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Isolation and Culture of a Thermophilic Fungus, *Melanocarpus albomyces*, and Factors Influencing the Production and Activity of Xylanase

By RAMESH MAHESHWARI* AND P. T. KAMALAM

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

An uncommon thermophilic fungus, *Melanocarpus albomyces*, was isolated from soil and compost by incubating samples in a glucose/sorbose/asparagine liquid medium, followed by enrichment culture in medium containing sugarcane bagasse as carbon source. The culture filtrate protein of the fungus grown in the presence of bagasse or xylose hydrolysed xylan and some other polysaccharides but cellulose was not hydrolysed. High extracellular xylanase (EC 3.2.1.8) activity was produced by cultures grown on xylose or hemicellulosic materials. The enzyme was induced in glucose-grown washed mycelia in response to addition of xylose or xylan but not by alkyl or aryl β-D-xylosides. Cultures produced higher enzyme yields in shaken flasks than in a fermenter. Gel-filtration chromatography of culture filtrate protein showed the presence of two isoenzymes of xylanase, whose relative proportions varied with the carbon source used for growth. The extent of hydrolysis of heteroxylans or the hemicellulosic fraction of bagasse by culture filtrate protein preparations was greater when the cultures had been grown on bagasse rather than xylose as the inducing substrate. The activity of xylanase preparations was increased when an exogenous β-glucosidase was added.

INTRODUCTION

There is increasing interest in fungi which can produce high levels of enzymes capable of degrading plant cell-wall polysaccharides. Such enzymes are useful as specific tools for elucidating the structure of plant cell walls, and are also required for evolving biodegradative methods for the conversion of biomaterials containing cellulose and hemicellulose into monosaccharides from which single-cell protein, single-cell oil or ethanol could be produced (Flickinger, 1980; Fall *et al.*, 1984). During a search for thermophilic fungi with biodegradative capability, an uncommon fungus, *Melanocarpus albomyces* (Cooney & Emerson) von Arx, was isolated for the first time from India. In this paper we report the isolation and culture of this fungus, and also describe the induction of xylanase in cultures and the conditions which influence its production and activity. The fungus can rapidly produce high levels of extracellular xylanase in culture medium incorporating bagasse, a surplus by-product of the sugar-milling industry, and it is one of the most active producers of xylanase discovered so far.

METHODS

Soil collection. Samples of soil from forests and compost were collected from different places in India. These were brought to the laboratory in small polyethylene bags and stored at 4 °C for several months before use.

Isolation of fungi. Soil (1–2 g) was incubated in 100 ml of a medium (pH 7·0), which contained (g l^{-1}): glucose, 5; L-sorbose, 10; L-asparagine, 1; K$_2$HPO$_4$, 1; MgSO$_4$.7H$_2$O, 0·5; streptomycin sulphate, 0·03 and rose bengal, 0·06. After 3 d growth at 50 °C on a gyratory shaker (240 r.p.m.), the fungal growth was transferred to fresh medium of the above composition except that the sugars were replaced with 1% (w/v) sugarcane bagasse, which served as a source of plant cell-wall polysaccharides, containing about 36% cellulose and 26% hemicellulose (Paturau, 1982). The fungal growth was subcultured after 4 d. Finally, after 7 d, individual fungi were isolated at 50 °C by plating a suitably diluted sample of the culture on YpSs agar (Cooney & Emerson, 1964) containing streptomycin sulphate and rose bengal at the concentrations mentioned above.

Culture of Melanocarpus albomyces. Strain IIS 68 (see Results) was used for all the experiments unless otherwise stated. A semi-synthetic medium was used, containing (g l^{-1}): glucose, 20; urea, 0·5; KH_2PO_4, 0·6; K_2HPO_4, 0·4; $MgSO_4.7H_2O$, 0·5 and yeast extract, 1. The pH of the medium before autoclaving was 6·0. A suspension of mycelia scraped from agar cultures stored at room temperature for 4–6 months was added to the sterile medium. The fungus was grown in 150 ml medium in 500 ml Erlenmeyer flasks on a gyratory shaker at 40 °C, unless stated otherwise. Peptone (0·5%, w/v) was added to the medium when bagasse was used as inducing substrate as it tended to increase xylanase yield by about 1·5-fold.

Xylanase (EC 3.2.1.8) production. Xylose or bagasse (1·5%, w/v) was used as inducing substrate. For studies of growth and enzyme production, cultures were initiated by adding a 5% (v/v) mycelium inoculum from a 48 h culture grown in glucose/urea medium as above.

Xylanase production was also studied in a Microferm fermenter (New Brunswick), using 3 l medium. A 5% (v/v) mycelial inoculum was added and the starting pH of the culture was adjusted to 6·0 by addition of 4M-HCl.

Enzyme assays. To determine cellulase activity, 1 ml reaction mixture containing approximately 50 mg (1 × 6 cm strip) Whatman no. 1 filter paper was incubated with culture filtrate in sodium/potassium phosphate buffer (pH 6·0, 50 mM) for 30 min at 50 °C. The glucose released was quantified by the glucose oxidase–peroxidase method (McComb & Yushok, 1957). Cellulase activity on carboxymethylcellulose (Na salt) was determined using 0·5 ml 1% (w/v) substrate. The reducing end-groups liberated were measured by the Nelson–Somogyi method (Somogyi, 1952).

Xylanase activity was determined with larchwood xylan as substrate. A 2 ml reaction mixture containing 0·2 ml 1% (w/v) xylan and a sample of 100-fold diluted culture filtrate was incubated in sodium/potassium phosphate buffer (pH 6·0, 50 mM) for 30 min at 50 °C. The reducing end-groups liberated were measured as above.

β-Glucosidase and β-xylosidase activity in culture solutions were assayed using *p*-nitrophenyl β-D-glucopyranoside and *p*-nitrophenyl β-D-xylopyranoside, respectively. The reaction mixture (1 ml) contained culture filtrate and 0·5 ml 1 mM substrate in phosphate buffer (pH 6·0, 50 mM), and was incubated for 30 min at 50 °C. Reaction was stopped by adding 2 ml 5% (w/v) Na_2CO_3 and the absorbance of the solution was measured at 400 nm. β-Xylosidase activity was also tested with methyl β-D-xyloside as substrate, measuring the formation of xylose by the Nelson–Somogyi method.

One unit of enzyme activity on a polysaccharide substrate was defined as 1 µmol reducing end-groups released min^{-1} under the above assay conditions. One unit of β-glucosidase activity was defined as that amount of enzyme which produced 1 µmol *p*-nitrophenol min^{-1} under the assay conditions.

Polysaccharide degradation. The activity of culture filtrate protein towards polysaccharides was determined by measuring the generation of reducing sugars, and is expressed as percentage saccharification, calculated as [reducing sugar (mg) × 0·9 × 100]/[initial substrate (mg)]. (This equation is for hexoses, but is a close enough approximation for pentoses.) The protein preparation was obtained by precipitation of culture filtrates with 80% saturated ammonium sulphate, and was desalted before use, as described below.

Column chromatography of xylanase. To culture filtrates (3 l) from 4-d-old cultures grown on xylose or bagasse, ammonium sulphate was added to 80% saturation with constant stirring. The preparation was allowed to stand overnight at 4 °C, and the precipitated material was collected by filtering through a Celite bed. When suspended in distilled water it gave a dark brown solution containing about 80% of the original xylanase activity. This solution was treated with ammonium sulphate at 40% saturation. The resulting precipitate was dissolved in water and the dark solution was desalted in batches by chromatography on a Sephadex G-25 column (106 × 2·5 cm). The active fractions were combined; they contained about 60% of the original xylanase activity. The solution was lyophilized, and the dry powder was dissolved in ammonium acetate buffer (pH 6·2, 75 mM). The enzyme solution was applied to a column (14 × 2·7 cm) of DEAE-Sephadex A-50 (pH 6·2) and eluted with the same buffer. The pigments were adsorbed by the ion-exchanger, whereas the enzyme was eluted in the buffer wash and was almost free of colour. The appropriate fractions were pooled and lyophilized. A portion (21 mg) was dissolved in water and subjected to gel-filtration chromatography on a Sephadex G-75 column (100 × 2 cm) eluted with water at 10 ml h^{-1}; 3 ml fractions were collected. Protein was monitored by absorbance at 280 nm.

Biochemicals. These were mostly purchased from Sigma. Araban and galactan were from Koch-Light. Gel-filtration and other chromatography media were from Pharmacia. Cellulase and β-glucosidase were purified from culture filtrates of *Sporotrichum thermophile* by K. M. Bhat in this laboratory. Sugarcane bagasse was obtained from Mandya Sugar Mills, Karnataka, India. It was air-dried, milled and sieved through a 60 mesh screen (250 µm) before use.

RESULTS

Identification of the fungus

Colonies of *M. albomyces* were distinguishable from other thermophilic fungi present (*Penicillium duponti, Thermomyces lanuginosus, Malbranchea pulchella* var. *sulfurea*) on YpSs agar

isolation medium by their white mycelia and lack of sporogenous hyphae. Three colonies of *M. albomyces* were obtained: one from soil (IIS 66) and two from compost (IIS 67 and IIS 68). The colonies were composed of two distinct hyphal types: thin, thread-like aerial hyphae and prostrate hyphae constricted at the septa to form chains of oval cells with thickened walls (arthroconidia).

When grown singly on YpSs agar at 40 °C, the isolates produced ascocarp-like structures after about 3–4 weeks, but these contained no spores. When the three isolates were grown together on YpSs agar (Fig. 1 *a*), dark lines of fruiting bodies appeared in about 10–14 d where the edges of the mycelium of isolate IIS 67 crossed those of isolates IIS 66 or IIS 68, showing that the fungus was heterothallic. Fruiting bodies did not form between isolates IIS 66 and IIS 68, which thus belong to the same mating type, whereas IIS 67 is of the opposite mating type. Figs 1 (*c*) and 1 (*d*) show stages in the formation of an ascocarp. The mature ascocarp was spherical and dark brown. It lacked an ostiole (Fig. 1 *e*). Each ascocarp contained numerous asci, which oozed out when the ascocarp was ruptured by mechanical pressure on the cover slip (Fig. 1 *f*). The young ascus was kidney-shaped and contained eight ascospores, arranged as two tetrads (Fig. 1 *g, h*). The mature ascospores were oval and dark brown (Fig. 1 *i*). The morphology of our isolates resembled the description given by Cooney & Emerson (1964) for the thermophilic fungus *Myriococcum albomyces*, but with the difference that the ascocarps they observed generally contained fewer asci or were completely sterile. von Arx (1981) has reassigned *Myriococcum albomyces* as *Melanocarpus albomyces*.

Growth and physiological characteristics

Nitrogen source. Strain IIS 68 was cultured in media containing $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$, $NaNO_3$, L-asparagine or urea at equivalent nitrogen concentration (0·023%), with 2% (w/v) glucose as carbon source. Measurement of mycelial yield (dry wt per 50 ml) after 4 d, when growth had more or less ceased, showed that the ammonium compounds produced poor growth (5–12 mg) and the pH of the culture medium dropped to approximately 4·2. Better growth occurred in medium containing L-asparagine (112 mg), urea (148 mg) or $NaNO_3$ (164 mg). The pH of these media either did not change significantly during growth (asparagine and urea), or it rose to 7·5 ($NaNO_3$). These results suggested that the fungus did not tolerate acidic conditions which presumably resulted from the absorption of NH_4^+ and counter-transport of H^+, as has been shown for *Penicillium cyclopium* (Roos & Luckner, 1984). Urea was chosen as the nitrogen source for subsequent culture as it produced satisfactory yields and the mycelia grew as small pellets (approximately 1–3 mm in diameter).

Temperature. In liquid shaken cultures at 30, 40 and 50 °C, the rate of growth (measured as dry weight per 20 ml of culture) was fastest at 50 °C, reaching a maximum (149 mg) on day 2, after which the dry weight declined rapidly, being 90 mg on day 4. At 40 °C, the maximal mycelial yield was 126 mg, on day 2, after which the dry weight again declined. At 30 °C, the yield was maximal (176 mg) on day 3 and remained the same on day 4 when the experiment was terminated. The fact that growth rate was maximal at 50 °C, confirmed the thermophilic character of the isolate.

Hemicellulose-degrading activity

As the fungus was isolated after enrichment culture in medium containing bagasse, it was assumed that it could degrade some isolated polysaccharide constituents of plant cell walls. The fungus was cultured in a bagasse/urea medium for 4 d, and the culture filtrate was tested for its ability to hydrolyse polysaccharides (Table 1). Of the polysaccharides tested, only starch, lichenan and xylan were appreciably degraded. The inability of the fungus to degrade cellulose was confirmed by growing it in a medium containing filter paper supplemented with 0·5% (w/v) peptone. Microscopic examination at intervals showed that although the cellulose fibres were fragmented and shorter fibres were generated within 24 h, these were not solubilized till day 6. The final cellulase activity of culture filtrates was very low: less than 0·01 µmol glucose min^{-1} ml^{-1} was liberated.
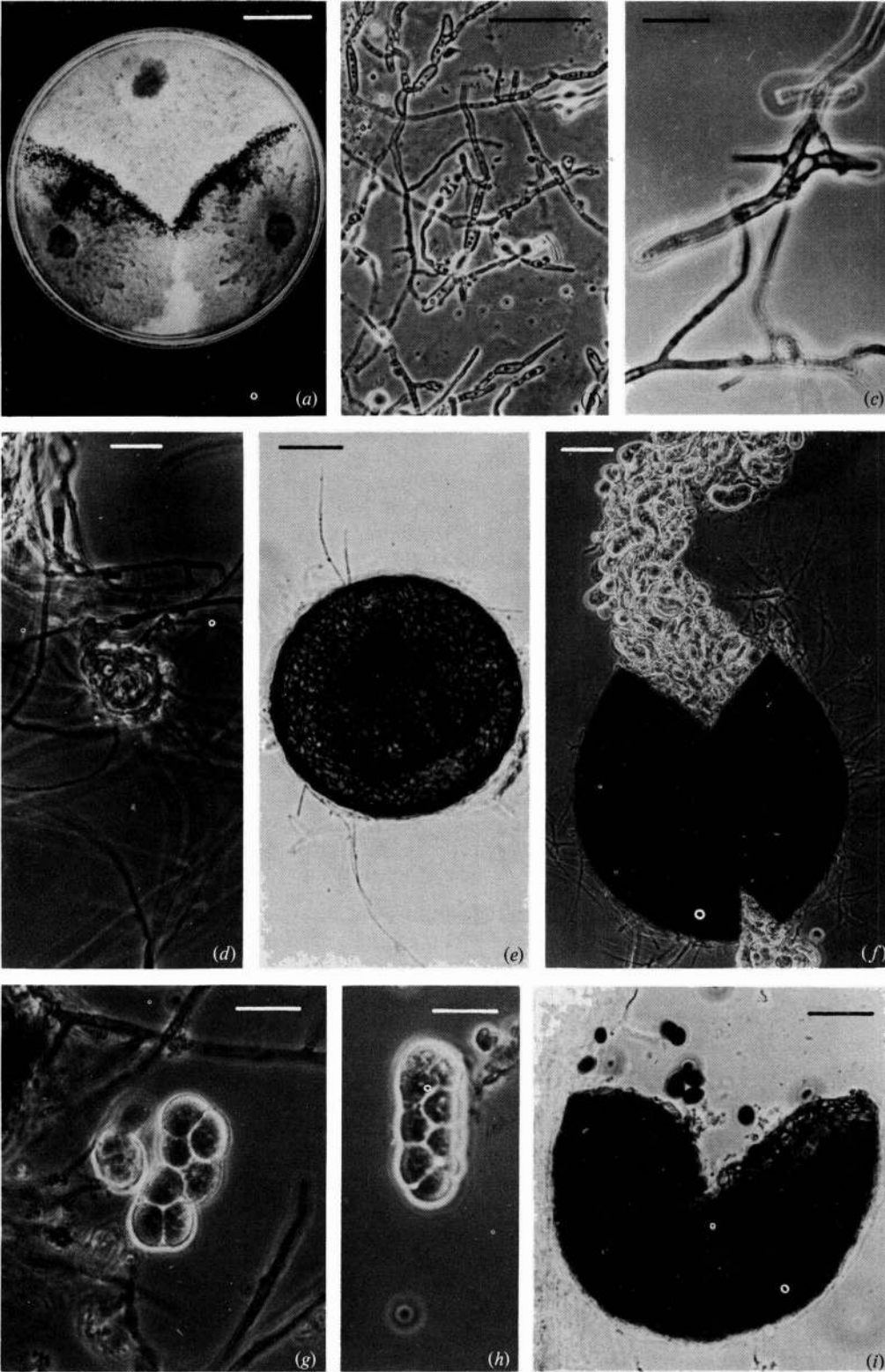
Table 1. *Activity of M. albomyces culture filtrates on some polysaccharides*

Reaction mixture (5 ml) consisting of 50 mg substrate and 5 mg culture filtrate protein from cultures grown on bagasse (4 d) in sodium/potassium phosphate buffer (pH 6·0, 50 mM) was incubated for 46 h at 50 °C without shaking. The data are typical values from two experiments.

Substrate	Predominant residue	Backbone linkage(s)	Percentage saccharification
Araban	Arabinose	α-1,5; α-1,3	6·4
Cellulose	Glucose	β-1,4	2·8
Dextran	Glucose	α-1,6	7·7
Galactan	Galactose	β-1,4; β-1,3	1·0
Lichenan	Glucose	β-1,3; β-1,4	37·4
Mannan	Mannose	β-1,4	1·0
Polygalacturonic acid	Galacturonic acid	α-1,4	0
Starch	Glucose	α-1,4	46·0
Xylan	Xylose	β-1,4	29·0

Effect of carbon sources on production of xylanase

As the culture filtrates had high activity towards xylan, which is the dominant hemicellulose present in most types of fibrous plant material, attention was focused on xylanase. The production of extracellular xylanase by *M. albomyces* cultured in the presence of some mono-, di- and polysaccharides was studied to determine whether the enzyme was synthesized constitutively or produced only in response to certain carbon sources. The enzyme was produced in significant amounts only in media containing xylose or xylan (Table 2). Unlike results obtained with some other fungi (Dekker & Richards, 1976; Mishra *et al.*, 1984), cellulose induced very low levels of xylanase. Our results suggested that xylanase in this fungus is induced only by xylosic material; the induction of enzyme by araban was taken to be due to the presence of xylose residues in this material (Dekker & Richards, 1976). The culture filtrates which were active on xylan were tested for β-xylosidase activity, but none was detected.

The inducibility of xylanase in *M. albomyces* was further tested by studying the response of cultures to the delayed addition of xylose or hemicellulosic material (bagasse). Cultures initiated in medium with glucose as the carbon source showed low levels (1·0–0·5 units ml^{-1}) of xylanase (day 1 or day 2) or none at all (day 3). However, 24 h after the addition of 2% (w/v) xylose to a separate set of flasks on day 2, the cultures had an enzyme level of 7 units ml^{-1}. A greater induction response (12 units ml^{-1}) occurred in the same time when 2% (w/v) bagasse was added. These results confirmed the inducibility of xylanase in the fungus.

Xylanase induction in washed mycelia

The time-course and the amount of induced enzyme formed suggested that this system might be useful for the study of the induction phenomenon using washed mycelia. Mycelia from 2-d-old cultures grown in glucose/urea medium were collected by filtration, washed with distilled water and the effect of different constituents of the growth medium on xylanase induction by xylose (0·5%, w/v) was tested. Extracellular xylanase was induced by xylose in mycelia incubated with unsupplemented buffer components (0·4 units ml^{-1} after 4 h at 40 °C), and the amount produced increased with cumulative additions of urea (0·6 units ml^{-1}), MgSO$_4$ (0·7 units

Fig. 1. *Melanocarpus albomyces.* (*a*) An agar plate inoculated with three isolates, IIS 67, 68 and 66, at approximately 12, 4 and 8 o'clock positions, respectively. Dark lines of fruiting bodies are visible between IIS 67 and 66 and between IIS 67 and 68. Bar, 5 mm. (*b*) Prostrate mycelia. Bar, 100 μm. (*c*) An early stage in development of an ascocarp (presumably plasmogamy). Bar, 20 μm. (*d*) Protoperithecium-like structure. Bar, 20 μm. (*e*) Surface view of ascocarp. Bar, 50 μm. (*f*) Ruptured ascocarp liberating numerous asci. Bar, 50 μm. (*g*) A young ascus with clumped ascospores. Bar, 50 μm. (*h*) An ascus showing eight ascospores. Bar, 20 μm. (*i*) A cracked mature ascocarp and dark oval ascospores. Bar, 50 μm.

Table 2. *Production of xylanase by M. albomyces in media containing different carbon sources*

Carbohydrates were tested at 0·5% (w/v). Enzyme activity was measured after 5 d growth. The data for the monosaccharides, the disaccharides and xylan are means of four experiments; the other data are means of two experiments.

Carbon source	Xylanase (units ml^{-1})
Monosaccharides	
Glucose	0·5
Xylose	10·7
Disaccharides	
Cellobiose	0·2
Lactose	0·8
Polysaccharides	
Araban	4·0
Cellulose (Sigmacell)	1·2
Chitin	0·7
Galactan	0·5
Inulin	0·8
Lichenan	0·4
Pectin	0·5
Starch	0·5
Xylan	12·0

ml^{-1}) and yeast extract + peptone (0·9 units ml^{-1}). Therefore, all the above components were included in the induction medium. When, in a separate experiment, washed mycelia grown for 2 d on the glucose/urea medium were resuspended in this medium with glucose replaced by xylose or xylan (each 0·5%, w/v), and incubated for 4 h at 40 °C, 0·6 and 1 unit xylanase ml^{-1}, respectively, was induced. Methyl β-D-xyloside and p-nitrophenyl β-D-xyloside were ineffective as inducers, as were cellobiose and carboxymethylcellulose (all at 0·5%, w/v). The level of induced enzyme produced in short-term incubations was equal to or higher than that produced by some micro-organisms during long-term culture on xylan or other substrates (Baker *et al.*, 1977; Biely *et al.*, 1980; Zalewska-Sobczak & Urbanek, 1981; Yoshioka *et al.*, 1981).

Culture conditions for xylanase production

Temperature. A study of xylanase production at 30, 40 and 50 °C showed that maximal stable enzyme activity was obtained at 40 °C (Fig. 2). This temperature was therefore used for all studies on xylanase production.

Carbon and nitrogen concentration. To determine the optimal concentrations of carbon (inducer) and nitrogen, xylose was tested at 0·5, 1·0, 1·5 and 2·0% (w/v) with urea at 0·05, 0·1, 0·2 and 0·4% (w/v) in all possible combinations. An activity of >8 units ml^{-1} was produced in all cases but a combination of 1·5% (w/v) xylose and 0·2% (w/v) urea produced the maximal yield of xylanase (22·5 units ml^{-1} in 4 d).

Time. The increase in xylanase activity in xylose medium paralleled growth, and activity was maximal on day 3 or day 4 (Fig. 3). With bagasse as inducer, the maximal xylanase activity was again obtained on day 3 or day 4. With either xylose or bagasse as inducer the pH of the culture medium rose to nearly 8·8 by the end of growth.

Inducing substrates. The yield of xylanase with bagasse or xylose as the inducing substrate was generally similar, particularly if bagasse was used in combination with 0·5% (w/v) peptone. This was so despite the fact that, judged visually, the mycelial growth with bagasse was only about one-tenth of that in xylose medium. The average xylanase yield from 7–10 experiments in shake flask cultures was 15 units ml^{-1} with 1·5% (w/v) bagasse or xylose.

With 1·5% (w/v) bagasse, the effect of supplementary addition of a soluble carbohydrate (0·5%, w/v) on xylanase yield was studied. Addition of glucose, starch or xylose increased enzyme production by up to 1·3-fold over the bagasse control (11·8 units ml^{-1}), and addition of
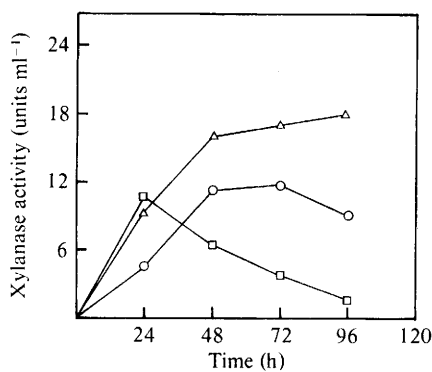
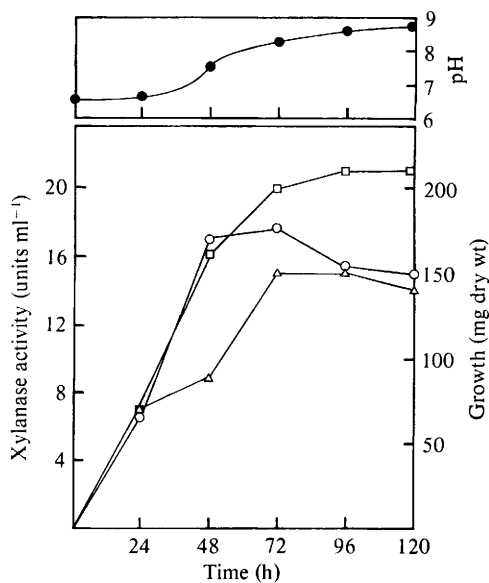Fig. 2. Extracellular xylanase activity in shake flask cultures incubated at 30 °C (O), 40 °C (△) and 50 °C (□).

Fig. 3. Growth, xylanase production and pH change in liquid shake cultures. Culture flasks (250 ml) contained 50 ml medium. Growth (O) and xylanase production (□) in medium containing 1·5% (w/v) xylose. △, Xylanase production in medium containing 1·5% (w/v) bagasse. ●, pH change in medium containing bagasse.

xylan nearly doubled the enzyme yield (21·2 units ml⁻¹), but addition of cellobiose, glycerol or lactose had no significant effect on the enzyme yield. However, in all cases the use of an additional carbon source resulted in more growth, as judged visually. A direct correlation between growth (mycelial crop) and enzyme productivity was, therefore, not found.

Xylanase production in a fermenter

Using the culture medium developed for the production of xylanase in shake flask cultures, the production of enzyme was studied in a fermenter. Bagasse (1·5%, w/v) was used as substrate for all experiments in view of its very low cost. As seen from Fig. 4, a higher rate of air-flow gave faster rate of xylanase production. With agitation at 400 r.p.m. and an air-flow of 1·21 min⁻¹, maximal enzyme (nearly 12 units ml⁻¹) was produced in 3 d. The pH of the culture medium rose during the enzyme production phase and the final pH ranged from 7·3 to 8·3. Maintaining the pH at 6·0 by the automatic addition of acid did not result in higher yield of xylanase. A noteworthy, but unexplained feature was that the growth of the fungus in the fermenter appeared, judged visually, to be better than that in shaken flask cultures. It was judged that growth reached a maximum on day 3. During the early phase of fermentation, the bagasse remained mostly settled in the fermentation jar. At around 72 h, the bagasse was mixed with mycelial growth and it became largely layered on the surface of the medium, resisting mixing.

Xylanase iosenzymes

To determine the number of extracellular xylanase iosenzymes produced by *M. albomyces*, the culture filtrate protein was fractionated by gel-filtration chromatography as described in Methods (Fig. 5). A small peak of activity (I) eluted immediately after the void volume of the column and it was well separated from a larger peak of xylanase (II) which eluted later. This showed that at least two xylanase isoenzymes, differing in molecular size, were produced by the
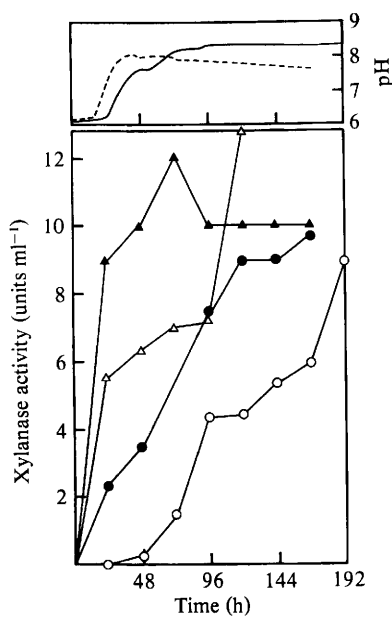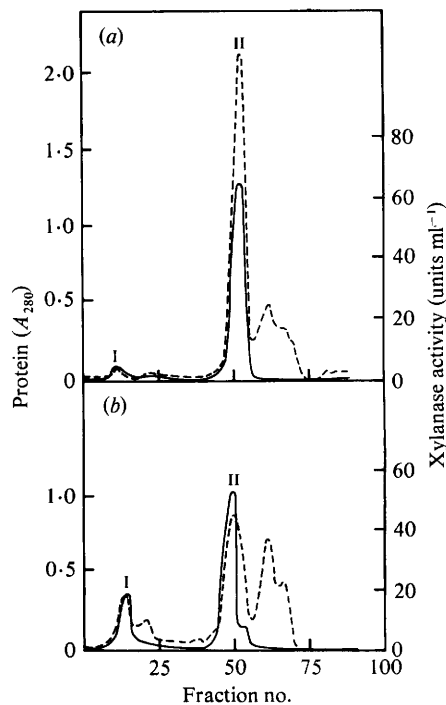
Fig. 4. Xylanase production and pH changes in a fermenter with different rates of air-flow and agitation. $\bigcirc$, $0 \cdot 21 \, min^{-1}$, 240 r.p.m.; $\bullet$, $1 \cdot 01 \, min^{-1}$, 240 r.p.m.; $\triangle$, $1 \cdot 21 \, min^{-1}$, 240 r.p.m.; $\blacktriangle$, $1 \cdot 21 \, min^{-1}$, 400 r.p.m.; ——, pH change with $\bigcirc$; ---, pH change with $\blacktriangle$.

Fig. 5. Elution profile of xylanase activity in gel-filtration chromatography (Sephadex G-75) of culture-filtrate protein from (a) xylose-grown, and (b) bagasse-grown cultures under identical conditions. ——, activity; ---, protein. The column was eluted with water at $10 \, ml \, h^{-1}$, and 3 ml fractions were collected.

fungus. Repetition of the experiment with different preparations of culture filtrate proteins gave similar results. The proportions of xylanase I and II differed considerably between xylose- and bagasse-grown cultures (Fig. 5). From the areas under the curves of enzyme activity, it was estimated that relative to culture filtrate protein, the fungus produced six times more xylanase I on bagasse than on xylose. On the other hand, the amount of xylanase II was 1·3-fold more in xylose- than in bagasse-grown cultures.

As the relative proportions of xylanase I and II in culture filtrates differed depending on the inducing substrates used (xylose or bagasse), the hemicellulolytic activity of the two culture filtrates was compared. Larchwood xylan, oat-husk xylan and hemicellulose fraction from bagasse (5 mg each) were incubated with 5 mg culture filtrate protein (after DEAE-Sephadex chromatography), without shaking, in pH 6·0 phosphate buffer (5 ml) for 24 h. The percentage saccharification of hemicelluloses by xylose-culture filtrate ranged from 5 to 6, while that by bagasse-culture filtrate ranged from 6·7 to 8·2. The results indicated that the mixture of two isoenzymes in appropriate proportions may have a synergistic action.

Effect of cellulase and β-glucosidase on xylanase activity

In several fungi xylanases are co-induced with cellulases and β-glucosidases (Dekker & Richards, 1976; Wolter et al., 1980; Zalewska-Sobczak & Urbanek, 1981). Moreover, in some fungi cellulases or β-glucosidase have cross-activity on xylan (Toda et al., 1971; Kanda et al., 1976; Shepherd et al., 1981; Sadana et al., 1984). Inasmuch as M. albomyces did not produce significant cellulase or β-glucosidase activities, it was of interest to determine if its xylanase

Table 3. *Effect of cellulase and β-glucosidase addition on xylanase activity*

The concentrations (ml^{-1}) of the enzymes were as follows: xylanase, 0·08 unit; β-glucosidase, 1·0 unit; cellulase (assayed on carboxymethylcellulose), 0·04 unit. The data are average values from two experiments.

Enzyme present	Relative activity on xylan
Xylanase	1·0
β-Glucosidase	0·06
Cellulase	1·0
Xylanase + β-glucosidase	2·5
Xylanase + cellulase	2·8
Xylanase + β-glucosidase + cellulase	3·1

activity would be greater if contaminated with the above enzymic activities. Therefore the activity of a partially purified preparation of xylanase of *M. albomyces* was measured in the presence of cellulase and β-glucosidase from *Sporotrichum thermophile*, at concentrations judged from experience to be in the normal range. Both enzymes resulted in increased production of reducing sugars in reaction mixtures containing a fixed amount of xylanase (Table 3).

DISCUSSION

There are very few reports of the isolation of *Melanocarpus albomyces* (Tansey & Brock, 1978). Cooney & Emerson (1964) commented that because of its thermophilic habit of growth and lack of air-borne conidia, this fungus (syn. *Myriococcum albomyces*) may not be widespread in occurrence. We have isolated this fungus from only two out of some 100 samples of compost or soil collected from different places in India, although the same isolation procedure was not used in all cases. We added L-sorbose and rose bengal to the isolation medium on the assumption that they would help to retard the development of the more abundant and faster-growing thermophilic species which commonly occur in soil. Both these compounds have been successfully used to reduce the colony spread of *Neurospora* (Tatum *et al.*, 1949; Gochenaur, 1964). We also used L-asparagine rather than ammonium salts as the nitrogen source, based on our experience that growth of several thermophilic fungi in medium containing ammonium salts is very poor in weakly buffered media because the medium becomes too acid (Gupta & Maheshwari, 1985). The use of special procedures may, therefore, prove rewarding in the isolation of uncommon or new species of thermophilic fungi of potential importance in biotechnology.

How good a producer of xylanase is *M. albomyces*? Quantitative comparison of xylanase production by different micro-organisms is not immediately possible for two chief reasons. First, a uniform substrate has not been adopted for the measurement of xylanase activity by various laboratories (Ghose & Bisaria, 1984). Second, the correct measurement of the intended activity is difficult if contaminating enzymes are present. This point is illustrated by the observation of Dekker (1983) that hydrolysis of heteroxylan in 24 h by hemicellulolytic enzymes of *Trichoderma reesei* was greater when exogenous β-xylosidase from *Aspergillus niger* was added. This effect was similar to the potentiation of cellulase activity by exogenous β-glucosidase found by Joglekar *et al.* (1983). Similarly, exogenous β-glucosidase from *Sporotrichum thermophile* enhanced the activity of xylanase from *M. albomyces* in a short-term enzyme assay (Table 3). This behaviour emphasizes that if xylanase production is accompanied by cellulases, β-D-glucosidases and/or β-D-xylosidases, an unreal high xylanase activity may be measured in the culture solution. However, since *M. albomyces* did not produce significant amounts of cellulase, β-glucosidase or β-xylosidase, it stands out as an exceptionally active producer of xylanase. Its extracellular xylanase activity (maximum value about 23 units ml^{-1}) was some 2–4 times higher than the values reported for some fungi which also produced some of the other enzyme activities mentioned above (Paice *et al.*, 1978; John *et al.*, 1979; Yoshioka *et al.*, 1981; Dekker, 1983;

Mishra *et al.*, 1984). Moreover, *M. albomyces* produced high levels of xylanase in a short time when induced by sugarcane bagasse, a cheap material available in bulk. Most previous studies have used commercial xylan, presumably because this gave the best yields (Iizuka & Kawaminami, 1965; Baker *et al.*, 1977; John *et al.*, 1979; Biely *et al.*, 1980; Yoshioka *et al.*, 1981; Mishra *et al.*, 1984).

Another noteworthy feature of *M. albomyces* is that the monomeric unit of the polysaccharide (xylose) was an effective inducer of xylanase. This is different from the situation in the yeast *Cryptococcus albidus* (Biely *et al.*, 1980; Biely & Petrakova, 1984) and in the *Streptomyces* sp. studied by Nakanishi *et al.* (1976). The rapid induction of the enzyme in washed mycelia shows that *M. albomyces* should be a suitable organism for investigations on the regulation of xylanase in mycelial fungi, providing an opportunity to study the regulation of synthesis of xylanase isoenzymes in response to different inducing substrates, particularly plant cell-wall poly-saccharides and xylose-oligosaccharide fragments derived therefrom by enzymic cleavage.

REFERENCES

VON ARX, J. A. (1981). *The Genera of Fungi Sporulating in Pure Culture*. Vaduz: J. Cramer.

BAKER, C. J., WHALEN, C. H. & BATEMAN, D. F. (1977). Xylanase from *Trichoderma pseudokoningii*: purification, characterization and effects on isolated plant cell walls. *Phytopathology* **67**, 1250–1258.

BIELY, P. & PETRAKOVA, E. (1984). Novel inducers of the xylan-degrading enzyme system of *Cryptococcus albidus*. *Journal of Bacteriology* **160**, 408–412.

BIELY, P., KRATZKY, Z., VRSANKA, M. & URMANI-COVA, D. (1980). Induction and inducers of endo-1,4-β-xylanase in the yeast *Cryptococcus albidus*. *European Journal of Biochemistry* **108**, 313–321.

COONEY, D. G. & EMERSON, R. (1964). *Thermophilic Fungi. An Account of their Biology, Activities and Classification*. San Francisco: W. H. Freeman.

DEKKER, R. F. H. (1983). Bioconversion of hemicellulose: aspects of hemicellulase production by *Trichoderma reesei* QM 9414 and enzymic saccharification of hemicellulose. *Biotechnology and Bioengineering* **25**, 1127–1146.

DEKKER, R. F. H. & RICHARDS, G. N. (1976). Hemicellulases: their occurrence, purification, properties and mode of action. In *Advances in Carbohydrate Chemistry and Biochemistry* **32**, 277–352.

FALL, R., PHELPS, P. & SPINDLER, D. (1984). Bioconversion of xylan to triglycerides by oil-rich yeasts. *Applied and Environmental Microbiology* **47**, 1130–1134.

FLICKINGER, M. C. (1980). Current biological research in conversion of cellulosic carbohydrates into liquid fuels: how far have we come? *Biotechnology and Bioengineering* **23**, 27–48.

GHOSE, T. K. & BISARIA, V. S. (1984). *Measurement of Hemicellulase Activities (Part I - Xylanases)*. New Delhi: Biotechnology Commission, International Union of Pure & Applied Chemistry.

GOCHENAUR, S. E. (1964). A modification of the immersion tube method for isolating soil fungi. *Mycologia* **56**, 921–923.

GUPTA, S. D. & MAHESHWARI, R. (1985). Is organic acid required for nutrition of thermophilic fungi? *Archives of Microbiology* **141**, 164–169.

IIZUKA, H. & KAWAMINAMI, T. (1965). Studies on the xylanase from Streptomyces. Part I. Purification and some properties of xylanase from *Streptomyces xylophagus* nov. sp. *Agricultural and Biological Chemistry* **29**, 520–524.

JOGLEKAR, A. V., KARANTH, N. G. & SRINIVASAN, M. C. (1983). Significance of β-D-glucosidase in the measurement of exo-β-D-glucanase activity of cellulolytic fungi. *Enzyme and Microbial Technology* **5**, 25–29.

JOHN, M., SCHMIDT, B. & SCHMIDT, J. (1979). Purification and some properties of five endo-1,4-β-D-xylanases and a β-D-xylosidase produced by a strain of *Aspergillus niger*. *Canadian Journal of Biochemistry* **57**, 125–134.

KANDA, T., WAKABAYASHI, K. & NISIZAWA, R. (1976). Xylanase activity of an endo-cellulase of carboxy-methyl-cellulase type from *Irpex lacteus* (*Polyporus tulipiferae*). *Journal of Biochemistry* **79**, 989–995.

MCCOMB, R. B. & YUSHOK, W. D. (1957). Colorimetric estimation of D-glucose and 2-deoxy-D-glucose with glucose oxidase. *Journal of the Franklin Institute* **265**, 417–422.

MISHRA, C., KESKAR, S. & RAO, M. (1984). Production and properties of extracellular xylanase from *Neurospora crassa*. *Applied and Environmental Microbiology* **48**, 224–228.

NAKANISHI, K., YASUI, T. & KOBAYASHI, T. (1976). Inducers of xylanase production by *Streptomyces* sp. *Journal of Fermentation Technology* **54**, 801–807.

PAICE, M. G., JURASEK, L., CARPENTER, M. R. & SMILLE, L. B. (1978). Production, characterization and partial amino acid sequence of xylanase A from *Schizophyllum commune*. *Applied and Environmental Microbiology* **36**, 802–808.

PATURAU, J. M. (1982). *By-products of the Cane Sugar Industry: an Introduction to their Industrial Utilization*, Series 3, p. 33. Amsterdam: Elsevier Scientific.

ROOS, W. & LUCKNER, M. (1984). Relationship between proton extrusion and fluxes of ammonium ions and organic acids in *Penicillium cyclopium*. *Journal of General Microbiology* **130**, 1007–1014.

SADANA, J. C., LACHKE, A. H. & PATIL, R. V. (1984). Endo-(1,4)-β-D-glucanases from *Sclerotium rolfsii*. Purification, substrate specificity and mode of action. *Carbohydrate Research* **133**, 297–312.

SHEPHERD, M. G., TONG, C. C. & COLE, A. L. (1981). Substrate specificity and mode of action of the cellulases from the thermophilic fungus *Thermoascus aurantiacus*. *Biochemical Journal* **193**, 67–74.

SOMOGYI, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry* **195**, 19–23.

TANSEY, M. R. & BROCK, T. D. (1978). Microbial life at high temperatures. Ecological aspects. In *Microbial Life in Extreme Environments*, chapter 5. Edited by D. J. Kushner. London: Academic Press.

TATUM, E. L., BARRATT, R. W. & CUTTER, V. M., JR (1949). Chemical induction of colonial paramorphs in *Neurospora* and *Syncephalastrum*. *Science* **109**, 509–511.

TODA, S., SUZUKI, H. & NISIZAWA, K. (1971). Enzymatic properties and the substrate specificities of *Trichoderma* cellulases with special reference to their activity towards xylan. *Hakko Kogaku Zasshi* **49**, 499–521.

WOLTER, K., HIGHLEY, T. & EVANS, F. (1980). A unique polysaccharide and glycoside degrading enzyme complex from the wood decay fungus, *Poria placenta*. *Biochemical and Biophysical Research Communications* **97**, 1499–1504.

YOSHIOKA, H., CHAVANICH, S., NILUBOL, N. & HAYASHIDA, S. (1981). Production and characterization of thermostable xylanase from *Talaromyces byssochlamydoides* YH-50. *Agricultural and Biological Chemistry* **45**, 2425–2432.

ZALEWSKA-SOBCZAK, J. & URBANEK, H. (1981). Cellulose and xylan-degrading enzymes of *Fusarium arenaceum*. *Archives of Microbiology* **129**, 247–250.