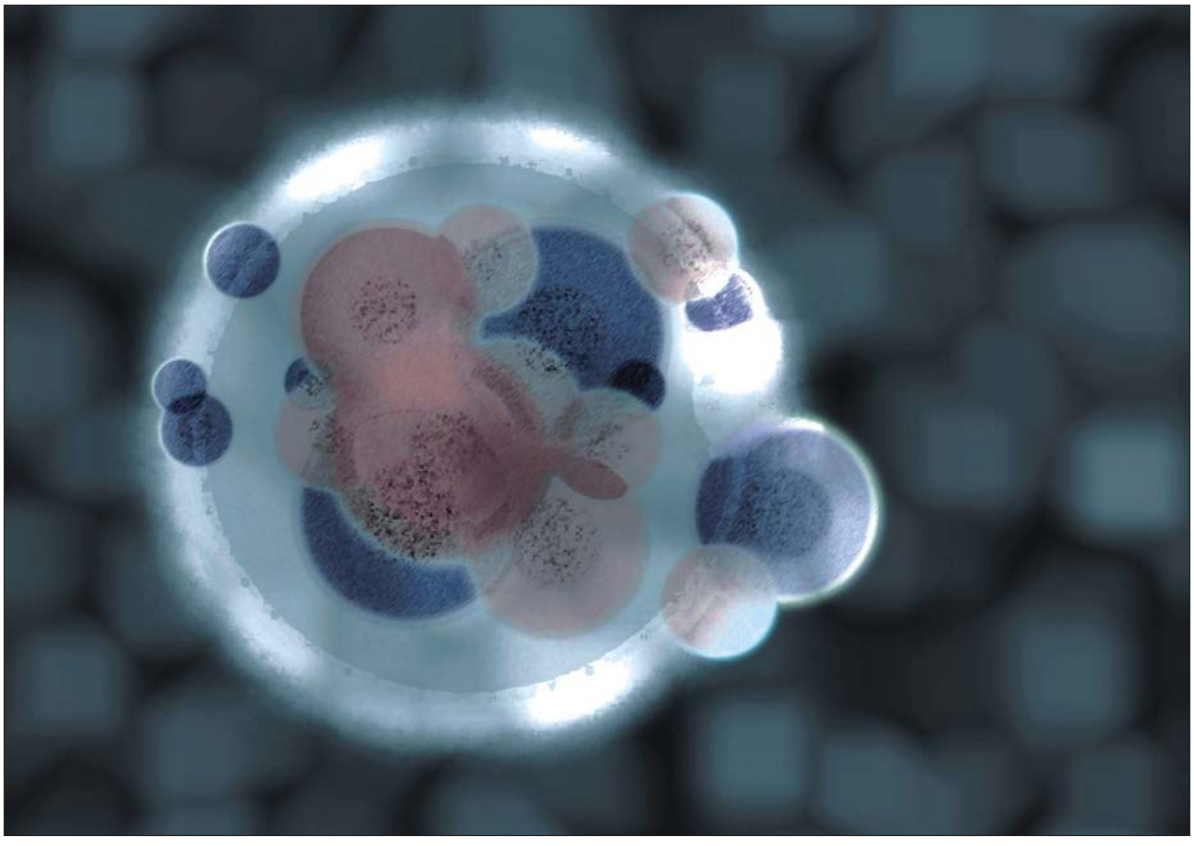

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Isolation and Some Properties of New Xylanase from the Intestine of a Herbivorous Insect (Samia Cynthia Pryeri)

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Abstract: Xylan the major portion of the hemicellulose of plant cell walls are heterogeneous polysaccharides. Xylanases are enzymes obtained from different species of microorganisms that degrade the xylosidic linkages of xylans backbone producing xylose with other monoresidues. In this study, xylanase producing strains were isolated from intestine of a herbivorous insect at Rajshahi University Campus. The strains were isolated on xylan agar media and screened by ß-xylanolysis method. Zymogram analysis was confirmed the xylanolytic activity. The strains was identified to be *Aeromonas sp.* and xylanase enzyme was detected in the culture supernatant of the strain. Xylanase enzyme was purified from culture supernatant of *Aeromonas sp.* by ammonium sulfate precipitation, gel filtration on Sephadex G-100 followed by ion exchange chromotography on DEAE-cellulose. In DEAE cellulose column chromatography, three protein peaks F-1a, F-1b and F-1c were appeared. Among these peaks, only F-1b showed xylanase activity and the degree of purification attained 64.54 fold. The purified enzyme gave single band on SDS-polyacrylamide gel electrophoresis indicating its homogeneity. The enzyme gave maximum activity against xylan as substrate at pH 7.0 and temperature at 55° C. The Km value of xylanase was found to be 0.91% using the oat spelt xylan as substrate. The xylanase hydrolyzed strongly oat spelt xylan and birch wood xylan but didnot hydrolyze cellulose, carboxymethyl cellulose and starch. Xylose was detected as the hydrolysis products of oat spelt xylan by the xylanase.

Key words: *Aeromonas sp.*, xylanase, zymogram, Km

INTRODUCTION

Plant cell walls consist of three major components: cellulose, hemicellulose (xylan) and lignin. Herbivorous and xylophagous insect intestines contain various symbiotic microorganisms. The role of these microorganisms in the digestion of food (wood compounds) in the intestine of the insects has been postulated (Mannesmann, 1972). The evidence has been given by isolating bacteria producing cellulases and lignalolytic enzymes from the intestines of these insects. In addition, these isolated bacteria have been tested for their ability to produce cellulases and lignalolytic enzymes in vitro. Recently, the degradation of hemicelluloses present in food was done by hemicellulases (xylanase and mannanse) and that gut microorgamisms were solely responsible for the production of these enzymes. Hong TY and Meng M (Hong, 2003) isolated the microorganism from garden soil in Taiwan.

Xylans are polysaccharides composed of β-1, 4-lined xylopyanose units. They are mostly highly branched and in tight association with other biopolymers. Xylans belong to the major constituents plant cell walls. As the most abundant hemicelluloses, they accout for more than 30% of the dry weight of terrestrial plants. Xylans thus belong to the main food source of farm animals and also represent a major component of the raw material for many industrial processes ranging from baking to paper production.

Several enzymes are involved in the hydrolysis of xylan polymers, of which the most important are the endo-1, 4 – β-xylanases (EC 3.2.1.8) (Reilly, 1981). These enzymes degrade xylan to short-chain xylo-oligosaccharides of verying lengths. They can be grouped into two families F and G based on hydrophobic cluster analysis and sequence homology, corresponding to families 10 and 11 in the numerical classification of glycosyl hydrolaes. Xylanases are already use feed and food industry as well as in pulp and paper industry and they have a potential in future industrial processes modifying plant matter for various applications (Biely, 1985). By using organic solvents the direction of reaction can be reversed, this makes possible the synthesis of complex carbohydrates in a very specific manner. The use of enzymes in industrial processes offers great advantages. Often the process can be run with less chemicals under less harsh conditions and with less disturbing side reactions. However, experimental evidence on xylanase production and xylanase activities by these microorganisms have not yet been investigated. So, our

laboratory, investigations were focused on herbivorous insects. An arabino-xylan degrading strain was isolated from larvae of a leaf-eating insects, *Samia cynthia pryeri* and identified as *Aeromonas sp*.

In the present study, it was found that xylanases are produced extracellularly by Intestine of a Herbivorous insect. The present study describes the purification and characterization of xylanase enzyme from selected strain.

MATERIALS AND METHODS

Collection of bacterial sample: For the screening xylanase-producing bacteria cowdungs (herbivorous insect) were collected from the Rajshahi University Campus.

Media and Culture conditions: Nutrient agar media, MacConkey agar media and Xylose lysine Deoxycholate agar were used as a solid medium through out the work. Xylan agar plates were used for the isolation and identification of the suspected bacteria and the bacteria were cultured at 37°C.

Isolation and Characterization of bacteria: All the xylanase-producing bacterial strains which were isolated by their growth on xylan agar media as clear zones and xylanolytic properties were characterized according to the biochemical tests described in the "Bergey's Manual of Determinative Bacteriology".

Biochemical tests used to characterize the isolated strains

Catalase test: Catalase test was performed to determine the ability of the organism to liberate gas (O_2) from hydrogen peroxide (H_2O_2) by enzymatic (catalase) degradation.

Oxidase test: Oxidase is an enzyme generally found in aerobes. The redox dye, tetramethyl-para-phenyline-diamine-dihydrocholoride was reduced to a deep purple colour by the microorganism.

Urease test: The enzyme urease produced by certain bacteria is capable of decomposing urea to ammonia which causes alkaline reaction. Urease test requires Christensen's urea agar media containing peptone 1.0 g, sodium chloride 5.0 g, potassium dihydrogen phosphate 2.0 g, glucose 1.0 g, phenol 6.0 ml and agar 20 g.

Citrate utilization test: The test organism was cultured in a medium which contains sodium acetate, an ammonium salt and the indicator bromophenol blue.

Fermentation test: This test is used to differentiate the organisms that ferment carbohydrate. Oxidation fermentation medium containing peptone 2.0 g, sodium chloride 5.0 g, dipotassium hydrogen phosphate, bromothymol blue 3 ml and agar 2.5 g is used in the fermentation test of carbohydrates.

Purification steps of xylanase

Preparation of crude enzyme extract: Cultivation of xylanolytic bacteria to extract xylanase 100 ml of xylan broth was taken in a 250 ml conical flask, a single colony of the isolated strain was inoculated in the xylan broth media in the conical flask by sterile loop and incubated at 37°C for 48 hours with slow shaking. When the strain grown vastly this culture was transferred into 500 ml volumetric flask and clear supernatant was collected after centrifugation at 8000 X g for 15 minutes at 4°C.

Ammonium sulfate precipitation: The clear supernatant was collected and adjusted to (100%) saturation by adding solid ammonium sulfate [$(NH_4)_2SO_4$]. The ammonium sulfate precipitates were then centrifuged at 8000 X g for 15 min at 4°C. The precipitate was collected and dissolved in minimum volume of deionized water and dialyzed against 50 mM sodium phosphate buffer pH 7.0 for 24 h at 4°C. After centrifugation, the clear supernatant was used as crude enzyme extract and insoluble materials were discarded.

Gel filtration: The crude extract was after dialysis was applied to Sephadex G-100 column (1.5X50 cm) which was equilibrated with 50 mM sodium phosphate buffer at pH 7.0 containing 1M NaCl. The column was washed with 300 ml of the same buffer at a fiow rate of 15 ml h^{-1}. 2 ml fractions were collected. The enzyme passed through the column and separated with three peak.

DEAE-cellulose chromatography: The active fraction from Sephadex G-100 column chromatography were pooled, concentrated and dialyzed against 50 mM sodium phosphate buffer, pH 7.0 and then put on a DEAE-cellulose column (4.0 X 15 cm) equilibrated with 50 mM sodium phosphate buffer at pH 7.0. The column was washed by five bed volumes of the same buffer and then elution was done with a step wise of sodium phosphate buffer, pH 7.0, from 0 M to 0.25 M. Xylanase activity was detected in the second peak, which was eluted at about 0.1 M sodium phosphate buffer, pH 7.0.

Assay for xylanase activity: Xylanase activity was assayed by measuring the amount of reducing sugars liberated from oat spelt xylan in 50 mM sodium phosphale

buffer pH 7.0 at 55° C as described in the section 2.3.3. The reducing sugars formed were measured by the Dinitro-salisylic acid (DNS) method (Miller 1972). One unit of xylanase was defined as the amount of enzyme which liberated 1 μmole of xylose per minute.

Determination of protein concentration: Protein concentration was determined following the method of Lowry et al. (Lowry, 1951) using BSA as the standard.

Electrophoresis: Protein-containing fractions were analyzed by Slab electrophoresis with SDS polyacrylamide gel as described by Laemmli method (Laemmli, U. K., 1970). The stacking and resolving gels were 5 and 12% acrylamide, respectively. Proteins were stained with Coomassie brilliant blue R-250.

Zymogram analyses: Zymogram analyses were done by the method of John et al. (John, 1990).

Effect of temperature on the activity of xylanase: The activity of the purified xylanase was determined at different temperature ranging from 20°C to 80° C using oat spelt xylan as substrate.

Effect of pH on the activity of xylanase: The activity of purified xylanase was determined at different pH values (50 mM sodium acetate buffer at pH 4.0 to 5.5, 50 mM sodium phosphate buffer at pH 6.0 to 8.0 and 50 mM Tris-HCl buffer at pH 8.5 to 9.0) ranging from 4.0 to 9.0.

Determination of substrate specificity on xylanase activity: To determine the substrate specificity of xylanase towards substrates including birch wood xylan, cellulose, carboxy methyl-cellulose, starch were used as substrates in the assay instead of oat spelt xylan.

Determination of K_m Value of xylanase: The initial velocity is equal to the amount of product formed per unit time. The initial velocity (Vi) is determined by quantitatively measuring the amount of one of the products at various time intervals (Robyt and White, 1990).

Thin-layer chromatography (TLC): Hydrolysis products produced by the action of xylanase were characterized by TLC using Merck Silica Gel with the solvent system of butanol-2-propanol-water-acetic acid (7:5:4:2, v/v). The sugars on the plates were detected by heating at 110° C for 30 min after they were sprayed with a mixture of aniline-diphenylamine-acetone-85% phosphoric acid (0.4:0.3:20:3, w/w).

Chemicals: D-xylose was obtained from Wako Pure Chemicals Industries Ltd. Sephadex-G100, DEAE-cellulose were obtained from Fluka Chemicals, Ltd, Switzerland. Bovine serum albumin was purchased from Sigma Chemicals Co., U. S. A. Other chemicals were of analytical grade.

RESULTS

Isolation and characterization of xylanase-producing bacteria: For a preliminary experiment of our study, bacterial samples were collected from intestine of a herbivorous insect soil. β-xylanolytic clear and transparent zone (on the xylan agar plate) producing bacterial strains were collected according to the method described in the materials and methods section. When β-xylanolytic was observed on the xylan agar plate after 36 to 48 h incubation at 37° C, β-xylanolytic bacterial strains were collected and were then further purified by pure culture technique. Isolated bacterial colonies from herbivorous insect were primarily screened by staining method, microscopic examination, oxidase and calalase test. After primary isolation, several other morphological and biochemical tests were performed to characterize the isolated bacteria. It was found that the strains were xylanolytic and were found to be *Aeromonas* by final identification. The best growth and clear zone production of the bacteria was observed on xylan agar plate, peptone water without NaCl and nutrient agar plate supplemented with starch for 24 h incubation at 37° C. On xylan agar plate, the colonies were circular, transparent and the color of the colonies was white. The results of various physicochemical tests of the isolated xylanase-producing *Aeromonas* strains have shown in the Table 1.

Purification of xylanase

Gel filtration: The ammonium sulfate (100%) precipitated of the crude enzyme extract from the bacteria was dialyzed against 50 mM sodium- phosphate buffer, pH 7.0 , was applied on to the column of

Table 1: Biochemical and physiological characteristics of the isolated strain, *Aeromonas*

Characteristic	Result
Growth in nutrient broth at 37°C	+
Growth in KCN broth	+
Fermentation of mannitol and lactose	+
Gram staining	+
Xylanase activity	+
Indole	+
Oxidase	+
Catalase	+
Urease	+
Citrate utilized	--
Gas from glucose	--
Growth in peptone water without NaCl	+
Motility	+

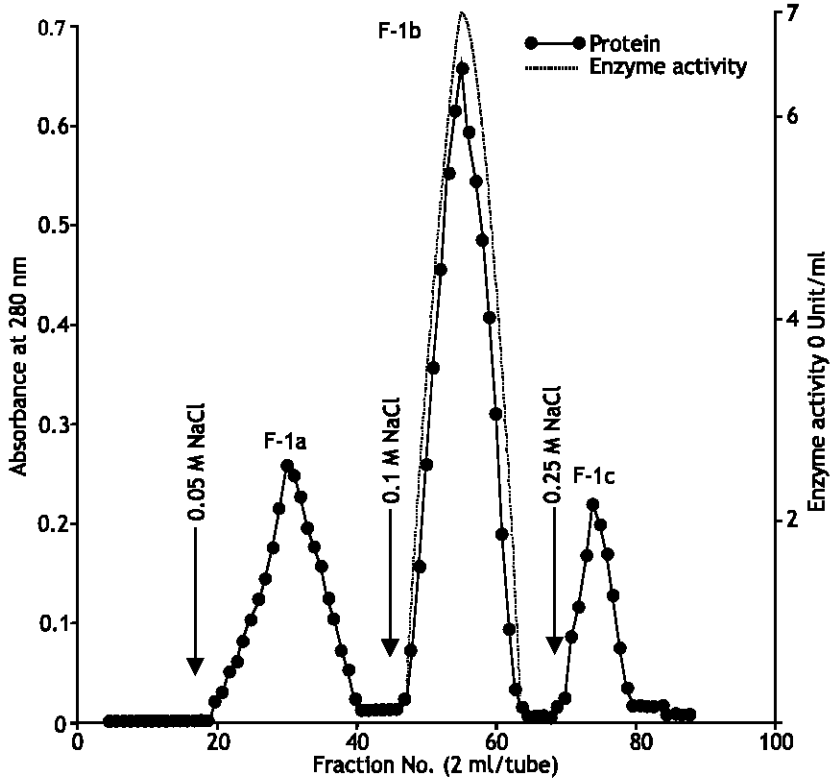

Fig. 1: Ion exchange chromatography of F-1 fraction (7.79 mg protein) on DEAE-cellulose column at 4°C. Size of column: (1.5x28cm), Buffer: 50 mM phosphate buffer, p[11] 7.0, Flow rate: 25 ml h^{-1}

Table 2: Summary of purification of xylanase

Steps	Total Protein (mg)	Total activity (units)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	475.00	650.00	1.37	100.00	1.00
Ammonium sulfate saturated	120.00	375.00	3.13	57.69	2.28
After gel filtration on Sephadex G-100	7.79	315.00	40.43	48.46	29.51
DEAE-cellulose ion exchange chromatography	1.27	112.72	88.45	17.34	64.56

Table 3: Substrate specificity of xylanase

Substrate	Relative hydrolysis activity (%)
Oat spelt xylan	100
Birch wood xylan	111
Cellulose	0
Carboxymethyl cellulose	0
Starch	0

sephadex G-100 which was previously equilibrated with 50mM sodium- phosphate buffer, pH 7.0. at 4° C. The protein was eluted from the column with the same buffer. The crude protein extract was eluted as two main peaks namely F-1 and F-2 (Data not shown). It was found that only F-1 fraction contained the xylanase activity while the F-2 peak showed very low activity. Hence F-2 fraction was not used for further investigation. The active fraction F-1 pooled separately and their purity was checked by slab gel electrophoresis. As shown in the Fig. 2, F-1 fraction gave more then one band indicating that it contained some other proteins.

DEAE- cellulose chromatography of F-1 fraction: All the active fractions (peak F-1) from gel filtration combined together and concentrated and was dialyzed against 50 mM sodium phosphate buffer, pH 7.0 for over night. After centrifugation, the clear supernatant was applied to DEAE-cellulose column, which was previously equilibrated with the same buffer. The protein was eluted step-wisely with increasing concentration of the NaCl with the same buffer. As shown in (Fig. 1), the components of F-1 fraction were separated into three peaks F-1a, F-1b and F-1c with the buffer containing 0.05M, 0.1M, 0.25M NaCl gradients respectively. The enzymatic activity of all these fraction were investigated and it was found that only the fraction F-1b contained xylanase activity while the fraction F-1a, and F-1c possessed no xylanase activity. The active fraction (F-1b) from DEAE-cellulose chromatography were homogenous on slab gel electrophoresis and showed single protein

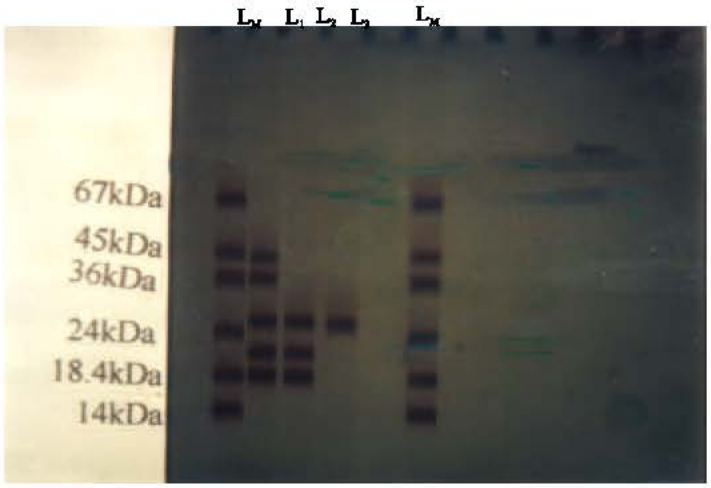

Fig. 2: Photographic representation of SDS-Polyacrylamide slab gel electrophoresis of xylanase during purification steps on 10% gel. Lane 1: crude encyme extract, Lane 2: fraction from gel filtration, Lane 3: (Purified xylanase) fraction from DEAE-cellulose column, Lane M: Molecular mass markers: Lysozyme (14KDa), β-lactoglobulin (18.4 kDa), Trypsinogen (24 kDa), pepsin (36 kDa) Albumin (C. egg white)-45 kDa, Albumin (BSA), 67 kDa

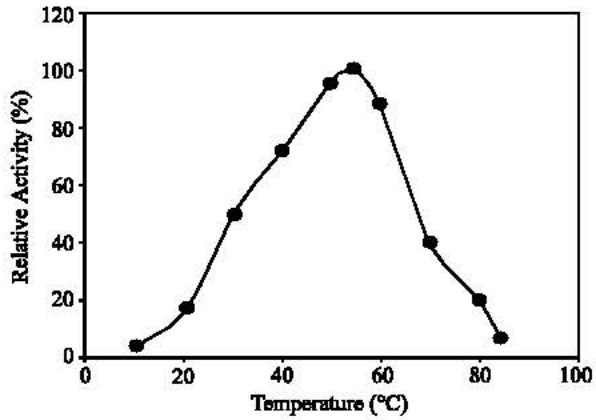

Fig. 3: Effect of temperature on the activity of xylanase. Enzyme solution in 50 mM phosphate buffe pH 7.0 were incubated at various temperatures for 10 min and the activities were measure as described in Materials and Method. The highest activity was expressed as 100%

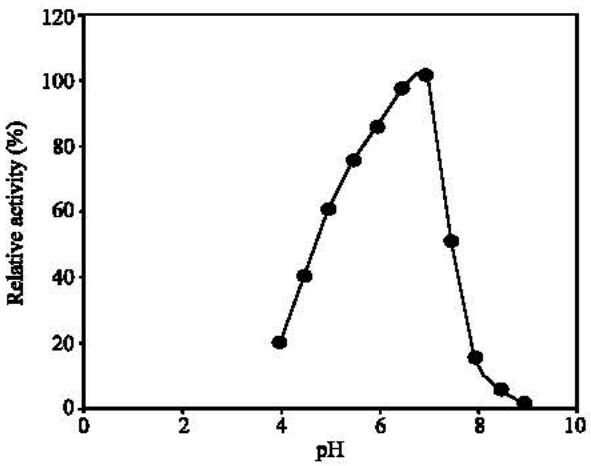

Fig. 4: The effect of pH on the activity of xylanase. The buffers were 50 mM Na-acetate buffer pH 4.0-5.5, 50 mM Na-phosphate buffer pH 6-8.0, 50 mM Tris-HCL buffer pH 8.5-9.0. The highest activity was expressed as 100%

band (Fig. 2). At the final steps, the specific activity and purification fold of the purified xylanase was 88.45 U/mg of protein and 17.34 respectively (Table 2).

Effect of temperature on the activity of xylanase: The effect of temperature on activity of xylanase against xylan was examined in the temperature range of 20° C –90°C. The enzyme showed maximum activity around 50-55°C. From the graph it was shown that optimum temperature was observed at 55°C. With further rise of the temperature the activity of the enzyme was decreased more sharply and 94% of the activity was destroyed at 85°C (Fig. 3). The xylanase obtained from *Aeromonas sp* strain no. 212 showed maximum activity at 50°C reported by Dung *et al.* (1991). Xylanase from *Aeromonas caviae* W-61 showed maximum activity at 55°C was repoted by Kilunga, *et al.* (1991).

Effect of pH on the activity of xylanase: To determine the optimal pH of the enzyme activity against xylan, 50 mM sodium acetate buffer at pH 4.0 to 5.5 and 50 mM sodium phosphate buffer at pH 6.0 to 8.0

Table 4: Some Properties of xylanases from bacterial strains

Strain	Molecular mass (kDa)	Optimum		Metal ions		K_m	Reference
		pH	temperature	Activator	Inhibitor		
Aeromonas sp. Strain no C_3	24.5	7.0	55	Ca^{2+},Mg^{2+},Fe^{2+}	Hg^{2+}, EDTA	9.1 mg ml^{-1}	This Study
Aeromonas sp. Strain no –212	23	5.0-7.0	60	ND	ND	ND	Dung *et.al.* (1991)
Aeromonas caviae W-61	22	7.0	55	ND	ND	ND	Dung *et al.* (1991)
Aeromonas caviae ME-1	20.1	7.0	50	ND	ND	9.4 mg ml^{-1}	Kilunga *et al.* (1991)
Bacillus sp. Strain 41M-1	36	9.0	50	Ca^{2+}, $Fe^{2+}Zn^{2+}$	Hg^{2+}, N-bromosuccinomide	3.3 mg ml^{-1}	Satoshi N *et al.* (1995)
Fibriobactor succinogenes S 85	53.7	7.0	39	C^{a2+},Mg^{2+}	Hg^{2+} EDTA	2.6 mg ml^{-1}	Allan. M *et al.* (1992)
Bacillus sp. Strain-W-1	21.5	6.0	65	Fe^{2+},Ca^{2+}	Hg^{2+},Cu^{2+}	4.5 mg ml^{-1}	Okazaki *et al.* (1985)
Bacillus Pumilus strain IPO	24	6.5	45-50	ND	ND	ND	Panbangred *et al.* (1985)

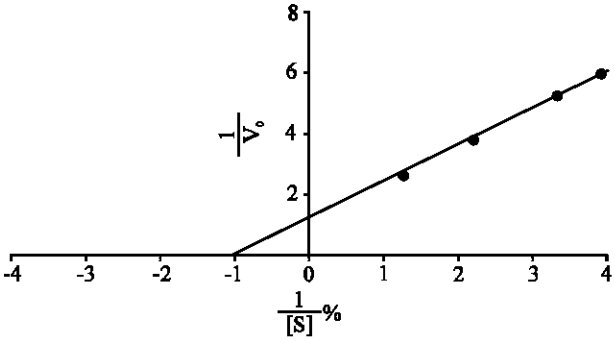

Fig. 5: A Lineweaver-Burk double reciprocal plot for the determination of K_m value of purified xylanase enzyme

and 50 mM Tris-HCl buffer at pH 8.5 to 9.0 were used. The optimal pH of the enzyme activity was 7.0 when the activity was measured over a pH range from 4.0 to 9.0 (Fig. 4). The xylanase from *Aeromonas sp* strain no 212 showed maximum activity at pH 5 7. reported by Dung, *et al.* (1991). *Aeromonas caviae* ME-1 produced xylanase exhibited maximum activity at pH 7.0 reported by Kilunga, *et al.* Xylanase from *Bacillus sp* W1-2 showed maximum activity at pH 7.0~9.0 reported by Wataru Okazakaki *et al.* (1985). Allan Matte *et al.* (1992) reported that xylanases (1 and 2) produced by *Fibrobactor succinogenes* S85 showed optimum activity at pH 7.0 and 6.3 respectively.

Substrates specificity on xylanase activity: The substrates specficity of purified xylanase was studied using various polysaccharides as the substrates and the results obtained were summarized in the Table 3. Purified xylanase hydrolyzed strongly oat spelt and birch wood xylan. Howover, xylanase did not hydrolyze cellulose, carboxymethyl cellulose, starch. Thus it was a true xylanase.

Determination of Km of xylanase: A Lineweaver-Burk's double reciprocal plot of the data showed the Km value for xylanase enzyme against oat spelt xylan as substrate to be 0.91% in Fig. 5. Kilunga *et al.* (Kilunga, 1991) reported a Km value of 0.94% at pH 7.0 for *A. caviae* ME-1.

Analysis of enzymatic hydrolysis products from oat spelt xylan by TLC: Analysis of reducing sugars by xylanase acting on oat spelt xylan showed that the final products was only xylose. Under prolonged incubation, the quantities of xylose increased (Data not shown). These results indicate that xylanase of *Aeromonas* was endoxylanases.

DISCUSSION

In our study, we have isolated bacterial strains from Intestine of a Herbivorous insect, which degraded β-1, 4 xylans and to belong to *Aeromonas* genus. In the primary step of our study, the xylanase-producing bacteria were isolated from the collected samples by screening procedure from their colonical growth as clear zone and xylanolytic properties on xylan agar plate and zymography. The xylanase activity in each strain was confirmed by measuring the amount of reducing sugars liberated from xylan by the DNS method (Miller, 1972) using crude extract. After isolation of xylanase-producing strains, the strains was identified as *Aeromonas* on the basis of catalase, oxidase, gram-staining reaction, morphological and cultural characteristics and different

biochemical tests. Under microscope the cells were arranged in singly, in pairs or chains, the cells were straight, rod shaped with rounded ends to coccoid. They were motile and gram-negative in staining reaction. They grew well on simple laboratory medium in the temperature ranges from 35-41°C and optimally at 36°C. Then xylanase was purified to homogeneity from culture fluid of *Aeromonas sp.* The molecular mass of purified xylanase was estimated to be 25 kDa on SDS-PAGE under reducing conditions. Similar result was obtained on xylanase from *Bacillus sp.* XE (Dedeire-Gosselin, 1992) and the molecular mass was 22kDa. The specific activity of purified xylanase was 88.45 and the purification fold was 17.34. The xylanase had no activity on cellulose, carboxymethyl cellulose, or starch; thus they are true xylanases. These properties were also found in the reported xylanase 1 (Dung, 1991) and other xylanases from *B. pumillus* (W. Panbangred, 1983) and *B. substilis* R. Bernier, 1983). Xylanase from *Aeromonas sp.* was endoxylanases and cleaved oat spelt xylan randomly to form xylose. These properties are similar to xylanase V from *Aeromonas caviae* ME-1 (Kubata, 1993) but different xylanase from *Aeromonas sp.* (Ohkoshi, 1984). Table 4 shows some properties of xylanase from A. caviae W-61 from *Aeromonas sp.*, *Bacillus sp* and *Fibriobactor succinogenes.* Table 4 also shows some properties of xylanase from *Aeromonas sp.* and those from *Aeromonas sp.* including molecular mass, optimum pH, optimum temperature. Among various xylanases of *Aeromonas sp.* and *Bacillus sp.* reported previously, some differences were found with molecular weight, optimal temperatures, pH and metal ions for the enzyme activity. So, it is concluded that the xylanase isolated from intestine of herbivorous insect is new type of xylanase.

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