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Influence of pH and Cellic® CTec2 enzymes dose on the glucose yield after enzymatic hydrolysis of cellulose at 50 °C

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Abstract: *Influence of pH and Cellic® CTec2 enzymes dose on the glucose yield after enzymatic hydrolysis of cellulose at 50 °C.* Cellulose obtained by the Kürschner-Hoffer method from the wood of 3-year-old poplar (*Populus trichocarpa*) was subjected to enzymatic hydrolysis. Cellic® CTec2 enzymes (Novozymes, Denmark) were used. The enzymatic hydrolysis was tested within the conditions recommended by the manufacturer and the literature. The process was carried out at 50 °C at various pH – 4.8, 5.0, 5.5 and enzymes doses - 25, 50 and 100 mg per 100 mg of the dry mass of cellulose. The process was ended after 24 h. The hydrolysates were analysed by high-performance liquid chromatography (HPLC) to determine the glucose content, and then the highest glucose yield. The highest glucose yield was obtained for pH 4.8 and 100 mg of enzymes per 100 mg of the dry mass of cellulose – 72 %.

Keywords: the enzymatic hydrolysis of cellulose, Cellic® CTec2 enzymes, cellulose

INTRODUCTION

In the process of hydrolysis of cellulose, the β -1,4-glycosidic bond is cleaved as a result of the attack and the attachment of a water molecule (Figure 1). This reaction is catalyzed by diluted or concentrated acids or by enzymes (cellulases) (Hamelinck et al. 2005).

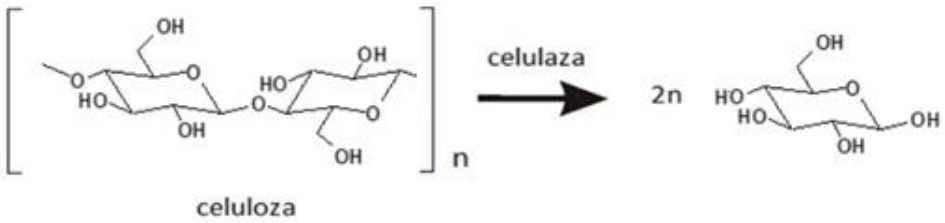

Figure. 1. A simplified diagram of the enzymatic hydrolysis of cellulose to glucose.

The first reports of cellulose saccharification date back to 1819, when the French chemist Braconnot used concentrated or diluted sulfuric acid. From the obtained hydrolysate, he managed to isolate D-glucose (Braconnot 1819). This method wasn't applied in the industry due to the high consumption of acid. Other scientists tried to investigate this subject using various acids, concentrations, and reaction times and temperatures. At some point, they established that the long-time hydrolysis leads to the degradation of simple sugars. Acid hydrolysis began to be used on an industrial scale (Rowe and Pearl 1961; Wenzel 1954 and 1959).

The use of concentrated acids is still expensive. The use of diluted acids requires high temperatures and enhance process costs (Duff and Murray 1996). Acid hydrolysis has also other disadvantages, such as the use of environmentally harmful reagents or need of disposing chemicals in an appropriate way. The acids cause not only the degradation of cellulose to glucose but also the further conversion of simple sugars (Prosiński 1984). The degradation of simple sugars is a serious problem as it reduces the obtainable yield. Additionally, the use of acids causes corrosion of industrial equipment.

The usage of enzymes is an alternative to acid-catalysed enzymatic hydrolysis. Enzymatic hydrolysis doesn't cause the degradation or formation of derivatives of simple

sugars (Chang et al. 2000; Gharpuray et al. 1983). As a result, it allows achieving a better yield than acid hydrolysis (Sánchez and Cardona 2008). Enzymes are environmentally friendly. They also are non-corrosive to the equipment. The whole process can be carried out in not very high temperatures with atmospheric pressure. It's very important when thinking about big industrial processes of the enzymatic hydrolysis. Low temperature and no need for artificial pressure don't generate additional high cost. Especially, that enzymes themselves are expensive. It will be necessary to recover the enzymes left after enzymatic hydrolysis if want the mentioned process to be cost-effective. The disadvantages of using enzymes as catalyst include the high price, specific performance (universal enzymes do not exist) and the fact that enzymatic hydrolysis of native material is a slow process due to the complex structure of the material (Pan et al. 2006). The efficiency of enzymatic hydrolysis is also influenced by many compounds, which most often inhibit this process. This drawback can be caused by compounds that occur naturally in water or arise during pretreatment of raw material e.g. wood treatment with high temperature, acids or alkalis as pretreatment (Ximenes et al. 2011; Mood et al. 2013). Also, hemicelluloses decay products (hemicelluloses often accompany cellulose in nature), such as pentoses and hexoses and their further derivatives (dehydration products) - furfural and hydroxymethylfurfural, as well as levulinic acid and formic acid inhibits enzymatic hydrolysis catalysed by enzymes (Hayes et al. 2005). Soluble carbohydrates such as glucose, cellobiose, oligoglucose compounds (Gusakov and Sinitsyn 1992; Kim 2018) contribute to inhibition of the enzymatic hydrolysis reaction. The enzymatic hydrolysis of cellulose is an environmentally friendly, but very sensitive process.

Considering all the provided information, it is very important to determine the exact parameters of the enzymatic hydrolysis of cellulose with the use of specific enzymes. Taking into account the manufacturer's guidelines (Novozymes 2010), the enzymatic hydrolysis was performed at various conditions, such as temperature, pH and the amount of Cellic® CTec2 enzymes (Novozymes, Denmark). This investigation aimed to check the exact pH and enzymes dose of enzymatic hydrolysis of cellulose at 50 °C with use of Cellic® CTec2 enzymes from the range provided by the enzymes manufacturer.

MATERIALS

Cellulose

The Kürschner-Hoffer method was used to obtain cellulose from the 3-year-old poplar wood (*Populus trichocarpa*) (Kürschner and Hoffer 1929). Three cycles of heating in the presence of nitrogen acid and ethanol mixture were applied on wood particles. The mass of 3 g of wood was used to obtain cellulose. All reagents were approximately calculated to the amount of wood.

Enzymes

In this investigation industrial enzymes - Cellic® CTec2 (Novozymes, Denmark) were used. It aims to degrade cellulose to glucose. According to manufacturer application sheet, it is a blend including cellulases (endo and exoglucanases), β-glucosidase and hemicellulases. Any specific enzymes are not mentioned. The manufacturer recommends a pH between 5.0 and 5.5 and a temperature in the range of 45-50 °C during enzymatic hydrolysis of cellulose (Novozymes 2010). According to the manufacturer's information, these enzymes are characterized by high conversion efficiency - also with a high content of the material - and tolerance to inhibitors and high stability. They are designed to work with a variety of materials, they are not specialized to work with one biomass.

Enzymatic hydrolysis

The enzymatic hydrolysis of the cellulose was performed in a 25 cm^3 glass flask. The mass of the material corresponding to 100 mg of the dry mass of cellulose, 12.5 cm^3 citrate buffer, 0.1 M sodium azide solution, 25 % enzymes solution and distilled water (to the 25 cm^3 volume) were added to the glass flask. The pH values 4.8, 5.0 and 5.5 were tested. For each of them, 25, 50 and 100 mg per 100 mg of enzymes per the dry mass of cellulose were used. The temperature was kept at a level of 50 °C. From each glass flask, 1 cm^3 sample was taken after 24 hours. Before HPLC analysis collected samples in a hermetic test tubes were placed in a freezer at -20 °C to stop the process of enzymatic hydrolysis. To determine the process parameters allowing for obtaining the highest glucose yield after the enzymatic hydrolysis process the hydrolysates were subjected to chromatographic analysis to determine the glucose content in the hydrolysates. All enzymatic hydrolysis tests were done in triplicate.

HPLC analysis (High-Performance Liquid Chromatography)

The samples taken after 24 h of enzymatic hydrolysis were prepared to HPLC analysis by warming them to room temperature. Then, the samples were put for 15 min at 95 °C in a water bath to denatured the enzymes. In the next step, the samples were centrifuged for 10 min on a laboratory centrifuge at 12 000 rpm. At the end step before HPLC analysis the samples were filtered by using a nylon syringe filter of 0.2 μm. After this preparation samples were subjected to HPLC analysis.

The analysis of glucose content after enzymatic hydrolysis was carried out using a Shimadzu liquid chromatograph with a differential refractive detector (RID-10A), control module (CBM-20A), oven (CTO-20A), degasser (DGU-20A) and pump (LC-20AD). Chromatographic data were developed using LC Solution v.1.21 SP1 software. Phenomenex columns Luna NH2 (universal column) connected with a guard column was used for the analysis. Analysis conditions: flow rate of 1.5 cm^3/min, oven temperature 50 °C, eluent acetonitrile-distilled water ($80:20_{v/v}$), injection volume 20 μL. To prepare a calibration curve a series of standard glucose solutions of known concentration were analysed. Calibration curve used for the quantitative determination of the glucose obtained in the hydrolysates. The determined value of the calibration curve coefficient (slope) was $(1.230 \pm 0.014) \times 10^{-6}$, and the correlation coefficient R^2=0.9994. By substituting the glucose peak area of a sample of unknown concentration in the calibration curve equation the concentration of glucose in that sample was obtained. To calculate the glucose mass the obtained glucose concentration was multiplied by the total volume of the sample (25 cm^3).

Each sample mass (mass of cellulose) was multiplied by factor 1.11 to calculate the theoretical mass of glucose (TMG), The factor 1.11 is the ratio of the glucose mass ($C_6H_{12}O_6$ - 180.16 u) to the mass of the glucopyranose residue ($C_6H_{10}O_5$ - 162.14 u) - the water molecule is attached to the glucopyranose ring in a hydrolysis process. Finally, the glucose yield after enzymatic hydrolysis was calculated by dividing the obtained mass of glucose in the hydrolysate after enzymatic hydrolysis by TMG.

RESULTS

According to manufacturer's application sheet, the recommended parameters to obtain maximum efficiency of enzymatic hydrolysis with use of Cellic® CTec2 enzymes are pH in the range between 5.0 and 5.5, a temperature from 45 to 50 °C and enzymes load 30 $\%_{w/w}$ (g enzyme/g cellulose) (Novozymes 2010). As recommended in this study pH values 5.0 and 5.5 and temperature 50 °C were tested. Also, various amounts of enzyme, not only loadings recommended by the manufacturer, but also higher were tested. This was to check the maximum glucose yield after enzymatic hydrolysis of cellulose with use of Cellic® CTec2 enzymes (Novozymes, Denmark). As reported by other researchers, the glucose yield after enzymatic

hydrolysis at pH 4.8 gets high values (Asem 2012; Gao et al. 2013). Therefore, enzymatic hydrolysis of cellulose at pH 4.8 has been also carried out. Based on the same research it was decided not to investigate lower pH values due to the low efficiency of enzymatic hydrolysis.

The obtained results of glucose yield after enzymatic hydrolysis at 50 °C with 3 various doses of the enzymes (25, 50 and 100 mg per 100 mg of the dry mass of cellulose) at pH 4.8, 5.0 and 5.5 are shown in Figures 1, 2 and 3.

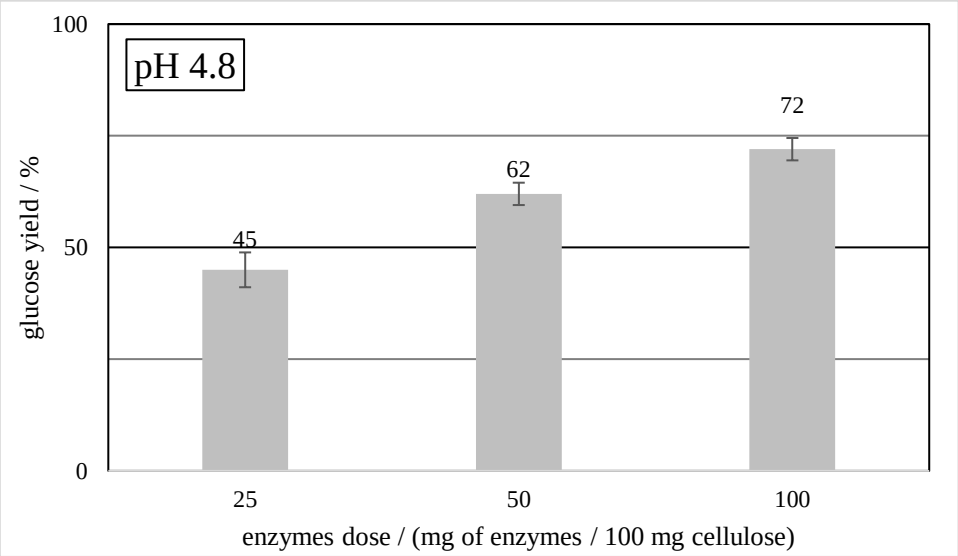

Figure 1. The glucose yield after 24 h of the enzymatic hydrolysis performed at 50 °C and pH 4.8.

The analysis of the hydrolysates after 24 hours of enzymatic hydrolysis at pH 4,8 showed significant differences in glucose yield after enzymatic hydrolysis resulting from the amount of enzyme. Based on the data (Figure 1), the highest glucose yield after enzymatic hydrolysis at pH 4,8 was found for 100 mg of enzymes per 100 mg of cellulose. It was 16 and 60 % higher than with the use of 50 and 25 mg of enzymes per 100 mg of the dry mass of the cellulose, respectively. The lower the amount of the enzymes used the lower the glucose yield.

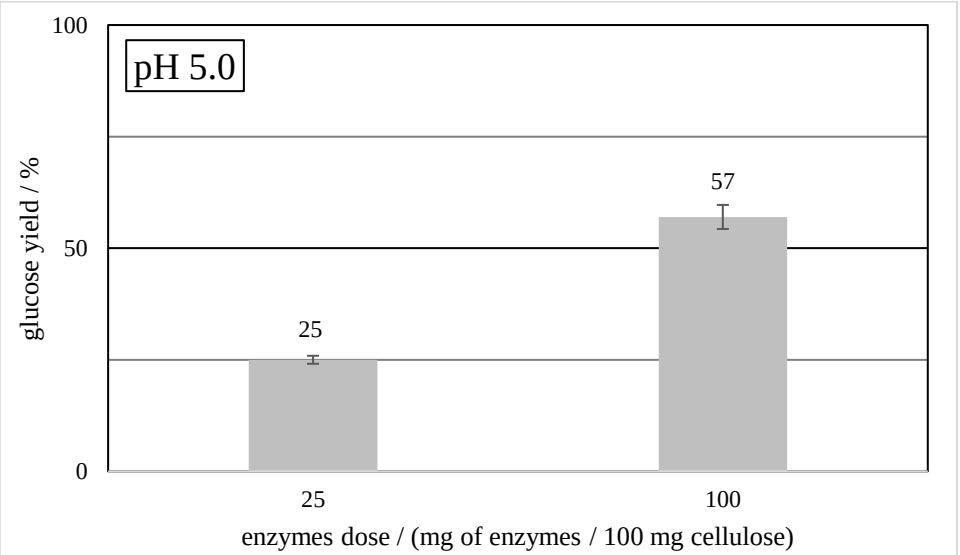

Figure 2. The glucose yield after 24 h of the enzymatic hydrolysis performed at 50 °C and pH 5.0.

Figure 2 presents the obtained data for the glucose yield after 24 h of the enzymatic hydrolysis performed at 50 °C and pH 5.0 with the use of 25 and 100 mg of the enzymes per 100 mg of the dry mass of the cellulose. The glucose yield after 24 h of enzymatic hydrolysis performed at 50 °C and pH 5.0 was the highest for the enzymes amounts of 100 mg per 100 mg dry mass of cellulose. The glucose yield with the use of the 25 mg enzymes was 56 % lower from the highest result at these conditions (50 °C, pH 5.0). The presented data for glucose yield after 24 h of the enzymatic hydrolysis at pH 5,0 are significantly lower in a comparison with obtained glucose yield after the enzymatic hydrolysis at pH 4.8.

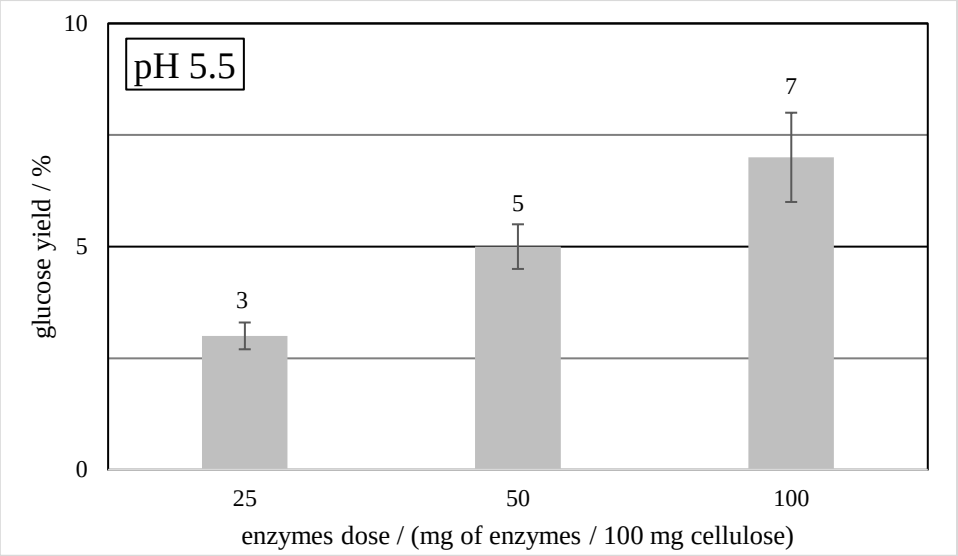

Figure 3. The glucose yield after 24 h of the enzymatic hydrolysis performed at 50 °C and pH 5.5.

The data presented in Figure 3 show that after 24 h of the enzymatic hydrolysis of the cellulose at 50 °C and pH 5.5, the highest glucose yield was obtained for the amount of 100 mg of the enzymes per 100 mg of the dry mass of the cellulose. These results differ from the yield obtained for the pH 5.0 and 4.8. The glucose yield after the enzymatic hydrolysis performed at 50 °C and pH 5.5 are strongly lower. When compere the highest glucose yield after the enzymatic hydrolysis at pH 5.5 with the glucose yield after the enzymatic hydrolysis at pH 4.8 and 5.0 and the same enzymes dose (100 mg of the enzymes per 100 mg of the dry mass of the cellulose) they are over 10 and over 8 times lower, respectively.

The highest glucose yield after 24 h of the enzymatic hydrolysis of the cellulose carried out at 45 °C was obtained for pH 4.8 and 100 mg of enzymes per 100 mg of the dry mass of the cellulose for all used conditions (Figure 1, 2 and 3). This result differs significantly from the glucose yields obtained for pH 5.5 and also for the pH 5,0 when compare all the enzymes doses used. The lowest glucose yield after 24 h of the enzymatic hydrolysis of the cellulose at 45 °C was obtained after process performed at pH 5.5 with use of 25 and 100 mg of the enzymes per 100 mg of the dry mass of the cellulose. In no case a maximum glucose yield after enzymatic hydrolysis was reached. Many pieces of research confirm that the enzymatic hydrolyses carried out with the use of the Cellic® CTec2 enzymes (Novozymes, Denmark) obtain the highest efficiency at pH 4.8 (Asem 2012; Cannella et al. 2012; Gao et al. 2013; Lan et al. 2013). Many reaserchers perform enzymatic hydrolysis of cellulose itself or a cellulose in lignocellulosic materials at temperature 50 °C (Ramos et al. 2015; Cannella et al. 2012; Lan et al. 2013).

CONCLUSION

1. The glucose yield after 24 h of the enzymatic hydrolysis of the cellulose carried out at 50 °C depends on the pH in tested range 4.8-5.5. The lower the pH, the higher the glucose yield.
2. The glucose yield after 24 h of the enzymatic hydrolysis of the cellulose carried out at 50 °C depends on the enzymes dose (25, 50 and 100 mg per 100 mg of the dry mass of the cellulose). The higher the enzymes dose, the higher the glucose yield.
3. Further investigation of glucose yield after enzymatic hydrolysis with use of Cellic® CTec2 enzymes with prolonged process time is needed to test how the enzymes work.
4. Further investigation of a glucose yield after enzymatic hydrolysis with use of Cellic® CTec2 enzymes at pH between 5.0 and 5.5 is needed.

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Streszczenie: *Wpływ pH i ilości enzymu Cellic® CTec2 na wydajnośc glukozy po hydrolizie enzymatycznej celulozy w 50 °C.* Celulozę otrzymaną metodą Kürschnera-Hoffera z drewna 3-letniej topoli *Populus trichocarpa* poddano hydrolizie enzymatycznej. Zastosowano enzymy Cellic® CTec2 (Novozymes, Dania). Hydrolizę enzymatyczną badano w warunkach zalecanych przez producenta i literaturę. Proces prowadzono w temperaturze 50 °C przy różnym pH - 4,8, 5,0, 5,5 i dawkach enzymu - 25, 50 i 100 mg na 100 mg suchej masy celulozy. Proces został zakończony po 24 godzinach. Hydrolizaty analizowano metodą wysokosprawnej chromatografii cieczowej (HPLC) w celu określenia zawartości glukozy, a następnie największej wydajności glukozy. Największą wydajność uzyskano dla pH 4,8 i 100 mg enzymu na 100 mg suchej masy celulozy - 72%.

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