World Journal of Agricultural Sciences 9 (2): 167-172, 2013
ISSN 1817-3047

DOI: 10.5829/idosi.wjas.2013.9.2.1713

# Pectinases from a New Strain of *Cladosporium cladosporioides* (Fres.) De Vries Isolated from Coffee Bean

*[1]Sabrina Carvalho Bastos, [1]Carlos José Pimenta, [1]Disney Ribeiro Dias,*
*[2]Sara Maria Chalfoun, [1]Caroline Lima Angélico and [1]Lucas Silva Tavares*

[1]Department of Food Science, Federal University of Lavras/UFLA
[2]Empresa de Pesquisa Agropecuária de Minas Gerais / EPAMIG
Caixa Postal 37, 37.200-000, Lavras, MG, Brazil

**Abstract:** To examine tThe optimal conditions of cultivation for *Cladosporium cladosporioides* to produce and partially purify pectin methylesterase (PME) and polygalacturonase (PG) were examined under laboratory conditions . Activities of PME and PG were evaluated after 10, 15 and 20 days of incubation at 28°C in rice solid medium. Enzyme extraction was carried out by homogenization in buffer solutions at different pH (4, 5 and 6) followed by precipitation with ammonium sulfate [$(NH_4)_2SO_4$] at 20, 40 and 60% saturation. After 10 days of incubation, the highest activities for PG (105.5 U/mL) and PME (1480 U/mL) in the crude extract was observed. Benzoate buffer (pH 4.0) was the best buffer for enzymes extraction. The final yield of 108.7% for PME and 10.6% for PG and a purification index of 14.2 and 1.4 for PME and PG, respectively, was obtained after saturation with 60%. The ideal conditions for obtained and pre-purification of PME and PG were 10 days of incubation, extraction with benzoate buffer pH 4.0 and precipitation with 60% of $(NH_4)_2SO_4$. This is the first report on of pectinases pectinolytic enzymes produced by from *C. cladosporioides* and provides information about growth conditions for production and pre-purification of PME and PG.

**Key words:** Pectinolytic enzymes · Filamentous fungi · Pectin · Coffee fermentation

## INTRODUCTION

Pectinolytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants. Pectins are a family of covalently linked galacturonic acid-rich plant cell wall polysaccharides. Galacturonic acid (GalA) comprises approximately 70% of pectin and all the pectic polysaccharides contain galacturonic acid linked at the O-1 and the O-4 position [1]. Pectinases are of major importance in the beverage industry due to their ability to improve pressing and clarification of concentrated fruit juices and they are extensively used in the food industry in the processing of fruits and vegetables, in the production of wine, the extraction of olive oil and fermentation of tea, coffee and cocoa [2-4]. Pectinolytic enzymes are widely distributed in higher plants and microorganisms. Almost all the commercial preparations of pectinases are produced from fungal sources. These enzymes are responsible for the degradation of the long and complex molecules of pectin that occur as structural polysaccharides in the middle lamella and the primary call walls of young plant cells [1, 5].

Filamentous fungi have been used for more than 50 years in for the production of industrial enzymes in industry and most of them produce various enzymes simultaneously. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes including pectin methylesterase (PME), polygalacturonase (PG) and pectin lyase (PL). Other microorganisms have been reported as pectinolytic enzymes producers, including other fungi such as *A. japonicus*, *Fusarium moniliforme*, *Neurospora*

**Corresponding Author:** Disney Ribeiro Dias, Department of Food Science Federal University of Lavras/UFLA Caixa Postal 37, 37.200-000, Lavras, MG. Brazil. Tel: +55 35 38295256, Fax: +55 35 38291391,

*crassa*, *Rhizopus stolonifer*, *Penicillium frequentans* and *P. occitanis*, yeasts such *Aureobasidium pullulans*, *Rhodotorula* sp., *Mrakia frigida* and *Kluyveromyces marxianus* and bacteria such *Lactobacillus lactis* subsp. *cremoris*, *Paenibacillus amylolyticus* and *Bacillus* spp. [3, 6-8].

The fungi fungus *Cladosporium cladosporioides* can easily grow on different substrates (including cassava root, coffee fruits and industrial food residues) and produces pectinases during its metabolism [9, 10]. Its presence on fruits and coffee beans is beneficial because its absence would leave the fruit susceptible to microorganisms that are harmful to quality [10, 11].

The present study aimed to investigate the optimal time for the cultivation of *C. cladosporioides* to achieve the maximum production of polygalacturonase and pectin methylesterase. In addition, the study also aimed to partially characterize the enzymes using ammonium sulfate [$(NH_4)_2SO_4$]. To the best of our knowledge, this is the first report on the production of pectinases by *C. cladosporioides*.

## MATERIALS AND METHODS

**Inoculum and Growth Conditions:** *Cladosporium.* cladosporioides, belonging to the Ffilamentous Fungi fungiCulture Collection of the Department of Food Science, previously isolated from coffee beans in the South of Minas Gerais State, Brazil, was kindly obtained from Culture Collection of the Department of Food Science and maintained in PDA medium (2% potato broth, 2% dextrose and 2% agar) was grown in the semi-solid rice medium. Rice was chosen due to its composition: it is rich in carbohydrates, is a source of pectin and has low levels of PME and PG (the enzymes under investigation). To obtain this semi-solid substrate, a mixture of parts of water (70 ml) and rice (70 g) was prepared, which was autoclaved at 110°C for 40 minutes. Therefore the semi-solid rice medium was used for growing the tested fungus.

The inoculum consisted of $10^7$ spores of *C. cladosporioides* per gram of culture medium. Quantification of the resulting suspension was performed by counting the spores in the Neubauer chamber. The sterilized and cooled culture medium was inoculated and maintained under growing conditions (controlled humidity and a temperature of 28°C) for 10, 15 and 20 days to investigate a relationship between the production of pectinases and the cultivation time. After that cultivation periods, samples were collected in order to assess pectinolytic activity (PG and PME).

**Enzyme Activity:** The enzymatic extract was obtained using the procedure described by Buescher and Furmanski [11, 12]. PG activity was determined by measuring the reducing groups released from polygalacturonic acid according to the method described by Schwan *et al*. [3] with minor modifications. One unit of PG activity (U/mL) was expressed in çmol of galacturonic acid released per minute of reaction under the test conditions.

PME activity was determined by the titration of carboxyl groups released by pectin de-esterification due to the effect of enzyme action [12, 13]. One unit of PME activity (U/mL) was defined as the ability of the enzyme to catalyze pectin demethylation corresponding to 1 çmol NaOH per minute under the assay conditions. The enzymatic activities of PG and PME were also determined in the purification steps.

**Protein Quantification:** Protein concentrations were measured according to Phutela *et al*. [4] to determine specific activities (U/mg) of pectinases.

**Effect of Ph on Enzymatic Enzymes Extraction:** It is known that most pectinases are stable in slightly acidic conditions (pH 4.0-6.0). In order to characterize the enzyme action at different pH, the following three buffer solutions were tested: sodium benzoate (pH 4.0), sodium citrate (pH 5.0) and sodium phosphate (pH 6.0). The crude enzyme extraction was performed according to Gummadi and Panda [13, 14]. The cultures (15 g) were homogenized in a Stomacher® containing 90 ml of buffer solution, which resulted in a crude enzyme extract. The obtained extract was centrifuged at 15000 ×*g* for 15 minutes at 4°C. Two enzyme fractions were obtained and labeled as supernatant 1 (S1) and precipitate 1 (P1), which were assayed for total proteins, PG and PME. The S1 fraction was used for subsequent enzymatic precipitation.

**Enzyme Precipitation:** The S1 fraction obtained previously were precipitated with $(NH_4)_2SO_4$ at 20%, 40% and 60% saturation [14, 15]. After adding $(NH_4)_2SO_4$, the sample was carefully homogenized and chilled at 4°C for 1 h, before centrifuging at 15000 ×*g* for 15 min at 4°C. The two fractions obtained were labeled as supernatant 2 (S2) and precipitate 2 (P2). The P2 fraction was resuspended in a four volumes of 50 mM Tris-HCl buffer pH 7.0 and transferred to dialysis membranes (cut-off 18 kDa). The membranes were immersed in 50 volumes of the same buffer and dialysis occurred for 24 h at 4°C, with buffer solution replacement every 8 h. After the dialysis, the two fractions obtained, supernatant 3 (S3) and

precipitate 3 (P3) were collected and the amount of total protein, polygalacturonase (PG) and pectin methylesterase (PME) activities were determined as described previously.

**Statistical Analysis:** For each cultivation time, buffer solution and for the 3 levels of enzyme saturation, a completely randomized design with five replications was performed. The data were analyzed by analysis of variance ($p < 0.05$) and the means were compared by Scott-Knott test at using the software SISVAR® 5•1 [15, 16].

## RESULTS

The results showed in Fig. 1a indicate that the highest enzymatic enzyme activity was obtained at 10 days of cultivation and the average values were 105.5 U/mL for PG and 1480 U/mL for PME. Enzymatic Enzyme activity gradually decreased during the incubation time and on day 20 the activity of PG and PME fell down to 32.4 U/mL and 526.7 U/mL, respectively (Fig. 1a).

With regard to buffer and pH, the results showed that the benzoate buffer with a pH value of 4.0 was better for the extraction of both enzymes. The values presented in Fig. 1 shows the highest activities of PME (Fig. 1b, 3300 U/mL) and PG (Fig. 1c, 163.4 U/mL) from benzoate buffer P1 fraction. PME activities were also detected in benzoate buffer S1 (Fig. 1b, 1671.4 U/mL) and in the citrate buffer S1 fraction (Fig. 1b, 964.3 U/mL). Fig. 1c shows the results of PG activities found in the benzoate S1 (52.6 U/mL), citrate S1 (26.8 U/mL), citrate P1 (39.8 U/mL) and phosphate S1 (50 U/mL). PME activity was not detected in the citrate buffer (S1) or in the phosphate buffer fractions (S1 and P1), whereas PG activity was not detected only in the phosphate buffer P1 fraction. Based on these results, the ideal pH value and buffer solution combination, which resulted in the maximum activity of PG, was the benzoate buffer at pH 4.0. For this reason, this buffer was employed in later stages of enzymatic assays.

According to theseis results, there was a higher enzyme activity in benzoate buffer (pH 4.0) fractions (Fig. 1b and 1c). Among the fractions analyzed, the enzymatic activity found in the benzoate P1 fraction was higher than that in the benzoate S1 for both enzymes (PME and PG). However, because of the presence of insoluble cell fragments and the insoluble pellet of P1 fraction, the benzoate buffer S1 was chosen for enzymatic enzymes precipitation with $(NH_4)_2SO_4$.

The average results obtained for the PME and PG activity after precipitation of benzoate buffer S1 with $(NH_4)_2SO_4$ at 20%, 40% and 60% saturation are shown in Fig. 1d. The 60% saturation presented the highest results for both pectinases, 1454.1 (U/mL) and 11.3 (U/mL) for PME and PG, respectively. It was not possible to

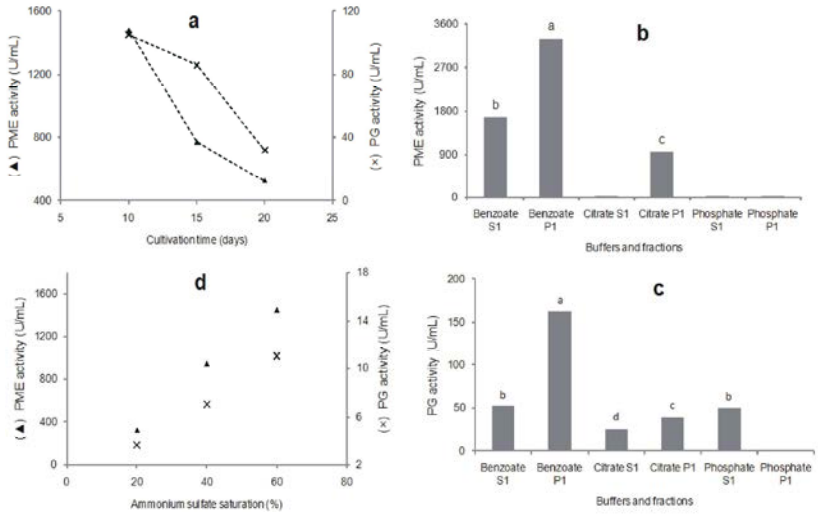


Fig. 1: PME and PG activities from C. cladosporioides during cultivation, extraction and pre-purification stages. 1a) Averages of PME and PG activities during cultivation periods; 1b) Averages of PME activities of different buffers an fraction (S: supernatant; P: precipitate); 1c) Averages of PG activities of different buffers an fraction (S: supernatant; P: precipitate); and 1d) PME and PG activities under 20, 40, and 60% saturation of ammonium sulphate. For 1a and 1b, averages followed by the same letter do not differ according to Scott-Knot test at 5% probability.

Table 1: Purification steps for PME from *C. cladosporioides* growth for 10 days at 28°C in the rice solid media.

| Purification Step | Total Protein (mg) | Enzimatic Activity (U/mL) | Especific activity (U/mg) | Yield (%) | Purification Index |
|---|---|---|---|---|---|
| Initial Culture | 42 | 1340 | 31.9 | 100 | 1 |
| Benzoate S1 | 7 | 1671.4 | 238.7 | 124.7 | 7.5 |
| Benzoate S3 | 3.1 | 0 | 0 | 0 | 0 |
| Benzoate P1 | 11.6 | 3300 | 284.5 | 246.3 | 8.9 |
| Benzoate P3 | 3.2 | 1454.1 | 454.4 | 108.7 | 14.2 |

Table 2: Purification steps for PG from *C. cladosporioides* growth for 10 days at 28°C in the rice solid media.

| Purification Step | Total Protein (mg) | Enzymatic Activity (U/mL) | Especific activity (U/mg) | Yield (%) | Purification Index |
|---|---|---|---|---|---|
| Initial Culture | 42 | 107.6 | 2.6 | 100 | 1 |
| Benzoate S1 | 7 | 52.6 | 7.5 | 48.9 | 2.9 |
| Benzoate S3 | 3.1 | 0 | 0 | 0 | 0 |
| Benzoate P1 | 11.6 | 163.4 | 14.1 | 151.8 | 5.5 |
| Benzoate P3 | 3.2 | 11.4 | 3.6 | 10.6 | 1.4 |

recovery a precipitated in 20% and 40% $(NH_4)_2SO_4$ after the dialysis. The values obtained for the enzymatic activity in 20% and 40% $(NH_4)_2SO_4$ (Fig. 1d) refer to the supernatant fraction (S3).

The data for recovery and the purification index of *C. cladosporioides* enzyme purification steps are shown in Tables 1 and 2. Our study revealed yields from the enzyme extraction step of 246•3% and 151•8% for the PME and PG, respectively, in the benzoate buffer P3 (fraction after dialysis). The values of purification index resulted in a further purification of the PME during the step of enzymatic precipitation with $(NH_4)_2SO_4$, reaching a purification index of 14.2. For the PG enzyme, the final purification index achieved was 1.4, lower than that found in enzymatic extraction step, which was 5.5.

## DISCUSSION

There was a rapid increase in colony diameter from 3rd to 10th day of incubation and the colonies filled the entire substrate surface on day 10th. After that the growth declined, which may be due to limited oxygen supply, since the culture surface was covered with a thin layer of mycelium.

In the present work, the optimum time for PME and PG production by *C. cladosporioides* was 10 days. The reason for the decrease in pectinolytic activity observed after 10 days of cultivation may be related to factors such as the consumption of some essential compound (*i.e.*, oxygen or nutrients), the presence of an inhibitor, loss of water, or metabolite accumulation [16, 17]. It may be possible that a mechanism for enzymatic regulation in fungi could consist of a blocking enzymatic activity when a certain relation between the enzyme concentration and the cell number is achieved [5, 13, 14].

The decrease on enzyme activity after 10th day suggests that cells with 15 and 20 days of fungal cultivation no longer had the full physiological capacity to germinate and grow and the enzyme secretion is growth–related. A similar result was found by Martin *et al*. [17, 18] using industrial food residuals as a substrate for pectinase production by *Penicillium* sp. EGC5. Their results showed a PG production peak on the 8th day of cultivation. In contrast of that finds Schwan and Rose [2] reported that the maximum production of PG in *Kluyveromyces marxianus* occurred only during cell growth and was no longer detected when the culture reached the stationary phase. Also and Phutela *et al*. [4] reported maximum production of PME and PG by *Aspergillus fumigatus* on solid substrate after 2 days of incubation (48 h); however, there was a decline in enzymatic enzymes activity on the 5th day suggesting the repression of pectinolytic enzyme synthesis or the inhibition of their activity by one or more metabolic products of enzymatic pectin degradation. According to those results the optimum period for enzymatic production varied widely according to the microorganism and culture medium used.

The optimum pH found for *C. cladosporioides* in this study was within the range established for other fungi, which typically have a maximum activity under acidic conditions [4]. Castilho *et al*. [18, 19] evaluated the influence of extraction parameters on the activity of pectinases from *Aspergillus niger* obtained under solid state fermentation. Their work showed that the most suitable solvent for pectinase extraction was acetate buffer at pH 4.0.

The purification index obtained for the purification steps was significant when compared with the results of other authors [20, 21] and high enzymatic activity and a higher rate of purification was found in benzoate buffer

(pH 4.0) P1 fraction. It was possible to obtain a partial purification of PME and PG enzymes of *C. cladosporioides* using $(NH_4)_2SO_4$, which was added to the supernatant remaining after the enzymatic extraction step and yielded 108•7% for PME and 10•5% for PG on P3 fraction. For the same fraction the purification index were 14•2 and 1•4, respectively for PME and PG.

The rResults presented in this present study are the first report on pectinases from *C. cladosporioides* and demonstrated that the ideal conditions for production and pre-purification of PME and PG were 10 days of incubation, extraction with benzoate buffer pH 4.0 and precipitation with 60% of $(NH_4)_2SO_4$ saturation. To the best of the authors knowledge, this is the first report on the production of pectinases by *C. cladosporioides*

## ACKNOWLEDGEMENTS

The authors thank the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Apoio à Pesquisa do Estado de Minas Gerais (FAPEMIG) for financial support and scholarships.

## REFERENCES

1. Jayani, R.S., S. Saxena and R. Gupta, 2005. Microbial pectinolytic enzymes: a review. Process Biochemistry, 40: 2931-2944.
2. Schwan, R.F. and A.H. Rose, 1994. Poligalacturonase production by Kluyveromyces *marxianus*: effect of medium composition. Journal of Applied Bacteriology, 76: 62-67.
3. Schwan, R.F., R. Cooper and A.E. Wheals, 1997. Endo polygalacturonase secretion by Kluyveromyces marxianus and other cocoa pulp-degrading yeasts. Enzyme and Microbial Technology, 4: 234-244.
4. Phutela, U., V. Dhuna and S. Sandhu, 2005. Pectinase and polygalacturonase production by a thermophilic Aspergillus fumigatus isolated from decomposting orange peels. Brazilian Journal of Microbiology, 36: 63-69.
5. Kashyap, D.R., P.K. Vohra, S. Chopra and R. Tewari, 2001. Applications of pectinases in the commercial sector: a review. Bioresource Technology, 77: 215-227.
6. Karam, N.E. and A. Belarbi, 1995. Detection of polygalacturonase and pectinesterases in lactic acid bacteria. World Journal of Microbiology and Biotechnology, 11: 559-563.
7. Maldonado, M.C., S. Cáceres, E. Galli and A.R. Navarro, 2002. Regulation of the production of polygalacturonase by Aspergillus niger. Folia Microbiologica, 47: 409-412.
8. Fujii, K., T. Sugimura and K. Nakatake, 2010. Ascomycetes with cellulolytic, amylolytic, pectinolytic and mannanolytic activities inhabiting dead beech (Fagus crenata) trees. Folia Microbiologica, 55: 29-34.
9. Sule, I.O. and G.P. Oyeyiola, 2012. Fungal population in the root region of cassava cultivar TMS 30572. World Journal of Agricultural Sciences, 8: 73-79.
10. Pereira, R.T.G., L.H. Pfenning and H.A. Castro, 2005. Characterization and dynamic of colonization of Cladosporium cladosporioides (Fresen.) de Vries in coffee fruits (Coffea arabica L.). Ciencia e Agrotecnologia, 29: 1112-1116.
11. Silva, C.F., L.R. Batista, L.M. Abreu, E.S. Dias and R.F. Schwan, 2008. Succession of bacterial and fungal communities during natural coffee (Coffea arabica) fermentation. Food Microbiology, 25: 951-957.
12. Buescher, R.W. and R.J. Furmanski, 1978. Role of pectinesterase and polygalacturonase in the formation of woolliness in peaches. Journal of Food Science, 43: 264-266.
13. Silva, E.G., M.F. Borges, C. Medina, R.H. Piccoli and R.F. Schwan, 2005. Pectinolytic enzymes secreted by yeasts from tropical fruits. FEMS Yeast Research, 5: 859-865.
14. Gummadi, S.N. and T. Panda, 2003. Purification and biochemical properties of microbial pectinases: a review. Process Biochemistry, 38: 987-996.
15. Dias, D.R., D.M. Vilela, M.A.P. Silvestre and R.F. Schwan, 2008. Alkaline protease from Bacillus sp. isolated from coffee bean grown on cheese whey. World Journal of Microbiology and Biotechnology, 24: 2027-2034.
16. Ferreira, D.F., 2008. Sisvar: a program for statistical analysis and teaching. Revista Symposium, 6: 36-41.
17. Mmdula, S. and R. Am'thamj, 2011. Pectinase production in solid state fermentation by Aspergillus niger using orange peel as substrate. Global Journal of Biotechnology & Biochemistry, 6: 64-71.

18. Martin, N., S.R. Souza, R. Silva and E. Gomes, 2004. Pectinase production by fungal strains in solid-state fermentation using agro-industrial bioproduct. Brazilian Archives of Biology and Technology, 47: 813-819.
19. Castilho, L.R., R.A. Medronho and T.L.M. Alves, 2000. Production and extraction of pectinases obtained by solid state fermentation of agro-industrial residues with Aspergillus niger. Bioresource Technology, 71: 45-50.
20. Yadav, S., G. Anand, A.K. Dubey and D. Yadav, 2012. Purification and characterization of an exo-polygalacturonase secreted by Rhizopus oryzae MTCC 1987 and its role in retting of Crotalaria juncea fibre. Biologia, 67: 1069-1074.
21. Thakur, A., R. Pahwa, S. Singh and R. Gupta, 2010. Production, purification and characterization of polygalacturonase from Mucor circinelloides ITCC 6025. Enzyme Research, pp: 1-7.