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SHORT CONTRIBUTION

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Detection of ferulic acid esterase production by *Bacillus* spp. and lactobacilli

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Abstract The production of feruloyl esterase activity by *Bacillus* spp. and lactobacilli can be detected in an agar-plate assay. The assay involves the substitution of the main carbon source in specific agar with ethyl ferulate. A number of *Bacillus* spp., predominantly *B. subtilis* strains, were found to exhibit feruloyl esterase activity by this method. Of the examined lactobacilli, *Lb. fermentum* (NCFB 1751) showed the highest level of ferulic acid esterase activity. The enzyme was released from harvested cells by sonication and showed pH and temperature optima of 6.5 and 30 °C respectively.

Introduction

Ferulic acid is the major phenolic acid found esterified to carbohydrates in the plant cell wall. Feruloyl esterase activity, resulting in the cleavage of such ester cross-linkages, was first described by MacKenzie et al. (1987). Subsequently, phenolic-acid-releasing enzymes have been reported for the bacteria *Streptomyces olivochromogenes* (Faulds and Williamson 1991) and *Fibrobacter succinogenes* (McDermid et al. 1990) and for the fungi *Penicillium* spp. (Castanares et al. 1992; Donaghy and McKay 1995), *Neocallimastix* spp. (Borneman et al. 1990, 1991, 1992), *Schizophyllum commune* (MacKenzie and Bilous 1988) and *Aspergillus* spp. (Tenkanen et al. 1991; Faulds and Williamson 1993, 1994; McCrae et al. 1994).

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Many quantitative methods for the measurement of feruloyl esterase activity are in use. These include gas chromatography (Borneman et al. 1990), high-performance liquid chromatography (MacKenzie and Bilous 1988; Castanares et al. 1992) and capillary zone electrophoresis (Donaghy and McKay 1995). Donaghy and McKay (1994) have also developed a screening assay for the detection of microbial phenolic acid esterases. However, some drawbacks were experienced with this method; the agar medium did not always support microbial growth and also, once the inoculated plate was flooded with the pH-sensitive dye, it could not be incubated further if esterase activity was absent.

The aim of this study was to develop further this earlier screening assay for microbial phenolic acid esterases. The study was primarily interested in bacteria, particularly gram-positive bacteria, capable of producing phenolic acid esterases. No reports had indicated the production of such esterases by this group of bacteria.

Materials and methods

Microorganisms screened

A total of 80 *Bacillus*-type strains were screened for feruloyl esterase activity. These included *B. brevis*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. fimus*, *B. licheniformis*, *B. macerans*, *B. megaterium*, *B. polymyxa*, *B. mycoides*, *B. sphaericus* and *B. subtilis* strains. All strains were grown on nutrient agar for 24 h at 30 °C prior to screening. A further 50 gram-positive bacteria including *Enterococcus divars*, *Enterococcus faecium*, *Enterococcus faecalis*, *Lactobacillus delbruickii*, *Lactobacillus farciminus*, *Lactobacillus fermentum*, *Lactobacillus leichmannii*, *Lactobacillus reuteri*, *Lactobacillus sake*, *Lactococcus lactis*, *Lactococcus* spp., *Leuconostoc mesenteroides*, *Leuconostoc* spp., *Pediococcus* spp. and *Propionibacterium* spp. were also screened. These strains were grown on De Man, Rogosa, Sharpe (MRS) agar (Oxoid) for 24 h at 30 °C (7% CO_2) prior to screening.

Screening assay

Bacillus spp. were screened on an agar medium consisting of (g/l) $(NH_4)_2SO_4$ 1.3, KH_2PO_4 0.37, $MgSO_4 \cdot 7H_2O$ 0.25, $CaCl_2 \cdot 2H_2O$

0.07, $FeCl_3$ 0.03, yeast extract 1.0 and bacteriological agar 20, pH 6.5. Each agar plate was supplemented with 0.3 ml ethyl ferulate (10% v/v in dimethylformamide) at the plate-pouring stage. This supplement was immediately mixed, by swirling, with the agar medium to ensure a homogeneous distribution (a cloudy haze) throughout the plate. Dried (3 h 25 °C) assay plates were streaked with a heavy inoculum from nutrient agar plates (24 h growth) and incubated for a maximum of 3 days at 30 °C. The formation of a clearing zone around the point of inoculation indicated feruloyl esterase production.

A similar technique was used to screen the remaining gram-positive bacteria. The agar medium used for these bacteria consisted of MRS agar (Oxoid) with glucose omitted, pH 6.5, supplemented with ethyl ferulate as before.

Detection of phenolic acid esters and their corresponding acids by capillary zone electrophoresis (CZE)

Ferulic acid and its methyl and ethyl esters were detected using the P/ACE 5510 capillary electrophoresis system fitted with a photo diode-array detector (Beckman Instruments, UK). (Donaghy and McKay 1994).

Enzyme production in liquid culture

Bacteria that demonstrated feruloyl esterase production in the screening assay were grown in liquid culture containing methyl ferulate as the main carbon source.

Bacillus spp., grown overnight in nutrient broth (Oxoid), were harvested, washed (three times) and resuspended in sterile deionized water. Strains were inoculated (0.2 ml) into 200-ml shake flasks containing 100 ml growth medium consisting of (g/l) $(NH_4)_2SO_4$ 1.3, KH_2PO_4 0.37, $MgSO_4 \cdot 7H_2O$ 0.25, $CaCl_2 \cdot 2H_2O$ 0.07, $FeCl_3$ 0.02, yeast extract 1.0. Each flask was supplemented with 5 ml methyl ferulate (1%w/v in dimethylformamide) and incubated on a rotary shaker (120 rpm) at 30 °C for 48 h. Lactic acid bacteria, which screened positive for feruloyl esterase, were similarly treated, the growth medium containing (g/1) peptone 10, Lab Lemco powder 8, yeast extract 4, 1 ml sorbitan mono-oleate, dipotassium hydrogen phosphate 2, $CH_3CONa \cdot 3H_2O$ 5, triammonium citrate 2, $MgSO_4 \cdot 7H_2O$ 0.2. Each 100 ml flask was supplemented with 5 ml methyl ferulate (1%w/v in dimethylformamide).

Cells were harvested (after 48 h) from growth media by centrifugation at 20 000 g for 20 min and washed (three times) with sterile deionized water. An equal volume of glass beads (106 μm) (Sigma) and breaking buffer (0.01 M TRIS/HCl pH 7.0; NADP 0.038%w/v and EDTA 0.4%w/v) was added to the cells. The ice-cold cells were sonicated for 3 min (10-s bursts with 30-s intervals). The crude enzyme supernatant was recovered by centrifugation at 20 000 *g* for 20 min. This crude enzyme preparation was used in subsequent enzyme assays.

Esterase assays

Feruloyl/*p*-coumaroyl esterase activities were assayed by CZE (Donaghy and McKay 1995). The synthetic esters methyl ferulate, methyl coumarate and a water-soluble carbohydrate ferulic acid ester: 2-*O*-[5-*O*-(*trans*-feruloyl)-β-L-arabinofuranosyl] D-xylopyranose (FAX) (MacKenzie and Bilous 1988), were used as assay substrates. One unit of enzyme activity was defined as the amount that releases 1 μmol ferulic acid/min and specific activity is given in units/mg protein. Protein was measured by the method of Bradford (1976) with a commercial protein assay kit (Pierce, Rockford, Ill., USA).

The pH optimum of the feruloyl esterase of *Lb. fermentum* was determined at 30 °C with methyl ferulate as the substrate. Buffers (0.05 M) used were sodium acetate (pH 4.2–5.6) (2-(*N*-morpholino)ethanesulphonic acid (MES; pH 5.3–6.4) and 1,3-bis[tris(hydroxymethyl)methylamino]propane (BISTRIS propane; pH 6.5–8.2). The temperature optimum was determined at pH 6.5 (BISTRIS propane buffer). Residual activity, defined as the percentage activity remaining at test temperatures or pH, relative to the activity at 30 °C and pH 6.5 (100%), was assayed (for up to 1 h) after pre-incubation of the enzyme at the test temperature and pH for 15 min.

Results

The agar screening assay developed involves the substitution of the main carbohydrate component of selective agars with the synthetic phenolic ester ethyl ferulate. Ethyl ferulate, at a final concentration of 0.1% v/v, resulted in an opaque haze in both the modified MRS and

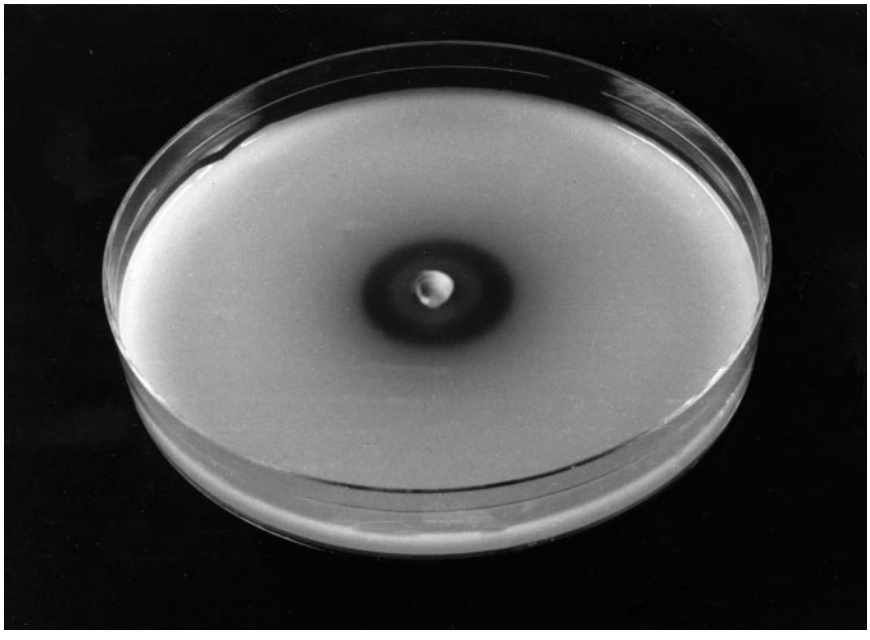

Fig. 1 Feriloyl esterase assay plate showing a zone of clearing around a culture of *Lactobacillus fermentum*

Table 1 *Bacillus* spp. and lactic acid screened for feruloyl esterase activity. *FMCC* Food Microbiology Culture Collection, Newforge Lane, Belfast, UK. + Zone of clearing < 10 mm, + + zone of clearing 10–20 mm, + + + zone of clearing > 20 mm

Culture	Activity
Bacillus subtilis FMCC 193	+
Bacillus subtilis FMCC 267	+ + +
Bacillus subtilis FMCC PL-1	+ + +
Bacillus subtilis FMCC 511	+ + +
Bacillus subtilis NCIMB 11034	+ + +
Bacillus subtilis NCIMB 3610	+ + +
Bacillus pumilis ATCC 7661	+ + +
Bacillus sphaericus ATCC 14577	+ +
Bacillus licheniformis ATCC 14580	+ +
Lactobacillus leichmanni NCIMB 7854	+
Lactobacillus farciminis NCIMB 11717	+
Lactobacillus fermentum NCFB 1751	+ + +
Lactobacillus fermentum NCIMB 2797	+ +
Lactobacillus reuteri NCIMB 11951	+ +

bacillus media without inhibiting microbial growth. Of the 130 bacteria screened, 14 produced zones of clearing (Fig. 1) around their inoculum (Table 1). The formation of ferulic acid in this cleared zone, resulting from deesterification of the ester substrate, was confirmed by CZE. *B. subtilis* strains produced the largest clearing zones on the bacillus medium while only *Lactobacillus* strains gave cleared zones on the ester-substituted MRS medium. *Lb. fermentum* (NCFB 1751) consistently gave the highest level of feruloyl esterase activity on the screening assay.

The *Bacillus* spp. and the *Lb. fermentum* strain that gave the largest zones of clearing on the screening media were grown in liquid culture containing methyl ferulate as one of the main carbon sources. Feruloyl esterase production by the cultures was confirmed by the resultant formation of ferulic acid in the culture broth (Table 2). In each culture broth methyl ferulate was not detectable after 24 h. Despite concentration and diafiltration (10 kDa cut-off membrane) of the culture broths, there was no evidence of feruloyl esterase activity in the culture broth.

The "crude" phenolic acid esterase from cells of *Lb. fermentum* and *Bacillus* spp. had feruloyl and *p*-coumaroyl esterase activity. In addition, ferulic acid was released from the "natural" substrate FAX by the enzyme preparation (Table 3). The pH and temperature optima for the feruloyl esterase (with methyl ferulate as the substrate) were 6.5 and 30 °C respectively. Enzyme preparations from the *B. subtilis* strains and the *B. pumilis* strain also released ferulic acid from the methyl ester and from FAX.

Table 2 Ferulic acid produced in methyl-ferulate-containing liquid medium. Values are the means of triplicate experiments and varied by less than 7% about the mean in all cases

Culture/strain	Ferulic acid formed (μg ml^{-1})	
	24 h	48 h
L. fermentum NCFB1751	53.7	134.2
B. subtilis NCIMB 3610	123.3	75.9
B. subtilis NCIMB 11034	104.4	38.0
B. subtilis FMCC 267	9.5	28.5
B. subtilis FMCC 511	142.3	208.7
B. subtilis FMCC PL1	113.8	37.9
B. pumilis ATCC 7661	104.4	47.4

Discussion

The incorporation of a synthetic ester into an agar medium has been used previously to screen microbial cultures for phenolic acid esterase production (Donaghy and McKay 1994). However, this earlier method failed to support the growth of many microorganisms, particularly bacteria. The agar screening method described here uses medium that promotes the growth of specific groups of bacteria, for example MRS for the culture of lactic acid bacteria. Positive cultures were easily identified by zones of clearing (deesterification of the incorporated synthetic ester) around the inoculum. Plates could be incubated for up to 3 days and continually monitored for activity, unlike the earlier screening assay, which involved flooding agar plates with a pH-sensitive dye.

Few bacterial phenolic acid esterases have been identified previously: *Fibrobacter succinogenes* (McDermid et al. 1990) and *Streptomyces* spp. (Johnson et al. 1988; Faulds and Williamson 1991). Although the literature cites a number of esterase-producing *B. subtilis* strains (Higerd and Spizizen 1973; Higerd 1977; Williams et al. 1990 and Chen et al. 1992, 1995) this is the first report of the ability of *B. subtilis* strains and a *B. pumilis* strain to deesterify phenolic esters. Similarly, there have been no reports of phenolic acid esterase

Table 3 Specific feruloyl/*p*-coumaroyl esterase activity from sonicated cells. Values are the means of triplicate experiments and varied by less than 10% about the mean. *NT* not tested, *FAX* 2-*O*-[5-*O*-(*trans*-feruloyl)-β-L-arabinofuranosyl] D-xylopyranose

Culture	Product yield (mU mg protein^{-1})		
	Methyl ferulate	FAX	Methyl coumarate
L. fermentum NCFB 1751	11.71	2.99	8.35
B. subtilis NCIMB 3610	3.35	1.26	NT
B. subtilis NCIMB11034	1.85	ND	NT
B. subtilis FMCC 267	34.14	8.37	NT
B. subtilis FMCC 511	31.06	3.77	NT
B. subtilis FMCC PL1	19.89	2.71	NT
B. subtilis ATCC 7661	1.29	2.59	NT

production by lactobacilli. However, El Soda et al. (1986) demonstrated the ability of *Lb. caesei*, *Lb. plantarum*, *Lb. brevis* and *Lb. fermentum* to release short-chain fatty acids from chromogenic esters. Furthermore, Gobbetti et al. (1997) have shown the co-existence of two esterases in *Lb. fermentum*, a different strain from that used in this study. One of the esterases was associated with the cytoplasm while the second was cell-surface-associated. Our study made no attempt to differentiate between a cytoplasmic or cell-surface-based esterase. However, the absence of activity in concentrated supernatant would indicate an enzyme that was intracellular or tightly bound to the cell surface membrane.

The temperature and pH optima (30 °C and 6.5) respectively of the feruloyl esterase in this study were similar to those recorded for the previous cell-surface-associated esterase of *Lb. fermentum*.

Phenolic acid esterases have considerable potential as biocatalysts for the food and allied industries. Their importance in the modification of plant-based food texture, the baking industry, the generation of chemical feedstocks from food waste and their potential use in animal nutrition have all been alluded to in the literature. The source of phenolic acid esterases identified in this study is interesting because food-processing enzymes from *B. subtilis* are already in use. Furthermore, *Lb. fermentum* is commonly used in the dairy industry as a cheese starter culture. Therefore, both bacteria may represent a source of phenolic acid esterases for use in the food industry.

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