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BIOCATALYSIS

Improving the Efficiency of the Bioconversion of Plant Raw Materials with Mutant Cellulases of *Penicillium verruculosum*

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Abstract—Plant biomass is the main type of organic material on Earth. The efficiency of biocatalytic conversion of plant raw materials determines the cost of their biotechnological processing to produce commercially valuable products such as organic alcohols and acids, carbohydrates, and hydrocarbons. New recombinant *Penicillium canescens* strains that produce not only their own enzyme complex but also heterologous cellulases (i.e., mutant and wild-type cellobiohydrolase I (CBH I) and endoglucanase II (EG II) of *P. verruculosum*) are constructed. Enzymatic agents (EAs) prepared on the basis of recombinant strains of *P. canescens* are found to be more active in the hydrolysis of crushed aspen wood. Yields of glucose and reducing sugars are observed 24–72 h after hydrolysis with EAs prepared in recombinant strains to be from 48 to 52 and 60 to 64%, respectively, higher than those for hydrolysis with EAs prepared in the initial recipient strain. The presence of N45A and N194A site-specific mutations introduced to reduce surface glycosilation thus results in a substantial increase in the yields of desired CBH I and EG II.

Keywords: bioconversion, plant raw materials, enzymatic agents, cellulases, recombinant strains, *Penicillium*

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INTRODUCTION

Plant biomass is the main type of organic material on Earth. The total amount of plant biomass is equal to approximately one trillion tonnes [1], with its annual increment in the world being approximately 50 billion tonnes [2, 3]. The biotechnological processing of plant raw materials allows us to obtain biofuels and such commercially important chemical products as organic alcohols, acids, carbohydrates, and hydrocarbons [4].

The biocatalytic conversion or enzymatic hydrolysis of cellulose as a main component of plant raw materials to form technical sugars is a key step of processing [5]. Complex mixed enzymatic agents (EAs) based on cellulases prepared in the producers of such genera as *Trichoderma*, *Penicillium*, *Acremonium*, and *Humicola* fungal strains are used for the effective hydrolysis of cellulose [6–8]. EAs are composed of key enzymes (mixed in various ratios) that participate in the hydrolysis of cellulose: endoglucanases (EGs) that are responsible for the degradation of cellulose to form small fragments and cellobiohydrolases (CBHs) that convert cellulose and EG products of degradation to cellobiose [9]. Optimizing mixed EAs by changing the composition of cellulase complex secreted by producer strains allows us to improve the biocatalytic ability of EAs. The catalytic activity of individual enzymes can also be increased by protein engineering applied to cellulases, opening up new opportunities to increase the total activity of the entire cellulase complex [10].

Fungi of the genus *Penicillium* are industrial producers of cellulases [11]. The search for new strains of fungi of this genus, including recombinant ones that produce highly active cellulase enzyme complexes, is thus an important task. Protein engineering of N-glycosylation sites of the main cellulases of *P. verruculosum* (e.g., CBH I and EG II) is one way of increasing their catalytic activity [12, 13]. It is known that the N45, N194 and N388 sites of CBH I are glycosylated, and N-linked glycans are high-mannose oligosaccharides of $(Man)_{0–13}(GlcNAc)_2$ general structure or GlcNAc residues [12], while EG II, N42, and N194 sitesre a glycosylated and N-linked glycans are $(Man)_{1–9}(GlcNAc)_2$ high-mannose oligosaccharides [13]. Mutant forms of CBH I N45A and EG II N194A of *P. verruculosum*, where substituting the asparagine residue of an N-glycosylation site for alanine residue removed N45 and N194 N-glycosylation sites from the structures of CBH I

and EG II, respectively, were characterized by 20–60% higher catalytic activities toward soluble (β-glucan) and insoluble (microcrystalline cellulose and crushed aspen wood) substrates, compared to wild-type forms. In this work, recombinant strains of *P. canescens* that produce mutant and wild-type forms of heterologous CBH I and EG II cellulases of *P. verruculosum* along with their own enzyme complex are constructed, and the catalytic activities of EAs prepared on the basis of these strains toward various substrates including crushed aspen wood are compared.

EXPERIMENTAL

Amplifying Genes and Constructing Recombinant Strains

Genes that code the CBH I and EG II of *P. verruculosum* B151 and producers based on them were obtained as described in [12, 13]. The enzyme producers were prepared via the ligase independent cloning (LIC) of *cbh I* and *eg II* genes of mutant and wild-type forms [14] into a shuttle vector containing nucleotide sequences that corresponded to the promoter region of the xylanase A gene of *P. canescens* and the terminator region of the EG III gene of *P. canescens*, along with the genetic elements needed for replication in *E. coli* cells [15] with their subsequent transformation into *P. canescens* cells [16].

Preparing Dry EAs

Producer strains were cultured in 3 L fermentors (Prointech, Moscow, Russia) in a medium containing 30 g L^{-1} of beet pulp, 50 g L^{-1} of peptone (DIA-M, Russia) and 25 g L^{-1} of KH_2PO_4 (Helicon, Russia) at 30°C and pH values of 4.8 to 5.0 for 6 days, and the culture broth was concentrated and freeze-dried to obtain EAs. Dry EAs were thus prepared on the basis of the initial *P. canescens* PCA-10 strain (PCA-10 EA), the recombinant strains that produce wild-type forms of CBH I and EG II (PCA-10 CBH I and PCA-10 EG II EAs, respectively), and those that produce mutant forms of CBH I and EG II (PCA-10 CBH I N45A and PCA-10 EG II N194A EAs, respectively).

Substrates

Microcrystalline cellulose (MCC) (PH-101; Sigma, United States), carboxymethylcellulose (CMC) (Sigma, United States), β-glucan from barley (Megazyme, Australia) and xylan (Sigma, United States) were used as substrates to determine the enzymatic activity of EAs. Aspen wood crushed in an AGO-2 laboratory planetary ball mill to particle sizes of 10–50 μm served as the substrate to determine the hydrolytic ability of EAs.

Electrophoresis under Denaturing Conditions

SDS electrophoresis was performed on plates coated with stacking (4%) and separating (12%) polyacrylamide gels. The enzyme solutions were treated with 1% sodium dodecyl sulfate and 5% β-mercaptoethanol at 100°C for 15–20 min. Coomassie-Brilliant Blue G-250 dye (Helicon, Russia) was used to stain the protein bands in gels. A protein marker (no. 26610, Thermo Scientific, United States) ranging from 14.4 to 116.0 kDa was applied as a molecular weight reference.

Activity of Enzymes

The Somogyi–Nelson technique was used to determine the activity of enzymes toward such polysaccharide substrates as MCC, CMC, β-glucan and xylan in terms of the initial rates of formation of reducing sugars (RSes) [17, 18]. Hydrolysis was performed at pH 5.0 and a temperature of 40°C (with MCC) or at pH 5.0 and a temperature of 50°C (with CMC, β-glucan, and xylan). The amount of enzyme that led to the formation of 1 μmole of RSes in 1 min at a substrate concentration of 5 g L^{-1} was considered a unit of activity. The resulting activity values were divided by the mass of protein in 1 g of EAs to calculate the specific activities. The content of protein in EAs was found using the Lowry method [19] with bovine serum albumin as a standard.

Enzymatic Hydrolysis

Aspen wood crushed in an AGO-2 laboratory planetary ball mill to particle sizes of 10–50 μm was enzymatically hydrolyzed in microtubes with constant stirring at 50°C. The total volume of the reaction mixture was 1.5 mL, the substrate concentration calculated per dry substance was 100 mg mL^{-1}, and the EA concentration (the total amount of protein) was 0.5 mg mL^{-1}. The weighed substrate samples were introduced into microtubes and a calculated amount of 0.1 M sodium acetate buffer (pH 5.0) and 250 μL of solution containing the required amount of EA were added. Hydrolysis was performed with an excess of the β-glucosidase of *Aspergillus niger* (0.015 mg mL^{-1}) to prevent inhibition of the enzymes by cellobiose [20]. Samples (100 μL) were taken 24, 48, and 72 h after the start of hydrolysis and the concentrations of glucose and RSs were determined. The glucose concentration was found using the glucose oxidase/peroxidase method with a Photoglucose kit (OOO Impact, Russia) and D-glucose (Reakhim, Russia) for calibration. The Somogyi–Nelson technique was used to determine RSes [17, 18]. All measurements were repeated three times, and the results were analyzed with the Data Analysis add-on in Microsoft Excel 2010.

RESULTS AND DISCUSSION

Comparing the Compositions and Specific Activities of EAs

The component composition and the specific activity toward a range of substrates such as CMC, β-glucan, xylan, and MCC are important characteristics that determine the biocatalytic activity of EAs used for bioconversion of plant raw materials [21, 22].

The component compositions were compared via the SDS electrophoresis of EAs prepared on the basis of the initial *P. canescens* PCA-10 strain (PCA-10 EA), the recombinant strains that produce wild-type forms of CBH I and EG II (PCA-10 CBH I and PCA-10 EG II EAs, respectively), and those that produce mutant forms of CBH I and EG II (PCA-10 CBH I N45A and PCA-10 EG II N194A EAs, respectively). Figure 1 shows an electrophoretogram of these EAs. Heterologous CBH I (67 kDa) and EG II (39 kDa) enzymes, indicated by arrows in the electrophoretogram, are observed in the EAs prepared on the basis of recombinant strains and not in the EAs obtained from the initial strain. It should also be noted that the mutant CBH I and EG II forms are characterized by lower molecular weights than those of the corresponding wild-type forms. This is due to the removal of N-glycosylation sites from the structures of CBH I and EG II, which leads to the removal of N-linked glycans from the protein globule surface and thus a reduction in molecular weight. It should be noted that the molecular weights of CBH I and EG II calculated from the amino acid sequences were 52 and 35 kDa, respectively, and the values experimentally determined for the wild-type forms of CBH I and EG II were 67 and 39 kDa, respectively. The difference between the molecular weights was due to the glycosylation of enzymes (the peptide linker is mainly O-glycosylated in the case of CBH I) [20].

The prepared EAs are characterized by rather high levels of expression of heterologous CBH I and EG II enzymes. The fractions of heterologous CBH I and EG II in secreted complexes were determined by analyzing the SDS electrophoretogram using the GelAnalyser 2010a program software. The fractions of CBH I (in the case of CBH I-containing EAs) and EG II (in the case of EG II-containing EAs) were from 44 to 59 and from 20 to 58% of all secreted proteins, respectively. The prepared EAs may therefore be used to isolate homogeneous CBH I and EG II and examine their properties. It should be noted that the use of EAs obtained from the culture broths of strain producers in the bioconversion of plant raw materials is economically more advantageous than the use of pure enzymes. The costs of obtaining EAs are less than those of preparing the individual enzymes. In addition, there are enzymes that are characterized by different substrate features in the composition of EAs and are capable of hydrolyzing various components of plant raw materials, thereby impoving the efficiency of bioconversion. However, further studies of the composition and properties of EAs prepared on the basis of the *P. canescens* PCA-10 strain are required, since this strain produces an extracellular protease like penicillopepsin [23], and the latter can influence the activity of EAs.

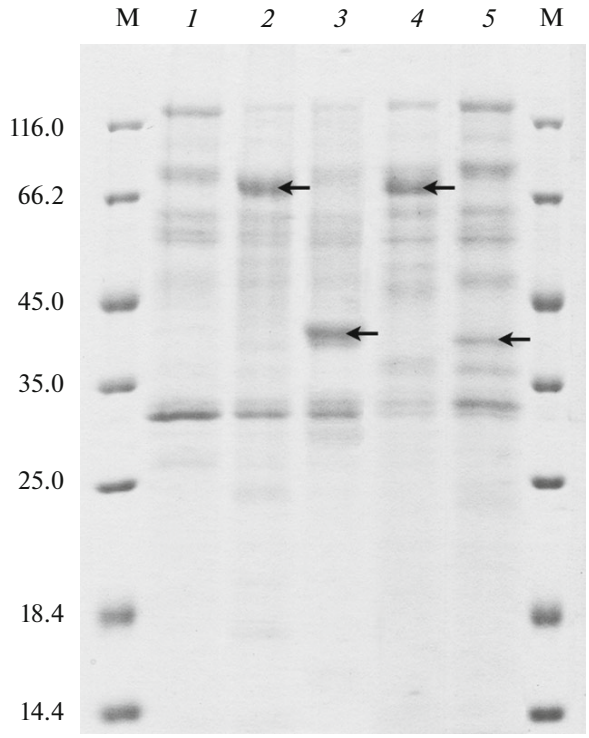

Fig. 1. SDS electrophoretogram of EAs prepared on the basis of such *P. canescens* strains as (*1*) PCA-10 EA, (*2*) PCA-10 CBH I EA, (*3*) PCA-10 EG II EA, (*4*) PCA-10 CBH I N45A EA and (*5*) PCA-10 EG II N194A EA, where M is a marker and the arrows indicate the positions of heterologous CBH I (in *2* and *4*) and EG II (in *3* and *5*) enzymes.

The specific activities of EAs obtained from the recombinant strains and the initial strain toward different substrates are summarized in the table. The activity toward MCC indicates the EAs can hydrolyze the crystalline form of cellulose, with CBH being mainly considered to manifest activity toward MCC [24, 25]. The emergence of mutant and wild-type forms of CBH I in the composition of EAs ensures an increase in the specific activities of EAs prepared from recombinant strains toward MCC. The activity towards CMC and β-glucan characterizes the ability of enzymes to hydrolyze the amorphous form of cellulose [24, 26]. The data in the table show that the emergence of mutant and wild-type forms of EG II in the composition of EAs considerably enhances the specific activities of EAs toward CMC and β-glucan.

The activity towards xylan indicates that EAs can hydrolyze hemicellulose components of plant raw materials [27]. The activity of all EAs obtained from

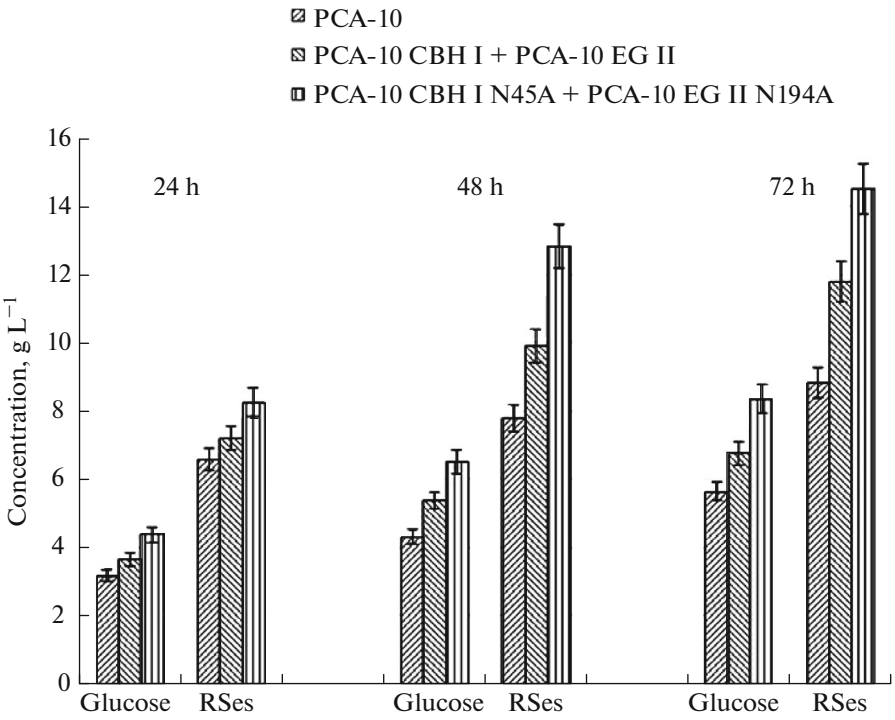

Fig. 2. Yields of glucose and RSes obtained after hydrolyzing crushed aspen wood with EAs prepared on the basis of various *P. canescens* strains at a temperature of 50°C, a pH value of 5.0, a substrate concentration of 100 g L^{-1}, and an EA (or EA complex) concentration of 0.5 g L^{-1} for 24, 48, and 72 h.

recombinant strains toward xylan is less than that of EAs prepared on the basis of the initial strain. This is due to the presence of the promoter region of the xylanase A gene of *P. canescens* as a regulatory element in plasmids used for transformation. Inserting target genes such as CBH I and EG II into the genome of the recipient strain leads to partial substitution of the *xylA* gene that codes xylanase A for the *cbhI* or *egII* genes that code CBH I and EG II, respectively. The activity of EAs toward xylan is thus reduced.

Enzymatic Hydrolysis of Aspen Wood

Crushed aspen wood was hydrolyzed to compare the biocatalytic activities of EAs. The simultaneous action of enzymes characterized by different substrate specificities, and especially cellulolytic enzymes such as CBH I and EG II, was noted above to be necessary for the effective hydrolysis of plant raw materials (mainly, cellulose) [9, 28]. Double EA complexes containing PCA-10 CBH I and PCA-10 EG II or PCA-10 CBH I N45A and PCA-10 EG II N194A were therefore used for the bioconversion of crushed aspen wood. The ratio between EAs in the complexes, calculated from the protein content, was 1 : 1. The concentration of EA complex in the reaction mixture was 0.5 mg mL^{-1} (calculated for the total amount of protein). EA obtained on the basis of the initial *P. canescens* PCA-10 strain (PCA-10 EA) was used as a control.

EA complex containing mutant forms of CBH I N45A and EG II N194A was more active than the control EA and the EA complex containing wild-type forms of CBH I and EG II (Fig. 2). The yield of glucose 24–72 h after the start of the bioconversion of crushed aspen wood in the presence of the PCA-10 CBH I N45A-PCA-10 EG II N194A EA complex was from 48 to 52 and 20 to 24% higher than those obtained with the control PCA-10 EA and the PCA-10 CBH I-PCA-10 EG II EA complex, respectively. The yield of RSs 24–72 h after the start of the bioconversion of crushed aspen wood in the presence of the PCA-10 CBH I N45A-PCA-10 EG II N194A EA complex was from 60 to 64 and from 22 to 30% higher than those obtained with the control PCA-10 EA and the PCA-10 CBH I-PCA-10 EG II EA complex, respectively.

EAs prepared on the basis of new recombinant strains of *P. canescens* that contain the mutant and wild-type forms of such heterologous cellulases as CBH I and EG II of *P. verruculosum* are thus characterized by higher biocatalytic activity than that of EAs obtained from the initial recipient strain. It should be noted that the approach to designing new recombinant strains with altered compositions of secreted enzyme complex is widely used in the preparation of highly biocatalytically active EA complexes [29]. For example, adding EG IV of *T. reesei* to the composition of

Specific activities of EAs prepared from various *P. canescens* strains

Producer strain	Specific activity, U per g of protein				Protein content, mg per g of EA
	MCC	CMC	β-glucan	xylan	
PCA-10	7.9 ± 0.6	41 ± 3	36 ± 2	9749 ± 585	191 ± 10
PCA-10 CBH I	10.7 ± 0.7	52 ± 3	42 ± 3	2427 ± 136	330 ± 18
PCA-10 EG II	8.6 ± 0.6	2441 ± 122	3630 ± 182	2898 ± 145	434 ± 24
PCA-10 CBH I N45A	7.4 ± 0.6	56 ± 3	48 ± 3	1217 ± 73	310 ± 19
PCA-10 EG II N194A	5.8 ± 0.4	3018 ± 150	4104 ± 208	1476 ± 91	164 ± 8

cellulolytic complex secreted by *P. verruculosum* allowed us to improve the efficiency of the hydrolysis of MCC and crushed aspen wood by 20 and 10%, respectively [30]. Meanwhile, adding β-glucosidase to the composition of cellulolytic complex secreted by *T. reesei* reduced the cellobiose-induced inhibition of cellulase activity, while further optimization of the complex composition allowed us to raise the efficiency of bioconversion by several times [29, 31]. The heterologous expression of cellulases of thermophilic organisms as components of complexes secreted by *T. reesei*, *Aspergillus oryzae*, and *Humicola insolens* also allow us to perform enzymatic hydrolysis at higher temperatures and increase the extent of hydrolysis [32].

CONCLUSIONS

The efficiency of the biocatalytic conversion of plant raw materials determines the cost of obtaining technical sugars and their subsequent use in the production of such commercially valuable products as organic alcohols and acids, carbohydrates, and hydrocarbons. The search for new recombinant fungal strains that produce highly biocatalytically active EAs will allow us to improve the efficiency of the hydrolysis of plant raw materials.

The designing of new recombinant strains of *P. canescens* that produce not only their own enzyme complex but also mutant and wild-type forms of such heterologous cellulases as CBH I and EG II of *P. verruculosum* allows us to increase the biocatalytic activity of prepared EAs. EAs obtained on the basis of recombinant strains of *P. canescens* are more active in the bioconversion of crushed aspen wood than ones prepared from the initial strain. Adding mutant forms of CBH I N45A and EG II N194A of *P. verruculosum* that are characterized by emhanced catalytic activities to the composition of secreted complex also improves the hydrolytic ability of EAs.

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