Biochemistry (Moscow), Vol. 68, No. 12, 2003, pp. 1313-1319. Translated from Biokhimiya, Vol. 68, No. 12, 2003, pp. 1631-1638.

Recombinant Endo-β-1,4-xylanase from *Penicillium canescens*

O. A. Sinitsyna[1]*, A. V. Gusakov[1], O. N. Okunev[2], V. A. Serebryany[3], E. A. Vavilova[3], Yu. P. Vinetsky[3], and A. P. Sinitsyn[1]

[1]Faculty of Chemistry, Lomonosov Moscow State University, Moscow 199899, Russia; fax: (7-095) 939-0997; E-mail: oasinitsyna@enzyme.chem.msu.ru
[2]Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino 142292, Moscow Region, Russia; fax: (095) 923-3602
[3]Institute of Genetics, Moscow 113545, Russia

Received March 24, 2003
Revision received April 21, 2003

Abstract—Recombinant endo-β-1,4-xylanase (Xyl-31_{rec}, 31 kD, p*I* 8.2-9.3, the tenth family of glycosyl hydrolases) was isolated from the culture liquid of *Penicillium canescens* (strain with the amplified homologous xylanase gene) by chromatofocusing on Mono P and hydrophobic chromatography on phenyl-Superose. It is shown that the biochemical and kinetic parameters, substrate specificity, stability, and other properties of the recombinant and native enzymes are almost the same. It was found that Xyl-31_{rec} can be used for biobleaching of cellulose, the recombinant *P. canescens* strains providing a high yield of extracellular Xyl-31_{rec} (up to 800-900 U/ml of culture liquid) and not secreting cellulases.

Key words: endo-β-1,4-xylanase, chromatofocusing, hydrophobic chromatography, recombinant strain, biobleaching of cellulose

The mycelial fungus *P. canescens* is an efficient producer of certain enzymes participating in cleavage of natural polysaccharides. Endo-β-1,4-xylanase with molecular mass 31 kD (Xyl-31) is one of these enzymes [1, 2]. Growing on medium with sugar beet pulp, the natural strain of *P. canescens* secrets xylanase with activity up to 100 U/ml into the culture liquid. Recently a gene encoding Xyl-31 has been cloned [2]. Analysis of the amino acid sequence based on the nucleotide sequence showed that Xyl-31 belongs to the tenth family of glycosyl hydrolases, and the protein molecule does not have an adsorption (substrate-binding) domain. Using plasmid transformation, highly active producers of Xyl-31 were obtained; these multi-copy strains (e.g., PCA-Xyl-26 strain) secreted xylanase activity up to 800-900 U/ml [2], which is of principal interest for industrial production of Xyl-31. It should be noted that *P. canescens* does not secret cellulases hydrolyzing insoluble cellulose [2]; along with the high xylanase activity, this makes enzyme preparations produced by the recombinant *P. canescens* strains promising for bleaching of cellulose pulp during primary cellulose production.

The goal of this work was to isolate recombinant xylanase (Xyl-31_{rec}) secreted by multi-copy *P. canescens* strain PCA-Xyl-26 and to study its properties compared with those of the native enzyme (Xyl-31_{nat}) isolated and described by us earlier [3].

MATERIALS AND METHODS

Enzyme preparations. Filtrates of culture liquids of *P. canescens* wild strain PCA 10 and multi-copy recombinant strain PCA-Xyl-26 with the amplified homologous Xyl-31 gene were used in this study; the strains were grown on medium with sugar beet pulp and peptone [2]. Preparations were obtained in the Institute of Biochemistry and Physiology of Microorganisms (Pushchino, Moscow Region). Xylanase activity of the culture liquid produced by the PCA 10 strain was 92 U/ml and that of the PCA-Xyl-26 strain was 820 U/ml. Their activity towards the filter paper (AFP) was 0.5 and 0.6 U/ml, respectively. Xylanase activity was determined using xylan of birch wood as the substrate. Hydrolysis was monitored by the Shomogyi–Nelson reducing sugars assay. The method for activity estimation was described earlier [3].

A liquid enzyme preparation Ecopulp X200 from Primalco (Finland) exhibited xylanase activity of 2500 U/ml.

Isolation and purification of recombinant xylanase [3-5]. The enzyme preparation was salted out with ammonium sulfate (80% saturation at 0°C). The precipitate formed dur-

* To whom correspondence should be addressed.

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ing 12 h (4°C) was centrifuged at 11,000 rpm for 30 min at 4°C. Desalting and replacement of buffers were performed by low-pressure GPC on columns with P4 and P6 Biogels from Bio-Rad (USA) using an Econo System chromatograph from Bio-Rad with the flow rate 1 ml/min.

To isolate xylanase, an FPLC liquid chromatograph from Pharmacia (Sweden) was used. During isolation the protein concentration was determined spectrophotometrically at 280 nm. Chromatofocusing (ion-exchange chromatography at low ionic strength with pH gradient elution) was performed on a Mono P column (5 × 200 mm, gel volume 4 ml) from Pharmacia equilibrated with the initial buffer (0.075 M Tris/AcOH, pH 9.3). For the first step of chromatofocusing, a pH gradient was pre-assigned by sequential passage of Polybuffers through the column: PB 96 with pH 6.0 and PB 74 with pH 3.0, the flow rate being 0.5-0.6 ml/min. For the second step, the pH gradient was pre-assigned by passage of PB 96 with pH 7.7, the flow rate being 0.6 ml/min.

Hydrophobic chromatography was performed on a column with phenyl-Superose (5 × 50 mm, gel volume 1 ml) from Pharmacia with an ionic strength gradient elution of the bound protein. The gradient was pre-assigned by a linear change of ammonium sulfate concentration from 100% buffer A (1.7 M $(NH_4)_2SO_4$/0.05 M NaH_2PO_4, pH 7.0) to 100% buffer B (0.05 M NaH_2PO_4, pH 7.0), the flow rate being 0.5 ml/min.

The protein concentration was determined according to Lowry [6] using BSA as the standard and spectrophotometrically at 280 nm.

Biochemical characteristics of xylanase, its activity against various carbohydrate-containing substrates, chromatography of the products of hydrolysis of glucurono- and arabinoxylan, adsorbability, stability, and pH and temperature dependence of its activity were studied as described in [3].

Bio-bleaching ability of the enzyme. The method is based on determination of the aromatic lignin fragments released into solution by the action of enzymes [7]. Wet eucalyptus cellulose with 18% dry substance content was used as the substrate. Cellulose pulp (0.22 g) was placed in a 20-ml vessel, 2 ml of enzyme solution in the corresponding buffer was added, and a vessel was placed in a shaker (200 rpm) thermostatted at 50°C. Bio-bleaching was performed for 2 h, then the reaction mixture was centrifuged at 12,000 rpm, and in the supernatant optical adsorption was measured at 237 nm. In the control, buffer solution was used instead of enzyme solution. To evaluate bio-bleaching efficiency, the value of $\Delta A_{237} = A_{237\ \text{sample with enzyme}} - A_{237\ \text{control}}$ was used: the higher ΔA_{237} the more efficient is bio-bleaching. Enzyme dilution was fitted so that ΔA_{237} be not more than 1.5 units at the supernatant dilution 5-6 times.

RESULTS AND DISCUSSION

Isolation of homogeneous Xyl-31_{rec}. SDS-PAGE of the culture liquid proteins of *P. canescens* PCA-Xyl-26 strain gave an intense protein band in the area 30 ± 2 kD (in Fig. 1 this area is framed); this indicates that the content of xylanase in the preparation is significant.

Xylanase was purified according to the following procedure. PCA-Xyl-26 preparation pre-purified from non-protein admixtures (insoluble substances, carbohydrates, pigments, etc.) was chromatofocused on Mono P (Fig. 2a). Xyl-31_{rec} was eluted in the beginning of the pH gradient (pH 9.0-7.8) in the first major fraction (a series of hatched peaks in Fig. 2a). Along with Xyl-31_{rec}, the fraction contained some minor proteins. Subsequent purification of Xyl-31_{rec} consisted of two steps: chromatofocusing of the "rough" xylanase fraction on Mono P in the range of pH 9.3-7.5 (Fig. 2b) and hydrophobic chromatography on phenyl-Superose (Fig. 2c). The fraction eluted from phenyl-Superose in the salt gradient 23-18% contained Xyl-31_{rec} homogeneous according to SDS-PAGE (Fig. 3a).

Biochemical parameters of Xyl-31_{rec}. Molecular mass (31 kD), alkaline isoelectric point, and the presence of several isoforms (p*I* 8.2-9.3, Fig. 3b) of Xyl-31_{rec} coincided with analogous parameters of the native enzyme (isolation and properties of Xyl-31_{nat} are described in [3]).

Substrate specificity. The properties of Xyl-31_{rec} are typical of endo-β-1,4-xylanases (Table 1): it hydrolyzed sol-

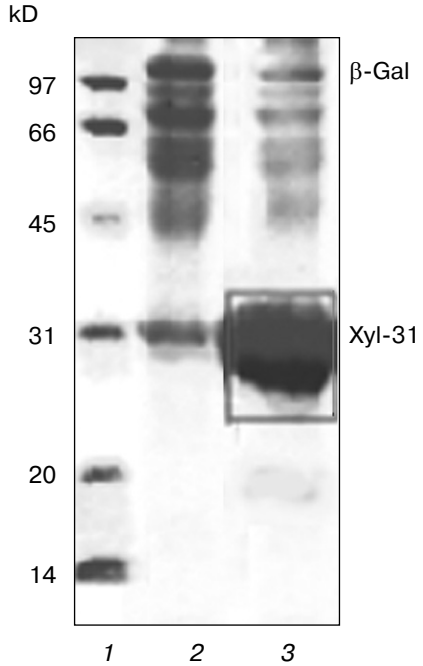

Fig. 1. SDS-PAGE of enzyme complexes secreted by *P. canescens* PCA 10 wild strain (*2*) and *P. canescens* PCA-Xyl-26 strain with the amplified Xyl-31 gene (*3*); *1*) standard markers for SDS-PAGE.

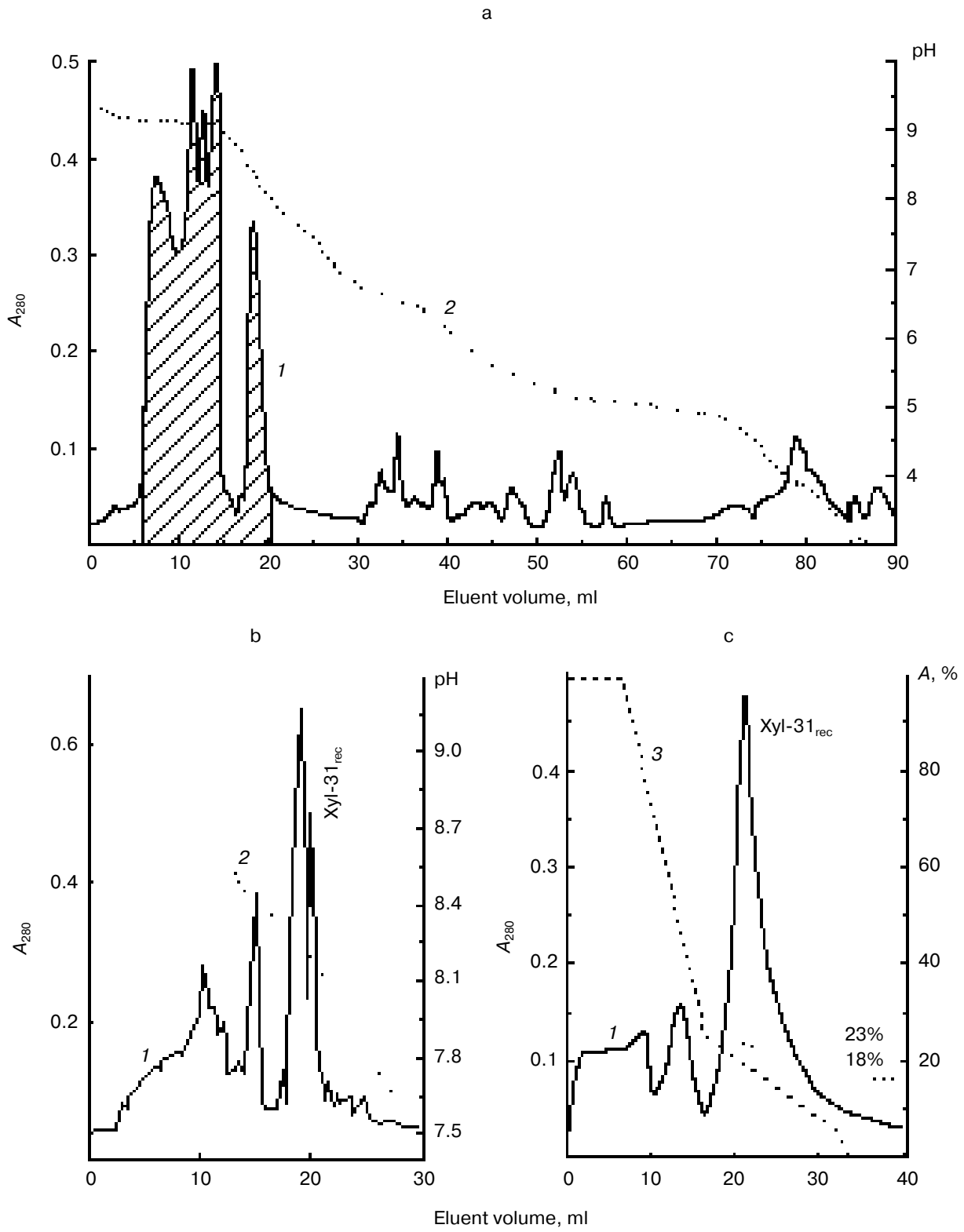

Fig. 2. Isolation of Xyl-31$_{rec}$ from *P. canescens* PCA-Xyl-26 enzyme complex: a) chromatofocusing of the initial enzyme complex on Mono P; b) chromatofocusing of the xylanase-rich fraction on Mono P; c) hydrophobic chromatography on phenyl-Superose. *1*) Protein, A_{280}; *2*) pH at the exit from the column; *3*) ammonium sulfate gradient.

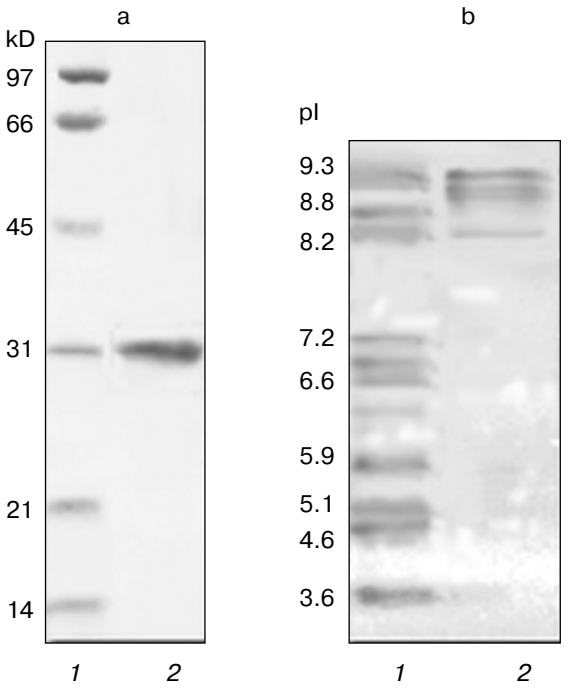

Fig. 3. Biochemical parameters of Xyl-31_{rec}: a) SDS-PAGE; b) IEF. *1*) Standard markers for SDS-PAGE and IEF; *2*) Xyl-31_{rec}.

uble polymeric xylans (glucurono- and arabinoxylan), but its activity against *p*-nitrophenyl-saccharide derivatives (excluding *p*-NP-cellobioside) was negligible; this is typical of xylanases belonging to the tenth family of glycosyl hydrolases [8]. Xyl-31_{rec} hydrolyzed some soluble cellulose derivatives (CMC, RBB-CMC, and ROD-CMC) and β-1,4-glucans and β-glucan from oat and lichenan. It should be noted that Xyl-31_{rec} did not hydrolyze insoluble cellulose (FP, MCC). The native enzyme exhibited similar properties: the values of the "relative enzyme activities" (ratio of specific Xyl-31_{rec} activity and specific Xyl-31_{nat} activity) are close to unity for all tested substrates (Table 1).

Hydrolysis of glucurono- and arabinoxylan by Xyl-31_{rec} was studied in detail. Data on change in MMD of these substrates in the course of hydrolysis are presented in Fig. 4a. The average molecular mass of both substrates significantly decreases at the initial step of hydrolysis (10-20 min), whereas the relative content of low-molecular-weight products in the reaction mixture was insignificant (after 1 h hydrolysis the area of a peak corresponding to the low-molecular-weight products was only 8-10% of the total area of the chromatographic profile). The degree of completeness of hydrolysis of glucurono- and arabinoxylan by the action of Xyl-31_{rec} was 43 and 31%, respectively (Fig. 4c). The yield of oligosaccharides was significantly higher than the yield of monomer (xylobiose > xylotriose > xylose, Fig. 4b). The data indicate that like the native enzyme Xyl-31_{nat} [2], Xyl-31_{rec} is an endo-β-1,4-xylanases having the ordered mechanism of hydrolysis.

Adsorption ability. Xyl-31_{rec} was negligibly adsorbed on MCC (the distribution coefficient K_d = 0.009 liter/g) as well as on insoluble glucuronoxylan from beech wood (K_d = 0.038 liter/g); similar to Xyl-31_{nat}, this indicates that there is no adsorption domain in the composition of the enzyme

Table 1. Specific (U/mg) and relative (Xyl-31_{rec}/Xyl-31_{nat}) activities of Xyl-31_{rec} against various substrates (50°C, pH 5.0)

Substrate	Activity	
	specific	relative
CMC	1.2	0.95
RBB-CMC*	**2.8**	0.89
ROD-CMC*	0.6	0.84
FP	0.01	1.00
MCC	0	—
Laminarin	0.2	1.08
β-Glucan	**2.0**	0.92
Lichenan	0.8	0.95
Curdlan	0.05	0.96
Xyloglucan	**7.0**	1.10
Glucuronoxylan	**29.4**	1.05
Arabinoxylan	**24.1**	1.01
Arabinan	0.4	0.97
Arabinogalactan	0.5	0.95
Galactomannan	0.7	0.85
Galactan	0	—
PGU	1.1	0.93
p-NP-β-D-cellobioside*	**6.4**	1.21
p-NP-β-D-lactopyranoside*	0	—
p-NP-α-D-glucopyranoside*	0	—
p-NP-β-D-glucopyranoside*	0	—
p-NP-α-D-galactopyranoside*	0.03	—
p-NP-β-D-galactopyranoside*	0.03	1.25
p-NP-α-D-mannopyranoside*	0	—
p-NP-β-D-mannopyranoside*	0	—
p-NP-α-D-xylopyranoside*	0.02	0.75
p-NP-β-D-xylopyranoside*	0	—
p-NP-α-L-arabinopyranoside*	0	—
p-NP-α-L-arabinofuranoside*	0.2	1.19
p-NP-glucuranoside*	0	—

* Activity against these substrates was measured at 40°C.

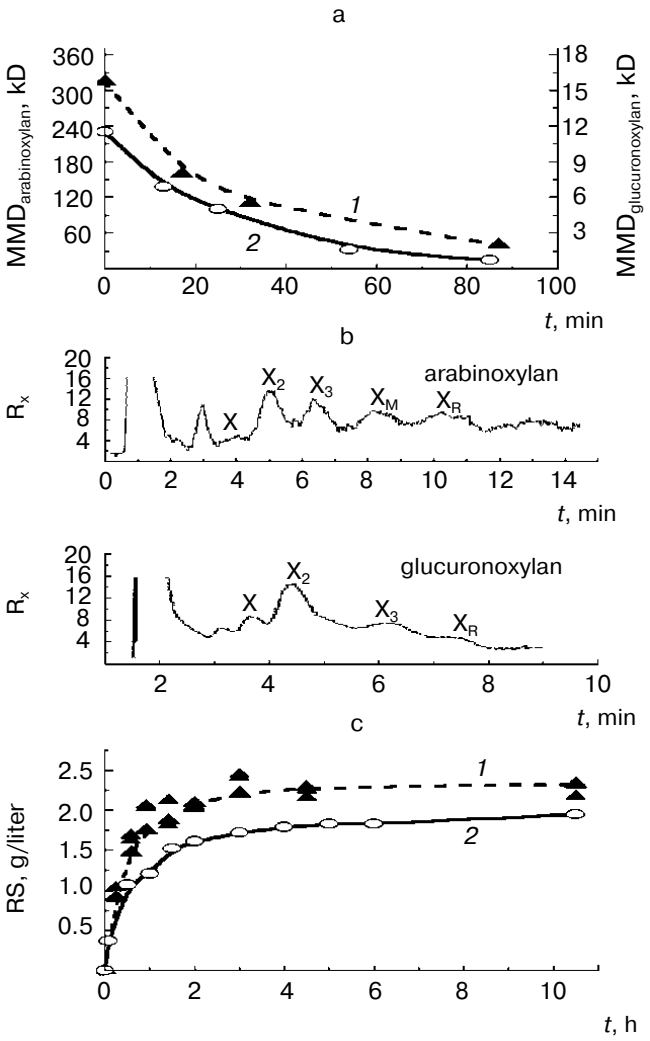

Fig. 4. Hydrolysis of glucurono- and arabinoxylan by Xyl-31_{rec}: a) change in the average mass of the substrates determined by high-pressure GPC; b) composition of the final products of hydrolysis determined by HPLC; c) kinetic curves of RS accumulation during complete hydrolysis. *1*) Glucuronoxylan; *2*) arabinoxylan.

molecule. It should be noted that for significantly adsorbing enzymes having an adsorption domain the K_d values are tens or hundreds of times higher than those mentioned above [9].

pH and temperature dependence of xylanase activity. Xyl-31_{rec} exhibited the maximal activity at pH 5.4 and Xyl-31_{nat} at pH 5.9 (Fig. 5a). Like the native enzyme, Xyl-31_{rec} lost most (to 80%) of the initial activity at pH < 4.0 and pH > 7.5.

The temperature dependence of Xyl-31_{rec} activity is presented in Fig. 5b: compared with that of Xyl-31_{nat}, the optimum is slightly shifted to higher temperature (from 55 to 60°C), and a "shoulder" appears in the low-temperature area (30-40°C). Both Xyl-31_{rec} and Xyl-31_{nat} exhibited the maximal activity in a rather narrow temperature range: $T_{opt} \pm 10$°C.

Stability of Xyl-31_{rec} was studied at pH from 4 to 6 and 40-60°C. Stability of Xyl-31_{nat} was relatively low: it lost from 40% (pH 5.0) to 70% (pH 4.0) of its initial activity after 4 h incubation at 35°C and 80% of its activity after the same period of time at 45°C; the enzyme was completely inactivated at pH 4.0 and 6.0 during 1 h at 55°C.

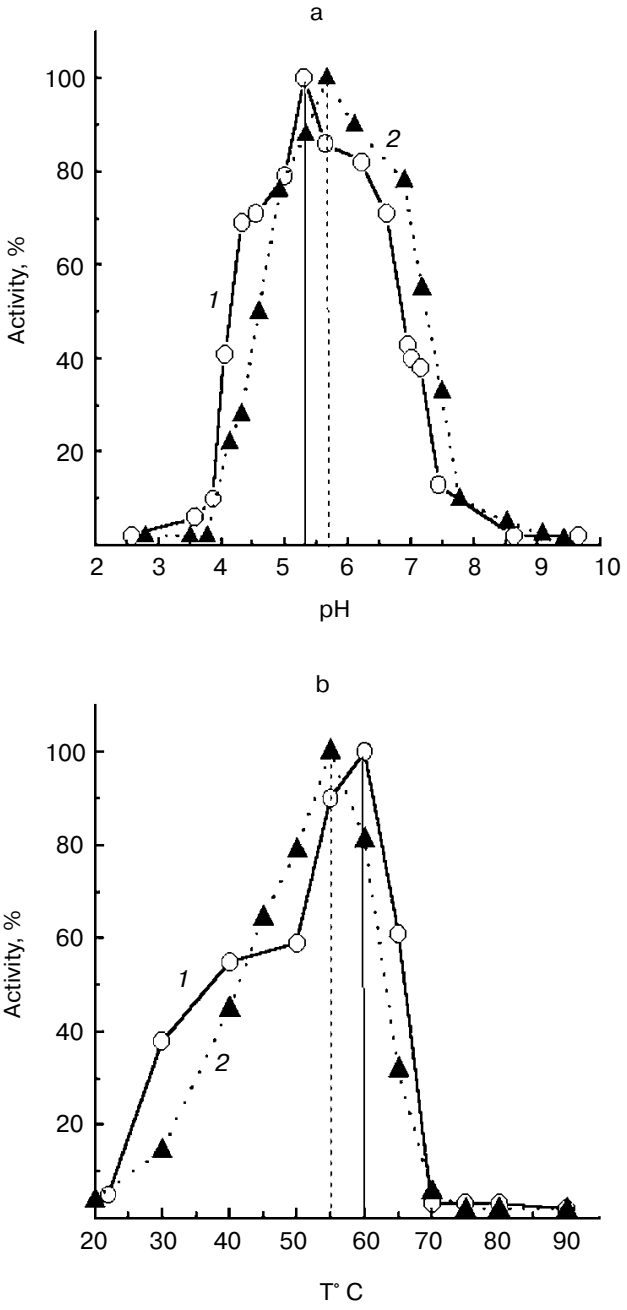

Fig. 5. pH (a) and temperature (b) dependence of Xyl-31_{rec} (*1*) and Xyl-31_{nat} (*2*) activities determined by hydrolysis of glucuronoxylan from birch wood. Conditions: 50°C (a), pH 5.0 (b).

Stability of Xyl-31_{rec} was somewhat higher: it lost only 40% of its initial activity in all the range of pH (4-6) after 3 h incubation at 40°C and 60% of its initial activity at pH 5.0 and 6.0 (close to pH_{opt}) after 3 h at 50°C. At pH 4.0 it was completely inactivated after 40 min. For Xyl-31_{rec}, 60°C appeared to be the temperature limit: the enzyme was completely inactivated after 5-10 min. Thus, Xyl-31_{rec} (like Xyl-31_{nat}) was most stable at pH 5.0 and 40-50°C.

Half-inactivation time of the enzymes was determined from kinetic curves of enzyme inactivation at various pH and temperatures. The results are presented in Table 2.

Ability of Xyl-31_{rec} for biobleaching of cellulose pulp. The essence of biobleaching of cellulose by xylanases is in hydrolysis of the secondary xylan and removal of the residual aromatic chromophore products of lignin degradation bound to the secondary xylan. Initial treatment of wood results in lignin degradation and hydrolysis of xylans, and then re-polymerization of low-molecular-weight products of xylan degradation results in formation of the secondary xylan "uptaking" aromatic fragments of lignin degradation. On the surface of cellulose pulp the secondary xylan forms an insoluble film decreasing whiteness of cellulose due to the presence of the chromophore groups [10]. To evaluate the efficiency of biobleaching, in our laboratory a method was developed based on treatment of unbleached cellulose pulp by enzyme preparations with subsequent detection of releasing chromophore groups by increasing optical absorption in solution (ΔA_{237}, see "Materials and Methods") [7].

Culture liquids produced by PCA-Xyl-26 and PCA 10 strains and also homogeneous Xyl-31_{nat} and Xyl-31_{rec} were used for biobleaching. A commercial xylanase preparation Ecopulp X200 (xylanase of *Trichoderma longibrachiatum* from Primalco) recommended for industrial biobleaching of cellulose pulp was chosen for comparison [11]. Biobleaching was performed under the following conditions: protein concentration in all cases 0.005 mg/ml, two pH values (5 and 7), 50°C.

The results are presented in Fig. 6. Efficiency of biobleaching with homogeneous Xyl-31_{nat} and Xyl-31_{rec} was very high and that with the cultural liquid produced by PCA-Xyl-26 strain and xylanase preparation Ecopulp X200 somewhat lower. Efficiency of biobleaching with the cultural liquid produced by PCA 10 strain was significantly lower than with other preparations. It should be noted that for all enzyme preparations, efficiency of biobleaching at pH 5.0 was ~1.5 times higher than at pH 7.0. Thus, the data indicate that it is possible to use efficiently enzyme preparations based on Xyl-31_{rec} for biobleaching of cellulose pulp.

Table 2. Comparison of properties of Xyl-31_{rec} and Xyl-31_{nat}

Parameters		Enzyme	
		Xyl-31_{nat}	Xyl-31_{rec}
Hydrolyzed bonds		Internal β-1,4-$(Xyl)_n$	Internal β-1,4-$(Xyl)_n$
Mechanisms of action		"Endo", ordered	"Endo", ordered
K_m, g/liter	Glucuronoxylan	3.4 ± 0.8	4.1 ± 0.9
	Arabinoxylan	2.0 ± 0.1	2.3 ± 0.1
Degree of complete hydrolysis, %[a]	Glucuronoxylan	40	43
	Arabinoxylan	35	31
Biobleaching, ΔA_{237}[b]	pH 5.0 (50°C)	2.70	2.75
	pH 7.0 (50°C)	1.65	1.75
K_d, liter/g[c]	MCC	0.011	0.009
	Xylan from beech wood	0.035	0.038
Activity optimum	pH	5.9	5.4
	T°C	55	60
Thermal stability: half-inactivation time, min[d]	40°C	180 / >180 / >180	>180 / >180 / >180
	50°C	15 / 180 / 130	20 / 120 / 60
	60°C	1 / 5 / 2	1 / 4 / 2

[a] Substrate concentration 5 g/liter, enzyme concentration 0.03 mg/ml.
[b] Enzyme concentration 0.005 mg/ml, treatment for 2 h.
[c] The distribution coefficient of adsorption on insoluble substrate.
[d] Determined by activity loss on hydrolysis of glucuronoxylan from birch wood at pH 4.0/5.0/6.0.

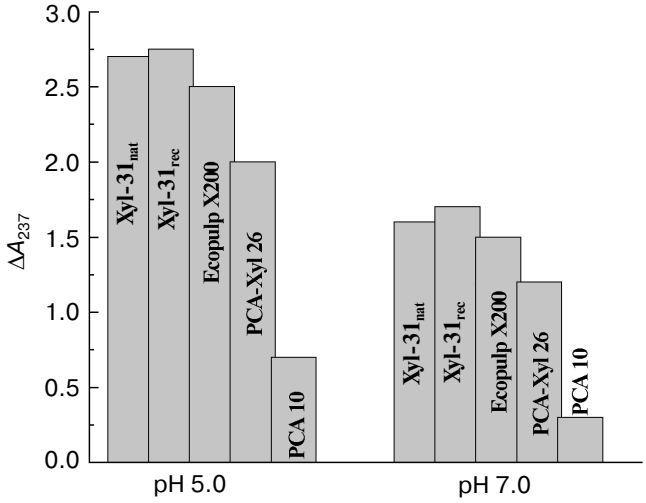

Fig. 6. Efficiency of biobleaching of cellulose pulp with homogeneous enzymes (Xyl-31$_{rec}$ and Xyl-31$_{nat}$) and enzyme preparations. Conditions: protein concentration in all cases 0.005 mg/ml, 200 rpm, 50°C.

Recombinant endo-β-1,4-xylanase was isolated from *P. canescens* for the first time, and the properties of the native and recombinant enzymes were shown to be almost the same (Table 2). Thus, it was proved that mycelial fungus *P. canescens* is suitable for homologous gene expression in order to obtain recombinant enzymes with the high yield (xylanase activity of multi-copy *P. canescens* strains is increased more than eightfold). Enzyme preparations obtained using multi-copy *P. canescens* strains producing recombinant endo-β-1,4-xylanase can be used for biobleaching of cellulose pulp.

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