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Characterization of pectin lyase produced by an endophytic strain isolated from coffee cherries

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Aims: The effect of endophytic bacterial activity on the quality of coffee beverage was studied.
Methods and Results: A survey of the micro-organisms in coffee cherries was performed before harvesting, and their growth on the main nutrients available in coffee cherries was determined *in vitro*.
Conclusions: Many endophytic bacteria were isolated from surface-sterilized coffee cherries. One of the pectinolytic strains was physiologically and phenotypically characterized, and was tentatively identified by partial 16S rDNA sequencing as *Paenibacillus amylolyticus*. This endophytic strain produced an extracellular pectinase with maximal activity at 40°C and pH 7·9, and was thermostable up to 45°C. EDTA and metal ions had little effect on pectin lyase activity. K_m and V_{max} values were 4·6 mg ml^{-1} and 94·0 10^{-8} mol min^{-1} ml^{-1}, respectively.
Significance and Impact of the Study: Pectin lyases have been found in fungi but rarely in bacteria, and this isolate is a promising tool for regulation studies of these enzymes.

INTRODUCTION

Endophytic bacteria are defined as those bacteria that can be isolated from surface-disinfected plant tissue or extract from within the plant, but which do not visibly harm the plant (Hallmann *et al.* 1997). Endophytes may have several beneficial effects in host plants and are used in the biological control of plant pathogens, for enhancing agronomic plant characteristics such as increased drought tolerance and nitrogen efficiency, as bioherbicides, and as pharmacological agents (Bacon and Hinton 1997).

Besides invading plants through natural openings or wounds, endophytic bacteria appear actively to penetrate plant tissues using hydrolytic enzymes such as cellulase and pectinase (Reinhold-Hurek and Hurek 1998). Numerous endophytic bacteria, such as *Azoarcus* sp. (Hurek *et al.* 1994) and *Pseudomonas fluorescens* (Quadt-Hallmann *et al.* 1997), produce pectinolytic enzymes.

Pectinases have widespread applications in the food and textile industries (Alkorta *et al.* 1998; Henriksson *et al.* 1999). Among the known pectinases, pectin lyase (E.C. 4.2.2.10) (PL) is the only pectic enzyme that can cleave the α-1,4 bonds of highly esterified pectins without prior action of other enzymes (Alanã *et al.* 1991). This enzyme is thus well suited for de-gumming of natural fibres (Baracat-Pereira *et al.* 1994), plant tissue maceration and fruit juice wastewater treatment (Kashyap *et al.* 2000). Pectin lyase is commonly produced by fungi (Prade *et al.* 1999) and is also produced by some bacteria, such as *Bacillus* spp. (Kashyap *et al.* 2000) and *Pseudomonas marginalis* (Hayashi *et al.* 1997).

Endophytic bacteria are ubiquitous in most plant species. Despite the importance of coffee in agricultural economies, the only reported endophytic bacterium isolated from coffee to date is *Acetobacter diazotrophicus*, which was isolated from roots and stems (Jimenez-Salgado *et al.* 1997). It is believed that the present work is the first record of isolation of an endophytic bacterium from healthy coffee cherries. This is also the first report of pectin lyase production by a strain of *Paenibacillus amylolyticus*.

MATERIALS AND METHODS

Media and cultural conditions

Nutrient broth and nutrient agar were used for isolation and culturing. A solid mineral medium containing: 7·0 g l^{-1}

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K_2HPO_4; 2·0 g l^{-1} KH_2PO_4; 1·0 g l^{-1} $(NH_4)_2SO_4$; 1·1 g l^{-1} $MgSO_4.7H_2O$; 1·5 g l^{-1} agar, at pH 7·0, enriched with 0·6 g l^{-1} yeast extract and 3 g l^{-1} citric pectin (Sigma) as the sole carbon source, was used for selection of pectinolytic strains. Basal medium (BM) containing 7·0 g l^{-1} K_2HPO_4; 2·0 g l^{-1} KH_2PO_4; 1·0 g l^{-1} $(NH_4)_2SO_4$; 1·1 g l^{-1} $MgSO_4.7H_2O$; and 0·6 g l^{-1} yeast extract, at pH 7·0, was used to verify the utilization of different carbon sources to aid in the characterization of the pectinolytic isolate. For these studies, 3 g l^{-1} of each carbon source was added to the BM. To establish pectinolytic isolate vitamin requirements, yeast extract was omitted from the BM and 50 mg l^{-1} of each vitamin (thiamin, calcium pantothenate, riboflavin and pyridoxine) was added. For enzyme assays, the isolate was cultured in BM enriched with 3 g l^{-1} citric pectin (Sigma). Bacteria were grown at 25°C on a rotary shaker (150 rev min^{-1}).

Sampling and surface sterilization

Ripe cherries from coffee plant (*Coffea arabica* L.) cultivar Catuaí Vermelho, grown under field conditions, were collected in Araponga, Minas Gerais, Brazil. The samples were maintained at 4°C until processing. Individual cherry samples were pre-washed in running tap water, brushed with soapy water and rinsed thoroughly in tap water. All subsequent steps were performed aseptically. The cherries were shaken twice on a rotary shaker in sterile tap water and once in sterile 0·05 mol l^{-1} potassium phosphate buffer, pH 7·0 (PB). Cherry samples were surface-sterilized by immersion in 70% ethanol for 1 min, soaked for 5 min under vigorous shaking in 5% sodium hypochlorite containing 0·01% Tween 80, and then flamed after immersion in 70% ethanol for 30 s. Cherries were then left in sterile PB for 15 min and the sterilization steps described above were repeated. Finally, the cherry samples were rinsed four times in sterile distilled water. Surface sterility was checked by incubating each cherry sample in a test tube containing 10 ml nutrient broth for 72 h. Test tubes in which growth occurred were discarded.

Isolation and preservation of endophytes

The surface-sterilized ripe coffee cherries were opened aseptically and the coffee beans were incubated in nutrient broth for 96 h. Endophytes were isolated from tubes showing growth on nutrient agar. Bacterial isolates were randomly selected from single colonies and re-streaked until pure cultures were obtained. Individual strains were cultured for 18–24 h in nutrient broth and maintained at – 80°C on a nutrient broth/30% glycerol mix.

Pectinolytic strain characterization

Colony morphology and pigment production on nutrient agar were observed after 24 h of growth. Cell shape and spore formation were determined by Gram staining. Gram characterization was confirmed by the presence of L-alanine aminopeptidase in the cell wall (Bactident reagent, Merck). Motility was observed by the hanging-drop method (Murray *et al.* 1994). Catalase activity was determined by bubble production in a 3% (v/v) H_2O_2 solution. Oxidase production was determined by oxidation of *p*-aminodimethylaniline dihydrochloride (Bacto oxidase strips). Casein, starch, and esculin hydrolysis, nitrate reduction, phenylalanine deamination, citrate and malonate utilization, acetylmethylcarbinol and indole production, and carbohydrate fermentation, were evaluated according to Smibert and Krieg (1994). Hydrogen sulphide production was verified on triple sugar iron agar (Merck). Further physiological characterization was carried out by evaluating utilization of cellobiose, cellulose, fructose, galactose, glucose, lactose, maltose, mannose, pectin, ribose, starch, sucrose, xylose, casein or hydrolysed casein as sole carbon sources.

The pectinolytic strain was tentatively identified by phylogenetic analysis of a partial 16S rDNA sequence at Fundação André Tosello, Campinas, Brazil (http://www.fat.org.br). A 16S rDNA gene fragment was amplified by PCR with p27f and p1525r primers, homologous to conserved regions of bacterial 16S rRNA genes. Purified 16S rDNA fragments were used as sequencing templates. Sequencing was carried out using a model ALFexpress automatic DNA sequencer (Pharmacia, Uppsala, Sweden). The sequencing primers were 10f (forward) and 765f (forward), which are internal primers to the 16S rRNA gene. The partial 16S rDNA sequence was aligned with previously published 16S rRNA gene sequences obtained from the GenBank and RPD (Ribossomal Database Project) databases. Distance matrices were calculated according to the Jukes–Cantor model (Jukes and Cantor 1969). Construction of a neighbor-joining phylogenetic tree was done according to Saitou and Nei (1987).

Enzyme assay

Pectin lyase activity was determined spectrophotometrically (A_{235}), according to Albersheim (1966), in the culture supernatant fluid harvested at the end of the log phase of growth. The reaction mixture consisted of 1·0 ml 2·5% citric pectin (Sigma), apple pectin (Sigma) or polygalacturonic acid (Sigma) in 80 mmol l^{-1} phosphate buffer (pH 6·8) and 1·5 ml culture supernatant fluid. Aliquots of 0·5 ml of the reaction mixture were added to 4·5 ml 0·01N HCl at 0 and 20 min of incubation at 40°C. One unit of PL activity unit (U) was defined as nanomoles of unsaturated uronides formed per millilitre of culture supernatant fluid per minute. Polygalacturonase (PG) activity was measured spectrophotometrically (A_{540}), using polygalacturonic acid as substrate and 3,5-dinitrosalicylic acid and D(+)galacturonic acid as standards (Miller 1959). Pectate lyase (PAL) activity was determined spectrophotometrically (A_{230}) using polygalacturonic acid as substrate (Moran *et al.* 1968). The pH of maximum PL activity was determined using sodium/acetate/potassium phosphate/Tris buffer, at a final concentration of 50 mmol l^{-1}, in the pH range of 3·5–10·5. The culture supernatant fluid was dialysed (membrane MWCO 12–14 000, Spectrum, Houston, TX, USA) for pH determination, and for evaluation of EDTA and metal ion effects on PL activity.

RESULTS

Isolation and characterization of pectinolytic strain

Among the endophytic and pectinolytic bacteria isolated from ripe, healthy coffee cherries, isolate F 7–4 produced the greatest hydrolysis zone in solid medium, and was further characterized.

Isolate F 7–4 produced extracellular PL but not extracellular PAL or PG. It was a facultatively anaerobic, motile and Gram-positive bacterium. Cells were rod-shaped, with cylindrical spores in swollen sporangia. The cells were Gram-negative at early stages of growth but became Gram-positive as the culture aged. Cell-wall L-alanine aminopeptidase was absent. Colonies were flat, smooth and circular. No soluble pigment was produced on nutrient agar. Isolate F 7–4 had catalase activity but no oxidase activity. Casein, starch and esculin were hydrolysed. Nitrate was reduced to nitrite. Phenylalanine was not deaminated. Citrate and malonate were not utilized. Acetylmethylcarbinol, indole and hydrogen sulphide were not produced. Acid, but no gas, was produced from D-glucose. Isolate F 7–4 utilized cellobiose, cellulose, fructose, galactose, glucose, lactose, maltose, mannose, pectin, ribose, starch, sucrose and xylose as carbon sources. Neither casein nor hydrolysed casein was used as sole carbon source. However, the casein hydrolysis test was positive. Isolate F 7–4 required yeast extract as a growth promoter in BM when glucose or pectin was used as sole carbon source. It also showed growth when thiamin, riboflavin, calcium pantothenate or pyridoxine replaced yeast extract.

The partial 16S rDNA sequence of isolate F 7–4 showed 96% similarity to the 16S rDNA sequence of *Paenibacillus amylolyticus* and 90–95% similarity to the 16S rDNA sequences of *P. pabuli*, *P. macquariensis* and *P. lautus* strains. Phylogenetic analysis (Fig. 1) grouped isolate F 7–4 with the *P. amylolyticus* type strain (Nakamura 1984; Ash *et al.* 1994 emend; Shida *et al.* 1997) . Phenotypic characteristics were in good agreement with those described by Shida *et al.* (1997) for *P. amylolyticus*. Isolate F 7–4 has been deposited as *Paenibacillus amylolyticus* CCT7128 at Coleção de Culturas Tropical, Fundação André Tosello, Campinas, Brazil.

Pectin lyase properties

Pectin lyase from isolate F 7–4 catalysed the cleavage of pectin from different sources, but was more active with citric pectin than apple pectin and showed little activity with polygalacturonic acid (Table 1). Maximal PL activity was observed at 40°C and the enzyme was fully thermostable up to 45°C. Above this temperature, activity was quickly lost and

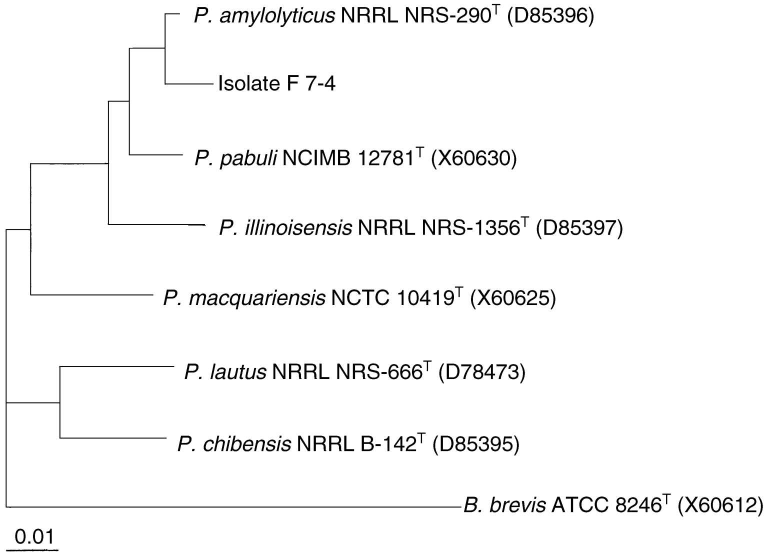

Fig. 1 Phylogenetic tree based on the alignment of 16S rRNA partial sequence of isolate F 7–4 and *Paenibacillus amylolyticus*, *P. chibensis*, *P. illinoisensis*, *P. lautus*, *P. macquariensis* and *P. pabuli*. *Brevibacillus brevis* was used as an out-group. Accession numbers between parenthesis are from GenBank database

Table 1 Substrate specificity of isolate F 7–4 pectin lyase

Substrate at 1% (w/v)*	Relative activity (%)†
Pectin (citrus)	100
Pectin (apple)	39
Polygalacturonic acid	4

*In 80 mmol l^{-1} potassium phosphate buffer, pH 6·8.
†Activity was measured using the standard assay procedure.
100% activity = 197·09 units ml $^{-1}$.

Table 2 Effect of EDTA and metal ions on isolate F 7–4 pectin lyase activity

Compound*	Final concentration (mmol l^{-1})	Relative activity† (%)
None	–	100
EDTA	2	107
$CaCL_2$	2	97
	10	97
	50	84
	100	76
Cu SO_4	2	95
$ZnSO_4$	2	95
$MgSO_4$	2	99
$MnCl_2$	2	102
$CoSO_4$	2	97
Mix*	2	95

*Each metal ion at 2 mmol l^{-1}.
†100% activity = 226·75 units ml^{-1}.

the enzyme was inactivated at 60°C. Pectin lyase activity was observed in the pH range 5·6–8·6, with optimum activity at 7·9. Addition of EDTA and metal ions to the reaction mixture had little effect on PL activity. A weak inhibitory effect was only seen in the presence of $CaCl_2$ at 50 and 100 mmol l^{-1} (Table 2). K_m and V_{max} values, calculated by double reciprocal plots (Lineweaver and Burk 1934), were 4·6 mg ml^{-1} and 94·0 10^{-8} mol min^{-1} ml^{-1}, respectively.

DISCUSSION

Endophytic bacteria have been found in a variety of tissue types within numerous plant species, as reviewed by Kobayashi and Palumbo (2000), suggesting a ubiquitous existence in most if not all higher plants. Nevertheless, there is only one report on endophytic bacteria isolated from coffee roots and stems (Jimenez-Salgado *et al.* 1997). Here, an endophytic bacterium was isolated from surface-sterilized coffee cherries for the first time.

It was not surprising that pectinolytic micro-organisms were isolated from ripe cherries, as coffee cherry mucilage consists largely of pectic substances (Wootton 1963). However, according to Tsuyumu and Chatterjee (1984), pectin lyases have been detected in various fungi, but rarely in bacteria. As far as is known, isolate F 7–4 is the first strain of *P. amylolyticus* that produces extracellular PL.

Pectin lyase produced by isolate F 7–4 showed the same substrate specificity as *Pseudomonas fluorescens* (Schlemmer *et al.* 1987). Enzyme thermostability, the effect of temperature on PL activity, and pH of maximal PL activity, were similar to results reported for other bacterial and fungal PLs (Itoh *et al.* 1982; Parini *et al.* 1988; Silva *et al.* 1993). However, unlike *Bacillus* spp. (Kashyap *et al.* 2000), isolate F 7–4 PL was not activated by calcium ions. The K_m value for isolate F 7–4 PL is not significantly different from that reported for pectin lyase from *Ps. fluorescens* (3·2 mg ml^{-1}) (Schlemmer *et al.* 1987).

Pectin lyase was produced by isolate F 7–4 after 18 h of growth on a simple mineral medium, with only 0·3% pectin as inducer and carbon source. The enzyme has potential for use in studies on plant tissue maceration, natural fibre de-gumming and fruit juice wastewater treatment. The isolate is a promising tool for PL regulation studies, as pectinases and cellulases of some bacterial endophytes are induced for penetration into host plants but never after internal tissue colonization, as reviewed by Hallman *et al.* (1997).

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