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The Influence of Cellulose Type on Enzymatic Hydrolysis Efficiency

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Cellulose is an important structural component of the primary cell walls of green plants. It is mainly used for paper production, as well as for the production of a wide-range of other products. Cellulose properties depend on the lengths of the polymer chains with the number of glucose units. As the bonds between the glucose molecules are very strong, the process of cellulose degradation as compared with the other polysaccharides is relatively difficult.

Several types of cellulose are known depending on the types of trees and their structures. Their degradation is carried out in several ways from which enzyme technology is one of the more sustainable. The efficiency of enzymatic hydrolysis regarding lingo-cellulosic materials is a key step in the development of bio-refineries.

The aim of this study was to examine the possibilities and conditions for the enzymatic degradation of crude cellulose originating from three species of trees (coniferous, deciduous trees and eucalyptus). The structures of the deciduous and conifers are different. The ability of cellulose fibers for degradation is largely dependent on the relationships between the amorphous and crystalline regions. For the hydrolysis of cellulose enzyme cellulase was used. Enzymatic hydrolysis of cellulose is strongly dependent on the pH of the solution, therefore its optimum was found and the experiments were carried out within a narrow area of pH values. Within the so-called "acidic" medium, processes are much faster than in neutral or basic media. Based on comparisons between the reaction yields the type of cellulose with a more effective and fast degradation to glucose was determined.

1. Introduction

Climate changes, as observed over recent years, have been mainly due to increased concentrations of greenhouse gases, the most of which is carbon dioxide (Brethauer and Wyman, 2010). The issue is actual at the global level therefore an increased interest in the development of alternative fuels is understandable. Economically, environmentally and strategically the most significant amongst alternative fuels is ethanol. It is generally obtained by the fermentation of sucrose from sugar beets and molasses or glucose, which is present in crops based on starch (maize, wheat ...) (Yamashita et al., 2008). Over the last few years stocks based on raw starch have become insufficient for meeting the growing demand for fuels. In addition, excessive use of food crops for fuel production has led to environmental problems in terms of forests being depleted in order to obtain additional agricultural land. Therefore, bioethanol is produced from the cheapest and most widespread biomass in the world, cellulose, which represents the most promising replacement for fossil fuels. It is found in wood, paper, agricultural residues, municipal waste, energy crops... Cellulosic materials can be converted further into a number of important industrial chemicals but they are mainly a basis for bioethanol production (Samayan and Schall, 2010). Conversion of cellulose into ethanol requires two stages. The first one is hydrolysis of polysaccharides into sugars and the second fermentation of sugars to ethanol.

Generally lignocellulosic materials are comprised of three main components: cellulose, hemicellulose and lignin (Datta et al., 2010). Cellulose is a crystalline polymer consisting of glucose units linked by β-1,4-glycosidic linkages. Molecules of cellulose are therefore linear. Approximately 1200 to 1400 molecules of

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cellulose are linked together with hydrogen bonds forming fibers and microfibrils. This structure gives cellulose its high strength (Zavrel et al., 2009). Due to its high resistance cellulose represents a major technological problem. The reaction can be catalysed by diluted acid (Dussán et al., 2014) or the cellulase enzymes. The porosity of cellulose materials (accessible surface areas), the crystallinity of cellulose fibers, as well as lignin and hemicellulose content, influence their effectiveness (Sun and Cheng, 2002). The presence of the latter, blocks the access of enzyme cellulase to cellulose, thereby decreasing efficiency during hydrolysis. Pre-treatment of cellulosic materials, which increases the porosity thus reducing crystallinity and the removals of lignin and hemicellulose is required processing step before the hydrolysis reaction. Consequently, biological, physical, chemical and physicochemical methods for cellulose pretreatment have been developed but only some of them are effective (Zhao et al., 2009). Thus, for example, cellulose after grinding remains insoluble material with β-1, 4-glycosidic bonds but it contains many amorphous areas (Zhao et al., 2005). Pre-treatment has to meet certain requirements. It should enhance the formation of sugars to avoid degradations of hydrocarbons, to prevent the formation of by-products and to be focused on the first two stages, which are necessary for the production of bioethanol. Cellulose is a crystalline structure in which the molecules are closely linked with hydrogen bonds and Van der Waals forces. Such a structure makes it difficult to access chemicals and enzymes. We strive for the highest possible conversion of cellulose to glucose, as this would allow the profitable production of bioethanol. Experiments were performed with crude cellulose from three types of trees. Optimal conditions were determined for cellulose decomposition and compared the efficiencies between hydrolysis reactions in all three cases.

2. Experimental

2.1 Materials

In this work the cellulose of three tree species was used: coniferous, deciduous trees and eucalyptus, because they are the more prevalent in the market. Coniferous and deciduous cellulose differ by size and shape of the fibers. A coniferous cellulose fiber is (2 - 4) mm long with a thickness of (0.02 - 0.07) mm. A deciduous cellulose fiber is short (1 mm) and thin (0.03 mm). Neither of both species cause problems during dispersion and can be easily transported in slurry form (Albrecht et al., 2003). Otherwise, coniferous have greater content of lignin, whilst the contents of pure cellulose in both types are approximately equal.

All chemicals: microcrystalline cellulose (20 μm, Aldrich), citric acid (w ≥ 99.5 %, Sigma-Aldrich), sodium phosphate dodecahydrate (w ≥ 99 %, Kemika Zagreb), glucose (puriss, Sigma-Aldrich), GOG -PAP glucose reagent (Roche / Hitachi), acetone (w ≥ 99.5 %, Chem-Lab), acetic acid (w ≥ 99.8 %, Fluka), phosphoric acid (w = 85 %, Kemika Zagreb), cellulase from Aspergillus sp. (Sigma) that are commercially available, were used without further purification.

2.2 Equipment

Reactor system EasyMaxTM

Experiments were carried out within reactor system EasyMaxTM. It is a system of two temperature-controlled 100 mL reactors, which are easy to use, effective, enabling repeatability, lower the cost of experimental work and provide high quality information. A highly accurate heating-cooling system controls the temperature of the reaction mixture that is possible within the range of -28 °C to 183 °C. The system works reliably even in the cases of highly exothermic reactions, when maintaining a constant temperature due to kinetic measurements is especially important. In addition the temperature rotational frequency of the stirrer and the addition of chemicals are automated. During the reaction EasyMaxTM records the values of process parameters that can be transferred to other media for further processing.

UV-VIS spectrophotometer

The concentration of glucose was measured using a Varian UV-VIS spectrophotometer.

2.3 Methods

Pre-treatment of cellulose

The crude cellulose was treated before performing the hydrolysis. From amongst several types of pre-treating processes, we first selected the physical one, that is, the mechanical grinding where we reduce the cellulose crystalline areas. For this purpose a simple kitchen grinder was used. Afterwards the cellulose fibers were homogenised using a homogeniser.

In addition, an acid pre-treatment was implemented, which is very effective with concentrated acids (H_2SO_4 and HCl), but due to their toxicity and corrosiveness their use is rather avoided (Sun and Cheng, 2002). After the reaction, acids have to be further recycled. It is better to use diluted acids, where high reaction speeds are achieved as well as improved efficiency. Temperature of pre-treatment was also meaningful. At low temperatures low yields of hydrolysis are expected due to sugar decomposition. Moderate or high temperatures are favorable. An acid pretreatment with 85 % phosphoric acid (H_3PO_4) was used. 3 g of ground cellulose was added to 50 mL of phosphoric acid and stirred for 2 hours at temperature, ϑ = 50 °C and a rotational frequency of the stirrer f_m = 200 min^{-1}. Then, the solution was quickly poured into ice water, which was vigorously stirred. When the cellulose was precipitated, it was separated from the suspension by centrifugation. For drying purposes the cellulose was further washed for three times with water and finally twice with acetone.

Afterwards, the solution was quickly poured into iced water which was then stirred. When the cellulose was eliminated, it was separated from the suspension by centrifugation. For drying purposes the cellulose was further washed three times with water and finally twice with acetone.

We also tried to pre-treat the cellulose with ionic liquid BMIMCl. 0.25 g of cellulose powder and 5 g BMIMCl was left for 2 h within an ultrasonic bath at a temperature, ϑ = 90 °C. 100 mL of water was added, and placed back in an ultrasonic bath for 2 h. The solution was filtered and the remaining pulp was dried in air and then in an exicator.

Experimental work

The reactions of hydrolysis were carried out according to the following procedure. 0.1 g of dry cellulose was added to 50 mL of buffer. The solution was heated up to a temperature of ϑ = 45 °C, at rotational frequency of the stirrer f_m = 200 min^{-1}. After reaching the temperature steady-state, we added 500 μL of the enzyme cellulase. After 24 h of hydrolysis the sample was taken for analysis of the resulting glucose.

3. Results and discussion

3.1 Eukaliptus

This study started by searching for the best type of cellulose (amongst three selected) and the conditions for the more efficient course of the hydrolysis reaction. The first set of experiments was carried out with eucalyptus cellulose, which had previously been treated mechanically or thermally. The reaction of hydrolysis was carried out at a temperature ϑ = 45 °C and the rotational frequency of the stirrer f_m = 200 min^{-1}. In each experiment a volume of 500 μL of cellulase was added to the solution. Increasing the amount of enzyme could increase the hydrolysis conversion but at the same time would increase the process cost. According to the literature reports (Sun and Cheng, 2002), cellulase enzyme dosages in hydrolysis vary from 7 to 33 FPU/g substrate which was consistent with our value. The duration of reaction was t = 24 h. We determined the conversions of the reaction in the cases of ground and boiled cellulose. Citric acid (c = 0.1 mol/L) and sodium phosphate (c = 0.2mol/L) were used for the preparation of the buffer. Each reaction was replicated three times. Table 1 shows the absorbance calculated concentrations of glucose and proper conversions (average of three repeated batches).

Table 1: Conversion of eucalyptus cellulose – mechanical and thermal treatment

Pre-treatment	$c_{glucose}$/mmol/L	X/ %
grounded	5.57	5.02
boiled (3 h) + grounded	6.34	5.71

3.2 Conifers

The process was repeated with coniferous cellulose. In this case a series of reactions regarding hydrolysis of cellulose were performed, which had been pre-treated with phosphoric acid. The conversions of the coniferous cellulose after three different pre-treatment methods are given in Table 2.

Table 2: Conversion of coniferous cellulose – mechanical, thermal and chemical treatment

Pre-treatment	$c_{glucose}$/mmol/L	X/ %
grounded	4.87	4.38
boiled	6.29	5.66
H_3PO_4	12.61	11.35

From two series of experiments (Tables 1 and 2) it was evident that the conversions were approximately the same, regardless of whether the cellulose was previously grounded or boiled. The conversion of acid pretreated cellulose was almost twice higher. Yet in all cases the conversions were too low (4 % to 11 %) for the possible continuation of the fermentation under the same conditions. Vibratory ball milling has been found to be most effective in breaking-down the cellulose crystallinity and improving the digestibility (Sun and Cheng, 2002). As we didn't have such equipment, an ordinary kitchen mill was used in our case. We supposed at least a similar effect regarding the extensiveness of crystallinity regions. The obtained average size of the particles after grinding was (2 - 3) mm.

Searching for the best conditions for cellulose conversion was continued by the hydrolysis of coniferous cellulose, which was homogenised this time. A slight difference was observed between the homogenised cellulose and non-homogenised one (Table 3) but the conversions were again much lower than was expected.

Table 3: Conversion of coniferous cellulose - homogenization

Pre-treatment	c_{glucose}/mmol/L	X/ %
non-homogenized	7.24	7.24
homogenized	8.75	8.76

Having determined that the homogenisation of the cellulose had not contributed to higher conversions, the cellulose of the deciduous trees was not tested under the same conditions. According to the similar structure of all three types of cellulose, low yields were expected in this case also.

Therefore it was concluded from the preliminary experiments that low amounts of glucose production were achieved. As almost complete saccharification of raw paper sludge was reported in different sources (Sun and Cheng, 2002), our results could be attributed to incorrect choices of pretreatment parameters. The fact that we were dealing with raw cellulose, indicated us to further testing. Many factors could affect the enzymatic hydrolysis of cellulose. Amongst others pH could improve the reaction yield.

3.3 Microcrystalline cellulose

We started with a new set of experiments with a sodium acetate buffer. This time the hydrolysis reaction was initially performed on microcrystalline cellulose, which is clean, without lignin and hemicellulose. Under the same process conditions (the duration of the reaction, t = 24 h) for all three repetitions high conversions were achieved but with slightly worse reproducibility - Table 4.

Table 4: Conversion of microcrystalline cellulose

Microcrystalline celullose	c_{glucose}/mmol/L	X/ %
1. experiment	9.71	87.47
2. experiment	9.28	83.53
3. experiment	9.58	81.72

3.4 Comparison

The next experiments were performed under the same conditions (sodium acetate buffer) with celluloses of all three trees species. The cellulose had been pre-treated with phosphoric acid. Each reaction was repeated three times. The changed process conditions enabled promising conversions of all three types of cellulose. They are shown in Table 5.

Table 5: Conversion of eucalyptus, coniferous and deciduous cellulose - sodium acetate buffer

Cellulose type	c_{glucose}/mmol/L	X/ %
eucalyptus	10.45	94.04
	10.52	94.67
	10.12	91.07
coniferous	8.69	79.27
	8.91	80.22
	9.65	86.85
deciduous	9.15	82.33
	9.71	87.44
	9.69	87.30

High conversions of all three types of cellulose (including microcrystalline cellulose) confirmed the selected process conditions. The repetitions showed small deviations (max ± 3.7), which could be explained by the fact that simple kitchen grinder was used for cellulose instead of the proper grinding mill.

Comparisons between the average conversions of coniferous, deciduous, eucalyptus, as well of microcrystalline cellulose, are shown in Figure 1.

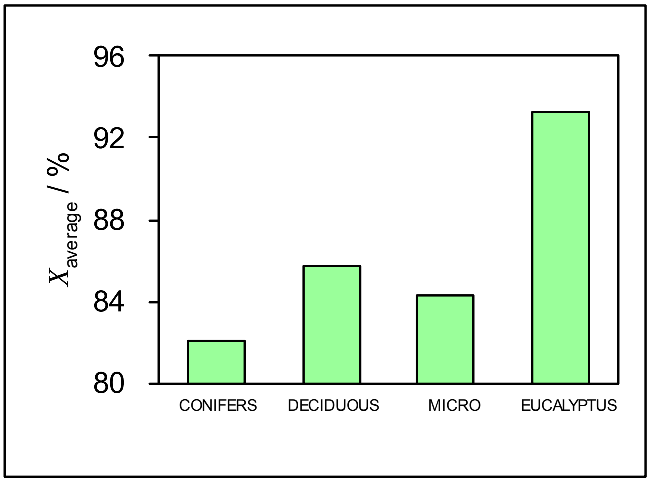

Figure 1: Average conversion of cellulose versus type of cellulose

The differences in Figure 1 can be explained by the chemical composition of the selected three wood species. Wood is essentially composed of cellulose, hemicellulose, lignin, and extractives. Each of these components contributes to fiber properties. Considering only cellulose, which is the main chemical component of the fiber wall and contributes (39 – 45) % of the woods' dry weight, it is well known that deciduous trees have higher content of cellulose compared to coniferous (Qiang et al., 2010). Among deciduous trees fast-growing eucalyptus has even 56 % of its dry weight in the form of cellulose. Cellulose fibers lenght is also decisive. Slow growing coniferous produce long fibers with high strength. The shorter fibers from rapidly growing deciduous trees are lower in strength and thus more receptive for the pre-treatment processes. Therefore, 93 % of eucalyptus cellulose was converted to sugars in our study. This explains the fact that eucalyptus is one of the major promising cellulose feedstocks for ethanol production in the long term.

We have observed in the literature, that ionic liquids can also be used for the pre-atment of cellulose. They are non-volatile moletn salts with high solubilities, low melting points and environmentally very useful. Thus we tested the conversion of coniferous cellulose in the case of pre-treatment with ionic liquid type BMIMCl. All other conditions of the hydrolysis were the same as in the pre-treatment with phosphoric acid. About 21 % conversion was achieved, thus we didn't continue the research in this direction.

4. Conclusion

Cellulose is the most abundant and cheapest biomass in the world and for that reason one of the better alternative raw materials for the production of bio-ethanol fuel. The production process takes place in two stages - hydrolysis of cellulose to sugars is performed first, followed by sugar’s fermentation into ethanol. For the highest possible efficiency of fermentation, pre-treatment of crude cellulose or optimally performed hydrolysis reaction is very important. Cellulose can be found in different sources, including trees. With our research we examined the suitability of eucalyptus, coniferous and deciduous cellulose for further processing into ethanol. Based on the performed experiments we determined the optimal conditions for the pre-treatment of cellulose and further reactions of hydrolysis. We found out that the differences between the investigated three types of cellulose were not great but the highest conversion (93 %) was reached with eucalyptus cellulose that is in agreement with the theory of their structures. The process of pre-treatment followed by hydrolysis of shorter cellulose fibers of deciduous trees was more efficient than that of coniferous.

The final conclusion from this study is that cellulase enzymes can hydrolyse previously pretreated raw eucalyptus cellulose at mild temperature (ϑ = 45 °C), resulting in significant increases in the amount of potential sugar production and also in potential ethanol production.

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