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Extracellular mannanases and galactanases from selected fungi

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SUMMARY

Eight thermophilic fungi were tested for production of mannanases and galactanases. Highest mannanase activities were produced by *Talaromyces byssochlamydoides* and *Talaromyces emersonii*. Mannanases from all strains tested were induced by locust bean gum except in the case of *Thermoascus aurantiacus*, where mannose had a greater inducing effect. Locust bean gum was also the best inducer of β-mannosidase and galactanase except in the case of *T. emersonii* where galactose was a better inducer of both these enzymes. Highest mannanase activity was produced by *Talaromyces* species when peptone was used as nitrogen source whereas sodium nitrate promoted maximum production of this enzyme by *Thielavia terrestris* and *T. aurantiacus*. The pH optima of mannanases from the thermophilic fungi were in the range 5.0–6.6 and contrasted with the low pH optimum (3.2) of the enzyme from *Aspergillus niger*. Galactanases had pH optima in the range 4.3–5.8. The mannanase from *T. emersonii* and the galactanase from *T. terrestris* were most thermostable, each retaining 100% activity for 3 h at 60 °C.

INTRODUCTION

The family of polymers comprising hemicellulose is an abundant renewable raw material and, therefore, represents a potential alternative source of fuels and chemicals [9]. Some hardwood species contain up to 35% hemicellulose [41]. The major hemicellulose components of wood are galactomannans (softwood), the glucomannans (hardwoods), arabinogalactans (larch wood) and soft- and hardwood xylans [41]. In contrast to the starch-containing cereal grains, galactomannan is the main storage carbohydrate in leguminous seeds [16]. For example, galactomannan constitutes 18–20% of the dry weight of guar and locust beans [26]. Mannans are a major part of the dry weight of many palm seeds, causing thickening of the endosperm wall and conferring the characteristic hardness on the palm kernel [29]. Mannanases and galactanases, therefore, may have potential as important supplementary enzymes to cellulases in the conversion of wood polysaccharides to hexoses. Mannanases and galactanases may be used to hydrolyse mannans and galactans of coffee bean in the coffee production process and these enzymes also have potential applications for processing other plant materials [17,19].

A variety of mesophilic microbial species produce mannanases and galactanases. *Sclerotium rolfsii* and *Rhizopus niveus* produce a combination of galactanases and mannanases [19,37]. Galactanase was found to be produced by *Penicillium expansum* [23] and *Botrytis cinerea* [36]. Fungal β-1,5-mannanases or β-mannosidases have been shown to be produced by *Aspergillus* species [5,7,12,31,35], *Chrysosporium lignorum* [15], *Polyporus* species [38], *Tremella fusiformis* [33], *Tyromyces palustris* [20] and *Trichoderma* species [15,32].

There are substantial commercial advantages in carrying out enzymic reactions at higher temperatures [40]. Enzymatic digestion at increased temperatures (above 60–65 °C) may reduce microbial contamination of the material being processed [6]. In addition, higher temperatures increase the rate of substrate digestion and additionally increase the solubility of the polymeric substrates, such as carbohydrates, rendering them more amenable to enzymatic attack. Considerable research has been devoted to identifying more thermostable industrial enzymes and a number of thermophilic fungi producing more thermostable cellulases have been identified [8,11,21,24,27,28]. However, research has not been carried out to identify sources of thermostable mannanases and galactanases. Since, in many of the potential applications, these latter enzymes might be used in combination with cellulase, we have screened a variety of cellulase producing thermophilic fungi as possible future commercial sources of mannanase and galactanase activity. *Aspergillus niger* was included in the study because its mannanase has been well characterised.

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MATERIALS AND METHODS

Organisms and culture conditions

Aspergillus niger NRRL 337, *Thielavia terrestris* NRRL 8126 and ATCC 26917, *Thermoascus aurantiacus* ATCC 26904 and ATCC 28082 and *Sporotrichum cellulophilum* ATCC 20493 were maintained on potato-dextrose agar slopes. *Talaromyces byssochlamydoides* NRRL 3658 and *Talaromyces emersonii* NRRL 3221 and NRRL 18080 were maintained on malt agar (Difco, Detroit). Stock cultures were incubated for 96 h at 37 °C for *A. niger* and *T. terrestris* and at 45 °C for *T. aurantiacus*, *S. cellulophilum* and *Talaromyces* spp. and then stored at 4 °C for a maximum of one month.

The basic mineral medium for enzyme production studies contained (per l) KH_2PO_4, 2 g; $CaCl_2 \cdot 2H_2O$, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; trace metal solution [1], 1 ml. For initial screening of fungal cultures for mannanases and galactanases, this medium was supplemented with 10 g/l locust bean gum (Sigma, St. Louis); 2 g/l $NaNO_3$; 1 g/l corn steep solids and 20% (v/v) potato extract. Potato extract was prepared by boiling 300 g diced potato in 500 ml water for 1 h. The extract was recovered by straining through cheesecloth and the volume made up to 1 l with water. The same liquid medium was used for preparation of inoculum except 5 g/l locust bean gum was used instead of 10 g/l. For studies of the effect of carbon and nitrogen sources on enzyme production, the basic mineral medium was supplemented with carbohydrates (10 g/l) and nitrogenous constituents (1 g/l) as indicated in the results section. The pH of all culture media was adjusted to 5.5.

Liquid cultures were incubated in 250 ml Erlenmeyer flasks containing 80 ml of medium. The flasks were stoppered with cotton plugs. Cultures were incubated at 45 °C on an orbital shaker set at 200 rpm. The seed culture medium was inoculated with mycelial mat (1 cm^2) from stock culture slopes and incubated for 30 h. Enzyme production flasks were then inoculated with 2 ml of this 30 h inoculum culture and incubated for 48 h. In preliminary production studies, biomass concentrations and enzyme activities produced in flasks containing 50 and 80 ml media were identical. Because 9.0 ml water was observed to evaporate from both culture volumes in 48 h, the 80 ml volume was used for further studies.

Cell free supernatants for determination of enzyme activity were prepared by centrifugation of the culture at $10\,000 \times g$ for 10 min. Biomass determinations were carried out by drying the mycelium on preweighed Whatman No. 1 filter paper to constant weight at 80 °C. Filtered mycelia were washed with water prior to drying to remove soluble medium constituents. Dry weight determinations using uninoculated media indicated insoluble medium constituents arising from corn steep solids amounted to <0.1 mg/ml and could thus be neglected.

Measurement of enzyme activity

Mannanase was assayed using locust bean galactomannan as the substrate. Galactomannan was prepared by ethanol precipitation of an aqueous solution of locust bean gum (Sigma, St. Louis) followed by redissolving and freeze drying according to the method of Halmer et al. [18]. Locust bean gum or carob flour is ground kernel endosperms of tree pods of *Ceratonia siliqua* L. Purified galactomannan contains 70% mannose, 28.5% galactose and 1% ribose and consists of a β-1,4-mannan backbone chain with one galactose branch on every fourth mannose. The mannanase assay mixture contained 0.5 ml of 1% (w/v) galactomannan, prepared in 0.1 M sodium acetate buffer, pH 5.8 and 0.5 ml of suitably diluted culture fluid. The reaction mixture was incubated at 50 °C for 30 min. Reducing sugars produced due to enzyme activity were determined as mannose reducing equivalents using the dinitrosalicylic acid (DNS) method of Miller [30].

Galactanase was assayed using galactan prepared from gum arabic (Aldrich, Milwaukee). Culture filtrate (0.5 ml) was incubated with 0.5 ml solution of 1% (w/v) galactan prepared in 0.1 M sodium acetate buffer, pH 5.8. The reaction mixture was incubated at 50 °C for 30 min. Reducing sugars produced were determined as galactose reducing equivalents using the DNS method.

The β-mannosidase activity was determined by incubation of 0.5 ml of culture fluid with 0.5 ml of a 2 mM solution of *p*-nitrophenyl-β-D-mannopyranoside (Sigma) prepared in 0.1 M sodium acetate buffer, pH 5.8. The reaction mixture was incubated at 50 °C for 30 min and stopped by addition of 2 ml of 2% (w/v) sodium carbonate solution. Released *p*-nitrophenol was determined by absorbance at 420 mm.

One unit (U) of mannanase or galactanase activity is defined as the amount producing 1 μmol of product in 1 min. One unit of β-mannosidase is defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol in 1 min.

To determine the effect of pH on enzyme activity, assays were carried out in 0.05 M sodium citrate–0.1 M sodium phosphate buffer at various pH values. For thermostability studies, enzyme solutions were incubated at various temperatures for periods up to 3 h and residual activity then determined.

RESULTS

Mannanase, β-mannosidase and galactanase activities were determined on cell free supernatants of the fungal cultures after a 48 h incubation in the culture screening

TABLE 1

Mannanase, β-mannosidase and galactanase activity in fungal culture filtrates

Organism	pH of culture fluid at harvest (48 h)	Enzyme activity (U/ml)		
		Mannanase	β-Mannosidase	Galactanase
Talaromyces byssochlamydoides NRRL 3658	6.6	52.4	0.005	0.36
Talaromyces emersonii NRRL 3221	6.3	43.1	0.002	0.38
Talaromyces emersonii ATCC 18080	7.5	0.6	0.001	0.02
Thielavia terrestris NRRL 8126	6.7	12.5	0.020	0.15
Thielavia terrestris ATCC 26917	6.5	2.8	0.005	0.12
Thermoascus aurantiacus ATCC 26904	6.8	0.1	0.040	0.03
Thermoascus aurantiacus ATCC 28082	7.2	0.0	0.005	0.02
Sporotrichum cellulophilum ATCC 20493	6.3	4.8	0.037	0.10
Aspergillus niger NRRL 337	6.4	5.2	0.020	0.02

medium (Table 1). Highest mannanase activities, 52.4 and 43.1 U/ml, were detected in cultures of *T. byssochlamydoides* NRRL 3658 and *T. emersonii*, NRRL 3221, respectively. Activities from other cultures ranged from 0–30% of these values. Relatively low levels of β-mannosidase (≤0.04 U/ml) and galactanase (≤0.38 U/ml) were detected in all cultures.

Six fungal strains, *T. byssochlamydoides* NRRL 3658, *T. emersonii*, NRRL 3221, *T. terrestris* NRRL 8126 *T. aurantiacus* ATCC 26904, *S. cellulophilum* ATCC 20493 and *A. niger* NRRL 337 (for comparison) were selected for further investigation. Effect of carbon sources on enzyme production was studied by incorporating locust bean gum, galactose, mannose, glucose and glycerol as the carbon source (10 g/l) in the basic mineral medium containing $NaNO_3$ (1 g/l) as nitrogen source. Enzyme activities in the culture supernatants, after a 48 h incubation, are presented in Table 2. Mannanases from all of the strains tested, with the exception of *T. aurantiacus*, were best induced by locust bean gum with relatively

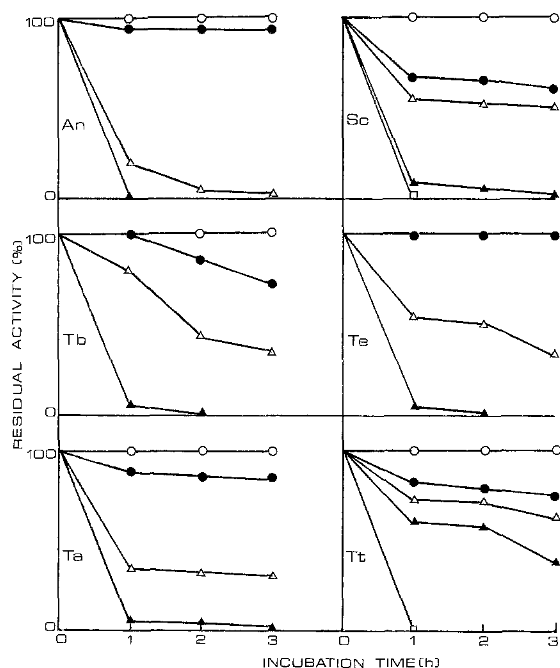

Fig. 1. Effect of temperature on stability of mannanase. ○, 55°C; ●, 60°C; △, 65°C; ▲, 70°C; □, 75°C. An, *A. niger*; Sc, *S. cellulophilum*; Tb, *T. byssochlamydoides*; Te, *T. emersonii*; Ta, *T. aurantiacus*; Tt, *T. terrestris*.

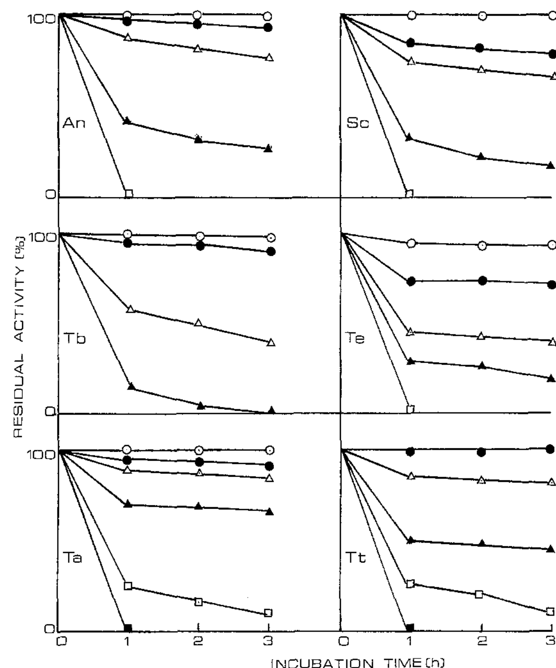

Fig. 2. Effect of temperature on stability of galactanase. ○, 55°C; ●, 60°C; △, 65°C; ▲, 70°C; □, 75°C. An *A. niger*; Sc, *S. cellulophilum*; Tb, *T. byssochlamydoides*; Te, *T. emersonii*; Ta, *T. aurantiacus*; Tt, *T. terrestris*.

TABLE 2

Effect of carbon source on enzyme production

Carbon source (g/l)	Mannanase (U/ml CFS)	β-Mannosidase (U/ml CFS $\times 10^{-3}$)	Galactanase (U/ml $\times 10^{-2}$)	Biomass dry wt. (mg/ml)
Talaromyces byssochlamydoides NRRL 3658				
Locust bean gum	23.9	4.0	12.0	3.1
Mannose	0.1	1.5	6.0	3.9
Galactose	0.1	1.2	6.0	3.6
Glucose	0.1	2.0	3.5	4.7
Glycerol	0.0	2.0	1.1	1.0
T. emersonii NRRL 3221				
Locust bean gum	19.7	1.0	13.0	4.0
Mannose	0.2	1.1	1.0	3.3
Galactose	0.2	2.7	9.5	3.5
Glucose	0.1	1.4	7.1	3.8
Glycerol	0.0	0.1	0.1	1.3
Thielavia terrestris NRRL 8126				
Locust bean gum	6.0	5.0	10.0	3.8
Mannose	0.1	0.6	7.2	3.3
Galactose	0.1	0.9	4.6	2.9
Glucose	0.1	0.7	6.0	3.0
Glycerol	1.1	1.7	4.8	2.2
Thermoascus aurantiacus ATCC 26904				
Locust bean gum	0.5	20.0	1.8	2.8
Mannose	2.0	0.5	0.1	2.2
Galactose	0.2	3.5	8.0	2.9
Glucose	0.1	0.6	5.2	3.5
Glycerol	0.0	2.0	1.7	1.6
Sporotrichum cellulophilum ATCC 20493				
Locust bean gum	1.0	2.0	9.9	3.4
Mannose	0.0	0.5	2.6	4.8
Galactose	0.1	0.5	4.1	3.9
Glucose	0.0	0.6	2.3	4.1
Glycerol	0.0	0.4	2.3	1.7
Aspergillus niger NRRL 337				
Locust bean gum	1.5	35.0	9.0	4.5
Mannose	0.0	1.6	4.0	4.0
Galactose	0.0	1.1	4.4	4.2
Glucose	0.1	0.2	4.3	4.3
Glycerol	0.0	1.0	3.2	1.6

Nitrogen source: $NaNO_3$, 1 g/l. Incubation time: 48 h.

low levels of enzymes detected on the other growth substrates. In the case of *T. aurantiacus*, mannose had a greater inducing effect than locust bean gum. This latter substrate was also the most potent inducer of β-mannosidase activity in *A. niger*, where the best alternative carbon source only produced 4.5% of the locust bean gum inducible enzyme. While locust bean gum was also the optimal inducer of β-mannosidase in *T. aurantiacus*, *S. cellulophilum*, *T. terrestris* and *T. emersonii*, the alternative carbon sources induced 17.5, 33, 34 and 50% respectively of the locust bean gum inducible level. In the case of *T. emersonii*, highest β-mannosidase activity was induced on galactose as the carbon source. Galactose induced maximum galactanase activity (8×10^{-2} U/ml) in *T. aurantiacus* cultures whereas maximum galactanase activity was observed with all other organisms in cultures containing locust bean gum, with activities ranging from $1.8–13.0 \times 10^{-2}$ U/ml. However, in many cases, alternative carbon sources resulted in production of at least 40–50% of the maximum level induced.

In order to study the effect of the nitrogen source on mannanase and galactanase production, locust bean gum

TABLE 3

Effect of nitrogen source on mannase and galactanase production

Organism	Nitrogen source	Mannanase (U/ml)	Galactanase (U/ml × 10^{-2})	Biomass dry weight (mg/ml)
Talaromyces byssochlamydoides NRRL 3658	$NaNO_3$	1.5	9.0	4.5
(Carbon inducer: locus bean gum)	NH_4Cl	1.3	2.0	4.3
	Urea	1.6	2.0	3.9
	Corn steep solids	1.2	1.0	4.8
	Yeast extract	1.0	3.0	5.0
	Peptone	2.7	3.0	5.0
T. emersonii NRRL 3221	$NaNO_3$	19.7	13.0	4.0
(Carbon inducer: locus bean gum)	NH_4Cl	9.0	2.0	4.0
	Urea	9.4	2.0	4.1
	Corn steep solids	8.4	8.0	4.2
	Yeast extract	10.5	4.0	4.6
	Peptone	49.2	6.2	3.5
Thielavia terrestris NRRL 8126	$NaNO_3$	6.0	10.0	
(Carbon inducer: locus bean gum)	NH_4Cl	2.7	5.0	3.0
	Urea	2.6	15.0	3.3
	Corn steep solids	4.9	7.0	3.7
	Yeast extract	3.9	10.0	3.5
	Peptone	5.2	2.0	3.7
Thermoascus aurantiacus NRRL 26904	$NaNO_3$	1.5	9.3	2.8 (2.7)[a,b]
(Carbon inducer: mannose for	NH_4Cl	0.1	8.2	3.3 (2.9)
mannanase, galactose	Urea	1.0	2.8	2.1 (2.0)
for galactananse)	Corn steep solids	0.0	6.0	3.3 (3.0)
	Yeast extract	0.1	10.1	4.3 (3.6)
	Peptone	0.0	9.2	4.1 (3.4)
Sporotrichum cellulophilum ATCC 20493	$NaNO_3$	1.0	10.0	3.4
(Carbon inducer: locus bean gum)	NH_4Cl	0.2	9.0	3.4
	Urea	2.4	8.0	3.0
	Corn steep solids	0.8	10.0	4.6
	Yeast extract	2.3	10.0	3.3
	Peptone	2.0	8.0	4.5
Aspergillus niger NRRL 337	$NaNO_3$	1.5	9.0	4.5
(Carbon inducer: locus bean gum)	NH_4Cl	1.3	2.0	4.3
	Urea	1.6	2.0	3.9
	Corn steep solids	1.2	1.0	4.8
	Yeast extract	1.0	3.0	5.0
	Peptone	2.7	3.0	5.0

Carbon source: locust bean gum 10 g/l. [a] Biomass with mannose; [b] biomass with galactose.

(10 g/l) was used as the carbon source except in the case of *T. aurantiacus* where mannose (10 g/l) and galactose (10 g/l) were used to induce mannanase and galactanase, respectively (Table 3). Highest mannanase activities were produced by *T. emersonii* (49.2 U/ml), *T. byssochlamydoides* (2.7 U/ml) and *A. niger* (2.7 U/ml) when peptone was incorporated as the nitrogen source. These activities were 69–150% higher than those observed with the next best nitrogen source. Maximum mannanase was produced by *T. terrestris* (6.0 U/ml) and *T. aurantiacus* (1.5 U/ml) in media containing sodium nitrate, and in the latter case, no activity was observed when a complex nitrogen source was used. Relationship between nitrogen source and galactanase production was less clear except in the case of *A. niger* and, perhaps, *T. emersonii* where highest activities were observed in media containing sodium nitrate.

The pH optima for activity of the mannanases and galactanases from the six fungi are presented in Table 4. Mannanase pH optima were in the range 5.0–6.6 with the notable exception of the *A. niger* enzyme which had a pH optima of 3.2. A narrower range (4.3–5.8) of pH optima

TABLE 4

pH optima for activity of mannases and galactanases

Organism	pH optima	
	Mannanases	Galactanases
Talaromyces byssochlamydoides	6.6	5.2
Talaromyces emersonii NRRL 3221	6.0	4.0
Thielavia terrestris NRRL 8126	6.0	5.8
Thermoascus aurantiacus ATCC 28082	5.0	4.2
Sporotrichum cellulophilum ATCC 20493	6.2	4.5
Aspergillus niger NRRL 337	3.2	4.3

was observed for the galactanases. The effects of temperature on mannanase and galactanase stability are illustrated in Figs. 1 and 2 respectively. The mannanase from *T. emersonii* was most thermostable, retaining 100% activity for 3 h at 60 °C and 53% activity after 1 h at 65 °C. While the enzyme from *A. niger* retained 91% activity after 3 h at 60 °C, the enzyme was 80% denatured after 1 h at 65 °C. *T. terrestris* produced the most thermostable galactanase, which retained 100% activity for 3 h at 60 °C. The biphasic nature of some of the stability curves may be due to multiple enzyme components having different stability properties or to the destruction of protective substances.

DISCUSSION

The results indicate that the β-1,4-mannanase from 5 of the 6 fungal strains examined is induced by the mannan containing substrate, locust bean gum, while mannose induced the enzyme from *T. aurantiacus*. In separate studies, we have likewise observed that locust bean gum induces production of mannanase by *Bacillus brevis*, *B. polymyxa* and *B. subtilis* whereas a variety of carbon sources, including glucose and glycerol, promoted mannanase production by *B. licheniformis* [3]. Both inductive and constitutive mannanases have been reported [10].

β-Mannosidase activity produced by all fungal strains was relatively low. McCleary [25] reported that β-mannosidase activity tends to be low in microbial cultures and is often unstable. Mannanase was produced by *C. lignorum* cultured on cellulose substrate [15] and the brown rot fungus, *T. palustris*, produced β-D-mannanase when cultivated on glucose [29]. The β-D-mannosidase was produced in cultures of *T. fusiformis* [33] and *T. palustris* [20] containing glucose as the carbohydrate source. The β-D-mannosidase of *A. niger* was induced by mannose [12]. In general, β-D-galactanases are inducible [10,13,36].

Mannanase produced by *T. palustris* had a low pH optimum (3.5–3.8), similar to the *A. niger* enzyme. The pH optima for the mannanases for *R. niveus*, *B. subtilis* and *Aeromonas* sp. were in the range 5.5–6.0, similar to the values for the enzymes for the five thermophilic fungi [1,13,19,20]. Based on our results, fungal galactanases generally have lower pH optima than mannanases. *B. subtilis* galactanase has a higher pH optimum of 6.0–7.0 [14].

It was noted that mannanase from *T. emersonii* was most thermostable, retaining 100% activity at 60 °C for 3 h. Mannanase from *Rhizopus niveus* and *Aeromonas* sp. -F25 have temperature optima for activity of 40 °C [1,19] and appears to be considerably less stable than the mannanases from the six fungi examined. Mannanase from *B. subtilis*, which is completely inactivated at 70 °C after 15 min [13], is, therefore, less stable than the enzymes produced by the five thermophilic fungal species. A thermostable mannanase is also produced by *Tyromyces palustris* [20]. Additionally, *T. emersonii* produces unusually thermostable cellulolytic enzymes [8,24,27] and *T. aurantiacus* and *T. terrestris* produce thermostable cellulases [39]. *S. cellulophilum*, *T. byssochlamydoides* and *T. terrestris* produce xylanases with temperature optima in the range 70–80 °C [11,42]. *T. aurantiacus* produced highly thermostable arabanase and arabinosidase enzymes [22].

The galactanase produced by *B. subtilis* had an optimum temperature for activity of 55–60 °C and was inactivated at 65–70 °C within 15 min [14]. All of the galactanases described in this report were found to be more thermostable than this bacterial enzyme. Of the fungi examined, *T. emersonii* NRRL 3221 is probably the organism to be examined further for commercial potential in production of mannanase. Highest mannanase titers were observed in cultures of this strain and the enzyme manifested greatest enzyme stability. *T. terrestris* NRRL 8126 was identified as a possible industrial source of galac-

tanase. Highest galactanase activity and maximum thermostability were observed in culture filtrates of this organism. Further comparative studies are required to confirm these conclusions. The more temperature stable mannanases and galactanases will now be purified and characterised.

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