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CHARACTERISATION AND EFFECTS OF A XYLANASE ENZYME PREPARATION EXTRACTED FROM *THERMOMYCES LANUGINOSUS* CULTURES

J. KUTASI[1], Á. BATA[1], E. BRYDL[2], P. RAFAI[2*] and V. JURKOVICH[2]

[1]Dr Bata Research and Development LLC, H-2364 Ócsa, Pesti úti major, Hungary;
[2]Department of Animal Hygiene, Faculty of Veterinary Medicine, Szent István University, H-1078 Budapest, István u. 2, Hungary

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This paper describes the production of an enzyme preparation from the fungus *Thermomyces lanuginosus*. Thermal resistance, pH stability and lignocellulolytic activity of the enzyme preparation high in xylanase were studied on a variety of grains and forages. The enzyme preparation preserved more than 70% of its original xylanase activity for 4 and 1 h at 60 and 70 °C, respectively. The xylanase activity remained over 80% when the preparation was incubated for 30 min at pH 4.5. *In vitro* digestibility studies indicated that the enzyme digested 7.5, 8.5 and 8.0% of the dry matter (DM) of barley meal, wheat bran and oat meal samples, respectively. When applying 60-min incubation, 7.5, 7.3 and 8.4% of DM of the oat straw, alfalfa hay and triticale straw was digested, respectively. When the time of digestion was increased to 360 min, the sunflower hull showed 15.8% DM digestibility.

Key words: *Thermomyces lanuginosus*, xylanase, lignocellulolytic activity, thermal and pH stability

Polysaccharidase enzymes have been used since the early 1960s as feed additives in poultry and pig nutrition and much later (from the beginning of the 'nineties) also in cattle nutrition for the improvement of feed conversion efficiency and other production traits. Enzyme additives may be mixed to the feeds either directly, usually in 0.01–0.1% concentration, in order to aid the digestive processes in the (fore)stomach(s) and intestines, or they may be used for enzymatic pre-treatment of cereals and/or forages for enhancing degradation of non-starch polysaccharides (NSP). These enzymes are produced industrially by fungal cultures (Van de Mierop and Ghesquiere, 1998). The main components of the enzyme mixtures are cellulase, xylanase, 1,4-beta-endoglucanase, β-glucosidase, α-amylase and α-galactosidase. Of these enzymes, xylanase predominates in most of the preparations (Van de Mierop and Ghesquiere, 1998; Pack and Bedford, 1999).

*Corresponding author; E-mail: prafai@univet.hu; Fax: +36 (1) 478-4243

Of the imperfect fungi, *Aspergillus oryzae* and *A. niger*, or *Trichoderma viridis* and *Humicola insolens* have been known to produce fibrolytic enzymes in submerged cultures (Gomes et al., 1993*a*; Bakalova et al., 1996). These fungi, however, may be cultured with equal success in solid-state media (e.g. mould-bran medium, Lisching et al., 1993; Alam et al., 1994). In this case the product may be dried presuming the enzymes produced are heat resistant. The xylanase enzyme produced by the best known thermal resistant fungus, *H. insolens*, may preserve most of its activity at 60 °C (pH 6.0), but at temperatures higher than this the activity fades away within minutes (Schülien et al., 1992). Xylanase enzymes produce maximum activity at pH 6.0–7.0. It follows that strong acidity prevalent in the stomach of monogastric animals (pH 3.0–4.0) will destroy the overwhelming majority of the enzymatic activity (Lisching et al., 1993).

According to the investigations of Purkarthofer et al. (1993) and Bennet et al. (1998), the thermophilic filamentous, imperfect fungus, *Th. lanuginosus*, produces cellulase-free extracts high in xylanase (2840 IU/ml) and low in β-xylosidase (1.8 IU/ml), α-glucosidase (1.3 IU/ml) and α-arabinosidase (1.0 IU/ml) activity. The strain of *Th. lanuginosus* used in these experiments has been deposited at the National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary) under the identification code NCAIM 001288.

In our laboratory an attempt was made to produce an enzyme product from the fungus *Th. lanuginosus* high in xylanase activity using submerged cultures for further study. This paper reports the thermal and pH stability of the enzyme preparation and provides data on its lignocellulolytic activity on cereals and forages.

Materials and methods

Production of the enzyme extract

The fungus *Th. lanuginosus* was cultured by submerged fermentation on a corn cob mixture (CCM) at 50 °C according to methods described in the relevant literature (Schülien et al., 1992; Gomes et al., 1993*b*; Purkarthofer et al., 1993; Alam et al., 1994; Hoq et al., 1994; Hoq an Deckwer, 1995; Bennet et al., 1998). In our case one litre of the medium contained 30.0 g CCM, 30 g yeast extract, 2.0 g ammonium sulphate, 3.0 g magnesium sulphate, 0.3 g calcium chloride, 0.5 g iron sulphate, 10 g di-hydrogen potassium sulphate, 5.0 g di-hydrogen potassium phosphate. Two hundred ml of this medium was measured into Erlenmeyer flasks of 500 ml and then autoclaved at 121 °C for 45 min. The pH was adjusted to pH 6.0. Each culture medium was inoculated by a culture of *Th. lanuginosus* grown on a potato-dextrose agar-slant (Becton and Dickinson Laboratories) for 10 days at 50 °C. Flasks containing the inoculated medium were shaken by an orbital shaker (Braun, Certomat S-II) at 150 rpm and 50 °C for 6 days. When completed, the fungal broth culture was centrifuged for 10 min at

3000 rpm and then 1 litre of the clear supernatant was added to 100 g yeast-extract and spray-dried at 115 °C inlet and 60 °C outlet temperature by a Mini Spray Dryer (Büchi, B-191, Switzerland) according to the method described by Caesar and Mrsa (1996) and Bennet et al. (1998). The average xylanase activity of this air-dry enzyme preparation (EP) was 2660 IU/g. [IU: one unit of xylanase activity was expressed as μmol of reducing (xylose equivalent) sugar released in one min.]

Measurement of enzyme activity

Since the relevant literature (Gomes et al., 1993*b*; Purkarthofer et al., 1993) has shown that the extract of the *Th. lanuginosus* cultures contains negligible quantities of polysaccharide enzymes other than xylanase, these experiments were restricted to measuring only the xylanase activity in the following way.

To prepare the enzyme solution (ES), 10 g EP was dissolved in 100 ml of 0.05 mol phosphate buffer (pH 6.5) and was homogenised by stirring for 30 min with a magnetic stirrer. After 5 min of sedimentation the supernatant was centrifuged for 10 min at 3000 rpm, then the xylanolytic activity was measured from the supernatant based on the method described by Bailey et al. (1992). Accordingly, 0.02 ml of the diluted ES was added to 2.0 ml oat-spelt xylane preparation of 1.0% (Sigma X 0550) and then incubated for 15 min at pH 6.5 and 50 °C. After centrifuging (3000 rpm for 5 min) the supernatant was used for assaying the quantity of reducing sugars by the method of Miller (1959). To 1 ml of supernatant 3 ml DNA (2-hidroxy-3.5-dinitrobenzoic acid) reagent was added and then boiled for 5 min. After cooling to room temperature, the absorbance of the solution was measured spectrophotometrically (Specol 11, Carl Zeiss, Jena) at 540 nm.

Examination of the thermal and pH stability of the EP

0.2 ml ES of 266 IU/ml activity dissolved in 1.8 ml of 0.05 M citrate-phosphate buffer was incubated either at constant temperature (50 °C) with pH 4.5, 5.5 and 6.5 for 15 min, or at invariant pH of 6.5 with temperatures of 60, 70 and 80 °C for 60–360 min (Gomes et al., 1993*a*; Lisching et al., 1993; Alam et al., 1994; Purkarthofer and Steiner, 1995). After incubation the residual activity was measured as indicated earlier.

Lignocellulolytic activity of the EP on grain and forage substrates

One gram of barley-, wheat-, oat- and rye-meal samples was soaked at 50 °C either in 100 ml of ES that contained citrate-phosphate buffer (enzyme activity: 266 IU/ml) or in dilutions of ES, where the enzyme activity was 133 and 66 IU/ml, respectively. After 5 to 30 min incubation the quantities of reducing sugars were measured as described above (Miller, 1959). The lignocellulolytic

activity was estimated as percent of xylose equivalent (M: 150.1 g) reducing sugars expressed in % of the dry matter (DM) of the sample. The results were compared to lignocellulolytic activity of a commercial enzyme preparation (CEP). In this case 10 g CEP was dissolved in 100 ml 0.05 mol phosphate buffer to obtain 226 IU/ml enzyme activity. This solution was then used for soaking the grain samples in identical conditions described above.

Forage samples (triticale straw, oat straw, alfalfa hay and sunflower hulls) were treated in the same way with the difference that straw samples were incubated for 5 to 60 min, and samples of the sunflower hulls were incubated for 5 to 360 min.

Results

Thermal stability

The enzyme extract preserved more than 70% of its activity for 2 h when incubated at 60 °C, and it decreased to below 50% only after 6 h of incubation (Fig. 1). The extract incubated at 70 °C kept more than 70% of its activity for 1 h showing a sharp decline afterwards. Incubation at 80 °C proved deleterious to the enzyme activity, because the extract lost approx. 50 and 80% of its activity at the end of the 1st and 2nd hour of incubation, respectively, and there was no indication of enzymatic activity after the 4th hour of the experiment.

pH stability

At pH 4.5 the extract retained the majority of its enzymatic capacity for 30 min, while the activity was halved by the 60th min of incubation (Fig. 2). At this pH the enzymatic activity was practically lost after 120 min of incubation. At pH 6.5, good activity was measured even after 3 h of incubation.

Experiments with different grains

More than 50% of the digestible dry matter content of the rye bran (Fig. 3), oat grits (Fig. 4), barley groats (Fig. 5) and wheat bran (Fig. 6) samples was dissolved within 5 min by the enzyme extracts studied. Maximum degradation was obtained by about the 15th min of the experiment and only in few cases went the digestion until min 30. In three of the four grains studied the enzyme extract produced more dissolved dry matter than the commercial enzyme preparation. In case of the barley (Fig. 5, 7.5 vs. 6.5%) and rye (Fig. 3, 8.4 vs. 7.1 %) samples the difference proved modest, while the in case of oat grits the difference was considerable (Fig. 4, 8.0 vs. 6.0 %). The dry matter digestion of the commercial enzyme preparation on wheat bran samples (Fig. 6) was better than that of the experimental preparation.

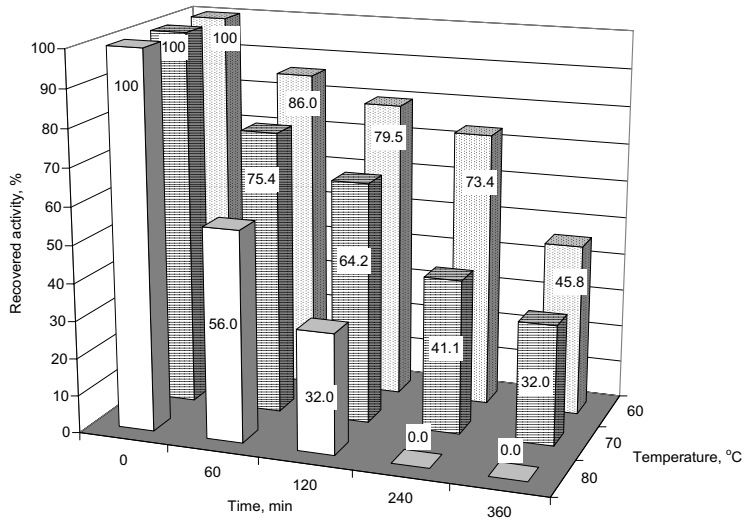

Fig. 1. Thermal resistance of the xylanase enzyme from *Thermomyces lanuginosus* at pH 6.5

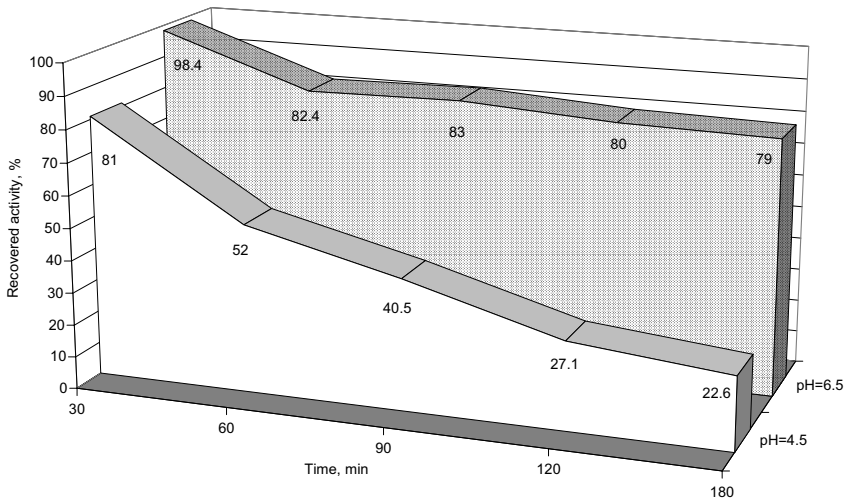

Fig. 2. Change of activity of xylanase enzyme from *Thermomyces lanuginosus* at different pH and at 50 °C

Experiments with forages and sunflower hulls

The experimental enzyme extract started to degrade the organic material of oat straw, triticale straw, alfalfa hay and sunflower seed hulls after 5 min of digestion (Fig. 7). The concentration of the dissolved dry matter gradually increased until min 30 of incubation and in case of the sunflower, alfalfa, oat and

triticale samples it reached 5.6, 5.4, 6.6 and 7.5%, respectively. Beyond min 30 there was some further increment (Fig. 7).

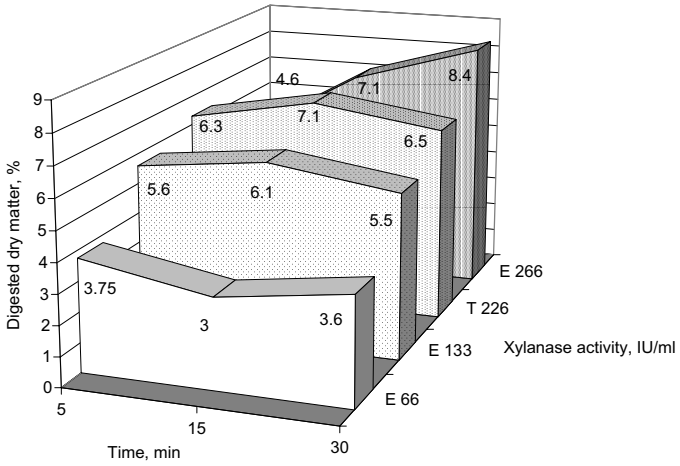

Fig. 3. Dry matter digestion of the xylanase produced in the present research at different activities (E) on rye bran substrate (pH: 6.5, temperature: 50 °C) in comparison with a commercial product (T)

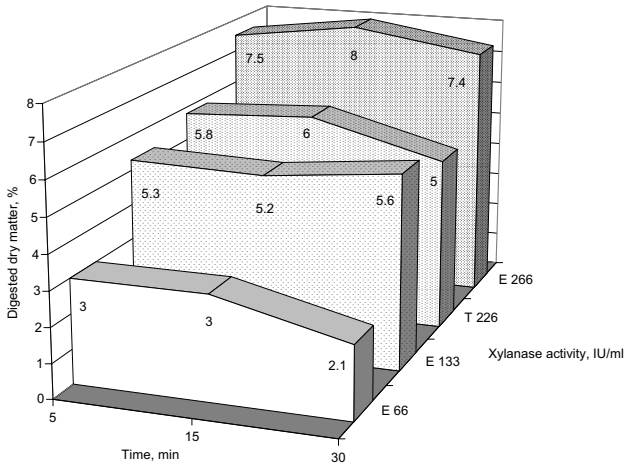

Fig. 4. Dry matter digestion of the xylanase produced in the present research at different activities (E) on oat meal substrate (pH: 6.5, temperature 50 °C) in comparison with a commercial product (T)

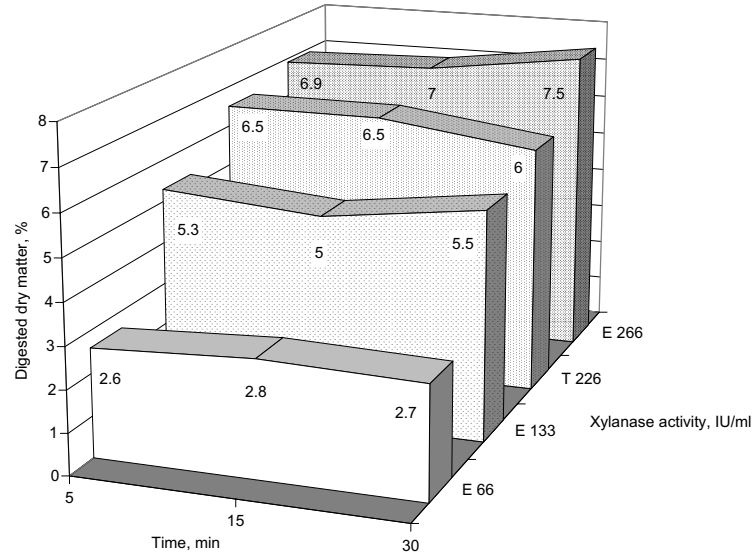

Fig. 5. Dry matter digestion of the xylanase produced in the present research at different activities (E) on barley meal substrate (pH: 6.5, temperature 50 °C) in comparison with a commercial product

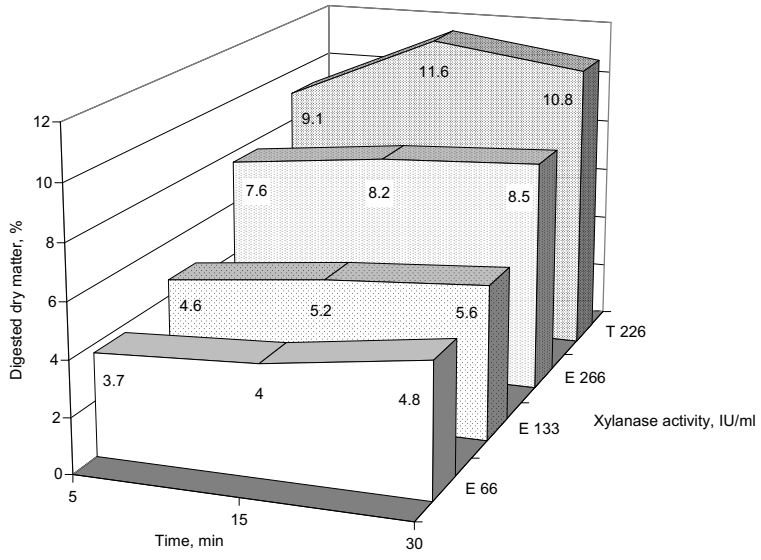

Fig. 6. Dry matter digestion of the xylanase produced in the present research at different activities (E) on wheat bran substrate (pH: 6.5, temperature 50 °C) in comparison with a commercial product

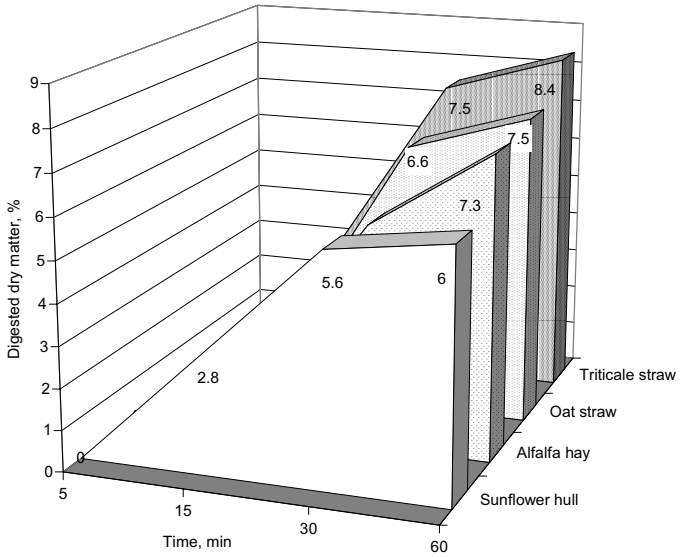

Fig. 7. Lignocellulolytic activity of xylanase from *Thermomyces lanuginosus* (activity: 266 IU/ml, pH: 6.5, temperature: 50 °C) on forages

Sunflower seed hull samples were set for longer enzymatic incubation. As shown in Fig. 8, the concentration of the dissolved dry matter was 9.7, 14.3 and 15.8% at 90, 210 and 360 min, respectively.

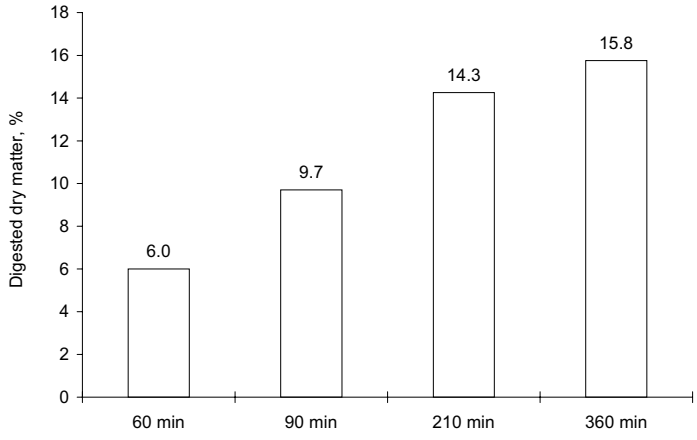

Fig. 8. Lignocellulolytic activity of xylanase from *Thermomyces lanuginosus* (activity: 266 IU/ml, pH: 6.5, temperature: 50 °C) on sunflower hull substrate

Discussion and conclusion

Thermal stability examinations indicated that the enzyme preparation has appropriate heat resistance. From this it follows that during the manufacturing of dry fermentation product, the enzyme preparation may be kept at 60 °C for as long as 2 to 3 h without considerable loss of enzyme activity. Owing to this the water content of the end product can be minimised. The enzyme preparation may be dried at 70 and 80 °C, however, at these temperatures the length of drying should be reduced to 60 and 30 min, respectively.

In acid environment the enzyme preparation preserves its enzymatic activity only for 30 to 60 min. In monogastric animals the preparation may only be effective if it passes through the stomach with considerable speed. This may be the case with poultry (Bedford and Morgan, 1997; Pack and Bedford, 1999; Silversides, 1999).

Comparative study of grain samples revealed that the enzyme preparation works in a similar way as the commercial product studied in these experiments. Differences were found with wheat bran and oat grits. These differences might be attributed to the presence of auxiliary enzymes in our preparation and cellulase in the commercial product which was not measured in the present experiments. It has been reported (Gomes et al., 1993*a*; Gomes et al., 1993*b*; Purkarthofer et al., 1993; Alam et al., 1994; Hoq et al., 1994), that under appropriate culturing conditions, such as those applied in the present studies, the *Th. lanuginosus* fungal strains produce considerable quantities of xylanase with and without the presence of amylase and cellulase, respectively. The presence of amylase with no or negligible amount of cellulase in preparations rich in xylanase may either bring down or improve the digestive capacity of xylanase depending on the substrate quality (Gomes et al., 1993*b*; Purkarthofer et al., 1993; Alam et al., 1994). This may explain our findings that our enzyme extract had inferior and superior polysaccharidase activity on wheat bran and oat grit samples, respectively, in comparison with that of the commercial product.

Due to their high lignin content, forages and sunflower seed hulls contain considerably less available xylane than grains (Gomes et al., 1993*b*; Purkarthofer et al., 1993). This explains the relatively slow and less efficient lignocellulolytic activity. However, applying longer incubation, such as they were soaking in the aqueous enzyme preparation, polysaccharides might become available for the enzymes. This was observed in our experiment with the sunflower seed hulls, where the fungal enzyme digested three times more dry matter in 6 h than during a 1-h incubation.

The laboratory findings indicate that the enzyme extract of *Th. lanuginosus* fungal culture with dominant quantities of xylanase is especially suitable for enzyme supplementation of barley, rye and oat based feed mixtures for poultry and monogastric animals.

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