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CHARACTERIZATION OF ALKALINE XYLANASES FROM *BACILLUS PUMILUS*

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ABSTRACT

Alkaline xylanases produced by four different strains of *Bacillus pumilus* were characterized. The optimal pH and temperature were pH 9.0 and 60ºC for strain 13_a, and pH 8.0 and 55ºC for strains 5_2, 5_{14}, and 4_a. Under these conditions the following activities were found after 10 min in the presence of 1% xylan (birchwood): 328 $U.ml^{-1}$, 131 $U.ml^{-1}$, 90 $U.ml^{-1}$, and 167 $U.ml^{-1}$, respectively, for the four strains. The enzymes were stable at 40ºC, with 40% of the xylanase activity remaining after 2 hours for the enzymes of strain 5_2 and 60% for the other three strains. Stability at 50ºC was improved by addition of glycerol. Taking into account the conditions under which kraft pulps are bleached during the manufacture of paper, xylanases from *B. pumilus* exhibit favorable potential for application to bleaching in the paper making process.

Key words: xylan, xylanase, *B. pumilus*, kraft pulp bleaching

INTRODUCTION

Bleaching in the kraft papermaking process consists in decolorizing and removing the highly colored residual lignin from washed pulp. Most present bleaching sequences are based on elemental chlorine (Cl_2), chlorine dioxide, and alkaline extraction of the pulp (19). Viikari *et al.* (21) reported on the ability of xylanase to facilitate subsequent chemical bleaching of kraft pulps, known as xylanase prebleaching. This results in a lower chlorine dosage, a lower chemical cost, and lower chloro-organic concentrations in pulp and effluent. This discovery led to extensive further studies (20, 22).

Although many bacteria and fungi have been studied for xylanase production (1, 3, 14, 18), several xylanases commercially available are active at a neutral or acidic pH and their optimum temperature for activity is below 45ºC. Enzymes which are active under alkaline conditions have great potential for industrial applications as a bleaching process without any need for changes in pH or temperature (18).

In previous investigations (4, 7, 8, 9) we had isolated four microorganisms, identified as different strains of *B. pumilus,* which were able to produce xylanase under alkaline conditions (8). The bacteria are aerobic, Gram-positive, catalase-positive and oxidase-negative, and rod-shaped. Although the strains were identified as *B. pumilus* using the criteria in Bergey's Manual of Systematic Bacteriology, differences were observed in morphology and in some tests that don't have an effect on the final results, but which indicate that they belong to different strains. All microorganisms are capable of growing at 40ºC; however, the 13_a strain grows at 55ºC. Enzyme production was observed at pH 8.0 to 11.0, but higher levels were observed at pH 10.0, which are appropriate conditions for the bleaching process. In this report, the characterization of xylanases produced by *B. pumilus* is described. The enzymatic assays were performed at pH and temperature ranges close to those at which kraft pulps are bleached.

MATERIALS AND METHODS

Microorganism.

B. pumilus strains 5_2, 5_{14}, 13_a, and 4_a were isolated from wood decomposition material and were maintained in a

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previously described media (12) containing xylan as the carbon source, which contained (g/L): birchwood xylan (Sigma), 10.0; peptone, 1.0; Tween 80, 1.0; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; urea, 0.3; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; agar-agar, 20.0; and solutions of the following salts (mg/L): $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; and $CoCL_2$, 2.0. The pH was adjusted to 10.0 with 2 N NaOH. The cultures were grown at 45ºC for 48 hours.

Inoculum preparation. The cultures were transferred to 250 ml Erlenmeyer flasks, containing 50 ml of the liquid media described above, and incubated at 45ºC in a rotary shaker (250 rpm) during 20 hours. Cell concentrations of the different cultures were adjusted to 3% T (transmittance) in relation to distilled water at 600 nm.

Enzyme production.

The initial number of cells for fermentation was equivalent to $10^8.ml^{-1}$. For xylanase production, the bacteria were grown in the media described, with optimized xylan and peptone concentrations for each strain as shown in Table 1. These concentrations were determined by means of response surface methods in a previous study (7). Fermentation was carried out in shake flasks under the same inoculum conditions (20 h, 45ºC, and 250 rpm). The fermented media was centrifuged for 15 min at 12000 x g to the assay activity.

Enzyme activity assay.

Xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8. xyl) activity was assayed using birchwood xylan 1% solution as the substrate, as described by Bailey *et al.* (2), and the amount of reducing sugars released was determined by the dinitrosalicylic acid method (13).

Filter paper cellulase (FPase) activity was assayed as an indicator of overall cellulolytic activity and determined according to IUPAC recommendations (10), using Wathman No. 1 filter paper (50 mg) as a substrate in 100 mM glycine-NaOH buffer, pH 10.0.

One unit of enzyme activity was defined as 1µmol of xylose or glucose equivalents produced per minute under the given conditions.

Birch xylan solution.

A 1g sample of birch xylan in 80 ml of 100 mM buffer, as indicated subsequently, was heated to boiling, cooled by stirring, diluted to 100 ml with buffer and kept at –20ºC.

Effect of pH and temperature on xylanase activity.

The effect of pH on xylanase activity was studied in the following buffers (100 mM): sodium phosphate, pH 8.0; glycine-NaOH, pH 9.0 and pH 10.0; and carbonate-bicarbonate, pH 11.0. The reaction mixture containing 0.9 ml of xylan solution and 0.1 ml of the crude enzyme preparations was incubated at 35-55ºC or until activity declined, and enzyme activity was determined for different times.

Effect of temperature and pH on xylanase stability.

The remaining xylanase activity was determined after preincubation of the crude enzyme preparations at 30-60ºC in buffers of optimal activity, without substrate, for 1, 2, 4 and 6 h. The effect of pH on xylanase stability was measured over the pH range of 8.0 to 11.0 at a defined optimal temperature. After incubation, residual activity was determined under optimal assay conditions for each strain.

Protein measurement.

Protein concentration was measured by the method of Sedmak and Grossberg (16). Bovin serum albumin was used as a standard. The results were used to calculate specific activity.

Kinetic determinations.

K_m and $V_{máx.}$ values were determined from Lineweaver-Burk plots (11), using xylan concentrations varying from 0.5 to 3.0% (w/v).

RESULTS AND DISCUSSION

Previous results

Throughout preliminary studies were used the original Mandels and Stenberg medium (12) that contains 1% xylan. In order to determine the ideal concentrations of xylan and peptone for maximum xylanase production, was used response surface analysis (7). The optimization study was relevant because with this medium, the levels of enzymes could be increased as compared to a conventional medium. Moreover, the strains showed different requirements for xylan or peptone, as shown in Table 1.

Table 1. Ideal concentration of xylan and peptone in the fermentation media, and xylanase activity for different strains of *B. pumilus* obtained from wood material decomposition. (Tests carried out at 45ºC and pH 10).

B. pumilus strains	Optimized Medium			Original Medium*
	xylan (g/l)	Peptone (g/l)	xylanase activity ($U.ml^{-1}$)	xylanase activity ($U.ml^{-1}$)
5_2	30.0	6.0	33	3
5_{14}	58.3	6.0	9	2.5
13_a	10.0	10.0	23	1.5
4_a	50.0	10.0	25	4

* xylan = 10 g/l; peptone = 1.0 g/l

Was also defined that the time for enzyme production must be around 16h, corresponding to log phase half. After 20 h, the cells start fast sporulation, resulting in cellular lysis. When cellular lysis occurs, proteases are liberated in to the medium with subsequent enzyme hydrolysis.

Finally, in our previous studies the molecular weight of the main protein bands of crude enzyme preparation was estimated to be about 78,900, 63,800, 24,500 and 15,500 Da (9).

Cellulase production from* B. pumilus *strains

Fpase activity was assayed as an indicator of overall

cellulolytic activity in crude enzyme preparations, obtained from cultivations at pH 10.0 and 45ºC. The cellulolytic activity found was lower than 0.01 UPF/ml for all strains.

Xylanase solutions for industrial uses must be cellulase-free. Contaminating cellulase in commercial xylanase preparations can result in a loss of fiber strength (15). When cellulase activity is very low, culture filtrate can be used for treating pulp without further purification (23). Our results indicate that enzymes produced by *B. pumilus* strains meet this requirements.

Enzyme characteristics. Effects of temperature and pH on activity and stability

Crude xylanases from *B. pumilus* strains were tested for the effect of pH and temperature on activity. Initially, activity was determined after incubation for 5, 10, and 20 min. Data obtained after 10 min are shown in Table 2. Maximum xylanase activity was found after 10 min at pH 9.0 and 60ºC for strain 13_a (328 $U.ml^{-1}$), and pH 8.0 and 55ºC for strains 5_2, 5_{14} and 4_a (131 $U.ml^{-1}$, 90 $U.ml^{-1}$ and 167 $U.ml^{-1}$, respectively). At pH 10.0, which was used during initial assays, enzymatic activities were very low and showed little variation in the temperature range studied. At pH 11.0, xylanase activity decreased rapidly, reaching insignificant levels.

Several reports have revealed that the optimum pH for the activity of xylanolytic enzymes produced by other bacterias does not usually exceed pH 7.0, as in the case of enzymes from *Bacillus* sp BP-23 (5), *Bacillus* sp (23) and *Thermoanabacterium* sp (17). In these cases, maximum enzymatic activity was observed, at pH 5.5 to 7.5, pH 7.0, pH 6.0 to 7.5, and pH 5.4, respectively. These bacteria were able to produce active enzymes at temperatures between 45ºC and 60ºC, and only *T. maritima* produces xylanases which are highly thermostable at 95ºC. Comparable enzymatic levels obtained in this study for *B. pumilus* 5_2 (482 U/ml) and 4_a (393 U/ml) were observed for *T. maritima* (585 U/ml) and *Thermoanabacterium* sp (393 U/ml). However, the optimum pH range for xylanase activity of *B. pumilus* was higher (pH 8.0 to pH 9.0). The latter are more suitable for application of enzymes at different stages of the bleaching process without the need for changes in pH.

Table 2. Effects of temperature and pH on the xylanolytic activity of *B. pumilus* strains after 10 min of incubation.

pH	strain	Enzyme Activity ($U.ml^{-1}$)						
		35ºC	40ºC	45ºC	50ºC	55ºC	60ºC	65ºC
8	5_2	47	66	86	118	131		
	5_{14}	37	48	62	80	90		
	13_a	144	181	189	220	241		
	4_a	80	102	123	145	167	156	
9	5_2	42	63	79	100	95	108	
	5_{14}	28	37	50	58	66	79	
	13_a	135	157	211	279	325	328	351
	4_a	57	79	95	100	108		
10	5_2	9	13	15	15	10		
	5_{14}	27	17	22	18	16		
	13_a	42	56	66	92	52		
	4_a	22	33	50	41	30		
11	5_2	1	1	1	0.1	0.1		
	5_{14}	3	4	3	3	3		
	13_a	3	1	0.4	1	0.4		
	4_a	3	3	1	3	3		

After the enzymes were preincubated at several temperatures and pHs during different periods of time, the xylanase activity tests were carried out under the optimal conditions determined for each *B. pumilus* strain. According to results, the enzymes were reasonably stable at 40ºC. The enzymes from *B. pumilus* 5_2 retained around 40% of their original activity after 2 h, while 60% was retained by the other strains (Fig. 1a). However, activity decreased gradually over time, with 30% of the activity remaining for strain 5_2, 50% for strain 5_{14} and 40% for strains 13_a and 4_a after 6 h (results not shown).

At 50ºC and 60ºC the enzymes were denaturated very rapidly, which is not appropriate for industrial purposes. However, according to literature, this problem is solved by an addition of a 50% w/w glycerol solution (1). Our results confirmed this statement since the stability of the xylanases from *B. pumilus* strains 5_2, 13_a and 4_a could be improved by glycerol, and there were increases from 8% to 56%, from 15% to 36% and from 25% to 66%, respectively, after 2 h at 50ºC for these strains (Fig. 2). On the other hand, this protective effect of glycerol was not observed in enzymes from *B. pumilus* 5_{14}.

The results obtained after incubation of the enzymes at different pH values did not show variations in xylanase activity after 2 h at pH 8.0 and pH 9.0 for enzymes of the several strains (Fig. 1b). However, at pH 10.0 the activity decreased rapidly and inactivation was observed at pH 11.0.

Kinetic determinations

Kinetic parameters of the four *B. pumilus* xylanases in birchwood xylan are summarized in Table 3. The crude enzymes produced from different bacterial strains showed quite different K_m and V_{max} values for the same substrate under the conditions studied. Substrate concentration is one of most important factors which determine the velocity of enzyme reactions. The xylanases from *B. pumilus* 5_2 and 5_{14} required lower substrate concentration to reach the V_{max} for catalysis, whereas the strains 13_a and 4_a required higher substrate concentration (Table 3). Therefore, considering the V_{max} attained by the enzymes, the xylanases from *B. pumilus* 13_a and 4_a show a higher catalytic power, and consequentetly could show a higher technology efficience.

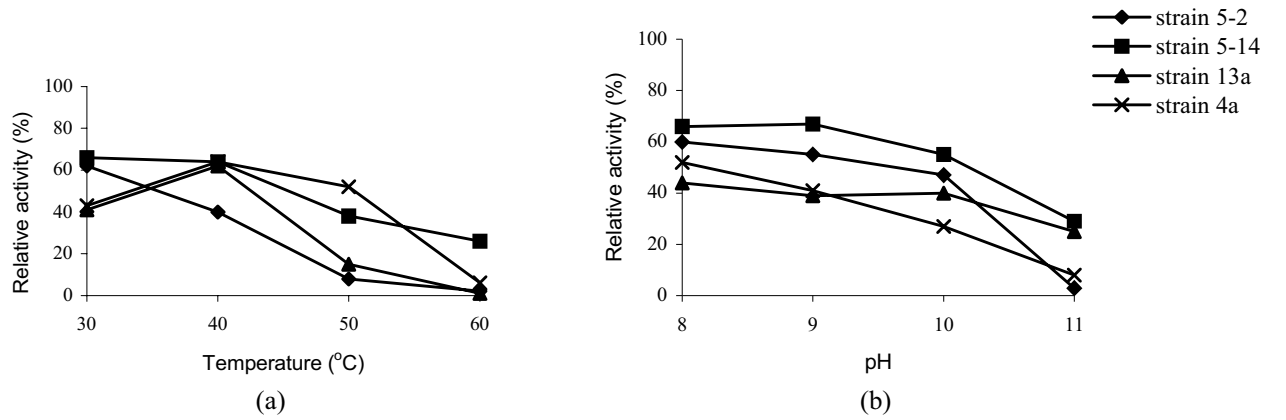

Figure 1. Xylanase activity remaining after incubation for 2 h at different temperatures **(a)**, and after 2 h at different pH values **(b)**.

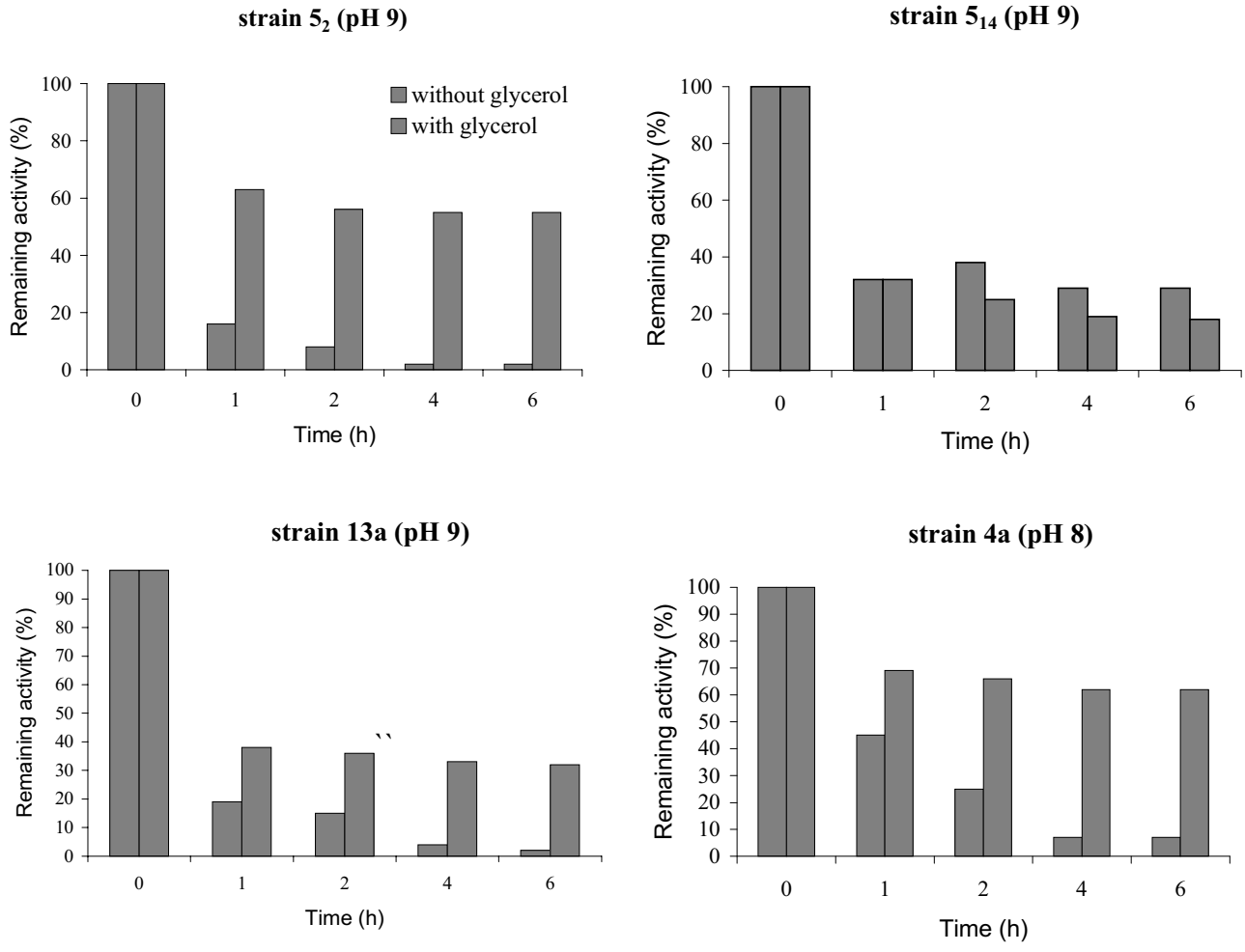

Figure 2. Effect of 50% glycerol on xylanase stability at 50ºC on birch xylan of enzymes from different *B. pumilus* strains.

Table 3. Kinetic parameters of *B. pumilus* xylanases in birchwood xylan.

B. pumilus strains	pH	Temperature (ºC)	K_m (mg/ml)	$V_{máx.}$ (µmol/ml/min)
5_2	9.0	55	8.9	178.57
5_{14}	9.0	55	1.1	112.36
13_a	9.0	60	33.3	1666.67
4_a	8.0	60	71.4	1428.57

CONCLUSIONS

The conditions used in our study on the activity and stability of xylanases from *B. pumilus* strains were based on the temperature, pH and time used in the kraft pulp bleaching. Taking into account industrial conditions such as dosage between 2 and 5 units (U) per gram of dry pulp, alkaline pH, temperature around 50ºC and 2 h for reaction (6), the enzymes from *B. pumilus* exhibit favorable potential for application to the bleaching of kraft pulps.

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RESUMO

Caracterização de xilanases alcalinas de *Bacillus pumilus*

Xilanases alcalinas produzidas por quatro diferentes linhagens de *Bacillus pumilus* foram caracterizadas. O pH e temperatura ótimos para máxima atividade enzimática foram pH 9.0 e 60ºC para o isolado 13_a, e pH 8.0 e 55ºC para os isolados 5_2, 5_{14} e 4_a. Nessas condições, as seguintes atividades foram encontradas após 10 min na presença de 1% de xilana (bétula): 328 $U.ml^{-1}$, 131 $U.ml^{-1}$, 90 $U.ml^{-1}$ e 167 $U.ml^{-1}$, respectivamente, para os quatro isolados. As enzimas foram estáveis à 40ºC, com 40% de atividade remanescente de xilanase após 2 horas para as enzimas do isolado 5_2 e 60% para os outros três isolados. Estabilidade a 50ºC foi melhorada com a adição de glicerol. Considerando-se as condições em que as polpas kraft são branqueadas durante a fabricação de papel, as xilanases de *B. pumilus* mostraram potencial favorável para aplicação no branqueamento no processo de fabricação de papel.

Palavras-chave: xilana, xilanase, *B. pumilus,* polpas kraft branqueadas

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