Chapter

Cellulases from Mycelial fungi *Penicillium verruculosum* as a Real Alternative to Trichoderma Enzymes in Industrial Hydrolysis of Cellulosic Biomass

Olga G. Korotkova, Alexandra M. Rozhkova, Ekaterina A. Rubtsova and Arkady P. Sinitsyn

Abstract

Abstract The possibility of using the recipient strain *Penicillium verruculosum* B1-537 (ΔniaD) as a producer of laboratory and industrial enzymes was considered. The advantage of this strain is its ability to secrete a basic cellulase complex consisting of cellobiohydrolases, endoglucanases, and β-glucosidase, which exceeds in its hydrolytic ability the enzyme complex of *Hypocrea* (*Trichoderma*) strains. Using the expression system, the basic complex of cellulases of the recipient strain *Piptochaetium verruculosum* B1-537 (ΔniaD) was supplemented with new (booster) enzymes that are necessary to increase its hydrolytic activity. Enzyme preparations adapted to the processing of various types of renewable plant biomass were obtained.

Keywords: *Penicillium verruculosum*, *Trichoderma*, cellulase complex, cellulose bioconversion, renewable plant biomass, industrial enzymes

1. Introduction

Currently, the attention of scientists is focused on the depletion of reserves of fossil energy sources. Rapid population growth provides high energy needs for fossil resources such as coal, natural gas, and crude oil [1]. The burning of fossil fuels poses a threat to the environment due to the emission of greenhouse gases, which, in turn, lead to global climate change. As for renewable energy sources, fuels and products derived from cellulosic waste do not harm the environment and can be considered as an alternative to fossil energy sources. Plant waste not only supports economic development but also creates an ecologically friendly environment for the production of energy and biochemicals [2–10].

Biomass cellulose is a plant polysaccharide and is an almost inexhaustible source of renewable raw materials that can be converted enzymatically into glucose. In turn,

glucose is a raw material for microbiological processes of obtaining liquid and gaseous fuels (ethanol, butanol, etc.), organic and amino acids, feed protein, and many other useful products of microbiological synthesis [11]. Coniferous and deciduous wood and its waste are of particular interest [12].

An important stage of bioconversion of cellulose-containing biomass, which prevents its commercial use, is the enzymatic conversion of cellulose into glucose. Natural wood and other lignocellulose materials are resistant to enzymes due to the crystalline structure of cellulose and the presence of lignin and hemicellulose-protecting cellulose fibers [12]. The reactivity of natural crystalline cellulose (for example, cotton) or lignocellulose (wood of various species, grass straw) during the enzymatic conversion is low, which is accompanied by extremely low yields of glucose and other sugars. Effective enzymatic hydrolysis of cellulose-containing biomass requires its pretreatment to increase reactivity by destroying the crystal structure of cellulose and completely or partially removing the lignin. As methods of pretreatment, gamma irradiation, mechanical grinding in a ball mill, treatment with mineral acids (sulfuric, phosphoric), cadoxene, and alkali delignification are used [12].

The influence of such factors on the efficiency of enzymatic conversion as changes in the degree of crystallinity of cellulose (determined by X-ray diffraction), the availability of cellulose surface to enzyme molecules (measured by such methods as protein adsorption, as well as by thermal desorption of nitrogen-hydrogen mixture), the size of cellulose particles (by dispersion analysis using optical microscopy), and the degree of polymerization of cellulose (determined by the viscosity of cellulose solutions in cadoxene) was investigated. Based on the results of these studies, the influence of these parameters on the efficiency of enzymatic cellulose conversion was quantified—it linearly depends on a decrease in the degree of crystallinity and an increase in the surface area available to the enzyme molecules, but it does not depend much on the geometric size of cellulose particles and its degree of polymerization [13].

Deep cleavage of crystalline cellulose to soluble sugars (glucose) is carried out by a complex of cellulolytic enzymes, including endoglucanases (EG), cellobiohydrolase (CBH), and β-glucosidase (BGL). The activity of the components of this complex and their interaction determine the action of the enzymes on cellulose-containing substrates [14].

Many research groups are looking for new effective producers of cellulases and hemicellulases. Research is being conducted to improve existing strains of microorganisms in order to increase the production of various cellulases and reduce their cost. In this chapter, we compare the hydrolytic potential of multi-enzyme cellulose complexes produced by different strains of fungi and evaluate the role of different enzymes in the hydrolysis of pretreated cellulose-containing substrates.

2. Enzymatic complex for deep destruction of cellulose-containing biomass

Enzymatic hydrolysis allows the destruction of plant raw materials to monosaccharides without significant energy costs and anthropogenic impact on the environment. Since plant biomass is a complex substrate that includes polysaccharides of various compositions, a complex of enzymes of various specificities is necessary for its deep destruction, which carries out cooperative hydrolysis of its constituent

components. In industrial biotechnology, various microscopic fungi are widely used as producers of such enzyme complexes. They are the main source of commercial cellulase preparations produced on an industrial scale in different countries of the world. For a long time, it was believed that cellulolytic fungi belonging to the genus *Trichoderma* are the undisputed leaders in the secretion of the most active cellulases [15–19]. Commercial cellulase preparations based on mutant strains of *Trichoderma reesei* (synonym of *Hypocrea jecorina*) are most common on the enzyme market. Therefore, it is not surprising that most of the research and developments on the bioconversion of lignocellulose raw materials (primarily for the production of second-generation bioethanol) were based on the use of *T. reesei* cellulases. However, in recent years, more and more publications demonstrating that there are alternatives to *T. reesei*-based enzyme preparations have appeared [16–18, 20–22]. In particular, it is related to cellulases produced by fungi from the genera *Penicillium* and *Myceliophthora*. Modern developments aimed at obtaining cellulose preparations of a new generation are characterized by using methods of protein and genetic engineering; recombinant (modified) enzymes with improved properties from various microorganisms are included in the composition of these preparations.

The leading role of mutant or recombinant strains of fungi of the genus *Trichoderma* (*T. reesei, T. viride, T. longibrachiatum*) is explained, firstly, by their high secretory ability, and, secondly, by the variety of enzymes produced with different substrate specificity [23–26]. Therefore, it is not surprising that biocatalysts (enzyme preparations) of cellulases based on *Trichoderma* fungi are produced in many countries by leading manufacturers of industrial enzymes, in particular, Novozymes (Denmark), Genencor and Danisco Division (USA), Iogen (Canada), PrimAlko (Finland), Röhm Gmbh (Germany), EnMex (Mexico), Meiji Seika Kaisha Ltd. and Shin Nihon Chemical Co. (Japan), etc. At the same time, the search for new producers of enzymes suitable for the hydrolysis of plant raw materials, as well as an increase in the overall activity and balance in the component composition of already known enzyme complexes, is still an actual task of modern biotechnology.

Strains of fungi of the genera *Penicillium*, *Acremonium*, *Chrysosporium*, *Chaetomium*, and *Humicola* can become a worthy alternative to strains of the genus *Trichoderma* since the enzyme complexes produced by them are not inferior in hydrolytic ability and sometimes are superior to those obtained with the help of known strains of *Trichoderma* [27–31].

Promising producers of highly active cellulase complexes are some species of fungi from the genus *Penicillium*. Cellulases from *Penicillium*, as a rule, surpass *T. reesei* enzymes in the rate of hydrolysis and glucose yield from various cellulose-containing substrates at the same dosage of protein concentration or cellulase activity (**Table 1**). Almost all the authors note a relatively high level of beta-glucosidase activity in enzyme preparations based on penicillin. This property is one of the main advantages of fungi of the genus *Penicillium* over *Trichoderma*. As a consequence, the gain of penicillin enzymes in glucose output sometimes reaches a 5-fold size (**Table 1**) [17, 18, 32–34]. Only after the addition of an excess of exogenous BGL, *T. reesei* preparations are able to provide comparable glucose yields. However, in some cases, even with the addition of BGL cellulase from *T. reesei* could not compete with cellulase complexes from *Penicillium* [17, 18, 20]. For example, with the same cellulase activity on filter paper in the reaction mixture (10 U/g of substrate), the preparation of *T. reesei* RUT-C30, enhanced with the addition of aspergilli BGL (6 U/g), was inferior in depth of hydrolysis of microcrystalline cellulose (MCC) after 48 hours to the preparation of *P. pulvirrolum* F-2220 (the difference

Cellulase producer	Substrate	Difference, times*
Penicillium sp.	Abies alba pretreated by steam explosion or organosolve	1.6–3.6 [17, 18]
Penicillium sp.	Poplar wood pretreated by organosolve	1.4–2.0 [17, 18]
Penicillium sp.	Red maple wood pretreated by organosolve	1.5–2.1 [17, 18]
P. brasilianum	Spruce wood pretreated by steam explosion	2.1 [18]
Panicum echinulatum	Bleached eucalyptus kraft pulp	1.1 [17, 18]
P. funiculosum	Crushed corn cobs	1.1–1.5 [18]
P. occitanis	Stipa tenacissima	1.8 [18]
P. oxalicum	Corn cobs alkali pretreated	2.0–2.2 [32]
P. oxalicum	Microcrystalline cellulose	3.6 [33]
P. pinophilum	Spruce wood pretreated by steam explosion	3.1 [17, 18]
Piptochaetium verruculosum	Microcrystalline cellulose	1.3–1.7 [18]
P. verruculosum	Cellulose from eucalyptus	1.3–3.9 [17, 18]
P. verruculosum	Coniferous wood pretreated by organosolve	1.3–5.1 [17, 18]
P. verruculosum	Wheat straw alkali pretreated	1.6 [34]

**The ratio of product concentrations (usually glucose) obtained by hydrolysis of cellulose-containing substrates under the action of enzyme preparations based on fungi Penicillium and T. reesei.*

Table 1.
Comparison of the hydrolytic ability of cellulase complexes from fungi of the genus Penicillium and T. reesei.

was 25%), whereas in the hydrolysis of pretreated spruce wood and wheat straw, both preparations showed comparable results [20].

Sequencing and annotation of the genomes of *P. decumbens* 114-2, *P. funiculosum* NCIM 1228, and *Piptochaetium verruculosum* TS63-9 have shown that these species of fungi differ in a richer set of enzymes that catalyze the cleavage of components of lignocellulose materials compared with *T. reesei* [35–37]. It especially concerns cellulases with a cellulose-binding module (CSM) and hemicellulases. Analysis of *P. decumbens* 114-2 secretome obtained by cultivation on a medium with wheat bran showed that it was richer in carbohydrates than that obtained on a medium with glucose [35]. In the secretome of *P. funiculosum* NCIM 1228, 113 enzymes acting on carbohydrates were detected by quantitative mass spectrometry, of which 92 were glycoside hydrolases [37]. Apparently, the genomes and secretomes, which are characterized by a high content of glycoside hydrolases and their accompanying enzymes, are also characteristics of other species of fungi of the genus *Penicillium*. Another reason for the high efficiency of cellulase complexes based on *Penicillium* fungi is the high specific activity of their key components, namely CBH I and CBH II, compared to the corresponding *T. reesei* enzymes. The difference can reach 2–2.5 times. In particular, this was demonstrated in the example of CBH *P. funiculosum*, *P. pulvirrolum*, *P. verruculosum*, and *P. canescens* [18–20, 38, 39]. One of the reasons for such high specific activity in the case of CBH I and CBH II of *P. verruculosum* is the optimal distribution of N-bound glycans on the surface of the catalytic domain of these enzymes [40, 41].

One of the properties that negatively affect the activity of cellulases during the hydrolysis of lignocellulose substrates is their unproductive adsorption on lignin [42–48]. During the hydrolysis of MCC, enzymes from *P. verruculosum* are significantly less inhibited by two types of lignin artificially added to the reaction system

than cellulases from five different *T. reesei* preparations. At the same time, the lignin regenerated from the solution in the organosolve caused a stronger inhibition (by 11–84%) than the residual lignin obtained by exhaustive enzymatic hydrolysis of pretreated wood (8–58% inhibition). Similar results demonstrating a lower negative effect of lignin on *P. verruculosum* cellulase compared to *T. reesei* enzymes were obtained in the work [34].

In the example of enzymes from *P. oxalicum*, it was shown that the degree of unproductive adsorption on lignin decreases in the raw of CBH – xylanase – EG – BGL, and mainly electrostatic interactions are responsible for the binding of xylanase and EG to lignin, whereas, in the case of CBH and BGL, hydrophobic interactions have a decisive influence. CBM in CBH plays a noticeable role in binding to the lignin [48]. The changes in the properties of lignin, caused by the pretreatment of lignocellulose raw materials, had a noticeable effect on the adsorption of *P. oxalicum* enzymes, as in the work [46]. Thus, as a result of pretreatment with hot water, lignin from corn waste got an increased ability to bind proteins [48].

Despite the high hydrolytic potential, commercial cellulase preparations from penicillins have not yet become widespread, although they are produced on an industrial scale by the company Adisseo in France (Rovabio ® Excel based on *P. funiculosum*) [36] and in China (enzyme preparations based on *P. decumbens*) [35]. Perhaps this is due to the fact that the existing strains of *Penicillium* are still inferior to the best industrial strains of *T. reesei* in extracellular protein secretion [17, 18]. One of the reasons may be the insufficiently aggressive marketing policy of the producers of these enzymes.

2.1 Recombinant strains of *Penicillium verruculosum*—Highly effective producers of cellulases

It is possible to increase the effectiveness of complex cellulase preparations both by improving the properties of individual enzymes and by optimizing the component composition of preparations. Protein engineering allows to change in the properties of already known individual enzymes, significantly increasing their activity and operational stability [49–51]. Since the composition and structure of various types of cellulose-containing raw materials can vary quite a lot depending on the source and type of pretreatment, the component composition of the enzyme preparations used for their hydrolysis should correspond to the composition of the raw materials.

The selection of the composition optimal for processing a particular type of raw material preparation is carried out by comparing the hydrolytic ability of different mixtures of individual enzymes [31, 52]. Genetic engineering of cellulase producers with a subsequent screening of the obtained mutants allows the creation of new highly effective strains of microorganisms secreting a complex of enzymes of optimal composition. At the same time, classical nondirectional mutagenesis also remains an effective tool for obtaining such producers.

The wild strain of *P. verruculosum* WA30 was isolated about 40 years ago. He was identified as a producer of a cellulase complex with high activity in relation to MCC. The productivity of the wild strain was low and was about 7 g/l of secreted protein in the culture fluid [53]. On the basis of the wild strain WA30 by the method of mutagenesis using nitrosoguanidine and ultraviolet irradiation with subsequent selection *P. verruculosum* strains were obtained. The protein concentration in the culture fluid reached 30–40 g/l [54, 55]. The synthesis of extracellular cellulolytic enzymes of *P. verruculosum* is induced by cellulose and gentiobiose, and in most cases, it is subject

to catabolic repression by glucose. The peculiarity of the obtained *P. verruculosum* mutants with a high level of extracellular cellulase secretion is that, during the mutagenesis, catabolic repression was reduced. Changes in pH during fermentation can influence the composition of the secreted cellulase complex. Some side activities, such as xylanase and amylase, become higher at higher pH values.

The enzyme complex secreted by *P. verruculosum* contains more than 20 enzymes differing in biochemical and catalytic properties. The main cellulases secreted by the control highly productive strains B1-221-151 (contracted B151) and B1-537 are CBH I (Cel7A), CBH II (Cel6A), EG I (Cel7B), EG IIa (Cel5A), EG IIb (Cel5B), EG III (Cel12A), and BGL (Cel3A). The content of CBH I is about 35%, CBH II is 21–33%, the content of these EGases varies from 2 to 5%, and the percentage of BGL is 3–4% [38, 56, 57]. In addition to cellulases, the enzyme complex also includes other carbohydrases—xyloglucanases, xylanases, α-galactosidase, and glucoamylase (a total of 18–30% of the total protein). The molecular weights of enzymes vary from 19 to 120 kDa and isoelectric points from 2.0 to 5.8. For most of these cellulases, the pH-optima of activity is in the pH range of 4.0–5.0 [38]. As a result of the limited proteolysis of the linker *P. verruculosum* cellulases, having CBM (CBH I, CBH II, EG I), as a rule, is represented by high-molecular (full-size) and low-molecular forms, the latter being the catalytic domain of a specific enzyme without CBM [38].

As mentioned above, CBH I and CBH II of *P. verruculosum*, when acting on crystalline cellulose, are significantly superior in hydrolytic ability to both CBH from *T. reesei* (of which CBH I is actually the "gold standard" in the study of properties of the cellulases [58]); at the same time, even the low molecular weight form of CBH II from *P. verruculosum* is comparable in effectiveness to the full-size CBH I from *T. reesei* [18]. This, together with a higher level of BGL activity, leads to a higher potential of *P. verruculosum*-based enzyme preparations compared to traditional commercial *T. reesei* preparations [17, 18, 34].

2.2 Hydrolytic potential of *P. verruculosum* cellulase complex in comparison with industrial analogs

One of the important criteria for comparing the effectiveness of enzyme preparations intended for the biodegradation of cellulose-containing raw materials is their specific activity on a number of substrates—soluble and insoluble polysaccharides (FP, MCC, CMC, xylan), synthetic substrates (pNPG, pNPX), and oligosaccharides (cellobiose). These activities on the example of the enzyme preparations obtained with the help of fungi of the genus *Trichoderma* (all the preparations Accelerase and Cellic CTec) and *Penicillium* as producers are shown in **Table 2**.

The presented data make it possible to characterize the individual components of the enzyme complex and their balance in the composition of the preparation, which mainly determines the effectiveness of their action in the hydrolysis of a substrate of complex composition, which is plant biomass.

Activity on filter paper characterizes the overall activity of enzyme preparations in relation to insoluble cellulose [59]. On average, it was 0.6–1.2 U/mg of protein, while the commercial preparation Accelerase1000 was characterized by the increased activity of 1.7 U/mg, and Accelerase XY had extremely low activity on filter paper.

MCC activity illustrates the ability of enzyme preparations to hydrolyze highly ordered crystalline zones of cellulose (crystallites).

Preparations of Cellic CTec-1 and Cellic CTec-2 had the same MCC activity (0.3 U/mg protein); preparations of Accelerase 1500 and DUET, as well as *P. verruculosum*

Preparation	FP	MCC	CMC	pNPG	Cellobiose	Xylan	pNPX
	50°C, pH 5.0	40°C, pH 5.0	50°C, pH 5.0	40°C, pH 5.0	40°C, pH 5.0	50°C, pH 5.0	40°C, pH 5.0
Accelerase 1000	1.7	1.2	12.3	3.6	2.7	2.4	<0.01
Accelerase 1500	1.2	0.8	10.5	3.8	2.3	1.7	<0.01
Accelerase XY	<0.1	0.1	0.8	0.6	0.4	92.5	0.3
Accelerase DUET	1.1	0.8	7.9	3.1	2.2	14.0	1.3
Cellic CTec-1	0.7	0.3	10.9	2.8	2.4	1.5	<0.01
Cellic CTec-2	0.6	0.3	15.6	5.5	4.0	28.2	<0.01
Piptochaetium verruculosum B151	0.9	0.7	18.3	1.7	0.7	30.3	<0.01
P. verruculosum F10	0.2	1.1	5.9	45.5	60.2	0.8	<0.01

Table 2.
Specific activities (units/mg of protein) of enzyme preparations obtained with the help of fungi of the genus Trichoderma *and* Penicillium *as producers.*

B151, were characterized by activity of 0.7–0.8 U/mg of protein; while Accelerase XY corresponded to a low level of MCC activity (0.1 U/mg of protein), and Accelerase 1000 had the highest level (1.2 U/mg of protein) compared to other drugs.

The activity of preparations on CMC demonstrates their ability to hydrolyze less ordered, amorphous zones of cellulose [60, 61]. The hydrolysis of these regions of the substrate is carried out mainly by endoglucanases, which hydrolyze internal β-1,4-glucosidic bonds remote from the ends of the cellulose polymer chain, with the formation of fragments of the polymer substrate chain and cellooligosaccharides, which is accompanied by a significant decrease in the degree of substrate polymerization and, as a result, a decrease in its viscosity (which is especially important in the first stages of the bioconversion process). Reducing the viscosity of the reaction mixture is a very significant factor in the industrial implementation of enzymatic hydrolysis processes since increased viscosity can lead to a significant decrease in the efficiency of some production stages (for example, due to a decrease in heat transfer rates, mixing, mass transfer, etc.). The considered commercial enzyme preparations, on average, were characterized by CMC activity of 8–12 U/mg of protein, while Accelerase XY corresponded with a low level of activity (0.8 U/mg of protein), and the *P. verruculosum* B151 preparation had the highest value (18, 3 U/mg protein) compared to other preparations.

In general, the specific activities of the *P. verruculosum* B151 preparation in relation to various substrates, which characterize the effectiveness of individual components of multienzyme complexes, are comparable with similar activities of commercial *Trichoderma* preparations. However, the specific activities given in **Table 2** were determined from the initial rates of hydrolysis of the corresponding substrates, and their comparison is not sufficient to compare the hydrolytic activity of enzyme preparations, since during long-term hydrolysis of biomass, the processes of enzyme inactivation, their inhibition by lignin (for due to unproductive adsorption of enzymes on it), as well as inhibition of enzymes by the reaction products.

For a more detailed consideration, **Table 3** shows the data characterizing the results of testing enzyme preparations during long-term (exhaustive) hydrolysis of biomass of various types [62]. Pretreated corn stalks, bagasse, and coniferous and deciduous wood were used as substrates. The selected substrates differ in composition, including the content of cellulose and hemicelluloses (xylans), as well as the content of lignin. The content of xylans decreased in the series: corn stalks, bagasse, aspen wood, and pine wood. In addition, MCC was used, which made it possible to evaluate the effectiveness of cellulase enzyme preparations in relation to a lignin-free substrate [63].

The hydrolytic efficiency of enzyme preparations based on fungi of the genus *Trichoderma* and *Penicillium* was evaluated by the formation of reducing sugars (RS) at various dosages of enzyme preparations (**Table 3**).

The enzyme preparations Accelerase 1000, Accelerate 1500, and Accelerate DUET had approximately the same efficiency of hydrolysis (RS yield) of pretreated corn stalks and bagasse, shredded pine, and aspen wood. The effectiveness of these preparations can be taken as an "average level".

Accelerase XY, which is essentially a xylanase preparation, turned out to be the least effective among those studied for the hydrolysis of pretreated corn stalks, shredded pine and aspen wood, and MCC but proved to be competitive in the hydrolysis of pretreated bagasse.

Cellic CTec-1 and Cellic CTec-2 preparations had approximately equal activity values for FP and MCC, which turned out to be less than the corresponding values for Accelerase 1000, Accelerase 1500, and Accelerase DUET preparations (**Table 2**). At the same time, Cellic CTec-2 was characterized by increased activities in relation to CMC, pNPG, and cellobiose, compared to Cellic CTec-1, as well as Accelerase 1000,

Preparation	**RS yield (mg/ml)**				
	Pretreated with steam explosion		**Grind wood**		**MCC**
	Corn stalks	**Bagasse**	**Pine**	**Aspen**	
Accelerase 1000	37.8-38.0-38.5	19.6-25.8-40.0	22.4-25.3-30.6	21.5-34.3-41.3	26.0-52.2-76.8
Accelerase 1500	33.9-36.5-38.9	17.6-24.4-34.6	19.9-24.5-29.1	19.3-35.2-40.9	27.0-50.6-77.3
Accelerase XY	18.2-18.3-18.3	18.7-19.5-32.8	3.6-4.9-8.6	11.8-13.9-17.3	2.9-4.8-5.8
Accelerase DUET	34.3-34.8-36.9	20.3-31.7-43.0	20.4-24.5-29.5	25.9–41.5-49.6	25.3-53.5-75.6
Cellic CTec-1	23.3-26.3-26.3	12.0-21.8-32.0	12.1-14.7-22.6	9.2-18.9-23.0	16.2-30.2-60.8
Cellic CTec-2	48.8–53.2-54.4	28.5-37.0-50.5	24.5-29.2-31.6	27.6–40.7-48.4	35.5-73.7-84.4
Piptochaetium verruculosum B151 + F10	48.1-51.0-52.2	32.2-34.5-48.6	27.3-29.0-32.4	29.8-44.9-52.3	32.5-58.0-75.1

Table 3.
Yields of RS as a result of 48-hour hydrolysis of biomass with the enzyme preparations obtained using fungi of the genus Trichoderma and Penicillium. The dosage of enzyme preparations is 2, 5, and 10 mg of the preparation protein per 1 g of dry matter of the cellulose-containing substrate, 50°C, pH 5.0. The substrate concentration was 100 mg/ml on a dry matter basis.

Accelerase 1500, and Accelerase DUET. In addition, Cellic Ctec-2 also had high xylanase activity. Such an advantage of Cellic CTec-2 in relation to Cellic CTec-1 and other studied commercial preparations, revealed in a comparison of their activities in terms of initial hydrolysis rates, was also observed during long-term biomass hydrolysis. Cellic CTec-2 has proven to be the most effective for hydrolyzing pretreated corn stalks and bagasse, ground pine wood, and MCC. The overall effectiveness of the Cellic CTec-1 preparation in long-term biomass hydrolysis was lower than the efficiencies of the Accelerase1000, Accelerase1500, and Accelerase DUET preparations, despite comparable values of the main corresponding activities (**Table 2**). Thus, among the studied commercial preparations, Cellic CTec-2 was the most effective for the hydrolysis of the considered biomass samples.

According to the data given in **Table 2**, the *P. verruculosum* B151 enzyme preparation had increased activities for FP, MCC, CMC, and xylan compared to the commercial Cellic CTec-2 preparation. At the same time, the BGL activity of the *P. verruculosum* B151 preparation was lower; therefore, the combined preparation *P. verruculosum* B151 + *P. verruculosum* F10 in a ratio of 9:1 for the protein was used in the hydrolysis experiments. The combination of enzyme preparations B151 + F10 exceeded the effectiveness of the preparation Cellic CTec-2 with prolonged hydrolysis of crushed aspen wood, both preparations hydrolyzed pine wood in the same way; with the hydrolysis of pretreated corn stalks and bagasse, the effectiveness was slightly lower than the result of Cellic CTec-2, but nevertheless higher than the effectiveness of the other investigated commercial preparations.

As can be seen from **Table 3**, the increase in the depth of hydrolysis of pretreated corn stalks (48 hours of hydrolysis) with an increase in the dosage of the most effective enzyme preparations (Cellic CTec-2 and B151 + F10) turns out to be very insignificant, which indicates the achievement of the maximum degree of enzymatic conversion of this substrate and its high reactivity (and high efficiency of the pretreatment processing of this type of biomass). Pretreated bagasse and crushed aspen wood were characterized by a greater increase in the yield of RS than crushed pine wood with an increase in the dosage of enzyme preparations, which is explained by the increased content of lignin in pine wood compared to other substrates under consideration. Also in pine, unlike bagasse, there are pitch and hardly hydrolyzable galactomannan. For the hydrolysis of bagasse, which contains readily available polysaccharides (arabinoxylan and xylan), the presence of xylanase, which is present in penicillin preparations, is important.

The maximum values of the RS increase also allow us to characterize and compare the effectiveness of the studied enzyme preparations conveniently analyzed by the results of saccharification of the MCC model substrate. It was shown that the degree of hydrolysis of MCC by all enzyme preparations except Accelerase XY turned out to be significantly higher than the maximum degree of hydrolysis of various types of biomass provided by them. At the same time, the maximum increase in the RS yield per unit mass of the consumed enzyme preparation corresponded to the Cellic CTec-2 and *P. verruculosum* B151 + F10 enzyme preparations, which demonstrated the highest efficiency in the hydrolysis of various types of biomass.

The effectiveness of the action of enzyme complex that performs the bioconversion of plant biomass is its most important characteristic, which determines the feasibility of its use in biotechnology and the resulting economic benefit. This characteristic, in turn, generalizes such important properties of the preparation as specific activities for a number of substrates and the limiting degrees of conversion of plant materials during long-term hydrolysis.

A comparative study of commercial enzyme preparations based on *Trichoderma* and an enzyme preparation based on *P. verruculosum* indicates that it is not inferior to commercial *Trichoderma* biocatalysts or surpasses them in hydrolytic activity in the processes of saccharification of various types of plant materials. This allows us to state that biocatalysts obtained on the basis of *P. verruculosum* producer strains can be a competitive alternative when scaling up biotechnological processes of bioconversion of renewable plant materials.

2.3 Methods for increasing the efficiency of bioconversion of plant materials under the action of an enzyme complex produced by *P. verruculosum*

An auxotrophic mutant B1-537 (ΔniaD) was obtained for genetic engineering manipulations based on the highly productive strain *P. verruculosum* B151. It has a defective nitrate reductase gene, which makes it possible to use it as a basis for obtaining recombinant producers of homologous and heterologous proteins by selecting transformants on the medium with nitrate [55–57, 64].

The expression of the gene of the target enzyme can be carried out under the control of various "promoter-terminator" systems selected by the researcher. This may be a proprietary system inherent in the target protein gene, or a promoter and terminator of another gene. To express various genes in the strain B1-537 (ΔniaD), a promoter and a transcription terminator of the inducible CBH I gene *P. verruculosum* (*cbh1*) [55–57, 65, 66], which is a major component of the cellulase complex of the fungus *P. verruculosum*, were used. The CBH I expression system makes it possible to obtain enzyme preparations with a high content of the target protein. For example, on the basis of strain B1-537 (ΔniaD) using the promoter of the c*bh1* gene, a recombinant strain F10 was created—a superproducer of highly active BGL *A. niger*. The enzyme preparations based on this strain contain up to 80% of the heterologous enzyme from the total secreted protein [56, 62, 63]. The strain F10 contained a large number of copies of the aspergillus BGL gene under the *cbh 1* promoter, whereas the expression of *P. verruculosum*'s own enzymes (and primarily CBH I) was largely suppressed. However, in many other cases, the use of the *cbh1* gene promoter made it possible to obtain recombinant *P. verruculosum* strains capable of secreting a largely preserved cellulase complex of this fungus together with homologously or heterologously expressed additional enzymes of various specificity to enhance the action of cellulases during hydrolysis of various types of cellulose-containing raw materials (or for use in other biotechnological processes), examples are given in **Table 4**.

One of the approaches to obtain recombinant strains is so-called "fusion construct", consisting, for example, of sequentially connected genes encoding CBH I, EG II *P. verruculosum*, and BGL *A. niger*, expressed under the promoter and terminator cbh1 [65]. The use of such a construct made it possible to obtain an enzyme preparation enriched with the three most important components for cellulose hydrolysis (**Table 4**). The use of this preparation together with the initial preparation P. *verruculosum* B151 led to an increase in the yield of sugars during the hydrolysis of pretreated pine and aspen wood by up to 70% [65].

Another example, as described in the previous chapter, is the composite preparation of *P. verruculosum*, which was obtained on the basis of strains B151 and F10 and which, during hydrolysis of various types of lignocellulose raw materials, provided a sugar yield comparable to that demonstrated by modern commercial enzyme preparations based on a new generation of mutant strains of *T. reesei* (Accelerase 1500

Cloned genes	Expressed enzymes	Content in the preparation, %*
xylA P. canescens	Xylanase A	18 (0) [55]
manB T. reesei	Mannanase B	45–70 (0) [55]
egl1 T. reesei	EG I	3 (0) [55]
eglIV T. reesei	LPMO (EG IV)	15 (0) [55]
cbhII T. reesei	CBH II	2 (0) [55]
xyl3 T. reesei	Xylanase III	17 (0) [55]
bgl1 A. niger	BGL I	21 (0) [56]
cbh1 P. verruculosum	CBH I	66 (20) [65]
cbh1 P. verruculosum + egII P. verruculosum	CBH I + EG II	56 + 19 (20 + 3) [65]
cbh1 P. verruculosum + egII P. verruculosum + bgl1 A. niger	CBH I + EG II + BGL I	37 + 34 + 12 (20 + 3 + 0) [65]
eglIV T. reesei	LPMO (EG IV)	24 (0) [65]
eglIV T. reesei + bgl1 A. niger	LPMO (EG IV) + BGL I	1 + 18 (0 + 0) [57]
cbh1 P. verruculosum + egII P. verruculosum + bgl1 A. niger + eglIV T. reesei	CBH I + EG II + BGL I + LPMO (EG IV)	24 + 35 + 10 + 1 (20 + 3 + 0 + 0) [57]

**The content of enzymes in % of the total protein content in the preparation obtained using the corresponding recombinant strain of P. verruculosum is given. In parentheses the content of the expressed enzymes in the control preparation P. verruculosum B151 is indicated.*

Table 4.
Using the promoter and terminator of the cbh1 gene to produce recombinant strains and enzyme preparations of Piptochaetium verruculosum with homologously or heterologously expressed enzymes.

and DUET, Cellic CTec1 and CTec2) [62]. In most cases, the composite preparation of *P. verruculosum* was superior to the indicated preparations of *T. reesei* in terms of effectiveness.

To obtain the strains with a moderate level of expression of target recombinant enzymes alternative expression systems can be used. This type includes, for example, a constitutive expression system based on the histone gene promoter hist4.1 [64]. Its use makes it possible to increase the production of the target protein without significantly changing the composition of the main secreted enzyme complex. Using an expression system based on a histone promoter, the B1_PrHist enzyme preparation was obtained with heterologous *A. niger* BGL, the content of which was about 13% of the total secreted protein. This preparation provided a higher yield of glucose (by 10–21%) during the hydrolysis of MCC and crushed aspen wood compared to the control preparation *P. verruculosum* B151. Together with the heterologous *A. niger* BGL under the control of a histone promoter, the expression of *P. verruculosum*'s own CBH I was increased, as a result, the enzyme preparation obtained on the basis of the recombinant strain demonstrated a 29–100% gain in sugar yield compared to the control preparation [57].

Thus, both of the genetic engineering approaches discussed above, in which the target protein is expressed either under the control of a strong inducible (*cbh1*) promoter or under the control of a weaker constitutive (*hist4.1*) promoter, can significantly increase the hydrolytic ability of secreted cellulase complexes. The choice of one or another approach largely depends on the specific application of the final

enzyme preparation in a particular biotechnological process. In the case of enzymatic hydrolysis of cellulose, it depends on the type of plant material and the method of its pretreatment.

Cellulases are an example of enzymes that are extremely demanded in practice and are used in Europe, the USA, and a number of other countries to implement the ideas of bioeconomics, implying the replacement of non-renewable fossil raw materials and chemical processes of its processing with alternative biotechnological processes with integrated use of renewable raw materials and various wastes. In particular, with the use of cellulases in a number of countries (USA, Italy, Brazil), the concept of biofactory (biorefinery) has been practically implemented, consisting in the realization of large-scale processes of enzymatic conversion of renewable lignocellulose plant biomass (agricultural waste) into sugars, followed by the production of alcohols (second-generation biofuels) from them [67]. Other biotechnological processes based on the use of lignocellulose raw materials have been tested and are close to practical implementation in order to obtain solvents, organic and amino acids, biologically active substances, monomers for the synthesis of various polymers, etc. In addition, cellulases are traditionally used in the textile industry, pulp and paper industry, and various branches of the food industry, such as brewing, alcohol, and bakery, as well as feed additives in poultry and animal husbandry [68].

3. Conclusions

Cellulolytic enzymes are found in various living systems, but the main destroyers of cellulose are microorganisms, primarily microscopic aerobic fungi, as well as various types of aerobic and anaerobic bacteria. Fungi from the genus *Trichoderma* are the most widely used industrial producers of cellulases in different countries, from which intensive research on cellulases began.

Using the methods of mutagenesis, selection, and genetic engineering, highly productive *T. reesei* strains capable of secreting up to 100 g/l of extracellular protein were obtained. Some species of fungi from the genus *Penicillium* (in particular *P. verruculosum*) have great prospects as an alternative to *T. reesei*, producing cellulase complexes whose enzymes have significantly higher specific activity compared to *T. reesei* cellulases. Highly productive mutant strains of *P.verruculosum* capable of secreting up to 60 g/l of extracellular protein were obtained. Based on the *P. verruculosum* strain, a host-vector system has been created that allows the expression of homologous and heterologous genes and the production of enzyme preparations, the qualitative and quantitative composition of which is appropriately optimized in terms of ensuring its maximum activity in relation to various types of cellulose-containing plant raw materials.

Abbreviations

EG	endoglucanase
CBH	cellobiohydrolase
BGL	β-glucosidase
LPMO	lytic polysaccharide monooxygenase
CBM	cellulose binding domain

MCC	microcrystalline cellulose
CMC	carboxymethyl cellulose
FP	filter paper
RS	reducing sugars
pNPG	*p*-nitrophenyl-β-D-glucoside
pNPX	*p*-nitrophenyl-β-D-xyloside

Author details

Olga G. Korotkova[1], Alexandra M. Rozhkova[1*], Ekaterina A. Rubtsova[1] and Arkady P. Sinitsyn[2]

1 Federal State Institution "Federal Research Centre "Fundamentals of Biotechnology" of the Russian Academy of Sciences", Moscow, Russian Federation

2 Lomonosov Moscow State University, Moscow, Russian Federation

*Address all correspondence to: amrojkova@yahoo.com

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