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ENZYMATIC ACTIVITY OF SOME INDUSTRIALLY-APPLIED CELLULOLYTIC ENZYME PREPARATIONS

AKTYWNOŚĆ ENZYMATYCZNA PRZEMYSŁOWYCH PREPARATÓW CELULOLITYCZNYCH

Abstract: Enzymatic hydrolysis is the essential step in the production of 2nd generation biofuels made from lignocellulosic biomass, i.e. agricultural or forestry solid wastes. The enzyme-catalysed robust degradation of cellulose and hemicellulose to monosaccharides requires the synergistic action of the independent types of highly-specific enzymes, usually offered as ready-to-use preparations. The basic aim of the study was to experimentally determine the enzymatic activity of two widely industrially-applied, commercially available cellulolytic enzyme preparations: (i) Cellic® CTec2 and (ii) the mixture of Celluclast® 1.5L and Novozyme 188, in the hydrolysis of pre-treated lignocellulosic biomass, i.e. (a) energetic willow and (b) rye straw, or untreated (c) cellulose paper as well, used as feedstocks. Before the hydrolysis, every kind of utilized lignocellulosic biomass was subjected to alkaline-based (10% NaOH) pre-treatment at high-temperature (121°C) and overpressure (0.1 MPa) conditions. The influence of the type of applied enzymes, as well as their concentration, on the effectiveness of hydrolysis was quantitatively evaluated, and finally the enzyme activities were determined for each of tested cellulolytic enzyme preparations.

Keywords: cellulolytic enzymes, lignocellulose, alkaline-based pre-treatment, hydrolysis, industrial-scale lignocellulosic biomass degradation

Introduction

Nowadays the world economic development, the depletion of fossil fuels reserves, as well as threat of global warming, contribute to increased demand for utilization of renewable energy sources [1]. Plant biomass represents a very valuable renewable energy source on the world-wide market. It is utilized primarily for the production of heat and biofuels, i.e. bioethanol and biodiesel, for the transport sectors. Recently, an increasing emphasis is putted on the development of effective methods for production of 2nd generation biofuels, i.e. those derived from lignocellulosic raw materials [2, 3]. The market demand for biofuels in European countries has been originated by substantial changes in European Union legislation [4]. According to basic regulations of Renewable Energy Directive (2009/28/WE), all member countries of European Union are obliged to increase the proportion of energy produced from renewable sources in the total reckoning of energy

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consumption, with up to 20%, as well as to achieve a minimum of 10% renewables in the transport sector by year of 2020 as restrict deadline. Moreover, the greenhouse gases emission intensity which is associated with fuels utilization, must be reduced by 6% before 2020, in comparison to 2010. The Renewable Energy Directive were transposed into Polish legislation and the consolidated version of Polish law on the monitoring and control of fuel quality was finally published in November of 2014 as the Announcement of the Republic of Polish Marshal of the Sejm (OJ 2014, item. 1728) [5].

Production of biofuels from lignocellulosic biomass-sources provides indisputable environmental and economic benefits, mainly related to reduction of greenhouse gases emissions, and utilization of agricultural and forestry solid wastes [6]. In addition, such approach does not compete with food manufacturing processes, as it was observed in the case of 1st generation biofuels produced from starch-based biomass-sources [7, 8]. However, due to the complex structure and uneven, heterogonous polysaccharide fractions of lignocelluloses, their robust processing into biofuels is rather problematic and still requires improvements in methodologies [2, 9].

The commonly known bottleneck in 2nd generation biofuels production technologies is the yield of enzyme catalysed hydrolysis of cellulose and hemicelluloses (i.e. major components of lignocelluloses, besides lignin) into easy-fermentable monosaccharides, mainly glucose and xylose. The unquestionable advantages of enzymatic hydrolysis are, if compared to acid-catalysed ones, lower consumption of media (e.g. water, energy), lower costs of waste managements, and no need to exploit corrosion resistant equipment [10]. In addition, acid-catalysed hydrolysis caused formation of highly-toxic by-products, which highly inhibit the growth and the activity of microorganisms further applied for monosaccharides fermentation. In general, hydrolysis must be preceded by pre-treatment of lignocellulosic biomass in order to increase its susceptibility for rapid enzymatic digestion [11], and the essential enzymatic degradation of cellulose and hemicelluloses into monosaccharides requires synergistic action of independent types of enzymes [12].

The basic aim of the study was to experimentally determine the enzymatic activity of two widely industrially-applied, commercially available cellulolytic enzyme preparations: (i) Cellic® CTec2 and (ii) the mixture of Celluclast® 1.5L and Novozyme 188, in the hydrolysis of pre-treated lignocellulosic biomass i.e. (a) energetic willow and (b) rye straw or untreated (c) cellulose paper as well, used as feedstocks.

Materials and methods

Enzyme preparations

Hydrolysis of lignocellulose biomass was conducted using two commercially available cellulolytic enzyme preparations with widen industrial applicability, both manufactured by Novozymes (Denmark): (i) Cellic® CTec2 and (ii) Celluclast® 1.5L supplemented with Novozyme 188. Cellic® CTec2 (abbr. CTec2) is a new generation enzyme cocktail containing all enzymes necessary for saccharification of cellulose and hemicelluloses. Unlike Celluclast® 1.5L (abbr. C1.5L), the CTec2 preparation contains increased concentration of β-glucosidase and xylanases, and achieves high conversion of cellulose even in the presence of inhibitors of cellulases. C1.5L contains a broad spectrum of cellulases (including mainly complex of endoglucanases and cellobiohydrolases) isolated from *Trichoderma reesei*. Product of lignocellulose degradation catalysed by C1.5L is a mixture of glucooligomers, cellobiose and glucose. As it is well known that cellobiose

inhibits cellulases activity [13], therefore the preparation was supported by Novozyme 188 (abbr. N188), with high β-glucosidase activity to provoke the final hydrolysis of disaccharide molecules into glucose. According to information provided by the manufacturer, CTec2 preparation delivers an average ca. 1.8 times increased performance improvement when used on many different feedstocks, if compared to cellulolytic coctails previously developed by Novozymes [14]. General compositions of enzyme cocktails used in experiments has been summarised in Table 1. Detailed data, i.e. concentrations and values of activities of particular enzyme-based ingredients of the preparations/cocktails are a trade secret and such data are not made officially public by the manufacturer.

Table 1

Main composition of studied enzyme preparations

Enzyme preparations	Abbreviation	Enzymatic qualitative composition
Cellic® CTec2	CTec2	endoglucanases, cellobiohydrolases, xylanases, β-glucosidase
Celluclast® 1.5L	C1.5L	endoglucanases, cellobiohydrolases, xylanases
Novozyme 188	N188	β-glucosidase

Pre-treatment of lignocellulosic biomass

Pre-treatment of lignocellulosic biomass were carried out in 1000 cm^3 screw-cap glass bottles. 20 g of dry matter of biomass were mixed with 500 cm^3 of 10% (w/v) NaOH, and obtained suspensions were placed in autoclave (T = 121°C, $p_{overpressure}$ = 0.1 MPa) for 40 minutes. After cooling, the supernatant was gently decanted, whilst the sludge was washed three times with 200 cm^3 of distilled water, then neutralized with 0.1 M HCl, and finally dried for 7 days at room temperature. The mass of insoluble solids after pre-treatment equalled to values 9.7 ± 0.5 g of dry matter of ray-straw and 10.2 ± 0.3 g in the case of energetic willow biomass. Prior to the pre-treatment, ray straw biomass was cut into 10-20 mm small pieces, whilst energetic willow was crushed in a mill into particles with the size of 2-15 mm. Untreated cellulose paper was cut with scissors into ca. 10 x 10 mm pieces, before hydrolysis.

Enzyme catalysed hydrolysis

Enzyme catalysed hydrolysis of all studied feedstocks was performed in 300 cm^3 Erlenmeyer flasks maintained in a water-bath shaker (T = 45°C, 150 rpm). Each of reaction mixture was prepared by mixing 5 g of dry mass of feedstock (pre-treated lignocellulose biomass or untreated cellulose paper) with 100 cm^3 of citrate buffer (pH = 5.5), then appropriate amount of studied enzyme preparation was added to initiate process of enzymatic hydrolysis. The hydrolysis reaction was carried out for 50 hours.

Depending on the experiment variant, the total enzyme concentration in reaction mixture equalled to 3.0, 6.0 or 10.0% w/w (g of enzyme preparation/g of dry feedstock). The dosage of CTec2 was chosen basing on the recommendations given by the manufacturer [15]. The dosages of C1.5L and N188 (in meaning of total enzyme concentration) were equilibrated to dosages of CTec2, i.e. 3.0, 6.0 10.0%, to clearly show the differences in estimated activities of both compared enzyme preparations. The detailed data concerning amount of studied enzyme preparations are summarised in Table 2.

Table 2

The dosages of enzyme preparations used for the hydrolysis

Enzyme preparations	Mass of enzyme preparations	Concentration of enzyme preparations
	[g]	[%]w/w*
CTec2	0.15	3.0
	0.30	6.0
	0.50	10.0
C1.5L + N188	0.10 + 0.05 (in total: 0.15)	2.0 + 1.0 (in total: 3.0)
	0.20 + 0.10 (in total: 0.30)	4.0 + 2.0 (in total: 6.0)
	0.32 + 0.18 (in total: 0.50)	6.4 + 3.6 (in total: 10.0)

* g of enzyme preparation/g of dry feedstock

The samples of reaction mixture (1.0 cm^3) were harvested to Eppendorf microtubes at appropriate time intervals (6 time-points during first 5 hours of reaction, and then 2 time-points after 24 h as well as 48 h of reaction) and were immediately placed on crushed ice in order to stop the reaction. After cooling the samples were centrifuged (10 000 rpm, 10 min, 4°C) to remove all solid rests of non-digested feedstock) and next supernatant was filtered with single-use syringe filters (0.2 μm; polypropylene housing, nylon membrane), and finally stored in the freezer (–18°C) until analysis was performed.

Analytical methods

The course of enzyme-catalysed hydrolysis of lignocelulosic feedstocks and cellulose paper was monitored by determination of the total concentration of reducing sugars (i.e. products of hydrolysis, mainly glucose and xylose) in the harvested samples of reaction mixtures. The colorimetric method, which involves reaction of reducing sugars with 3,5-dinitrosalicylic acid (DNS method), under alkaline conditions, at 100°C, has been applied for quantitative analysis of reducing sugars [16]. The absorbance of the chromogenic product, i.e. 3-amino-5-nitrosalicylic acid, was measured at 550 nm using *GENESYS™ 20* spectrophotometer (*Thermo Fisher Scientific,* USA). The calibration curve has been prepared with glucose used as the standard reducing monosaccharide to estimate the calibration curve equation (1), as following:

$$A = 0.2278 \cdot C_{rs} - 0.0286 \quad (R^2 = 0.997) \tag{1}$$

where C_{rs} means the total concentration [mg/cm^3] of reducing monosaccharides in the sample, whilst A is the absorbance of that sample at 550 nm.

Results and discussion

In order to observe the differences in enzymatic activities of CTec2 and C1.5L + N188 mixture, two independent types of cellulosic feedstocks, i.e. lignocellulosic biomass (energetic willow and ray straw composed mainly of cellulose, hemicellulose and lignin) and cellulose paper composed in 100% of cellulose, were used as a substrate in our

experiments. Application of different raw materials varying in lignin content has enabled us to study the general influence of lignin content on the yield of enzymatic digestion of polysaccharides.

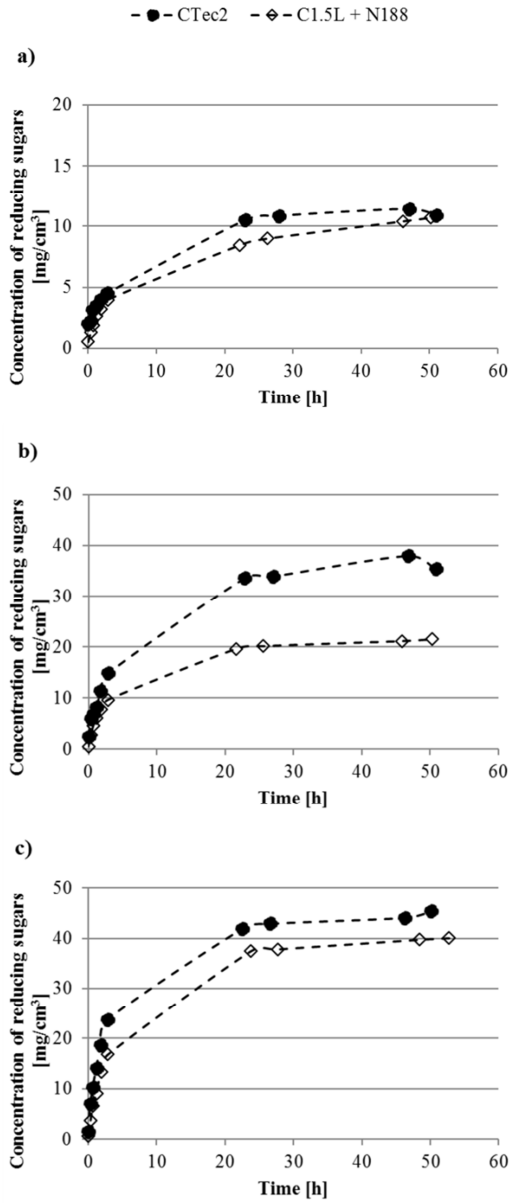

Fig. 1. Exemplary time courses of enzyme-catalysed hydrolysis of three studied feedstocks: energetic willow (a), ray straw (b) and cellulose paper (c) noted for both investigated enzyme preparations (i.e. CTec2 and C1.5L + N188)

The time courses of values of the reducing sugars concentrations during the enzymatic hydrolysis of all studied feedstocks, i.e. pre-treated lignocellulose biomass (energetic willow and ray straw) as well as cellulose paper, catalysed by CTec2 and C1.5L + N188 mixture, have been presented in Figure 1. The presented results include data of enzyme-catalysed hydrolysis obtained for the highest studied concentration of enzyme preparations equalled to 10% w/w (g of enzyme preparation/g of dry feedstock). However, similar relationships were observed for the other enzyme amounts (data not shown).

The experimental data show that the hydrolysis proceeded faster when CTec2 was used as catalyst. Such effect has been observed for all studied feedstocks used as substrate, with the highest differences noted for hydrolysis of ray straw (Fig. 1b). In the case of applied C1.5L + N188 mixture, after ca. 20 hours the significant slowdown in monosaccharide release from ray straw biomass can be observed. It can be hypothesized as being a negative effect caused by the hindered access of enzyme molecules to the substrate, or by adsorption of enzymes molecules on lignin chains, as well as it can also be associated with inhibition of enzymes by products or instability of enzyme molecules in reaction conditions [17, 18].

In the case of energetic willow used as feedstock, the progress of reaction (Fig. 1a) was significantly lower if compared to the other studied substrates. Even after 48-50 hours, values of the concentration of reducing sugars in samples of reaction mixtures do not exceeded 11 mg/cm^3, without any significant difference for used CTec2 or C1.5L + N188 preparations.

The final concentration of monosaccharides hydrolytically released from cellulose paper reached after 50 hours of the enzymatic process (Fig. 1c) equalled to 45.40 mg/cm^3 in the case of using CTec2. This value corresponds to almost 90% conversion of cellulose into glucose. The value of 40.03 mg/cm^3 obtained for the hydrolysis supported with C1.5L + N188 mixture relates to ca. 80% conversion of polysaccharide into glucose.

Based on experimental data of time-changes in reducing sugars concentration, the initial rates of hydrolytic degradation of cellulosic feedstocks may be determined by graphical method. The values of such kinetic parameters were found as a slope of the line tangent to the initial reaction curve, i.e. for a short period after the start of reaction, and have been summarized in Table 3.

Table 3

Values of initial reaction rates determined for studied enzyme preparations and cellulosic feedstocks

Enzyme preparations	**Concentration of enzyme preparations**	**Initial reaction rate**		
		cellulose paper	**ray straw**	**energetic willow**
	[%] w/w*	**[mg/cm^3/h]**		
CTec2	3.0	2.83	1.75	0.15
CTec2	6.0	5.79	2.95	0.54
CTec2	10.0	10.30	4.62	1.32
C1.5L + N188	3.0	1.39	1.39	0.71
C1.5L + N188	6.0	4.27	2.38	1.26
C1.5L + N188	10.0	6.58	4.18	2.31

* g of enzyme preparation/g of dry feedstock

It can be clearly concluded that the studied reaction proceeded faster according to increased concentration of enzyme preparations in the reaction mixtures in the case of both studied sets of enzymes. Such effect resulted in increased final concentration of reducing

sugars in samples harvested from reaction mixtures with higher level of added cellulolytic enzymes.

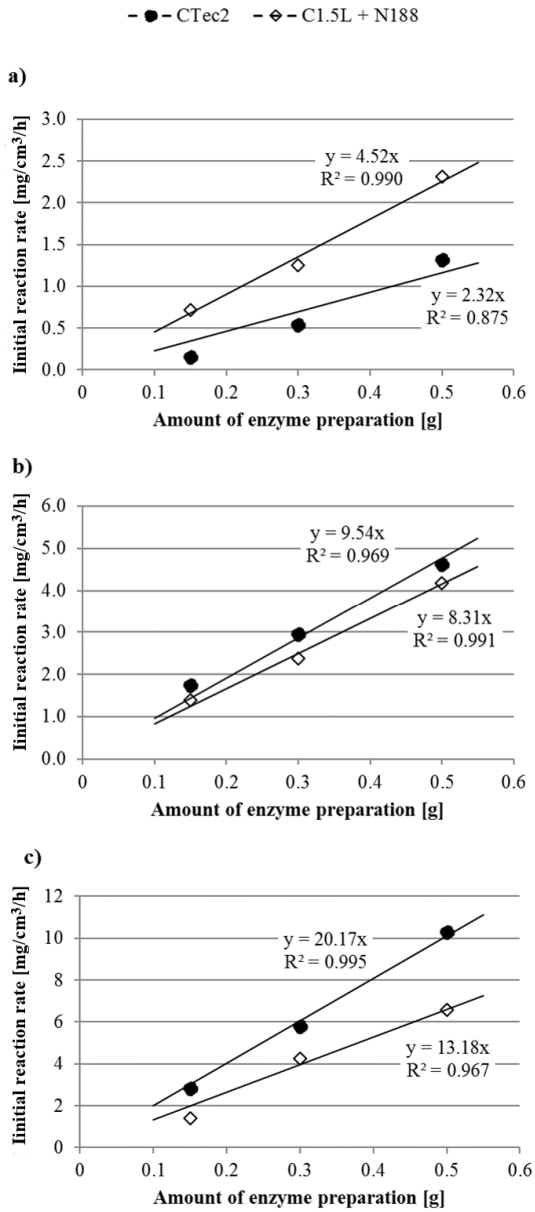

Fig. 2. Results of the linear regression of the experimentally obtained values of the initial reaction rates in the function of amount of CTec2 and C1.5L + N188 enzyme preparations used individually towards all types of studied feedstocks

In the case of hydrolysis of cellulose paper and rye straw, the highest values of initial reaction rate, i.e. 10.30 and 4.62 mg/cm^3/h respectively, were found for CTec2. However, hydrolysis of energetic willow was more efficient if mixture of C1.5L + N188 has been applied as catalyst, than CTec2. These results indicated that although CTec2 is a better choice for cellulose hydrolysis, its advantage over C1.5L depends on the lignocellulose feedstock.

The plot of initial rates of enzyme-catalysed degradation of cellulosic feedstocks versus mass of applied enzyme preparations, supported with results of linear regression analysis of experimental data, have been presented in Figure 2. As it has been revealed, values of the initial reaction rate increased linearly with the total enzyme amount in the case of all investigated enzyme-substrate sets. Linear regression coefficients were interpreted as values of the enzymatic activities of investigated lignocellulosic enzyme preparations, i.e. CTec2 and C1.5L + N188 as well, towards all three studied feedstocks, i.e. energetic willow, ray straw and cellulose paper. The enzymatic activity was expressed as the concentration of reducing sugars (in [mg/cm^3]) released from a given feedstock, per 1 g of a given enzyme preparation during 1 minute of hydrolysis. The comparison of estimated values of enzymatic activities, which characterize studied enzyme cocktails used towards all three lignocellulosic substrates, at the conditions of used methodology, have been compared in Figure 3.

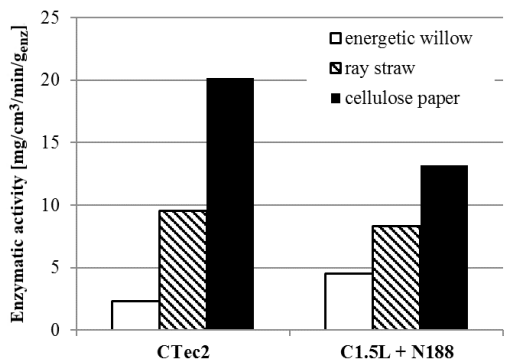

Fig. 3. The comparison of the enzymatic activity values estimated for CTec2 and C1.5L + N188 mixture used as catalysts of hydrolytic degradation of energetic willow, ray straw and cellulose paper

Taking into account the results obtained for enzyme-catalysed degradation of pre-treated lignocellulosic materials, i.e. energetic willow and ray straw, it can be stated, that hydrolytic digestion of ray straw is noticeably more effective than hydrolysis of energetic willow both in the case of CTec2, as well as the mixture of C1.5L + N188, applied as enzyme preparation. Higher activity of CTec2 exhibited toward pre-treated lignocellulosic feedstocks were additionally proved by analysis of the progress of reactions, as well as over 3.5 times and ca. 2 times higher concentration of reducing monosaccharides released from ray straw in comparison to monosaccharaides released from biomass of energetic willow if they were degraded by CTec2 and C1.5L + N188 respectively, after 50 hours of the process carried out with 10% w/w concentration of enzyme preparations (as showed in Figure 1a,b).

We hypothesized that lower enzymatic activity of CTec2 noted for the pre-treated biomass of energetic willow used as substrate, than in the case of the pre-treated ray straw, is related to the differences in level of lignin within those two different biomass-based feedstocks. Basing on standard NREL procedure [19] we have found, that the pre-treated biomass of energetic willow contained ca. 45% w/w of lignin, whilst pre-treated ray straw only ca. 25% w/w of lignin. As it was reported earlier in the literature, e.g. [17, 20], cellulolytic enzyme cocktails which were used in our experiments have distinct ability to adsorption on lignin molecules. Despite of significantly higher hydrolytic efficiency of CTec2 than C1.5L enzyme cocktail, CTec2 shows higher affinity towards lignin and therefore significant number of active enzyme molecules remained unproductively adsorbed to the solid residues during hydrolysis, what finally resulted in lower yield of enzyme-catalysed degradation of feedstocks containing more lignins.

Considering the data obtained for enzymatic hydrolysis of cellulose paper, CTec2 preparation exhibited clearly higher activity in comparison to C1.5L + N188 enzyme cocktail. In our opinion, such effect may be recognized as confirmation of the information given by the manufacturer, that CTec2 preparation is more efficient and less susceptible to enzyme inhibition caused by cellobiose than C1.5L.

Conclusions

The enzymatic activity of industrially-applied enzyme-preparations, i.e. CTec2 and the C1.5L + N188 mixture, commercially offered for lignocellulosic biomass saccharification has been estimated, compared and discussed. Enzyme preparations/cocktails have been tested in the small-scale reaction system in which energetic willow and ray straw, both pre-treated with NaOH at high-temperature and overpressure conditions, as well as untreated cellulose paper, were used as model feedstocks. Based on the results of our studies, it can be concluded that efficiency of enzyme catalysed hydrolysis depends not only on applied catalyst but also on the general type of raw material used as substrate. In the case of degradation of pre-treated ray straw, as well as cellulose paper, the highest enzymatic activity was found for CTec2. Whereas if pre-treated biomass of energetic willow was used as a substrate the best results have been obtained for C1.5L + N188 mixture. Furthermore, we hypothesised that such effects were associated with the differences in the percentage content of lignin fraction in the total mass of studied pre-treated feedstocks.

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