Identification and Characterisation of a Pectinolytic Enzyme from *Paenibacillus xylanolyticus*

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Pectinolytic enzymes play an important role in the processing of lignocellulosic materials because of their ability to improve the access of cellulases to their substrate by removing pectins. The strain *Paenibacillus xylanolyticus* 2-6L3 was isolated from mature compost obtained from agro-industrial wastes, and the enzyme pectate lyase from *P. xylanolyticus* 2-6L3, named PaenxylPel, was partially purified and subjected to structural and functional characterisation. The enzyme exhibited an optimum temperature between 60 and 70 °C and optimal pH value of 9.0 for its pectinase activity on pectin from citrus fruit. PaenxylPel showed a thermoresistance and pH resistance higher than those of other pectate lyases so far described, with half-lives of 48 and 24 h at 60 and 70 °C, respectively, a retention of around 80% of activity after 96 h at 40 and 50 °C, and a half-life of about 15 days at pH 8.0. PaenxylPel followed Michaelis-Menten kinetics toward pectin from citrus fruit, pectin from sugar beet pulp, high-ester pectin extracted from citrus peel (> 50% esterified), and polygalacturonic acid (PLA). The ability to act on both PLA and highly methylated pectins, together with a double peak in the graph of optimum pH at pH 5 and 9, suggest that pectate lyase from *P. xylanolyticus* shows an unusual activity, combining traits of pectate lyase and pectin lyase. This is the first manuscript on the pectinolytic activity of *P. xylanolyticus*.

Keywords: Paenibacillus; Pectate lyase; Pectin

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INTRODUCTION

Pectinolytic enzymes, or pectinases, are a group of enzymes involved in the breakdown of pectin, a polysaccharide present in the cell walls of plants, fruits, and vegetables. In plants, they are involved in cell wall extension and growth (Ward and Moo-Young 1989), the softening of some plant tissues during maturation, and the decomposition of dead plant materials (Sakai 1992). They are also responsible for the spoilage of fruits and vegetables (Collmer and Keen 1986; Lang and Dörnenburg 2000).

Pectinolytic enzymes are broadly distributed in plants and are also produced by both prokaryotic and eukaryotic microorganisms, mostly fungi that synthesize acid pectinases and yeasts (Jayani *et al.* 2005; Arunachalam and Asha 2010). The majority of studies are regarding pectinases from *Erwinia* and *Bacillus* within the bacteria, pectinases from *Aspergillus* within fungi, and pectinases from *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* within yeasts. Many pectic enzymes are recombinantly expressed in *Escherichia coli* (Soriano *et al.* 2000); Wang *et al.* 2011; Damak *et al.* 2013).

Pectinolytic enzymes have an important role in the processing of lignocellulosic materials. In fact, bioconversion of agro-industrial cellulosic wastes, such as orange peels, citrus peels, rotten bananas, and grapefruit peels, requires synergistic actions of both pectinolytic and cellulolytic enzymes (Adeleke *et al*. 2012). Regarding ethanol production from cellulosic materials containing pectin, pectinolytic enzymes have been applied to pretreatment of various lignocellulosic biomasses to improve ethanol production (Spagnuolo *et al*. 1997; Hossain *et al.* 2011). In particular, Berlin *et al.* (2007) reported that pectins limit the access of cellulases to their substrate and demonstrated that improvement in hydrolysis is achieved using pectinases. Edwards *et al.* (2011) reported the engineering of an *Escherichia coli* strain with pectate lyase from *Erwinia chrysanthemi* and demonstrated that the recombinant strain was able to produce ethanol with a yield two-fold higher than that of the wild-type strain.

Among pectinolytic enzymes, lyases, classified into polysaccharide lyase family 1 (CAZY 2014), are depolymerising enzymes that cleave the α 1-4 glycosidic linkages in polygalacturonate or pectin by a trans-elimination reaction generating 4,5-unsaturated oligogalacturonates (Jayani *et al*. 2005). Pectin lyases or endo-polymethylgalacturonate lyase (EC 4.2.2.10) preferentially cleave glycosidic bonds in highly methyl esterified substrates, are stimulated by cations, and generally have an optimum pH close to 5.5 (Pedrolli and Carmona 2009); pectate lyases are specific for demethylated or low-esterified forms of pectin, have an optimum pH from 8.0 to 9.8 (Nagel and Wilson 1970), and have an absolute requirement for calcium ions (Jayani *et al.* 2005). Depending on the mode of action, pectate lyases are classified as exo-PL (EC 4.2.2.9) that catalyse the substrate cleavage from the non-reducing end and are specific for pectate, but not for citrus pectin; or endo-PL (EC 4.2.2.2), which are more abundant than exo-PL and cleave the chain randomly (Jayani *et al*. 2005). Pectate most generally is used for endo pectate lyase studies because the enzymatic activity on pectate is higher than that on commercial high-methoxyl pectin (about 70% esterified).

In this manuscript, a pectate lyase produced by a strain of *Paenibacillus xylanolyticus* isolated from raw compost materials was characterised determining optimal temperature and pH for its activity on pectin from citrus fruit, its Michaelis-Menten constants toward pectin from citrus fruit, pectin from sugar beet pulp, high-ester pectin extracted from citrus peel (> 50% esterified), and polygalacturonic acid (PLA), thermoresistance and pH resistance.

EXPERIMENTAL

Isolation and Selection of Pectinolytic Bacteria

Pectinolytic microorganisms were isolated in the Campania region from mature compost obtained from agro-industrial wastes consisting of pomace with kernel (65%), liquid sewage sludge from industrial processing of potatoes and carrots (22%), and borland from the distillation of molasses (13%) (Pepe *et al*. 2013). Pectinolytic isolates were detected on solid medium composed of 2 g L^{-1} citrus pectin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 1 g L^{-1} yeast extract, 50 mL L^{-1} standard salt solution (in 1 L: 5 g K_2HPO_4; 2.5 g $MgSO_4$; 2.5 g NaCl; 0.05 g $Fe_2(SO_4)_3$; and 0.05 g $MnSO_4$), with 1 mL L^{-1} trace elements solution (in 1 L: 0.05 g $K_2MoO_4 \cdot 5H_2O$; 0.05 g $Na_2B_4O_7 \cdot 10H_2O$; 0.05 g $Co(NO_3)_2 \cdot 6H_2O$; 0.05 g $MnSO_4$; 0.05 g $CdSO_4$; 0.05 g $ZnSO_4 \cdot 7H_2O$; 0.05 g $CuSO_4 \cdot H_2O$; and 0.1 g $FeCl_3$), and 20 g L^{-1} bacteriological agar, pH 7.0. After incubation

at 28 °C for 7 d, the plates were flooded with a 1% hexadecyltrimethylammonium bromide (Sigma-Aldrich) solution. After 20 to 30 min, pectinase activity was detected by the presence of a clear halo surrounding the colony (Egamberdiyeva 2007; Pepe *et al*. 2013).

In order to perform a preliminary comparison of the pectinase production by the selected pectinolytic strains, a semi-quantitative agar spot method was carried out. Briefly, bacterial cells were suspended in Ringer solution (Sigma-Aldrich) at a concentration approximately $1.5x10^8$ CFU mL^{-1} and spotted on solid agar medium in triplicate. The enzymatic activity was recorded as the "indices of relative enzyme activity = diameter of clearing or halo zone/colony diameter" (Saini and Tewari 2012), and pectinase activity was reported as I_{PEC} (index of pectinase enzyme activity).

Screening on Liquid Media

The liquid medium adopted for analysis of pectinase production levels contained 0.5 g L^{-1} pectin from citrus fruit (Sigma-Aldrich), 1 g L^{-1} NH_4NO_3, 1 g L^{-1} yeast extract, 50 mL L^{-1} standard salt solution (as described above), and 1 mL L^{-1} trace elements solution (also as described above).

Inoculum Preparation and Submerged Fermentation

The bacterial strains were pre-inoculated by dissolving a single colony in 3 mL of liquid medium having the composition described in the previous paragraph and incubated overnight at 37 ºC. Cultures were further grown in 100-mL plugged Erlenmeyer flasks, each containing 20 mL of medium and inoculated with volumes of pre-inoculum corresponding to 0.1 optical density (O.D.).

The inocula were incubated at 37 °C on a rotary shaker at 225 rpm for 48 h. From time to time, samples of the liquid culture were withdrawn and used to measure $O.D._{600nm}$ and extracellular pectinase activity. The results of these determinations reported in the figures and tables correspond to mean values of three independent experiments, each of which was performed in duplicate.

Molecular Identification of Selected Pectinolytic Strains

Total genomic DNA of selected strains was extracted and purified using InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA) according to the supplier's recommendations. Two synthetic oligonucleotide primers, fD1 and rD1, were used to amplify the 16S rRNA gene (Pepe *et al.* 2011). The PCR amplification fragment was purified in agarose 1.5% (wt/vol) by gel electrophoresis using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. The DNA sequencing was conducted at Primm srl (Milan, Italy). The DNA sequences were determined and analysed as previously reported (Amore *et al*. 2013) and compared to the GenBank nucleotide data library using the Blast software (NCBI 2014) to determine their closest phylogenetic relatives.

The partial 16S rRNA gene sequences of the two selected strains 2-6L3 and 1-6H5 were deposited in the GenBank database under accession numbers KF923402 and KF923403, respectively.

Determination of Protein Concentration

Protein concentrations of crude enzyme preparations were determined by the Bradford method using Bio-Rad reactive (München, Germany) following the procedure suggested by the supplier. Bovin serum albumin (BSA) was used for the standard curve.

Pectinase Assay

Pectinase activity was determined using pectin from citrus fruit as a substrate as described in Okafor *et al.* (2010). The reaction mixture, containing equal amounts (0.5 mL) of 1% pectin prepared in sodium acetate buffer 0.05 M (pH 5.5) and suitably diluted crude enzyme, was incubated at 50 °C in a water bath for 30 min. The reaction was stopped with 1.0 mL of dinitrosalicylic acid solution (Miller 1959), and the mixture was boiled for 5 min and cooled. The absorbance at 540 nm was measured using a spectrophotometer. The amount of reducing sugar released was quantified using galacturonic acid as a standard. The enzyme activity (IU) was calculated as the amount of enzyme required to release one micromole (1 µmol) equivalent of galacturonic acid per minute under the assay conditions.

Enzyme Identification

Protein fractionation

Culture media were harvested based on the optimal pectinase production time by sedimentation of cells through centrifugation at 5,000 ×g for 10 min at 4 °C. Secreted proteins in the supernatant were filtered through Whatman filter paper. Filtrates were concentrated with ultrafiltration devices with a molecular weight cut-off of 10 kDa (Millipore S.p.A., Vimodrone, Italy).

Zymogram analyses

To identify the proteins putatively responsible for pectinase activity of the 2-6L3 strain, zymogram analysis was performed following a method similar to that reported by Schneider *et al*. (2010) with some modifications. Semi-denaturing gel electrophoresis was carried out by loading non-denatured and non-reduced samples on a SDS polyacrylamide gel (12.5%) with 0.1% pectin from citrus fruit. After electrophoresis, the proteins in the gel were renaturated *in situ* by removing SDS through washing in Tris-HCl and isopropanol buffers. After renaturation, the zymogram was incubated at 40 °C for 3 h to allow the enzymatic degradation of pectin. Subsequently, the gel was stained with 0.05% Ruthenium red for 10 min and destained with water until pectinase activity could be detected as colourless bands against a purple background.

Protein identification by mass spectrometry

Slices of interest from the semi-denaturing PAGE were cut and digested *in situ* after extensive destaining with 0.1 M NH_4HCO_3, pH 7.5 and acetonitrile, reduction of disulphide bonds for 45 min in 100 µL of 10 mM dithiothreitol, 0.1 M NH_4HCO_3, pH 7.5, and carboxyamidomethylation of thiols for 30 min in the dark by addition of 100 µL of 55 mM iodoacetamide dissolved in the same buffer. Enzymatic digestion was performed by adding to each slice 100 ng of proteomic-grade trypsin in 10 µL of 10 mM NH_4HCO_3, pH 7.5 for 2 h at 4 °C. The buffer solution was then removed, and 50 µL of 10 mM NH_4HCO_3, pH 7.5 was added and incubated for 18 h at 37 °C. Peptides were extracted with 20 µL of 10 mM NH_4HCO_3, 1% formic acid, and 50% acetonitrile at room temperature.

Peptide mixtures were filtered on 0.22-µm PVDF membranes (Millipore) and analysed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on a 6520 Accurate-Mass Q-TOF LC/MS (liquid chromatography mass spectrometry) System (Agilent Technologies, Palo Alto, CA) equipped with a 1200 high-performance liquid chromatography (HPLC) system and a chip cube (Agilent Technologies). After loading, the peptide mixture was concentrated and washed in in a 40-nL enrichment column (Agilent Technologies), with 0.1% formic acid in 2% acetonitrile as the eluent.

The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies) at a flow rate of 400 nL/min, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 80% over 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 m/z) followed by MS/MS scans of the five most abundant ions in each MS scan. The MS/MS spectra were measured automatically when the MS signal was over the threshold of 50,000 counts. Double and triple charged ions were preferably isolated and fragmented over single charged ions. Raw data from nano LC-MS/MS analyses transformed in *mz.data* format and were used to query non-redundant protein databases with a licensed version of MASCOT 2.1 (Matrix Science, Boston, USA). Additional search parameters consisted of a peptide mass tolerance set at 10 ppm, and a fragment mass tolerance of 0.6 Da, which will allow for up to 3 missed cleavages, carbamidomethylation of cysteines as fixed modification, oxidation of methionine, and cyclisation of N-term Q to pyro-Glu as variable modifications. Only doubly and triply charged ions were considered. The ions' score was -10 log(P), where P is the probability that the observed match is a random event. The threshold above which the individual ion's score indicated identity or extensive homology ($p < 0.05$) can vary from search to search. In our searches, on average, individual ion scores > 25 indicated identity or extensive homology ($p < 0.05$). Protein scores are derived from ion scores as a nonprobabilistic basis for ranking protein hits (Matrix Science 2013). Trypsin, dithiothreitol, iodoacetamide, and NH_4HCO_3 were purchased from Sigma-Aldrich. Trifluoroacetic acid (TFA)-HPLC grade was from Carlo Erba (Milan, Italy). All other reagents and solvents were of the highest purity available from Baker.

Protein Purification

Proteins secreted by *P. xylanolyticus* after 24 h of growth were precipitated from the filtered medium (after cell removal by centrifugation at 5,000 ×g for 10 min) with 100% ammonium sulphate at 4 °C and centrifugation at 10,000 ×g for 30 min. The precipitate was resuspended in 0.02 M Tris-HCl pH 7.5 and loaded on a HiTrap Phenyl FF high sub column (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A (0.02 M Tris–HCl, 1.5 M $(NH_4)_2SO_4$, pH 7.5), and the proteins were eluted isocratically with buffer B (0.02 M Tris-HCl pH 7.5). Fractions containing activity were combined and concentrated on an Amicon (10 KDa MW cutoff).

Effects of pH and Temperature

The optimum temperature was determined by incubating the enzyme (30 min) at temperatures ranging from 30 to 70 °C and using the pectinase assay procedure described in "Pectinase Assay" paragraph.

The thermal stability of the enzyme was studied by incubation at 40, 50, 60, and 70 °C. Enzyme samples were withdrawn periodically (every 30 min) and assayed to determine the residual enzyme activity, performing incubation (30 min) at the optimum temperature (60 °C) using the pectinase assay procedure described above.

To determine the optimum pH, the substrate, pectin from citrus fruit, was dissolved in 20 mM sodium acetate/HCl (pH 2 to 3); 20 mM sodium acetate (pH 4 to 5); 20 mM sodium phosphate (pH 6 to 8) and 20 mM sodium carbonate/bicarbonate (pH 9 to 11) as reported by Sukhumsiirchart *et al.* (2009). The incubation (30 min) was performed at the optimum temperature (60 °C).

The stability of the enzyme at various pH values was determined by incubating the enzyme at the desired pH (2.0 to 11.0) at room temperature. Enzyme samples were withdrawn periodically and assayed to determine the residual enzyme activity, performing incubation (30 min) at 60 °C using the pectinase assay procedure described above.

Substrate Specificity

The activity of the pectate lyase towards polygalacturonic acid was tested under standard assay conditions (Miller 1959). First, 50 µL of suitably diluted enzyme solution was incubated in the presence of 450 µL of 0.25% (w/v) polygalacturonic acid (PLA; Sigma-Aldrich) in 50 mM Tris-HCl buffer (pH 8.0) for 5 min. The reaction was stopped with 0.75 mL of dinitrosalicylic acid solution and subsequently boiled for 5 min, cooled on ice, centrifuged, and the absorbance read at 540 nm. The specific enzyme activity was calculated as the amount of enzyme required to release one micromole (1 µmol) equivalent of galacturonic acid per minute under the assay conditions.

Pectate lyase activity was also determined using pectins with different degrees of esterification: low ester pectin extracted from citrus peel, high ester pectin extracted from citrus peel, partly amidated low ester pectin from citrus peel, and pectin from sugar beet pulp (Megazyme International Ireland, Co. Wicklow, Ireland) following manufacturer instructions.

The activity was monitored spectrophotometrically by measuring the increase in absorption at 235 nm of the reaction mixture. First, 1 mL sample of substrate solution, 0.1mM $CaCl_2$, and 0.1% substrate in 20 mM Tris-HCl (pH 8) was pre-incubated for 5 min at 40 °C; then, 0.5 mL of suitably diluted enzyme was added and incubated at 40 °C for 15 and 30 min. One Unit of enzyme activity is the amount of enzyme required to release one micromole of product in 1 min under the described assay conditions.

Determination of V_{max} and K_M

For determination of the Michaelis-Menten constants, K_M, and V_{max}, the activity assay was performed at substrate concentrations from 0.025 gL^{-1} to 0.4 gL^{-1} of pectin from citrus fruit, pectin from sugar beet pulp, and PLA; and from 0.025 gL^{-1} to 10 gL^{-1} of high-ester pectin extracted from citrus peel. The enzyme activities were measured using the described assay procedure. The experiments were performed in triplicate and the reported values are the average of the values of three experiments.

RESULTS AND DISCUSSION

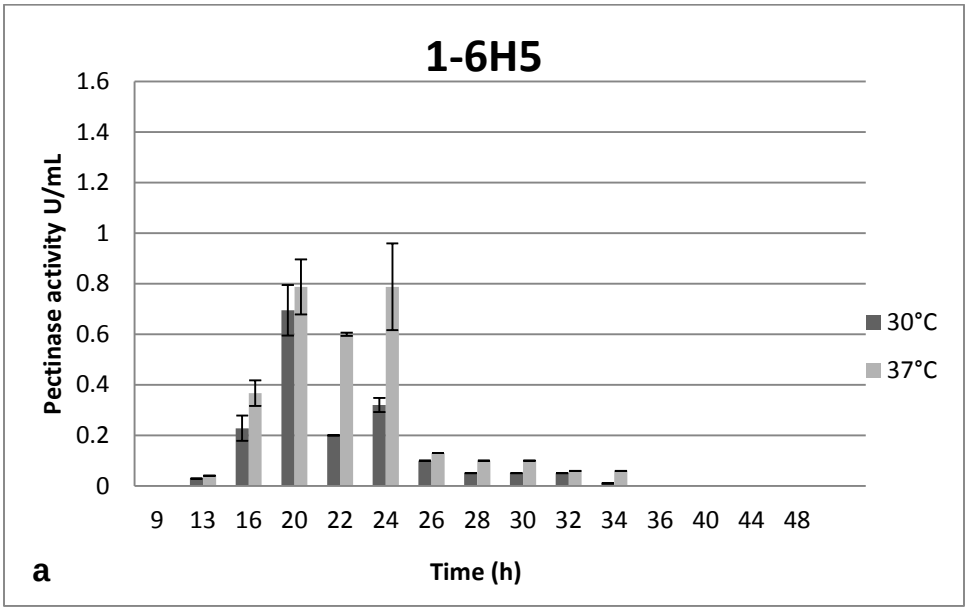
Selection and Identification of Pectinolytic Microorganisms

Seventy-two bacterial strains isolated from mature compost obtained from agro-industrial wastes were screened for their pectinolytic activity on solid medium. From this preliminary selection, two pectinolytic strains, that showed the higher activity with an I_{PEC} values ≥ 10, were chosen for further characterisation.

The two selected strains 2-6L3 (I_{PEC} = 12) and 1-6H5 (I_{PEC} = 10) were identified by sequencing of the 16S rRNA gene. Results showed that both the strains belonged to *Paenibacillus xylanolyticus* (accession number KF923402 and KF923403).

Screening of Pectinolytic Microorganisms in Liquid Medium

A further screening of the two selected microorganisms, *P. xylanolyticus* 1-5H6 and *P. xylanolyticus*, 2-6L3 was performed by cultivating each in liquid medium containing 0.2% pectin from citrus fruit and assaying culture supernatants for pectinase activity production. Time courses of pectinase activity production by the strains *P. xylanolyticus* 1-5H6 (Fig. 1a) and *P. xylanolyticus* 2-6L3 (Fig. 1b) were monitored at 30 °C and 37 °C. Increasing the growth temperature from 30 to 37 °C led to a 2-fold and 6-fold increase of pectinase activity production at 24 h for *P. xylanolyticus* 1-5H6 (Fig. 1a) and *P. xylanolyticus* 2-6L3 (Fig. 1b), respectively. No difference in pectinase activity level was observed by changing pectin concentration in the culture medium (data not shown).



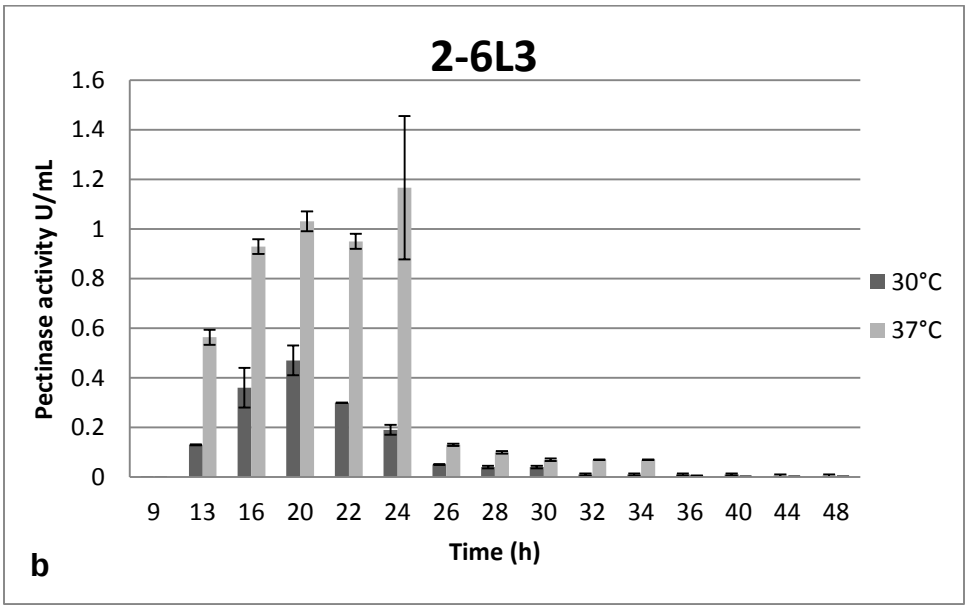


Fig. 1. Time course of production of pectinase activity assayed on pectin from citrus fruit for the strains 1-5H6 (**a**) and 2-6L3 (**b**) at 30 and 37 °C

P. xylanolyticus 2-6L3 was found to be the more productive strain with a maximum of activity of 1.16 UmL^{-1} between 20 and 24 h of growth (Fig. 1b), while the *P. xylanolyticus* 1-5H6 strain showed a maximum activity of 0.78 UmL^{-1} at 24 h (Fig. 1a). Further studies were therefore focused on pectinolytic enzymes from *P. xylanolyticus* 2-6L3. This is the first manuscript on pectinolytic activity of *P. xylanolyticus*. This microorganism was described for the first time by Rivas *et al*. (2005) as a new strain able to hydrolyse xylan. Recent studies were performed on polysaccharides-hydrolysing enzymes of *Paenibacillus* such as endo-glucanase, β-glucosidase, and xylanase (Park *et al*. 2012; 2013a,b), but pectinase has not yet been characterised from *P. xylanolyticus*.

Enzyme Identification

The proteins putatively responsible for pectinase activity of *P. xylanolyticus* 2-6L3 strain were tentatively identified after a fractionation on a semi-denaturing SDS-PAGE containing 0.1% pectin from citrus fruit where samples from the supernatant of the cell cultures were loaded without any denaturing treatment. An activity halo was visualised (Fig. 2a), and in correspondence to this halo, two different gel slices detected on the gel stained by Coomassie Blue (Fig. 2b) were excised and subjected to protein identification after *in situ* digestion and LC-MS/MS analysis of the peptide mixtures. Raw data were used to search the non-redundant NCBI database with no taxonomic restriction, with the MS/MS ion search program on a MASCOT server as described below.

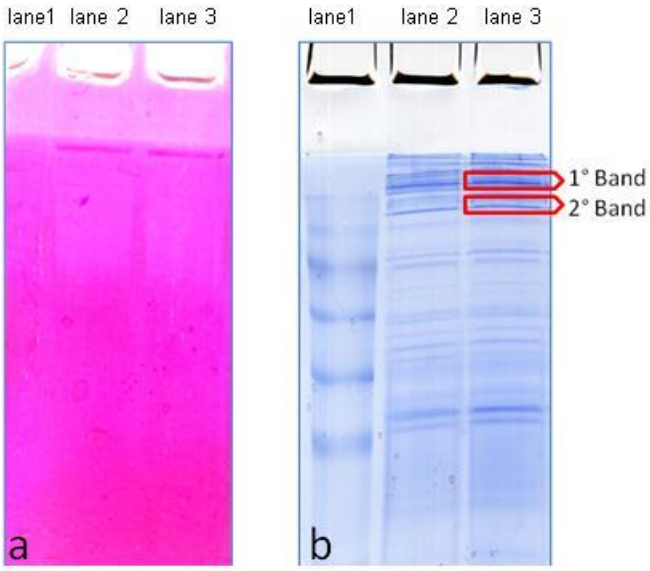


Fig. 2. Analyses of supernatant of *P. xylanolyticus* 2-6L3 strain by SDS-PAGE without denaturating treatments containing 0.1% pectin from citrus fruit stained with 0.05% Ruthenium red for pectinase activity detection (a) and with Coomassie Blue (b). Lane 1, protein molecular weight marker; lanes 2 and 3, culture supernatant

Several proteins were identified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analyses, and among these, three proteins putatively involved in pectin hydrolysis were identified in the first band (Fig. 2b): 9 peptides matched to peptides present in the sequence of a α-N-arabinofuranosidase from *Bacillus pumilus* SAFR-032 (identification number NCBI: gi 157693268); 7 peptides matched to peptides present in the sequence of a β-galactosidase from *Bacillus pumilus* ATCC 7061

(identification number NCBI: gi 194016271), and 4 peptides matched to peptides present in the sequence of a pectate lyase from *Bacillus sp*. (identification number NCBI: gi 4589753).

Enzyme Characterisation

The enzyme with pectate lyase activity from *P. xylanolyticus* 2-6L3, named PaenxylPel, was partially purified (Fig. 3) and subjected to characterisation. The estimated molecular weight deduced from SDS-PAGE was around 37,000 Da (Fig. 3), similar to the molecular masses, from 37 to 44 kDa, so far reported for other bacterial pectate lyases, *i.e.*, those from *Bacillus pumilus* BK2 (Klug-Santner *et al*. 2006), *Erwinia chrysanthemi* (Preston *et al*. 1992), *Bacillus pumilus* BS22, *Bacillus subtilis* BS66, and *Bacillus fusiformis* BS90 (Ouattara *et al*. 2010).

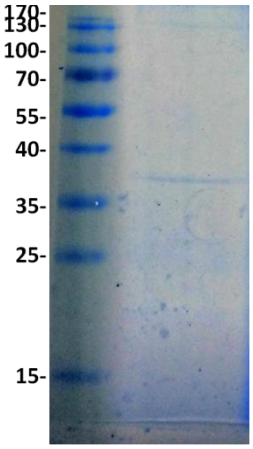


Fig. 3. SDS-PAGE profiling of the purified pectate lyase. Lane 1: protein molecular weight marker; lane 2: purified pectate lyase

An optimum temperature range of 60 to 70 °C was detected (Fig. 4a), in agreement with the optimum temperature found for other bacterial pectate lyases reported in literature (Ouattara *et al*. 2010; Soriano *et al.* 2006). When the effect of pH on the activity of PaenxylPel was investigated (Fig. 4b), an optimal pH of 9.0 was detected, which is in agreement with the optimum pH of other pectate lyases published in the literature (Klug-Santner *et al.* 2006). However, it is worth noting that PaenxylPel shows 60% of activity at pH 5.0. To the best of our knowledge, this is the first report of a pectate lyase with two optimum pHs. Yuan *et al*. (2012) reported recombinant pectate lyase from *Xanthomonas campestris* with optimum pH of 9.0 that retained more than 45% of its maximum activity from pH 3.0 to 12.0.

The thermoresistance and pH resistance of PaenxylPel were also determined (Table 1 and 2).

Table 1. Thermo-resistance of PaenxylPel

Temperature (°C)	$T_{1/2}$ (days)
40	7
50	6
60	2
70	1

Table 2. The pH resistance of PaenxylPel

pH	$T_{1/2}$ (days)
2	1
3	1
4	2
5	5
7	6
8	6
9	15
10	1
11	1

Table 1: Temperature resistance was studied by incubating the enzyme at 40 °C, 50 °C, 60 °C and 70 °C. The samples withdrawn were assayed for residual activity performing incubation (30 min) at 60 °C. Table 2: Results were determined by incubating the enzyme at the desired pH (from 2.0 to 11.0) at room temperature. At different times, samples were withdrawn and immediately assayed for residual activity performing incubation (30 min) at 60 °C.

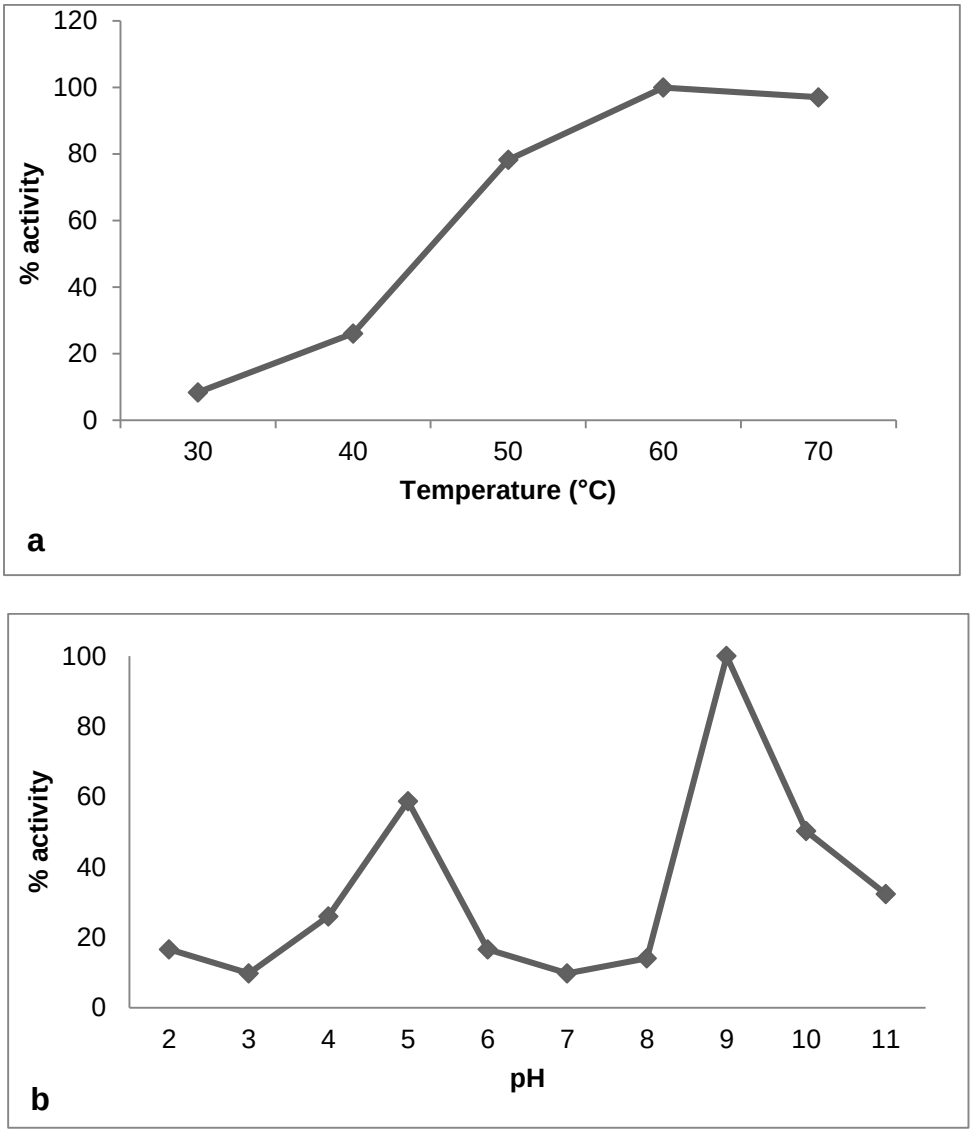


Fig. 4. Effect of temperature (a) and pH (**b**) on the pectate lyase activity of the strain 2-6L3. The pectate lyase activity was measured at the temperatures ranging from 30 to 70 °C, and pH ranging from 2 to 11

PaenxylPel showed a thermoresistance and pH resistance higher than those of the other pectate lyases so far described. In particular, at 60 °C, the half-life of PaenxylPel was 48 h, whilst the pectate lyases PL from *B. pumilus BK2* described by Klug-Santner *et al.* (2006), Apel from *B. subtilis* described by Liu *et al.* (2012), and the three extracellular pectate lyases (Pels) produced by *bacilli* described by Ouattara *et al*. (2010) showed a half-life of about 5, 20, and 30 min, respectively. Moreover, the pectate lyase of this study exhibited a half-life of around 24 h at 70 °C, which was higher than that of pectate lyases described by Liu *et al.* (2012) and Ouattara *et al.* (2010). The thermoresistence of PaenxylPel at 50 °C was higher than other pectate lyases reported in literature; indeed it showed a retention of around 80% of activity after 96 h at 50 °C, whilst pectate lyase from non-sporulating *Amycolata* sp. described by Bruhlmann (1995) lost its activity after 1 h of incubation at 50 °C.

Table 3. Values of K_M and V_{max} of Pectate Lyase from *P. xylanolyticus* and other Bacterial Pectate Lyases towards Pectins

ENZYME	PLA		PECTIN from citrus fruit		PECTIN from sugar beet pulp		HIGH ESTER PECTIN (>50% esterification)		LOW ESTER PECTIN (< 50% esterification)		REFERENCE
	K_m (gL^{-1})	V_{max}	K_m (gL^{-1})	V_{max}	K_m (gL^{-1})	V_{max}	K_m (gL^{-1})	V_{max}	K_m (gL^{-1})	V_{max}	
PL from *Bacillus pumilus BK2*	0.24	0.72 $gl^{-1}min^{-1}$					1.34	0.21 $gl^{-1}min^{-1}$	0.35	0.35 $gl^{-1}min^{-1}$	Klug-Santner *et al*. 2005
PaenxylPel from *Paenibacillus xylanolyticus*	0.20	0.74 $gl^{-1}min^{-1}$	0.10	1,953 $gl^{-1}min^{-1}$	0.36	0.79 $gl^{-1}min^{-1}$	1.07	0.22 $gl^{-1}min^{-1}$			This study
Pel-22from *Bacillus pumilus* BS22	0.045	1.41 $\mu molmin^{-1}mg^{-1}$							0.043	1.45 $\mu molmin^{-1}mg^{-1}$	Ouattara *et al*. 2010
Pel-66 from *Bacillus subtilis* BS66	0.1	667 $\mu molmin^{-1}mg^{-1}$							0.1	714 $\mu molmin^{-1}mg^{-1}$	Ouattara *et al*. 2010
Pel-90 from *Bacillus fusiformis* BS90	0.125	909.9 $\mu molmin^{-1}mg^{-1}$							0.111	1136.36 $\mu molmin^{-1}mg^{-1}$	Ouattara *et al*. 2010
pelA from *Azospirillum irakense*	0.076	23 $\mu molmin^{-1}mg^{-1}$									Bekri *et al*. 1999
PL D from *Xanthomonas campestris*	4.9	30.1 $\mu molmin^{-1}mg^{-1}$									Yuan *et al*. 2012

As regards to pH resistance, the enzyme exhibited a half-life of about 15 days at pH 9.0, which is higher than the pectate lyase described by Klu-Santner *et al.* (2006) that showed a half-life of around 38 h.

The hydrolysing ability of PaenxylPel was tested *versus* pectins with different degrees of esterification, and all these compounds were hydrolyzed by the enzyme. The enzyme followed Michaelis-Menten kinetics towards pectin from citrus fruit, pectin from sugar beet pulp, high-ester pectin extracted from citrus peel (> 50% esterified), and PLA. The values of K_M and V_{max} of pectate lyase from *P. xylanolyticus* 2-6L3 towards the abovementioned pectins are reported in Table 3.

The enzyme showed lyase activities on both polygalacturonic acid and highly methylated pectin with K_M and V_{max} similar to the PL from *Bacillus pumilus* BK2 reported by Klug-Santner *et al.* (2006).

These data suggest that pectate lyase in *P. xylanolyticus* shows an unusual activity by combining traits of pectate lyase and pectin lyase. These results are in agreement with those on other pectate lyases published by Soriano *et al.* (2000) and Boland *et al*. (2010). Soriano *et al.* (2000) studied pelA, from the strain *Paenibacillus sp.* BP-23, actives on pectins of any degree of esterification. Boland *et al.* (2010) described two pectinases from *P. amylolyticus*, PelA and PelB, that are pectate lyases that show a remarkable pectin lyase activity. The peculiar double peak observed in the graph of optimum pH at (pH 9 and 5) for PaenxylPel can be explained by the ability of the enzyme to work as both pectate lyase, typically characterised by optimum alkaline pH, and pectin lyase, typically exhibiting an optimum acidic pH.

CONCLUSIONS

1. In this study, the strain *Paenibacillus xylanolyticus* 2-6L3 was isolated from mature compost obtained from agro-industrial wastes, and the enzyme pectate lyase from *P. xylanolyticus* 2-6L3, named PaenxylPel, was purified to apparent homogeneity and subjected to structural and functional characterisation.
2. PaenxylPel exhibited an optimum temperature of 60 to 70 °C and optimal pH value of 9.0 for its pectinase activity on pectin from citrus fruit.
3. PaenxylPel showed a thermoresistance and pH resistance higher than those of the other pectate lyases so far described, with a half-life of 48 and 24 h at 60 and 70 °C, respectively, a retention of around 80% of activity after 96 h at 40 and 50 °C, and a half-life of about 15 days at pH 8.0.
4. PaenxylPel follows Michaelis-Menten kinetics towards pectin from citrus fruit, pectin from sugar beet pulp, high ester pectin extracted from citrus peel (> 50% esterified), and polygalacturonic acid (PLA).

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