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Synergistic action of thermophilic pectinases for pectin bioconversion into D-galacturonic acid

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ABSTRACT

Large amounts of pectin-rich biomass are generated worldwide yearly, which can be hydrolysed by pectinases to obtain bio-based chemical building blocks such as D-galacturonic acid (GalA). The aim of this work was to investigate thermophilic pectinases and explore their synergistic application in the bioconversion of pectic substrates into GalA. Two exo-polygalacturonases (exo-PGs) from *Thermotoga maritima* (TMA01) and *Bacillus licheniformis* (BLI04) and two pectin methylesterases (PMEs) from *Bacillus licheniformis* (BLI09) and *Streptomyces ambofaciens* (SAM10) were cloned and expressed in *Escherichia coli* BL21 (DE3), purified and fully characterised. These pectinases exhibited optimum activity at temperatures above 50 °C and good stability at high temperature (40–90 °C) for up to 24 h. Exo-PGs preferred non-methylated substrates, suggesting that previous pectin demethylation by PMEs was necessary to achieve an efficient pectin monomerisation into GalA. Synergistic activity between PMEs and exo-PGs was tested using pectin from apple, citrus and sugar beet. GalA was obtained from apple and citrus pectin in a concentration of up to 2.5 mM after 4 h reaction at 50 °C, through the combined action of BLI09 PME with either TMA01 or BLI04 exo-PGs. Overall, this work contributes to expand the knowledge of pectinases from thermophiles and provides further insights into their application in the initial valorisation of sustainable pectin-rich biomass feedstocks.

1. Introduction

Large amounts of pectin-rich agro-industrial biomass such as apple pomace, citrus processing waste and sugar beet pulp are generated, mainly from the sugar and juice industries. A small fraction of this biomass is used as a pectin source for the food industry, whilst most of it is sold as low cost animal feed [1,2]. Pectin is a complex and heterogeneous polymer formed by various substructures including homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) [3,4]. Although the ratio between pectin substructures is variable and depends on the source, in most cases, HG accounts for around 65% of the molecule. HG is formed by a backbone of a α-1,4-D-galacturonic acid (GalA) that can be methylated in the carboxyl groups and acetylated at *O-2* or *O-3* positions. Thus, GalA being one of the main components released after pectin hydrolysis [5,6].

Owing to pectin complexity, several pectinolytic enzymes are involved in its natural degradation. Among the most important are polygalacturonases (PGs) and pectin methylesterases (PMEs) which are the main focus of this study. PGs catalyse the cleavage of (1→4)-α-D-glycosidic bonds releasing oligalacturonates by endo-PGs and monomeric GalA by exo-PGs as products. PMEs catalyse the de-esterification of methyl ester groups in pectin backbones releasing methanol and acting prior to PGs, which in turn prefer non-methylated pectin substrates. Additionally, pectin acetylesterases remove acetyl groups being an important enzyme class specially for highly acetylated pectin substrates [6–8]. The application of pectinases depends on their biochemical properties. For instance, acidophilic pectinases are useful in food and beverage industry, whereas alkaline are used in textile industry, paper making, coffee and tea fermentations, and treatment of pectic industrial residues [7,9,10].

Pectinases represent around 25% of the enzymes global market; however, most of them originate from mesophilic microorganisms, having restricted thermal stability [8,10,11]. Thermophilic pectinases, which have been scarcely reported, exhibit optimal activity and great stability at high temperatures, with the potential to be used in a wide range of more demanding industrial processes compared to their mesophilic counterparts [8,12]. Thermophilic pectinases could be used in a combined manner to depolymerise pectin-rich biomass allowing its

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depolymerisation into compounds such as GalA via sustainable biomass recycling within a circular economy context. In addition, enzymatic hydrolysis is the preferred method for pectin bioconversion since acid pre-treatment is less sustainable and degrades most of the GalA [13].

GalA from biomass is a key chemical used for the synthesis of a number of valuable compounds (SM Fig. A.1). It may be converted into L-galactonic acid, a precursor for L-ascorbic acid [1] and keto-deoxy sugars with potential in the synthesis of medicinal and other compounds [14]. Also, GalA can be oxidized (at C1) to 1,6 diacid galactaric acid (mucic acid), a precursor of furanedicarboxylic acids (FDCA) and adipic acid which are used for the production of polyesters, nylon and other bio-based polymers [15–18]. Recently, Cardenas-Fernandez et al. [19] described the enzymatic upgrading of GalA to 7-keto-octuronic acid, a rare C8 uronic acid.

The aim of this research was to study thermophilic PMEs and exo-PGs and explore the synergistic application of these enzymes in the bioconversion of pectin into GalA. To achieve this, molecular cloning as well as biochemical characterisation of pectinases were performed. Then, the selected enzymes were assayed in a synergistic reaction in order to hydrolyse apple, citrus and sugar beet pectin. The results from this work demonstrated the ability of our thermophilic pectinases to act on different substrates, confirming their potential to catalyse pectin degradation and bioconversion.

2. Materials and methods

2.1. Materials

All chemicals used in this research were of analytical grade and purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Sugar beet pectin was supplied by CP Kelko UK Ltd (Leatherhead, UK). According to the manufacturers, the molecular weight of polygalacturonic acid (product 81325) is 25–50 kDa, of apple (product 93854) and citrus pectin (product P9135) 30–100 kDa, and of sugar beet pectin (product 9000–69–5) no less than 60 kDa.

2.2. Molecular cloning and enzymes expression

Enzyme protein sequences annotated with the Pfam domain families of PGs (PF00295) and PMEs (PF01095) were retrieved from bacterial genomes using Pfam 32.0 libraries (https://pfam.xfam.org/). Genomic DNA of selected microorganisms was already available in our laboratory. Genes including two exo-PGs from *Thermotoga maritima* DSM 3109 (TMA01, ID: Q9WYR8) and *Bacillus licheniformis* DSM 13 (BLI04, ID: Q65F26), as well as two PMEs from *Bacillus licheniformis* DSM 13 (BLI09, ID: Q65F39) and *Streptomyces ambofaciens* DSM 40053 (SAM10, ID: A0A0K2AQ74) were cloned using the primers described in SM Table A.1. Signal peptides were analysed through the program SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) and removed from the genes before amplification. PCR was carried out using Phusion® High-Fidelity PCR Master Mix with GC Buffer kit (New England Biolabs, Hitchin, UK) and 100 ng of bacterial DNA per 50 µL of reaction. The reaction was performed as follows: 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min and then a final extension at 72 °C for 10 min (C1000 Touch Thermal Cycler, BioRad, UK). PCR products were purified from the gel using Monarch® DNA Gel Extraction Kit (New England Biolabs, Hitchin, UK) and ligated to the pET-29a (+) or pET29a (+): *SacB-Sap*I plasmids [20] using T4 DNA ligase (New England Biolabs, Hitchin, UK). The ligated mixtures were transformed into *E. coli* NovaBlue and the recombinant plasmids were extracted using QIAprep® Spin Miniprep Kit (Qiagen, Manchester, UK) and sent for sequencing (Eurofins Genomics). Then, the plasmids were transformed into the expression host *E. coli* BL21(DE3). All the recombinant enzymes were cloned with a C-terminal His_6-Tag.

For enzymes expression, *E. coli* BL2 (DE3) cells harbouring the recombinant plasmids were grown in 10 mL of LB broth containing 0.05 g/L kanamycin and incubated at 37 °C overnight. The overnight starter culture was used to inoculate 300 mL of the same medium. Cultivation was carried out in 2 L baffled shake flasks at 37 °C and 250 rpm in a shaker incubator (Climo-shaker ISF1-X, Kuhner, Switzerland) until the OD_{600} reached 0.3 – 0.5. At this point, the cells were induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Post induction, cells were incubated at 25 °C and 250 rpm up to 24 h. The cells were harvested by centrifugation (8,500g at 4 °C for 10 min), the cell pellet was re-suspended in the purification binding buffer (Section 2.4.1.), and disrupted by sonication (MSE Soniprep 150 sonicator, Sanyo, Japan) with 20 cycles of 10 s ON and 15 s OFF at 12 µm amplitude. Subsequently, the cell suspension was centrifuged (18,000g at 4 °C for 15 min) and the clarified cell lysate was recovered and kept at 4 °C for protein quantification, SDS-PAGE, enzymatic activity assay and purification.

2.3. Pectinolytic activity assays

2.3.1. Exo-polygalacturonase

The exo-PG activity was measured by quantifying reducing groups expressed as GalA units according to the method described by Miller [21]. A volume of 0.5 mL of enzyme solution (0.5 µg/mL TMA01 and 4 µg/mL BLI04) was added to 0.5 mL of 0.5% (w/v) polygalacturonic acid in 20 mM sodium phosphate buffer at pH 6.5. After 15 min of incubation at 50 °C (Thermomixer™ C, Eppendorf, UK), 0.5 mL of 3,5-Dinitrosalicylic acid (DNS) reagent were added. The mixture was heated at 100 °C for 15 min and then centrifuged at 10,000g for 5 min. The reducing groups were quantified in the supernatant at 540 nm (CLARIOstar Plus Microplate Reader, BMG LabTech, Germany) in 96-well plates. A blank reaction was carried out following the procedure describe above using only buffer instead of enzyme solution. The molar extinction coefficient (ε) of 0.1266 mM^{-1} cm^{-1} was calculated using GalA as standard (SM Fig. A.2). One unit (U) of exo-PG activity was defined as the amount of enzyme that releases 1 µmol of GalA equivalent per min at pH 6.5 and 50 °C.

2.3.2. Pectin methylesterase

Two methods were used to determine the PME activity. The first based on methanol quantification reported by Held et al. [22] was used for all the assays except for optimum temperature experiments. A volume of 0.2 mL of enzyme solution (6 µg/mL BLI09 and 9 µg/mL SAM10) was added to 0.3 mL of 0.5% (w/v) apple pectin in 20 mM sodium phosphate buffer at pH 7. After 15 min of incubation at 50 °C, the reaction was cooled down on an ice bath, centrifuged at 10,000g for 3 min and the supernatant was recovered. The colorimetric reaction for methanol quantification was carried out in a 96-well plate where 25 µL of the supernatant, 175 µL of 50 mM HEPES buffer pH 7, 10 µL of 36 mg/mL Fluoral-P and 10 µL of 20 U/mL alcohol oxidase (AO) were mixed. The mixture was incubated at room temperature for 30 min and methanol was quantified at 410 nm in a plate reader. A blank reaction was carried out following the procedure described above using only buffer instead of enzyme solution. The molar extinction coefficient (ε) of 0.0388 mM^{-1} cm^{-1} was calculated using formaldehyde as standard (SM Fig. A.3). One unit (U) of PME activity was defined as the amount of enzyme that releases 1 µmol of methanol (oxidised to formaldehyde) equivalent per min at pH 7 and 50 °C.

The second PME activity assay was a colorimetric method using bromothymol blue as a pH indicator [23] and was used for optimum temperature determination. A volume of 0.2 mL of enzyme solution (6 µg/mL BLI09 and 9 µg/mL SAM10) was added to 0.3 mL of 0.5% (w/v) apple pectin in 5 mM sodium phosphate buffer at pH 7.6. After 15 min of incubation at 50 °C, the reaction was heated at 100 °C for 10 min. Subsequently, 100 µL of 0.04% bromothymol blue were added and 200 µL of the mixture were used for the measurement in a 96-well plate at 620 nm in a plate reader. A blank reaction was carried out following the

procedure described above using only buffer instead of enzyme solution. The molar extinction coefficient (ε) of 0.3858 mM^{-1} cm^{-1} was calculated using GalA as standard (SM Fig. A.4). One unit (U) of PME activity was defined as the amount of enzyme that releases 1 μmol of GalA equivalent per min at pH 7.6 and 50 °C.

2.4. Enzymes purification and characterisation

2.4.1. Purification

All the enzymes were purified by affinity chromatography using a His-Tag Ni-affinity resin (Ni-NTA Agarose, Thermo Fisher Scientific Inc, UK). Buffers containing 10, 50 and 500 mM Imidazole were used as binding, washing and elution buffer, respectively. These buffers were prepared in 20 mM sodium phosphate buffer containing 300 mM NaCl at pH 6.5 for exo-PGs and at pH 7 for PMEs. The purified enzymes were precipitated with ammonium sulphate powder to a final saturation of 70% (w/v) and stored at 4 °C to be used for characterisation assays.

2.4.2. Enzymes characterisation

2.4.2.1. Optimum pH. The optimum pH of the pectinases was determined at 50 °C using 0.5% (w/v) polygalacturonic acid (for exo-PGs) and apple pectin (for PMEs) in the following 20 mM buffers: sodium acetate (pH 4 and 5), sodium phosphate (pH 6–7), TRIS–HCl (pH 8 and 9) and sodium bicarbonate (pH 10). The exo-PG and PME activities were determined following the standard assay procedures described in Section 2.3. In the case of the PME activity, it was performed by the method based on methanol quantification. The relative activity was expressed as a percentage of the maximum activity.

2.4.2.2. Effect of metal ions. The influence of the metal ions Na^{1+}, Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} at 1 mM on the activity of pectinases was investigated. In the case of Mn^{2+}; 0.1, 0.25, 0.5 and 0.75 mM were also tested. The enzymes were pre-incubated for 15 min at room temperature in 20 mM sodium phosphate buffer at pH 6.5 (for exo-PGs) and pH 7 (for PMEs) containing the respective metal ion salt (NaCl, $CaCl_2$, $MnCl_2$, $ZnSO_4$ or $MnCl_2$). Exo-PG and PME activities were measured according to the procedure described in Section 2.3. For PME activity, the method based on methanol release was used. The relative activity was calculated as the percentage of activity compared with a control assay in absence of metal ion.

2.4.2.3. Effect of temperature. The optimum temperature was determined at temperatures from 40 °C to 100 °C in 10 °C increments using 0.5% (w/v) polygalacturonic acid (for exo-PGs) and apple pectin (for PMEs) in 20 mM sodium phosphate buffer at pH 6.5 and 7 for exo-PGs and PME, respectively. For exo-PGs, the assays were performed after the pre-incubation of the enzymes with 0.25 mM Mn^{2+} for 15 min at room temperature or in absence of this ion. By using this data, the effect of Mn^{2+} on the amount of GalA released was also calculated. The exo-PG and PME activities were determined following the standard assay procedure mentioned in Section 2.3. and the relative activity was expressed as a percentage of the maximum activity. The PME activity was assessed following the standard assay procedure by the method using the pH indicator.

The thermal stability was tested by incubating the enzymes in 20 mM HEPES pH 6.5 and 7 for exo-PGs and PMEs, respectively from 20 °C to 100 °C in 10 °C increments for up to 24 h in a Thermomixer C fitted with a ThermoTop heating lid (Thermomixer™ C, Eppendorf, UK). In the case of exo-PGs, they were incubated either in presence of 0.25 mM Mn^{+2} or in absence of this ion. Aliquots of the enzymes solution were taken periodically and cooled down on ice. Then, exo-PG and PME activities were measured under the standard assay conditions at 50 °C, as detailed in Section 2.3. and expressed as residual activity. The PME activity was measured by the method based on methanol quantification.

2.4.2.4. Substrate specificity. To determine substrate specificity of exo-PGs; polygalacturonic acid (non-esterified) as well as citrus pectin and apple pectin (both esterified, SM Table A.2) were used as substrates. The experiments were performed pre-incubating the enzymes with 0.25 mM Mn^{2+} at optimum conditions of activity for each enzyme. The exo-PG activity was measured following the standard assay procedure detailed in Section 2.3.1. The relative activity was expressed as the percentage of the activity with polygalacturonic acid which was considered as 100 %.

2.4.2.5. Kinetic studies. Kinetic parameters were determined by measuring the initial velocity of reaction at different concentrations of substrates: polygalacturonic acid from 20 to 400 μM for exo-PGs and apple pectin from 5 to 350 μM for PMEs. The reactions were performed under optimum conditions of activity for each enzyme. The kinetic data obtained was analysed by non-linear regression using GraphPad Prism 8 software. The curve from the plot of initial velocity versus substrate concentration was fitted to the equation of the generally used substrate inhibition model (Eq. 1) as well as to the Eq. 2, that belongs to a new model of substrate inhibition. In this new model, it is assumed that a number of molecules of substrate (n) binds simultaneously to the enzyme during the inhibition process. The value of n is determined from the analysis of the data [24]. The total concentration (*[Et]*) of each enzyme used in the reactions was kept constant and this value was used to determine the *kcat* through "kcat" analysis tool of the software (Eq. 3). In the case of multimeric enzymes, it is important to note that due to *[Et]* is the concentration of enzyme catalytic sites, this value is larger than the concentration of enzyme molecules.

$$V_0 = Vmax[S]/(Km \quad + [S](1 + [S]/Ki)) \tag{1}$$

$$\boldsymbol{V_0 = Vmax[S]/(Km \quad + [S](1 + [S]^n/Ki^n))} \tag{2}$$

$$\boldsymbol{V_0 = [Et] \quad kcat \quad [S]/(Km \quad + [S])} \tag{3}$$

Where: V_0 is the initial velocity of the enzyme (μmol/min mL), *Vmax* is the maximum enzyme velocity, *Km* is the Michaelis-Menten constant (μM), *[S]* is the substrate concentration (μM), *Ki* is the substrate inhibition constant (μM), *n* is the number of molecules of substrate that binds to the inhibitor site, *[Et]* is the concentration of enzyme catalytic sites (μmol/mL) and *kcat* is the turnover number defined as the number of times each enzyme site converts substrate to product per unit time (1/s).

2.5. Synergistic activity between pectin methylesterases and exo-polygalacturonases

Synergistic activity between PMEs and exo-PGs was carried out using 0.5% (w/v) apple pectin, citrus pectin and sugar beet pectin in 20 mM sodium phosphate buffer pH 7. Composition of pectin substrates is detailed in SM Table A.2 and for GalA yield (%) calculations, 74% GalA content was used for apple and citrus pectin. For the reactions, a volume of the enzyme solution (PME plus exo-PG) containing 0.25 mM $MnCl_2$ was added to 0.5 mL of each substrate. In the case of synergistic activity between BLI09 PME and TMA01 or BLI04 exo-PGs, the mixture was incubated at 50 °C. Regarding synergistic activity between SAM10 PME and TMA01 or BLI04 exo-PGs, the incubation was performed at 40 °C for 1 h and then increased to 50 °C for 24 h. In both cases, samples were taken periodically. Blank reactions were prepared without addition of enzyme solution. Furthermore, reactions adding only TMA01 or BLI04 exo-PG were used as controls. The enzymatic reactions were stopped at indicated times using 0.1% trifluoroacetic acid (TFA) and the mixtures were centrifuged at 18,000g for 15 min. Then, the supernatants were recovered and used for methanol and GalA quantification according to the procedure described in Section 2.6.

In addition, GalA inhibition effect on TMA01 and BLI04 exo-PGs was determined. GalA at final concentrations ranging from 1.5 to 6.5 mM

Table 1
. Thermophilic pectinases successfully cloned and expressed.

Bacteria	Type	EC number	UniProtKB	Enzyme code	Plasmid	Gene length (bp)	Full-length protein (aa)	Signal peptide (aa)	Molecular Weight (kDa)
Thermotoga maritima DSM 3109	Exo-PG	3.2.1.67	Q9WYR8	TMA01	pET-29a (+)	1347	448	ND	50.48
Bacillus licheniformis DSM 13	Exo-PG	3.2.1.67	Q65F26	BLI04	pET29a (+):*SacB-Sap1*■	1311	436	ND	48.14
Bacillus licheniformis DSM 13	PME	3.2.1.11	Q65F39	BLI09	pET29a (+):*SacB-Sap1*■	954	317	ND	35.11
Streptomyces ambofaciens DSM 40053	PME	3.2.1.11	A0A0K2AQ74	SAM10	pET29a (+):*SacB-Sap1*■	1128	375	31	40.68

ND: not detected. ■ Dobrijevic et al. [20]

were tested and residual activity was expressed as the percentage of activity compared with a control assay without addition of GalA.

2.6. Methanol and galacturonic acid quantification from the synergistic activity

Methanol quantification was carried out according to the procedure described in Section 2.3.2. using 25 μL of the synergistic reactions between PMEs and exo-PGs. GalA was analysed following the method reported by Ward et al. [25] using Ion Chromatography System (ICS 5000 +, Thermo Scientific, Hemel Hempstead, UK) equipped with a Dionex Aminopac™ PA1 anion exchange column 4 × 250 mm fitted with a Dionex Aminopac™ PA1 guard column 4 × 50 mm. Analysis was carried out using as mobile phase 5% (v/v) 1 M sodium acetate (electrochemical detection grade, Fisher Scientific, UK) and 95% (v/v) Milli Q water at 0.25 mL/min for 8 min at 30 °C. An external standard calibration curve of GalA was used for quantitative analysis, which was performed by measuring the peak area. The retention time of GalA was 4 min

3. Results and discussion

3.1. Enzymes cloning, expression and purification

Two exo-PGs (TMA01 and BLI04) along with two PMEs (BLI09 and SAM10) were cloned and successfully expressed in *E. coli* BL21(DE3) (SM Fig. A.5). The presence of a signal peptide was identified only in SAM10 PME which was removed before cloning. Enzymes information such as EC number, gene and protein length and molecular weight are detailed in Table 1 [26–29].

All the studied enzymes were expressed in their soluble form and purified using affinity Ni-NTA chromatography. SM Fig. A.5A shows the expression and purification of TMA01 and BLI04 exo-PGs, showing a single band of ~ 55 kDa for both enzymes. TMA01 is a tetrameric enzyme of 212 kDa formed by monomers of around 50.5 kDa [27], while BLI04 is a monomeric enzyme with a molecular weight of 48.1 kDa [29]. SM Fig. A.5B shows the expression and purification of BLI09 and SAM10 PMEs with bands of ~ 35 and 40 kDa, respectively. BLI09 has been reported as a protein of 35.1 kDa [30] and according to the information of UniProtKB, SAM10 has a size of 40.7 kDa.

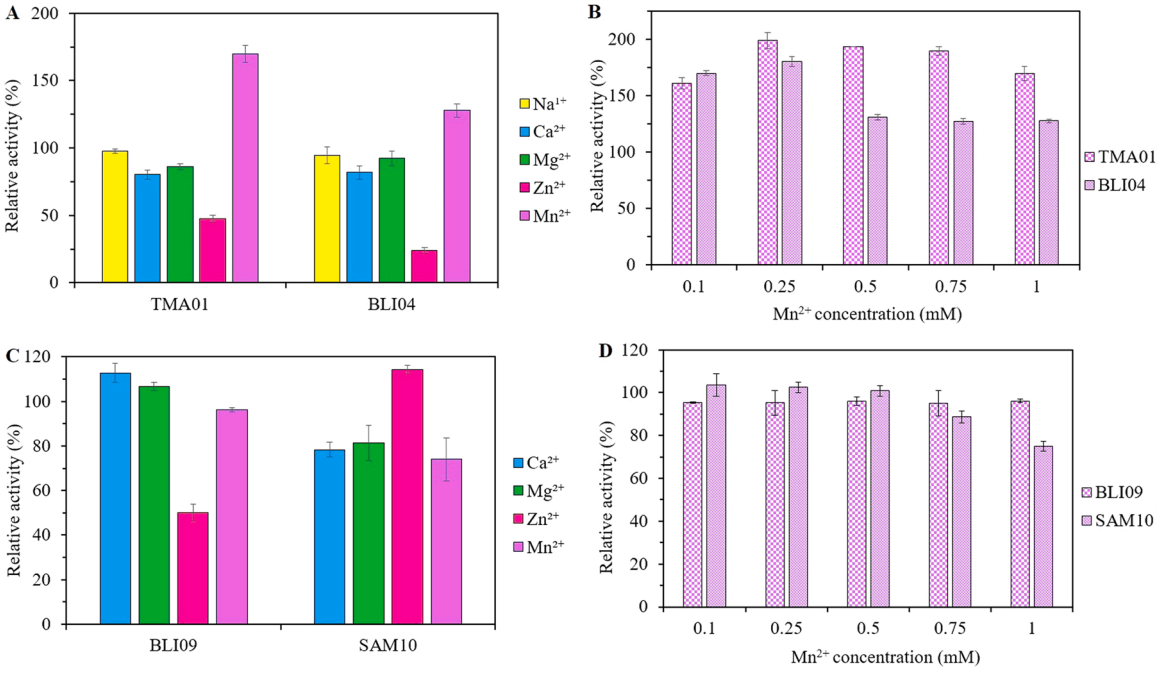

Fig. 1. Effect of metal ions on the activity of purified pectinases. (A) 1 mM and (B) different Mn^{2+} concentrations on TMA01 and BLI04 exo-PGs. (C) 1 mM and (D) different Mn^{2+} concentrations on BLI09 and SAM10 PMEs. The enzymes were pre-incubated with the metal ions at room temperature for 15 min. The exo-PG activity was determined at pH 6.5 and 50 °C, while the PME activity at pH 7 and 50 °C with the method based on methanol quantification. The relative activity was expressed as the percentage of activity compared with a control without metal ion. Error bars represent one standard deviation from the mean (n = 2).

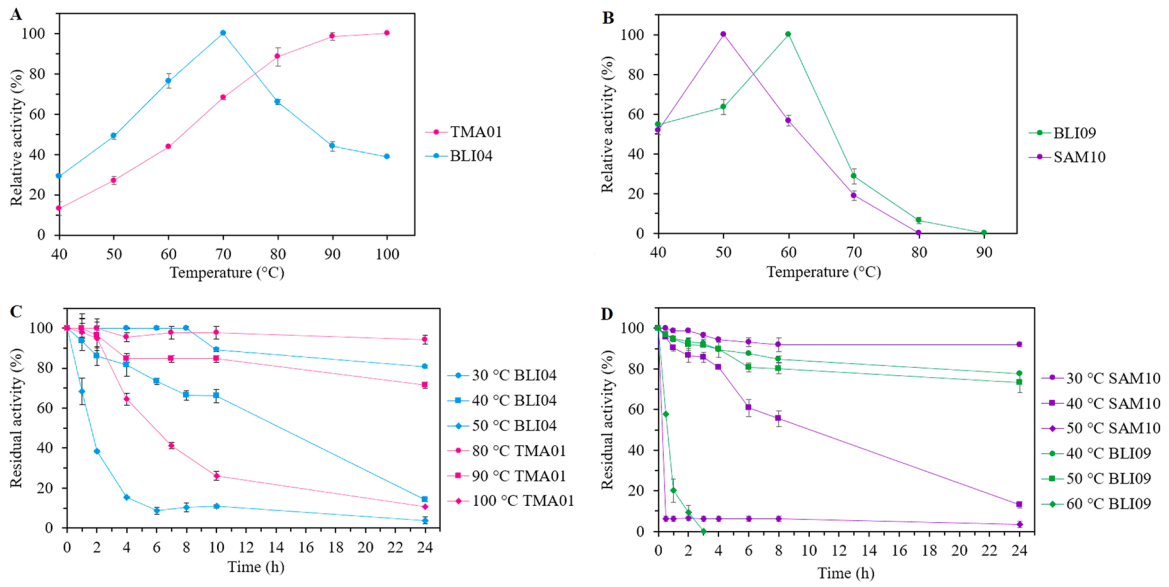

Fig. 2. Effect of temperature on the activity of purified pectinases. Optimum temperature of (A) TMA01 and BLI04 exo-PGs and (B) BLI09 and SAM10 PMEs. Thermal stability of (C) TMA01 and BLI04 exo-PGs and (D) BLI09 and SAM10 PMEs. The experiment was performed at pH 6.5 and 7 for exo-PGs and PMEs, respectively. In thermal stability assays, after the indicated time, the exo-PG activity was determined by DNS method and the PME activity by the method using the pH indicator, both at 50 °C. The relative activity was expressed as percentage of the maximum activity, whereas that the residual activity was expressed as the percentage of the starting activity. Error bars represent one standard deviation from the mean (n = 2).

3.2. Biochemical and kinetic characterisation

Determination of optimum conditions of activity of enzymes is fundamental prior to their application in biocatalysis. This was especially important in our research since we used these enzymes in synergistic action. In this sense, biochemical and kinetic characterisation of the pectinases were carried out.

3.2.1. Optimum pH

TMA01 exo-PG exhibited maximum activity between pH 7 and 8, and maintained approximately 60% of its optimum activity at pH 6.5, 9 and 10 (SM Fig. A.6A). BLI04 exo-PG was active in a pH range from 4 to 10, exhibiting maximum activity at pH 8. In addition, a second peak of activity at pH 5 was observed, where the enzyme kept 60% of its maximum activity (SM Fig. A.6A). Previous publications have also described similar pH profile for pectinases from other *Bacillus* strains [31,32].

The effect of pH on the activity of BLI09 and SAM10 PMEs was very similar. An optimum pH of 7 was identified for both PMEs. They preserved about 60 % of their highest activity at pH 6, while at pH 8 and above the enzymatic activity was less than 30% (SM Fig. A.6B).

3.2.2. Effect of metal ions

The effect of several metal ions on the activity of TMA01 and BLI04 exo-PGs was tested initially at 1 mM (Fig. 1A). A significant increase on the activity of both enzymes was observed in presence of Mn^{2+}. Ca^{2+} produced a slight decrease on the exo-PGs activity, this might be due to its interaction with blocks of non-esterified GalA residues from pectin which results in gel formation [33]. An inhibitory effect of Zn^{2+} was observed in both enzymes. Additionally, different Mn^{2+} concentrations were tested in order to find the optimum value (Fig. 1B). The best concentration was 0.25 mM, which increased the activity by 2 and 1.8-fold for TMA01 and BLI04, respectively.

Likewise, the effect of metal ions on the activity of BLI09 and SAM10 PMEs was determined at 1 mM (Fig. 1C). No significant activity improvement was observed for either enzyme, however, Zn^{+2} decreased BLI09 PME activity by 50%. Aiming to establish a cascade enzymatic reaction by coupling the activity of exo-PG and PMEs, the effect of Mn^{+2} (an exo-PG activity enhancer) on PMEs activity was also examined (Fig. 1D). Optimum Mn^{+2} concentration (0.25 mM) for our exo-PGs did not have a negative effect on PMEs activity.

3.2.3. Effect of temperature

Most of the PGs and PMEs from different microbial sources have an optimum temperature range between 30 °C and 50 °C [9,34]. Therefore, they might not be able to be used in bioprocesses in which high temperatures are required. The optimum temperature for exo-PGs was around 90–100 °C for TMA01 and 70 °C for BLI04 (Fig. 2A). We also found that the temperature activity profile for both exo-PGs was the same either in presence or in absence of 0.25 mM Mn^{2+}. However, in presence of this ion, the higher was the temperature, the higher was the percentage of GalA released (SM Fig. A.7). Thus, for TMA01, at 90 °C and 100 °C, 65 % of the total GalA was released in presence of Mn^{2+}, whereas in absence only 45 % (SM Fig. A.7A). For BLI04, at optimum temperature, 38 % of GalA was released in presence of Mn^{2+}, whilst in absence only 22 % (SM Fig. A.7B).

Regarding PMEs, BLI09 presented optimum activity at 60 °C, while SAM10 at 50 °C keeping around 60% of its maximum activity at 60 °C (Fig. 2B). The scant studies about *Streptomyces* pectinases have also indicated optimum temperatures around this value [35].

Additionally, the majority of pectinases from different sources have good stability up to 50 °C [36]. Thermal stability of TMA01 and BLI04 exo-PGs are presented in Fig. 2C. TMA01 showed remarkable stability at high temperatures, retaining 100 % and 80 % of its activity at 80 °C and 90 °C after 24 h. Even after 4 h of incubation at 100 °C, TMA01 kept around 65 % of its activity. BLI04 exhibited great stability at 30 °C retaining approximately 80 % of its activity after 24 h of incubation. At 40 °C, the activity was around 65 % after 10 h, whereas the enzyme was almost deactivated after 6 h at 50 °C.

About PMEs thermal stability (Fig. 2D), BLI09 exhibited good stability up to 50 °C, maintaining around 80 % of its activity after 24 h of incubation. However, the enzyme was almost deactivated after 2 h at 60 °C. SAM10 remained more than 90 % of activity at 30 °C after 24 h of incubation. At 40 °C, this PME kept around 50 % after 8 h. The metal ion Mn^{2+} did not affect the thermal stability profile of exo-PGs and PMEs (data not shown).

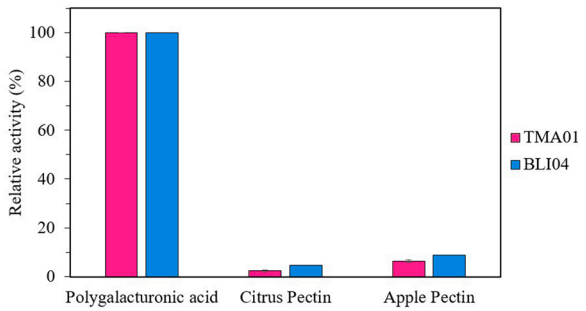

Fig. 3. Effect of the degree of pectin methylation on the activity of purified TMA01 and BLI04 exo-PGs. Polygalacturonic acid (non-esterified), citrus pectin (low esterification degree) and apple pectin (high esterification degree). Mn^{2+} (0.25 mM) was added into the reaction and the enzymes were pre-incubated for 15 min with the ion. The exo-PG activity was determined at optimal conditions for each enzyme: TMA01, pH 8 and 90 °C; and BLI04, pH 8 and 70 °C. The relative activity was expressed as the percentage of the activity with polygalacturonic acid which was considered as 100 %. Error bars represent one standard deviation from the mean (n = 2).

3.2.4. Substrate specificity

The effect of the degree of pectin methylation on the activity of TMA01 and BLI04 exo-PGs was evaluated (Fig. 3). Both exo-PGs were active on polygalacturonic acid (non-esterified); however, they exhibited low activity towards both citrus and apple pectin (esterified).

3.2.5. Kinetic studies

Typically, kinetic data from enzymes fit to the Michaelis-Menten model; however, inhibition could be observed at high substrate concentrations. Substrate inhibition can happen because the substrate binds to an alternate site of the enzyme resulting in an inactive enzyme-substrate complex [37]. Kinetic parameters of the exo-PGs and PMEs were calculated using polygalacturonic acid and apple pectin, respectively; substrate inhibition was observed in all the enzymes. Two different inhibition models were used (Eqs. 1 and 2) and the analysis was done with GraphPad Prism 8 using the extra-sum-of-squares F test [24, 37]. Kinetic data was successfully fitted to the Eq. 2 [24] since this model described better the almost complete inhibition that was observed in all the pectinases (Fig. 4). The values of *kcat* were calculated through the Eq. 3. The regression coefficients (R^2) of Eqs. 2 and 3 were 0.99.

All the kinetic parameters of the exo-PGs and PMEs from this study as well as the comparison with kinetic information of other thermophilic pectinases are summarised in Table 2. From this table, we can see that previously published data of TMA01 and BLI04 exo-PGs reported a Michaelis-Menten kinetics [27,29]. The previous calculated parameters of TMA01 were similar to those determined in this study, but parameters of BLI04 were completely different. In our study, BLI04 presented a *Km* higher and the *Vmax* and *kcat* values were 44-fold using the same substrate. TMA 01 has been reported as a tetrameric enzyme. Thus, according to our substrate inhibition model, it was predicted that up to eight molecules of substrate (n = 8) bind to the enzyme in the inhibition process. BLI04 has been reported as a monomeric enzyme, however our model predicted that up to six molecules of substrate (n = 6.1) bind it during the inhibition. Comparison of kinetic parameters between TMA01 and BLI04 exhibited that *Vmax* and *kcat* values were considerably higher in TMA01 (7.3 and 7.6-fold, respectively). Besides, this enzyme presented smaller *Km* and *Ki* values, showing that the substrate affinity was higher but also the inhibitory effect was slightly stronger. Regarding kinetic information of thermophilic exo-PGs which has been previously reported, most of the studies found that these enzymes

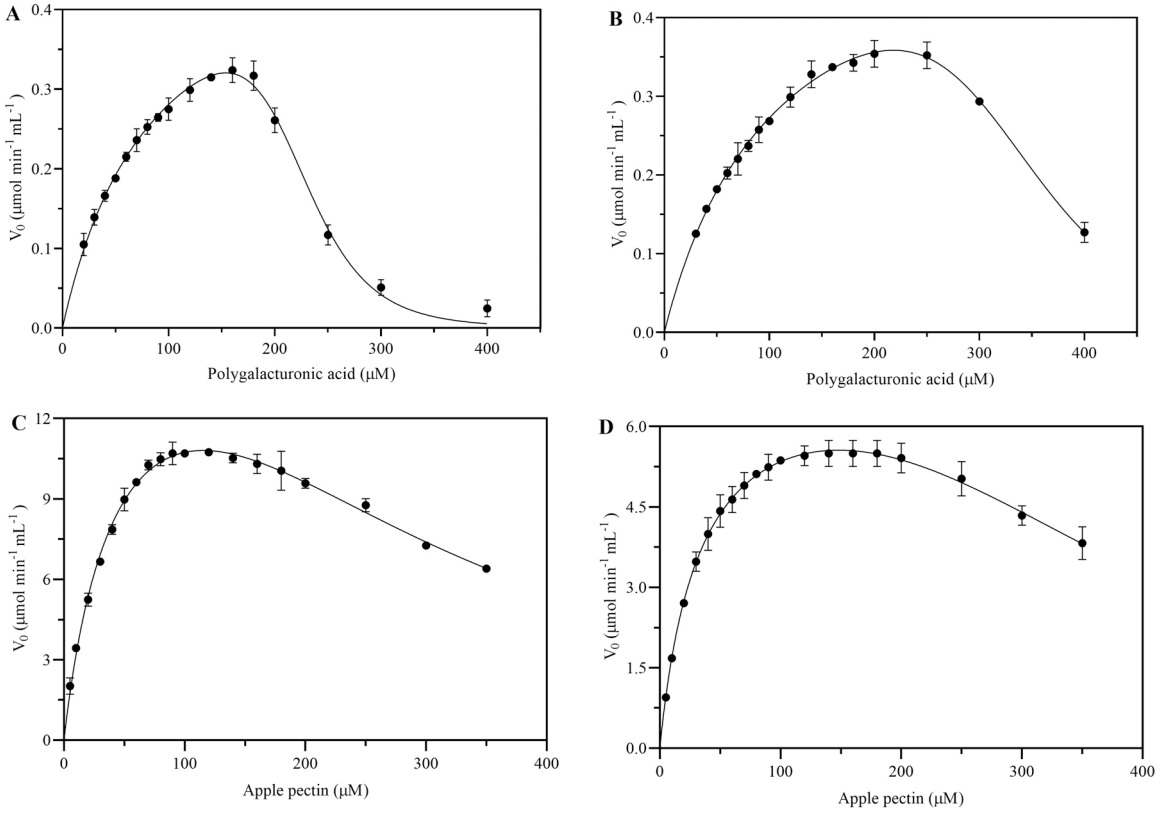

Fig. 4. Substrate inhibition kinetics of (A) TMA01 and (B) BLI04 exo-PGs using polygalacturonic acid as substrate, and (C) BLI09 and (D) SAM10 PMEs using apple pectin. The enzymes velocity was measured at optimum conditions: TMA01, pH 8 and 90 °C; BLI04, pH 8 and 70 °C; BLI09, pH 7 and 60 °C and SAM10, pH 7 and 50 °C. Kinetic data were analysed by non-linear regression using GraphPad Prism 8 software. The parameters are detailed in Table 2. Error bars represent one standard deviation from the mean (n = 2).

Table 2
. Comparative kinetic information of thermophilic exo-PGs and PMEs using polygalacturonic acid and apple pectin, respectively as substrates.

Bacteria	Enzyme	Vmax (μmol/min mg)	Km (μM)	kcat (1/s)	kcat/Km (1/s μM)	Ki (μM)	n	Reference
Exo-PGs								
Thermotoga maritima	TMA01[a*]	1027.67 ± 57.31	86.75 ± 9.14	858.85 ± 6.77	9.90 ± 0.74	217.20 ± 3.91	8.00 ± 0.52	This study
Bacillus licheniformis	BLI04[b*]	141.41 ± 5.05	105.90 ± 7.55	112.75 ± 0.82	1.06 ± 0.11	330.84 ± 5.19	6.06 ± 0.39	This study
Thermotoga maritima	PelB[c■]	1170	60	936	15.6	NA	NA	[26]
Bacillus licheniformis	BlExoPG[d■]	3.20	86.67	2.58	0.03	NA	NA	[28]
Caldiceellulosiruptor bescii	PelA[e■]	384.6	8	ND	ND	NA	NA	[38]
Thermotoga thermophilus	TtGH28[f■]	ND	8.77 ± 0.64	10.6 ± 0.3	1.21 ± 0.09	NA	NA	[37]
Rhodothermus marinus	RmGH28[g*]	ND	0.67 ± 0.09	6.02 ± 0.17	8.94 ± 1.03	71 ± 6	NA	[15]
PMEs								
Bacillus licheniformis	BLI09[h*]	2785.14 ± 158.32	44.19 ± 4.51	1562.10 ± 12.95	35.35 ± 2.87	272.90 ± 17.14	1.80 ± 0.16	This study
Streptomyces ambofaciens	SAM10[i*]	801.32 ± 28.48	32.85 ± 2.84	523.22 ± 4.89	15.93 ± 1.72	379.19 ± 11.26	2.63 ± 0.34	This study
Clostridium thermocellum	*Ct*PME[i■]	6.22	58.46 ± 7.69	3.68	0.06	NA	NA	[39]
Erwinia chrysanthemi	Mutant JL25[h■]	1062.16	11.23 ± 3.54	655 ± 104	58.32	NA	NA	[40]

Reactions were performed at a90 °C, b70 °C, c80 °C, d60 °C, e72 °C, f40 °C, g25 °C, h60 and i50. *Substrate inhibition kinetics, ■Michaelis-Menten kinetics, ND: not determined, NA: not applicable, n: number of molecules of substrate that binds to the inhibitor site. Errors represent one standard deviation about the mean (n = 2).

exhibited a Michaelis-Menten kinetics [27,29,38,39]. Additionally, we can see in Table 2 that exo-PGs from *Thermotoga maritima* presented the highest *Vmax* values. The values of *kcat* were also remarkably higher in exo-PGs from *Thermotoga maritima* as well as in the BLI04 exo-PG from this study.

Regarding PMEs, our substrate inhibition model predicted that up to two and three molecules of substrate (n = 1.8 and 2.63) bind to BLI09 and SAM10 PMEs, respectively during the inhibition process. Comparison of kinetic parameters between BLI09 and SAM10 showed that *Vmax* and *kcat* were higher in BLI09 (3.5 and 3-fold, respectively). Kinetic information about PMEs is scarce. Rajulapati and Goyal [40] determined the kinetic parameters of a thermophilic and thermostable PME from *Clostridium thermocellum* and found *Vmax* and *kcat* values notably smaller than those from this study, as well as a higher *Km* value. Chakiath et al. [41] reported the kinetic parameters of an engineered PME from *Erwinia chrysanthemi* (JL25) which were closed to those from SAM10.

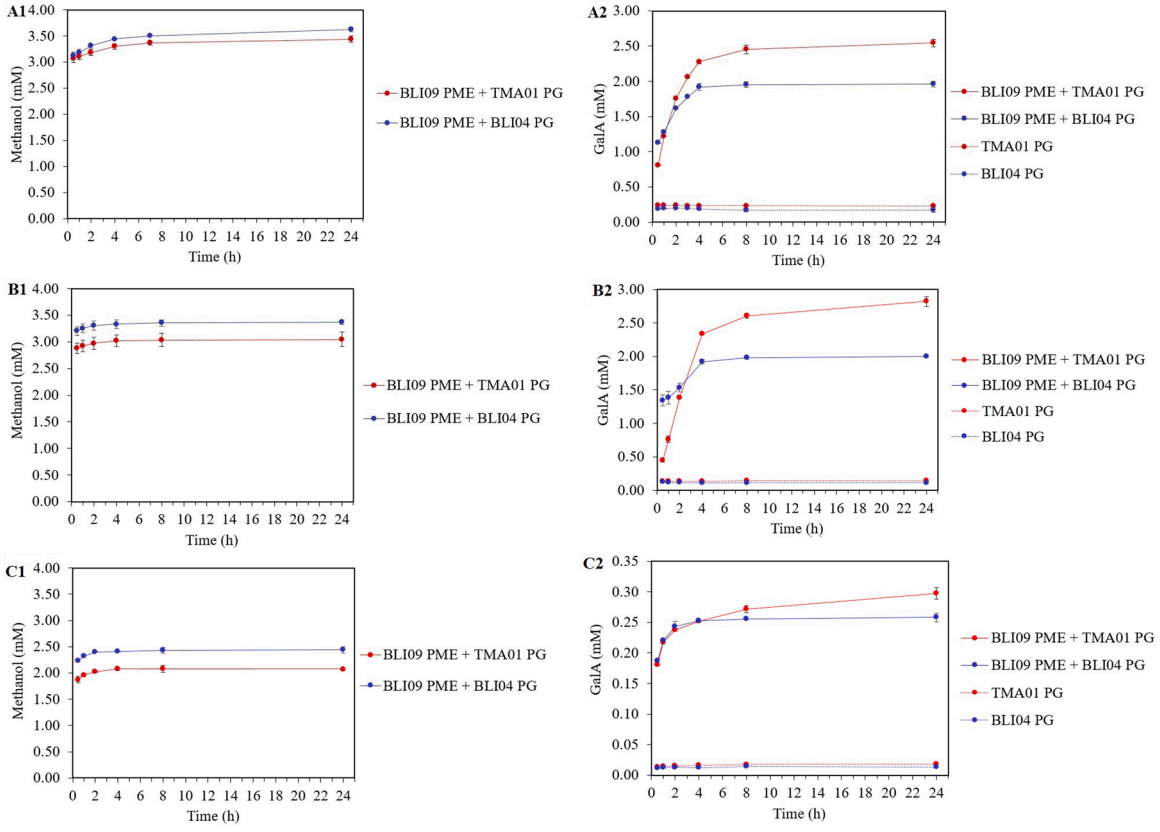

Fig. 5. Synergistic activity between BLI09 PME and either TMA01 or BLI04 exo-PGs using different pectic substrates. (A) Apple pectin, (B) citrus pectin and (C) sugar beet pectin. (A1), (B1) and (C1) methanol and (A2), (B2) and (C2) GalA quantification. The reactions were carried out at 50 °C using 0.5 % of each substrate in 20 mM phosphate buffer at pH 7; and 9, 0.5 and 2 U/mL of BLI09, TMA01 and BLI04, respectively. Methanol was quantified by using alcohol oxidase and Fluoral-P and GalAc by ICS. Error bars represent one standard deviation from the mean (n = 2).

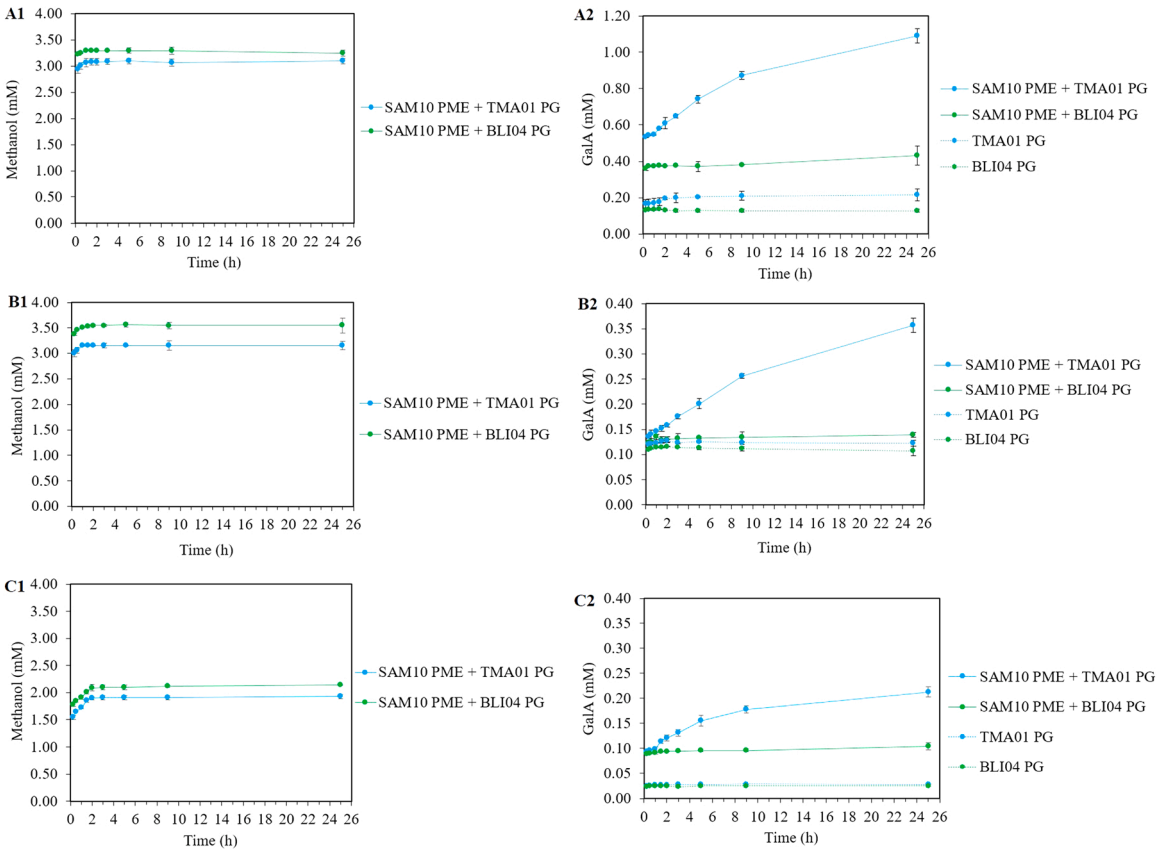

Fig. 6. Synergistic activity between SAM10 PME either with TMA01 or BLI04 exo-PGs using different pectin substrates. (A) Apple pectin, (B) citrus pectin and (C) sugar beet pectin. (A1), (B1) and (C1) methanol and (A2), (B2) and (C2) GalA quantification. The reactions were carried out at 40 °C for 1 h and at 50 °C for 24 h, using 0.5 % of each substrate in 20 mM phosphate buffer at pH 7; and 18, 1 and 1 U/mL of SAM10, TMA01 and BLI04, respectively. Methanol was quantified by using alcohol oxidase and Fluoral-P and GalAc by ICS. Error bars represent one standard deviation from the mean (n = 2).

3.3. Synergistic activity between PMEs and exo-PGs

The presence of ester groups in pectin hampers the activity of exo-PGs on the HG backbone in pectin hydrolysis [42,43]. As observed in Fig. 3, TMA01 and BLI04 exo-PGs exhibited activity only towards polygalacturonic acid (non-esterified substrate) and showed negligible activity towards apple and citrus pectin (esterified substrates). Therefore, to achieve an efficient pectin hydrolysis and GalA release by exo-PGs, pectin demethylation by PMEs is required before the exo-PGs can act on the natural pectins.

Thus, four synergistic enzymatic reactions were run by combining the activity of the PMEs and exo-PGs characterised in this study: BLI09 and SAM10 PMEs were tested in combination with either TAM01 and BLI04 exo-PGs. Compatible operational reaction conditions (pH and temperature) were established in order to enhance enzymes synergistic activity, even if optimum conditions were compromised. Hence, the reactions between BLI09 PME with either TMA01 or BLI04 exo-PGs were carried out at 50 °C and pH 7. Whilst reactions between SAM10 PME with either TMA01 or BLI04 exo-PGs were performed at pH 7 and at 40 °C for 1 h for demethylation followed by an increase in temperature to 50 °C. These two-step temperature reactions were set up in order to favour catalytic activity of SAM10 PME (unstable at 50 °C) and both exo-PGs. As described in Section 3.2.2., Mn^{2+} enhanced significantly exo-PGs activity and did not alter PMEs action, thus it was added into the reactions. Synergistic activity using apple, citrus and sugar beet pectin was monitored by quantifying methanol and GalA release (Section 2.6. and SM Fig. A.8).

Initially, 4.5 and 9 U/mL of BLI09 and SAM10 PMEs, respectively were tried for the synergistic assays (SM Fig. A.9). Then, PMEs concentration were adjusted to achieve the maximum demethylation at the beginning of the synergistic reactions. Subsequently, different concentrations of exo-PGs were tested in order to enhance GalA release during the first hours of reaction until reach a plateau was reached. Thereby, the final enzymatic concentration used for the synergistic experiments between BLI09 PME with either TMA01 or BLI04 exo-PGs were 9, 0.5 and 2 U/mL, respectively. Whereas, 18 U/mL of SAM10 PME and 1 U/mL of TMA01 and BLI04 were used for the synergistic reactions between SAM10 PME with either TMA01 or BLI04 exo-PGs.

Synergistic reactions between BLI09 PME with either TMA01 or BLI04 exo-PGs using different pectin substrates are presented in Fig. 5. Methanol levels were similar for apple (Fig. 5A1) and citrus pectin (Fig. 5B1) reaching around 3.5 mM per around 0.04 mM of substrate. These findings are consistent with data presented in SM Table A.2. which shows similar methylation (%) for both substrates. Additionally, a lower concentration of methyl groups was released from sugar beet pectin (around 2.5 mM per 0.04 mM of substrate) (Fig. 5C1). This result is in agreement with low methylation of sugar beet pectin in comparison with apple and citrus pectin (SM Table A.2). With regard to GalA, concentrations of 2.5 mM (29 % yield) and 2 mM (23 % yield) were achieved for BLI09 PME and either TMA01 or BLI04 exo-PG, respectively; after 4 h of the synergistic reaction using apple and citrus pectin (Fig. 5 A2 and B2, respectively). Although sugar beet pectin was demethylated by BLI09 PME, TMA01 and BLI04 exo-PGs were not able to hydrolyse this substrate and only around 0.25 mM GalA was obtained after 24 h (Fig. 5 C2). This might due to the presence of acetyl groups hinder the activity of exo-PGs. A pectin acetylesterase which removes acetyl groups, could improve GalA release from sugar beet pectin [6]. Notice that for all cases, no GalA was detected in the blank reaction where PME was not added.

Concerning the synergistic reactions between SAM10 PME with

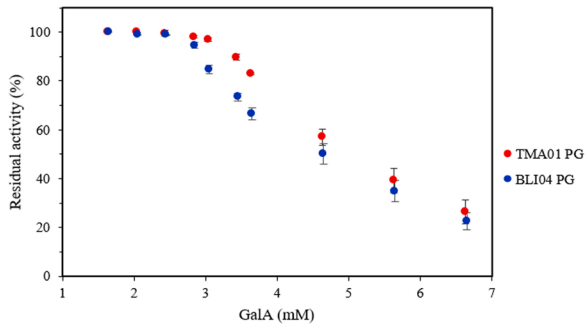

Fig. 7. GalA inhibition effect on TMA01 and BLI04 exo-PGs. GalA: D-galacturonic acid, exo-PGs: exo-polygalacturonases.

either TMA01 or BLI04 exo-PGs, concentrations of released methyl groups were also similar for apple and citrus pectin (Fig. 6A1 and B1). In the case of sugar beet pectin, around 20% less methyl groups were released by SAM10 PME in comparison with BLI09 PME (Fig. 6C1). It could be due to a lower tolerance of this enzyme to acetyl esters with respect to BLI09 [30]. Although methyl groups were released by both PMEs, GalA released in the all synergistic reactions with SAM10 PME was below 1 mM after 24 h (Fig. 6A2, B2 and C2). This could be due to PMEs mechanism and methyl groups distribution in each source within the complex pectin structure. PMEs can remove methyl ester groups from HG in pectic substrates either in a blockwise (processive or single-chain manner) or in a random (multiple chain) mechanism [44–50]. PMEs with a blockwise demethylation pattern create fragments of nonmethylesterified GalA, where exo-PGs can act and release monomers of GalA from the non-reducing end (SM Fig. A.10). Otherwise, when substrates are demethylated in a random way, it is less likely that exo-PGs would act and therefore hindering the GalA release (SM Fig. A.11). It could be the reason why higher concentrations of GalA were obtained in the synergistic reactions of exo-PGs with BLI09 PME than with SAM10 PME. This is in agreement with a report that suggested that BLI09 PME can follow a blockwise demethylation pattern [30]. Thereby, we can hypothesise that SAM10 PME removes methyl groups following a random mechanism, since low GalA concentrations were obtained in all synergistic reactions with this enzyme (SM Fig. A.10 and A.11.

). In addition, it has been reported that sugar beet pectin is highly branched containing arabinan and galactan lateral branches, which may also hinder exo-PG activity [51,52].

Finally, the results of GalA inhibition effect on exo-PGs showed that TMA01 and BLI04 were inhibited by GalA at concentrations over 3 and 2.5 Mm, respectively (Figs. 6 and 7). These findings are in agreement with the maximum concentration of GalA released as a result of the synergistic activity (Fig. 5A2 and 5B2).

4. Conclusions

This work contributes to the knowledge of thermophilic pectinases providing additional and more detailed information about kinetic parameters, operational conditions and stability. Likewise, characterisation results were fundamental to the subsequent application of the enzymes in a synergistic action. Synergistic activity between PMEs and PGs allowed a better understanding of the catalytic mechanisms of these enzymes as well as a more efficient pectin hydrolysis leading to GalA release. Based on our results, the best synergistic action to obtain GalA was achieved combining BLI09 PME and either TMA01 or BLI04 PGs using apple and citrus pectin. Additionally, these findings provide further insights to valorise pectin-rich renewable feedstocks to obtain GalA within a circular economy context.

CRediT authorship contribution statement

Carol N. Flores-Fernández: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Funding acquisition. **Max Cárdenas-Fernández:** Conceptualization, Methodology, Investigation, Writing – review & editing, Visualization, Supervision, Project administration. **Gary J. Lye:** Conceptualization, Writing – review & editing, Supervision, Project administration. **John M. Ward:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.enzmictec.2022.110071.

References

[1] J. Kuivanen, H. Dantas, D. Mojzita, E. Mallmann, A. Biz, N. Krieger, D. Mitchell, P. Richard, Conversion of orange peel to L-galactonic acid in a consolidated process using engineered strains of *Aspergillus niger*, AMB Express 4 (2014) 1–8, https://doi.org/10.1186/s13568-014-0033-z.

[2] M.C. Edwards, J. Doran-Peterson, Pectin-rich biomass as feedstock for fuel ethanol production, Appl. Microbiol. Biotechnol. 95 (2012) 565–575, https://doi.org/10.1007/s00253-012-4173-2.

[3] A.G.J. Voragen, G.J. Coenen, R.P. Verhoef, H.A. Schols, Pectin, a versatile polysaccharide present in plant cell walls, Struct. Chem. 20 (2009) 263–275, https://doi.org/10.1007/s11224-009-9442-z.

[4] J. Harholt, A. Suttangkakul, H.V. Scheller, Biosynthesis of pectin, Plant Physiol. 153 (2010) 384–395, https://doi.org/10.1111/j.1399-3054.2006.00834.x.

[5] D. Gawkowska, J. Cybulska, A. Zdunek, Structure-related gelling of pectins and linking with other natural compounds: a review, in: Polymers, 10, Basel,, 2018, p. 762, https://doi.org/10.3390/polym10070762.

[6] E. Bonnin, C. Garnier, M.C. Ralet, Pectin-modifying enzymes and pectin-derived materials: applications and impacts, Appl. Microbiol. Biotechnol. 98 (2014) 519–532, https://doi.org/10.1007/s00253-013-5388-6.

[7] N. Sharma, M. Rathore, M. Sharma, Microbial pectinase: sources, characterization and applications, Rev. Environ. Sci. Biotechnol. 12 (2012) 45–60, https://doi.org/10.1007/s11157-012-9276-9.

[8] S.S. Dhiman, R. Mahajan, J. Sharma, Chapter 26: Pectinases of Thermophilic Microbes, in: T. Satyanarayana, Y. Kawarabayasi, J. Littlechild (Eds.), Thermophilic microbes in environmentaland industrial biotechnology, biotechnology of thermophiles, Springer Science+Business Media Dordrech, 2013, pp. 689–710, https://doi.org/10.1007/978-94-007-5899-5.

[9] R.S. Jayani, S. Saxena, R. Gupta, Microbial pectinolytic enzymes: a review, Process Biochem. 40 (2005) 2931–2944, https://doi.org/10.1016/j.procbio.2005.03.026.

[10] K.C. Sudeep, J. Upadhyaya, D.R. Joshi, B. Lekhak, D.K. Chaudhary, B.R. Pant, T. R. Bajgai, R. Dhital, S. Khanal, N. Koirala, V. Raghavan, Production, characterization, and industrial application of pectinase enzyme isolated from fungal strains, Fermentation 6 (2020) 59, https://doi.org/10.3390/fermentation6020059.

[11] M. Cerreti, K. Markošová, M. Esti, M. Rosenberg, M. Rebroš, Immobilisation of pectinases into PVA gel for fruit juice application, Int. J. Food Sci. Technol. 52 (2017) 531–539, https://doi.org/10.1111/ijfs.13309.

[12] P. Turner, G. Mamo, E.N. Karlsson, Potential and utilization of thermophiles and thermostable enzymes in biorefining, Microb. Cell Fact. 6 (2007) 9, https://doi.org/10.1186/1475-2859-6-9.

[13] D.S. Leh, A. Biz, D.H.F. de Paula, P. Richard, A.G. Gonçalves, M.D. Noseda, D. A. Mitchell, N. Krieger, Conversion of citric pectin into D-galacturonic acid with high substrate loading using a fermented solid with pectinolytic activity, Biocatal. Agric. Biotechnol 11 (2017) 214–219, https://doi.org/10.1016/j.bcab.2017.07.003.

[14] M.G. Wiebe, D. Mojzita, S. Hilditch, L. Ruohonen, M. Penttilä, Bioconversion of D-galacturonate to filamentous fungi, BMC Biotechnol. 10 (2010) 63. ⟨http://www.biomedcentral.com/1472-6750/10/63⟩.

[15] K.C. Wagschal, J. Rose Stoller, V.J. Chan, D.B. Jordan, Expression and characterization of hyperthermostable exopolygalacturonase RmGH28 from *Rhodothermus marinus*, Appl. Biochem. Biotechnol. 183 (2017) 1503–1515, https://doi.org/10.1007/s12010-017-2518-0.

[16] Y. Taguchi, A. Oishi, H. Iida, One-step synthesis of dibutyl furandicarboxylates from galactaric acid, Chem. Lett. 37 (2007) 50–51, https://doi.org/10.1246/cl.2008.50.
[17] C. Lavilla, A. Alla, A.M. De Ilarduya, E. Benito, M.G. García-Martín, J.A. Galbis, S. Muñoz-Guerra, Carbohydrate-based polyesters made from bicyclic acetalized galactaric acid, Biomacromolecules 12 (2011) 2642–2652, https://doi.org/10.1002/pola.25930.
[18] H. Zhang, X. Li, X. Su, E.L. Ang, Y. Zhang, H. Zhao, Production of adipic acid from sugar beet residue by combined biological and chemical catalysis, ChemCatChem 8 (2016) 1500–1506, https://doi.org/10.1002/cctc.201600069.
[19] M. Cárdenas-Fernández, F. Subrizi, D. Dobrijevic, H.C. Hailes, J.M. Ward, Characterisation of a hyperthermophilic transketolase from *Thermotoga maritima* DSM3109 as a biocatalyst for 7-keto-octuronic acid synthesis, Org. Biomol. Chem. 19 (2021) 6493–6500, https://doi.org/10.1039/d1ob01237a.
[20] D. Dobrijevic, L.A. Nematollahi, H.C. Hailes, J.M. Ward, pET expression vector customized for efficient seamless cloning, Biotechniques 69 (2020) 384–387, https://doi.org/10.2144/btn-2020-0101.
[21] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (1959) 426–428, https://doi.org/10.1021/ac60147a030.
[22] M.T. Held, G.E. Anthon, D.M. Barrett, The effects of bruising and temperature on enzyme activity and textural qualities of tomato juice, J. Sci. Food Agric. 95 (2015) 1598–1604, https://doi.org/10.1002/jsfa.6990.
[23] J.A. Salas-Tovar, A.C. Flores-Gallegos, J.C. Contreras-Esquivel, S. Escobedo-García, J.A. Morlett-Chávez, R. Rodríguez-Herrera, Analytical methods for pectin methylesterase activity determination: a review, Food Anal. Methods. 10 (2017) 3634–3646, https://doi.org/10.1007/s12161-017-0934-y.
[24] T.E. Bapiro, A. Sykes, S. Martin, M. Davies, J.W.T. Yates, M. Hoch, H.E. Rollison, B. Jones, Complete substrate inhibition of cytochrome P450 2C8 by AZD9496, an oral selective estrogen receptor degrader, Drug Metab. Dispos. 46 (2018) 1268–1276, https://doi.org/10.1124/dmd.118.081539.
[25] D.P. Ward, P. Hewitson, M. Cárdenas-Fernández, C. Hamley-Bennett, A. Díaz-Rodríguez, N. Douillet, J.P. Adams, D.J. Leak, S. Ignatova, G.J. Lye, Centrifugal partition chromatography in a biorefinery context: optimisation and scale-up of monosaccharide fractionation from hydrolysed sugar beet pulp, J. Chromatogr. A 1497 (2017) 56–63, https://doi.org/10.1016/j.chroma.2017.03.003.
[26] J. Parisot, V. Langlois, V. Sakanyan, C. Rabiller, Cloning expression and characterization of a thermostable exopolygalacturonase from *Thermotoga maritima*, Carbohydr. Res. 338 (2003) 1333–1337, https://doi.org/10.1016/S0008-6215(03)00165-4.
[27] L.D. Kluskens, G.J.W.M. Van Alebeek, J. Walther, A.G.J. Voragen, W.M. De Vos, J. Van Der Oost, Characterization and mode of action of an exopolygalacturonase from the hyperthermophilic bacterium *Thermotoga maritima*, FEBS J. 272 (2005) 5464–5473, https://doi.org/10.1111/j.1742-4658.2005.04935.x.
[28] T. Pijning, G. van Pouderoyen, L. Kluskens, J. van der Oost, B.W. Dijkstra, The crystal structure of a hyperthermoactive exopolygalacturonase from *Thermotoga maritima* reveals a unique tetramer, FEBS Lett 583 (2009) 3665–3670, https://doi.org/10.1016/j.febslet.2009.10.047.
[29] D.E. Evangelista, E.A. de Araújo, M.O. Neto, M.A.S. Kadowaki, I. Polikarpov, Biochemical characterization and low-resolution SAXS structure of an exo-polygalacturonase from *Bacillus licheniformis*, N. Biotechnol. 40 (2018) 268–274, https://doi.org/10.1016/j.nbt.2017.10.001.
[30] C. Remoroza, M. Wagenknecht, H.C. Buchholt, B.M. Moerschbacher, H. Gruppen, H.A. Schols, Mode of action of Bacillus licheniformis pectin methylesterase on highly methylesterified and acetylated pectins, Carbohydr. Polym. 115 (2015) 540–550, https://doi.org/10.1016/j.carbpol.2014.09.016.
[31] T. Kobayashi, Y. Hatada, N. Higaki, D.D. Lusterio, T. Ozawa, K. Koike, S. Kawai, S. Ito, Enzymatic properties and deduced amino acid sequence of a high-alkaline pectate lyase from an alkaliphilic *Bacillus* isolate, Biochim. Biophys. Acta - Gen. Subj. 1427 (1999) 145–154, https://doi.org/10.1016/S0304-4165(99)00017-3.
[32] W. Nasser, F. Chalet, J. Robert-Baudouy, Purification and characterization of extracellular pectate lyase from *Bacillus subtilis*, Biochimie 72 (1990) 689–695, https://doi.org/10.1016/0300-9084(90)90053-J.
[33] J.-P. Vincken, H.A. Schols, R.J.F.J. Oomen, M.C. McCann, P. Ulvskov, A.G. J. Voragen, R.G.F. Visser, If Homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture 1, Plant Physiol. 132 (2003) 1781–1789, https://doi.org/10.1104/pp.103.022350.in.
[34] S.L. Gonzalez, N.D. Rosso, Determination of pectin methylesterase activity in commercial pectinases and study of the inactivation kinetics through two potentiometric procedures, Ciência e Tecnol. Aliment 31 (2011) 412–417, https://doi.org/10.1590/s0101-20612011000200020.
[35] Y.A. Ramírez-Tapias, C.W. Rivero, C.N. Britos, J.A. Trelles, Biocatalysis and agricultural biotechnology alkaline and thermostable polygalacturonase from *Streptomyces halstedii* ATCC 10897 with applications in waste waters, Biocatal. Agric. Biotechnol. 4 (2015) 221–228, https://doi.org/10.1016/j.bcab.2014.12.004.
[36] E. Favela-Torres, T. Volke-Sepúlveda, G. Viniegra-González, Production of hydrolytic depolymerising pectinases, Food Technol. Biotechnol. 44 (2006) 221–227.
[37] S.N. Gummadi, T. Panda, Analysis of kinetic data of pectinases with substrate inhibition, J. Microbiol. Biotechnol. 13 (2003) 332–337.
[38] K. Wagschal, J. Rose Stoller, V.J. Chan, C.C. Lee, A.A. Grigorescu, D.B. Jordan, Expression and characterization of hyperthermostable exo-polygalacturonase TtGH28 from *Thermotoga thermophilus*, Mol. Biotechnol. 58 (2016) 509–519, https://doi.org/10.1007/s12033-016-9948-8.
[39] Y. Chen, D. Sun, Y. Zhou, L. Liu, W. Han, B. Zheng, Z. Wang, Z. Zhang, Cloning, expression and characterization of a novel thermophilic polygalacturonase from *Caldicellulosiruptor bescii* DSM 6725, Int. J. Mol. Sci. 15 (2014) 5717–5729, https://doi.org/10.3390/ijms15045717.
[40] V. Rajulapati, A. Goyal, Molecular cloning, expression and characterization of pectin methylesterase (CtPME) from *Clostridium thermocellum*, Mol. Biotechnol. 59 (2017) 128–140, https://doi.org/10.1007/s12033-017-9997-7.
[41] C. Chakiath, M.J. Lyons, R.E. Kozak, C.S. Laufer, Thermal stabilization of *Erwinia chrysanthemi* pectin methylesterase A for application in a sugar beet pulp biorefinery, Appl. Environ. Microbiol. 75 (2009) 7343–7349, https://doi.org/10.1128/AEM.01010-09.
[42] C. Lara-Espinoza, E. Carvajal-Millán, R. Balandrán-Quintana, Y. López-Franco, A. Rascón-Chu, Pectin and pectin-based composite materials: beyond food texture, Molecules 23 (2018) 942, https://doi.org/10.3390/molecules23040942.
[43] M. Dimopoulou, K. Alba, G. Campbell, V. Kontogiorgos, Pectin recovery and characterization from lemon juice waste streams, J. Sci. Food Agric. 99 (2019) 6191–6198, https://doi.org/10.1002/jsfa.9891.
[44] F. Micheli, Pectin methylesterases: cell wall enzymes with important roles in plant physiology, Trends Plant Sci. 6 (2001) 414–419, https://doi.org/10.1016/S1360-1385(01)02045-3.
[45] M. Fries, J. Ihrig, K. Brocklehurst, V.E. Shevchik, R.W. Pickersgill, Molecular basis of the activity of the phytopathogen pectin methylesterase, EMBO J. 26 (2007) 3879–3887, https://doi.org/10.1038/sj.emboj.7601816.
[46] L.M. Kent, T.S. Loo, L.D. Melton, D. Mercadante, M.A.K. Williams, G.B. Jameson, Structure and properties of a non-processive, salt-requiring, and acidophilic pectin methylesterase from *Aspergillus niger* provide insights into the key determinants of processivity control, J. Biol. Chem. 291 (2016) 1289–1306, https://doi.org/10.1074/jbc.M115.673152.
[47] G. Limberg, R. Körner, H.C. Buchholt, T.M.I.E. Christensen, P. Roepstorff, J. D. Mikkelsen, Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. niger*, Carbohydr. Res. 327 (2000) 293–307, https://doi.org/10.1016/S0008-6215(00)00067-7.
[48] S.A. Mohamed, T.M.I.E. Christensen, J.D. Mikkelsen, New polygalacturonases from *Trichoderma reesei*: characterization and their specificities to partially methylated and acetylated pectins, Carbohydr. Res. 338 (2003) 515–524, https://doi.org/10.1016/S0008-6215(02)00398-1.
[49] F. Goubet, A. Ström, P. Dupree, M.A.K. Williams, An investigation of pectin methylesterification patterns by two independent methods: capillary electrophoresis and polysaccharide analysis using carbohydrate gel electrophoresis, Carbohydr. Res. 340 (2005) 1193–1199, https://doi.org/10.1016/j.carres.2005.01.037.
[50] E. Bonnin, A. Le Goff, R. Körner, J. Vigouroux, P. Roepstorff, J.F. Thibault, Hydrolysis of pectins with different degrees and patterns of methylation by the endopolygalacturonase of *Fusarium moniliforme*, Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol. 1596 (2002) 83–94, https://doi.org/10.1016/S0167-4838(02)00207-8.
[51] M. Cárdenas-Fernández, M. Bawn, C. Hamley-Bennett, P.K.V. Bharat, F. Subrizi, N. Suhaili, D.P. Ward, S. Bourdin, P.A. Dalby, H.C. Hailes, P. Hewitson, S. Ignatova, C. Kontoravdi, D.J. Leak, N. Shah, T.D. Sheppard, J.M. Ward, G.J. Lye, An integrated biorefinery concept for conversion of sugar beet pulp into value-added chemicals and pharmaceutical intermediates, Faraday Discuss 202 (2017) 415–431, https://doi.org/10.1039/c7fd00094d.
[52] M. Cárdenas-Fernández, C. Hamley-Bennett, D.J. Leak, G.J. Lye, Continuous enzymatic hydrolysis of sugar beet pectin and L-arabinose recovery within an integrated biorefinery, Bioresour. Technol. 269 (2018) 195–202, https://doi.org/10.1016/j.biortech.2018.08.069.