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ORIGINAL ARTICLE

Enhancement of the enzymatic cellulose saccharification by *Penicillium verruculosum* multienzyme cocktails containing homologously overexpressed lytic polysaccharide monooxygenase

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Abstract

The gene *lpmo1* encoding *Penicillium verruculosum* lytic polysaccharide monooxygenase (PvLPMO9A) was sequenced and homologously overexpressed in *P. verruculosum* B1-537 (*ΔniaD*) auxotrophic strain under the control of the *cbh1* gene promoter in combination with either the *cbh1* signal sequence (sCBH1-X series of samples) or the native *lpmo1* signal sequence (sLPMO1-X series). Three enzyme samples of the sCBH1-X series were characterized by a lower overall content of cellobiohydrolases (CBHs: 26–45%) but slightly higher content of endoglucanases (EGs: 17–23%) relative to the reference B1-537 preparation (60% of CBHs and 14% of EGs), while the PvLPMO9A content in them made up 9–21% of the total secreted protein. The PvLPMO9A content in four enzyme preparations of the sLPMO1-X series was much higher (30–57%), however the portion of CBHs in most of them (except for sLPMO1-8) decreased even to a greater extent (to 21–42%) than in the samples of the sCBH1-X series. Two enzyme preparations (sCBH1-8 and sLPMO1-8), in which the content of cellulases was substantially retained and the portion of PvLPMO9A was 9–30%, demonstrated the increased yields of reducing sugars in 48-h saccharification of Avicel and milled aspen wood: 19–31 and 11–26%, respectively, compared to the reference cellulase cocktail.

Keywords Lytic polysaccharide monooxygenase · Homologous expression · *Penicillium verruculosum* · Cellulase · Saccharification

Introduction

The biotechnological production of second-generation biofuels from renewable lignocellulosic biomass has been implemented on an industrial scale in the last few years in Italy, USA and Brazil [1, 2]. The most laborious stage of this technology is enzymatic saccharification of cellulose, which is catalyzed by a multienzyme system of hydrolytic cellulases, including endo-1,4-β-glucanases (EGs), exo-cellobiohydrolases (CBHs) and β-glucosidases (BGLs) [3]. Rather recently, metal-dependent chitin- and cellulose-oxidizing enzymes, called lytic polysaccharide monooxygenases (LPMOs), have been discovered [4–6]. LPMOs act as auxiliary activities (AA) to hydrolytic cellulases, enhancing the rate of cellulose destruction and boosting the yield of sugars in the enzymatic reaction [7–11]. For this reason, LPMOs have been introduced into modern commercial cellulase preparations produced by leading enzyme manufacturing companies [12].

Penicillium verruculosum is an efficient producer of highly-active extracellular cellulase multienzyme cocktails [13, 14]. LPMOs from *Thielavia terrestris* and *Trichoderma reesei* have previously been cloned and heterologously expressed in *P. verruculosum* in order to enhance the hydrolytic performance of its cellulases [9, 15]. At the same time, our earlier attempts to isolate a native LPMO from this fungus failed, although some experimental data

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indicated the presence of a minor LPMO activity in *P. verruculosum* multienzyme cocktails [9]. It should be noted that none of LPMOs from *Penicillium* species has been characterized up date, although the genes and translated amino acid sequences of one LPMO from *P. oxalicum* and four LPMOs from *P. rubens* are present in the CAZy database (http://www.cazy.org/AA9_all.html).

In this paper, a gene encoding the *P. verruculosum* LPMO I (PvLPMO9A) was sequenced and homologously overexpressed in this fungal host using different constructions of the expression plasmids, and the performance of the secreted crude multienzyme preparations in saccharification of cellulosic materials was investigated.

Materials and methods

Microorganisms and the growth conditions

Penicillium verruculosum B1-537 strain [16, 17] was used as a host strain for transformation and as a source for genomic DNA preparation. This host strain was deficient by the nitrate reductase gene (*ΔniaD*) for auxotrophic selection of recombinants. The *Escherichia coli* MachI strain (Thermo Fisher Scientific Inc., Waltman, MA, USA) was used for bacterial transformation and isolation of new plasmid constructs containing target genes.

The medium for screening of fungal clones contained 40 g/l cellulose, 15 g/l KH_2PO_4, 10 g/l of wheat bran (pH 5.0).

Cloning of the *lpmo1* gene

The nucleotide sequence corresponding to the full-length *lpmo1* gene was amplified by polymerase chain reaction (PCR). In order to amplify the *lpmo1* gene, two pairs of oligonucleotide primers (CBH1-LPMO1-LIC5/CBH1-LPMO1-LIC3 and LPMO1-LIC5/LPMO1-LIC3) were designed:

CBH1-LPMO1-LIC5	GGCAACAGCAGGAGCTCATGGTTTTGTGCAAAACATCATTATTGAC
CBH1-LPMO1-LIC3	AGAGGAGGGCGACACAGTTAAAAGACAGTAGTGGTGATGACGGTAGTC
LPMO1-LIC5	CAAACAGAAGCAACCGACACAATGCCTTCTACTAAAGTCGCTGCCCT
LPMO1-LIC3	GAGGAGAAGCCCGGTTAAAAGACAGTAGTGGTGATGACGGTAGTC

The PCR was carried out using a freshly prepared genomic DNA of the fungus *P. verruculosum* (Dneasy® Plant Mini Kit, QIAGEN, Valencia, CA, USA), the derived primers, Long polymerase mix (LPM, Thermo Fisher Scientific Inc., Waltham, MA, USA) and MyCycler equipment (Bio-Rad Laboratories, Hercules, CA, USA) according to the following protocol: 3 min at 95 °C, followed by 25 cycles of 1.5 min at 95 °C, 1 min at 50 °C, 2 min at 68 °C, 10 min at 4 °C. The resulting PCR product was purified from agarose gel using QiAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA). The PCR products were sequenced, and identified as the *lpmo1* gene of *P. verruculosum*.

Construction of expression plasmids and generation of mutants

A full *lpmo1* gene (1040 bp) encoding the PvLPMO9A (LPMO I) was cloned into a modified pUC19 vector, pCBHI, containing the promoter and terminator regions of the *cbh1* gene of *P. verruculosum*, by Ligation Independent Cloning method (LIC-method) as previously described [17, 18]. As an alternative, the polynucleotide sequence of the *lpmo1* gene without a signal sequence (977 bp) was cloned into the vector pCBHI-SS, that is, pCBHI [17] with an additive sequence encoding the CBH I signal sequence (SS) fused to the 3′-end of the *cbh1* promoter region (see Fig. 1).

The PCR-products (1040 bp or 977 bp) and linearized pCBHI or pCBHI-SS vectors were treated with T4 DNA polymerase (Thermo Fisher Scientific Inc., Waltman, MA, USA) in the presence dATP and dTTP (Thermo Fisher Scientific Inc., Waltman, MA, USA), respectively. The treated inserts were ligated into the treated vectors by mixing 50 ng of vector with 150 ng of insert. The mixture was incubated for 30 min at 22 °C and then transformed into *E. coli* MachI competent cells using a standard transformation protocol [19]. Thus, plasmids pLPMOI and pCBHI-LPMOI, containing the complete *lpmo1* gene and *lpmo1* gene fused to the *cbh1* signal sequence were obtained. The absence of mutations, additional insertions or deletions in the cassettes

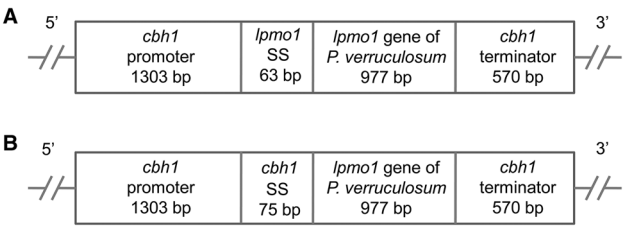

Fig. 1 Construction of pCBHI and pCBHI-SS vectors for cloning the *lpmo1* gene into *P. verruculosum* B1-537 recipient strain under the control of the *cbh1* gene promoter in combination with either the native *lpmo1* signal sequence (**a**) or the *cbh1* signal sequence (**b**)

containing the *lpmo1* gene was confirmed by sequencing in both directions by the method described by Sanger et al. [20].

Obtaining enzyme preparations

The *P. verruculosum* B1-537 (*ΔniaD*) host strain was co-transformed with the obtained pLPMOI or pCBHI-LPMOI plasmid together with pSTA10 plasmid taken at a standard ratio (10:1, μg), using the transformation protocol described elsewhere [21]. The pSTA10 plasmid contained a nitrate reductase gene, allowing carrying out the selection of transformants in the medium with 10 mM $NaNO_3$.

Screening of transformants was carried out in shake flasks (total volume 500 ml, fermentation volume 100 ml). The *P. verruculosum* B1-537 (*ΔniaD*) host strain was used as a control. As a result of primary screening, several recombinant *P. verruculosum* strains of the sCBH1-X and sLPMO1-X series secreting the PvLPMO9A, visible as a remarkable band on the SDS-PAGE gel, were selected. The enzyme identification in the protein bands was carried out by MALDI-TOF mass spectrometry (MS) peptide fingerprinting using the online FindPept tool (https://web.expasy.org/findpept/) as described elsewhere [13, 22].

Supernatants of the culture filtrates were freeze-dried on a VirTis BenchTop 2K ES freeze dryer (SP Scientific,Warminster, PA, USA).

Enzyme activity assays

Enzyme activities against carboxymethylcellulose (CMC) and birchwood xylan (Sigma, St. Louis, MO, USA) were determined by analyzing reducing sugars (RS) released from 5 g/l substrates after 5 min (CMC) or 10 min (xylan) of the enzymatic reaction at pH 5.0 and 50 °C as described elsewhere [23]. Avicelase activity was assayed by analyzing RS released after 60 min of the enzymatic reaction with 5 g/l Avicel (microcrystalline cellulose from Vitek Company, Russia) at pH 5.0 and 40 °C as described elsewhere [24]. Reducing sugars were analyzed by the Nelson-Somogyi method [25]. The standard calibration plot was made using different concentrations of glucose (Helicon, Russia) in the range of 0.01–0.2 g/l. The enzyme quantity that catalyzed the formation of 1 μmol RS (in glucose equivalents) per 1 min was defined as the enzyme activity unit. Protein concentration was determined by the modified Lowry method [26].

Analysis of the enzyme component composition

Analysis of the component composition of multienzyme cocktails produced by recombinant *P. verruculosum* strains overexpressing PvLPMO9A was carried out essentially as described elsewhere [16, 27]. In brief, HPLC fractionation of enzymes was carried out on a FPLC Pharmacia system using Source 15Q and MonoQ HR 5/5 columns (Pharmacia, Uppsala, Sweden). Each protein fraction was analyzed for different enzyme activities and protein content using the Lowry assay [26], as described in the preceding section, and it was also characterized by SDS-PAGE in a 12% gel using a Mini Protein Cell system (Bio-Rad Laboratories, Hercules, CA, USA).The enzyme components in fractions were identified based on the obtained activities and MALDI-TOF MS peptide fingerprinting of the corresponding protein bands from a gel [13, 22]. Identified proteins having purity higher than 90% in the respective fractions, according to the SDS-PAGE data, were considered as individual enzymes, and the content of the corresponding enzymes in the initial crude multienzyme preparation was calculated as the ratio of the protein content in those fractions to the total protein content in the initial preparation. Some protein fractions contained more than one enzyme (purity less than 90%) identified by its substrate specificity and MALDI-TOF MS. In such case, we first estimated the enzyme share in a fraction based on the intensity of its band on the electrophoretic gel, and then, knowing the protein content in this fraction and the enzyme share, its content in the initial preparation was calculated.

Cellulose saccharification

Avicel (Vitek Company, Russia) and aspen wood, pretreated by milling on an impeller mill Mikrosilema IM-450 (Monolitstroy, Russia) as described elsewhere [28], were treated in 2-ml test tubes at pH 5.0 and 40 °C by composite multienzyme cocktails containing 85% of the enzyme preparation under study and 15% of F10 preparation as a source of BGL as described elsewhere [9]. The F10 preparation contained the *Aspergillus niger* BGL (~80% of the total protein) heterologously expressed in *P. verruculosum* B1-537 host strain, while the rest of proteins (~20%) represented cellulases of *P. verruculosum* [16, 29]. In all cases, the substrate concentration was 100 g/l, and the total protein dosage in the reaction mixture was 5 mg/g substrate. In Avicel saccharification experiments, 5 mM gallic acid was added to the reaction system as an electron donor to LPMO. After 6, 24 and 48 h of the enzymatic reaction, 0.1-ml aliquots of reaction mixture were taken, centrifuged, and then the RS concentration was determined in the supernatant by the Nelson-Somogyi method [25]. The experiments were carried out in triplicate.

Results and discussion

PvLPMO9A expression

The *lpmo1* gene encoding LPMO I was sequenced and homologously overexpressed in *P. verruculosum* B1-537 auxotrophic strain using two constructions of the expression plasmids, one of which used the *cbh1* gene promoter and the full-sized *lpmo1* gene and the other used a native *cbh1* signal sequence instead of the *lpmo1* signal sequence. The native *cbh1* signal sequence was fused directly upstream the *lpmo1* gene encoding a mature LPMO I (PvLPMO9A) protein (Fig. 1).

The *lpmo1* gene consists of 1040 bp and contains one intron. The nucleotide sequence of the gene was deposited in the GenBank with an accession number MK158950. A translated amino acid sequence contains 328 residues. The first 21 residues (MPSTKVAALSAVLALASTVAG) represent a signal peptide; the N-terminal sequence of the mature protein starts from a histidine residue (HGYVQN...), like in other LPMOs [6, 10]. The alternative signal peptide sequence from CBH I, used for the expression of PvLPMO9A (Fig. 1), was MSALNSFNMYKSALILGSLLATAGA [30].

Three clones of the sCBH1-X series (sCBH1-8, sCBH1-14, sCBH1-17) and four clones of the sLPMO1-X series (sLPMO1-1, sLPMO1-4, sLPMO1-8, sLPMO1-9) of fungal transformants were selected for further analysis and testing (sCBH1- and sLPMO1—denote the respective signal sequences used in the expression plasmids). SDS-PAGE data for the respective multienzyme preparations are shown in Fig. 2. Bands on the SDS-PAGE image marked by an arrow correspond to the secreted PvLPMO9A, as MALDI-TOF MS peptide fingerprinting showed. The MS analysis also confirmed the N-terminal peptide sequence of the mature PvLMPO9A for both constructions of the expression plasmids. Peaks in the mass spectra of the enzyme tryptic digests with m/z of 6393 and 6464 matched the specific tryptic peptide 1 HGY...CHK 58 of the mature protein; the second mass corresponded to the peptide modified by acrylamide at Cys56 residue, a typical CYS_PAM modification in such kind of MALDI-TOF MS analysis. Moreover, the observed peaks with m/z 6407 and 6478, differed by 14 Da from the above mentioned peaks, matched the same N-terminal peptide (without and with CYS_PAM modification), in which the N-terminal histidine residue is methylated. So, both recombinant forms of PvLPMO9A expressed with different signal peptides were methylated incompletely. The methylation of the N-terminal histidine that coordinates the copper ion in the enzyme active site is rather typical for cellulose-active LPMOs, however the role of this modification is unclear [6, 10]. It should be noted that we have previously observed similar partial His1 methylation in the case of recombinant LPMO from *T. terrestris*, also expressed in *P. verruculosum* [9], while the native LPMO from *Myceliophthora thermophila* studied in the same work was found to exist only in the methylated form, since the N-terminal peptide with unmodified His1 residue was not observed in the mass spectrum of the enzyme tryptic digest. Thus, incomplete methylation of N-terminal histidines in recombinant LPMOs, homologously or heterologously expressed by *P. verruculosum*, seems to be a typical feature of this fungal host.

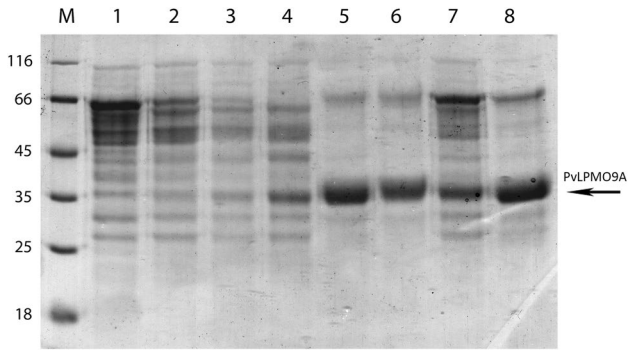

Fig. 2 SDS-PAGE of crude enzyme preparations based on recombinant strains of *P. verruculosum* overexpressing the *lpmo1* gene. *M* molecular markers (shown in kDa); 1 B1-537 (control); 2 sCBH1-8; 3 sCBH1-14; 4 sCBH1-17; 5 sLPMO1-1, 6 sLPMO1-4; 7 sLPMO1-8; 8 sLPMO1-9

The specific activities of the enzyme preparations under study against cellulosic substrates (Avicel, CMC) and xylan are shown in Table 1. Using a previously developed method for assaying the component composition of cellulase multienzyme cocktails [16, 27], the content of major enzymes was estimated in each sample (Table 2). It should be noted that CBH I, CBH II and EG I of *P. verruculosum* are usually found in a culture liquid in two forms, a high-molecular-weight and low-molecular-weight form, the second one (without a cellulose-binding module) being formed as a result of limited proteolysis of the enzyme linker [13]. For simplicity, the total content of both forms for each of the mentioned enzymes is given in Table 2.

The enzyme preparations based on sCBH1-X series of transformants were generally characterized by lower overall content of CBHs (26–45%) but slightly higher content of EGs (17–23%) relative to the control B1-537 sample, in which CBHs and EGs represented 60 and 14% of the total secreted protein, respectively (Table 2). For sLPMO1-X series, the content of CBHs decreased even to a greater extent (to 21–42%), while the content of EGs (7–14%) was closer to that in the control preparation. The content of BGL and Xyl I for both series of transformants also varied compared to the reference B1-537 sample. The specific activities of the preparations against Avicel, CMC and xylan (Table 1)

Table 1 Specific activities (U/g protein) of enzyme preparations produced by recombinant *P. verruculosum* strains overexpressing PvLPMO9A

Sample	Avicel	CMC	Xylan	Total content of CBHs (%)[a]	Total content of EGs (%)[a]
sCBH1-8	239 ± 12	12100 ± 600	18200 ± 900	45	23
sCBH1-14	160 ± 9	10800 ± 500	18400 ± 900	34	17
sCBH1-17	94 ± 5	10300 ± 500	15700 ± 800	26	20
sLPMO1-1	82 ± 4	2100 ± 100	5200 ± 300	21	9
sLPMO1-4	89 ± 5	2600 ± 100	2500 ± 100	24	9
sLPMO1-8	267 ± 14	7800 ± 400	14400 ± 700	42	14
sLPMO1-9	109 ± 6	2000 ± 100	6400 ± 300	25	7
B1-537 control	380 ± 19	11900 ± 600	20100 ± 900	60	14

[a]Data from Table 2

Table 2 Component composition (%) of multienzyme preparations produced by recombinant *P. verruculosum* strains overexpressing PvLPMO9A

Sample	CBH I	CBH II	EG I	EG IIa	EG IIb	EG III	BGL	Xyl I	LPMO I	Total cellulases[a]
sCBH1-8	20	25	5	9	6	3	3	3	9	71
sCBH1-14	12	22	4	9	2	2	3	3	15	54
sCBH1-17	13	13	7	6	5	2	2	2	21	48
sLPMO1-1	17	4	2	3	2	2	1	1	50	31
sLPMO1-4	19	5	3	2	3	1	1	< 1	48	34
sLPMO1-8	26	16	2	5	4	3	2	2	30	58
sLPMO1-9	19	6	2	3	1	1	1	1	57	33
B1-537 control	33	27	5	4	3	2	3	3	< 1	77

Enzyme designations: CBH I, two forms of Cel7A; CBH II, two forms of Cel6A; EG I, two forms of Cel7B; EG IIa, Cel5A; EG IIb, Cel5B; EG III, Cel12A; BGL, Cel3A; Xyl I, Xyn11A; LPMO I, PvLPMO9A studied in this work. High-molecular-weight and low-molecular-weight forms of CBH I, CBH II and EG I as well as other cellulases are described elsewhere [13]

[a]Total content of CBHs, EGs and BGL

fairly well correlated with a total content of CBHs, EGs and Xyl I (Table 2) in them; the coefficient of determination R^2, calculated with the assumption of a linear dependence between the parameters, was found to be 0.975, 0.779 and 0.955, respectively. The lower value of R^2 for the pair "CMCase activity—total content of EGs" was the result of different specific activities of the four major endoglucanases [13] and their different shares in the *P. verruculosum* multienzyme cocktails (Table 2).

As regarding to the PvLPMO9A content in the samples, it was much higher in the sLPMO1-X series of the enzyme preparations (30–57%) compared to the sCBH1-X series (9–21%); this is also clearly visible on the electrophoregrams (Fig. 2). Thus, using a native signal sequence of the *lpmo1* gene for the expression of PvLPMO9A has proven to be more effective than using the *cbh1* signal sequence. It should be noted that *P. canescens* xylanase A, *P. verruculosum* EG IIa and *P. funiculosum* dextranase have previously been expressed in *P. verruculosum* B1-537 strain under the control of a strong *cbh1* gene promoter using the native signal sequences of the enzymes mentioned; the secretion of the target proteins reached 31–36% of the total protein in culture broths [17, 31, 32]. However, Gouka et al. [33] reported that using the *glaA* signal sequence for the expression of foreign proteins in *Aspergillus* species under the control of the *Aspergillus glaA* gene promoter provides better results than using the native signal sequences of those proteins because of their improved translocation and folding. Therefore, in this work we used both possible variants of PvLPMO9A expression, that is, with and without a native signal sequence of the *lpmo1* gene, in the latter case replacing it by a signal sequence of the *cbh1* gene. Regarding the influence of the signal peptide structure on the expression of other LPMOs, systematic studies on this subject are rather scarce. The codon-optimized native signal sequence of *T. reesei* LPMO9A (Cel61A) was found to provide better secretion of this enzyme by *Pichia pastoris* than the more commonly used signal sequence of the alpha-mating factor (α-MF) from *Saccharomyces cerevisiae* [34]. In another work, twelve signal peptides with reported high secretion efficiency were screened to assess the extracellular production of the chitin-active LPMO10A (CBP21) from *Serratia marcescens* in *E. coli* [35]. Only four signal peptides, including those from PelB, CBHI, SacB and XCs, increased the

protein production level of CBP21, the PelB being the most efficient, while two another signal peptides improved the release of the recombinant chitinase Chi92 from *Aeromonas veronii* [35]. The reported data confirm a paradigm that a suitable signal peptide for one protein may not be efficient for another protein [36].

Enzymatic saccharification of cellulosic substrates

Avicel and milled aspen wood were hydrolyzed by the enzyme preparations under study with the addition of F10 preparation enriched by a BGL activity [16, 29]. The ratio between cellulase samples and F10 preparation was 85:15 by protein, as that optimized in our previous studies [16, 37], while maintaining the total protein dosage in the reaction system at the level of 5 mg/g substrate.

The results of Avicel hydrolysis are shown in Fig. 3. In the presence of 5 mM gallic acid as an electron donor to PvLPMO9A, the enzyme preparations sCBH1-8 and sLPMO1-8 demonstrated superior performance over the control B1-537 sample at each time point, the RS concentration after 48 h being higher by 19 and 31%, respectively, relative to the control. The sCBH1-14 sample exhibited the performance close to that of the reference sample only in the first period of the reaction (6 and 24 h), while the final RS yield was lower than in the control. The rest of samples containing homologously overexpressed PvLPMO9A could not compete with the reference B1-537 multienzyme cocktail by the saccharification efficiency. To identify the role of LPMO activity in improving the efficiency of cellulose saccharification, the hydrolysis of Avicel was also carried out by the sLPMO1-8 preparation in the absence of gallic acid. The removal of the electron donor led to a decrease in the final RS concentration by 19% (see bars above the sLPMO1-8* legend in Fig. 3). These results show that the PvLPMO9A was indeed active and the increase in the sugar yields for the best preparations containing this enzyme was substantially due to its presence in the cocktails.

In hydrolysis of milled aspen wood (Fig. 4), the picture was rather similar to that observed with Avicel as a substrate, except the superiority of sCBH1-8 and sLPMO1-8 samples over the reference preparation was less pronounced: by 11 and 26% considering the final RS concentration.

Data shown in Figs. 3 and 4, together with those presented in Table 2, demonstrate that sCBH1-8 and sLPMO1-8 multienzyme preparations based on recombinant *P. verruculosum* strains, in which the secretion of major cellulases was substantially retained (at the level of 58–71% of the total protein) relative to the reference strain (77%) and which additionally secrete PvLPMO9A at a moderate level

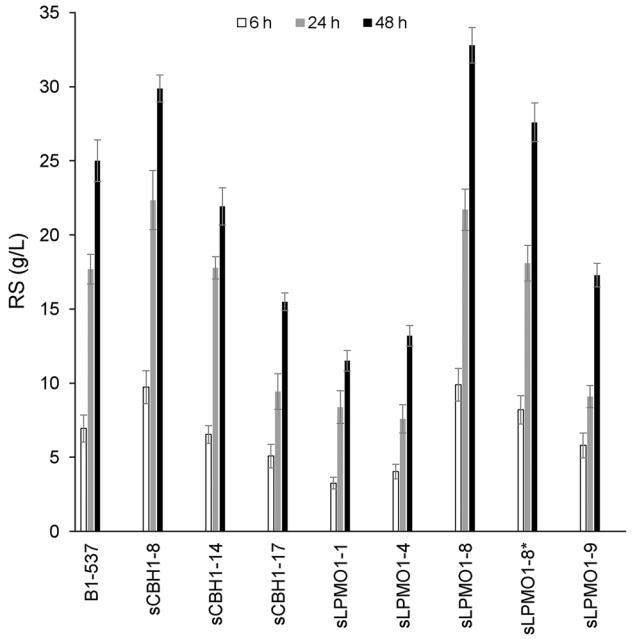

Fig. 3 Yields of reducing sugars (RS) in hydrolysis of Avicel (100 g/l) by composite multienzyme cocktails containing 85% of the enzyme preparations produced by recombinant *P. verruculosum* strains overexpressing PvLPMO9A and 15% of F10 preparation as a source of BGL, pH 5.0, 40 °C, 5 mM gallic acid, enzyme loading 5 mg protein/g substrate. B1-537, control (reference sample consisting of 85% of B1-537 preparation and 15% of F10 preparation); sLPMO1-8*, data for sLPMO1-8 sample in the absence of gallic acid

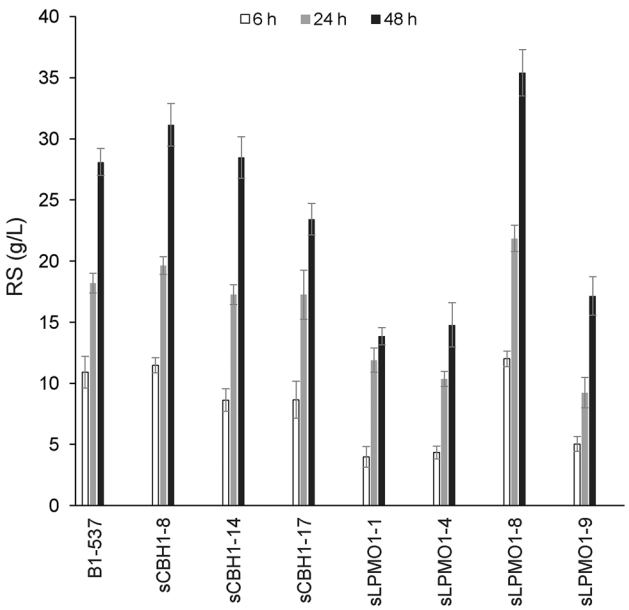

Fig. 4 Yields of reducing sugars (RS) in hydrolysis of milled aspen wood (100 g/l) by composite multienzyme cocktails containing 85% of the enzyme preparations produced by recombinant *P. verruculosum* strains overexpressing PvLPMO9A and 15% of F10 preparation as a source of BGL, pH 5.0, 40 °C, enzyme loading 5 mg protein/g substrate. B1-537, control (reference sample consisting of 85% of B1-537 preparation and 15% of F10 preparation)

(9–30%), provide the highest yields of RS in hydrolysis of both cellulosic and lignocellulosic substrate. The positive effects observed for sCBH1-8 and sLPMO1-8 samples in comparison with the reference B1-537 preparation were largely the result of the synergistic cooperation between cellulolytic enzymes and PvLPMO9A, the latter acting as an auxiliary activity to hydrolytic cellulases, as already reported in other publications [8, 9, 11, 38, 39]. At the same time, the enzyme preparations, in which the total content of cellulases was < 50%, exhibited a poor performance in saccharification of both cellulosic substrates despite the high content of PvLPMO9A in them, that is, the extra high PvLPMO9A secretion by sLPMO1-1, sLPMO1-4 and sLPMO1-9 strains caused a detrimental effect on the secretion of cellulases, resulting in a poor hydrolytic performance of the respective multienzyme cocktails. Nevertheless, the latter mentioned samples with PvLPMO9A content of ~ 50% or higher may be used as blends to the traditional cellulase preparations not having their own LMPO. Their using as the blends has at least one advantage: it allows the optimization of the ratio between cellulase and LPMO activities. Hu et al. [11] showed that the optimal ratio between cellulase enzymes and LPMO depends on the cellulosic feedstock and the loading of solids in the reaction system. In particular, for steam pretreated poplar and corn stover the optimal LPMO content in the multienzyme cocktail decreased from 20 to 3% and from 6.7 to 2%, respectively, with increasing the solids' loading from 2 to 20% [11]. Thus, a variety of obtained in this work cellulase preparations, containing the PvLPMO9A, opens up broad prospects for further optimization of multienzyme cocktails for saccharification of lignocellulosic materials.

Conclusions

In the present work, a variety of recombinant *P. verruculosum* strains secreting cellulase multienzyme cocktails containing the homologously overexpressed PvLPMO9A were obtained using two different constructions of the expression plasmids, based on the *cbh1* gene promoter in combination with either the *cbh1* signal sequence (sCBH1-X series of samples) or the native *lpmo1* signal sequence (sLPMO1-X series). Three enzyme samples of the sCBH1-X series were characterized by lower overall content of CBHs but slightly higher content of EGs relative to the reference *P. verruculosum* B1-537 preparation, while the PvLPMO9A content in them made up 9–21% of the total secreted protein. The PvLPMO9A content in four enzyme preparations of the sLPMO1-X series was much higher (30–57%), however the portion of CBHs in most of them (except for sLPMO1-8) decreased even to a greater extent than in the samples of the sCBH1-X series. At least two new promising recombinant strains of *P. verruculosum* overexpressing the native PvLPMO9A were developed. The secreted multienzyme cocktails based of these strains (sCBH1-8 and sLPMO1-8), in which the content of major cellulases was substantially retained and the portion of PvLPMO9A was 9–30%, demonstrated the increased yields of reducing sugars in saccharification of Avicel and milled aspen wood: by 19–31 and 11–26%, respectively, compared to the reference cellulase cocktail.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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