Production of cutinase by *Thermomonospora fusca* ATCC 27730

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W.F. FETT, C. WIJEY, R.A. MOREAU AND S.F. OSMAN. 1999. Ten strains belonging to various *Thermomonospora* species were tested for their ability to hydrolyse the insoluble plant polyester cutin. One strain, the thermophile *T. fusca* ATCC 27730, was found to produce a highly inducible cutinase when grown in broth medium containing purified apple cv. Golden Delicious cutin. Apple pomace, tomato peel, potato suberin and commercial cork were also shown to induce cutinase production. Addition of glucose to the culture medium either at the beginning of fermentation or after 2 days of incubation in the presence of apple cutin led to repression of cutinase production. The cutinase was active against a wide range of cutins, including those isolated from other apple cultivars as well as tomato, cucumber, grapefruit, and green pepper. Cutinase activity in the induced culture supernatant fluids exhibited a half-life of over 60 min at 70 °C and a pH optimum of 11·0. Some potential applications for cutinases are discussed.

INTRODUCTION

The surface of most aerial plant parts are covered by a protective layer called the cuticle. The cuticle is composed of chloroform-soluble waxes and the insoluble biopolyester cutin. Cutin is composed primarily of C_{16} and C_{18} hydroxy- and epoxyfatty acids held together via ester bonds together with phenolic material (Walton 1990). In contrast, in tissues which undergo secondary growth (e.g. woody stems, roots and underground storage organs), the protective barrier is composed of suberin which occurs between the primary cell wall and the plasmalemma in cork cells. Suberin is also formed as a protective barrier by wound periderm. Suberin is a more complex polymer than cutin and consists of aliphatic domains resembling cutin and aromatic domains resembling lignin. The lipid component consists primarily of C_{12} to C_{34} fatty acids, fatty alcohols, omega-hydroxy acids and dicarboxylic acids (Walton 1990). The aromatic domain of suberin obtained from potato wound periderm was recently reported to consist primarily of a covalently linked, hydroxycinnamic acid-derived polymeric matrix (Bernards *et al.* 1995). These water impermeable barriers protect against water loss and attack by parasites and plant pathogens, and control gas exchange.

Many fungi (primarily plant pathogens) have been reported to produce cutin-degrading enzymes (cutinases) which may assist in the penetration of the plant cuticle (Kolattukudy 1985). In contrast, very few bacteria appear to produce cutinase (Fett *et al.* 1992a, b). Only two bacterial cutinases, one produced by the filamentous plant pathogen *Streptomyces scabies* (Lin and Kolattukudy 1980) and the other by the plant epiphyte *Pseudomonas mendocina* strain ATCC 55613 (Sebastian *et al.* 1987; Sebastian and Kolattukudy 1988; Gray *et al.* 1995), have been purified and characterized. With few exceptions, microbial cutinases are monomeric proteins with molecular masses of approximately 25–30 kDa and belong to the class of serine esterases. Cutinases represent a bridge between esterases, which act on soluble substrates, and lipases, which exhibit interfacial activation requiring a lipid/water interface for activity. The most studied and best characterized of all microbial cutinases is produced by the plant pathogenic fungus *Nectria haematococca* (*Fusarium phaseoli* f. sp. *pisi*). Studies done by X-ray crystallography indicate that this cutinase does not have a 'flap' extending over the active site, in contrast to lipases, thus explaining the lack of need for interfacial activation (Martinez *et al.* 1992).

There are few reports demonstrating microbial degradation of the more complex substrate suberin. *Nectria haematococca*

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can be induced to produce a suberinase when grown in the presence of potato suberin, and this suberinase appears to be identical to cutinases produced by the same fungus (Fernando *et al.* 1984). The ability to degrade suberin has also been reported for the fungi *Rosellinia desmazieresii* (Ofong and Pearce 1994), *Armillaria mellea* (Zimmerman and Seemuller 1984) and *Mycena meliigena* (Schultz *et al.* 1996).

The eubacterial genus *Thermomonospora* comprises primarily aerobic, thermophilic, spore-forming actinomycetes which are commonly found in manures, composts and overheated fodders (McCarthy 1989). Most *Thermomonospora* species are moderate thermophiles exhibiting good growth at 50 °C with some strains capable of growth up to 60 °C. The thermophiles *T. fusca* and *T. curvata* produce cellulose- and xylose-degrading enzymes (Wilson 1988; Bachmann and McCarthy 1991; Irwin *et al.* 1994; Stutzenberger 1994; Lin and Stutzenberger 1995) as might be expected, based on their natural habitats. In this study, the ability of *Thermomonospora* species to produce cutin-degrading enzymes or cutinases was determined.

MATERIALS AND METHODS

Bacterial strains

The bacteria tested for cutinase production were as follows: *Thermomonospora alba* NRRL B-16963 (= NCIMB 10169) (type strain); *T. chromogena* ATCC 43196 (type strain); *T. curvata* ATCC 19995 (type strain); *T. formosensis* ATCC 49059 (type strain); *T. fusca* ATCC 27730 (type strain), NRRL B-11456, and YX-5p; *T. mesophila* ATCC 27303 (type strain); *T. mesouviformis* ATCC 27644 (type strain); and *Thermomonospora* sp. NRRL B-16962. All ATCC strains were obtained from the American Type Culture Collection (Rockville, MD, USA). The NRRL strains were obtained from Dr Dave Lebeda at the USDA, ARS, National Center for Agriculture Utilization Research (Peoria, IL, USA). *Thermomonospora fusca* strain YX-5p was obtained from Dr David B. Wilson, Cornell University (NY, USA).

Bacteria were grown for long-term storage in Bacto tryptone-yeast extract liquid medium (TYE) (Difco) (pH adjusted to 8·0 before sterilization) for 2–3 d at or near their optimum temperature for growth (30–50 °C). An equal volume of 40% (v/v) glycerol was added and after mixing, the suspensions were transferred to sterile conical centrifuge tubes and stored at − 80 °C.

Preparation of cutins and potato suberin

Cutin was prepared from mature fruits of apple cultivars Golden Delicious, Red Delicious and Granny Smith as well as cucumbers, grapefruit, green peppers and tomatoes purchased at a local supermarket as described previously (Gerard *et al.* 1993). The same method was used to obtain suberin-enriched preparations from periderm (skins) of potatoes purchased at a local supermarket.

Screening for esterase and cutinase activities

Bacteria were screened for esterase activity using TYE liquid medium supplemented with 0·4% (w/v) apple cv. Golden Delicious cutin adjusted to pH 8·0 before sterilization. Inoculum consisted of frozen (−80 °C) stock culture which had been thawed, subjected to centrifugation and the pellet washed once with TYE. Culture tubes (20 × 150 mm) containing 5 ml TYE with or without cutin, or 125 ml Erlenmeyer flasks containing 20 ml of TYE with or without cutin, were inoculated with 0·1 or 0·2 ml washed inoculum, respectively. Cultures were incubated at or near the optimum temperature for growth (30–50 °C) of each strain with shaking (250 rev min^{-1}). Culture fluids were periodically removed (100 μl for esterase assays, 1 ml for cutinase assays) during the 7 day incubation period. The fluids were clarified by centrifugation and stored at −20 °C until the end of the experiment.

Esterase activity was determined by a spectrophotometric assay with *p*-nitrophenyl butyrate (PNB) as substrate (Sebastian *et al.* 1987) as previously described (Fett *et al.* 1992a). Cutinase activity was determined using apple cv. Golden Delicious cutin as substrate. Clarified culture fluid was filter-sterilized (0·2 μm) and added (0·5 ml) to assay tubes containing 30 mg ground cutin (20 mesh) and 0·5 ml of 50 mmol l^{-1} potassium phosphate buffer, pH 8·0, with or without the non-ionic detergent *n*-octylglucoside (35 mmol l^{-1}) (Sigma) (Fett *et al.* 1992a). Assay tubes were incubated at 30 °C for 18 h with shaking (125 rev min^{-1}). After incubation, 0·5 ml glacial acetic acid was added to each tube, the tube contents mixed and then 3 ml $CHCl_3$ added. Tubes were shaken vigorously and then centrifuged to allow for separation of the aqueous and $CHCl_3$ layers. The $CHCl_3$ layer was removed and taken to dryness under a stream of nitrogen. The residue was taken up in 1 ml $CHCl_3$:MeOH (85 : 15), filtered through glass wool into glass autosampler vials, and taken to dryness under a stream of nitrogen. Finally, 1 ml $CHCl_3$:MeOH (85 : 15) containing 0·01% butylated hydroxytoluene (Sigma) was added to each vial. Released cutin monomers were separated and quantified by HPLC as previously described (Gerard *et al.* 1992) with two modifications: a ChromSep 5 μm LICHROSORB Diol cartridge system (3 × 100 mm) (Chrompack, Inc., Raritan, NJ, USA) was substituted for the Si 60 silica cartridge system, and a shortened linear gradient was used. The gradient solvents were A, hexane:acetic acid (1000 : 1, v/v) and B, isopropanol. The linear 20 min gradient timetable was: 0 min, 99·3/0·7 (%A:%B); at 15 min, 70/30; at 16 min, 99·3/0·7 through 20 min. Quantification of cutin hydrolysis was based on a standard curve of area units *vs*

mass for 16-hydroxypalmitic acid (Sigma). The identity of released cutin monomers was confirmed by GC/MS analyses (Fett *et al.* 1992a). Percentage hydrolysis was calculated by dividing total released cutin monomers (in mg) by 30 mg (total cutin initially present in the assay tube) and multiplying by 100. Controls consisted of culture fluid from uninduced cultures, heated (100 °C, 10 min) culture fluids and cutin plus buffer alone.

Cutinolytic esterase induction/repression

Various fatty acids, a fatty alcohol and triglycerides, as well as various cutin- or suberin-containing natural products, were compared with apple cv. Golden Delicious cutin for their ability to induce production of cutinolytic esterase by *T. fusca* strain ATCC 27730. The fatty acids tested were palmitate, 16-hydroxypalmitate and ricinoleic acid (12-hydroxy-9-octadecenoic acid) (Sigma). Also tested were cetyl alcohol (1-hexadecanol), castor oil, olive oil (Sigma), cutin hydrolysate, apple pomace, corn fibre, corn bran, tomato peel and potato suberin. Apple pomace and corn fibre were kindly supplied by Knause Foods (Biglerville, PA, USA) and Cargill Co. (Dayton, OH, USA), respectively. Corn bran (ultrafine grade) was kindly supplied by Lauhoff Grain Co. (Danville, IL, USA).

Cutin hydrolysate was prepared from apple cv. Golden Delicious cutin as follows. Cutin (30 mg) was suspended in 1·5 mol l^{-1} KOH in methanol (2 ml) and shaken at room temperature for 20 h. The mixture was then acidified by adding HCl (1 mol l^{-1}) and extracted twice with $CHCl_3$ (2 volumes each time). The combined $CHCl_3$ extracts were then taken to dryness under a stream of nitrogen. The apple pomace and corn fibre were dried to approximately 5% moisture content then ground in a Wiley mill (20 mesh) before use. The ground corn fibre was either used untreated, or it was treated as follows. One sample was stirred vigorously in water for 1 h at room temperature in Milli-Q purified water. A second sample was suspended in purified water and heated in an autoclave (121 °C, 15 min). Both treated samples were then centrifuged, the pelleted corn fibre washed twice with purified water, and air-dried. Tomatoes (unwaxed) were purchased from a local supermarket. Tomato peels were isolated by cutting the tomatoes in quarters, placing in boiling distilled water for about 5 min, cooling in a cold water-bath, and stripping away the peels by hand. The peels were then washed once with distilled water, freeze-dried, and ground in a Wiley mill (20 mesh followed by 60 mesh).

The various materials were incorporated into the growth medium (TYE) (25 ml per 125 ml Erlenmeyer flask) before sterilization (121 °C, 15 min). Before use, aqueous preparations of cutin hydrolysate, 16-hydroxypalmitate, palmitate and cetyl alcohol were sonicated to obtain fine suspensions. Starter culture (2 ml) of strain ATCC 27730 grown for 2 d in TYE (50 °C, 250 rev min^{-1}) was added to each flask and cultures were incubated at 50 °C with shaking (250 rev min^{-1}). Duplicate flasks were used per natural product for each experiment and experiments were repeated once. Samples of culture fluids were periodically removed up to 7 d after inoculation, clarified by centrifugation and tested for esterase activity as described above. Active culture fluids were tested for cutinase activity by the HPLC method as described above using apple cv. Golden Delicious cutin as the substrate and 50 mmol l^{-1} glycine-NaOH, pH 11·0, as the buffer with no detergent added.

The ability of glucose to repress the production of cutinolytic esterase by *T. fusca* ATCC 27730 was determined. In one set of experiments, glucose was added, before autoclaving, to the apple cutin-containing medium to give a final concentration of 1% (w/v). In a second set of experiments, a concentrated filter-sterilized glucose solution was added to growing cultures after 2 d of incubation to give a concentration of 1% (w/v). In order to rule out an inhibitory effect of lowered culture pH due to growth on glucose as the primary carbon source, experiments were repeated with glucose and $CaCO_3$ (final concentration of 20 g l^{-1}) added to the medium before autoclaving. Experiments were carried out in 125 ml Erlenmeyer flasks containing 25 ml TYE with duplicate flasks per treatment as described above. Esterase values for clarified culture fluids were determined by the spectrophotometric assay. Controls consisted of media alone and cutin-containing media devoid of glucose. Each experiment was repeated once.

Activity against additional cutins

Culture fluid of *T. fusca* ATCC 27730 grown for 5 d in TYE supplemented with apple cv. Golden Delicious apple cutin was tested for enzyme activity against various cutins. Cutinase assays were done in duplicate as described above in 50 mmol l^{-1} glycine/NaOH buffer, pH 11·0. No detergent was added. The protein content of the culture fluid was determined to be 3·1 mg ml^{-1} by the Pierce BCA protein assay carried out according to manufacturer's instructions (Pierce, Rockford, IL, USA) with bovine serum albumin as standard. The experiments were repeated once.

Temperature stability and pH optimum

Samples (1 ml) of active culture supernatant fluids were added to 13 × 100 mm test tubes (duplicate tubes per temperature) and the test tubes were placed in a controlled temperature water-bath set at 50, 60, 70, 80 or 90 °C. Aliquots of heated culture fluids were removed after 15, 30, 45 and 60 min and were immediately placed on ice. Esterase and cutinase activities of the heated samples, as well as unheated control samples, were determined as described above. Cutin-

ase activity was determined only for the samples heated for 60 min. For cutinase assays, the substrate used was apple cv. Golden Delicious cutin and the buffer was 0·1 mol l^{-1} potassium phosphate, pH 8·0. Each experiment was repeated once.

To determine the pH optimum for cutinase activity, the following buffers (all 0·1 mol l^{-1}) were used: sodium citrate (pH 5·0), sodium phosphate (pH 6·0, 7·0, 8·0) and glycine-NaOH (pH 9·0, 10·0, 11·0). Cutinase activity was determined by incubating 0·5 ml samples of culture supernatant fluid with 0·5 ml buffer and 30 mg apple cv. Golden Delicious cutin as described above. Controls consisted of buffer and cutin alone as well as heated (100 °C, 10 min) culture supernatant fluid plus buffer and cutin. Duplicate assays per pH were run and the experiment was repeated once.

RESULTS

The ability of culture fluids to hydrolyse the ester bond of the colourless substrate PNB is presumptive evidence of the presence of cutinolytic esterase (cutinase) (Kolattukudy *et al.* 1981). Hydrolysis of PNB can be followed by determining the increase in absorbence at 405 nm due to the release of the coloured product p-nitrophenol. Bacteria were tested for production of esterase in liquid media containing apple cv. Golden Delicious cutin by this method. Only *T. fusca* strain ATCC 27730 exhibited esterase activities of > 500 nmol min^{-1} ml^{-1} with or without cutin added to the media over the 7 day incubation period. Esterase activity by this bacterium was highly inducible, with strain ATCC 27730 exhibiting a 450-fold increase in production in the presence of apple cutin (27 nmol min^{-1} ml^{-1} without cutin in the media *vs* 12 150 nmol min^{-1} ml^{-1} with cutin).

The cutin-degrading ability of the esterase-containing clarified culture fluids obtained from *T. fusca* ATCC 27730 grown in the presence of apple cv. Golden Delicious cutin was confirmed. Culture fluids were incubated in the presence of cutin from apple cv. Golden Delicious, and the release of cutin monomers was monitored by HPLC. The culture fluids exhibited cutinase activity against both types of cutin as indicated by the release of fatty acid monomers typical of apple and tomato cutins (Holloway 1982). Monomer identity was confirmed by GC/MS. Addition of the non-ionic detergent *n*-octylglucoside (final concentration of 35 mmol l^{-1}) had no effect on cutinase activity of the culture fluids. The culture fluids of *T. fusca* ATCC 27730 were active not only against apple cv. Golden Delicious cutin, but also against cutins of the apple cvs Red Delicious and Granny Smith, and cutins isolated from cucumber, grapefruit, green pepper and tomato (Table 1). Specific activity was highest against apple cv. Golden Delicious cutin. Controls contained no significant amounts of cutin monomers.

The ability of selected compounds, as well as various cutin and/or lipid-containing natural products, to induce cutinolytic esterase production by *T. fusca* ATCC 27730 was tested (Table 2). The individual fatty acids and cutin hydrolysate were inactive as inducers of cutinase production and were inhibitory to growth of *T. fusca* ATCC 27730 at the higher of the two concentrations tested. Cetyl alcohol was also inactive as an inducer. Untreated and treated corn fibre (0·4% w/v), corn bran (0·4% w/v), castor oil (0·4% and 0·04% v/v) and olive oil (0·4% and 0·04% v/v) were strongly inhibitory towards growth of strain ATCC 27730. However, apple pomace, tomato peel, potato suberin and commercial cork all acted as inducers of cutinolytic esterase activity. Final pH values of the cultures usually ranged from 7·2 to 8·5. Cutinase activity in clarified culture fluids from cultures grown in media containing apple cutin, apple pomace, tomato peel, potato suberin and commercial cork was confirmed by HPLC using apple cv. Golden Delicious cutin as the substrate and 50 mmol l^{-1} glycine-NaOH, pH 11·0, buffer. The hydrolysis values were similar, ranging from 17 to 28% as determined by HPLC using a standard curve of area units per mass for 16-OH palmitate.

The production of cutinolytic esterase in culture fluids of *T. fusca* ATCC 27730 grown in TYE supplemented (0·4% w/v) with apple cv. Golden Delicious cutin, apple pomace, tomato peel or commercial cork was followed over 7 d of incubation at 50 °C. Results of a representative experiment are shown in Fig. 1. Cutinolytic esterase activity of culture fluids increased most quickly and to the highest levels with tomato peel as the inducer. Apple cutin and apple pomace were roughly equivalent as inducers, while potato suberin and commercial cork were the least effective inducers.

Table 1 Substrate specificity of the cutinase produced by *Thermomonospora fusca* ATCC 27730 grown in the presence of apple cv. Golden Delicious cutin

Substrate	% Hydrolysis	Specific activity (% Hydrolysis mg^{-1} protein)
Cutins:		
Apple cv.		
Golden Delicious	16·0 ± 1·0*	10·4 ± 0·6
Red Delicious	7·9 ± 0·4	5·1 ± 0·3
Granny Smith	14·0 ± 0·1	9·0 ± 0·0
Cucumber	14·0 ± 0·1	6·2 ± 1·3
Grapefruit	6·6 ± 0·5	4·3 ± 0·1
Green pepper	12·4 ± 1·7	8·0 ± 1·1
Tomato	10·7 ± 0·1	6·9 ± 0·0

*Data shown are average values of the means from two experiments ± standard error.

Table 2 Ability of various natural products to induce cutinolytic esterase production by *Thermomonospora fusca* ATCC 27730

Inducer	Concentration	Relative esterase activity (%)	
		Expt. 1 (day 6)	Expt. 2 (day 7)
Tomato peel	0·4% (w/v)	453*	199
Apple pomace	0·4% (w/v)	106	95
Apple cutin (cv. Golden Delicious)	0·4% (w/v)	100	100
Potato suberin	0·4% (w/v)	42	39
Commercial cork	0·4% (w/v)	28	36
Cetyl alcohol	0·29 mM	<1	<1
Cutin hydrolysate	8 μh/ml	<1	<1
	80 μg/ml	no growth	no growth
Palmitate	0·029 mM	<1	<1
	0·29 mM	no growth	no growth
16-OH palmitate	0·029 mM	<1	<1
	0·29 mm	no growth	no growth
Ricinoleic acid	0·029 mM	<1	<1
	0·29 mM	no growth	no growth
Corn fibre (untreated)			
	0·04% (w/v)	<1	<1
	0·4% (w/v)	no growth	no growth
Corn bran	0·04% (w/v)	<1	<1
	0·4% (w/v)	no growth	no growth
Castor oil	0·04% (v/v)	no growth	no growth
	0·4% (v/v)	no growth	no growth
Corn oil	0·04% (v/v)	no growth	no growth
	0·4% (v/v)	no growth	no growth
Olive oil	0·04% (v/v)	no growth	no growth
	0·4% (v/v)	no growth	no growth

*Values shown are the mean values for each experiment.

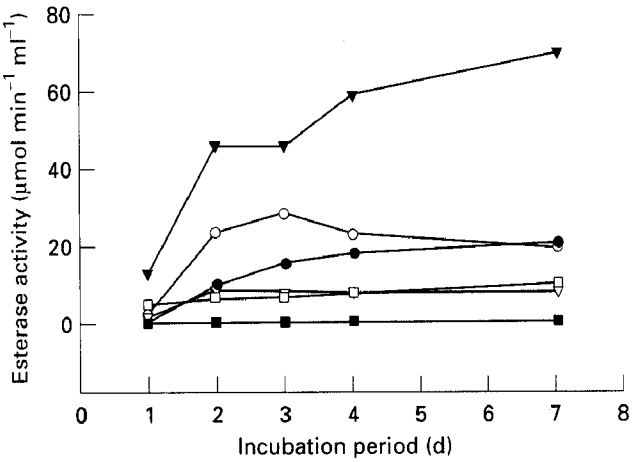

Fig. 1 Extracellular esterase activity of culture fluids of *Thermomonospora fusca* ATCC 27730 grown in TYE broth (pH 8·0) supplemented (0·4% w/v) with apple cv. Golden Delicious cutin (●), apple pomace (○), tomato peel (▼), commercial cork (▽) potato suberin (□), media alone (■). Values shown are the mean values for one representative experiment

Addition of glucose (1% w/v) to the medium before inoculation strongly inhibited cutinolytic esterase production over the 7 day incubation period (Fig. 2). The pH of the glucose-containing cultures had dropped to 5·9 by day 7 compared with 8·2 for the cutin-containing cultures (no glucose added) and 8·4 for the control cultures with media alone. To determine whether the lowering of the pH was the cause of the lowered cutinolytic esterase production, the medium was supplemented with $CaCO_3$, a solid buffering agent, in additional experiments. The final pH (at 7 days) of the inoculated $CaCO_3$-containing media with glucose was the same as the controls (pH 8·4), but the esterase values were still very low (Fig. 2). When glucose (final concentration of 1%, w/v) was added to cultures already growing in the presence of apple cutin at day 2 of incubation, the production of esterase was significantly reduced thereafter (Fig. 2).

Esterase activity in active culture supernatant fluids was very stable at 50 and 60 °C (Fig. 3), retaining approximately 90% of activity after 1 h of heating. The half-life at 70 °C was 60 min whereas little activity remained after heating at 80 or 90 °C for 15 min or longer. Cutinase activity dem-

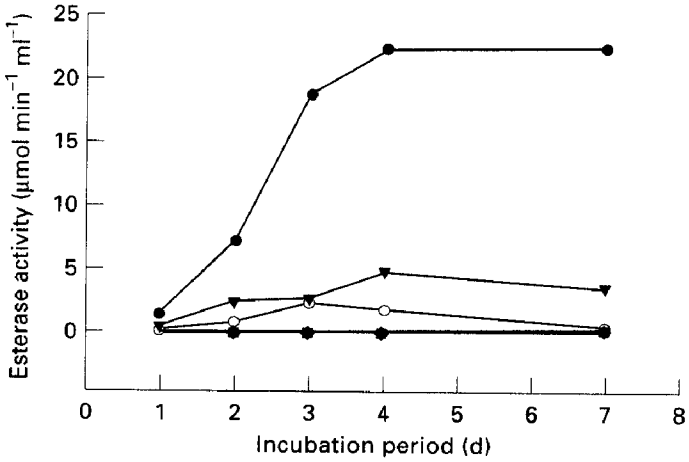

Fig. 2 Glucose repression of cutinolytic esterase synthesis by *Thermomonospora fusca* ATCC 27730 grown in TYE broth (pH 8·0) supplemented (0·4% w/v) with apple cv. Golden Delicious (GDA) cutin. GDA alone (●), GDA + glucose (Day 0) (◆), GDA + $CaCO_3$ + glucose (Day 0) (○), GDA + glucose (Day 2) (▼), media alone (■). Values shown are the mean values for one representative experiment

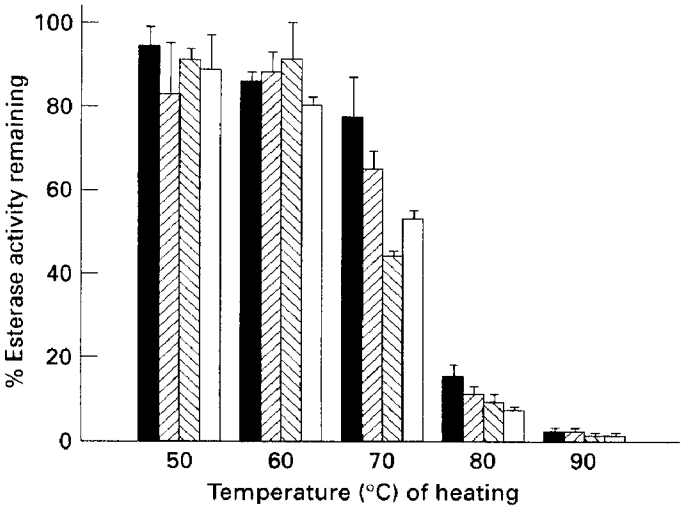

Fig. 3 Heat stability of esterase activity in induced culture supernatant fluids of *Thermomonospora fusca* ATCC 27730. Values shown are the average mean values for two experiments ± S.E. Length of heating: (■), 15 min; (▨), 30 min; (▧), 45 min; (□), 60 min

onstrated half-lives of over 60 min at 50, 60 and 70 °C, but activity was significantly reduced after heating at 80 and 90 °C for 1 h (Fig. 4). With the buffers used, the *T. fusca* cutinase exhibited two pH optima with optimal activity at pH 11·0 (Fig. 5). Controls contained only minor amounts of cutin monomers. These values were subtracted out to give the final values shown in Fig. 5.

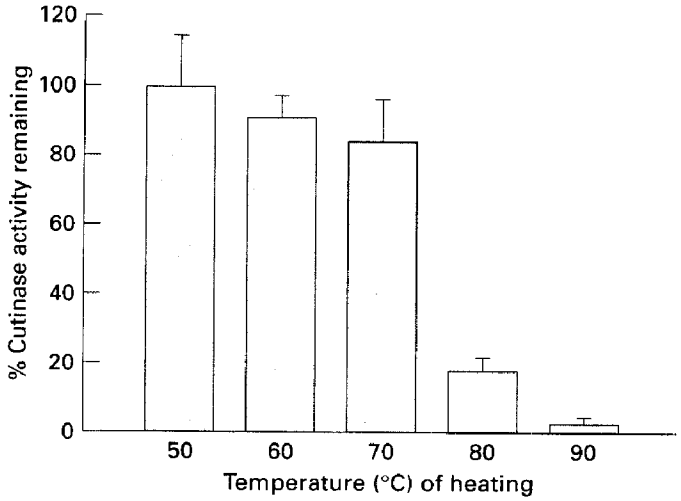

Fig. 4 Heat stability of cutinase activity in induced culture supernatant fluids of *Thermomonospora fusca* ATCC 27730. Culture fluids were heated for 60 min. Apple cv. Golden Delicious cutin was used as the substrate. Values shown are the average mean values for two experiments ± S.E.

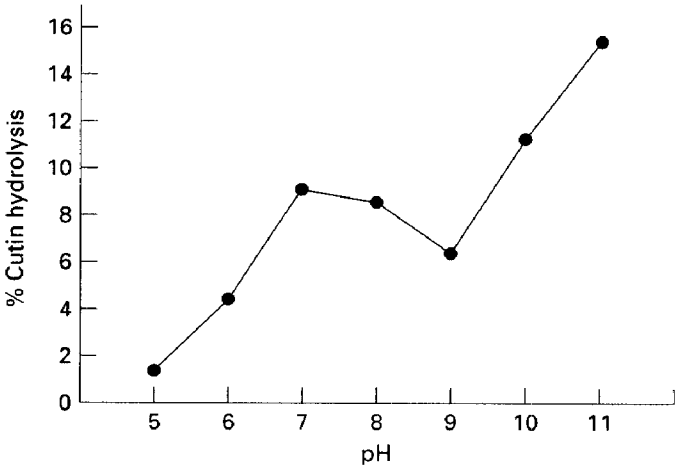

Fig. 5 Optimum pH for cutinase activity of induced culture supernatant fluids of *Thermomonospora fusca* ATCC 27730 using apple cv. Golden Delicious cutin as the substrate. Values shown are the mean values for one representative experiment

DISCUSSION

Thermomonospora species play an important role in the primary degradation of organic material (McCarthy 1989). To accomplish this they produce an array of extracellular enzymes including cellulases and xylanases (Wilson 1988; Bachman and McCarthy 1991). Another enzyme which might assist these bacteria in the degradation of plant materials is cutinase. As there was no prior report of cutin degradation by this group of bacteria, we decided to determine whether the *Thermomonospora* contained members with this ability.

Of the 10 strains examined, only *T. fusca* strain ATCC 27730 was positive for esterase production under the conditions tested. Two additional strains of *T. fusca* were negative for this enzyme activity. Cutinase activity was confirmed by an HPLC method which separated the released cutin fatty acid monomers (Gerard *et al.* 1992) using various cutins as substrates, as well as by GC/MS analysis.

The cutinase produced by strain ATCC 27730 was highly inducible by apple cv. Golden Delicious cutin with very low constitutive levels, as found for other bacterial cutinases (Sebastian and Kolattukudy 1988, 1992b; Fett *et al.* 1992a). Inducers other than purified cutin were identified. The inducers were apple pomace, tomato peel, potato suberin and commercial cork. Apple pomace is the material left after pressing apples for juice or after pressing peel and core wastes resulting from the preparation of apples for canning, drying and freezing. Surprisingly, both corn fibre and corn bran were inhibitory towards bacterial growth. Corn fibre is a by-product of the industrial wet milling process and is composed of cell wall material from corn hulls (pericarp) plus residual starch. Corn bran is a similar by-product from the industrial dry milling process. The inhibitory activity of corn fibre was not diminished by stirring or autoclaving in water, indicating that the inhibitory substance(s) were not highly water soluble.

Strain ATCC 27730 is similar to the previously reported cutinase-producing bacterium *Pseudomonas* sp. ATCC 53553 (Sebastian *et al.* 1987) in that cutinase was not induced by addition of cutin-hydrolysate to the medium, differing from the well studied fungal cutinases (Lin and Kolattukudy 1978). Cutinase production by strain ATCC 27730 also differed from fungal cutinase production in that low levels of the cutin monomer 16-OH palmitate and cetyl alcohol were not active as inducers. Fungal cutinase production appears to require only an aliphatic chain with a primary alcohol group (Lin and Kolattukudy 1978), but this was not true for cutinase production by strain ATCC 27730.

Cutinase synthesis by strain ATCC 27730 was also subject to repression by glucose. Addition of glucose at the beginning of incubation or after 2 d of incubation in the presence of apple cutin caused almost complete inhibition or cessation, respectively, of cutinase production. Synthesis of fungal cutinase is also repressed by the presence of glucose in the medium (Lin and Kolattukudy 1978; Bajar *et al.* 1991).

Under the experimental conditions used, the *T. fusca* cutinase was similar in heat stability to the cutinase produced by the mesophyllic bacterium *Ps. mendocina* ATCC 55613, and both enzymes were considerably more heat-stable than fungal cutinase (Sebastian *et al.* 1987). Thermostable enzymes are necessary for the many industrial reactions carried out at high temperatures (above 50 °C). Significant cost savings can be realized because of the longer storage stability of thermostable enzymes, as these enzymes are more resistant to most chemical denaturants than their mesophilic counterparts. Additional cost savings can be made by the higher reaction rate at increased temperature (Ng and Kenealy 1986). As heat stability of an enzyme is dependent on experimental conditions (e.g. pH and protein concentration) (Lauwereys *et al.* 1991), the actual heat stability in any potential commercial application would have to be determined under those specific sets of conditions.

The *T. fusca* cutinase exhibited a very basic pH optimum (pH 11·0). Most of the fungal cutinases also have a basic pH optimum (pH 9·0) (Kolattukudy 1985), although fungal cutinases with pH optima below 7·0 have been reported (Koller and Parker 1989; Trail and Koller 1993). The bacterial cutinase produced by *Ps. mendocina* ATCC 55613 has a broad pH optimum of 8·5–10·5 when tested against apple cutin (Sebastian and Kolattukudy 1988). The pH optimum of the cutinase produced by *Streptomyces scabies* has not been reported.

The activity of potato suberin and commercial cork as inducers of cutinase production by strain ATCC 27730 suggests that the cutinase may also have suberin-degrading activity. To our knowledge, there are no reports of bacterial enzymes with suberinase activity.

The cutinase inducers apple pomace, tomato peels and potato skins are all agricultural by-products which are either disposed of or used for low value animal feeds. Thus, they represent potential low cost inducers in industrial fermentations for the preparation of microbial cutinases for commercial applications. Several potential commercial applications for cutinases of mesophiles have been patented, including their use for increasing uptake of agricultural chemicals by plants (Iwasaki and Hioki 1988; Kolattukudy and Poulose 1996a), increasing the water permeability across the surface membrane of harvested fruits and vegetables, allowing for more rapid dehydration and for uptake of sweeteners, flavour enhancers, preservatives, etc. (Poulose and Boston 1996), and as components of cleaning agents (Kolattukudy and Poulose 1996b). The use of thermostable cutinases from thermophiles may afford cost savings for the various applications stated above. Currently, we are in the process of purifying and characterizing the cutinase produced by *T. fusca* to determine its commercial potential.

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REFERENCES

Bachmann, S.L. and McCarthy, A.J. (1991) Purification and cooperative activity of enzymes constituting the xylan-degrading sys-

tem of *Thermomonospora fusca*. *Applied and Environmental Microbiology* **57**, 2121–2130.

Bajar, A., Podila, G.K. and Kolattukudy, P.E. (1991) Identification of a fungal cutinase promoter that is inducible by a plant signal via a phosphorylated trans-acting factor. *Proceedings of the National Academy of Sciences USA* **88**, 8208–8212.

Bernards, M.A., Lopez, M.L., Zajicek, J. and Lewis, N.G. (1995) Hydroxycinnamic acid-derived polymers constitute the polyaromatic domain of suberin. *Journal of Biological Chemistry* **270**, 7382–7386.

Fernando, G., Zimmermann, W. and Kolattukudy, P.E. (1984) Suberin-grown *Fusarium solani* f. sp. *pisi* generates a cutinase-like esterase which depolymerizes the aliphatic components of suberin. *Physiological Plant Pathology* **24**, 143–155.

Fett, W.F., Gerard, H.C., Moreau, R.A., Osman, S.F. and Jones, L.E. (1992a) Cutinase production by *Streptomyces* spp. *Current Microbiology* **25**, 165–171.

Fett, W.F., Gerard, H.C., Moreau, R.A., Osman, S.F. and Jones, L.E. (1992b) Screening of nonfilamentous bacteria for production of cutin-degrading enzymes. *Applied and Environmental Microbiology* **58**, 2123–2130.

Gerard, H.C., Fett, W.F., Osman, S.F. and Moreau, R.A. (1993) Evaluation of cutinase activity of various industrial lipases. *Biotechnology and Applied Biochemistry* **17**, 181–189.

Gerard, H.C., Osman, S.F., Fett, W.F. and Moreau, R.A. (1992) Separation, identification and quantification of monomers from cutin polymers by high performance liquid chromatography and evaporative light scattering detection. *Phytochemical Analysis* **3**, 139–142.

Gray, G.L., Power, S.D. and Poulose, A.J. (1995) Lipase from *Pseudomonas mendocina* having cutinase activity. U.S. Patent no. 5,389,536.

Holloway, P.J. (1982) The chemical constitution of plant cutins. In *The Plant Cuticle* ed. Cutler, D.F., Alvin, K.L. and Price, C.E. pp. 45–85. London: Academic Press.

Irwin, D., Jung, E.D. and Wilson, D.B. (1994) Characterization and sequence of a *Thermomonospora fusca* xylanase. *Applied and Environmental Microbiology* **60**, 763–770.

Iwasaki, T. and Hioki, Y. (1988) Enhancement of biocide. U.S. Patent no. 4,762,547.

Kolattukudy, P.E. (1985) Enzymatic penetration of the plant cuticle by fungal pathogens. *Annual Review of Phytopathology* **23**, 223–250.

Kolattukudy, P. and Poulose, A.J. (1996a) Enzymes as agricultural chemical adjuvants. U.S. Patent no. 5,545,547.

Kolattukudy, P. and Poulose, A.J. (1996b) Cutinase cleaning compositions. U.S. Patent no. 5,512,203.

Kolattukudy, P.E., Purdy, R.E. and Maiti, I.B. (1981) Cutinases from fungi and pollen. *Methods in Enzymology* **71**, 652–664.

Koller, W. and Parker, D.M. (1989) Purification and characterization of cutinase from *Venturia inaequalis*. *Phytopathology* **79**, 278–283.

Lauwereys, M., DeGeus, P., DeMeutter, J., Stanssens, P. and Matthyssens, G. (1991) Cloning, expression and characterization of cutinase, a fungal lipolytic enzyme. In *Lipases: Structure, Mechanism and Genetic Engineering* ed. Alberghina, L., Schmid, R.D. and Verger, R. pp. 243–251. New York: VCH Publishers.

Lin, T.S. and Kolattukudy, P.E. (1978) Induction of a biopolyester hydrolase (cutinase) by low levels of cutin monomers in *Fusarium solani* f. sp. *pisi*. *Journal of Bacteriology* **133**, 942–951.

Lin, T.S. and Kolattukudy, P.E. (1980) Isolation and characterization of a cuticular polyester (cutin) hydrolyzing enzyme from phytopathogenic fungi. *Physiological Plant Pathology* **17**, 1–5.

Lin, S.-B. and Stutzenberger, F.J. (1995) Purification and characterization of the major β-1,4-endoglucanase from *Thermomonospora curvata*. *Journal of Applied Bacteriology* **79**, 447–453.

Martinez, C., De Geus, P., Lauwereys, M., Matthyssens, G. and Cambillau, C. (1992) *Fusarium solani* cutinase is a lipolytic enzyme with a catalytic serine accessible to solvent. *Nature* **356**, 615–618.

McCarthy, A.J. (1989) *Thermomonospora* and related genera. In *Bergey's Manual of Systematic Bacteriology*, Vol. 4 ed. Williams, S.T., Sharpe, M.E. & Holt, J.G. pp. 2552–2572. Baltimore: Williams & Wilkins.

Ng, T.K. and Kenealy, W.R. (1986) Industrial applications of thermostable enzymes. In *Thermophiles: General, Molecular and Applied Microbiology* ed. Brock, T.D. pp. 197–215. New York: Wiley.

Ofong, A.U. and Pearce, R.B. (1994) Suberin degradation by *Roselinia desmazieresii*. *European Journal of Forest Pathology* **24**, 316–322.

Poulose, A.J. and Boston, M. (1996) Enzyme assisted degradation of surface membranes of harvested fruits and vegetables. U.S. Patent no. 5,510,131.

Schultz, E., Chamuris, G.P. and Dallabrida, S. (1996) Screening wood- and bark-inhabiting Basidiomycetes for esterase activity in liquid stationary culture. *Mycologia* **88**, 831–838.

Sebastian, J., Chandra, A.K. and Kolattukudy, P.E. (1987) Discovery of a cutinase-producing *Pseudomonas* sp. cohabiting with an apparently nitrogen-fixing *Corynebacterium* sp. in the phyllosphere. *Journal of Bacteriology* **169**, 131–136.

Sebastian, J. and Kolattukudy, P.E. (1988) Purification and characterization of cutinase from a fluorescent *Pseudomonas putida* bacterial strain isolated from the phyllosphere. *Archives of Biochemistry and Biophysics* **262**, 77–85.

Stutzenberger, F. (1994) Extracellular enzyme production by *Thermomonospora curvata* grown on bagasse. *Journal of Industrial Microbiology* **13**, 35–42.

Trail, F. and Koller, W. (1993) Diversity of cutinases from plant pathogenic fungi: purification and characterization of two cutinases from *Alternaria brassicicola*. *Physiological and Molecular Plant Pathology* **42**, 205–220.

Walton, T.J. (1990) Waxes, cutin and suberin. In *Methods in Plant Biochemistry*, Vol. 4. *Lipids, Membranes and Aspects of Photobiology* ed. Harwood, J.L. and Bowyer, J.R. pp. 105–158. San Diego: Academic Press.

Wilson, D.B. (1988) Cellulases of *Thermomonospora fusca*. *Methods in Enzymology* **160**, 314–323.

Zimmerman, W. and Seemuller, E. (1984) Degradation of raspberry suberin by *Fusarium solani* f. sp. *pisi* and *Armillaria mellea*. *Phytopathologische Zietschrift* **110**, 192–199.