiose and in the oxidation cellobiose is converted to cellobionic acid. (Westermark et al., 1974) Also, other accessory enzymes that prevent for example oligomer inhibition are usually used in the enzyme cocktails (Neumüller et al., 2014).

In addition, swollenins and expansins have been studied to affect to the degradation of cellulose (Kallioinen, 2014). Expansins are proteins in plants that enlarge cell wall structure and polysaccharides without hydrolytic activity (Eibinger et al., 2016; Kim et al., 2009). While they do not break down the cell wall structure, they may induce amorphogenesis, which can result in loosening it up. This is believed to increase the accessibility of the enzymes in enzymatic hydrolysis which profits the conversion rate. It is proved that glucose conversion can be increased by introducing expansins into the solution with cellulases. (Kim et al., 2009) Although expansins and swollenins are not cleaving the glycosidic bonds like enzymes they might have a big role in the hydrolysis rate.

3.2 Hemicellulolytic enzymes

Hemicellulose can be depolymerized with enzymes like cellulose. Unlike cellulose, hemicellulose is a group of different kind of polysaccharides which makes its enzymatic hydrolysis more complicated. These polysaccharides consist of multiple different monosaccharides, sugars acids, and non-carbohydrate subunits. Therefore many different enzymes are needed to depolymerize hemicellulose. (Kallioinen, 2014) The raw material also affects to the enzymes needed since different raw materials have different hemicellulose composition. Even though the branched structure of hemicellulose brings difficulties to the depolymerization, it is easier degrade than crystalline cellulose (Decker et al., 2009). The hemicellulolytic enzymes are usually not needed for lignocellulosic biomass due to the pretreatment but can be used to enhance the hydrolysis. In pretreatment the pentoses are usually degraded into the liquid phase and can be separated before the enzymatic hydrolysis in solid liquid separation. Nevertheless, enzymatic hydrolysis can also be done with mixed pentoses and hexoses which needs to be considered in the enzyme cocktail.

In enzymatic hydrolysis of hemicellulose, the enzymes act mainly on the backbone chain which is for example xylan, glucomannan or glucan chain (Kallioinen, 2014). The main linkages that the backbone structure has are β-(1 → 3, 1 → 4)-linked xylosyl, β-(1 → 4)-linked mannosyl, and β-(1 → 3, 1 → 4)-linked glucosyl residues (Frommhagen et al., 2015). For cleaving xylose from hemicellulose structure there are many different kind of xylanase enzymes. These xylanases have specific dominant linkages which they act on i.e., endo-1,4-β-xylanase. For each linkage in the chain structure there is an enzyme. (Decker et al., 2009) Some of the most usual hemicellulolytic enzymes and their dominant linkages used in the depolymerization of hemicellulose are presented in Table 2.

Table 1. Hemicellulolytic enzymes and their dominant cleaving linkages (Decker et al., 2009).

β-xylanases	**Dominant linkages**
Endo-1,4-β-xylanase (EC 3.2.1.8)	(1→4)-β-D-xyloside
Endo-1,3-β-xylosidase (EC 3.2.1.32)	(1→3)-β-D-xylosyl
1,4-β-xylosidase (EC 3.2.1.37)	Reducing end (1→4)-β-D-xylosyl
Xylan 1,3-β-xylosidase (EC 3.2.1.72)	Non-reducing end (1→3)-β-D-xylosyl
Glucuronoarabinoxylan endo-1,4-β-xylanase (EC 3.2.1.136)	(1→4)-β-D-xylosyl adjacent to glucuronosyl substituted xylose
Oligosaccharide reducing-end xylanase (EC 3.2.1.156)	Reducing end (1→4)-β-D-xylosyl

Glycosyl hydrolases	**Dominant linkages**
β-mannosidase (EC 3.2.1.25)	Non-reducing end (1→4)-β-D-mannosyl
Mannan endo-1,4-β-mannosidase (EC 3.2.1.78)	(1→4)-β-D-mannosyl
Arabinogalactan endo-1,4-β-galactosidase (EC 3.2.1.89)	(1→4)-β-D-galactosyl
Arabinan endo-1,5-β-L-arabinosidase (EC 3.2.1.99)	(1→5)-β-L-arabinosyl
Mannan 1,4-mannobiosidase (EC 3.2.1.100)	Non-reducing end (1→4)-β-D-mannosyl
Galactan 1,3-β-galactosidase (EC 3.2.1.145)	Non-reducing end (1→3)-β-D-galactosyl

In addition of the backbone depolymerizing enzymes there are debranching enzymes which work in synergy with the enzymes presented in the Table 2 above. Even though their depolymerization does not decay the backbone chain they increase the accessibility of the backbone enzymes to their substrates. (Decker et al., 2009) The debranching enzymes are presented in Table 3.

Table 2. Hemicellulolytic debranching enzymes and their dominant linkages (Decker et al., 2009).

Debranching enzymes	**Dominant linkages**
Acetyl xylan esterase (EC 3.1.1.72)	Xylose-*O*-acetyl
Ferulic acid esterase (EC 3.1.1.73)	Arabinose-*O*-feruloyl
Acetyl esterase (EC 3.1.1.6)	Xylose-*O*-acetyl
Xylan α-1,2-glucuronosidase (EC 3.2.1.131)	(1→2)-α-D-glucorunosyl
α-glucuronidase (EC 3.2.1.139)	(1→2)-α-D-glucorunosyl
α-N-arabinofuranosidase (EC 3.2.1.55)	(1→3,5)-α-L-arabinosyl

3.3 Enzyme cocktails and commercial enzymes

Usually, multiple different enzymes are needed in enzymatic hydrolysis of cellulose and these mixtures are called enzyme cocktails. In the hydrolysis enzymes work in synergy due to the complex structure of cellulose. Endoglucanase and cellobiohydrolases work in so called endo-exo-synergy. (Kallioinen, 2014) Endo-exo-synergy roughly means that EG cleaves internal linkages creating new sites for CBHs which can only cleave the end chains. Although, there are studies showing that these two enzymes have even more synergy actions than just endo-exo-synergy during the hydrolysis. (Olsen et al., 2017) Also, CBH I and CBH II work in synergy choosing mainly to degrade the chains form different ends. Their activity has been detected even when working sequentially. (Väljamäe et al., 1998) Other enzymes also have been detected to work in synergy which improves the rate of hydrolysis. The optimal enzyme cocktails have high synergy and optimal amount of each enzyme to reach high conversions.

In optimal enzyme cocktails there can be such as 3-16 different enzyme components. The number of components depend on the raw material and pretreatment. (Kallioinen, 2014) The cocktails are designed to a specific raw material structure to have improved synergy and hydrolysis rate. There are multiple commercial enzyme producers that produce these cocktails which contain different enzymes for specific need. The producers can also design an optimal enzyme cocktail for their customer's needs. Many enzyme manufacturers provide enzyme solutions for different industrial areas. Few enzyme manufacturers and their

cocktails for degradation of cellulose and hemicellulose for enzymatic hydrolysis of LCB is presented in Table 4.

Table 3. Manufacturers and their solutions for cellulolytic and hemicellulolytic enzyme cocktails for degradation of lignocellulosic biomass.

Manufacturer	Commercial cocktail	Properties	References
Novozymes	Cellic® CTec2	Blend of cellulases, β-glucosidases & hemicellulases. Designed for pretreated lignocellulosic biomass.	(Novozymes, 2022)
Novozymes	Cellic® CTec3 HS	Blend of cellulases and hemicellulases with proprietary enzyme activities. Flexible operating conditions.	(Novozymes, 2022)
BASF SE	Spartec™ CEL 100	Designed to hydrolyse non-starch polysaccharides, cellulose, and hemicellulose. Especially for corn biomass.	(BASF, 2022)
Sunsonzymes	SUN47	Designed for pretreatment of wood chips. Promotes water absorption and swelling.	(Sunsonzymes, 2022)
Enzyme supplies	Cellulase 15,000L	Liquid or powder enzyme cocktail designed for acid conditions (pH 4,5-6,0). Contains endoglucanases, cellobiohydrolase, and β-glucosidase. Can be modified with e.g., xylanases.	(Enzyme Supplies, 2022)
Noor Enzymes	BioConvert-ACEL	Cellulase and hemicellulase cocktail in liquid or powder form. Designed to degrade cellulose, xylan, arabinan, and other hemicellulolytic components. Optimal pH range is 4,5-6,5.	(Noor Enzymes, 2015)

Enzyme dosage can be described in many ways in enzymatic hydrolysis. Usually, manufacturers define the activity of the enzyme cocktails. The enzyme dosage can be given as activity per dry matter or substrate such as glucan in this case. It can be also defined as Filter Paper Unit (FPU). FPU is enzyme activity measurement which guidelines are set by Union of Pure Applied Chemistry (IUPAC). The measurement value of 2,0 mg of reducing sugar from 50 mg of filter paper in 4% conversion in 60 minutes is set as the intercept for calculating FPU. (Adney et al., 2008)

4 Enzymatic hydrolysis

Enzymatic hydrolysis is a hydrolysis process where enzymes degrade polymeric feeds into smaller particles or monomers. Usually, in enzymatic hydrolysis the raw material is a biomass but it can be done also to other materials such as plastics with right enzymes (Chen et al., 2021). This study focuses on enzymatic hydrolysis of lignocellulosic biomasses.

In enzymatic hydrolysis the enzymes presented in the Chapter 3 work in synergism degrading the raw material. Enzymatic hydrolysis is usually considered to be an option for acid-catalysed hydrolysis. Enzymatic hydrolysis has some advantages over acid-catalysed hydrolysis. The operating conditions in enzymatic hydrolysis are milder due to the optimal conditions of the enzymes. (Branco et al., 2018) The cellulase enzymes degrading the cellulose have usually optimal temperature from 40-50 ˚C and optimal pH around 4,5 to 5 (Vasić et al., 2021). The optimal operating conditions depend on the enzymes which are used in the hydrolysis but tend to have similar optimal ranges. Since acids are not used in enzymatic hydrolysis there is no corrosion risk in the process. The temperature is relatively low and easily achieved without excessive energy consumption. The process has relatively high product yield with proper optimization. The main advantage in enzymatic hydrolysis is the selectivity which can be gained with enzymes. With the selectivity higher amount of monomeric sugars can be produced unlike in acid hydrolysis the result can be either mixture with high amounts of oligomeric sugars or high amount of sugar degradation products. On the other hand, it is considered costly and relatively slow process compared to the acid-catalysed hydrolyses. (Branco et al., 2018).

Before the hydrolysis some sort of preparation of the biomass and a pretreatment is required. During the pretreatment most of the hemicellulose is hydrolyzed into the liquid and therefore can be removed before the enzymatic hydrolysis. The solids continue to the hydrolysis process where enzymes degrade the polymers and oligomers left. As a result, is a slurry where lignin and monomeric hexose sugars are. After that, separation and purification steps are usually needed to increase the purity of the products. A simplified schematic flow diagram of enzymatic conversion process is presented in Figure 4.

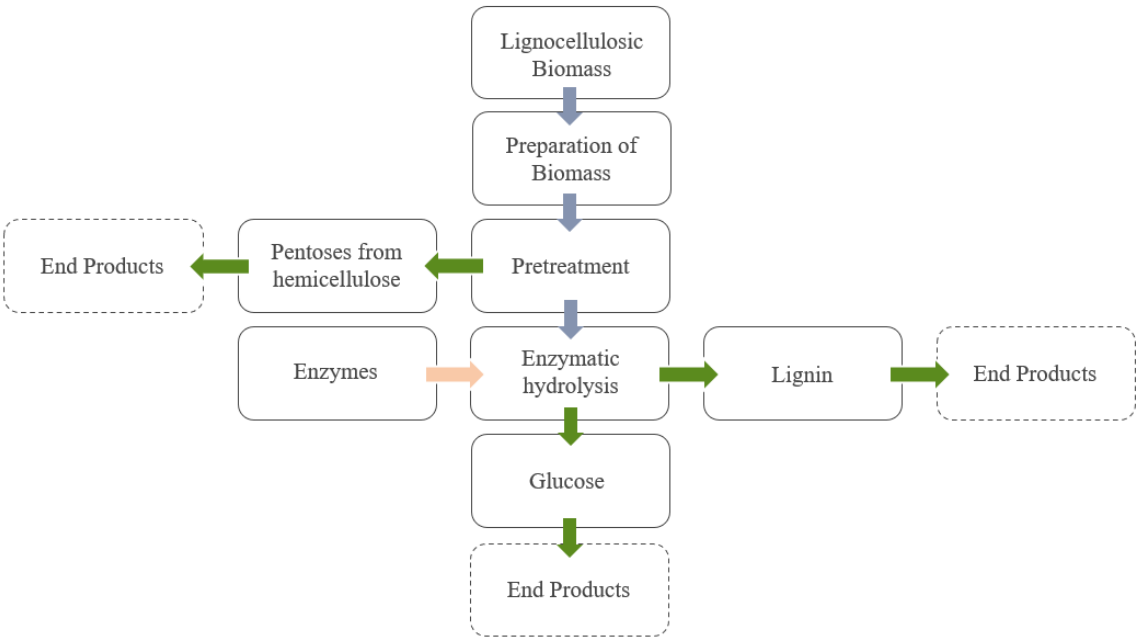

Figure 4. Simplified flow diagram of enzymatic conversion process (Vasić et al., 2021).

4.1 Pretreatment

Due to the structure of lignocellulosic biomass, it is resistant to chemical, mechanical and biological treatments. Thus, pretreatment is necessary to remove or weaken those natural defence systems. (Kallioinen, 2014) Pretreatment is expected to increase yield and hydrolysis rate (Harmsen et al., 2010). Non-pretreated LCB raw materials produce under 20 % of the theoretical quantity in enzymatic hydrolysis (Smits et al., 2014).

During pretreatment, the cellulose and hemicellulose components are treated to increase their accessibility to enzymes. Furthermore, pretreatment can also impact the accessibility by separating lignin and hemicellulose from cellulose. It may also influence the structure of crystalline cellulose by decreasing it, thus making it more easily hydrolyzed. (Kallioinen, 2014) Usually, pretreatment produces solid and liquid fractions. Solid fraction contains amorphous cellulose and lignin. Liquid fraction contains monomeric sugars from hemicellulose and degradation products of lignin. (Jing et al., 2009) The pretreatment methods can be divided into physical, biological, and chemical pretreatments. All of these three pretreatments can be combined to create an efficient pretreatment plan for the raw

material. (Kallioinen, 2014; Rahikainen, 2013) Usually, mechanical pretreatment that is included in physical pretreatment is carried out before any other pretreatments. In mechanical pretreatment the particle size is decreased which increases the surface area and eases the handling. Mechanical pretreatment can be such as chipping or milling. (Harmsen et al., 2010) The particle size of the biomass can have high effects on the hydrolysis and handling (Vasić et al., 2021). The main factors to consider when selecting a pretreatment method are the yield of carbohydrates, the formation of inhibitory by-products, and processing costs (Kallioinen, 2014). While several pretreatment methods have their own benefits and drawbacks, the choice of raw material and operating conditions affect greatly. Hence, optimizing the pretreatment process is important for each specific application. This chapter covers and introduces the most commonly used pretreatment methods for enzymatic hydrolysis of biomass.

4.1.1 Steam, ammonia fibre, and CO_2 explosion

Steam explosion is widely used pretreatment method due to its low chemical consumption. In steam explosion high-pressure steam is introduced to biomass in reactor. (Harmsen et al., 2010) It is considered to be the most environmental friendly and energy efficient pretreatment process (Shrotri et al., 2017). Steam explosion can be used for all raw materials, but the operation parameters may vary. For example, softwood steam explosion usually requires harsher conditions and added acid catalyst to achieve the desired result. (Rahikainen, 2013) In steam explosion the steam is introduced to the biomass and the temperature increases to 160-250˚C. During the treatment the steam is absorbed into the biomass structure. After the needed treatment time is reached the steam is released from the reactor. This generates the absorbed steam to expand resulting explosive decompression inside the biomass structure. (Harmsen et al., 2010; Kallioinen, 2014) The expansion of the structure causes hemicellulose degradation and lignin matrix disruption. The steam explosion affects to the cellulose mainly by decreasing the degree of polymerization and degree of crystallinity. In harsh steam explosion operating conditions condensation and repolymerization can occur. Also, degradation products of hemicellulose can form inhibitory

pseudo lignin products. (Xu et al., 2014) Structural difference of steam treated, and non-treated lignocellulosic biomass are represented in Figure 5.

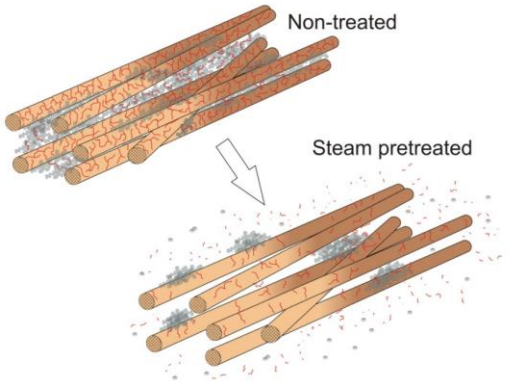

Figure 5. Nanometre-scale structures of steam pretreated and non-treated lignocellulose where long cylinders represent cellulose, red hemicellulose, and grey lignin (Rahikainen, 2013).

After steam explosion the degraded hemicellulose is in water soluble oligo- and monomers which can then be extracted (Rahikainen, 2013). Cellulose usually maintains its structure but slight depolymerization may occur in mild processing conditions. Although, in Atalla's study the crystallinity index of cellulose increased in higher temperature such as 200-245 ˚C (Atalla, 1991). Lignin on the other hand undergoes changes. (Shrotri et al., 2017) Steam explosion causes cleaving of β-O-4 linkages and C-C bonds in lignin structure (Chung & Washburn, 2016).

Ammonia fibre explosion or AFEX is a pretreatment with aqueous ammonia in high temperature and pressure like in steam explosion (Rahikainen, 2013). AFEX is usually done for moist biomass in temperatures between 60-100 ˚C with usual reaction time of 5-60 minutes. The process can be done in batch or flow through reactor. Approximately 95 % of the ammonia can be recovered as gas and be recycled back to the process. (Xu et al., 2014) The difference of AFEX compared to other pretreatment is that it does not produce separate liquid fraction (Kallioinen, 2014). Like other pretreatment processes AFEX increases the surfaces area. It also decrystalizes cellulose and removes hemicellulose which eases the enzymatic hydrolysis. It has also affects to the lignin structure. (Mosier et al., 2005)

CO_2 explosion is similar process to steam explosion and AFEX. In CO_2 explosion the high-pressure gas is carbon dioxide (Harmsen et al., 2010). It is studied that CO_2 explosion pretreated enzymatic hydrolysis yield is lower than with steam explosion or AFEX when the raw material is wood (Sun et al., 2002). But in Zheng et al. studies when the raw material was paper mix, sugarcane bagasse, and repulping waste of recycled paper it did not produce inhibitory compounds unlike in steam explosion. In addition in these cases the CO_2 explosion was more cost effective than AFEX. (Zheng et al., 1998) CO_2 can be also used in super critical CO_2 extraction but it is considered to be overly expensive (Harmsen et al., 2010).

4.1.2 Alkaline pretreatments

The alkaline pretreatment affects to the biomass mainly by removing lignin from the structure. During alkaline treatment hemicellulose is also solubilized. (Harmsen et al., 2010; Xu & Huang, 2014) Alkaline pretreatments are widely used in pulp and paper manufacturing such as Kraft pulping process (Kallioinen, 2014; Kim et al., 2016). Calcium hydroxide and sodium hydroxide are mainly used due to their efficiency and economic perspective but also ammonium, and potassium can be used (Kim et al., 2016; Zhang, 2021). Usually alkaline pretreatment chemicals are non-polluting and non-corrosive which is an advantage (Kim et al., 2016). Alkaline pretreatments can cause swelling of fibres which increases the surface area. It can also decrease the degree of polymerization, crystallinity, and can cleave bonds between lignin and hemicellulose. (Zhang, 2021) The ester linkages in lignin-carbohydrate complexes are cleaved by the alkali mediums and lignin is solubilized (Shah et al., 2021). In hemicellulose the intermolecular ester bonds are also saponified which causes the solubilization of xylan (Guangyin et al., 2017). Alkaline pretreatment is usually done in milder condition than acid pretreatment (Kim et al., 2016). Main disadvantage of alkaline pretreatment is the formation of irrecoverable salts and the incorporation of salts into the biomass (Xu et al, 2014). There are different kind of alkaline pretreatments such as ammonia recycle percolation, soaking in aqueous ammonia, low-liquid ammonia, low-moisture anhydrous ammonia, and technologies that utilize natrium hydroxide and calcium hydroxide. Also, AFEX can be included in alkaline pretreatments. (Kim et al., 2016) Soaking in aqueous

ammonia has had good results in lignin removal. It has been studied to remove up to 74 % of lignin from corn stover and at the same time retaining over 85 % of xylans and almost all glucan. (Xu et al., 2014)

4.1.3 Organosolv pretreatment

In organosolv process the lignin is removed from the biomass using organic solvent or mixture of organic solvents (Harmsen et al., 2010). Hemicellulose is hydrolyzed into soluble components which includes for example oligomers, monomers, and acetic acid. Cellulose is hydrolyzed to smaller fractions but remains mostly insoluble in pretreatment. Lignin degrades into lower molecular weight molecules which dissolve into the solvent. (Xu et al., 2014) The solvents used are usually ethanol, methanol, acetone, or ethylene glycol. One or multiple solvents can be used at the same time. (Harmsen et al., 2010) Usually, low molecular weight solvents such as ethanol are preferred due to their low boiling point (Xu et al., 2014). The process temperature and reaction time depends on the raw material and the solvent (Chundawat et al., 2010). Typically the temperature is between 170-220 ˚C and pH between 2-4 (Macfarlane et al., 2014; Xu et al., 2014). The cooking time is usually between 30-90 minutes, solvent concentration is 35-70 %, and solid ratio between 4:1 and 10:1 (w/v). Addition of acid into the process can increase xylose yield obtained from the pretreatment process. (Xu et al., 2014) Solvents are usually expensive and might have an effect to the enzymatic hydrolysis in the further steps, thus it must be at least partially removed (Harmsen et al., 2010). For example, ethanol is an inhibitory compound in enzymatic hydrolysis. Solvents are usually easy to recover with distillations. On the other hand, the process needs strict control due to the volatility of the solvents. (Xu et al, 2014) Organosolv process produces high quality lignin and is an efficient process for lignin removal. (Harmsen et al., 2010) One of the best known organosolv process is Alcell which uses ethanol-water mixture. The main advantage in this process compared to others is that it produces separate cellulose, hemicellulose, and lignin streams. (Macfarlane et al., 2014)

4.1.4 Liquid hot water pretreatment

Liquid hot water (LHW) process or solvolysis/autohydrolysis is a process where biomass is treated in water with high temperature and pressure (Harmsen et al., 2010; Mosier et al., 2005). The temperatures in the process are usually between 160-240 ˚C and the reaction time is from few minutes to hours. In the process water acts like a weak acid by releasing hydronium ion. This causes depolymerization of hemicellulose by hydrolysis of glycosidic linkages. Acidic compounds are released by this reaction which can catalyze hydrolysis of hemicelluloses and their oligomers. (Xu et al., 2014) According to Mosier et al. 40-60% of the biomass can be solved in this process. In the process 4-22% of cellulose, 35-60% lignin and all of the hemicellulose can be removed. LHW can be done in different reactors such as batch, co-current, counter current, and flow through reactor. (Mosier et al., 2005; Xu et al., 2014) In batch reactor the biomass is introduced to water and treated with the high temperature and held in for the needed reaction time. In flow through reactor the hot water is passing the stationary bed of lignocellulose. Although, LHW works similarly to DAH it forms less inhibitory sugar degradation products. In addition, LHW pretreatment does not need a washing step or neutralization after the process since the solvent is water. (Mosier et al., 2005) However, it has been noticed that the presence of extractives in the biomass can impact the effectiveness of LHW pretreatment. This was observed with samples of corn stover with varying levels of extractives that underwent LHW pretreatment. The sample with highest extractive content resulted in a lowest yield during enzymatic hydrolysis. (Li et al., 2016)

4.1.5 Acid catalyzed hydrolysis pretreatment

Acid catalyzed hydrolyses can be divided into dilute acid catalyzed hydrolysis (DAH) and concentrated acid catalyzed hydrolysis (CAH). Both can be used as a pretreatment for enzymatic hydrolysis. Acid catalyzed hydrolysis is studied as pretreatment of enzymatic hydrolysis in this chapter.

Acid catalyzed hydrolysis mainly effects to the reactivity, solubility of carbohydrates, lignin structure, and structural features of the substrates. During acid catalyzed hydrolysis occurs

cellulose decrystallization, fibre swelling, depolymerization, formation of monosaccharides, esterification, and re-polymerizations. The decrystallization starts with dissolution into the acid by swelling of the fibre caused by the permeated acid molecules. When the swelling is severe enough the cellulose chains separate from each other. By this mechanism the crystal structure is decayed, and the cellulose is in amorphous state. In amorphous state cellulose forms gelatin with the acid present in the solution. After this, introduction of water to the mixture causes rapid hydrolysis into glucose units. (Zhou et al., 2021) As a pretreatment for enzymatic hydrolysis the main factor is the increased surface area and accessibility to the cellulose chains not necessary the production of glucose. The main effect as pretreatment acid catalyzed hydrolysis mainly affects to the hemicelluloses by degrading them (Xu et al., 2014).

DAH is considered to be one of the most effective pretreatment method for lignocellulose. It can be done either in high temperature and with continuous flow or in low temperatures in batch process. (Harmsen et al., 2010) Usually, with lignocellulosic raw materials the temperature is higher than 200˚C (Zhou et al., 2021). Acid that is used is usually a mineral acid such as sulphuric acid, hydrochloric acid, or phosphoric acid (Harmsen et al., 2010; Zhou et al., 2021). Due to the acid consumption and monomeric sugar production DAH is preferred over CAH as a pretreatment. After hydrolysis the acid can be neutralized with lime without needing any separation methods. Usually, acid concentration in DAH is 0,1-5 %. Main disadvantage of DAH is the degradation of sugars. These sugar degradation by-products such as furfural, 5-hydroxymethylfurfural (5-HMF), formic, and levulinic acid can have inhibitory affects. (Zhou et al., 2021) The production pathways for the sugar degradation inhibitory by-products are presented in Figure 6.

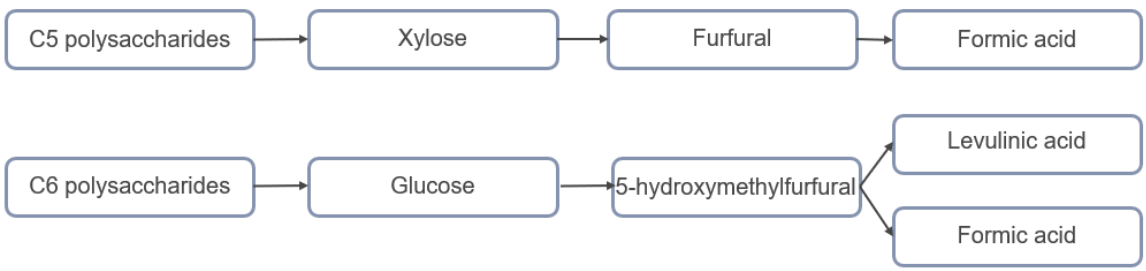

Figure 6. Pathways of formic acid and levulinic acid formation in acid catalyzed hydrolysis (Zhou et al., 2021).

In DAH the sugar degradation is a bigger concern since it is usually done in high temperatures which affects to the sugar degradation more than the acid concentration (Zhou et al., 2021). Also, formation of pseudo lignin is a concern in both DAH and CAH (Xu et al, 2014; Zhou et al., 2021). In CAH the same acids are used as in DAH. Due to the higher acid concentration the hydrolysis can be done in lower temperatures with higher yield of sugar. The high acid consumption is although costly and corrosion needs to be considered in the process design. In addition, the acid recovery is more difficult and significant amounts of gypsum is formed with lime in the neutralization. High acid concentrations also have a negative environmental impact. (Zhou et al., 2021) Due to these disadvantages DAH is often considered more suitable pretreatment method since sugar yield is not important at this stage of the process. The pretreatment methods with their advantages and disadvantages are concluded in Table 5.

Table 4. Advantages and disadvantages of the pretreatment methods. In the table (-) is negative character and (+) is positive character. (Harmsen et al., 2010)

Pretreatment	Potential sugar yield	Inhibitor formation	Residue formation	Chemical recycling	Low costs
Mechanical	-	++	++	++	+/-
Steam explosion	+	-	+	++	++
AFEX	++	++		-	
CO_2 explosion	+	+	++	++	-
Organosolv	++	++	+	-	-
LHW	++	-	++	++	+
DAH	++	-	-	-	+
CAH	++	-	-	-	-

4.2 Kinetics of enzymatic hydrolysis

Kinetics of cellulose degradation in enzymatic hydrolysis follows certain equations. The kinetics are presented in this chapter.

When endoglucanase cleaves cellulose producing cellobiose units the kinetics follow the Equation 1.

$$(C_6H_{10}O_5)_{\mathrm{n}} + H_2O \xrightarrow{r_1} C_{12}H_{22}O_{11} + (C_6H_{10}O_5)_{n-2} \quad (1)$$

Where,

r_1 Rate catalyzed by endoglucanase. (Scott et al., 2013)

The cellulose to cellobiose reaction with competitive glucose and cellobiose inhibition can be described with Equation 2.

$$r_1 = \frac{k_{1r}E_{1bC}SRC}{1 + \frac{G_2}{K_{1IG_2}} + \frac{G}{K_{1IG}}} \quad (2)$$

Where,

k_{1r} Reaction rate constant for cellulose to cellobiose (mL/mg*h)

E_{1bC} Bound concentration of EG/CBH on cellulose content in biomass ($mg_{protein}$/mL)

SR Substrate reactivity

C Cellulose concentration (mg/mL)

G_2 Cellobiose concentration (mg/mL)

K_{1IG2} Inhibition constant of cellobiose on enzymes for cellulose to cellobiose (mg/mL)

G Glucose concentration (mg/mL)

K_{1IG} Inhibition constant for glucose on enzymes for cellulose to cellobiose (mg/mL). (Zheng et al., 2009)

Substrate reactivity *SR* in Equation 2 can be calculated with Equation 3.

$$SR = \propto \frac{S}{S_0} \tag{3}$$

Where,

α Dimensionless constant

S Cellulose concentration at certain time (g/mL)

S_0 Initial cellulose concentration (g/mL). (Kadam et al., 2004)

β-glucosidase follows Equation 4 when cleaving cellobiose into glucose units.

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{r_2} 2\ C_6H_{12}O_6 \tag{4}$$

Where,

r_2 Rate catalyzed by β-glucosidase. (Scott et al., 2013)

The cellobiose to glucose reaction with competitive glucose inhibition can be described with Equation 5.

$$r_2 = \frac{k_{2r} E_{2fL} G_2}{K_{2M}\left(1 + \frac{G}{K_{2IG}}\right) + G_2} \quad (5)$$

Where,

k_{2r}	Reaction rate constant for cellobiose to glucose (mL/mg*h)
E_{2fL}	Concentration of free β-glucosidase enzyme in the solution when the lignin is contained in substrate ($mg_{protein}$/mL)
K_{2M}	Cellobiose saturation constant (mg/mL)
K_{2IG}	Inhibition constant of glucose on enzymes for cellobiose to glucose (mg/mL). (Zheng et al., 2009)

Straight hydrolysis route from cellulose to glucose by cellobiohydrolase or glycohydrolase follows Equation 6.

$$(C_6H_{10}O_5)_n + H_2O \xrightarrow{r_3} C_6H_{12}O_6 + (C_6H_{10}O_5)_{n-1} \quad (6)$$

Where,

r_3	Rate catalyzed by cellobiohydrolase or glycohydrolase. (Scott et al., 2013)

The cellulose to glucose reaction with competitive glucose and cellobiose inhibition follows Equation 7.

$$r_3 = \frac{k_{3r} E_{1bC} SRC}{1 + \frac{G_2}{K_{2IG_2}} + \frac{G}{K_{3IG}}} \quad (7)$$

Where,

k_{3r}	Reaction rate constant for cellulose to glucose (mL/mg*h)
K_{1IG2}	Inhibition constant for cellobiose on enzymes for cellulose to glucose (mg/mL)
K_{3IG}	Inhibition constants of glucose on enzymes for cellulose to glucose (mg/mL). (Zheng et al., 2009)

These reactions are simplified, and the relations of the enzymes and reaction rates are shown in Figure 7.

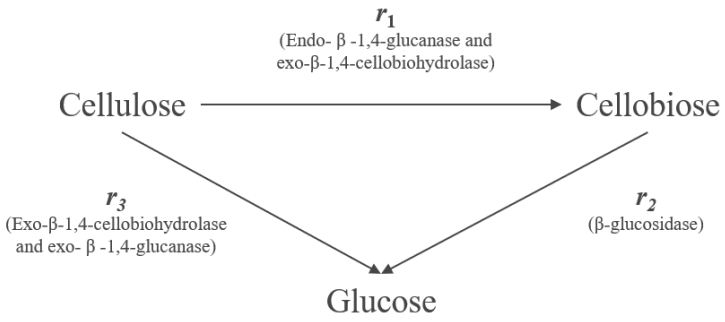

Figure 7. Simplified scheme of reactions and kinetics in enzymatic hydrolysis of cellulose (Kadam et al., 2004).

The adsorption of cellulase to cellulose can be described with Langmuir isotherm. It can be applied to cellulase by following Equation 8.

$$E_a = \frac{W_{max} K_P E_f}{1 + K_P E_f} \quad (8)$$

Where,

E_a Absorbed cellulase (mg or $\mu mol_{cellulase}/L$)

W_{max} Maximum cellulase adsorption

E_f Free cellulase (mg or $\mu mol_{cellulase}/L$)

K_P Dissociation constant.

The maximum cellulase adsorption W_{max} in Equation 8 can be calculated following Equation 9.

$$W_{max} = A_{max} S \quad (9)$$

Where,

A_{max} Maximum cellulase adsorption per gram of glucose (mg or $\mu mol_{cellulase}/g_{cellulase}$).

The dissociation constant K_P in Equation 8 can be calculated according to Equation 10.

$$K_P = \frac{E_a}{E_f S} \tag{10}$$

The maximum cellulase adsorption dissociation constants can be found in the literature to fit the Langmuir isotherm. (Zhang et al., 2004)

4.3 Degree of polymerization

Degree of polymerization (DP) describes the number of the basic structural units in the chain (Dimzon et al., 2012). In the reaction the distribution of the degree of polymerization is meaningful. The DP varies between raw materials in natural cellulose (Hallac et al., 2011). The degree of polymerization depends on the operation conditions, reactors and polymerization methods (Li, 2017). Also, with different measurement methods can give different DP results. The degree of polymerization of cellulose is usually already decreased during the pretreatment. Although, different pretreatment methods tend to have different affects to the DP. This affects to the cellulase since the number of available chain ends are increased. (Hallac et al., 2011) After the pretreatment there is usually soluble and insoluble cellulose chain present in the biomass. Insoluble cellulose usually has DP between 100-20 000 and soluble cellulose between 2-12. (Karimi et al., 2016) With different DP concentrations in the end product the successfulness of enzymatic hydrolysis can be determined. The monomeric sugars had DP of 1 which is the wanted result in enzymatic hydrolysis. If there is a higher amounts of other DP sugars such as oligomers the hydrolysis is not successful. Thus, the conversion and yield are lower than wanted.

4.4 Immobilized enzymes

In enzymatic hydrolysis enzymes can be fed to the reactor in two ways. They can be freely in the fluid or immobilized such as attaching them on a surface. In theory immobilized enzymes can be recovered and reused in high rate which decrease the consumption of enzymes. (Ingle et al., 2017) Free enzymes are more difficult to recycle than immobilized enzymes.

Enzymes are considered to be expensive and immobilized enzymes can be a solution for the recovery problems (Ingle et al., 2017). Free enzymes can at this state only be recovered with expensive and high maintenance membrane solutions (Pino et al., 2018). Immobilized enzymes are easier to separate from the hydrolyzed biomass due to the size or other properties of the carrier materials. The separated enzymes can then be recycled back to the process. Although, enzyme activity decreases in time the enzymes have been studied to retain their activity at relatively high levels after multiple hydrolysis cycles. (Ingle et al., 2017) In Cherian et al. study the enzyme retained 60 % of the original activity after five cycles (Cherian et al., 2015). Other benefits of having enzymes immobilized are that they are easier and more stable to storage (Ingle et al., 2017). In Sánchez-Ramírez et al. study the immobilization of the enzyme affected to the stability of the enzyme in storage. Free enzyme lost 70% of its activity in 28 days while immobilized enzyme lost 45% in the same time and conditions. (Sánchez-Ramírez et al., 2017) The activity loss can vary between different cocktails

In Ingle et al. study the cellulase enzymes were immobilized onto metal nanoparticles which were iron oxide nanoparticles. The carrier material was chosen by its magnetic properties to achieve easier recovery of the particles. The recovery of magnetic carriers can be done with magnetic fields. (Ingle et al., 2017) Also, other materials can be magnetized. For instance in Miao et al. study magnetic chitosan carrier was synthetized by anchoring Fe_3O_4 onto the chitosan (Miao et al., 2016). In Ingle et al. study sugar cane bagasse was hydrolyzed with immobilized enzymes. With free enzymes the conversion of cellulose to glucose was 78 % and with immobilized enzymes it was 72 %. (Ingle et al., 2017) The slight decrease of conversion can be seen with immobilized enzymes. In Cherian et al. study the enzymes were immobilized onto MnO_2 nanoparticles and the raw material was powdered sugar cane leaves. The immobilized enzymes were detected to have increased optimum pH and temperature

compared to the free enzymes. As a result the immobilized enzymes produced reducing sugars 20 g/L and free enzymes 24,29 g/L. (Cherian et al., 2015) Reducing sugar is a carbohydrate, such as glucose, that can act as reducing agent. (Nelson et al, 2013) Thus, free enzymes reached to higher conversion.

All though, immobilized enzymes have better recyclability they still have disadvantages. As shown in the results of the studies the conversions might be slightly lower than with free enzymes. Nevertheless, the magnetic nanoparticles have solved the problem of capturing the carriers from the flows without screening or filtering technologies. Although, in few cases there has been issues with the durability of the carrier materials. For example, silica coated magnetic nanoparticles can break under abrasion and the enzyme can flow out of the reactor without the carrier. (Garcia-Galan et al., 2011) In addition, all of the enzymes cannot be immobilized onto the same surfaces due to the differences of their working principles (Morais Júnior et al., 2021).

4.5 Inhibition

Enzymes have inhibitory factors which can prevent or restrict their actions and activity. Inhibition can happen in two main ways. Either the inhibitor blocks the active site hence the substrate cannot attach to the enzyme, or the inhibitor affects to the enzyme structure so that the active site is no longer suitable for the substrate to attach. Inhibitory factors can occur already in the pretreatment or in the enzymatic hydrolysis. Inhibitors can be compounds or conditions. Inhibition affects to the sugar yield and the enzyme consumption.

End product inhibition is one type of inhibition that occurs in enzymatic hydrolysis. Enzymatic hydrolysis is usually inhibited by sugars produced in the degradation of the polymers. Usually the inhibiting sugars are glucose, cellobiose, and xylose, but there can be for example galactose and arabinose also inhibiting the enzymes (Kadam et al., 2004). Cellobiose and glucose are considered to be strong inhibitors to cellulase and β-glucosidase. (Jing et al., 2009) They inhibit the enzymes by binding onto the active site leaving the enzyme unable to catalyze other chains (Pino et al., 2018). In Qing et al study was found that xylan, xylose, and xylooligomers also have a negative impact on the hydrolysis rate. The

most affective inhibitor of these was xylooligomer. (Qing et al., 2010) Although, according to Andrić et al. the product inhibition is rarely so severe that it causes drastic changes in the process (Andrić et al., 2010). The end product inhibition usually affects to the hydrolysis rate by decreasing it. End product inhibition affects to the reaction rates in enzymatic hydrolysis of cellulose is presented in Figure 8.

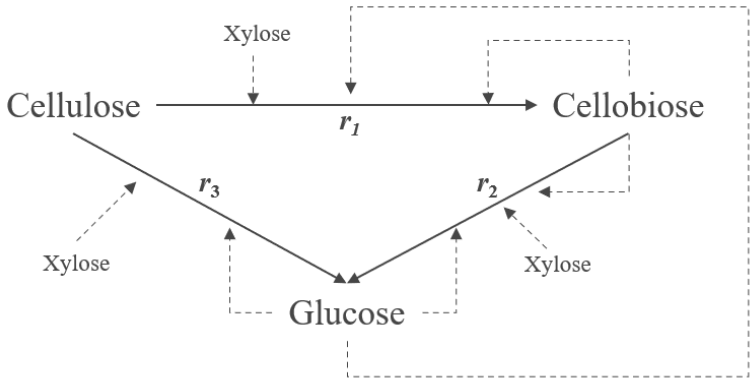

Figure 8. Reaction scheme for end product inhibition in enzymatic hydrolysis of cellulose. The inhibition pathways are presented with dashed arrows. (Kadam et al., 2004)

Different inhibitions can be detected from product formation during the process. Classical inhibition and end-product inhibition can be detected usually from the conversions since they behave differently. Classical inhibition is linear through the process or from the point the inhibition starts. End-product inhibition on the other hand increases the inhibition rate after a certain point and the reaction rate is decreased highly in the end of the process. The differences of product inhibition and classical inhibition affects to the product formation rate and yield are presented in Figure 9.

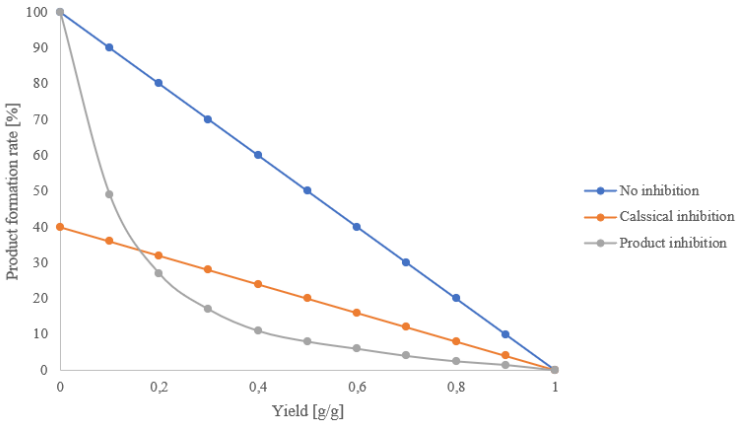

Figure 9. Product inhibition affect to yield, and product formation rate compared to classical inhibition, and no inhibition cases (Andrić et al., 2010).

As already mentioned sugar degradation products furfural, levulinic acid and 5-HMF have inhibitory affects to enzymatic hydrolysis (Zhou et al., 2021). In addition, there can occur degradation products such as furoic acid, acetic acid, and formic acid. (Jing et al., 2009; Klinke et al., 2004) Acetic acid is formed in hemicellulose degradation. Hemicellulose is linked to lignin with acetyl group which forms acetic acid in the degradation. Oxidation of D-glucose and D-mannose forms levulinic acid and oxidation of xylose produces formic acid. (Jing et al., 2009) Also, xylooligomers and glucooligomers are known to inhibit CBH. The oligomers are usually formed already in the pretreatment step and higher oligosaccharide accumulation is detected in high solid loading hydrolysis. The mechanism is yet unknown but in one theory it is presented that the oligomers mimic cellulose chain. This causes them binding more enzymes than cellobiose. (Xue et al., 2015) In Feng et al. study was detected that formic acid decreases the conversion rate and restricts the cellulase activity. With 0,5 g/L formic acid present in the hydrolysis the conversion rate decreased 19 %. In the same study was noticed that acetic acid did not affect to the hydrolysis rate by itself. Although, when it was added with formic acid a significant difference occurred. (Feng et al., 2012)

Lignin also acts as an inhibitor by binding to the enzyme. It also forms many inhibitory products in its degradation. Thus, lignin degradation is not wanted reaction in enzymatic hydrolysis. When lignin is solubilized, and cleaved phenols are formed. The main phenols that are formed in the lignin degradation are 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde, and syringic

acid. The formation of phenols occurs more in oxidative acidic conditions. (Klinke et al., 2004) The formation of phenolic groups depends on the raw material, pretreatment method, and the solid loading. Increasing the solid loading increases the phenol formation. (Qin et al., 2016) In Tejirian et al. study was found that oligomeric phenols are a major inhibitor for cellulase activity. Oligomeric phenols appeared to be more inhibitory than monomeric phenols. (Tejirian et al, 2011) In Ximenes et al. study the phenols were considered to be the most affective inhibitors to cellulase, β-glucosidase, and hemicellulolytic enzymes. (Ximenes et al., 2010). In Qin et al. study vanillin inhibition was detected to correlate to the cellulose concentration. Higher cellulose concentration caused lower vanillin inhibition. (Qin et al., 2016) On the other hand, inhibition mechanisms of phenolic compounds and acids are unknown and the results in the studies are diverse (Jing et al., 2009). In addition, in acid catalyzed pretreatment pseudo lignin particles or humins can be formed (Hu et al., 2012; Liu et al., 2022). These particles have sphere structure. The mechanics of pseudo lignin formation is yet unknown but sugar degradations products such as 5-HMF and furfural are believed to take a role in it. The pseudo lignin molecules act like lignin in enzymatic hydrolysis and bind irreversibly the enzymes. (Hu et al., 2012) The formation of by-products such as humins are affected by the reaction conditions. In acidic pretreatments the harsher conditions and longer reaction time create by-products with higher degree of polymerization. In too harsh acid pretreatment coke or hydrothermal carbon (HTC) can be formed. (Liu et al., 2022) In long run and especially in continuous operating mode coke can glogg pipes and coat surfaces. Thus, pretreatment needs to be optimized to not produce excessive amounts humins. In Table 6 is presented the degradation products of lignin and polysaccharides that are formed in acid pretreatment such as DAH or CAH which might have inhibitory properties.

Table 5. Degradation products of cellulose, hemicellulose, and lignin in acid pretreatment (Agrawal et al., 2021).

Lignin		Cellulose and Hemicellulose		
Phenolics	**Non-phenolic Aromatics**	**Acids**	**Aldehydes**	**Sugars**
Coniferyl aldehyde	Benzoic acid	Acetic acid	Furfural	Xylooligomers
Vanillin	Cinnamic acid	Formic acid	Hydroxymethyl	Glucooligomers
Ferulic acid		Levulinic acid		Cellobiose
4-hydroxy benzoic acid				

Inhibition effects of formic acid, acetic acid, furfural, and vanillin to the enzymatic hydrolysis with different concentrations are presented in Figure 10. In these inhibition tests the substrate loading was 6 % (w/v) and enzyme loading 15 FPU/$g_{substrate}$. The retention time was 96 h and temperature in the lab scale reactor 43 ˚C. (Feng et al., 2012) Unlike in Qin et al. study the vanillin in these concentration does not show significant inhibition which would effect to the conversion severely.

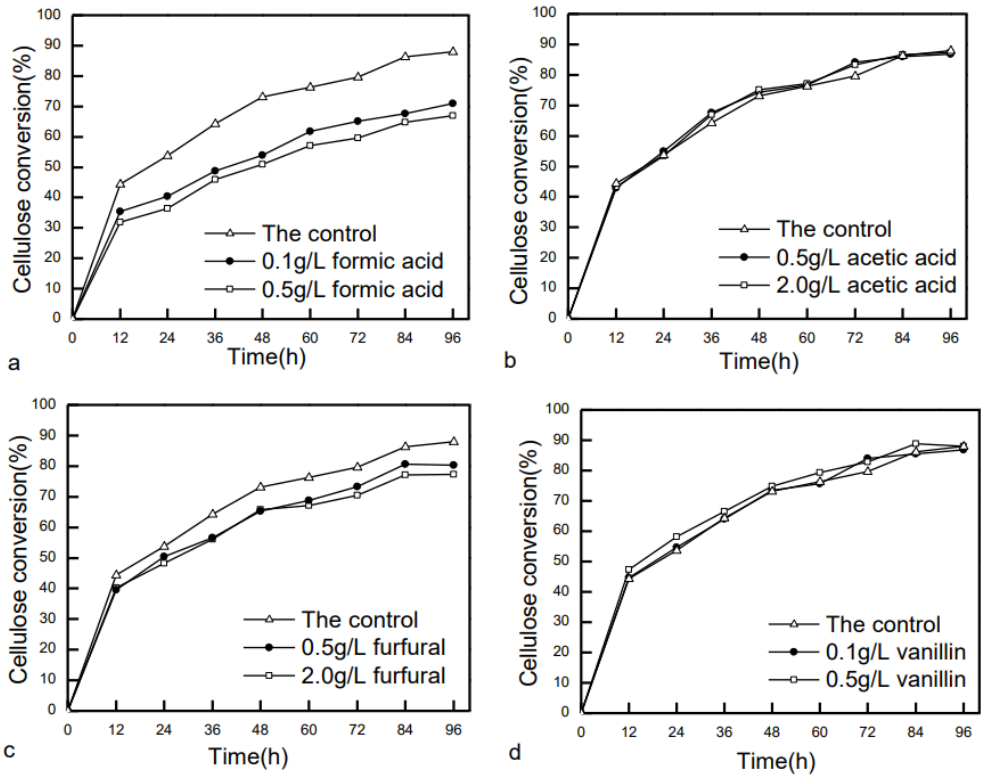

Figure 10. Inhibition effects of formic acid, acetic acid, and vanillin to the enzymatic hydrolysis of SE pretreated Lespedeza stalks (Feng et al., 2012).

In addition of the inhibiting compounds an inhibitor can be an operation condition such as temperature, pH, or solids loading. These are very important parameters for enzyme activity. In too low temperatures the enzyme activity decreases, and degradation rate is slow or almost zero. In too high temperatures the enzyme structure can denature which will irreversibly inhibit the enzyme. When the three-dimensional structure denatures the active site no longer matches the substrate structure. pH can have similar affect to the enzyme structure and activity, but it also might have an effect to the substrate structure. pH can also affect to a microorganism growth by creating better growing conditions to them which can cause inhibition through contamination. Optimal temperature and pH are different for each enzyme or enzyme cocktail. In addition, solids loading affects to the enzyme activity. With too high or too low substrate concentration enzymes cannot find the substrates efficiently. Also, other solids than the specific substrates hinder the enzyme activity. In addition, the contaminants usually inhibit enzymes. The inhibiting contaminants are presented in the chapter 4.8.

4.6 Additives in Enzymatic Hydrolysis

Additives can be used in enzymatic hydrolysis to increase the conversion. Additives can contribute to the conversion by decreasing possible inhibition and increasing the enzyme activity in the hydrolysis. In this chapter is studied additives that can be used in enzymatic hydrolysis.

One possible form of additive for enzymatic hydrolysis is proteins. In Smit et al. study was found that when protein containing extract is added in the enzymatic hydrolysis the enzyme dosage can be decreased. The biomass used in this study was straw and grass. It is assumed that the proteins are adsorbed to the lignin. Thus, lignin cannot attach to the enzymes which decreases the enzyme inhibition by lignin. With herbaceous biomass the protein containing extract can be prepared simply by washing the biomass with water and filtering the mixture. The extraction of protein only applies to the herbaceous biomass since for example woody biomass has no washable proteins. (Smit et al., 2014) With woody biomass the protein extract needs to be prepared in other ways. In Luo et al. the same phenomenon was studied with soy protein. The soy protein (SP) was added to the enzymatic hydrolysis of bamboo, eucalyptus, and pine. With Novozymes Celluclast 1,5L the conversion of the three raw

materials increased from 57 %, 54 % and 45 % to 87 %, 94 % and 86 % with addition 80 mg SP/g glucan. The enzyme loading was 24 mg enzyme/ g glucan and reaction time 72 hours. Also with 12,1 mg protein/g glucan enzyme dosage the conversions increased for eucalyptus and pine from 48 % and 41 % to 86 % and 79 % (Luo et al., 2019) Gong et al. has reached in their studies to similar results as Luo et al. with peanut protein (Gong et al., 2021). Results from enzymatic hydrolysis with soy protein addition (80mg SP/g glucan) with different raw materials and different enzyme dosages are presented in Table 7.

Table 6. Enzymatic hydrolysis conversions with soy protein addition of 80 mg SP/g glucan and hydrolysis time of 72 h (Luo et al., 2019).

Raw material	**Enzyme dosage (mg protein/ g glucan)**	**EC without SP (%)**	**EC with SP (%)**
Bamboo	24	57	87
Eucalyptus	24	54	94
Masson pine	24	45	86
Eucalyptus	12,1	48	86
Masson pine	12,1	41	79

Polymeric additives are another group of additives used in enzymatic hydrolysis. In Rocha-Martín et al. study was studied the effect of PEG4000 as an additive in enzymatic hydrolysis for corn stover, sugar cane straw, and Avicel raw materials. The enzymatic hydrolysis was done with 20 % of total solids and 72 h hydrolysis time and 4 mg protein/g glucan enzyme loading. PEG4000 can bind onto the lignin present in the hydrolysis and prevent lignin inhibition similarly as the earlier mentioned proteins. Thus, better yield and conversion can be reached in the process. It was noticed that addition of 5 g/l of PEG4000 the liquefaction time could be decreased up to 25 %. With Avicel, corns stover, and sugar cane straw the concentration of glucose was increased by 32 %, 6 %, and 9 % after 72h. The Avicel raw material proves that lignin inhibition reduction is not the only phenomenon occurring with PEG4000. It was also discovered that PEG4000 increased β-glucosidase and endoglucanase activity by 20 % and 60 % which increases the sugar yield. PEG4000 did not show any effect on the thermal stability on thermoresistance in the enzyme cocktails. In addition, surfactants have been studied to be enhancing enzymatic hydrolysis of biomass. Surfactants can

decrease surface tension and modify the biomass structure. In enzymatic hydrolysis mainly non-ionic surfactants are used which decrease the lignin inhibition. (Rocha-Martín et al., 2017) How different surfactants and additives affect to the hydrolysis are presented in Table 8.

Table 7. Additives used in different enzymatic hydrolysis with different raw materials and pretreatments to enhance the glucan conversion. (DAH= Dilute acid hydrolysis, SE= steam explosion, BSA=Bovine serum albumin, SMP =Skimmed milk powder)

Biomass (pretreatment)	Enzyme Dosage (Enzyme)	Solids Loading (%)	Hydrolysis Time (h)	Conversion without/with Additive (%) (Additive & Dosage)	Reference
Wheat straw (DAH)	10 FPU/g (Cellic CTec2)	5	120	79,8/82,6 (Tween 20 5,0g/L)	(Chen et al., 2018)
Poplar pulp (SE)	5,625 FPU/g (Cellic CTec2)	5	96	40/57,5 (TritonX-100 5 %)	(Alhammad et al., 2018)
Bamboo residue (DAH)	20 FPU/g_{glucan} (Cellic CTec2)	5	72	29,4/64,6 (PEG 4000 0,3 g/g_{lignin})	(Huang et al., 2022)
Bamboo residue (DAH)	20 FPU/g_{glucan} (Cellic CTec2)	5	72	29,4/61,6 (Tween 80 0,3 g/g_{lignin})	(Huang et al., 2022)
Wheat straw (DAH)	20 FPU/g (cellulase T.reesei)	10	72	67,4/71,4 (BSA 1 %/dry pulp)	(Vergara et al., 2021)
Wheat straw (DAH)	20 FPU/g (cellulase T.reesei)	10	72	67,4/75,7 (BSA 10 %/dry pulp)	(Vergara et al., 2021)
Wheat straw (DAH)	20 FPU/g (cellulase T.reesei)	10	72	67,4/73 (SMP 3 %/dry pulp)	(Vergara et al., 2021)

When additives are used in enzymatic hydrolysis the conversion improvement needs to be evaluated carefully. In addition, the effect to the purity of the end product needs to be considered. After the enzymatic hydrolysis the liquid and solid phases are separated, and the main question is where the additives end up. Large particles such as proteins might end up in the solid phase which means it can decrease the lignin purity. Decreased purity in the end products might need an additional separation step which can affect to the operating costs of the process.

4.7 Economics of enzymes

Enzymatic hydrolysis is often described as expensive process especially when it is compared to acid catalyzed hydrolysis. In 2010 Tolan et al. considered the cellulase costs to be over 50 % of the hydrolysis costs (Tolan et al., 2010). To the economics of enzymatic hydrolysis affects the raw material, pretreatment, enzyme dosage, operation parameters, total dry matter content, and many other factors. Enzymes are still usually listed to be the limiting factor when designing enzymatic hydrolysis process. In addition, the investment costs of enzymatic hydrolysis can be high since the process is slow and the needed tank volume is high. The economic evaluation of enzyme cost has inconsistency in the studies. With the decrease in enzyme production costs over time, cost evaluations are changing, and enzymes are becoming a more attractive option as their production prices become competitive. Usually, the costs of enzymes are evaluated for the bioethanol production since platform sugar production is not yet done commercially. The biofuel productions can give an understanding of the enzyme costs in enzymatic hydrolysis since it is usually a part of the process in biofuel production.

Pretreatment can affect to the enzyme costs. Efficient pretreatment can lower the enzyme dosage which decrease the costs. Also, the total solid loading affects to the enzyme activity which can affect to the dosage and costs of enzymes. According to Humbird et al. the enzyme costs can be decreased by on-site enzyme production (Humbird et al., 2011). In 2011 In Davis et al. report was calculated enzyme costs for biological renewable diesel blendstock (RDB). This process consists of DAH pretreatment, enzymatic hydrolysis, hydrocarbon bioconversion, and hydrotreating to paraffins. The capital costs of the total installed equipment costs would be approximately 230,9 million euros. The enzymatic hydrolysis and bioconversion part covering approximately 55 million euro. The minimum fuel selling price of the product being 1,03 €/l. It was estimated that enzyme cost would be 7,5 cent/l. (Davis et al., 2013) In Boakye-Boaten et al. study when *Miscanthus x giganteus* was converted into ethanol with enzymatic hydrolysis and fermentation the enzyme costs were 15,7 % of the total costs. Which made it the second largest contributor. In this case the enzymes were produced on-site. The total costs covered feedstock, enzymes, wastewater treatment, pretreatment, enzymatic hydrolysis and fermentation, recovery, utilities, boiler/generator, and storage costs. (Boakye-Boaten et al., 2017)

MetGen has evaluated costs of different level of optimization of enzymes in Asian biorefinery project. In 2020 non tailored enzymes costed approximately 180 €/ tonnes of biomass and after optimization and with onsite production the evaluated cost was approximately 25 €/tonnes of biomass. (MetGen, 2023) The enzyme costs which MetGen evaluated are presented in Figure 11.

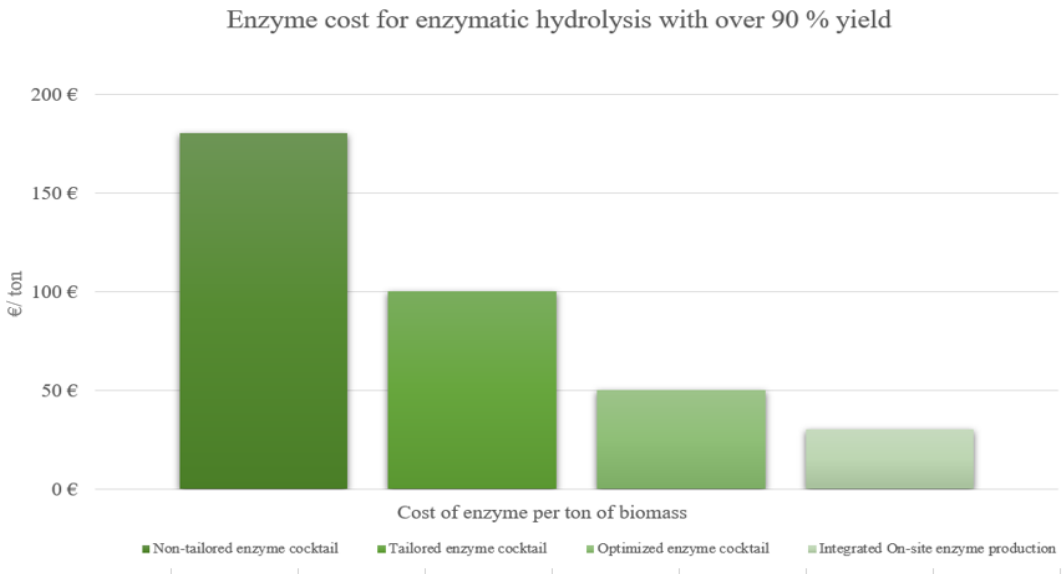

Figure 11. Enzyme cost development in Asian biorefinery project for enzymatic hydrolysis enzyme costs (MetGen, 2023).

4.8 Contamination

Mild process conditions are typically used in enzymatic hydrolysis to enhance enzyme activities, along with a long retention time in the reactor. These conditions do not only promote enzyme activities but also support the growth of other microorganisms, such as bacteria, yeast, and mold. These growing contaminants can usually consume the sugars and cause unwanted by-products which have an effect to the hydrolysis rate. (Retsina et al., 2021) The type of contamination can be basically anything. Usually, the contaminants coming with the raw materials are eliminated in the pretreatment. For example, steam explosion is very effective pretreatment method to remove all growing contaminants from the biomass. Therefore, the contamination that affects to the end product or hydrolysis rate is usually

introduced to the biomass after the pretreatment. The contamination can be introduced to the biomass from any surface such as containers, pipes, reactors, or pumps. Therefore, the most effective way to prevent contamination is careful cleaning and sterilization. In addition, air can contain contaminants which can be introduced to the biomass in the transportation, storage, or in the reaction tank air. For example, with filtering the air those air contaminants could be eliminated. Realistically, the contaminations cannot be avoided in industrial settings of enzymatic hydrolysis. The aim is to avoid severe contamination which affects to the hydrolysis rate or the purity of the products.

The contamination is usually detected from the conversion and some severe contaminations can be seen on top of the biomass such as mold. If the enzymatic hydrolysis is contaminated with bacteria, they usually produce acids such as lactic acid or acetic acid to the biomass. (Schell et al., 2007) To do so, bacteria consume sugars and metabolise acid compounds. One of the possible bacteria to contaminate the process is lactic acid bacteria (LAB). Some LAB can produce ethanol, glycerol, and carbon dioxide. (Muthaiyan et al., 2011) From these for example ethanol is known to inhibit cellulase activity which can be detected from the conversion. In Wu et al. study was detected that for each 10 g/l of added ethanol the enzymatic digestibility decreased approximately 7 %. It was also detected that in higher temperatures such as 50 ˚C the decline was higher compared to lower temperatures such as 38 ˚C. (Wu et al., 1997) LAB such as *Lactobacillus* sp. has been detected to significantly decrease sugar yield (Serate et al., 2015). In Muthaiyan et al. study the *Lactobacillus* was detected to also consume arabinose. In Iyer et al study was detected that over 90 g/l of lactic acid present in the hydrolysis can decrease over 50 % of the conversion compared to reference (Iyer et al, 1999). Acetic acid bacteria are also possible contaminants in the enzymatic hydrolysis due to its capability to metabolize glucose to acetic acid. (Muthaiyan et al., 2011)

4.9 Enzymatic hydrolysis process configurations

Enzymatic hydrolysis can be done in continuous or batch process. In the process the reactor type and number can change. It can be done in one or multiple steps to achieve the wanted conversion. Traditionally enzymatic hydrolysis is done in a simple continuously stirred batch

reactor. Besides normal continuously stirred tank there are other tank options like paddle blender reactor. The paddle blender tanks can be also used as prehydrolysis tanks for high solid loads. (McMillian, 2021)

The potential benefits of continuous operation are extensively researched in enzymatic hydrolysis to improve the process. Continuous operation can lead to higher product yield when compared to the tank volume or hydrolysis time. A smaller tank volume is needed with the same reaction time in continuous operation, or longer hydrolysis time in the same tank volume can increase conversion and thus yield. Cleaning time can also be reduced since the reactor does not have to be cleaned after each batch as in batch processing. Thus, water consumption is also decreased. Product inhibition is decreased due to continuous outflow of the product, which also improves process stability. In batch processing, there may be variation in the quality between batches, unlike in continuous operation the quality variations are less likely to be that notable. Fresh feed also improves the hydrolysis rate bringing new chains to the enzymes to degrade.

In continuous enzymatic hydrolysis there are a few problems to overcome. From the economical point of view the hydrolysis needs to be done in high solids loading with high conversion. The high solids loading increases the viscosity of the slurry which can cause pumpability and stirring problems. In addition, the risk of unhydrolyzed material flowing out of the reactor needs to be considered in the process configuration. Different process configurations for continuous and semi continuous enzymatic hydrolysis processes found in the literature are presented in this chapter.

4.9.1 Fed batch process

Fed batch process is started with dilute suspension of substrates. During the operation the substrates are fed to the reactor and the solids content is increased. (Chen et al., 2017) The feed of fresh biomass is done when the viscosity has decreased due to the hydrolysis. One of the main problems in enzymatic hydrolysis is that the solid concentration needs to be relatively high. Usually, enzymatic hydrolysis is done in 5-8% solids concentration to ensure enzyme activity. Although, it is suggested that it should be done in higher solid loadings to

be a cost-effective process. The lower solids concentrations are usually preferred due to the decreased enzyme action in high solid loads. Decreased enzyme activity decreases the yield again affecting to the cost-effectiveness. (González Quiroga et al., 2015)

One solution for that is the fed-batch process. (González Quiroga et al., 2015) Fed batch operation overcomes the high viscosity problem the process might have in the beginning. In fed batch systems the retention time is usually longer than in conventional batch process due to the addition of unhydrolyzed material. To overcome the longer retention time the enzyme dosage might be needed to increased which adds costs of the process. (Chen et al., 2017) Besides gradual feed of pretreated raw material the enzyme can be also added during the process and the enzyme composition can change during the feed (Smits et al., 2014). Nevertheless, fed batch process is not necessarily considered a fully continuous process since there are time when there is no feed or out flow.

4.9.2 Membrane bioreactor process

Enzymatic hydrolysis can be done in membrane bioreactor (MBR) which can be operated in batch, fed batch, or continuous modes. MBRs are combination reactors combining biological reaction and membrane separation unit. The membrane can be integrated into the biological reaction tank, or it can be a separate unit with recycling of the reaction slurry. The substrate concentrations as a function of time depends on the operation mode and it is presented in the Figure 12. (Pino et al., 2018)

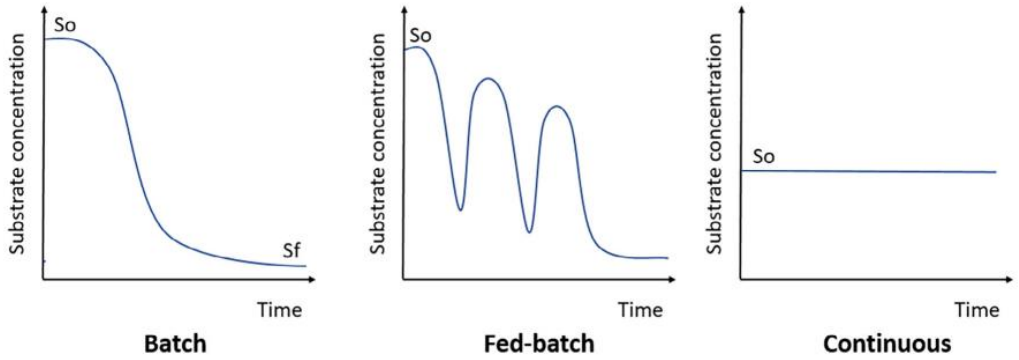

Figure 12. Substrate concentration as a function of time in batch, fed batch, and continuous MBR processes (Pino et al., 2018).

The advantages of MBR in enzymatic hydrolysis are that it can reduce the product inhibition. In MBR glucose passes through the membrane and therefore is removed from the area where enzymes are active. In MBR the enzyme use can also be optimized. With right membrane selection majority of the enzymes can be separated from glucose leaving them into the reactor. In MBR the immobilization of enzymes is also possible. The more effective use of enzymes in MBR compared to the conventional batch reduces the enzyme costs. (Pino et al., 2018) According to Sueb et al. the cost of membrane technology can be cheaper compared to chromatographic purification (Sueb et al., 2017).

In Ohlson et al. study was found that removing the glucose from reactor with UF membrane could increase the enzyme activity which was detected through the sugar yields. On the other hand, the reduced sugars were in very dilute form and the process could be considered a semi-continuous since the dry content matter was kept constant (10 % wt) by feeding the substrate. Also, the permeate was continuously replaced with buffer solution during the process. (Ohlson et al., 1984) In Adhikari et al. experiments the continuous MBR was also tested with ultrafiltration membrane module, but clogging was a major problem (Adhikari et al., 2014).

Compared to conventional reactors MBRs have a few disadvantages. Membranes are expensive and their fouling is a significant phenomenon which needs to be considered especially in continuous operation. Enzymes in enzymatic hydrolysis are usually in the same size range as the membrane pores and are considered as a possible fouling cause. Membrane changes or cleaning adds operating costs to the process. Also, in membrane selection the

shear stress the membrane might cause to the enzymes need to be studied to avoid enzyme activity loss. (Pino et al., 2018) The fouling of membrane affects to the purification and reaction kinetics, which might be important factor considering the viability of the process. (Sueb et al., 2017)

In National Renewable Energy Laboratory project McMillian has presented a possible design for continuous enzymatic hydrolysis. The design has three reactors which have their own microfiltration units. (McMillian, 2021) The process configuration is presented in Figure 13.

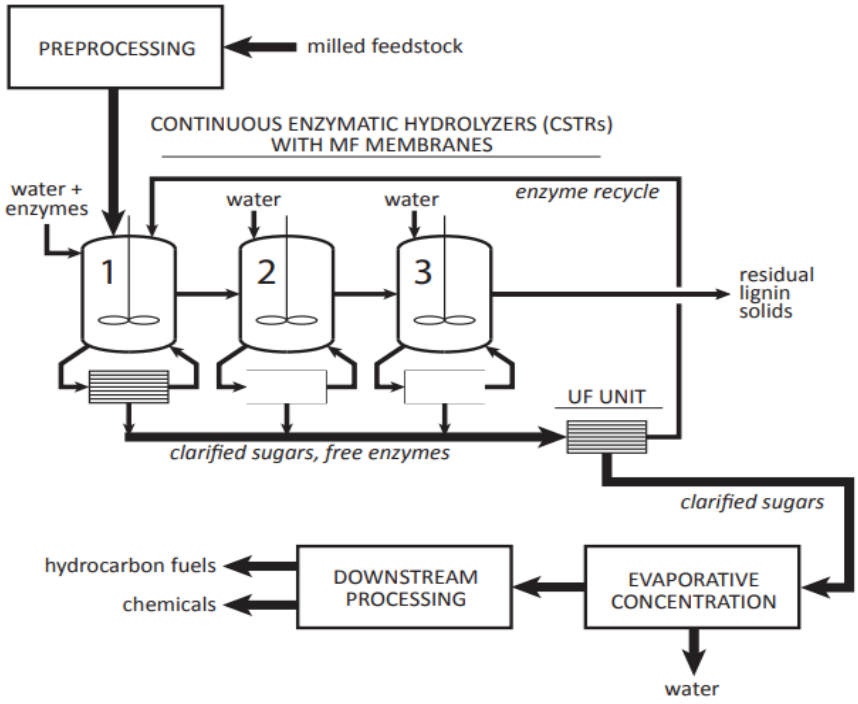

Figure 13. Schematic figure of process configuration of McMillian continuous enzymatic hydrolysis process (McMillian, 2021).

From the Figure 13 can be seen that to the first reactor is fed milled and pretreated feedstock with water and enzymes. The clarified sugars and free enzymes are separated with ultrafiltration and the lignin solids are recycled back to the first and second reactor. To the second reactor is also added water. From the second tank is separated the sugars and free enzymes while the lignin solids are recycled to second and third tank. To the third tank is added water. From the third tank is yet again separated the sugars and free enzymes and the

rest lignin residual is recycled back to the first tank or removed from the process. After the tanks own separation units there is ultrafiltration unit which separates the sugars and enzymes. Enzymes are recycled back to the first tank. Sugars are then concentrated and refined into products. The reactors are designed to operate at insoluble solids levels higher than 10 wt%. (McMillian, 2021) In this process is assumed that the enzymes end up in the liquid phase. Usually, in other processes it is assumed that the enzymes do not pass the membrane. To be noted this design is not in use yet. This process is still under research and has not been able to achieve the production targets wanted yet (Davis et al., 2022). In small scale mini-pilot systems with single-stage operation the sugar conversion has been 50 % with DAH pretreated feedstock with 10 % insoluble solids concentration. Also, 100 % enzyme recovery was achieved. (McMillian, 2021)

4.9.3 Continuous reactors in cascade

In Sjoede et al. patent is presented a continuous enzymatic hydrolysis for cellulosic biomass pulp. The biomass in pretreated and the preferred pretreatment is sulfite cooking. After the pretreatment the pulp contains at least 70 % of cellulose. The formed sulfonated lignin is separated into the liquid phase after the pretreatment. In the invention there is at least 2 reactors in cascade and the total solids content is preferably over 10 %. In the invention cellulosic biomass in added continuously into the process and hydrolyzed biomass in continuously removed from the process. In the Sjoede et al. invention is presented several different process configurations for the continuous enzymatic hydrolysis. These configurations are presented in the Figure 14. In the Figure 14 configuration A the reaction time is 16 hours in each reactor and 48 hours in total with three reactors. The preferred reaction time is the same as what is needed in batch reactor. The reaction time in first reactor is designed considering the pumpability of the removed slurry. Due to the pumpability issues the hydrolysis time needs to be long enough to decrease the viscosity. In the configuration B is added enzyme recycling to minimize the enzyme costs. The enzyme consumption can be decreased at least 40 % with this configuration. The solid liquid separation after the last tank separates the solid phase and liquid phase from the hydrolyzed biomass. The liquid

phase containing the enzymes is recycled back to the mixing tank where it is mixed into fresh unhydrolyzed feed. This configuration also decreases the end-product inhibition caused by glucose. In the configuration C is solid liquid separation after every hydrolysis tank. In this configuration approximately 60 % glucose conversion is reached in the first tank. The remaining unhydrolyzed 40 % is pumped to the second reactor. To the second reactor is added new enzymes as well as recovered enzymes from the first tank. Also, the reactor size is optimized to decrease as the unhydrolyzed material decreases. (Sjoede et al., 2015)

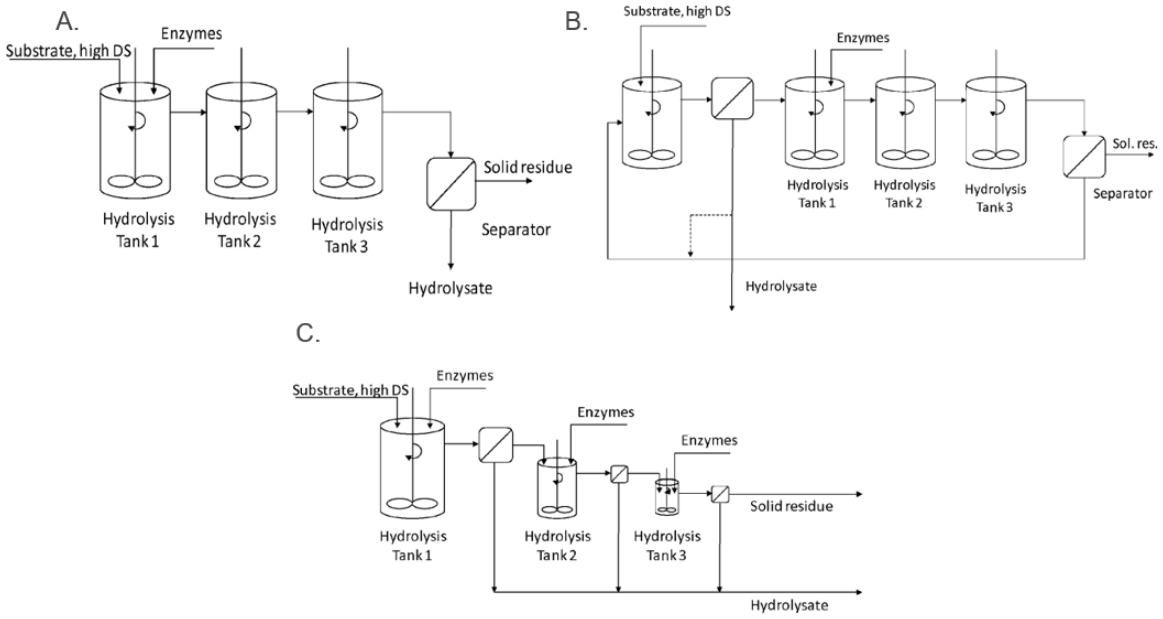

Figure 14. Figures of the process configurations. A. basic process configuration, B. added mixing tank with separation unit, C. added separation units to tanks with decreasing tank size. (Sjoede et al., 2015).

In Sjoede et al. example three reactors were set into cascade. The total volume of the reactors was 6,6 liters. The used raw material was bagasse pulp and temperature in the process was kept at 50 ˚C. The feed to the system was 144,1 g/h and the dry substrate concentration in the feed was 18,8 %. Enzyme load of Accelerase DUET enzyme was 0,173 g /$g_{dry\ substrate}$. The retention time in the three reactors in total was 46 hours. After 4-5 days the process

reached a steady state and the conversion in glucose outputs from each tank were 39,8%, 55% and 58,8%. (Sjoede et al., 2015)

Gonzales Quiroga et al. study was done a modelling with distributed feeding into a series of CSTRs. The distributed feeding was fed to the two or three first reactors. As a conclusion distributed feeding did not give an advantage in the conversion compared to the feeding only to the first reactor. Although, it can increase the substrate concentration in the process. (González Quiroga et al., 2010)

In Shin et al. invention is presented a continuous saccharification device for marine algae and biomass. The biomass can be pretreated if it is needed to increase the yield. In the invention the hydrolysis is done in a reactor or reactors in which high-pressure steam can be sprayed in. The pressure can be maintained in the reactor while feeding the fresh feed. In the invention is described multiple different configurations for the process apparatus which are presented in Figure 15. (Shin et al., 2016)

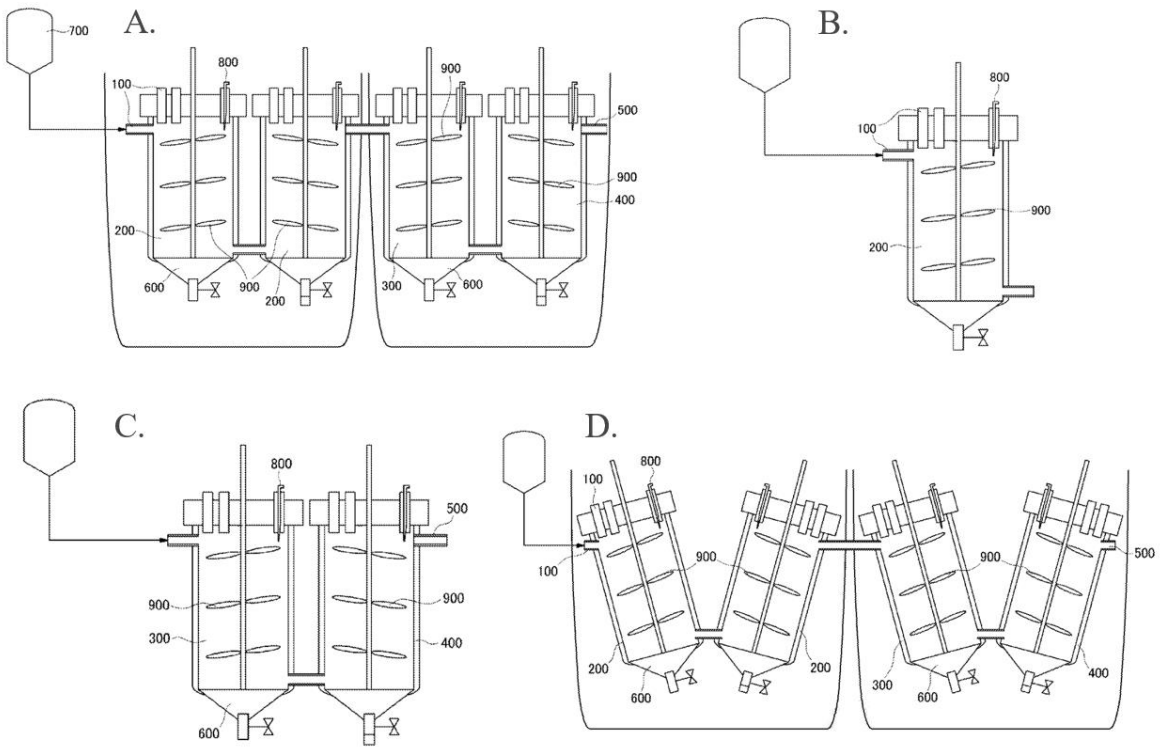

Figure 15. Continuous saccharification process configuration invented for marine algae and biomass in which 100: source input unit, 200: reactor, 300: downward reactor, 400: upward reactor, 500: reactant liquid discharge unit, 600: deposit removal unit, 700: cushion tank, 800: pressure control device, and 900: impeller (Shin et al., 2016).

In configuration B a simple reactor with top feed and bottom outlet is presented. The impellers can be adjusted in different slopes. In configuration C downward and upward reactors are joined to form a parallel unit. In configuration A parallel downward and upward reactors in which the impellers can also be adjusted in slope are presented. In configuration D the parallel downward and upward reactors are tilted. The temperature, retention time and pressure in each reactor are adjusted separately. (Shin et al., 2016)

Experimental part

The aim of this research is to study if the continuous enzymatic hydrolysis of biomass would reach the same conversion as in the batch process which is considered as reference in this study. By changing the batch process into a continuous process, the aim is to improve runnability, enhance the use of tank volume, and prevent contamination better. With continuous process the water consumption can be reduced since the tanks are more or less full all times unlike in batch process. In batch process the tanks must be cleaned and disinfected after each batch which consumes more water and time compared to continuous process where cleaning sequences can be reduced. With less water consumption the wastewater treatment capacity can also be reduced. With continuous process the contamination microorganisms have less or no time to grow when the biomass is changing all the time. The steady flow to the next process steps can ease for example solid liquid separation. In this experimental part is studied a pilot scale one step continuous enzymatic hydrolysis of biomass.

5 Materials and methods

The materials used in the experiments along with the trial set up and laboratory analysis are presented in this chapter.

5.1 Raw materials

Pretreated beech biomass slurry was used as raw material in the continuous enzymatic hydrolysis. Prior to the enzymatic hydrolysis, the pretreatments of hemihydrolysis, steam explosion, and washing were conducted. Through effective washing, all soluble sugars were removed from the slurry before the enzymatic hydrolysis. The raw material contained 66 % carbohydrates, 36 % lignin, and 0,85 % protein. The commercial enzyme manufacturer

supplied the optimized enzyme cocktail, which was in a liquid solution form. The dry matter content and pH of the slurry were adjusted before the trials to meet the requirements set by the enzyme manufacturer.

5.2 Trial set up

The trials were performed in pilot scale. The trial set up included feed container, reactor, product container, enzyme container, feed pump, and enzyme pump. The feed container had continuous mixing to prevent solid liquid separation of the feed slurry. The enzyme was mixed to the fresh feed by feeding it to the feed line with a small hose pump. The mixture of fresh feed and enzyme was fed to the enzymatic hydrolysis reactor with a screw pump. The mixture was fed to the bottom of the reactor through a valve which was located on the side of the reactor. The hydrolyzed material was flowing out of the reactor through an open valve which was located in the reactor wall, so there was no pump in the outlet line. Thus, when the fresh feed enters to the reactor the surface rises and same amount of slurry flows out of the reactor through the open valve. The set up was easier to control when the slurry was fed to the bottom since there was only one pump in the system. The process configuration is presented in Figure 16.

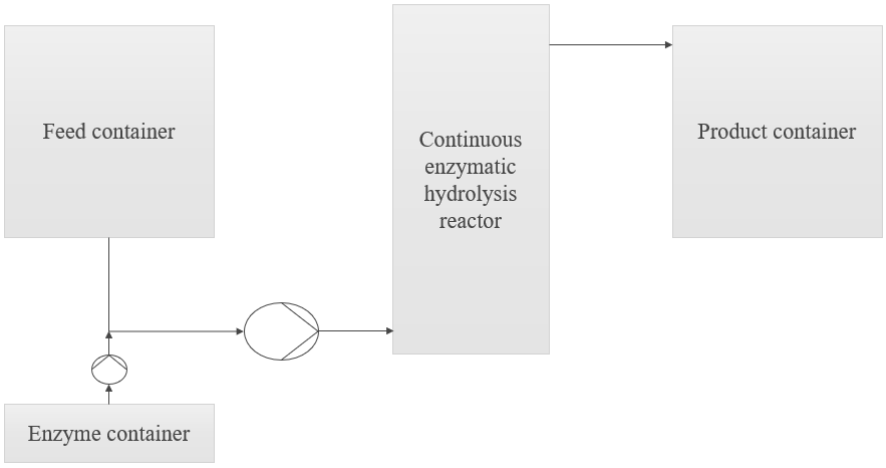

Figure 16. Schematic figure of the process configuration.

The continuous enzymatic hydrolysis was done in continuously stirred tank reactor. The design of the mixer was three pitch blades on a rotating shaft. The pitch blades create turbulent mixing in horizontal and vertical directions in the reactor. The reactor had water filled jacket for heating. The total hight of the reactor was 200 cm and the diameter 60 cm. The reactor had outlet flow valve on the side of it at 177,5 cm height. The reactor volume when the surface is approximately at 177,5 cm height is 502 litres. The inlet flow valve was at the lowest point possible on the side of the reactor. The reactor was covered on the top with aluminium foil to prevent evaporation of water and limit the contamination from above. Feeding to the bottom was selected since during hydrolysis the viscosity of the slurry decreases. The more viscose material, which in this case is fresh feed, tends to stay at the bottom of the reactor. Thus, it prevents unhydrolyzed material to flow out from the reactor. The biomass was mixed at constant speed at approximately 50 rpm. In addition, the feed contains some amount of shives which will also stay in the bottom of the reactor and create longer retention time for them. Thus, the degradation of the shives is increased and less shives are flown out of the reactor unhydrolyzed. This will also prevent the clogging of the outlet valve. Some of the biomasses required sieving before the enzymatic hydrolysis due to the high shives content. The continuous enzymatic hydrolysis reactor used in the trials is presented in Figure 17.

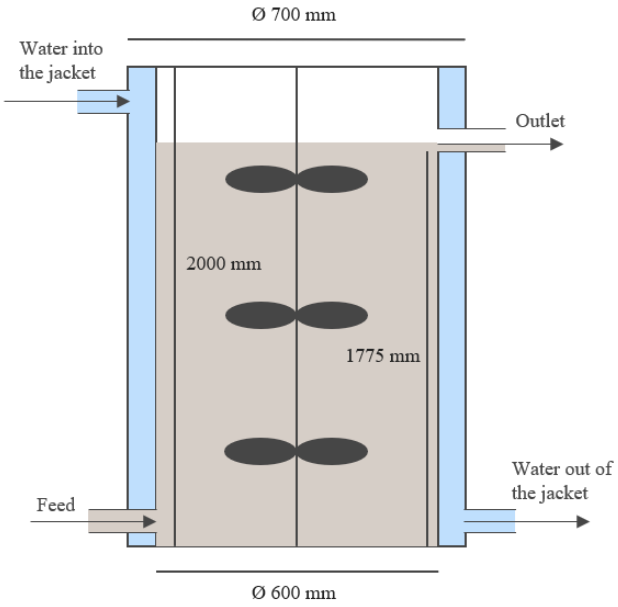

Figure 17. The continuous enzymatic hydrolysis reactor used in the trials.

With the same reactor a batch reference was conducted in which the continuous trials can be compared to. The volume in reference batch was 500 L and the valves were closed so there was no flow in or out of the reactor. Otherwise, the conditions remained the same as in continuous trials.

5.3 Operating parameters

The optimal hydrolysis conditions for temperature and pH were determined by the enzymes optimal activity range which were provided by the enzyme manufacturer. Temperature was kept between 50-55 ˚C and pH between 4,5-5,5 in which the enzyme activity is the highest. The pH in the feed container was adjusted to 5,5 to maintain the pH level in the reactor since the pH tends to decrease during enzymatic hydrolysis. The pH decreases mainly due to deacetylation of remaining acetylic groups in pulp slurry. The pH in feed container and reactor was adjusted with 25 % NaOH during the process if needed. The dry matter content was aimed to be 10 % in the slurry. The temperature was kept constant with the water filled jacket. The continuous enzymatic hydrolysis process was studied with different retention times. The retention time in the reactor was changed by adjusting the flow rate. The aim was

to maintain a constant dry matter content, enzyme dosage, pH, and temperature throughout the process.

5.4 Trial runs

The trial runs were conducted between 9/2022-4/2023. The trial set up had some major issues in the beginning of the trials and thus only three of the 19 conducted trials were successful and analysed. The three trials with their operating parameters are presented in Table 9.

Table 8. Trial run parameters.

Trial	**Flow (kg/h)**	**Reaction time (h)**	**Enzyme dosage (%)** (Enzyme solution/ Dry matter)	**Dry matter content (w/w %)**	**Batch time (h)**	**Total hydrolysis time (h)**
1	35	14,8	4,6	9	15,5	63
2	20	25,9	4,5	10	26	93
3	4,9	105,5	5	12	23,5	143,5

Every trial started with a batch operation mode to increase the conversion. Thus, the aim was to decrease the time required to achieve a steady state in the continuous process once the conversion has already increased during the batch start. The batch times varied between trials due to time restrictions. Before every continuous start a 0-hour sample was taken which gives a view of the start point from which the continuous process was started from. The 0-hour sample also represents the end point of the batch. The other samples were taken during the continuous processing which were analysed to see the development of the hydrolysis process.

In addition, a reference batch was conducted with enzyme dosage of 4,5 % which was the aimed dosage also in the continuous processes. The dry matter content in batch was 11 % and the total hydrolysis time was 116 h.

The samples taken from each trial are presented in Table 10. Two different times are presented with the samples. First is presented the time from the beginning of the batch start which is the total process time. The second time is from the beginning of the continuous start

which represents the continuous process time. For the reference batch is given one time since there is no change in the process.

Table 9. Samples taken from the three trials and reference batch with the total processing time and continuous processing time except in the batch.

Sample	**Trial 1** (Total time / continuous process time)	**Trial 2** (Total time / continuous process time)	**Trial 3** (Total time / continuous process time)	**Batch** (Total time)
1	15,5 h / 0 h	26 h / 0 h	23,5h / 0h	23 h
2	29,5 h / 14 h	49 h / 23 h	47 h / 23,5 h	46 h
3	39 h / 23,5 h	76 h / 50 h	72,5 h / 49 h	70 h
4	51,5 h / 36 h	103,5 h / 77,5 h	94,5 h / 71 h	91 h
5	64 h / 48,5 h	-	117,5 h / 94 h	116 h
6	-	-	142,5 h / 119 h	-

5.5 Sample preparation

The slurry samples were taken from the outlet of the reactor in continuous trials and from the surface of the reactor in the reference batch. First the sample was heated in boiling water for 10 minutes to denature the enzymes protein structure which will stop the hydrolysis reactions in the sample. Thus, the state of conversion at that time is captured from the sample. From the slurry sample solid and liquid phases were separated with centrifuge (OHAUS Frontier 5916). The liquid fraction was analysed after this. The solid phase was re-slurried into a significant amount of water to remove the remaining soluble sugars. The re-slurried sample was then again centrifuged to separate to solids from the washing liquid. From the centrifuge the solid sample was analysed. To ensure that the washing was efficient enough, brix from the liquid phase of the second centrifuge step was measured to be 0 %.

5.6 Analyses

The liquid and solid samples were analysed in the laboratory. The laboratory analysis for both samples are presented in Table 11.

Table 10. Analytical determinations for liquid and solid samples.

Liquid sample determinations	**Solid sample determinations**
Oxalic acid, HPLC+PDA	Dry matter content, Trockengehalt 105˚C
Citric acid, HPLC+PDA	Acid-insoluble lignin, gravimetric
Succinic acid, HPLC+PDA	Acid soluble lignin, UV 205 nm
Formic acid, HPLC+PDA	Protein, calculated
Acetic acid, HPLC+PDA	Glucose, acid hydrolysis and HPLC+RI
Levulinic acid, HPLC+PDA	Xylose, acid hydrolysis and HPLC+RI
Carboxylic acid, HPLC+PDA	Total nitrogen, Kjeldahl
2-Furoic acid, HPLC+PDA	
5-HMF, HPLC+PDA	
Furfural, HPLC+PDA	
Total furans, HPLC+PDA	
Lactic acid, HPLC+PDA	
Cellobiose (DP2), HPLC+RI	
Glucose, HPLC+RI	
Xylose, HPLC+RI	
Galactose, HPLC+RI	
Arabinose, HPLC+RI	
Mannose, HPLC+RI	
Fructose, HPLC+RI	
Total carbohydrates, HPLC+RI	
Soluble lignin, UV 205 nm	
Brix, Pen Refractometer	

5.6.1 Brix

Brix analysis is conducted to the liquid sample, and it is used to detect the conversion during the process. Brix is used to determine the total soluble solids but mainly it detects the sugar content in liquid samples. Brix detects sugars such as glucose, sucrose and fructose, and sugar alcohols such as sorbitol and mannitol. (Magwaza et al., 2015) The devise calculates the brix by passing light through the liquid and measuring the bending of the light. The bending of the light is caused by the sugars in the liquid and thus the sugar content can be determined. (Heney, 2021) In this process it is mainly used to detect the glucose content. Degrees of Brix or ˚Bx indicates 1 gram of sugar per 100 grams of aqueous solution (Beck, 2018). In this research 1% Brix means 1 g of glucose and xylose in 100 grams of water. The brix measurements were conducted in room temperature. With brix measurement the conversion can be calculated with Equation 11. This conversion gives a clue of the state of the process before the more accurate analysis from the liquid and solid samples.

$$Conversion, \% = \frac{B_I}{B_{max}} = \frac{B_t - B_0}{X_{CH} * c_{dry\ matter} * X_{CF} * \rho} \quad (11)$$

Where,

B_I	Brix increase
B_{max}	Maximum sugar brix in the determined dry matter content
X_{CH}	Total content of carbohydrates (In this glucose and xylose = 0,61)
B_T	Brix at moment t
B_0	Brix in the beginning
$c_{dry\ matter}$	Dry matter content in the slurry
X_{CF}	Conversion factor to convert polysaccharide to monosaccharide including water uptake during hydrolysis (For glucose 1,111 (Lee et al., 2021))
ρ	Density of the slurry, kg/l.

5.6.2 Carbohydrates from liquid sample

Cellobiose, glucose, xylose, galactose, arabinose, mannose, fructose, and total carbohydrates are determined from liquid sample with high performance liquid chromatograph (HPLC) and refractive index detector (RI). The HPLC used is a twin-line system with two column ovens, columns, and detectors. The models of the equipment are Waters e2695 Alliance Separation module and Waters 2414 Refractometer detector. The column is Bio-Rad Aminex HPX-87O and pre columns are Micro-Guard De-ashing and Carbo P columns. Carbohydrates are separated in analytical column and detected with refractive index (RI) detector. The separation of sugar compounds follows the principles of ion-moderated partitioning. In the column the bed is styrene-divinylbenzene sulfonated copolymer in particle size of 9 μm. Ultrapure water is used as an eluent in the determination. The samples are filtered with 0,45 μm syringe filter and Sep-Pak C18 cartridges before the HPLC analysis. The analysis is done with 30 minutes isocratic method with RI detector. The eluent flow through is 0,6 ml/min, columns oven temperature 80 ˚C, and injection volume 20 μl. RI detector temperature is 40 ˚C.

5.6.3 Soluble lignin

Soluble lignin from liquid sample is determined with an internal method. The measurement is done with spectrophotometer in wavelength 205 nm. It is based on the absorbance different of the sample and the reference sample which is water. Before the measurement the sample can be diluted if the absorption is not in range of 0,2-0,7 AU and filtrated if turbid. The measurement is done in 1 cm cuvettes. The dissolved lignin amount can be calculated with Equation 12 in mg/l.

$$Soluble\ lignin = \left(\frac{A}{a}\right) d \quad (12)$$

Where,

A	Absorbance
a	Absorptivity coefficient (in this 0,110 l/mg*cm)
d	Dilution factor.

5.6.4 Inhibitors

Oxalic acid, citric acid, succinic acid, formic acid, acetic acid, levulinic acid, carboxylic acid, 2-fucorin acid, 5-HMF, furfural, total furans (5-HMF and furfural), and lactic acid are determined from the liquid sample with HPLC and photodiode array detector (PDA). The equipment used are Waters e2695 Alliance separation module and Waters 2998 Photodiode Array detector. The column is Phenomex Rezex RHM-Monosaccharide and pre column is Security Guard AJO-4490. Before the analysis sample is diluted if needed and filtered with 0,45 μm syringe filter. In HPLC analysis is used 90 minutes isocratic methos with eluent flow of 0,4 ml/min and column oven temperature is set to 40 ˚C. The eluent used is 49-51 % (w/w) sulphuric acid. Injection volume is 20 μl. Inhibitors are detected with PDA detector with different wavelengths between 210 and 280 nm.

5.6.5 Acid soluble and insoluble lignin, and carbohydrates in solid sample

From solid samples the acid soluble lignin, acid insoluble lignin, and carbohydrates are detected with acid hydrolysis and HPLC. The sample is introduced to 72 % sulphuric acid. The hydrolysis is done in 30 ˚C temperature for one hour and is autoclaved in 121 ˚C for one hour. In the acid hydrolysis lignin is precipitated with acid, filtered, and determined

gravimetrically. The filtration is done with Munktell MGA 410132 filter. It can be calculated with equation 13.

$$Acid\ insoluble\ lignin, \% = \frac{(M * 100)}{W} \quad (13)$$

Where,

M Mass of dry insoluble lignin (g)

W Mass of total dry matter (g).

From the filtrate monosaccharides glucose and xylose are analysed with HPLC and RI detector similarly as from liquid samples which is mentioned earlier. Before the analysis the filtrate is diluted, filtered with C-18 and 0,45 μm filters. To be noted with acid hydrolysis the sample is neutralized before the analysis. Acid soluble lignin is also determined from the filtrate with spectrophotometer in wavelength of 205 nm the same way as from liquid sample which is mentioned earlier.

5.6.6 Dry matter content

The dry matter content of the solid sample determination is based on ISO 638-8:2021 standard. Approximately 2-5 g sample is dried in 105 ˚C and cooled down in exicator and then weighed. The dry matter content is calculated with Equation 14.

$$Dry\ matter\ content, \% = 100 * \left(\frac{S - C}{T}\right) \quad (14)$$

Where,

T Mass of wet sample, g

s Mass of dry sample and container, g

c Mass of container, g.

5.6.7 Total nitrogen and protein content

Total nitrogen (Kjeldahl) determination is based on SFS 5505:1988 standard. In addition of nitrogen content, the protein content can be determined with this analysis. The analysis is done in Foss Tecator 2520 Auto Lift, Foss Tecator Scrubber, and Foss Tecator Kjeltech 8400 devices. The nitrogen in the sample is degraded into ammonium with concentrated sulphuric acid with copper catalyst and Devarda's alloy. It is done in 420 ˚C temperature for one hour. This causes the nitrogen, nitrite, and nitrate to react into ammonium. The reaction follows Equation 15.

$$(CHNO) + H_2SO_4 \rightarrow CO_2 + SO_2 + H_2O + NH_4^+ \quad (15)$$

Natrium hydroxide is added to the sample to neutralise the sulphuric acid and release ammonium gas. The reactions follow Equations 16 and 17.

$$H_2SO_4 + 2\ NaOH \rightarrow 2\ Na^+ + SO_4^{2-} + 2\ H_2O \quad (16)$$

$$NH_4^+ + OH^- \overset{heat}{\longleftrightarrow} NH_3(g) + H_2O \quad (17)$$

Ammonia is then distilled with steam to boric acid which forms tetrahydroxyborate. This reaction follows Equation 18.

$$B(OH)_3 + NH_3 + H_2O \leftrightarrow NH_4^+ + B(OH)_4^- \quad (18)$$

Then it is distilled back with hydrochloric acid to detect the nitrogen content of the sample. This reaction follows Equation 19.

$$B(OH)_4^- + HCl \rightarrow Cl^2 + B(OH)_3 + H_2O \quad (19)$$

The nitrogen content can be calculated following the Equation 20.

$$N\% = \frac{(t - B) * N * 14{,}007 * 100}{m} \quad (20)$$

Where,

t	Volume of titrate, ml
B	Volume of titrate in reference, ml
N	Normality of titrate acid
m	Mass of the sample, mg.

Protein content can be calculated from Kjeldahl analysis also and it follows Equation 21.

$$Protein, \% = N\% * F \tag{21}$$

Where,

F Coefficient for nitrogen to protein. (In this F=6,25)

5.7 Calculations

In this chapter is presented how the laboratory determination results were handled or other calculations done to represent the results in a wanted form.

The conversion was determined form the liquid samples with HPLC. The glucose amount from the samples was given in mg/l from the HPLC analysis. This can be transformed into conversion with Equation 22.

$$Conversion, \% = \frac{c_{glucose}}{c_{0,glucan} * C_{dry\ matter} * \rho * X_{CF}} \tag{22}$$

Where,

c_{glucose} Glucose content in the sample, (mg/l)

$c_{0,\text{glucan}}$ Proportion of glucan in the fresh feed (In this 0,57)

$C_{\text{dry matter}}$ Dry matter of the fresh feed

ρ Density of the fresh feed (In this approximately 1,03 kg/l).

The conversion can also be determined from the solid sample. Since there is enough previous knowledge about the process the lignin amount can be compared to the figure in Appendix 2 to determine the conversion. The lignin amount increases in the solid sample during the hydrolysis and since the raw material composition is known the conversion can be determined. This can be done since the feed slurry composition in the trials is identical to the composition of the slurry from which the Appendix 2 figure is calculated.

Average retention times for the trials were calculated to see when the process has reached fully continuous operation. The continuous operation time was reached once the average retention time has reached the calculated retention time of the continuous process. The average retention time t_A which included the batch start were calculated with Equation 23.

$$t_A = x_c * t_R + \big((1 - x_c) * t_B\big) \tag{23}$$

Where,

x_c Changed proportion of slurry in reactor after the continuous operation has started

t_R Retention time of continuous process

t_B Batch time in the beginning of the process.

Retention time of continuous process can be determined with Equation 24.

$$t_R = \frac{V}{v} \tag{24}$$

Where,

V Volume of the reactor, L

v Flow rate, L/h.

6 Results and discussion

The results from the three somewhat successful trials are presented in this chapter. All in all, the trials had many difficulties. The main issue was to gain steady flow rate with the feed pump. The viscose feed raw material caused many issues, and the optimal flow rate was difficult to reach. The slow flow rates resulted in two issues: clogging of the feed line and separation in the valves. Furthermore, the use of a larger pump with valves to regulate the flow rate also led to separation occurring in the valves. The volume and hydrostatic pressure of the feed container appeared to have a significant impact on the flow. It was observed that as the surface level of the feed container decreased, the flow rate also decreased. Furthermore, the hydrostatic pressure within the enzymatic hydrolysis reactor had an influence on the feed flow rate when the feed was introduced at the bottom of the reactor. It was observed that pressure in the reactor decreased the flow rate compared to when the feed was fed without pressure. Also, the mixing speed in the reactor affected to the feed flow. When the mixing was too slow the dry matter started to settle causing the feed flow rate to decrease.

Partial circulation back to the feed container was also tested. The feed line was split into two lines in the circulation, with one line supplying the raw material to the reactor and the other returning back to the feed container. This resulted the feed line to block, causing the circulation line to remain open and enabling the entire slurry volume to flow back to the feed container. Thus, no raw material was fed to the reactor anymore. The slow feed flow rate resulted in as slow out flow rate. When the out-flow rate was too slow the outlet line started to build up dried slurry causing partial clogging. This decreased the outlet flow and increased the slurry volume in the reactor when the feed flow stayed constant. The feed raw material had variable amount of shives which caused a few problems. With small lines and pumps the shives caused clogging. Also, they might be one reason for the breakage of the hose pumps inner hose which broke the pump. Due to the unstable feed the enzyme dosage varied also. Thus, steady operation parameters were difficult to reach which needs to be considered while making conclusions of the analysis.

6.1 Conversion

Glucose conversions were determined from the liquid and solid samples. The liquid sample conversion results for Trials 1-3 are presented in Figure 18. The conversions are calculated according to Equation 22. The calculated conversion values are presented in Appendix 1. The first sample points are from the start of the continuous operation and at the same time the end of the batch start. The last sample points are the end points of each trial.

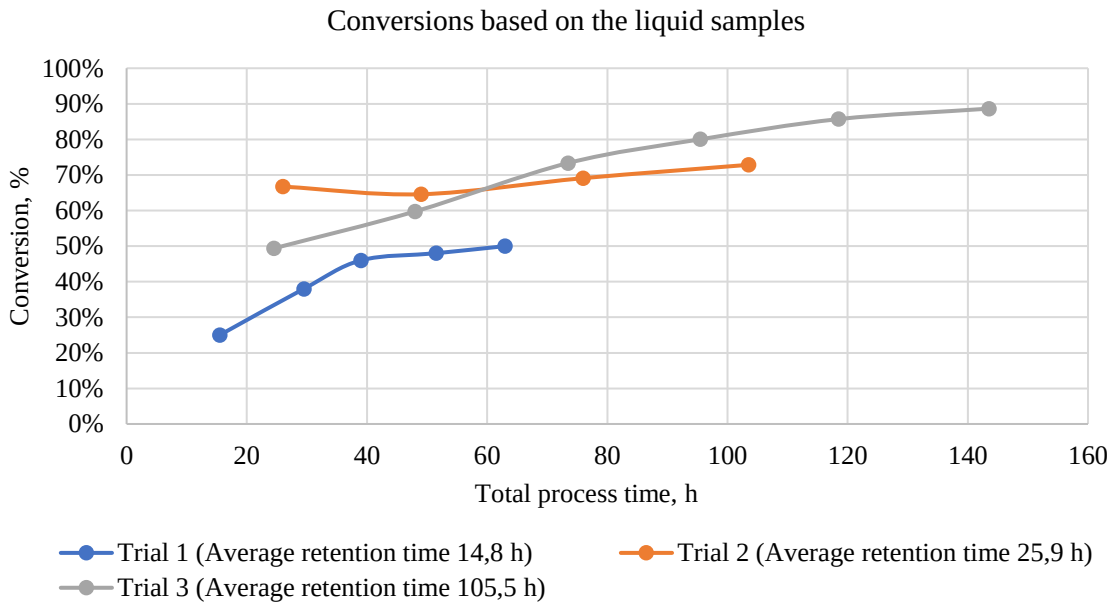

Figure 18. Conversions based on the liquid samples in Trials 1-3.

The conversion curves differ between the trials due to variations in the batch start and average retention times of the processes. In the Trial 1 which is marked as blue the average retention time was 14,8 h and it has the lowest conversions due to that. In addition, the first sample point may distort the curve. The first sample was taken at a point when the pH was too high which usually lowers the conversion. The optimal pH was 5 and the pH at the first point was 5,9. Thus, the conversion in the first point of Trial 1 could be higher if the pH was at the optimal level. The Trial 1 curve still exhibits signs of beginning to level out, even though it has not yet reached a steady state. The last and highest conversion calculated from liquid samples in the Trial 1 was 50 %.

In the Trial 2 which is marked as grey the average retention time was 25,9 h. From the curve can be seen that the process has relatively steady development and probably has almost reached the steady state. The batch in the beginning of Trial 2 was only one hour longer than the calculated average retention time in the continuous process which seems to level the curve quicker than in the other trials. Thus, as assumed the batch time should be optimized to be as long as the retention time in the continuous process to reach the steady state as fast as possible. The slight drop in the second sample point is most likely caused by too high flow rate in the beginning of continuous process since the adjustment of the feed pump took time. The last and highest conversion in liquid samples in Trial 2 was 73 %.

Trail 3 which is marked with orange had the longest average retention time of 105,5 h. Thus, the conversion in the end is at the highest when compared to the Trials 1 and 2. From the Trial 3 curve can be seen the development of the hydrolysis since the batch was short compared to the average retention time of the continuous process. Thus, the conversion follows more typical conversion curve to a batch process. It seems that the steady state is not reached yet but still the process has reached relatively high conversion. The highest and last conversion in liquid samples was 89 %. Although the flow rate is very slow and retention time is long the process seems to be able to produce relatively high conversion. It is to be noted that also the enzyme dosage in the Trial 3 was a bit higher than in the other trials.

The conversions can also be calculated from the solid samples. The solid samples are sometimes more accurate since the sample does not age similarly as the liquid sample. The conversion from solid sample is determined here by comparing the lignin content in the solid sample into the Appendix 2 figure from which the conversion can be determined. The conversions from solid samples in Trials 1-3 are presented in Figure 19. The calculated values of Figure 19 are presented in the Appendix 1.

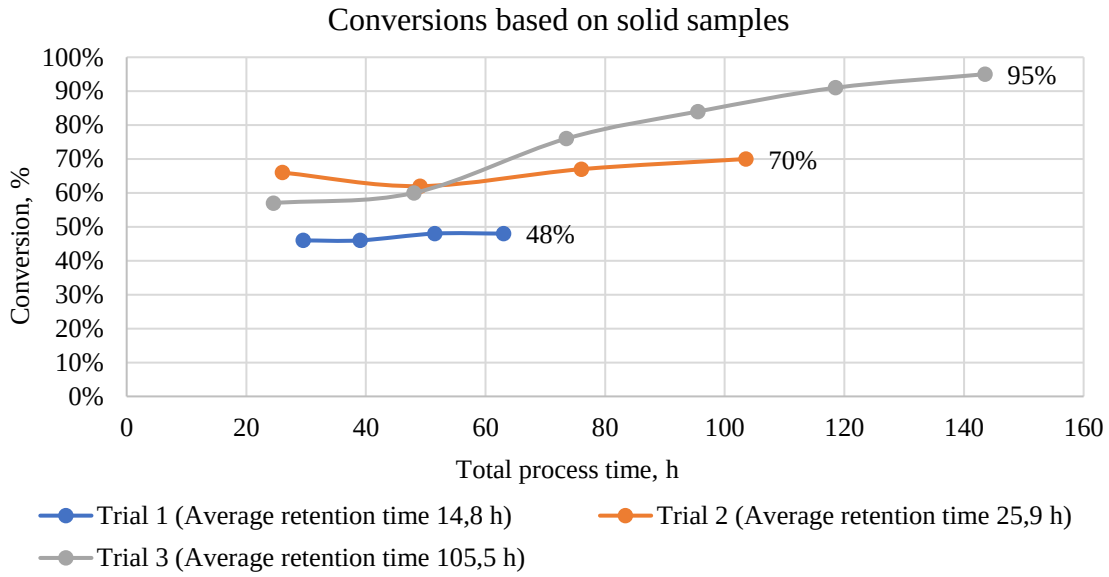

Figure 19. Conversions based on the solid samples in Trials 1-3.

As from Figures 18 and 19 can be seen that the conversions from liquid and solid samples differ from each other a bit. To take a closer look at the differences in the solid and liquid conversions in each Trial the Figures 20-25 are presented to support the Figures 18 and 19.

The conversions based on the solid and liquid samples in Trial 1 are presented in Figure 20.

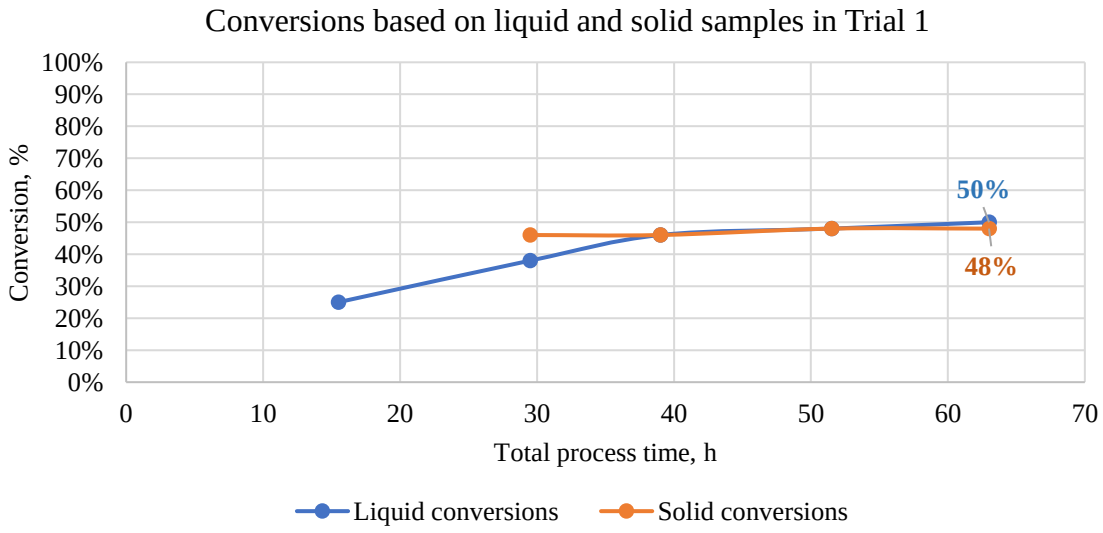

Figure 20. Conversions based on liquid and solid samples in Trial 1 in which the average retention time was 14,8 h.

In the Figure 20 the blue represents the calculated conversions from liquid samples and the orange represents the determined conversion from solid samples. As can be seen that the steady state seems to be reach at the third sample point. After that there is not much change in the conversions. The pH was too high in the beginning of the Trial 1 and the first solid sample was not analysed. The first sample point is not representative due to the high pH. Since the pH was too high the conversion in the first liquid sample is lower than it could be in the optimal pH. From the second samples can be seen that the conversion in liquid sample is still at a lower level than in the solid sample. Thus, the pH is probably still affecting to the liquid sample. Otherwise, the conversions are very close to each other. The end point conversion in liquid samples is 50 % and in solid samples 48 %. Thus, the results are in the same range and the slight difference can be explained with error in analysis or handling.

The average retention times were determined in the trials to see the development of the process. The batch in the beginning affected to the retention times which is considered when calculating the retention times according to the Equation 23. From the average retention times can be seen when the process has reached fully continuous operation after the batch start. At that point forward the batch start doesn't affect to the conversions. The conversions and average retention times in Trial 1 are presented in the Figure 21.

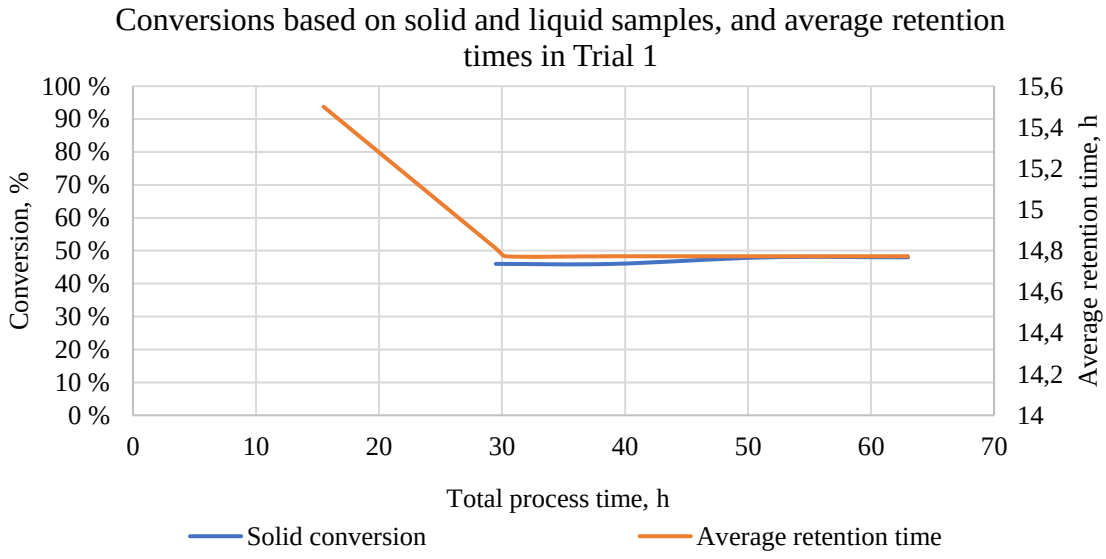

Figure 21. Conversions and average retention times in Trial 1 in which the average retention time in continuous operating was 14,8 h.

In Figure 21 the conversion is in the primary y-axis and the average retention time is on the secondary y-axis while the total process time is on the x-axis. From Figure 21 can be seen that the average retention time was higher in the beginning due to the longer batch time compared to the average retention time in the continuous operating. The batch time was 15,5 h and the calculated average retention time in the continuous operating was 14,8 h. The process reaches fully continuous operating at 30,3 h of total process time when it reaches the average retention time of 14,8 h. To be noted in the beginning of the continuous operation the pH was higher than optimal thus the first sample point conversion is left out from the figure. Once fully continuous operation is achieved and the average retention time is constant, the conversion appears to level out. In this trial, it could be said that the process has achieved a steady operating state, or at the very least, is very close to it.

Conversions based on liquid and solid samples in Trial 2 are presented in Figure 22. These are calculated in the same way as for the Trial 1.

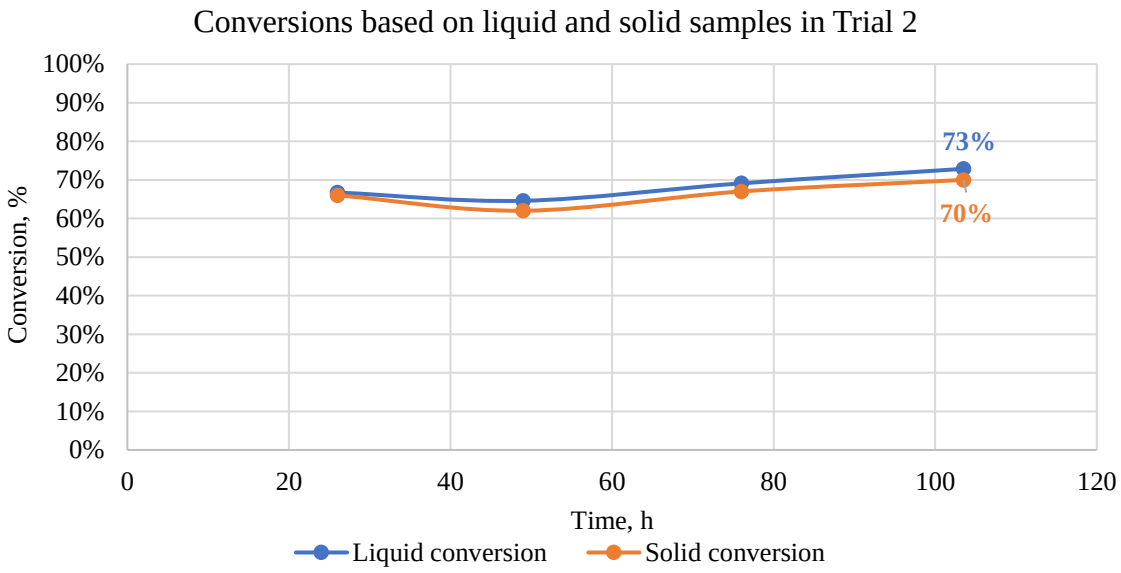

Figure 22. Conversions based on liquid and solid samples in Trial 2 in which the average retention time was 25,9 h.

In the Figure 22 the blue represents the calculated conversions from liquid samples and the orange represents the determined conversion from solid samples. It can be seen that in Trial 2 the liquid and solid sample conversions are pretty close to each other. The difference seen

in the curves can be explained with error in analysis or handling of the sample. It seems that the process is quite close to reaching the steady state and there is only slight change in the conversion in the end on the process.

The conversions and average retention times in Trial 2 are presented in Figure 23. These are calculated in the same way as in Trial 1.

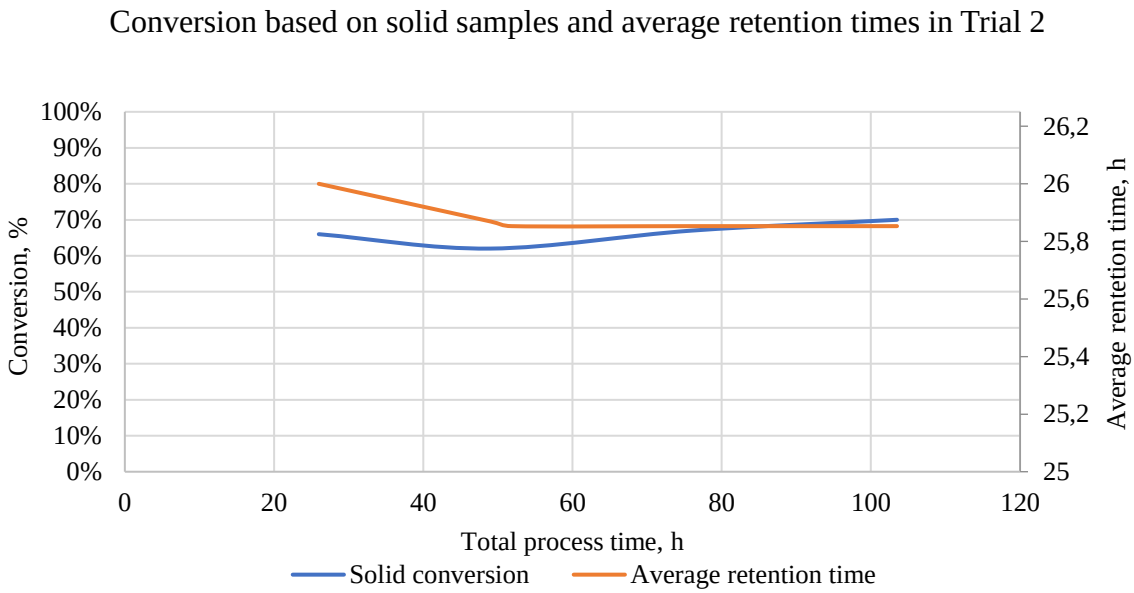

Figure 23. Conversions and average retention times in Trial 2 in which the average retention time in continuous operating was 25,9 h.

In Figure 23 conversion which is marked with blue is on the primary y-axis and the average retention times which is marked with orange is on secondary y-axis. The total process time is in the x-axis. Form the average retention time can be seen that the batch time is only slightly longer than the retention time in the continuous process. Thus, the levelling of the average retention time curve is rapid. The process reaches fully continuous operating at 51,9 h of total process time when it reaches the average retention time of 25,9 h. Despite the levelling of the average retention time, there is slight increase in the conversion. The process seems to be close to the steady state, but a longer trial would have been needed to determine at what level the conversion would have levelled out.

Conversions based on liquid and solid samples in Trial 3 are presented in Figure 24. These are calculated in the same way as for the Trial 1 and 2.

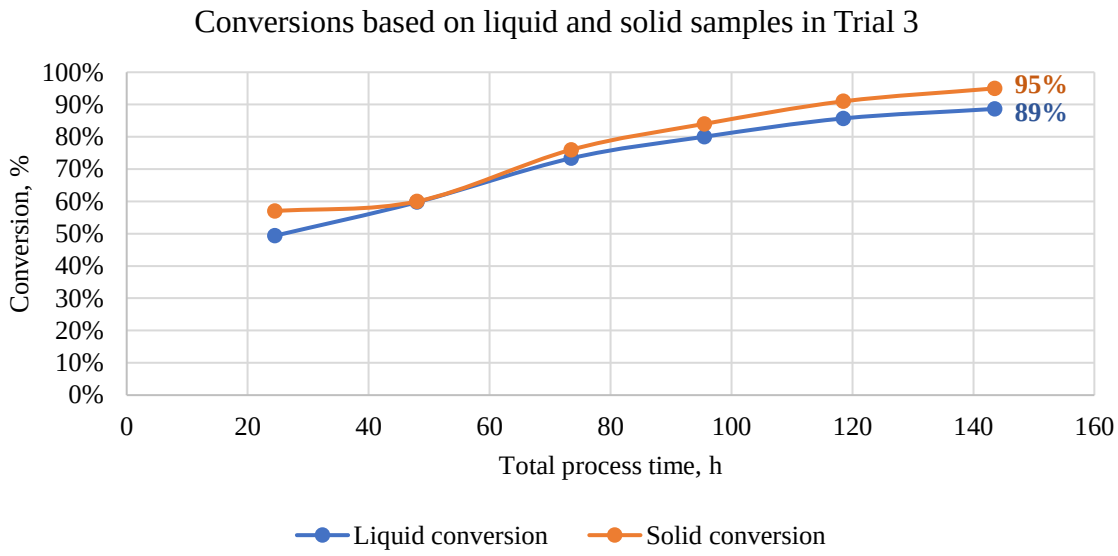

Figure 24. Conversions based on liquid and solid samples in Trial 3 in which the average retention time was 105,5 h.

In the Figure 24 the blue represents the calculated conversions from liquid samples and the orange represents the determined conversions from solid samples. Both conversions seem to follow similar curve except the first sample point conversions. This can be caused either by handling error or ageing of the sample. Overall, the solid conversions seem to be a bit higher than the liquid conversions. The end point conversion in liquid samples is 89 % and in solid sample 95 %. This suggests that the liquid samples have faced some aging before the analysis which might decrease the glucose amount in the liquid sample. Due to the differences in the conversions in solid and liquid samples the solid samples are more accurate. Storing liquid samples always carries a risk, whereas solid samples typically give more accurate results that are not dependent on the storage time.

The conversions and average retention times in Trial 3 are presented in Figure 25.

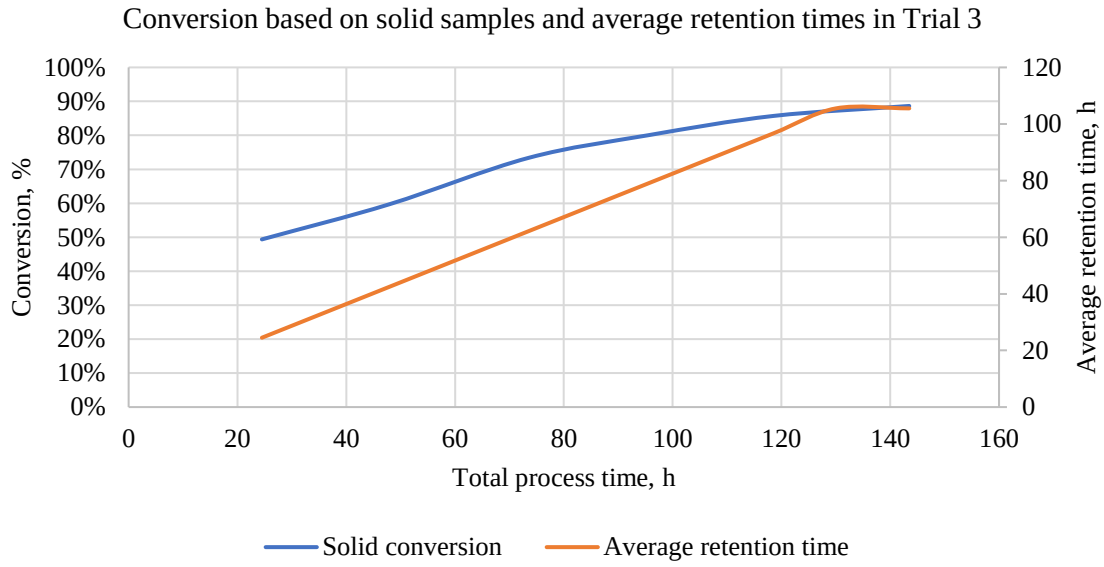

Figure 25. Conversions from solid samples and average retention times in Trial 3.

In Figure 25 conversion which is marked with blue is on the primary y-axis and the average retention times which is marked with orange is on secondary y-axis. The total process time is in the x-axis. From the Figure 25 can be seen that the batch start was shorter than the average retention time in the continuous process. While the average retention time increases the conversion increases also. The process reaches fully continuous operation at 130 h of total process time when the average retention time reaches 105,5 h. Once the average retention time is levelled, slight increase in the conversion can still be seen. However, if the process had continued, the high conversion in the end suggests that the conversion would have likely started to level out relatively soon. The batch start should have been longer with such slow flow rate so that the process would have reached steady state sooner.

In Figure 26 is compared the end points of the three conducted trials to the batch reference results. The calculated values of the Figure 26 are presented in Appendix 1.

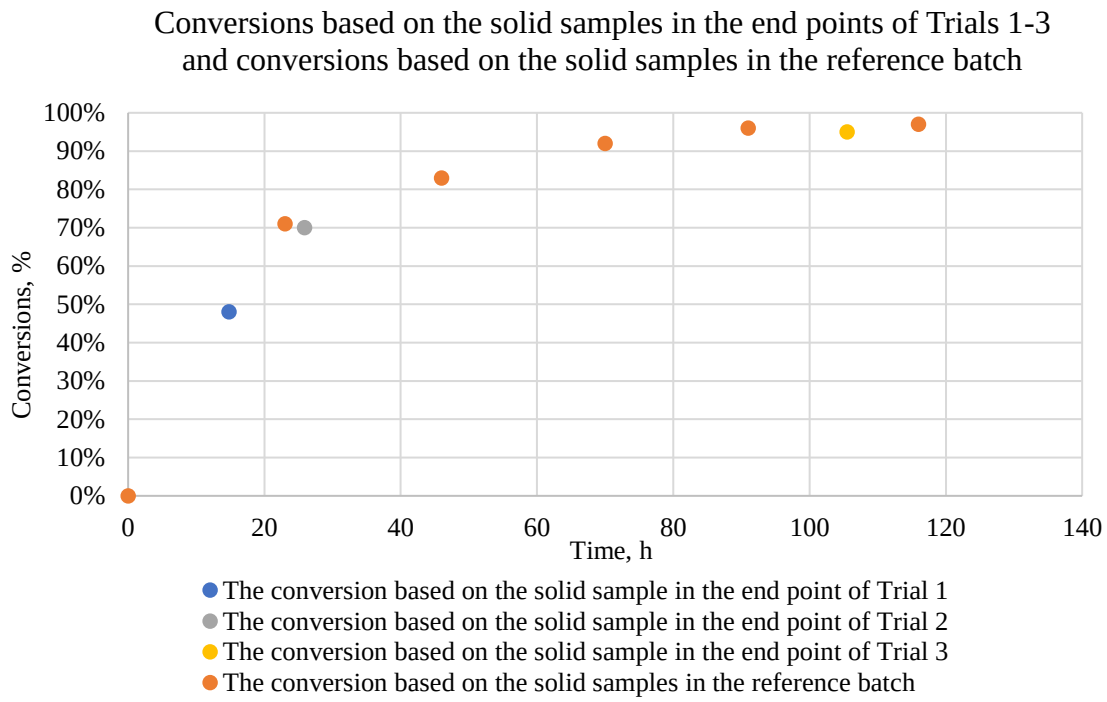

Figure 26. The conversions in the Trial 1-3 with average retention times in the continuous processes and the conversion in the batch process with total processing time. (Average retention time in Trial 1=14,8 h, Trial 2 =25,9 h, and Trial 3=105,5 h.)

The main results for the study can be seen in the Figure 26 when the continuous trials are compared to the batch process. The continuous trial points follow the same curve as the batch conversions. The Trial 1 conversions is on the curve while the Trials 2 and 3 are at slightly lower level. This can be explained with error in the sampling or analysis since the samples are analysed at different times. Nevertheless, the difference is so small that in can be stated that the conversions are at the same level as in the batch reference. Also, the Trial 3 has not reached the steady state which could possibly be at a bit higher level. All in all, the continuous process seems to end up at the same level results as the batch process. Thus, the continuous operating does not seem to affect or most importantly decrease the conversion when compared to a batch process.

To further optimize continuous operation, several factors must be taken into account, including enzyme dosage, retention time, and reactor tank volume. In order to minimize enzyme costs, the dosage should be kept as low as possible, but this can impact on the conversion rate. Additionally, the retention time has an influence on the conversion. On the other hand, the retention time also affects the size of the reactor tank, which is typically desired to be minimized to reduce costs and save space. Therefore, a comprehensive evaluation of these three factors is necessary to assess when the process in optimized.

6.2 Composition of solid samples

From the solids samples total lignin, total carbohydrate, and protein content was determined. The compounds can vary between the feeds. It is expected that the carbohydrates content decreases and lignin content increases in the solid samples during the hydrolysis. In an ideal situation the solid sample would not contain carbohydrates at all which would mean 100 % conversion to glucose. Although, that is realistically unreachable. The protein content increases in the process due to the enzyme addition. Also, with high conversions the protein content can increase since the carbohydrates in the solids sample are degraded and thus the sample contains less other solids which increases the proportion of protein in it. Most on the protein in the biomass ends up in the solid fraction after the hydrolysis which is why it is measured from the solid sample. The enzyme dosage can be also determined afterwards from the solid sample. The lignin, carbohydrates, and protein content in solid samples in Trial 1 are presented in Figure 27.

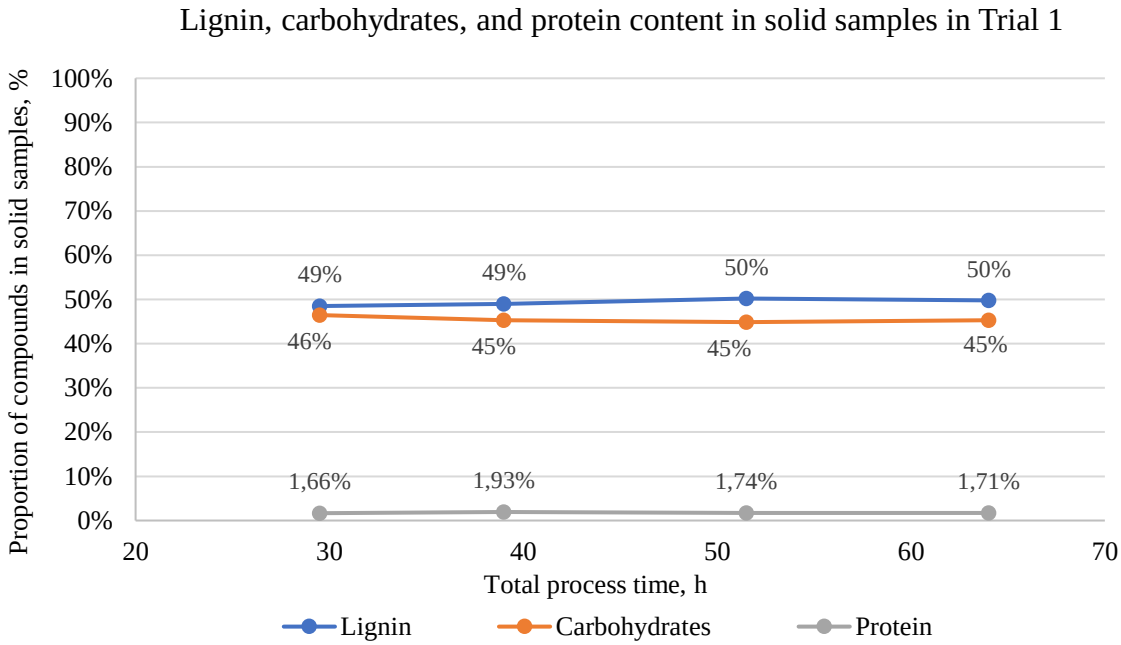

Figure 27. Lignin, carbohydrates, and protein proportions in solids samples in Trial 1.

From the solid sample compositions can be seen that the process is quite close to a steady state if not there yet. The lignin content and carbohydrate content has only slight change during the process and the last two sample points are almost identical. A longer trial would have been needed to see if the composition stays the same in long period of time or if there are quality variations. The protein content seems to be quite constant throughout the process.

The lignin, carbohydrates, and protein content in solid samples in Trial 2 are presented in Figure 28.

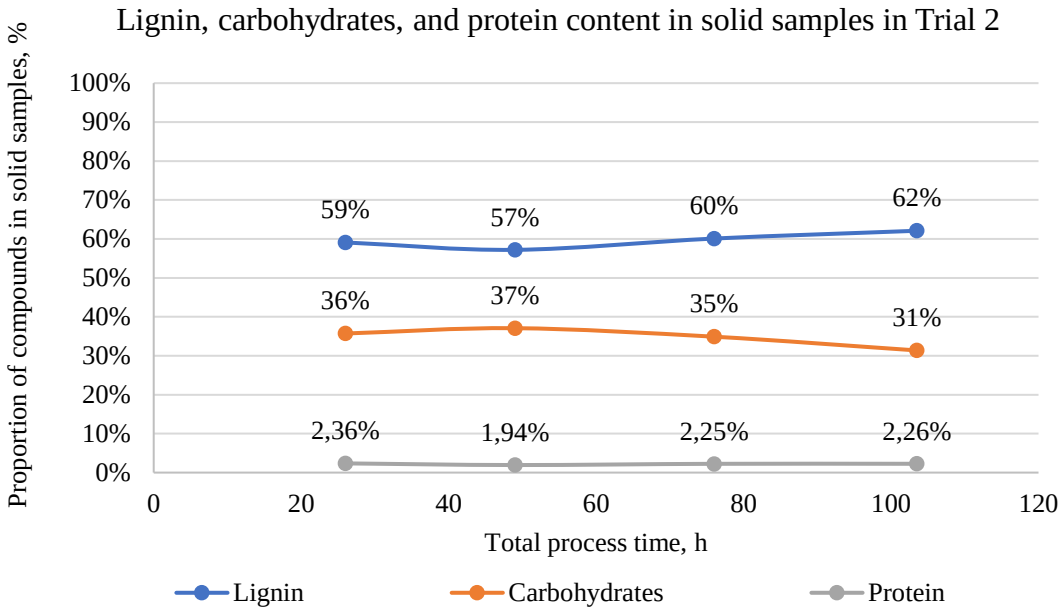

Figure 28. Lignin, carbohydrates, and protein proportions in solids samples in Trial 2.

The lignin content is increasing, and carbohydrates content is decreasing during the process in Trial 2. The slight difference from the overall trend in the second samples is probably caused by the transitioning from the batch to the continuous mode. The steady operation is difficult to gain quickly and here can be seen that more fresh feed is probably flowing into the reactor and less hydrolyzed material is flowing out in the second sample point. The process has not reached steady state since the composition of the samples are changing. A longer run would have been needed to see where the steady state composition would have levelled. The protein content is quite steady throughout the process.

The lignin, carbohydrates, and protein content in solid samples in Trial 3 are presented in Figure 29.

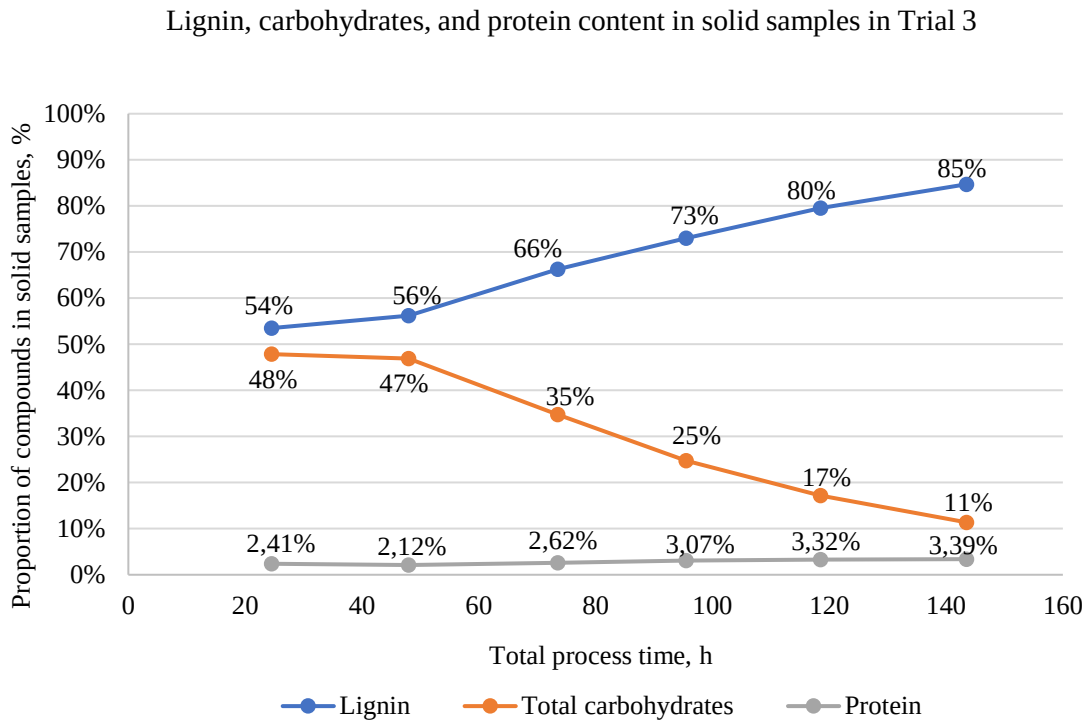

Figure 29. Lignin, carbohydrates, and protein proportions in solids samples in Trial 3.

The development of the hydrolysis can be seen well from the carbohydrates and lignin content. The lignin content in the last sample point is high and the carbohydrate content is relatively low. The process is not in steady state yet, but the composition suggests that the process should start levelling soon since the lignin purity is quite high. A longer trial would have been needed to determine in which composition the process would have levelled out. The protein content increases towards the end which is caused by the solid samples composition change. The carbohydrates are hydrolysed and the solids mass decreases. Thus, the proportion of protein increases even though the protein content in the outlet stays the same. In addition, the enzyme dosage in Trial 2 was higher than in the Trials 1 and 3 which can be seen from the slightly higher protein content throughout the process.

6.3 Sugar purity

In this chapter is evaluated the sugar and lignin purity based on the determinations. The sugar purity is important due to the later purification steps when high quality sugars are produced in enzymatic hydrolysis. Thus, the quality requirements set to the batch processing needs to be met also in the continuous operating mode. The soluble lignin contents were determined from the liquid samples and are presented in Figure 30 for each trial.

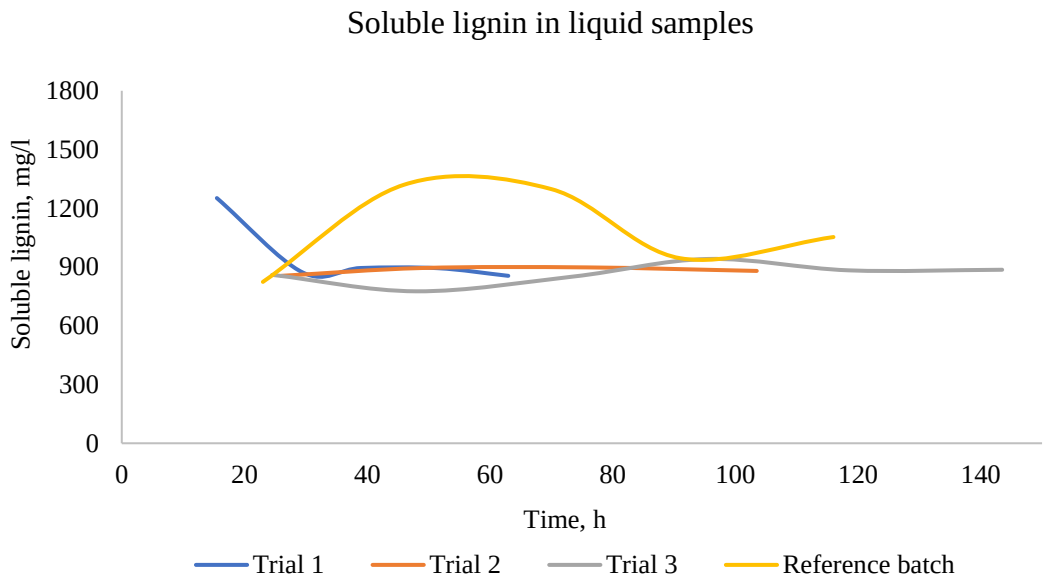

Figure 30. Soluble lignin content in the liquid samples in Trials 1-3 and batch reference.

The soluble lignin amount seems to stay between 800-900 mg/l nicely except in the beginning of the Trial 1 and in the reference batch. This is caused by the high pH which usually causes lignin solubilization to the liquid samples. In the reference batch the high amounts of soluble lignin can be explained by the timing of the sample collection. The pH was adjusted with NaOH before the sample was taken. The NaOH was added on to the reactor surface from which the sample was also taken from. Thus, the NaOH solubilize lignin on the slurry surface locally which then end up in the sample. Due to that the sample is not representative in the soluble lignin amount. In Trial 1 can be seen clearly that once the pH is settled to the optimal level the soluble lignin amount also decreases to the same levels in

which it is detected in other trials too. This indicates that enzymatic hydrolysis should be conducted at a slightly acidic pH, as lignin is undesired in the liquid phase. The liquid sugars are typically purified after hydrolysis for further use, and the presence of lignin can complicate the purification process. Therefore, having higher amounts of soluble lignin in the liquid phase is undesirable. From these results can be stated that continuous operating does not increase the amount of soluble lignin in the liquid sugar phase and thus does not affect to the purification steps.

6.4 Inhibition and contamination in the trials

Inhibitory compounds were also determined in the analysis from the liquid samples. The most important inhibitory degradation product from which the contamination can usually be detected is lactic acid. Lactic acid is formed by lactic acid bacteria when it uses glucose as energy source. Lactic acid concentrations in the samples are presented in the Figure 31.

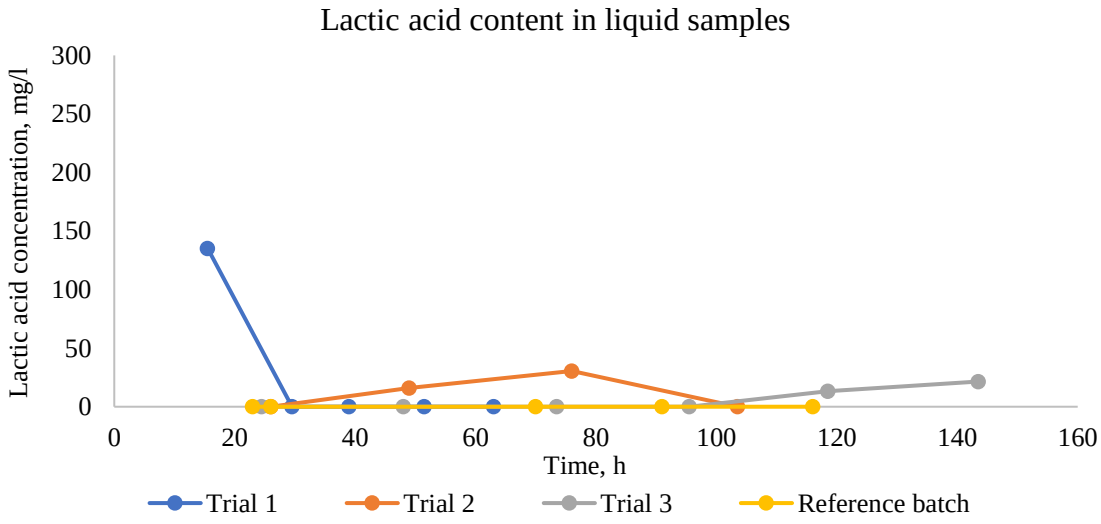

Figure 31. Lactic acid content in liquid samples in Trials 1-3 and reference batch.

From Trial 1 can be seen that the lactic acid concentration is at the highest in the beginning of the process. Once again, the high pH might be the reason. Since the pH is 5,9 which is

closer to neutral, the microorganisms can grow faster. In this case the lactic acid bacteria have grown faster and produced lactic acid into the reactor. Once the pH is settled to optimal 5, the lactic acid concentration drops to zero. In Trials 2 and 3 there are some amounts of lactic acid at some points of the process. One reason for that is the feed container. In the trials the feed container was in room temperature and the pH was a bit elevated to 5,5. This creates better conditions to lactic acid bacteria to grow. Also, in the reference batch there is no lactic acid which also supports the theory of the contamination in the feed container. Nevertheless, the concentrations are relatively low and does not seem to have great effect to the conversion. Thus, can be said that no severe contamination is appearing in the trials. In the future the contamination in the feed container needs to be considered. Ideally in continuous process there is no need for long wating times for the raw material after the pretreatment and before the enzymatic hydrolysis.

7 Conclusions

The aim of the thesis was to determine if a simple continuous enzymatic hydrolysis process would reach high enough conversions to be viable compared with a batch process. Simple one step continuous enzymatic hydrolysis trials were conducted to pretreated beech raw material in dry matter contents between 9-12 %. Even though, there were serious pumpability issues during the trials, three of the trials were successful. With 14,8 h, 25,9 h, and 105,5 h retention times 48 %, 70 %, and 95 % conversions were reached. The results were compared to a reference batch. As a conclusion the continuous process reaches the same conversions levels as the batch process. Thus, continuous processing does not seem to have any significant affect to the conversion in enzymatic hydrolysis.

Based on the results, it can be concluded that the process shows potential when implemented on a larger scale. Despite achieving the calculated average retention times in each process, it was observed that the conversions improved in all of the trials thereafter. This might be an indication that the conversion could be even higher than in the batch process if the trials would have been longer. Once the steady state can be reached with proper equipment, the conversion levelling can be studied in more detailed. The sugars and lignin showed comparable levels of purity to batch processing, suggesting that continuous processing does

not introduce any quality concerns for the products. However, it was discovered that the simple process design posed more operational challenges than anticipated when implemented on a small scale with slow flow rates, resulting major pumpability issues. These problems will not be an issue in a bigger scale where the flow rates are higher and the pipelines are bigger, thus they are less prone to glogg. The contamination in continuous operation was difficult to study since the trials ended up being shorter than planned due to the issues. Hence, the objective of reducing water consumption and enhancing contamination prevention could not be thoroughly investigated in these short trials. To comprehensively examine these phenomena in the continuous process, longer-duration trials will be required in the future. But it can be stated that water consumption can be reduced due to the lack of cleaning after each batch and thus wastewater capacity can be reduced. In the trials, the flow through the reactor was conducted from the bottom to the surface, which potentially offered advantages in terms of process operability. However, further research is necessary to determine whether a bottom-to-surface or surface-to-bottom flow configuration is more efficient for the process.

Various process configurations have been described in the literature for continuous enzymatic hydrolysis. It is important to note that the simple CSTR utilized in the trials cannot be directly compared to the literature due to the absence of similar results. Consequently, it can be concluded that many of the configurations discussed in the literature involve multiple steps or reactors in order to achieve the desired conversion. Although the topic has been extensively researched according to the literature, the implementation of these technologies on an industrial scale still lags behind. Hence, the experimental trials conducted in this study provide results that high conversions can be attained through a straightforward process design, thereby highlighting the potential for industrial-scale implementation of continuous enzymatic hydrolysis process.

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Appendix 1. Total process times, average retention times, conversions from liquid samples, lignin content in solid samples, and conversions from solid samples.

TRIAL 1				
Total process time, h	**Average retention time, h**	**Conversion based on liquid sample, %**	**Lignin content in solid sample, %**	**Conversion based on solid sample, %**
15,5	15,50	25 %	-	-
29,5	14,81	38 %	49 %	46 %
30,3	14,77	-	-	-
39	14,77	46 %	49 %	46 %
51,5	14,77	48 %	50 %	48 %
63	14,77	50 %	50 %	48 %
TRIAL 2				
Total process time, h	**Average retention time, h**	**Conversion based on liquid sample, %**	**Lignin content in solid sample, %**	**Conversion based on solid sample, %**
26	26	67 %	59 %	66 %
49	25,87	65 %	57 %	62 %
51,9	25,85	-	-	-
76	25,85	69 %	60 %	67 %
103,5	25,85	73 %	62 %	70 %
TRIAL 3				
Total process time, h	**Average retention time, h**	**Conversion based on liquid sample, %**	**Lignin content in solid sample, %**	**Conversion based on solid sample, %**
24,5	24,5	49 %	54 %	57 %
48	42,54	60 %	56 %	60 %
73,5	62,12	73 %	66 %	76 %
95,5	79,02	80 %	73 %	84 %
118,5	96,68	86 %	80 %	91 %
130,0	105,52	-	-	-
143,5	105,52	89 %	85 %	95 %
Reference batch (REF)				
Total process time, h	**Average retention time, h**	**Conversion based on liquid sample, %**	**Lignin content in solid sample, %**	**Conversion based on solid sample, %**
23	-	61 %	63 %	71 %
46	-	72 %	72 %	83 %
79	-	82 %	81 %	92 %
91	-	94 %	86 %	96 %
116	-	94 %	88 %	97 %

Appendix 2. Composition of solid sample compared to conversion.