Enzymatic Properties of the Xylanase Preparation from *Thermomyces lanuginosus*

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Xylanase preparation obtained by submersed fermentation of *Thermomyces lanuginosus* (sample of the commercial xylanase preparation obtained from »Krka«, p.o., Novo Mesto, Slovenia) was characterized. The preparation is apparently devoid of other proteins, as shown by SDS-electrophoresis. The enzyme exhibited the highest activity at pH about 7.0, and in the temperature range 60–70 °C. It was stable for 96 hours at pH between 5.0 and 9.0, and at temperatures up to 60 °C. Dithiobis-2-nitrobenzoic acid, *p*-hydroxymercuri benzoic acid and Hg^{2+} ions completely inhibited the enzyme, while Mn^{2+}, Fe^{2+} and β-mercaptoethanol enhanced the xylanase activity.

INTRODUCTION

In the last several years the production of microbial xylanase preparations has attracted great interest due to their potential application in the chemical, pharmaceutical and food industries. Xylanase preparations free of cellulase activity are of particular importance for the pretreatment of paper pulps decreasing the xylan content and, therefore, reducing the dependence on chlorine used for bleaching in the brightening process. Cellulase contaminations of xylanases primarily originate from the presence of cellulase-inducing contaminants in the growth medium[1], or from the substrate specificity of some glucanases hydrolyzing both substrates.[2,3]

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Xylans represent the major hemicellulose in hardwoods and about 40% of the softwood hemicellulose.[4] They possess a backbone composed of $\beta(1 \to 4)$ linked xylosyl residues but differ in side chains attached to it.[5]

Among xylanase producers procaryotes and fungi are of the greatest biotechnological importance.[6] There have been a number of papers describing isolation and purification procedures for xylanases from different mesophyllic (7–11) as well as thermophilic fungi.[12–15] Recently, the optimization of the xylanase production by *Thermomyces lanuginosus*, an excellent enzyme producer, has been reported.[16,17] In this paper, we describe some enzymatic properties of the xylanase preparation from the same strain of *Thermomyces lanuginosus* as a potential enzyme preparation to be used in paper industry.

MATERIALS AND METHODS

Xylanase produced by the *Thermomyces lanuginosus* strain deposited at the German type culture collection (DSM 5826, obtained from »Krka«, p.o., Novo Mesto, Slovenia) was used in this study. The fungus was grown as described by Purkarthofer *et al.*,[16] the culture filtrate was freeze-dried and used as the enzyme preparation.

Dithiobis-2-nitrobenzoic acid, *p*-hydroxymercuri benzoic acid, and β-mercaptoethanol were obtained from Serva, Heidelberg. Oat spelts xylan was from Sigma, St Louis. All other chemicals were purchased from Kemika, Zagreb and were of the highest purity grade available.

Electrophoresis was performed according to Laemmli.[18] Staining of polyacrylamide gels for the xylanase activity was essentially done as described by Morage *et al.*.[19] Polyacrylamide gel slabs containing 0.1% oat spelts xylan were prepared and electrophoresis was performed by the standard procedure.[18] Subsequently, gels were washed 2 times for 30 minutes in 50 mM Na-phosphate buffer pH 6.0 containing 25% isopropanol, and then 2 times for 30 minutes in the same buffer without isopropanol. Gels were then incubated 5 minutes at 60 °C and submerged in the 0.1% Congo Red solution for about 10 minutes. The excess of the dye was removed by washing in 1 M NaCl and the gel was transferred to 5% acetic acid.

Xylanase activity was determined by the method of Bailey *et al.*.[20] If the activity was measured in the presence of a potential effector, xylanase was pretreated with the indicated concentrations of the effector for 5–10 minutes, and the activity was assayed in a reaction mixture containing the same concentration of the effector compound.

Proteins were determined by the Lowry method.[21]

RESULTS AND DISCUSSION

Proteins found in the *Thermomyces lanuginosus* growth medium were analyzed by SDS-electrophoresis. As seen in Figure 1. only one protein band, migrating at the molecular mass of 25.5 Kda, could be detected. The staining of the gel for the xylanase activity revealed that the dominating band indeed represented the endoxylanase. No other xylan degrading bands could be detected in zymograms, indicating that *T. lanuginosus*, unlike some other fungi,[14,22,23] produces only one xylanase. Kitpreeghavanich *et al.*[24] estimated the molecular mass of 21 Kda for the same enzyme from another *T. lanuginosus* strain, indicating that different strains might secrete different xylanases. Such an observation has also been reported by Annand *et al.*.[15]

To examine the properties important for the practical use of the xylanase preparation, the pH activity profile and the optimal temperature were determined. Highest activity was recorded about pH 7.0 and in the temperature range 60–70 °C (not shown). Such results are in good agreement with the data already published by Gomes *et al.*.[17]

Among enzymatic properties of the highest importance for the application of the xylanase preparation is the enzyme stability. As it can be seen in Figure 2., xylanase is stable in the pH range from 5.0 to about 9.0 for 98 hours at room temperature. Relative stability was also recorded at higher pH values so that at pH 12 only about 30% of the activity was lost in a 3 day incubation. In contrast, xylanase is quickly inactivated at acidic pH and at pH 3.5 only about 10% of the original activity remained after 1 hour. The determined pH stability range is somewhat broader than the one reported

KDa

94.0
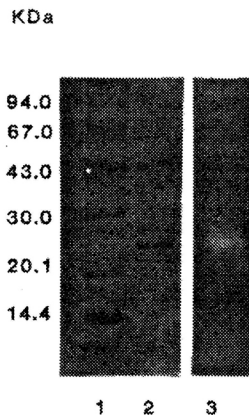67.0
43.0
30.0
20.1
14.4

1   2   3

Figure 1. SDS-electrophoresis of the xylanase preparation from *T. lanuginosus*. Lanes: 1. molecular mass standards; 2. xylanase preparation stained for proteins; 3. xylanase preparation stained for xylanolytic activity.
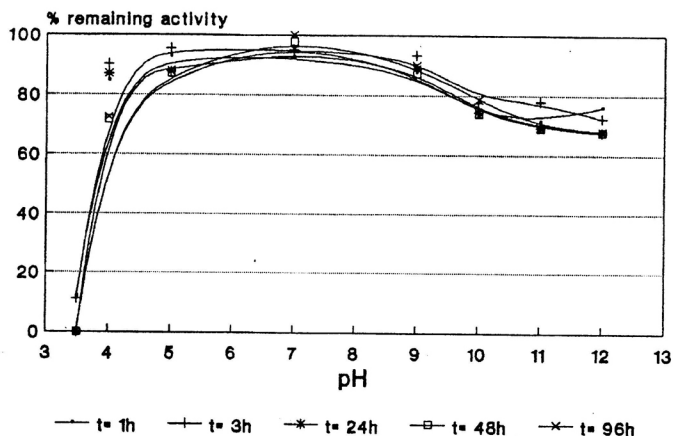
Figure 2. Inactivation of the *T. lanuginosus* endoxylanase at different pH. The enzyme preparation was incubated at different pH, at room temperature, for indicated time periods, and the xylanase activity was assayed.

previously for the other *T. lanuginosus* strain.[24] However, the stability determined in the acidic pH region is not as high as already reported for the enzyme from the same strain of the fungus[17] since, according to that report, xylanase was relatively stable even at pH 3.0.
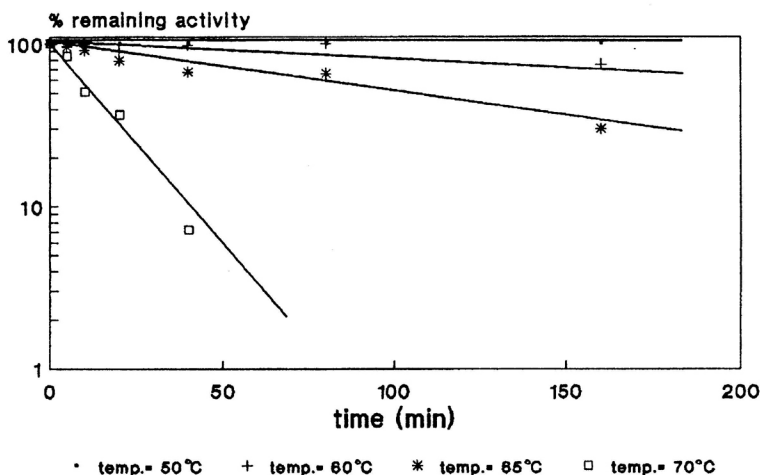

Figure 3. Heat inactivation of *T. lanuginosus* endoxylanase. The enzyme preparation was incubated at different temperatures, at times indicated aliquotes were transferred to cold buffer and the xylanase activity was assayed.

TABLE I

Influence of different effectors on the activity of endoxylanase from
T. lanuginosus.

Reagent	Concentration (mM)	% Activity
none		100
dithiobis-2-nitro benzoic acid	7.5	0
p-hydroxymercuri benzoic acid	15.0	0
Hg^{2+}	2.0	0
Mn^{2+}	1.0	137
Fe^{2+}	1.0	150
β-mercaptoethanol	10.0	148
Fe^{2+}+β-mercaptoethanol	0.8 / 10.0	181

Heat inactivation rate of the xylanase preparation is presented in Figure 3. It can be seen that the enzyme is stable at 50 °C and 60 °C over a period of 200 minutes. At 70 °C, the inactivation is rapid and the enzyme loses its total activity in about 80 minutes. In the report of Gomes et al.,[17] again a higher stability of xylanase has been described (only a 4% decrease of activity at 70 °C in 15 minutes). Since the experiment was performed at the same pH, the reason for this discrepancy is unknown. The dependence of the logarithm of the residual activity on the inactivation time follows a straight line (Figure 3.), again suggesting that there is only one enzyme species responsible for the xylanolytic activity of the preparation .

Influence of several potential effectors on the xylanase activity is summarized in Table I. Cysteine reacting reagents, dithiobis-2-nitrobenzoic acid and p-hydroxymercuribenzoic acid, were found to inhibit the enzyme, pointing to the importance of sulphydryl group(s) for the xylanase activity. This property contrasts very strongly with the report of Kitpreeghavanich et al.,[24] who found no inhibition with sulphydryl reagents, indicating that xylanases from different *T. lanuginosus* strains differ in their enzymatic properties, as well. Hg^{2+} ions were also found to inactivate the enzyme. In contrast, Mn^{2+}, Fe^{2+} and β-mercaptoethanol increased the activity, and the best results were achieved by the combined action of the latter two agents. Figure 4. shows changes of the xylanase activity caused by different concentrations of several effectors.

In conclusion, the presented data could be summarized to determine the optimal parameters for the application of the *Thermomyces lanuginosus* xylanase preparation. The recommended use of the enzyme should be in the neutral pH region, at 60 °C, in a buffer containing 0.8 mM Fe^{2+} ions and 10 mM β-mercaptoethanol. Further work is in progress to determine the
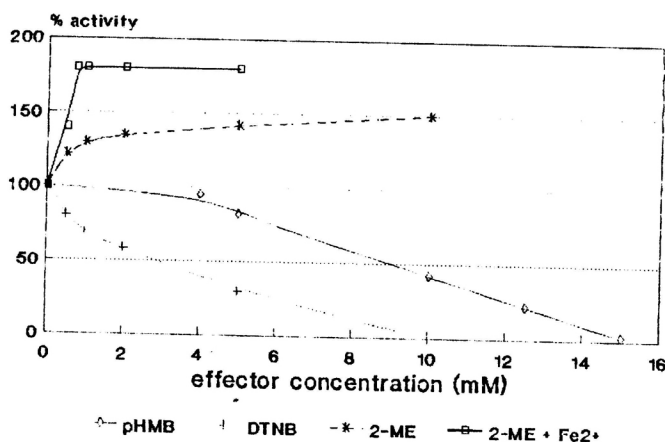
Figure 4. Influence of different effectors on the xylanase activity. Different concentrations of *p*-hydroxymercuribenzoic acid (pHMB), dithiobis-2-nitrobenzoic acid (DTNB), or β-mercaptoethanol (2-ME) were added to the reaction mixture as described in Materials and Methods. In addition, different concentrations of $FeCl_2$ were added to the reaction mixture containing 10 mM β-mercaptoethanol (2-ME+Fe^{2+}), and the xylanase activity was determined.

physico-chemical as well as enzymatic properties of purified *T. lanuginosus* xylanase which should give a better insight into application possibilities of this enzyme.

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SAŽETAK

Enzimska svojstva preparata ksilanze iz funga *Thermomyces lanuginosus*

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Provedena je karakterizacija preparata ksilanaze dobivenog iz podloge za uzgoj plijesni *Thermomyces lanuginosus* (komercijalni preparat »Krke«, p.o., Novo Mesto, Slovenija). SDS elektroforeza je pokazala da u preparatu nema drugih vidljivih proteinskih vrpci. Enzim je pokazivao najveću aktivnost pri pH oko 7.0 i u rasponu temperatura 60–70 °C. Bio je stabilan 96 sati pri pH između 5.0 i 9.0, te na temperaturama do 60 °C. Ditiobis-2-nitrobenzojeva kiselina, *p*-hidroksimerkuribenzojeva kiselina i Hg^{2+} ioni potpuno su inhibirali enzim, dok su Mn^{2+} ioni, Fe^{2+} ioni i β-merkaptoetanol pojačavali aktivnost ksilanaze.