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Production and Characterization of Alkaline Xylanases from *Bacillus* sp. Isolated from an Alkaline Soda Lake

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Abstract: An aerobic, alkaliphilic, xylanolytic bacterium was isolated from local Soda lake. The strains were isolated on xylan agar medium and screened by xylanolysis method. The bacterial strains (St_A, St_B, St_C and St_D) were identified based on morphological, biochemical and physiological characters. On the basis of these tests, the four strains were identified as belonging to the genus *Bacillus*. Alkaline Xylanases produced by four different strains of *Bacillus sp*. were also characterized. The optimal pH and temperature were pH 8.0 for and 50°C St_A and pH 8.5 and 55°C for strain St_D and pH 9.0 and 55°C for strains St_B and St_C. Under these conditions the following activities were found after 10 min in the presence of 0.7% oat spelt xylan; 302, 233, 189 and 207 U ml^{-1}, respectively, for the four strains. The enzymes were stable at 40-50°C, with 45% of the xylanases activity remaining after 2 hours for the enzymes of strain St_B and 55% for the other four strains. Taking into account the conditions under which Kraft pulps are bleached during the manufacture of paper, Xylanases from *Bacillus sp*. exhibit favorable potential for application to bleaching in the paper making process.

Key words: Xylan, xylanases, *Bacillus sp*., kraft pulp bleaching

INTRODUCTION

Biodegradation of xylan, a major component of plant cell walls, requires action of several enzymes, among which xylanases play a key role[1]. A wide variety of microorganisms are known to produce xylan-degrading enzymes. In recent years, important applications for xylanases in different industrial processes have been found. One major area of application is for the bleaching of Kraft pulp in the pulp and paper industries[2]. The use of Xylanases prior to the normal bleaching operation has been shown to significantly reduce the amount of chlorinated organic compounds formed during the bleaching process[3], thus reducing the risk of environmental pollution. Since the Kraft process of pulp and paper making is carried out at alkaline pH and high temperature, the use of alkaline xylanases with higher temperature, the use of alkaline xylanases with higher temperature optima is considered to be advantageous[4]. On the other hand, in the process of enzyme-assisted pulp bleaching, the incoming pulp has a higher temperature and an alkaline pH[5], making the use of thermostable alkaline xylanases very attractive. Recently the interest in xylanases has markedly increased due to the potential applications in pulping and bleaching processes using cellulase free preparations, in the food and feed industry, textile processes, the enzymatic saccharification of lignocellulosic materials and waste treatment[6]. From the application point of view, xylanases active and stable in the alkaline pH and at elevated temperature are very important. Most alkaliphilic and alkalitolerant microorganisms produce xylanases optimally active around neutrality[7].

Until now there have been very few reports on the production of alkaline xylanases[8]. Many xylanases-producing alkaliphilic microbial strains have been reported from different laboratories. However, the xylanases from most of these alkaliphilic strains have their optimum pH around neutrality[9]. The majority of alkaliphiles known so far were isolated from neutral soil samples. On the other hand, naturally occurring alkaline habitats are found scattered in different parts of the world[5]. Such habitats are expected to harbour novel micro-organisms that are adapted to living at alkaline pH. Extracellular enzymes produced by such organisms are likely to have their optimum pH for activity in the alkaline range. Such enzymes may find important application in different industrial processes. Until now there has been very little effort to isolate alkaliphiles from naturally occurring alkaline habitats. In this present study, the production of an alkaline xylanases by an alkaliphilic *Bacillus sp*. isolated from an alkaline soda lake and the properties of the crude enzyme are reported.

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MATERIALS AND METHODS

Microorganism: *Bacillus sp.* strains St_A, St_B, St_C and St_D, were isolated from soda water lake at Rajshahi Industrial Area and were maintained the medium as follows: xylan, 7 g l^{-1}, yeast extract, 1 g l^{-1}; NaCl, 5 g l^{-1}; K_2HPO_4, 1 g l^{-1}; $MgSO_4$, 0.2 g l^{-1}; $CaCl_2$, 0.1 g l^{-1}; and Na_2CO_3, 10 g l^{-1}. Sodium carbonate was sterilized separately and added to the rest of the medium to adjust the pH 9.0. The cultures were grown at 37°C for 48 h.

Isolation and screening: Sample from Soda lake in sterilized water were poured and spread onto agar plates A. These plates were incubated at 37°C for 2 days. The colonies that were found on the plates were transferred onto agar plates B, which were again incubated at 37°C for 2 day. Several xylanases-producing bacterial colonies were selected in the medium described above which produce clear zone in the plate. All colonies showing a clear zone on agar plates were further screened by growing them in liquid medium and assaying enzyme activity from the cell-free culture supernatant fluid.

Characterization of the bacterium: The morphological properties and taxonomic characteristics of the bacterium were studied by the methods in Bergey's manual of Systematic bacteriology[10]. The isolated bacterium was an aerobic, gram-positive, motile, spore-forming, rod-shaped organism. It showed a positive reaction for the production of catalase. Acid was produced from D-glucose, D-xylose and D-mannitol. Based on these characteristics, the bacterium was identified as belonging to the genus *Bacillus* according to Sneath[10].

Enzyme production: The medium (50 ml in 250 ml Erlenmeyer flasks) was inoculated with 1 ml of an overnight culture and incubated at 37°C with vigorous aeration in a shaker at 150 rpm for 2 days. Before assay, the cells were separated by centrifugation at 5000 g. The clear supernatant was used as crude enzyme preparation.

Partial purification of the xylanases: The cell-free supernatant fluid was precipitated with the addition of solid ammonium sulphate to 80% saturation. After centrifugation the pellet was suspended in a minimum volume of 50 mm glycine- NaOH buffer, pH 9 and dialysed against three changes of the same buffer. The dialysed crude enzyme preparation was used for all subsequent studies.

Enzyme activity assay: Xylanases (1,4-β-D-xylan xylanohydrolase EC 3.2.1.8. xyl) activity was assayed using oat spelt xylan 1% solution as the substrate as described by Bailey *et al.*[11] and the amount of reducing sugars released was determined by the dinitrosalicylic acid method[12]. One unit of enzyme activity was defined as 1μ mol of xylose equivalents produced per minute under the given conditions. Filter paper cellulase (FP ase) activity was assayed as an indicator of overall cellulolytic activity and determined according to IUPAC recommendations[13] using Wathman No. 1 filter paper as a substrate in 50 mM glycine-NaOH buffer, pH 10. One unit of enzyme activity was defined as 1 μ mol of xylose or glucose equivalents produced per minute under the given conditions.

Oat spelt xylan solution: A 1 g sample of oat spelt xylan in 80 ml of 50 mM buffer, as indicated subsequently, was heated to boiling, cooled by stirring, diluted to 100 ml with buffer and kept at 4 °C.

Effect of temperature and pH on xylanase activity: The reaction mixture containing 0.9 ml of xylan solution and 0.1 ml of the crude enzyme preparations was incubated at 30-80°C or until activity declined and enzyme activity was determined for different times. The effect of pH on xylanases activity was studied in the following buffers (50 mM): sodium phosphate, pH 7.0; Tris- HCL buffer 8.0; glycine-NaOH, pH 9.0 and 10.0.

Effect of temperature and pH on xylanase stability: Thermal stabilities of the xylanases were determined by pre-incubating the xylanases for 1 h at various temperatures ranging from 30 to 80°C. After the incubation, the reaction mixtures were cooled on ice for 5 min. Finally the residual activities were measured by dinitrosalicylic acid. The effect of pH on xylanases stability was measured over the pH range of 7.0 to 10.0 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 Lowary *et al.*[14]. Bovine serum albumin was used as a standard. The results were used to calculate specific activity.

Kinetic determinations: K_m and V_{max} values were determined from Lineweaver-Burk plots[15], using xylan concentrations varying from 0.5 to 3.0% (w/v).

Materials: D-Xylose and Dinitrosalicylic acid were obtained from Wako Pure Chemicals Industries Ltd. Oat spelt xylan was obtained from Fluka Co. Ltd. Bovine serum albumin was purchased from Sigma Chemicals Co.,U. S. A. Other chemicals were of analytical grade.

RESULTS

The organism: Xylanases from *Bacillus sp*. were identified by the previous studies[20] of our laboratory. Here the alkaline xylanases-producing *Bacillus* strains were isolated from Soda lake by screening procedure from their colonical growth as clear zone and xylanolytic properties on xylan agar plate (Fig. 1). The xylanases activity in each strain was confirmed by measuring the amount of reducing sugars liberated from xylan by the dinitro-salicylic acid (DNS) method using crude extract. It grows at pH 8-9 and produced a high level of xylanases activity both in solid and liquid media. The organism was rod-shaped, gram-positive, aerobic, motile and catalase-positive.

Enzyme production: Xylanases produced by *Bacillus* were growth-associated, reaching a maximum after 20 h. Enzyme production remained more or less the same up to 50 h while biomass started to gradually decline after 40 h. A high level of enzyme produced was observed when the organism was grown in media containing oat spelt xylan, wheat bran and birchwood xylem as carbon sources (Table 1). A significant amount of xylanase was also produced when starch, sucrose, arabinose, glucose and xylose were used as carbon sources (Table 1). However, compared with xylan and wheat bran, these sugars induced lower xylanases activity.

Cellulose production from *Bacillus sp*.: Filter paper cellulose activity was assayed as an indicator of overall cellulolytic activity in crude enzyme preparations, obtained from cultivations at pH 9.0 and 45°C. The cellulolytic activity found was lower than 0.001 UPF/ml for all strains. Xylanases solutions for industrial uses must be cellulase-free. Contaminating cellulase for in commercial Xylanases preparations can result in a loss of fiber strength[16]. When cellulase activity is very low, culture filtrate can be used for treating pulp without further purification[4]. Our results indicate that enzymes produced by *Bacillus* strains meet this requirements.

Enzyme characteristics

Effects of temperature on activity: Crude Xylanases from *Bacillus sp*. 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 xylanases activity was found after 10 min at pH 9.0 and 55°C for strain St_B (302 U ml^{-1}) followed by St_C, St_D and St_A (233, 189 and 207 U ml^{-1}). At pH 10.0, Xylanases activities decreased. Several reports have revealed that the optimum pH for the activity of xylanolytic enzymes produced by other bacteria does not usually exceed pH 7.0, as in the case of enzymes from *Bacillus sp*.[4] and *Thermoanabacterium*[17].

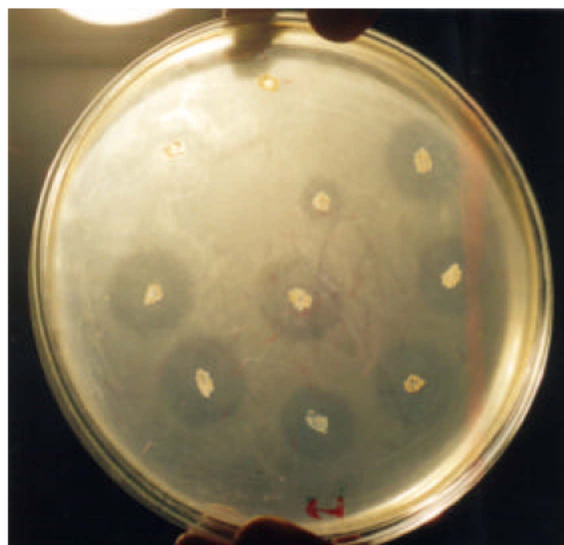

Fig. 1: Clear zone on xylan agar plate by xylanases degrading bacteria

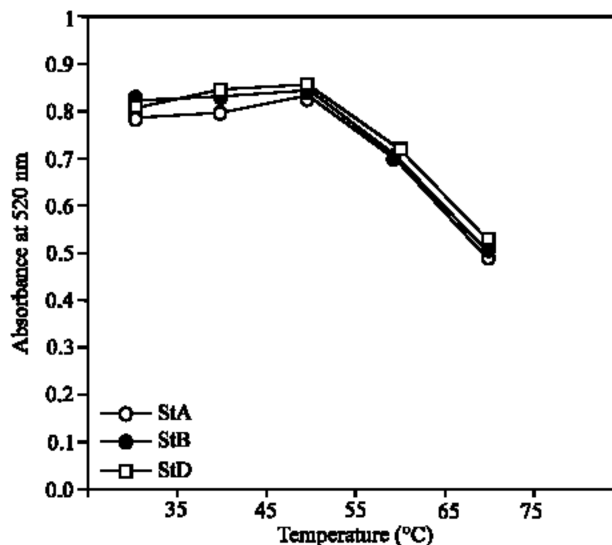

Fig. 2: Effect of temperature on Xylanases stability. The enzyme was incubated for 1 h at various temperatures and the residual activities were measured at 37°C

Effects of temperature and pH on activity and stability: Crude xylanases from *Bacillus* strains were stable at temperature 40-80°C shown in Fig. 2. At 80°C, xylanases lost 60% of activity. Crude xylanases from *Bacillus* strains were stable at pH 7-pH 9, when the pH stability was measured at pH 6 to pH 9 (Fig. 3).

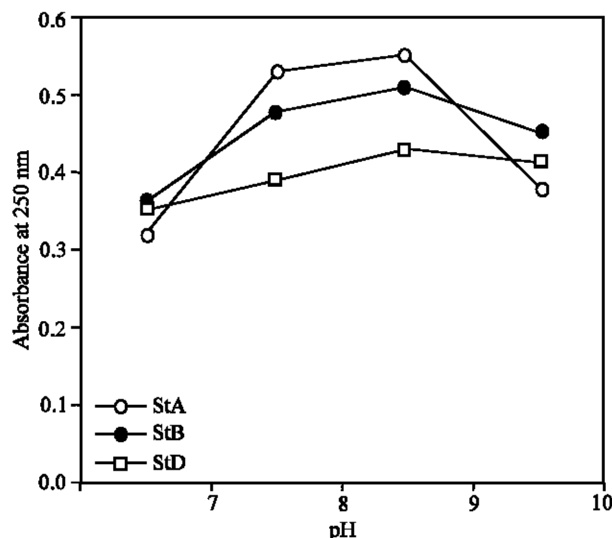

Fig. 3: Effect of pH on Xylanases stability. The enzyme was incubated for 1 h at various pH and the residual activities were measured at 37°C

Table 1: Effect of different carbon sources on xylanases production by *Bacillus sp.*

Sugar	Xylanases activity (U ml^{-1})
Oat spelt xylan	48
Birchwood xylan	37
Wheat bran	45
Starch	22
Sucrose	15
Arabinose	11
Xylose	5

Table 2: Effects of temperature and pH on the xylanolytic activity of *Bacillus sp.* strains after 10 min of incubation

		Enzyme activity (U. ml-1)					
pH	strain	35°C	40 °C	45°C	50°C	55°C	60°C
7	St_A	9	12	15	17	19	8
	St_C	26	18	23	25	28	11
	St_B	43	57	69	74	87	21
	St_D	24	35	51	55	63	19
8	St_A	48	67	88	117	132	92
	St_C	38	49	64	83	95	56
	St_B	114	113	119	122	141	99
	St_D	80	100	115	123	130	88
9	St_A	44	69	85	102	207	81
	St_C	30	64	70	98	233	79
	St_B	134	160	213	279	302	92
	St_D	59	89	98	103	124	68
10	St_A	2	3	3	4	5	1
	St_C	5	4	5	5	4	1
	St_B	4	4	3	6	7	3
	St_D	2	3	4	4	5	2

Table 3: Kinetic parameters of *Bacillus* xylanases in oat spelt xylan

Bacillus strains	pH	Temperature (°C)	Km (mg/ml)	Vmax (μmol/ml/min)
St_B	9.0	50	9.0	180
St_C	9.0	50	1.3	114
St_D	9.0	55	35	1668
St_A	9.0	55	72	1515

Kinetic determinations: Kinetic parameters of the four *Bacillus* Xylanases in oat spelt xylem 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 *Bacillus* St_B and St_C required lower substrate concentration to reach the V_{max} for catalysis, whereas the strains St_D and St_A required higher substrate concentration (Table 3). Therefore, considering the V_{max} attained by the enzymes, the Xylanases from *Bacillus* St_D and St_A show a higher catalytic power and consequently could show a higher technology efficience.

The Xylanases-producing alkaline bacterial strains *Bacillus sp.* were isolated from the Soda lake by screening procedure from their colonical growth as clear zone and xylanolytic properties on xylan agar plate. *Bacillus sp.* produced a high level of xylanases in the presence of oat spelt xylan and wheat bran as carbon sources. The xylanases that are thermostable and have their optimum pH in the alkaline range are considered to have good potential for application in the pulp and paper industry. This is because the use of such enzymes is expected to greatly reduce the need for costly pH and temperature readjustments before enzyme addition. Most xylanases known so far have their optimum pH around neutrality. Even xylanases produced by most alkaliphiles reported to date have their optimum pH around neutrality[18]. Nakamura *et al.*[19] reported the first alkaline xylanases produced by *Bacillus sp.* strain 41M-1 which had an optimum temperature and pH of 55°C and 9.0, respectively. Yang *et al.*[4] isolated an alkaliphilic *Bacillus sp.* VI-4 from a hard wood Kraft pulp which produced a xylanases having a pH optimum of 6-8.5 and a temperature of 55°C. The xylanases from *Bacillus* were a novel enzyme, being active at alkaline pH with an optimum at pH 9 and was stable over a broad pH range. It showed optimum activity at 55°C and good stability at 50-55°C at alkaline pH values. These are desirable properties for application in the pulp and paper industry as well as in other industrial processes. Further study of the enzyme might give information about the molecular basis of stability and activity of xylanases at alkaline pH and elevated temperature.

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