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# Heterologous expression of a *Penicillium purpurogenum* pectin lyase in *Pichia pastoris* and its characterization

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ABSTRACT

Lignocellulose is the major component of plant cell walls and it represents a great source of renewable organic matter. One of lignocellulose constituents is pectin. Pectin is composed of two basic structures: a 'smooth' region and a 'hairy' region. The 'smooth' region (homogalacturonan) is a linear polymer of galacturonic acid residues with $\alpha$-(1→4) linkages, substituted by methyl and acetyl residues. The 'hairy' region is more complex, containing xylogalacturonan and rhamnogalacturonans I and II. Among the enzymes which degrade pectin (pectinases) is pectin lyase (E.C. 4.2.2.10). This enzyme acts on highly esterified homogalacturonan, catalysing the cleavage of $\alpha$-(1→4) glycosidic bonds between methoxylated residues of galacturonic acid by means of $\beta$-elimination, with the formation of 4,5-unsaturated products. In this work, the gene and cDNA of a pectin lyase from *Penicillium purpurogenum* have been sequenced, and the cDNA has been expressed in *Pichia pastoris*. The gene is 1334 pb long, has three introns and codes for a protein of 376 amino acid residues. The recombinant enzyme was purified to homogeneity and characterized. Pectin lyase has a molecular mass of 45 kDa as determined by SDS-PAGE. It is active on highly esterified pectin, and decreases 40 % the viscosity of pectin with a degree of esterification ≥85 %. The enzyme showed no activity on polygalacturonic acid and pectin from citrus fruit 8 % esterified. The optimum pH and temperature for the recombinant enzyme are 6.0 and 50 °C, respectively, and it is stable up to 50 °C when exposed for 3 h. A purified pectin lyase may be useful in biotechnological applications such as the food industry where the liberation of toxic methanol in pectin degradation should be avoided.
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## Introduction

Lignocellulose is the major component of plant cell walls and it represents a great source of renewable organic matter. There is considerable interest in the exploitation of lignocellulosic materials as a source of food, fuels, and chemical feedstocks (Malherbe & Cloete 2002). It can be converted to value-added products through saccharification by lignocellulolytic enzymes. To achieve an efficient degradation process, a good understanding of the properties and specificity of these enzymes is required (Malherbe & Cloete 2002). Lignocellulose is composed of lignin, pectin, cellulose, and hemicelluloses. The focus of this work is on pectin, the least studied component, and of its biodegradation. Pectin is composed of two

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basic structures: a ‘smooth’ region and a ‘hairy’ region. The ‘smooth’ region (homogalacturonan) is a linear polymer of galacturonic acid residues with α-(1→4) linkages, substituted by methyl and acetyl residues. The ‘hairy’ region is more complex, containing xylogalacturonan and rhamnogalacturonans I and II. The main chain of rhamnogalacturonan I contains repeats of α-(1→4)-D-galacturonate and α-(1→2)-L-rhamnopyranose. The rhamnose residues are highly substituted with polysaccharides such as arabinan, galactan or arabinogalactan. Rhamnogalacturonan II has a backbone of homogalacturonan which is highly substituted by at least twelve different monosaccharides (Scheller *et al.* 2007).

The biodegradation of pectin is very complex and it is performed by a set of enzymes known as ‘pectinases’. Pectinases are used in the food industry (juice, wine, coffee, baby food, and olive oil production) (Kashyap *et al.* 2001), in textile processing (Hoondal *et al.* 2002), in water treatment (Tanabe *et al.* 1986), and in the pulp and paper industry (Bajpai 1999) among other applications. The group of enzymes involved in the degradation of the ‘smooth region’ (homogalacturonan) include de-esterifying enzymes i.e. pectin methyl esterases (E.C. 3.1.1.11) and pectin acetyl esterases (E.C. 3.1.1.6) which remove methoxyl and acetyl residues, respectively, yielding polygalacturonic acid. The other subclass of homogalacturonan-degrading enzymes are broadly termed as depolymerases; they break the α-1,4-linkages of the main chain either by hydrolysis (polygalacturonases, E.C. 3.2.1.15) or via a β-elimination mechanism (pectate lyases, E.C. 4.2.2.2, and pectin lyases, E.C. 4.2.2.10) (Yadav *et al.* 2009).

Pectin and pectate lyases show different substrate specificities. Pectin lyases degrade pectin polymers directly by a mechanism resulting in the formation of 4,5-unsaturated oligogalacturonides while pectate lyases, utilizing a similar mechanism, use polygalacturonic acid as substrate and are dependent on $Ca^{+2}$; thus, when acting on pectin, pectate lyases require the previous action of the de-esterifying enzymes (Lara-Márquez *et al.* 2011).

Pectin lyases (PL) are produced by a variety of microorganisms. Some PLs have been reported from bacteria and yeasts, but the majority of the PLs studied are produced by fungi (Yadav *et al.* 2009). Based on amino acid sequence similarities, PLs have been assigned to family 1 of the polysaccharide lyases according to the CAZY classification (Cantarel *et al.* 2009). The three-dimensional structure of PL has been determined (Mayans *et al.* 1997; Vitali *et al.* 1998); the enzyme is composed mainly of three parallel beta-sheets which coil into a right-handed cylinder called ‘parallel beta helix’ with a substrate binding cleft which includes a network of histidine and tryptophan residues. PLs have also been described in plants such as *Arabidopsis* (Cao 2012).

PLs have several biotechnological applications. It can be used in the fruit juice industry, since it degrades pectin without liberating methanol, a toxic product (Yadav *et al.* 2009) and also for the improvement of wine (Wu *et al.* 2007). Numerous industrial applications using pectinase preparations containing PL have been reviewed by Hoondal *et al.* (2002).

In this work, PL produced by the soft rot fungus *Penicillium purpurogenum* is studied. This fungus grows well on pectin-containing substrates such as sugar beet pulp and it secretes a variety of pectinolytic enzymes to the medium, among them one PL. The gene of this PL has been sequenced, expressed in *Pichia pastoris* and the enzyme has been characterized. The availability of a heterologously expressed PL may be useful for biotechnological applications of pectin lyases.

## Materials and methods

### *Microbial strains and culture conditions*

*Penicillium purpurogenum* ATCC strain MYA-38 was grow in Mandel’s medium as described by Hidalgo *et al.* (1992) using 1 % sugar beet pulp (obtained from IANSA, Chile) as carbon source. *Escherichia coli* TOP10 F′ was used for cloning of the cDNA of PL from *P. purpurogenum*. Heterologous expression was performed in *Pichia pastoris* GS115, supplied in the EasySelect™ Pichia Expression Kit (Invitrogen). The instructions from the manufacturers were followed in all procedures utilizing kits in this work.

### *DNA and RNA preparation*

Genomic DNA from *Penicillium purpurogenum* was extracted with the GeneJET™ Genomic DNA Purification kit (Fermentas) from fungus grown on 1 % glucose. Fungus grown on sugar beet pulp (1 %) and washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4) was utilized for total RNA extraction by means of the RNeasy Plant Mini Kit (Qiagen). mRNA was extracted from total RNA using the Absolutely mRNA kit (Stratagene). cDNA was prepared from mRNA by means of the First Choice RLM-RACE kit (Ambion).

### *Gene and cDNA sequencing strategy*

An analysis of the secretome of the fungus grown on sugar beet pulp by 2D-electrophoresis allowed the recognition of numerous spots. Peptide analysis of the spots was performed by mass spectrometry (nanoLC–ESI–MS/MS) and peptide matches and similarity searches were conducted with MASCOT server v2.2 and BLASTp (Navarrete *et al.* 2012).

The analysis of one of the spots displayed several peptides related to pectinases, such as pectin lyase. One of the peptides obtained, IVSGASNIIIQNIAITDINPK, when subjected to a BLAST search, showed 100 % identity to a peptide from a putative pectin lyase from *Neosartorya fischeri*. Using this last sequence, a multiple alignment was performed (using ClustalW) with other similar pectin lyase sequences. Regions of highly conserved sequence were used to design degenerate primers JE-CO1, JE-CO2, JE-CR1, and JE-CR2 (see Table 1). A nested PCR using these primers and genomic DNA as template yielded a single product of about 400 bp which was sequenced. Based on this sequence, primers PEC-E3 and PEC-I3 were designed and utilized (along with the antisense primers provided by the kit) for a 3′ RACE using the kit provided by Ambion (FirstChoice RLM-RACE), with cDNA as template. Thus, the complete downstream sequence was obtained. In order to obtain the 5′ region of the gene, the Genome Walker kit (Clontech) was used; primers RGWE and RGWI were designed and utilized with the kit primers AP1 and AP2 for nested PCR. A

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