Production and properties of alkaline xylanases from *Bacillus* sp. isolated from sugarcane fields

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Xylanolytic bacteria were isolated on oat spelt xylan agar medium and screened by the xylanolysis method. The three bacterial strains isolated from sugarcane fields, St_A, St_B and St_C were identified as *Bacillus* sp. based on morphological, biochemical and physiological characters. Some properties of alkaline xylanases produced by the three strains of *Bacillus* sp. were studied. Optimal pH and temperature were 9.0 and 55°C for strain St_A and 9.0 and 50°C for strain St_B and 8.0 and 55°C for strain St_C respectively. The enzymes were stable between 30 and 50°C and xylanase activity remained up to 2 h for the enzymes of the three strains. Effect of various carbon sources on xylanase production was studied. Enzyme activity was stimulated by β-mercaptoethanol and dithiothreitol, and decreased by isopropanol. These properties qualify the enzyme to be novel and exhibit favourable potential for application to bleaching in the paper and pulp industry.

Keywords: *Bacillus* sp., oat spelt xylan, sugarcane fields, xylanase.

SUGARCANE is an important cash crop in Andhra Pradesh (AP), India. On an average sugarcane residues after harvest account for 15–20 t/ha. These residues are majorly composed of cellulose and hemicellulose, the most abundant components of plant biomass. Bacterial degradation of hemicellulosic biomass in agricultural waste plays a vital role in carbon recycling.

Hemicellulose is the second most abundant plant fraction available in nature. It is a storage polymer in seeds, being also a structural component of cell walls in plants. Agricultural residues contain up to 40% hemicelluloses formed by pentose sugars[1]. Monomers of various hemicelluloses are useful in the production of different antibiotics, alcohols, animal feed and fuels[2]. Hemicelluloses consist of a mixture of hexosans, pentosans and polyuronides. Xylan is the most abundant of the hemicelluloses. It has a linear backbone of β-1,4-linked D-xylopyranose residues. There is great interest in the enzymatic hydrolysis of xylan due to possible applications in feed stock, chemical production and paper manufacturing[3].

Biodegradation of xylan requires action of several enzymes, among which xylanases play a key role[4]. A wide variety of microorganisms are known to produce xylanases that are involved in the hydrolysis of xylan[5–9]. Recently, 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, enzymatic saccharification of lignocellulosic materials and waste treatment[10–14]. Most of these processes are carried out at high temperatures, so that thermostable enzymes find applications[15]. Therefore, thermophilic organisms are of special interest as a source of novel thermostable enzymes[16–18].

Few reports are available on the production of alkaline xylanases[19]. Many xylanases producing alkaliphilic microbial strains have been reported from different laboratories. However, xylanases from most of these alkaliphilic strains have their optimum pH around neutrality[20]. Majority of alkaliphiles were isolated from neutral soil samples. On the other hand, naturally occurring alkaline habitats are found scattered in different parts of the world[21]. Such habitats are expected to harbour novel microorganisms 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 applications in different industrial processes. Until now there has been little effort to isolate alkaliphiles from naturally occurring alkaline habitats. In the present study, production of an alkaline xylanase by *Bacillus* sp. isolated from sugarcane fields and the properties of the crude enzyme are reported.

Bacillus sp. strains St_A, St_B and St_C were isolated from sugarcane fields around Tirupati, AP and were maintained on the oat spelt xylan medium as follows (in g/l): xylan, 7; yeast extract, 1; NaCl, 5; K_2HPO_4, 1; $MgSO_4$, 0.2; $CaCl_2$, 0.1, and Na_2CO_3, 10. Sodium carbonate was sterilized separately and added to the rest of the medium to adjust the pH to 9.0. The cultures were grown at 37°C for 48 h.

Soil suspensions in sterilized water were poured and spread onto nutrient agar plates. These plates were incubated at 37°C for 2 days. Colonies that were found on the plates were transferred onto oat spelt xylan agar plates, which were again incubated at 37°C for 3 days. Efficient bacterial isolates were selected on the basis of formation of clearing zones. 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.

Xylanolytic bacteria were identified and characterized by morphological and biochemical tests using *Bergey's Manual of Systematic Bacteriology*[22].

The medium (50 ml in 250 ml Erlenmeyer flasks) was inoculated with 2 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 4500 *g*. The clear supernatant was used as crude enzyme preparation.

The cell-free supernatant fluid was precipitated with the addition of solid ammonium sulphate to 70% saturation.

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After centrifugation, the pellet was suspended in a minimum volume of 50 mM glycine–NaOH buffer, pH 9.0 and dialysed against three changes of the same buffer. The dialysed crude enzyme preparation was used for all subsequent studies.

Xylanase (1,4-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.*[23] and the amount of reducing sugars released was determined by the method of Miller[24] using DNS reagent. One unit of enzyme activity was defined as 1 µmol of xylose equivalent produced per minute under the assay conditions.

Protein concentration was measured by the method of Lowry *et al.*[25] using bovine serum albumin as standard.

The effect of pH on activity of xylanases was measured by incubating 0.5 ml of the enzyme and 1.5 ml of different buffers, adjusted to a pH of 6.0–10.0, containing oat spelt xylan (0.5%). The buffers used were: sodium phosphate, pH 6.0 and 7.0; Tris-HCL buffer, pH 8.0, and glycine–NaOH, pH 9.0 and 10.0. The effect of pH on xylanase stability was measured over the pH range 5.0–13.0 for 24 h at 25°C. After incubation, residual activity was determined under optimal assay conditions for each strain.

The effect of temperature on enzyme activity was determined by performing the standard assay procedure for 10 min at pH 8.0 within a temperature range of 30–65°C. Thermostability was determined by incubation of crude enzyme at temperatures ranging from 30 to 80°C for 2 h. After incubation, the enzyme extracts were cooled on ice for 10 min. Finally the residual xylanase activities were measured using dinitrosalicylic acid reagent.

The effect of chemical reagents on xylanase activity was determined using the crude enzyme. Enzyme activity was measured in the presence of chemical reagents at concentrations of 1 and 3 mM.

The alkaline xylanase producing *Bacillus* strains were isolated from sugarcane fields by a screening procedure on the basis of clearing zones and xylanolytic properties on xylan agar plate. Xylanase activity in each strain was confirmed by measuring the amount of reducing sugars liberated from xylan with dinitrosalicylic acid reagent using crude extract. The organism grows at pH 8–9 and produced a high level of xylanase activity both in solid and liquid media. It was rod-shaped, Gram-positive, aerobic, motile and catalase-positive. Acid was produced from D-glucose, D-xylose and D-mannitol. Based on these characteristics, the bacteria were identified as belonging to the genus *Bacillus*, according to Sneath *et al.*[22].

Xylanases produced by *Bacillus* were growth-associated, reaching a maximum after 24 h. Enzyme production remained more or less the same up to 48 h, while biomass started to gradually decline after 36 h. High level of enzyme production was observed when the organism was grown in medium containing oat spelt xylan. A significant amount of xylanase was also produced when starch, sucrose, arabinose, glucose and xylose were used as carbon sources (Table 1). The increase in xylanase activity during later stages of growth might be due to the release of small amounts of xylanase from the aged cells entering into autolysis[26] and also due to the scarcity of insoluble xylan particles in the medium, which if present in the culture broth might bind the xylanases[27].

Optimum pH was found to be 9.0 for both St_A and St_B, and 8.0 for St_C (Figure 1 *a*). Enzyme activity at pH 8 and 9 was 79 and 89 IU/ml for St_A, 65 and 80 IU/ml for St_B, and 57 and 36 IU/ml for St_C respectively. At pH 10.0, xylanase activity decreased. Crude xylanases from *Bacillus* strains were stable between pH 7 and 11, when pH stability was measured at values 5 to 13 (Figure 1 *b*). Alkaline xylanases 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 pH and temperature readjustments before enzyme addition. Xylanases produced by most alkaliphiles reported to date have their optimum pH around neutrality. Nakamura *et al.*[19] reported the first alkaline xylanases produced by *Bacillus* sp. strain 41M-1, which had an optimum pH and temperature of 9.0 and 55°C respectively. Yang *et al.*[28]

Table 1. Effect of different carbon sources on xylanase production

Sugar	Xylanase activity (IU/ml)
Oat spelt xylan	52
Starch	25
Sucrose	18
Xylose	8
Arabinose	13

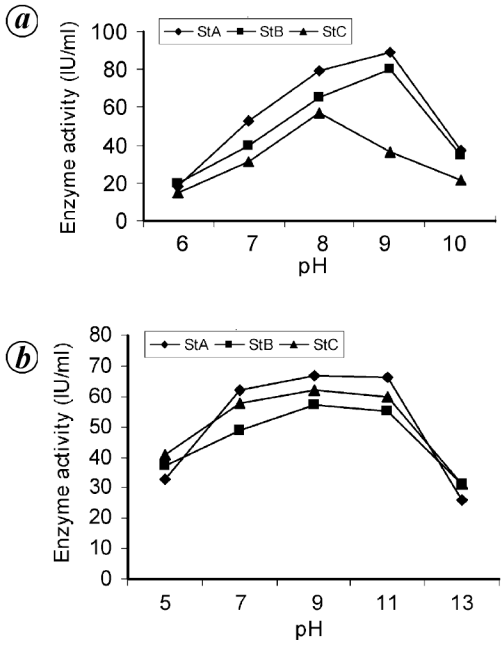

Figure 1. Effect of pH on activity (*a*) and stability (*b*) of xylanases.

isolated an alkaliphilic *Bacillus* sp. VI-4 from a hard wood Kraft pulp, which produced xylanases having an optimum pH of 6–8.5. Xylanases from *Bacillus* sp. were novel enzymes, being active at alkaline pH, with an optimum value at 9 and were stable over a broad pH range. Further study of the enzyme might help to understand the molecular basis of stability and activity of xylanases at alkaline pH and elevated temperature.

Xylanases from the three isolates exhibited a temperature profile with a sharp peak of maximal activity at 55°C for St_A and St_C, and 50°C for St_B (Figure 2 *a*). Enzyme activity at 55°C was 86 and 76 IU/ml for St_A and St_C respectively, and 64 IU/ml for St_B. It showed optimum activity at 50–55°C, and good stability at 30–50°C, at alkaline pH values. Thermal stability of xylanase is important property due to its potential applications in several industrial processes (Figure 2 *b*). Strains isolated by us could be a good source for biotechnological applications.

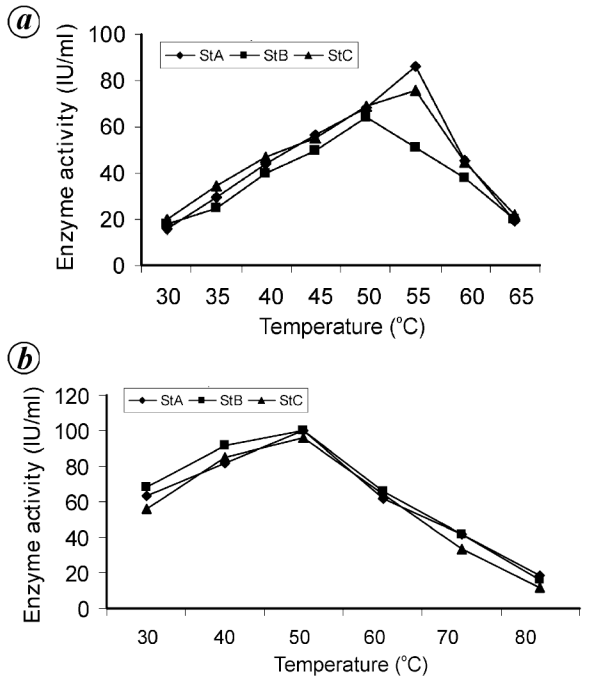

Figure 2. Effect of temperature on activity (*a*) and stability (*b*) of xylanases.

Table 2. Effect of several chemical reagents on xylanase activity

	Relative activity (%)	
Reagent	1 mM	3 mM
Control (none)	100	100
NaCl	115	120
β-Mercatoethanol	130	150
Dithiothreitol	125	135
Isopropanol	90	85

Control, without test compound, activity was considered as 100%.

The influence of different chemical reagents on xylanase activity is given in Table 2. The effects varied according to the reagents and their concentrations. NaCl at concentrations of 1 and 3 mM caused slight stimulation of the enzyme activity, perhaps due to alteration of enzyme conformation. As widely reported in the literature[29–32] enzymes can be modulated by interaction of cations with amino acid residues involved in their active sites. Such interactions can either increase (positive modulation) or decrease (negative modulation) enzyme activity. Nonetheless, Numao *et al.*[33] studying the human pancreatic α-amylase, found that some members of the amylase family require chloride for full catalytic activity. This characteristic property is due to the existence of conserved chloride ion-binding site located in domain A, which is an a/b barrel that contains the active site. Thus, not only cations but also anions can alter enzyme activity. The activity measured in the presence of reagents was relatively expressed in terms of activity of control.

The protein disulphide reducing reagents, β-mercaptoethanol and dithiothreitol caused high stimulation of xylanase activity, mainly at a concentration of 3 mM (35–50%). Such thiol compounds prevent oxidation of sulfhydryl groups and hence they are commonly added during enzyme purification[34]. Stimulation of xylanase activity by such disulphide reducing agents indicates that cysteine residues should be a part of the catalytic site in the enzyme structure. Similar effects were described for xylanases from *Bacillus* sp. strain SPS-0 (ref. 29), *Bacillus amyloliquefaciens*[30], and also *Thermomyces lanuginosus* DSM 5826 (ref. 31) in the presence of these compounds.

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Floral nectaries in some apple and pear cultivars with special reference to bacterial fire blight

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The structure of floral nectaries in some apple (*Malus domestica* Borkh.) and pear (*Pyrus communis* L.) cultivars, either susceptible or tolerant to fire blight caused by bacterium, *Erwinia amylovora* (Burrill) Winslow *et al.* was studied. The surface of nectaries is smooth in tolerant apple cv. 'Freedom' as well as in all the pear cultivars investigated. The surface of nectaries was wrinkled and striate in the susceptible apple cv. 'Sampion'. These features are favourable for the bacterium, because the nectar retained longer in the furrows provides a nutrient-rich environment. The nectary stomata in cv. 'Freedom' were meso- or hygromorphic, while those of cv. 'Sampion' were usually meso- or xeromorphic and hygromorphic stomata were rare. In pear cultivars, no hygromorphic stomata were observed. The nectary stomata of the tolerant pear cv. 'Beurré Bosc' were mostly mesomorphic or were slightly below the level of the epidermis. In susceptible pear cultivars, xeromorphic stomata were observed more frequently.

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