J Ind Microbiol Biotechnol (2008) 35:159–166
DOI 10.1007/s10295-007-0277-6

ORIGINAL PAPER

Overexpression of the *plg*1 gene encoding pectin lyase in *Penicillium griseoroseum*

Patrícia Gomes Cardoso · João Batista Ribeiro · Janaina Aparecida Teixeira · Marisa Vieira de Queiroz · Elza Fernandes de Araújo

Received: 1 July 2007 / Accepted: 27 October 2007 / Published online: 21 November 2007
© Society for Industrial Microbiology 2007

Abstract The pectin lyase (PL) is an industrially important enzyme since it is used for maceration and clarification in the process of fruit juice production in food industries. In order to increase the yields of pectin lyase we cloned the *plg*1 (pectin lyase 1) from *Penicillium griseoroseum* gene under the control of the strong constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*A) and the terminator region of the tryptophan synthetase (*trp*C) gene from *Aspergillus nidulans* (plasmid pAN52-Plg1) and transformed this construct into the *P. griseoroseum* strain PG63. One of the pAN52-Plg1 multi-copy transformants (strain 105) grown in culture medium containing glucose or sugar cane juice showed PL activities of 4,804 or 5,202 U ml^{-1} respectively, which represented 57- and 132-fold increases. In addition, the apparent specific activity of PL produced by this strain was much higher than the one observed for a commercial pectinase preparation. Evaluation of the extracellular proteins in the culture supernatant of strain 105 by SDS-PAGE showed the presence of a clear and strong band of approximately 40 kDa that probably corresponds to PL. The enzyme yields reported here demonstrate that the system we developed is able to express pectin lyase at levels comparable to, or exceeding, previously reported data.

P. G. Cardoso
Departamento de Biologia/Setor de Microbiologia,
Universidade Federal de Lavras, Lavras-MG, Brazil

J. B. Ribeiro
EMBRAPA Suínos e Aves, Concórdia-SC, Brazil

J. A. Teixeira · M. V. de Queiroz · E. F. de Araújo (✉)
Departamento de Microbiologia/BIOAGRO,
Universidade Federal de Viçosa,
Viçosa-MG CEP 36570-000, Brazil
e-mail: ezfa@ufv.br

Keywords *Penicillium griseoroseum* · Pectin lyase genes · Transformation · Overexpression · *Gpd* promoter

Introduction

Pectinases are a group of enzymes that hydrolyze pectin by different mechanisms and are divided into two broad classes: pectinesterases and depolymerases. The pectinesterases remove methoxy groups from methylated galacturonides. The depolymerases catalyze the cleavage of glycosidic bonds via hydrolysis (hydrolases) or via β-elimination (lyases) [42]. These enzymes are widely applied in fruit juice industries, in the processing of ramie, hemp, flax and jute fibers, tea, coffee, oil extraction, treatment of industrial wastewater, and in the making of paper [18, 19]. Pectin lyase is the most important enzyme involved in pectin depolymerization, since it is the only enzyme capable of cleaving the internal glycosidic bonds of highly methylated pectin, such as fruit pectin, without the prior action of other enzymes [1]. Thus, the development of PL overproduction systems is of great importance for the fruit juice industry.

Fungi of the genus *Penicillium* are of significant industrial importance and species of this genus are among the microorganisms able to produce the enzymatic complex involved in pectin degradation [35]. *Penicillium griseoroseum* CCT6421 has proven to be a good pectinase producer [4]. Multiple forms of pectin lyases are produced by filamentous fungi and several pectin lyase genes have been isolated and characterized, which constitute gene families in many fungal species, such as, *Aspergillus niger* [14, 15, 23, 24], *Aspergillus oryzae* [20, 21], and *P. griseoroseum* [6]. Two pectin lyase-encoding genes (*plg1* and *plg2*) from *P. griseoroseum* CCT6421 were isolated, characterized,

and their expression regulation evaluated by northern hybridization and RT-PCR analyzes. Both genes are regulated at transcriptional level, being induced by pectin and substantially repressed by glucose [6]. The requisite of induction by pectin for the expression of PL genes constitutes a major limitation to the enzymatic production process at industrial level due to the high cost of this substrate. However, addition of small amounts of yeast extract was proven to be effective for PL production in *P. griseoroseum* cultivated on sucrose [5].

Even though traditional genetic improvement strategies such as mutagenesis [10] and parasexual processes [40] have been shown to improve pectinase production for industrial purposes in several species of *Aspergillus* [8, 25], they have failed to work satisfactorily in *Penicillium expansum* and *P. griseoroseum*. Nevertheless, 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 [29, 39, 41]. Gene expression controlled by *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (GPD) and tryptophan synthetase expression signals has been accomplished in *Penicillium chrysogenum* [22], *Trichoderma reesei* [30], *Metarhizium anisopliae* [16], *Acremonium chrysogenum*, and *Sordaria macrospora* [32] among other filamentous ascomycetes.

Another example 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 plasmid pAN52-1-GFP under the control of *gpdA* promoter region and its subsequent efficient expression in *P. griseoroseum* [26]. This result opens new possibilities for more in-depth studies of the biological processes involved in the production and secretion of pectinases in this organism. For this purpose, our group has developed a specific strain (PG63) and efficient transformation systems for *P. griseoroseum* [31, 36].

With the aim of obtaining strains capable of overexpressing pectin lyase, we genetically modified *P. griseoroseum* PG63, a spontaneous mutant for the nitrate reductase gene, by introducing additional copies of *plg*1 under the control of its own promoter sequence as well as by inserting the *plg*1 gene controlled by *gpd*A promoter from *A. nidulans*. Both types of transformant strains were characterized with regards to PL activity and recombinant strains producing up to 132 times higher PL activity than the one observed for the control strain in the same conditions were observed.

Materials and methods

Strains, plasmids and growth conditions

Penicillium griseoroseum CCT6421 was isolated from forest trees at the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. Discs of mycelia cultivated on minimal medium were kept in water at 4°C for long-term storage. Initial inoculum was prepared from 5 day-old cultures grown at 25°C on minimal medium (MM) or complete medium (CM) [33]. The carbon sources tested for PL production were 1% (w/v) glucose (transformant strains), 1% (w/v) sucrose supplemented with 0.06% (w/v) yeast extract, 0.3% (w/v) citric pectin P-9135 (Sigma®) (PG63 strain), and sugar cane juice (both type of strains). The sucrose content of sugar cane juice of 17.8% (w/v) was determined by an enzymatic UV-method (Boehringer Mannheim, Mannheim®) and the culture medium was adjusted to contain 1% of this carbon source. *P. griseoroseum nia*D mutant (PG63) and plasmid pNPG1, containing the nitrate reductase (*nia*D) gene of *P. griseoroseum* as selective marker were developed by Pereira et al. [31]. Plasmid pPlg1, containing the complete sequence of *plg*1 gene from *P. griseoroseum* was constructed by Bazzolli et al. [6]. Plasmid pAN52-1-GFP carrying the SGFP-TYG version of the green fluorescent protein (GFP) under control of the *A. nidulans gpd*A promoter was kindly provided by Dr. Corinne Clavé, of the *Institut de Biochimie et de Genetique Cellulaires*, Bordeaux, France. Bacterial transformation was carried out using the method proposed by Inoue et al. [17]. Plasmid DNA isolation was performed as described by Sambrook et al. [37].

Construction of plasmid pAN52-Plg1

Plasmid pAN52-Plg1 was obtained by replacing the coding region of the GFP gene in vector pAN52-1-GFP by the coding region of *plg*1. This DNA fragment was generated by PCR amplification using primers P1 (5′AACTCCCACC ATGGAGATCGCC 3′) and P2 (5′CACAACTTGGA TCCGCGAGT 3′). These primers were designed to introduce an *Nco*I restriction site immediately prior to the translation initiation codon (ATG) of the *plg*1 gene and a *Bam*HI restriction site right after translation termination codon (TGA). The amplified *plg*1 gene was then directionally cloned into the *Nco*I and *Bam*HI sites of plasmid pAN52-1.

DNA extraction and Southern blot analysis

Genomic DNA of *P. griseoroseum* wild, mutant, and recombinant strains was prepared from fresh mycelia [38] and digested with *Sph*I and *Eco*RI restriction enzymes. The digestion products were separated in a 0.8% agarose gel and transferred to Duralon membranes (Stratagene), according to standard procedures [37]. In order to evaluate whether multiple integrations of the pectin lyase gene under the control of its own promoter sequence had occurred, the membrane was probed with a labeled DNA fragment containing the structural *plg*1 gene from *P. griseoroseum*. To

evaluate the pattern of integration of the plasmid pAN52-Plg1, the genomic DNA from recombinant strains was digested with *Eco*RI (a restriction enzyme that fails to cut the *gpd*A promoter sequence). Then, the membrane was probed with a labeled DNA fragment containing the *gpd*A promoter from *A. nidulans*. Probes were labeled using the Prime-it® Fluor Fluorescence Labeling Kit (Stratagene). Hybridization experiments were carried out overnight at 60°C in standard hybridization buffer, according to the manufacturer's instructions and signal detection was performed using the illuminator™ non-radioactive detection system (Stratagene), according to the product's manual.

Fungal transformation

Protoplasts of the strain PG63 were obtained as described by Balance and Turner [3], with some modifications. Briefly, 25 ml of CM [33] were inoculated with 10^7 spores from the mutant PG63 of *P. griseoroseum* and incubated for 20 h at 25°C with 150 rpm agitation. Mycelia were colleted by filtration and resuspended in 5 ml 0.6 M KCl pH 5.8 containing Glucanex (3 mg ml^{-1}). The reaction was incubated at 30°C for 3 h with gentle shaking. Protoplasts were separated from mycelia debris by filtration and washed three times with 1 M Sorbitol, 100 mM Tris–HCl pH 7,5, 50 mM $CaCl_2$ (STC) by centrifugation (5 min, 2,300 g). The collected protoplasts were resuspended in 0.5 ml of STC buffer and diluted to a final concentration of 10^8 protoplasts ml^{-1}. Protoplast transformation was based on Yelton [43] and Balance and Turner [3] methods, with some modifications. Approximately 10^7 protoplasts were incubated with 1 µg of pNPG1, 10 µg of pPlg1 or pAN52-Plg1 and 50 µl of 25% polyethylene glycol 6,000/50 mM $CaCl_2$. The mixture was incubated on ice for 20 min before the addition of 500 µl of the same PEG/$CaCl_2$ solution. After 20 min at 25°C, the protoplasts were plated on MM which contained sodium nitrate as the sole nitrogen source and 0.5 M sucrose. Cultures were incubated for 5 days at 25°C. Control transformations were performed under the same conditions described above but no DNA was added to the protoplast suspension. Mitotic stability was assessed by successive transfers to non-selective medium. Genetic stability of the *plg*1 gene and PL activity, were monitored for 12 months.

Pectinase production on solid medium and pectin lyase activity in liquid medium

Fungi were inoculated in mineral medium consisting of 2.0 g l^{-1} K_2HPO_4; 0.1 g l^{-1} $MgSO_4 \cdot 7H_2O$; 1.0 g l^{-1} $(NH_4)_2SO_4$; supplemented with 3 g l^{-1} citric pectin P-9135 (Sigma®), pH 7.2 and 15 g l^{-1} agar, and incubated for 5 days at 25°C. Agar discs (7 mm in diameter) containing mycelia from the colonies were transferred to Macllvaine buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid, pH 6.0), containing 0.25% (w/v) citric pectin P-9135 (Sigma®), 1.5% (w/v) agar and incubated for 48 h at 40°C. Iodine-potassium iodide solution (1.0 g iodine, 5.0 g potassium iodide and 330 ml of distilled water) was added in order to detect clearing zones.

To determine PL activity, the fungal mycelia were grown in liquid medium containing: 6.98 g l^{-1} K_2HPO_4; 5.44 g l^{-1} KH_2PO_4; 1.0 g l^{-1} $(NH_4)_2SO_4$; 1.1 g l^{-1} $MgSO_4 \cdot 7H_2O$; 0.6 g l^{-1} yeast extract; and 3.0 g l^{-1} pectin. Erlenmeyer flasks (125 ml) with 50 ml of medium were inoculated with 1.0 ml of a spore suspension (1×10^6 spores ml^{-1}) and incubated at 25°C with 150 rpm agitation. Pectin lyase activity was determined in the culture filtrate as described by Albersheim [2]. The reaction mixture was set by adding 1.5 ml of the culture filtrate to 1 ml of 2.5% (w/v) citrus pectin P-9135 (Sigma®) in 50 mM phosphate buffer, pH 6.8. After incubation for 30 min at 40°C, 0.5 ml of the reaction mixture was withdrawn and added to 4.5 ml of 0.01 N HCl. One unit of PL (U) activity was defined as nmoles of unsaturated products produced per min. The molar extinction coefficient of unsaturated product is 5.55×10^3.

Growth measurement

Mycelial mass production was quantified by collecting mycelium in a 400 mesh screen (37 jam pores), followed by drying at 105°C until obtaining constant mass [9].

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was conducted according to the method described by Laemmli [25] in a 12% acrylamide gel and 4.5% stacking gel containing 0.1% SDS. Samples were boiled at 100°C for 5 min in Tris-glycine buffer containing 2.3% SDS and 2% 2-β-mercaptoethanol. Electrophoresis was carried out at a constant current of 80 volts (10 mA) for 3 h using a running buffer of 0.24% SDS in Tris-glycine buffer. After electrophoresis, the gel was stained with 0.1% Coomassie brilliant blue R-250. Protein content was quantified by the method of Bradford [7], using bovine serum albumin as standard.

Results and discussion

In the present work we aimed at obtaining recombinant strains of *P. griseoroseum* able to overexpress pectin lyase for industrial application. In order to reach our objective we used two independent strategies.

The first strategy consisted in inserting additional copies of the *plg*1 gene, under the control of its own promoter sequence, into the genome of the PG63 strain of *P. griseoroseum*. The PG63 strain was isolated for sodium chlorate-resistance by our group and presents a 122-bp spontaneous deletion in the *nia*D gene, which makes this strain ideal for transformation experiments since reversion of this kind of mutation is very unlikely or impossible [31]. Other important feature of PG63 is that its PL and other pectinases production was not affected by mutation. Vigorous transformant strains were observed growing on selective medium 72 h after transformation experiment. An average of 1,500 *nia*D stable transformants were obtained with plasmids pNPG1 and pPlg1, being the transformation frequency of 40 transformants per microgram of pNPG1, which is a high frequency when compared to eight transformants obtained by heterologous transformation of the same fungus using one microgram of the plasmid pNH24 carrying the gene *nia*D from *Fusarium oxysporum* [36].

These transformants were analyzed for total pectinase production on solid medium by measuring the pectin degradation halo. Approximately 380 transformants showed higher pectinolytic activity when compared to wild and PG63 mutant strains. Measurements of PL activity from liquid medium containing sucrose and yeast extract revealed that the transformants showed at most 3.0 times higher PL activity than wild or PG63 mutant strains (Fig. 1a).

Eleven of these transformants were analyzed by Southern blot revealing that integration of the *niaD* gene mainly occurred at the resident locus characterizing occurrence of homologous integration in the most transformants (data not shown). Homologous integration of the gene marker (*nia*) is interesting for co-transformation experiments, since a lower percentage of transformants would show integrations at other regions in the genome that may be important for maintaining cell metabolism or be of biotechnological interest. When the same membrane was probed using *plg*1, transformants T487, T47, T34, T89 and T88 exhibited the same band profile as the wild type strain (Fig. 1b). This result showed these are not cotransfomants. These hypotheses are in agreement with the fact that their PL activities were similar to that observed for the wild type strain. Vector insertion out of the *plg*1 locus (ectopic integration) was observed for the transformants T86, T62, T119, T63, T7 e T13. The number of additional copies of the *plg*1 gene

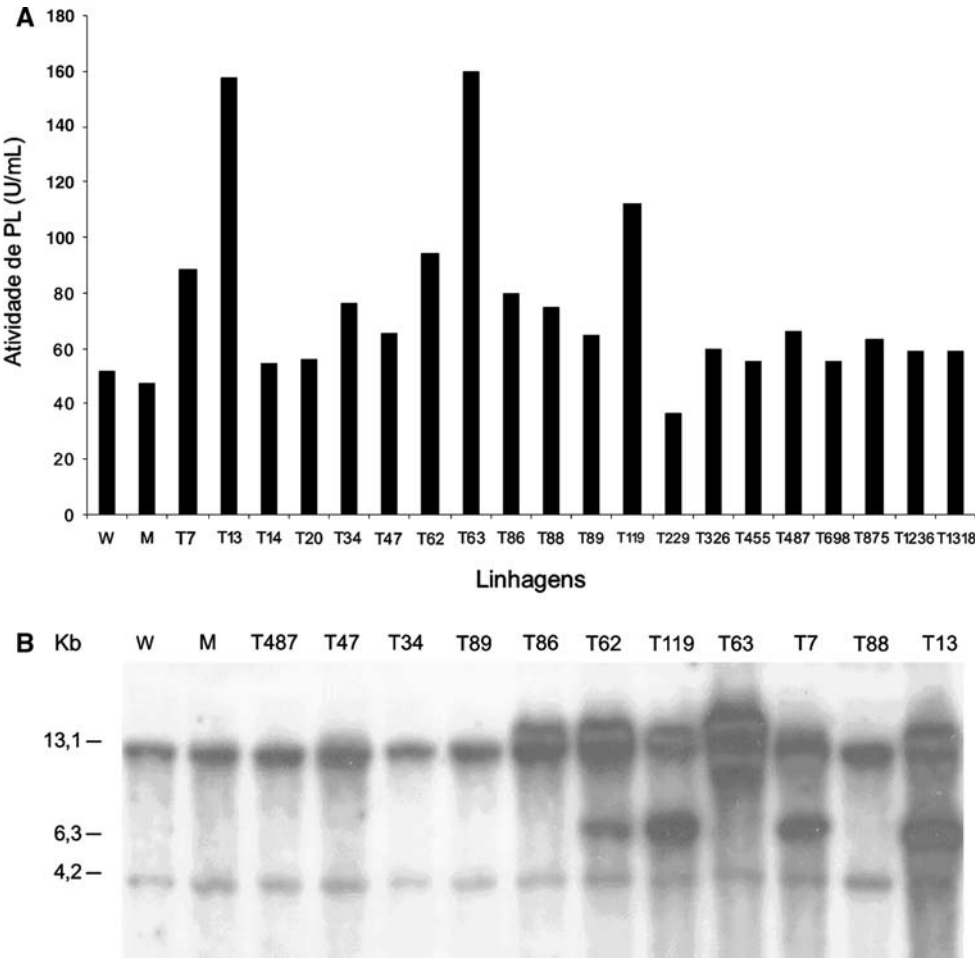

Fig. 1 Pectin lyase activity and hybridization analysis of *Penicillium griseoroseum* transformed with plasmid pPlg1. **a** Pectin lyase activity of the wild (*W*), PG63 (*M*), and transformant (*T*) strains of *P. griseoroseum* grown in sucrose-yeast extract medium for 48 h. **b** Hybridization analysis of the transformants genomic DNA digested with *Sph*I (no cleavage site in *plg*1 gene). The blot was probed with a DNA fragment containing the *plg*1 gene from *P. griseoroseum*. The sizes of the DNA fragments are indicated in kilobases (kb)

present in the genome of these transformants could not be precisely determined, thus making it impossible to correlate PL activity and gene copy number.

To explain the limited production of PL observed in these transformants, we hypothesized that the expression of the additional copies of *plg*1 could be affected by the integration site, or that the protein production in larger levels was limited by poor amount of regulatory proteins available. Several studies have reported a considerable increase in protein production resulting from introduction of multiple copies of genes of interest into fungi genomes [10, 26]. However, it has been noted that both copy number and site of integration greatly affect the expression of introduced genes [41]. For instance, no correlation between additional copy number of genes that codify polygalacturonase and enzyme activity was observed in transformants of *A. niger* [8]. The same was noted in transformants of *P. chrysogenum* [13], in which a transformant carrying only six copies of this gene showed 50% higher enzymatic activity than the one exhibited by a strain with nine copies.

The limited amount of specific transcription factors can also compromise the expression profile of a gene of interest. In fact, addition of multiple copies of the glucoamylase-encoding gene in *A. niger* resulted in titration of trans-acting regulatory protein(s), which actually resulted in reduced gene expression [41]. Thus, the low PL activity observed in the *plg*1 transformants of *P. griseoroseum* could also be explained by a lack of expression signals for the additional copies of the gene. One strategy that could be used to overcome this problem would be the overexpression of genes encoding specific regulatory factors, but this would require simultaneous genetic manipulations that are very difficult to achieve. An alternative approach for overexpression of the gene could be achieved by replacing the expression signals for sequences from highly expressed genes [11, 12, 41]. We chose this second approach and built a plasmid vector carrying *plg*1 under the control of the *gpd*A promoter and the terminator region of the tryptophan synthetase gene (*trp*C) from *A. nidulans*. Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in glycolysis and gluconeogenesis that may represent up to 5% of total content of soluble proteins in several organisms, thus indicating that this gene has a very strong promoter region [34].

The strategy used to construct the recombinant vector pAN52-Plg1 is summarized in Fig. 2. This plasmid, together with pNPG1, was also used to transform protoplasts of PG63 strain. Two hundred and fifty transformant strains showed themselves genetically stable after five

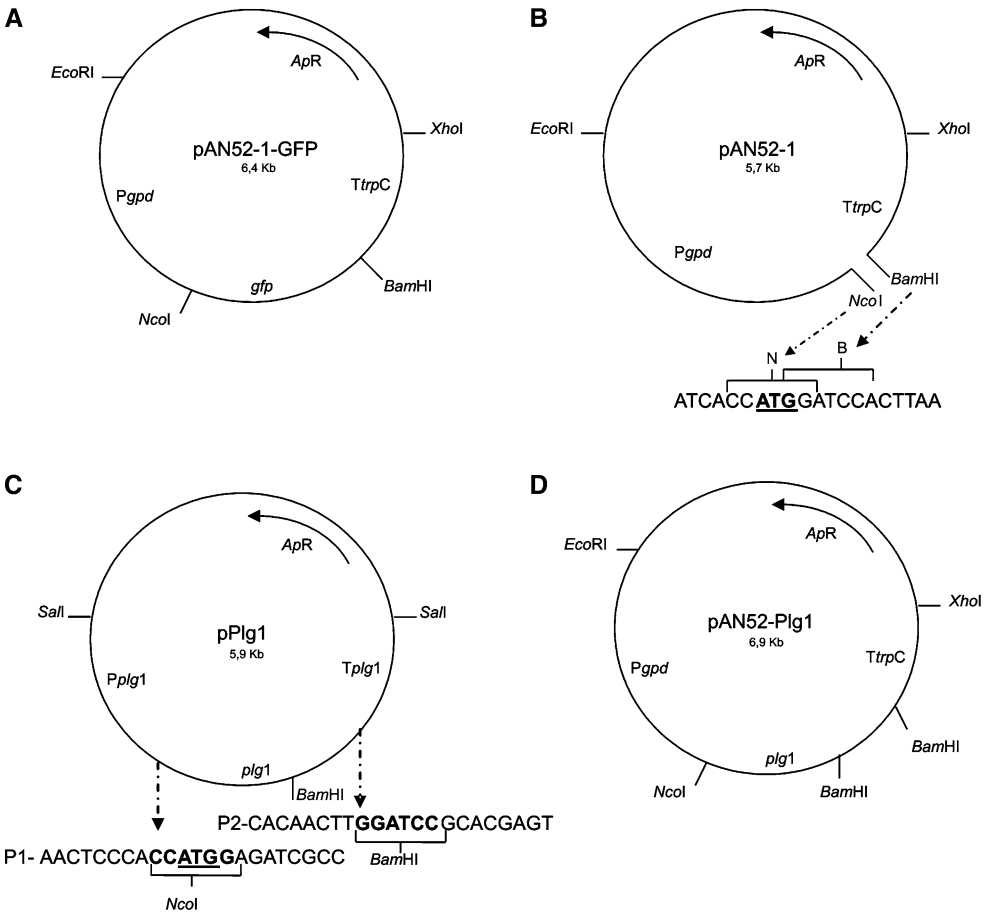

Fig. 2 Construction of plasmid pAN52-*Plg*1. Plasmid pAN52-Plg1 (D) was obtained by replacing the *gfp* gene in vector pAN52-1-GFP (A) by *plg*1 gene from *P. griseoroseum*. The *Nco*I–*Bam*HI 1000bp DNA fragment was generated by digesting the PCR-amplified DNA fragment containing the *plg*1 coding region from the plasmid pPlg1, using primers P1 and P2 (C). This DNA fragment was directionally ligated to the *Nco*I and *Bam*HI sites of pAN52-1 (B). Restriction sites and gene regions are indicated in the figure. Ap^R-ampicillin resistance; P*gpd* 5′-promoter region of the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase gene; T*trp*C-terminator region of the tryptophan synthetase gene from *A. nidulans*

successive transfers to non-selective medium. The transformants were grown in liquid medium containing glucose as the sole carbon source for 48 h, after which they were analyzed for PL activity. Five recombinant strains (30, 42, 73, 105, 149) showed PL overproduction (Table 1). These strains exhibited enzymatic activities ranging from 33 to 57 times higher than the one observed for PG63 strain grown in medium containing sucrose and yeast extract. The highest PL activity was produced by recombinant strain 105.

The presence of pectic substances in growth medium is required for PL production in wild type strains of *P. griseoroseum* [5, 27]. Alternative carbon sources, such as sugar cane juice, have been assayed with the objective of reducing production costs of microbial PGs and PLs [28]. We investigated the effect of different carbon sources in PL activity of the recombinant strain 105 (Table 2). The highest activity was obtained when the fungus was cultured in presence of sugar cane juice (5,202 U ml^{-1}) and the lowest one, in presence of pectin (1,190 U ml^{-1}). These PL activities obtained by fungi cultivation in presence of sugar cane juice are 132 times higher than the one observed for PG63 strain grown in the same carbon source. These results indicate that recombinant 105 is a promising strain for industrial production of pectin lyase, since sugar cane juice is an inexpensive and readily available carbon source in Brazil.

Table 1 Pectin lyase activities and biomass of some *P. griseoroseum* recombinant strains grown in mineral medium containing glucose and the mutant PG63 grown in mineral medium supplemented with sucrose and yeast extract

Strain	PL activity (U ml^{-1})	Biomass (g l^{-1})
PG63	83.4 ± 11.10	4.89 ± 1.92
30	3,676.0 ± 473.25[ab]	5.67 ± 2.12
42	2,777.0 ± 356.06[b]	5.18 ± 2.38
73	2,817.0 ± 532.45[b]	5.85 ± 2.26
105	4,804.0 ± 415.32[a]	4.85 ± 1.89
149	3,202.0 ± 387.18[b]	4.43 ± 0.95

The results represent arithmetical average ± standard-error of three independent experiments

[a, b] Values followed by the same letter are not different according to Tukey test (α = 0,05)

In order to further characterize the recombinant strains able to overproduce PL, we performed a Southern blot analysis of genomic DNA of these strains digested with *Eco*RI (a restriction enzyme that fails to cut the *gpd* promoter sequence) using a DNA fragment containing the *gpd*A promoter of *A. nidulans* as probe. We found that all five recombinant strains had at least two copies of the plasmid pAN52-Plg1 (Fig. 3). Since no hybridization signal was observed between the *A. nidulans* and *P. griseoroseum gpd* promoters, in the conditions used, we concluded that these two sequences share relatively low similarity. Fortunately, expression of *plg*1 was not hindered in spite of this low similarity.

Pectin lyase production was also evaluated by size fractionation in SDS-PAGE gel electrophoresis followed by Coomassie staining (Fig. 4). Total extracellular proteins

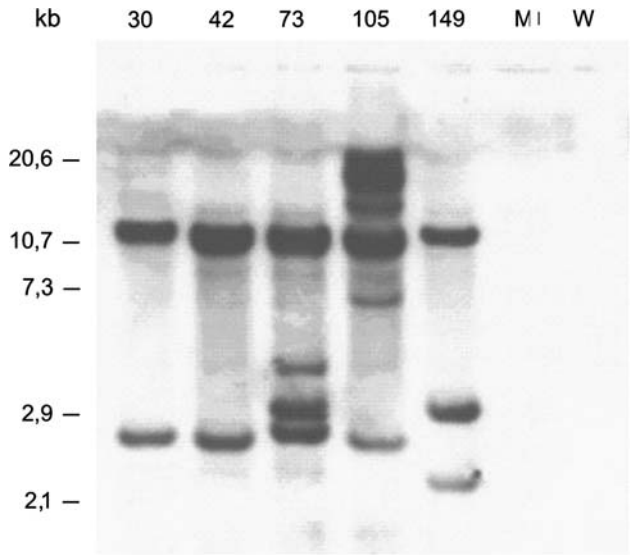

Fig. 3 Hybridization analysis of *Penicillium griseoroseum* recombinant strains transformed with the vectors pAN52-Plg1 and pNPG1. Genomic DNA from wild, mutant, and recombinant strains was digested with *Eco*RI (no site of cleavage into the probe sequence). The blot was probed with a DNA fragment corresponding to the *gpd*A promoter of *A. nidulans*. Letters and numbers represent wild strain (*W*), PG63 mutant strain (*M*), and recombinant strains (30, 42, 73, 105, 149). Sizes of the DNA fragments are indicated in kilobases (kb)

Table 2 Effect of different carbon sources on pectin lyase activity and growth rate of *P. griseoroseum* recombinant strain 105 and mutant PG63

Carbon source	PG63 PL activity (U ml^{-1})	PG63 Biomass (g l^{-1})	105 PL Activity (U ml^{-1})	105 Biomass (g l^{-1})
Glucose	ND	4.89 ± 0.93	4,804 ± 415.32	4.85 ± 1.85
Sucrose	ND	5.18 ± 1.95	4,403 ± 377.50	4.76 ± 1.73
Pectin	21.82 ± 8.15	0.60 ± 0.25	1,190 ± 232.65	0.62 ± 0.21
Sugar cane juice	39.40 ± 12.50	5.30 ± 1.84	5,202 ± 483.91	7.20 ± 3.35

The results represent arithmetical average ± standard-error of three independent experiments

ND not detected

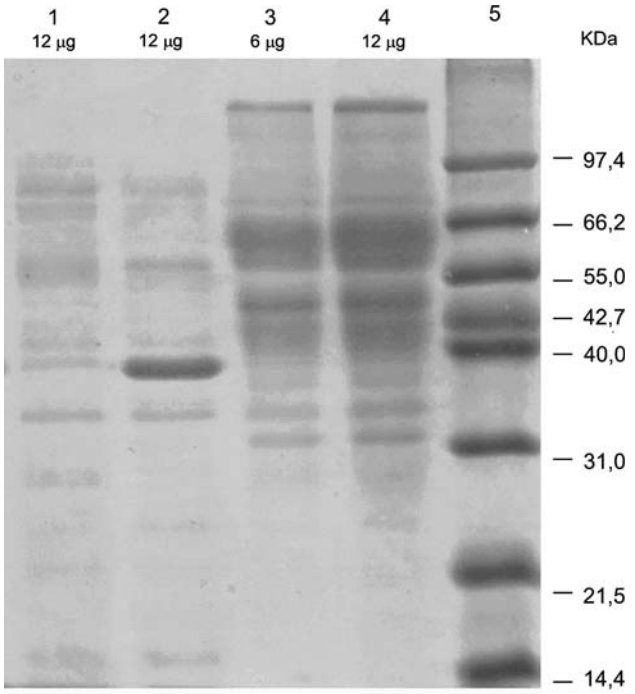

Fig. 4 Size fractionation in SDS-PAGE gel electrophoresis of the extracellular proteins secreted by the mutant strain PG63 (*lane 1*) and recombinant strain 105 (*lane 2*). A commercial pectinase preparation "citrus clear" (CC) (*lane 3* and *4*) was used for comparison. The proteins were electrophoresed in a 12% polyacrilamide gel and stained with Coomassie brilhant blue R-250. Protein sizes of the molecular size marker (*lane 5*) are indicated in kDa

were measured in culture supernatants of the recombinant strain 105 grown in presence of sucrose and the mutant PG63 grown in presence of pectin. For comparison, we used a commercial pectinase preparation (Citrus Clear®, Valley Research) as standard. Analysis of the gel revealed that strain 105 shows a single strong band with a molecular mass of approximately 40 kDa, similar to the molecular mass estimated for PL from the *plg*1 nucleotide sequence [6]. On the other hand, the commercial pectinase preparation showed several bands of moderate intensity, none of them presenting the same size of the putative pectin lyase produced by strain 105. These data suggest that PL production in the recombinant 105 predominates over other extracellular proteins, contrary to the situation observed for the commercial preparation. Moreover, the apparent specific activity of PL produced by this strain was 54 times higher than that detected in the commercial preparation (Table 3).

Taken together, our results show that recombinant strain 105 has an enormous potential to produce pectin lyase for application in industrial processes, such as textile and plant fiber processing, coffee and tea fermentation, oil extraction, industrial wastewater treatment, and paper making. Furthermore, it offers the advantage of producing great amounts of PL using sugar cane juice as the sole carbon source, which would lower production costs. Moreover, the PL enzyme has an estimated pI (9.46) higher than those reported for the *Aspergillus* PLs (varying from 3.65 to 5.9) [6, 23], which, in association with its working pH, renders great industrial value to this enzyme. Further experiments will be carried out in order to optimize the growth conditions of this strain for scaling up PL production in bench bioreactors.

Table 3 Comparison of protein concentration, pectin lyase activity and pectin lyase specific activity of culture supernatants from recombinant strain 105 grown in sucrose medium, mutant strain PG63 grown in pectin medium, and the commercial pectinase preparation "Citrus clear" from *Aspergillus*

Sample	Protein concentration ($\mu g\ ml^{-1}$)	PL activity ($U\ ml^{-1}$)	PL specific activity ($U\ \mu g^{-1}$)
PG63	10.37 ± 4.57	21.82 ± 3.75	2.10 ± 0.35
105	21.55 ± 9.35	4,403 ± 99.33	204.32 ± 13.58
PG63 $_{\text{concentrated (100-fold)}}$	1,217 ± 43.23	2,792 ± 116.44	2.29 ± 0.57
105 $_{\text{concentrated (100-fold)}}$	2,299 ± 65.96	468,057 ± 883.52	203.59 ± 19.26
"Citrus clear"	10,786 ± 109.38	40,949 ± 297.85	3.79 ± 1.05

The results represent arithmetical average ± standard-error of three independent experiments

Acknowledgments This research was supported by the Brazilian agencies Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

1. Alaña A, Gabilondo A, Hernando F, Moragues MD, Dominguez JB, Llama MJ, Serra JL (1989) Pectin lyase production by *Penicillium italicum* strain. Appl Environ Microbiol 55:1612–1616
2. Albersheim P (1966) Pectin lyase from fungi. In: Neufeld E, Ginsgurg V (ed) Methods in enzymology. Academic, New York, pp 628–635
3. Ballance DJ, Turner G (1985) Development of a high-frequency transforming vector for *Aspergillus nidulans*. Gene 36:321–331
4. Baracat MC, Valentim C, Muchovej JJ, Silva DO (1989) Selection of pectinolytic fungi for degumming of natural fibers. Biotechnol Lett 11:899–902
5. Baracat-Pereira MC, Coelho JLC, Minussi RC, Chaves-Alves VM, Brandio RL, Silva DO (1999) Cyclic AMP and low molecular weight effector(s) present in yeast extract are involved in pectin lyase production by *Penicillium griseoroseum* cultured on sucrose. Appl Biochem Biotechnol 76:129–142
6. Bazzolli DS, Ribon AO, Queiroz MV, Araújo EF (2006) Molecular characterization and expression profile of pectin-lyase-encoding genes from *Penicillium griseoroseum*. Can J Microbiol 52:1070–1077
7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
8. Bussink HJD, van den Hombergh JPTW, van den Ijssel PRLA, Visser J (1992) Characterization of polygalacturonase-overproducing *Aspergillus niger* transformants. Appl Microbiol Biotechnol 37:324–329

9. Calam CT (1969) The evaluation of mycelial growth. In: Norris JR, Ribbons DW (ed) Methods in Microbiology. Academic Press, London, pp 567–591
10. Daly R, Hearn MT (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J Mol Recognit 18(2):119–138
11. Fincham JR (1989) Transformation in fungi. Microbiol Mol Biol Rev 53:148–170
12. Gouka RJ, Punt PJ, van den Hondel CAMJJ (1997) Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. Appl Microbiol Biotecnol 47:1–11
13. Graessle S, Haas H, Friedlin E, Kürnsteiner H, Stöffler G, Redl B (1997) Regulated system for heterologous gene expression in *Penicillium chrysogenum*. Appl Environ Microbiol 63:753–756
14. Gysler C, Harmsen JA, Kester HC, Visser J, Heim J (1990) Isolation and structure of the pectin lyase D-encoding gene from *Aspergillus niger*. Gene 89:101–108
15. Harmsen JAM, Kusters-van Someren MA, Visser J (1990) Cloning and expression of a second *Aspergillus niger* pectin lyase gene (*pel*A): indications of a pectin lyase gene family in *Aspergillus niger*. Curr Genet 18:161–166
16. Inglis PW, Aragao FJL, Frazao H, Magalhães BP, Valadares-Inglis MC (2000) Biolistic co-transformation of *Metarhizium anisopliae* var. *acridum* strain CG423 with green fluorescent protein and resistance to glufosinate ammonium. FEMS Microbiol Lett 191:249–254
17. Inoue H, Nojima H, Okayama H (1990) High efficiency transformation of *Escherichia coli* with plasmids. Gene 96:23–28
18. Jayani RS, Saxena S, Gupta R (2005) Microbial pectinolytic enzymes: a review. Process Biochem 40:2931–2944
19. Kashyap DR, Vohra PK, Chopra S, Tewari R (2001) Applications of pectinases in the commercial sector: a review. Bioresour Technol 77:215–227
20. Kitamoto N, Yoshino-Yasuda S, Ohmiya K, Tsukagoshi N (2001a) Sequence analysis and overexpression of a pectin lyase gene (*pel*1) from *Aspergillus oryzae* KBN616. Biosci Biotechnol Biochem 65:209–212
21. Kitamoto N, Yoshino-Yasuda S, Ohmiya K, Tsukagoshi N (2001b) A second pectin lyase gene (*pel*2) from *Aspergillus oryzae* KBN616: its sequence analysis and overexpression, and characterization of the gene products. J Biosci Bioen 91:378–381
22. Kolar M, Punt PJ, van den Hondel CA, Schwab H (1988) Transformation of *Penicillium chrysogenum* using dominant selection markers and expression of an *Escherichia coli lac*Z fusion gene. Gene 62:127–134
23. Kusters-van Someren MA, Harmsen JA, Kester HC, Visser J (1991) Structure of the *Aspergillus niger pelA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet 20:293–299
24. Kusters-van Someren MA, Flipphi M, Graaff L, van den Broeck H, Kester HC, Hinnen A, Visser J (1992) Characterization of the *Aspergillus niger pelB* gene: structure and regulation of expression. Mol Gen Genet 234:113–120
25. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
26. Lopes FJF, Araujo EF, Queiroz MV (2004) Easy detection of green fluorescent protein multicopy transformants in *Penicillium griseoroseum*. Genet Mol Res 3:449–455
27. Minussi RC, Baracat-Pereira MC, Coelho JLC, Silva DO (1997) Methylxanthines as inducers of pectin lyase in *Penicillium griseoroseum* cultured on sucrose. Lett Appl Microbiol 24:369–372
28. Minussi RC, Soares-Ramos JRL, Coelho JLC, Silva DO (1998) Sugar-cane juice induces pectin lyase and polygalacturonase in *Penicillium griseoroseum*. Rev Microbiol 29:246–250
29. Nevalainen HKM, Te'o VSJ, Bergquist PL (2005) Heterologous protein expression in filamentous fungi. Trends Biotechnol 23:468–474
30. Penttilã M, Nevalainen H, Ratto M, Salminen E, Knowles J (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61:155–164
31. Pereira JF, Queiroz MV, Lopes FJF, Rocha RB, Daboussi MJ, Araújo EF (2004) Characterization, regulation, and phylogenetic analysis of the *Penicillium griseoroseum* nitrate reductase gene and its use as selection marker for homologous transformation. Can J Microbiol 50:891–900
32. Pöggeler S, Masloff S, Hoff B, Mayrhofer S, Kuck U (2003) Versatile EGFP reporter plasmids for cellular localization of recombinant gene products in filamentous fungi. Curr Genet 43:54–61
33. Pontecorvo G, Roper JA, Hemmons LM, MacDonald KD, Bufton AW (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5:141–238
34. Punt PJ, Dingemanse MA, Kuyvenhoven A, Soede RD, Pouwels PH, van den Hondel CA (1990) Functional elements in the promoter region of the *Aspergillus nidulans gpd*A gene encoding glyceraldehyde-3-phosphate dehydrogenase. Gene 93:101–109
35. Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J, van den Hondel CA (2002) Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol 20:200–206
36. Queiroz MV, Barros AO, Barros EG, Guimarães WV, Araújo EF (1998) Transformation of *Penicillium griseoroseum* nitrate reductase mutant with the *nia* gene from *Fusarium oxysporum*. Can J Microbiol 44:487–489
37. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning:a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, New York
38. Specht CA, Dirusso CC, Novotny CP, Ullrich RC (1982) A method for extracting high molecular weight deoxyribonucleic acid from fungi. Anal Biochem 119:158–163
39. Storms R, Zheng Y, Li H, Sillaots S, Martinez-Perez A, Tsang A (2005) Plasmid vectors for protein production, gene expression and molecular manipulations in *Aspergillus niger*. Plasmid 53:191–204
40. Varavallo MA, Queiroz MV, Lana TG, Brito ART, Gonçalves DB, Araújo EF (2007) Isolation of recombinant strains with enhanced pectinase production by protoplast fusion between *Penicillium expansum* and *Penicillium griseoroseum*. Braz J Microbiol 38:1–6
41. Verdões JC, Punt PJ, van den Hondel CAMJJ (1995) Molecular genetic strain improvement for the overproduction of fungal proteins by filamentous fungi. Appl Microbiol Biotechnol 43:195–205
42. Vries RP, Visser J (2001) *Aspergillus* Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. Microbiol Mol Biol Rev 65:497–522
43. Yelton MM, Hamer JE, Timberlake WE (1984) Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc Natl Acad Sci USA 81:1470–1474