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# Enzymatic Valorization of Lignocellulosic Biomass—The Influence of Deep Eutectic Solvents and Ionic Liquids on the Activity of Cellulolytic Enzymes

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**Abstract:** In recent years, there has been a steady increase in the interest in changing lignocellulose processing technologies from harmful and nonecological to more ecological and sustainable. Deep eutectic solvents (DESs) and ionic liquids (ILs) are green, efficient solvents/catalysts for biomass pretreatment as well as its further transformation. In many cases, they are used together with biocatalysts. Additionally, in processes where DESs and ILs are used for the pretreatment of biomass, before enzymatic transformation, traces of these solvents can influence the activity of biocatalysts. For this reason, it is important to evaluate the effect of novel, green solvents on the activity of enzymes widely applied in the biomass valorization processes. In this work, we present the impact of chosen DESs and ILs, most often applied in biomass pretreatment and transformation, on the enzymatic activity of popular cellulolytic enzymes and enzymatic preparations (namely, cellulases from *Aspergillus niger*, *Trichoderma reesei*, and Viscozyme L). The enzymatic activity was investigated in the process of transformation of cellulose or biomass to glucose. The results showed that many of the solvents tested had a negative effect on enzymatic activity, suggesting a separation of pretreatment and enzymatic processes.

**Keywords:** deep eutectic solvent; ionic liquid; cellulase; biomass processing; biomass enzymatic hydrolysis



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## 1. Introduction

The processing of lignocellulosic biomass is one of the most significant parts of the circular economy. It is a common and inexpensive resource, not only for biofuels (especially bioethanol), but also for valuable chemicals, such as saccharides and fine chemicals [1,2]. Traditional methods for chemical transformation of biomass include the application of mineral acids or alkaline solutions, which are considered hazardous and toxic. Due to this, according to the Sustainable Development Goals, as well as green chemistry principles, a rapidly growing trend of replacing these methods with more environmentally friendly methods (e.g., thermal processing, pyrolysis, application of ionic liquids (ILs), deep eutectic solvents (DESs), and/or biocatalysts) is being observed [3–6].

The physical and chemical properties of ILs and DESs are well known and have been discussed in detail in many scientific reports [7–10]. The most interesting, from an ecological and sustainable development point of view, is lower or zero toxicity, compared to the majority of organic solvents. The majority of DESs are considered even less hazardous solvents than classical ILs, and some of them are biodegradable, especially those called natural deep eutectic solvents (NADESs) [9,11,12]. Also, the cost of DES preparation, including the chemicals, the time needed, and the preparation steps required, is lower compared to that of ILs [13]. In addition, they can be recycled and reused in many

subsequent processes, without significant loss of their activity [14–16]. On the contrary, ILs have a lower volatility, higher thermal stability, and larger electrochemical window compared to DESs [7,17]. Although their physicochemical properties and advantages are well known, their introduction into industrial-scale processes is still under discussion and being researched. This also applies to the processes of economic and ecological valorization of biomass.

There are several biomass-processing steps where ILs and DESs can be used. If they are used for the pretreatment of biomass (goal: the dissolution of lignocellulosic fractions and the degradation of rigid structure), before enzymatic conversion, the problem of their impact on enzymatic activity should be taken into account. ILs, especially acidic ILs, can also be used as catalysts and/or solvents in the direct conversion of biomass into fine chemicals, for example, furfural and levulinic acid [3,18]. From an industrial and economic point of view, the thorough separation of these solvents after pretreatment would add additional steps, costs, and chemicals to the process. Therefore, in all variants of biomass processing, the compatibility between ILs and enzymes is an important issue to address.

Similarly, some DESs are receiving increasing attention because of their ability to dissolve lignin from plant material. Lignin can disturb effective biomass processing, especially via biological methods, due to possible interactions between the polymer and enzyme, and also steric hindrance between the biocatalyst and cellulose. There are some examples in the literature where the addition of some DESs or ILs increased the activity or stability of biocatalysts [19,20]. The outcome varied on the basis of both the solvent and the type of enzyme. Cellulase from *Stachybotrys microspora* showed 115.5% of its primary activity in the presence of 5% of 1-ethyl-3-methylimidazolium diethyl phosphate [emim][DEP] [21]. The addition of a DES (namely, 15% $v/v$ of ChCl:Gly) increased the activity of halophilic cellulase from *Aspergillus terreus (A. terreus)* by 40% [22]. In the same DES, cellulases from *Aspergillus* species retained 90% of their primary activity [23]. However, there are also opposite results, indicating a negative impact of ILs and DESs on the enzymatic activity of cellulolytic enzymes. The application of ILs such as [bmim][Cl], [emim][DEP], [bmim][HOOC], and [emim][$CH_3COO$] had an inhibitory effect on the cellulase from *Trichoderma reesei (T. reesei).* IL [emim][$CH_3COO$], which was the best IL in the delignification process, had the most significant negative influence on enzymatic activity. It decreased to about 60% of the original activity when 10% $w/v$ of IL was added [24]. Another research study showed that only 55% of the primary activity of cellulase from *Aspergillus niger (A. niger)* was retained, when 10% of [bmim][Cl] was added [25]. The adverse effects of DESs on cellulases have also been reported. In the presence of a mere 2.5% choline chloride:lactic acid DES, cellulase from *A. terreus* was almost completely inactivated after a 24 h incubation period. [26]. Studies have shown that the activity of cellulolytic enzymes and their stability in green solvents such as ILs and DESs are significant issues to address in order to achieve efficient biomass transformation technologies. On the one hand, a one-step process has the potential to save time and resources, but it can also become intricate if the solvent inactivates the biocatalyst [27]. On the other hand, even in the two-step process (pretreatment with ILs or DESs followed by enzymatic transformation), residual solvents, after the first step, can significantly decrease the activity of a biocatalyst in enzymatic hydrolysis. Sriariyanun et al. demonstrated that cellulases isolated from *Bacillus* sp. and *Brevibacillus* sp., originating from high-salinity soils, exhibited tolerance to ionic liquids (retaining approximately 95% of their original activity) and displayed enhanced potential for the one-pot processing of rice straw. Furthermore, the yield of sugars produced via enzymatic hydrolysis increased with the concentration of choline acetate. The same processes carried out with choline acetate IL and commercial Celluclast 1.5 L resulted in lower sugars yields compared to the control process. It was also shown that selecting the enzymes tolerant to the IL used for pretreatment could reduce the time and amount of resources required [28]. Therefore, it is very important to determine the tolerance of cellulolytic biocatalysts to chemicals (such as ILs and DESs) used for the pretreatment and processing of biomass before designing a process.

In this work, the influence of chosen DESs and ILs on the enzymatic activity of three cellulolytic biocatalysts was investigated. The application of biocatalysts in the transformation of lignocellulose is widely investigated. The Sustainable Development Goals require the use of ecological, biodegradable resources as well as solvents, catalysts and auxiliaries. In most cases, the enzymatic conversion of lignocellulose must be preceded by pretreatment. One of the new sustainable methods of pretreatment uses ILs and DESs. DESs, mostly applied in biomass treatment, are composed of choline chloride (ChCl) or betaine (Bet) as hydrogen bond acceptors (HBAs), and several hydrogen bond donors (HBDs) were used. Selected commercially available or easy-to-prepare ILs, used as solvents or catalysts in the transformation of biomass (1-butyl-3-methylimidazolium chloride [bmim][Cl], 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][$PF_6$]; 1-butyl-3-methyl-imidazolium tetrafluoroborate [bmim][$BF_4$], and methylimidazolium acetate [Hmim][OAc]) were also studied. Cellulases from *A. niger*, and *T. reesei*, as well as the cellulolytic preparation Viscozyme L, were investigated. It has been reported that fungal cellulases (especially from *A. niger* and *T. reesei*) are more effective in biomass conversion than bacterial ones [29]. Many results are available regarding the enzymatic transformation of different types of biomass. The selection of such biocatalysts allowed better discussion and interpretation of the results obtained for the biomass, DESs, and ILs that we tested. To the best of our knowledge, there are no reports available on the influence of DESs and ILs on the activity of Viscozyme L. Since this preparation contains various enzymes that act on different fractions of lignocellulose, it may serve as an attractive biocatalyst for biomass processing. The relative activity of cellulolytic biocatalysts was calculated on the basis of the amount of glucose, which was produced in the hydrolysis of microcrystalline cellulose in buffer, with the addition of 0–20% of ILs or DESs. The concentration of glucose was analyzed using high-performance liquid chromatography (HPLC), on the Rezex ROA-Organic Acid H+ (8%) (Phenomenex) column, with isocratic elution of 0.05 M $H_2SO_4$. The HPLC method was employed due to its repeatability and potential for automation. Microcrystalline cellulose was chosen as a model substrate, but the study was extended to use real, waste biomass, e.g., walnut wood, walnut shells and husks, grass, and sawmill chips. The waste from walnut trees and walnut processing is difficult to compost because it contains numerous tannins and phytotoxic substances. Other types of waste were chosen because of their common accessibility and high cellulose content. Two methods of biomass processing were investigated: pretreatment of biomass with DESs or ILs and subsequent enzymatic hydrolysis and direct enzymatic hydrolysis of biomass in the presence of DES/IL. To the best of our knowledge, there are no available reports regarding Viscozyme L activity in the presence of DESs and ILs.

## 2. Materials and Methods

### *2.1. Materials*

Choline chloride (98%), lactic acid (85%), 1-methylimidazole, cellulase from *A. niger* (0.8 U/mg), cellulase from *T. reesei* (0.7 U/mg), Viscozyme L (cellulolytic enzyme mixture), and microcrystalline cellulose (20 µm) were purchased from Sigma Aldrich (St. Louis, MO, USA). According to the biocatalysts supplier, one unit of enzyme will liberate 1 µmole of glucose from cellulose in 1 h at pH = 5.0 at 37 °C. Citric acid monohydrate, trisodium citrate, ethylene glycol, betaine (98%), and anhydrous glucose were from Avantor Chemicals (Gliwice, Poland). Urea, glycerin, and acetic acid (99%) were purchased from Chempur (Piekary Śląskie, Poland); levulinic acid (98%) and [bmim][Cl] (98%) were purchased from AcrosOrganics (Geel, Belgium). Malonic acid and [bmim][$PF_6$] (98%) were purchased from Apollo Scientific (Bredbury, UK), and [bmim][$BF_4$] (98%) was from Thermo Fisher Scientific (Waltham, MA, USA).

Several types of biomass (sawmill chips, grass, walnut wood, walnut leaves, walnut shells, walnut shells, and rye straw) were collected from local gardens or farmland in Silesia (Poland), air-dried, and ground in a Bosch ErgoMix homogenizer. The biomass was sieved,

and the particle size fraction ($0.2 < x \leq 0.6$ mm) was dried to a constant weight and used in the experiments.

### 2.2. Preparation of DESs

DESs were synthesized according to the method described by Kandanelli et al. (2018) [30]. Choline chloride (ChCl) or betaine (Bet) (25 g) was weighed to a round-bottom flask, and an adequate molar amount of HBD (lactic acid (Lac), levulinic acid (Lev), urea (U), glycerol (Gly), or ethylene glycol (Et)) was added (Table 1). The mixture was stirred at 70 °C until a clear and transparent liquid was obtained. The DESs were stored at room temperature and used without further purification.

**Table 1.** DESs prepared and applied in the study.

| HBA | HBD | Molar Ratio HBA:HBD | Abbreviation |
|---|---|---|---|
| Choline chloride | Lactic acid | 1:2 | ChCl:Lac |
| | Levulinic acid | 1:2 | ChCl:Lev |
| | Urea | 1:2 | ChCl:U |
| | Glycerol | 1:2 | ChCl:Gly |
| | Ethylene glycol | 1:2 | ChCl:Et |
| Betaine | Lactic acid | 1:2 | Bet:Lac |
| | Levulinic acid | 1:2 | Bet:Lev |
| | Urea | 1:1 * | Bet:U |
| | Glycerol | 1:2 | Bet:Gly |
| | Ethylene glycol | 1:3 * | Bet:Et |

* DES in the molar ratio HBA:HBD 1:2 was impossible to prepare using the standard procedure.

### 2.3. Preparation of [Hmim][OAc]

1-methylimidazole (0.027 mol) was added to the double-neck round-bottom flask in an ice bath. Glacial acetic acid (0.027 mol) was added dropwise to the stirred solution (with magnetic stirrer, 300 rpm) of 1-methylimidazole, at a rate of one drop per second. The synthesis was carried out using a reflux condenser. After the addition of the whole amount of acetic acid, the mixture was stirred for 2 h (300 rpm) at room temperature. At this point, a clear, light-yellow liquid was obtained. The IL was dried on the Schlenk line, stored at room temperature, and used without further purification. The yield of the obtained IL was above 99%.

### 2.4. Pretreatment of Biomass with DES or IL

The biomass was collected from a local garden in Silesia, Poland, and air-dried to a constant weight. Dry biomass (250 mg) was pretreated in a round-bottom flask with 5 mL of ChCl:Gly or [bmim][Cl] for 3 h at 90 °C, with mechanical stirring (400 rpm). After pretreatment, the biomass was filtered and washed with 25 mL of distilled water. The pretreated biomass was dried at 50 °C for 24 h. Processes were carried out in triplicate. Pretreated biomass was used for further enzymatic processing. Filtrates containing lignin dissolved in DES were analyzed via UV–Vis spectrophotometry to calculate the degree of delignification.

### 2.5. Enzymatic Hydrolysis of Cellulose or Biomass

A total of 100 mg of microcrystalline cellulose or biomass, 4 mL of 50 mM citrate buffer (pH = 5.0), and 0–20% *v*/*v* of DESs or ILs were added to the Falcon tubes. Then, 19 U of the chosen biocatalyst (cellulase from *A. niger*, cellulase from *T. reesei*, Viscozyme L) was added to the mixture. Hydrolysis was carried out in an Enviro-genie incubator at 50 °C, for 8 h, with stirring (70 rpm). Each process was carried out in triplicate. The samples were collected and filtered prior to HPLC analysis. The relative activities of biocatalysts were calculated based on the amount of glucose obtained after 8 h of the process. The amount

of glucose obtained in the same enzymatic process, without addition of DESs or ILs, was taken as a control (100% of relative activity).

### *2.6. Characteristics of Lignocellulosic Biomass*

Lignocellulosic biomass, before the determination of the contents of lignin, cellulose, and hemicelluloses, was extracted using Soxhlet's apparatus with 95% ethanol, for 6 h, to remove extractable ingredients. After extraction, the plant material was dried at 60 °C. The solvent was evaporated from the extract and weighed. The content of extractable ingredients was calculated based on the following Equation (1):

$$\%\text{extr} = \frac{\text{Mass of extracted ingredients}}{\text{Total mass of lignocellulose sample}} * 100\% \quad (1)$$

#### 2.6.1. Lignin Content Determination

The lignin content in the plant material was analyzed according to the method of Klason's lignin determination [31]. A total of 200 mg of dry biomass (after the removal of extractable ingredients) was placed in a round-bottom flask with 3 mL of 72% $H_2SO_4$. The mixture was kept at room temperature for 2 h, and then 115 mL of distilled water was added, in order to dilute the acid to a concentration of 3%. The mixture was boiled using a reflux condenser for 4 h. The suspension was left for sedimentation and cooling to room temperature. The precipitate (lignin) was filtered off and dried at 60 °C to a constant weight. The lignin content was calculated based on the following Equation (2):

$$\%\text{lig} = \frac{\text{Mass of lignin} \cdot (100\% - \%\text{extr})}{\text{Total mass of lignocellulose sample}} \quad (2)$$

where % lig—content of lignin in biomass; % extr—content of extractable ingredients in biomass.

#### 2.6.2. Cellulose and Hemicelluloses Content Determination

The cellulose and hemicelluloses content was analyzed according to the method described by Ioelovich M., due to its simplicity and low cost [32]. Firstly, 0.5 g of dry plant material (after the removal of extractable ingredients) was placed in a round-bottom flask containing 40 mL of distilled water, 0.5 g of sodium chlorate(III), and 1 mL of glacial acetic acid. The mixture was heated under reflux for 45 min, with magnetic stirring (300 rpm). Then, an additional 0.5 g of sodium chlorate and 1 mL of acetate buffer (pH = 3.5) were added, and the mixture was heated for another 45 min, with stirring. After being cooled to room temperature, the suspension was filtered, and the precipitate (holocellulose) was washed with hot distilled water (20 mL). The holocellulose was dried at 60 °C.

Dried holocellulose was placed in a round-bottom flask with 45 mL of 2% HCl. The mixture was heated to reflux for 2 h. After being cooled to room temperature, the suspension was filtered, and the precipitate (cellulose) was washed with hot distilled water (20 mL) and a 1% solution of sodium bicarbonate (20 mL). The cellulose was dried at 60 °C.

The cellulose content in the plant material was calculated based on the following Equation (3):

$$\%\text{cel} = \frac{\text{Mass of cellulose} \cdot (100\% - \%\text{extr})}{\text{Total mass of lignocellulose sample}} \quad (3)$$

where % cel—content of cellulose in biomass; % extr—content of extractable ingredients in biomass.

The hemicelluloses content was calculated based on the following Equation (4):

$$\%\text{hemi} = \frac{(\text{Mass of holocellulose} - \text{Mass of cellulose}) \cdot (100\% - \%\text{extr})}{\text{Total mass of lignocellulose sample}} \cdot 100\% \quad (4)$$

where % hemi—content of hemicelluloses in biomass; % extr—content of extractable ingredients in biomass.

### *2.7. Determination of the Degree of Delignification*

The lignin content in the samples after the pretreatment of biomass with a DES or IL was analyzed using the spectrophotometric method described by Skulcova et al. [33]. The sample was prepared through the mixing of 200 µL of the solution after pretreatment with DES or IL with 400 µL of DES/IL:water mixture (1:1 *v*/*v*). To the rest of the DES solution, 10 mL of water was added, which resulted in the precipitation of lignin. The mixture was centrifuged (10 min, 23 °C, 5000 rpm) and filtered off. Lignin was washed with 10 mL of distilled water, to remove solvent residues, and dried at 60 °C. The prepared lignin was used as a reference material for spectrophotometric determination of lignin content.

The absorbance of the diluted lignin samples was measured at 420 nm. Lignin concentration was determined based on calibration curves (curve equations: for lignin from grass, y = 0.1305x + 0.0083 and $R^2$ = 0.9956; for lignin from sawmill chips, y = 0.8876x + 0.016 and $R^2$ = 0.9996; for lignin from walnut wood, y = 2.466x − 0.0314 and $R^2$ = 0.9945, where x—lignin concentration [mg/mL], y—absorbance). The analysis was carried out in triplicate, against a blank sample consisting of diluted DES or IL. Biomass delignification degree (%del) was calculated based on the following Equation (5):

$$\%\text{del} = \frac{\text{Lignin concentration in sample}\cdot\text{DES or IL volume in delignification}}{\text{Lignocellulose mass}\cdot\text{total lignin content in biomass}}\cdot 100\% \quad (5)$$

### *2.8. HPLC Analysis*

The amount of glucose formed in the enzymatic hydrolysis of cellulose/biomass was analyzed using the HPLC method with a refractive index detector (RID). The system (Agilent 1200) was equipped with the Rezex ROA-Organic Acid H+ (8%) (Phenomenex) column (300 × 7.8 mm). The analysis was carried out at 60 °C, using a isocratic 5 mM solution of $H_2SO_4$, with a flow rate of 0.5 mL/min. Qualitative determination of glucose in the samples was carried out on the basis of the retention time, which was compared with the retention time of the standard glucose sample. The amount of glucose in the studied samples was calculated from the calibration curve (calibration curve equation: y = 583604x + 7587.5 and $R^2$ = 0.9994 where x—glucose concentration [mg/mL], y—the area of glucose peak).

## 3. Results and Discussion

According to the literature data, ILs and DESs can have an activating or inactivating effect on cellulolytic enzymes [19–22,24]. It depends on the type of biomass, as well as the source of the enzyme. To verify the literature data and expand the research to include solvents and biomasses that have not been studied in such depth so far, the effects of several ILs and DESs were studied. A preliminary study was conducted on the model microcrystalline cellulose. The relative activity of the studied enzymes was calculated on the basis of the obtained yield of glucose. Since different enzymatic preparations were applied, the maximum glucose yield, which could be obtained via the standard hydrolysis of cellulose (without the addition of ILs or DESs), in the conditions used in all the experiment, was determined (Figure 1). The amount of biocatalyst was recalculated in units. These values were taken as 100% of the activity of a particular enzyme/preparation.

In previous scientific studies, the amounts of enzymatic preparations are given in terms of mass or activity units; therefore, the influence of the same masses of biocatalysts, on the glucose yield was also determined (Figure 2) [34–36]. It corresponds to different values of units (Table 2). According to the biocatalyst supplier, one unit of enzyme will liberate 1 µmole of glucose from cellulose in 1 h at pH = 5.0 at 37 °C. The activities of particular commercial preparations were taken from the producer data.

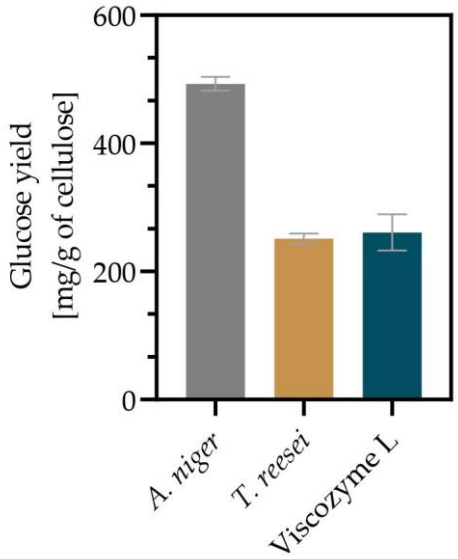


**Figure 1.** Control enzymatic hydrolysis of microcrystalline cellulose in the presence of biocatalysts (100 mg of cellulose, 19 U of biocatalyst, 50 °C, 8 h, 4 mL of 50 mM citrate buffer pH = 5.0).

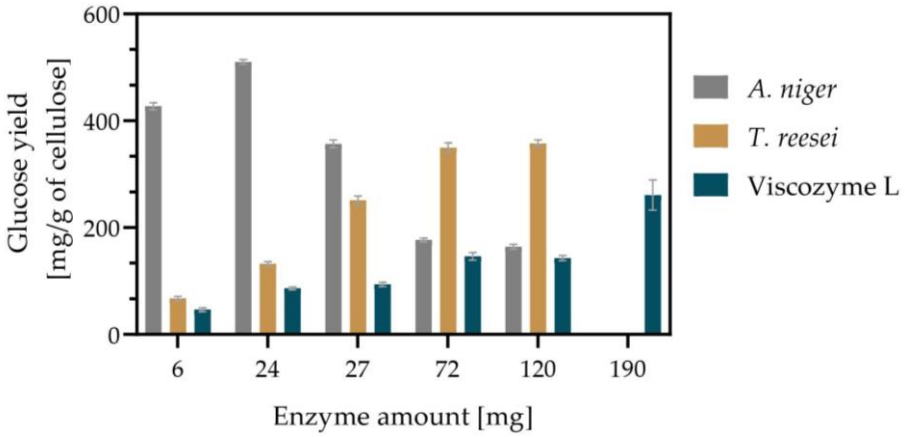


**Figure 2.** The glucose yield in the enzymatic hydrolysis of microcrystalline cellulose with cellulolytic biocatalysts depending on the mass of the added biocatalyst (100 mg of cellulose, 50 °C, 8 h, 4 mL of 50 mM citrate buffer, pH = 5.0).

**Table 2.** The amount of catalysts in mg and corresponding activities in units (according to producer data). The highlighted masses of the biocatalysts were applied in the experimental part of this work.

| **Biocatalyst** | **Mass [mg]** | | | | | |
|---|---|---|---|---|---|---|
| | 6 | 24 | 27 | 72 | 120 | 190 |
| | Corresponding activity [U] | | | | | |
| Cellulase from *A.niger* | 4.8 | 19.2 | 21.6 | 57.6 | 96.0 | 152.0 |
| Cellulase from *T. reesei* | 4.2 | 16.8 | 18.9 | 50.4 | 84.0 | 133.0 |
| Viscozyme L | 0.6 | 2.4 | 2.7 | 7.2 | 12.0 | 19.0 |

The study revealed that the highest yield of glucose can be obtained with 6–27 mg of cellulase from *A. niger*, which corresponds to 4.8–21.6 U, while, for other biocatalysts, a larger amount of enzyme is needed, and the yield of glucose is still lower than that for the cellulase from *A. niger.* In the case of Viscozyme L, it can be explained by the complex mixture of different enzymes, which do not act directly on cellulose. Since a much higher glucose yield was obtained in the presence of 24 mg (19 U) of cellulase from *A. niger*, we

decided to use the amounts of all biocatalysts that correspond to the same activity (19 U) for further studies.

### *3.1. The Influence of ILs on the Activity of Cellulolytic Enzymes in the Hydrolysis of Model Cellulose*

ILs, previously used in many investigations into biomass treatment (namely, [bmim][$BF_4$], [emim][OAc], [hmim][Oac], [bmim][$PF_6$], and [bmim][Cl]), were studied [37–40]. To reflect the environment of the enzymatic hydrolysis of biomass pretreated with ILs, we have added 10% *v*/*v* of ILs to the reaction mixture. We decided to test the impact of 10% *v*/*v* Il addition, in order to compare the obtained data with similar results already published for different ILs, and to expand the accessible dataset [25,41,42]. The process was performed using 50 mM citrate buffer (pH = 5.0), in the presence of the three most-commonly used cellulolytic biocatalysts (cellulases from *A. niger*, and *T. reesei*, as well as the cellulolytic preparation Viscozyme L). Viscozyme L contains β-glucanases, pectinases, hemicellulases, and xylanases, which should enhance the process of biomass conversion. The relative activities of the biocatalysts were calculated based on the amount of glucose obtained after 8 h of the process performed under the same conditions (Figure 3). Furthermore, for cellulase from *A. niger*, investigations were extended to check the effect of a larger amount of the studied ILs (Figure 4).

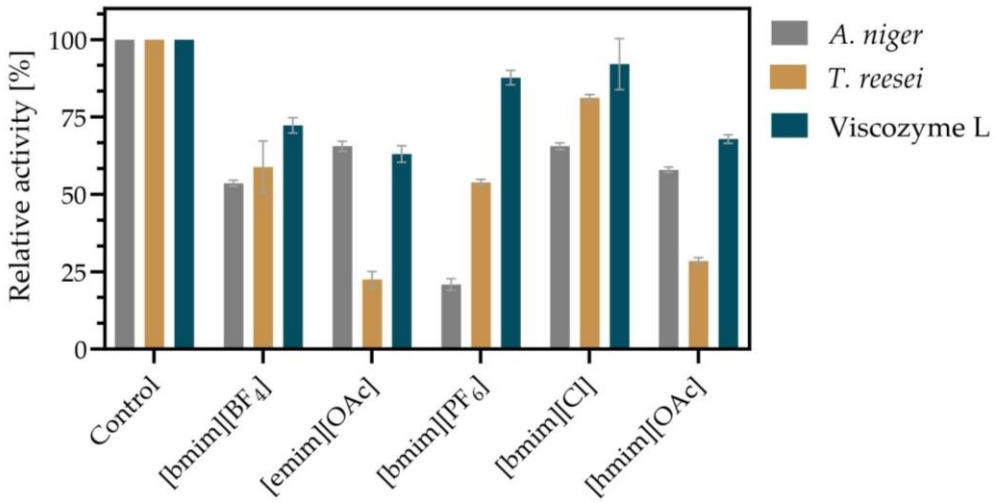


**Figure 3.** The relative activity of cellulolytic biocatalysts in the enzymatic hydrolysis of microcrystalline cellulose, performed with or without the addition of 10% of IL (100 mg of cellulose, 19 U of biocatalyst, 50 °C, 8 h, 3.6 mL of 50 mM citrate buffer, pH = 5.0); the glucose amount obtained in the process without IL was taken as 100% of the relative activity (control).

As can be seen in Figures 3 and 4, each of the applied ILs had a negative effect on the activity of all enzymes examined in the hydrolysis of cellulose. It can be caused by several factors. It is possible that ILs interfere with the enzyme–substrate interactions, occurring at the active site of enzymes [43]. Additionally, the nucleophilicity of anions in ILs makes them capable of creating hydrogen bonds with proteins, which may result in a change in conformation. A higher concentration of IL (20%) may even cause the denaturation of biocatalysts [44,45].

The [bmim][Cl] had a less-negative influence on the enzymatic activity, especially on Viscozyme L (the relative activity was 92.1%). It can be the result of the basicity of hydrogen bonds formed between the IL and enzyme. The high basicity of the H-bond causes a destabilization in the enzyme and lowers its activity. ILs containing the $[Cl]^-$ anion have a lower H-bond basicity than those with the $[OAc]^-$ anion, which results in a lower negative impact on cellulase activity [19]. Another factor that should be taken into consideration is the polarity of an IL. It has been reported that proteins are more stable in

ILs with higher polarity. Because of that, the IL with the $[PF_6]^-$ anion, which is the least polar of all the ILs applied in the experiments, has the most destructive effect on the activity of cellulase from *A. niger* (Figure 4) [44]. Based on the obtained results, it can be assumed that the one-pot process of pretreatment with ILs together with enzymatic hydrolysis is not recommended. A better method is to use ILs as pretreatment solvents and separate them before enzymatic hydrolysis.

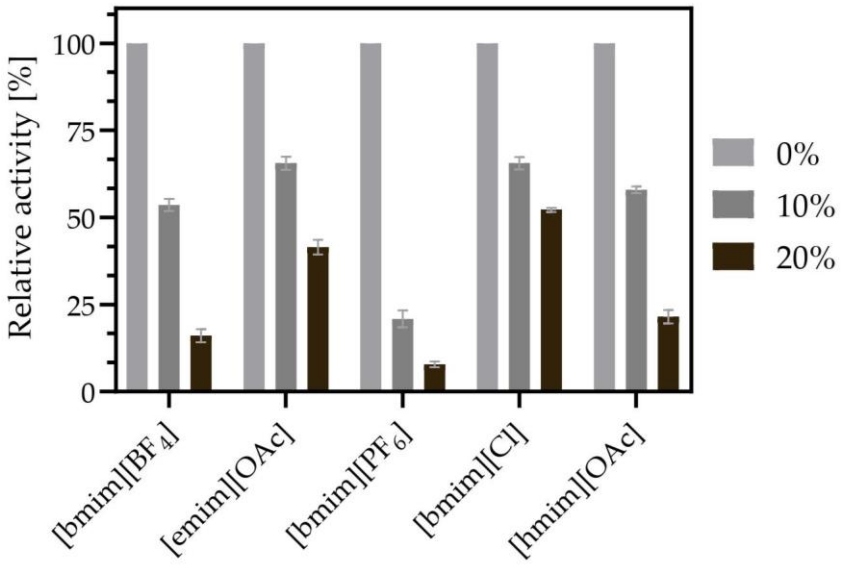


**Figure 4.** The relative activity of cellulase from *A. niger* in the enzymatic hydrolysis of microcrystalline cellulose, performed with 10% and 20% of IL (100 mg of cellulose, 21 mg of *A. niger* cellulase, 50 °C, 8 h, 50 mM citrate buffer, pH = 5.0); the glucose amount obtained in the process without IL was taken as 100% of the relative activity.

### *3.2. The Influence of DESs on the Activity of Cellulolytic Enzymes in the Hydrolysis of Model Cellulose*

DESs are considered even "greener" solvents, capable of dissolving different fractions of lignocellulose. Investigations aiming to determine the effect of DESs on the activity of cellulolytic enzymes were performed. DESs (10% *v*/*v*) were added to the enzymatic hydrolysis of cellulose. It was reported that in the presence of excess water (in most cases above 40%), DESs are prone to lose their hydrogen-bond-based structure and properties [46,47]. In the case of our research, the addition of 20% of DES to the aqueous buffer most likely led to its decomposition. However, the aim of this study was to check how the presence of DES residues (or components) after biomass pretreatment can affect the subsequent enzymatic hydrolysis. The DESs applied in our investigations are often used for biomass pretreatment before further transformation (e.g., enzymatic hydrolysis) [48–53]. Typically, pretreated biomass should be thoroughly washed before enzymatic hydrolysis to remove DES residues that may have an inactivating effect on the biocatalysts. It is important to determine whether this step can be limited, and a small amount of DES (or its components) may remain in the material. This would reduce the amount of water needed to wash the biomass. In this study, the impact of small amounts of different DESs on the activity of chosen biocatalysts was investigated. Figure 5A,B show the relative activity of biocatalysts in the presence of DESs (with choline chloride and betaine as the HBA, respectively), compared to the control sample (without DESs). Several HBDs were tested, namely, lactic acid (Lac), levulinic acid (Lev), glycerol (Gly), ethylene glycol (Et), and urea (U). The ratio of HBA:HBD is presented in Table 1.

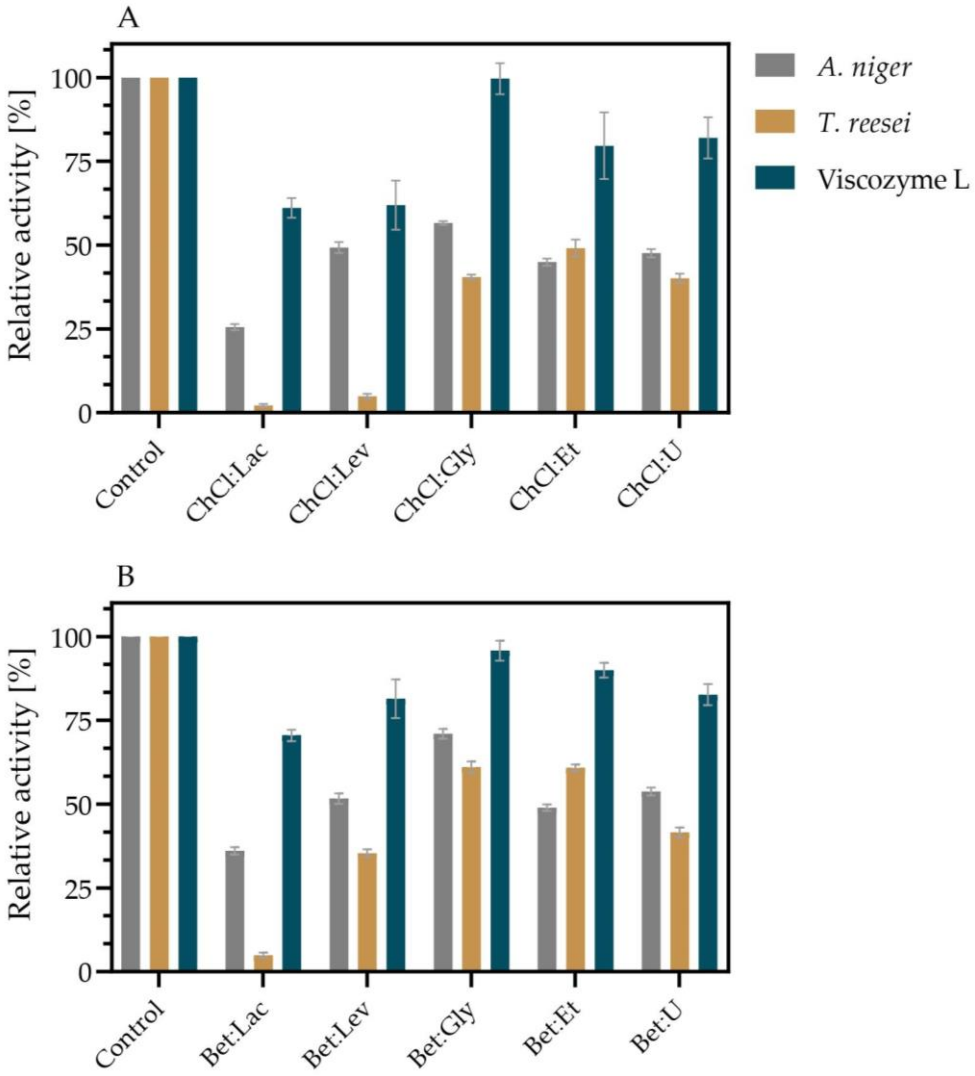


**Figure 5.** The relative activity of cellulolytic biocatalysts in the enzymatic hydrolysis of microcrystalline cellulose, performed with or without the addition of 10% of DESs based on choline chloride (**A**) and betaine (**B**) (100 mg of cellulose, 19 U of biocatalyst, 50 °C, 8 h, 3.6 mL of 50 mM citrate buffer, pH = 5.0); the glucose amount obtained in the process without DESs was taken as 100% of the relative activity (control).

DESs have a crucial impact on the activity of biocatalysts, which is usually negative. The decrease in the activities of the cellulases from *A. niger* and *T. reesei* is the most significant. The Viscozyme L preparation seems to be more resistant to DESs. It contains several enzymes that may not be as sensitive to added chemicals. However, it should be emphasized that in the presence of Viscozyme L, in general, less glucose can be produced from cellulose, compared to cellulase from *A. niger* (Figure 1). In almost all systems (except for ChCl:Et and Bet:Et), cellulase from *T. reesei* is the most sensitive enzyme, compared to the other biocatalysts. It is particularly visible in the DESs based on acids. In the presence of 10% of ChCl:Lac, the relative activity decreased to 2.1% of the initial value. Similar trends can be observed in the presence of ChCl:Lev. Data published in the literature suggest that in the presence of ChCl:Lac, the stability (especially the thermal stability) of cellulase from *T. reesei* also decreases [25]. Generally, the impact of HBD in DESs on enzymatic activity and stability is what is mostly discussed in the literature. We have proven that HBA is also important. DESs with betaine as the HBA seem to be less harmful to the enzymes. The most visible differences can be observed between the relative activity of cellulase from *T. reesei* in ChCl:Lev and Bet:Lev (4.9% and 35.3%, respectively). Betaine-based DESs have

been proven to enhance the activity of other enzymes, such as laccases, peroxidases, and cellulases. In addition, they can improve the thermal stability of biocatalysts [26–29]. In the case of Viscozyme L, the presence of ChCl:Gly and Bet:Gly does not significantly affect the enzymatic activity (the relative activity for ChCl:Gly is 99.7%; for Bet:Gly, it is 95.8%). The influence of different amounts of DESs, in the range of 0–20%, was also examined in the hydrolysis of cellulose with cellulase from *A. niger*. The results are presented in Figure 6.

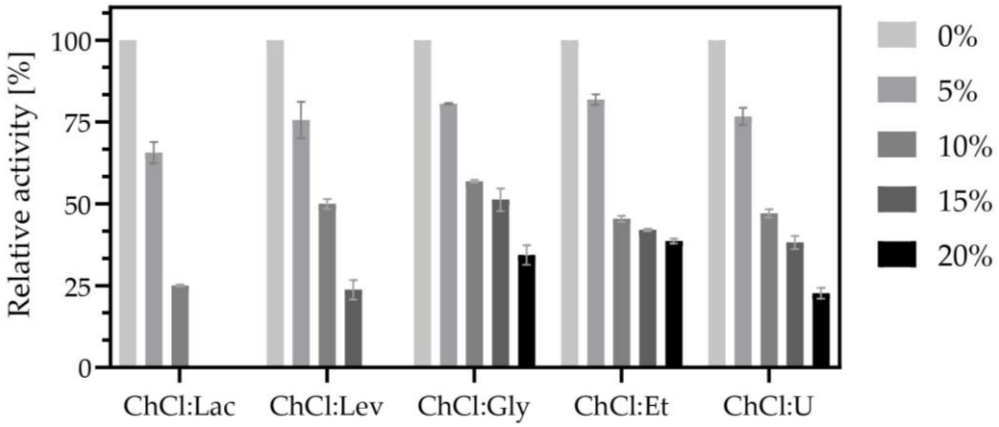


**Figure 6.** The relative activity of cellulase from *A. niger* in the enzymatic hydrolysis of microcrystalline cellulose, performed with different concentrations of DESs based on choline chloride (100 mg of cellulose, cellulase from *A. niger* 19 U, 50 °C, 8 h, 50 mM citrate buffer, pH = 5.0). The glucose amount obtained in the process without DESs was taken as 100% of the relative activity (control).

Changes in relative activity depend not only on the amount but also on the type of DES used. Dramatic changes are mostly visible in the case of ChCl:Lac. The addition of more than 15% (*v*/*v*) of that DES (Figure 6) caused the total inactivation of cellulase from *A. niger*. A similar phenomenon was observed in enzymatic hydrolysis with the addition of 20% of ChCl:Lev. It can be assumed that DESs with acidic HBD (or their components) lower the pH of the system, which makes it impossible to carry out the process under optimal conditions [54]. We have determined the pH values of the reaction medium (50 mM citrate buffer, pH 5.0) after the addition of 10% DESs and, in all cases, it decreased (ChCl:Lac to pH 3.10; Bet:Lac to pH 3.71; ChCl:Lev to pH 3.95; Bet:Lev to pH 4.38). The addition of other DESs (e.g., ChCl:Gly) did not change the pH. The results show differences in the pH values of the medium with 10% of DESs based on choline chloride vs. betaine. It may be a result of the nature of betaine (zwitterion nature). In the case of ChCl:Et, the addition of more than 10% of DES does not significantly change the relative activity. Out of all the evaluated solvents, those based on glycerin as the HBD showed the lowest impact on enzymatic activity (when up to 15% was added). Glycerin is often applied as a protein-stabilizing agent, so the negative influence of glycerin-based DESs is limited [55,56]. The results differ from those obtained by Gupta et al. [41]. The authors reported that the cellulase from *A. niger* retained 70% of its original activity, even with a 70% (*v*/*v*) addition of ChCl:Gly and ChCl:Et. In our experiments, a similar relative activity was obtained with the addition of only 5% of DESs. DESs may lower the stability of cellulase, which affects the efficiency of the process. In molecular dynamics simulations reported by Kovács et al., many factors influencing the activity of the enzyme due to DES were evaluated, including changes in enzyme structure, distances between amino acids within the catalytic triad, and hydrogen bonds creation between the biocatalyst and DES. The authors concluded that DES did not have any significant impact on enzymes, so the change in its enzymatic activity must originate from interactions between DES and the substrate [57]. DESs have also been reported to have no significant impact on cellulose morphology [58]. However, it has been proven using Raman spectroscopy that choline chloride in DES interacts with cellulose fibers through electrostatic forces, resulting in a change in charge on the surface of the cellulose [59]. The mechanism of enzymatic hydrolysis of cellulose is not fully defined. The

interaction between cellulases and cellulose begins with the adsorption of enzymes on the polymer surface, via hydrogen bonding, hydrophobic interactions, van der Waals forces, and also electrostatic charge [60,61]. On the surface of proteins, which bind to cellulose, charged amino acids are present, able to bind to C1 and C4 carbons in glucose units in this polymer [61]. If the cellulose surface charge is modified by DES, then the electrostatic interaction between cellulose and the enzyme could be disrupted, which may result in a lower efficiency in the biocatalytic reaction. This hypothesis requires further studies.

### *3.3. Biomass Composition*

The study on the enzymatic hydrolysis of the chosen lignocellulosic biomass was preceded by the determination of the content of three major components: cellulose, lignin, and hemicelluloses (Table 3).

**Table 3.** Cellulose hemicelluloses, and lignin content in biomass.

| Biomass Type | Cellulose Content [%] | Hemicelluloses Content [%] | Lignin Content [%] |
|---|---|---|---|
| Sawmill chips | 51.2 ± 0.2 | 18.3 ± 0.5 | 27.3 ± 0.4 |
| Grass | 32.4 ± 0.3 | 27.1 ± 0.6 | 18.7 ± 0.3 |
| Walnut wood | 42.7 ± 0.2 | 23.7 ± 0.7 | 29.5 ± 0.3 |
| Walnut leaves | 31.2 ± 0.4 | 30.1 ± 0.4 | 20.4 ± 0.2 |
| Walnut shells | 28.3 ± 0.5 | 21.4 ± 0.4 | 32.5 ± 0.5 |
| Walnut husks | 35.2 ± 0.1 | 27.9 ± 0.4 | 23.9 ± 0.6 |
| Rye straw | 27.1 ± 1.2 | 22.5 ± 1.0 | 21.4 ± 0.4 |

Among the biomass studied, the highest content of cellulose was determined in the wood (sawmill chips and walnut wood), as well as grass, walnut husks, and leaves. This biomass requires pretreatment due to the complicated access to the desired biopolymer. Since some types of biomass studied have not been widely explored, we performed enzymatic hydrolysis, using the biocatalysts previously applied to cellulose (Figure 7).

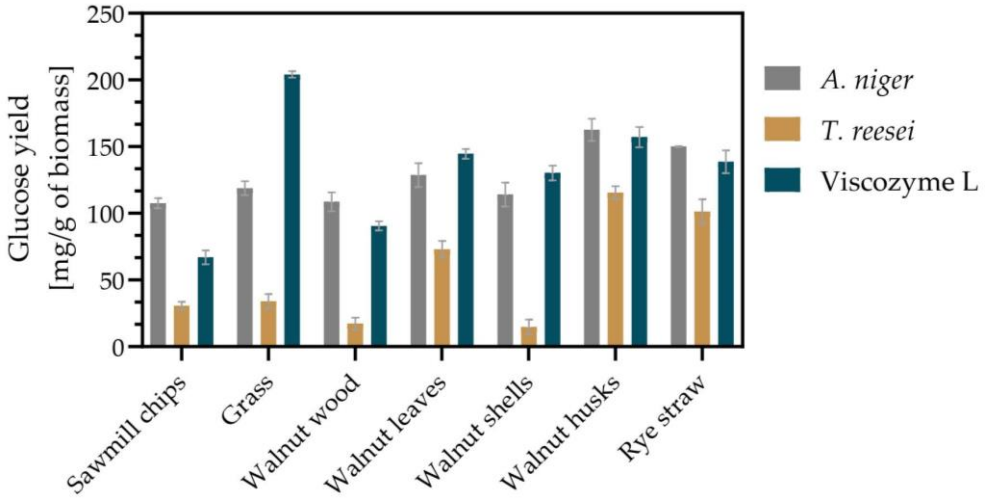


**Figure 7.** Glucose yield [mg/g of biomass] in the enzymatic hydrolysis of biomass, in the presence of selected biocatalysts (100 mg of biomass, biocatalyst 19 U, 50 °C, 8 h, 4 mL of 50 mM citrate buffer, pH = 5.0).

The highest yield of glucose, under the conditions used, and in the presence of cellulase from *A. niger* and Viscozyme L, can be obtained from grass, followed by walnut husks, walnut leaves, and rye straw. In the case of biomass hydrolysis, the significant increase in the glucose yield in the presence of Viscozyme L is connected with the specificity of the mixture of enzymes in this preparation. According to the information provided by the supplier, Viscozyme L contains arabanase, cellulase, β-glucanase, hemicellulase, and xylanase.

These enzymes are capable of hydrolyzing not only cellulose but also hemicelluloses, which makes the biomass transformation more efficient. Hemicelluloses are biopolymers that contain six-carbon and five-carbon sugars, including glucose [62]. During biomass processing with Viscozyme L, glucose can be produced via the hydrolysis of cellulose and also hemicelluloses, leading to a higher total glucose yield, compared to processes with *A. niger* and *T. reesei* cellulases. In this process, other saccharides, such as cellobiose, arabinose, and xylose are also produced. However, to compare the performance of each of the biocatalysts examined, we decided to measure glucose concentration exclusively. In the case of cellulose as a substrate, the other enzymes did not participate in the process (Figure 1). Three types of biomass (sawmill chips, walnut wood, and grass) were selected to evaluate the influence of pretreatment with the selected ILs and DESs on the glucose yield obtained in enzymatic hydrolysis with cellulase from *A. niger* (Figure 8). Waste grass and sawmill chips were selected due to their different structures and textures of lignocellulose and high cellulose contents. Additionally, these types of waste biomass are widely available. Walnut wood is another example of an abandoned hard biomass that is problematic to compost and process. We chose it to explore the possibility of transforming it into valuable products.

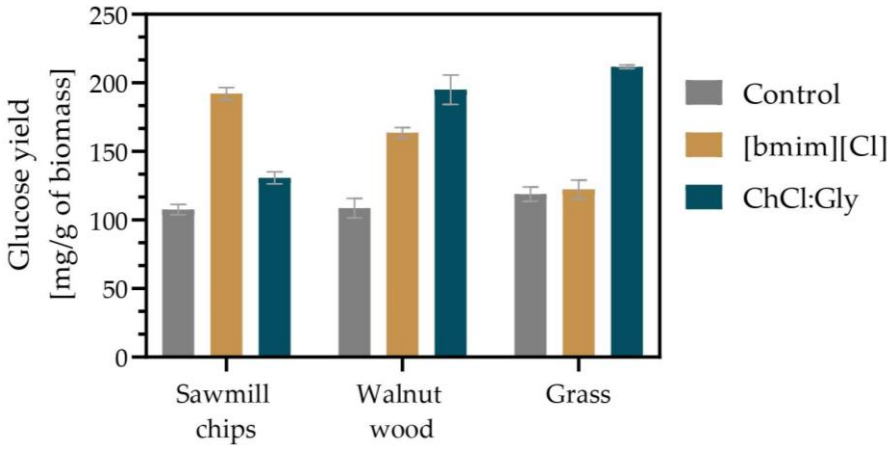


**Figure 8.** Glucose yield [mg/g of biomass] in enzymatic hydrolysis of biomass with *A. niger* cellulase, after pretreatment with [bmim][Cl] or [ChCl:Gly] (pretreatment: 250 mg of biomass, 90 °C, 3 h, 400 rpm; enzymatic hydrolysis: 100 mg of biomass, 50 °C, 8 h, 4 mL of 50 mM citrate buffer, pH = 5.0); the glucose yield obtained in the process without IL or DES was taken as a control.

The results obtained prove that pretreatment with [bmim][Cl] and ChCl:Gly positively influenced the yield of glucose obtained. In the case of sawmill chips and walnut wood, an increase as high as 100% in the glucose yield is observed. Even better effects can be observed for grass pretreated with DES. It is worth mentioning that the obtained results are not proportional to the degree of delignification obtained in the pretreatment with ChCl:Gly or [bmim][Cl], which was not the same for all the types of biomass. The most efficient removal of lignin was observed for grass (the degree of delignification with DES was 53.7% and with IL was 31.4%) and sawmill chips (43.5% with DES, 25.2% with IL). Less lignin was removed from walnut wood (12.0% with DES and 10.4% with IL). In this case, DES is more effective than IL in biomass delignification. In some reports, DESs and ILs are presented as equally effective solvents for lignin dissolution and extraction from plant material [58]. However, ILs can also interact with other biomass components—cellulose and hemicelluloses—resulting in their dissolution or even processing to other chemicals, e.g., furfural, 5-hydroxymethylfurfural, levulinic acid [63]. On the contrary, DESs based on choline chloride as HBAs are reported to be poor cellulose solvents and they do not react with biomass polymers other than lignin [58,64]. This may be the explanation for the lower glucose yields obtained in the enzymatic hydrolysis of biomass pretreated with [bmim][Cl] (in the cases of walnut wood and grass). Because of possible degradation of cellulose during pretreatment with IL, its amount in material is lower in the next step,

enzymatic hydrolysis. It can be assumed that ILs and DESs act not only by removing lignin but also by changing the structure of biomass, making it more susceptible to the action of cellulase from *A. niger*. Despite the fact that DESs are not suitable for cellulose dissolution, they can create hydrogen bonds with cellulose fibers and disturb the bonding within the rigid structure of the cellulose, which also refers to the properties of ILs [65]. Thus, though there was a low degree of biomass delignification, pretreatment with IL and DES increased the efficiency of enzymatic hydrolysis.

Additional experiments were carried out on grass, using ChCl:Gly and Viscozyme L, which gave the best results in the control experiment (Figure 7). Two methods were investigated: one step (I step)—grass was processed with Viscozyme L, in medium containing 10% $v/v$ of ChCl:Gly (added to citrate buffer); two steps (II step)—biomass was first pretreated with DES, at 90 °C, washed (until DES could not be detected in the material via analytical methods such as HPLC and UV–Vis analysis of the solution after washing), dried, and then subjected to enzymatic hydrolysis with Viscozyme L. The results are presented in Figure 9.

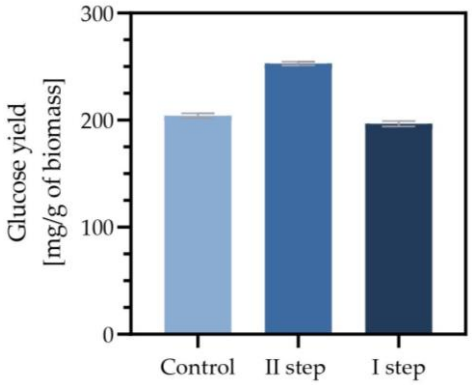


**Figure 9.** Glucose yield [mg/g of biomass] obtained in grass processing with ChCl:Gly and Viscozyme L (I step—100 mg of biomass, 19 U of Viscozyme L, 3.6 mL of 50 mM citrate buffer pH = 5.0, 10% $v/v$ of ChCl:Gly, 50 °C, 8 h); II step (pretreatment: 250 mg of biomass, 90 °C, 3 h, 400 rpm; enzymatic hydrolysis: 100 mg of pretreated biomass, 19 U of Viscozyme L, 50 °C, 8 h, 4 mL of 50 mM citrate buffer pH = 5.0.); the glucose yield obtained in the process without IL or DES was taken as a control.

The obtained results show that the one-step process with only 10% of DES added to the enzymatic hydrolysis is not sufficient to dissolve lignin and make the polysaccharides more susceptible to biocatalysts. Furthermore, the optimum temperature for the enzyme activity (50 °C) is too low to loosen the biomass structure. However, it is worth mentioning that in the presence of ChCl:Gly, the biocatalyst was not inactivated, as comparable glucose yields were obtained (204 mg/g for the control and 197 mg/g for the I step). An improvement in glucose yield was observed in the two-step process. Delignification enhances the access of the enzyme to cellulose and hemicelluloses. It also limits side reactions between the enzyme and lignin [34]. However, the question of the economic aspect of such a method has to be asked. The pretreatment of the biomass, which is followed by enzymatic transformation, usually requires a washing step, to remove the residual solvent. It generates additional costs for solvents, operations, and disposal of waste. In the experiments presented, since ChCl:Gly does not inactivate Viscozyme L, this step can be limited to the simple filtration of pretreated biomass. This way, two-step biomass processing would be more cost and time effective.

The results obtained with Viscozyme and DES prompted us to also check the effect of the pretreatment of the grass with [bmim][Cl], followed by enzymatic hydrolysis with Viscozyme L. Figure 10 presents the comparison of the results obtained in the enzymatic hydrolysis with Viscozyme L, using waste grass pretreated with DES (ChCl:Gly) and IL ([bmim][Cl]).

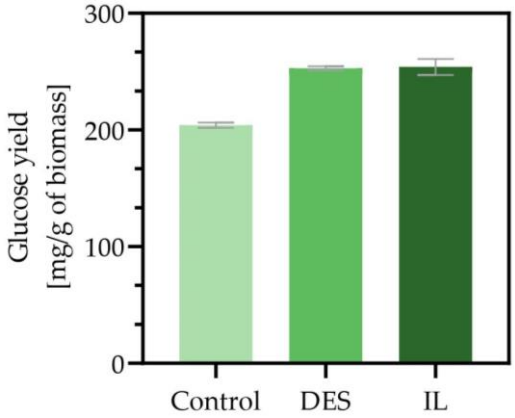


**Figure 10.** The glucose yield [mg/g biomass] obtained in the enzymatic hydrolysis of waste grass with Viscozyme L, preceded by pretreatment with ChCl:Gly or [bmim][Cl] (pretreatment: 250 mg of biomass, 5 mL of DES/IL, 90 °C, 3 h, 400 rpm; enzymatic hydrolysis: 100 mg of biomass, 19U of Viscozyme L, 50 °C, 8 h, 4 mL of 50 mM citrate buffer, pH = 50); the glucose yield obtained in the process without IL or DES was taken as a control.

Although [bmim][Cl] did affect the activity of Viscozyme L in the hydrolysis of microcrystalline cellulose (Figure 3), it seems to be useful for the pretreatment of biomass before enzymatic hydrolysis. The efficiency of pretreatment with DES and IL is similar. Hydrolysis of pretreated grass in the presence of Viscozyme L resulted in similar yields of glucose (252.9 mg/g and 254.0 mg/g for DES and IL, respectively). The positive effect of [bmim][Cl], similar to ChCl:Gly, is the effect of partial lignin dissolution and removal [66]. However, ILs can also dissolve hemicelluloses, which can influence the further enzymatic hydrolysis of cellulose. In addition, pretreatment can increase the contact surface area between cellulose and cellulases [67].

## 4. Conclusions

The sustainable development of chemicals, fuels, and energy production requires changes in resources and technologies. Lignocellulosic biomass transformation can result in obtaining many high-added-value products. The key factor is to develop ecological, efficient system of biorefineries. One of the interesting methods is the enzymatic transformation of biomass. However, it requires the pretreatment of lignocellulose. Novel, ecological methods of chemical pretreatment use green solvents, such as DESs or ILs, which can remain in the biomass taken to the enzymatic conversion. The influence of ILs and DESs on the enzymatic activity of cellulolytic biocatalysts depends on the structure of these solvents, as well as the structure and type of biomass. We have shown that in the case of model cellulose hydrolysis, the addition of the majority of evaluated ILs and DESs decreased the activity of the studied biocatalysts (cellulases from *A. niger*, *T. reesei*, and Viscozyme L). Among all the enzymes examined, Viscozyme L presented the highest tolerance for DESs and ILs studied. For some solvents, its relative activity remained almost unchanged (for ChCl:Gly 99.7%, Bet:Gly 95.8%, and [bmim][Cl] 92.1%).

In the case of biomass, where access to cellulose is difficult because of the presence of lignin and hemicelluloses, the pretreatment step is crucial. It was proven that DESs and ILs are useful solvents for pretreatment of lignocellulose, before enzymatic digestion. The removal of lignin (or even part of lignin) facilitates the contact between the biocatalyst and cellulose. Depending on the enzyme used and the type of biomass, glucose production can be significantly increased (even by 100%). It is worth pointing out that Viscozyme L seems to be a suitable biocatalyst for biomass processing because of the presence of different enzymes acting on the different biopolymers in lignocellulose. It improves the digestion of the plant cell wall, which results in better access to cellulose. Using this biocatalyst, it was possible to transform ~78% of the cellulose present in the pretreated biomass. Since ILs and DESs have a significantly lower impact on the natural environment compared to the

traditional chemicals used for the pretreatment of biomass, they are promising solvents for the ecological transformations of lignocellulose to valuable products.

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