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# Influence of pH and Cellic® CTec2 enzymes dose on the glucose yield after enzymatic hydrolysis of cellulose at 45 °C

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**Abstract:** *Influence of pH and Cellic® CTec2 enzymes dose on the glucose yield after enzymatic hydrolysis of cellulose at 45 °C.* The enzymatic hydrolysis with the use of industrial enzymes Cellic® CTec2 (Novozymes, Denmark) was carried out within the conditions recommended by the manufacturer and literature. Cellulose obtained by the Kürschner-Hoffer method from a wood of 3-year-old poplar (*Populus trichocarpa*) was used for the study. Three pH values of 4.8, 5.0 and 5.5 were applied. Also, three amounts of enzymes were used: 25, 50 and 100 mg per 100 mg of the dry mass of cellulose for each pH used. The temperature was 45 °C. Samples were taken after 24 h and subjected to chromatographic analysis to determine the glucose content in the hydrolysates, and then the process parameters allowing for the highest glucose yield after the enzymatic hydrolysis process. The highest glucose yield was obtained for pH 5.0 and 100 mg of enzymes per 100 mg of the dry mass of cellulose – 79 %.

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

## INTRODUCTION

The enzymatic hydrolysis of cellulose is performed with cellulases - an enzymes blend. Cellulases are highly specialised in the conversion of cellulose into glucose (Sánchez and Cardona 2008). They consist of endoglucanase, exoglucanase (also known as cellobiohydrolase) and β glucosidase. The mechanism of enzymatic hydrolysis of cellulose is based on the synergistic action of these enzymes: (Zhang and Lynd 2004; Zhang et al. 2006; Howard et al. 2003). Endo 1,4 β-glucanase breaks the cellulose chains at random places, which leads to a decrease in the polymerization degree (Oyekola 2004). As a result of breaking the β 1,4 glycosidic bond in cellulose, new ends of chains are formed and there are more places available for the activity of exoglucanase. It detaches cellobiose from the ends of the chain, and then β glucosidase hydrolyzes cellobiose to glucose. There are also reports that the activity of exoglucanase produces directly a small amount of glucose (Zhang et al. 2006; Heikinheimo 2002). The process diagram is shown in Figure 1.

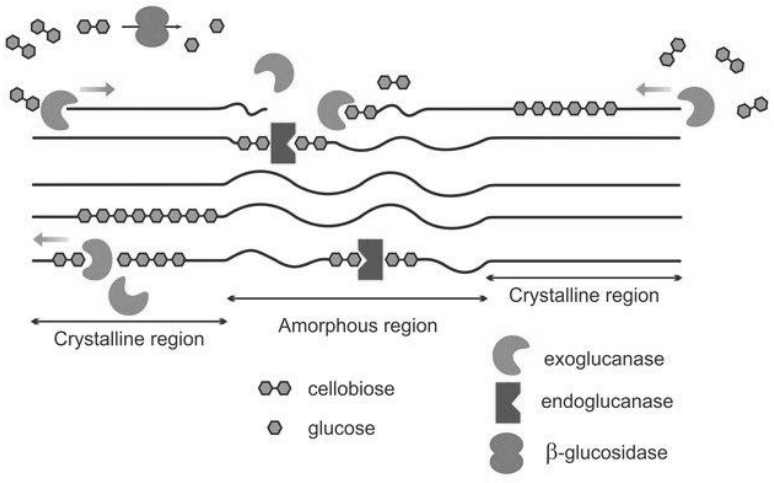


**Figure 1**. The mechanism of enzymatic hydrolysis of cellulose is based on the synergistic action of Endoglucanase, exoglucanase (also known as cellobiohydrolase) and β glucosidase (Akhtar et al. 2016).

Most of the industrial cellulolytic enzymes are obtained from the fungus *Trichoderma reeseii*. *T. reesei* produces a blend of cellulases, including at least two cellobiohydrolases, five

endoglucanases, β-glucosidase, and hemicellulases (Zhang and Lynd 2004). The cellulolytic enzymes can also be obtained from the fungus kingdom from *Aspergillus* genus. The companies that obtain cellulase industrially, such as Novozymes, Genencor and Iogen use Trichoderma or Aspergillus fungi (Himmel et al. 2007). Enzymes are environmentally friendly and non-corrosive to equipment. Among the disadvantages can be mentioned the high price and specific performance of enzymes - which can sometimes be considered a disadvantage as there are no universal enzymes (Pan et al. 2006).

The enzymatic hydrolysis of cellulose is a heterophasic system. Enzymes are dissolved in the liquid phase, while cellulose is in the solid phase. There are two groups of factors that can influence the enzymatic hydrolysis efficiency: factors related to enzymes and factors related to raw material. The factors related to the enzymes are the activity of enzymes, the amount of enzymes used, appropriate temperature and pH of the reaction medium (Alvira et al. 2010; Thompson et al. 1992; Fan et al. 1981; Mood et al. 2013).

The investigation was performed due to the fact that the nature of enzymes work is specific and complicated and a change of its activity results from even small change in the process parameters. The enzymatic hydrolysis was performed at various conditions, such as temperature, pH and the amount of enzymes - considering the condition range provided by manufacturer's application sheet (Novozymes 2010). The aim of this work was to check the exact pH and enzymes dose of enzymatic hydrolysis of cellulose at 45 °C with use of Cellic® CTec2 enzymes from the range provided by the enzymes manufacturer.

## MATERIALS

### Cellulose

Cellulose was obtained from the wood of a 3-year-old poplar (*Populus trichocarpa*) by the Kürschner-Hoffer method (Kürschner and Hoffer 1929). The mass of 3 g of wood particles was subjected to three cycles of heating in the nitrogen acid-ethanol mixture. All reagents were approximately calculated to the amount of wood used.

### Enzymes

Industrial enzymes Cellic® CTec2 (Novozymes, Denmark) were used in this investigation. It is a specialized cellulase complex designed to degrade cellulose into glucose. The blend includes cellulases (endo and exoglucanases), β-glucosidase and hemicellulases (the manufacturer does not provide information about specific enzymes). Recommended hydrolysis parameters are pH between 5.0 and 5.5 and temperature in the range of 45-50 °C (Novozymes 2010). According to the manufacturer's information, These enzymes are characterized by high conversion efficiency, high tolerance to inhibitors and also high stability. They are not produced for work with only one biomass, but with multiple kinds of lignocellulosic biomasses.

### Enzymatic hydrolysis

The enzymatic hydrolysis was made on never dried, wet material. The humidity of cellulose was determined by the oven-dry method. It was used to calculate the dry mass of cellulose. The mass of the material corresponding to 100 mg of the dry mass of cellulose was weighed in a 25 $cm^3$ glass flask. In next step 12.5 $cm^3$ citrate buffer solution was added to the flask. Then, 0.1 M sodium azide solution was added to each flask to prevent the growth of microorganisms during the process. Then, 25 $\%_{v/v}$ enzymes solution was added. In the end, the amount of distilled water calculated, so that the volume of the solution was 25 $cm^3$ was added to the flask. The temperature was applied for each of these variants at level 45 °C by placing the flasks in a water bath.

Three pH values of 4.8, 5.0 and 5.5 were investigated. For each of them, three amounts of enzymes were used: 25, 50 and 100 mg per 100 mg of the dry mass of cellulose.

From each flask, samples were taken after 24 hours and placed in a freezer at -20 °C in hermetic test tubes. This was to stop the process of enzymatic hydrolysis before HPLC analysis. The glucose content determination in the hydrolysates was done by the HPLC method. All enzymatic hydrolysis tests were done in triplicate.

HPLC analysis (High-Performance Liquid Chromatography)

Before the HPLC analysis, the samples were prepared warming to room temperature. In the next step, the samples were placed in a water bath for 15 min at 95°C to denature the enzymes. Then, the samples were centrifuged for 10 min on a laboratory centrifuge at 12 000 rpm. The end step of preparation was to filter samples by using a nylon syringe filter of 0.2 μm. The samples prepared by this method were subjected to HPLC analysis.

The analysis of glucose content in the hydrolysate was carried out using a Shimadzu liquid chromatograph with a control module (CBM-20A), differential refractive detector (RID-10A), oven (CTO-20A), degasser (DGU-20A) and pump (LC-20AD). Chromatographic data were developed using LC Solution v.1.21 SP1 software. Phenomenex column was used for the analysis - Luna NH2 (universal column) connected with a guard column. 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. Analysis of a series of standard glucose solutions of known concentration was used to prepare a calibration curve. Calibration curve allowed for the quantitative determination of the glucose obtained in the hydrolysates. The determined value of the calibration curve coefficient (slope) in this case was $(1.230 \pm 0.014) \times 10^{-6}$, and the correlation coefficient $R^2 = 0.9994$. The concentration of glucose in that sample was obtained by substituting the glucose peak area for a sample of unknown concentration to the calibration curve equation. The glucose mass was calculated by multiplying the obtained glucose concentration by the total volume of the sample (25 $cm^3$).

The theoretical mass of glucose (TMG) was calculated by multiplying the mass of cellulose by the factor 1.11 corresponding to 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 attached to the glucopyranose ring in a hydrolysis process was considered when determining the theoretical mass of glucose. The glucose yield after enzymatic hydrolysis was calculated by dividing the obtained mass of glucose in the hydrolyzate (after chromatographic analysis) by the theoretical mass of glucose.

RESULTS

The enzymes manufacturer recommends using the following enzymatic hydrolysis parameters: pH 5.0 - 5.5 and a temperature of 45-50 °C, to obtain maximum efficiency (Novozymes 2010). In this study, it was decided to test two pH values provided by the manufacturer, 5.0 and 5.5, and temperature 45 °C. It was also decided to test various amounts of enzyme, also higher than recommended by the manufacturer to check the maximum glucose yield after enzymatic hydrolysis of cellulose with use of Cellic® CTec2 enzymes (Novozymes, Denmark). Following not the manufacturer's application sheet, but the results of other researchers, the glucose yield after enzymatic hydrolysis at pH 4.8 was also investigated (Asem 2012; Gao et al. 2013). These studies have shown that the glucose yield after enzymatic hydrolysis of lignocellulosic materials after pretreatment reaches high values.

The results of the glucose yield after enzymatic hydrolysis at 45 °C with enzymes doses of 25, 50 and 100 mg per 100 mg of the dry mass of cellulose, for each of them at pH 5.0 and 5.5 and are presented in Figures 1, 2 and 3.

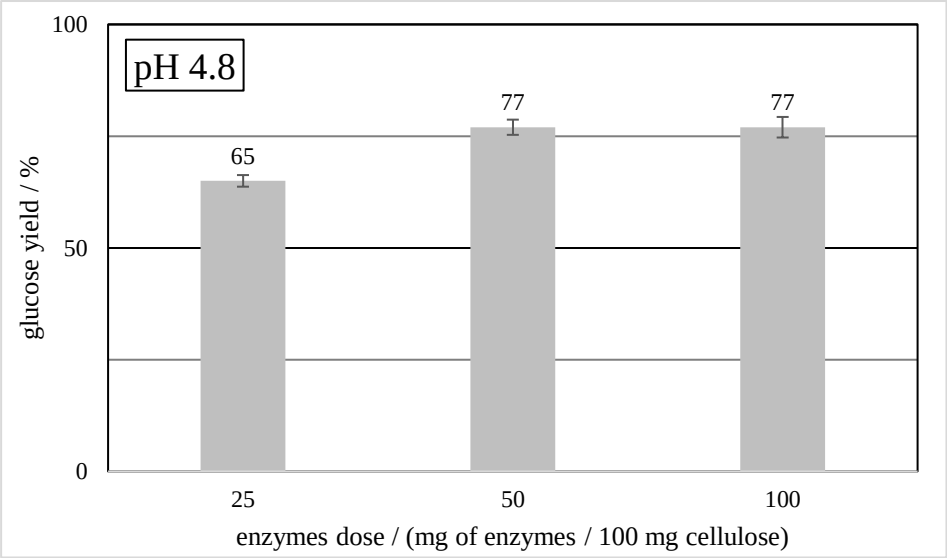


**Figure 2.** The glucose yield after 24 h of the enzymatic hydrolysis performed at 45 °C and pH 4.8.

The data presented in Figure 1 show that after 24 h of enzymatic hydrolysis of cellulose carried out at 45 °C and pH 4.8, the highest efficiency was obtained with the use of 50 and 100 mg of enzymes per 100 mg of the dry mass of cellulose. Comparing the results, it may be observed that mentioned results differ from the yield obtained after the enzymatic hydrolysis with the enzymes dose of 25 mg per 100 mg of the dry mass of cellulose. The glucose yield obtained in these conditions of enzymatic hydrolysis with the lowest dose of the enzymes was 16 % lower than other amounts of the enzymes used. Additionally, in no case the maximum glucose yield was observed. Certainly, a longer hydrolysis time is requested to test.

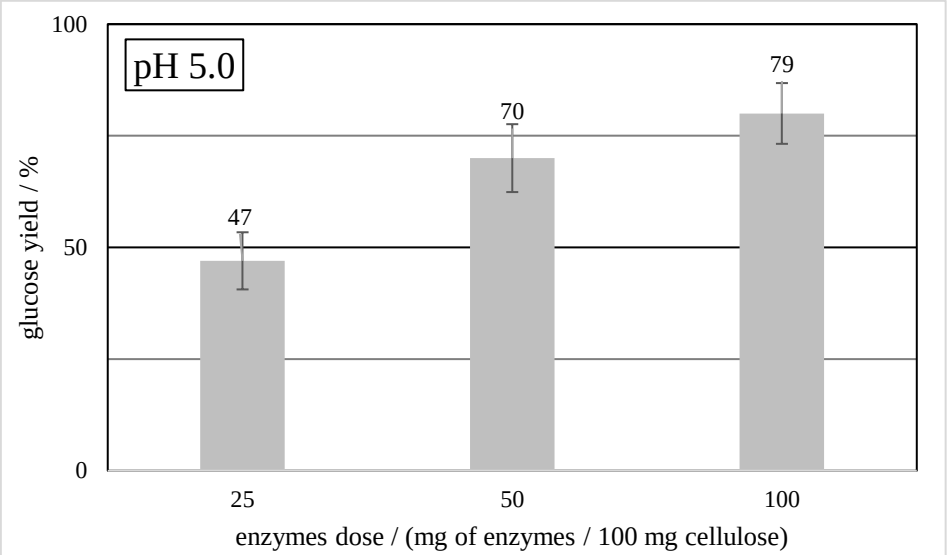


**Figure 3.** The glucose yield after 24 h of the enzymatic hydrolysis performed at 45 °C and pH 5.0.

The analysis of the hydrolysates for glucose content after 24 hours of enzymatic hydrolysis at pH 5.0 showed significant differences in glucose yield after enzymatic hydrolysis resulting from the amount of enzyme. Based on the data (Figure 2), it can be concluded that the glucose yield after enzymatic hydrolysis at pH 5.0 was the highest for 100 mg of enzymes per 100 mg of cellulose. In the process carried out with 25 and 50 mg

of enzymes per 100 mg of cellulose, the decrease in glucose yield is approx. 41 % and 13 %, respectively (compared to the efficiency in the process carried out with 100 mg of the enzymes per 100 mg of cellulose).

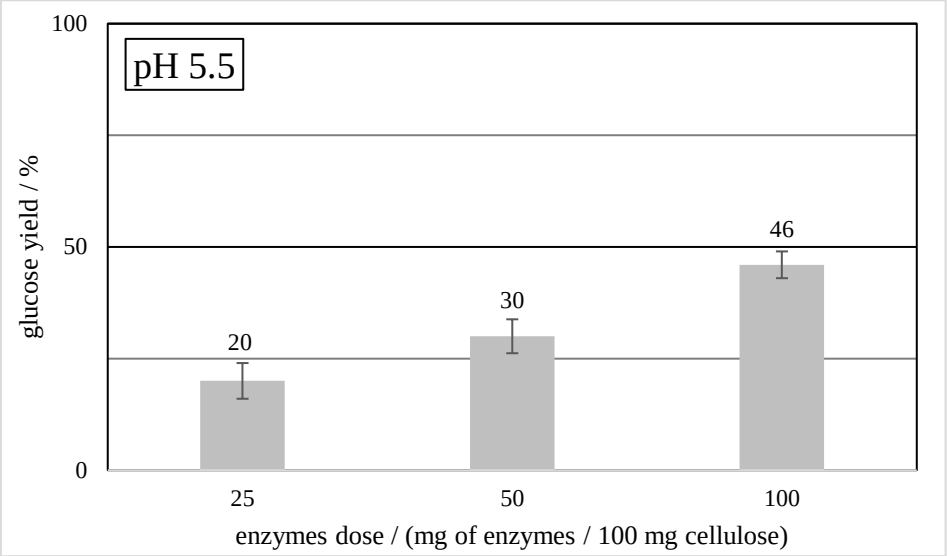


**Figure 4.** The glucose yield after 24 h of the enzymatic hydrolysis performed at 45 °C and pH 5.5.

Figure 3 presents the glucose yield after 24 h of enzymatic hydrolysis performed at 45 °C at pH 5.5. The highest glucose yield was for the amounts of enzymes 100 mg per 100 mg of the dry mass of cellulose. This yield was obtained at a level of 46 % and was over twice higher than this obtained for the lowest amount of enzymes used. Moreover, the results of glucose yields were the lowest of glucose yields obtained after enzymatic hydrolysis performed at all investigated pH.

The data presented in Figures 1, 2 and 3 show that the highest glucose yield after 24 h of enzymatic hydrolysis of cellulose carried out at 45 °C was obtained for pH 5.0 and 100 mg of enzymes per 100 mg of the dry mass of cellulose. However, this result does not differ significantly from the glucose yields obtained for pH 4.8 with the use of 50 and 100 mg of enzymes per 100 mg of the dry mass of cellulose. The lowest glucose yield after 24 h of enzymatic hydrolysis of cellulose carried out at 45 °C was obtained at pH 5,5 with the use of 25 and 100 mg of enzymes per 100 mg of the dry mass of cellulose. Based not only on the parameters provided by the enzymes manufacturer but also on the experiences of other researchers, in this study it was not decided to test a pH lower than 4.8. The same relationships were observed in other studies. Most of the found reports on enzymatic hydrolyses carried out with the use of the Cellic® CTec2 enzymes (Novozymes, Denmark) showed that enzymatic hydrolysis of cellulose and lignocellulosic biomass achieved the highest efficiency at pH 4.8, 5.0 or higher (Gao et al. 2013; Lan et al. 2013; Cannella et al. 2012).

## CONCLUSION

1. The pH in range 4.8-5.5 influence the glucose yield after 24 h of enzymatic hydrolysis of cellulose carried out at 45 °C with use of 25 and 50 mg of enzymes per 100 mg of the dry mass of cellulose. The lower pH, the higher the glucose yield.
2. Enzymes dose (25, 50 and 100 mg per 100 mg of the dry mass of cellulose) influence the glucose yield after 24 h of enzymatic hydrolysis of cellulose carried out at 45 °C and pH 5,0 and 5,5. The higher enzymes dose, the higher the glucose yield.

3. The enzymes dose influences the glucose yield after 24 h of enzymatic hydrolysis of cellulose carried out at 45 °C and pH 4,8 to some point. The higher enzymes dose (25 and 50 mg per 100 mg of the dry mass of cellulose), the higher glucose yield.
4. Enzymes dose 50 and 100 mg per 100 mg of the dry mass of cellulose doesn't influence the glucose yield after 24 h of enzymatic hydrolysis of cellulose carried out at 45 °C and pH 4,8. Both enzymes doses show the glucose yield at the level of 77 %.
5. Investigation of longer and shorter time of enzymatic hydrolysis of cellulose at 45 °C is needed to determine the conditions in which the highest glucose yield is obtained.
6. Investigation of pH between 5.0 and 5.5 for example 5.2 ora 5.3 during enzymatic hydrolysis of cellulose at 45 °C is needed to determine the conditions in which the highest glucose yield is obtained.

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**Streszczenie:** *Wpływ pH i ilości enzymu Cellic® CTec2 na wydajnośc glukozy po hydrolizie enzymatycznej celulozy w 45 °C.* Hydrolizę enzymatyczną z zastosowanie enzymów przemysłowych Cellic® CTec2 (Novozymes, Dania) przeprowadzono w warunkach zalecanych przez producenta i literaturę. Do badań wykorzystano celulozę otrzymaną metodą Kürschnera-Hoffera z drewna topoli trzyletniej *Populus trichocarpa*. Zastosowano trzy wartości pH: 4,8, 5,0 i 5,5. Zastosowano również trzy ilości enzymu: 25, 50 i 100 mg (na 100 mg suchej masy celulozy) dla każdego użytego pH. Temperatura wynosiła 45 °C. Próbki pobrano po 24 h i poddano analizie chromatograficznej w celu oznaczenia zawartości glukozy w hydrolizatach, a następnie parametrów procesu pozwalających na uzyskanie największej ilości glukozy po procesie hydrolizy enzymatycznej. Najwyższą wydajność glukozy uzyskano dla pH 5,0 i 100 mg enzymu na 100 mg suchej masy celulozy – 79 %.

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