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Investigation of Factors Affecting Xylanase Activity from *Trichoderma harzianum 1073 D3*

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ABSTRACT

In this study, some physiological conditions affecting the activity of xylanase enzyme produced from Trichoderma harzianum 1073 D3 were determined. In addition, stabilization of pH and temperature in liquid and semi-solid state cultivation media were investigated. It was concluded that for maximum xylanase activity, incubation at 60°C in an enzyme incubation medium with pH 5 that contained 1 % xylan was appropriate. The stability studies showed that the enzyme was relatively stable in the pH range 3-7 and retained more than 50 % of its original activity after four months.

Key words: *Trichoderma harzianum*, xylan, xylanase, lignocellulosic material, enzyme stability, metal ions

INTRODUCTION

Lignocellulosic materials are widespread in nature and xylan is a polysaccharide found in the hemicellulosic fraction of lignocellulose. Xylan is a potential significant resource for renewable biomass, which can be utilized as a substrate for the preparation of many products such as fuels, solvents and pharmaceuticals. Corn, wheat stem, barley shells and similar material contain xylan (Gomes et al., 1992; Gawande and Kamat, 1999; Beg et al., 2001; Subramaniyan and Prema, 2002). On the other hand, xylanases (E.C. 3.2.1.8) are needed for making use of hemicelluloses. For most bioconversion processes, xylan must first be converted to xylose or xylo-oligosaccharides. This can be done either by acid hydrolysis or by the use of xylanolytic enzymes (Chivero et al., 2001).

Xylanase enzyme deconstructs plant structural material by breaking down hemicellulose, a major component of the plant cell wall. Xylanases are produced from many different fungi and bacteria. Xylanase enzymes are used commercially in the pulp and paper, food, beverage, textile and animal feed industries (Tan et al., 1987; Wong et al., 1988; Li et al., 2000). Most commercial xylanases are produced by *Trichoderma*, *Bacillus*, *Aspergillus*, *Penicillium*, *Aureobasidium*, and *Talaromyces sp*. (Li et al., 2000).

In this study, some physiological conditions affecting the activity of xylanase enzyme produced from *Trichoderma harzianum 1073 D3*, namely pH, temperature, reaction time and substrate concentration were determined. In addition, the effect of metal ions and different carbon sources were determined and finally pH and temperature stabilities of the xylanase enzyme were studied both in liquid and semi-solid state cultivation media.

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MATERIALS AND METHODS

Microbial Strain

In the study, *Trichoderma harzianum 1073 D3*, which was isolated and classified from the microflora of Turkey by Marmara Research Center, was used for production of the xylanase enzyme. Stock cultures were maintained on potato dextrose agar at 4ºC.

Medium. Two different media were used for xylanase production. Liquid medium described by Kim et al. (1985) was used with some modification for growth and enzyme production. It contained (gl^{-1}); 0.5 Proteous-peptone, 0.3 urea, 0.2 KH_2PO_4, 0.3 $CaCl_2$, 0.2 Tween-80, 1.4 $(NH_4)_2SO_4$, 0.3 $MgSO_47H_2O$ and 1 % xylan (sigma) as carbon source. In 250 ml flasks, 100 ml of medium was sterilized in autoclave at 121ºC, 1.5 atm for 15 min.

Wheat bran was used for the semi-solid state cultivation medium, which contained 10 g wheat bran and 10 ml distilled water prepared in 250 ml Erlenmeyer flask. Semi-solid state medium was sterilized in autoclave at 121ºC, 1.5 atm for 2 h. After sterilization, trace element solution at 0.1 % concentration was added to both the media. The trace element solution contained (gl^{-1}); 0.05 $FeSO_4$, 0.014 $ZnSO_47H_2O$, 0.02 $CoCl_2$, 0.016 $MnSO_4$.

Inoculation. One ml spore suspensions containing 15.10^6 spores were inoculated into the growth media. The liquid medium was incubated at 30ºC for 13 days and agitated at 150 rpm, whereas the semi-solid state medium was incubated at 30ºC for 5 days.

Enzyme Activity. The enzyme activity was measured by modifying the method described by Khanna and Gauri (1993), using a 0.1 M sodium-acetate buffer with pH 5. The liquid culture filtrate was centrifuged at 7,200 rpm for 15 min, while the semi-solid medium was mixed with 100 ml distilled water at room temperature and agitated for 1 h and used as the enzyme sample. One ml of 1 % xylan solution (in 0.1 M, pH 5 sodium acetate buffer) and 0.5 ml enzyme sample were added to the reaction tubes and incubated for 30 min at 37ºC and then centrifuged at 7,200 rpm for 10 min. The amount of reducing sugar in the reaction tubes was measured using the Dinitrosalicylic Acid Method (DNS) described by Miller (1959). The absorbance was read at 550 nm using Jenway, 105 u.v. vis spectrophotometer. The amount of reducing sugar was calculated from the standard curve based on the equivalent glucose. One unit xylanase activity was described as the amount of enzyme producing 10 μg of reducing sugar in 1 ml medium in 1 minute under standard test conditions. The amount of protein was determined by Lowry Method (Lowry et al., 1951).

The optimum pH value for the reaction was determined by varying pH values of 0.1 M sodium acetate buffer between 4-6 while all other parameters were kept constant. After incubation at 37°C for 30 min, xylanase activity was determined as described above. In order to investigate the effect of substrate concentration, xylan ranging from 0.1 % to 1.5 % (in 0.1 M sodium acetate buffer) was added to the reaction medium, while all other parameters were kept constant. The effect of reaction time on enzyme activity was investigated by incubating the reaction media at 37°C for 20 to 60 min while keeping all other parameters constant. Finally, in order to investigate the effect of temperature, reaction media were incubated for 30 min at different temperatures between 20 and 70°C.

Temperature stability. Temperature stability was assessed by preincubating enzyme solutions for 2 h at 30, 40, 50, 60 and 70°C prior to addition of substrate and the determination of activity at 37°C.

pH stability. pH stability was determined by preincubating enzyme solutions at pH values of 3, 4, 5, 6 and 7 for 24 h prior to the addition of substrate and the determination of activity at pH 5.

Metal ions

The mixture of 1 ml xylan substrate, 0.5 ml dialyzed enzyme and 1 ml different metal ions (Mn^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, K^{2+}, Mg^{2+}) were incubated at 37°C for 30 min. After incubation, enzyme activities were determined. Ions were added in the assay buffer at a level of 1 mM. The percent activity was determined based on the reference sample, which did not contain any metal ion.

The mixture of 1 ml xylan substrate, 0.5 ml enzyme and 1 ml different carbon sources (glucose, galactose, fructose, lactose, xylose and sucrose) was incubated at 37°C for 30 min. After incubation, enzyme activities were determined. Carbon sources were added in the assay buffer at a level of 10 mM. The percent activity was

determined based on the reference sample, which does not contain any carbon source. Xylanase enzyme was kept at 4°C and enzyme activity was determined at certain time intervals for 120 days.

RESULTS

In this study, xylanase activities of liquid and semi-solid state media were 480.1 Uml^{-1} and 185.2 Ug^{-1}, respectively.

Fig. 1 shows the results on the effect of pH on xylanase activity. It was observed that the activity was maximum around pH 5. Different xylan concentrations (between 0.1 % and 1.5 %) were tested and it was concluded that the optimum xylan concentration, where xylanase activity reached its maximum value, was 1 % (Fig. 2). When the effect of reaction time on enzyme activity was investigated, it was concluded that xylanase activity was maximum at 50 min (Fig. 3). Also, experiments carried out at different temperatures showed that the optimum temperature for xylanase activity was 60°C (Fig. 4).

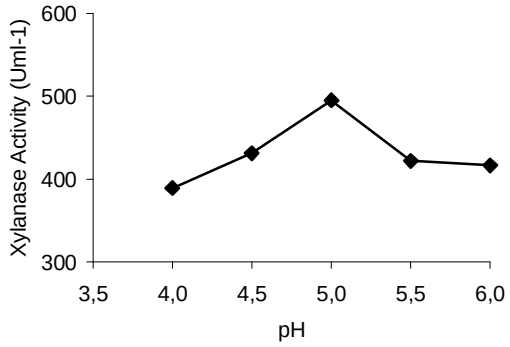

Figure 1 - Effect of pH on xylanase activity

Since *T. harzianum 1073 D3* xylanase is an enzyme with a potential technological significance, its stability is of great importance. In this respect, enzyme stability analyses produced in liquid and semi-solid state media were carried out. The results are presented in Fig. 5. For example, in liquid medium the enzyme kept 90% of its original activity after 2 h at 50°C and more than 65% at 60°C. Experimental results revealed that thermal stability of semi-solid state culture was higher than the liquid culture, similar to a previous study (Deschamps and Huet, 1985). Therefore, semi-solid state medium should be preferred in applications where high temperature stability was desired.

Similarly, the stabilities of xylanase enzyme at different pH values for the two different cultivation media were studied. It was concluded that enzyme produced in liquid medium was more stable when compared to the semi-solid state medium from pH point of view. The results are presented in Fig. 6. For example, the enzyme produced in liquid medium was relatively stable in the pH range 3.0-7.0 and kept 95% of its original activity after 24 h at room temperature (Fig. 6).

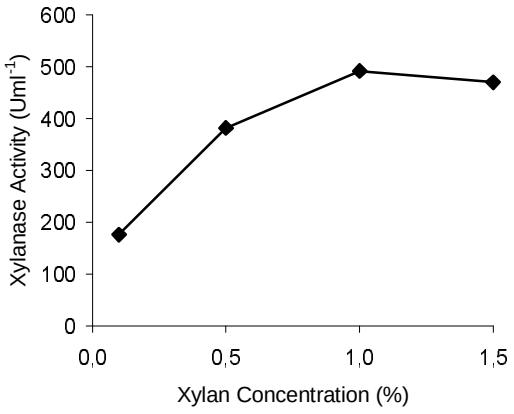

Figure 2 - Effect of xylan concentration on xylanase activity

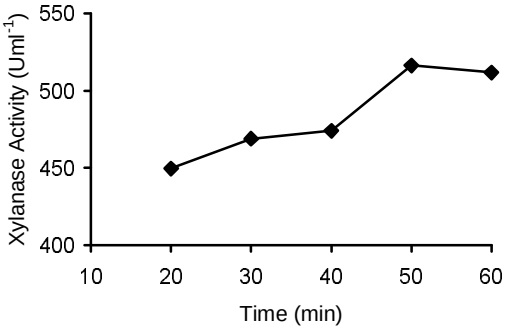

Figure 3 - Effect of reaction time on xylanase activity

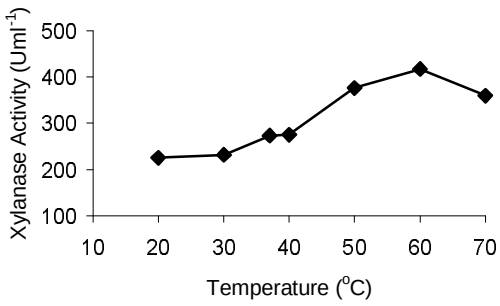

Figure 4 - Effect of temperature on xylanase activity

Referring to the potential use of the xylanase enzyme in pulp and paper industry, some metal ions occurring in pulp were included in this study. Among those metal ions, Mn^{2+}, Ca^{2+} and Zn^{2+} enhanced the xylanase activity whereas K^{2+} reduced the activity. Mg^{2+} and Cu^{2+} had no significant effect on the activity (Table 1).

In order to investigate the effect of sugars on the activity, six different sugars (glucose, galactose, fructose, xylose, lactose and sucrose) were studied. Among these, xylose was found to be increasing the enzyme activity slightly, whereas others decrease the activity (Table 1).

The results of the stability analysis revealed that the activity of the enzyme produced in liquid medium did not decrease for 30 days. The activity decreased to 89 % of the original activity after 45 days and 70 % after 75 days. It is a major advantage of this enzyme that it kept more than 50% of its original activity even after 4 months, which increased its importance in industrial applications (Fig. 7).

Table 1 - Activation and/or Inhibition of Xylanase Activity

Metal ions	Xylanase Activity (%)	Carbon Sources	Xylanase Activity (%)
none	100.0	none	100.0
Mn	107.6	glucose	76.4
Zn	103.9	galactose	75.8
Cu	99.9	xylose	103.2
Ca	108.6	fructose	81.0
K	96.6	lactose	96.7
Mg	99.8	sucrose	83.1

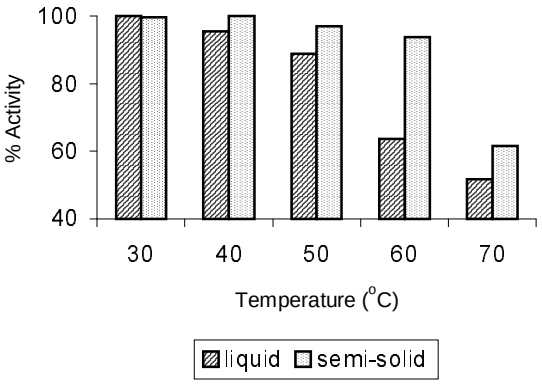

Figure 5 - Enzyme thermostability

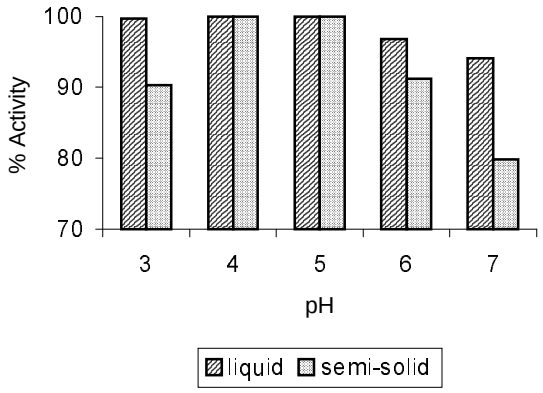

Figure 6 - Enzyme pH stability

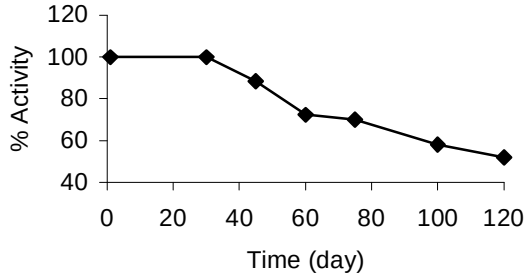

Figure 7 - Enzyme stability (liquid medium)
** Enzyme was kept at 4°C and activity was determined at 37ºC for 30 min.*

DISCUSSION

In order to maximize enzyme production and decrease production costs, substrates such as wheat bran are used to produce xylanase enzyme from different fungi. In similar studies, solid fermentation was used especially with *Trichoderma sp*. mainly due to economic reasons (Atev et al., 1987; Deschamps and Huet, 1985). Our experimental results revealed that xylanase activity was higher in liquid production medium. However, it should be noted that production time for the liquid medium was 13 days and during this period many batches could be carried out in semi-solid state medium. Therefore, both production media have their own advantages and disadvantages.

Our studies revealed that the xylanase activity was maximum around pH 5. In studies carried out with *Trichoderma sp.*, it was also concluded that the most suitable pH value for xylanase activity was 5 (Dekker, 1983; Gomes et al., 1992; Royer and Nakkas, 1991). The optimum pH was also around 5 in similar experiments on fungal xylanases (Milagres et al., 1993; Gandhi et al., 1994; Sigoillot et al., 2002). In some other studies, in order to determine xylanase activity generally pH was kept around 5 (Bailey et al., 1992). But optimum pH value was found out to be 3 in a study carried out with *T. reesei Rut C-30* (Couchon and LeDuyt, 1985). Results of similar experiments in literature reveal that the optimal pH for bacterial xylanases is, in general, slightly higher than the pH optima of fungal xylanases, which is parallel with our findings (Pham et al., 1998; Subramaniyan et al., 2000; Sá-Pereira et al., 2002).

Our results on optimum xylan concentration were similar with the results of the previous studies (Atev et al., 1987; Leathers et al., 1984; Deschamps and Huet, 1985; Keskar et al., 1989; Royer and Nakas, 1991; Gomes et al., 1993).

When the effect of reaction time was investigated, it was observed that xylanase activity was maximum at 50 min. As the enzyme-substrate interaction period increases, the amount of products also increases up to a certain point. There are reports on variation in incubation time for the determination of xylanase activity. This was due to the fact that microorganisms and xylan sources were different (Royer and Nakas, 1991; Deschamps and Huet, 1985; Atev et al., 1987; Dekker, 1983; Gomes et al., 1993).

Optimum temperature for xylanase activity was found out to be 60°C. Similarly, in other studies carried out with *Trichoderma sp*. it was concluded that the optimum temperature varied between 45 and 60°C (Dekker, 1983; Gomes et al., 1992). Besides, for fungal xylanases the optimum temperature was 60°C (Chandra and Chandra, 1995; Chandra and Chandra, 1996; Gandhi et al., 1994).

Similar to our findings, experimental results of Ghanem et al. for *Aspergillus terreus* (Ghanem et al., 2000) and Cesar and Mrsa for *T. lanuginosus* (Cesar and Mrsa, 1996) showed that Ca^{2+} enhanced xylanase activity. Mn^{2+} and Zn^{2+} ions had also positive effect on xylanase activity (Castro et al., 1997; Cesar and Mrsa, 1996).

RESUMO

Neste estudo algumas condições que afetam a atividade da enzima xylanase produzida a partir de *Trichoderma harzianum* 1073 D3 foram determinadas. A estabilização do pH e temperatura em cultivo líquido e semi-sólido foram avaliados. Foi concluído que para o máximo de atividade xylanase foi obtida com 1% de xylano a temperatura de 60ºC e pH 5.0.

Estudos de estabilidade demonstraram que a enzima foi relativamente estável na faixa de pH entre 3-7 e retém mais do que 50% de sua atividade original após 4 meses.

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