Effects of Pretreatment on the Biocatalysis of Renewable Resources

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Within a biorefinery platform several conversion steps such as pretreatment, saccharification, fermentation and downstream processing are necessary to obtain the final bio-based product(s) from lignocellulosic biomass. The structural composition of the biomass, especially the lignin content, determines the necessary pretreatment steps. To obtain sugar monomers, the hydrolysis of lignocellulosic biomass is an essential step. This work examines the impact of different pretreatments on the sugar release during biocatalysis. Even without prior pretreatment the biocatalysis of low lignin biomass achieves glucose yields of up to 93 %, while the biocatalysis of high lignin biomass requires an upstream hydrothermal procedure to achieve a glucose yield of 74 %.

Keywords: Biocatalysis, Lignocellulosic biomass, Pretreatment, Valorization

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1 Introduction

During the past decades the uncontrolled consumption of fossil-based resources has caused several environmental issues, such as greenhouse gas emissions, climate change and the reduction of natural resources [1]. Further, at present, the energy and organic chemical consumptions are growing incessantly due to the rapid increase of the world's population with improved standards of living [2]. As a consequence, the exploration of new renewable resources to produce energy and chemicals is becoming imperative. In this scenario, lignocellulosic biomasses have been recognized as emerging and sustainable alternative resources because of their relatively low cost, great abundance, and sustainable supply [3]. In addition, an important element in the transition towards a bio-based economy is the biorefinery facility. The International Energy Agency defines biorefining as the conversion of renewable raw materials into a spectrum of marketable products and bioenergy [4]. Among others, lignocellulosic biomasses (LCB) such as municipal green waste and herbaceous materials are particularly attractive as they are one of the major underused waste streams occurring in urban and agricultural areas respectively [5]. In order to use LCB in a biorefinery concept, physical, chemical and catalytic pretreatment steps followed by (bio)catalytic conversion steps are necessary. The first ones have great influence on the efficiency of the (bio)catalytic conversion steps.

The chemical composition of lignocellulosic biomass determines their potential as biorefinery feedstocks, even if it differs depending on the species, its maturity, and environmental conditions [5, 6]. Lignocellulose is mainly composed of cellulose (30–50 %), hemicellulose (20–35 %) and lignin (15–20 %) [7]. Cellulose is a linear polymer of *D*-glucose linked to each other by β-1,4 glycosidic bonds [8]. Because of its crystalline structure, cellulose is highly resistant to hydrolysis thus impeding efficient conversion of this polymer during biorefining processes [2]. Hemicellulose is an amorphous and branched polymer of five carbon (xylose and arabinose) and six carbon sugars (galactose, glucose and mannose) linked by β-1,4 and β-1,3 glycosidic bonds [2]. Due to their amorphous branched structure and low molecular weight, hemicellulose can be readily hydrolyzed [8]. Lignin is an amorphous heteropolymer network of three-dimensional polymers composed of three different methoxylated phenylpropane units (coniferyl alcohol, sinapyl alcohol and coumaryl alcohol) that are bonded together by different kinds of linkages [3]. It provides structural support and acts as natural, impermeable barrier to microbial attacks and oxidative stress on plant tissues [3, 8]. As reported by Langsdorf et al., slight differences can be observed with regards to the biomass type [5]. In particular, stems and leaves in municipal waste contain a lower amount of cellulose and hemicellulose and higher concentration of lignin compared to grassy material [5]. In the

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current study, two different lignocellulosic biomasses were considered for a high lignin content biomass (municipal green waste) and low lignin content biomass (perennial ryegrass). The overall structure made up of cellulose, hemicellulose, and lignin exist in complex carbohydrates linkages formed by hydrophobic and covalent interactions between lignin and carbohydrates. All the structure components in biomass cellulose-hemicellulose-lignin are responsible for the recalcitrant character of lignocellulose [7].

To overcome biomass recalcitrance, pretreatment is used for the isolation of cellulosic and hemicellulosic polysaccharides [7]. The main objectives of this process are the breakdown of cell wall structures, the reduction of crystallinity, particle size or degree of polymerization, the increase of porosity and accessibility, as well as the delignification in order to make the cellulose and hemicellulose more accessible to hydrolytic enzymes. Factors such as energy consumption and economics of a pretreatment process are also a key criterion for defining the viability of the process [9]. A generalized classification of pretreatment methods groups them into physical, chemical, biological or combinatorial pretreatment [3, 10].

The production of lignocellulosic value-added products is mainly divided into three steps: pretreatment, hydrolysis (also known as biocatalysis), and fermentation. Enzymatic hydrolysis [11, 12] is one of the most important unit operations, in which polysaccharides are hydrolyzed into monosaccharides that can be further converted to bioproducts. The enzymatic hydrolysis is the result of the synergistic action of multiple enzyme components having different mechanisms of action. The main enzymes employed are cellulases (endoglucanases, exoglucanases and β-glucosidases) and hemicellulases (xylanase, mannanase, arabinose, galactosidase etc.). In the first step of biocatalysis, the binding of the enzymes to the substrate takes place. The bound fraction of endo- and exoglucanases converts cellulose to cellobiose. On the contrary, the unbonded fraction of β-glucosidases converts cellobiose to glucose. The binding step is followed by a hydrolysis reaction, which is inhibited by hydrolysis products like cellobiose and glucose [13]. Nevertheless, the biocatalysis process is affected by several substrate-related factors, enzyme-related factors, presence of inhibitors and feedback inhibition [14].

The aims of this work are to compare different pretreatment methods and conditions on two biomasses with different lignin content and to assess the effects of the procedures on the following biocatalysis step. For this purpose, a physical pressing step and a chemical organosolv (OS) as well as a liquid hot water (LHW) method was chosen for the pretreatment of municipal green waste and perennial ryegrass. The present work demonstrates that by using different pretreatment procedures on different lignin-content biomass, differences in sugars released can be observed. As expected, the higher sugar amount was obtained by treating the biomasses with more severe processes. Finally, a kinetic study on the low lignin biomass was performed to assess the rate of the polymer degradation over time.

2 Materials and Methods

2.1 Material

Grass cutting from German ryegrass (*Lollium perenne*) was used as representative example for herbaceous lignocellulosic biomass with a low lignin content and was kindly provided by Julius Kühn-Institute, Braunschweig, Germany. A mixture of beech and pine wood chips, originating from local gardens, was used exemplary for recalcitrant lignocellulosic material with a high lignin content.

2.2 Mechanical Pretreatment

Biomass with a low lignin content was mechanically pretreated using either a tincture press (high pressure tincture press HP 2 H, Fischer Maschinenfabrik GmbH, Neuss, Germany) or screw press (Angel Juicer 7500, Luba GmbH, Bad Homburg, Germany). The tincture press operated at p = 440 bar for 20 min and the screw press, which is made up of two rotating cylinders, at 82 rpm. The wood chips as an example for biomass with high lignin content were shredded into particles with a size of about 5 mm and further milled to a particle size of about 200 µm. Biomass was dried in a drying cabinet (Memmert GmbH & Co. KG, Schwabach, Germany).

2.3 Hydrothermal Pretreatment

Biomass was pretreated hydrothermally in a high-pressure reactor BR-500 (Berghof Products + Instruments GmbH, Eningen, Germany). The reaction vessel consisted of polytetrafluorethylene (PTFE) and had a volume of 0.5 L. The solid/liquor ratio of all experiments was 1:10. Demineralized water was used as solvent for LHW and 50 % (w/w) ethanol for organosolv pretreatments. The reactor was heated using an electric heating jacket (Berghof Products + Instruments GmbH) with a holding time of 15 min at 180 °C. An overpressure of 5 bar N_2 was applied to the reactor vessel and the reaction mixture was stirred at 600 rpm. Solid and liquid fraction of the reaction mixture were separated via centrifugation. The residue was washed three times with demineralized water to remove the solvent and possible by-products of the hydrothermal pretreatment.

2.4 Biocatalysis

The pretreated low lignin content biomass was enzymatically hydrolyzed in 0.1 mM sodium-acetate buffer. To prevent microbial contamination, 0.02 % (w/v) sodium azide was added during hydrolysis for analytical purposes. If the hydrolysate is used for subsequent fermentations, this needs to be omitted and the substrate autoclaved instead. The

reaction was conducted at 50 °C and 50 rpm. Two different enzyme mixtures were employed. Ultraflo® Core and Ultraflo® Max (Novozymes A/S, Bagsvaerd, Denmark) were used in a ratio of 50:50 with an enzyme loading of 0.1 g enzyme solution per g biomass and a solid content of 50 g L^{-1}. Xylanase 2x and Pectinase L-40 (both ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) were used in a ratio xylanase:pectinase of 60:40. Xylanase 2x contained a mixture of endo-1,4-ß-D-xylanases and endo-1,3-ß-D-xylanase, Pectinase L-40 consisted of polygalacturonase. Enzyme loading was based on the protein concentration in the stock solutions, and they were tested using the Bradford (Coomassie Bradford Protein Assay Kit, ThermoFisher Scientific) assay. The protein concentrations were found to be 23.5 mg mL^{-1} for xylanase and 4.2 mg mL^{-1} for pectinase. The enzyme loading was set at 0.16 $g_{enzyme}g_{biomass}^{-1}$ with a biomass loading of 10 % (w/w). The hydrolysate samples were taken at 0, 2, 4, 8, 24, 48, 72 and 96 h and were thermally inactivated at 100 °C for 7 min, centrifuged and filtered. All the enzymatic hydrolysis were performed at higher enzyme loadings in order to get comparable results between the different biomasses. Thus, the experiments focused on the comparability without considering the optimization process as well as the economic evaluations. The enzymatic hydrolysis was carried out in duplicate, and the results have been analyzed as average values and corresponding standard deviation.

2.5 Analytical Methods and Data Processing

The carbohydrate composition of the pretreated materials was performed according to the protocol [15]. Sugar monomers were analyzed using a HPLC system (ESA Inc. 542 autosampler (Chelmsford, Massachusetts, USA), Azura pump P 6.1 L (Knauer GmbH, Berlin, Germany)) equipped with a refractive index detector and a BioRad Aminex HPX- 87H column (300×7.8 mm) (Hercules, California, USA). In the chromatographic analysis, the samples were diluted with deionized water, filtered, and thus 20 µL of each sample was injected into the chromatograph under the conditions of a column temperature (80 °C), and 2.5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1}. The yield of the enzymatic hydrolysis was calculated as the amount of sugar released over the total carbohydrate content in the biomass. The percentage was calculated as follows:

$$\text{yield}[\%] = \frac{C_S - C_{S0}}{\alpha_s C_{i0} M_{P0}} 100 \quad (1)$$

Where C_s is the concentration (g L^{-1}) of solubilized sugar in the supernatant, C_{S0} is the initial sugar concentration (g L^{-1}), α_s is the molecular weight of glucose to glucan monomer (α_s = 1.11), C_{i0} is the initial concentration (g L^{-1}) of insoluble solids, and M_{P0} is the initial mass fraction of the polymer in the insoluble solids.

3 Results and Discussion

Pretreatment of lignocellulosic material is necessary to obtain carbohydrates which can be applied for further uses such as carbon source for fermentations. Due to its varying composition, different strategies are necessary for different biomass types. As lignin plays an important role in the digestibility of lignocellulosic biomass, the lignin content can serve as a decision criterion to select an appropriate pretreatment method.

3.1 Pretreatment of Biomass with Low Lignin Content (Grass Biomass)

Herbaceous material is one example for a biomass with low lignin content. Therefore, the lignocellulosic structure is less recalcitrant. Due to a higher moisture content, the biomass can be pressed, resulting in a liquid and a solid fraction [16]. The pressing process can be carried out in decentralized facilities (e.g., on a farm) to obtain a solid fraction with reduced water content, resulting in a better shelf life and lower transport costs due to the lower weight. However, the press juice must be used immediately, e.g., as an additive for biogas fermentation. The solid press cake must be further pretreated, for example in a central biorefinery.

Grass cutting was pressed using either a screw press or a tincture press. This was followed by a hydrothermal pretreatment as described in Sect. 2.3 and [17] using water (LHW) or 50 % (w/w) ethanol (OS) as solvent. As can be seen in Fig. 1, the influence of a pressing step on the composition of the residue can be neglected. Neither for the native grass, which was not further pretreated after pressing, nor for the hydrothermally processed grass there is a significant difference in the composition of the dried biomass between substrates that were pressed by screw or tincture press or not pressed at all. Compared to the native composition, the hydrothermal procedure increases the share of cellulose in the residue. This effect is stronger for LHW pretreated grass, with an increase of up to 70 %, compared to an increase of up to 50 % for the organosolv pretreatment. At the same time, the LHW procedure reduces the share of hemicellulose in average by 50 %, while it does not change significantly during organosolv pretreatment. The percentage increase of lignin during the LHW pretreatment procedure can be explained by the removal of other soluble compounds during the pretreatment, leading to lignin to account for a bigger share. Dimos et al. report 58 % increase of the lignin share during hydrothermal pretreatment of cotton stalks, and 74 % decrease during an organosolv process using formic acid [18]. This increase of lignin does not occur during organosolv pretreatment with ethanol due to the increased solubility of lignin in this solvent compared to the solubility in water [19].

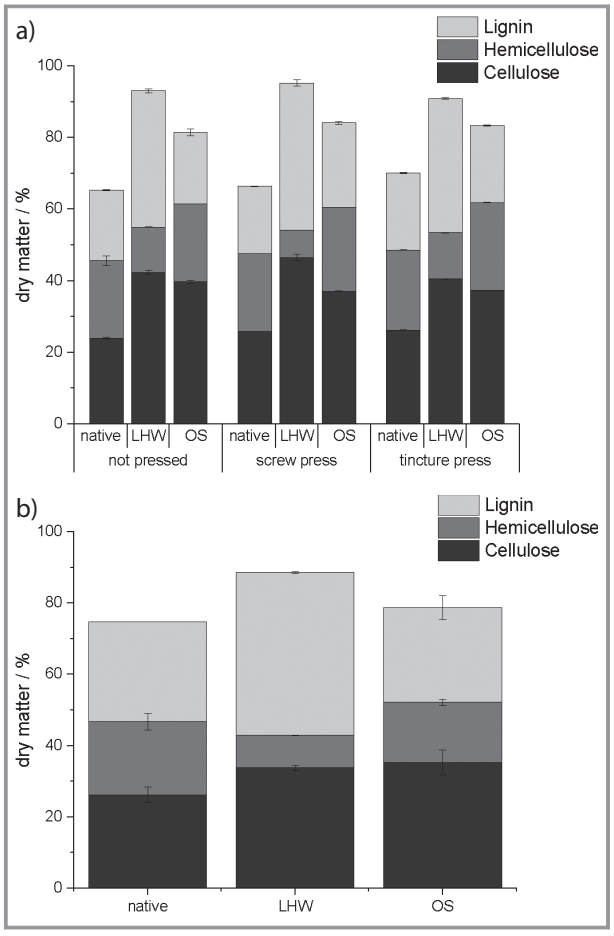

Figure 1. Influence of different pretreatment procedures on the composition of the dried biomass of grass cutting (A) and mixed wood chips (B). Native: no further pretreatment.

3.2 Pretreatment of Biomass with High Lignin Content (Woody Biomass)

Biomass with a high lignin content such as wood chips usually is more solid and also has a lower moisture content than grass cuttings. Consequently, a pressing step is either mechanically not possible (e.g., for the screw press) or does not yield a notable amount of press juice and was thus neglected in this work. However, the surface area of the material was increased by crushing and grinding to achieve a better yield in the processes used.

The effects of applying a hydrothermal procedure on biomass with a low lignin content could be confirmed for woody biomass with a lignin content of 30 %. In accordance with the findings for grass cutting, the hydrothermal pretreatment of wood chips increased the cellulose content in the residue by up to 35 %. In contrast to the previous shown results, there is no significant difference in the increase of cellulose content between LHW and organosolv pretreated biomass. The hemicellulose content decreases by 55 % and 18 % during the LHW and organosolv pretreatment, respectively. Kabir et al. report a hemicellulose decrease of 28.5 % during an organosolv process of forest residues [20]. The percentage increase of lignin previously described can also be observed, with an increase of 63 % for LHW pretreated wood chips. In contrast, during the organosolv procedure, the lignin content decreases by 5 %. Again, this can be explained by the solubility of lignin in ethanol [19].

3.3 Biocatalysis of Biomasses with Different Lignin Contents

Grass cutting was digested enzymatically in a native condition as well as after pressing with either a screw or a tincture press. All biomass samples were saccharified both right after cutting or pressing, respectively, and after drying to constant weight. Similar to commonly used enzyme preparations like Cellic CTec the enzyme mixture of Ultraflo® Max and Ultraflo® Core consists of *ß*-glucanases, xylanases and cellulases and is therefore promising for the degradation of lignocellulosic biomass. Weiermueller achieved a glucose yield of 87 % from grass cuttings with these enzymes [21].

As can be seen in Fig. 2, slightly higher conversion rates were achieved when the biomass was dried to constant weight at 50 °C prior to the enzymatic hydrolysis. The best result was obtained from screw pressed grass with a conversion rate of 82 % of the total amount of glucose based on the NREL-protocol, followed by native grass with 67 %. Biocatalysis of grass pretreated by tincture press yielded 55 % cellulose conversion. This can be explained by the influence of the pressing mechanism. The tincture press

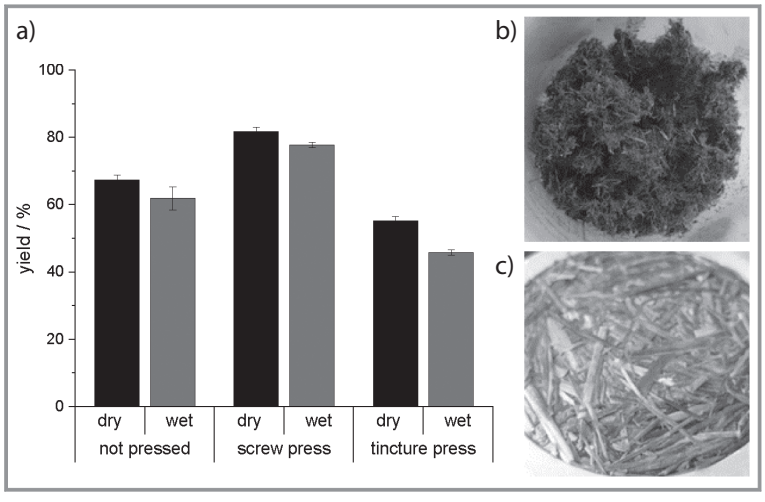

Figure 2. Conversion of cellulose during the biocatalysis step of untreated and pressed grass cuttings in wet or dry conditions (A). Press cake after pressing by screw press (B) or tincture press (C). Enzymatic hydrolysis was carried out with Ultraflo® Max (50 %) and Ultraflo® Core (50 %).

applies pressure of up to 440 bar, resulting in a strong compression of the biomass. Thus, the produced press cake is very dense and compact as can be seen in Fig. 2C. In the screw press however, the material is crushed between two rotating cylinders. The result is a very fluffy grass cut (Fig. 2A). The screw press thereby changes the integrity of the grass cuttings. Konan et al. also discuss the possibility of extrusion, breaking up the lignocellulose structure by the shearing forces of rotating screws, as a potential pretreatment procedure for lignocellulosic biomass [22]. The fibers are shredded and partially broken up. This results in a significantly enlarged surface. The pretreatment step aims to disrupt the cell wall as well as to reduce the particle size in order to make the cellulose and hemicellulose available to hydrolytic enzymes. According to this, the press cake obtained from the screw press is more accessible to the enzymes than the compact press cake from the tincture press.

Fig. 3 shows the influence of different pretreatment methods on the cellulose conversion during the biocatalysis process. A hydrothermal process step increases the cellulose conversion rate both for pressed and not pressed biomass. Regardless of the prior pretreatment method, the conversion rate of the pretreated biomass is on average 93 % with no significant difference between different pressing or hydrothermal procedures. Only the enzymatic hydrolysis of organosolv pretreated grass, which was pressed via tincture press, yields a lower amount of 81 %. The hydrothermal procedure increases the glucose yield for grass which was not pressed by 38 %, while the yield for screw pressed grass only increased by 13 % as the conversion rate for this pressing method was already at 81 % without an additional hydrothermal step. The hydrothermal process is a time- and energy-consuming procedure. Thus, the small increase of the glucose yield does not justify the expenditure. A pressing step with a screw press, which mechanically breaks up the structure of the biomass, is a sufficient pretreatment method for herbaceous biomass with a low lignin content of up to 20 %. A further hydrothermal procedure is economically not worthwhile.

For more recalcitrant biomass with a higher lignin content of around 30 %, however, the situation is different. Kirui et al. observed about twice as much lignin-cellulose interactions in woody biomass than in grasses. This leads to the mechanical strength and also the reduced accessibility for enzymes of biomass with a higher lignin content [23]. The cellulose conversion rate of chopped wood chips is only 14 %. This can be increased by 5 % if the biomass is milled prior to the enzymatic hydrolysis. The smaller particle size facilitates the access of saccharolytic enzymes to cellulose. Csiszar et al. examined the hydrolysis of linen and cotton powders by a cellulase after different ultrasound pretreatments. The authors demonstrated a preferential hydrolysis of smaller particles by cellulases as well as a higher saccharification rate of substrates with a smaller particle size [24]. Fernandes et al. found an increase in glucose yield during enzymatic hydrolysis of sugarcane bagasse of 76 % with particles smaller than 60 mesh compared to native particle size [25]. However, the achieved glucose yield of milled biomass particles is still unsatisfactory. An organosolv pretreatment of wood chips increases the glucose yield by 41.4 %, reaching a cellulose conversion rate of 74 %. This can be further increased to a final conversion rate of 91 % by milling the organosolv pretreated residue prior to the enzymatic hydrolysis to improve the cellulose accessibility (Fig. 4). An overview of different sugar yields obtained from various lignocellulosic feedstocks using different pretreatment processes and biocatalysis steps is given in Tab. 1.

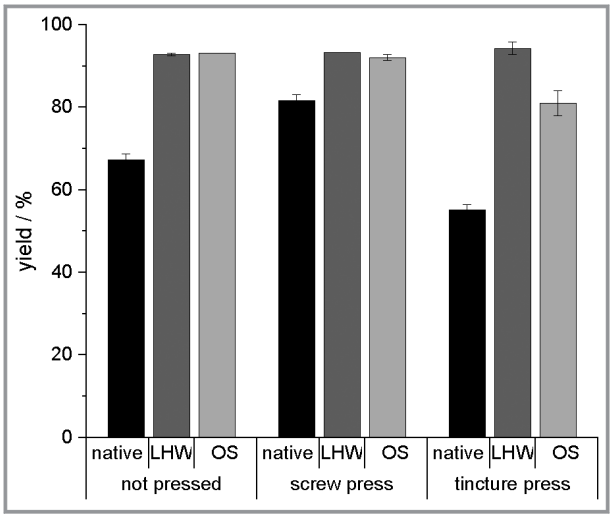

Figure 3. Conversion rate of cellulose after the biocatalysis step of differently pressed and hydrothermally pretreated grass cutting. Native: no further pretreatment. Enzymatic hydrolysis was carried out with Ultraflo® Max (50 %) and Ultraflo® Core (50 %).

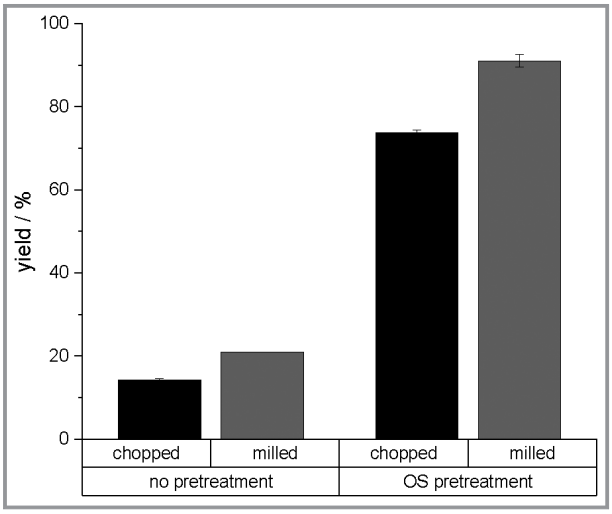

Figure 4. Conversion rate of cellulose after the biocatalysis step of differently pretreated wood chips. Enzymatic hydrolysis was carried out with Xylanase 2x (60 %) and Pectinase L-40 (40 %).

Table 1. Comparison of sugar yields obtained from different lignocellulosic feedstocks after pretreatment and biocatalysis using varying enzymes.

Substrate	Pretreatment	Biocatalysis	Yield [%]	Ref.
Eucalyptus wood chips	LHW and extrusion	CelliCTec2	79.6	[26]
		solid loading: 10 % (w/v)		
		enzyme loading: 20 mg $g_{cellulose}^{-1}$		
Sugarcane bagasse	LHW	Cellulase, Xylanase	55.6	[27]
		solid loading: 5 % (w/v)		
		enzyme loading: 15 FPU $g_{substrate}^{-1}$		
German ryegrass	LHW	Ultraflo Max, Ultraflo Core 1:1	87 (glucose)	[21]
		solid loading: 50 g L^{-1}		
		enzyme loading: 0.1 g $g_{dry\ matter}^{-1}$		
Sorghum sweet biomass	Organosolv	CelliCTec2	42.1 (total sugars)	[28]
		solid loading: 27.7 mg g_{solid}^{-1}		
		enzyme loading: 10 FPU $g_{dry\ matter}^{-1}$		
Poplar sawdust	Organosolv	CelliCTec2	78 (total sugars)	[29]
		solid loading: 2 % (w/v)		
		enzyme loading: 20 FPU $g_{cellulose}^{-1}$		
Perennial ryegrass	LHW, Organosolv	Ultraflo Max, Ultraflo Core 1:1	93 (glucose)	this work
		solid loading: 50 g L^{-1}		
		enzyme loading: 0.1 g $g_{dry\ matter}^{-1}$		
Mixed beech and pine wood chips	Organosolv	Xylanase 2x, Pectinase L-40 3:2	74 (glucose)	this work
		solid loading: 10 % (w/w)		
		enzyme loading: 0.16 g $g_{dry\ matter}^{-1}$		
Spruce and birch chips	Organosolv	CelliCTec2	69.1	[30]
		solid loading: 5 % (w/v)		
		enzyme loading: 10 mg g_{solids}^{-1}		
Energy crop	Organosolv	Cellulase	87.5	[31]
		solid loading: 5 % (w/v)		
		enzyme loading: 20 FPU $g_{dry\ maatter}^{-1}$		

3.4 Kinetic of the Biocatalysis of Low Lignin Content Biomass

The enzymatic hydrolysis of low lignin content biomass was conducted to study the sugar release over time. Fig. 5 reports the yield of glucose and xylose released as a function of the time. Sugars were released fast at the beginning of the hydrolysis and progressively reached a steady state. In particular, the glucose released changed little after 24 h, suggesting that the hydrolysis of glucan was almost completed. At the same time, the amount of xylose was still increasing thus indicating that the xylanase needs more time to completely hydrolyze the xylan.

The overall results were expected as the raw material was pretreated using a weak procedure, thus not completely disrupting the recalcitrant structure of lignocellulosic biomass and reducing the efficiency of the hydrolysis process. As mentioned above, the main goals of the pretreatment are the breakdown of the cell wall and the increase of accessibility for the enzymes. Harsh process methods with chemicals (acids, bases, organic solvents) are extensively used in industrial production and ensure higher sugar release during the enzymatic hydrolysis step. However, the use of strong conditions caused the formation of undesirable products which inhibit the fermentation process. For all these reasons, the physical and chemical pretreatment procedures are

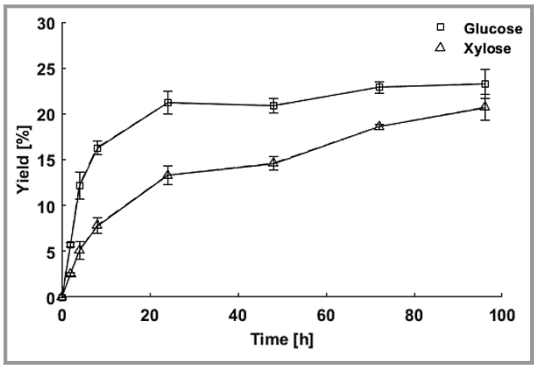

Figure 5. Glucose and xylose yield from mechanical pre-treated low lignin content biomass over time during biocatalysis. Enzymatic hydrolysis was carried out with Xylanases (60 %) and Pectinases (40 %). All experiments were performed in duplicates.

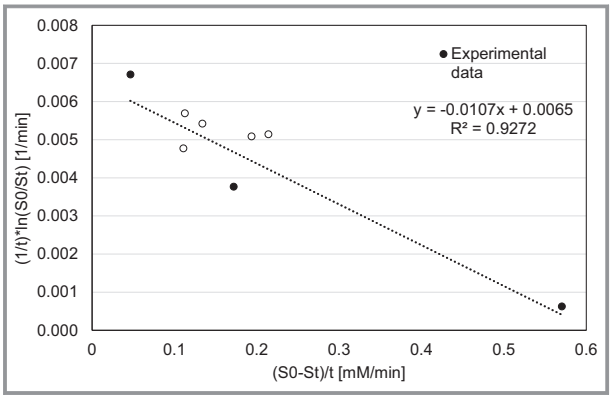

Figure 6. Integrated Michaelis-Menten method to determine the kinetic parameters V_{max} and K_M. Full circles: experimental data; empty circles: predicted data.

usually combined because no pretreatment technique alone can ensure the goals mentioned above [7].

Another explanation for the present result could be the high biomass loading. As reported by Niglio et al., the increase of biomass loading causes high viscosity that makes it more difficult for the solution to access and wet the biomass. Moreover, enzyme inactivation and product inhibition could hinder the enzymatic hydrolysis process [32].

In the usual single-substrate enzyme-catalyzed reactions (Eq. (2)), the relationship between the initial reaction rate and the substrate concentration assumes the form of a saturation curve (Fig. 5) [33]. A mathematical model to describe the kinetic is the Michaelis-Menten equation (Eq. (3)):

$$E + S \rightleftharpoons ES \rightarrow E + P \tag{2}$$

$$v = \frac{V_{max}[S]}{K_M + [S]} \tag{3}$$

Where v is the product formation rate, V_{max} is the maximum velocity achieved at the maximum substrate concentrations, and K_M is the substrate concentration at which the velocity is 50 % V_{max}.

The initial rate of reaction is calculated by correlating the substrate concentration or product concentration change and the reaction period insofar as their time course is estimated as a linear relationship [33]. In this work, as showed in Fig. 6, the linear range was found within the first 8 h. To experimentally define the kinetic parameters V_{max} and K_M, the Michaelis-Menten equation is modified using the integration method. The integration method was applied by assuming that the reverse reaction is negligible, and that the product does not affect the reaction rate [33]. According to this, it is possible to integrate the Michaelis-Menten equation (Eq. (2)):

$$\int_{S_0}^{S_t} \frac{[S] + K_M}{[S]} = -\int_0^t V_{Max}\mathrm{d}t \tag{4}$$

$$\frac{1}{t}\ln\frac{S_0}{S_t} = -\frac{1}{K_M}\frac{(S_0 - S_t)}{t} + \frac{V_{Max}}{K_M} \tag{5}$$

Eq. (4) can be recognized as an equation of a line in which the data are plotted as $(S_0 - S_t)/t$ versus $(1/t)\ln(S_0/S_t)$.

Since the calculation is based on the decreasing substrate concentration over time, the substrate concentration was calculated as:

$$S_{(t)} = \text{Total carbohydrate content (NREL)} - c(t)_{glu} - c(t)_{xyl} \tag{6}$$

The resulting curve (Fig. 6) is a line with a negative slope. From Eq. (3), V_{max} and K_M result to be 0.6 mmol min^{-1} and 93.4 mmol L^{-1}, respectively. The spectrum of experimentally determined K_M values of enzymes varies depending on the experimental setup and the substrate. Typical K_M values of cellulases range from 0.45 to 6.7 mmol L^{-1} for the model substrate carboxymethyl cellulose [34, 35]. For xylanases, K_M values between 0.03 mmol L^{-1} for beechwood xylan [36] and 7.7 mmol L^{-1} for birchwood xylan [36, 37] are reported. Polygalacturonases display a slightly lower range of K_M values from 0.02 mmol L^{-1} to 0.6 mmol L^{-1} [38, 39]. The K_M value of the enzyme mixture of Xylanase 2x and Pectinase L-40 used in the present experiments is in the same order of magnitude as the reported literature data for these kinds of enzymes. The slightly higher K_M value, signifying a lower affinity between enzymes and substrate, can be explained by the different substrates used for the experiments.

4 Conclusion

Lignocellulosic biomass is a potential carbon source for various purposes. However, a pretreatment procedure is indispensable prior to its use. Complex carbohydrates are broken down into fermentable monosaccharides by saccharolytic enzymes. Depending on the lignin content of the biomass,

an additional hydrothermal process is necessary to break up the recalcitrant structures. Cellulose from grass biomass can be converted with an efficiency of 93 % without prior hydrothermal pretreatment. In contrast to this, the cellulose conversion rate of woodier biomass such as wood chips without a hydrothermal processing is 14 %, whereas an organosolv procedure increases the cellulose conversion rate to 74 %.

The overall results demonstrated that the saccharification step depends on the pretreatment process used, as it aims to disrupt the biomass structure to make the biomass more accessible for the hydrolytic enzymes. To make a biorefinery process more efficient, lignocellulosic biomass should be separated according to its lignin content. In this way, the energy consuming hydrothermal pretreatment is only applied to biomass which requires this procedure for an effective saccharification. Finally, kinetic studies were carried out on the grass material over time. With this regard, the time-course of the enzymatic hydrolysis revealed that after 24 h the hydrolysis reached the steady state. In order to study the kinetic parameters, the Michaelis-Menten equation was integrated by considering the time window where product concentration and the reaction time had a linear relationship. The parameters of V_{max} and K_M were estimated to be 0.6 mmol min^{-1} and 93.4 mmol L^{-1} respectively, and are in line with the data reported in literature.

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Symbols used

C_{i0}	[g L^{-1}]	Initial sugar concentration of insoluble solids
C_S	[g L^{-1}]	Concentration of solubilized sugar in the supernatant
C_{S0}	[g L^{-1}]	Initial sugar concentration
K_M	[mmol L^{-1}]	Michaelis-Menten constant
M_{P0}	[–]	Initial mass fraction of the polymer in the insoluble solids
p	[bar]	Pressure
v_{max}	[mmol min^{-1}]	Maximum velocity achieved at the maximum substrate concentrations
v	[mmol min^{-1}]	Product formation rate
α_s	[–]	Molecular weight of glucose to glucan monomer

Abbreviations

FPU	Filter Paper Unit
HPLC	High-Performance Liquid Chromatography
LCB	Lignocellulosic biomass
LHW	Liquid-Hot-Water
NREL	National Renewable Energy Laboratory
OS	Organosolv
PTFE	Polytetrafluorethylene
rpm	Revolution per minute

References

[1] H. Y. Leong, C.-K. Chang, K. S. Khoo, K. W. Chew, S. R. Chia, J. W. Lim, J.-S. Chang, P. L. Show, *Biotechnol. Biofuels* **2021**, *14 (1)*, 87. DOI: https://doi.org/10.1186/s13068-021-01939-5

[2] S. K. Maity, *Renewable Sustainable Energy Rev.* **2015**, *43*, 1427–1445. DOI: https://doi.org/10.1016/j.rser.2014.11.092

[3] V. B. Agbor, N. Cicek, R. Sparling, A. Berlin, D. B. Levin, *Biotechnol. Adv.* **2011**, *29 (6)*, 675–685. DOI: https://doi.org/10.1016/j.biotechadv.2011.05.005

[4] F. Cherubini, *Energy Convers. Manage.* **2010**, *51 (7)*, 1412–1421. DOI: https://doi.org/10.1016/j.enconman.2010.01.015

[5] A. Langsdorf, M. Volkmar, D. Holtmann, R. Ulber, *Bioresour. Bioprocess.* **2021**, *8 (1)*. DOI: https://doi.org/10.1186/s40643-021-00367-5

[6] S. Mohapatra, C. Mishra, S. S. Behera, H. Thatoi, *Renewable Sustainable Energy Rev.* **2017**, *78*, 1007–1032. DOI: https://doi.org/10.1016/j.rser.2017.05.026

[7] A. A. Houfani, N. Anders, A. C. Spiess, P. Baldrian, S. Benallaoua, *Biomass Bioenergy* **2020**, *134*, 105481. DOI: https://doi.org/10.1016/j.biombioe.2020.105481

[8] H. P. Vu, L. N. Nguyen, M. T. Vu, M. A. H. Johir, R. McLaughlan, L. D. Nghiem, *Sci. Total Environ.* **2020**, *743*, 140630. DOI: https://doi.org/10.1016/j.scitotenv.2020.140630

[9] A. R. Mankar, A. Pandey, A. Modak, K. K. Pant, *Bioresour. Technol.* **2021**, *334*, 125235. DOI: https://doi.org/10.1016/j.biortech.2021.125235

[10] B. Kumar, N. Bhardwaj, K. Agrawal, V. Chaturvedi, P. Verma, *Fuel Process. Technol.* **2020**, *199*, 106244. DOI: https://doi.org/10.1016/j.fuproc.2019.106244

[11] K. Vasić, Ž. Knez, M. Leitgeb, *Molecules (Basel, Switzerland)* **2021**, *26 (3)*. DOI: https://doi.org/10.3390/molecules26030753

[12] J. S. van Dyk, B. I. Pletschke, *Biotechnol. Adv.* **2012**, *30 (6)*, 1458–1480. DOI: https://doi.org/10.1016/j.biotechadv.2012.03.002

[13] F. Fenila, Y. Shastri, *Resour.-Effic. Technol.* **2016**, *2*, S96–S104. DOI: https://doi.org/10.1016/j.reffit.2016.11.006

[14] A. K. Amit, M. Nakachew, B. Yilkal, Y. Mukesh, *Res. J. Chem. Environ.* **2018**, *22 (7)*, 62–67.

[15] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP)*, Technical Report **2012**.

[16] T. Sieker, A. Neuner, D. Dimitrova, N. Tippkötter, E. Heinzle, H.-J. Bart, R. Ulber, *Chem. Ing. Tech.* **2010**, *82 (8)*, 1153–1159. DOI: https://doi.org/10.1002/cite.201000088

[17] J. Weiermüller, A. Akermann, W. Laudensack, J. Chodorski, L. M. Blank, R. Ulber, *Bioresour. Technol.* **2021**, *336*, 125262. DOI: https://doi.org/10.1016/j.biortech.2021.125262

[18] K. Dimos, T. Paschos, A. Louloudi, K. G. Kalogiannis, A. A. Lappas, N. Papayannakos, D. Kekos, D. Mamma, *Fermentation* **2019**, *5 (1)*, 5. DOI: https://doi.org/10.3390/fermentation5010005

[19] M. Parot, D. Rodrigue, T. Stevanovic, *Bioresour. Technol. Rep.* **2022**, *17*, 100880. DOI: https://doi.org/10.1016/j.biteb.2021.100880
[20] M. M. Kabir, K. Rajendran, M. J. Taherzadeh, I. Sárvári Horváth, *Bioresour. Technol.* **2015**, *178*, 201–208. DOI: https://doi.org/10.1016/j.biortech.2014.07.064
[21] J. Weiermueller, *Prozessintensivierung auf Basis nachwachsender Rohstoffe*, Dissertation, Technical University Kaiserslautern **2022**.
[22] D. Konan, E. Koffi, A. Ndao, E. C. Peterson, D. Rodrigue, K. Adjallé, *Energies* **2022**, *15 (9)*, 3002. DOI: https://doi.org/10.3390/en15093002
[23] A. Kirui, W. Zhao, F. Deligey, H. Yang, X. Kang, F. Mentink-Vigier, T. Wang, *Nat. Commun.* **2022**, *13 (1)*, 538. DOI: https://doi.org/10.1038/s41467-022-28165-3
[24] E. Csiszar, Z. Szabo, O. Balogh, E. Fekete, K. Koczka, *Ultrason. Sonochem.* **2021**, *78*, 105711. DOI: https://doi.org/10.1016/j.ultsonch.2021.105711
[25] É. S. Fernandes, D. Bueno, F. C. Pagnocca, M. Brienzo, *ChemistrySelect* **2020**, *5 (25)*, 7627–7631. DOI: https://doi.org/10.1002/slct.202001008
[26] D. Tian, F. Shen, G. Yang, S. Deng, L. Long, J. He, J. Zhang, C. Huang, L. Luo, *Fuel* **2019**, *252*, 589–597. DOI: https://doi.org/10.1016/j.fuel.2019.04.155
[27] Y. Zhang, X. Di, J. Xu, J. Shao, W. Qi, Z. Yuan, *Chem. Eng. Commun.* **2019**, *206 (6)*, 772–780. DOI: https://doi.org/10.1080/00986445.2018.1525365
[28] S. P. Joy, C. Krishnan, *Ind. Crops Prod.* **2022**, *177*, 114409. DOI: https://doi.org/10.1016/j.indcrop.2021.114409
[29] Q. Chu, W. Tong, J. Chen, S. Wu, Y. Jin, J. Hu, K. Song, *Biotechnol. Biofuels* **2021**, *14 (1)*, 136. DOI: https://doi.org/10.1186/s13068-021-01988-w
[30] L. Matsakas, C. Nitsos, D. Vörös, U. Rova, P. Christakopoulos, *Energies* **2017**, *10 (3)*, 263. DOI: https://doi.org/10.3390/en10030263
[31] X. Tan, Q. Zhang, W. Wang, X. Zhuang, Y. Deng, Z. Yuan, *Fuel* **2019**, *249*, 334–340. DOI: https://doi.org/10.1016/j.fuel.2019.03.117
[32] S. Niglio, A. Procentese, M. E. Russo, G. Sannia, A. Marzocchella, *BioEnergy Res.* **2019**, *12 (2)*, 312–324. DOI: https://doi.org/10.1007/s12155-019-09969-6
[33] K. Miyanaga, H. Unno, in *Comprehensive Biotechnology*, Elsevier, Amsterdam **2011**.
[34] R. D. Bhaumik, A. P. Sudhir, R. B. Subramanian, *Biotechnol. Rep.* **2015**, *6*, 85–90. DOI: https://doi.org/10.1016/j.btre.2014.11.004
[35] R. Rawat, S. Kumar, B. S. Chadha, D. Kumar, H. S. Oberoi, *Antonie van Leeuwenhoek* **2015**, *107 (1)*, 103–117. DOI: https://doi.org/10.1007/s10482-014-0308-z
[36] B. Enkhbaatar, C.-R. Lee, Y.-S. Hong, S.-K. Hong, *Appl. Biochem. Biotechnol.* **2016**, *180 (2)*, 349–360. DOI: https://doi.org/10.1007/s12010-016-2103-y
[37] H. Liao, S. Sun, P. Wang, W. Bi, *J. Ind. Microbiol. Biotechnol.* **2014**, *41*, 1071–1083.
[38] F. Abe, H. Minegishi, T. Miura, T. Nagahama, R. Usami, K. Horikoshi, *Biosci., Biotechnol., Biochem.* **2006**, *70 (1)*, 296–299. DOI: https://doi.org/10.1271/bbb.70.296
[39] S. K. Niture, A. Pant, A. R. Kumar, *Eur. J. Biochem.* **2001**, *268 (3)*, 832–840. DOI: https://doi.org/10.1046/j.1432-1327.2001.01959.x

