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Comparative study of saccharification of biomass by various cellulolytic enzymes

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Abstract : In this paper a comparative study of saccharification of cellulose-rich switchgrass biomass by various enzyme preparations (Accelerase 1500, GC 220, NZ50013 and CTec 2) has been carried out. Furthermore, an effect of supplementary β-glucosidase on activity of enzymes and yield of sugars has been studied. It was found that adding of β-glucosidase to enzyme preparations improves cellulolytic activity and increases the yield of glucose after enzymatic hydrolysis of the cellulosic biomass. In order to evaluate the yield of glucose (Y) from cellulosic substrate after its cleavage by different enzymatic preparations, a combined parameter of cellulolytic activity, $P=L^2/D$, has been proposed; where L is used level of cellulolytic activity expressed in FPU per 1g substrate; and D is used doze of the enzyme expressed in mg proteins per 1g substrate. There is a directly proportional dependence between yield of glucose and combined parameter of cellulolytic activity. Due to high squared correlation coefficient ($R^2 = 0.934$), the regression equation $Y = k P$ can be used to predict the yield of glucose from the biomass hydrolyzed by different enzyme preparations.

Keywords : Biomass; Enzyme preparations; Parameter of cellulolytic activity; Yield of glucose; Correlation.

INTRODUCTION

Considerable attention in recent years has been given to renewable plant biomass, which can be used as a feedstock for production of glucose and other valuable bioproducts[1, 2]. The non-edible plant raw materials are attributed to abundant, renewable and inexpensive biomass types. The total amount of such biomass that accumulates annually in the world is estimated in 10 billion tons at least[3]. Only in USA annual accumulation of the lignocellulosic biomass is of around 1 billion tons.

The conversion technology of non-edible plant biomass into fermentable sugars, mainly glucose, is based on enzymatic saccharification of the pretreated biomass. As known, initial biomasses are highly recalcitrant to enzymatic hydrolysis due to dense inaccessible structure, increased crystallinity and low content of cellulose, barrier properties of lignin and non-productive sorption of enzymes by non-cellulosic components. Therefore, a special pretreatment of initial biomass is required in order to remove

ligno-hemicellulose matrix and make the cellulosic component of the biomass more accessible to cellulolytic enzymes[3-5].

The saccharification of the pretreated biomass is performed by means of cellulolytic enzymes that can be synthesized by some fungi, bacteria and protozoans[6]. Nevertheless special microfungi (*e.g. Trichoderma, Aspergillus, etc.*) are the most widespread source of cellulolytic enzymes. Recently, the large biotechnological companies have achieved a great success in development of novel commercial enzyme preparations having high concentration of proteins, increased activity, and decreased cost. Known types of such enzyme preparations are Accellerase-1500 and GC-220 of DuPont/Genencor; and Celluclast 1.5 L, Cellic CTec 2 and Cellic CTec 3 of Novozymes, *etc.* Recommended conditions of the enzymatic hydrolysis are the following: temperature 45 to 50 °C, pH = 4.5 to 5.0, and conventional loading of the enzyme (level of cellulolytic activity) 10 to 20 FPU per 1 g of solid substrate.

As is know, to perform conversion of cellulosic component of biomass into glucose the cellulolytic complex can consist of three main enzyme groups[7]: (1). Endo-1,4-β-glucanases (EN) cleave chemical glycoside bonds in the amorphous domains of cellulose fibrils, and as a result produce small particles with reduced degree of polymerization; (2). Exo-1,4-β-glucanases (EX) or cellobiohydrolases attack the reducing or non-reducing ends of the depolymerized cellulose particles with forming mainly cellobiose and some cellodextrins; (3). β-glucosidases (BG) hydrolyze the cellobiose and cellodextrins into glucose. These three groups of enzymes act synergistically because endo-acting enzymes generate new chain ends for the exo-acting enzymes, which release the cellobiose and cellodextrins that are converted into glucose by β-glucosidases.

Each enzymatic group comprises several cellulases. It has been shown that the cellulase system of *Trichoderma reesei* contains Cel5A and Cel7B endoglucanases, Cel7A and Cel6A exoglucanases, and some other enzymes[8]. From *Trichoderma viride* six endoglucanases and two exoglucanases were isolated[9]. Individual cellulase group – only EN or EX, and their binary mixtures, are not able to carry out an efficient conversion of cellulose into glucose. Implementation of saccharification requires all three enzymatic groups – EN, EX and BG, which can be presented in different proportions in various enzyme preparations[8, 10].

Detailed studies have shown that various enzyme preparations under the equal enzyme loading and other hydrolysis conditions give different yield of glucose from the same pretreated biomass. Despite the abundant investigations, the main causes of this phenomenon still are not clear. One of these causes may be different content and different specific activity of cellulases and BG in the enzyme cocktails leading to different final cellulolytic activity of various preparations. For this purpose, a comparative study of saccharification of cellulose-rich biomass by various enzyme preparations having a different composition and cellulolytic activity was carried out. Furthermore, the effect of supplementary β-glucosidase on activity of enzymes and yield of sugars has been studied.

EXPERIMENTAL

Material

One of widespread energy crops – switchgrass, supplied from Nott Farms (Canada), has been used as an initial biomass. The biomass sample was knife-milled and screened through a sieve to obtain the fraction of 2-3 mm.

Pretreatment

The initial biomass was pretreated by nitric acid/alkaline (NA) method[11]. The biomass sample was treated with 4-5 wt.% nitric acid at 110-115 C for 30 min acid, washed and then extracted with boiling 1-2 wt.% sodium hydroxide for 30 min. The pretreated biomass slurry was neutralized with 1 wt.% sulfuric acid up to pH 6, washed and squeezed on vacuum glass-filter up to a final solids content about 20-25 wt.%.

Analysis of chemical composition

The chemical composition of biomass samples was determined by conventional and standard methods of chemical analysis[11, 12]. Contents of three main

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TABLE 1 : Chemical composition of the biomass samples

Biomass	Cellulose, %	Hemicelluloses, %	Lignin, %
Initial	38	27	19
Pretreated	88	5	6

TABLE 2 : Characteristics of enzymes

Enzyme	Abbreviation	Composition	*Activity, U/ml	Concentration, mg/ml
Accelerase 1500	AC	EN/EX/BG	115	100
GC 220	GC	EN/EX/BG	300	250
CTec 2	CT	EN/EX/BG	500	270
NZ50013	NZ	EN/EX	80	240
NZ188	BG	BG	620	210

***Note: Activity of cellulases AC, GC, CT and NZ was in FPU/ml; Activity of β-glucosidase NZ188 was in CBU/ml.**

polymeric components – cellulose, hemicelluloses and lignin – in the initial and pretreated switchgrass are shown in TABLE 1. The initial switchgrass contained a relative low amount of cellulose, 38 wt. % only. After NA-pretreatment, a main amount of lignin, hemicellulose and other non-cellulosic components has been removed, resulting in a significant increase in cellulose content in the pretreated biomass.

Enzyme preparations and enzymatic hydrolysis

The following enzyme preparations have been used: Accelerase 1500 and GC 220 (DuPont Ind. Biosciences, Wilmington, DE, USA); NZ188, NZ50013 and Cellic CTec 2 (Novozymes A/S, Bagsvaerd, Denmark). Activity of enzymes was measured in accordance with Ghose protocols[13]. Concentration of enzymes was determined by BCA protein assay[14]. Some characteristics of the enzymes are shown in TABLE 2.

The pretreated cellulose-rich biomass was hydrolyzed with various enzyme preparations and their mixtures with β–glucosidase NZ-188. The used level cellulolytic activity of various enzymes was 10 to 20 FPU per 1 g of solid sample. Enzymatic hydrolysis was carried out in 50-mL polypropylene tubes. The sample containing 1 g of the solid matter and 10 ml of 50 mM acetate buffer (pH=4.8) was put into the tubes, and then enzyme or its mixture was added. Further, an additional volume of the buffer was supplemented to achieve concentration of the biomass 50 g/l (C_b). The tubes closed with covers were placed in a shaker incubator at 50°C and shaken at 150 rpm for 24 h. Finally, the tubes were centrifuged in order to separate the sugar solution (hydrolyzate) from the residual biomass. Concentration of the sugar (C_s, g/l) in the hydrolyzate was determined by the by HPLC-apparatus of Agilent Technologies 1200 Infinity Series. The Amines HPX-87H column was used. Main conditions of the analysis were: temperature 45°C; mobile phase 0.005 M sulfuric acid; flow rate 0.6 ml/min. The sample of hydrolyzate was preliminary filtered through 0.45 μm Nylon filter and degassed. Yield of the sugar after enzymatic hydrolysis of the biomass was calculated by the equation:

$$\text{Yield (\%)} = 100\% \ (C_s/C_b)$$

where C_s is concentration of the sugar (g/l), and C_b = 50 g/l is concentration of the biomass.

RESULTS AND DISCUSSION

The cellulolytic activity of various enzyme preparations, as well as mixtures thereof with β-glucosidase NZ188 (BG) supplemented in an amount of 15 vol. % to volume of the enzyme, is shown in TABLE 3. It was found that the supplement of BG to CT does not change the cellulolytic activity of the enzymatic cocktail. On the other hand, the additive of BG to EN/EX cellulase complex (NZ) causes a four-fold enhance in cellulolytic activity. When BG is added to AC or GC enzymes, a moderate activity increase (1.4 times) is observed.

The obtained results confirm the conclusion of other researchers that content and/or activity of β-glucosidase in the enzyme preparation may affect the result of FPU assay[13, 15]. The absence or lack of

TABLE 3 : Activity of various enzymes and their mixtures with β-glucosidase

Enzyme and Enzyme/BG mixture	Activity, FPU/ml	Relative Activity
NZ	80	1
NZ/BG	350	4.3
AC	115	1
AC/BG	170	1.5
GC	300	1
GC/BG	410	1.4
CT	500	1
CT/BG	500	1

β-glucosidase, as well as low activity of β-glucosidase in enzyme preparations lead to formation of cellobiose and also cellodextrins after hydrolysis of filter paper, resulting in lowering of FPU. Adding of β-glucosidase to the enzyme promotes conversion of cellobiose and some cellodextrins into glucose, and thus improves the FPU of the enzyme preparation.

The positive impact of the β-glucosidase (BG) additive is also observed, when the enzymatic cleavage of the pretreated biomass was studied using mixtures of various enzyme preparations with 15 vol. % of BG (NZ188) (Figure1). As can be seen, the NZ preparation, containing a complex of EN/EX converts cellulose of the pretreated biomass mainly into cellobiose, while the yield of glucose was low. Other enzyme preparations - AC and GC, containing EN, EX and BG can turn cellulose of the biomass mainly into glucose and considerable amount of cellobiose, probably due to lack of content or activity of BG. Moreover, the formed cellobiose can inhibit the activity of exo- and endoglucanases, thus hampers the further enzymatic cleavage of cellulose[16, 17]. It has been reported that β-glucosidase is a low-stable enzyme and gradually loses its activity under conditions of enzymatic hydrolysis, at temperature 50°C and pH 5[18]. Therefore, an excess of β-glucosidase is required to prevent the preservation of a large amount of cellobiose inhibitor.

Adding of β-glucosidase NZ188 (BG) to enzyme preparations promotes transformation of cellobiose into glucose. As a result, there is a complete disappearance of cellobiose and increase in the yield of glucose.

For example, the BG supplement to NZ enzyme drastic increases the yield of glucose from 5 to 72% after enzymatic hydrolysis of the biomass. An exception was CT (CTec 2) enzyme preparation, since the additive of BG to this preparation had no effect on the yield of glucose. This indicates that CTec 2 contains a sufficient large amount of active β-glucosidase.

As is known, the yield of glucose after hydrolysis of cellulosic substrate by certain enzyme correlates with the used level of cellulolytic activity expressed in FPU/g substrate. However, when the hydrolysis of the same substrate is carried out by different enzyme preparations, the value of FPU/g does not determine the yield of glucose (Figure 2). For example, at equal activity level, 15 FPU/g, the various enzymes produce from the cellulosic biomass different amounts of glucose, which varies in a wide range from 5 to 75%.

This phenomenon can be explained by different

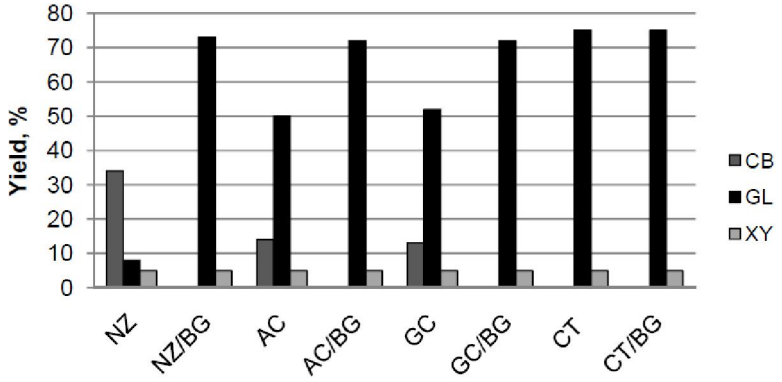

Figure 1 : Yield of cellobiose (CB), glucose (GL) and xylose (XY) after saccharification of NA-pretreated biomass using various enzyme preparations and their mixtures with β-glucosidase at a constant level of cellulolytic activity, 15 FPU/g biomass

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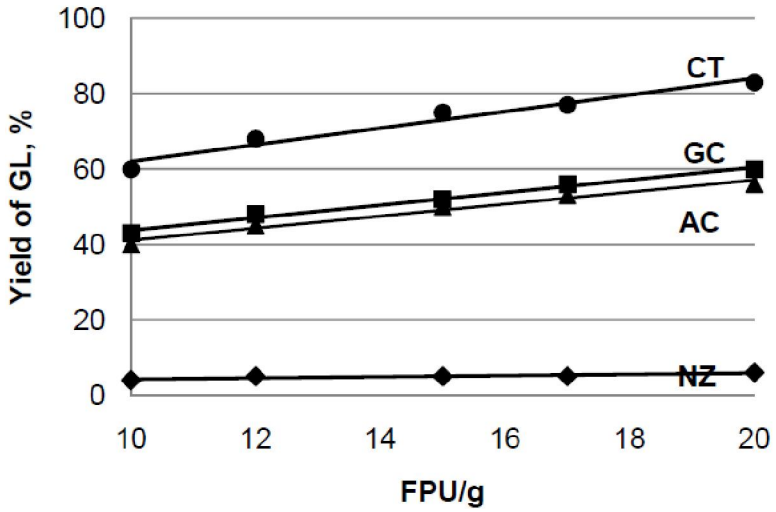

Figure 2 : Dependence of glucose yield on the used level of cellulolytic activity after hydrolysis of the pretreated biomass by different enzyme preparations: NZ, AC, GC and CT

TABLE 4 : Example of combined parameter of cellulolytic activity and yield of glucose

Enzyme and Enzyme/BG mixture	L, FPU/g	D, mg/g	P	Y, %
NZ	10	30	3	5
NZ/BG	15	9	25	71
AC	20	17	23	58
AC/BG	10	6	17	50
GC	15	12	19	52
CT	15	8	28	75

generate different amounts of glucose and other sugars.

In order to evaluate the yield of glucose (Y) from cellulosic substrate after its cleavage by different enzymatic preparations, a combined parameter of cellulolytic activity, $P=L^2/D$, has been proposed; where L is used level of cellulolytic activity expressed in FPU per 1g substrate; and D is used doze of the enzyme expressed in mg proteins per 1g substrate (TABLE 4).

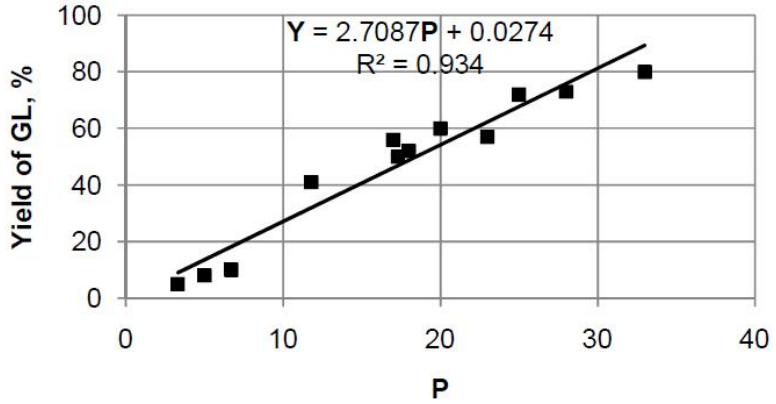

Figure 3 : Correlation between glucose yield and combined parameter of cellulolytic activity for different enzyme preparations (NZ, NZ/BG, AC, AC/BG, GC, GC/BG, CT and CT/BG) used for enzymatic hydrolysis of the pretreated biomass

proportions and different activities of EN, EX and BG enzymatic groups, as well as by the presence of different types and amounts of some auxiliary agents (promoters, stabilizers, surfactants, preservatives and others) in various enzyme preparations. Thus, despite the use equal FPU/g, the different enzyme preparations at the same hydrolysis conditions will

As can be seen from Figure 3, there is a directly proportional dependence between and yield of glucose (Y) and combined parameter of cellulolytic activity (P). Due to high squared correlation coefficient ($R^2 = 0.934$), the regression equation $Y = k P$ can be used to predict the yield of glucose from the biomass hydrolyzed by different enzyme preparations.

CONCLUSION

In this paper a comparative study of saccharification of cellulose-rich switchgrass biomass by various enzyme preparations (Accelerase 1500, GC 220, NZ50013 and CTec 2) has been carried out. Furthermore, an effect of supplementary β-glucosidase on activity of enzymes and yield of sugars has been studied. It was found that adding of β-glucosidase to enzyme preparations improves cellulolytic activity and increases the yield of glucose after enzymatic hydrolysis of cellulosic biomass. An exception was CTec 2, since the supplementary β-glucosidase dose to this enzyme had no effect on the yield of glucose.

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