

ORIGINAL ARTICLE

Improved pectinase production in *Penicillium griseoroseum* recombinant strains

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Abstract**Aims:** To obtain recombinant strains of *Penicillium griseoroseum* that produce high levels of pectin lyase (PL) and polygalacturonase (PG) simultaneously.**Methods and Results:** A strain with high production of PL was transformed with the plasmid pAN52p_{gpg2}, containing the gene encoding PG of *P. griseoroseum*, under control of the *gpd* promoter gene from *Aspergillus nidulans*. Southern blot analysis demonstrated that all strain had at least one copy of pAN52p_{gpg2} integrated into the genome. The recombinant strain *P. griseoroseum* T20 produced levels of PL and PG that were 266- and 27-fold greater, respectively, than the wild-type strain. Furthermore, the extracellular protein profile of recombinant T20 showed two protein bands of c. 36 and 38 kDa, associated with PL and PG, respectively.**Conclusions:** This recombinant strain T20 produces PL and PG using carbon sources of low costs, and an enzyme preparation that is free of cellulolytic and proteolytic activities.**Significance and Impact of the Study:** PL and PG play an important role in the degradation of pectin. Owing to their use in the juice and wines industries, there is a growing interest in the inexpensive production of these enzymes. This work describes an efficient system of protein expression and secretion using the fungus *P. griseoroseum*.**Introduction**

Pectin lyase (PL) EC 4.2.2.10 and polygalacturonase (PG) EC 3.2.1.15 are pectinolytic enzymes involved in depolymerizing pectin, one of the carbohydrates present in the plant cell wall. These enzymes act by cleaving the α -1,4-glycosidic links of polygalacturonic acid and pectic acid, respectively (Vries and Visser 2001; Yadav *et al.* 2009). Pectic enzymes are relevant to industrial use, comprise about 10% of the world's enzyme preparation production and may be employed in the steps of preparing fruit pulp as maceration or extraction of juice and in clarifying wines and juices, among other applications in the food industries (Kashyap *et al.* 2001; Jayani *et al.* 2005).

The production of enzymes for industrial interest has been conducted efficiently and safely with genetically modified fungal strains, such as *Aspergillus* spp. (Bo *et al.* 2003; Panagiotou *et al.* 2008; Matsushita-Morita *et al.*

2010). The fungus *Penicillium griseoroseum* CCT6421, isolated at the Universidade Federal de Viçosa, has shown promise for the production of pectinases (Baracat *et al.* 1989; Pereira *et al.* 2002).

In this context, our group isolated and characterized two genes encoding PL (*plg1* and *plg2*) and two genes encoding PG (*p_{gpg2}* and *p_{gpg1}*) in *P. griseoroseum* (Ribon *et al.* 2002a,b; Bazzolli *et al.* 2006) and studied their regulation (Bazzolli *et al.* 2008; Ribon *et al.* 2009). Genes *plg1* and *p_{gpg2}* are single copies in the genome of *P. griseoroseum* and make the greatest contribution to the production of PL and PG. Nevertheless, both genes encoding PL and PG in *P. griseoroseum* are naturally regulated at the transcriptional level and are induced by pectin and repressed by glucose.

Improvements at the level of gene expression could be achieved by replacing the regulator signals of the genes of interest by sequences from highly expressed genes (Kolar

et al. 1988; Belshaw *et al.* 2002; Nevalainen *et al.* 2005). An instance of successful application of this strategy was the cloning of the gene SGFP-TYG coding for the green fluorescent protein of *Aequorea victoria* into the pAN52-1-GFP under the control of *gpdA* promoter region and its subsequent efficient expression in *P. griseoroseum* (Lopes *et al.* 2004).

In this work, we aim to obtain recombinant strains of *P. griseoroseum* to produce PL and PG in high concentrations with no need for induction by pectin. Additionally, we evaluated culture conditions using cost-efficient carbon sources such as commercial sucrose and sugar cane juice. The crude enzyme preparation was produced without the production of proteases or cellulases, which are undesirable in textile and food industries.

Materials and methods

Microorganisms and plasmids

The strains used in this work were wild-type *P. griseoroseum* CCT6421, isolated at the Universidade Federal de Viçosa, the mutant strain *P. griseoroseum* PG63 *niaD*, obtained from Pereira *et al.* (2004), and the recombinant strains *P. griseoroseum* T105 and T146, which overproduce PL (Cardoso *et al.* 2008) and PG (Araújo *et al.* 2007), respectively, were used. For cotransformation of the recombinant strain T105, pAN7.1, which contains the *hph* gene (Punt *et al.* 1987), and pAN52*pgg2*, which contains the *pgg2* gene under control of the *gpd* promoter gene and terminator region of the *trpC* gene from *Aspergillus nidulans* were used (Araújo *et al.* 2007). The recombinant strains *P. griseoroseum* T105 and *P. griseoroseum* T146 were used as controls for the production of PL and PG, respectively.

Fungal transformation

The transformation was based on the methodology established by Queiroz *et al.* (1998). Production of protoplasts was based on the method of Dias *et al.* (1999) with modifications. The recombinant strain *P. griseoroseum* T105 was cultured on solid complete medium for 18–20 h, and 800 mg of its mycelium was added to 5 ml of 10 mmol l⁻¹ phosphate buffer containing 0.6 mol l⁻¹ KCl and 30 mg of 'Lysing Enzymes' L1412 (Sigma, St Louis, MO). The mixture was incubated at 80 rev min⁻¹ and 30°C for 3–4 h. The protoplasts were filtered through sterile gauze and washed three times in 1 mol l⁻¹ Sorbitol, 100 mmol l⁻¹ Tris-HCl and 50 mmol l⁻¹ CaCl₂ (STC), by centrifugation at 2300 g for 5 min. The sediment was resuspended in STC to obtain 10⁷ protoplasts per ml. Then, a mixture of 5 µg of plasmid DNA pAN7.1 and 5 µg of plasmid DNA pAN52*pgg2* were prepared in 200 ml of

solution 10⁷ protoplasts per ml and 50 µl of 50% (w/v) polyethylene glycol (PEG) 6000. The mixture was incubated on ice for 20 min, and 0.5 ml of 50% (w/v) PEG solution and kept at 25°C for 20 min. The mixture was plated on minimal medium with 0.5 mol l⁻¹ sucrose and incubated at 25°C for 24 h. After 24 h, 5 ml of a minimal semi-solid medium containing 200 mg ml⁻¹ of hygromycin B was added to the mixture at plate and incubated at 25°C for more 5 days. These transformants were genetically stable, after five successive transfers to a nonselective medium.

Growing conditions

To maintain the cultures and obtain conidia, the fungal strains were cultured on solid complete medium containing 6.0 g l⁻¹ NaNO₃, 1.52 g l⁻¹ KH₂PO₄, 0.52 g l⁻¹ MgSO₄·7H₂O, 0.52 g l⁻¹ KCl, 0.01 g l⁻¹ FeSO₄, 0.01 g l⁻¹ ZnCl₄, 10.0 g l⁻¹ glucose, 2.0 g l⁻¹ peptone, 1.5 g l⁻¹ hydrolysed casein, 2.0 g l⁻¹ yeast extract, 15.0 g l⁻¹ agar and 1.0 ml of vitamins solution (Pontecorvo *et al.* 1953) at 25°C for 7 days. The inoculum was prepared in a sterile solution containing 0.2% (v/v) Tween-80.

The culture filtrate was obtained by cultivating 10⁶ conidia per ml of wild-type *P. griseoroseum*, PG63 and recombinant strains in mineral-buffered minimal medium containing 6.98 g l⁻¹ K₂HPO₄, 5.44 g l⁻¹ KH₂PO₄, 1.0 g l⁻¹ (NH₄)₂SO₄, pH 6.8, supplemented with 1.1 g l⁻¹ MgSO₄·7H₂O. In each experiment, the buffered mineral medium was supplemented with different carbon sources and inducers, such as 10 g l⁻¹ glucose, 10 g l⁻¹ sucrose, 10 g l⁻¹ sucrose and 0.6 g l⁻¹ yeast extract; 10 g l⁻¹ commercial sucrose; 10 g l⁻¹ sugar cane juice and 3.0 g l⁻¹ citrus pectin (P-9135) (Sigma) and 0.6 g l⁻¹ yeast extract in a volume of 50 ml in 125 ml Erlenmeyer flask. Fungi were grown at 25°C in a rotary shaker at 150 rev min⁻¹ for 24, 48, 72, 96 and 120 h. The culture filtrates were used to directly estimate PG and PL activities, and the mycelia were collected to quantify total mycelial dry mass. The sucrose content of 178 g l⁻¹ sugar cane juice was determined by an enzymatic UV-method (Boehringer Mannheim, Mannheim, Germany), and the culture medium was adjusted for 10 g l⁻¹ of this carbon source.

DNA extraction and Southern blot analysis

To evaluate the pattern of integration of the pAN52*pgg2*, the genomic DNA was extracted, digested and probed. Total DNA from mycelium of wild-type *P. griseoroseum* PG63 and the recombinant strains obtained here was extracted, according to Specht *et al.* 1982, with modifications. The mycelium was ground in liquid nitrogen and transferred to an Eppendorf tube containing 400 µl of

extraction buffer (50 mmol l⁻¹ Tris pH 8.0, 20 mmol l⁻¹ EDTA pH 8.0, 250 mmol l⁻¹ NaCl, 1% SDS), which was incubated at 70°C for 30 min. Three hundred and fifty microlitres of 5 mol l⁻¹ potassium acetate was added to mixture and incubated in an ice bath for 30 min. The mixture was centrifuged (10 000 g/10 min), and the supernatant was transferred to a new tube. A half volume of phenol–chloroform was added under slight agitation, in the ice bath and then centrifuged (13 000 g/5 min). The upper layer was transferred to a new tube and added to half its volume of isopropanol then incubated at -20°C for 2 h. After the mixture was centrifuged at 13 000 g for 5 min and the nucleic acid washed with 70% ethanol, it was dried at room temperature and resuspended in autoclaved milliQ water. Two microlitres of RNase was added (10 mg ml⁻¹ stock concentration) and incubated at 37°C for 30 min.

Total DNA was cleaved with the restriction enzyme *EcoRI* to analyse the integration of the *pgg2* and *plg1* genes. *EcoRI* does not cleave the coding region of either gene *pgg2* or gene *plg1*. DNA fragments were separated on 0.8% gel, which was then treated with denaturing (1.5 mol l⁻¹ NaCl, 0.5 mol l⁻¹ NaOH) and neutralization (0.5 mol l⁻¹ Tris–HCl pH 7.0, 1.5 mol l⁻¹ NaCl) solutions for 1 h, followed by DNA transfer to a nylon membrane (Duralon-UV™; Stratagene, La Jolla, CA). The hybridization (Southern 1975) occurred for 18 h at 65°C. The membrane was washed twice with 2× SSC and 0.1% SSC for 20 min and twice with 1× SSC and 0.1% SDS for 10 min at 65°C. The Illuminator™ Non-radioactive Detection System kit (Stratagene) was used for detection. The probes containing the coding regions of the gene *pgg2* and gene *plg1* were marked using the 'Gene Images random prime labeling module' kit from Amersham Pharmacia Biotech (São Paulo, Brazil).

Enzymatic trials of PL, PG, protease and cellulase

PL activity was determined in the culture filtrates as described by Albersheim and Killias (1962) with some modifications. The reaction mixture was set by adding 1.5 ml of the culture filtrate to 1 ml 2.5% (w/v) citric pectin P-9135 (Sigma) in 50 mmol l⁻¹ phosphate buffer, pH 6.8. After incubating for 30 min at 40°C, 0.5 ml of the reaction mixture was added to 4.5 ml of 0.01 mol l⁻¹ HCl, and it was measured at 235 nm. One unit of PL (U) activity was defined as the nmoles of unsaturated products ($\Delta^{4,5}$ -D-galacturonate) produced per minute. The molar extinction coefficient of the unsaturated product was $\epsilon = 5550 \text{ mol l}^{-1} \text{ cm}^{-1}$ (Albersheim 1966).

PG activity was determined in a reaction mixture containing 0.5 ml of the culture filtrate and 1.5 ml substrate solution (100 mmol l⁻¹ sodium acetate buffer,

pH 4.8, 2.5 mol l⁻¹ NaCl, 1.0% (w/v) polygalacturonic acid P-3889 – Sigma). The reaction was incubated for 20 min at 40°C. Reducing sugars released were measured using the method reported by Miller (1959).

Protease activity was measured by the azocasein assay described by Rajmohan *et al.* (2002) with modifications. One hundred microlitres of each culture filtrate was added to 0.5 ml of reaction mixture containing 0.5% (w/v) azocasein in 0.1 mol l⁻¹ citrate buffer, pH 6.0. After incubation at 37°C for 2 h, the reaction was stopped by adding one volume of 10% (w/v) trichloroacetic acid and placing it on ice for 15 min. The samples were clarified by centrifugation at 5000 g for 10 min, and the absorbance of the supernatant was measured at 366 nm. Protease P-5147 (Sigma) was used as a standard.

Cellulase activity in the culture filtrates was determined using a reaction mixture consisting of 4.0 ml of culture filtrate and a cellulose strip (3 × 1 cm) and incubating for 22 h at 40°C. Release of reducing sugars was measured using the method described by Miller (1959).

Determination of total protein and SDS-polyacrylamide gel electrophoresis

To investigate the PG and PL production, the culture filtrates of strains were size fractionated by SDS-PAGE. Quantifying total protein was performed using the method described by Bradford (1976), using bovine serum albumin to construct the standard curve. The extracellular protein profile of the strains was analysed by electrophoresis on a denaturing polyacrylamide gel (SDS-PAGE). The separation gel (12.5% acrylamide) and stacker gel (4% acrylamide) were made as described by Laemmli (1970). Samples were denatured in buffer (60 mmol l⁻¹ Tris–HCl pH 6.8, 10% glycerol, 2.3% SDS, 2% β -mercaptoethanol, 1% bromophenol blue) at 100°C for 10 min. The gel was run at 60 V for 4 h in buffer (72 mmol l⁻¹ Tris–HCl pH 8.5, 576 mmol l⁻¹ glycine, 0.24% SDS), stained for 2 h (using 45% methanol, 9% acetic acid, 0.1% Coomassie Brilliant Blue R-250) and bleached (7.5% acetic acid, 25% methanol) for 12 h. The samples were concentrated to 5 μg of total protein, using three volumes of acetone per volume of sample. After 12 h, samples were centrifuged at 10 000 g, and the precipitate was resuspended in 50 ml of autoclaved deionized water.

Results

Overexpression of *plg1* and *pgg2* genes in recombinant strains *Penicillium griseoroseum*

Five hundred and twenty-five transformants were selected from a transformation efficiency of 105 transformants per microgram of plasmid DNA pAN7.1. Of these, 200 strains

were evaluated for the production of PG and PL in liquid buffered mineral medium. Among these transformants, 16 strains showed high production of PG and PL and were tested for mitotic stability. Then, four strains were selected for further analysis (Table 1). The strains T10 and T20 showed the highest production of PG and PL, as evaluated by analysis of means according to Tukey's test using a significance level of 5%. Strain T20 was selected for use in different culture conditions for production of PG and PL. Strains wild *P. griseoroseum* PG63 and recombinants T105 and T146 were used as controls.

To confirm that the increased production of PG and PL was because of the integration of expression vectors used in cotransformation, the DNA of these strains was digested with *EcoRI* and hybridized with the respective coding regions of genes *pgg2* and *plg1*, as shown in Fig. 1. All recombinant strains contained at least one ectopic integration of pAN52pgg2. The strain *P. griseoroseum* T105 was used for analysis of gene integrations *plg1* (Fig. 1a) because the other recombinant strains were derived from this strain (with the exception of recombinant strain T146).

Production of PL and PG by recombinant strain *Penicillium griseoroseum* T20

Although T20 does not require induction for production of PL and PG, the production of these enzymes was assessed in response to carbon sources other than pectin. Wild-type *P. griseoroseum* and PG63 were used as a control. The highest activities of PL ($3485 \pm 171 \text{ U ml}^{-1}$) and PG ($15\,065 \pm 1280 \text{ U ml}^{-1}$) were observed when using sugar cane juice or sucrose (Table 2). The supernatant of T20 showed PG activity 27 times greater than the activity of PG detected in the supernatant of wild-type

P. griseoroseum when cultured in 10 g l^{-1} sucrose. PL activity in the supernatant from strain T20 was 266 times greater than the PL activity of the supernatant of the wild-type strain when cultured in 10 g l^{-1} sugar cane juice. Furthermore, the secretion of extracellular proteins was efficient, producing about 10 mg total protein per litre of culture medium for the recombinant strain T20 when cultured on sucrose for a period of 48 h (Table 2).

Strains PG63 and T20 were cultured for a period of 120 h, and the culture supernatants were evaluated for cellulase and protease activity. The strains did not show protease or total cellulase activities under the conditions evaluated (data not shown). The study also showed an increase in total protein concentration during the culture period, which was observed for all strains. Strain T20 had a production of about $17.8 \mu\text{g ml}^{-1}$ total protein after 120 h of culture, while strain PG63 produced $9.4 \mu\text{g ml}^{-1}$ (Table 3).

To check the expression of PL and PG enzymes produced by strain T20, the protein profile was assessed by SDS-PAGE. Figure 2 shows that the protein profile of this strain included two clear bands, one *c.* 38 kDa and another *c.* 36 kDa. The 38-kDa band was associated with PG and also detected in the culture supernatant of recombinant strain T146. The 36-kDa band was associated with PL and detected in the culture supernatant of recombinant strain T105. The recombinant strains T105 and T146 were used as control for production of PL and PG, respectively.

Discussion

Previous experiments have shown that the efficiency of transformation varies widely and seems to be dependent on both the organism and the conditions used. This

Table 1 Activity of polygalacturonase (PG) and pectin lyase (PL) and dry mycelia mass of wild-type *Penicillium griseoroseum*, PG63 and recombinants, after a 48-h culture. Results represent arithmetic means \pm standard deviation of three repetitions

Strains	PG activity (U ml^{-1})	PL activity (U ml^{-1})	Dry mycelial mass (g l^{-1})
<i>P. griseoroseum</i> ^P	167 ± 30.30	77.60 ± 13.80	1.60 ± 0.00
PG63 ^P	186 ± 58.10	90.01 ± 11.10	1.50 ± 0.40
<i>P. griseoroseum</i> ^G	102 ± 81.82	3.94 ± 1.80	6.61 ± 2.29
PG63 ^G	104 ± 27.89	3.24 ± 1.90	4.59 ± 0.90
T105 ^G	139 ± 92.97	1543.54 ± 179.40	5.47 ± 0.41
T146 ^G	2200 ± 165.58	4.17 ± 3.50	4.97 ± 1.92
T07* ^G	3241 ± 652.12^{ab}	1961.23 ± 812.35^{bc}	5.00 ± 2.32
T10* ^G	3490 ± 380.18^a	2292.35 ± 681.61^{ab}	5.64 ± 1.60
T20* ^G	3286 ± 295.96^a	2981.58 ± 820.43^a	6.18 ± 2.38
T33* ^G	2790 ± 921.36^{ab}	998.84 ± 381.98^c	5.14 ± 1.24

^{a,b,c}Values followed by the same letters do not differ significantly according to Tukey's test ($\alpha = 0.05$).

^PCulture medium containing 3.0 g l^{-1} citric pectin and 0.6 g l^{-1} yeast extract.

^GCulture medium containing 10 g l^{-1} glucose.

*Recombinant strains derived from recombinant strain *P. griseoroseum* T105.

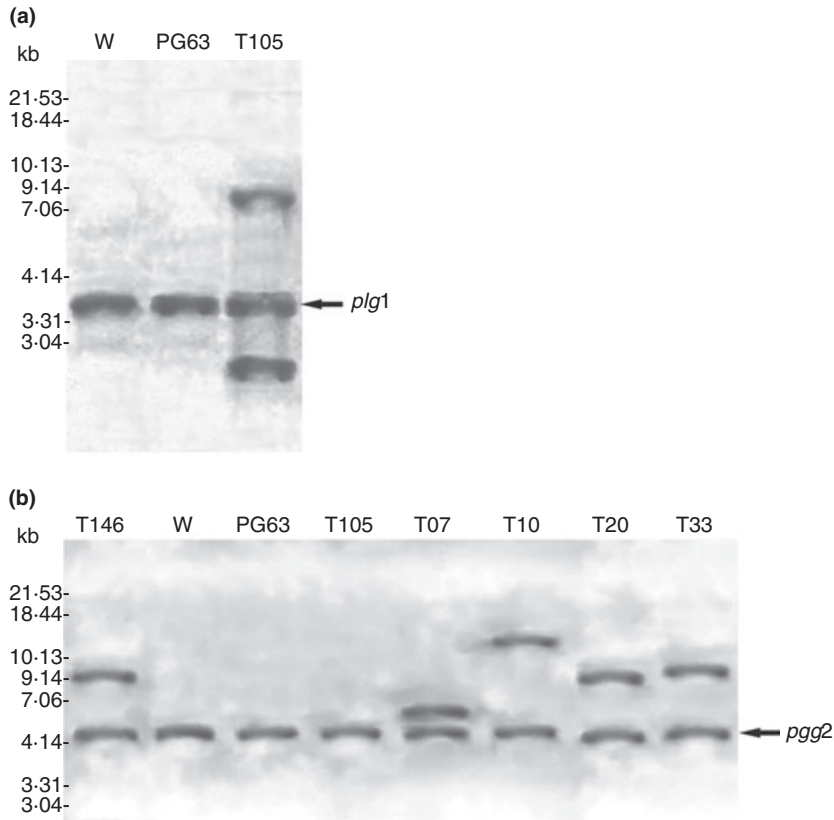


Figure 1 Analysis of copy number the genes *plg1* and *pgg2* for recombinant strains. Autoradiography of total DNA hybridization of recombinant strains (T), *Penicillium griseoroseum* wild type (W) and PG63. Total DNA was digested with *EcoRI* enzyme and hybridized with the DNA fragment corresponding to the coding region of *plg1* (a) and *pgg2* (b) of *P. griseoroseum*. The bands correspond to the copy of the gene *plg1* (a) and gene *pgg2* (b) under control of the endogenous promoter (arrows). The other bands correspond to additional copies of genes *plg1* and *pgg2* under control of the *gpd* promoter of *Aspergillus nidulans*.

paper describes an efficiency of transformation of 105 transformants per microgram of integrative plasmid DNA pAN7.1. Such a high efficiency of transformation was similar to that reported by Kolar *et al.* (1988) using *Penicillium chrysogenum* and by Guo *et al.* (2009) using *Tremella fuciformis*. The high efficiency of transformation is directly related to the type of plasmid, integrative or autonomous replication and can be improved with knowledge of how different types of plasmids behave in the recipient cell. In various studies with microorganisms aiming to produce a secondary metabolite or protein, vector integration was the common choice, as it increased the stability of the changes to the genome (Van den Hombergh *et al.* 1997; Belshaw *et al.* 2002; Daly and Hearn 2005). When genetically modified strains are desired in industry, it is preferred that strains producing the protein of interest are mitotically stable. In such cases, the use of integrative plasmids is recommended. Our results show heterologous integrations of at least one copy of the genes *plg1* and *pgg2*, both under control of the *gpd* promoter of *A. nidulans*, corresponding to the integration of pAN52*plg1* (Cardoso *et al.* 2008) and pAN52*pgg2* (Araújo *et al.* 2007), respectively. The recombinants showed a 27-fold increase in the production of PG and a 266-fold increase in the production of

PL compared with wild-type *P. griseoroseum*, when cultured with carbon sources other than pectin.

Alternative carbon sources, such as sugar cane juice, have been assayed with the object of reducing the production costs of microbial PGs and PLs. The approach of pectinase production, using carbon sources of low cost and other substances to replace the natural inducer, pectin, has been used by several researchers (Brumano *et al.* 1993; Baracat-Pereira *et al.* 1994; Minussi *et al.* 1996; Fawole and Odunfa 2003; Patil and Dayanand 2006). However, these authors had little success, because the genes encoding pectinases in filamentous fungi undergo catabolite repression by simple sugars such as glucose and sucrose (Kusters-Van Someren *et al.* 1991; Bazzolli *et al.* 2006; Trigui-Lahiani and Gargouri 2007). The replacement of the endogenous promoter of genes encoding pectinases in *P. griseoroseum* by a strong and constitutive promoter, such as the promoter of the *gpd* gene of *A. nidulans*, increased production of PG and PL, using carbon sources such as sucrose and sugar cane juice (see Table 2). For the recombinant strains, all the alternative carbon sources provided the highest PL and PG activity that pectin, and also highest mycelial dry mass (data not shown). Given the high production of sugar cane and its processing technology, the use of this carbon

Table 2 Activity of pectin lyase (PL) and polygalacturonase (PG), and total protein of wild-type *Penicillium griseoroseum*, PG63 and recombinants T146, T105 and T20 cultured with different carbon sources for a period of 48 h. Results represent arithmetic mean ± standard deviation of three experimental repetitions

	PL activity (U ml ⁻¹)			PG activity (U ml ⁻¹)			Total protein (mg l ⁻¹)								
	W	PG63	T146	T105	T20	W	PG63	T146	T105	T20	W	PG63	T146	T105	T20
Glucose	4.7 ± 1.8	3.2 ± 1.9	6.6 ± 2.5	13466 ± 190.9	13610 ± 29.5	137 ± 30	186 ± 58	3228 ± 827	191 ± 28	6528 ± 1750	1.4 ± 0.4	1.4 ± 0.5	5.4 ± 1.3	6.2 ± 0.5	5.8 ± 0.3
Sucrose	7.7 ± 4.0	14.7 ± 0.5	23.0 ± 4.7	1946.1 ± 125.9	1672.1 ± 393.7	392 ± 150	353 ± 101	9023 ± 2723	357 ± 166	10 567 ± 5093	3.7 ± 0.7	2.2 ± 0.9	6.7 ± 0.1	6.3 ± 0.6	6.6 ± 1.1
Commercial sucrose	3.4 ± 2.5	13.4 ± 2.7	16.6 ± 4.5	1967.4 ± 70.6	1445.6 ± 283.2	407 ± 114	360 ± 71	11 341 ± 4985	341 ± 184	9256 ± 4048	3.7 ± 2.09	2.6 ± 1.9	5.3 ± 2.8	6.4 ± 1.7	7.6 ± 1.1
Sucrose + YE	15.9 ± 6.0	52.3 ± 4.6	28.2 ± 1.2	11259 ± 281.1	2571.2 ± 197.8	580 ± 143	519 ± 152	7878 ± 562	546 ± 36	8423 ± 483	6.0 ± 3.0	3.5 ± 2.3	10.9 ± 0.2	9.4 ± 0.3	10.8 ± 1.0
Sugar cane juice	13.1 ± 3.5	35.3 ± 3.7	35.7 ± 3.1	2880.7 ± 177.7	3485.5 ± 171.1	1313 ± 150	1304 ± 293	16 730 ± 7144	2188 ± 465	15 065 ± 1280	5.5 ± 0.5	4.2 ± 2.7	8.4 ± 0.2	8.2 ± 1.9	10.1 ± 0.8
Pectin + YE	77.6 ± 138	90.0 ± 11.1	67.2 ± 4.3	465.7 ± 126.3	576.8 ± 198.9	1349 ± 93	1389 ± 221	2628 ± 195	1306 ± 77	2802 ± 383	1.1 ± 2.7	1.0 ± 0.6	5.2 ± 0.1	4.4 ± 0.2	4.9 ± 0.4

W, wild-type *P. griseoroseum*; YE, 0.6 g l⁻¹ yeast extract.

Table 3 Total protein of strains PG63 and recombinants T20 cultured for a period of 120 h. Results represent arithmetic mean ± standard deviation of three experimental repetitions

Culture period (h)	Total protein (μg ml ⁻¹)	
	PG63	T20
24	ND	0.32 ± 0.58
48	0.02 ± 0.58	5.75 ± 0.30
72	3.49 ± 0.54	11.02 ± 1.38
96	7.90 ± 3.16	14.25 ± 3.56
120	9.38 ± 0.23	17.80 ± 3.24

PG63 = cultured in 3.0 g l⁻¹ citric pectin supplemented with 0.6 g l⁻¹ yeast extract; T20 = cultured in 10 g l⁻¹ glucose.

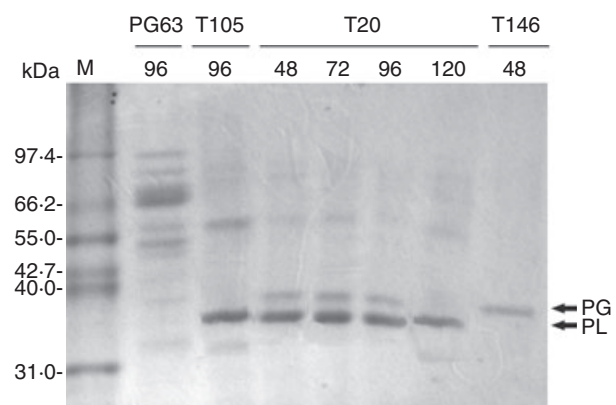


Figure 2 Extracellular protein profile of strain PG63 and recombinant strains T105, T20 and T146 after varying hours of culture. PG63 (96 h of culture) and recombinant T105 (96 h of culture), recombinant T20 (48–120 h of culture) and T146 (48 h of culture) by SDS-PAGE. Supernatants of cultures were concentrated and applied to a 12.5% denaturing polyacrylamide gel. The gel was stained with Coomassie Blue R-250. M – mean protein molecular weight marker of Promega (3.5 μg; Promega, Madison, WI, USA). The arrows indicate the position of the proteins PG – polygalacturonase (c. 38 kDa) and PL – pectin lyase (c. 36 kDa).

source as substrate for microbial enzyme production shows great potential and low cost. Replacing the regulatory region eliminates the need for induction and catabolite repression, which has been considered an obstacle to the production of pectinases when using alternative sources of carbon. This is interesting because the recombinant strain T20 can be cultured under repressive conditions for wild-type pectinase and, therefore, only synthesizes PL and PG instead of other pectinases, such as pectinesterase, which are undesirable in the juice industry. Furthermore, the amount of total protein produced was higher when sucrose or sugar cane juice was used (Table 2).

Our preliminary data suggest that once synthesis of PG and PL protein by strain T20 has occurred, these proteins are stable in the culture supernatant for a period of

120 h. In the textile industry, the presence of cellulases may weaken fibres, and the presence of proteases can reduce the stability of pectinase during the extraction and clarification processes of juices and wines (Punt *et al.* 2002; Wang *et al.* 2005; Cardoso *et al.* 2010). Therefore, an optimal enzyme preparation for use in the textile and food industries would have little or no cellulases or proteases. T20 does not produce cellulases or proteases under conditions of pectinase production, thus resulting in a purer product for application in industry.

In addition to the above features of T20, our research group further evaluated that the PL and PG enzymes encoded by genes *plg1* and *pgg2* work in a wide pH range, have high thermal stability and can be stored for a period of at least 2 months without loss of enzyme activity. These features indicate that the PL and PG enzymes produced by recombinant strain T20 are promising for applications in industrial processes of clarifying fruit juices with acidic pH, such as tomato, orange, apple and mango. Take together, our data suggested a potential application of these recombinant strains to the production of pectinases for use in the food industry; besides this, the fungus *P. griseoroseum* represents an efficient system of protein expression and secretion for use as host strains.

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