Journal of Industrial Microbiology, 4 (1989) 173–180
Elsevier
SIM 00173

Comparative study of some microbial arabinan-degrading enzymes

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Received 2 May 1988
Revised 26 August 1988
Accepted 1 September 1988

Key words: Arabinan-degrading enzyme; Arabanase; *p*-Nitrophenyl-α-L-arabinosidase

SUMMARY

A variety of thermophilic organisms and *Bacillus* species were screened in shake flask culture for arabanase and *p*-nitrophenyl-α-L-arabinosidase activities. Highest arabanase activity was produced by strains of *Thielavia terrestris* and *Sporotrichum cellulophilum*. *Thermoascus aurantiacus* and several *Bacillus* species were most active producers of arabinosidase. Arabinosidases from *Bacillus* strains had pH optima in the range 5.9–6.7. pH optima of fungal arabinosidases ranged from ≤ 2.9 to 6.7. *Bacillus* arabanases had neutral pH optima, whereas fungal arabanases had pH optima in the range 3.7–5.1. In general, arabinosidases were found to be relatively thermostable, retaining > 70% activity for 3 h at 60°C. The *T. aurantiacus* enzyme retained 98% activity at 70°C after 3 h. *Bacillus* arabanases were relatively unstable. All fungal arabanases except the *T. aurantiacus* enzyme were fully denatured at 70°C after 3 h.

INTRODUCTION

The arabinans are associated with pectic substances located in the primary cell wall and intracellular regions of higher plants [1]. These polysaccharides are primarily made up of a 1,5-α-linked arabinose polymeric main chain containing some 1,3-α-linked single unit arabinose side chains [3]. Water-soluble arabinan, present in apple juice, consists of an arabinose backbone polymer containing 1,5-α-linkages with monomeric and dimeric 1,2- and 1,3-linked arabinose side chains [2]. Arabinogalactans of cell wall matrices or cell reserve polysaccharide materials and arabinoxylans associated particularly with the hemicellulose fraction of woody tissue [4] contain arabinose side chains attached to a galactan and xylan backbone respectively.

Endogenous plant enzymes, which modify these arabinose-containing polysaccharides, have been implicated, together with other polysaccharases, in plant growth and development [28] and plant pathogenesis [5,8–10]. Endo-1,5-α-L-arabinase (EC 3.2.1.99) is produced by bacteria, including strains

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of *Bacillus subtilis* and *Clostridium felsineum* [15] and by various fungi [17,18], including *Botrytis cinerea, Coniothyrium diplodiella, Gloesporium khaki* and *Sclerotinia sclerotiorum*. α-L-Arabinosidases (EC 3.2.1.55) are capable of hydrolysing both 1,3- and 1,5-α-L-arabinofuranosyl linkages [20,23]. They remove arabinose units from the non-reducing end of the arabinan chain. They are produced by bacteria including *B. subtilis* [12] and *Cellulomonas* species [24], *Streptomyces purpurescens* [25] and fungi including *Panaeobus* species and *Aspergillus* species [27].

In apple juice extraction, using commercial pectinase preparations, the presence of α-L-arabinosidase activity can sometimes cause haze problems. Enzymic removal of the 1,2- and 1,3-linked arabinose side chains of water-soluble apple juice arabinan causes the residual unbranched chains to associate, forming insoluble micelles which are responsible for the haze [31]. These hazes can be removed by using endo-1,5-L-arabinases. Arabinan hydrolases may also be used in combination with other enzymes in food and vegetable liquefaction processes and in the recovery of sucrose or starch from beet and potato respectively [30]. Facultative anaerobic bacterial strains isolated from the rumens of animals often produce arabinan-degrading enzymes and these organisms may have application in the bioconversion of plant residues into chemical feedstocks [33].

We have carried out a comparative study on the production of arabinan-degrading enzymes by *Aspergillus niger*, some *Bacillus* species and some thermophilic micro-organisms.

MATERIALS AND METHODS

Maintenance media

All *Talaromyces* strains were maintained on malt agar (Difco). *Thermomyces lanuginosus* was maintained on PYG medium (Difco Bacteriological Peptone, 1.25 g/l; yeast extract, Sigma, 1.25 g/l; glucose, 3 g/l; Difco Bacto-Agar, 20 g/l). Potato dextrose agar (glucose, 20 g/l; Difco Bacto-agar, 15 g/l; potato extract, 1 litre) was used as maintenance medium for the remaining fungal strains. *Thermoactinomyces* species were maintained on potato-carrot agar (potato, 300 g/l; carrots, 25 g/l; Difco Bacto-agar, 15 g/l). *Thermomonospora* strains were maintained on CMC-salt-agar medium (CMC, 10 g/l; $MnSO_4 \cdot 7H_2O$, 2.5 g/l; yeast extract, Sigma, 0.5 g/l; KH_2PO_4, 2.7 g/l; Na_2HPO_4, 5.3 g/l; NaCl, 0.2 g/l; $MgSO_4 \cdot H_2O$, 0.2 g/l; $CaCl_2$, 0.05 g/l; Difco Bacto-agar, 15 g/l). *B. licheniformis* strains NRRL-1264 and ATCC 27811 and *B. stearothermophilus* strains were maintained on brain heart infusion agar (Difco). All remaining *Bacillus* strains were maintained on nutrient agar (Difco). Temperature of incubation of cultures on maintenance media was 45°C for *S. cellulophilum, Thermomyces lanuginosus, Thielavia* strains, *Thermoactinomyces vulgaris* ATCC 21364, *Thermomonospora fusca, B. licheniformis* ATCC 27811 and *B. stearothermophilus* strains. All other strains were maintained at 37°C.

Fermentation media for enzyme production

Medium A (per 100 ml): beet pulp, 1.0 g; potato extract, 50 ml; corn steep solids, 0.1 g; yeast extract, 0.1 g; basal salts: $NaNO_3$, 2 g/l; KH_2PO_4, 2 g/l; $CaCl_2$, 0.3 g/l; $MgSO_4 \cdot 7H_2O$, 0.3 g/l; $FeSO_4 \cdot 7H_2O$, 0.5 mg/l; $ZnCl_2$, 0.14 mg/l; $MnSO_4 \cdot H_2O$, 0.156 mg/l; $CoCl_2 \cdot 6H_2O$, 0.2 mg/l; $CuSO_4 \cdot 5H_2O$, 0.2 mg/l; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.04 mg/l; pH of medium, 4.0.

Medium B (per 100 ml): beet pulp extract, 15 ml; diced potato, 25 g; corn steep solids, 0.1 g; yeast extract, 0.1 g; basal salts: as for Medium A; pH of medium, 4.0.

Medium C: beet pulp extract, 10 ml; bacteriological peptone (Difco), 0.5 g; KCl, 0.05 g; K_2HPO_4, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; pH of medium, 7.0.

Beet pulp extract was prepared by extraction of 100 g beet pulp with 2 litres of 0.5% NaOH at 100°C for 1 h, recovery and neutralization of the filtrate to pH 7.0 with HCl. The filtrate was then made up to 1 litre. Potato extract was prepared by boiling 150 g diced potato in 250 ml water for 2 h. The extract was recovered by filtration through cheesecloth and made up to 500 ml.

250 ml Erlenmeyer flasks containing 100 ml of medium were sterilised and loop inoculated from maintenance agar slopes. *B. circulans, B. megateri-*

um, B. brevis, B. coagulans, B. licheniformis ATCC 14580 and *B. subtilis* were cultivated at 37°C on an orbital shaker set at 150 rpm for enzyme production. All other strains were incubated at 45°C. These were shaken at 225 rpm because of the more viscous nature of the fungal cultures. *Bacillus* strains were incubated for 72 h. All other strains were cultured for 120 h. In preliminary investigations on enzyme production versus time, maximum enzyme activities were generally observed at the latter incubation times. Cell-free supernatants containing the enzyme activity were prepared by centrifugation at 3100 × *g* for 10 min.

Enzyme assays

Beet arabinan was prepared essentially using the method of Hirst and Jones [11]. In summary, 50 g ground sugar beet pulp was extracted with 400 ml of 2.5% (w/v) calcium hydroxide at 100°C for 12 h. The clarified supernatant was acidified to pH 4 using acetic acid and held overnight. Insoluble material was removed and the arabinan was purified by repeated ethanol precipitation and resuspension in deionized water. Finally, the arabinan was recovered from an aqueous solution by freeze drying. The reaction mixture, total volume 1 ml, for assay of arabanase contained arabinan 5 mg, and enzyme in 0.01 M sodium citrate phosphate buffer, pH 5.2. Enzyme reactions were carried out at 50°C for 1 h. Reducing sugar produced due to enzyme activity was determined using the dinitrosalicylic acid method of Bernfeld [5]. A standard curve was prepared using arabinose. One unit of enzyme activity produces 1 μM of arabinose reducing equivalents per min at 50°C.

The reaction mixture for determination of arabinosidase contained 1.25 μM *p*-nitrophenyl-α-L-arabinofuranoside and enzyme in 1 ml of 0.01 M sodium citrate-phosphate buffer, pH 5.2. Enzyme reactions were carried out for 30 min at 50°C and the reaction was stopped by adding 2 ml of 2% (w/v) Na_2CO_3. Absorbance due to production of *p*-nitrophenol was determined at 420 nm and compared with a *p*-nitrophenol standard curve. One unit of enzyme activity produces 1 μM *p*-nitrophenol per min.

Enzyme assays were carried out at diluted enzyme concentrations giving initial rate values. Assays were reproducible within a given assay and between separate assays.

For pH versus enzyme activity determinations, the pH of the enzyme-substrate reaction mixture was varied as indicated. pH-adjusted citrate-phosphate buffer, final assay concentration of 0.01 M, was used in this experiment. The effect of temperature on enzyme stability was determined following incubation of the enzyme with 0.05 M sodium citrate-phosphate buffer, pH 5.2 for up to 3 h. Residual activity was then measured.

RESULTS

Cell-free supernatant arabanase and *p*-nitrophenyl-α-L-arabinosidase activities observed in shake flask cultures are presented in Table 1. Highest arabanase activity was produced by *Thielavia terrestris* strains NRRL 8126 (0.4 U/ml) and ATCC 26917 (0.35 U/ml) and *S. cellulophilum* (0.33 U/ml). Highest *p*-nitrophenyl-α-L-arabinosidase activities were detected in cultures of *B. subtilis* NRRL NRS-744a and *B. subtilis* NRRL B-356, *B. licheniformis* NRRL NRS-1264 and *Thermoascus aurantiacus* ATCC 26904. *B. circulans* produced *p*-nitrophenyl-α-L-arabinosidase activity but no arabanase activity. Microbial strains screened which exhibited < 0.8 U/ml of both arabanase and arabinosidase are listed at the bottom of Table 1. Neither activity was detected in cultures of *B. megaterium* ATCC 14581, *B. brevis* ATCC 8186, *B. coagulans* ATCC 7050, *B. subtilis* strains NRRL B-972 and NRRL B-941.

Organisms giving the highest enzymatic activities were selected in order to carry out preliminary comparative studies on the enzymes. pH optima for *p*-nitrophenyl-α-L-arabinosidases and arabanases are presented in Fig. 1a and b and Fig. 2a and b respectively. *p*-Nitrophenyl-α-L-arabinosidases from the *Bacillus* strains have pH optima in the range 5.9–6.7. A much broader spectrum of pH optima was observed with enzyme from the fungi, ranging from 6.7 for *S. cellulophilum* to a very low pH optimum of ⩽ 2.9 for *Talaromyces byssochalamydoides*. Ara-

Table 1

Arabanase and *p*-nitrophenyl-α-L-arabinosidase activities in culture cell-free supernatants (A, B and C denote media used for enzyme production)

Organism	Number	Enzyme activity (U/ml)[a]	
		arabanase	arabinosidase
Thermoascus aurantiacus	ATCC 28082	0.02 A	0.110 A
Thermoascus aurantiacus	ATCC 26904	0.11 B	0.200 A
Talaromyces emersonii	NRRL 3221	0.03 A	0.080 A
Talaromyces byssochalamydoides	NRRL 3658	0.11 A	0.080 A
Thielavia terrestris	NRRL 8126	0.40 A	0.100 A
Thielavia terrestris	ATCC 26917	0.35 A	0.070 A
Sporotrichum cellulophilum	ATCC 20493	0.33 A	0.005 B
Aspergillus niger	NRRL 337	0.11 B	0.160 A
Bacillus licheniformis	NRRL NRS-1264	0.04 B	0.200 B
Bacillus circulans	ATCC 4513	0.00 C	0.120 C
Bacillus licheniformis	ATCC 14580	0.03 C	0.080 C
Bacillus subtilis	NRRL B 544	0.05 C	0.160 C
Bacillus subtilis	NRRL B 209	0.03 C	0.080 C
Bacillus subtilis	NRRL B 365	0.03 C	0.090 C
Bacillus subtilis	NRRL B 356	0.04 C	0.200 C
Bacillus subtilis	NRRL NRS 744a	0.04 C	0.200 C
Bacillus subtilis	NRRL B-1466	0.04 C	0.150 C

[a] The following strains produced < 0.8 U/ml of both arabanase and arabinosidase: *Talaromyces emersonii* ATCC 28080, *Thermomyces lanuginosus* ATCC 34626, *Thermoactinomyces vulgaris* strains ATCC 21364 and NRRL 5790, *Thermomonospora fusca* ATCC 27730, *Thermomonospora viridis* NRRL B-3044, *Bacillus licheniformis* ATCC 27811, *Bacillus stearothermophilus* strains ATCC 29609 and ATCC 21365, *Bacillus megaterium* ATCC 14581, *Bacillus brevis* ATCC 8186, *Bacillus pumilus* ATCC 72, *Bacillus coagulans* ATCC 7050, *Bacillus cereus* ATCC 14893 and *Bacillus subtilis* strains NRRL (B-207, B-357, B-360, B-363, B-447, B-645, B-941, B-972, B-1471, B-3749, B-4219, B-14195, B-14206, B-14393) and ATCC (6051, 6633, 9943, 14307, 21331, 21394).

banases from the *Bacillus* strains had pH optima in a narrow range, pH 7.0–7.3, while the fungal arabanases had more acidic pH optima in the range 3.7–5.1.

In order to determine the effect of temperature on enzyme stability, residual enzyme activity was determined following incubations of each of the enzymes for up to 3 h at temperatures ranging from 40 to 80°C. The results are presented in Figs. 3 and 4. Comparison of residual *p*-nitrophenyl-α-L-arabinosidase activities indicate that, with the exception of the *S. cellulophilum* enzyme, all other enzymes retained greater than 70% activity for 3 h at 60°C. In particular, the enzyme from *Thermoascus aurantiacus* manifested exceptional thermal stability, retaining 100%, 98% and 72% residual activity after 3 h at 60°C, 70°C and 80°C respectively. The *Bacillus* arabanases were all relatively unstable with enzyme activity being denatured after 1 h at 40 or 50°C. Temperature stability varied among fungal arabanases. The enzyme from *S. cellulophilum* was least stable, retaining 15% residual activity when incubated at 60°C for 3 h. Arabanases from *Thermoascus aurantiacus* and *Talaromyces byssochalamydoides* retained 100% activity after a 3 h incubation at 60°C. With the exception of the *Thermoascus aurantiacus* enzyme, which retained 33% residual activity at 70°C after 3 h, all the other arabanases were fully denatured at 70°C after 3 h.

DISCUSSION

Many strains of *Bacillus*, isolated from the rumen

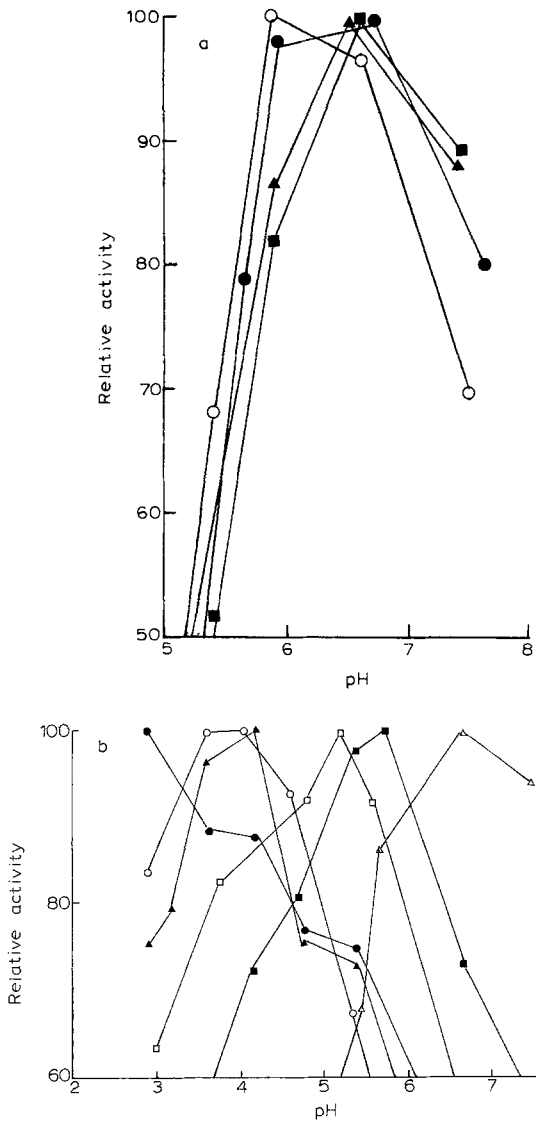

Fig. 1 (a) Effect of pH on activity of *p*-nitrophenyl-α-L-arabinosidases of *Bacillus* strains. ○, *B. licheniformis* ATCC 14580; ●, *B. licheniformis* NRRL NRS 1264; ▲, *B. subtilis* NRRL B356; ■, *B. subtilis* NRRL NRS 744a. (b) Effect of pH on activity of *p*-nitrophenyl-α-L-arabinosidases of fungal strains. ○, *A. niger*; ●, *Talaromyces byssochalamydoides*; □, *Thielavia terrestris* NRRL 8126; ■, *T. terrestris* ATCC 26917; △, *S. cellulophilum*; ▲, *Thermoascus aurantiacus* ATCC 26904.

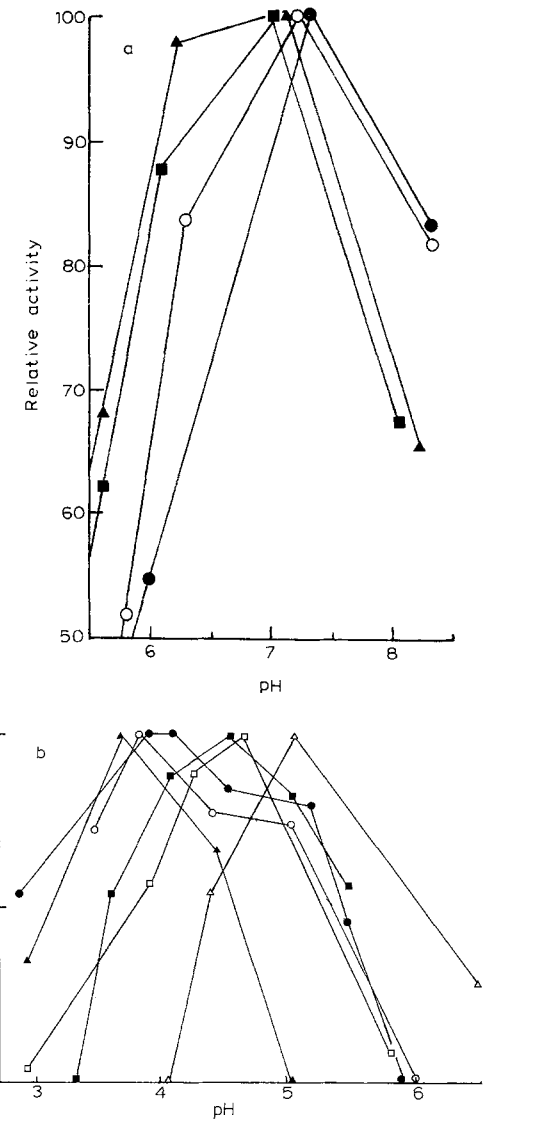

Fig. 2. (a) Effect of pH on activity of arabanases of *Bacillus* strains. ○, *B. licheniformis* ATCC 14580; ●, *B. licheniformis* NRRL NRS 1264; ▲, *B. subtilis* NRRL B356; ■, *B. subtilis* NRRL NRS 744a. (b) Effect of pH on activity of arabanases of fungal strains. ○, *A. niger*; ●, *Talaromyces byssochalamydoides*; □, *Thielavia terrestris* NRRL 8126; ■, *T. terrestris* NRRL 26917; △, *S. cellulophilum*; ▲, *Thermoascus aurantiacus* ATCC 26904.

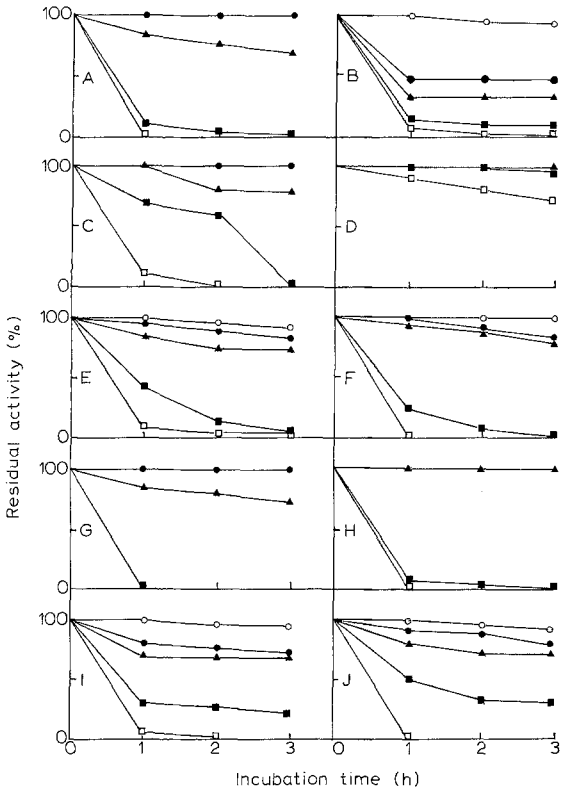

Fig. 3. Effect of incubation temperature on stability of *p*-nitrophenyl-α-L-arabinosidases. ○, 40°C; ●, 50°C; ▲, 60°C; ■, 70°C; □, 80°C. A, *A. niger;* B, *S. cellulophilum;* C, *Talaromyces byssochalamydoides;* D, *Thermoascus aurantiacus* ATCC 26904; E, *Thielavia terrestris* NRRL 8126; F, *T. terrestris* ATCC 26917; G, *B. licheniformis* NRRL NRS 1264; H, *B. licheniformis* ATCC 14580; I, *B. subtilis* B356; J, *B. subtilis* NRRL NRS 744a.

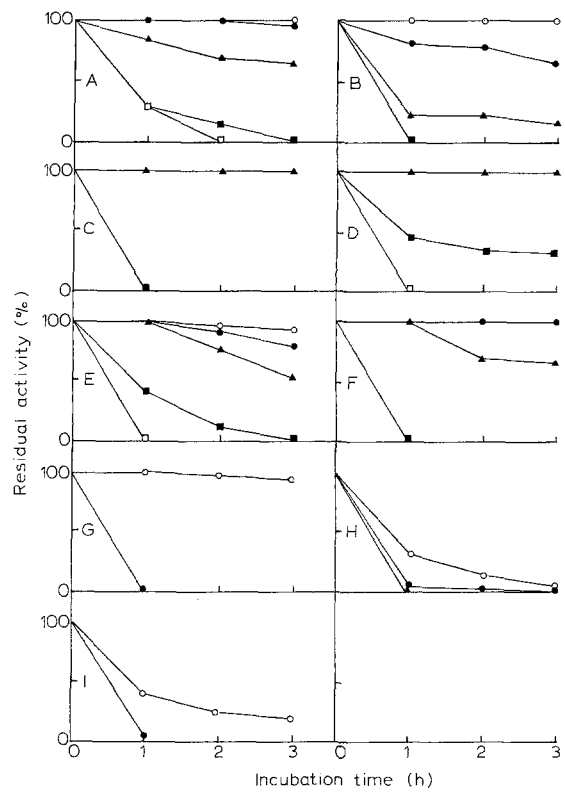

Fig. 4. Effect of temperature on stability of arabanases. ○, 40°C; ●, 50°C; ▲, 60°C; ■, 70°C; □, 80°C. A, *A. niger;* B, *S. cellulophilum;* C, *Talaromyces byssochalamydoides;* D, *Thermoascus aurantiacus* ATCC 26904; E, *Thielavia terrestris* NRRL 8126; F, *T. terrestris* ATCC 26917; G, *B. licheniformis* NRRL NRS 1264; H, *B. licheniformis* ATCC 14580; I, *B. subtilis* NRRL B356.

contents of animals fed on hay, produce arabanase and arabinosidase activity under anaerobic culture conditions [33]. A variety of other rumen microorganisms from the genera *Eubacterium, Ruminococcus, Butyrivibrio* and *Bacteroides* also exhibited arabanase and arabinosidase activity [32]. As observed in our experiments, activity levels tend to vary widely within a species. This was also observed with arabinosidase activities of a variety of species of *Corticium rolfsii* [21].

The relatively high pH optima observed for bacterial arabinan-degrading enzymes are consistent with the findings of other workers. The enzymes from *B. macerans* [34], *B. subtilis* [19], *Streptomyces purpurescens* [24], *Clostridium felsineum* [14] and *Streptomyces massasporeus* [16] had pH optima of 7.0, 6.5, 6.5, 5.6 and 5.0 respectively. A variety of fungi [6,13] produced arabinosidases in the pH range 3.0–6.0. As was observed in the case of *S. cellulophilum,* a number of fungi produced arabinosidases having pH optima of less than 3.0. The enzymes from *Corticium rolfsii* [18], *Poria viporaria* [7] and *Lentinus edodes* [7] all had pH optima of 2.5 while *Rhodotorula flava* [26] exhibited a pH optimum of 2.0. Very few arabanases have been characterised. The pH optima for arabanases from *A. niger* [19] and *B. subtilis* F-11 [15] were reported to be 4.0 and 6.0 respectively. Comparative data on thermostabilities are not available in the literature.

The pH or temperature characteristics of en-

zymes degrading arabinose containing polysaccharides will vary depending on application. Where prolonged incubations of plant tissue extracts are required for enzymatic hydrolysis it is desirable that incubation temperature is maintained above 65°C to minimise microbial spoilage [29]. Enzyme digestion temperatures for extraction or clarification of fruit juice material are generally lower than those required for recovery of carbohydrate extracts from tissues such as cereals and tubers. For processes such as recovery of maximum extract from beet pulp or potato, higher digestion temperatures would be more effective. In general, the more acidic properties of fungal arabinan-degrading enzymes are more suitable for digestive extraction of plant materials.

Selected enzymes from this work will now be purified and more fully characterised.

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