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Characterization and Identification of Xylanase Producing Bacterial Strains Isolated from Soil and Water

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Abstract: In the present study, the four bacterial strains were isolated from soil and water at Rajshahi region. The strains were isolated on xylan agar media and screened by xylanolysis method. The bacterial strains (St_1, St_2, St_3 and St_4) were characterized and identified based on morphological, biochemical and physiological characters. On the basis of these tests, the four strains were initially identified as belonging to the genus *Bacillus*. *Bacillus* strains were capable of growing in xylan medium at pH 7.0 and produced 55 IU (μ mol xylose min^{-1} ml^{-1}) of xylanase when cultivated in neutral medium at pH 7.0. Maximal enzyme activity was obtained by cultivation in oat spelt xylan, but high enzyme production was also obtained on wheat bran. With cultivation at pH 7.0 and assays performed in phosphate buffer, maximal activity was observed at pH 7.0; with phosphate buffer, maximal activity was between pH 6 and 7. The xylanase temperature optimum at pH 7.0 was 50°C. The enzyme could hydrolyze the oat spelt xylan and birch wood xylan effectively but did not hydrolyze cellulose, carboxymethyl-cellulose and starch. *Bacillus* strains also showed drug resistance to ampicilline, amoxycilline, cotromosilline and chlorophenical and sensitive to tetracycline and erythromycin. Plasmid DNA was isolated from the xylanase-producing multi-drug resistant strain. Agarose gel electrophoresis of the isolated plasmid DNA showed the presence of a plasmid of about 32 kb in size.

Key words: *Bacillus sp.*, xylanase, plasmid, antibiotic resistant

INTRODUCTION

Ligocellulose constitutes the major portion of plant cell walls and is mainly composed of lignin, cellulose and hemicellulose. Xylan, which comprises 20-35% by weight of wood and agricultural wastes, is the major component of the hemicellulose portion. Xylan consists of a backbone of β-1, 4-xylopyranoside residues which are commonly substituted with acetyl, arabinose and 4-O-methylglucuronose residues. A wide variety of microorganisms are known to produce xylan-degrading enzymes[1]. The enzymatic cleavage of β-1, 4-xylosidic linkages is performed by xylanases (β-1,4-xylan-xylanohydrolase, EC 3.2.1.8). Xylanases occur widely in bacteria, yeasts and fungi. Many microorganisms are known to produce different types of xylanases, the nature of the enzymes varies between different organisms. Many reports on xylanases from *Clostridium* sp.[2] *Streptomyces* sp.[3] *Aspergillus* sp.,[4] and other microorganism are available. A *Bacillus* sp. strain AR-009, an alkaliphile isolated from an alkaline soda lake reported by Amare Gessesse[5]. An alkaliphilic bacterium, also *Bacillus* sp. strain K-1, produces alkali-stable xylanases without cellulase and one of them has a strong affinity for insoluble xylan[6]. Xylanase have important applications in the pulp and paper industry[7]. They can also be used to increase the digestibility of animal feed stock and in the baking and brewing industries[8]. Xylanase may also be used to prepare materials for scientific research, besides being research materials themselves. Well-characterized xylanases may be useful for the characterization of polysaccharides and plant cell walls. Although many bacteria have been studied for xylanase production are alkaliphiles[9] only very few produced xylanase in neutral medium[10]. Recently, an alkali tolerant strain of *Bacillus sp.* BP-23 from rice field soil was isolated[11]. So, in this study, the characteristics of a *Bacillus sp.* reported that are isolated from soil and water. The organism is capable of growing M9-xylan medium at pH 7.0 and producing high level of xylanase when cultivated in neutral medium.

MATERIALS AND METHODS

Bacterial strain: *Bacillus* sp. used in this study, were isolated from the soil and water from the Rajshahi University Campus during the month of June and July in 2003.

Xylan broth: 5 gm of oat spelt xylan, 1 g sodium nitrate, ($NaNO_3$), 1.6 g disodium monohydrogen phosphate

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(Na_2HPO_4. $12H_2O$), 0.9 g potassium dihydrogen phosphate (KH_2PO_4), 0.5 g magnesium sulphate ($MgSO_4$. $7H_2O$), 0.5 g potassium chloride (KCl), 5 g polypeptone were dissolved in 800 ml of distilled water and pH was adjusted to 7.0. The volume was adjusted to 1000 ml with distilled water. The medium was sterilised by autoclaving.

Luria bertani (LB) broth: 10 g Bacto-trypton, 5 g bacto-yeast extract and 10 g sodium chloride were dissolved in 800 ml of distilled water and pH was adjusted to 7.0 by pH meter with 5N sodium hydroxide solution. This volume was adjusted to 1000 ml with distilled water. The medium was sterilized by autoclaving.

Peptone water (broth) without NaCl: 20 gm peptone was dissolved in 800 ml of distilled water and pH was adjusted to 7.0 with 5 N sodium hydroxide solution. The volume was adjusted to 1000 ml with distilled water. The medium was sterilized by autoclaving.

Nutrient agar media: 10 g bacto-trypton, 5g bacto yeast extract and 10 g sodium chloride were dissolved in 800 ml distilled water and pH of the medium was adjusted to 7.0. Then 15 g agar was added and the volume was made 1000 ml with distilled water and sterilized by autoclaving.

Bacterial sample collection: For the screening of xylanase-producing bacteria soil and water were collected from the University Campus. 0.2 g of soil and water were dissolved in 100 ml sterile distilled water in two separate 100 ml volumetric flask and shaked vigorously. 0.2 ml of diluted sample solution was then spread on two different xylan agar plates for isolation and rapid identification of the xylanase-producing bacteria from their colonical growth as clear one and xylanolytic properties. After incubation at 30°C for 2 days, colonies showing clearing zones on M-9 xylan medium were further purified. Cell suspensions were plated on the same medium using serial dilution with sterile water to obtain isolated colonies. Twenty five isolates were screened for xylanase activities by cultivating each in M-9 xylan medium for two days, the highest xylanase-producing strains (St_3) were selected for further investigation.

Isolation of bacteria: All the xylanase-producing bacterial strains were isolated by their growth on xylan agar media as clear zone and xylanolytic properties were characterized according to the biochemical tests described in the "Bergey's Manual of Determinative Bacteriology, Eighth edition, "Text book of" C. H. Collins and "Monica Cheesbrough".

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[12]. The isolated bacterium was an aerobic, gram-positive, motile, spore-forming, rod-shaped organism (0.7 μm by 2.9 to 4.6 μm). 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[12].

Enzyme detection: Xylanase activity was assayed by measuring the amount of reducing sugars liberated from xylan by the 3, 5-dinitrosalicylic acid method[13]. Briefly, 40 µl of the enzyme preparation was added to 160 µl of a 0.5% oat spelt xylan suspension in 100 mM sodium phosphate buffer (pH 7.0). The reaction was carried out at 3°C for 10 min. The enzyme was inactivated by adding 400 µl of the 3, 5 dinitrosalicylic acid reagent and boiling for 5 min. A_{520} was measured immediately after 2.5 ml of water was added to the mixture. One unit was defined as the amount of enzyme which produced reducing sugars equivalent of µ mol of xylose per min under the condition describe above.

Antibiotic resistance test: The antibiotic resistance of *Bacillus* strains were isolated from the selected strains containing of xylanolytic activity using the disc diffusion method of Bauer and Kirly[14]. A 16 h broth cultures of the strains when grown at 30°C and was spread on nutrient agar plate. Then the ampicilline, amoxycilline, cotrimoxazole tetracycline erythromycin and chlorophenical antibiotic discs were distributed in different conc. (25, 50 and100 µg ml^{-1}) on plates and kept the plates at 4°C for 4 h, so that the antibiotic can diffuse on the agar media. The plates were then incubated at 30°C for 16 h and the growth of the bacteria was observed. The presence of a clear zone around the disc was the index of sensitivity to the antibiotic. The absence of such a clear zone or the presence of some colonies within the clear zone indicated that the collected strains were resistant to that antibiotic.

This culture was then subjected for the extraction of plasmid DNA according to Holmes and Quigley. The extracted plasmid DNA was then purified with polyethylene glycol (PEG) according to Meyers *et al.*[15].

RESULTS

Morphological and colonial characterization: Colonies of isolates on nutrient agar and selective media

were circular, convex, translucent, yellow or white, dry or shining with a slimy mucoid consistency. All of the strains were grown colonies of isolates on nutrient agar and selective media were circular, convex, translucent, yellow or white, dry or shining with a slimy mucoid consistency. positive, short rods, non-sporing and motile by peritrichous flagella (Table 1).

Table 1: Colonial and morphological characteristics of the isolates

Tests/Strains	St_1	St_2	St_3	St_4
Colony characteristics:				
Growth at 48 h	moderate	abundant	abundant	moderate
Colony pigmentation	yellow	white	white	white
Colony type	dry	wet	dry	dry
Morphological characters:				
Gram strain	+	+	+	+
Cell Shape	short rods	straight rod	short rod	short rod

Biochemical tests: It was found that, five strains were xylanolytic and were found to be *Bacillus* by final identification. The best growth and clear zone production of the bacteria was observed on xylan agar plate, peptone water (broth) without NaCl and nutrient agar plate supplemented with starch for 24 h incubation at 30^0C. Most of the samples produced clear zone around the colonies on xylan agar plate. All isolates were catalase and oxidase positive and utilized aspartic acid as sole carbon source. The strains could not grow on a medium without N_2 source. The strains showed negative urease test because they are unable to decompose urea to ammonia, which causes alkaline reaction. The isolated strains showed negative citrate utilization test and showed positive indole test. Results of the biochemical test have shown in the Table 2.

Effect of culture conditions on growth and xylanase production: Figure 1 shows the growth and xylanase activity using 0.7% xylan as the substrate at pH 7.0 maximal xylanase activity was observed after 2 days of cultivation. Xylanase activity was assayed at pH 7.0 and activities were measured as 30 and 25 IU ml^{-1} for pH 6.0 and 7.0 culture filtrates, respectively. When comparing various xylan concentrations (0.5, 0.7 and 1%) as substrates, the highest activity was detected with 0.7% xylan at pH 7.0 on day 2. Activities were similar with 1% xylan, but the cultivation time was longer (4-5 days, data not shown). Addition of 0.2% Tween 80 to 1% xylan culture at pH 7.0 did not affect enzyme production. Cultivation temperature did affect enzyme production. The highest enzyme production was observed at 30 and 25°C was the next most suitable temperature for production (Fig. 2). Activity decreased rapidly in cultures grown at 37°C.

Effect of pH and temperature on crude xylanase: The optimum reaction pH was determined with crude culture

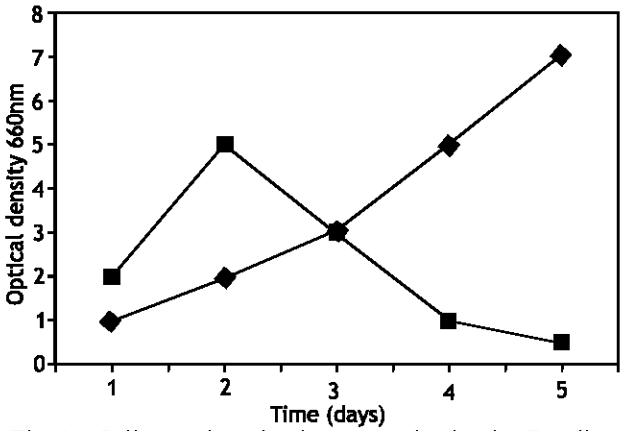

Fig. 1: Cell growth and xylanase production by *Bacillus sp.* Enzyme activity was assayed in phosphate buffer at pH 7.◆ OD 660 nm and ■ IU^{-1}

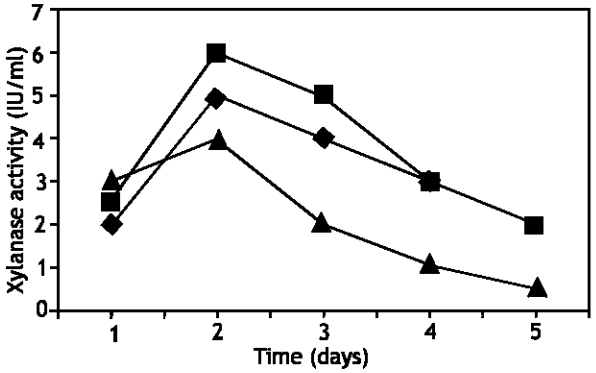

Fig. 2: Effect of cultivation temperature on xylanase production by *Bacillus sp.* ▲ 25°C, ■ 30°C, ◆ 37°C

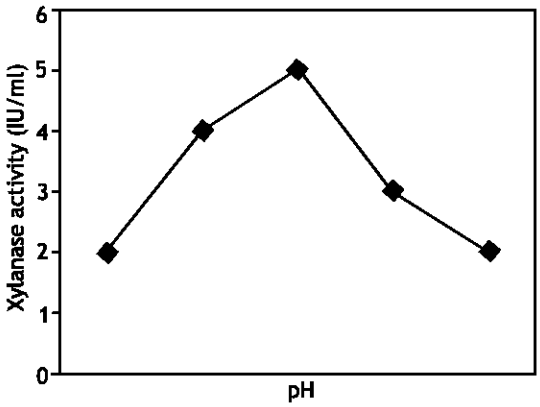

Fig. 3: Effect of pH on the activity of *Bacillus* xylanase produced on 0.7% oat spelt xylan

supernatant solution collected from day 2 cultures grown at pH 7.0. xylanase activity was detected over a wide pH range from 5 to 9 (Fig. 3); pH values of 6 to 8 were most suitable for xylanase activity. the optimum temperature for crude xylanase activity from cells grown at pH 7.0 was 50°C (Fig. 4).

Table 2: Biochemical tests for the identification of 4 collected strains

Sample No.	Motility test	Urease test	Citrate utilization test	Indole test	Xylanase activity	Fermentation test		Comments
						Mannitol	Lactose	
St_1	+	-	-	+	+	+	+	*Bacillus*
St_2	+	-	-	+	+	+	+	*Bacillus*
So_3	+	-	-	+	+	+	+	*Bacillus*
So_4	+	-	-	+	+	+	+	*Bacillus*

+ = Positive test - = Negative test

Table 3: The effects of various carbon and nitrogen sources on xylanase production by *Bacillus*

Source	(%)	Day 2 (IU ml^{-1})	Day 3 (IU ml^{-1})
Carbon			
Oat spelt xylan	0.5	15.0	12.0
Birchwood xylan	0.5	11.0	8.7
Oat spelt xylan	0.7	21.0	18.0
Oat spelt xylan	1.0	14.0	11.0
Wheat bran	0.7	8.0	17.0
Malt extract	0.7	7.0	6.0
D-Glucose	0.7	6.5	5.2
D-xylose	0.7	13.0	11.5
Nitrogen			
Yeast extract + peptone	0.7	18.0	21.0
Yeast extract	0.7	19.0	17.7
Peptone	0.7	16.0	15.2
Corn steep liquor	0.7	16.0	22.0

Table 4: Antibiotic resistance of isolates

	St_1	St_2	St_3	St_4
Tetracycline µg ml^{-1}				
25 µg ml^{-1}	-	-	-	-
50 µg ml^{-1}	-	-	-	-
100 µg ml^{-1}	-	-	-	-
Erythromycin				
25 µg ml^{-1}	-	-	-	-
50 µg ml^{-1}	-	-	-	-
100 µg ml^{-1}	-	-	-	-
Ampicilline µg ml^{-1}				
25 µg ml^{-1}	+	+	+	+
50 µg ml^{-1}	+	+	+	+
100 µg ml^{-1}	-	-	-	-
Amoxycilline µg ml^{-1}				
25 µg ml^{-1}	+	+	+	+
50 µg ml^{-1}	+	+	+	+
100 µg ml^{-1}	-	-	-	-
Chlorophenical µg ml^{-1}				
25 µg ml^{-1}	+	+	+	+
50 µg ml^{-1}	+	+	+	+
100 µg ml^{-1}	-	-	-	-
cotrimoxazole µg ml^{-1}				
25 µg ml^{-1}	+	+	+	+
50 µg ml^{-1}	+	+	+	+
100 µg ml^{-1}	-	-	-	-

Effect of carbon and nitrogen sources: The effects of various carbon and nitrogen sources on production of xylanase by bacillus are given in the Table 3. Both day 2 and 3 levels are presented. Oat spelt xylan (0.7) and finely ground wheat bran (2.5%) were the best carbon-sources for enzyme production. Oat spelt xylan was a much better carbon source than that from birch wood xylan. among the three sugars tested, only xylose resulted in significant activity. Corn steep liquor was the best nitrogen source for enzyme production. The addition of corn steep liquor

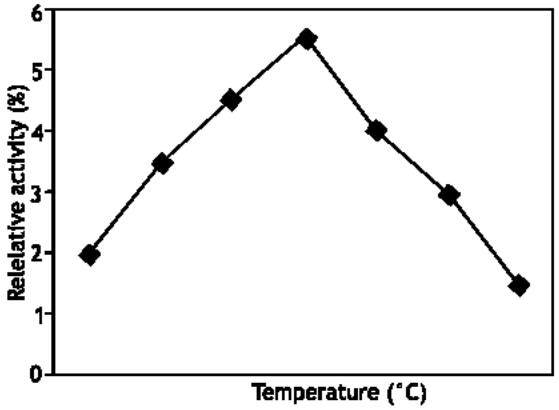

Fig. 4: Effect of temperature on *Bacillus* crude xylanase at pH 7. Relative activity is expressed as a percentage of the medium

Fig. 5: Plasmid profile of xylanase-producing Bacillus strain (St_1). The marker DNA was shon in Lane 1 and the isolated plasmid DNA in lane 2

to 0.5% oat spelt xylan substrate enhanced enzyme production.

Antibiotic resistance: The antibiotic resistance of the isolates is shown in the Table 4. All strains were sensitive to tetracycline and erythromycin at 25 and 50 μg ml^{-1} except ampicilline, amoxycilline, chlorophenical and cotrimoxazole which were sensitive only when the concentration was increased to 100 μg ml^{-1}. However, these same strains were resistance to ampicilline, amoxycilline, cotrimoxazole and chlorophenical upto 25 and 50 μg ml^{-1} and sensitive upto μg ml^{-1} antibiotic concentration.

Plasmid profile of *Bacillus* strain: All strains were found to harbor plasmids of at least 30-32 kilobases (kbp; Fig. 5). The plasmid DNA isolated from xylanase producing strain by boiling method described in materials and methods. After electrophoresis, the gel was stained in ethidium bromide solution (0.5 μg ml^{-1}) for 20 min. Then the gel was washed with tap water and placed on an UV-transsilluminator and finally photographed by a camera. Plasmid profile showed that plasmid DNA was about 32 kb in size.

DISCUSSION

There are many reports have been published alkaline xylanase from alkaliphic *bacillus sp.*[16]. As far my knowledge, xylanase from neutral *Bacillus sp.* is very rare. So, the types of xylanase is very interesting. In our study, we have isolated bacterial strains from damp soil and water, which degraded β-1, 4 xylans and to belong to *Bacillus* 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. The xylanase activity in each strain was confirmed by measuring the amount of reducing sugars liberated from xylan by the DNS method[13] using crude extract. After isolation of xylanase-producing strains, the strains was identified as *Bacillus* on the basis of catalase, oxidase, gram-staining and different biochemical tests. It was also observed that, the strains were resistant to four antibiotics i.e. ampicilline, amoxycilline, cotromosilline and chlorophenical and were sensitive to two antibiotics tetracycline and erythromycin.

In the next step of our study, xylanase-encoding plasmid DNA from xylanase-producing and multi-drug resistant *Aeromonas* bacteria was isolated by Mini-Scale boiling method. The purified plasmid DNA was subjected to agarose gel electrophoresis with reference to a marker DNA (λDNA Hind III digested), a single plasmid of about 32 kb in size was measured (Fig. 5). Toshiaki Kudo reported that plasmid pAX1 from *Aeromonas* sp. No 212 was isolated from transformants producing xylanase and the xylanase gene was located in a 6.0 kb HindIII fragment[18].

Due to limitation of our laboratory facilities, it was not possible to characterize the plasmid in details. Further study is now going on to digest the plasmid DNA with different restriction enzymes, which will help us to establish a complete restriction map of the plasmid. From this restriction map we may characterize the plasmid structure of the xylanase-producing *Bacillus*.

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