

## Design of Highly Efficient Cellulase Mixtures for Enzymatic Hydrolysis of Cellulose

Alexander V. Gusakov,<sup>1</sup> Tatyana N. Salanovich,<sup>1</sup> Alexey I. Antonov,<sup>1</sup> Boris B. Ustinov,<sup>1</sup>  
Oleg N. Okunev,<sup>2</sup> Richard Burlingame,<sup>3</sup> Mark Emalfarb,<sup>3</sup> Marco Baez,<sup>3</sup> Arkady P.  
Sinitsyn<sup>1</sup>

<sup>1</sup>*Department of Chemistry, M. V. Lomonosov Moscow State University, Moscow 119899, Russia;*

<sup>2</sup>*Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences,  
Pushchino, Moscow Region 142292, Russia*

<sup>3</sup>*Dyadic International, Inc., 140 Intracoastal Pointe Drive, Suite 404, Jupiter, Florida 33477-  
5094*

*Correspondence to:* Mark Emalfarb, Dyadic International, Inc. [memalfarb@dyadic-group.com](mailto:memalfarb@dyadic-group.com)

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Received September 8, 2006; Accepted December 28, 2006

**Abstract:** An extremely highly active cellobiohydrolase (CBH IIb, or Cel6B) was isolated from *Chrysosporium lucknowense* UV18-25 culture filtrate. The CBH IIb demonstrated the highest ability for a deep degradation of crystalline cellulose amongst a few cellobiohydrolases tested, including *C. lucknowense* CBH Ia, Ib, IIa, and *Trichoderma reesei* CBH I and II. Using purified *C. lucknowense* enzymes (CBH Ia, Ib, and IIb; endoglucanases II and V;  $\beta$ -glucosidase, xylanase II), artificial multienzyme mixtures were reconstituted, displaying an extremely high performance in a conversion of different cellulosic substrates (Avicel, cotton, pretreated Douglas fir wood) to glucose. These mixtures were much or notably more effective in hydrolysis of the cellulosic substrates than the crude multienzyme *C. lucknowense* preparation and other crude cellulase samples produced by *T. reesei* and *Penicillium verruculosum*. Highly active cellulases are a key factor in bioconversion of plant lignocellulosic biomass to ethanol as an alternative to fossil fuels.

**Keywords:** cellobiohydrolase; cellulase; *Chrysosporium lucknowense*; endoglucanase; *Penicillium* sp.; *Trichoderma reesei*

## INTRODUCTION

Bioconversion of renewable lignocellulosic biomass to ethanol as an alternative to liquid fuels has attracted an intensive attention of researchers since 1970's, when the oil crisis broke out because of decreasing the output of petroleum by OPEC (Bungay, 1981; Olsson and Hahn-Hägerdal, 1996; Zaldivar et al., 2001; Galbe and Zacchi, 2002). Ethanol has been widely used as a 10% blend to gasoline in the USA or as a neat fuel for vehicles in Brazil. The importance of fuel bioethanol will increase in parallel with skyrocketing prices for oil and gradual depletion of its sources.

The fuel alcohol production from corn increased in the USA very quickly since the end of 1970's and reached about 4 billion gallons in 2005 displacing 2% of the total demand of fossil fuel. According to the roadmap of the U.S. Department of Energy ([http://www.doe.genomestolife.org/roadmap/pdf/GenomicsGTL\\_Roadmap\\_highres.pdf](http://www.doe.genomestolife.org/roadmap/pdf/GenomicsGTL_Roadmap_highres.pdf)), later in this century the bioethanol may displace 15-100% of fossil fuel and its production in the country may reach 30-200 billion gallons. The growing interest for bioethanol is also observed in other countries of America, Europe and Asia. Since corn or other grains as a feedstock may not satisfy the growing demands for alcohol because of a limited yield of grain from one unit of land area, plant lignocellulosic biomass is considered to be an alternative (non-starch) renewable feedstock for bioethanol production.

The major polysaccharides comprising different lignocellulosic residues are cellulose and hemicelluloses (Olsson and Hahn-Hägerdal, 1996; Pérez et al., 2002). The enzymatic hydrolysis of these polysaccharides to soluble sugars (and finally to monomers: glucose, xylose, and other hexoses and pentoses) occurs under the action of different enzymes acting in concert (Clarke, 1997; Pérez et al., 2002). Endo-1,4- $\beta$ -glucanases (EG) and exo-cellobiohydrolases (CBH)

catalyze the hydrolysis of insoluble cellulose to cellooligosaccharides (cellobiose as a main product), while  $\beta$ -glucosidases (BGL) convert the oligosaccharides to glucose. Xylanases together with other accessory enzymes ( $\alpha$ -L-arabinofuranosidases, feruloyl and acetylxyylan esterases,  $\beta$ -xylosidases, etc.) catalyze the hydrolysis of hemicelluloses.

Regardless of the type of cellulosic feedstock, the cost and hydrolytic efficiency of enzymes are major factors that restrict the commercialization of the biomass bioconversion processes (Nieves et al., 1998; Himmel et al., 1999; Galbe and Zacchi, 2002). The enzyme production costs are tightly connected with a productivity of enzyme-producing microbial strain and the final activity (protein) yield in the fermentation broth (Duff and Murray, 1996; Nieves et al., 1998; Himmel et al., 1999). The hydrolytic efficiency of a multienzyme complex in the process of lignocellulose saccharification depends both on properties of individual enzymes and their ratio in the multienzyme cocktail.

Filamentous fungi are the major source of cellulases and hemicellulases. Mutant strains of *Trichoderma* sp. (*T. viride*, *T. reesei*, *T. longibrachiatum*) have long been considered to be the most productive and powerful destroyers of crystalline cellulose (Mandels and Sternberg, 1976; Nieves et al., 1998; Galbe and Zacchi, 2002). Two cellobiohydrolases (CBH I and II) are the major *T. reesei* enzymes, which act synergistically with two major endoglucanases: EG I and II (Nidetzky et al., 1994; Teeri, 1997; Foreman et al., 2003). Owing to extensive financial U.S. governmental support and efforts of enzyme producing companies, the production costs of *Trichoderma* cellulases have been reduced dramatically in the last few years (see for instance <http://www.report2005.novozymes.com/>). However, one of the major *Trichoderma* drawbacks is the low level of BGL activity (Duff and Murray, 1996; Nieves et al., 1998), leading to incomplete conversion of cellobiose to glucose in the cellulose hydrolysis process.

In spite of the success, achieved in the last decades in the fields of finding out the mechanisms of enzymatic cellulose degradation and cellulase production, the task of finding new highly active cellulases remains topical. Such enzymes could be used for a rational design of highly efficient multienzyme mixtures capable to carry out fast and complete biodegradation of lignocellulosic materials. Then, using modern genetic techniques, the optimal multienzyme cocktail could be expressed in a highly productive microbial host.

Data have been reported on superior performance of *Penicillium sp.* cellulases in hydrolysis of microcrystalline cellulose and different lignocellulosic substrates, compared to the performance of various *Trichoderma sp.* preparations (Castellanos et al., 1995; Berlin et al., 2005a; Kurabi et al., 2005). High level of the BGL activity in *Penicillium sp.* samples provided practically complete conversion of intermediate cellobiose to glucose. Recently, highly productive *P. verruculosum* strains have been developed, which provide the protein concentrations up to 47 g/L in the end of fermentation (Solov'eva et al., 2005). Such protein yields are comparable or exceed the respective parameters for the best *Trichoderma sp.* strains (35-40 g/L) (Durand et al., 1988; Foreman et al., 2003).

*Chrysosporium lucknowense* is another perspective fungus, superproducer of cellulases and hemicellulases (U.S. Patent No. 6,015,707, 2000; Int. Patent WO 01/79558, 2001; U.S. Patent No. 6,573,086, 2003). Previously, we have reported data on highly active CBH I of *C. lucknowense* that was more thermostable than the CBH I of *T. reesei* (Gusakov et al., 2005). *C. lucknowense* also secretes at least five different endoglucanases, the EG II (51 kDa) being the most active (Bukhtojarov et al., 2004).

In this paper, we report the properties of two new cellobiohydrolases (CBH Ib and Iib) from *C. lucknowense*, one of which (CBH Iib) was characterized by an extremely high activity

against crystalline cellulose. This enzyme displayed a pronounced synergism with other *C. lucknowense* cellulases, and it was used for designing the highly effective multienzyme mixtures for cellulose hydrolysis, whose hydrolytic performance was better than the performance of *T. reesei* and *P. verruculosum* crude multienzyme preparations.

## MATERIALS AND METHODS

### Enzymes

Culture filtrates produced by the *C. lucknowense* mutant strains UV18-25, UV18 $\Delta$ Cbh1#10 and Xyl2-18 (from Dyadic International, Inc., USA) were used for isolation of individual enzymes. Commercial enzyme preparation Celoviridin G20x produced by the *T. reesei* mutant strain 18.2 KK (from Berdsky Fermentation Plant, Russia) was used for purification of *T. reesei* CBH I (Cel7A) and CBH II (Cel6A). Crude commercial preparations BioAce (*T. reesei*), NCE-L600 (*C. lucknowense*) from Dyadic International, Inc., USA, and two laboratory *P. verruculosum* multienzyme samples (#6 and #151) from the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, were used in cellulose saccharification experiments. Highly purified BGL (cellobiase) from *Aspergillus japonicus* produced by NPO Biotekhnika (Russia), having specific cellobiase activity 50 U/mg protein (pH 5.0, 40°C), was used in the experiments on hydrolysis of insoluble cellulose.

### Enzyme Purification

The enzyme purification was carried out by chromatography on a Pharmacia FPLC system (Sweden). Cellobiohydrolases and endoglucanases were isolated from a *C. lucknowense* UV18-

25 culture filtrate. BGL and Xyl II (xylanase II) were isolated from culture filtrates produced by the *C. lucknowense* UV18ΔCbh1#10 and Xyl2-18 mutant strains, respectively.

In all cases, the first purification stage was anion-exchange chromatography on a Source 15Q column (40 mL volume). The column was equilibrated with 0.02 M Bis-Tris-HCl buffer, pH 6.8. The initial culture filtrate was preliminary desalted and transferred into the starting buffer by gel-filtration on Acrylex P4 (Reanal, Hungary). The sample (400 mg of protein) was applied to the Source 15Q column, and the elution was carried out with a gradient of 0-1 M NaCl at a flow rate of 10 mL/min.

The first protein fraction after the Source 15Q, eluted at 0.05 M NaCl and having high Avicelase activity, was subjected to hydrophobic interaction chromatography on a Source 15 Isopropyl column (Pharmacia, Sweden). The column was equilibrated with 1.7 M ammonium sulfate in 50 mM Na-acetate buffer, pH 5.0. Proteins were eluted with a reversed linear gradient of 1.7-0 M ammonium sulfate at a flow rate of 4 mL/min. The protein fraction with the highest activity against Avicel (eluted at the salt concentration of 0.30-0.35 M) contained the homogeneous protein with a molecular mass of 70 kDa (CBH IIb, see Fig. 1).

The protein fraction after the Source 15Q, eluted at 0.22 M NaCl and having the activity against Avicel and 4-nitrophenyl  $\beta$ -D-cellobioside, was further purified by chromatofocusing on a Mono P HR 5/20 column (Pharmacia, Sweden). The column was equilibrated with 0.025 M Na-formate buffer, pH 4.0. Proteins were eluted with a gradient of pH 4.5-3.0 (using Polybuffer 74) at a flow rate of 0.5 mL/min. Homogeneous 60 kDa CBH Ib was obtained as a result of chromatofocusing (Fig. 1).

The *C. lucknowense* BGL was isolated from the protein fraction after the Source 15Q (eluted at 0.10 M NaCl) containing the highest activity against 4-nitrophenyl  $\beta$ -D-

glucopyranoside and cellobiose. The fraction was subjected to the hydrophobic interaction chromatography as described above, the homogeneous BGL with a molecular mass of 106 kDa and *pI* 4.8 was eluted at 1.3 M of ammonium sulfate. The specific activity of the BGL toward 4-nitrophenyl  $\beta$ -D-glucopyranoside and cellobiose was found to be 11 and 26 U/mg of protein, respectively (40°C, pH 5.0).

The homogeneous Xyl II (24 kDa, *pI* 7.9) was obtained after the anion-exchange chromatography followed by the hydrophobic interaction chromatography as described above and gel-filtration on a Superose 12 HR 10/30 column (Pharmacia, Sweden). Elution at the last chromatographic stage was performed with 0.1 M Na-acetate buffer, pH 5.0, at a flow rate of 0.3 mL/min. The Xyl II had specific xylanase activity of 395 U/mg of protein (50°C, pH 5.0, birchwood xylan as a substrate).

The *C. lucknowense* CBH Ia (Cel7A, 65 kDa), CBH IIa (Cel6A, 43 kDa), EG II (Cel5A, 51 kDa), EG V (Cel45A, 25 kDa), EG VI (Cel6C, 47 kDa) were purified as described elsewhere (Bukhtojarov et al., 2004; Gusakov et al., 2005).

The *T. reesei* CBH I (Cel7A) and CBH II (Cel6A) were purified from Celloviridin G20x as described elsewhere (Gusakov et al., 2000; Markov et al., 2005). The purification scheme combined chromatofocusing on a Mono P column with hydrophobic interaction chromatography on Source 15 Isopropyl and gel-filtration on a Superose 12 column.

The enzyme purity was characterized by SDS-PAGE and isoelectrofocusing. SDS-PAGE was carried out in 12% gel using a Mini Protean II equipment (Bio-Rad Laboratories, USA). Isoelectrofocusing was performed on a Model 111 Mini IEF Cell (Bio-Rad Laboratories, USA). Staining of protein was carried out with Coomassie Blue.

## **MALDI-TOF and Tandem TOF/TOF Mass Spectrometry of Peptides**

The in-gel tryptic digestion of the protein bands after the SDS-PAGE was carried out essentially as described by Smith (1997). Trypsin (Promega, modified, 5 µg/mL) in 50 mM NH<sub>4</sub>HCO<sub>3</sub> was used for a protein digestion. The resulting peptides were extracted from a gel with 20% aqueous acetonitrile containing 0.1% trifluoroacetic acid and subjected to MALDI-TOF mass spectrometry (James, 2001). Selected peptides from the mass spectra of the tryptic digests of the CBH Ib and IIb were analyzed by tandem mass spectrometry in order to determine their sequences *de novo*. Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Germany) was used in the MS experiments.

## **Enzyme Activity Assays**

CMCase activity was measured by assaying reducing sugars released after 5 min of enzyme reaction with 0.5% carboxymethylcellulose (CMC, medium viscosity, Sigma, USA) at pH 5.0 and 50°C (Sinitsyn et al., 1990). Enzyme activities against barley β-glucan (Megazyme, Australia) and birchwood xylan (Sigma, USA) were determined in the same way as the CMCase activity, except the incubation time was 10 min. Avicelase activity was determined by analyzing reducing sugars released after 60 min of enzyme reaction with 5 mg/mL Avicel PH 105 (Serva, Germany) at pH 5.0 and 40°C (Sinitsyn et al., 1990; Gusakov et al., 2005). Reducing sugars were analyzed by the Somogyi-Nelson method (Somogyi, 1952). Filter paper activity (FPA) was determined as recommended by Ghose (1987). Activities against 4-nitrophenyl glycosides (Sigma, USA) were determined at pH 5.0 and 40°C as described elsewhere (Gusakov et al., 2005). Cellobiase activity was assayed at pH 5.0 and 40°C by measuring the initial rate of glucose release from 2 mM cellobiose by the glucose oxidase – peroxidase method (Sinitsyn et

al., 1990). All activities were expressed in International Units, i.e. one unit of activity corresponded to the quantity of enzyme hydrolyzing one  $\mu\text{mol}$  of substrate or releasing one  $\mu\text{mol}$  of reducing sugars (in glucose equivalents) per one minute.

### **Enzymatic Hydrolysis of Cellulosic Substrates**

The enzymatic hydrolysis of cellulosic substrates was carried out at pH 5.0 under magnetic stirring. Avicel PH 105 (Serva, Germany), cotton pretreated with acetone-ethanol mixture (1:1) for two days in order to remove wax from the surface of cellulose fibers, and Douglas fir wood pretreated by organosolv (Berlin et al., 2005a; Kurabi et al., 2005) were used as substrates. The experiments on progress kinetics of Avicel hydrolysis by purified individual cellobiohydrolases and experiments on synergistic interaction between *C. lucknowense* cellulases (with cotton as a substrate) were carried out at 40°C. The substrate concentration in those experiments was 5 mg/mL. In order to eliminate the effect of product (cellobiose) inhibition on the kinetics and to convert all cellooligosaccharides to glucose, the hydrolysis was carried out in the presence of purified BGL from *A. japonicus*, which was extra added to the reaction system in excessive quantity (0.5 U/mL). The experiments on enzymatic saccharification of Avicel, cotton, and pretreated Douglas fir wood by combinations of purified *C. lucknowense* enzymes and crude multienzyme preparations were carried out at 50°C. The concentration of Avicel and pretreated wood in those experiments was 50 mg/mL, while the concentration of cotton was 25 mg/mL.

A typical experiment was carried out in the following way. A weighed amount of cellulosic substrate (by dry weight) was placed into a 2-mL plastic test tube, then 0.5-1 mL of 0.05 M Na-acetate buffer, containing 1 mM  $\text{NaN}_3$  to prevent microbial contamination, was added, and the substrate was soaked in the buffer for 1 h. Then, the tube was placed into a thermostated water

bath, located on a magnetic stirrer, and suitably diluted enzyme solution in the same buffer was added to the substrate suspension in order to adjust the total volume of the reaction system to 2 mL and to start the hydrolysis. The tube was hermetically closed with a lid, and the hydrolysis was carried out under magnetic stirring. At definite time of the reaction, an aliquot of the suspension (0.05-0.1 mL) was taken, diluted, centrifuged for 3 min at 15,000 rpm, and the concentrations of glucose and reducing sugars in supernatant were determined by the glucose oxidase – peroxidase and Somogyi-Nelson methods (Somogyi, 1952; Sinitsyn et al., 1990). In those cases, when glucose was a single product of the reaction, the degree of substrate conversion (for Avicel and cotton, which represented pure cellulosic substrates) was calculated using the following equation:

$$\text{Conversion (\%)} = \frac{\text{Glucose concentration (mg/mL)} \times 100\%}{\text{Initial substrate concentration (mg/mL)} \times 1.11}$$

The kinetic experiments were carried out in duplicates. Protein concentration was the measure of enzyme loading in the reaction system. In the case of purified enzymes, the protein concentration was calculated from the UV absorption at 280 nm using enzyme extinction coefficients predicted by the ProtParam tool (<http://www.expasy.ch/tools/protparam.html>). For crude multienzyme preparations, the protein concentration was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

## RESULTS

### Purification and Properties of Cellobiohydrolases

Previously (Bukhtjarov et al., 2004; Gusakov et al., 2005), we have described isolation and properties of two cellobiohydrolases from *C. lucknowense*, belonging to families 7 and 6 of

glycoside hydrolases: CBH I (Cel7A) and CBH II (Cel6A) – see classification into families elsewhere (Henrissat, 1991; Henrissat and Bairoch, 1996; <http://afmb.cnrs-mrs.fr/CAZY/>). In this paper, using different types of chromatography, we isolated two new cellobiohydrolases from *C. lucknowense*. They were homogeneous according to the data of SDS-PAGE and isoelectrofocusing (Fig. 1); their molecular masses were found to be 60 and 70 kDa, pI 3.8 and 5.6, respectively. Peptide mass fingerprinting using MALDI-TOF mass spectrometry indicated that these proteins are different from the above-mentioned cellobiohydrolases (Cel6A and Cel7A) as well as from other *C. lucknowense* enzymes. Subsequent *de novo* sequencing of tryptic peptides from the new cellobiohydrolases, using tandem TOF/TOF mass spectrometry (MS/MS), followed by the BLAST search in the SWISS-PROT (UniProtKB) database showed that the 60 and 70 kDa proteins display sequence similarity to cellobiohydrolases from the glycoside hydrolase families 7 and 6 (Table I). So, they were classified as Cel7B (CBH Ib) and Cel6B (CBH IIb), respectively.

Specific activities of the CBH Ib and IIb toward different substrates are given in Table II. For a comparison, the activities of previously isolated *C. lucknowense* cellobiohydrolases (now named as CBH Ia and CBH IIa) are also given there. The CBH Ib and IIb displayed maximum activity at pH 4.7 and 5.0. Both enzymes were stable during 24 h incubation at pH 5.0 and 50°C. Study of the enzyme adsorption on Avicel, carried out at pH 5.0 and 6°C as reported elsewhere (Gusakov et al., 2005), revealed that only the CBH IIb (as well as the previously described CBH Ia) possesses a cellulose-binding module (CBM).

Figure 2 shows the progress kinetics of Avicel hydrolysis by the all purified *C. lucknowense* cellobiohydrolases, where the enzymes were equalized by protein concentration (0.1 mg/mL). For a comparison, hydrolysis of Avicel by the purified *T. reesei* CBH I and II was

carried out under the same conditions. In order to eliminate the effect of product (cellobiose) inhibition on the kinetics, the hydrolysis was carried out in the presence of purified BGL from *A. japonicus*, added to the reaction system in excessive quantity (0.5 U/mL). The highest hydrolysis rate was observed in the case of *C. lucknowense* CBH IIb: 3.2 mg/mL of glucose, i.e. 58% cellulose conversion was achieved after 5 days of hydrolysis. The *T. reesei* CBH I and II as well as the *C. lucknowense* CBH Ia (all these enzymes have a CBM) were notably less effective (the yield of glucose after 5 days was 2.8, 2.0, 2.5 mg/mL, which corresponded to the cellulose conversion degree of 50, 36 and 46%, respectively). As expected, the *C. lucknowense* cellobiohydrolases without CBM (CBH Ib and IIa) had the lowest ability to hydrolyze Avicel: only 23 and 21% cellulose conversion was achieved after the same time of reaction.

### **Synergism between *C. lucknowense* Enzymes**

Ability of the most active *C. lucknowense* cellobiohydrolases (CBH Ia and IIb) to hydrolyze native crystalline cellulose (cotton) was studied. Since the synergism between cellobiohydrolases and endoglucanases in hydrolysis of crystalline cellulose is a well-known phenomenon (Wood and McCrae, 1979; Henrissat et al., 1985; Kleman-Leyer et al., 1996), each CBH was also tested on cotton together with one of the major *C. lucknowense* endoglucanases: EG II (Cel5A, 51 kDa), EG V (Cel45A, 25 kDa), and EG VI (Cel6C, 47 kDa), of which only the EG II has a CBM (Bukhtojarov et al., 2004). Synergism between CBH I and II of *T. reesei* has also been documented (Nidetzky et al. 1994; Medve et al., 1994). So, a possible synergistic interaction between CBH Ia and IIb was also studied. The experiments were carried out at pH 5.0 and 40°C for 140 h.

As an example, the progress kinetics of cotton hydrolysis by combinations of CBH IIb with other *C. lucknowense* enzymes are shown in Figure 3. Glucose yields obtained after 140 h of cotton hydrolysis under the action of individual cellobiohydrolases and endoglucanases and their combinations are summarized in Table III. The coefficient of synergism ( $K_{syn}$ ) was calculated as a ratio of experimental glucose concentration (column 2 of Table III) to the theoretical sum of glucose concentrations (column 3).

As seen from Table III, individual enzymes could not hydrolyze cotton effectively. The CBH IIb provided the highest glucose yield after 140 h of hydrolysis: 1.18 mg/mL, which corresponded to the substrate conversion degree of 21%. Both cellobiohydrolases displayed a pronounced synergism with all endoglucanases under study, the highest glucose yields (4.1-4.7 mg/mL) were achieved for combinations of CBH Ia or CBH IIb with EG II, the coefficient of synergism being varied in the range of 2.6-2.8. A strong synergism ( $K_{syn} = 2.75$ ) was also observed between CBH Ia and CBH IIb. In fact, the combination of two cellobiohydrolases (1:1 by weight) with BGL provided practically complete conversion (98.6%) of cotton cellulose to glucose after 140 h of hydrolysis.

### **Hydrolysis of Different Substrates by Combinations of *C. lucknowense* Enzymes and Crude Fungal Multienzyme Preparations**

In the previous section, the combination of cellobiohydrolases Ia and IIb or each of them in combination with EG II provided the most efficient hydrolysis of cotton. So, we tried to design a mixture of *C. lucknowense* enzymes, which could be successfully used for efficient hydrolysis of cellulosic substrates to glucose. Together with the above-mentioned enzymes, the *C. lucknowense* BGL was used for this purpose. The total protein concentration in the reaction

system was 0.5 mg/mL, the multienzyme mixture (*C.l.* combination #1) is described in Table IV. Avicel (50 mg/mL) and cotton (25 mg/mL) were used as substrates representing pure crystalline cellulose in these experiments. Sample of Douglas fir wood pretreated by organosolv (50 mg/mL) was taken as an example of real lignocellulosic feedstock that may be used for bioconversion to ethanol (Berlin et al., 2005a; Kurabi et al., 2005). A few crude cellulase preparations (diluted so that the protein concentration in the reaction system would also be 0.5 mg/mL) were taken for a comparison in these studies. Those preparations were: NCE-L600 representing the crude multienzyme *C. lucknowense* sample, Celoviridin G20x and BioAce representing typical *T. reesei* crude cellulase samples, and two *P. verruculosum* multienzyme preparations (#6 and #151). Their specific activities toward different substrates are given in Table V. Since the *T. reesei* preparations were deficient by BGL, the hydrolysis experiments with them were carried out also in the presence of extra added *A. japonicus* BGL (0.5 U/mL).

The progress kinetics of cotton, Avicel and Douglas fir wood hydrolysis are shown in Figures 4-6. It should be noted that in all cases (except for the NCE-L600 and *T. reesei* preparations without supplementation with BGL) the concentrations of glucose and reducing sugars after 24-72 h of hydrolysis in a concrete experiment were practically the same, i.e. glucose made up >96% of the total soluble sugars. So, the glucose yield can be taken as reliable criterion in comparison of the hydrolytic efficiency of different multienzyme samples.

In hydrolysis of cotton (Fig. 4), the combination #1 of purified *C. lucknowense* enzymes provided much higher glucose yield after 72 h of the reaction (23.4 mg/mL, i.e. 84% degree of substrate conversion) than all crude multienzyme samples, for which the glucose yields varied from 4.2 mg/mL (NCE-L600) to 13.5 mg/mL (*P. verruculosum* #151). In hydrolysis of Avicel (Fig. 5), the *C.l.* combination #1 was also superior, although the difference with other

multienzyme samples was lower than in the case of cotton. In the case of pretreated Douglas fir wood (Fig. 6), the *C.l.* combination #1 was the winner in the first 24 h of hydrolysis, however after 72 h of the reaction the best results (30.7 mg/mL of glucose) provided the *P. verruculosum* #151 sample.

Unlike Avicel and cotton, the pretreated wood sample contained not only cellulose (~85%) but also 13% of lignin and 2% of hemicellulose (Berlin et al., 2005a; Kurabi et al., 2005). The artificial *C.l.* combination #1 was composed of only cellulases; all of them, except for the BGL, having CBM. All other multienzyme samples possessed not only cellulase but also xylanase and other types of carbohydrase activity, i.e. they contained non-cellulase accessory enzymes. This may explain relatively lower efficiency of the *C.l.* combination #1 on pretreated Douglas fir compared to the *P. verruculosum* #151 preparation (Fig. 6). So, in the next series of experiments (Fig. 7) the composition of *C. lucknowense* mixtures was varied, while the total protein concentration in the reaction system was maintained at the same level of 0.5 mg/mL (Table IV). In two experiments (*C.l.* combinations #2 and #4), the highly active *C. lucknowense* Xyl II (Xyn11A) was added to the above-mentioned four enzymes. Since a synergism between tightly and loosely adsorbed cellulases has been described (Sinitzyn et al., 1986), EG V or EG V together with CBH Ib (both enzymes have no CBM) were used in the *C.l.* combinations #3 and #4.

As can be seen from Figure 7, the initial rate of glucose formation decreased in a row from *C.l.* combination #1 to combination #4, however the glucose yield after 2-3 days of hydrolysis increased in the same row. The Xyl II demonstrated only slight positive effect on the glucose yield, while the EG V or EG V together with CBH Ib provided a very notable increase in the product concentration after 72 h of the reaction (37 and 41 mg/mL, respectively) compared to the

*C.l.* combination #1 (29 mg/mL), i.e. the combinations #3 and #4 performed much better than all crude multienzyme samples including the *P. verruculosum* #151 (Fig. 6).

## DISCUSSION

Two new cellobiohydrolases (Ib and Iib) were isolated from *C. lucknowense* (Fig. 1), which were different from the previously described CBH Ia and CBH Iia (Bukhtojarov et al., 2004; Gusakov et al., 2005). Thus, this fungus secretes at least four cellobiohydrolases encoded by different genes, two of them belonging to the GH6 and two other enzymes – to the GH7 family (Table II). It should be noted that the most studied fungus *T. reesei* has only two cellobiohydrolases: I (Cel7A) and II (Cel6A) (Nidetzky et al., 1994; Teeri, 1997; Foreman et al., 2003). Other fungi, such as *Humicola insolens*, also secrete two cellobiohydrolases (Cel7A and Cel6A, Schülein, 1997), while *Phanerochaete chrysosporium* produces at least seven different cellobiohydrolases, of which six enzymes belong to the GH7 family (Covert et al., 1992; Muñoz et al., 2001). All the enzymes mentioned, except for the *C. lucknowense* CBH Ib, Iia and *P. chrysosporium* CBH 1-1 (Cel7A), possess CBM.

The *C. lucknowense* CBH Iib demonstrated the highest ability for a deep degradation of crystalline cellulose amongst a few cellobiohydrolases tested, including three other *C. lucknowense* enzymes (CBH Ia, Ib, Iia) and CBH I and II of *T. reesei* (Fig. 2). In hydrolysis of cotton, both *C. lucknowense* cellobiohydrolases having a CBM (Ia and Iib) displayed a pronounced synergism with three major endoglucanases from the same fungus (EG II, EG V, EG VI) as well as a strong synergy with each other (Table III). Whereas synergistic interaction between endoglucanases and cellobiohydrolases has been known for a long time (Wood and McCrae, 1979; Henrissat et al., 1985; Kleman-Leyer et al., 1996), the synergy between

cellobiohydrolases has been disputed. It has been clearly demonstrated for CBH I and II of *T. reesei* (Nidetzky et al. 1994; Medve et al., 1994), *H. insolens* (Boisset et al., 2000) as well as for bacterial exocellulases (Barr et al., 1996). The explanations for this phenomenon have been given by Barr et al. (1996), Väljamäe et al. (1998) and Boisset et al. (2000).

Using four purified *C. lucknowense* enzymes, an artificial cellulase complex was constructed (*C.l.* combination #1) that demonstrated an extremely high ability to convert different cellulosic substrates to glucose (Figs. 4-6). This multienzyme mixture was much (or notably) more effective in hydrolysis of crystalline cellulose than the crude *C. lucknowense* preparation NCE-L600 and other crude cellulases produced by *T. reesei* and *P. verruculosum*. In 72-h hydrolysis of a lignocellulosic substrate (pretreated Douglas fir wood), the *C.l.* combination #1 conceded in the hydrolytic efficiency only to the *P. verruculosum* #151 preparation.

The *C.l.* combination #1 was composed by 96% of two cellobiohydrolases and EG II, the enzymes with strong adsorption on crystalline cellulose. The activity of tightly adsorbed cellulases is known to decrease gradually in the course of hydrolysis of insoluble cellulose as a result of the enzyme limited mobility along the substrate surface or unproductive binding – so called pseudoinactivation (Gusakov et al., 1985; Gusakov et al., 1992; Eriksson et al., 2002). Synergism between tightly and loosely adsorbed cellulases has been previously described (Sinitsyn et al., 1986). Such kind of synergism may be explained by the possibility for the loosely binding cellulases (enzymes without CBM) to destroy obstacles hindering the processive action of the tightly adsorbed cellobiohydrolases (Sinitsyn et al., 1986; Eriksson et al., 2002), thus helping them to move to the next cellulose reactive sites. So, in one set of experiments (Fig. 7) the pretreated wood sample was hydrolyzed by different mixtures of purified *C. lucknowense* enzymes, to which cellulases lacking a CBM were included (EG V or EG V in combination with

CBH Ib). Indeed, two *C.l.* combinations (#3 and #4), containing weakly adsorbed enzymes, provided a notable enhancement of the glucose yield after 72 h of the enzymatic reaction in comparison with the *C.l.* combination #1. The *C.l.* combinations #3 and #4 were also more effective than the *P. verruculosum* #151 preparation.

The low performance of the crude *C. lucknowense* preparation (NCE-L600) in hydrolysis of different cellulosic substrates deserves a special attention. It may be explained by the low total content of different cellobiohydrolases in the sample (35-40% of the total protein content).

Moreover, two of four *C. lucknowense* cellobiohydrolases (Ib and IIa) lack a CBM, while two other enzymes (CBH Ia and IIb) also partially lose the CBM because of a protease action in the course of fermentation (Gusakov et al., 2005). In the case of crude *T. reesei* samples

(Celloviridin G20x and BioAce) the total content of the CBH I and II was 65-70% while the EG I and II comprised ~15% of the total protein (Markov et al., 2005); all the enzymes mentioned possess CBM. So, the efficiency of the crude *T. reesei* samples was higher than that for the NCE-L600, but lower than that for the combinations of the best purified *C. lucknowense* cellulases.

The high hydrolytic efficiency of the *Penicillium sp.* cellulases in hydrolysis of different cellulosic materials reported previously (Castellanos et al., 1995; Berlin et al., 2005a; Kurabi et al., 2005) was also confirmed by the results of the present studies. In particular, the *P.*

*verruculosum* #151 multienzyme sample provided superior performance over both *T. reesei*

preparations in most cases. The difference between the *P. verruculosum* and *T. reesei* enzymes becomes more evident in the case of real lignocellulosic substrate (pretreated Douglas fir wood,

Fig. 6). High level of the BGL activity, highly active and well-balanced cellulase complex and its lower susceptibility to the negative influence of lignin (Berlin et al., 2005b; Kurabi et al., 2005)

seem to be the major factors for superior performance of the *P. verruculosum* #151 preparation.

Different strains of *Trichoderma sp.* have long been considered to be the most efficient destroyers of cellulose. To a large extent, this opinion was based on a pioneering work of Mandels and Sternberg, who collected and screened 14,000 fungi (Mandels and Sternberg, 1976). Even recently this opinion is predominated amongst specialists working in the field of lignocellulose bioconversion (Nieves et al., 1998; Himmel et al., 1999; Galbe and Zacchi, 2002). However, in wild (or even mutant) fungal strains, which are subjected to screening for cellulase or other types of activities, the secreted multienzyme cocktail may be non-optimal for application in a biotechnological process. The most active cellulases may be expressed at a level that is insufficient for highly effective cellulose hydrolysis or the secreted cellulase complex may be not well balanced by individual enzymes. The examples presented above show that, while the NCE-L600 produced by the *C. lucknowense* mutant strain demonstrated quite mediocre results in cellulose saccharification, the artificially designed multienzyme mixtures based on selected cellulases from the same microbial producer provided the superior results in the hydrolysis process. The *C. lucknowense* mutant strains (including UV18-25) have been developed to produce enzymes for textile, pulp and paper, detergent and other applications, but not for the enzymatic saccharification of cellulose; these strains can also be used for a high-level production of homologous and heterologous proteins (U.S. Patent No. 6,015,707, 2000; Int. Patent WO 01/79558, 2001; U.S. Patent No. 6,573,086, 2003). The best *C. lucknowense* mutant strains secrete 50-80 g/L of extracellular protein, while the viscosity of the fermentation medium being low. The full fungal genome of the *C. lucknowense* has been sequenced in 2005 (see [http://www.dyadic-group.com/wt/dyad/pr\\_1115654417](http://www.dyadic-group.com/wt/dyad/pr_1115654417)), and now the genome annotation is carried out. Both previous experimental and novel genomic data indicate that a wide variety of not only cellulases but also hemicellulases and other accessory enzymes are produced (or may be

potentially produced) by this fungus. So, if to express the desired optimal cocktail of *C.*

*lucknowense* own cellulases together with accessory enzymes, that could make up >90% of the secreted protein, the potential of this fungus as a producer of enzymes for saccharification of lignocellulosics would be extremely high.

Two other examples shown above (*P. verruculosum* #151 and #6) together with earlier publications (Castellanos et al., 1995; Berlin et al., 2005a; Kurabi et al., 2005) demonstrate that the secreted cellulase complexes, whose performance in lignocellulose hydrolysis process is already better than that of *T. reesei*, exist even now. Moreover, modern *P. verruculosum* strains (Solov'eva et al., 2005) are comparable to the best *T. reesei* strains in terms of cellulase (protein) productivity and yield, although the cost of production of *Penicillium* enzymes seems to be higher at this moment.

The authors thank Dr. Alex Berlin and Prof. John Saddler from the Department of Wood Science, University of British Columbia, Vancouver, Canada, for providing the sample of Douglas fir wood pretreated by organosolv.

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**Table I.** Identification of peptides in the isolated *C. lucknowense* proteins using MALDI-TOF MS/MS.

| Enzyme         | m/z    | Peptide*           | BLAST identification**   | UniProtKB No. |
|----------------|--------|--------------------|--|---------------|
| Protein 60 kDa | 1133.6 | HEYGTNIGSR         | 118 <b>HEYGTNIGSR</b> 127<br>(cbh1.2 <i>Humicola grisea</i> – GH7)           | O94093        |
|                | 1829.9 | MGNQDFYGPGLTVDTSK  | 291 <b>LGNTDFYGPGLTVDT</b> 305<br>(cbhB <i>Aspergillus niger</i> – GH7)      | Q9UVS8        |
| Protein 70 kDa | 1061.4 | LFANDYYR           | 127 <b>LWANNYYR</b> 132<br>(Avicelase 2 <i>Humicola insolens</i> – GH6)      | Q9C1S9        |
|                | 1990.0 | HYIEAFSPLLNSAGFPAR | 367 <b>KYIEAFSPLLNAAGFPA</b> 383<br>(CBH II <i>Neurospora crassa</i> – GH6)  | Q872J7        |
|                | 2073.5 | NGKQPTGQQQWGDWCNVK | 381 <b>SGKQPTGQQQWGDWCNV</b> 394<br>(CBH II <i>Trichoderma reesei</i> – GH6) | P07987        |

\* Since the MS/MS can not distinguish between Leu and Ile residues (they have the same masses), there may be ambiguity in the appropriate positions of the identified peptides.

\*\* Residues conserved in the *C. lucknowense* enzymes are shown in **bold**.

**Table II.** Specific activities (U/mg of protein) of purified cellobiohydrolases from *C. lucknowense* toward different substrates at pH 5.0 and 40°C.

| Enzyme  | Mol. mass (kDa) | Cat. domain designation | CBM presence | Avicel | CMC* | Barley $\beta$ -glucan* | 4-Nitrophenyl $\beta$ -D-cellobioside | 4-Nitrophenyl $\beta$ -D-lactoside |
|---------|-----------------|-------------------------|--------------|--------|------|-------------------------|---------------------------------------|------------------------------------|
| CBH Ia  | 65              | Cel7A                   | Yes          | 0.21   | 0.1  | <0.1                    | 0.021                                 | 0.12                               |
| CBH Ib  | 60              | Cel7B                   | No           | 0.12   | 0.3  | <0.1                    | 0.020                                 | 0.09                               |
| CBH IIa | 43              | Cel6A                   | No           | 0.08   | 1.1  | 2.0                     | 0                                     | 0                                  |
| CBH IIb | 70              | Cel6B                   | Yes          | 0.22   | 0.2  | 0.2                     | 0                                     | 0                                  |

\* Activity was determined at 50°C.

**Table III.** Synergism between *C. lucknowense* cellulases in hydrolysis of cotton cellulose (5 mg/mL) at pH 5.0 and 40°C in the presence of 0.5 U/mL of *A. japonicus* BGL. In all cases the CBH concentration was 0.15 mg/mL, the EG concentration was 0.05 mg/mL.

| Enzyme           | Glucose concentration after<br>140 h, experimental<br>(mg/mL) | Glucose concentration after<br>140 h, theoretical* | $K_{syn}$ |
|------------------|---|--|-----------|
| CBH Ia           | 0.81  | –  | –         |
| CBH IIb          | 1.18  | –  | –         |
| EG II            | 0.64  | –  | –         |
| EG V             | 0.70  | –  | –         |
| EG VI            | 0.40  | –  | –         |
| CBH Ia + EG II   | 4.05  | 1.45   | 2.79      |
| CBH Ia + EG V    | 3.68  | 1.51   | 2.44      |
| CBH Ia + EG VI   | 3.93  | 1.21   | 3.25      |
| CBH IIb + EG II  | 4.72  | 1.82   | 2.59      |
| CBH IIb + EG V   | 3.81  | 1.88   | 2.03      |
| CBH IIb + EGVI   | 4.05  | 1.58   | 2.56      |
| CBH Ia + CBH IIb | 5.47  | 1.99   | 2.75      |

\* Calculated as a sum of glucose concentrations obtained under the action of individual enzymes.

**Table IV.** Composition of artificial multienzyme combinations based on purified *C. lucknowense* enzymes. The total protein concentration in the reaction system was 0.5 mg/mL, the concentration of each component is given in mg/mL.

| Combination | CBH Ia | CBH Ib | CBH IIb | EG II | EG V | BGL  | Xyl II |
|-------------|--------|--------|---------|-------|------|------|--------|
| #1          | 0.2    | 0      | 0.2     | 0.08  | 0    | 0.02 | 0      |
| #2          | 0.2    | 0      | 0.2     | 0.07  | 0    | 0.02 | 0.01   |
| #3          | 0.2    | 0      | 0.2     | 0.04  | 0.04 | 0.02 | 0      |
| #4          | 0.1    | 0.1    | 0.2     | 0.03  | 0.04 | 0.02 | 0.01   |

**Table V.** Specific activities (U/mg of protein) of multienzyme preparations toward different substrates at pH 5.0 and 50°C.

| Preparation                 | Filter paper | CMC  | Xylan | Cellobiose* |
|-----------------------------|--------------|------|-------|-------------|
| Celloviridin G20x           | 0.60         | 10.4 | 4.5   | 0.28        |
| BioAce                      | 0.55         | 12.6 | 1.8   | 0.13        |
| <i>P. verruculosum</i> #151 | 0.83         | 15.3 | 35.3  | 0.64        |
| <i>P. verruculosum</i> #6   | 0.61         | 15.8 | 36.3  | 0.71        |
| NCE-L600                    | 0.25         | 12.2 | 4.8   | 0.07        |
| <i>C.l.</i> combination #1  | 1.10         | 6.6  | 0     | 1.05        |

\* Activity was determined at 40°C.

## Captions to figures

**Figure 1.** SDS-PAGE (A) and isoelectrofocusing (B) of purified cellobiohydrolases from *C. lucknowense*. Lanes: 1, markers with different molecular masses; 2 and 5, CBH Ib; 3 and 6, CBH IIb; 4, markers with different *pI*.

**Figure 2.** Progress kinetics of Avicel (5 mg/mL) hydrolysis by purified cellobiohydrolases (0.1 mg/mL) in the presence of purified *A. japonicus* BGL (0.5 U/mL), 40°C, pH 5.0.

**Figure 3.** Synergism between CBH IIb and other *C. lucknowense* purified enzymes during hydrolysis of cotton cellulose (5 mg/mL) in the presence of purified *A. japonicus* BGL (0.5 U/mL), 40°C, pH 5.0. The CBH and EG concentration was 0.15 and 0.05 mg/mL, respectively. Experimental data for the pairs of enzymes are shown with open symbols (continuous curves); the theoretical sums of glucose concentrations obtained under the action of individual enzymes are shown with filled symbols (dotted lines).

**Figure 4.** Progress kinetics of cotton (25 mg/mL) hydrolysis by combination #1 of purified *C. lucknowense* enzymes and different crude cellulase preparations at protein loading of 0.5 mg/mL, 50°C, pH 5.0 (see text and Table V for details).

**Figure 5.** Progress kinetics of Avicel (50 mg/mL) hydrolysis by combination #1 of purified *C. lucknowense* enzymes and different crude cellulase preparations at protein loading of 0.5 mg/mL, 50°C, pH 5.0 (see text and Table V for details).

**Figure 6.** Progress kinetics of hydrolysis of pretreated Douglas fir wood (50 mg/mL) by combination #1 of purified *C. lucknowense* enzymes and different crude cellulase preparations at protein loading of 0.5 mg/mL, 50°C, pH 5.0 (see text and Table V for details).

**Figure 7.** Progress kinetics of hydrolysis of pretreated Douglas fir wood (50 mg/mL) by different combinations of purified *C. lucknowense* enzymes at protein loading of 0.5 mg/mL, 50°C, pH 5.0 (see text and Table IV for details).













