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Cellulase-free xylanase production from an alkalophilic *Bacillus* species

H. Balakrishnan, M. Dutta-Choudhury, M.C. Srinivasan & M.V. Rele*

An alkalophilic *Bacillus* (NCL-87-6-10, NCIM 2128), with a high productivity for extracellular xylanase (EC 3.2.1.8) and free of cellulase, was isolated from soil containing coconut fibre detritus. When grown on a wheat bran/yeast extract medium in submerged culture for 48 h, it produced 100 to 120 IU of enzyme activity per ml. The crude enzyme consists of two fractions of apparent mol sizes of approx 10.4 and 29 kDa in the proportion of 90:10, as determined by native gel exclusion chromatography. Optimum activity of the xylanase was at 60°C and pH 8.0. A two-fold increase in enzyme activity was obtained when reducing agents, thioethanol and dithiothreitol, were included in the assay.

Key words: Alkalophilic *Bacillus*, cellulase-free xylanase, hemicellulase.

Xylanases free of cellulolytic activity have several applications in the paper and pulp industry and have recently received considerable attention as their use means that the application of Cl_2 and ClO_2 for biobleaching can be reduced (Viikari *et al.* 1990; Kruss & Koljonen 1991; Kruss *et al.* 1991). Naturally-occurring microbial strains capable of secreting xylanases free of cellulase activity would be attractive for such applications. A *Chainia* sp. (NCL 82-5-1) discovered in our laboratory was one of the earliest strains to be identified with such a characteristic (Srinivasan *et al.* 1984, 1986; Bastawade *et al.* 1990). Other reports of actinomycete strains secreting high activity xylanase free of substantial cellulase activity include a thermotolerant *Streptomyces* (Keskar *et al.* 1989), *Saccharomonospora* (Roberts *et al.* 1990) and *Streptomyces roseiscleroticus* (Grabski & Jeffries 1991). The advantages offered by xylanases active and stable at alkaline pH for paper and pulp industry applications have been emphasized by Zamost *et al.* (1991). Srinivasan *et al.* (1988) patented a process for the manufacture of such a xylanase from an alkalophilic *Bacillus* and the crude broth was substantially free of cellulase activity. The present report deals with cultural studies on enzyme production by this *Bacillus* strain in submerged culture and the preliminary characterization of its xylanases.

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Materials and Methods

Strain Isolation

The culture was isolated from a soil sample containing decomposing coconut detritus collected at Calicut, Kerala, on a medium containing wheat bran (2% w/v), that had been autoclaved and washed to free it of the associated starch, yeast extract (0.1% w/v) and Na_2CO_3 (1% w/v), which had been sterilized separately. Plates were incubated at 28°C and single colonies were evaluated for growth and xylan clearance on alkaline nutrient agar plates supplemented with xylan. Following purification and preliminary evaluation of the isolates in shake flasks, one of the strains of alkalophilic *Bacillus*, designated NCL 87-6-10, was taken for detailed investigations.

Enzyme Production

The enzyme was produced in shake flasks on a wheat bran/yeast extract medium containing 3% (w/v) wheat bran, 1% (w/v) yeast extract and 0.5% (v/v) Tween 80, supplemented with 1% (w/v) Na_2CO_3 added after being separately autoclaved.

Xylanase Assay

The enzyme was assayed routinely by the dinitrosalicylic acid method of Sumner (Bernfeld 1955) while the Somogyi Nelson method (Collmer *et al.* 1988) was used for a few samples for comparative evaluation. Xylose was used as the standard and the activity expressed as IU (μmol sugar/min.ml). Two grams of oat spelts xylan (Sigma) was suspended in 100 ml of 50 mM

KH_2PO_4/KOH buffer, pH 8 and stirred for 16 h followed by centrifugation. The soluble fraction corresponded to 0.7% (w/v).

The reaction mixture (1 ml) contained 0.5 ml of soluble xylan substrate and 0.5 ml of appropriately diluted enzyme in phosphate buffer, pH 8. The reaction mixture was incubated at 50°C for 30 min and the reaction terminated by adding 1 ml of dinitrosalicylic acid. After boiling for 5 min, the colour was read at 540 nm after dilution with 10 ml of water. When assayed by the Somogyi-Nelson method, the activity obtained was approximately 25% of that shown by the dinitrosalicylic acid method. Similar observations have been recorded by Grabski & Jeffries (1991).

Cellulase Assay

The cellulase assay was carried out as above, using 1% carboxymethyl cellulose (CMC) at both pH 8.0 and pH 4.8. Filter paper activity was determined by incubating Whatman No. 1 filter paper (50 mg) with the culture filtrate at both pH 8.0 and pH 4.8. One unit of enzyme corresponded to one μmol of reducing sugar produced per minute under the assay conditions.

β-Xylosidase Assay

This was carried out according to Berghem & Petterson (1973) using *p*-nitrophenyl-β-D-xylopyranoside (*p*NP-X). However, the incubation was for 30 min instead of 10 min as in the standard procedure.

Glucose Isomerase Assay

Glucose isomerase was estimated by the colorimetric method of Takasaki & Tanabe (1966). The fructose produced was estimated by the cysteine-carbazole method as modified by Marshall & Kooi (1957).

Xylose Isomerase Assay

Xylose isomerase was estimated according to Slein (1955) using the cysteine-carbazole method.

Molecular Weight

Molecular weights were determined on a Sephadex G-50 column according to Andrews (1965). Aprotinin (6.5 kDa), cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa) and serum albumin (66 kDa) were used as standards.

Paper Chromatography

Paper chromatography was carried out using *n*-butanol/acetic acid/water (3:1:1, by vol). The products of xylan hydrolysis were detected using *p*-anisidine hydrochloride reagent.

Treatment with Various Chemical Reagents and Metal Ions

Diluted culture filtrate/ammonium sulphate precipitates and dialysed enzyme were pre-incubated with several additives at different concentrations for 30 min and the activity then determined by the standard procedures.

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was carried out according to Davis (1964). Xylanase bands were detected by slicing the gel into eight equal parts and estimating each section by the conventional methods.

Results and Discussion

Strain Characterization

The isolate was an aerobic, Gram-positive rod, forming endospores after 2 to 3 days. Colonies of the alkalophilic *Bacillus* (NCL, 87-6-10) appear rhizoidal with a prominent clearance zone beyond the growing margin of the colony when grown on a nutrient agar medium containing xylan, thereby indicating copious extracellular growth-associated secretion of xylanase. Microscopic examination indicated chains of Gram-positive bacilli in the young culture while older cultures showed terminally-borne endospores.

The optimal growth was above pH 9.0 and the presence of sodium (added as Na_2CO_3) was essential. When K_2CO_3 was used to make the pH alkaline, addition of 0.5% NaCl was obligatory to support growth (Figure 1). These observations agree with those of Kitada *et al.* (1987), who explained the role of Na^+ as being essential for maintenance of the internal pH balance as well as for nutrient uptake in an alkalophilic bacillus.

Fermentation

Optimum Concentration of Wheat Bran. Maximum activity of 75 IU/ml was obtained in 48 h when the organism was grown in the wheat bran/yeast extract medium with wheat bran at 3 to 5% (w/v); the activity was significantly lower when the concentration was either 1% or 7%. Under the optimal conditions, the final pH was 10.0. Inoculum containing 1% (w/v) wheat bran gave higher activity in shake cultures.

Carbon Sources. Various xylan-rich substrates were evaluated for xylanase production (Table 1). Xylanase secretion was optimal in media containing pure xylan. Rice bran, wheat bran and corncobs gave approximately half the activity of pure xylan in the absence of Tween 80.

Nitrogen Sources. Cells grown for 48 h with tryptone or yeast extract (1% w/v) gave the highest activity (85 to 100 IU/ml)

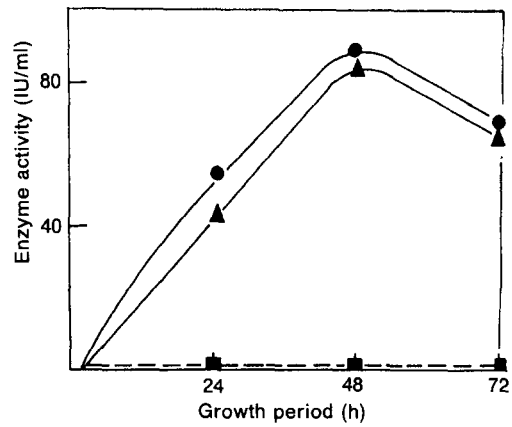

Figure 1. Requirement of Na^+ for growth and enzyme production (means of triplicate experiments) in medium with 3% (w/v) wheat bran and 1% (w/v) yeast extract. ●—1% (w/v) Na_2CO_3; ▲—1% (w/v) K_2CO_3 and 0.5% (w/v) NaCl; ■—1% (w/v) K_2CO_3.

Table 1. Xylanase production by *Bacillus* sp. growing on agricultural wastes at 3% (w/v) after 48 h*.

Agricultural waste	Maximum xylanase activity (IU/ml)
Wheat bran (control)	52
Rice bran	51
Corncobs (untreated)	45
Bagasse (untreated)	15
Xylan	100

* All flasks contained yeast extract at 1% (w/v). Values based on three replicates.

in 48 h. Yeast extract could be effectively substituted by whole baker's yeast. Other nitrogen sources, such as Proflo (Trader's protein, Buckeye Oilseed Products Company, Memphis, USA) and casein, gave lower yields (Table 2). Urea or malt extract supported little or no enzyme secretion.

Effect of Surfactants. Among the various surfactants tried, Tween 80 gave a significant increase in enzyme production, while sodium dodecyl sulphate (SDS), Tween 20 and Triton X 100 gave comparatively lower activities. Tween 80 at 0.5 to 1% (v/v) gave twice the enzyme secretion found in the controls where no surfactant was added (Figure 2). The effect could be due either to better enzyme secretion or release of adsorbed enzyme from the wheat bran.

Optimised Media for Enzyme Production. Supplementing wheat bran (3% w/v) medium with tryptone (1 to 3% w/v) and Tween 80 (0.5 to 1% v/v) gave the highest activities of xylanase secretion, ranging from 130 to 170 IU/ml in 48 h and the productivity ranged from 2700 to 3500 IU/l.h.

Table 2. Xylanase production by *Bacillus* sp. growing with different nitrogen sources after 48 h*.

Nitrogen source	Maximum xylanase activity (IU/ml)
Yeast extract (control)	90
Baker's yeast	98
Tryptone	120
Peptone	74
Casein	51
Proflo†	25
Pharma-media*	47
Malt extract	9
Soybean	33
Coconut milk	18

* All flasks contained wheat bran 3% (w/v) as carbon source in addition to the nitrogen source added on equivalent nitrogen to 1% (w/v) yeast extract.
† Traders protein, Memphis, TN, U.S.A.

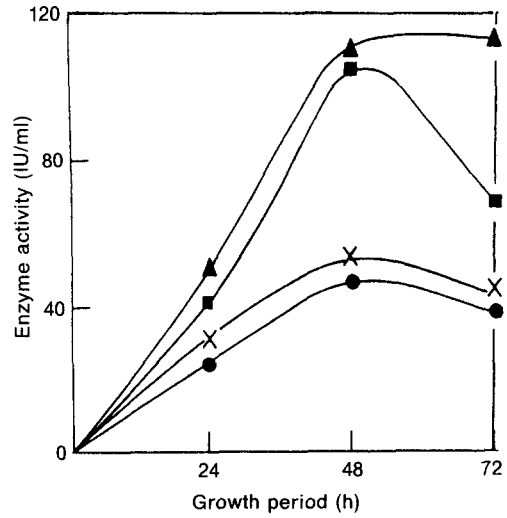

Figure 2. Effect of 0.1% (x), 0.5% (■) and 1% (▲) (v/v) Tween 80 on xylanase production (means of triplicate experiments) in medium with 3% (w/v) wheat bran and 1% (w/v) yeast extract. ●—Control with no Tween 80.

Specificity of Culture Broth. The culture broth was virtually free of cellulase activity (ratio of xylanase:cellulase 2430:1). Amylase, β-xylosidase and specific glucose isomerase were not detected. However, a low activity of a specific extracellular xylose isomerase was observed (41 IU/l) when the organism was grown on xylose (1% w/v). Intracellular glucose isomerase/xylose isomerase were detected in sonicated cell extracts (glucose isomerase:90 IU/l; xylose isomerase:32 IU/l) when grown on 1% (w/v) xylose.

Properties of the Xylanase

Optimum pH and Temperature. The enzyme was active between pH 7 and 9, with optimum activity at pH 8.0. At pH 9.0, 75% activity was observed, while at pH 5.0 only 10 to 15% of activity was evident. Under standard assay conditions at pH 8.0, the optimum temperature was at 60°C, while at 35 or 70°C only 25% of the activity was observed.

pH and Temperature Stability. Ten-fold diluted broth had greater pH and temperature stability compared with the undiluted culture filtrate. Maximum stability of the enzyme was observed in the pH range 6 to 8 and at 50°C.

Molecular Size. Molecular size was determined on Sephadex G-50. Two distinct peaks of xylanase activity were detected, corresponding to apparent molecular sizes of approx 29 and 10.4 kDa, in the proportion of 10:90 (w/w). PAGE also resulted in two distinct peaks of activity which differed considerably in their mobility as well as molecular weight.

Products of Hydrolysis. Hydrolysis of oat spelts xylan was carried out at pH 8.0 for 5 min to 24 h at 50°C. Different ratios of xylan to enzyme were tried (20 mg of xylan was

incubated with 2 to 20 IU of enzyme). Xylobiose and xylose, as products of hydrolysis, were detected by paper chromatography (as described under Materials and Methods) even at early stages of hydrolysis (5 min) and at all enzyme concentrations studied. They were the predominant end-products after 24 h hydrolysis, during which 70% of the xylan was hydrolysed. Spots consistent with the presence of tri- and higher oligo-saccharides were also present but always in relatively small amounts. Since there was no β-xylosidase activity, these observations suggest the possible presence of an exo-xylanase component.

Estimates of K_m. The K_m value for soluble xylan was determined to be 1.37 mg/ml, indicating the enzyme to be a powerful xylanase. The enzyme was also capable of acting on insoluble xylan.

Effect of Reducing Agents on Enzyme Activity. Various reducing agents (Table 3) were tested for their effect on enzyme activity in the assay mixture; enzyme activity increased progressively with increasing concentrations of the reducing agent. The possible existence of a dimer with partial activity was ruled out by running PAGE in the presence of thioethanol. Two bands of xylanase having similar mobilities to the untreated enzyme were obtained, indicating the absence of a dimer.

Effect of Various Additives on Xylanase Activity. *p*-Chloromercuric benzoate (PCMB) at 0.5 mM inhibited the enzyme totally. Thioethanol at 10 mM reactivated the PCMB-treated enzyme and gave a final activity of 170%. However, iodoacetamide, 5-5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and N-ethylmaleimide (NEM) did not inhibit the enzyme even at 10 mM (Table 4). We have made similar observations with the purified endoxylanase II from *Chainia* (Bastawde *et al.* 1990). These results agree with those obtained for cellulases (Eriksson & Petterson 1968; Clarke & Yaguchi 1985; Wilson 1992). It has been postulated that Hg^{2+} binds with either tryptophan or carboxylated amino acids involved in either catalytic activity or binding. Hence, the presence of a reactive cysteine residue would have to be confirmed with the purified enzyme. Urea (6 M) inhibited the activity by 60%, while EDTA and SDS showed marginal inhibition at the concentrations tested.

Table 3. Activation of xylanase activity by reducing agents.

Reducing agent	Concentration in assay mixture (mM)	Activation* (%)
Thioethanol	0.5	16
	1.5	58
	2.5	81
Cysteine	0.5	–
	2.5	44
Dithiothreitol	1.0	19
	25	98

* 100% enzyme activity varied from 80 to 100 IU/ml in different experiments.

Table 4. Effect of various additives on xylanase activity.

Added compound	Concentration* (M)	Inhibition† (%)
EDTA	0.01	15
SDS	0.017	40
Iodoacetamide	0.001 to 0.01	–
N-Bromosuccinimide	0.0005 to 0.005	100
PMSF	0.001	11
DTNB	0.01	–
NEM	0.005	12
Urea	6	60
DEP	0.01	44

* The diluted enzyme was incubated with various additives as indicated in the table before assaying.
† 100% enzyme activity varied from 70 to 93 IU/ml in different experiments.

Effect of Metal Ions on Xylanase Activity. Various metal ions added at 5 mM failed to activate the enzymes. Hg^{2+} at 0.1 mM inhibited the enzyme up to 85%. This could be reversed by adding thioethanol (10 mM). Fe^{2+} inhibited the enzyme at 5 mM by 58%. The enzyme could not be reactivated by complexing with *o*-phenanthroline but could be reactivated easily by adding thioethanol (10 mM). Other metal ions had marginal inhibitory effect or none at all.

Discussion

Our studies on the alkali-stable cellulase-free xylanase from an obligately alkalophilic *Bacillus* have brought out several aspects of scientific as well as commercial interest. This discovery of a strain showing such high activities of xylanase secretion virtually free of cellulase among alkalophiles is perhaps novel and the fact that the enzyme can be produced on relatively inexpensive natural raw materials, including abundantly available crop residues and agricultural by-products such as cereal brans, makes the process technology for xylanase manufacture using this strain feasible. While we have indicated the possibility of an exoxylanase in the culture filtrates, further investigations are needed to definitely indicate its presence. Further work to understand the synthesis and regulation of the enzyme protein, its secretion and characterization is in progress.

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