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Partial purification and characterisation of a xylanase enzyme produced by a micro-organism isolated from selected indigenous fruits of Zimbabwe

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Abstract

Aerobic bacteria and fungi isolated from *Ziziphus mauritiana, Scierocarya birrea* fruits and a cattle compost were screened for production of endo-xylanase enzyme. Xylanolytic activity was found in 10 of the 88 isolates obtained. Two best endo-xylanase enzyme producers (SB-9a and TC-17d) were selected for further investigations. The two isolates were classified as belonging to the genus *Bacillus*. The endo-xylanase enzymes from both isolates were optimally active at pH 8 and stable over a pH range of 6.0–9.0. The optimum temperature for xylanase activity, assayed at pH 8 was 60°C. The endo-xylanase from isolate SB-9a was stable at 50°C, maintaining over 50% of its activity for 1 h at pH 8. The endo-xylanase from isolate TC-17d was less stable, maintaining about 20% of its activity for 20 mm at 50°C and pH 8. Endo-xylanase activity for isolate SB-9a was inhibited by Hg^{2+}, Ag^{+} and Mn^{2+} ions while Fe^{3+}, K +, Nat, Ca^{2+}, and Cu^{2+} ions stimulated xylanase activity. The endo-xylanase enzyme from isolate SB-9a was partially purified by ammonium sulphate precipitation, and gel filtration chromatography. It had a specific activity of 308 nkat/mg protein. This enzyme could have potential uses in biotechnological applications such as in pulp, paper and food manufacture due to its high specific activity and alkaline pH optima.

1. Introduction

Indigenous fruits and forests of Zimbabwe form an invaluable source of micro-organisms for potential biotechnological use. These microbial sources have not been fully investigated to date. *Ziziphus mauritiana (masau)* and *Sclerocarya birrea* subspecies *caffra (mapfura)* fruits, for example, are among indigenous fruits that are laden with microbes. They ferment naturally following ripening. The composition of the fruits and the hot, arid environments in which they grow make them an attractive source of micro-organisms.

Besides their potential as sources of micro-organisms, these fruits form an important dietary component of the rural people of Zimbabwe. The fruits can be eaten fresh when ripe or can be preserved by drying for later use. Thus they contribute towards household food security.

Cattle composts also have an abundance of micro-organisms. They are characterised by high internal temperatures and hence may provide thermophilic micro-organisms which are of particular interest in industrial processes. Micro-flora from these fruits and a cattle compost were screened for the production of xylanolytic enzymes.

Xylan constitutes 20–30% of the weight of wood and agricultural wastes. Thus xylan is a potential significant resource for renewable biomass which can be utilised as a substrate for the preparation of many products such as fuels, solvents and pharmaceuticals. For most bioconversion processes, xylan must first be converted to xylose or xylo-oligosaccharides. This can be done either by acid hydrolysis or by the use of xylanolytic enzymes.

Several thermophilic and alkaliphilic bacteria, actinomyces and fungi producing xylanases have been isolated and the enzymes characterised (Cesar & Mrsa, 1996; Holtz, Kaspari & Klemme, 1991; Magnuson & Crawford, 1997; Murty & Chandra, 1992; Dahlberg, 1996). However, there is still need for the isolation of novel strains capable

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of producing, in simple media, the necessary enzymes, which are stable under high pH and temperature conditions, as well as possessing high specific activity.

This paper focuses on screening of *Ziziphus mauritiana (masau), Sclerocarya birrea (mapfura)* fruits and cattle compost for aerobic bacteria producing xylanolytic enzymes.

2. Materials and methods

2.1. Sample collection and isolation of pure cultures

Ziziphus mauritiana (masau) and *Sclerocarya birrea (mapfura)* ripe and fresh fruits were collected from Muzarabani and Mberengwa areas of Zimbabwe, respectively. They were stored at 4°C until needed for microbial screening. A cattle-compost manure was also used as a source of microorganisms. Pure cultures were obtained from these sources by streaking 3–4 times on agar plates.

2.2. Cultivation medium

The microbial cultures were grown in a complex, modified Ml 62 growth medium consisting of (per litre): tryptone (LAB M), 2.5 g; yeast extract (DIFCO), 2.5 g; oat-spelt xylan (SIGMA), 0.5% (w/v), a buffer solution, 100 ml [containing (g/l); KH_2PO_4, 5.44 g; $Na_2HPO_4.2H_2O$, 40 g] and a base solution, 100 ml. The base solution was made up as follows (per litre): nitriloacetic acid (SIGMA). 1.0 g; NaOH (SIGMA), 0.2 g; $CaSO_4.2H_2O$ (SIGMA), 0.4 g; $MgCl_2.6H_2O$, 2.0 g; Fe-citrate (0.05 M), *5* ml (Degryse, Glandorff & Pierard, 1978). The growth media was sterilized at 121°C for 15 mm before use.

The isolates were stored at −80°C, in the above growth medium containing 50% glyerol (v/w) but without xylan. For cultivation of micro-organisms on agar plates, the growth medium was supplemented with 3.0% (w/v) agar (DIFCO).

2.3. Preparation of pre-cultures

Pre-cultures were prepared by inoculating 50 ml of modified M162 medium in 250 ml Erlenmeyer flasks with a loopful of the pure strain. The pre-cultures were incubated overnight at 37°C and 150 rpm in a shaker incubator (New Brunswick Scientific, Innova 4300). The pre-cultures were microscopically examined for purity before inoculation for enzyme production.

2.4. Shake flask cultivations

Ten millilitres of the pre-culture were used to inoculate 100 ml of modified M162 medium in 500 ml Elernmeyer flasks. The experiments were carried out in a shaker incubator (New Brunswick Scientific, Innova 4300) at 37°C and 150 rpm for 24 h for bacterial isolates and at 30°C and 150 rpm for 72 h for fungal isolates.

Culture samples were collected at 1 or 2 h intervals during cultivations. The samples were spun immediately after collection at 15 000 rpm for 10 mm in a microfuge. Supernatants were frozen in 1.5 ml Eppendorf tubes at −20°C until required for further analysis.

2.5. Primary screening procedure

The harvested culture of each isolate, grown in modified Ml 62 medium, was centrifuged in a microfuge at 15 000 rpm for 10 mm. The cell-free culture supernatants were used as the enzyme source for screening purposes. To screen for xylanolytic activity, agar (DIFCO), 12%, was supplemented with 2–3 g/l of oat-spelt xylan (SIGMA X-0627) and 100 ml of 25 mM phosphate buffer and autoclaved at 121°C for 15 min. Agar plates were prepared using this medium. When the medium was solidified, a sterile 6 mm borer was used to make wells. Samples of the cell-free culture supernatants (50 μl) were poured into the wells and the plates incubated at 40°C for approximately 24 h. Enzymatic hydrolysis of the surrounding xylan in the agar plates produced clear zones in the medium in a milky/cloudy background. Controls of heat killed (140°C/20 min) culture supernatants were included. Results were recorded as xylanase positive or negative and the size of the clearing zone measured for xylanase positive strains.

2.6. Secondary screening procedure

Cultures that were endo-xylanase positive in the primary screening exercise underwent a secondary screening procedure where the culture supernatants were assayed for reducing sugars by the DNS stopping method (Bailey, Biely & Poutanen, 1992; Miller, 1959). Glucose was used as a standard. Enzyme activity was expressed as nanokatals/ml.

2.7. Fermentor cultivations

All bioreactor experiments were carried out in a 1.5-l fermenter (Applikon, ADI 1030) with a working volume of 1 l. The fermenter vessel, head plate, accessories and medium were autoclaved at 121°C for 45 min. Modified Degryse M162 medium was used. Fermenter conditions were: agitation speed 800 rpm, air flow rate of 30 1/h, temperature of 40°C and initial pH of 8.0. pH was monitored by measuring the pH of samples off line using a pH meter (JENWAY 3010). Foaming was prevented by addition of sterile anti-foam 289 (SIGMA) as necessary. A 10% pre-culture was used as the inoculum.

Samples were collected at 2 h intervals and treated as stated for the shake flask samples.

2.8. Endo-xylanase activity assay

Endo-1-4-β-xylanase activity was assayed using the DNS-stopping method at 50°C with 1% (w/v) oat-spelt xylan as substrate in 20 mM potassium phosphate buffer, pH 7 (Bailey et al., 1992). The crude enzyme sample (200 μl) was mixed with 1.8 ml of 1% (w/v) oat-spelt xylan in a 20 mM sodium phosphate buffer, pH 7, pre-warmed to 50°C. The mixture was incubated for 5 min at 50°C followed by addition of 3 ml of DNS reagent. The tubes were vortexed and placed in a boiling water-bath for 5 min and then chilled in water. Absorbance of the solutions was measured at 540 nm. Glucose was used as a standard.

2.9. Protein assay

Proteins were assayed by the bicinchoninic acid (BCA) method after Smith et al. (1986) using a Sigma assay kit according to the manufacturer's instructions.

2.10. Enzyme purification

2.10.1. Separation of cells and concentration of culture liquid

The culture broth from the fermenter was centrifuged at 8000 *g* in an ultracentrifuge for 10 min. The supernatant was micro-filtered through a 0.45 μm Whatman membrane mounted on a Buchner apparatus.

2.10.2. Ammonium sulphate precipitation

Proteins were precipitated by adding 60% (w/v) $(NH_4)_2SO_4$ for 4 h at 4°C with shaking. The mixture was centrifuged at 22 000 *g* for 30 min in an ultracentrifuge. The pellet was resuspended in 5 ml of 20 mM Tris/HC1 buffer, pH 8, and the suspension filtered through a 0.45 μm Whatman membrane filter. The filtrate was dialysed overnight with 10 mM Tris/HCl buffer, pH 8. The enzyme was concentrated by freeze drying and stored at −20°C.

2.10.3. Gel filtration chromatography

Sephadex G-75 gel filtration matrix was prepared as recommended by the manufacturer (Pharmacia Biotech). One millilitre of 450 mg/ml of enzyme sample was applied on a Sephadex G-75 column (3 × 40 cm). 20 mM Tris/HCl buffer was passed through the column at a flow rate of 0.4 ml/min. A UV detector (Bio-Rad) was used to determine protein levels (at 280 nm) of the fractions collected by a Bio-Rad Fraction collector (Model 2128). The fractions exhibiting xylanase activity were pooled, dialysed against 10 mM Tris/HC1 buffer, pH 8 and concentrated by lyophilization.

2.11. Characterisation of the crude and purified endo-xylanase

2.11.1. Effects of temperature on endo-xylanase activity

Xylanase activity was assayed at specified temperatures (from 30–75°C) and pH 8. The oat-spelt xylan substrate was pre-warmed and incubated at the specified temperature and the enzyme added at that temperature. The rest of the assay was performed as described above. Where necessary, the enzyme was diluted in 20 mM Tris/HC1 buffer, pH 8. For temperature stability, 1 ml of 10 mg/ml of the enzyme solution in 20 mM Tris/HCl buffer was incubated at specified temperatures (30–60°C) for 1 h and samples taken at 10 min intervals to assay for residual activity. Samples were kept frozen until required for assays.

2.11.2. Effects of pH on endo-xylanase activity

Xylanase activity of the enzyme samples was assayed in different 20 mM buffers of pH range 4–10 at a temperature of 50°C. Oat-spelt xylan 1% (w/v) was dissolved in the respective buffers and the xylanase activity assay carried out as previously described. pH stability was determined by diluting the crude enzyme with the same buffer solutions (50% v/v), then incubating at 30°C for 24 h. Samples were taken at 4 h intervals and assayed for residual xylanase activity. Samples were kept frozen until required for assays. The experiment was done in duplicate.

2.11.3. Effect of metal ions and enzyme inhibitors on endo-xylanase activity

Solutions of the following salts and compounds (5 mM) were prepared: NaC1, KC1, $ZnSO_4$, $MgCl_2$, $FeCl_3$, $CuSO_4$, $MnCl_2$, $CaCl_2$, $HgCl_2$, EDTA, $AgNO_3$, Iodoacetamide (SIGMA), Ethylenediaminetetraacetic acid (EDTA) and phenylmethyl-sulphonyl-fluoride (PMSF). Iodoacetamide, EDTA and PMSF were prepared by dissolving the appropriate amounts of each compound in dimethyl sulfoxide (DMSO). The crude enzyme solution was diluted with each of these compounds to give concentrations of 4 mM and 1 mM of either compound or chemical. The mixture was incubated at 30°C for 30 min. This enzyme/metal ion mixture (200 μl) was immediately used in the assay for xylanolytic activity described above. A control, in which Tris/HCl buffer replaced metal ion solutions was set up. The degree of inhibition or stimulation of enzyme activity was expressed as a percentage of the initial activity in the control experiment. The experiment was done in duplicate.

2.12. SDS gel electrophoresis

SDS gel electrophoresis was performed according to the method of Laemmli (1970).

3. Results and discussion

3.1. Primary screening

Pure cultures (88) were obtained from *Ziziphus mauritiana* and *Sclerocarya birrea* fruits and a cattle-compost sample. Following the primary screening procedure, 10 isolates were found to be endo-xylanase positive. The culture supernatants of these isolates gave a clear zone of between 10 and 20 mm in diameter when incubated at 40°C in agar containing xylan as shown in Table 1. Fruits and plant material form a suitable carbon source for micro-organisms. The presence of micro-organisms on these fruits, capable of degrading polymers such as xylan, can therefore be expected. Endo-xylanase positive strains were isolated from all the three microbial sources screened. Of the xylanase-positive isolates, five were from *S. birrea,* four from the cattle compost and one from *Z. Mauritiana*. Fewer xylanase positive strains were isolated from *Z. mauritiana.* The dry fruits had an abundance of fungi. However, the fungal isolates from the dry fruits were xylanase negative. *S. birrea* fruits when fresh, are more fleshy and less acidic than *Z. mauritiana* fruits which creates it a better habitat for bacteria. Isolates SB-9a and SB-9b, isolated from *S. birrea* fruits, grew in alkalinic conditions pH 8–9 (results not shown).

3.2. Secondary screening

The results for endo-xylanase production with time during secondary screening, using submerged culture, are shown in Fig. 1. Two isolates, SB-9a and TC-17d isolated from *S. birrea* and a cattle compost, respectively, were selected as the best producers of endo-xylanase enzyme.

3.3. Morphology of cultures SB-9a and TC-17d

The isolates SB-9a and TC-17d were rod shaped, Gram-positive, aerobic, motile, catalase positive and formed endospores. On the basis of these properties, the two isolates were classified as strains of the *Bacillus* genus (Claus & Berkeley, 1986).

Table 1
Xylanase-positive cultures obtained from *Z. mauritana, S. bierrea* fruits and a cattle compost sample

Culture code	Source of culture	Xylan clearing zone (mm)
TC-21a	Cattle compost	10
TC-l3	Cattle compost	10
TC-17d	Cattle compost	19
TC-38	*Z. mauritiana*	15
SB-3	*S. birrea*	12
SB-9b	*S. birrea*	18
SB-9a	*S. birrea*	20
TC-21	Cattle compost	17
SB-14	*S. birrea*	15
SB-15	*S. birrea*	13
HR-68 (control culture)	Chimanimani hot spring	14

3.4. Enzyme purification

The results of the purification of endo-xylanase from the culture supernatant of isolate SB-9a are shown in Table 2. Specific activity was increased by about 35 fold after gel filtration.

Xylanases have been purified to homogeneity using standard purification procedures (Cesar & Mrsa, 1996; John, Schmid & Schmid, 1979; Magnuson & Crawford, 1997; Murty & Chandra, 1992). Most purification protocols consist of 3–4 major steps. In this study, the endo-xylanase enzyme, from a *Bacillus* isolate, was partially purified in two major steps, consisting of concentration of proteins by ammonium sulphate precipitation and Sephadex G-75 gel filtration. Two peaks were detected from the collected fractions from the column (Fig. 2). Endo-xylanase activity was found in the second peak only.

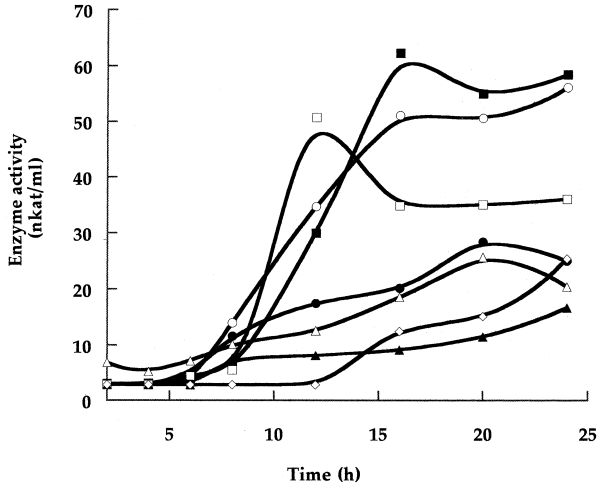

Fig. 1. Endo-xylanase enzyme production levels of seven micro-organisms isolated from *Z. mauritiana, S. birrea* and a cattle compost. The isolates were cultivated in M162 medium, pH 6.5 and at 37°C. —◇—, TC-38; —●—, HR-68; —○—, SB-9b; —■—, SB-9a; —△—, TC-21a; —▲—, TC-21; —□—, TC-17d.

Table 2
Purification of endo-xylanase from isolate SB-9a

Step	Total protein (mg)	Total activity (nkat/ml)	Specific activity (nkat/mg)	Yield (%)	Purification factor
Culture supernatant	764	6677	8.74	100	1.0
Ammonium sulphate ppt	492	4970	10.1	74.4	1.2
Gel filtration (G-75)	1.0	30.8	30.8	4.6	35.2

Enzyme loss during purification may be attributed to the proteins strongly adsorbing to the gel-filtration matrix. Such losses are, however, normal with most protocols (Cesar & Mrsa, 1996; Chen, Chen & Lin, 1997; Magnuson & Crawford, 1997).

The SDS gel picture (Fig. 3) shows the partially purified xylanase enzyme in lanes 2 and 3. A zymogram analysis would have proved whether the major protein band was responsible for the reported xylanase activity or not.

3.5. Properties of the crude and purified enzyme

3.5.1. Effect of pH and pH stability of the endo-xylanase enzyme

Both isolates produced endo-xylanase enzyme which had an optimum pH of activity at pH 8.0 (Fig. 4). The crude endo-xylanase enzymes were stable in the pH range 6.0–9.0. However, the crude enzyme from isolate SB-9a was more stable than that of isolate TC-17d (Fig. 5). It was more stable between pH values 7 and 8 retaining over 95% of activity after 1 h of incubation at 30°C

The purified endo-xylanase from isolate SB-9a was also stable in the pH range 6.0–9.0. However, it was maximally stable at pH 7, retaining 100% of its activity up to 24 h at 30°C. At pH 8, over 90% of activity was maintained after 24 h (figure not shown). The enzyme was not active or stable at pH 4 and 10.

3.5.2. Optimum temperature and temperature stability of the endo-xylanase enzyme

The endo-xylanase enzyme from isolates SB-9a had an optimum temperature of 60°C (Fig. 6). There was no observed difference in optima between the crude and the pure enzyme.

The purified endo-xylanase from SB-9a was most stable at temperatures 30, 40 and 50°C (Fig. 7). At 30 and 40°C, over 80% activity was maintained for 1 h. At 50°C, over 90% activity was maintained up to 20 min.

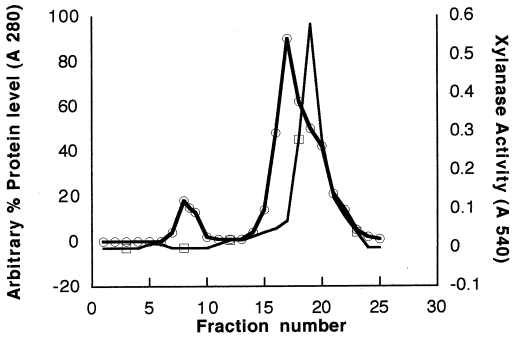

Fig. 2. Elution profile of proteins on Sephadex G-75 gel filtration medium as shown by absorbance at 280 nm. Fractions 18–22 which had xylanase activity were pooled and concentrated as described in the text.

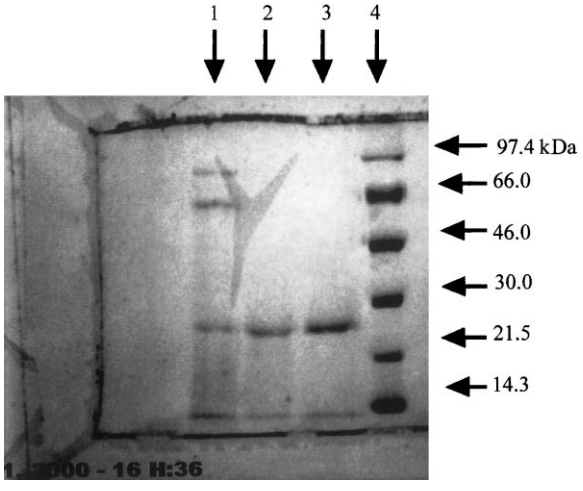

Fig. 3. SDS PAGE of the endo-xylanase enzyme from isolate SB-9a. Lane 1: Ammonium sulphate precipitated crude enzyme, Lanes 2 and 3 are a duplicate of the G 75 partially purified enzyme, Lane 4: Molecular weight markers.

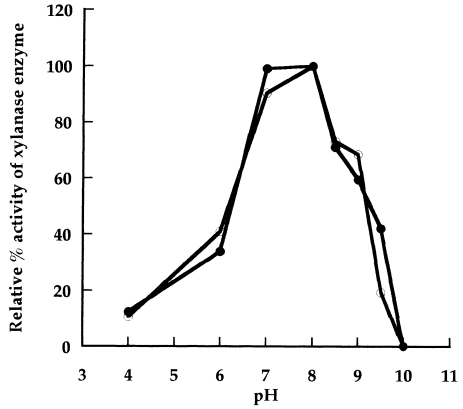

Fig. 4. Determination of the optimum pH for the crude endo-xylanase enzyme activity. The following buffers were used: pH 4–7, 20 mM sodium citrate; pH 7.5–8.5, 20 mM Tris/HC1; pH 9–10, 20 mM sodium hydrogen carbonate. —●—, TC-17d; —○—, SB-9a.

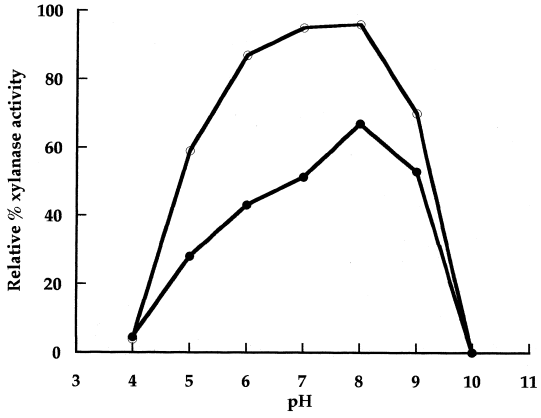

Fig. 5. pH stability of the crude endo-xylanase enzyme from isolates TC-17d and SB-9a. The enzyme was incubated in the specified buffer for 1 h at 30°C after which xylanase activity was determined. —●—, TC-17d; —○—, SB-9a.

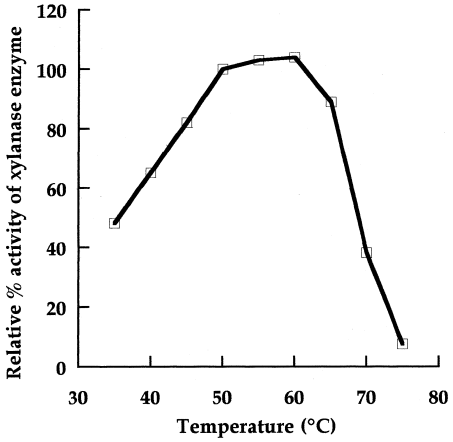

Fig. 6. Optimum temperature of activity of the purified endo-xylanase enzyme from isolate SB-9a assayed in Tris/HCl buffer at pH 8.

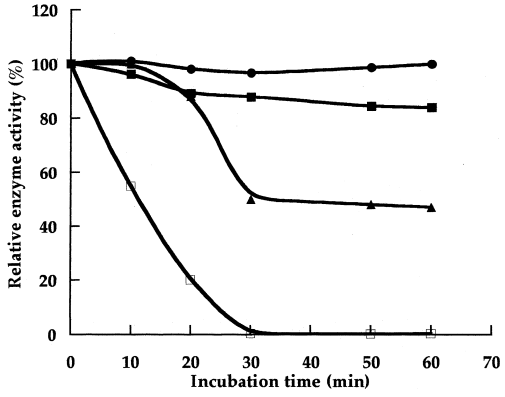

Fig. 7. Temperature stability of the purified endo-xylanase enzyme from isolate SB-9a. The enzyme was incubated at specified temperatures and residual activity assayed in Tris/HC1 buffer, pH 8. —●—, 30°C; —■—, 40°C; —▲—, 50°C; —□—, 55°C.

At 55°C over 50% of activity was maintained for 10 min. The endo-xylanase enzyme from TC-17d was less stable than that of SB-9a. Less than 20% of activity of the enzyme from TC-17d remained after 20 min at 50°C (results for TC-17d not shown).

3.5.3. Effect of metal ions, activators and inhibitors on endo-xylanase

Xylanase activity was reduced by Mg^{2+}, Ag^{2+}, and Hg^{2+} (Table 3). Ag^{2+} and Hg^{2+} ions completely inhibited the enzyme at 4 mM concentrations. Manganese ions reduced activity to less than 40% at 1 mM. The inhibition of the enzyme was generally obtained with ions which reacted with sulphydryl groups such as Hg^{2+} ions, suggesting that there was an important cysteine residue in or close to the active site of the enzyme. Other workers have reported similar results (Bastawde, 1987; Cesar & Mrsa, 1996; Sreenath & Joseph 1982). However the stimulation by iodoacetamide is unexpected as it may inhibit sulphydryl groups. Such anomalies have been reported by other workers (Breccia Sineriz, Baigori, Custro & Hatti-Kaul, 1998; Cesar & Mrsa, 1996)

Table 3
The effect of metal ions and enzyme inhibitors on xylanase activity after exposure of the enzyme for 30 min at 30°C to each of the compounds

	% Residual activity	
Ion/inhibitor	4 mM	1 mM
None	100	100
$AgNO_3$	0.0	20.6±0.13
$HgCl_2$	0.0	2.42±0.01
$MnCl_2$	29.8±0.01[a]	39.1±0.08
KCl	257±0.07	131±0.08
Citric acid	78.4±0.12	95.0±0.11
$FeCl_3$	136±0.00	115±0.07
$CuCl_2$	116±0.01	114±0.05
EDTA	171±0.21	94.3±0.09
$MgCl_2$	29.8±0.04	39.1±0.03
Iodoacetamide	86.3±0.06	96.0±0.03
$CaCl_2$	85.9±0.02	109±0.04
Nacl	189±0.01	122±0.01
$CoCl_3$	85.7±0.02	87.6±0.01
PMSF	68.3±0.18	78.5±0.25

[a] ± = Standard deviation of two determinations

Metal ions which stimulated xylanase activity were Fe^{+3}, K^{+}, Na^{+}, Ca^{+2}, Cu^{2}, Cu^{2+}. It is not certain from the study whether these ions/agents could be binding to the enzyme, causing conformational changes and resulting in increased enzyme activity, or whether the xylanase requires a metal ion on the active site. Further work using more inhibitors, their analogues and combinations thereof would be necessary to ascertain the mode of action of the xylanase.

4. Conclusion

Indigenous Zimbabwean fruits could serve as a source of micro-organisms that produce xylanase enzymes. Such enzymes could be used in food and related industrial applications.

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