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Short contribution

Production and characterization of xylanase from *Bacillus thermoalkalophilus* grown on agricultural wastes

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Summary. *Bacillus thermoalkalophilus* isolated from termite-infested mound soils of the semi-arid zones of India had the ability to produce good amounts of xylanase(s) from cheap agricultural wastes. Of the two hemicellulosic substrates tested, bagasse was found to be the better inducer for xylanase production. Alkali treatment of bagasse and rice husk had varied effects on enzyme production. The enzyme preparation had activity optima at 60° C and 70° C and a half-life of 60 min at 65° C. The enzyme was stable for 24 h over a pH range of 4.0–6.0, while maximum activity was observed at pH 6.0–7.0. Enzyme production and activity were inhibited by the end-product of xylan hydrolysis, xylose.

Introduction

Agricultural wastes can serve as an abundant and inexpensive source of fixed carbon for the production and utilization of cellulase(s) and xylanase(s). Whilst information regarding production and characterization of these enzymes from fungal and bacterial systems is exhaustive, examples of the practical utilization of microbial degradation of cellulosic and/or hemicellulosic material is scanty. The search for promising microorganisms capable of producing substantial amounts of these enzymes for efficient and economical degradation of agricultural wastes has been on for a long time. With similar objectives, we screened the microflora of termite-infested soil from the semi-arid zones of India, as termite nests play a key role in recycling nutrients and maintaining soil fertility leading to better crop production (Breznak 1982; Varma et al. 1989).

This communication deals with the production and partial characterization of xylanase produced by a unique isolate which was thermoalkalophilic. The isolate was a potent producer of xylanase when grown on agricultural wastes, rice husk and bagasse.

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Materials and methods

Characterization of the isolate. The organism was isolated from live termite-mound soils of semi-arid zones infested with the higher termite *Odontotermes obesus*. The isolate was screened and identified by the methods of Gordon et al. (1973) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974) as a new species of the genus *Bacillus* (Sarkar 1987).

Selection of carbon substrate. For preliminary screening, various carbon sources were added at the rate of 0.5% to a modified Dubos' salt medium (g/l): K_2HPO_4, 0.5; KH_2PO_4, 0.5; KCl, 0.5; NH_4NO_3, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; yeast extract (Difco, Detroit, Mich, USA), 0.5. The pH was adjusted to 9.0 (Dubos 1928). One percent of logarithmically growing cells (10^8) were inoculated and cultivated in shake-culture flasks (100 ml medium in 250-ml culture flasks) at 60° C with constant shaking (200 rpm) in a New Brunswick Scientific (Edison, NJ, USA) water-bath shaker.

Treatment of substrates. Lignocellulosic substrates, rice husk and bagasse were milled (mesh size = 1.7 mm) and washed thoroughly in distilled water to remove the residual dust. For delignification, they were soaked in 2 N NaOH for 24 h and steamed for 1 h. After the alkali treatment, the material was repeatedly washed with distilled water until neutral and oven-dried.

Crude enzyme preparation. After 24 h aerobic cultivation under the specified growth conditions cells were harvested by centrifugation at 6000 *g* for 10 min. Supernatant per se was used for time-course production studies. The crude xylanase(s) was precipitated using ammonium sulphate (70% saturation) and dissolved in 50 m*M* potassium phosphate buffer, pH 7.0. The enzyme solution was dialysed against the same buffer for 24 h at 4° C and stored at 0° C (no significant loss of activity was seen even after 7 month's storage). This was suitably diluted and used as the enzyme source for further characterization studies.

Enzyme assay. Xylanase activity was assayed using 1% oat spelt xylan (Fluka, Neu-Ulm, FRG) as substrate, suspended homogeneously in 50 m*M* potassium phosphate buffer, pH 7.0 unless otherwise stated, by ultrasonic treatment. Suitably diluted enzyme (50 µl) was added to 1 ml substrate and incubated at 60° C for 30 min with constant shaking. The reducing sugar liberated was quantified by following the 3,5-dinitrosalicylic acid (Merck, Darmstadt, FRG) method (Miller 1959). One unit (U) of xylanase was defined as the amount of enzyme required to release 1 µmol reducing sugar as xylose equivalent in 30 min under the standard incubation conditions. All assays were done in triplicate.

Results and discussion

Organism

Cells were Gram-positive, aerobic, spore-forming motile rods (4 μm × 0.8 μm), occurring singly or in short chains. The spores were ellipsoidal and central to subterminal in position. The specific growth rate of this bacterium was found to be 0.28 h^{-1} when grown under optimum conditions. The isolate grew best at 60° C and pH 9.0.

Effect of carbon source on xylanase production

The production of xylanase(s) during the growth of the isolate on different carbon sources is illustrated in Fig. 1. The results indicate that bagasse was a better source for xylanase synthesis than rice husk or xylan. Whereas the cells grown on xylose expectedly did not produce the enzyme because of catabolite repression, the result with carboxymethylcellulose as carbon source is contrary to a report of Rho et al. (1982), where it was shown to induce enzyme synthesis. The slowness of enzyme production up to 36 h in the cells grown on bagasse and xylose further supports the assumption of catabolite repression. The appearance of activity after 36 h could be due to the consumption of xylose, the catabolite repressor, by the organism as a preferential carbon source. Comparatively higher production of xylanase(s) after 36 h could be explained by the fact that consumption of xylose resulted in a higher amount of cells which in turn yielded more xylanase(s).

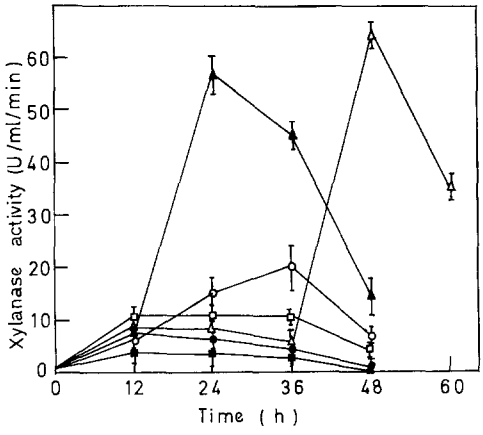

Fig. 1. Xylanase production by *Bacillus thermoalkalophilus* growing on various carbon sources: ○—○, rice husk; ●—●, xylose; ▲—▲, bagasse; △—△, bagasse and 0.25% xylose; ■—■, carboxymethylcellulose; □—□, xylan. U = units

Effect of pretreatment on enzyme production

Table 1 indicates that pretreatment of the carbon sources, rice husk and bagasse, had varied effects on enzyme synthesis. Maximum enzyme production with treated rice husk was about four times that with the untreated sample. However, a decreasing tendency was invariably observed with treated bagasse over untreated. The increased value in delignified rice husk could be due to easier access of the hemicelluloses to the organism, whereas the tendency towards lower activity could be due to bleaching, as has been observed by others (Stewart et al. 1983; Ghosh and Deb 1988).

Effect of pH on activity and stability of the enzyme

The pH was adjusted with 50 m*M* citrate-phosphate buffer (pH 3.0–9.0) or 50 m*M* carbonate-bicarbonate buffer (pH 9.0–11.0). The enzyme was most active in the pH range 6.0–7.0 (Fig. 2). Berenger et al. (1985) generalized that most bacterial xylanases have optimum activity in the acido-neutral range of pH 4.0–7.0. Stability of the enzyme was investigated in buffer solutions of various pH values (pH 3.0–9.0). One millilitre of the enzyme was incubated in 2 ml of appropriate buffer for 24 h at 4° C. After the incubation period the residual activities were measured. The enzyme was stable in the pH range 4.0–6.0, but was progressively unstable at pH values below 4.0 and above 6.0 (Fig. 2). However, the loss in activity was rather low, i.e. only 10–20% of the total activity was lost in the latter cases.

Effect of temperature on activity and stability of the enzyme

The optimum temperature for activity was determined by varying the incubation temperature. The tempera-

Table 1. Time course of production of xylanase in treated and untreated hemicellulosic wastes

Substrates	Xylanase activity (units/ml)			
	Incubation (h)			
	12	24	36	48
Rice husk (U)	4.30 ± 0.42	14.80 ± 0.61	19.90 ± 0.10	6.70 ± 0.20
Rice husk (T)	50.40 ± 2.01	82.30 ± 1.08	57.10 ± 1.51	14.00 ± 0.12
Bagasse (U)	3.20 ± 0.20	56.90 ± 1.00	45.80 ± 2.51	14.70 ± 0.48
Bagasse (T)	7.70 ± 0.10	45.30 ± 0.51	31.30 ± 0.51	6.80 ± 0.20

T = treated; U = untreated

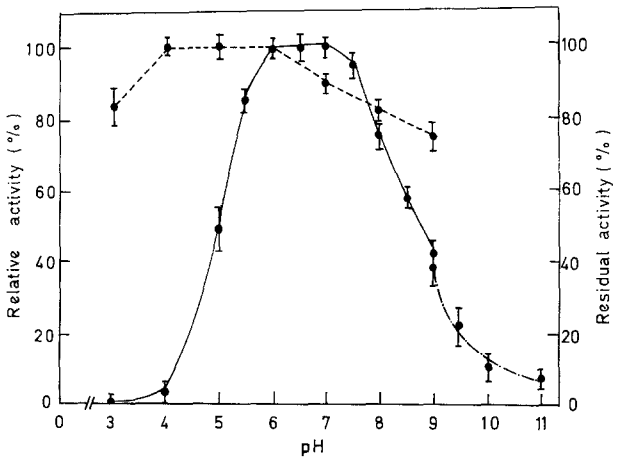

Fig. 2. Effect of pH on xylanase enzyme at 60° C. Xylanase activity: ● — ●, 50 m*M* citrate phosphate buffer; ●-·-·-·-●, 50 m*M* carbonate-bicarbonate buffer. Stability of xylanase after 24 h in 50 m*M* citrate phosphate buffer is expressed as residual activity, ●-----●

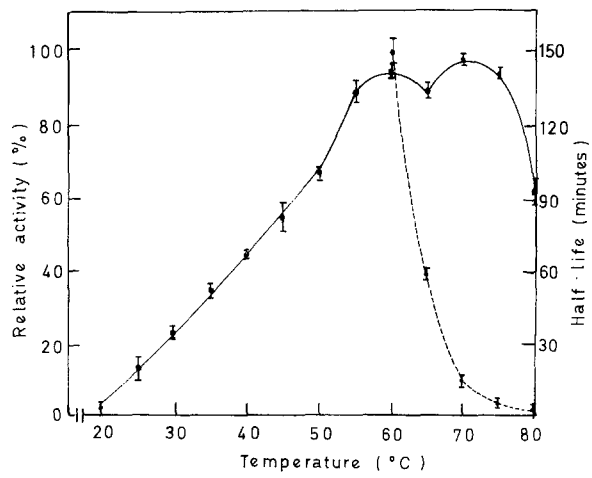

Fig. 3. Effect of temperature on xylanase at pH 7.0: ● — ●, activity; ● — - - ●, stability of xylanase expressed as half-life of enzyme

ture-activity profile gave a curve with two peaks at 60° C and 70° C (Fig. 3). At 80° C, nearly 40% of the activity was lost while practically no activity was seen below 25° C. The two peaks may be due to the presence of two xylanases as multiple xylanases are common in microorganisms (Dekker 1985). The thermal stability of the enzyme was measured by incubating the enzyme solutions in 50 m*M* potassium phosphate buffer, pH 7.0, at various temperatures. The residual activities were monitored at intervals and the half-lives calculated. The half-life at 60° C was 150 min, whereas at 80° C it was only 3 min (Fig. 3). This enzyme could be classified as relatively thermostable (Klyosov 1988) for all practical purposes.

End-product inhibition

Figure 4 illustrates the inhibition of xylanase activity by the end-product, xylose. The standard assay mixture was supplemented with different concentrations of xylose (0.0–7.5 mg/ml) and the residual activity was measured. Fifty percent of the activity was lost with the addition of 2.8 mg xylose, while only 5% of the activity remained with 7.5 mg. Small amounts, viz 0.3 mg, did enhance the activity though not to a great extent (maximum 10%).

The strain isolated from the termite-infested soil is a good producer of xylanase(s) when grown on bagasse under thermophilic and alkalophilic conditions. The enzyme(s) was stable for 1 h at 65° C (pH 7.0 without substrate) and for 24 h in the pH range 4.0–6.0, whereas the activity was maximal at 60° C and 70° C at neutral pH. Besides being a good source of thermostable enzyme (thermophilic microorganisms are one of the best sources for thermostable enzymes: Horikoshi and Akiba 1982; Wiegel and Ljungdahl 1986), the thermoalkalophilic nature of this bacterium has the added advantage of minimizing contamination in the fermentor. Xylose, the end-product of xylan hydrolysis, inhibited both enzyme production and activity.

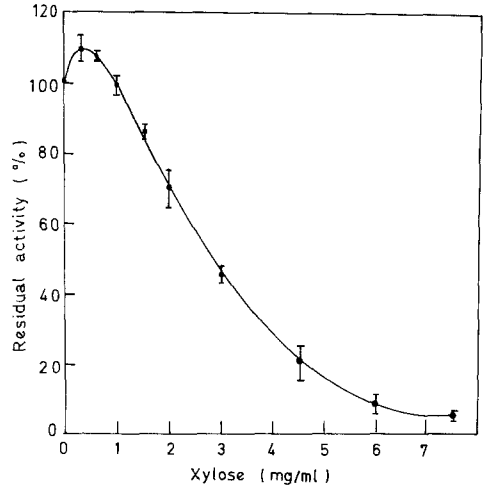

Fig. 4. End-product inhibition of xylanase activity. Different concentrations of xylose were added to the incubation mixture and residual activity was measured at 60° C and pH 7.0

Amongst many microorganisms known to produce xylanases, the members of the family Bacillaceae are considered excellent sources (Bernier et al. 1983). *Bacillus thermoalkalophilus* justifies the above view as it produces good amounts of xylanase from natural agricultural wastes. Earlier we reported that this organism is also a rich source of cellulase (Sarkar 1987). The thermoalkalophilic nature of this organism predicates an interest to manipulate it physiologically and genetically for academic and industrial purposes.

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