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PRODUCERS, BIOLOGY, SELECTION, AND GENETIC ENGINEERING

The Production of Highly Effective Enzyme Complexes of Cellulases and Hemicellulases Based on the *Penicillium verruculosum* Strain for the Hydrolysis of Plant Raw Materials

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Abstract—Methods for the production and analysis of cellulase and hemicellulase enzyme preparations of various compositions based on the *Penicillium verruculosum* carbohydrase complex and intended for the effective hydrolysis of different types of cellulose-containing materials (CCMs) have been developed. New recombinant strains of *P. verruculosum* producing multienzyme carbohydrase complexes with increased activities of cellulases (due to the expression of endo-β-1,4-glucanases I and IV and cellobiohydrolase II from *Trichoderma reesei*) and hemicellulases (due to the expression of endo-β-1,4-xylanases from *P. canescens* and *T. reesei* and endo-β-1,4-mannanase from *T. reesei*) were constructed. The hydrolytic efficiency of the enzyme preparations (EPs) produced by the new recombinant strains during continuous hydrolysis of three CCM types (milled aspen, depitched pine wood, and milled bagasse) was studied. It was shown that new EPs containing recombinant proteins and retaining their own basic cellulase complex are characterized by the highest hydrolytic ability, exceeding that of the EP based on the original *P. verruculosum* strain. The recombinant enzyme preparations were highly stable; the optimal pH and temperature values for cellulase, xylanase and mannanase activities were in the range of 3.5–5.5 and 50–80°C, respectively.

Keywords: cellulases, cellulose-containing materials, enzymatic hydrolysis, hemicellulases, *Penicillium verruculosum*

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INTRODUCTION

Renewable plant biomass comprises the main body of organic material on Earth and is an inexhaustible source of raw material and energy [1]. That is why the development of efficient methods for its use is an important and crucial problem for modern biotechnology. Current methods of biomass use are mainly based on the enzymatic degradation of plant material with polysaccharide components to form oligo- and monosaccharides, which can be further converted into various widely used products (spirits, organic and amino acids, polymers, food additives, etc.) by microbial or chemical synthesis [2–4]. The microbial and chemical production of these substances is fairly well studied [4, 5]; however, the enzymatic hydrolysis (saccharification) of cellulose-containing materials (CCMs) is a limiting stage that restrains their industrial use [6].

CCM samples differ in composition and structure (for example, various species of perennial and annual plants and the products of their conversion) [7]. That is why an enzyme preparation (EP) with a composition optimal for a particular CCM type is required to reach the maximum hydrolysis yield of this CCM type. Modern producers of industrial enzymes make standard EPs that are not adapted to the maximum effective hydrolysis of various CCM groups [8]. The majority of industrial EPs are obtained using various strains of the *Trichoderma* fungus (*T. reesei*, *T. viride*, *T. longibrachiatum*, etc.), which are currently the main

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Abbreviations: RS—reducing sugars, GX—glucuronoxylan, GM—galactomannan, GPC—gel-permeating chromatography, IEF—isoelectric focusing, CL—culture liquid, CMC—carboxymethylcellulose, XYL—endoxylanase, MCC—microcrystalline cellulose, PAAG—polyacrylamide gel, pNPG—*p*-nitrophenyl-β–D-glucopyranoside, PCR—polymerase chain reaction, DM—dry matter, EP—enzyme preparation, CBH—cellobiohydrolase, CCMs—cellulose-containing materials, EG—endoglucanase, EP—electrophoresis, FPLC—fine protein liquid chromatography, ManB—mannanase B, SDS—sodium dodecyl sulfate.

industrial producers of cellulases and hemicellulases [9–11]. This is mainly caused by the high secretory capacity of *Trichoderma* strains; however, EPs of this microorganism have a number of shortcomings, the most significant of which is low CCM hydrolytic ability [12, 13]. Unlike *Trichoderma*, *Penicillium* fungi synthesize cellulase enzymatic complexes of a more balanced composition [14] that are able to hydrolyze cellulose efficiently [8, 15].

The purpose of the work was to study the enzymatic activities (particularly those of cellulase and hemicellulase) of constructed recombinant clones of the *Penicillium verruculosum* strain in order to select a universal basis for the creation of new EPs capable of efficient hydrolysis of various CCM types.

METHODS

Enzyme preparations. EPs were obtained by freeze drying the culture media (CM) of the initial *P. verruculosum* B1-221-151 strain and the recombinant strains based on it. They contain the following heterologous genes: *xylA* of the endo-β-1,4-xylanase A from *P. canescens* (XylA preparations), *xyl3* of the endo-β-1,4-xylanase III from *T. reesei* (XylIII preparations), *manB* of the endo-β-1,4-mannanase B from *T. reesei* (ManB preparations), *eglIV* of the endo-β-1,4-glucanase IV from *T. reesei* (EGIV preparations), *eglI* of the endo-β-1,4-glucanase I from *T. reesei* (EGI preparations), and *cbhII* of the cellobiohydrolase II from *T. reesei* (CBHII preparations). Fermentation was performed at the Institute of Biochemistry and Physiology of Microorganisms (Pushchino) in media containing MCC and glucose as the main components of the culture medium (see "Culturing of *P. verruculosum* transformants...").

Substrates. EP activities were determined using the following substrates: *p*-nitro-phenyl-β-D-glucopyranoside (pNPG), birch glucuronoxylan, sodium salt of carboxymethylcellulose (CMC), galactomannan (all preparations from Sigma, United States), and microcrystalline cellulose (MCC) (MK Tsentr, Dzerzhinsk, Russia). Enzymatic hydrolysis (saccharification) was performed using milled aspen, depitched pine wood, milled bagasse, and MCC milled in an orbicular planetary activator mill (provided by GosNIIsintezbelok, Moscow).

Other reagents. Polymerase chain reaction (PCR) was performed using a mixture of high-fidelity and processive polymerases—Long polymerase mix, 10× Long polymerase PCR buffer + $MgCl_2$, and dNTP mix, T4 polymerase, and T4 ligase (Thermo Scientific, United States). Isolation, preparative obtainment, and purification of DNA were performed using Qiagen kits (United States).

Polyacrylamide gel (PAAG) plates (70 × 80 × 0.75 mm) for electrophoresis in denaturing conditions (SDS-PAGE) containing concentrating (4%) and resolving (12%) gels and for isoelectrofocusing (IEF) in 4% PAAG (125 × 65 × 0.75 mm) were made using reagents and kits by Reanal (Hungary), Sigma, and Bio-Rad (United States). Protein staining in the gels was performed using Coomassie Brilliant Blue R-250 (Ferrak, Germany) in 25% trichloroacetic acid (Poch, Poland). The studied enzymatic preparations were treated with 1% SDS and 5% β-mercaptoethanol at 100°C for 15 min before electrophoresis. The protein mixtures SM0431 (14.4–116 kDa) and SM0441 (19–117 kDa) (Thermo Scientific) were used as the molecular weight markers for SDS-PAGE. The IEF Calibration Kit (pI 2.5–6.5) (Pharmacia, Sweden) was used as a standard for IEF. Lowry reagents and buffer solutions were made using reagents of AR and ACS grades (Reakhim, Russia; MP Biomedicals Inc., France; Sigma, United States).

PCR conditions and the obtainment of genetic constructs. PCR was performed in a My Cycler amplifier (Bio-Rad). PCR with genomic DNA was performed under the following conditions: primary denaturation at 95°C for 5 min; denaturation at 95°C for 1.5 min; primer annealing at 50–55°C for 1 min; elongation at 68°C for 1.5–2.0 min (depending on the length of the polynucleotide chain of a gene); 25 cycles. PCR with plasmid DNA was performed as follows: primary denaturation at 95°C for 45 s; denaturation at 95°C for 30 s; primer annealing at 50–55°C for 1 min; elongation at 68°C for 1.5–2.0 min depending on the length of the polynucleotide chain of a gene, 20–25 cycles.

The obtained PCR product was cloned by independent ligation [16]. It was isolated from the agarose gel and purified using the Qiagen kit. Then the PCR product and linearized vector pUC-CBHI were treated with T4 DNA polymerase in the presence of deoxyadenosine triphosphate (dATP) and deoxythymidine triphosphate (dTTP) (Thermo Scientific) [16], respectively. Ligation of the insert (150 ng) and pUC-CBHI vector (50 ng) was performed by mixing and incubation for 30 min at 22°C; after that *E. coli* MACHI cells (Invitrogen, United States) were transformed with the ligation mixture according to the standard protocol described in [17].

Staining of agar medium with CMC and amorphous cellulose by Congo red. In order to perform the primary screening, the transformants were grown in Petri dishes with the minimal culture medium containing CMC (for transformants bearing EGI and EGIV genes) or amorphous cellulose (for transformants bearing CBHII) for 24 h at 30°C. Then 5 mL of 0.1% Congo red (AR, Reakhim) solution was added into a Petri dish and incubated for 30 min. Then the dye was removed, and the dish was filled with 1 M NaCl (GPR), which also was removed in 30 min. Consequently, the CMC and amorphous cellulose hydrolysis zones remained unstained. We used the minimal culture medium of the following composition: 1.5 g/L KH_2PO_4 (AR), 0.5 g/L KCl (GPR), 0.5 g/L $MgSO_4$ ·

$7H_2O$ (GPR), 1 g/L CMC or amorphous cellulose, 50 mg/mL H_3BO_3 (AR), 400 mg/mL $CuSO_4 \cdot 5H_2O$ (GPR), 800 mg/mL $FeSO_4 \cdot 7H_2O$ (AR), 800 mg/mL $MnSO_4 \cdot 2H_2O$ (AR), 800 mg/mL $Na_2MoO_4 \cdot 2H_2O$ (AR), 800 mg/mL $ZnSO_4 \cdot 7H_2O$ (AR), 20 g/L bacterial agar, and 10 mM $NaNO_3$ (GPR). The inorganic components of the culture medium were from Labtekh (Russia), Reakhim, Khimmed, MP Biomedicals Inc., and Sigma.

Culturing of *P. verruculosum* transformants and preparative obtainment of EPs. The transformants were cultured in rotating flasks in the medium standard for *P. verruculosum* including the following components (g/L): MCC—40, wheat bran—10, yeast extract—10, glucose—10, KH_2PO_4—15, $(NH_4)_2SO_4$—5, $MgSO_4 \cdot 7H_2O$—0.3, and $CaCl_2 \cdot 2H_2O$—0.3. The EPs of *P. verruculosum* were obtained in one-liter reactors in the same medium. The fermentation time for both cases was 144 h. The culture medium from the reactor was centrifuged, filtered through fiberglass, and ultraconcentrated (10000 Da). Then the ultraconcentrates were freeze-dried.

Fermentation (see above) was performed using glucose (Roquette Pharma, France), wheat bran (Enzim, Ukraine), MCC (MTs-Tsentr, Russia), yeast extract (Lesaffre, France), and reagents from Labtekh, Reakhim, Khimmed, MP Biomedicals Inc., and Sigma as the inorganic components of the culture media.

Protein concentration was determined by the modified Lowry method [18, 19]. The EP of *P. verruculosum* (protein content of 899 mg/g) was used as a calibrating solution. Measurements were performed using a Cary 50-Scan spectrophotometer (Varian, United States).

Determination of biochemical properties of the enzymes. Analytical IEF of proteins and SDS-PAGE in 12% gel were made using a Model 111 IEF Cell instrument (Bio-Rad) and Mini Protean system (Bio-Rad), respectively, according to the instruction manuals.

EP activity assay. The endoglucanase, cellobiohydrolase, xylanase, and mannanase activities were determined using CMC, MCC, glucuronoxylan, and galactomannan (Sigma) as the substrates, respectively, by the initial speed of formation of reducing sugars (RS) by the Somogyi-Nelson method [20–22]. The amount of the enzyme that provides formation of 1 μmol of RC per 1 min (pH 5.0) at a substrate concentration of 5 g/L and temperature of 50°C was taken as an activity unit.

Cellobiase (β-glucosidase) activity was determined using pNPG (Sigma) as the substrate by the initial speed of formation of *p*-nitrophenol [22]. The amount of the enzyme that provides formation of 1 Mmol of *p*-nitrophenol per 1 min at pH 5.0 and 40°C was taken as an activity unit.

Mass-spectrometric analysis of trypsin digested proteins. Fragments of stained protein bands after SDS-PAGE with a size of about 1 mm^2 were cut and placed into 0.5 mL plastic tubes; after that, the proteins were trypsin digested [23]. A-cyano-4-hydroxycinnamic acid (Sigma) was used as a matrix for MALDI-TOF mass spectrometry. The target cell was covered with 0.5 μL of saturated matrix solution (in 0.1% trifluoroacetic acid (Sigma)), 30% acetonitrile (Kriokhrom, Russia), and 0.5 μL of the sample. The mixture was air dried for 10–20 min. Mass spectrometric studies were performed at the Laboratory of Physical Organic Chemistry, Department of Chemistry, Lomonosov Moscow State University, using an ULTRAFLEX instrument (Brucker Daltonics, United States).

Temperature and pH optimums of the EPs. The temperature optimum was determined by the measurements of enzymatic activity in a temperature range of 30–80°C (pH 5.0) while the pH optimum was measured in a pH range of 2.5 to 8.0 (50°C). Solutions with certain pH values were made using the buffer system on the basis of 0.1 M acetic, 0.1 M boric, and 0.1 M phosphoric acids.

Stability studies. The EPs were incubated in 0.1 M Na-acetate buffer, pH 5.0, at 50 and 60°C. In the course of incubation, aliquots were taken, in which the residual enzymatic activity was determined using the specific substrates. The results were presented as the dependence of the residual activity (% from the initial one) on the incubation time at the selected temperature.

CCM hydrolysis was performed in a thermostated cell (50°C) placed on a shaker. The substrate concentration in the reaction mixture was 100 g/L (on a dry basis). The reaction was performed in 0.1 M acetate buffer containing 1 mM NaN_3 and ampiox antibiotic (20 μL, concentration of 1 g/mL, Ferein, Russia) at a rotation of 250 rpm. The final volume of the reaction mixture was 20 mL. The calculated volume of the studied EP was added to the reaction mixture to reach a concentration of 5 mg of protein/g of substrate on a dry basis. The *P. verruculosum* F10 EP, which included heterologous β-glucosidase from *Aspergillus niger* (the total β-glucosidase of the preparation was 35263 U/g; the protein content was 775 mg/g) as the main component, was also added to the reaction mixture. The *P. verruculosum* F10 strain was obtained by heterologous cloning of the β-glucosidase from *A. niger* into the *P. verruculosum* 537 strain, which is an auxotroph and unable to consume nitrate nitrogen due to the mutation in the nitrate reductase gene (*niaD*). The *P. verruculosum* 537 strain resulted from mutagenesis of the B1-221-151 strain. The protein concentration of the F10 EP in the reaction mixture was 0.88 mg/g of dry substrate (which is the equivalent of an additional 40 U of β-glucosidase activity per 1 g of dried substrate). The qualitative and quantitative compositions of the enzymatic complexes from the *P. verruculosum* B1-221-151 and *P. verruculosum* 537 were the same.

Hydrolysis lasted for 2 days. The reaction cell was a tank with a lid with the volume of 50 mL; additional mixing of the reaction mixture was provided by a stainless steel stir bar (a cylinder with a diameter of 7 mm and a height of 10 mm). Aliquots were taken from the reaction mixture after 3, 24, and 48 h and centrifuged for 3 min at 11200 *g*. The concentrations of RS and glucose were measured in the supernatant. The RS concentration was measured by the method of Somogyi-Nelson, and the glucose concentration was detected by the glucose oxidase-peroxidase test [24]. Hydrolytic activities of the preparations were detected as the yields of glucose and RS expressed in g/L.

Chromatographic fractionation of the EPs. For analytic separation, a dry EP was dissolved in 0.1 M Na-acetate buffer (pH 5.0) up to a concentration of 20 g/L and centrifuged (11200 g, 10 min). The supernatant was desalted by gel permeation chromatography in a column packed with the Bio Gel P6 (Bio-Rad) (the volume of 10 mL) equilibrated with 0.02 M bis-tris-HCl, pH 6.8.

Ion exchange chromatography of the desalted EPs was made by Fine Protein Liquid Chromatography (FPLC; Pharmacia, Sweden) in a column filled with Source 15 Q (Pharmacia, 1.6 × 0.5 cm, volume of 1 mL). The FPLC system consisted of two P-500 pumps, a mixer, an injector, a column with an anion exchange carrier, an ultraviolet flow detector (280 nm), a recorder, and a fraction collector. A sample containing 10 mg of protein was applied to a column equilibrated with 0.02 M bis-tris-HCl, pH 6.8. The unbound protein was washed by the start buffer; the bound protein was eluted by NaCl ionic gradient from 0 up to 0.4 M at a flow speed of 1 mL/min and a gradient volume of 40 mL.

Hydrophobic chromatography was performed using a Source 15 Iso carrier (Pharmacia, 1.6 × 0.5 cm, volume of 1 mL). Elution was performed using a medium-pressure liquid chromatograph (see above). Protein was applied to the column in the starting 50 mM Na-acetate buffer containing 1.7 M $(NH_4)_2SO_4$ and then eluted with the linear descensive gradient of ammonium sulfate from 1.7 M to 0.

The composition of the fractions was controlled by SDS-PAGE. The concentrations of the individual enzymes in the chromatographic fractions were determined spectrophotometrically as the ratio of the absorbance of a fraction at 280 nm (A_{280}) and the average specific extinction coefficient of cellulases, which was taken to be equal to 2.0. The content of each enzyme was counted as the mass fraction of an enzyme in a chromatographic sample in a total protein amount in a sample and expressed in percents.

RESULTS AND DISCUSSION

The initial B1-221-151 strain was obtained as a result of sequential mutagenesis of the wild strain WA 30. Studies of the composition and properties of the secreted enzymatic complex of the micelial fungus *P. verruculosum* B1-221-151 showed that the mutant possessed high cellulase activity [8, 14, 15]; however, the hemicellulase activity was low [25, 26]. This fact indicates that this EP cannot perform efficient hydrolysis of raw materials; the access of cellulases to cellulose is obstructed because of the presence of the surrounding hemicellulose matrix. That is why it was necessary to increase the content of hemicellulases in order to increase the hydrolytic efficiency of the EPs secreted by *P. verruculosum*. It was also reasonable to increase the total cellulase activity.

Before the experiments on the enzymatic complexes of the new recombinant strains of *P. verruculosum*, we studied the relationship between the composition of an EP and its hydrolytic activity towards various substrate types [8, 27, 28]. The composition of plant raw material is known to vary significantly [7]. It requires individual selection of the optimum EP composition for the efficient hydrolysis of a certain raw material. That is why we decided to create several recombinant strains producing multienzyme complexes with various ratios of cellulase and hemicellulase activities on the basis of the recipient strain *P. verruculosum* 537 ($niaD^-$).

Xylans are the main hemicellulose components of the plant cell wall and the second most frequent natural polysaccharide after cellulose. That is why the EPs meant for the hydrolysis of CCMs containing significant amounts of hemicelluloses (for example, corn stems, sugarcane bagasse, aspen wood) should contain increased contents of xylanases. In order to increase the xylanase activity of the initial *P. verruculosum* strain, we selected the endo-β-1,4-xylanase A from *P. canescens* and the endo-β-1,4-xylanase III from *T. reesei*.

Mannans are the second most frequent component of plant hemicellulose after xylans. Mannan content (namely, gluco- and galactomannans) is highest in pine wood. EPs intended for the hydrolysis of this CCM type should evidently possess activity towards gluco- and galactomannans. In order to increase the mannanase activity of the initial *P. verruculosum* strain, we selected the endo-β-1,4-mannanase B from *T. reesei*.

The cellulase activity of the initial *P. verruculosum* strain was increased by the use of the endo-β-1,4-glucanase IV, endo-β-1,4-glucanase I and cellobiohydrolase II from *T. reesei*.

Creation of expression constructs and transformation of the recipient strain. Fragments corresponding to the following target genes were amplified by PCR using genomic DNAs of *P. canescens* and *T. reesei* as

Table 1. Activities of the enzymes and protein concentrations in culture medium of transformants with the heterologous CBH II

Transformant number	Avicelase (U/mg)	CMCase (U/mg)	β-Glucosidase (U/mg)	Protein (method of Lowry) (mg/mL)
1	0.62 ± 0.03	9.4 ± 0.5	1.7 ± 0.1	6.3 ± 0.3
2	***0.74 ± 0.04***	***7.8 ± 0.4***	***2.2 ± 0.1***	***8.4 ± 0.4***
3	0.27 ± 0.01	5.5 ± 0.3	1.7 ± 0.1	3.4 ± 0.2
4	***0.70 ± 0.04***	***9.6 ± 0.5***	***2.2 ± 0.1***	***9.2 ± 0.5***
5	0.23 ± 0.01	4.8 ± 0.2	0.9 ± 0.1	3.5 ± 0.2
6	0.45 ± 0.02	13.9 ± 0.7	2.3 ± 0.1	4.6 ± 0.2
7	0.29 ± 0.01	2.8 ± 0.1	0.1 ± 0.01	2.8 ± 0.1
8	0.46 ± 0.02	14.2 ± 0.7	1.6 ± 0.1	4.9 ± 0.3
9	0.44 ± 0.02	8.1 ± 0.4	1.5 ± 0.1	3.8 ± 0.2
10	***0.83 ± 0.04***	***8.4 ± 0.4***	***1.6 ± 0.1***	***8.8 ± 0.4***
11	0.55 ± 0.04	10.4 ± 0.5	1.6 ± 0.1	8.4 ± 0.4
12	0.14 ± 0.01	12.1 ± 0.6	1.7 ± 0.1	6.8 ± 0.3
13	0.62 ± 0.03	6.4 ± 0.3	0.9 ± 0.1	3.4 ± 0.2
14	0.38 ± 0.01	11.9 ± 0.6	1.7 ± 0.1	8.4 ± 0.4
15	0.14 ± 0.01	6.7 ± 0.3	1.1 ± 0.1	5.3 ± 0.3
16	**0.75 ± 0.04**	**6.5 ± 0.3**	**0.7 ± 0.1**	**3.4 ± 0.2**
17	0.52 ± 0.03	11.2 ± 0.6	1 ± 0.1	5.7 ± 0.3
18	0.57 ± 0.04	11.2 ± 0.6	1.8 ± 0.1	8.7 ± 0.4
19	0.63 ± 0.04	11.1 ± 0.6	1.6 ± 0.1	8.3 ± 0.4
20	0.20 ± 0.01	11.3 ± 0.6	1.6 ± 0.1	8.1 ± 0.4
21	0.54 ± 0.03	10.5 ± 0.5	3.1 ± 0.2	4.5 ± 0.2
22	**0.77 ± 0.04**	**7.5 ± 0.4**	**0.3 ± 0.1**	**7.6 ± 0.4**
23	**0.73 ± 0.04**	**9.8 ± 0.5**	**1.7 ± 0.1**	**7.1 ± 0.4**
24	**0.71 ± 0.04**	**10.7 ± 0.5**	**1.4 ± 0.1**	**6.9 ± 0.4**
25	***0.84 ± 0.04***	***11.8 ± 0.6***	***1.8 ± 0.1***	***8.4 ± 0.4***
26	**0.75 ± 0.04**	**9.1 ± 0.5**	**1.3 ± 0.1**	**7.7 ± 0.4**
B1 221-151 (control)	0.64 ± 0.03	13.6 ± 0.7	2.2 ± 0.1	8.9 ± 0.5

matrixes and isolated: *xylA* of the endo-β-1,4-xylanase A (XylA) from *P. canescens*, *xyl3* of the endo-β-1,4-xylanase III (XylIII) from *T. reesei*, *manB* of the endo-β-1,4-mannanase B (ManB) from *T. reesei*, *eglIV* of the endo-β-1,4-glucanase IV (EGIV) from *T. reesei*, *eglI* of the endo-β-1,4-glucanase I (EGI) from *T. reesei*, *cbhII* of the cellobiohydrolase II (CBHII) from *T. reesei*. Then the fragments were cloned into a vector containing nucleotide sequences corresponding to the promoter and terminator of the cellobiohydrolase I (*cbhI*) from *P. verruculosum* and the necessary genetic elements for replication in *E. coli* cells.

The *E. coli* MachI competent cells were transformed with expression plasmids. Then we performed a number of cotransformations of the *P. verruculosum* 537 (niaD$^-$) recipient strain using the created plasmids together with pSTA10 plasmid bearing the gene of nitrate reductase, which (*niaD*) provided complementation of the defective nitrate reductase gene in the

Table 2. Recombinant strains and enzyme preparations made on their basis

Strain	Promoter/terminator	Signal peptide	Gene	Enzyme preparations (clone designation within parenthesis)
B1/CBHI_ XylA	*cbhI* *(P. verruculosum)*	*xylA* *(P. canescens)*	*xylA* *(P. canescens)*	XylA–(1–8)
B1/CBHI_ Man	*cbhI* *(P. verruculosum)*	*manB* *(T. reesei)*	*manB* *(T. reesei)*	ManB–(1–8)
B1/CBHI_ EGIV	*cbhI* *(P. verruculosum)*	*cbhI* *(P. verruculosum)*	*eglIV* *(T. reesei)*	EGIV–(1–8)
B1/CBHI_ EGI	*cbhI* *(P. verruculosum)*	*cbhI* *(P. verruculosum)*	*egl1* *(T. reesei)*	EGI–(1–4)
B1/CBHI_CBHII	*cbhI* *(P. verruculosum)*	*cbhI* *(P. verruculosum)*	*cbhII* *(T. reesei)*	CBHII–(1–4)
B1/CBHI_ Xyl3	*cbhI* *(P. verruculosum)*	*xyl3* *(T. reesei)*	*xyl3* *(T. reesei)*	XylIII-1

recipient strain, in order to provide positive selection of transformants in a medium containing sodium nitrate. The stability of transformants was tested by four sequential passages to a selective medium containing sodium nitrate as the nitrogen source. Stable transformants retaining the initial activity were screened further.

Obtainment of recombinant producer strains (on the example of transformants with the heterologous CBH II from *T. reesei*). Transformants were cultured in shake flasks with the standard medium for *P. verruculosum* (see section "Methods"). Target (avicelase, by hydrolysis of MCC) and basic (CMCase and β-glucosidase) activities and protein concentration were measured in the culture medium (Table 1). The culture medium obtained using the initial *P. verruculosum* B1-221-151 strain was used as the control.

Transformants were divided into three groups according to the ratio between the target and basic activities. *The first group* included the transformants with a target (avicelase) activity that does not surpass this activity in the control (see Table 1, body type). These transformants were evidently of no further interest. *The second group* included transformants with high target activity but low basic enzyme activity (Table 1, **bold type**). Maintenance of the basic activity at the level of the initial strain (or its insignificant decrease in comparison with the control) is a necessary condition for the hydrolytic efficiency of the preparations. That is why transformants of the second group were also excluded from further study. *The third group* contained transformants with lower target activity (though it surpassed the control value) that retained the activities of basic enzymes at the level of the initial strain (see Table 1, ***boldface italic type***). These transformants were the most promising; they were used for further obtainment of EPs in one-liter bioreactors.

Comparison of the target (avicelase) and other (CMCase and β-glucosidase) activities of the transformants showed that transformants #2, 4, 10, and 25 are the most promising: their avicelase activity ranged from 0.7 to 0.8 U/mg (0.6 U/mg in the control), and CMCase and β-glucosidase activities ranged from 7.8 to 11.8 U/mg and from 1.6 to 2.2 U/mg, respectively (13.6 and 2.2 U/mg in the control, respectively).

Expression of the target protein in the selected transformants (#2, 4, 10, and 25) was confirmed by MALDI-TOF mass spectrometry. For this purpose the culture media of the corresponding transformants underwent SDS-PAGE; gel samples corresponding to the target recombinant enzyme CBHII by molecular weight were cut, trypsin-digested, and submitted for mass spectrometry. The analysis of the results and the search for target peptides were performed using Brucker DataAnalysis software. The data on tryptic peptides were compared with theoretical peptide sequences of CBHII from *T. reesei* obtained using PeptideMass software (http://expasy.org/tools/peptide-mass.html). The results (data not shown) allowed us to conclude that CBHII from *T. reesei* presented in the culture media of the selected *P. verruculosum* trasnformants.

The recombinant *P. verruculosum* strains producing other target proteins—XylIII, XylA, ManB, EGIV, and EGI—were obtained and selected in a similar way. The expression of the target genes was

Table 3. Specific activities of EPs containing recombinant enzymes towards various substrates (EPs are divided into groups according to the recombinant enzymes; target activities of the EP groups are in bold.)

Preparations	Specific activity (U/mg)			Protein content in recombinant EPs (mg/g)
	activity	recombinant EPs	control (*P. verruculosum* B1-221-151)	
XylA-1–8	**Xylanase**	22.5–69.6	12.9	336–737
	Avicelase	0.1–0.21	0.3	
	CMCase	1.2–2.9	13.0	
	β-Glucosidase	0.5–3.3	1.1	
ManB-1–8	**Mannanase**	14.7–53.7	Absent	378–755
	Xylanase	1.3–9.7	12.9	
	Avicelase	0.05 –0.15	0.3	
	CMCase	2.1–17.0	13.0	
	β-Glucosidase	0.3–1.1	1.1	
EGIV-1–8	Xylanase	12.5–33.3	12.9	620–950
	Avicelase	0.1–0.4	0.3	
	CMCase	4.9–10.9	13.0	
	β-Glucosidase	0.8–1.6	1.1	
GI-1–4	Xylanase	9.1–17.2	12.9	685–940
	Avicelase	0.2–0.3	0.33	
	CMCase	10.7–24.2	13.0	
	β-Glucosidase	1.0–1.2	1.1	
CBHII-1–4	Xylanase	15.2–21.5	12.9	751–916
	Avicelase	0.25–0.35	0.3	
	CMCase	14.7–19.8	13.0	
	Glucosidase	1.1–1.5	1.1	
XylIII-1	**Xylanase**	20.8	12.9	807
	Avicelase	0.1	0.3	
	MCase	18.0	13.0	
	β-Glucosidase	1.1	1.1	

proved. A total of 33 recombinant strains were selected.

Obtainment of recombinant EPs. The selected recombinant *P. verruculosum* strains were fermented in one-liter bioreactors as described in “Methods.” The EPs were obtained by the removal of fungal biomass from the culture media and subsequent freeze drying. A total of 33 dry EPs were obtained from 6 producer clones (Table 2).

The molecular weights and pI of the recombinant enzymes were determined: ~39 kDa and 2.8 for the EGIV from *T.reesei*; ~55 kDa and 4.6 for the EGI from *T.reesei*; ~55 kDa and 5.7 for the CBHII from *T. reesei*; ~31 kDa and 8.5 for the XylA from *P. canescens*; ~38 kDa and 9.1 for the XylIII from *T. reesei*; and ~50 kDa and 4.55 for the endo-β-1,4-ManB from *T. reesei*, respectively.

The results of SDS-PAGE (data not shown) indicate that expression of the target genes slightly decreases expression of the CBHI gene of *P. verruculosum* in a number of cases. It is connected with the use of flanking regions of the *cbhI* gene, which in some cases results in partial substitution of the *cbhI* gene for the gene of the heterologous protein during homologous recombination by double crossing-over.

Data characterizing the activities of the obtained EPs containing recombinant cellulases and hemicellulases towards various substrates are shown in Table 3.

As can be seen from Table 3, the target activities of the EPs varied within a wide range. The activity values

Table 4. pH and temperature optimums and EP stability

Target gene		*xylA P. canescens*		*xylIII T. reesei*	*manB T. reesei*		*eglI T. reesei*	*eglIV T. reesei*	*cbhll T. reesei*	
Preparation name		XylA-3	XylA-4	XylIII-1	ManB-6	ManB-2	EGI-4	EGIV-2	CBHII-2	CBHII-3
Substrate activity		GX		GX	GM	GM	CMC	CMC	CMC	CMC
pH optimum ($pH_{50\%}$)		5.5 (4.7–7.1)	5.5 (4.2–6.7)	3.5 (2.5–6.5)	3.5 (2.5–7)	3.5 (2.5–6.5)	4.0–4.3 (2.8–5.8)	4.0–4.9 (2.5–5.9)	4.9 (2.5–5.7)	4.9 (2.5–5.8)
Temperature optimum ($T_{50\%}$) (°C)		50 (30–58)	54 (35–60)	(37–67)	80 (60–85)	80 (60–85)	60 (44—71)	60 (43—76)	56 (38–67)	59 (40–66)
Residual activity after 3 h of incubation (pH 5.0) (%)	50°	47	30	34	76	42	75	61	90	95
	60°	N.s.*	N.s.	N.s.	75	33	52	45	17	17
Half-inactivation time (pH 5.0), min	50°	130	85	150	>180	120	>180	>180	>180	>180
	60°	4.4	4.6	7	>180	53	>180	100	95	70

* N.s. is not stable.

for a number of preparations exceeded by several times those for the control EP obtained using the initial *P. verruculosum* B1-221-151 strain: specific xylanase activity increased 2–5 times, CMCase activity (characterizing endoglucanase activity) increased 2 times, and avicelase (activity towards MCC characterizing cellobiohydrolase activity) increased 3–4 times; mannanase activity, which was absent in the initial preparation, reached a significant level in the corresponding recombinant enzymatic preparations.

pH and temperature optimums of activity and stability of the recombinant EPs. The optimum pH and temperature for the target enzymes and their stability are important parameters that are used to compare EPs and considered in biotechnological processes. That is why the pH and temperature optimums towards various substrates—CMC, MCC, glucuronoxylan (GX), and galactomannan (GM)—and stabilities were determined for recombinant EPs possessing the maximum target activity and at the same time retaining all types of the basic enzymatic activity at the level of the initial strain (Table 4).

As can be seen from Table 4, the pH optimum values of the xylanase, mannanase, CMCase, and avicelase activities of the EPs lay in the weak acid region (pH 3.5–5.5) while the temperature optimums for various activities varied from 50 to 80°C. The considered EPs had wide overlapping ranges of pH and temperature, in which over half of the maximum activity exhibited at the optimum values of each parameter was observed: $pH_{50\%} = 2.5–7.1$ and $T_{50\%} = 30–85$°C.

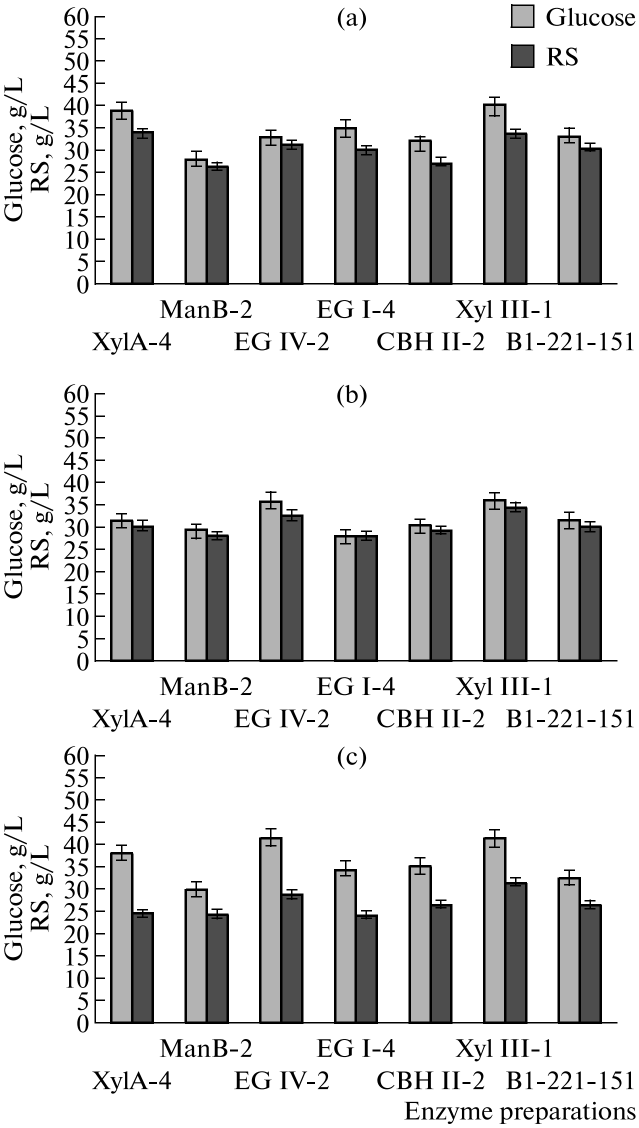
Hydrolysis of cellulose-containing plant materials. Hydrolytic ability is the most important property of an EP, defining the economic feasibility of its industrial use. This ability was estimated as the maximum conversion of various CCMs during their hydrolysis by the studied EPs (yields of RS and glucose at complete hydrolysis). The enzymatic degradation of various natural CCMs (milled aspen, milled depitched pine wood, and milled bagasse) was performed for 48 h at 50°C and the EP content of 5 mg of protein/1 g of dry substrate. In order to reveal the full hydrolytic potential of the studied EPs and to increase the conversion ratio of CCMs due to the destruction of oligosaccharide hydrolysis products, the recombinant *P. verruculosum* EP F10 was added into the reaction mixture. The additional preparation contained the heterologous β-glucosidase from *A. niger* as the main component. The addition corresponded to 40 U of β-glucosidase activity per 1 g of dry substrate.

Samples with measured concentrations of RS and glucose were taken in 3, 24, and 48 h. The B1-221-151 EP obtained from the initial *P. verruculosum* strain was used as the control (figure).

Aspen wood, used as the hydrolysis substrate, has high contents of cellulose (40–55%) and hemicellulose (up to 40%) and a relatively low content of lignin (18–25%) [7]. Aspen cellulose fibrils are surrounded by the hemicellulose matrix, which mostly consists of various xylans; therefore, their destruction requires high xylanase activity of the EPs. In fact, EPs XylA-4 and XylIII-1, obtained on the basis of strains with cloned xylanase genes, demonstrates the most hydrolytic effect for milled aspen. The best out of these preparations provided the formation of approximately 42 g/L of RS and 40 g/L of glucose for 2 days. The control *P. verruculosum* B1-221-151 EP provided 35 g/L of RS and 33 g/L of glucose under the same conditions.

Pine wood, like aspen, contains a significant amount of cellulose (45–50%), but differs in its higher content of lignin (25–35%) and lower content of hemicellulose (up to 30%) [7]. Conifer hemicelluloses are represented by xylans and galactoglucomannans (up to 10–15%). Consequently, we can assume that the EP containing ManB will be highly efficient in th hydrolysis of milled pine wood. However, according to the experimental data, the XylIII-1 and EGIV-2 EPs were the best EPs for the hydrolysis of milled depitched pine wood: they were able to form 38 g/L of RS and 35 g/L of glucose in the best case. The corresponding values for the control *P. verruculosum* B1-221-151 EP were 32 g/L of RS and 30 g/L of glucose. This result can be explained by the fact that the cellulase and xylanase activities of the ManB-2 EP are lower than those of the XylIII-1 and EGIV-2, which causes a decrease in its total hydrolytic activity. This occurs despite the additional content of mannanase, which is able to hydrolyze galactoglucomannans of soft wood.

The cellulose content in bagasse is lower than in trees (~40%). However, sugar cane is an annual plant, which is why its biomass contains a lot of xylans (>30%) and a small amount of lignin (not more than 25%) [7]. The XylIII-1 and EGIV-2 EPs were the most efficient in bagasse hydrolysis: they were able to form 42 g/L of RS and 32 g/L of glucose in the best case. The corresponding values for the control *P. verruculosum* B1-221-151 EP were 32 g/L of RS and 27 g/L of glucose under the same conditions.



Yields of RS (g/L) and glucose (g/L) from hydrolysis of various CCMs (100 g/L). EP protein amount of 5 mg/1 g of the substrate in the presence of β-glucosidase preparation F10 (40 U of β-glucosidase activity per 1 g of the substrate, hydrolysis of 48 h, 50°C, pH 5.0): a—aspen; b—pine; c—bagasse.

Composition of the recombinant EPs. The efficiency of CCM hydrolysis depends significantly on the composition of the multienzyme complex and the cooperation of individual enzymes inside it. Quantitative analysis of the EP composition reveals the correlation between its hydrolytic efficiency and the content

Table 5. EP compositions*

Preparation \ Component	Cellobiohydrolase I from *P.verruculosum*	Cellobiohydrolase II from *P.verruculosum*	Cellobiohydrolase II from*T. reesei*	Endo-β-1,4-glucanases from *P.verruculosum*	Endo-β-1,4-glucanase I from *T. reesei*	Endo-β-1,4-glucanase IV from *T. reesei*	Endo-β-1,4-mannanase from *T. reesei*	Endo-β-1,4-xylanases from *P.verruculosum*	Endo-β-1,4-xylanase from *T. reesei*	Endo-β-1,4-xylanase A from *P. canescens*	Xyloglucanases from *P.verruculosum*	β-Glucosidase from *P.verruculosum*
XylA-4	35	12	–	10	–	–	–	2	–	**18**	2	2
XylIII-1	25	14	–	25	–	–	–	5	**17**	–	3	4
ManB-2	11	6	–	2	–	–	**70**	–	–	–	–	1
ManB-6	13	18	–	10	–	–	**45**	2	–	–	2	1
CBHII-2	44	12	**2**	27	–	–	–	7	–	–	4	4
EGIV-2	32	28	–	10	–	**15**	–	6	–	–	3	4
EGI-4	39	21	–	24	**3**	–	–	1	–	–	3	1
B1-221-151 (control)	35	34	–	16	–	–	–	4	–			

* Values for target enzymes are in bold.

of individual enzymes and further optimizes the cellulase and hemicellulase composition in order to reach maximum hydrolytic efficiency.

The EP compositions were studied using two-stage chromatographic fractionation with the following detection of the contents of individual enzymes in the obtained fractions (Table 5).

Data characterizing the compositions of recombinant EPs allow interpretation of their hydrolytic efficiencies towards various CCMs. The EPs that simultaneously contain recombinant (heterologous) enzymes and retain their own basic cellulase complex—namely, the XylA-4 (18% of the recombinant xylanase A, 47% of cellobiohydrolases, and 10% of endoglucanases), XylIII-1 (17% of the recombinant xylanase III, 39% of cellobiohydrolases, and 25% of endoglucanases), and EGIV-2 (15% of the recombinant endoglucanase IV, 60% of cellobiohydrolases, and 10% of endoglucanases) EPs—were the most efficient in hydrolysis. The contents of cellobiohydrolases and endoglucanases in the control *P. verruculosum* EP were 69% and 16%, respectively.

CONCLUSIONS

Consequently, we developed a method for the obtainment and analysis of properties of recombinant cellulase and hemicellulase EPs of various compositions based on the cellulase complex from *P. verruculosum*, which is a universal base for new EPs optimized for the efficient hydrolysis of certain CMMs of various natures and compositions. Using this method, we created new recombinant *P. verruculosum* strains producing multienzyme carbohydrase complexes with increased cellulase (due to the expression of the endo-β-1,4-glucanases I and IV and cellobiohydrolase II from *T. reesei*) and hemicellulase (due to the expression of the endo-β-1,4-xylanases from *P. canescens* and *T. reesei* and endo-β-1,4-mannanase from *T. reesei*) activities. EPs obtained on the basis of these strains possessed high stability, with pH and temperature optimums of 3.5–5.5 and 50–80°C, respectively. We showed that the most hydrolytic ability towards various CCMs (milled aspen, depitched pine wood, and milled bagasse) is shown by EPs that simultaneously contain recombinant (heterologous) proteins and retain their own basic cellulase complex, namely the XylA-4, XylIII-1, and EGIV-2 preparations. The hydrolytic abilities of these EPs surpassed that of the control EP obtained using the initial *P. verruculosum* strain.

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