Novel thermophilic pectinases for recycling sustainable biomass

Carol Nathali Flores Fernández

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DECLARATION

I, Carol Nathali Flores Fernández, confirm that the work presented in my thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Large amounts of pectin-rich biomass are produced mainly from juice and sugar industries. Pectinases represent an economical and eco-friendly alternative to depolymerise it in comparison with chemical treatments, for both obtaining bio-based chemicals and improving industrial bioprocess. This thesis aimed to identify novel thermophilic pectinases, carry out their functional characterisation, and explore the synergistic activity between pectin methylesterases (PMEs) and exo-polygalacturonases (exo-PGs) for pectin bioconversion into galacturonic acid (GalA). Also, the work was focused on the co-expression of genes encoding a PME and exo-PG in a single host for a cost-effective pectin bioconversion into GalA. Finally, the synergistic activity between PMEs and pectate lyases (PGLs) for improving pectin depolymerisation, useful in several industrial processes, was also explored.

A total of seven genes encoding thermophilic bacterial pectinases were successfully cloned, and the enzymes expressed included two exo-PGs (TMA01 and BLI04), two PMEs (BLI09 and SAM10) and three PGLs (TMA14, TFU19 and TFU20). Mn^{2+} significantly increased the exo-PGs activity (2-fold) and did not affect PMEs action allowing its inclusion in the synergistic reactions. Both exo-PGs and PMEs exhibited high activity and stability between 40 and 90 °C up to 24 h. The synergistic reactions between BLI09 PME paired either with TMA01 or BLI04 exo-PGs using apple and citrus pectin were the most successful for pectin bioconversion, releasing around 2.5 mM GalA (29% yield). GalA release by pectinases is important in industry since it is a key chemical for the synthesis of a number of valuable compounds. In addition, enzymes allow to release this compound in a sustainable biocatalytic process.

Four co-expression plasmids containing a PME and exo-PG were constructed in pETDuet-1, in which the gene's cloning order as well as the presence of a T7 terminator behind the second gene affected the pectinases expression levels. TMA01 and BLI04 exo-PGs were well expressed in all the constructs, but BLI09 PME was better expressed cloned downstream the exo-PGs in MCS-2 and with the presence of its own T7 terminator behind. Thus, the most successful co-expression plasmids were the constructs 3 and 4 (pETDuet-TMA01-BLI09 and

pETDuet-BLI04-BLI09, respectively) which allowed the release of around 3 mM GalA (35% yield) from apple and citrus pectin. GalA release was limited by product inhibition since this compound had an inhibitory effect on both exo-PGs from around 3 mM.

Finally, the three PGLs were characterised showing Ca^{2+} dependant activity, while Mn^{2+} significantly improved the activity of TFU20 (2.5-fold). All the PGLs exhibited optimum activity and stability between 50 and 90 °C and Ca^{2+} improved their thermal stability. The three PGLs were able to depolymerise pectin, but the main peak of 650 kDa observed in both non-esterified and esterified substrates was depolymerised only in the non-esterified. These results evidenced the need of a synergistic action of PGLs with PMEs for improving esterified pectin depolymerisation. Thus, the smallest oligogalacturonates (oligoGalA) because of this main peak depolymerisation in apple, citrus and sugar beet pectin were obtained from synergistic reactions between SAM10 PME paired either with TMA14 (4 – 10 kDa) or TFU20 (8 – 25 kDa).

Overall, this work provided optimum conditions of activity of novel thermophilic pectinases, which are fundamental to set up compatible operational conditions especially in synergistic reactions. Synergistic activity between pectinases allowed the release of GalA and co-expression systems made the pectin bioconversion process more cost-effective. Synergistic activity also improved esterified pectin depolymerisation, useful and applicable to several industries. These findings provided further insights for recycling sustainable biomass within a context of biorefineries and circular economy.

IMPACT STATEMENT

This research has identified and fully characterised several novel thermophilic pectinases. Likewise, it has demonstrated the utility of these enzymes, which acting in a synergistic manner allowed to obtain valuable bio-based chemicals such as GalA from underused biomass feedstocks. In addition, as a result of this research, methods such as enzymes co-expression for a cost-effective production and application of these pectinases have been developed. Finally, the synergistic activity of these pectinases has improved pectin depolymerisation which could be useful in several fields of industry. The potential benefits of this research will have impact in academia, environment and industry.

In academia, the discovery of novel thermophilic pectinases contributes to extending the knowledge in this field in which mesophilic enzymes are mostly reported. Likewise, the study of the applicability of these enzymes is vital to understand their importance in the biocatalytic processes such as native pectin depolymerisation. All this knowledge provides further insights for the development of related research projects. Moreover, the dissemination of this research in scientific events where the industrial sector is also involved could facilitate the establishment of a collaborative relationship between academia and industry.

From an environmental perspective, pectin-rich biomass is produced in larger amounts mainly from juice and sugar industries and in some causes the excess is causing pollution issues. The bioconversion of this biomass using pectinases represent a promising alternative for recycling it as well as to reduce the excessive amounts that produce environmental pollution. This approach could be applied for the recycling of different kinds of biomass using a variety of enzymes. Furthermore, with the increasing emphasis in green chemistry, the biocatalytic processes using enzymes such as pectinases are becoming more important since they are environmentally sustainable.

In industry, the results from this research could give a number of future benefits. Juice and sugar industries which are the main producers of pectin-rich biomass could develop lines of business using pectinases for recycling it to obtain

added-value products. Moreover, industries related to the biorefineries sector could exploit this biomass which is sold as a low-cost material or mainly discarded by other industries. Regarding enzymes co-expression, the development of this methodology will contribute to reduce the enzymes production and purification costs. On the other hand, the pectinases from this research could be incorporated in industrial processes in which pectin depolymerisation is crucial to improve quality and yield such as in juice, sugar, and textile industry as well as in paper and wine making.

Overall, this research provides to the scientific community and industrial sector additional insights to valorise and exploit biomass feedstocks through biocatalytic processes with economic and environmental benefits. The dissemination through congresses, conferences, seminars, meetings, and publications not only for researchers will give more opportunities for multidisciplinary collaborations in this field.

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All authors contributed to the conception and the main idea of the work. Carol N. Flores-Fernández carried out the experiments, wrote the manuscript and elaborate figures and tables. Max Cárdenas-Fernández supervised the work, provided feedback and additional scientific information, and revised the manuscript. Gary J. Lye and John M. Ward also reviewed and revised the text. All authors read and approved the final version of the work.

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ABBREVIATIONS

Ace*f*A	3-C-carboxy-5-deoxy-L-xylofuranose or Aceric acid
AF	Arabinofuranosidase
Api*f*	Apiofuranose
Ara	Arabinose
Ara*f*	Arabinofuranose
Ara*p*	Arabinopyranose
CexCons	Co-expression construct
CV	Column volume
Dha*p*	3-Deoxy-D-lyxo-hept-2-ulo-pyranosaric acid
Exo-PG	Exo-polygalacturonase
FDCA	Furanedicarboxylic acids
Fuc*p*	Fucopyranose
Gal	Galactose
GalA	Galacturonic acid
Gal*p*	Galactopyranosyl
GH	Glycoside hydrolases
Glc*p*A	Glucopyranuronic acid
HG	Homogalacturonan
His6-tag	6x Histidine-tagged
IPTG	Isopropil-β-D-1-tiogalactopiranósido
kDa	Kilo Dalton
Kdo*p*	3-Deoxy-D-manno-oct-2-ulopyranosonic acid
MeFuc*p*	2-*O*-methylfucopyranose

MeXyl*p*	2-*O*-methyl-xylopyranose
Rha	Rhamnose
PAE	Pectin acetylesterase
PCR	Polymerase chain reaction
PG	Polygalacturonase
PGL	Pectate lyase
PL	Pectin lyase
PME	Pectin methylesterase
POS	Pectic-derived oligosaccharides
RGAE	Rhamnogalacturonan acetylesterase
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
RGH	Rhamnogalacturonan hydrolase
RGL	Rhamnogalacturonan lyase
Rha*p*	Rhamnopyranose
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SBP	Sugar beet pulp
XG	Xylogalacturonan
Xyl*p*	Xylopyranose

CHAPTER 1 INTRODUCTION

1.1 Overview of pectin-rich sustainable biomass

A large amount of pectin-rich agroindustrial by-products is generated annually, mainly from sugar and juice industries. The cell walls of pectin-rich biomass contain 12–35% pectin on a dry weight basis (Hoondal *et al.*, 2002; Edwards and Doran-Peterson, 2012). Agro-by-products such as citrus peel and apple pomace are utilised as a source of pectin which is used as a natural ingredient in food industry for its gelling, thickening and stabilising properties. However, pectin-rich by-products such as sugar beet pulp are low cost feedstocks used as animal feed, and other from other sources are not processed causing pollution problems (Voragen *et al.*, 2009; Bhatia, Sharma and Alam, 2016).

Pectin-rich biomass constitutes a promising sustainable feedstock to produce bio-based chemicals in a circular economy context, however the current methods to hydrolyse it and produce these molecules involve the use of acidic, alkaline and chelating agents. The use of pectinases represents an alternative for the hydrolysis and biotransformation of pectin-rich biomass feedstocks with economic and ecological advantages (Pedrolli *et al.*, 2009; Benoit *et al.*, 2012; Minzanova *et al.*, 2018; Vastano *et al.*, 2019). One of the most important compounds obtained as a result of pectin bioconversion is GalA, because it is a precursor of a number of valuable compounds in industry such as L-galactonic acid, L-ascorbic acid, keto-deoxy sugars, galactaric acid, adipic acid, furanedicarboxylic acids as well as polyesters, nylon and other bio-based polymers (Taguchi, Oishi and Iida, 2007; Lavilla *et al.*, 2011; Kuivanen *et al.*, 2014; Zhang *et al.*, 2016; Wagschal *et al.*, 2017).

1.1.1 The composition of pectin

Pectin is a complex heteropolymer and consists of a mixture of heterogeneous, branched and high molecular weight polysaccharides (Voragen *et al.*, 2009). Although pectin from different sources shares some structural and chemical characteristics, a number of them such as D-Galacturonic acid (GalA) content and degree of esterification change according to the plant species, the physiological stage of the plant and the tissue (Benoit *et al.*, 2012; Minzanova *et*

al., 2018). Pectin structure is a composite of several substructures such as homogalacturonan (HG), xylogalacturonan (XG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Figure 1.1) (Vincken *et al.*, 2003; Wong, 2008; Pedrolli *et al.*, 2009; Voragen *et al.*, 2009).

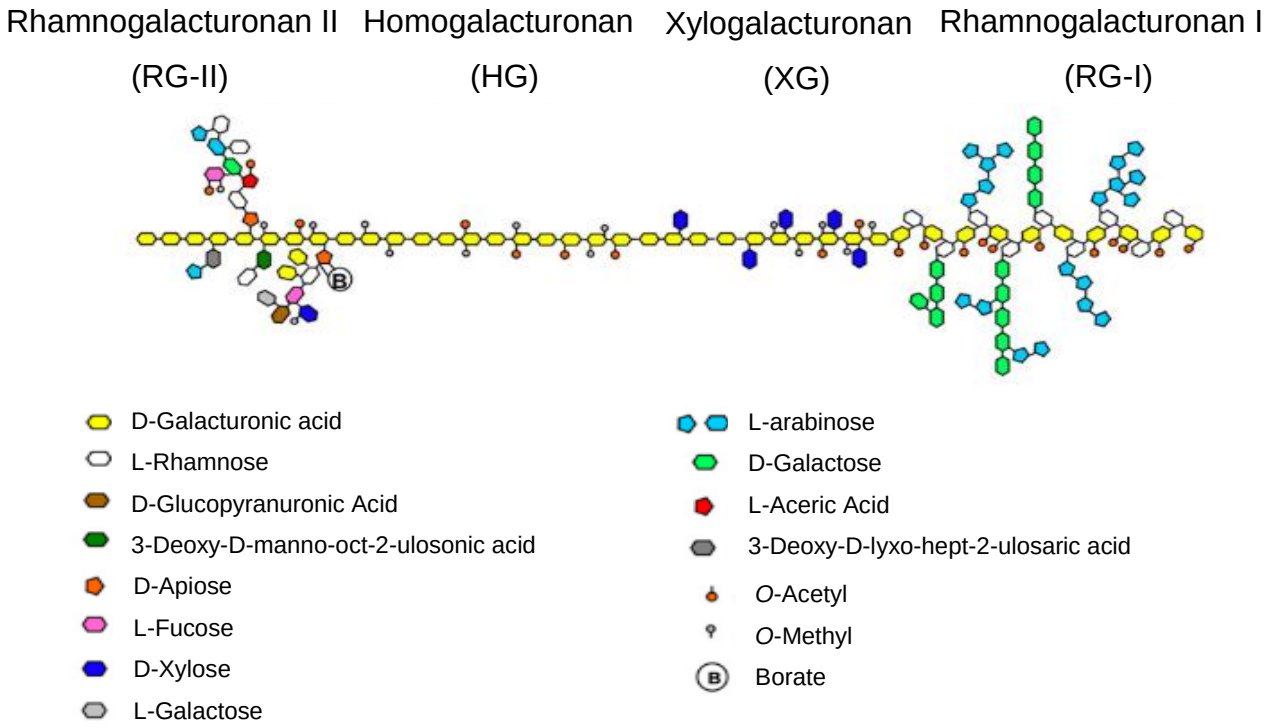

Figure 1.1. Schematic structure of pectin. Adapted from Harholt, Suttangkakul and Scheller (2010).

The ratio between pectin substructures is variable. However, in most cases, HG is the major polysaccharide accounting for 60-65%, RG-I constitutes from 20 to 35% and XG and RG-II each less than 10% (Harholt, Suttangkakul and Scheller, 2010; Gawkowska, Cybulska and Zdunek, 2018). A brief description of pectin substructures is mentioned below (Figure 1.2).

HG is a linear homopolymer formed by (1→4)-linked α-D-galacturonic acid with the carboxyl group (C6) methyl esterified at various degrees up to 80%. In some plants such as sugar beet, acetyl substitution also may be found at *O*-2 and *O*-3 positions. The amount and distribution of methyl ester and acetyl groups vary according to the plant source. The presence of ester groups in HG has a deep impact on pectin-gelation properties. Thus, a high-methyl esterified pectin forms

a gel at pH <3.5 and in the presence of at least 55% sugar, while a low-methyl esterified pectin can form gel in the presence of divalent cations, such as calcium, in a wide range of pH and without sugar. In commercial formulations, the degree of esterification depends on the processing conditions (Wong, 2008; Voragen *et al.*, 2009; Bonnin, Garnier and Ralet, 2014; Remoroza *et al.*, 2015).

XG is a branched heteropolymer formed by HG with β-D-Xylopyranose (Xyl*p*) substitutions at *O*-3 position of some GalA residues. Depending on the source, XG may also be methylated at the carboxylic acid groups of GalA residues, as found in apple pectin (Wong, 2008; Voragen *et al.*, 2009; Bonnin, Garnier and Ralet, 2014).

RG-I is a heteropolymeric backbone composed of the repeating disaccharide (1→2)-linked α-L- rhamnose-(1→4)-linked α-D-galacturonic acid with both residues in the pyranose form. The GalA residues may be methyl esterified as well as different degree of acetyl substitutions may be found at the *O*-2 and *O*-3 positions depending on the source of pectin. The L-Rhamnopyranose residues are also highly substituted mainly at the C4, but sometimes at the C3 position, with neutral side chains of arabinan, galactan and arabinogalactan ranging from 20 to 80% depending on the source. (Wong, 2008; Pedrolli *et al.*, 2009; Voragen *et al.*, 2009; Bonnin, Garnier and Ralet, 2014; Gawkowska, Cybulska and Zdunek, 2018).

Finally, RG-II is a highly substituted and branched HG with side chains containing 12 uncommon sugars including Ace*f*A (3-C-carboxy-5-deoxy-L-xylofuranose or Aceric acid), D-Api*f* (D-apiofuranose), L-Ara*f* (L-Arabinofuranose), L-Ara*p* (L-Arabinopyranose), D-Dha*p* (3-Deoxy-D-lyxo-hept-2-ulo-pyranosaric acid), L-Fuc*p* (L-Fucopyranose), D-Glc*p*A (D-Glucopyranuronic Acid), Gal*p* (galactopyranosyl), GalA*p*, Kdo*p* (3-Deoxy-D-manno-oct-2-ulopyranosonic acid), MeFuc*p* (2-*O*-methylfucopyranose), 2-O-MeXyl*p* (2-*O*-methyl-xylopyranose), and L-Rha*p* residues. The substitutions may be found at the C2 and C3 positions of GalA residues (Wong, 2008; Bonnin, Garnier and Ralet, 2014; Gawkowska, Cybulska and Zdunek, 2018).

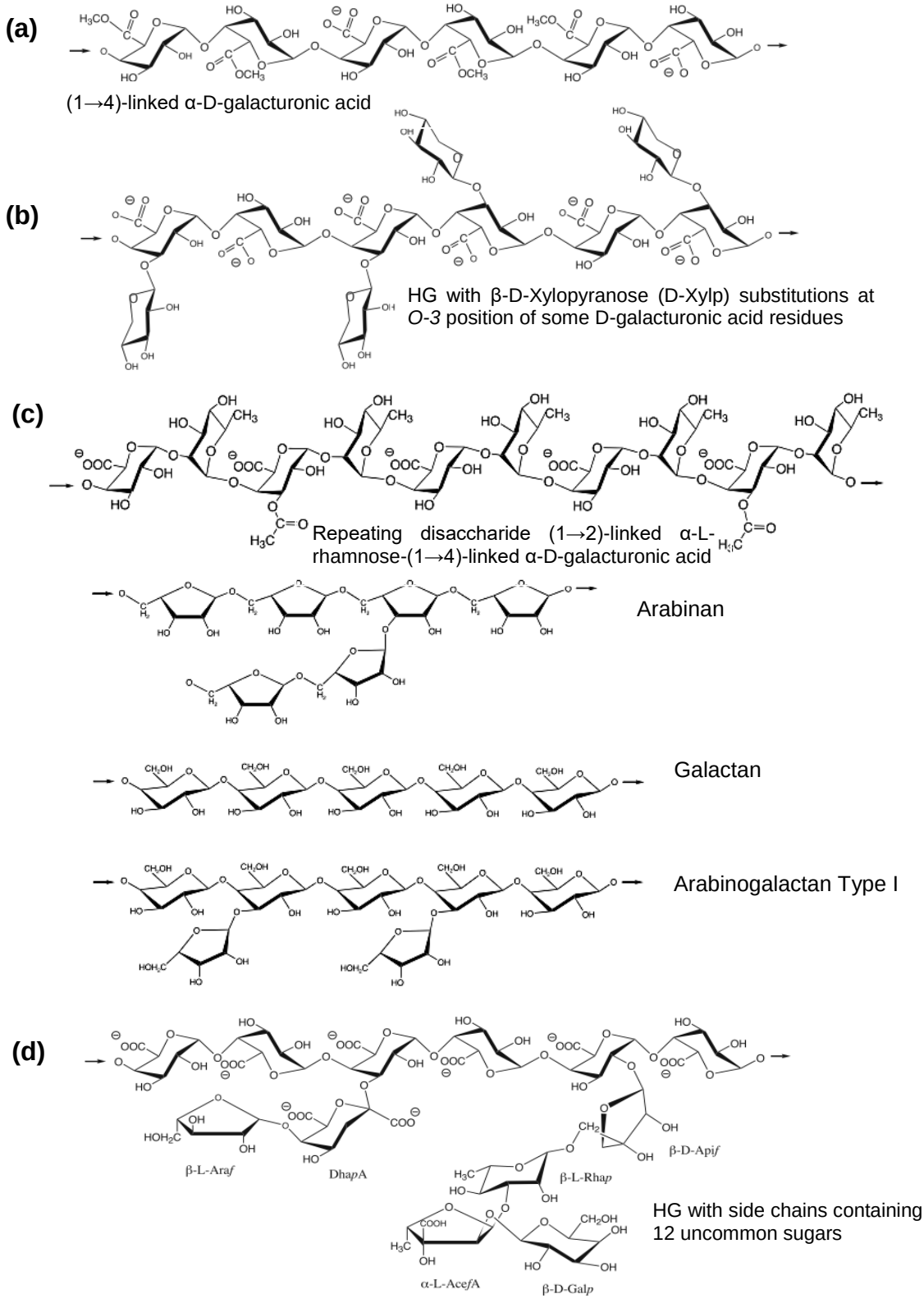

Figure 1.2. Structure of pectin. **(a)** HG formed by (1→4)-linked α-D-galacturonic acid, **(b)** XG formed by HG with β-D-Xylopyranose (D-Xyl*p*) substitutions at *O*-3 position of some D-galacturonic acid residues, **(c)** RG-I formed by the repeating disaccharide (1→2)-linked α-L- rhamnose-(1→4)-linked α-D-galacturonic acid. Neutral side chains of arabinan, galactan and arabinogalactan could be linked at C4 positions of L-rhamnose, and **(d)** RG-II formed by a branched HG with side chains containing 12 uncommon sugars (Wong, 2008).

1.1.2 Sugar beet pulp

About 250 million tonnes of sugar beet are produced worldwide, annually. Most of them are produced in Europe, where more than 112 million tonnes were grown in 2016. Sugar beet is one of the most important raw materials used for sugar production and as a result of its processing, a huge amount of sugar beet pulp (SBP) residues is generated (Richard and Hilditch, 2009; Cárdenas-Fernández *et al.*, 2018). SBP is the main by-product of sucrose extraction, and it is a rich source of pectin, about 20% pectin on a dry weight basis. After sugar extraction, this by-product is dried from 18–23 to 88% w/w dry solids in a high energy consuming process. Finally, it is pelleted and sold as a low value animal feed incurring significant drying and transportation costs. SBP is well-suited for enzymatic saccharification of the remaining polysaccharides such as pectin. The resultant sugars could be used for chemical conversion to other value-added products (Chakiath *et al.*, 2009). Therefore, SBP abundance and low cost making it a sustainable biomass feedstock for the production of chemical and pharmaceutical intermediates within a biorefinery context (Ward *et al.*, 2015; Hamley-Bennett, Lye and Leak, 2016; Cárdenas-Fernández *et al.*, 2017; Gawkowska, Cybulska and Zdunek, 2018).

Sugar beet pectin structure mainly consists of HG and RG-I backbones where polymeric methylated and acetylated GalA is linked with intermittent blocks of alternating rhamnose (Rha) – GalA residues. The degree of acetylation is higher compared to pectin from other sources (around 35%) with acetyl groups present in both HG and RG-I (Leijdekkers *et al.*, 2013). From Rha residues start unbranched galactan and highly branched arabinan side chains where both GalA and arabinose (Ara) can be feruloylated (Ward *et al.*, 2015; Cárdenas-Fernández *et al.*, 2018). The arabinan content represents 20–25% of the SBP dry matter (Leijdekkers *et al.*, 2013). The most abundant monosaccharides are Ara (23%) and GalA (14.4%), while other monosaccharides such as galactose (Gal), Rha, xylose and mannose are in 6.2, 2.4, 1.7 and 1.0%, respectively (Hamley-Bennett, Lye and Leak, 2016). In general, pectin of different sources is used as a gelling agent; however, sugar beet pectin is a poor gel-forming agent due to its high degree of acetylation at the hydroxyl groups at C2 and C3, hence this application is limited. Nevertheless, sugar beet pectin can be used as a

thickener or emulsifier due to acetyl esters on pectin contributes to its role as emulsion stabilizer (Cárdenas-Fernández *et al.*, 2017, 2018).

1.1.3 Citrus processing waste

The current annual world production of citrus fruits is greater than 100 million tonnes of which oranges constitute about 55 million tonnes. In the European Union, orange production is around 6.5 million tonnes (USDA, 2019). Large quantities of citrus processing waste are produced annually in the world, around 10 million tonnes by the orange processing industry alone (Kuivanen *et al.*, 2014). Citrus peel, main residue of citrus processing waste, contains about 25-35% pectin on a dry mass basis and is used as animal feed as well as raw material for pectin production for the food industry (Richard and Hilditch, 2009; Banerjee *et al.*, 2016). Nonetheless, only a relatively small fraction of citrus processing waste is used as a pectin source for food industry and its use as animal feed is seldom economic since the process demands high energy consumption (Kuivanen *et al.*, 2014).

For those reasons, most attempts to produce higher-value products from citrus processing waste have involved the extraction of pectin and the use of pectinases. Pectin from citrus peel comprises mainly highly methyl esterified HG and RG-I (Yapo *et al.*, 2007) whose conversion into biofuels and value-added products is the subject of much current interest.

1.1.4 Apple pomace

Annually approximately 70 million tonnes of apples are produced worldwide which are required to manufacture apple juice, cider, jam, and vinegar; generating large volumes of residue, known as apple pomace (about 4 million tonnes). Apple pomace contains about 15% pectin on a dry mass basis and the same as other by-products rich in pectin is used as low cost animal feed as well as a source of pectin for food industry (Voragen *et al.*, 2009; Banerjee *et al.*, 2016). In addition, it has been used as a raw material for microbial fermentation where the targets are metabolic products of specific microorganisms such as citric acid, lactic acid, enzymes and biopolymers. Nevertheless, apple pomace may also be valorised by hydrolysis of pectin into high value-added products. In

addition, the valorisation of apple pomace will reduce environmental impact and meet the requirement of sustainable development of the large-scale apple processing industry (Shalini and Gupta, 2010; Perussello *et al.*, 2017).

1.2 Introduction to pectinases

1.2.1 Classification of pectinases

Owing to the structural complexity of pectin, several kinds of pectinases are involved in its degradation. This diverse group of enzymes includes glycoside hydrolases (GHs), polysaccharide lyases and carboxylic ester hydrolases (Table 1.1). Physiologically, carboxylic ester hydrolases have great impact on functional properties of pectin since ester groups hinder the association abilities of pectin and obstruct pectin-depolymerising enzymes action (GHs and polysaccharide lyases) (Jayani, Saxena and Gupta, 2005; Sharma, Rathore and Sharma, 2012; Satyanarayana, Kawarabayasi and Littlechild, 2013; Bonnin, Garnier and Ralet, 2014).

Nevertheless, the most widely used classification of pectinases is based on the substructure or functional group of pectin used as a substrate (Figure 1.3). Thus, polygalacturonases (PGs) catalyse the cleavage of (1→4)-α-D-glycosidic bonds in HG and belong to GHs family 28. PGs are divided into endo-PGs (EC 3.2.1.15) and exo-PGs (EC 3.2.1.67 and EC 3.2.1.82). Endo-PGs catalyse random cleavage of HG and different forms of these enzymes have different tolerances to methyl and acetyl esterification or Xyl*p* substitutions of the substrate (Figure S 1, Appendix 1). The first type of exo-PGs (EC 3.2.1.67) hydrolyse the α-1,4-glycosidic bond from the non-reducing end producing monogalacturonate (Figure S 2, Appendix 1), whereas the second type of exo-PGs (EC 3.2.1.82) hydrolyse the α-1,4-glycosidic bond from the non-reducing end releasing digalacturonate (Figure S 3, Appendix 1) (Hasegawa and Nagel, 1968; He and Collmer, 1990; Martens-Uzunova *et al.*, 2006; Bonnin, Garnier and Ralet, 2014). Exo-PGs are unable to degrade unsaturated or methyl-esterified substrates, but they are tolerant for xylose substitutions (Voragen *et al.*, 2009).

Table 1.1. Classification of pectinases according to their mechanism of activity. Adapted from Bonnin, Garnier and Ralet (2014).

Enzyme	EC number	Substrate	Product
Glycoside Hydrolases			
Endo-polygalacturonase (Endo-PG)	3.2.1.15	HG	OligoGalA
Exopoli-D-galacturonase[a] (Exo-PG)	3.2.1.67	HG	GalA
Exo-poly-α-digalacturonosidase[a] (Exo-PG)	3.2.1.82	HG	$GalA_2$
Rhamnogalacturonan hydrolase (Endo-RGH)	3.2.1.171	RG-I	OligoRG-I
Rhamnogalacturonan galacturonohydrolase (Exo-RGH)	3.2.1.173	RG-I	GalA
Rhamnogalacturonan rhamnohydrolase (Exo-RGH)	3.2.1.174	RG-I	Rha
Xylogalacturonan hydrolase	3.2.1.-	XG	Xylosylated OligoGalA
Arabinan endo-1,5-α-L-arabinosidase	3.2.1.99	Arabinan	Oligoarabinosides
Exo-arabinosidase	3.2.1.-	Arabinan	Arabinobiose
Non-reducing end α-L-arabinofuranosidase (AF)	3.2.1.55	Oligoarabinosides	Arabinose
Arabinogalactan endo-β-1,4 galactanase	3.2.1.89	Arabinogalactan Type I	Oligogalactosides
Exo-β-1,4 galactanase	3.2.1.-	Galactan	Galactose or galactobiose
β-galactosidase	3.2.1.23	Oligogalactosides	Galactose
Polysaccharide Lyases			
Pectate lyase[a] (Endo-PGL)	4.2.2.2	HG	Unsaturated OligoGalA
Pectate disaccharide-lyase[a] (Exo-PGL)	4.2.2.9	HG	Unsaturated digalacturonate
Pectate trisaccharide-lyase[a] (Exo-PGL)	4.2.2.22	HG	Unsaturated trigalacturonate
Pectin lyase (Endo-PL)	4.2.2.10	HG	Unsaturated OligoA

Table 1.1

Continued

Enzyme	EC number	Substrate	Product
Rhamnogalacturonan endolyase (Endo-RGL)	4.2.2.23	RG-I	Unsaturated OligoRG-I
Rhamnogalacturonan exolyase (Exo-RG-I)	4.2.2.24	RG-I	Unsaturated disaccharide
Carboxylic ester hydrolases			
Pectin methylesterase (PME)	3.1.1.11	Pectin	Pectate acid + methanol
Pectin acetylesterase (PAE)	3.1.1.6	Pectin	Pectin/Pectate + acetic acid
Rhamnogalacturonan acetylesterase (RGAE)	3.1.1.86	RG-I/HG	Acetic acid
Feruloylesterase	3.1.1.73	Feruloylated oligosides	Oligosides + ferulic acid

[a] act on non-methylated HG.

Lyases involved in pectin breakdown are divided in pectate lyases (PGLs, use non-methylated HG as a substrate) and pectin lyases (PLs, act on methyl esterified HG). These enzymes belong to polysaccharide lyase family 1. PGLs and PLs, the same as PGs, cleave (1→4)-α-D-glycosidic bonds in HG backbone but those release unsaturated products through transelimination reaction (Section 6.1, Figures S 4 – S7). PGLs require Ca^{2+} for their activity and are classified as endo-PGLs (EC 4.2.2.2) and exo-PGLs (EC 4.2.2.9 and EC 4.2.2.22). Endo-PGLs catalyse the eliminative cleavage of (1→4)-α-D-glycosidic linkages to give oligosaccharides with 4-deoxy-α-D-galact-4-enuronosyl groups at their non-reducing ends (Figure S 4, Appendix 1). The first type of exo-PGLs (EC 4.2.2.9) also known as pectate disaccharide-lyase catalyse the eliminative cleavage of an unsaturated disaccharide from the reducing end of non-esterified HG (Figure S 5, Appendix 1), whereas the second type of exo-PGLs (EC 4.2.2.22) also called pectate trisaccharide-lyase catalyse eliminative cleavage

of unsaturated trigalacturonate as the major product from the reducing end although disaccharides and tetrasaccharides may also be removed (Figure S 6, Appendix 1). On the other hand, PLs are classified only as endo-PLs (EC 4.2.2.10) which catalyse the eliminative cleavage of (1→4)-α-D-glycosidic bonds in methyl esterified HG to give oligosaccharides with 4-deoxy-6-O-methyl-α-D-galact-4-enuronosyl groups at their non-reducing end (Figure S 7, Appendix 1) (Mayans *et al.*, 1997; Shevchik *et al.*, 1999; Berensmeier *et al.*, 2004).

Rhamnogalacturonan-degrading enzymes act on RG-I. These enzymes are classified in Rhamnogalacturonan Hydrolases (RGHs), Rhamnogalacturonan Lyases (RGLs) and Rhamnogalacturonan Acetylesterases (RGAEs). RGHs belong to GHs family 28 and are divided into Endo-RGHs (EC 3.2.1.171) and Exo-RGHs (EC3.2.1.173 and EC 3.2.1.174). Endo-RGHs cleave α-D-GalpA-(1→2)-α-L-Rhap glycosidic bonds with initial inversion of anomeric configuration releasing oligosaccharides with β-D-GalA at the reducing end (Kofod *et al.*, 1994; Azadi *et al.*, 1995; Petersen, Kauppinen and Larsen, 1997). The first type of Exo-RGHs (EC 3.2.1.173) hydrolyse α-D-GalpA-(1→2)-α-L-Rhap linkages in rhamnogalacturonan oligosaccharides with initial inversion of configuration releasing GalA from the non-reducing end of rhamnogalacturonan oligosaccharides (Mutter, Beldman, *et al.*, 1998), while the second type of Exo-RGHs (EC3.2.1.174) catalyse the hydrolysis of the α-L-Rha-(1→4)-α-D-GalA bond in rhamnogalacturonan oligosaccharides with initial inversion of configuration releasing β-L-rhamnose from the non-reducing end of rhamnogalacturonan oligosaccharides (Mutter *et al.*, 1994; Mutter, Beldman, *et al.*, 1998). RGLs belong to polysaccharide lyase family 11 and are also classified into Endo-RGLs (EC 4.2.2.23) and Exo-RGL (EC 4.2.2.24). Endo-RGLs catalyse the eliminative cleavage of α-L-rhamnopyranosyl-(1→4)-α-D-galactopyranosyluronic acid bonds leaving L-rhamnopyranose at the reducing end and 4-deoxy-4,5-unsaturated D-galactopyranosyluronic acid at the non-reducing end (Mutter *et al.*, 1996; Mutter, Colquhoun, *et al.*, 1998). Exo-RGLs catalyse the eliminative cleavage of α-L-rhamnopyranosyl-(1→4)-α-D-galactopyranosyluronic acid linkages containing α-L-rhamnopyranose at the reducing end and 4-deoxy-4,5-unsaturated D-galactopyranosyluronic acid at the

non-reducing end (the product of Endo-RGLs). The products are the disaccharide 2-*O*-(4-deoxy-β-L-threo-hex-4-enopyranuronosyl)-α-L rhamnopyranose and the shortened rhamnogalacturonan oligosaccharide containing one 4-deoxy-4,5-unsaturated D-galactopyranosyluronic acid at the non-reducing end (Ochiai *et al.*, 2009). RGAEs (EC 3.1.1.86) belong to carboxylic ester hydrolase family 12 and remove 2-O-acetyl- or 3-O-acetyl groups of GalA in RG-I and HG (Kauppinen *et al.*, 1995; Mølgaard, Kauppinen and Larsen, 2000; Bonnin, Garnier and Ralet, 2014).

Pectin methylesterases or pectinesterases (PMEs) (EC 3.1.1.11) belong to carboxylic ester hydrolase family 8 and catalyse the de-esterification of methyl ester groups of the carboxyl groups (C6) of GalA residues realising methanol (Figure S 8, Appendix 1). In general, these enzymes act before other pectinases such as PGs and PGLs which prefer non-esterified substrates (Pedrolli *et al.*, 2009; Bonnin, Garnier and Ralet, 2014).

Additionally, pectinases involved in the removal of acetyl groups as well as in XG, arabinan and galactan degradation have been described. Pectin acetylesterases (PAEs) (EC 3.1.1.6) remove acetyl groups of GalA residues releasing acetate, whereas xylogalacturonan hydrolases (EC 3.2.1.-) cleave glycosidic bonds between galacturonate residues in XG (Wong, 2008; Pedrolli *et al.*, 2009). Degradation of arabinan comprises enzymes which belong to various families of GHs such as Arabinan endo-1,5-α-L-arabinosidases (EC 3.2.1.99), Exo-arabinosidases and Arabinofuranosidases (EC 3.2.1.55) (Inácio, Lopes Correia and de Sá-Nogueira, 2008; Bonnin, Garnier and Ralet, 2014). Similarly, the degradation of arabinogalactan and galactan also requires different GHs enzymes such as Arabinogalactan endo-β-1,4 galactanases (EC 3.2.1.89), Exo-β-1,4 galactanases and β-Galactosidases (EC 3.2.1.23). As side chains sometimes carry ferulic acid, Feruloylesterases (EC 3.1.1.73) are also required (Bonnin, Garnier and Ralet, 2014).

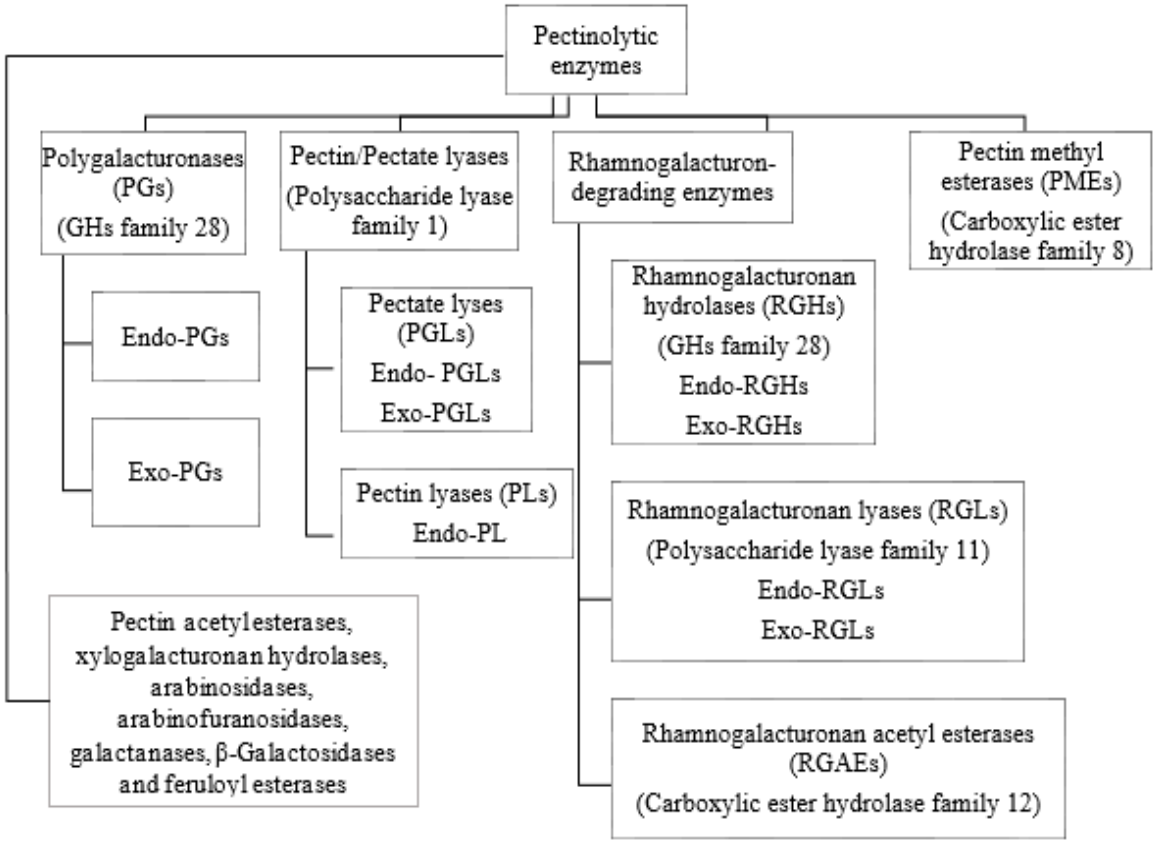

Figure 1.3. Classification of pectinases according to the structure or functional group of pectin used as a substrate.

1.2.2 Relevance of pectinases in the commercial sector

The importance of pectinases in industry varies according to their physical and chemical properties. Acidophilic pectinases are useful in food and beverage industry for extraction, clarification, and liquefaction of fruit juices; improving stability and visual characteristics in red wine; as well as for the saccharification of biomass. On the other hand, alkaline pectinases are widely used in textile industry, paper making, enhance tea quality, removing the mucilage coat from the coffee beans and treatment of pectic industrial residues (Hoondal *et al.*,

2002; Jayani, Saxena and Gupta, 2005a; Hakur and Upta, 2012; Reis *et al.*, 2014). In addition, pectinases are useful for the production of bioactive pectin-derived oligosaccharides (Benoit *et al.*, 2012; Minzanova *et al.*, 2018) and enzymatic cocktails for animal feed (Hoondal *et al.*, 2002).

Thus far, the study of pectinases has been focused on mesophilic microorganisms such as *Aspergillus, Erwinia* and *Bacillus* species. *Aspergillus* and *Erwinia* pectinases have exhibited optimum activity between 30 and 50 °C and restricted thermal stability (Hinton *et al.*, 1989; Saarilahti *et al.*, 1990; Leeuwen *et al.*, 1992; Kofod *et al.*, 1994; Mutter *et al.*, 1994; Shevchik *et al.*, 1996; Parenicova *et al.*, 2000; Kazemi-Pour, Condemine and Hugouvieux-Cotte-Pattat, 2004; Chakiath *et al.*, 2009). In contrast, some *Bacillus* species produce multiple pectinases (Pickersgill *et al.*, 1994; Hatada *et al.*, 1999; Kapoor *et al.*, 2000; Ochiai *et al.*, 2007) which are mainly alkaline and have shown optimum activity up to 80 °C and thermal stability up to around 70 °C (Singh, Plattner and Diekmann, 1999; Hatada, Kobayashi and Ito, 2001; Takao *et al.*, 2002; Berensmeier *et al.*, 2004; Mei *et al.*, 2013).

It has been estimated that pectinases account for 25% of the global food enzymes sales. The most commercial and well-known pectinases are HG degrading enzymes (Jayani, Saxena and Gupta, 2005a; Sharma, Rathore and Sharma, 2012; Satyanarayana, Kawarabayasi and Littlechild, 2013; Bonnin, Garnier and Ralet, 2014). Single pectinases or enzyme cocktails are commercially available (Parenicova *et al.*, 1998), as shown in Table 1.2. Most of the commercialised pectinases are produced by the mesophilic fungi *Aspergillus niger* and are active in acidic pH range and display their maximum activity and thermal stability up to 50 °C (Martens-Uzunova *et al.*, 2006; Cerreti *et al.*, 2017; Sudeep *et al.*, 2020). Only a very small number of industrial alkaline pectinases are produced by *Bacillus* species, which are more active and stable at higher values of temperature (Evangelista *et al.*, 2018).

Table 1.2. Commercial pectinases. Adapted from Evangelista *et al.* (2018).

Commercial Enzyme	Source	Type	Optimum temperature	Optimum pH	Company	Application
Pectinex® Ultra SPL	*A. aculeatus*	PG PL PME	50	4.5	Novozymes	Food and beverage
Klerzyme® 150	*A. niger*	PG	50	4.5-5.0	DSM Food Specialties	Food and beverage
Panzym® YieldMASH XXL	*A. niger*	PG PME	50	4.5	Eaton's Begerow	Food and beverage
Panzym® Smash XXL	*Aspergillus* sp.	PL	45	5.0	Eaton's Begerow	Food and beverage
Pectinase PE-500	*A. niger*	PME, PG, PL	45-50	3.5-5.5	Jiangsu Boli Bioproducts	Food and beverage
Pectinase	*A. niger*	PG	50	3.5	Parchem	Food and beverage
Pectinase	*A. niger*	PME, PG, PL	Activity up to 55	3.0-5.5	Biocon	Food and beverage
Pectinase	*A. aculeatus*	PME, PG, PL	Activity up to 55	4.5-9.0	Biocon	Food and beverage
Polygalacturonase	*A. niger*	PG	Activity up to 60	4.0-6.0	Biocon	Food and beverage
Pectate lyase	*Bacillus* sp.	PGL	60	10.0	Novozymes	Treatment of cellulosic material and detergents
Pectate lyase	*B. licheniformis*	PGL	65	10.0	Novozymes	Treatment of cellulosic material and detergents

1.2.3 Exploration to thermostable pectinases

The study of thermostable pectinases is becoming more relevant in bioprocesses and the research in this field is increasing. These enzymes have developed structural and physiological properties to exhibit optimal activity and stability at high temperatures, thereby may be applied in a wide range of industrial processes in which their mesophilic counterparts are unable to be used. Furthermore, thermostable enzymes are relevant in biocatalysis due to a number of advantages such as better solubility of substrates, longer operational stability of the enzymes with lower activity losses during processing, higher tolerance to organic solvents and reduction of microbial contamination issues (Parisot *et al.*, 2003; Turner, Mamo and Karlsson, 2007; Satyanarayana, Kawarabayasi and Littlechild, 2013). Thermostable enzymes are produced mainly by thermophiles, nevertheless some mesophiles have also been reported as thermostable enzymes producers.

Up to now, only a few thermostable pectinases have been discovered, among them from *Thermotoga maritima*, *Pseudothermotoga thermarum*, *Rhodothermus marinus, Caldicellulosiruptor* species and *Clostridium thermocellum*.

The hyperthermophilic bacterium *Thermotoga maritima* MSB8 contains at least two pectinases which are the Exo-PG PelB and the PGL PelA. The Exo-PG PelB (EC 3.2.1.67) (locus Tm0437) catalyses the hydrolytic release of mono-GalA from the non-reducing end of non-methylated and saturated HG, but shows low activity with XG. In contrast with the majority of PGs, the absence of signal peptide and the detection of pectinolytic activity in the cell fraction supported that Exo-PG PelB is an intracellular enzyme. In the study of its the kinetic parameters, substrates from digalacturonate to octagalacturonate as well as polygalA were tested, and it was observed that with an increasing degree of polymerization, the substrate affinity rose significantly up to reach its maximum value (the same for octagalacturonate and polyGalA). This preference for polymeric substrates seems to be in conflict with its cytoplasmic character and may be due to conformational changes in the substrate, thereby facilitating binding to the substrate-binding cleft (Parisot *et al.*, 2003; Kluskens *et al.*, 2005;

Drone *et al.*, 2007). Moreover, the crystal structure of Exo-PG PelB revealed a unique tetramer in solution, an unusual oligomerization in PGs (Pijning *et al.*, 2009). The 3D structure analysis showed that this enzyme contains the eight amino acids conserved in all thermophilic PGs, which are N237, D239, D260, D261, H296, G297, R327 and K329. Among them, the aspartate residues (D239, D260, D261) are import in the catalytic process, D239 acts as a catalytic acid protonating the oxygen of the scissile glycosidic linkage, while D260 and D261 activate a conserved water molecule proposed to attack the anomeric C1 carbon of the substrate. The residues N237, H296, G297, R327 and K329 play a role in substrate binding. Furthermore, the structural conservation in thermophilic PGs extends to residues Y362 and the G302-S303 cis-peptide motif (Kluskens *et al.*, 2005; Drone *et al.*, 2007; Pijning *et al.*, 2009) (Figure 1.4).

Figure 1.4. Alignment showing the comparison between the amino acids sequences of thermophilic exo-PGs with the mesophilic enzyme from *Aspergillus niger* (A). T, *Thermotoga maritima*; R, *Rhodothermus marinus*; B, *Bacillus licheniformis*; C, *Caldicellulosiruptor bescii* and P, *Pseudothermotoga thermarum*. Residues conserved in all thermophilic exo-PGs are in blue squares. It is observed that R327, K329 and Y362 from thermophilic pectinases are not conserved in the mesophilic enzyme from *Aspergillus niger*. The numbers of the residues belong to the enzyme from *Thermotoga maritima*.

On the other hand, the Exo-PGL PelA (EC 4.2.2.22) (locus Tm0433) is a pectate disaccharide-lyase that catalyses the eliminative cleavage of an unsaturated trigalacturonate from the reducing end of non-methylated HG, although a slight formation of unsaturated digalacturonate has been identified. The presence of signal peptide and its secretion to the medium support that Exo-PGL PelA is an extracellular enzyme, similar to other PGLs. In addition, Ca^{2+} is essential for the catalytic activity and thermal stability of PGLs such as PelA. The study of the mode of action of this pectinase on oligogalacturonate (oligoGalA demonstrated a parallel production of unsaturated trigalacturonate and GalA (n−3). The fact that the saturated counterproduct GalA(n−3) is formed in equivalent amounts indicates that PelA follows a multiple-chain attack, as opposed to a single-chain–multiple attack mode that would result in the immediate degradation of the saturated intermediate GalA(n−3) (Kluskens *et al.*, 2003; Kim, 2014; Kim and Cheong, 2015). The conserved residues located in the substrate-binding cleft of Exo-PelA are R224, D144, D166 and D179. The three aspartate residues which form a complex between PGLs and Ca^{2+} are conserved in all PGLs. Exo-PGL PelA is also a tetrameric enzyme. To date, most of PGLs studied are from mesophiles and have been characterised as monomeric enzymes and oligomerization is considered a stabilisation factor in thermophilic enzymes (Kluskens *et al.*, 2003).

The hyperthermophilic bacterium *Pseudothermotoga thermarum* DSM 5069 produces the Exo-PG TtGH28 (EC 3.2.1.67) (locus Theth0397). The amino acid sequence alignment between Exo-PG TtGH28 and the Exo-PG PelB from *Thermotoga maritima* MSB8 showed that all eight conserved residues in PGs are conserved in these both enzymes. In addition, other active side residues in Exo-PG PelB such as cis-peptide motif G302-S303 and residues E106, W214, K266, E304, R324, Y362, K387 are also conserved in Exo-PG TtGH28 (Exo-PG PelB numbers). Exo-PG TtGH28 has been characterised as a dimeric enzyme and it was found that the substrate affinity increased with an increasing degree of polymerization (Wagschal *et al.*, 2016).

Rhodothermus marinus is a hyperthermophilic bacterium that expresses the Exo-PG RmGH28 (EC 3.2.1.67). The amino acid sequence alignment of this enzyme exhibited that it contains the eight conserved residues in PGs, as well

shares other catalytic amino acids with Exo-PG PelB from *Thermotoga maritima* (Wagschal *et al.*, 2017).

Besides, some pectinases from *Caldicellulosiruptor* species have been studied. The thermophilic Exo-PG CbPelA (EC 3.2.1.67) from *Caldicellulosiruptor bescii* DSM 6725 was cloned, expressed and characterised. This pectinase was also able to hydrolyse methylated pectin (48 and 10% relative activity on 20–34% and 85% methylated pectin, respectively). Exo-PG CbPelA showed catalytic behaviour similar to Exo-PG PelB from *Thermotoga maritima*, both enzymes share the eight catalytic and substrate-binding sites of PGs as well as Y299 and the G238-S239 cis-peptide motif, which is believed to play an important role in the anchoring of the oriented galacturonate unit to the catalytic sites (Chen *et al.*, 2014). Furthermore, the structure and the catalytic mechanism of a PGL from the hyperthermophilic *Caldicellulosiruptor bescii* have been determined (Alahuhta *et al.*, 2011, 2013, 2015). Likewise, the thermostable PGL Pel-863 (EC 4.2.2.2) (locus Calkro 0863) from *Caldicellulosiruptor kronotskyensis* was biochemically characterised, and the application for pectin containing biomass degradation was also studied. The quaternary structure of purified recombinant Pel-863 analysed through gel filtration chromatography showed that this enzyme exists as monomer and homotrimer (Su *et al.*, 2015).

Clostridium thermocellum has been identified as an important source of industrial enzymes including thermostable pectinases. For instance; cloning, expression and characterization of three cellulosomal pectinases, two variants of PL1 (PL1A and PL1B) and PL9, from this bacterium have been reported. PL1A, PL1B and PL9 displayed activity toward poly-GalA and pectin from citrus fruits (Chakraborty *et al.*, 2015). Likewise, the PME *Ct*PME was cloned, expressed and characterised. This enzyme showed high activity towards citrus pectin with >85% methyl esterification (Rajulapati and Goyal, 2017).

Some thermophilic pectinases from mesophilic species from *Bacillus* have been investigated. Although *Bacillus* pectinases are mainly alkaline (optimum pH around 8-11), some of them have exhibited optimum activity at acidic pH (Hoondal *et al.*, 2002). In particular, the biochemical characterisation and structure of the thermostable Exo-PG BlExoPG (EC 3.2.1.67) from *Bacillus*

licheniformis DSM 13 has been determined. BlExoPG displayed optimum catalytic activity at pH 6.5, retaining considerable activity at neutral and slightly alkaline pH. Similar behaviour was previously observed for Exo-PG PelB from *Thermotoga maritima*. The gel filtration chromatography analysis indicated that this pectinase exists in monomeric form in solution (Evangelista *et al.*, 2018).

In addition, the production, purification and characterisation and mode of action of the PME BliPME from *Bacillus licheniformis* DSM 13 on highly methyl esterified and acetylated pectin have been reported. BliPME was able to generate pectin with low degree of methyl esterification releasing up to 100 and 73% of the methyl ester groups from lime and sugar beet pectin, respectively, creating blocks of non-methyl esterified GalA residues. Other kinds of pectinases such as the alkaline PGL Pel-7 and Pel-15 from *Bacillus* spp. strains have been cloned, purified and characterised (Kobayashi, Hatada, *et al.*, 1999; Kobayashi, Koike, *et al.*, 1999; Hakamada *et al.*, 2005)

Similarly, *Streptomyces* strains have also been studied as thermostable pectinases producers. Thus, the production and characterization of a thermostable pectinase from *Streptomyces* sp. QG-11-3 isolated from a sample of decaying coconut fibres was carried out (Beg *et al.*, 2000). Furthermore, *Streptomyces sp.* RCK-SC has been found to produce an alkaline thermostable pectinase (Kuhad, Kapoor and Rustagi, 2004). Ramírez-tapias *et al.* (2015) reported the study of an alkaline and thermostable PG from *Streptomyces halstedii* ATCC 10897 with applications in waste water treatment.

Table 1.3 summarises thermostable enzymes produced by thermophilic and some mesophilic bacteria that have been partially studied.

Table 1.3. Thermostable pectinases previously studied.

Enzyme	Microorganism	Gen size (bp)/ MW (kDa)	Optimum pH	Optimum temperature (°C)	Reference
Polygalacturonases					
Exo-PG (EC 3.2.1.67)	*T. maritima* MSB8 DSM 3109	1365 51.3[a]	6	95	Parisot *et al.* (2003)
Exo-PG PelB (EC 3.2.1.67)	*T. maritima* MSB8 DSM 3109	1341 212[d]	6.4	80	Kluskens *et al.* (2005)
Exo-PG (EC 3.2.1.67)	*T. maritima* MSB8 DSM 3109	1365 51.3[a]	6	85	Drone *et al.* (2007)
Exo-PG TmPG (EC 3.2.1.67)	*T. maritima* MSB8 DSM 3109	1347 212[d]	6.4	80	Pijning *et al.* (2009)
Exo-PG CbPelA (EC 3.2.1.67)	*Caldicellulosiruptor bescii* DSM 6725	NI 50	5.2	72	Chen *et al.* (2014)
Exo-PG TtGH28 (EC 3.2.1.67)	*Pseudothermotoga thermarum* DSM 5069	1548 117.0[b]	NI	NI	Wagschal *et al.* (2016)
Exo-PG RmGH28 (EC 3.2.1.67)	*Rhodothermus marinus*	1413 52.7[a]	NI	NI	Wagschal *et al.* (2017)
Exo-PG BlExoPG (EC 3.2.1.67)	*B. licheniformis* DSM 13	1311 bp 48.14a	6.5	60	Evangelista *et al.* (2018)

Table 1.3

Enzyme	Microorganism	Gen size (bp)/ MW (kDa)	Optimum pH	Optimum temperature (°C)	Reference
Lyases					
PGL F1 (PelA) (EC 4.2.2.22)	*T. maritima* MSB8 DSM 3109	1104 151.2[d]	9	90	Kluskens *et al.* (2003)
PGL F1 (PelA) (EC 4.2.2.22)	*T. maritima* MSB8 DSM 3109	1104 bp 40.6 [a]	6.5	85-95	Kim (2014); Kim and Cheong (2015)
Endo-PGL Pel-863 (EC 4.2.2.2)	*Caldicellulosiruptor kronotskyensis* DSM 18902	NI 148[c]	9	70	Su *et al.* (2015)
Endo-PGL (EC 4.2.2.2) 1. PL1A 2. PL1B 3. PL9	*Clostridium thermocellum* DSM 1237	1. 906 2. 1059 3. 867 1. 34[a] 2. 40[a] 3. 32[a]	8.5 9.8	50 60	Chakraborty *et al.* (2015)
Endo-PGL Pel103 (EC 4.2.2.2)	*Bacillus* sp. KSM-P103	1308 33[a]	10.5	60-65	Hatada *et al.* (1999)

Continued

Table 1.3

Continued

Enzyme	Microorganism	Gen size (bp)/ MW (kDa)	Optimum pH	Optimum temperature (°C)	Reference
Endo-PGL Pel-7 (EC 4.2.2.2)	*Bacillus* sp. KSM-P7	909 33[a]	10.5	60-65	Kobayashi *et al.* (1999)
Endo-PGL Pel-4A (EC 4.2.2.2)	*Bacillus sp* P-4-N	NI 37.7[a]	11	60	Kobayashi *et al.* (2000)
Endo-PGL Pel-4B (EC 4.2.2.2)	*Bacillus sp* P-4-N	NI 37[a]	11.5	70	Hatada, Kobayashi and Ito (2001)
PGL PL47	*Bacillus sp.* TS 47	1326 50[a]	NI	70	Takao *et al.* (2002)
Exo-PGL PelA (EC 4.2.2.22)	*Bacillus licheniformis* 14A	NI 38[a]	11	69	Singh, Plattner and Diekmann (1999); Berensmeier *et al.* (2004)
Endo-PGL Pel-15 (EC 4.2.2.2)	*Bacillus* sp. KSM-P15	594 20.9[a]	10.5	50-55	Kobayashi, Koike, *et al.* (1999); Hakamada *et al.*(2005)
Endo-PGL (EC 4.2.2.2)	*Bacillus pumilus* BK2	NI 37[a]	8.5	70	Klug-Santner *et al.* (2006)

Table 1.3

Continued

Enzyme	Microorganism	Gen size / MW (kDa)	Optimum pH	Optimum Temperature (°C)	Reference
1. Endo-RGL YesW 2. Exo-RGL YesX	*Bacillus subtilis* 168	NI 1. 68[a] 2. 77[a]	8 8.5	60 65	Ochiai *et al.* (2007)
Pectin methylesterases					
PME BliPME (EC 3.1.1.11)	*Bacillus licheniformis* DSM13	954 35.1[a]	8	50	Remoroza *et al.* (2015)
PME *Ct*PME (EC 3.1.1.11)	*Clostridium thermocellum*	900 35.5[a]	8.5	50	Rajulapati and Goyal (2017)
Other pectinases					
Pectinase	*Streptomyces* sp. QG-11-3	NI	3	60	Beg *et al.* (2000)
Pectinase	*Streptomyces sp.* RCK-SC	NI	8	60	Kuhad, Kapoor and Rustagi (2004)
Pectinase	*Bacillus pumilus* dcsr1	NI	10.5	50	Sharma and Satyanarayana (2006)
Pectinase	*Bacillus halodurans* M29	1017 39[a]	10	80	Mei *et al.* (2013)
PG	*Streptomyces halstedii* ATCC 10897	NI 48[a]	12	50	Ramírez-tapias *et al.* (2015)

[a]monomer, [b]homodimer, [c]homotrimer, [d]homotetramer NI: No information

1.3 Potential of thermophilic pectinases in industrial biocatalysis

Thermophilic pectinases have a remarkable industrial potential as they can be used in bioprocesses to obtain bio-based chemicals such as monosaccharides and functional oligosaccharides from pectin-rich biomass at high or moderate temperatures, in which their mesophilic counterparts show low activity and poor stability or are inactive at such conditions. Furthermore, these enzymes may improve some industrial processes such as in sugar, and food and beverage industries (Sharma, Rathore and Sharma, 2012).

1.3.1 Ara and GalA release from pectin-rich biomass

The production of monosaccharides by enzymatic hydrolysis of pectin-rich biomass such as apple, citrus and sugar beet pectin is a promising step towards increasing the value of this polymer. Ara and GalA constitute the major monosaccharides present in these biomass which are interesting molecules for further conversion into building blocks which can be subsequently transformed into high-value bio-based chemicals or materials, like polyesters, polyamides or plasticizers (Leijdekkers *et al.*, 2013; Ward *et al.*, 2015). Leijdekkers *et al.*(2013) studied enzymatic saccharification of SBP for producing GalA and Ara, in which selected commercial pectinases with optimum activity of 45 °C were able to release 79% of the GalA and 82% of the Ara as monomers while simultaneously degrading 17% of the cellulose. The use of thermostable pectinases in similar studies may improve the releasing of these monosaccharides.

Cárdenas-Fernández *et al.* (2018) hydrolysed sugar beet pectin released as a result of sugar beet pulp fractionation by steam explosion using a novel recombinant thermostable α-L-arabinofuranosidase from *Geobacillus thermoglucosidasius*. Monomeric Ara and GalA backbone obtained as products were separated by tangential flow ultrafiltration with 92% Ara recovery. Bawn *et al.*, (2018) synthetised L-glucoheptulose, a high value pharmaceutical compound, from Ara via a coupled reaction using a thermostable transketolase and transaminase. Figure 1.5 shows the importance of GalA to the synthesis of valuable compounds.

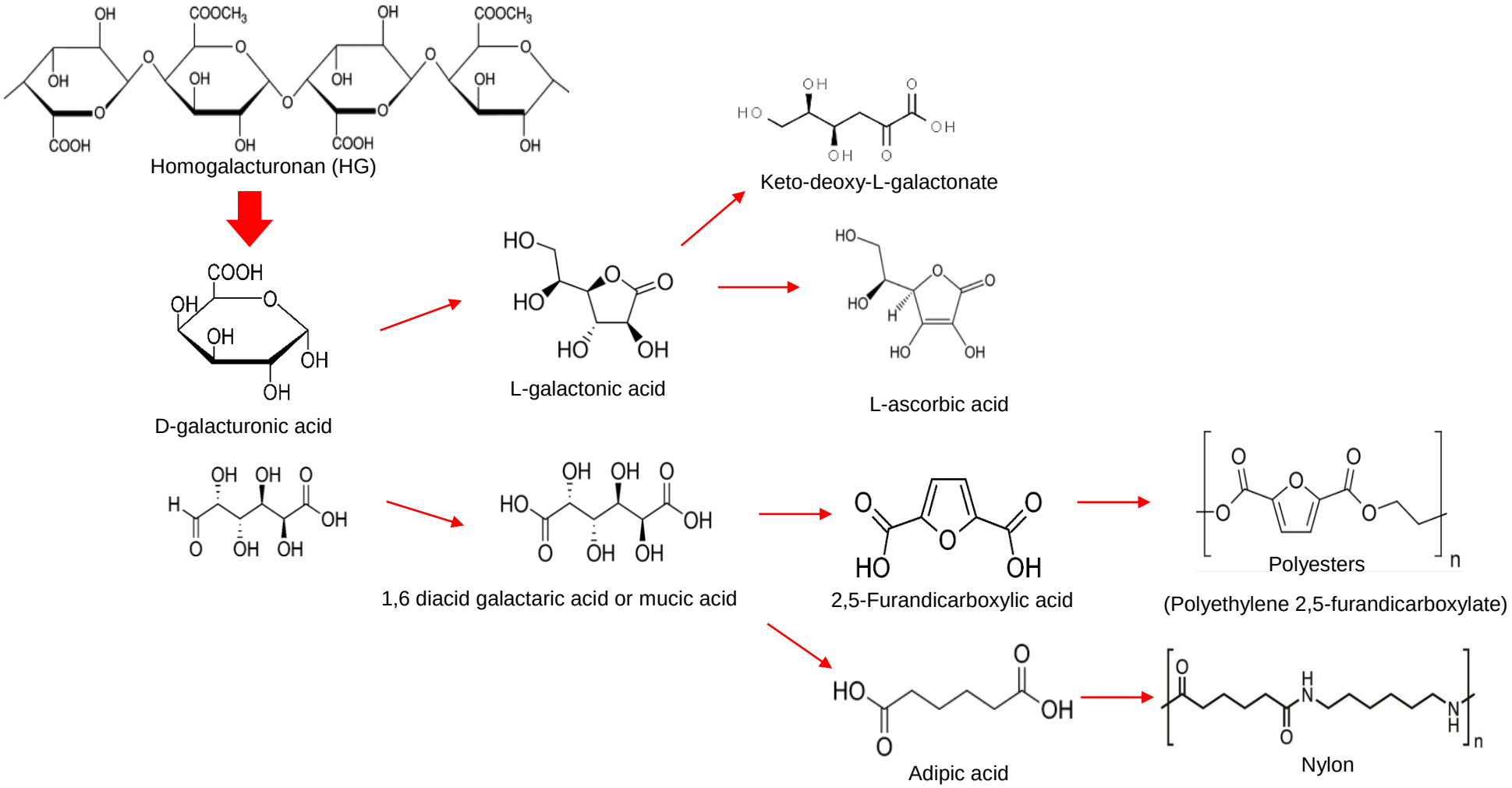

Figure 1.5. Galacturonic acid as biomass-derived platform chemical to the synthesis of several valuable compounds.

GalA is a potentially valuable biomass-derived platform chemical that can be converted to L-galactonic acid, a precursor for L- ascorbic acid (Kuivanen et al., 2014). Moreover, the C1 carbon of GalA can be readily oxidized by enzymatic or chemical methods to galactaric acid also known as mucic acid. The galactaric acid has been reported as metal ion chelator (Abbadi et al., 1999; Wagschal et al., 2017) and precursor of furanedicarboxylic acids (FDCA), which have been considered by the US Department of Energy as one of the top 12 building-block chemicals. FDCA can be used as substitutes of terephthalic acid in the production of polyesters or for the synthesis of other bio-based polymers (Taguchi, Oishi and Iida, 2007; Lavilla et al., 2011; Wagschal et al., 2017). Moreover, galactaric acid is a precursor of adipic acid which is used for nylon production (Kiely, Chen and Lin, 2000; Zhang et al., 2016). Thus, Zhang et al. (2016) reported the production of adipic acid directly from sugar beet pultp by combination of biological and chemical catalysis, thereby enabling the development of a green route to adipic acid from renewable biomass.

1.3.2 Enhancement of industrial bioprocesses

1.3.2.1 Sugar industry

In sugar industry, sugar beet processing involves slicing the roots into long, thin strips, called cossettes and releasing the sucrose from the cells by continuous-counter current diffusion with hot water. In the diffuser, the cossettes are kept in contact with hot water for about 1 h to diffuse the sucrose juice from the beet cells (Asadi, 2007; Chakiath *et al.*, 2009). During this process, the water builds up inside the cells increasing osmotic pressure. At the same time, the heat denatures the cell wall and deforms the protoplasm becoming more permeable, thereby components of the juice diffuse through the membrane owing to the increase in pressure. Denaturation of the cell wall is crucial in the diffusion process, and it may be improved under the influence of physical, chemical or enzymatic conditions resulting in a high-purity juice and low sugar loss to pulp. The standard conditions for the diffusion process are pH from 5.8 to 6.5 and temperature from 70 to 73 °C, which contribute to denaturation and prevent microbiological activity (Asadi, 2007). In this context, thermostable acidophilic pectinases may be used to enhance sugar release the during diffusion process

in sugar industry. These enzymes are able to degrade pectin from the cell wall of beet cells improving denaturation which may result in a more efficient process.

1.3.2.2 Food and beverage industry

Thermophilic acidic pectinases may be useful in extraction and clarification of fruit juices and wines, steps which can be improved at elevated temperatures. For instance, considering the thermal stability and activity, as well as slightly acidic pH optimum of the exo-PG from *Thermotoga maritima*, it could be successful especially for fruits with high pectin content which are poorly soluble and viscous at normal processing temperatures (Kluskens *et al.*, 2005; Pijning *et al.*, 2009). In the same way, the high thermo-activity and methylated pectin hydrolysis of exo-PG CbPelA from *Caldicellulosiruptor bescii* DSM 6725 suggest that it has potential applications in the food industry (Chen *et al.*, 2014).

In addition, pectinases may be used to modulate functional properties of pectin. In food industry, as a result of industrial pectin extraction from citrus peel and apple pomace, a high-methyl esterified pectin is obtained, which form a gel at pH < 3.5 and in the presence of at least 55% sugar (Bonnin, Garnier and Ralet, 2014). Subsequently, pectin is de- methyl esterified by alkali treatment, but this could affect the molecular weight of pectin due to depolymerisation of the backbone (Remoroza *et al.*, 2015). Optionally, PMEs may decrease the degree of methylation giving as a product low-methoxy pectin which is able to form gel in the presence of divalent cations, such as calcium, in a wide range of pH and without sugar. Likewise, PAEs may improve functional properties of pectin with high degree of acetylation such as in sugar beet pectin, where de-methyl esterification by using PMEs after de-acetylation by PAEs could create blocks of non-methyl esterified GalA residues and thereby enhance the gelling properties in the presence of Ca^{2+} ions. In particular, the thermostable BliPME PME from *Bacillus licheniformis* DSM 13 showed high activity in sugar beet pectin releasing up to 73% of the methyl ester groups (Bonnin, Garnier and Ralet, 2014; Remoroza *et al.*, 2015).

1.3.2.3 Textile industry

Thermophilic alkaline pectinases are more competitive than mesophilic due to pectic substances becoming more soluble at high temperature in alkaline conditions and processes such as degumming and retting of natural fibres and textile scouring demand applications of alkaline pectinases (Su *et al.*, 2015; Evangelista *et al.*, 2018). PGL Pel-863 from *Caldicellulosiruptor kronotskyensis* DSM 18902 was able to remove most of pectin in hemp fibre with less damage compared to alkaline degumming. In addition, pre-digestion with Pel-863 improved glucose and xylose yield by 14.2 and 31.6% respectively for corn stalk, 6.5 and 55% for rice stalk compared with sole action of Novozymes Cellic CTec2 (Su *et al.*, 2015). Sharma and Satyanarayana (2006) studied the production of an alkaline and thermophilic pectinase by *Bacillus pumilus* dcsr1, which was cellulase-free and selectively degraded only the non-cellulosic gummy material of the fibre, thereby this enzyme could find application in fibre processing industry. Besides, the applicability of a PGL from *Bacillus pumilus* BK2 for the bio-scouring process was tested on a cotton fabric and this enzyme was able to remove up to 80% of pectin from the outer layer of the cotton and a uniform result was obtained (Klug-Santner *et al.*, 2006).

1.4 Synergistic activity between pectinases and their co-expression for biomass depolymerisation

Most of the publications about pectinases have been focused on studying one specific kind of pectinase. These studies have reported cloning of the gene encoding the enzyme as well as its partial purification and characterisation (Parisot *et al.*, 2003; Kluskens *et al.*, 2005; Remoroza *et al.*, 2014, 2015; Rajulapati and Goyal, 2017; Evangelista *et al.*, 2018). Regarding applications, the authors have tested this specific pectinase in different substrates describing its potential uses in some fields of industry such as food and beverage as well as textiles (Su *et al.*, 2015; Oumer and Abate, 2017; Haile, Masi and Tafesse, 2022).

On the other hand, some studies have reported the synergistic activity between pectinases for pectin-biomass depolymerisation. In these studies, the used

enzymes have been commercial pectinases, available as individual enzymes which have been mixed in different ratios, or as cocktails containing different kinds of pectinases. Combo *et al.* (2013) used a commercial PME and endo-PG for sugar beet pectin hydrolysis to obtain pectic-derived oligosaccharides (POS) with potential applications in medical and food industry, and Leijdekkers *et al.* (2013) used a commercial pectinases cocktail for sugar beet pulp conversion into GalA and Ara. Furthermore, pectinases have been mixed in different ratios with other biomass degrading enzymes such as cellulases, xylanases and cutinases for the hydrolysis of agricultural residues to improve processes in the textile and food industries (Xia *et al.*, 2020; Degani, 2021; Olawuyi *et al.*, 2022).

With respect to co-expression of enzymes, it has been carried out with different purposes such as reducing the enzymes production cost, developing whole cell biocatalysis, and enhancing soluble and extracellular expression of enzymes (Roongsawang *et al.*, 2010; Kumar *et al.*, 2015; Zhu *et al.*, 2015; Su *et al.*, 2017). Carbohydrate degrading enzymes such as xylanases and cellulases has been co-expressed in order to use this enzymatic cocktail to improve animal feed (Roongsawang *et al.*, 2010). Likewise, isoamylases genes has been co-expressed in order to depolymerase starch-rich biomass (Panpetch, Field and Limpaseni, 2018). Pectinases have been co-expressed in and single vector in *E. coli* with other enzymes such as laccases and xylanases to reduce the enzymes production cost for exploiting lignocellulosic-rich biomass (Kumar *et al.*, 2015).

1.5 Critical appraisal of the published literature

As described in Section 1.1.1, pectin is a complex polymer and consists of different substructures. Regarding pectin-rich sustainable biomass feedstocks, million tonnes of pectin-rich by-products are produced annually and most of them are sold as low-cost animal feed (Sections 1.1.2 – 1.1.4).

Due to the complexity of pectin structure, there are multiple enzymes involved in its breakdown (Section 1.2.1). Pectinases find a great number of applications principally in food and beverage industries and represent a relevant percentage of the global food enzymes sales. Nonetheless, most of the studies about these enzymes have been focused on mesophilic microorganisms such as species

from *Aspergillus* and *Erwinia*. Pectinases in enzymatic cocktails or single enzymes are currently commercially available, predominantly enzymes from *Aspergillus* sp. (Section 1.2.2). Therefore, there is an increased need for thermophilic pectinases, the discovery of these novel enzymes can be achieved by using a gene mining approach in both thermophilic and some mesophilic microorganisms which have been reported to be able to produce thermophilic enzymes.

The few studies that have addressed thermophilic pectinases (Section 1.2.3) have emphasised molecular cloning, partial characterisation studies, catalytic mechanism, and structure. However, there remains a need for further biochemical characterisation of these enzymes which is equally important alongside exploring their application in the hydrolysis of pectin-rich biomass feedstocks.

Hence, the challenge here lies in utilising these renewable biomass feedstocks such as apple pomace, citrus processing waste and sugar beet pulp in order to obtain value-added products, reduce environmental impact and recycle biomass. Thermophilic pectinases have the potential to degrade these structurally complex biomass feedstocks, releasing monosaccharides and oligosaccharides that may be used as chemical precursors and pharmaceutical intermediates (Section 1.3.1).

Furthermore, thermophilic pectinases may be used to improve some industrial processes in which their mesophilic counterparts are inactive (Section 1.3.2). To date, studies about the application of these enzymes in industrial processes have been scarcely reported (Klug-Santner *et al.*, 2006; Sharma and Satyanarayana, 2006; Su *et al.*, 2015). These few studies provide initial insights into the promising applications of thermophilic pectinases.

Most of the studies about pectinases have been focused in one specific enzyme and they have only reported partial characterisation of it. Thus, these studies have important limitations since pectin is a complex polysaccharide that needs the synergistic action of different kinds of pectinases to be depolymerised. In addition, full characterisation of enzymes is necessary to set up compatible operational conditions in the synergistic reactions. Moreover, in the reported

studies about synergistic action of pectinases, commercial enzymes have been used. As described above, most of the commercial pectinases come from mesophilic fungi, and they have shown restricted activity and stability in a broad range of pH and temperature. Finally, although different carbohydrate degrading enzymes have been co-expressed with different purposes, to the best of our knowledge no studies about co-expression of pectinases for obtaining valuable bio-based chemicals such as GalA have been published (Section 1.4).

1.6 Aim and objectives

The aim of this research was to identify and study novel thermophilic pectinases as well as explore their application for recycling sustainable biomass feedstocks and for improving pectin depolymerisation in industry. The specific objectives of the project are:

1. To identify putative pectinases and select the thermophilic candidates for cloning and expression. In addition, to carry out their functional characterisation and the exploration of the synergistic activity between exo-PGs and PMEs for pectin bioconversion into GalA.
2. To co-express an exo-PG and a PME in a single plasmid and host for a cost-effective pectin bioconversion into GalA.
3. To carry out functional characterisation of PGLs as well as explore their synergistic activity with PMEs to improve esterified pectin depolymerisation.

CHAPTER 2 MATERIALS AND METHODS

2.1 Chemicals and media

Chemicals used in this research were of analytical grade and purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Enzymes and reagents for molecular techniques were purchased from New England Biolabs (Hitchin, UK) unless otherwise noted.

2.2 Genome mining of pectinases-encoding sequences and phylogenetic analysis

Bacteria used in this study come from Prof. John Ward's microbial collection. Pfam domain families of pectinases were identified through the option QUERY PFAM BY KEYWORD by using their EC number (Table 1.1). Identified Pfams were: PF00295 (PGs and RGHs, GHs family 28); PF00544 and PF03211 (PGLs and PLs); PF01095 (PMEs); PF18370 (RGLs beta-sheet domain); PF09284 (RGLs B, N-terminal); PF14686 (RGLs, Polysaccharide lyase family 4 domain II); PF14683 (RGLs, Polysaccharide lyase family 4 domain III) and PF13472 (RGAEs-GDSL, like Lipase/Acylhydrolase family). Predicted protein sequences from thermophilic microorganisms genomes were functionally annotated by scanning the sequences against Pfam 32.0 libraries (https://pfam.xfam.org/) by using hmmscan (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan). To identify putative pectinases, sequences annotated with the all previously identified Pfams were retrieved. Regarding mesophilic bacterial genomes, only the Pfams: PF00295 (PGs and RGHs, GHs family 28), PF00544 and PF03211 (PGLs and PLs), and PF01095 (PMEs) were used to retrieve pectinases sequences.

Whilst for phylogenetic analysis purposes, the screening of pectinases was also performed by using homology-based approach. BLAST searches by using blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) as well as HMMER trough phmmer (https://www.ebi.ac.uk/Tools/hmmer/search/phmmer) were carried out by using as query sequences those retrieved from thermophiles, in order to identify their closest thermophilic and/or mesophilic homologues. UniProtKB

(https://www.uniprot.org/) was used to obtain functional information of all the identified enzymes.

All the retrieved sequences were subjected to a multiple sequence alignment and a phylogenetic analysis by using ClustalW and MEGA-X, in order to evaluate the evolutionary relationships between thermophiles and mesophiles pectinases.

2.3 Molecular cloning techniques of individual enzymes

The presence of putative signal peptides in the genes encoding pectinases was analysed through the program SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/). All pectinases were cloned removing the signal peptides. PCR primers were designed by using SnapGene 4.2.11 software (Table S 1, Appendix 4). The genes of interest were amplified using Phusion® High-Fidelity PCR Master Mix with GC Buffer kit and 100 ηg of bacterial DNA per 50 µL of reaction. PCR conditions were 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing for 30 s and extension at 72 °C for 1 min; then a final extension at 72 °C for 10 min (C1000 Touch Thermal Cycler, BioRad, UK). PCR products were visualised by gel electrophoresis, isolated and purified via Monarch® DNA Gel Extraction Kit. The purified PCR products and expression vectors were digested, and cohesive ended PCR products were ligated into the plasmids using T4 DNA ligase. The plasmids pET-29a (+), pET29a (+): *SacB-SapI* and pET29a (+): *SacB-BsaI* (Dobrijevic *et al.*, 2020) (Table S 1, Appendix 4) were used for cloning the pectinases, all with a C-terminal His6-tag (Figures S 9 – S16, Appendix 5) . The ligated mixtures were transformed into *E. coli* NovaBlue (DE3) which were grown at 37 °C overnight in agar plates containing LB, 10% sucrose and 0.05 g L^{-1} kanamycin. Single colonies of *E. coli* NovaBlue (DE3) harbouring the recombinant plasmid were grown in 10 mL of LB broth supplemented with 0.05 g L^{-1} kanamycin at 37 °C overnight. Plasmids were extracted using QIAprep® Spin Miniprep Kit (Qiagen, Manchester, UK) and sent for sequencing to confirm the coding sequence of the enzymes. Then, the plasmids were transformed into the expression host *E. coli* BL21(DE3).

2.4 Construction of co-expression plasmids containing a PME and exo-PG

Four co-expression plasmids containing a PME and exo-PG were constructed in pETDuet-1 (Merck-Novagen, Darmstadt, Germany) (Table S 2, Appendix 4) In co-expression constructs 1 and 2, BLI09 PME was cloned in the multiple cloning site 1 (MCS-1) and either TMA01 or BLI04 exo-PGs in MCS-2. In co-expression constructs 3 and 4, the cloning order of the genes was inverted with respect to co-expression constructs 1 and 2. Thus, in these constructs either TMA01 or BLI04 were cloned in MCS-1 and BLI09 in MCS-2. The genes encoding BLI09, TMA01 and BLI04 were amplified from pET29a_SacB_SacI-BLI09, pET-29a-TMA01 and pET29a_SacB_SacI-BLI04, respectively using the primers described in Table S 2 (Appendix 4). The genes were cloned in the MCS-1 using the restriction enzymes *BamH*I and *Not*I and in the MCS-2 using *Nde*I and *Xho*I.

The flowchart of the construction of co-expression constructs 1 and 2 is outlined in Figure 2.1. Genes encoding TMA01 and BLI04 were cloned in MCS-2 of the pETDuet-1 with a C-terminal S-tag, giving as a result pETDuet-TMA01 and pETDuet-BLI04, respectively. These plasmids were transformed into *E. coli* NovaBlue(DE3) using 0.05 g L^{-1} ampicillin. After plasmids extraction, the gene encoding BLI09 was cloned in MCS-1 of the resulting plasmids mentioned above with an N-terminal His6-tag. The constructed plasmids containing the genes of both pectinases were named as co-expression constructs 1 and 2 (pETDuet-BLI09-TMA01 and pETDuet-BLI09-BLI04, respectively). A schematic representation of the construction of these co-expression constructs is shown in Figures S 23 and S 24 (Appendix 7).

Similarly, Figure 2.2 shows the flowchart of the construction of co-expression constructs 3 and 4. In this case, the gene encoding BLI09 was cloned in MCS-2 of the pETDuet-1 with a C-terminal S-tag giving as a result pETDuet-BLI09. Then, the genes encoding either TMA01 or BLI04 were cloned in MCS-1 with an N-terminal His6-tag. The constructed plasmids possessing the genes of both pectinases were named as co-expression constructs 3 and 4 (pETDuet-TMA01-BLI09 and pETDuet-BLI04-BLI09, respectively). A schematic representation of

the construction of these co-expression constructs is shown in Figures S 25 and S 26 (Appendix 7). All the co-expression constructs were transformed again into *E. coli* NovaBlue(DE3). Then, they were extracted and validated by sequencing. Finally, the constructs were transformed into *E. coli* BL21(DE3) for expression.

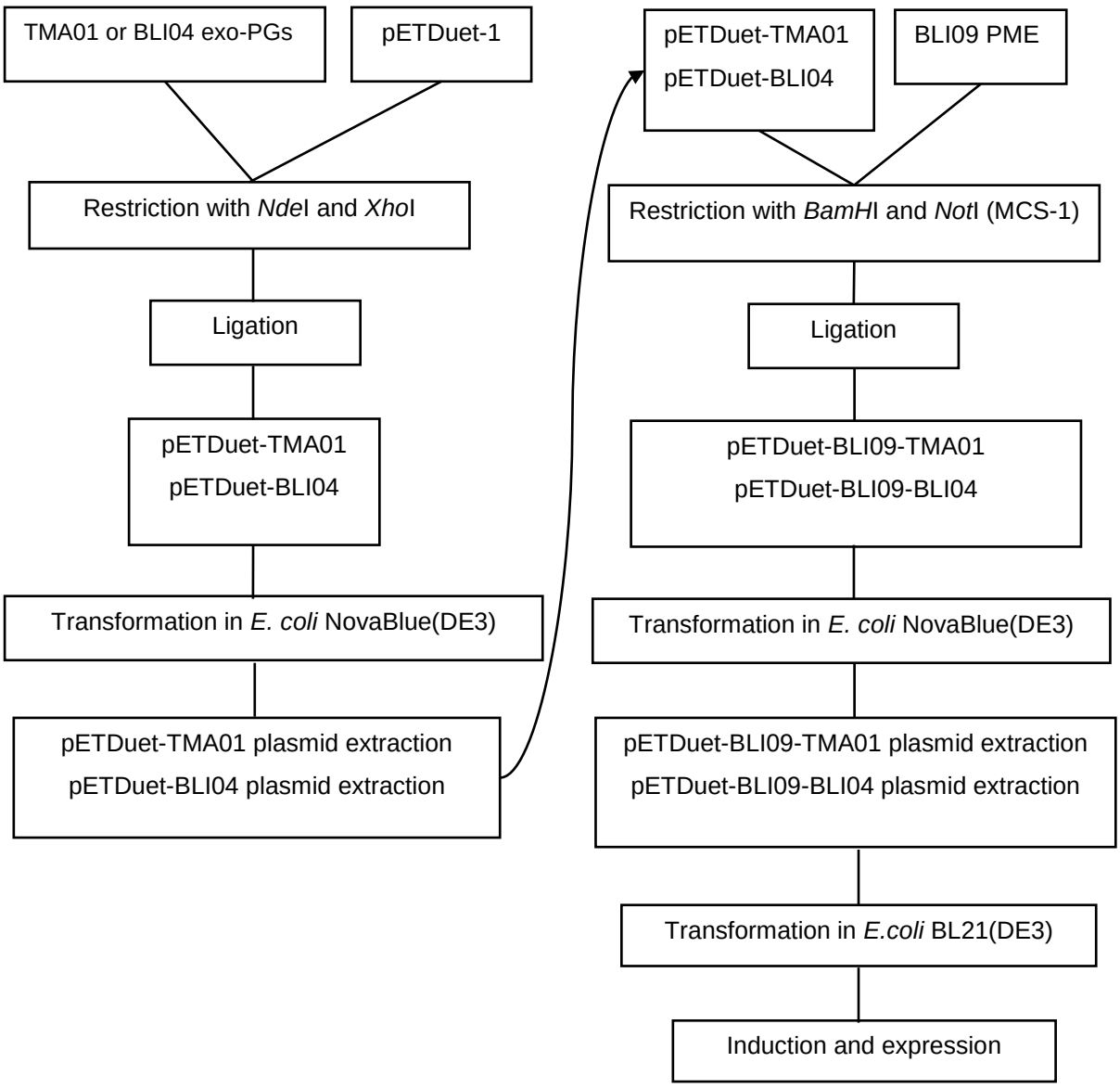

Figure 2.1. Flowchart of the construction of the co-expression constructs 1 and 2.

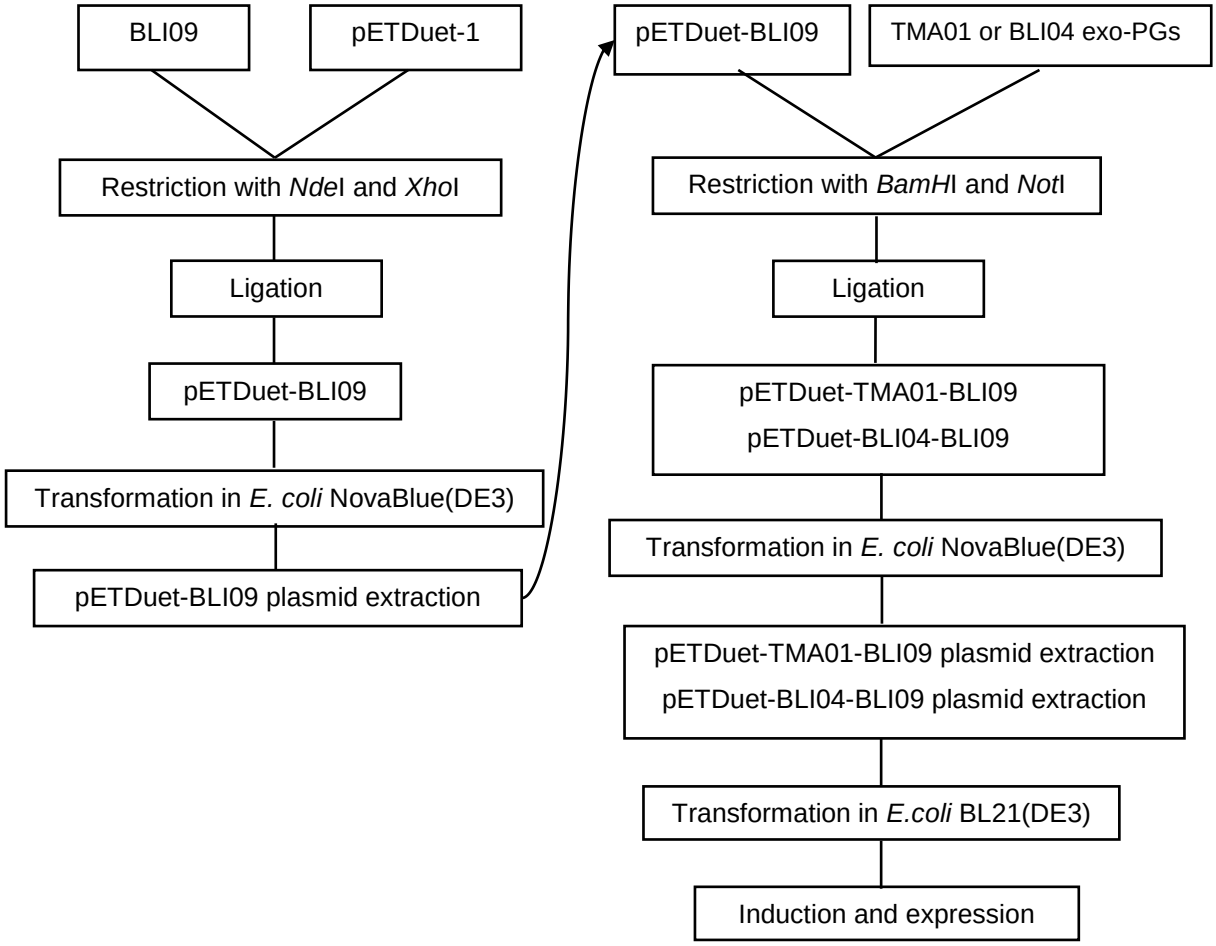

Figure 2.2. Flowchart of the construction of the co-expression constructs 3 and 4.

2.5 Protein expression

2.5.1 Individual enzymes

For individual enzymes expression, *E. coli* BL21(DE3) cells harbouring the recombinant plasmids were grown in 10 mL of LB broth containing 0.05 g L^{-1} kanamycin at 37 °C overnight. The overnight starter culture (3 mL) 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 OD600 reached 0.3 – 0.5, at which point the cells were induced by addition of IPTG to a final concentration of 0.5 mM. Post induction, cells were incubated at 25 °C and 250 rpm until 24 h. The cells were

harvested by centrifugation (8 500 g at 4 °C for 10 min), the cell pellet was re-suspended in a buffer solution 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 (18 000 g at 4 °C for 15 min) and the clarified cell lysate was recovered and kept at 4 °C for SDS-PAGE, protein quantification, enzymatic activity assay and purification.

2.5.2 Co-expression constructs

E. coli BL21(DE3) cells harbouring the co-expression constructs were grown in 10 mL of LB broth containing 0.05 g L^{-1} ampicillin at 37 °C overnight. The rest of the process was carried out as described in Section 2.5.1. Clarified cell lysates were kept at 4 °C for SDS-PAGE, protein quantification, enzymatic activity assay and purification.

2.6 Purification of thermostable pectinases

2.6.1 Individual enzymes

Gravity-flow purification by affinity chromatography was carried out using an open tubal column containing a His_6-Tag Ni-affinity resin (Ni-NTA Agarose, Thermo Fisher Scientific Inc, UK) with a column volume (CV) of 3 mL was used. Purification buffers used for each kind of pectinases are described in Table 2.1. Prior purification, the Ni-NTA column was pre-equilibrated with 5 CV of equilibration buffer. The *E. coli* BL21(DE3) cell pellets of 50 mL of culture containing the recombinant plasmids were re-suspended in 2.5 mL of equilibration buffer. The cells were disrupted, and the cell lysate was recovered by centrifugation (18 500 g for 20 min) and loaded into the column. Subsequently, the washing buffer was used to wash out non- specific binding proteins. Finally, proteins were eluted with the elution buffer and then precipitated with ammonium sulphate powder to a final saturation of 70% (w/v). The precipitated enzymes were recovered by centrifugation and stored at 4 °C as a suspension in 70% (w/v) ammonium sulphate until analysis. The cell lysate as well as the loading, washing and elution fractions were tested for protein, SDS-PAGE, and enzyme activity assays.

Table 2.1. Buffers used for pectinases purification by affinity chromatography using a His_6-Tag Ni-affinity resin.

Purification buffer	Exo-PGs	PMEs	PGLs*
Equilibration	Buffer A1 + 10 mM imidazole	Buffer A2 + 10 mM imidazole	Buffer A3 + 10 mM Imidazole
Washing	Buffer A1 + 50 mM imidazole	Buffer A2 + 50 mM imidazole	Buffer A3 + 50 mM Imidazole
Elution	Buffer A1 + 500 mM imidazole	Buffer A2 + 500 mM imidazole	Buffer A3 + 500 mM Imidazole

Buffer A1: 50 mM sodium phosphate buffer + 300 mM NaCl, pH 6.5.
Buffer A2: 20 mM sodium phosphate buffer + 300 mM NaCl, pH 7.
Buffer A3: 20 mM Tris-HCl buffer + 300 mM NaCl, pH 8.
*TFU19 PGL was purified using as washing buffer, buffer A3 + 20 mM imidazole.

2.6.2 Co-expression constructs

Pectinases cloned in MCS-1 of pETDuet-1 with an N-terminal His6-tag were purified by affinity chromatography using a His_6-Tag Ni-affinity resin. Purification was carried out following the procedure described in Section 2.6.1. Whereas that, pectinases cloned in MCS-2 with a C-terminal S-tag were purified by affinity chromatography using a S-protein agarose (Merck-Novagen, Darmstadt, Germany). For it, an open tubal column containing a S-protein agarose with a column volume (CV) of 2 mL was used. Prior purification, the resin was pre-equilibrated with 3 CV of bind/wash buffer (20 mM sodium phosphate + 150 mM NaCl + 0.1% Triton X-100 pH 7). The *E. coli* BL21(DE3) cell pellets of 50 mL of culture containing the co-expression constructs were re-suspended in 2.5 mL of bind/wash buffer. The cells were disrupted and the cell lysate was recovered by centrifugation and loaded into the column. The lysate and the S-protein agarose were mixed thoroughly and were incubated at room temperature on an orbital shaker for 45 min. Subsequently, the non- specific binding proteins were washed out using the bind/wash buffer by repeating centrifugation and resuspension of the S-protein agarose in the buffer. Finally, proteins were eluted by mixing 3 M $MgCl_2$ with S-protein agarose and incubating the mixture at room temperature for 10 min. This final elution step was carried out twice. The elution fractions were desalted and concentrated by using Amicon ®Ultra-

15 centrifugal filters (Merck, Carrigtwohill, Ireland). The cell lysate as well as the loading, washing and elution fractions were tested for protein and SDS-PAGE assays. Protein quantification of pectinases in co-expression constructs was carried out as described in Section 2.9.1, but also through the SDS-PAGE gels analysis using the software ImageJ (https://imagej.nih.gov/ij/).

2.7 Characterisation of thermostable pectinases

2.7.1 Optimum pH

Exo-polygalacturonases: the optimum pH of purified exo-PGs (~ 0.25 U mL^{-1}) was determined at 50 °C using 0.5% (w/v) polyGalA in the following 50 mM buffers: sodium acetate (pH 4 and 5), sodium phosphate (pH 6 and 7), Tris–HCl (pH 8 and 9) and sodium bicarbonate (pH 10-12). The exo-PG activity was determined following the standard assay procedure (Section 2.9.3.1).

Pectin methylesterases: the optimum pH of purified BLI09 and SAM10 PMEs (~ 9 U mL^{-1} and ~ 4.5 U mL^{-1}, respectively) was determined at 50 °C using 0.5% (w/v) apple pectin. The buffers used were the same as for exo-PGs but at 20 mM. The PME activity assay was performed based on methanol quantification using AO and Fluoral-P (Section 2.9.3.2).

Pectate lyases: the optimum pH of purified PGLs (~ 0.25 U mL^{-1}) was determined at 50 °C using 0.5% (w/v) polyGalA. The buffers used were the same as for exo-PGs but at 20 mM. The PGL activity assay was determined following the standard assay procedure (Section 2.9.3.3).

2.7.2 Effect of ions

Exo-polygalacturonases: the influence of various ions such as Na^{1+}, Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} at 1, 5 and 10 mM was investigated. In the case of Mn^{2+}; 0.1, 0.25, 0.5 and 0.75 mM were also tested. The purified enzymes (0.25 U mL^{-1}) were pre-incubated for 15 min at room temperature in 50 mM sodium phosphate pH 6.5 containing the respective ion salt (NaCl, $CaCl_2$, $MnCl_2$, $ZnSO_4$ or $MnCl_2$). Exo-PG activity was measured at pH 6.5 and 50 °C according to the standard assay procedure (Section 2.9.3.1). The relative activity was

calculated as the percentage of activity compared with a control assay in absence of any ion.

Pectin methylesterases: the influence of Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} at 1 mM was investigated. In addition, concentrations of 0.5 and 1.5 mM Ca^{2+} and Mg^{2+} were tested for BLI09 as well as 0.5 and 1.5 mM Zn^{2+} for SAM10. In order to assess the effect of Mn^{2+} on PMEs activity, concentrations of 0.1, 0.25, 0.5 and 0.75 of this ion were tried. The purified enzymes (BLI09, ~ 9 U mL^{-1} and ~SAM10, 4.5 U mL^{-1}) were pre-incubated for 15 min at room temperature in 20 mM sodium phosphate pH 7 containing the respective ion salt. PME activity was measured following the standard assay procedure based on methanol release using AO and Fluoral-P (Section 2.9.3.2) and expressed as the relative activity.

Pectate lyases: As PGLs activity depends on Ca^{2+}, concentrations of 0.25, 0.6, 0.75 and 1 mM of this ion were tested. Based on these results, the effect of Mn^{2+}, Zn^{2+} and Mg^{2+} was also assayed at 1 mM in presence of the optimum Ca^{2+} concentration. In addition, for Mn^{2+} and Mg^{2+}, concentrations of 0.25,0.5, 0.75 were evaluated. The purified enzymes (0.25 U mL^{-1}) were pre-incubated for 15 min at room temperature in 20 mM Tris-HCl pH 8 containing the respective ion salt. PGL activity was measured at pH 8 and 50 °C according to the standard assay procedure (Section 2.9.3.3). The relative activity was calculated as the percentage of activity compared with a control assay in absence of any ion but in presence of the optimum Ca^{2+} concentration.

The effect of individual ions (Mn^{2+} or Ca^{2+}) on enzymatic activity was tested for significance using the Student's t-test at a significance level of $p = 0.05$. In addition, the significance level ($p = 0.05$) of various Ca^{2+} concentrations on enzyme activity was determined using one-way ANOVA.

2.7.3 Optimum temperature

Exo-polygalacturonases: the optimum temperature was determined by assaying purified enzymes (~ 0.25 U mL^{-1}) at temperatures from 40 to 100 °C in 10 °C increments using 0.5% (w/v) polyGalA in 50 mM sodium phosphate pH 6.5. The assay was 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. The exo-PG

activity was determined following the standard assay procedure (Section 2.9.3.1) and the relative activity was expressed as a percentage of the maximum activity. By using this data, the effect of Mn^{2+} on the amount of released product (GalA) was also calculated. The µmoles of released GalA were expressed as a percentage of the total amount of this compound that can be obtained using 0.5% (w/v) polyGalA as substrate (14.3 µmoles).

Pectin methylesterases: the optimum temperature was determined by assaying the purified enzymes (BLI09, ~0.30 U mL^{-1} and SAM10, ~ 0.15 U mL^{-1}) at temperatures from 40 to 90 °C in 10 °C increments using 0.5% (w/v) apple pectin in 20 mM sodium phosphate pH 7.6. The PME activity was assessed following the standard assay procedure by the method using the pH indicator (Section 2.9.3.2) and the relative activity was expressed as a percentage of the maximum activity.

Pectate lyases: the optimum temperature was determined by assaying the purified enzymes (0.25 U mL^{-1}) at temperatures from 40 to 100 °C in 10 °C increments using 0.5% (w/v) polyGalA in 20 mM Tris-HCl pH 8. The PGL activity was assessed following the standard assay procedure (Section 2.9.3.3) and the relative activity was expressed as a percentage of the maximum activity.

2.7.4 Thermal stability

Exo-polygalacturonases: the thermal stability was tested by incubating the enzymes (~4 U mL^{-1}) in 50 mM sodium phosphate pH 6.5, either in presence of 0.25 mM Mn^{+2} or in absence of this ion, from 20 to 100 °C in 10 °C increments for up to 24 h. Aliquots of 30 µL of enzymes solution were taken at indicated time points and cooled down on ice. Then, exo-PG activity was measured under the standard assay conditions at 50 °C, as detailed in Section 2.9.3.1, and expressed as residual activity. The thermal stability assay at 70 °C was also tried measuring the exo-PG activity at 70 °C.

Pectin methylesterases: the thermal stability was assayed by incubating the enzymes (BLI09, ~90 U mL^{-1} and SAM10, ~22.5 U mL^{-1}) in 50 mM HEPES pH 7 from 20 to 70 °C for up to 24 h. Aliquots of 50 µL of enzymes solution were

taken at indicated time points and cooled down on ice. Then, PME activity was measured by the method based on methanol quantification under the standard assay conditions (Section 2.9.3.2) and expressed as residual activity.

Pectate lyases: the thermal stability was determined by incubating the enzymes (~1.5 U mL^{-1}) in 20 mM Tris-HCl pH 8, either in presence of 0.6 mM Ca^{+2} or in absence of this ion, from 40 to 70 °C for up to 24 h. Aliquots of 45 µL of enzymes solution were taken at indicated time points and cooled down on ice. Then, PGL activity was measured following the standard assay conditions (Section 2.9.3.3) and expressed as residual activity.

2.7.5 Kinetic studies

Kinetic parameters were determined by measuring the reaction velocity at different concentrations of substrates: polyGalA from 10 to 400 µM for exo-PGs and PGLs, and apple pectin from 5 to 350 µM for PMEs. The reactions were performed under optimum conditions of activity for each enzyme (pH, temperature, and ions concentration). The kinetic data obtained were analysed by non-linear regression using GraphPad Prism 8 software. For exo-PGs and PMEs, the curve from the plot of initial velocity versus substrate concentration was fitted to the equation of the generally used substrate inhibition model (Equation 2.1) as well as to the Equation 2.2, that belongs to a new model of substrate inhibition. In this new model, a simultaneous binding of n molecules of substrate during the inhibition process is assumed. The value of n is determined from the analysis of the data (Bapiro *et al.*, 2018). The total concentration of each enzyme used in the reactions was kept constant and this value was used to determine the *kcat* through “kcat” analysis using the software (Equation 2.3). 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. For PGLs, the curve from the plot of initial velocity versus substrate concentration was fitted to the Michalis-Menten equation (Equation 2.4). Meanwhile, the *kcat* for these enzymes was determined in the same way as for exo-PGs and PMEs using the Equation 2.3.

$$V_0 = Vmax[S]/(Km + [S](1 + [S]/Ki)) \quad \text{(Equation 2.1)}$$

$$V_0 = Vmax[S]/(Km + [S](1 + [S]^n/Ki^n)) \quad \text{(Equation 2.2)}$$

$$V_0 = [Et]\ kcat\ [S]/(Km + [S]) \quad \text{(Equation 2.3)}$$

$$V_0 = Vmax\ [S]/(Km + [S]) \quad \text{(Equation 2.4)}$$

Where: V_0 is the enzyme velocity (µmol min^{-1} mL^{-1}), *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^{-1}) and *kcat* is the turnover number defined as the number of times each enzyme site converts substrate to product per unit time (s^{-1}).

2.7.6 Substrate specificity

Exo-polygalacturonases: to determine substrate specificity of exo-PGs; 0.5% polyGalA (non-esterified) as well as citrus pectin and apple pectin (all esterified, Table 2.2) were used as substrates. The experiments were performed using 0.25 U mL^{-1} of each enzyme at optimum conditions (pH, temperature, and ions concentration) of activity. The exo-PG activity was measured following the procedure detailed in Section 2.8.3.1. The relative activity was expressed as the percentage of the activity with polyGalA which was considered as 100%.

Pectate lyases:

Substrate specificity determined by reducing sugar quantification: to determine substrate specificity of PGLs; 0.5% polyGalA, as well as citrus, apple, and sugar beet pectin (Table 2.2) were used as substrates. The experiments were performed using ~ 0.25 U mL^{-1} of each enzyme at optimum conditions (pH, temperature, and ions concentration) of activity. The PGL activity was

measured following the procedure detailed in Section 2.9.3.3. The relative activity was expressed as the percentage of the activity with polyGalA which was considered as 100%.

Substrate specificity determined by gel filtration chromatography (GFC): the substrate specificity reactions were incubated up to 4 h and samples were taken at 30 min, 1 h, 2 h and 4 h. These samples were analysed by GFC following the procedure detailed in Section 2.9.7. This assay allowed to determine the molecular weight distribution of the oligogalacturonates produced because of the different substrates depolymerisation by the PGLs action.

Table 2.2. Composition of pectin from different sources used as substrates.

Composition (%)	**Apple pectin**	**Citrus pectin**	**Sugar beet pectin**
Esterification	55-75*	N.R*	55*
Methylation	58▪	55 ▪	35*
Acetylation	1.2▪	1.6 ▪	20*
Galacturonic acid	≥ 74*	≥ 74*	≥ 65*

N.R: not reported (defined as low esterified), *from the manufacturer, ▪from by (Ma *et al.*, 2020).

2.7.7 Product inhibition assays

2.7.7.1 Effect of methanol and galacturonic acid on pectin methyl esterases and exo-polygalacturonases

The effect of methanol and GalA on BLI09 PME as well as in TMA01 and BLI04 exo-PGs was determined in co-expression systems 2 plasmids (pETDuet-TMA01-BLI09 and pETDuet-BLI04-BLI09). Methanol and GalA were tested at final concentrations from 0.2 to 20 mM. Clarified lysates of each plasmid of co-expression systems 2 were pre-incubated for 10 min at room temperature in 20 mM sodium phosphate pH 7 containing the respective methanol or GalA concentration in a final volume of 500 µL. Pectinases (PME and exo-PG) activities were measured by adding the previous mixture to 500 µL of 0.5%

(w/v) apple pectin. The reactions were carried out at 50 °C for 30 min at 300 rpm. Methanol and GalA were quantified as described in Sections 2.9.4 and 2.9.5, respectively. These quantified amounts were used for pectinases activity calculations, where the total amount of methanol or GalA quantified in each reaction was subtracted by the added amount. The residual activity was expressed as percentage compared with a control assay without addition of methanol or GalA. Graphs plotted show residual PME or exo-PGs activity (Y-axis) vs methanol or GalA total concentration in the reactions (X-axis). The methanol or GalA total concentration resulted from the sum of methanol or GalA produced in the control reaction plus the amount added to each reaction.

2.7.7.2 Effect of acetic acid on pectin methyl esterases and exo-polygalacturonases

The effect of acetic acid was determined in enzymes from co-expression constructs 3 and 4. Acetic acid was tested at final concentrations from 0.2 to 10 mM. Clarified lysates of each co-expression systems 2 plasmid were pre-incubated for 10 min at room temperature in 100 mM sodium phosphate pH 7 containing the respective acetic acid concentration in a final volume of 500 µL. Pectinases activities were measured under the same conditions mentioned in Section 2.7.7.2 using apple pectin. Methanol and GalA quantifications were carried as described in Sections 2.9.4 and 2.9.5, respectively. These calculated amounts were used for pectinases activity calculations. The residual activity was calculated as the percentage of activity compared with a control assay without addition of acetic acid.

2.7.7.3 Effect of acetic acid on pectate lyases

The effect of acetic acid was tested in TMA14 and TFU20 PGLs. Acetic acid was tested at final concentrations from 0.2 to 10 mM. Purified enzymes were pre-incubated for 10 min at room temperature in 20 mM Tris-HCl pH 8 containing 0.6 mM Ca^{2+} and the respective acetic acid concentration in a final volume of 200 µL. PGL activity was measured according to the procedure described in Section 2.9.3.3. The residual activity was calculated as the percentage of activity compared with a control assay without addition of acetic acid.

2.8 Synergistic action of pectinases

2.8.1 Exo-PGs and PMEs individually expressed and as co-expressed enzymes

Different concentrations of PMEs were tested using 0.5% (w/v) apple pectin in 20 mM sodium phosphate pH 7, in order to find the optimum concentration to reach maximum de-methylation during the first hours of the synergistic reactions. For these reactions, 0.5 mL of enzyme solution containing the purified PMEs was added to 0.5 mL of substrate, then the mixture was incubated at 50 °C and 300 rpm until 24 h. Blank reactions were prepared without the addition of enzymes solution. Samples were taken periodically and used for methanol quantification following the procedure described in Sections 2.8.4.

Synergistic activity between PMEs and exo-PGs was carried out with the purified individually expressed enzymes and the clarified lysates of the co-expression constructs using 0.5% (w/v) apple pectin, citrus pectin, and sugar beet pectin in 20 mM sodium phosphate pH 7. For the reactions, 0.5 mL of enzyme solution containing the PME and exo-PG (enzymatic activities in Tables 2.3 and 2.4) with 0.5 and 2 mM $MnCl_2$ for TMA01 and BL04 exo-PGs, respectively; were added to 0.5 mL of each substrate. In the case of synergistic activity between BLI09 PME and the exo-PGs, the mixture was incubated at 50 °C and 300 rpm. Regarding synergistic activity between SAM10 PME and the exo-PGs, the incubation was performed at 40 °C and 300 rpm for 1 h and then increased to 50 °C. Incubation was until 24 h and reactions adding only TMA01 or BLI04 exo-PGs were used as controls and blank reactions were prepared without addition of enzymes solution. Samples were taken periodically and used for methanol and GalA quantification according to the procedures described in Sections 2.9.4 and 2.9.5. For GalA yield (%) calculations, 74% GalA content was used for apple and citrus pectin (manufacturer information).

Table 2.3. Activity of purified PMEs and exo-PGs individually expressed used for the synergistic reactions.

Individually expressed enzymes (U mL^{-1})							
BLI09 PME + Exo-PGs				**SAM10 PME + Exo-PGs**			
BLI09 + TMA01		BLI09 + BLI04		SAM10 + TMA01		SAM10 + BLI04	
BLI09	TMA01	BLI09	BLI04	SAM10	TMA01	SAM10	BLI04
9	0.5	9	2	18	1	18	1

PME: pectin methylesterases and exo-PGs: exo-polygalacturonases.

Table 2.4. Activity of pectinases in the clarified lysates of co-expression constructs used for the synergistic reactions.

Co-expression constructs (U mL^{-1})							
1		**2**		**3**		**4**	
pETDuet-BLI09-TMA01		pETDuet-BLI09-BLI04		pETDuet-TMA01-BLI09		pETDuet-BLI04-BLI09	
BLI09*	TMA01	BLI09*	BLI04	TMA01	BLI09*	BLI04	BLI09*
4.4	0.5	11	2	0.5	40.8	2	86

*The activity of BLI09 PME was calculated in a clarified lysate volume containing 0.5 and 2 U mL^{-1} of TMA01 and BLI04 exo-PGs, respectively. PMEs: pectin methylesterases, exo-PGs: exo-polygalacturonases.

2.8.2 Exo-PGs and PMEs as co-expressed enzymes along with a PAE and AF

Before testing the synergistic reactions of the co-expression constructs with PAE21 from *Bacillus licheniformis*, the capacity of this enzyme to deacetylate sugar beet pectin was evaluated. For this assay, 0.5 mL of enzyme solution containing 0.022 U mL^{-1} of PAE21 were added to 0.5 mL of 0.5% (w/v) sugar beet pectin in 100 mM sodium phosphate pH 7. The mixture was incubated at 50 °C at 300 rpm until 24 h. Blank reactions were prepared without addition of enzyme solution. Samples were taken periodically, and the enzymatic reactions were processed as mentioned above. Acetic acid was quantified according to the procedure described in Section 2.9.6.

Synergistic activity between exo-PGs and PMEs as co-expressed enzymes along with PAE21 was carried out only with co-expression constructs 3 and 4, using 0.5% (w/v) sugar beet pectin in 100 mM sodium phosphate pH 7. For the

reactions, 0.5 mL of enzyme solution containing the PME and exo-PGs (enzymatic activity in Table 2.4) along with 0.022 U mL^{-1} of PAE21 were added to 0.5 mL of substrate. The mixture was incubated at 50 °C until 24 h at 300 rpm. Similarly, synergistic activity between exo-PGs and PMEs as co-expressed enzymes along with 0.022 U mL^{-1} of PAE21 and 0.1 U mL^{-1} of an AF (Cárdenas-Fernández *et al.*, 2018) was tested. Blank reactions were prepared without the addition of enzymes solution. Samples were taken periodically and used for methanol, GalA and Ara, and acetic acid quantification according to the procedures described in Sections 2.9.4, 2.9.5 and 2.9.6, respectively. For GalA yield (%) calculations, 65% GalA content was used for sugar beet pectin.

2.8.3 PGLs, PMEs and a PAE individually expressed

2.8.3.1 Synergistic activity determined by reducing sugars quantification

The first assays for testing the synergistic activity between PMEs and PGLs individually expressed were carried out using 0.5% (w/v) apple pectin in 20 mM sodium phosphate pH 7. For these reactions, 9 and 18 U mL^{-1} of BLI09 and SAM10 PMEs, respectively with different U mL^{-1} of PGLs were used (Table 2.5). Enzyme solution volume containing the pectinases units was added to 0.5 mL of the substrate. Optimum concentrations of ions such as Ca^{2+} and Mn^{2+} were added according to the PGLs units used for the reactions (Table 2.6). Synergistic reactions with BLI09 PME were incubated at 50 °C and 300 rpm. While those with SAM10 PME at 40 °C and 300 rpm for 1 h and then increased to 50 °C. Blank reactions were prepared without addition of enzymes solution. The incubation time was up to 24 h, samples were taken periodically and used for pectin depolymerisation analysis through methanol and reducing sugars quantification. Methanol was quantified according to the procedure described in Section 2.9.4. Reducing sugars were quantified using 150 µL of the samples taken periodically and adding 75 µL of 3,5-Dinitrosalicylic acid (DNS). The mixtures were heated at 100 °C for 15 min and centrifuged at 10 000 g 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 (200 µL system).

2.8.3.2 Synergistic activity determined by GFC

Synergistic activity between PMEs and PGLs was assayed using 0.5% (w/v) apple pectin, citrus pectin, and sugar beet pectin in 20 mM sodium phosphate pH 7. Reactions were carried out following the procedure described in Section 2.8.3.1. Control reactions using only PGLs and polyGalA were also assayed. The reactions were incubated up to 24 h and samples were taken periodically. Methanol was quantified as described in Section 2.8.4 and, in this case, pectin depolymerisation was analysed by GFC following the procedure described in Section 2.9.7, through the determination of molecular weight distribution of the produced oligogalacturonates.

Table 2.5. Activity of PMEs and PGLs individually expressed used for the synergistic reactions.

Synergistic	**PME (U mL^{-1})**		**PGL (U mL^{-1})**		
activity	BLI09	SAM10	TMA14	TFU19	TFU20
Reducing sugars	9	18	0.25	0.5	0.1
GFC	9	18	0.25 and 1	-	0.1 and 0.5

DNS: 3,5-Dinitrosalicylic acid and GFC: gel filtration chromatography.

Table 2.6. Ions concentrations for synergistic reactions based on pectate lyases activity.

Ion (mM)	**PGLs (U mL^{-1})**				
	TMA14		TFU19	TFU20	
	0.25	1	0.5	0.1	0.5
Ca^{2+}	0.6	2.4	1.2	0.24	1.2
Mn^{2+}	0.25	1	-	0.2	1

Similarly, synergistic activity between PMEs, PGLs and a PAE was carried out using 0.5% (w/v) sugar beet pectin in 100 mM sodium phosphate pH 7. For these reactions, 9 and 18 U mL^{-1} of BLI09 and SAM10 PMEs, respectively were used as well as 1 and 0.5 U mL^{-1} of TMA14 and TFU20 PGLs, respectively. Meanwhile, 0.022 U mL^{-1} of PAE21 were used for the synergistic reactions. The enzyme solution containing the three enzymes was added to 0.5 mL of the

substrate and the reactions were processed as mentioned above. Methanol, acetic acid, and pectin depolymerisation were determined following the procedures described in Sections 2.9.4, 2.9.6 and 2.9.7.

2.9 Analytical methods

2.9.1 Protein assay

The total protein concentration was determined based on the Bradford assay (Bradford, 1976) using Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories Inc., Hemel Hempstead, UK). The assay was performed following the micro assay protocol using 250 µL of Bradford reagent and 5 µL of sample. After 5 min of incubation at room temperature, the absorbance was measured at 595 nm and translated into protein concentration based on the calibration curve elaborated using bovine serum albumin (BSA) (Figure S 17, Appendix 6).

2.9.2 SDS-PAGE analysis

SDS-PAGE analysis was carried out on an XCell SureLock Mini-Cell system using Novex™ 10% Tris-Glycine Mini Gels, WedgeWell™ format and NuPAGE™ MOPS SDS running Buffer (Thermo Fisher Scientific Inc, UK). The samples were mixed with Laemmli 2x Concentrate Sample Buffer (Sigma-Aldrich, UK) and heated to 98 °C for 10 min in a Thermal Cycler (C1000 Touch™ Thermal Cycler, Bio-Rad Laboratories Inc., UK). Following that, 15 µg of total protein were loaded in each lane and 5 µL of PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Fisher Scientific Inc, UK) were used as protein molecular weight marker. The gels were run at 200 V for about 30 min and then were stained with InstantBlue™ Protein Stain (Expedeon Ltd, Cambridge, UK). Finally, the gels were visualized and analysed on an AlphaImager Mini system (Bio-Techne (ProteinSimple), Oxford, UK).

2.9.3 Enzymatic activity assay

2.9.3.1 Exo-PG activity

Exo-PG standard assay was performed following the method described by Miller (1959) with some modifications (Kashyap *et al.*, 2000; Mei *et al.*, 2013; Kim, 2014). In this method, the activity is measured by quantifying reducing

groups expressed as GalA units. The substrate, 0.5 mL of 0.5% (w/v) polyGalA in 50 mM sodium phosphate pH 6.5, was pre-incubated at 50 °C for 5 min. Then, 0.5 mL of enzyme solution in the buffer mentioned above were added and after 15 min of incubation (Thermomixer™ C, Eppendorf, UK), 0.5 mL of DNS were incorporated into the reaction. The mixture was heated at 100 °C for 15 min and then centrifuged at 10 000 g for 5 min. The reducing groups were quantified in the supernatant at 540 nm in 96-well plates. A blank of reaction was carried out following the procedure described above using only buffer instead of enzyme solution. The enzymatic activity was calculated according to Equation 2.4, where molar extinction coefficient (ε) was calculated from the calibration curve using GalA as standard (Figure S 18, Appendix 6).

$$U\ mL^{-1} = \frac{Abs}{time} x\ \frac{1}{\varepsilon} x \frac{V\ total}{V\ enzyme} x\ df \qquad \text{(Equation 2.5)}$$

Where: time, 15 min; V total, 1.0 mL; V enzyme, 0.2 mL; ε, 0.127 mM^{-1} cm^{-1} and *df*, dilution factor of the enzyme solution

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.

2.9.3.2 PME activity

Methanol quantification: several methods for PME activity based on methanol quantification have been described (Salas-Tovar *et al.*, 2017). Wojciechowski and Fall (1996) reported a method in which methanol is oxidised to formaldehyde mediated by alcohol oxidase (AO). Then, formaldehyde reacts with Fluoral-P (4-amino-3-penten-2-one) producing 3,5-diacetyl-1,4-dihydrolutidine (DDL) which is detected at 410 nm (Figure 2.1). The reaction occurs at room temperature and pH around 7 that is compatible with AO and Fluoral-P stability (Salas-Tovar *et al.*, 2017).

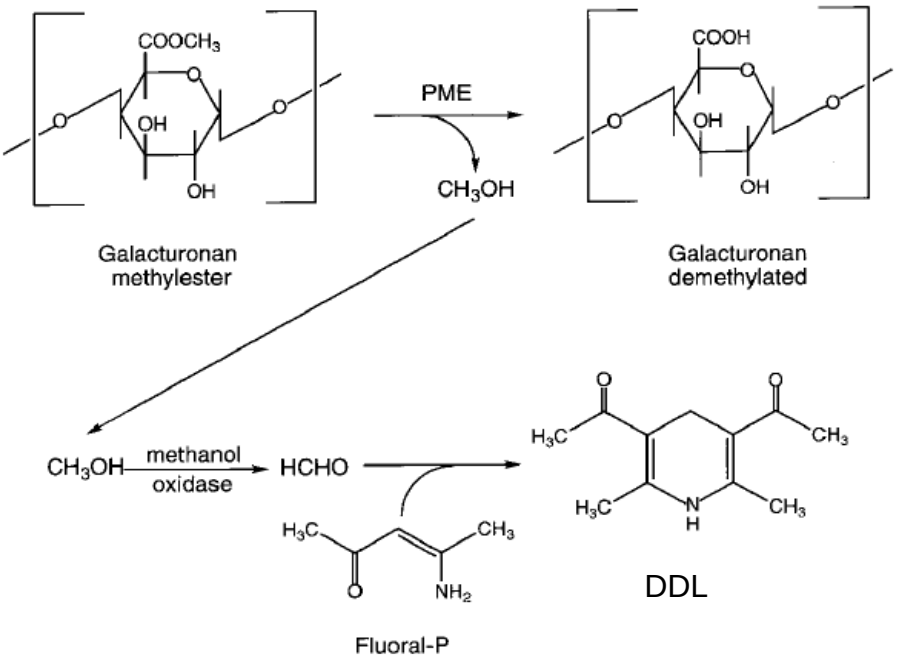

Figure 2.3. Pectin methylesterase activity based on methanol quantification (Wojciechowski and Fall, 1996).

Thus, PME standard assay was carried out following the method reported by Wojciechowski and Fall (1996) with some modifications (Anthon and Barrett, 2004; Held, Anthon and Barrett, 2015). The substrate, 0.3 mL of 0.5% (w/v) apple pectin in 20 mM sodium phosphate buffer pH 7, was pre-incubated at 50 °C for 5 min. Then, 0.2 mL of enzyme solution were added and after 15 min of incubation, the reaction was cooled down on an ice bath for 7 min. The mixture was centrifuged at 10 000 g for 3 min and the supernatant was recovered. The colorimetric reaction 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^{-1} Fluoral-P (in Milli Q water) and 10 µL of 20 U mL^{-1} AO (in 50 mM HEPES buffer pH 7) were mixed. The mixture was incubated at room temperature for 30 min and DDL produced was quantified at 410 nm. A blank of reaction was carried out following the procedure described above using only buffer instead of enzyme solution. The enzymatic activity was calculated according to Equation 2.5, where ε was calculated from the calibration curve using formaldehyde as standard (in 50 mM HEPES buffer pH 7) (Figure S 19, Appendix 6).

$$U\ mL^{-1} = \frac{Abs}{time} x\ \frac{1}{\varepsilon} x \frac{Vtotal}{Venzyme} x\ df1\ x\ df2 \qquad \text{(Equation 2.6)}$$

Where: time, 15 min; V total, 0.5 mL; V enzyme, 0.2 mL; ε, 0.0388 mM^{-1} cm^{-1}; df1, dilution factor of the enzyme solution and df2, dilution factor of the colorimetric reaction.

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.

Colorimetric method by using a pH indicator: the use of a pH indicator provides an easy, fast and cost-effective alternative for PME activity analysis; however, this method is less sensitive than that based on methanol quantification. In this assay, once PMEs hydrolyse methyl esters, the produced protons could be detected by a pH indicator (Salas-Tovar *et al.*, 2017). Hagerman and Austin (1986) established the use of bromothymol blue which acquires different conformations when pH varies among 6 and 7.6 (yellow to blue). This method is performed at pH 7.6 and the reaction is detected at 620 nm. The quantification is carried out through the elaboration of a standard curve of GalA.

In this work, the colorimetric assay was carried out to determine optimum temperature of PMEs since the method based on methanol quantification is unable to be applied at high temperatures. To the standard assay, the substrate, 0.3 mL of 0.5% (w/v) apple pectin in 5 mM sodium phosphate buffer pH 7.6, was pre-incubated at 50 °C for 5 min. Then, 0.2 mL of enzyme solution in the buffer mentioned above were added and after 15 min of incubation, the reaction was heated at 100 °C for 10 min. Subsequently, 100 µL of 0.04 % bromothymol blue (in 5 mM sodium phosphate buffer pH 7.6) were added and 200 µL of the mixture were used for the measurement of the pH change, produced because of the released protons, which was quantified in a 96-well plate at 620 nm. A blank of reaction was carried out following the procedure described above using only buffer instead of enzyme solution. All the solutions (substrate, enzyme, and pH indicator) were adjusted to pH 7.6 with NaOH

before the assay was started. The enzymatic activity was calculated according to Equation 2.6, where ε was calculated from the calibration curve using GalA as standard (in 5 mM sodium phosphate buffer pH 7.6) (Figure S 20, Appendix 6).

$$U\ mL^{-1} = \frac{Abs}{time} x\ \frac{1}{\varepsilon} x \frac{V\ total}{V\ enzyme} x\ df \qquad \text{(Equation 2.7)}$$

Where: time, 15 min; V total, 0.5 mL; V enzyme, 0.2 mL; ε, 0.3858 mM^{-1} cm^{-1} and *df*, dilution factor of the enzyme solution.

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.

2.9.3.3 PGL activity

PGL standard assay was carried out similar to exo-PG activity, by quantifying reducing groups released in the reaction and expressed as GalA units. The substrate, 0.3 mL of 0.5% (w/v) polyGalA in 20 mM Tris-HCl pH 8, was pre-incubated at 50 °C for 5 min. Then, 0.2 mL of enzyme solution containing 0.6 mM Ca^{2+} were added and after 15 min of incubation, 0.25 mL of DNS were incorporated into the reaction. The mixture was heated at 100 °C for 15 min and then centrifuged at 10 000 g for 5 min. The reducing groups were quantified in the supernatant at 540 nm in 96-well plates. A blank of reaction was carried out following the procedure described above using only buffer instead of enzyme solution. The enzymatic activity was calculated according to Equation 2.7, where ε was calculated from the calibration curve using GalA as standard (Figure S 18, Appendix 6).

$$U\ mL^{-1} = \frac{Abs}{time} x\ \frac{1}{\varepsilon} x \frac{Vtotal}{Venzyme} x\ df \quad \text{(Equation 2.8)}$$

Where: time, 15 min; V total, 0.5 mL; V enzyme, 0.2 mL; ε, 0.127 mM^{-1} cm^{-1} and *df*, dilution factor of the enzyme solution

One unit (U) of PG activity was defined as the amount of enzyme that releases 1 µmol of GalA equivalent per min at pH 8 and 50 °C.

2.9.3.4 PAE activity

PAE activity standard assay was measured according to the method reported by Remoroza *et al.* (2014) using 4-nitrophenol acetate (pNPA). The substrate, 30 µL of 10 mM pNPA in DMSO with 270 µL of 100 mM sodium phosphate pH 7, at 50 °C for 3 min. Then, 0.2 mL of enzyme solution were added. After 5 min of incubation at 50 °C at 500 rpm, the reactions were put on ice for 7 min and centrifuged at 10 000 g for 3 min at 4 °C. The released p-nitrophenol was quantified in the supernatant at 405 nm in 96-well plates. A blank of reaction was carried out following the procedure described above using only buffer instead of enzyme solution. The enzymatic activity was calculated according to Equation 2.8, where ε was calculated from the calibration curve using p-nitrophenol as standard (Figure S 21, Appendix 6).

$$U\ mL^{-1} = \frac{Abs}{time} x\ \frac{1}{\varepsilon} x\ \frac{Vtotal}{Venzyme}\ x\ df \quad \text{(Equation 2.9)}$$

Where: time, 5 min; V total, 0.5 mL; V enzyme, 0.2 mL; ε, 8.3424 mM^{-1} cm^{-1} and *df*, dilution factor of the enzyme solution

One unit (U) of PAE activity was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol equivalent per min at pH 7 and 50 °C.

2.9.3.5 AF activity

AF activity was carried out following the method described by Cárdenas-Fernández *et al.* (2018) using p-nitrophenyl-α-L-arabino- furanoside (p-NP-Ara) as substrate. The substrate, 195 µL of 2mM p-NP-Ara in 0.1 M Tris-HCl pH 7 was pre-incubated at 37 °C for 2 min. Then, 5 µL of enzyme solution were added and incubated at 37 °C and 750 rpm. After 5 min of incubation, 400 µL of 0.2 M sodium borate pH 9.8 were added and the absorbance measured at 405 nm (Aquarius spectrophotometer, Cecil Instruments, UK). The ε of the released p-nitrophenol was 18.5 mM^{-1} cm^{-1} and one unit (U) of AF was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol equivalent per minute at pH 7 and 37 °C.

2.9.4 Methanol quantification

Samples from the synergistic reactions taken at indicated times were centrifuged at 10 000 g for 3 min and the supernatants were used for methanol quantification. In a 96-well plate; 25 µL of the supernatant, 175 µL of 50 mM HEPES pH 7,10 µL of 36 mg mL^{-1} Fluoral-P and 10 µL of 20 U mL^{-1} AO were mixed. The mixture was incubated at room temperature for 30 min and the absorbance was measured at 410 nm. A calibration curve using formaldehyde as standard (ε = 0.0388 mM^{-1} cm^{-1}) was used for calculations (Figure S 19, Appendix 6).

2.9.5 GalA and Ara quantification

Synergistic reactions samples were taken at indicated times and stopped using 0.1% trifluoroacetic acid (TFA). The mixtures were centrifuged at 18,000×g for 15 min and the supernatants were recovered. GalA was analysed following the method reported by Ward *et al.* (2015) using Ion Chromatography System (ICS 5000+, Thermo Scientific, Hemel Hempstead, UK) equipped with a Dionex Aminopac™ PA1 anion exchange column 4×250mm 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^{-1} 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 (Figure S 27, Appendix 8). Ara was analysed by the same ICS system using as mobile phase 15mM KOH (electrochemical detection grade, Fisher Scientific, UK) according to the method reported by Cárdenas-Fernández *et al.*, (2018). An external standard calibration curve of Ara was used for quantitative analysis, which was performed by measuring the peak area. The retention time of Ara was 7 min (Figure S 28, Appendix 8).

2.9.6 Acetic acid quantification

Samples from the synergistic reactions taken at indicated times were centrifuged at 10 000 g for 3 min and the supernatants were used for acetic acid quantification. Acetic acid was quantified using the Megazyme acetic acid kit K-

ACETRM 04/20 (Megazyme, Wicklow, Ireland). For this assay, the microplate procedure was followed using 210 µL samples. A calibration curve using acetic acid as standard (ε = 3.7541 mM^{-1} cm^{-1}) was used for calculations (Figure S 22, Appendix 6).

2.9.7 Determination of molecular weight distribution of depolymerised pectin

Synergistic reactions samples were taken at indicated times and stopped by heating at 100 °C for 6 min. The samples were centrifuged at 18 500 g for 30 min, and the supernatants were used for evaluating pectin depolymerisation through the determination of molecular weight distribution by GFC. GFC was carried out with a HPLC (Dionex 3000 Ultimate, Thermo Scientific, Hemel Hempstead, UK) using a column Polysep 4000 (Phenomenex, Cheshire, UK) fitted in the oven at 40 °C and RI detection (Refractomax 5000). MilliQ water was used as a mobile phase at 0.4 mL min^{-1}. Molecular weight distribution of depolymerised pectin because of the synergistic reactions was determined by an external standard method using ReadyCal Kit PEO/PEG 238-969 000 Da (PSS Polymer Standards Service, Mainz, Germany). The ReadyCal Kit was injected first in each experiment and the calibration curve was obtained by plotting the molecular weight of each standard of the calibration kit against their respective retention time peak. An exponential regression curve was then applied to the graph and the molecular weight of depolymerised pectin was calculated using the regression curve equation and the samples retention times peak.

CHAPTER 3 THERMOPHILIC PECTINASES AND THEIR SYNERGISTIC ACTION FOR PECTIN BIOCONVERSION INTO D-GALACTURONIC ACID

3.1 Introduction

Thermophilic pectinases represent an economically and environmentally advantageous alternative to depolymerise the complex structure of pectin. Among pectin substructures, HG which generally accounts the highest percent of the molecule, is a backbone of GalA that can be methylated and/or acetylated (Section 1.1.1). Thus, GalA is one of the main released components after pectin depolymerisation. GalA is a key chemical used for the synthesis of several valuable compounds such as L-galactonic acid, L-ascorbic acid, keto-deoxy sugars, mucic acid, FDCA, adipic acid, polyesters, nylon, and other bio-based polymers (Section 1.3.1).

Among the most important pectinases are PGs, PGLs, PMEs and PAEs (Section 1.2.1). PMEs catalyse the de-esterification of methyl ester groups in pectin releasing methanol and acting prior to PGs and PGLs, which in turn prefer demethylated substrates. Additionally, PAEs remove acetyl groups being an important enzyme class specially for highly acetylated pectic substrates (Remoroza *et al.*, 2014). The application of pectinases depends on their biochemical properties such as pH, temperature, ions concentration and substrate specificity as well as of their kinetic parameters.

Pectinases represent an important group of enzymes in the global market accounting around 25%. However, most of them are from mesophilic microorganisms and have restricted thermal activity and stability (Section 1.2.2). Thermophilic pectinases, which are scarcely reported, could be used to depolymerise the large amounts of pectin-rich biomass generated annually mainly from the sugar and juice industries (Turner, Mamo and Karlsson, 2007; Satyanarayana, Kawarabayasi and Littlechild, 2013; Kuivanen *et al.*, 2014). Moreover, these enzymes could be applied in a synergistic manner to catalyse pectin bioconversion into valuable compounds such as GalA via sustainable biomass recycling within the context of biorefineries and the circular economy.

3.2 Aims and objectives

The aim of this chapter was to identify putative pectinases in bacterial genomes and select thermophilic candidates for molecular cloning and expression. Additionally, to carry out their functional characterisation and exploration of the synergistic activity between PMEs and exo-PGs for pectin bioconversion into GalA. The key objectives of the chapter are outlined below:

1. To identify putative pectinases from thermophilic and mesophilic bacteria using a genome mining approach.
2. To select the thermophilic candidates for cloning and expression through a phylogenetic analysis from thermophilic and mesophilic bacteria pectinases.
3. To carry out molecular cloning and gene expression of the selected thermophilic pectinases.
4. To purify and functionally characterise two exo-PGs and two thermophilic PMEs.
5. To assess the synergistic action between exo-PGs and PMEs individually expressed using apple, citrus and sugar beet pectin as substrates.

3.3 Results and discussion

3.3.1 Identification of putative pectinases from thermophilic and mesophilic bacteria through genome mining

The genomes of 25 thermophilic bacteria were analysed to identify sequences annotated with the Pfam domain families of pectinases such as PGs, PMEs and PGL/PL (Table 3.1). A total of nine putative pectinases were retrieved and in the case of *Themotoga maritima* DSM 3109 and *Thermobispora bispora* DSM 43833, different kinds of pectinases were identified.

The same analysis was performed on the genomes of mesophilic bacteria. A total of 131 putative pectinases were retrieved as specified in Table 3.2. Up to three kinds of different pectinases were identified in several mesophiles including *Bacillus licheniformis* DSM 13, *Bacillus pumilus* DSM 27, *Clostridium acetobutylicum* DSM 792, *Pectobacterium carotovorum* DSM 30168, *Dickeya*

dadantii 3937, *Dickeya chrysanthemi* DSM 4610, *Flavobacterium johnsoniae* DSM 2064, *Lechevalieria aerocolonigenes* DSM 40034, *Streptomyces curacoi* DSM 40107 and *Xanthomonas campestris* DSM 3586.

Table 3.1. Pectinases from the genomes of thermophilic microorganisms retrieved by using Pfam domain families. UniProtKB accession number of the proteins is shown. Bacteria used in this study come from Prof. John Ward's microbial collection.

Bacteria	**PG**	**PME**	**PGL/PL**
Archaeoglobus fulgidus (from our collection)	NI	NI	NI
Caldanaerobacter subterraneus subsp. *tengcongensis* DSM 15242	NI	NI	NI
Deinococcus geothermalis DSM 11300	NI	NI	NI
Deinococcus radiodurans (from our collection)	NI	NI	NI
Deinococcus radiophilus DSM 20551	NI	NI	NI
Geobacillus stearothermophilus 10 DSM 13240	NI	NI	NI
Geobacillus stearothermophilus DSM 22	NI	NI	NI
Pyrococcus furiosus (from our collection)	NI	NI	NI
Rubrobacter xylanophilus DSM 9941	NI	NI	NI
Saccharomonospora viridis DSM 43017	NI	NI	NI
Sulfolobus solfataricus (from our collection)	NI	NI	NI
Sulfolobus acidocaldarius (from our collection)	NI	NI	NI
Sulfolobus shibatae (from our collection)	NI	NI	NI
Thermaerobacter marianensis DSM 12885	NI	NI	NI
Thermaerobacter subterraneus DSM 13965	NI	NI	NI
Thermincola ferriacetica DSM 14005	NI	NI	NI
Thermococcus litoralis (from our collection)	NI	NI	NI
Thermobifida fusca DSM 43792 (TFU)	NI	NI	Q47MW8 Q47TM3
Thermobispora bispora DSM 43833 (TBIS)	NI	D6Y4C1	D6Y7Z0 D6Y6H1
Thermomonospora curvata DSM 43183 (TCUR)	NI	NI	D1A899 D1A3U6
Thermoplasma acidophilum DSM 1728	NI	NI	NI
Thermotoga maritima DSM 3109 (TMA)	Q9WYR8	NI	Q9WYR4
Thermus aquaticus (from our collection)	NI	NI	NI
Thermus thermophiles DSM 7039	NI	NI	NI
Thermus thermophiles DSM 579	NI	NI	NI

PG: polygalacturonase, PME: pectin methylesterase and PGL/PL: pectate lyase/pectin lyase. NI: not identified.

Table 3.2. Pectinases from the genomes of mesophilic bacteria retrieved using Pfam domain families. UniProtKB accession number of the proteins is showed. Bacteria used in this study come from Prof. John Ward's microbial collection.

Bacteria	PG	PME	PGL/PL
Bacillus licheniformis DSM 13 (BLI)	Q65F26	Q65F39	Q8GCB2 Q65G96 A0A1Q9FIY7 A0A447SED5 Q65EF5
Bacillus pumilus DSM 27 (BPU)	W8QZW7	A0A1Q9BBJ0	D8X181
Tannerella forsythia ATCC 43037 (TFO)	A0A1D3UFD3	NI	NI
Burkholderia pyrrocinia DSM 10685 (BPY)	A0A2Z5N7G1 A0A2Z5N708 A0A118PXM8	NI	NI
Clostridium acetobutylicum DSM 792 (CAC)	Q97D05 Q97M45	Q97DU8	Q97KW2
Clostridium beijerinckii DSM 791 (CBE)	A0A1S8P9R8 A0A1S8SBS7 A0A1S8S9T0 A0A1S8QP81	NI	NI
Pectobacterium carotovorum DSM 30168 (PCA)	A0A330J753 Q7M0W7	A0A1M5TQ95 A0A330JA38	A0A1M5RKS5 A0A1M5Q3A5 A0A1M5NQI0 Q47465
Dickeya dadantii 3937 (DDA)	E0SDK6	Q47474 P0C1A9	P0C1A5 P0C1A3 E0SAZ3 E0SJQ6 E0SG38 E0SKU1 E0SM75
Dickeya chrysanthemi DSM 4610 (DCH)	Q8KKH7 Q9K5A2 Q9K5A1 P15922	P0C1A8	P11073 P04960 P0C1A2 P18209 P0C1A4 O50325
Flavobacterium daejeonense DSM 17708 (FDA)	A0A0Q0RRK0 A0A0Q0RM76 A0A0N8VME2 A0A0Q0SGD8 A0A0Q0VYY8 A0A0Q0RR74 A0A0Q0S1L4 A0A0Q0X5D1 A0A0Q0SFJ8 A0A0Q0S3Y3 A0A0Q0SG09 A0A0N8VPC6	A0A0Q0WT32 A0A0Q0WZX1 A0A0Q0RR80 A0A0N8VME9 A0A0Q0W549	NI
Flavobacterium defluvii DSM 17963 (FDE)	A0A1M5VJV3	A0A1M5VKS6	NI

PG: polygalacturonase, PME: pectin methylesterase and PGL/PL: pectate lyase/pectin lyase. NI: not identified.

Table 3.2.

Continued

Bacteria	PG	PME	PGL/PL
Flavobacterium flevense DSM 1076 (FFL)	A0A1M7BGU8 A0A1M6ZM68 A0A1M7B5F0 A0A1M7E6Q2 A0A1M7D1L6 A0A1M7CFV1 A0A1M7FN05 A0A1M6ZXP0 A0A1M6ZLM9 A0A1M7GHU0 A0A1M6ZNJ7 A5FF89 A5FF90	A0A1M6ZM80 A0A1M7D0X7 A0A1M6ZJV8 A0A1M6ZLF4	NI
Flavobacterium johnsoniae DSM 2064 (FJO)	A0A1J7CFJ6 A0A1J7CJM7 A0A1J7BN84 A0A1J7CF78 A0A1J7CLI4 A0A1J7CFB1	A5FCH1 A0A1J7CHB2 A5FC12	A0A1J7C7A9
Lechevalieria aerocolonigenes DSM 40034 (LAE)	A0A0F0GF26 A0A0F0GLI4	A0A0F0H2I0	A0A0F0GER1
Pseudomonas fluorescens DSM 50090 (PFL)	A0A1T2YGX8 A0A370XKS8	NI	Q59671
Streptomyces ambofaciens DSM 40053 (SAM)	NI	A0A0K2AQ74	A0A0K2APS5 A0A0K2ASG2 A3KK49
Streptomyces avermitilis DSM 46492 (SAV)	NI	Q829N4 Q829N3	Q829N5 A0A4D4M8J8 Q829M8
Streptomyces curacoi DSM 40107 (SCU)	A0A117PAF7 A0A124H655	A0A117PLH5	A0A117P4P5 A0A124H7W0 A0A124H7W2 A0A117P5D8 A0A117PI11 A0A117PLG7 A0A117P5S3 A0A117PLM8
Vibrio fluvialis DSM 19283 (VFL)	NI	A0A109X3N6	NI
Xanthomonas campestris DSM 3586 (XCA)	Q8P582 Q8P8H5	Q8PE60 Q8P8H6	Q8P6Z9 Q8PCR9

PG: polygalacturonase, PME: pectin methylesterase and PGL/PL: pectate lyase/pectin lyase. NI: not identified.

3.3.2 Phylogenetic analysis of putative pectinases and selection of thermophilic candidates for cloning and expression

The relationship between pectinases from mesophilic and thermophilic bacteria was analysed through the construction of phylogenetic trees of the protein sequences using the ClustalW and MEGA X bioinformatic tools. The phylogenetic analysis was carried out to select pectinases from mesophiles most closely related to those from thermophiles. Pectinases (PGs, PMEs and PGLs/PLs) from *Aspergillus* and *Erwinia* species were included into the trees as they have been broadly studied and reported as mesophilic enzymes. The phylogenetic analysis was performed as independent trees for PGs, PMEs and PGLs/PLs.

Figure 3.1 shows the relationship between PGs from mesophilic and thermophilic bacteria. BPU W8QZW7 and BLI04 Q65F26 from *Bacillus* species were homologous to RMAR D0MEP1 from *Rhodothermus marinus* and TMA01 Q9WYR8 from *Thermotoga maritima* (Cluster A). FDA A0A0Q0RM76, FFL A0A1M7BGU8 and FJO A0A1J7CF78 from *Flavobacterium* species exhibited high homology to CBES B9MNB8 from *Caldicellulosiruptor bescii* (Cluster B). CAC Q97M45 and CBE A0A1S8S9T0 from *Clostridium* species were homologous to PTHE F7YW05 from *Pseudothermotoga thermarum* (Cluster C). Mesophilic PGs from *Aspergillus* and *Erwinia* were grouped in separated clusters with other enzymes from mesophilic bacteria. BLI04 and TMA01 were studied in this work.

Figure 3.2 illustrates the phylogenetic relationship between PMEs from mesophiles and thermophiles. PMEs from *Streptomyces* species (SAV Q829N4, SCU A0A117PLH5, SAM10 A0A0K2AQ74 and SAV Q829N3) were homologous to TBIS D6Y4C1 from *Thermobispora bispora* (Cluster D). Interestingly, BLI09 Q65F39 from *Bacillus licheniformis* was associated in the same cluster with several PMEs from thermophiles including CHYD E4QA11 from *Caldicellulosiruptor hydrothermalis*, CKRO E4SCS2 from *Caldicellulosiruptor kronotskyensis*, COWE E4Q6N9 from *Caldicellulosiruptor owensensis* and THSP A0A0F6AK54 from *Thermotoga* sp. Strain RQ2 (Cluster

E). Mesophilic enzymes such as PCA A0A1M5TQ95, DDA P0C1A9 and DCH P0C1A8 from *Erwinia* species exhibited high homology to RMAR D0MF90 from the thermophilic bacterium *Rhodothermus marinus* (Cluster F). SAM10 and BLI09 were studied in this work.

Figure 3.3 shows the phylogenetic analysis of pectin degrading lyases from mesophilic and thermophilic bacteria. Several lyases from *Streptomyces species* (SCU A0A124H7W2, SCU A0A117PI11, SCU A0A117PLG7, SAM A0A0K2APS5 and SAV Q829N5) were homologous to TBIS D6Y7Z0 from *Thermobispora bispora* and TFU20 Q47TM3 from *Thermobifida fusca* (Cluster G). FJO A0A1J7C7A9 from *Flavobacterium johnsoniae* exhibited high homology to CTHE A3DJL9 from *Clostridium thermocellum* (Cluster H). SCU A0A117P4P5 and SCU A0A124H7W0 from *Streptomyces curacoi* were homologous to TMA14 Q9WYR4 from *Thermotoga maritima* and TCUR D1A3U6 from *Thermomonospora curvata* (Cluster I). Similarly, lyases from *Streptomyces* (SAM A0A0K2ASG2 and SCU A0A117P5D8) and *Bacillus* (BLI Q8GCB2 and BPU D8X181) showed high homology to those from TFU19 Q47MW8 from *Thermobifida fusca* and CTHE A3DHF2 from *Clostridium thermocellum* (Cluster J). Finally, lyases from S*treptomyces* species (SAM A3KK49, SAV Q829M8 and SCU A0A117PLM8), *Bacillus licheniformis* (BLI Q65EF5) and *Lechevalieria aerocolonigenes* (LAE A0A0F0GER1) were homologous to CKRO E4SDD0 from *Caldicellulosiruptor kronotskyensis*, CBES B9MKT4 from *Caldicellulosiruptor bescii* and TBIS D6Y6H1 from *Thermobispora bispora* (Cluster K). Mesophilic lyases from *Aspergillus* and *Erwinia* were grouped in separated clusters with other enzymes from mesophilic bacteria. TFU20, TMA14 and TFU19 were studied in this work.

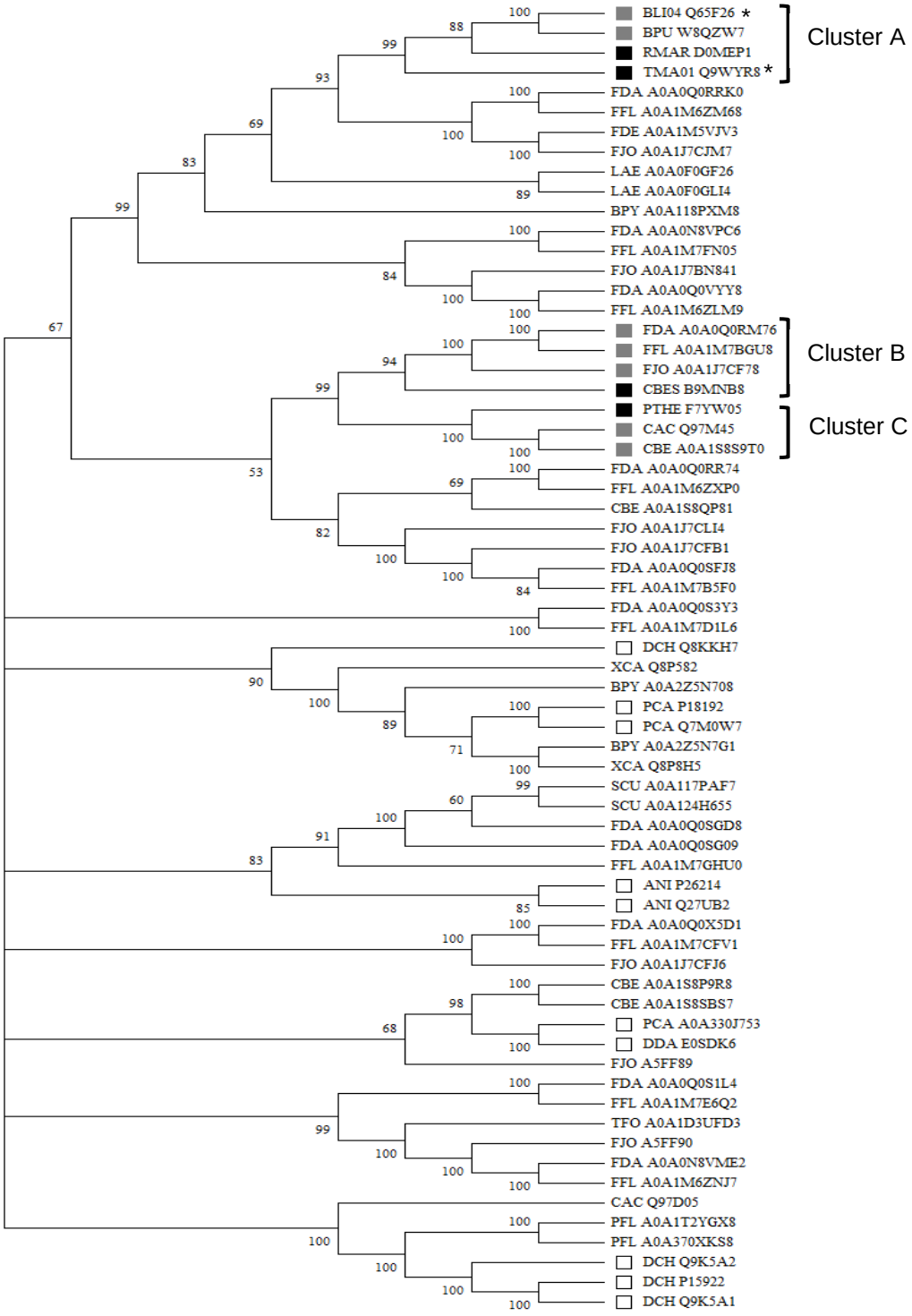

Figure 3.1. Phylogenetic relationship between PGs from mesophilic and thermophilic bacteria. The tree was constructed using the Neighbor-Joining method and the confidence scores (percent) of a bootstrap test of 1000 replicates are indicated in the branching nodes. Labels at tree terminals indicate the strain and UniProtKB accession numbers. (■) PGs from thermophiles, (■) PGs from mesophiles closely related to those from thermophiles and (□) PGs from *Aspergillus* and *Erwinia* species. (*) PGs studied in this work. Phylogenetic tree was constructed as described in Section 2.2 in Chapter 2.

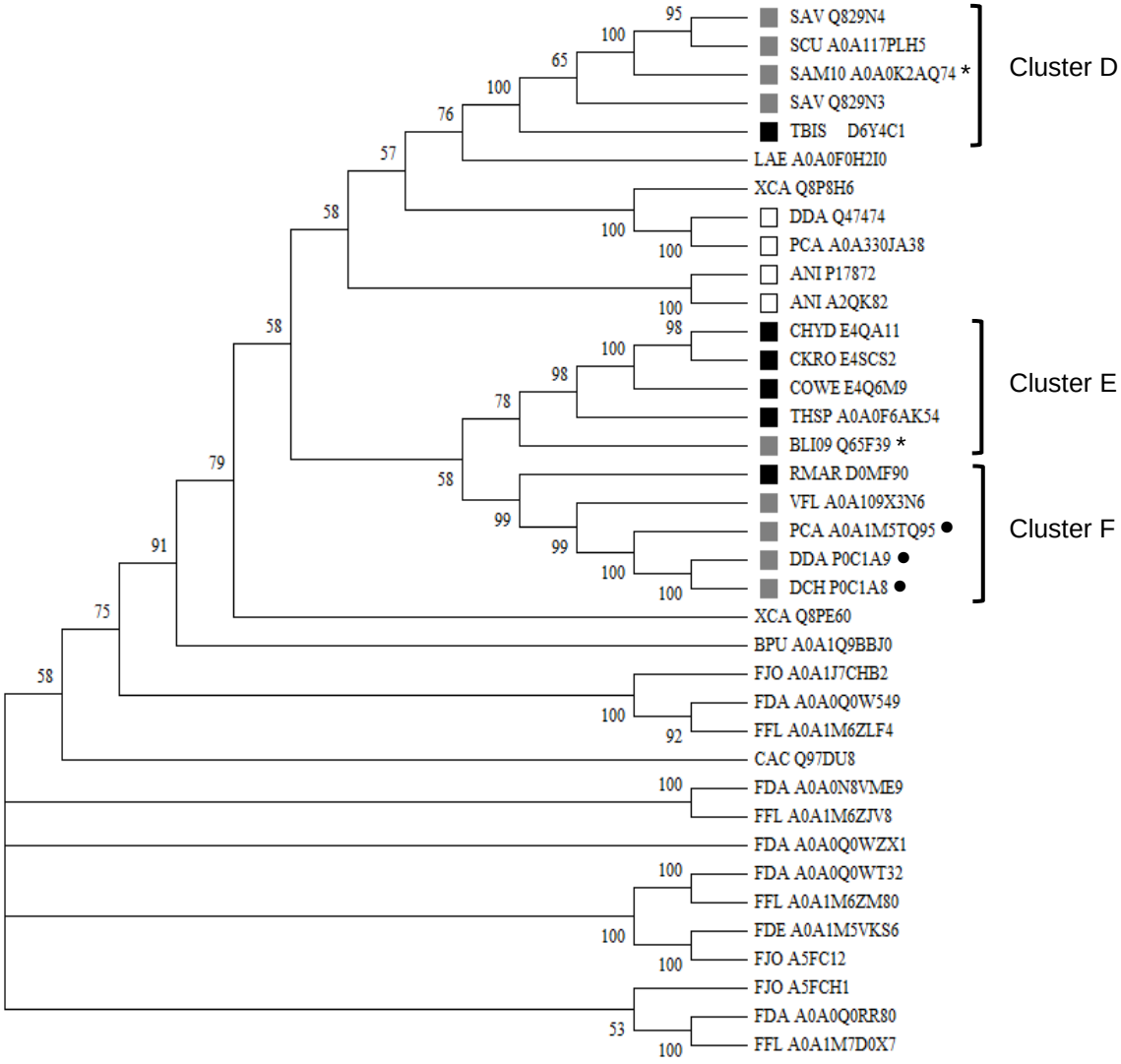

Figure 3.2. Phylogenetic relationship between PMEs from mesophilic and thermophilic bacteria. The tree was constructed using the Neighbor-Joining method and the confidence scores (percent) of a bootstrap test of 1000 replicates are indicated in the branching nodes. Labels at tree terminals indicate the strain and UniProtKB accession numbers. (■) PMEs from thermophiles, (■) PMEs from mesophiles closely related to those from thermophiles and (□) PMEs from *Aspergillus* and *Erwinia*, (●) PMEs from *Erwinia* closely related to a PME from a thermophilic bacterium. (*) PMEs studied in this work. Phylogenetic tree was constructed as described in Section 2.2 in Chapter 2.

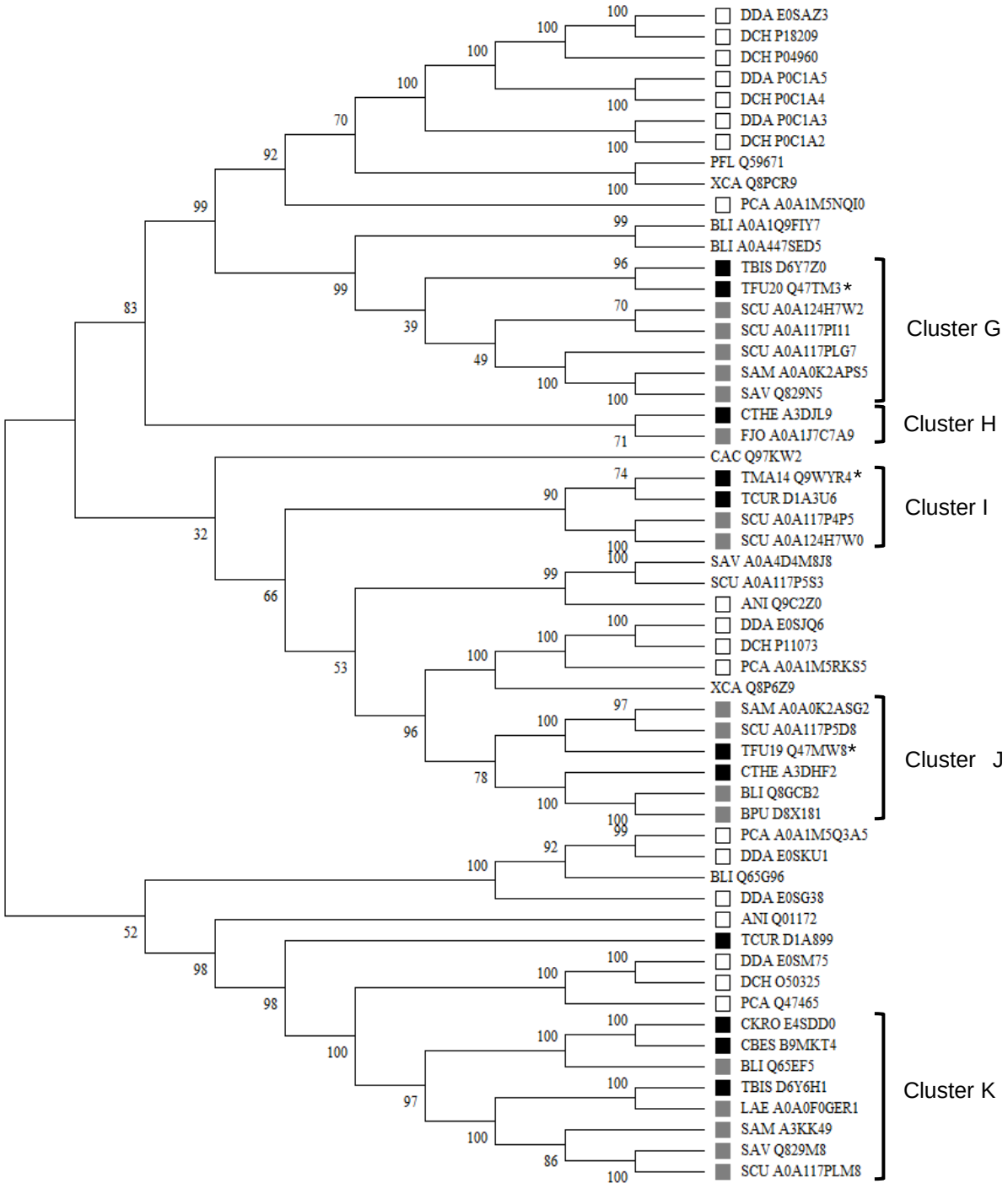

Figure 3.3. Phylogenetic relationship between PGLs/PLs from mesophilic and thermophilic bacteria. The tree was constructed using the Neighbor-Joining method and the confidence scores (percent) of a bootstrap test of 1000 replicates are indicated in the branching nodes. Labels at tree terminals indicate the strain and UniProtKB accession numbers. (■) PGLs/PLs from thermophiles, (■) PGLs/PLs from mesophiles closely related to those from thermophiles and (□) PGLs/PLs from *Aspergillus* and *Erwinia*. (*) PGLs studied in this work. Phylogenetic tree was constructed as described in Section 2.2 in Chapter 2.

According to the phylogenetic analysis presented above, 29 pectinases from mesophiles closely related to those from thermophiles were identified. From these, 22 belonged to *Bacillus* and *Streptomyces* species which were selected as candidates for cloning and expression, along with the nine pectinases identified previously from thermophiles (Table 3.3).

Table 3.3. Candidate thermophilic pectinases selected for cloning and expression.

From thermophiles	**From mesophiles**
PGs	
Q9WYR8 *Thermotoga maritima* DSM 3109	W8QZW7 *Bacillus pumilus* DSM 27 Q65F26 *Bacillus licheniformis* DSM 13
PMEs	
D6Y4C1 *Thermobispora bispora* DSM 43833	Q829N4 *Streptomyces avermitilis* DSM 46492 Q829N3 *Streptomyces avermitilis* DSM 46492 A0A117PLH5 *Streptomyces curacoi* DSM 40107 A0A0K2AQ74 *Streptomyces ambofaciens* DSM 40053 Q65F39 *Bacillus licheniformis* DSM 13
PGLs	
Q47MW8 *Thermobifida fusca* DSM 43792 Q47TM3 *Thermobifida fusca* DSM 43792 D6Y7Z0 *Thermobispora bispora* DSM 43833 D6Y6H1 *Thermobispora bispora* DSM 43833 D1A899 *Thermomonospora curvata* DSM 43183 D1A3U6 *Thermomonospora curvata* DSM 43183 Q9WYR4 *Thermotoga maritima* DSM 3109	A0A117P4P5 *Streptomyces curacoi* DSM 40107 A0A124H7W0 *Streptomyces curacoi* DSM 40107 A0A0K2ASG2 *Streptomyces ambofaciens* DSM 40053 A0A117P5D8 *Streptomyces curacoi* DSM 40107 Q8GCB2 *Bacillus licheniformis* DSM 13 D8X181 *Bacillus pumilus* DSM 27 Q65EF5 *Bacillus licheniformis* DSM 13 A3KK49 *Streptomyces ambofaciens* DSM 40053 Q829M8 *Streptomyces avermitilis* DSM 46492 A0A117PLM8 *Streptomyces curacoi* DSM 40107 A0A124H7W2 *Streptomyces curacoi* DSM 40107 A0A117PI11 *Streptomyces curacoi* DSM 40107 A0A117PLG7 *Streptomyces curacoi* DSM 40107 A0A0K2APS5 *Streptomyces ambofaciens* DSM 40053 Q829N5 *Streptomyces avermitilis* DSM 46492

PGs: polygalacturonases, PMEs: pectin methylesterases and PGLs: pectate lyases.

3.3.3 Cloning and gene expression of thermophilic pectinases

From the 31 candidate thermophilic pectinases selected in Table 3.3 (Section 3.3.2), seven genes were successfully cloned and the expressed enzymes included: two exo-PGs, TMA01 from *Thermotoga maritima* DSM 3109 and BLI04 from *Bacillus licheniformis* DSM 13; two PMEs, BLI09 from *Bacillus licheniformis* DSM 13 and SAM10 from *Streptomyces ambofaciens* DSM 40053; and three PGLs, TMA14 from *Thermotoga maritima* DSM 3109, and TFU19 and TFU20 from *Thermobifida fusca* DSM 43792 (Table 3.4). The rest of the selected genes were not cloned due to the strains were not available or unspecific PCR products were obtained. The primers used in this study are described in Table S 1 (Appendix 4), overhangs were added to include the specific restriction site for cloning. The seven pectinases were well expressed in *E. coli* BL21(DE3). In this work, as mentioned in Section 2.3, all the enzymes were cloned removing the signal peptides and a C-terminal His6-tag was included. No signal peptide was detected in TMA01 and BLI04 exo-PGs as well as in BLI09 PME.

Table 3.4. Thermophilic pectinases successfully cloned and expressed.

Bacteria	Type	UniProtKB	Enzyme code	EC number	Gene length (bp)	Full-length protein (aa)	Signal peptide (aa)	Molecular Weight (kDa)
Thermotoga maritima DSM 3109	Exo-PG	Q9WYR8	TMA01	3.2.1.67	1347	448	ND	50.48
Bacillus licheniformis DSM 13	Exo-PG	Q65F26	BLI04	3.2.1.67	1311	436	ND	48.14
Bacillus licheniformis DSM 13	PME	Q65F39	BLI09	3.2.1.11	954	317	ND	35.11
Streptomyces ambofaciens DSM 40053	PME	A0A0K2AQ74	SAM10	3.2.1.11	1128	375	31	40.68
Thermotoga maritima DSM 3109	PGL	Q9WYR4	TMA14	4.2.2.22	1104	367	27	40.61
Thermobifida fusca DSM 43792	PGL	Q47MW8	TFU19	4.2.2.2	1539	512	32	53.62
Thermobifida fusca DSM 43792	PGL	Q47TM3	TFU20	4.2.2.2	1350	449	25	49.89

Exo-PG: exo-polygalacturonase, PME: pectin methylesterase and PGL: pectate lyase. ND: not detected.

3.3.4 Purification of individually cloned and expressed thermophilic exo-PGs and PMEs

Pectinases were cloned individually including a C-terminal His6-tag and for characterisation purposes, they were fully purified by affinity chromatography by using a His_6-Tag Ni-affinity resin as specified in Section 2.6.1 in Chapter 2. All the studied enzymes were expressed in their soluble form.

Figure 3.4 confirms the expression and purification of the two exo-PGs, TMA01 and BLI04. TMA01 is a tetrameric enzyme of 212 kDa formed by monomers of 50.5 kDa (Kluskens *et al.*, 2005) and BLI04 is a monomeric enzyme with a molecular weight of 48.1 kDa (Evangelista *et al.*, 2018). Figures 3.4 A and 3.4 B reveal purified proteins of ~ 55 kDa that correspond to TMA01 and BLI04, respectively. No enzymatic activity was observed in loading and washing fractions. After the purification process, around 95% of both PGs was recovered. Enzymatic activities of TMA01 and BLI94 were 33 and 24 U mL^{-1}, respectively. Protein concentration for each enzyme was 0.2 mg mL^{-1}, thus specific activities of 165 and 120 U mg^{-1} were calculated for TMA01 and BLI04, respectively.

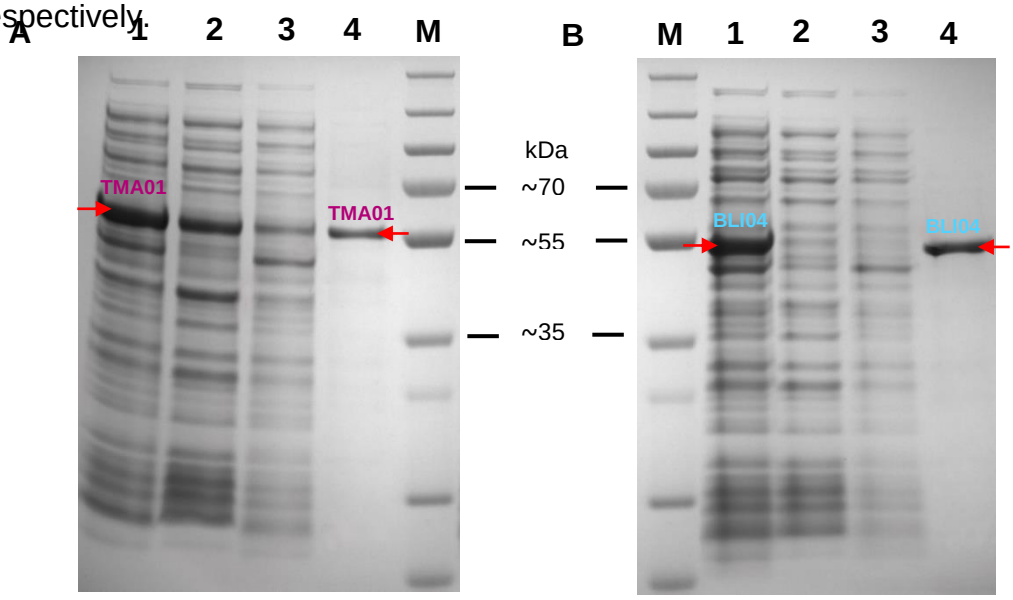

Figure 3.4. SDS-PAGE analysis of affinity chromatography purification from (A) TMA01 and (B) BLI04 PGs. Lanes: 1, clarified cell lysate; 2, loading; 3, washing and 4, elution. Fifteen micrograms of protein were load per lane. M represents molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa). Molecular weights of TMA01 and BLI04 are 50.5 and 48.1 kDa, respectively. Enzymes purification and SDS-PAGE analysis were carried out as described in Sections 2.6.1 and 2.9.2 in Chapter 2, respectively.

Figure 3.5 reveals the expression and purification of the two PMEs, BLI09 and SAM10. BLI09 has a calculated molecular mass of 35.1 kDa (Remoroza *et al.*, 2015) which is similar to the single band showed in Figure 3.5 A. Concerning SAM10, a purified protein between 35 and 55 kDa can be observed in Figure 3.5 B which confirms the molecular weight of 40.7 kDa reported by UniProtKB. No enzymatic activity was detected in loading and washing fractions. After the purification process, around 90% of both PMEs was recovered. Enzymatic activities of BLI09 and SAM10 were 9000 and 2018 U mL^{-1}, respectively. Protein concentration for BLI09 and SAM10 was 0.5 and 0.26 mg mL^{-1}, respectively, thus specific activities of 18 000 and 7 760 U mg^{-1} were calculated for BLI09 and SAM10, respectively.

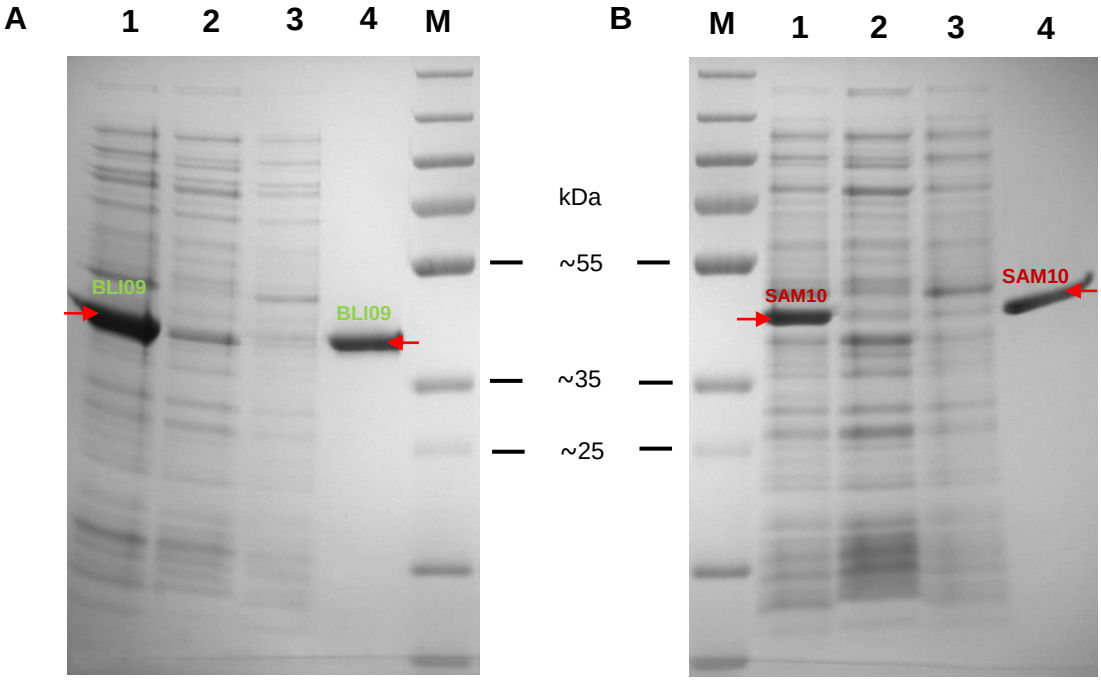

Figure 3.5. SDS-PAGE analysis of affinity chromatography purification from (A) BLI09 and (B) SAM10 PMEs. Lanes: 1, clarified cell lysate; 2, loading; 3, washing and 4, elution. Fifteen micrograms of protein were loaded per lane. M represents molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa). Molecular weights of BLI09 and SAM10 are 35.1 and 40.7 kDa, respectively. Enzymes purification and SDS-PAGE analysis were carried out as described in Sections 2.6.1 and 2.9.2 in Chapter 2, respectively.

3.3.5 Functional characterisation of thermophilic exo-PGs and PMEs

3.3.5.1 Influence of pH on the activity

Exo-polygalacturonases: on the basis of the pH of activity, pectinases can be classified in acidic or alkaline (Satyanarayana, Kawarabayasi and Littlechild, 2013). The pH of PGs varies markedly depending on the microbial source. Pectinases from fungi have an optimum pH range between 3 and 5.5. In general, bacterial PGs work more efficiently close to neutral and alkaline pH values, especially those from some *Bacillus* strains which are alkaline pectinases (Shevchik *et al.*, 1999; Jayani, Saxena and Gupta, 2005; Pedrolli *et al.*, 2009; Gonzalez and Rosso, 2011; Sharma, Rathore and Sharma, 2012). However, some bacterial thermophilic PGs such as from *Caldicellulosiruptor bescii* have exhibited an optimum pH of 5.2 (Chen *et al.*, 2014).

TMA01 exhibited maximum activity between pH 7 and 8, and notably maintained approximately 60% of its optimum activity at pH 6.5, 9 and 10 (Figure 3.6 A). Some previous studies have not reported activity above pH 7 as well as have suggested an inhibitory effect of phosphate ions from phosphate buffer (Parisot *et al.*, 2003; Kluskens *et al.*, 2005), which was not found in our study. Regarding BLI04, it was active in a pH range from 4 to 10, exhibiting higher activity at pH 8 (Figure 3.6 B). Furthermore, what stands out in Figure 3.6 B is a second peak of activity at pH 5, where the enzyme kept 60% of its maximum activity, which dropped sharply at pH 6 to only 20%. Previous publications have described similar results, showing two peaks of activity in pH assays for pectinases from *Bacillus* strains (Nasser, Chalet and Robert-Baudouy, 1990; Kobayashi, Hatada, *et al.*, 1999). In addition, BLI04 retained almost 65% of its optimum activity at pH 7, 9 and 10 demonstrating that it prefers alkaline pH values as most *Bacillus* enzymes.

Pectin methylesterases: it has been reported that most of these enzymes are active in a pH range between 4 and 8, being more acidic those from fungi than from bacteria (Jayani, Saxena and Gupta, 2005; Gonzalez and Rosso, 2011). Thus, PME from *Erwinia* and *Bacillus* have exhibited an optimum pH around 8 (Sharma, Rathore and Sharma, 2012; Kavuthodi and Sebastian, 2018).

As illustrated in Figure 3.7, the effect of pH on BLI09 and SAM10 activity was similar. The influence of pH was tested based on methanol quantification method described in Section 2.9.3.2 in Chapter 2 in a range from 5 to 10. An optimum pH of 7 was identified for both PMEs. Negligible activity was measured at pH 5, but they preserved about 60% of their highest activity at pH 6. At pH 8 and above the enzymatic activity of these PMEs was less than 30%.

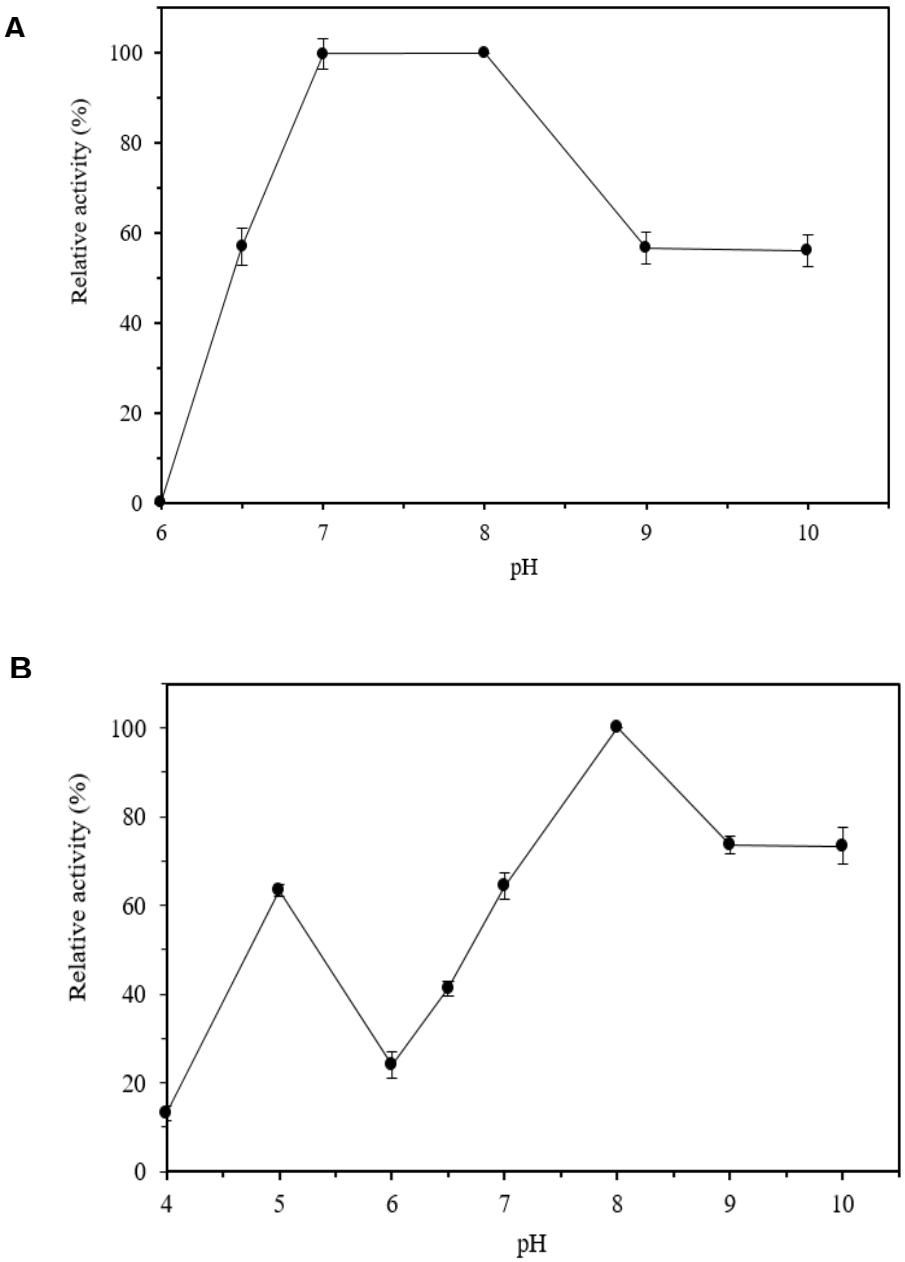

Figure 3.6. Influence of pH on the activity of purified (A) TMA01 and (B) BLI04 exo-PGs (~ 0.25 U mL^{-1}). The experiment was carried out using 0.5% (w/v) polyGalA and the reactions were incubated at 50 °C for 15 min. The relative activity was expressed as a percentage of the maximum activity. Error bars represent one standard deviation from the mean (n = 2). The assay was performed as described in Sections 2.7.1 and 2.9.3.1 in Chapter 2. PolyGalA: polygalacturonic acid.

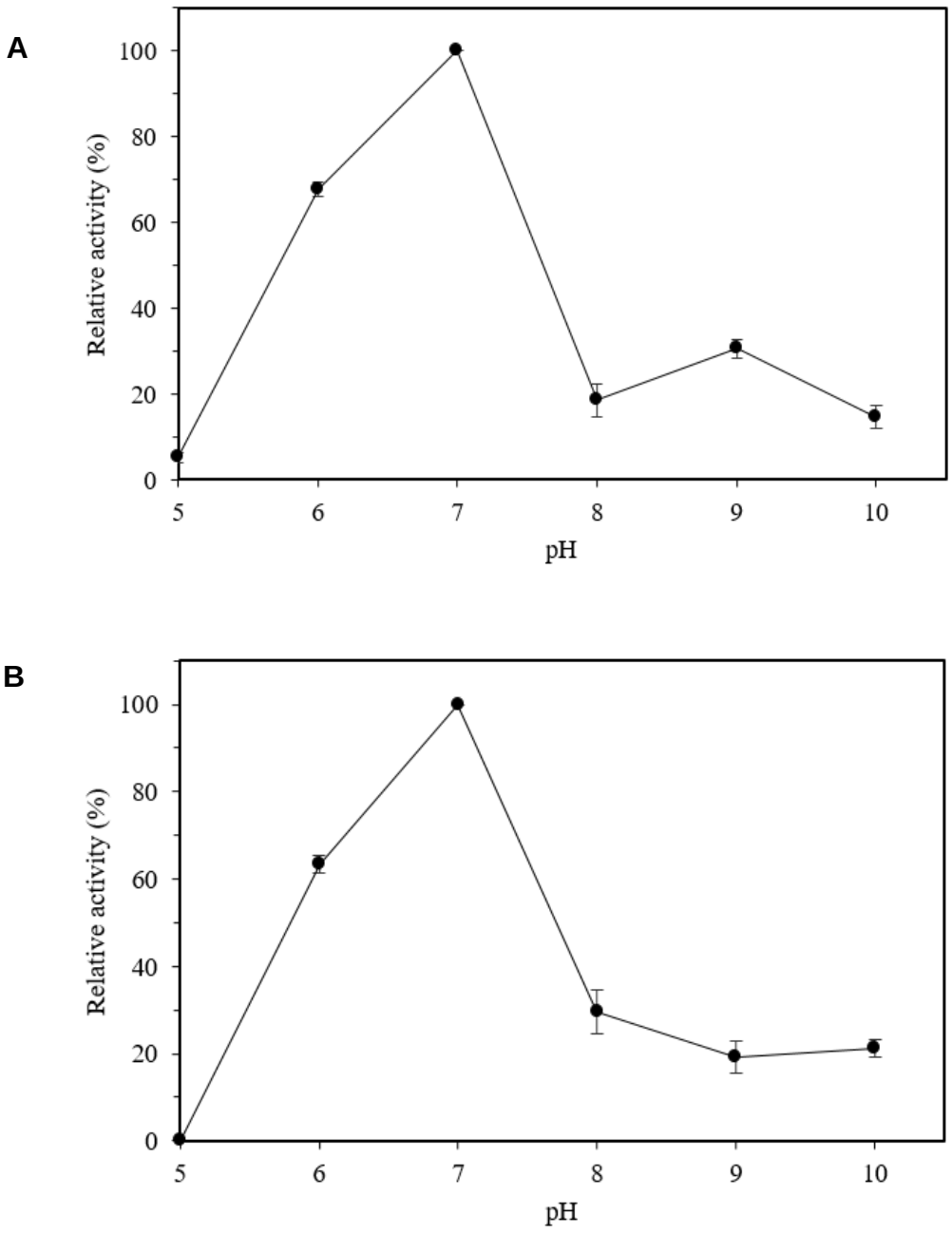

Figure 3.7. Influence of pH on the activity of purified (A) BLI09 (~ 9 U mL^{-1}) and (B) SAM10 (~ 4.5 U mL^{-1}) PMEs. The experiment was carried out using 0.5% (w/v) apple pectin and the reactions were incubated at 50 °C for 15 min. The PME activity was determined based on the methanol quantification using AO and Fluoral-P. The relative activity was expressed as a percentage of the maximum activity. Error bars represent one standard deviation from the mean (n = 2). The assay was performed as described in Sections 2.7.1 and 2.9.3.2 in Chapter 2.

3.3.5.2 Influence of ions on the activity

Exo-polygalacturonases: the effect of several ions on TMA01 and BLI04 activity (~ 0.25 U mL^{-1}) is presented in Figures 3.8 and 3.9, respectively. These ions were selected since some studies have reported that they affect pectinases activity (Vincken *et al.*, 2003; Chen *et al.*, 2014; Rehman *et al.*, 2015; Evangelista *et al.*, 2018). Similar results were obtained for both enzymes. As Figure 3.8 A shows, there was a significant increase in TMA01 activity in presence of 1 mM Mn^{2+} (1.7-fold) (Table S 3, Appendix 9). Nevertheless, the enzyme was inhibited by around 50% of its activity at 5 and 10 mM Mn^{2+}. To find the optimum Mn^{2+} concentration to enhance the activity of TMA01, different concentrations of this ion were assessed. Figure 3.8 B reveals that 0.25 mM Mn^{2+} was the best concentration to stimulate the activity of this exo-PG (2-fold). Figure 3.9 A provides the results of the effect of the ions on BLI04 activity. The activity of this enzyme was also significantly stimulated by Mn^{2+}(1.2-fold) (Table S 4, Appendix 9). However, compared with TMA01, the increase of BLI04 activity was the same in presence of 1, 5 and 10 mM Mn^{2+} concentrations. Figure 3.9 B shows that 0.25 mM Mn^{2+} was also the optimum concentration to improve BLI04 activity (1.8-fold).

Concerning the other ions, Na^{1+} and Mg^{2+} did not affect the activity of both exo-PGs. On the other hand, as Ca^{2+} concentration increased more inhibition of the enzyme's activity was observed. Falling in the exo-PG activity by Ca^{2+} might be due to the interaction between this ion and blocks of non-esterified GalA residues resulting in gels formation (Vincken *et al.*, 2003). In addition, the enzymes were inhibited by Zn^{2+} (Figures 3.8 A and 3.9 A). Variable findings about the effect of ions on the exo-PGs activity have been reported, but to the best of our knowledge the positive effect of Mn^{2+} on their activity have not been observed (Kapoor *et al.*, 2000; Chen *et al.*, 2014; Rehman *et al.*, 2015; Evangelista *et al.*, 2018).

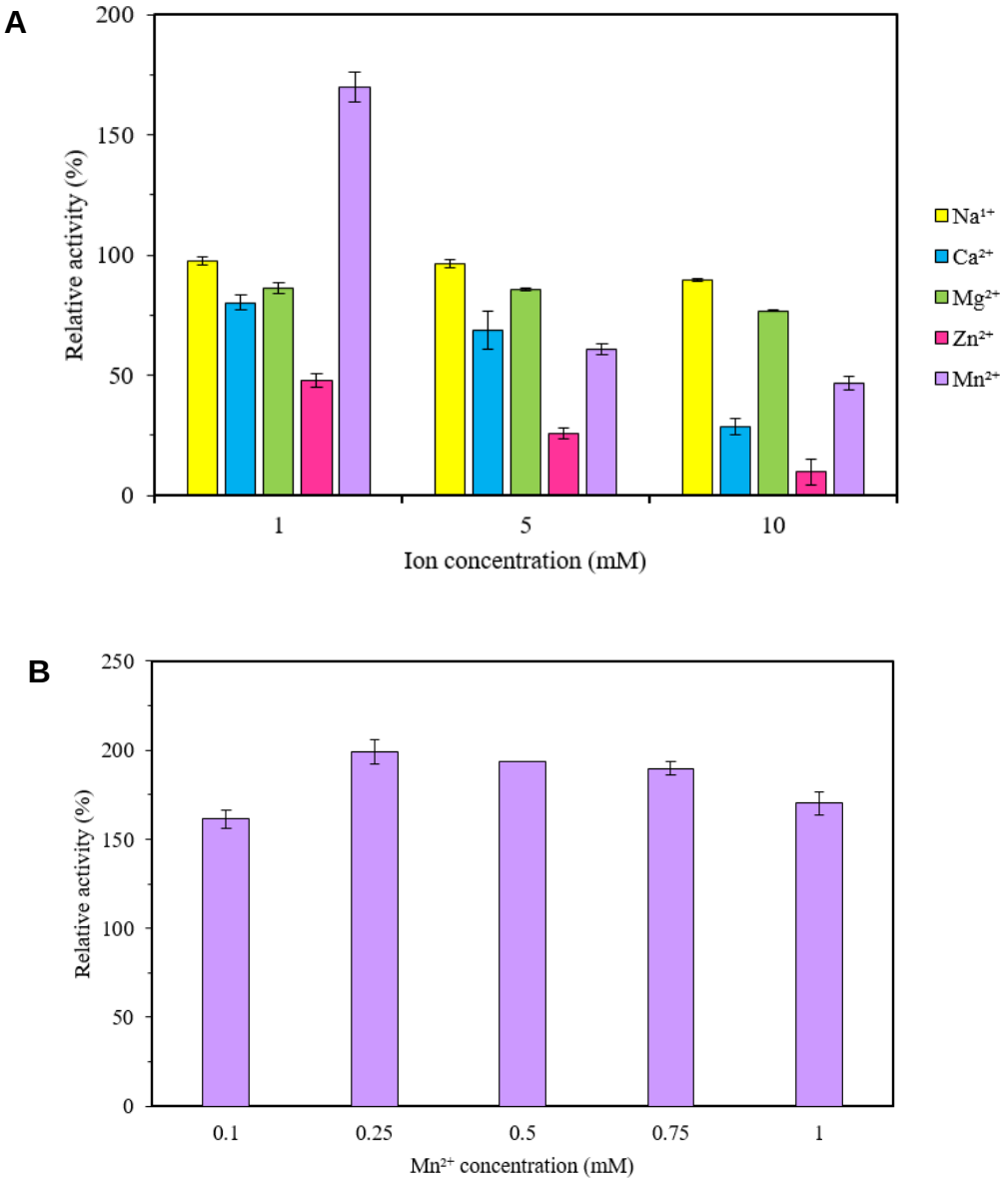

Figure 3.8. Influence of ions on the activity of purified TMA01 exo-PG (~ 0.25 U mL^{-1}). (A) Ions at different concentrations and (B) Mn^{2+} at different concentrations. The enzymes were pre-incubated with the ions at room temperature for 15 min. The exo-PG activity was determined using 0.5% (w/v) polyGalA at pH 6.5 and 50 °C. The relative activity was expressed as the percentage of activity compared with a control without ion. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.2 and 2.9.3.1 in Chapter 2. PolyGalA: polygalacturonic acid.

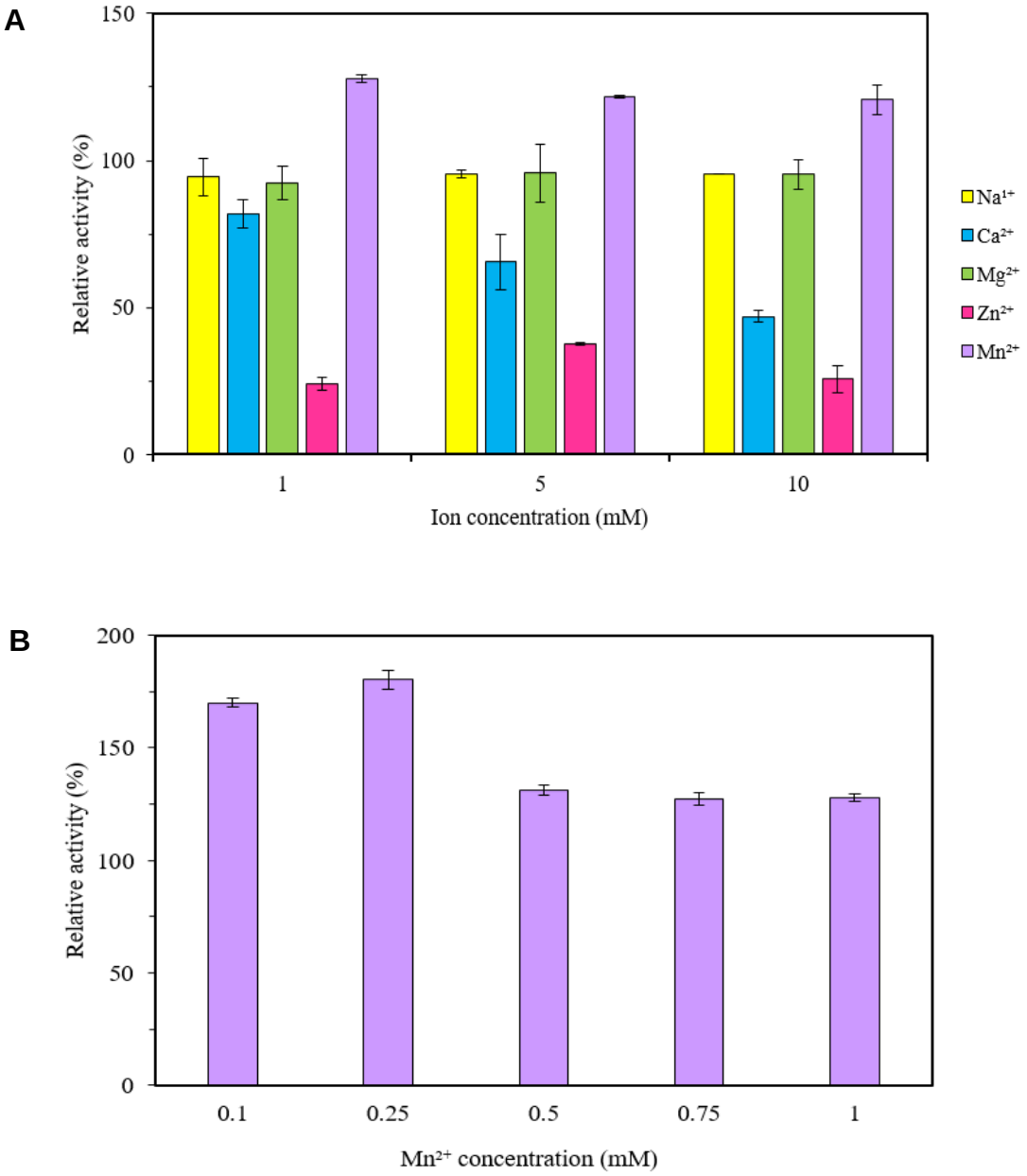

Figure 3.9. Influence of ions on the activity of purified BLI04 exo-PG (~ 0.25 U mL^{-1}). (A) Ions at different concentrations and (B) Mn^{2+} at different concentrations. The enzymes were pre-incubated with the ions at room temperature for 15 min. The exo-PG activity was determined using 0.5% (w/v) polyGalA at pH 6.5 and 50 °C. The relative activity was expressed as the percentage of activity compared with a control without ion. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.2 and 2.9.3.1 in Chapter 2. PolyGalA: polygalacturonic acid.

Pectin methylesterases: the influence of various ions (1 mM) on BLI09 PME activity (~ 9 U mL^{-1}) is set out in Figure 3.10. From Figure 3.10 A, we can see that Mn^{2+} did not affect the enzyme, whereas that Zn^{2+} inhibited until 50% of its maximum activity. As Ca^{2+} and Mg^{2+} produced a slight stimulation on the activity, three different concentrations of these ions were evaluated to determine if they were able to enhance the enzyme in a higher degree. However, the findings presented in Figure 3.10 B revealed that only 1 mM Ca^{2+} and Mg^{2+} produced a small increase on the activity. With regard to Ca^{2+}, demethylation of pectin by PMEs could create blocks of non-methylated GalA residues, enhancing its gelling properties in presence of high concentrations of this ion (Remoroza *et al.*, 2015). Figure 3.11 provides the results obtained from the effect of ions (1 mM) on SAM10 PME activity (~ 4.5 U mL^{-1}). As shown in Figure 3.11 A, the activity decreased until around 80% by Ca^{2+}, Mg^{2+} and Mn^{2+}. In contrast to BLI09 activity which was notably inhibited by Zn^{2+}, SAM10 activity was slightly enhanced by this ion. Figure 3.11 B presents the effect of three Zn^{2+} concentrations on SAM10 activity and although no important effect on the activity was found, the activity was slightly higher with 0.5 and 1 mM than with 1.5 mM.

Figure 3.12 presents the influence of various Mn^{2+} concentrations on BLI09 and SAM10 PMEs activity. As shown in previous results, this ion produced a significant enhancement on TMA01 and BLI04 exo-PGs activity. Hence, for subsequent reactions involving multienzymatic preparations of PMEs and exo-PGs, the assessment of the effect of Mn^{2+} on PMEs is fundamental. From Figures 3.12 A and 3.12 B, we can see that the different Mn^{2+} concentrations did not affect the activity of the PMEs. Moreover, at 0.25 mM Mn^{2+}, which was the optimum concentration for exo-PGs, the activity of both PMEs kept around 100%. These results suggest that this ion can be included in reactions where the synergistic activity between PMEs and exo-PGs be assessed. Similar to exo-PGs, a small number of studies have reported the influence of ions on PMEs activity and variable results have been described (Shevchik et al., 1996; Rajulapati and Goyal, 2017).

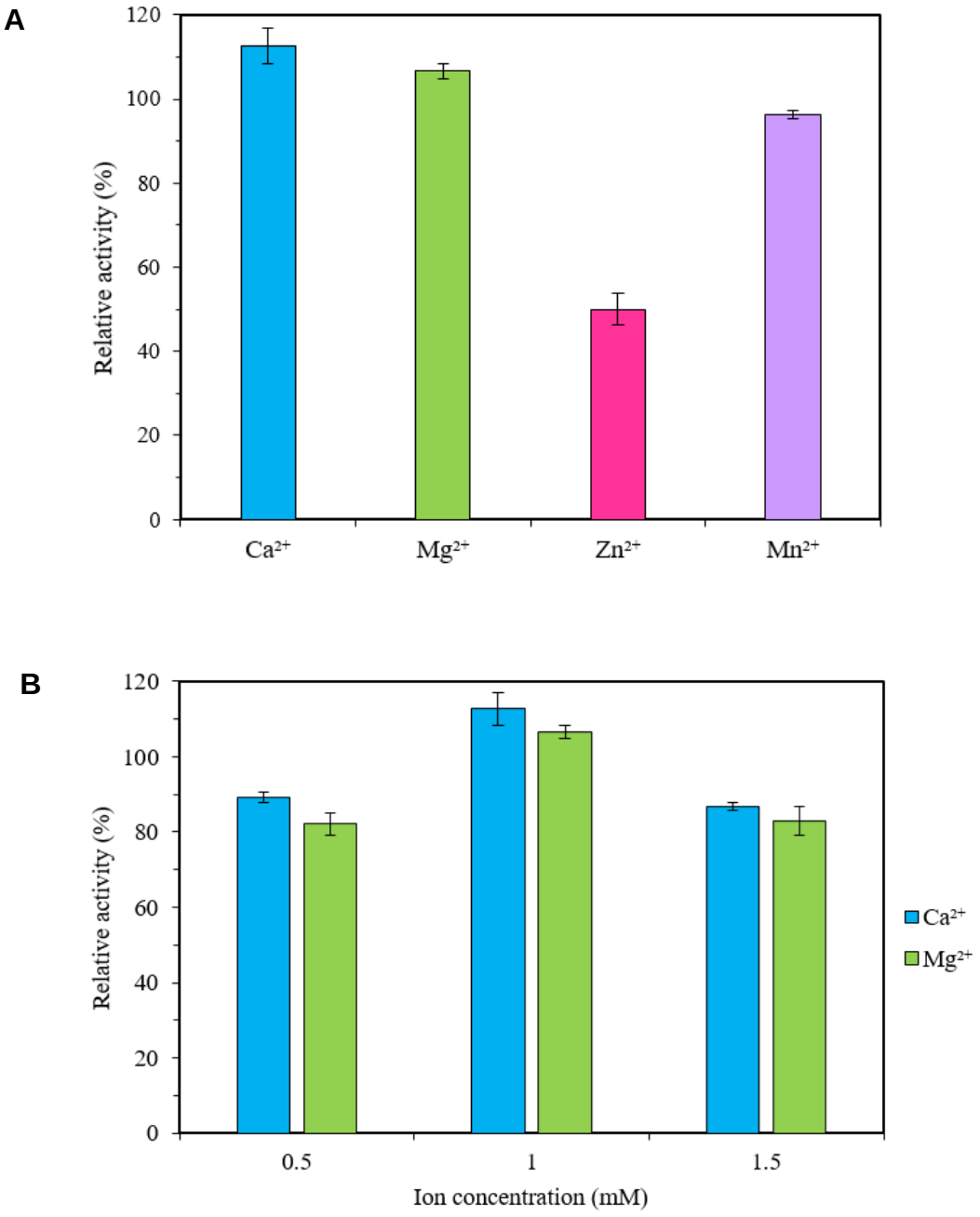

Figure 3.10. Influence of ions on the activity of purified BLI09 PME (~ 9 U mL^{-1}). (A) Ions at 1 mM and (B) Ca^{2+} and Mg^{2+} at different concentrations. The enzymes were pre-incubated with the ions at room temperature for 15 min. The PME activity was measured based on the methanol quantification method using 0.5% (w/v) apple pectin at pH 7 and 50 °C. The relative activity was expressed as the percentage of activity compared with a control without ion. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.2 and 2.9.3.2 in Chapter 2.

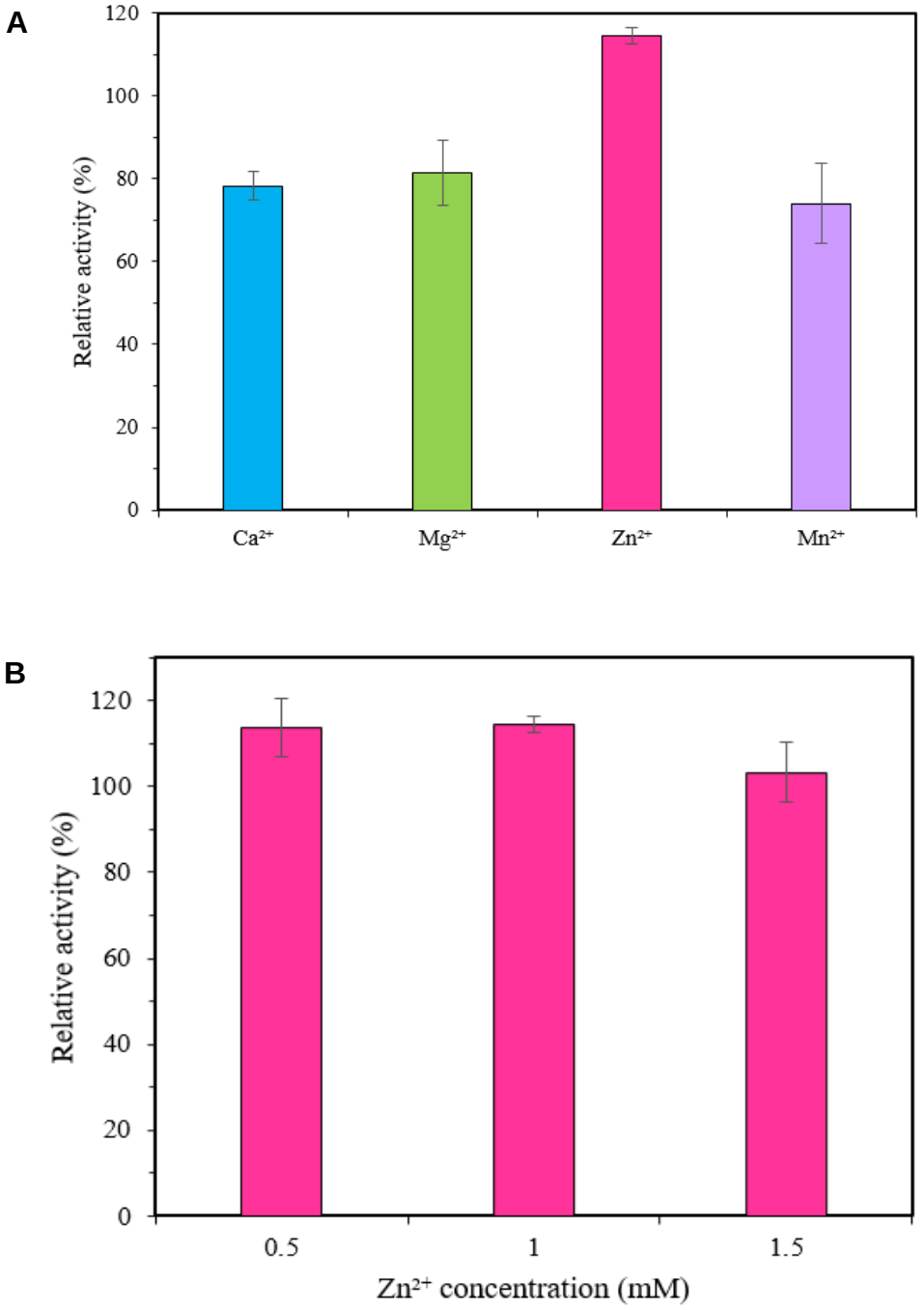

Figure 3.11 . Influence of ions on the activity of purified SAM10 PME (~ 4.5 U mL^{-1}). (A) Ions at 1 mM and (B) Zn^{2+} at different concentrations. The enzymes were pre-incubated with the ions at room temperature for 15 min. The PME activity was measured based on the methanol quantification method using 0.5% (w/v) apple pectin at pH 7 and 50 °C. The relative activity was expressed as the percentage of activity compared with a control without ion. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.2 and 2.9.3.2 in Chapter 2.

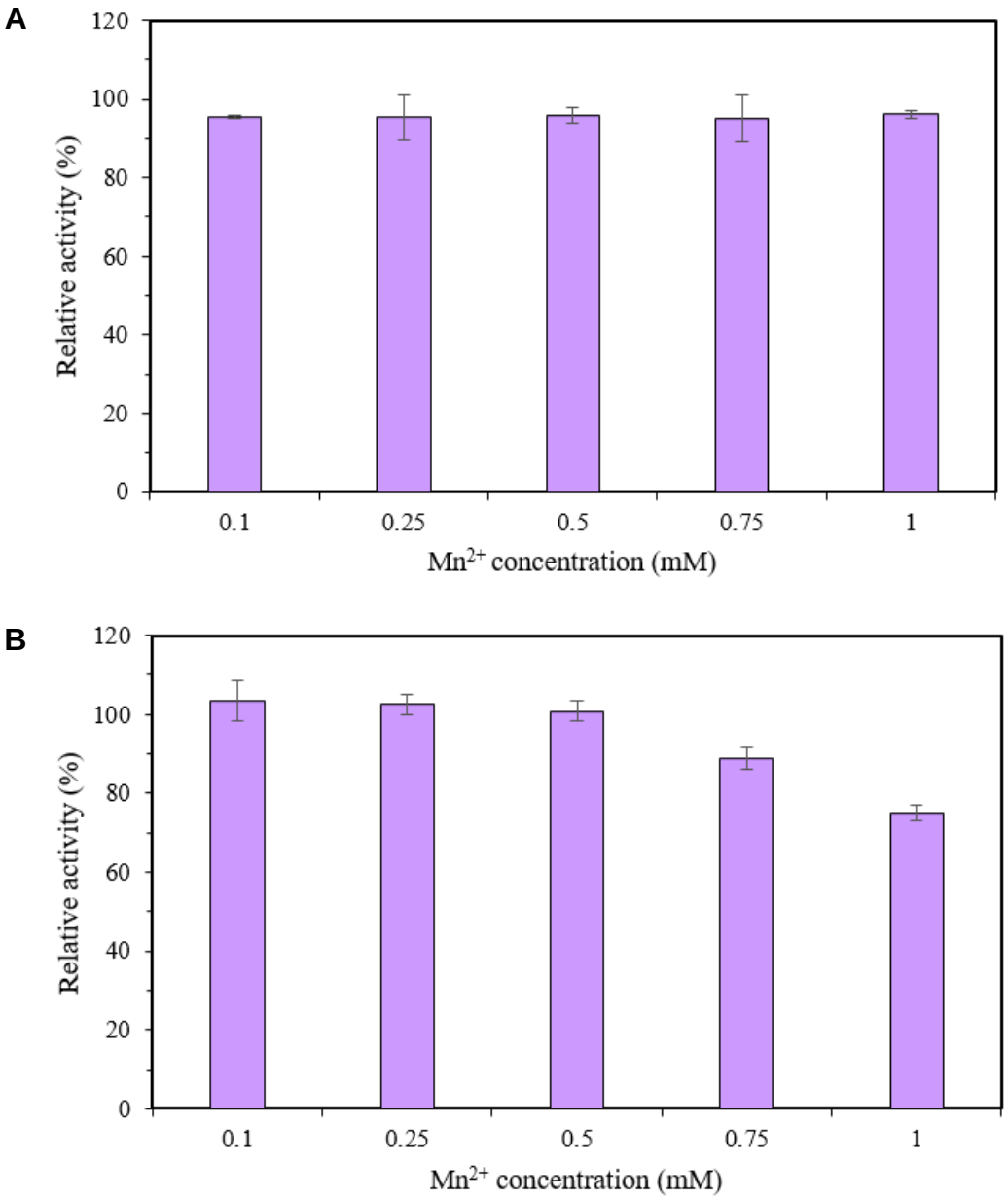

Figure 3.12. Influence of Mn^{2+} at different concentrations on the activity of purified (A) BLI09 (~ 9 U mL^{-1}) and (B) SAM10 (~ 4.5 U mL^{-1}) PMEs. The enzymes were pre-incubated with the ions at room temperature for 15 min. The PME activity was measured based on the methanol quantification method using 0.5% (w/v) apple pectin at pH 7 and 50 °C. The relative activity was expressed as the percentage of activity compared with a control without ion. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.2 and 2.9.3.2 in Chapter 2.

3.3.5.3 Influence of temperature on the activity

Exo-polygalacturonases: most of the exo-PGs from different microbial sources have exhibited optimum temperature between 30 and 50 °C (Jayani, Saxena and Gupta, 2005; Gonzalez and Rosso, 2011). Therefore, they are not able to be used in bioprocesses in which high temperatures are required. As mentioned in Section 1.2, only a few pectinases with considerable activity and stability at high temperatures have been investigated. In this study, the influence of the temperature on the exo-PGs activity was assessed in presence and in absence of 0.25 mM Mn^{2+}, as this ion had a notable effect on their activity.

TMA01 exhibited maximum activity at 90-100 °C, reaching a plateau at these temperature values as shown in Figure 3.13 A. In this figure, it was also noted that the activity profile at different temperatures was the same in presence and in absence of 0.25 mM Mn^{2+}. Although Mn^{2+} did not produce changes on TMA01 activity profile, it improved the product release (GalA). As the temperature increased, a higher difference in the percentage of released GalA was observed. Thus, at 90-100 °C, 65% of GalA was released in presence of Mn^{2+}, whereas in absence of the ion only 45% (Figure 3.13 B).

Figure 3.14 A presents the effect of temperature on BLI04. As detailed in this figure, BLI04 showed an optimum temperature of 70 °C, keeping 67 and 45% of its maximum activity at 80 and 90 °C, respectively. Similar to TMA01, Mn^{2+} did not affect the activity profile of BLI04 at different temperatures (Figure 3.14 A), but the ion increased the percentage of product release (GalA) (Figure 3.14 B). At optimum temperature, 38% of GalA was released in presence of the Mn^{2+}, whilst in absence of the ion only 22%.

Pectin methylesterases: most of the PMEs from different microbial sources have exhibited optimum activity between 40 and 50 °C, including those which have been commercialised in the market (Jayani, Saxena and Gupta, 2005; Gonzalez and Rosso, 2011). Figure 3.15 provides the results of the effect of temperature on BLI09 and SAM10. PME activity was measured by using the method based on the use of pH indicator as detailed in Section 2.8.3.2. As can

be seen from Figure 3.15 A, BLI09 presented optimum activity at 60 °C, but this value fell sharply to around 30% at 70 °C. Figure 3.15 B details the influence of temperature SAM10 activity. This PME exhibited maximum activity at 50 °C, keeping around 60% of it at 60 °C. The scant number of studies about pectinases from *Streptomyces* has indicated that these enzymes show their maximum around 50 °C (Kuhad, Kapoor and Rustagi, 2004; Ramírez-Tapias *et al.*, 2015).

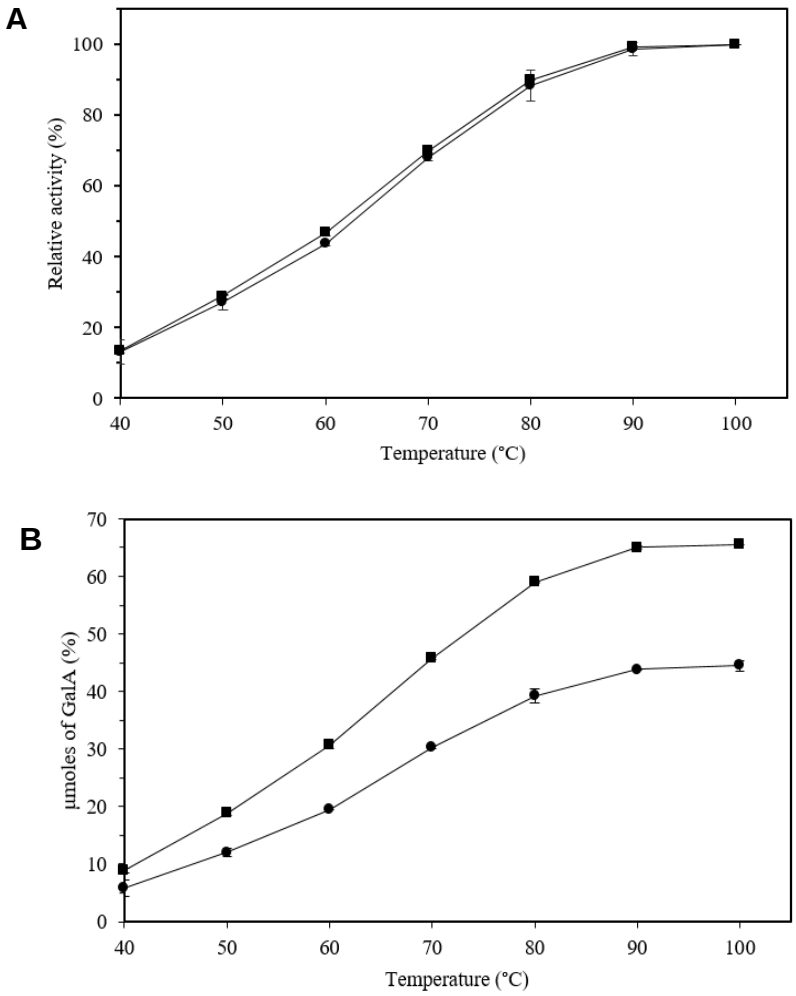

Figure 3.13. Influence of temperature and the presence of Mn^{2+} at different temperatures on the activity of purified TMA01 exo-PG (~ 0.25 U mL^{-1}). (A) Effect of the temperature (■) in presence of 0.25 mM Mn^{2+} and (●) in absence of Mn^{2+}. (B) Effect of Mn^{2+} on product release at different temperatures, (■) in presence of 0.25 mM Mn^{2+} and (●) in absence of Mn^{2+}. The enzyme was pre-incubated with the ion for 15 min at room temperature and the reactions were performed using 0.5% (w/v) polyGalA at pH 6.5. The relative activity was expressed as a percentage of the maximum activity, while the µmoles of GalA were expressed as a percentage of the total amount of product that can be released using 0.5% (w/v) polyGalA as substrate (14.3 µmoles of GalA). Error bars represent one standard deviation from the mean (n = 2). The assay was performed as described in Sections 2.7.3 and 2.9.3.1 in Chapter 2. PolyGalA: polygalacturonic acid.

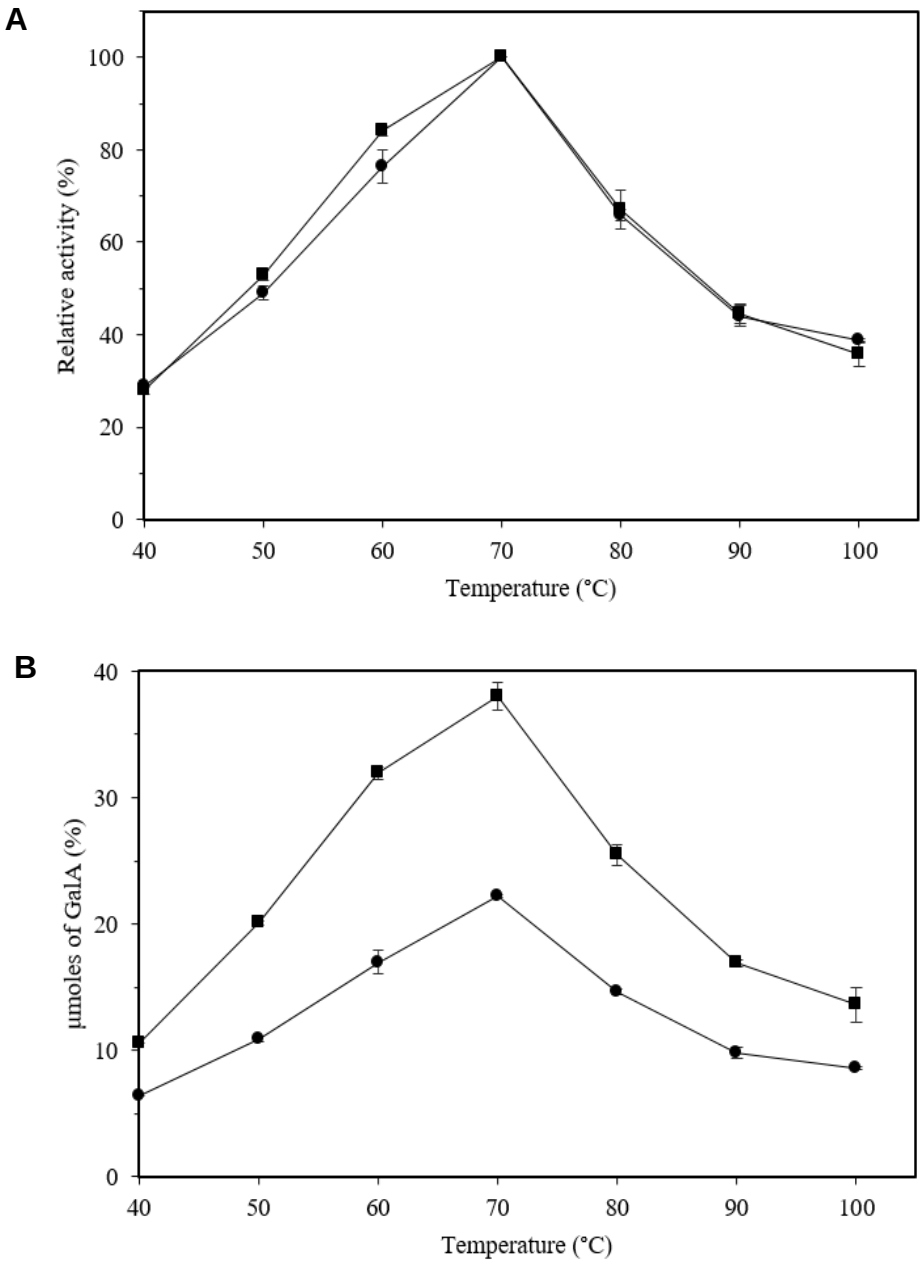

Figure 3.14. Influence of temperature and the presence of Mn^{2+} at different temperatures on the activity of purified BLI04 exo-PG (~ 0.25 U mL^{-1}). (A) Effect of temperature (■) in presence of 0.25 mM Mn^{2+} and (●) in absence of Mn^{2+}. (B) Effect of Mn^{2+} on product release at different temperatures, (■) in presence of 0.25 mM Mn^{2+} and (●) in absence of Mn^{2+}. The enzyme was pre-incubated with the ion for 15 min at room temperature and the reactions were performed using 0.5% (w/v) polyGalA at pH 6.5. The relative activity was expressed as a percentage of the maximum activity, while the µmoles of GalA were expressed as a percentage of the total amount of product that can be released using 0.5% (w/v) polyGalA as substrate (14.3 µmoles of GalA). Error bars represent one standard deviation from the mean (n = 2). The assay was performed as described in Sections 2.7.3 and 2.8.3.1 in Chapter 2. PolyGalA: polygalacturonic acid.

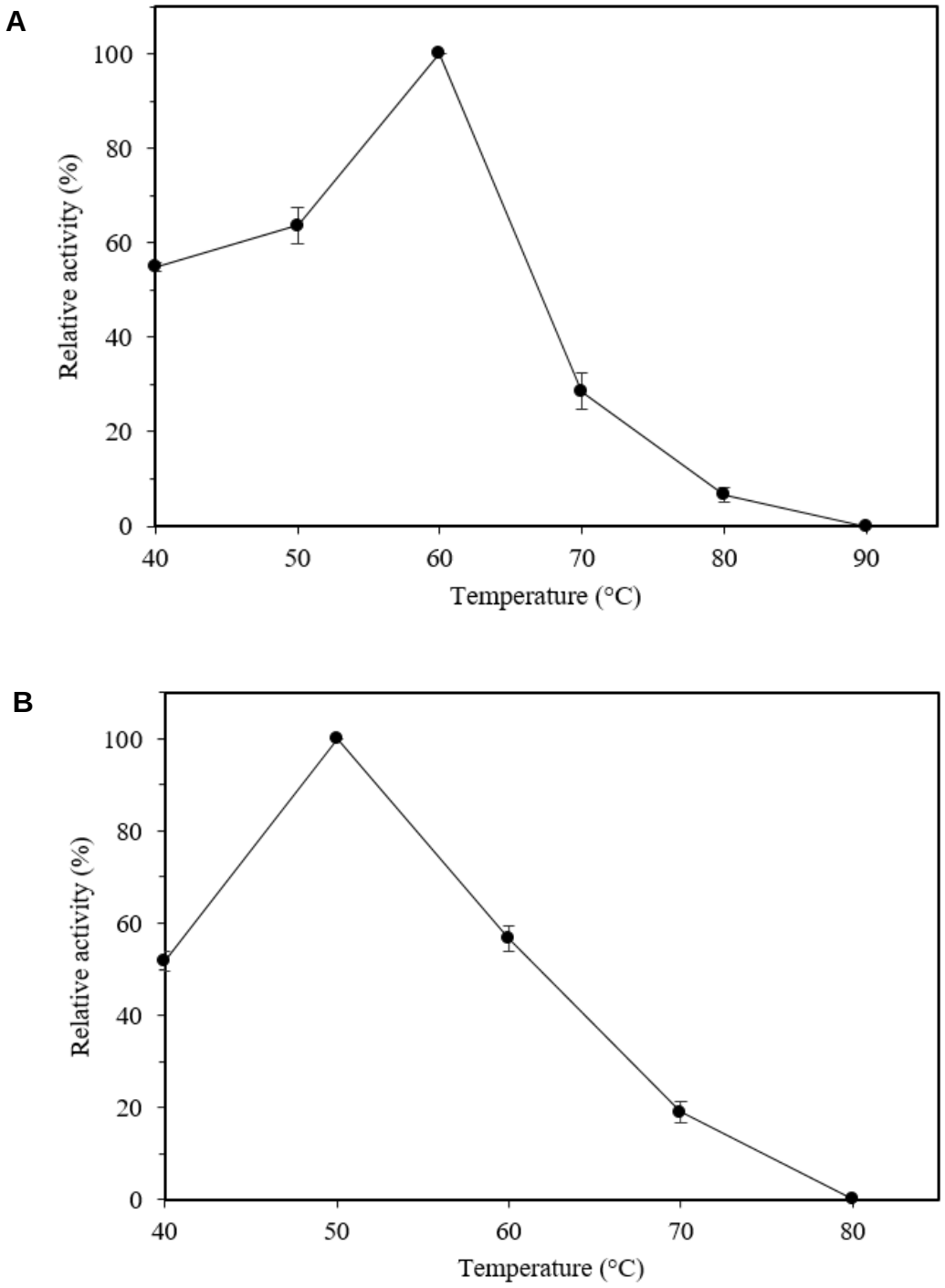

Figure 3.15. Influence of temperature on the activity of purified (A) BLI09 (~ 0.30 U mL^{-1}) and (B) SAM10 (~ 0.15 U mL^{-1}) PMEs. The reactions were performed using 0.5% (w/v) apple pectin at pH 7.6 and the reactions were incubated for 15 min. The PME activity was determined by the method using a pH indicator. The relative activity was expressed as a percentage of the maximum activity. Error bars represent one standard deviation from the mean (n = 2). The assay was performed as described in Sections 2.7.3 and 2.9.3.2 in Chapter 2.

3.3.5.4 Thermal stability of pectinases

The majority of pectinases commercialised in the market including PGs and PMEs from different sources have exhibited stability up to 50 °C (Favela-Torres, Volke-Sepúlveda and Viniegra-González, 2006).

Exo-polygalacturonases: Thermal stability assays of TMA01 and BLI04 were performed in presence and in absence of 0.25 mM Mn^{2+} to test if this ion had influence on the stability (Figure 3.16). What is striking about the data in Figure 3.16 A is the great stability of TMA01 at high temperatures. In absence of Mn^{2+}, the enzyme retained approximately 100% of activity after 24 h of incubation at 70 and 80 °C. Moreover, TMA01 maintained 72% of activity after 24 h of incubation at 90 °C. Even at 100 °C, after 4 h of incubation at this temperature, the enzyme kept 65% of activity. The presence of Mn^{2+} did not affect the stability of the enzyme. Figure 3.16 B details thermal stability data of BLI04. The enzyme exhibited great stability at 20 °C and 30 °C retaining approximately 80% of its activity after 24 h of incubation. At 40 °C, the activity decreased gradually until around 65% after 10 h of incubation, whereas at 50 °C the remaining activity was 68% after 1 h of incubation and it fell to around 40% after 2 h. The ion Mn^{2+} did not have a major influence on the stability of this enzyme.

As described in Section 1.3.2.1 in Chapter 1, pectinases may play a fundamental role to enhance the sucrose release in sugar industry. During diffusion process, standard conditions are pH from 5.8 to 6.5 and temperature from 70 to 73 °C. Thus, to determine the potential application of TMA01 in this bioprocess, the thermal stability assay at 70 °C was also performed testing the enzymatic activity at 70 °C. Figure 3.17 reveals that TMA01 exhibited great thermal stability and activity at 70 °C and pH 6.5. The enzyme retained 100% of activity after 24 of incubation at these conditions. The presence of Mn^{2+} did not considerably affect the stability. Our findings showed that this enzyme may have a great potential to be used in sugar industry.

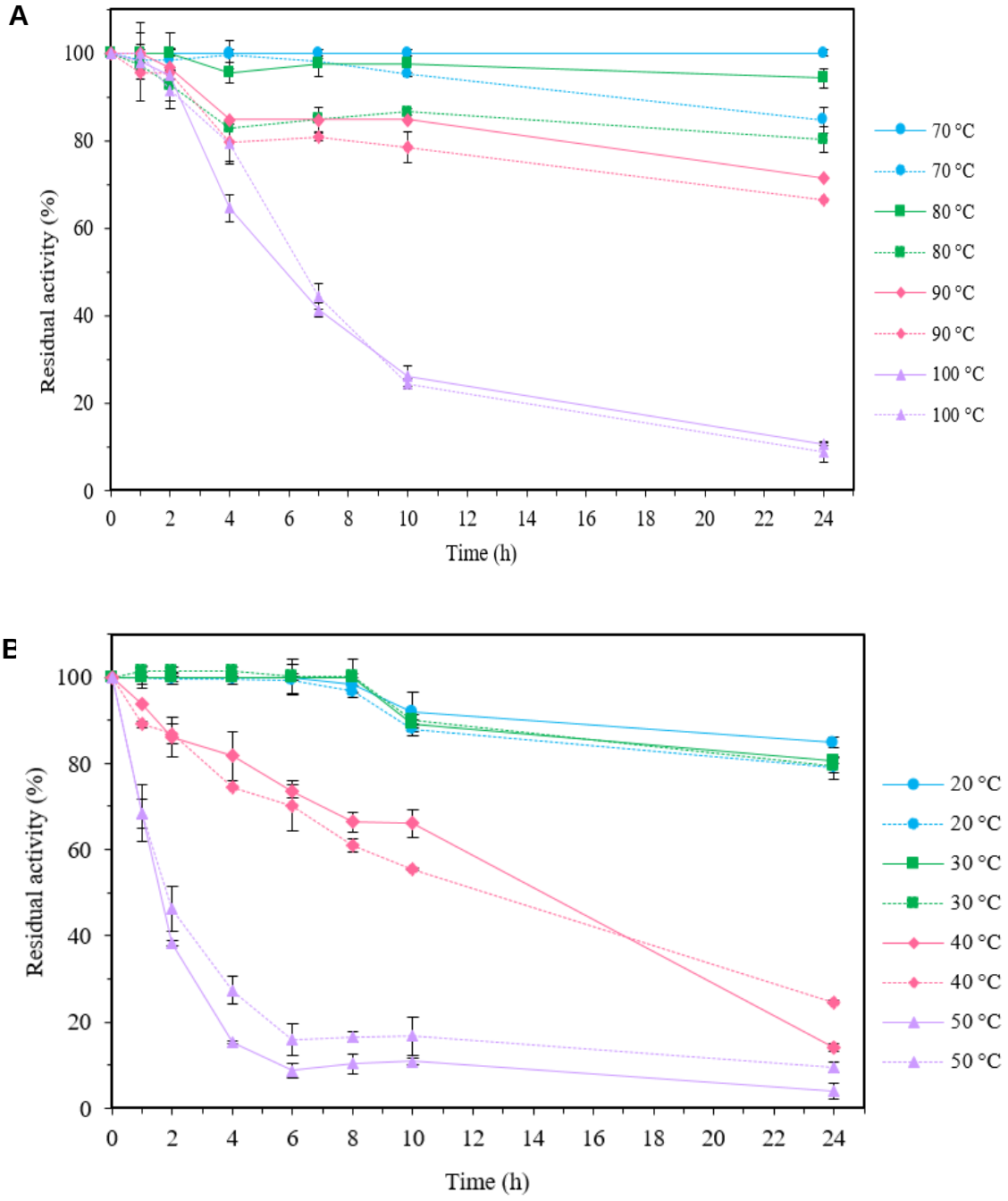

Figure 3.16. Thermal stability of purified (A) TMA01 and (B) BLI04 exo-PGs (~4 U mL^{-1}), (···) in presence of 0.25 mM Mn^{2+} and (–) in absence of Mn^{2+}. The enzymes were pre-incubated with the ion for 15 min at room temperature. After the indicated time of incubation, the exo-PG activity was determined using 0.5% (w/v) polyGalA at pH 6.5 and 50 °C. The residual activity was expressed as the percentage of the starting activity. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.4 and 2.9.3.1 in Chapter 2. PolyGalA: polygalacturonic acid.

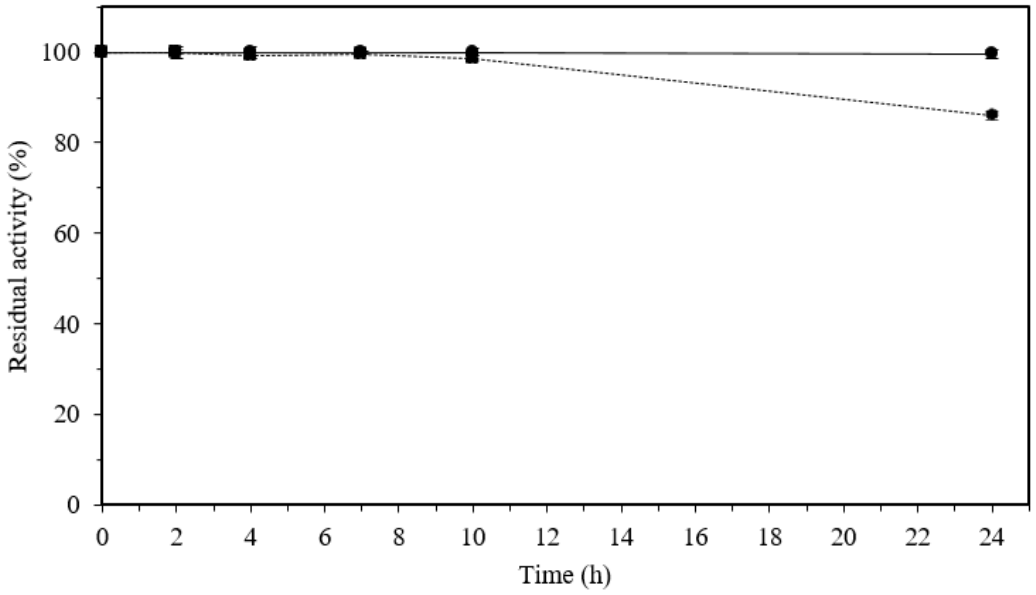

Figure 3.17. Thermal stability and activity of TMA01 exo-PG (~4 U mL^{-1}) at 70 °C, (…) in presence of 0.25 mM Mn^{2+} and (—) in absence of Mn^{2+}. The enzyme was pre-incubated with the ion for 15 min at room temperature. After the indicated time of incubation, the exo-PG activity was determined using 0.5% (w/v) polyGalA at pH 6.5 and 70 °C. The residual activity was expressed as the percentage of the starting activity. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.4 and 2.9.3.1 in Chapter 2. PolyGalA: polygalacturonic acid.

Pectin methylesterases: similar to exo-PGs, thermal stability of BLI09 and SAM10 was tested in presence and in absence of 0.25 mM Mn^{2+}, but the presence of this ion did not have major effect on the thermal stability of both PMEs (Figure 3.18). Figure 3.18 A shows that BLI09 was very stable up to 50 °C, maintaining around 80% of activity up to after 24 h of incubation. However, its thermal stability dropped at 60 and 70 °C retaining around 50 and 20% of activity, respectively after 30 min of incubation.

From Figure 3.18 B we can see that SAM10 remained more than 90% of activity at 20 and 30 °C after 24 h of incubation. At 40 °C, this PME kept 80% of activity after 4 h of incubation and around 50% after 8 h. The enzyme did not exhibit thermal stability at 50 °C.

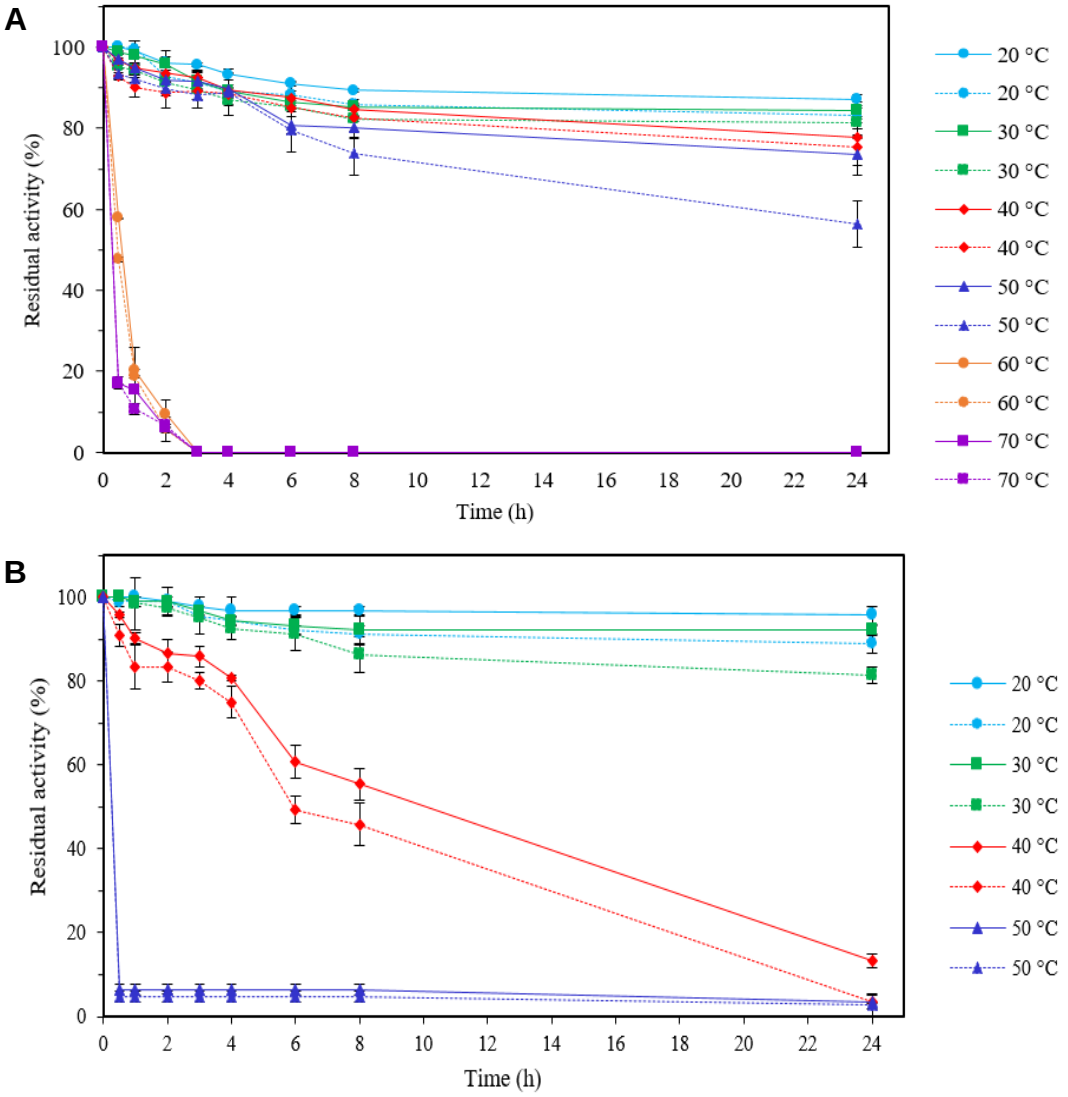

Figure 3.18. Thermal stability of purified (A) BLI09 (~90 U mL^{-1}) and (B) SAM10 (~22.5 U mL^{-1}) PMEs, (···) in presence of 0.25 mM Mn^{2+} and (–) in absence of Mn^{2+}. After the indicated time of incubation, the PME activity was determined using 0.5% (w/v) apple pectin at pH 7 and 50 °C based on methanol quantification method. The residual activity was expressed as the percentage of the starting activity. Error bars represent one standard deviation from the mean (n = 2). The assay was performed as described in Sections 2.7.4 and 2.9.3.2 in Chapter 2.

3.3.6 Kinetic characterisation of thermophilic exo-PGs and PMEs

Typically, kinetic data from an enzyme fit to the Michaelis-Menten equation. Nevertheless, several enzymes do not follow this kinetics model and inhibition is observed at high substrate concentrations. This substrate inhibition kinetics occurs in around 20% of the enzymes (Reed, Lieb and Nijhout, 2010). It might happen because the substrate binds to an alternate site of the enzyme and the resulting enzyme substrate complex is inactive. Also can occur when the enzyme has multiple active catalytic sites and the presence or absence of substrate at those sites alters the catalytic activity (Gummadi and Panda, 2003).

Exo-polygalacturonases: kinetic parameters of TMA01 and BLI04 were calculated, and a substrate inhibition kinetics was observed in both enzymes. However, the commonly accepted substrate inhibition model (Equation 2.1, Section 2.7.5 in Chapter 2) fitted poorly to the data. Hence, other previously reported substrate inhibition models (not shown) were tried. Comparison between the models was done in GraphPad Prism 8 using the extra-sum-of-squares F test (Gummadi and Panda, 2003; Bapiro *et al.*, 2018). Thus, the kinetic data were successfully fitted to the Equation 2.2 (Section 2.7.5 in Chapter 2) owing to this model describes better the almost complete inhibition that was observed in both exo-PGs at high substrate concentrations (Figure 3.19) (Bapiro *et al.*, 2018). The *kcat* values were calculated using the Equation 2.3 (Section 2.7.5 in Chapter 2). The protein concentration and molecular weight used for TMA01 were 0.506 µg mL^{-1} and 50.48 kDa, respectively; while for BLI04 3.96 µg mL^{-1} and 48.14 kDa, respectively. The regression coefficients (R^2) of Equations 2.2 and 2.3 were 0.99.

The kinetic parameters values are summarised in Table 3.5. The *Vmax* of TMA01 was 1027.67 µmol min^{-1} mg^{-1}; while the *Km* and *kcat* were 86.75 µM and 858.85 s^{-1}, respectively. This enzyme has been reported as a tetramer, thus up to seven molecules of substrate (n = 7.5) were predicted to bind it during the inhibition process. Similar kinetic parameters have been reported for this enzyme using polyGalA as substrate, but fitting the data to a Michaelis-Menten kinetics (Kluskens *et al.*, 2005).

Likewise, the *Vmax* of BLI04 was 141.41 µmol min^{-1} mg^{-1}; while the *Km* and *kcat* were 105.90 µM and 112.75 s^{-1}, respectively. This enzyme has been reported as a monomeric enzyme, however up to six molecules of substrate (n = 6.06) were predicted to bind to the enzyme during the inhibition process. A Michaelis-Menten kinetics and different kinetic parameters have been reported for this enzyme (Evangelista *et al.*, 2018).

Comparison of the kinetic parameters between TMA01 and BLI04 is also observed in Table 3.5. The *Vmax* of TMA01 was notably higher (7.3-fold) as well as this enzyme presented a smaller *Km,* which means that it exhibited more affinity towards polyGalA. The *Ki* value (concentration of substrate required to decrease the maximal rate of the reaction to half of the uninhibited value) was smaller in TMA01, showing that the substrate inhibitory effect was slightly stronger in this enzyme. The *kcat* value was considerably higher in TMA01 (7.6-fold).

Kinetic information of previously reported thermophilic exo-PGs (Table 3.5) shows that most of them have exhibited a Michaelis-Menten kinetics (Kluskens *et al.*, 2005; Chen *et al.*, 2014; Wagschal *et al.*, 2016; Evangelista *et al.*, 2018). Additionally, we can see that exo-PGs from *Thermotoga maritima* presented the highest *Vmax* values, while exo-PGs from *Caldicellulosiruptor bescii*, *Thermotoga thermophilus* and *Rhodothermus marinus* exhibited the highest affinity to polyGalA. The *kcat* values were higher in exo-PGs from *Thermotoga maritima* including TMA01 as well as in BLI04, these last two enzymes from this study.

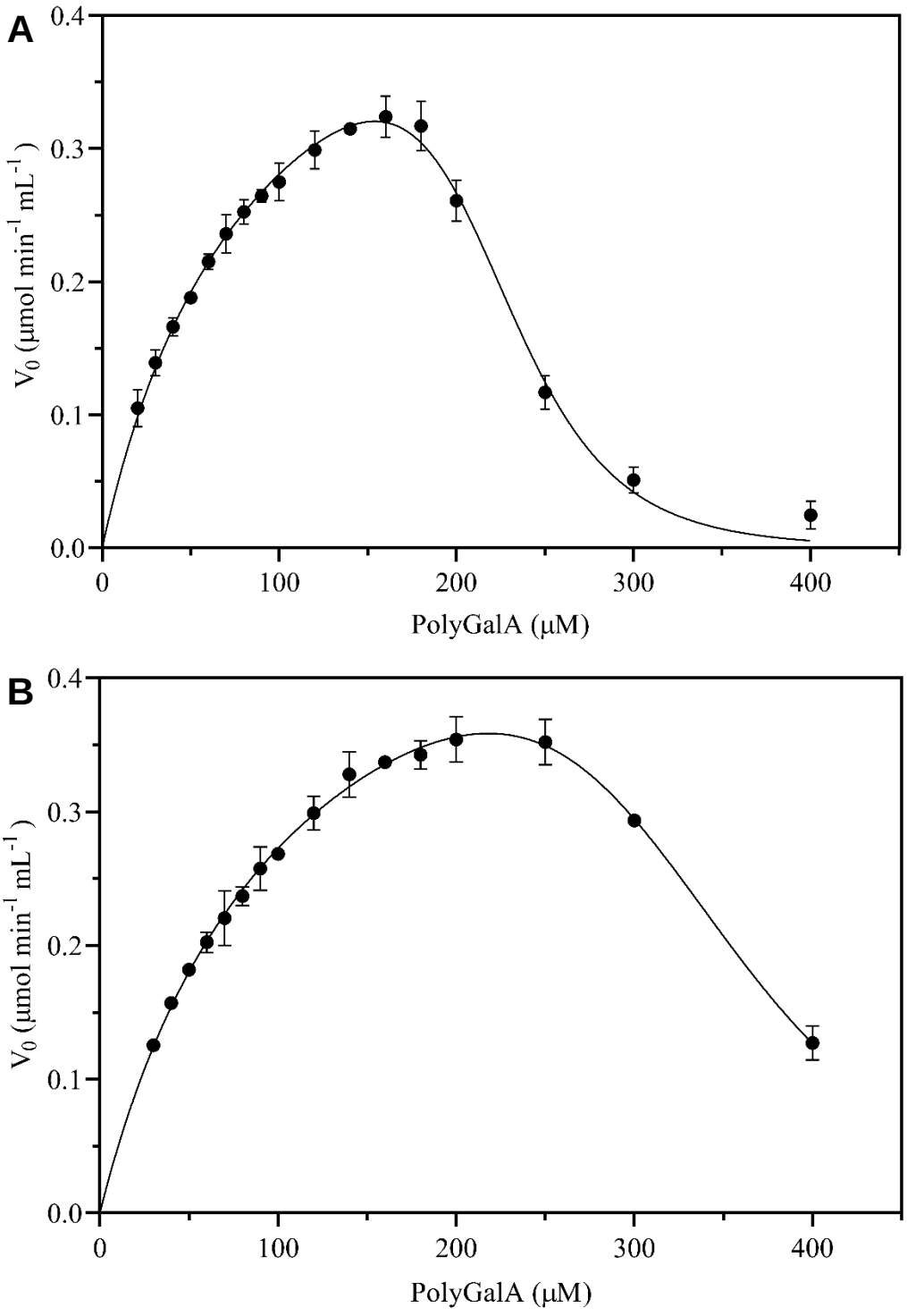

Figure 3.19. Substrate inhibition kinetics of (A) TMA01 and (B) BLI04 exo-PGs using polyGalA as substrate. The enzymes velocity was measured at optimum conditions: TMA01, pH 8 and 90 °C and BLI04, pH 8 and 70 °C; and using 0.25 mM Mn^{2+} for both enzymes. Kinetic data were analysed by non-linear regression and successfully fitted to Equation 2.2 (Section 2.7.5 in Chapter 2) using GraphPad Prism 8. The parameters are detailed in Table 3.5. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Section 2.7.5 in Chapter 2. PolyGalA: polygalacturonic acid.

Table 3.5. Kinetic parameters of TMA01 and BLI04 exo-PGs and comparative kinetic information with other thermophilic exo-PGs using polyGalA as substrate. Kinetic parameters of TMA01 and BLI04 were calculated using Equations 2.2 and 2.3 (Section 2.7.5 in Chapter 2).

Bacteria	**Enzyme**	***Vmax*** **(μmol min^{-1} mg^{-1})**	***Km*** **(μM)**	***kcat*** **(s^{-1})**	***kcat/Km*** **(s^{-1} μM^{-1})**	***Ki*** **(μM)**	**n**	**Reference**
Thermotoga maritima	TMA01[a]*	1027.67 ± 57.31	86.75 ± 9.14	858.85 ± 6.77	9.90 ± 0.74	217.20 ± 3.91	7.50 ± 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	Kluskens *et al.* (2005)
Bacillus licheniformis	BlExoPG[d]▪	3.20	86.67	2.58	0.03	NA	NA	Evangelista *et al.* (2018)
Caldicellulosiruptor bescii	PelA[e]▪	384.6	8	ND	ND	NA	NA	Chen *et al.* (2014)
Thermotoga thermophilus	TtGH28[f]▪	ND	8.77 ± 0.64	10.6 ± 0.3	1.21 ± 0.09	NA	NA	Wagschal *et al.* (2016)
Rhodothermus marinus	RmGH28[g]*	ND	0.67 ± 0.09	6.02 ± 0.17	8.94 ± 1.03	71 ± 6	NA	Wagschal *et al.* (2017)

Reactions were performed at [a]90 °C, [b]70°C, [c]80 °C, [d]60 °C, [e]72 °C, [f]40 °C and [g]25 °C. *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).

Pectin methylesterases: a substrate inhibition kinetics was observed in BLI09 and SAM10. Similar to exo-PGs, the commonly used substrate inhibition model (Equation 2.1, Section 2.7.5 in Chapter 2) fitted poorly to the data, and other previously reported substrate inhibition models (not shown) were tried (Gummadi and Panda, 2003; Bapiro *et al.*, 2018). The kinetic data were well fitted to the Equation 2.2 (Section 2.7.5 in Chapter 2) as shown in Figure 3.20. The *kcat* values were calculated using Equation 2.3 (Section 2.7.5 in Chapter 2). The protein concentration and molecular weight used for BLI09 were 6.19 µg mL^{-1} and 35.11 kDa, respectively; while for SAM10 9.06 µg mL^{-1} and 40.68 kDa; respectively. The regression coefficients (R^2) of Equations 2.2 and 2.3 were 0.99.

The kinetic parameters values are detailed in Table 3.6. The *Vmax* of BLI09 was 2785.14 µmol min^{-1} mg^{-1}; while the *Km* and *kcat* values were 44.19 µM and 1562.10 s^{-1}, respectively. It has been predicted that up to two molecules of substrate (n = 1.8) bind to the enzyme during the inhibition process. Similarly, the *Vmax* of SAM10 was 801.32 µmol min^{-1} mg^{-1}; while the *Km* and *kcat* values were 32.85 µM and 523.22 s^{-1}, respectively. It has been predicted that up to three molecules of substrate (n = 2.63) bind to the enzyme during the inhibition process.

Comparison of kinetic parameters between BLI09 and SAM10 (Table 3.6) shows that the *Vmax* of BLI09 was higher (3.5-fold), but the *Km* value was slightly smaller in SAM10. The *Ki* value was smaller in BLI09 showing that the substrate inhibitory effect was slightly stronger in this enzyme. The *kcat* value was higher in BLI09 (3-fold).

Kinetic information about thermophilic PMEs is scarce. Rajulapati and Goyal (2017) determined the kinetic parameters of a thermophilic PME from *Clostridium thermocellum* and found *Vmax* and *kcat* values notably smaller than those from this study, as well as presented a higher *Km* value. Chakiath *et al.* (2009) carried out mutagenesis of the pmeA PME from *Erwinia chrysanthemi* to improve its thermal stability. Kinetic parameters values of this mutant enzyme called JL25 were close to those of SAM10 from this study.

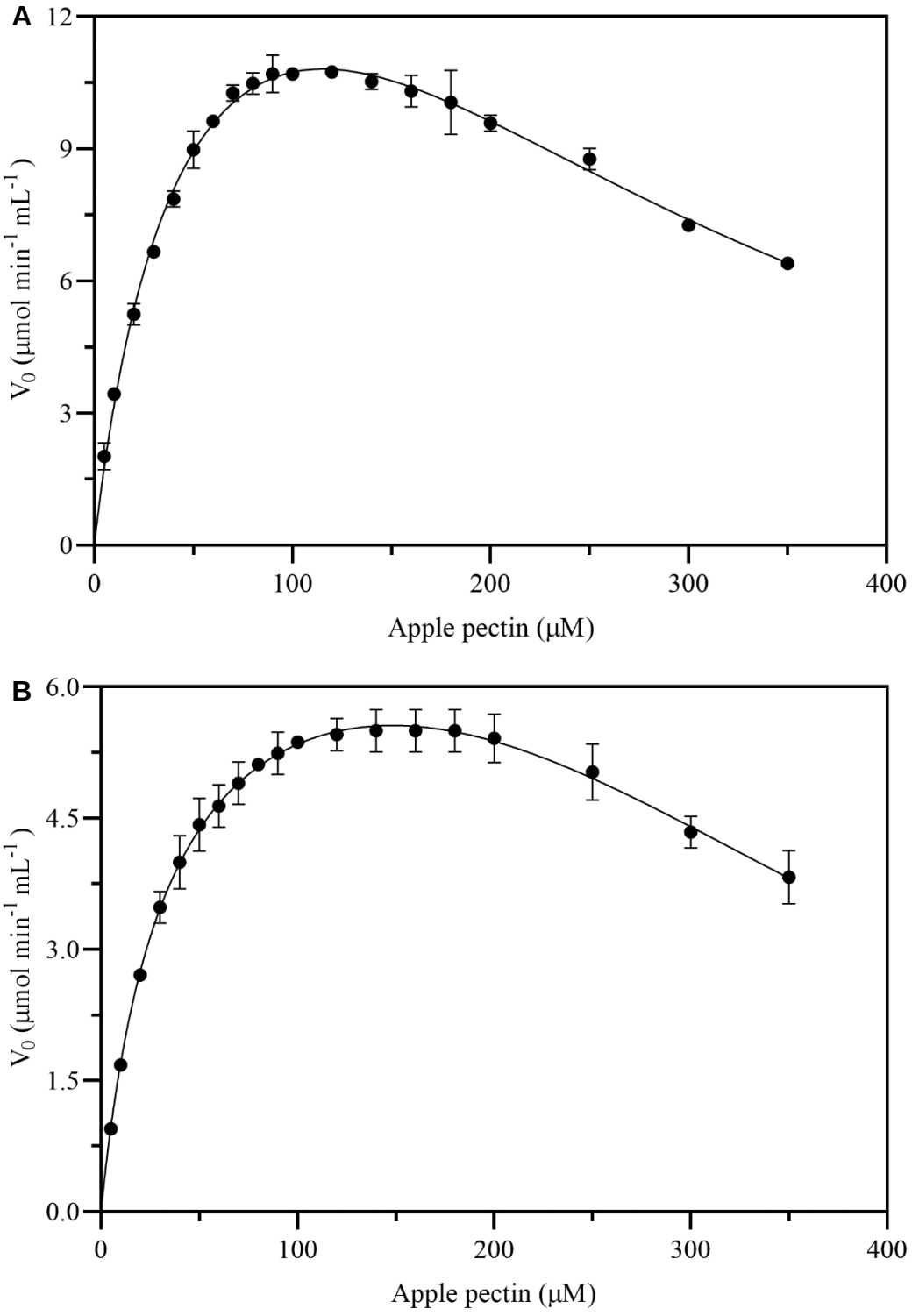

Figure 3.20. Substrate inhibition kinetics of (A) BLI09 and (B) SAM10 PMEs using apple pectin as a substrate. The enzymes velocity was measured at optimum conditions: BLI09, pH 7 and 60 °C and SAM10, pH 7 and 50 °C. Kinetic data were analysed by non-linear regression and successfully fitted to Equation 2.2 using GraphPad Prism 8 software. The parameters are detailed in Table 3.6. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Section 2.7.5 in Chapter 2.

Table 3.6. Kinetic parameters of BLI09 and SAM10 PMEs and comparative kinetic information with other thermophilic PMEs using apple pectin as substrate. Kinetic parameters of BLI09 and SAM10 were calculated with Equations 2.2 and 2.3 (Section 2.7.5 in Chapter 2).

Bacteria	Enzyme	*Vmax* (μmol min^{-1} mg^{-1})	*Km* (μM)	*kcat* (s^{-1})	*kcat/Km* (s^{-1} μM^{-1})	*Ki* (μM)	n	Reference
Bacillus licheniformis	BLI09[a*]	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[b*]	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[b▪]	6.22	58.46 ± 7.69	3.68	0.06	NA	NA	Rajulapati and Goyal (2017)
Erwinia chrysanthemi	Mutant JL25[a▪]	1062.16	11.23 ± 3.54	655 ± 104	58.32	NA	NA	Chakiath *et al.* (2009)

Reactions were performed at [a]60 °C and [b]50 °C. *Substrate inhibition kinetics, ▪Michaelis-Menten Kinetics, 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).

3.3.7 Substrate specificity studies for exo-PGs

The effect of the degree of pectin methylation on the activity of TMA01 and BLI04 was evaluated (Figure 3.21). Both exo-PGs were active on non-esterified polyGalA; however, they exhibited low activity towards both esterified citrus and apple pectin. These results showed that exo-PGs hydrolyse less than 10% of the pectin with a degree of esterification greater than 50%, demonstrated that prefer non-esterified substrates (Kluskens *et al.*, 2005; Chen *et al.*, 2014; Evangelista *et al.*, 2018).

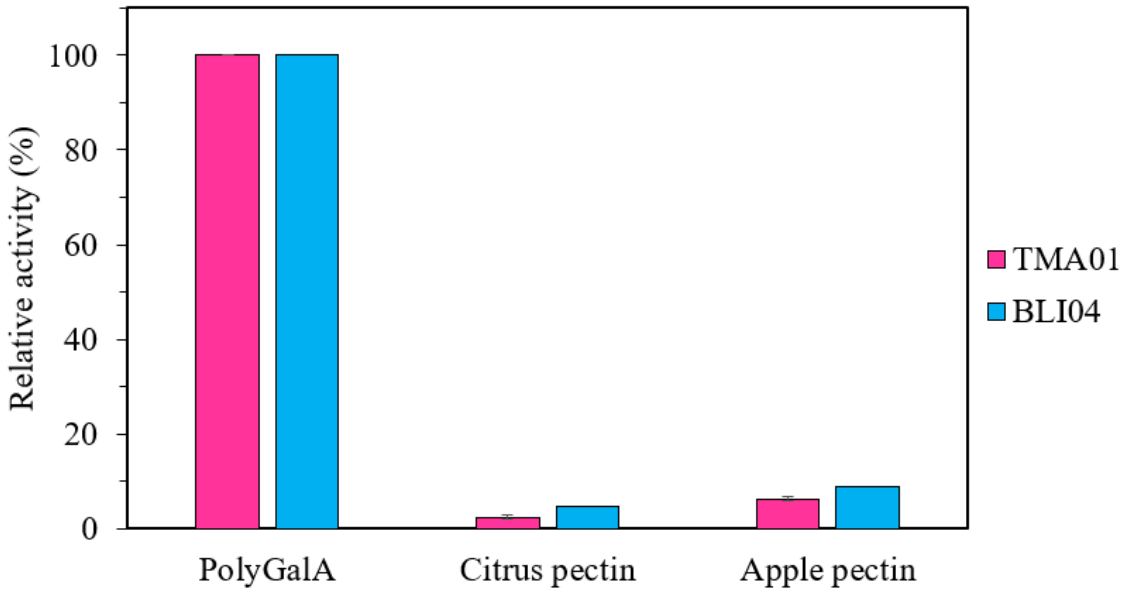

Figure 3.21. Effect of the degree of pectin esterification on the activity of purified TMA01 and BLI04 exo-PGs. The experiment was performed using ~0.25 U mL^{-1} of each enzyme which were pre-incubated for 15 min with 0.25 mM Mn^{2+}. The PG activity was determined at optimum conditions for each enzyme: TMA01, pH 8 and 90 °C; and BLI04, pH 8 and 70 °C; and 0.25 mM Mn^{2+} for both enzymes. The relative activity was expressed as the percentage of the activity with polyGalA which was considered as 100%. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.6 and 2.9.3.1 in Chapter 2. Exo-PGs: exo-polygalacturonases, PolyGalA: polygalacturonic acid.

3.3.8 Synergistic action between exo-PGs and PMEs individually expressed

As observed in Figure 3.21, TMA01 and BLI04 exo-PGs hydrolyse non-esterified substrates as polyGalA, exhibiting negligible activity towards the esterified ones such as apple and citrus pectin. The presence of ester groups in

pectin hampers the activity of exo-PGs on the HG backbone, consequently the release of GalA (Lara-Espinoza *et al.*, 2018; Dimopoulou *et al.*, 2019). Therefore, to achieve an efficient pectin hydrolysis and GalA release by exo-PGs, demethylation by PMEs is previously required.

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 either with TMA01 or BLI04 exo-PGs. Compatible operational reaction conditions (pH, temperature, and ions concentration) were established to enhance enzymes synergistic activity, even if optimum conditions were compromised. Hence, the reactions between BLI09 paired either with TMA01 or BLI04 were carried out at 50 °C and pH 7. Whilst reactions between SAM10 paired either with TMA01 or BLI04 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 to favour catalytic activity of SAM10 (unstable at 50 °C) and both exo-PGs. As described in Section 3.3.5.2, Mn^{2+} significantly enhanced the exo-PGs activity and did not alter PMEs action, thus this ion was added into the reactions. Synergistic activity using apple, citrus and sugar beet pectin was monitored by quantifying the two released products, methanol and GalA as described in Sections 2.8.1 and 2.8.5 in Chapter 2, respectively.

Initially, 4.5 and 9 U mL^{-1} of BLI09 and SAM10, respectively were tried for the synergistic assays (Figure 3.22). 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 to enhance GalA release during the first hours of reaction until a plateau was reached. Thereby, for the synergistic reactions between BLI09 with both exo-PGs, 9 U mL^{-1} of BLI09 were used and 0.5 and 2 U mL^{-1} of TMA01 and BLI04, respectively. Whereas for the synergistic reaction between SAM10 with the exo-PGs, 18 U mL^{-1} of SAM10 were used and 1 U mL^{-1} of TMA01 and BLI04 (Table 2.3, Section 2.8.1 in Chapter 2).

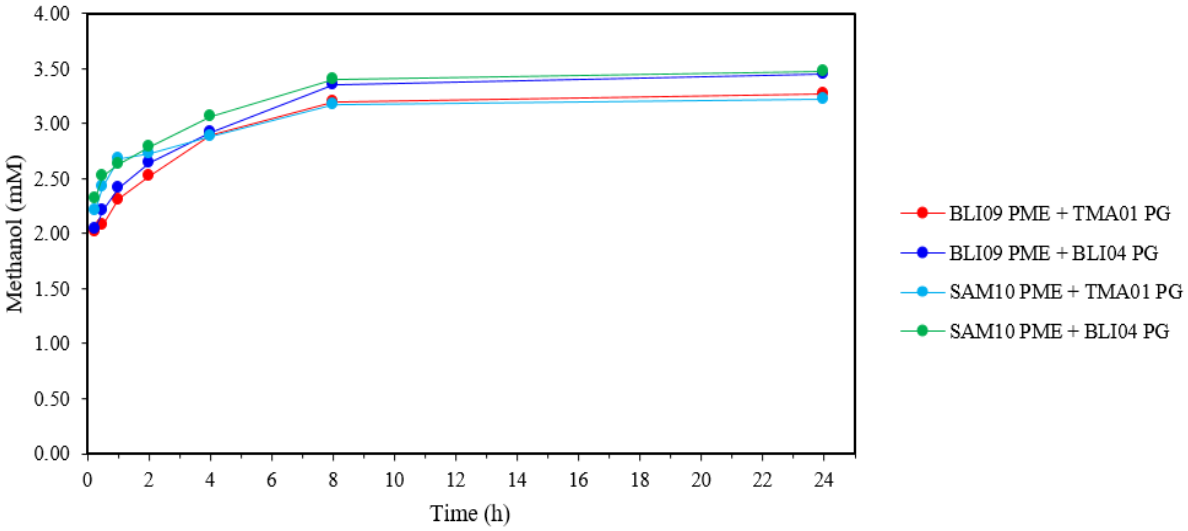

Figure 3.22. Methanol quantification because of the activity of BLI09 and SAM10 PMEs. PMEs units used in the reaction were 4.5 and 9 U mL^{-1} of BLI09 and SAM10, respectively. The reactions were carried out at pH 7 and 50 °C up to 24 h using 0.5% apple pectin. The assay was performed as described in Section 2.8.1 and methanol was quantified following the procedure described in Section 2.9.4, both in Chapter 2. Error bars represent one standard deviation from the mean (n = 2).

Synergistic reactions between BLI09 paired either with TMA01 or BLI04 using apple, citrus and sugar beet pectin are presented in Figures 3.23, 3.24 and 3.25, respectively. Methanol levels were similar for apple (Figure 3.23 A) and citrus pectin (Figure 3.24 A) reaching around 3.5 mM per around 0.04 mM of substrate. These findings are consistent with data presented in Table 2.2, Section 2.7.6 in Chapter 2, which shows similar methylation (%) for both substrates. Additionally, a lower concentration of methyl groups was released from sugar beet pectin (around 2 mM per 0.04 mM of substrate) (Figure 3.25 A). This result agrees with low methylation of sugar beet pectin in comparison with apple and citrus pectin (Table 2.2, Section 2.7.6 in Chapter 2). Regarding GalA, concentrations of 2.5 (29% yield) and 2 mM (23% yield) were achieved for BLI09 paired either with TMA01 or BLI04, respectively after 4 h of the synergistic reaction using apple and citrus pectin (Figures 3.23 B and 3.24 B, respectively). The higher GalA concentration released in the reactions with TMA01 may be due to a higher tolerance of this enzyme towards methyl groups (Ralet, Crépeau and Bonnin, 2008). Although sugar beet pectin was demethylated by BLI09, TMA01 and BLI04 were not able to hydrolyse this substrate and only around 0.25 mM GalA was obtained after 24 h (Figure 3.25 B). This might be due to the presence of acetyl groups hinders the activity of

exo-PGs. A PAE which removes acetyl groups, could improve GalA release from sugar beet pectin (Bonnin, Garnier and Ralet, 2014). Notice that for all cases, no GalA was detected in the blank reactions where PMEs were not added.

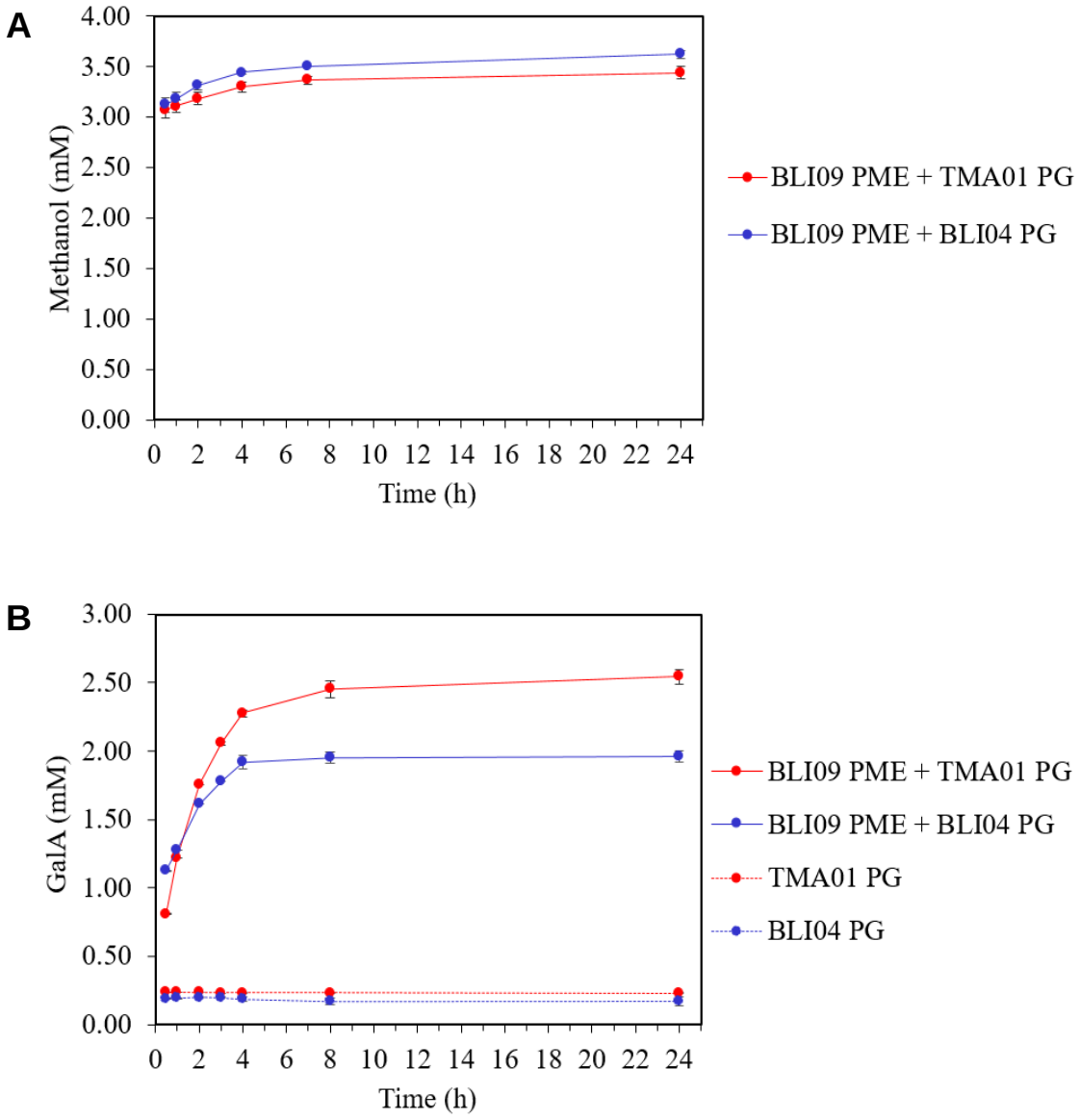

Figure 3.23. Synergistic activity between BLI09 PME either with TMA01 or BLI04 exo-PGs using apple pectin. (A) Methanol and (B) GalA quantification. The reactions were carried out using 0.5% substrate in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm up to 24 h. The U mL^{-1} of BLI09, TMA01 and BLI04 used were 9, 0.5 and 2, respectively (Table 2.3). The assay was performed as described in Section 2.8.1 in Chapter 2. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively in Chapter 2. Error bars represent one standard deviation from the mean (n = 2).

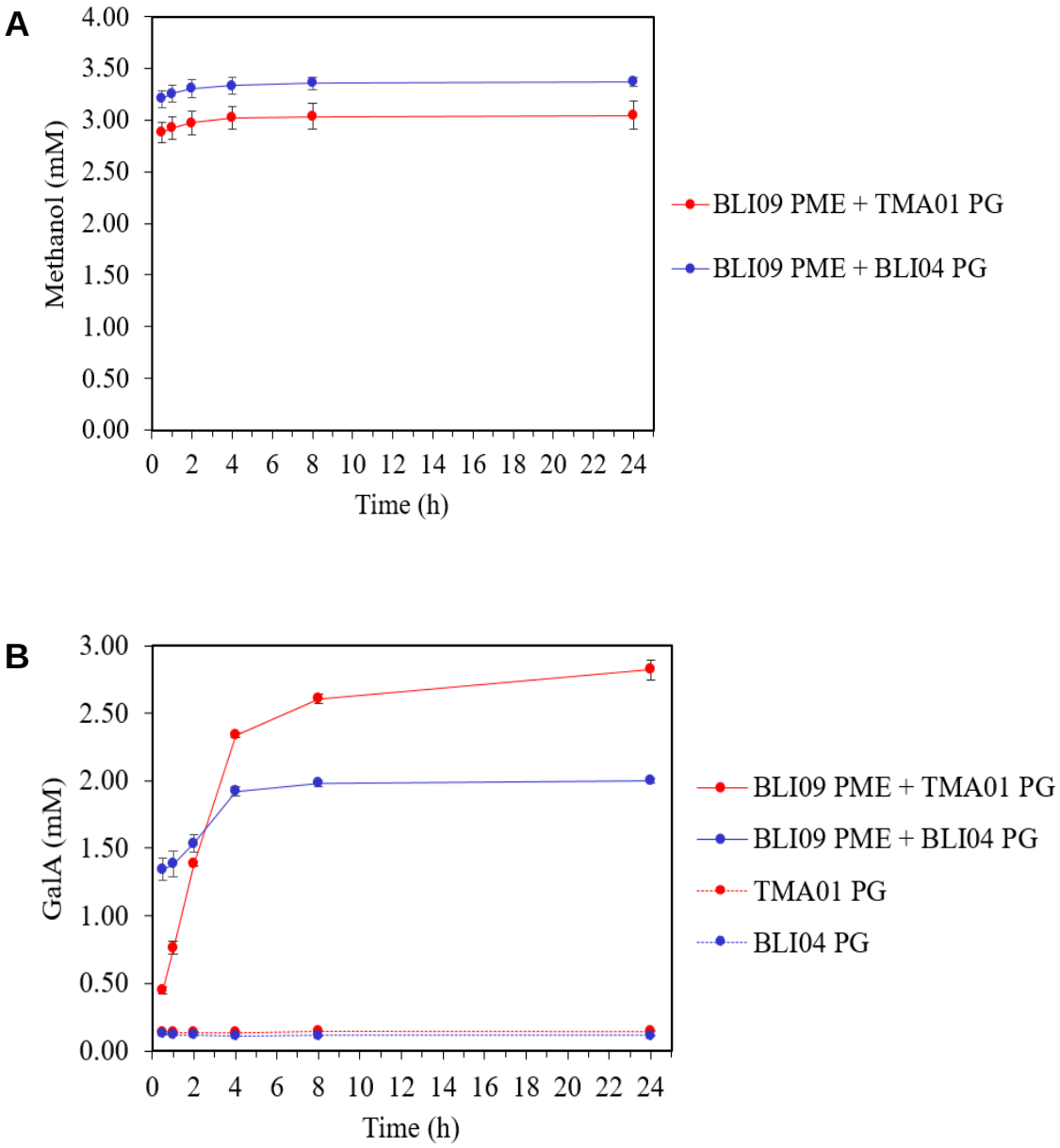

Figure 3.24. Synergistic activity between BLI09 PME either with TMA01 or BLI04 exo-PGs using citrus pectin. (A) Methanol and (B) GalA quantification. The reactions were carried out using 0.5% of substrate in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm up to 24 h. The U mL^{-1} of BLI09, TMA01 and BLI04 used were 9, 0.5 and 2, respectively (Table 2.3). The assay was performed as described in Section 2.8.1 in Chapter 2. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively in Chapter 2. Error bars represent one standard deviation from the mean (n = 2).

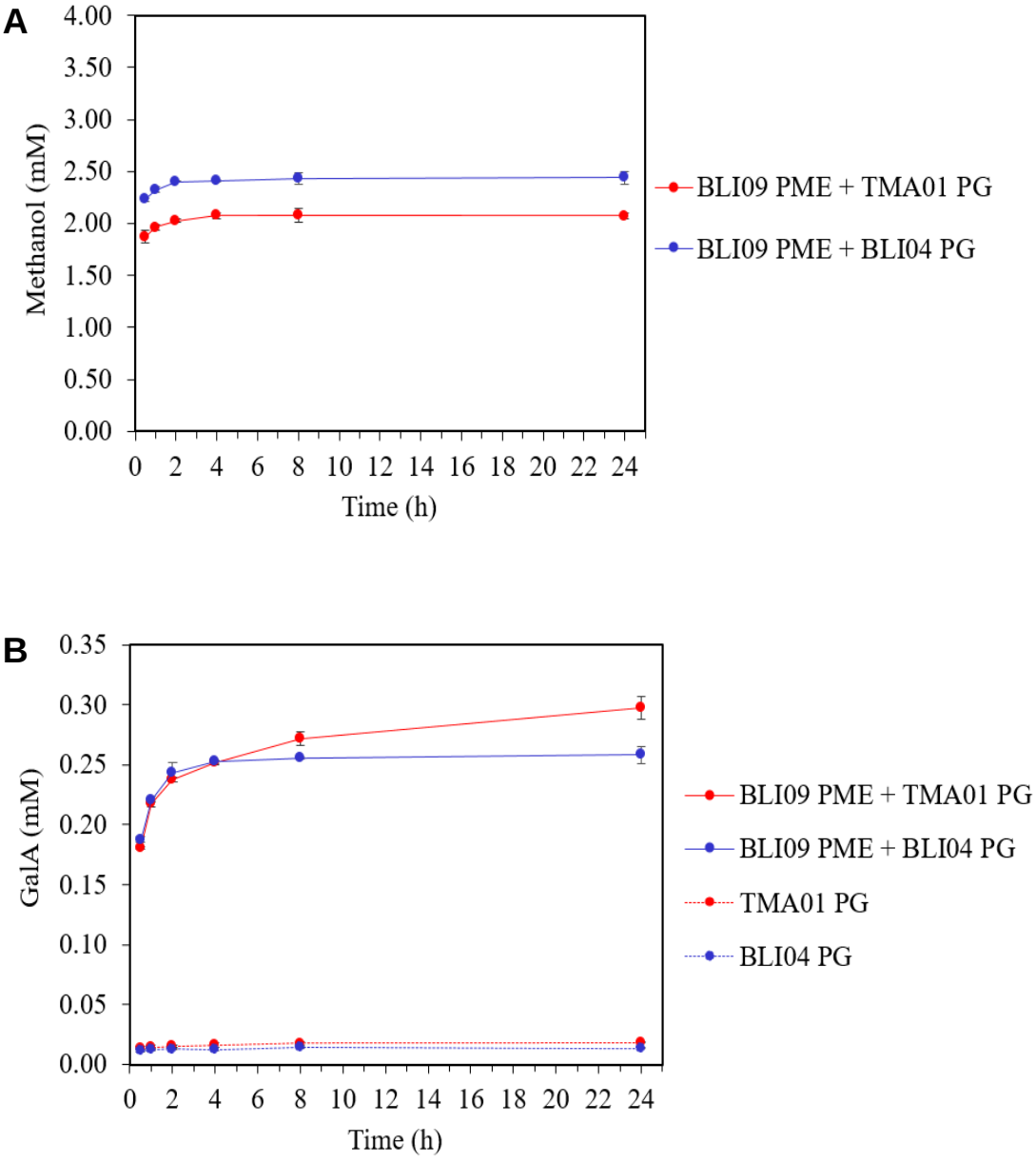

Figure 3.25. Synergistic activity between BLI09 PME either with TMA01 or BLI04 exo-PGs using sugar beet pectin. (A) Methanol and (B) GalA quantification. The reactions were carried out using 0.5% of substrate in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm up to 24 h. The U mL^{-1} of BLI09, TMA01 and BLI04 used were 9, 0.5 and 2, respectively (Table 2.3). The assay was performed as described in Section 2.8.1 in Chapter 2. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively in Chapter 2. Error bars represent one standard deviation from the mean (n = 2).

Synergistic reactions between SAM10 paired either with TMA01 or BLI04 using apple, citrus and sugar beet pectin are shown in Figures 3.26, 3.27 and 3.28, respectively. Released methanol was also similar for apple and citrus pectin (Figure 3.26 A and 3.27 A). In the case of sugar beet pectin, around 20% less methanol was released by SAM10 in comparison with BLI09 (Figure 3.28 A). It could be due to a lower tolerance of SAM10 to sugar beet pectin acetyl groups with respect to BLI09 (Remoroza *et al.*, 2015). Although both PMEs demethylated pectin, released GalA in all synergistic reactions with SAM10 was below 1 mM after 24 h (Fig. 6A2, B2 and C2). This could be due to methyl groups distribution within the complex pectin structure in each source and PMEs mechanism of action. Regarding PMEs mechanism, they 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) manner (Limberg *et al.*, 2000; Micheli, 2001; Bonnin *et al.*, 2002; Mohamed, Christensen and Mikkelsen, 2003; Goubet *et al.*, 2005; Fries *et al.*, 2007; Kent *et al.*, 2016). PMEs with a blockwise demethylation pattern create fragments of non-methylesterified GalA, where exo-PGs can act and release GalA monomers from the non-reducing end (Figure 3.29). Otherwise, when substrates are demethylated in a random way, it is less likely that GalA release can occur since the remaining methyl groups hinder the exo-PGs activity (Figure 3.30). The reason why higher concentrations of GalA were obtained in the synergistic reactions of exo-PGs with BLI09 than with SAM10 might be due to BLI09 acts on a blockwise manner (Remoroza *et al.*, 2015). Thereby, we can hypothesise that SAM10 removes methyl groups following a random mechanism, since low GalA concentrations were obtained in all synergistic reactions with this enzyme. In addition, it has been reported that sugar beet pectin is highly branched containing arabinan and galactan lateral branches, which may also hinder the exo-PG activity (Cárdenas-Fernández *et al.*, 2017, 2018).

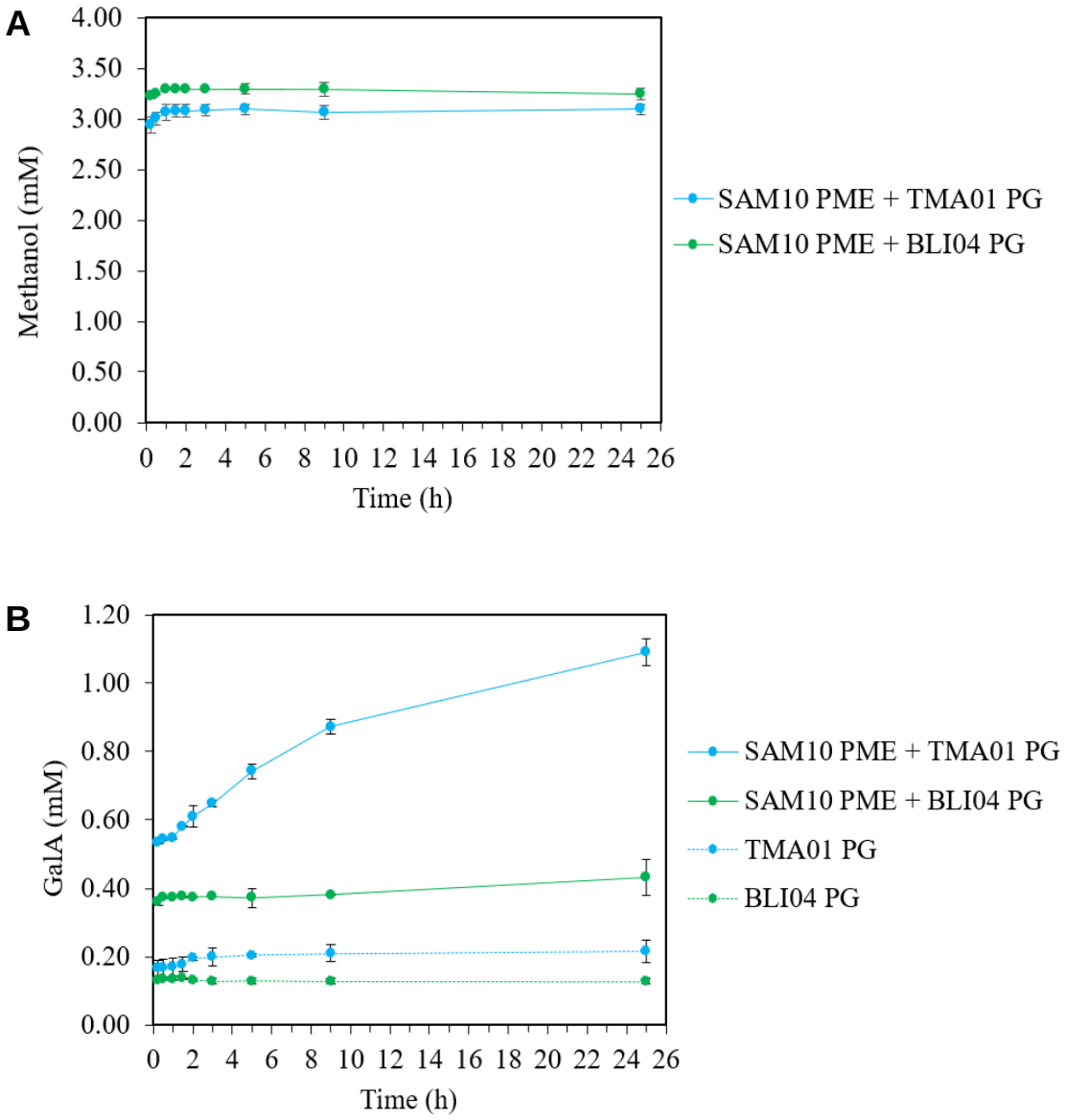

Figure 3.26. Synergistic activity between SAM10 PME either with TMA01 or BLI04 exo-PGs using apple pectin. (A) Methanol and (B) GalA quantification. The reactions were carried out using 0.5% of substrate in 20 mM phosphate buffer pH 7 at 40 °C for 1 h and 50 °C at 300 rpm up to 24 h. The U mL^{-1} of SAM10 and both exo-PGs used were 18 and 1, respectively (Table 2.3). The assay was performed as described in Section 2.8.1 in Chapter 2. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively in Chapter 2. Error bars represent one standard deviation from the mean (n = 2).

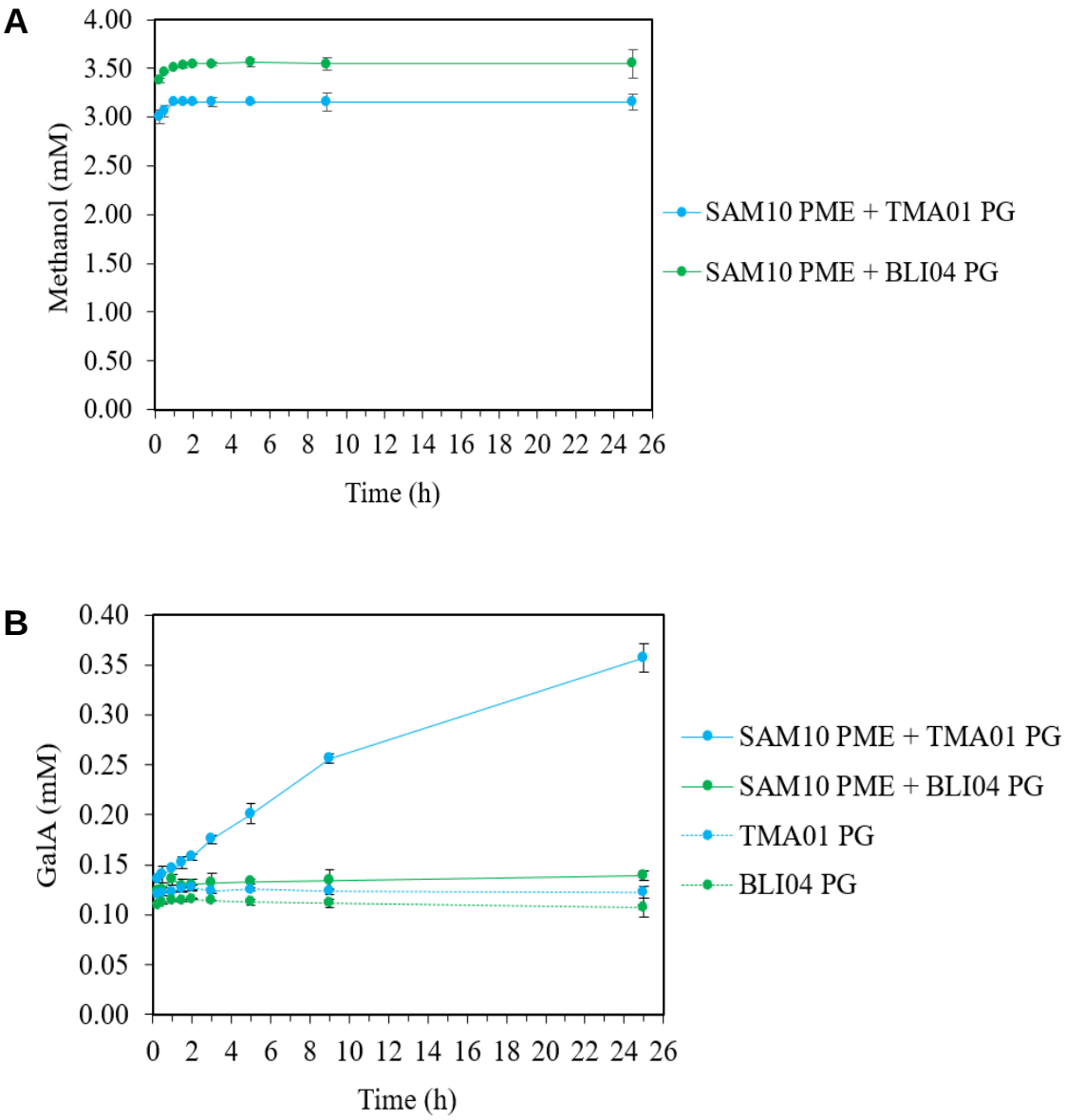

Figure 3.27. Synergistic activity between SAM10 PME either with TMA01 or BLI04 exo-PGs using citrus pectin. (A) Methanol and (B) GalA quantification. The reactions were carried out using 0.5% of substrate in 20 mM phosphate buffer pH 7 at 40 °C for 1 h and 50 °C at 300 rpm up to 24 h. The U mL^{-1} of SAM10 and both exo-PGs used were 18 and 1, respectively (Table 2.3). The assay was performed as described in Section 2.8.1. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively in Chapter 2. Error bars represent one standard deviation from the mean (n = 2).

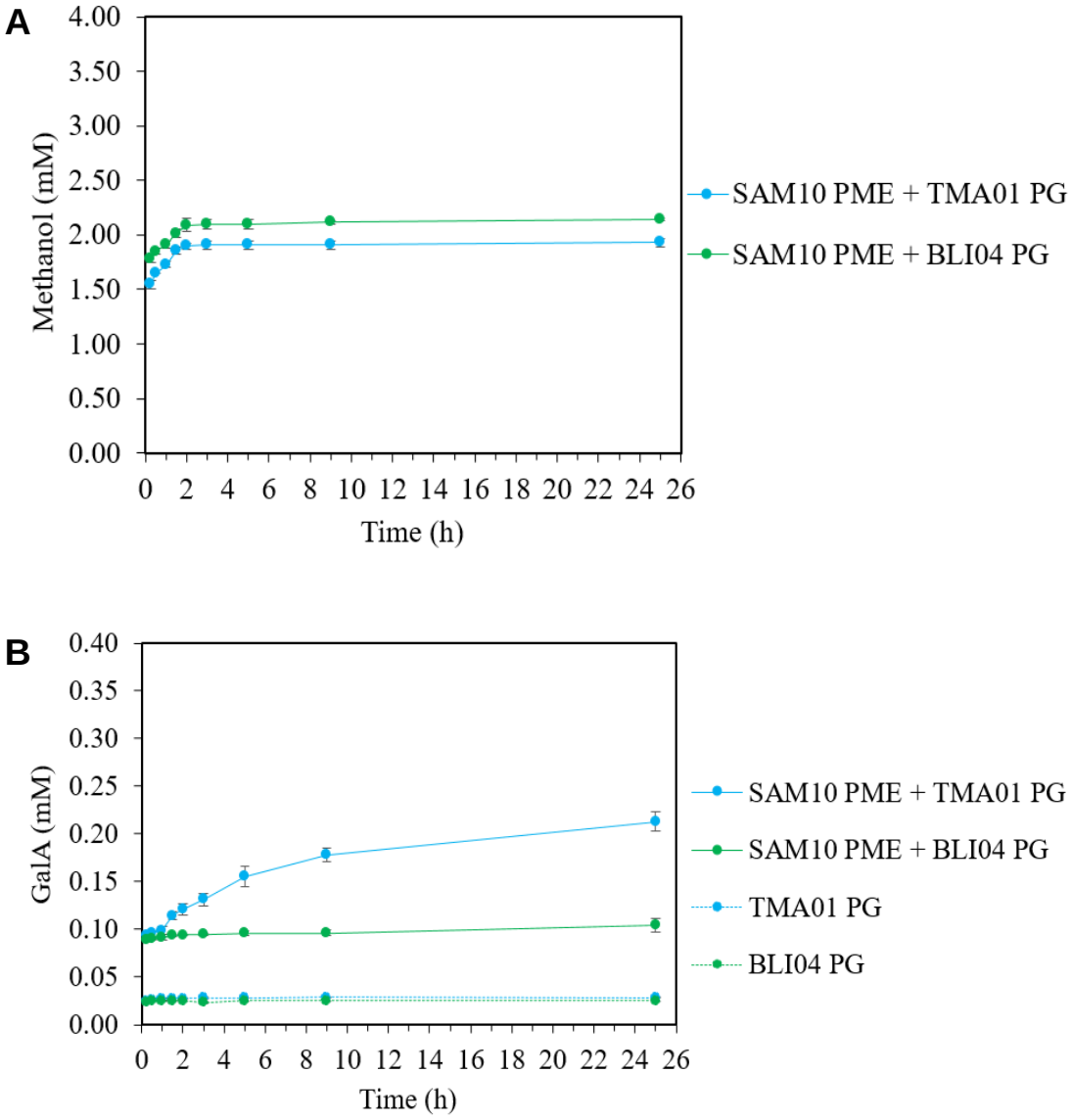

Figure 3.28. Synergistic activity between SAM10 PME either with TMA01 or BLI04 exo-PGs using sugar beet pectin. (A) Methanol and (B) GalA quantification. The reactions were carried out using 0.5% of substrate in 20 mM phosphate buffer pH 7 at 40 °C for 1 h and 50 °C at 300 rpm up to 24 h. The U mL^{-1} of SAM10 and both exo-PGs used were 18 and 1, respectively (Table 2.3). The assay was performed as described in Section 2.8.1. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively in Chapter 2. Error bars represent one standard deviation from the mean (n = 2).

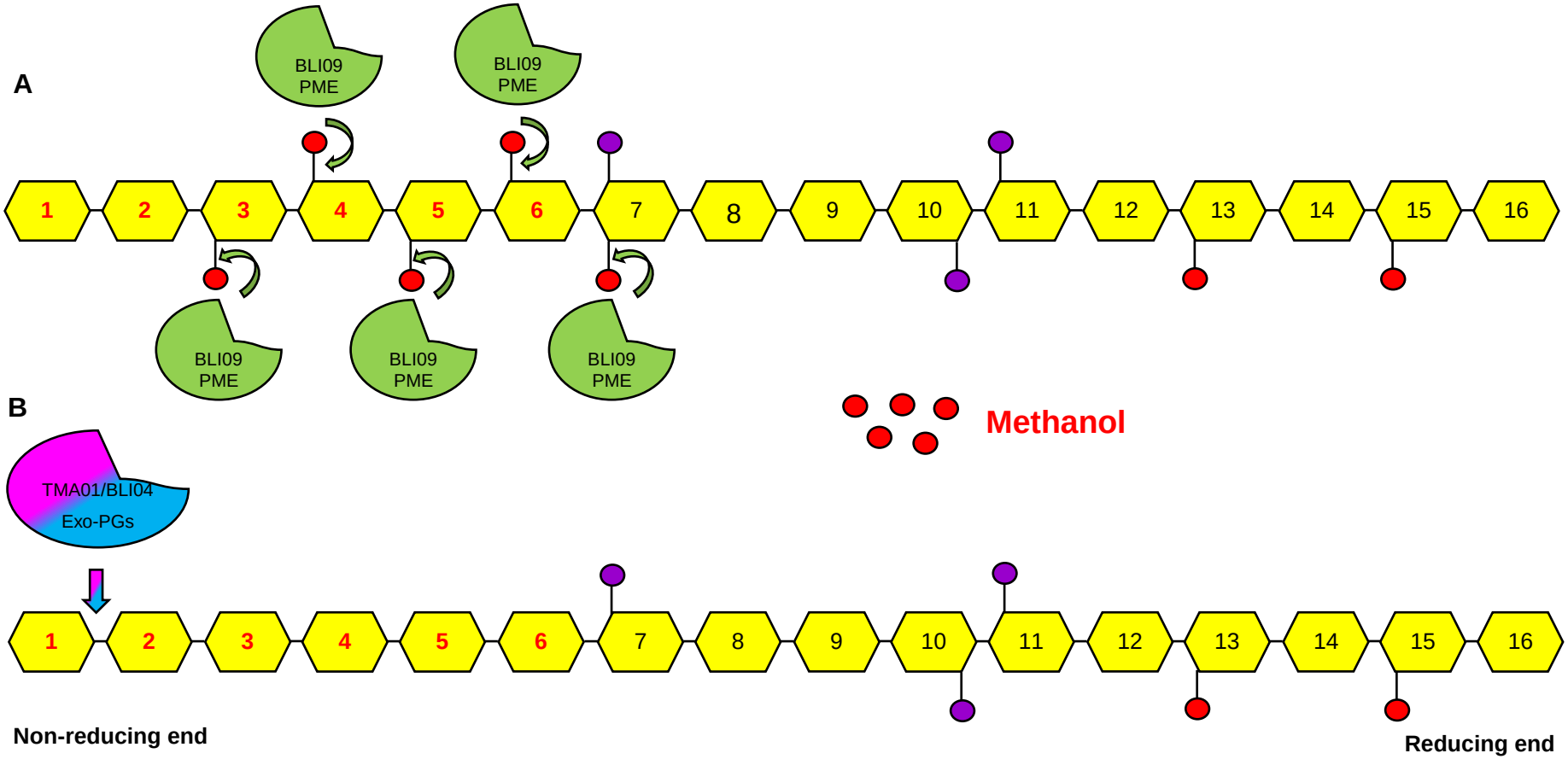

Figure 3.29. Synergistic action between BLI09 PME either with TMA01 or BLI09 exo-PGs on the HG backbone in pectin. (A) Blockwise demethylation pattern of BLI09 PME producing blocks of non-methylated pectin. (B) Exo-PGs action in the non-reducing end of demethylated pectin in a blockwise manner, leading to monomeric GalA release at high concentrations. GalA monomers highlighted in red (from 1 to 6) can be released. (⬡) GalA, (⚲) methyl groups and (⚲) acetyl groups. PME: pectin methylesterase, exo-PG: exo-polygalacturonase, HG: homogalacturonan and GalA: galacturonic acid.

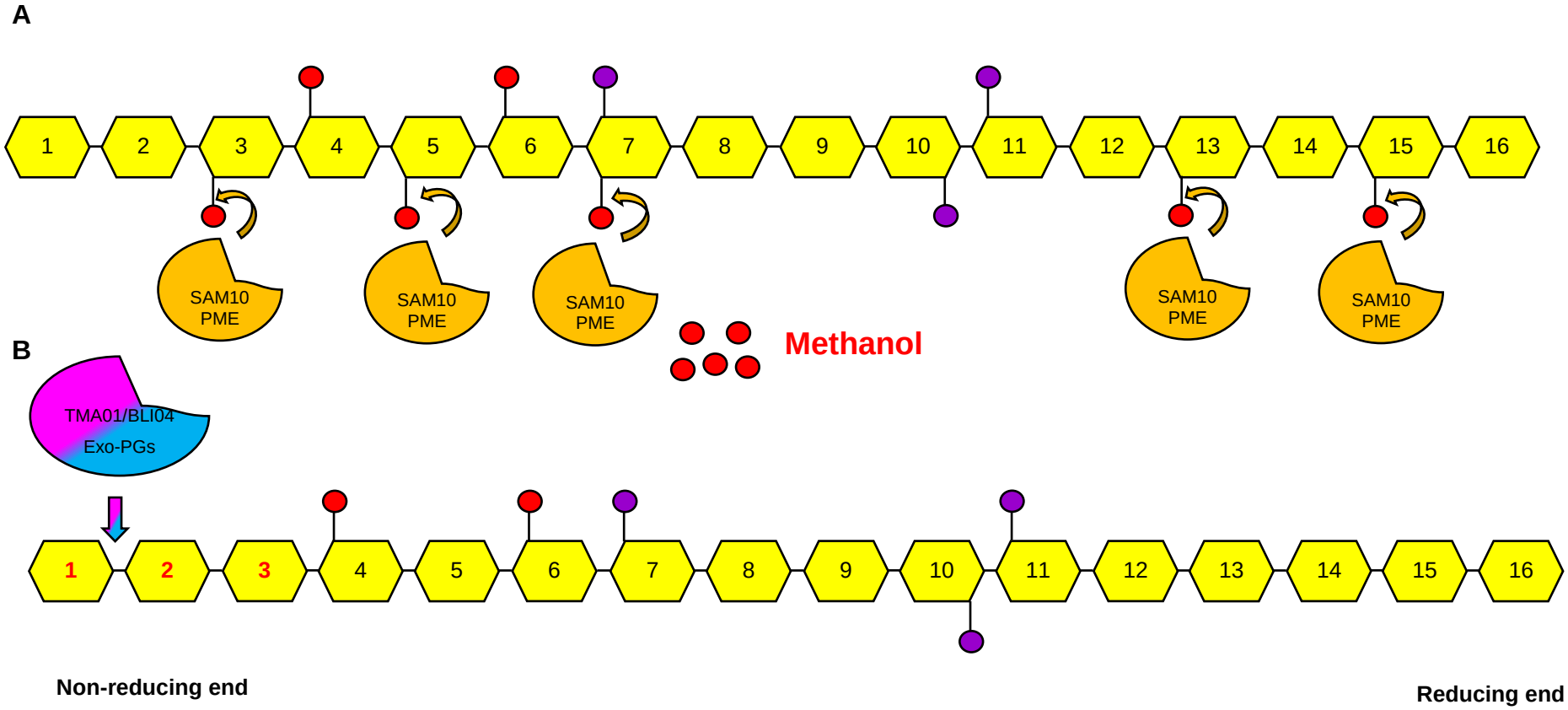

Figure 3.30. Synergistic action between SAM10 PME either with TMA01 or BLI09 exo-PGs on the HG backbone in pectin. (A) Random demethylation pattern of SAM10 PME which does not produce blocks of non-methylated pectin. (B) Exo-PGs action in the non-reducing end of pectin demethylated in a random way, leading to monomeric GalA release at low concentrations. GalA monomers highlighted in red (from 1 to 3) can be released. (⬡) GalA, (🍄) methyl groups and (🍄) acetyl groups. PME: pectin methylesterase, exo-PG: exo-polygalacturonase, HG: homogalacturonan and GalA: galacturonic acid.

3.4 Conclusions

The aim of this chapter was to identify and select bacterial thermophilic pectinases for molecular cloning and expression, carry out functional characterisation and explore the synergistic activity between PMEs and exo-PGs for pectin bioconversion into GalA. The analysis of the bacterial genomes from our collection allowed me to identify nine thermophilic pectinases from thermophiles; and according to the phylogenetic analysis, 29 from mesophiles which were closely related to those from thermophiles. From all of them, a total of seven pectinases were successfully cloned and expressed. Among them are two exo-PGs (TMA01 and BLI04), two PMEs (BLI09 and SAM10) and three PGLs (TMA14, TFU19 and TFU20). The functional characterisation assays from the two exo-PGs and the two PMEs showed that they exhibited maximum activity at pH from 7 to 8. In addition, 0.25 mm Mn^{2+} increased the activity of both exo-PG by around 2-fold and did not affect PMEs activity, indicating that this ion can be used in the synergistic reactions between these two kinds of pectinases. Exo-PGs and PMEs showed optimum activity between 50 and 90 °C. TMA01 presented great thermal stability up to 90 °C, BLI04 and BLI09 up to 50 °C and SAM10 up to 40 °C. The four pectinases exhibited a substrate inhibition kinetics. Substrate specificity assays using non-esterified and esterified substrates confirmed that exo-PGs prefer those non-esterified, evidencing the need of a synergistic activity between PMEs and exo-PGs.

Synergistic reactions between BLI09 and SAM10 PMEs either with TMA01 or BLI04 exo-PGs were run setting up compatible operational conditions, even if optimum activity of one of the enzymes was compromised. From the four synergistic reactions carried out using apple, citrus and sugar beet pectin, the most successful for demethylation and GalA release were those between BLI09 paired either with TMA01 or BLI04 using apple and citrus pectin. These reactions allowed release 3.5 mM methanol as well as 2.5 and 2 mM GalA in the synergistic reactions with TMA01 and BLI04, respectively. Low GalA concentrations were released using sugar beet pectin which could be associated with the high percentage of acetyl groups in this substrate. Otherwise, in the synergistic reactions of SAM10 paired either with TMA01 or

BLI04, although SAM10 demethylated pectin releasing the same methanol concentrations as BLI09, low GalA concentrations were released using the three substrates. These low concentrations of GalA released can be explained by the random demethylation pattern of SAM10 which leave methyl groups that hinder exo-PGs activity.

This work contributes to the knowledge of thermophilic pectinases providing information about operational conditions, stability, and kinetic parameters. Likewise, characterisation results were fundamental to the subsequent application of the enzymes in a synergistic action. Synergistic activity between PMEs and exo-PGs allowed a better understanding of the catalytic mechanisms of these enzymes as well as a more efficient pectin hydrolysis leading to GalA release. Additionally, these findings provide further insights to valorise pectin-rich renewable feedstocks through bio-based compounds obtaining (Flores-Fernández *et al.*, 2022).

In the following chapter, co-expression systems containing both a PME and an exo-PG for a cost-effective pectin bioconversion into GalA will be addressed.

CHAPTER 4 CO-EXPRESSION OF THERMOPHILIC PECTINASES IN A SINGLE HOST FOR COST-EFFECTIVE PECTIN BIOCONVERSION INTO D-GALACTURONIC ACID

4.1 Introduction

Pectin is a complex polymer composed of several substructures, thus different kinds of pectinases are involved in its degradation (Voragen *et al.*, 2009). Among the most important are PGs and PMEs. In addition, in substrates such as sugar beet pectin, PAEs and AFs are also relevant due to the high percent of acetyl groups and Ara content (Bonnin, Garnier and Ralet, 2014). These enzymes act in a synergistic manner for pectin bioconversion in bio-based chemicals such as GalA. AFs remove Ara residues from arabinan side chains in pectin while PMEs and PAEs remove methyl and acetyl groups, respectively. Meanwhile, exo-PGs catalyse the GalA release in demethylated and deacetylated pectin (Voragen *et al.*, 2009; Remoroza *et al.*, 2014; Cárdenas-Fernández *et al.*, 2018).

In general, genes encoding enzymes are cloned separately in an adequate plasmid and the enzymes are expressed and produced individually in a host cell. Then, they are purified and can be mixed in different ratios according to their applications. This individual enzymes production demands extra cost and time for the bioprocess (Roongsawang *et al.*, 2010). Thus, co-expression of enzymes in a single host represents a cost-effective alternative for enzymes production increasing their applicability in biocatalysis. Another additional advantages of co-expression include reduction in possibilities of improper folding, ease of simultaneous purification and even the use of the clarified lysates as such (Kumar *et al.*, 2015). Among the main drawbacks of co-expression of enzymes could be included the difficulty to control the suitable ratio between the co-expressed enzymes as well as their interactions. In addition, the genes' cloning order is a crucial factor within the existing strategies for enzymes co-expression, since it affects the expression levels (Zhu *et al.*, 2015; Chen, Huang and Zhang, 2017).

Two main strategies for the co-expression of enzymes in a single host such as *E. coli* have been reported and these include the use of a single or multiple plasmids. In the first strategy, two or more genes are cloned and expressed in a single plasmid and three options are possible. Firstly, all the genes could be under the control of a single promoter and terminator; secondly, each gene can be under the control of its own promoter and all share one terminator; and thirdly, each gene be under the control of its own promoter and its own terminator. pETDuet-1 is one of the most used plasmids for this strategy. It contains two MCSs and each cloned gene is under the control of its own T7 promoter and both share one T7 terminator (Held, Yaeger and Novy, 2003). In this strategy, expression levels depend on the presence or absence of promoters and terminators as well as of the cloning order of the genes. In the second strategy, the genes are cloned and expressed in multiple plasmids. The plasmids should have different origins of replication and resistance markers. One of the drawbacks in the strategy is the maintenance of the multiple plasmids by the host, due to the high metabolic and bioenergetic burdens that it involves. Also, copy number and expression level of one plasmid is influenced by the presence of the others (Lan *et al.*, 2006; Romier *et al.*, 2006; Kumar *et al.*, 2015; Chen, Huang and Zhang, 2017).

To the best of our knowledge, co-expression of pectinases has not been reported yet. The co-expression of PMEs and exo-PGs in a single plasmid and in *E. coli* as well as their synergistic activity with other pectinases including AFs and PAEs represent a promising alternative to improve GalA release from pectin rich-biomass.

4.2 Aims and objectives

The aim of this chapter was to co-express a thermophilic PME and exo-PG in a single plasmid and host for a cost-effective pectin bioconversion into GalA. Also, to evaluate the addition of a PAE and AF into the synergistic reactions to improve GalA release from sugar beet pectin. The key objectives of the chapter are outlined below:

1. To construct co-expression plasmids containing a thermophilic PME and exo-PG in a single plasmid and evaluate the effect of the cloning order of the genes in the pectinases expression in *E. coli* BL21(DE3).
2. To purify the pectinases from the co-expression constructs and determine their activity and protein concentration to compare the values with those from the individually expressed enzymes.
3. To assess the synergistic action between a PME and exo-PG as co-expressed enzymes using apple, citrus and sugar beet pectin.
4. To clone and express a PAE from *Bacillus licheniformis* to remove acetyl groups from sugar beet pectin.
5. To assess the synergistic action between a PME and exo-PG as co-expressed enzymes along with a PAE and AF to improve GalA release from sugar beet pectin.
6. To evaluate product inhibition of methanol, GalA and acetic acid on the PME and exo-PG from the co-expression constructs.

4.3 Results and discussion

4.3.1 Construction, expression, and purification of co-expression plasmids containing a PME and exo-PG

As described in Section 3.3.8 (Chapter 3), PMEs and exo-PGs act on a synergistic way for an efficient GalA release from pectin. According to the results from this section, synergistic reactions between BLI09 PME paired either with TMA01 or BLI04 exo-PGs were the most successful for GalA release. For that reason, co-expression plasmids containing both were constructed in pETDuet-1, which has MCSs for two target genes cloning and expression (Held, Yaeger and Novy, 2003). Thus, four co-expression plasmids were constructed and the effect of the cloning order of the genes in pectinases expression was evaluated. In co-expression constructs 1 and 2 (pETDuet-BLI09-TMA01 and pETDuet-BLI09-BLI04, respectively), BLI09 was cloned in MCS-1 and TMA01 and BLI04 in MCS-2 (Figures 4.1 A and 4.1 B). In co-expression constructs 3 and 4 (pETDuet-TMA01-BLI09 and pETDuet-BLI04-BLI09, respectively) the cloning order of the genes was inverted and TMA01 and BLI04 were cloned in MCS-1 and BLI09 in MCS-2 (Figures 4.2 A and 4.2 B).

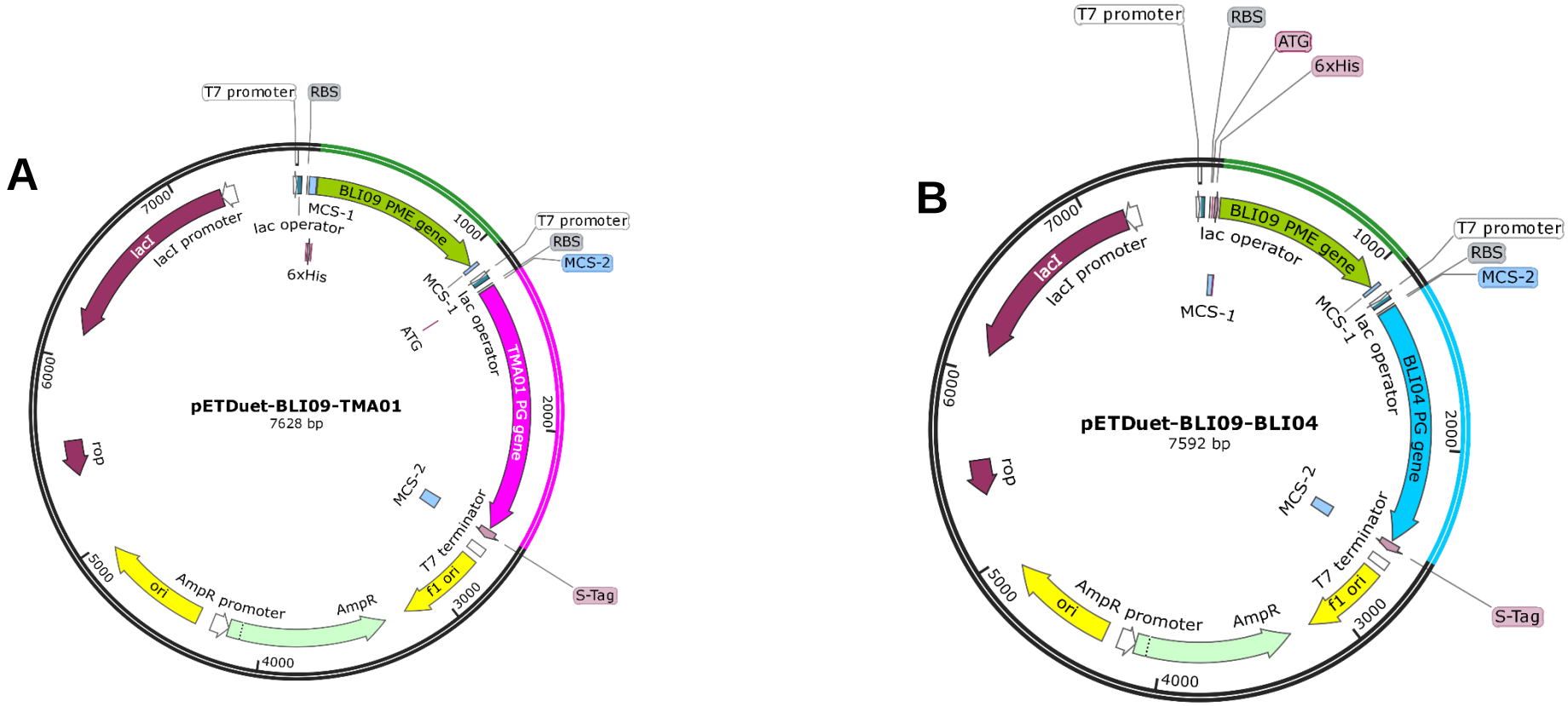

Figure 4.1. Co-expression constructs 1 and 2 containing a PME and exo-PG in pETDuet-1 where the PME was cloned in MCS-1 and the exo-PG in MCS-2. (A) Co-expression construct 1 (pETDuet-BLI09-TMA01), BLI09 is in MCS1 (green) and TMA01 in MCS2 (pink). (B) Co-expression construct 2 (pETDuet-BLI09-BLI04), BLI09 is in MCS1 (green) and BLI04 in MCS2 (light blue). Co-expression plasmids were constructed as described in Section 2.4 in Chapter 2 and plotted using SnapGene 4.2.11 software. PME: pectin methylesterase, exo-PG: exo-polygalacturonase, MCS: multiple cloning site.

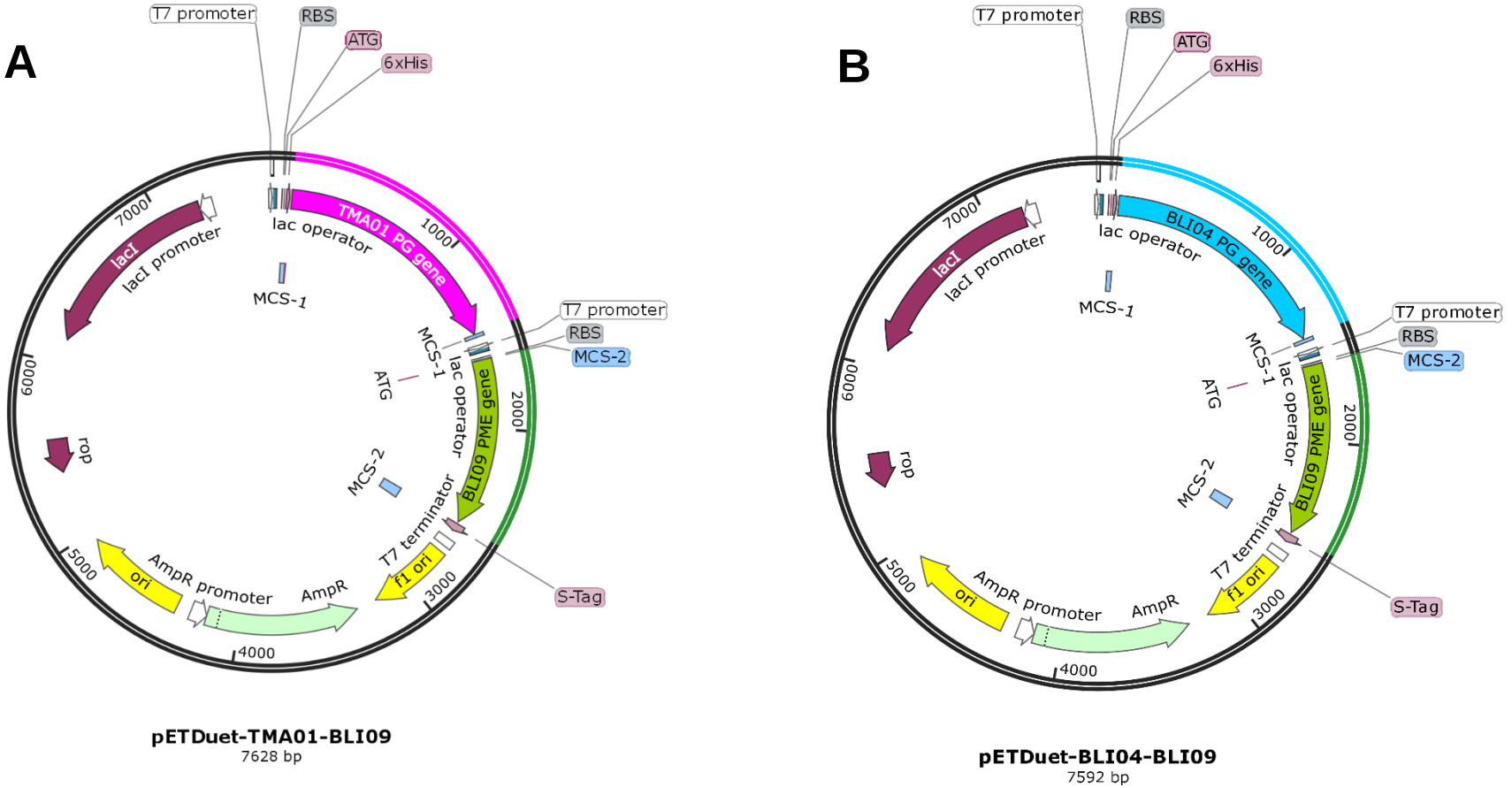

Figure 4.2. Co-expression constructs 3 and 4 containing a PME and exo-PG in pETDuet-1 where the exo-PG was cloned in MCS-1 and the PME in MCS-2. (A) Co-expression construct 3 (pETDuet-TMA01-BLI09), TMA01 is in MCS-1 (pink) and BLI09 in MCS-2 (green). (B) Co-expression construct 4 (pETDuet-BLI04-BLI09), BLI04 is in MCS-1 (light blue) and BLI09 in MCS-2 (green). Co-expression plasmids were constructed as described in Section 2.4 in Chapter 2 and plotted using SnapGene 4.2.11 software. PME: pectin methylesterase, exo-PG: exo-polygalacturonase, MCS: multiple cloning site.

All the co-expression constructs were expressed in *E. coli* BL21(DE3), and the enzymes in their soluble form and as separate proteins (Figure 4.3). In co-expression constructs 1 and 2, TMA01 and BLI04 showed good expression levels, but BLI09 cloned upstream the exo-PGs in MCS-1 and without a T7 terminator behind showed low expression. On the other hand, in co-expression constructs 3 and 4, both exo-PGs presented good expression, and BLI09 cloned downstream the exo-PGs in MCS-2 and with a T7 terminator behind exhibited improved expression levels in comparison with co-expression constructs 1 and 2. Overall, co-expression constructs 3 and 4 presented better expression of both pectinases. These results suggested that in co-expression constructs 1 and 2, the presence of a T7 promoter for each gene ensured the good expression of TMA01 and BLI04 although BLI09 expression was low. In addition, the presence of only one T7 terminator did not cause that BLI09 low expression affects that of TMA01 or BLI04. While in co-expression constructs 3 and 4, a T7 promoter for each gene also seems to have ensured the good expression of TMA01 and BLI04 as well as of BLI09. Moreover, the high expression of TMA01 and BLI04 and the presence of the T7 terminator behind BLI09 seem to have positively influenced its expression. Overall, the cloning order of the genes was fundamental for BLI09 expression. Also, the presence of a T7 terminator behind this enzyme seems to have been necessary to enhance its expression level. However, for TMA01 and BLI04 exo-PGs expression, these previously mentioned factors seem not to have been essential. From these findings, as co-expression constructs 3 and 4 successfully expressed BLI09 and both exo-PGs, not other strategies such as the insertion of a T7 terminator behind BLI09 gene were tested to improve the expression of this enzyme in co-expression constructs 1 and 2.

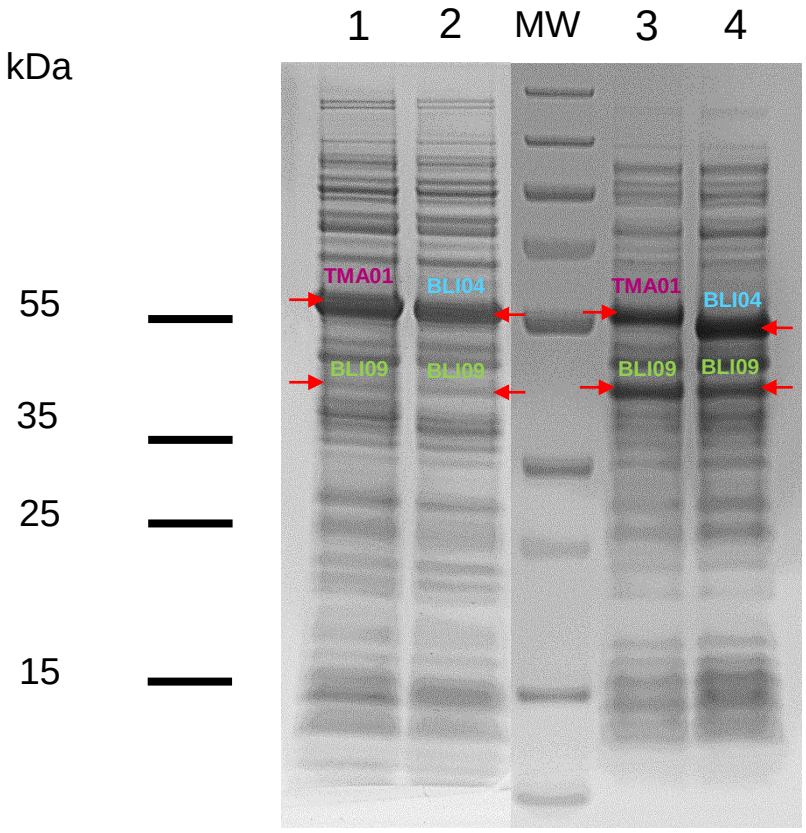

Figure 4.3. SDS-PAGE showing the co-expression of BLI09 PME paired either with TMA01 or BLI04 exo-PGs in pETDuet-1 and *E. coli* BL21(DE3). Lanes: 1, co-expression construct 1 (pETDuet-BLI09-TMA01) and 2, co-expression construct 2 (pETDuet-BLI09-BLI04). In co-expression constructs 1 and 2, BLI09 was cloned in MCS-1 and either TMA01 or BLI04 in MCS2. Lanes: 3, co-expression construct 3 (pETDuet-TMA01-BLI09) and 4, co-expression construct 4 (pETDuet-BLI04-BLI09). In co-expression constructs 3 and 4, either TMA01 or BLI04 were cloned in MCS-1 and BLI09 in MCS2. MW, molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa). SDS-PAGE was carried out as described in Section 2.9.2 in Chapter 2.

The pectinases from all the co-expression constructs were purified by affinity chromatography. Enzymatic activity, protein concentration and specific activity were determined for each one of the purified co-expressed enzymes. Purification of pectinases from co-expression constructs 1 and 2 is presented in Figure 4.4. In both constructs BLI09 was cloned with a N-terminal His_6-tag and purified using a His_6-Tag Ni-affinity resin, while TMA01 and BLI04 were cloned with a C-terminal S-tag and purified using a S-protein agarose. Similarly, purification of pectinases from co-expression constructs 3 and 4 is shown in Figure 4.5. TMA01 and BLI04 were

cloned with a N-terminal His6-tag and purified using a His_6-Tag Ni-affinity resin, while BLI09 was cloned with a C-terminal S-tag and purified using a S-protein agarose. In addition, as presented in Figures 4.4 and 4.5, no BLI09 binding to TMA01 or BLI04 was observed through purification, thus these results suggested no interaction between the two enzymes.

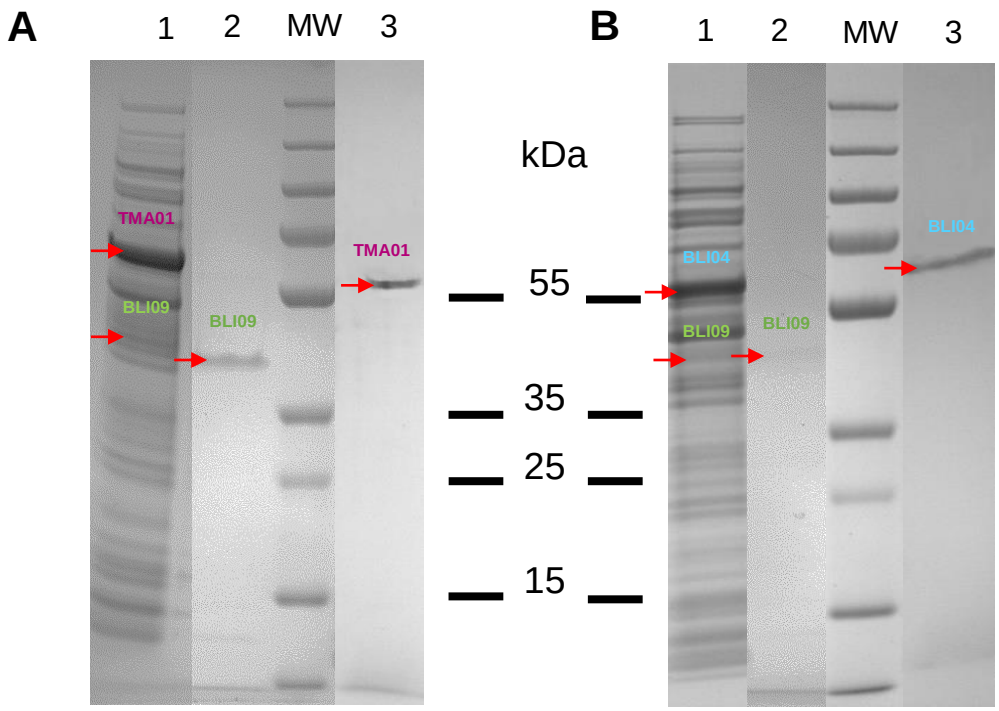

Figure 4.4. SDS-PAGE showing affinity chromatography purification of pectinases from plasmids of co-expression constructs 1 and 2. (A) Co-expression construct 1 (pETDuet-BLI09-TMA01): BLI09 was cloned in MCS-1 with a N-terminal His_6-tag and TMA01 in MCS-2 with a C-terminal S-tag. Lanes: 1, clarified lysate; 2, BLI09 and 3, TMA01. (B) Co-expression construct 2 (pETDuet-BLI09-BLI04): BLI09 was cloned in MCS-1 with a N-terminal His_6-tag and BLI04 in MCS-2 with a C-terminal S-tag. Lanes: 1, clarified lysate; 2, BLI09 and 3, BLI04. MW, molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa). Co-expression construct purification and SDS-PAGE were carried out as described in Sections 2.6.2 and 2.9.2 in Chapter 2, respectively.

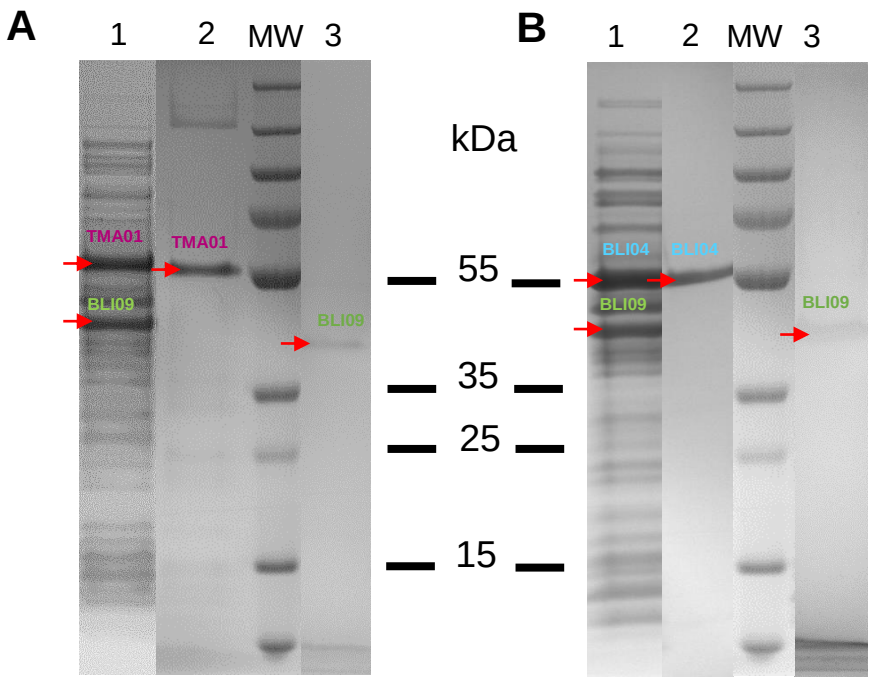

Figure 4.5. SDS-PAGE showing affinity chromatography purification of pectinases from plasmids of co-expression constructs 3 and 4. (A) Co-expression construct 3 (pETDuet-TMA01-BLI09): TMA01 was cloned in MCS-1 with a N-terminal His6-tag and BLI09 in MCS-2 with a C-terminal S-tag. Lanes: 1, clarified lysate; 2, TMA01 and 3, BLI09. (B) Co-expression construct 4 (pETDuet-BLI04-BLI09): BLI04 was cloned in MCS-1 with a N-terminal His6-tag and BLI09 in MCS-2 with a C-terminal S-tag. Lanes: 1, clarified lysate; 2, BLI04 and 3, BLI09. MW, molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa). Co-expression constructs purification and SDS-PAGE were carried out as described in Sections 2.6.2 and 2.9.2 in Chapter 2, respectively.

Comparison of enzymatic activity, protein concentration and specific activity between pectinases from the co-expression constructs is presented in Table 4.1. Enzymatic activities and protein concentrations were 8 and 9.5-fold higher for BLI09 in co-expression constructs 3 and 4 respect to co-expression constructs 1 and 2, respectively being the highest value in co-expression construct 3 with 2556 U mL^{-1} and 0.142 mg mL^{-1}. Regarding TMA01 and BLI04, similar enzymatic activities and protein concentrations were determined in all co-expressed constructs ranging from 23 and 36 U mL^{-1} and 0.182 and 0.228 mg mL^{-1}. Finally, specific activities of BLI09, TMA01 and BLI04 were similar in all the co-expression constructs with values of 17 800, 166 and 118 U mg^{-1}, respectively

Table 4.1. Comparison of enzymatic activity, protein concentration and specific activity between pectinases (BLI09, TMA01 and BLI04) from the co-expression constructs.

Co-expression construct activity (U mL^{-1})				
Enzyme	**1**	**2**	**3**	**4**
	pETDuet-BLI09-TMA01	pETDuet-BLI09-BLI04	pETDuet-TMA01-BLI09	pETDuet-BLI04-BLI09
PME	BLI09 (MCS-1): 319	BLI09 (MCS-1): 125	BLI09 (MCS-2): 2556	BLI09 (MCS-2): 1188
Exo-PG	TMA01 (MCS-2): 36	BLI04 (MCS-2): 23	TMA01 (MCS-1): 31	BLI04 (MCS-1): 28
Co-expression construct protein (mg mL^{-1})				
Enzyme	**1**	**2**	**3**	**4**
	pETDuet-BLI09-TMA01	pETDuet-BLI09-BLI04	pETDuet-TMA01-BLI09	pETDuet-BLI04-BLI09
PME	BLI09 (MCS-1): 0.018	BLI09 (MCS-1): 0.007	BLI09 (MCS-2): 0.142	BLI09 (MCS-2): 0.067
Exo-PG	TMA01 (MCS-2): 0.221	BLI04 (MCS-2): 0.200	TMA01 (MCS-1): 0.182	BLI04 (MCS-1): 0.228
Co-expression construct specific activity (U mg^{-1})				
Enzyme	**1**	**2**	**3**	**4**
	pETDuet-BLI09-TMA01	pETDuet-BLI09-BLI04	pETDuet-TMA01-BLI09	pETDuet-BLI04-BLI09
PME	BLI09 (MCS-1): 17 722	BLI09 (MCS-1): 17 857	BLI09 (MCS-2): 18 000	BLI09 (MCS-2): 17 331
Exo-PG	TMA01 (MCS-2): 163	BLI04 (MCS-2): 115	TMA01 (MCS-1): 170	BLI04 (MCS-1): 0.123

PME: pectin methylesterase, exo-PG: exo-polygalacturonase.

Table 4.2 provides information of enzymatic activity and protein concentration of individually expressed pectinases (Section 3.3.4 in Chapter 3). Comparing this information with that from co-expressed enzymes (Table 4.1), enzymatic activity and protein concentration for BLI09 were 3.5 and 7.5-fold lower in co-expression constructs 3 and 4, respectively with respect to the individually expressed enzyme. The lower protein concentration of BLI09 in the co-expression constructs could be explained by the major overload to the host and as consequence the total protein synthesis limitation in comparison with the individual expression. In addition, some factors such as mutual interaction between the recombinant enzymes are absent in the individual expression (Roongsawang *et al.*, 2010; Kumar *et al.*, 2015). Specific activity of BLI09 in the co-expression constructs was similar to in the individual expression. Meanwhile, enzymatic activities, protein concentrations and specific activities of TMA01 and BLI04 in all the co-expression constructs were similar to the values in individual expression.

Table 4.2. Enzymatic activity, protein concentration and specific activity of BLI09, TMA01 and BLI04 individually expressed as described in Section 3.3.4 in Chapter 3.

Individual expressed enzymes	**Activity (U mL^{-1})**	**Protein (mg mL^{-1})**	**Specific activity (U mg^{-1})**
BLI09 PME	9 000	0.5	18 000
TMA01 exo-PG	33	0.2	165
BLI04 exo-PG	24	0.2	120

PME: pectin methylesterase, exo-PG: exo-polygalacturonase.

4.3.2 Synergistic action between an exo-PG and PME as co-expressed enzymes

In Section 3.3.8 in Chapter 3, it was found that synergistic reactions between exo-PGs with BLI09 PME were the most successful for GalA release due to the blockwise demethylation pattern of this enzyme favoured exo-PGs activity. To achieve a cost-effective enzymes production for pectin bioconversion into GalA, co-expression plasmids containing BLI09 paired either with TMA01 or BLI04 were constructed and expressed in a single vector and host (Section 4.3.1). The four constructed co-expression plasmids were tested using substrates with

different degree of esterification (Table 2.2, Section 2.7.6 in Chapter 2) and the reactions were performed at compatible conditions for both enzymes (pH 7 and 50 °C). The activity of pectinases from the co-expression constructs used in the synergistic reactions is presented in Table 2.4, Section 2.8.1 in Chapter 2. These values were calculated based on the exo-PGs activity used in the synergistic reactions with individually expressed enzymes (Table 2.3, Section 2.8.1 in Chapter 2). Thus, 0.5 and 2 U mL^{-1} of TMA01 and BLI04, respectively were used. The activity of BLI09 was calculated in the volume containing the U mL^{-1} of the exo-PGs mentioned above and it was 4.5 and 9.5-fold higher in the co-expression constructs 3 and 4 than in individual expression (9 U mL^{-1}). In the case of the co-expression systems, clarified lysates were used. While in individual expression, the purified enzymes were tested.

Figures 4.6, 4.7 and 4.8 present the synergistic activity of the four co-expression constructs using apple, citrus and sugar beet pectin, respectively. Methanol release using all the co-expression constructs was similar for apple (Figure 4.6 A) and citrus pectin (Figure 4.7 A) reaching around 4 mM after 4 h of reaction. Methanol levels were slightly higher using the co-expression constructs 3 and 4. These findings agree with data presented in Table 2.2, Section 2.7.6 in Chapter 2, which shows similar methylation for these two substrates (55-58%). In sugar beet pectin, methanol release was also similar using all co-expression constructs but lower than for apple and citrus pectin (2 mM after 4 h of reaction) (Figure 4.8 A). This could be explained by the lower methylation of this substrate in comparison with apple and citrus pectin (35% in comparison with 58 and 55%, respectively). With respect to GalA, similar concentrations were released using the four co-expression constructs for apple (Figure 4.6 B) and citrus pectin (Figure 4.7 B) after 24 h of reaction. But interestingly, considerably higher concentrations were quantified after 4 h of reaction using the co-expression constructs 3 and 4, which allowed to release 3 (35% yield) and 2.5 mM (29% yield) GalA, respectively. In sugar beet pectin, GalA release was lower in comparison with apple and citrus pectin. After 24 h of reaction, only between 0.1 and 0.2 mM were released using all the co-expression constructs (Figure 4.8 B). Overall, co-expression constructs 3 and 4 were the most efficient for GalA release from apple and citrus pectin.

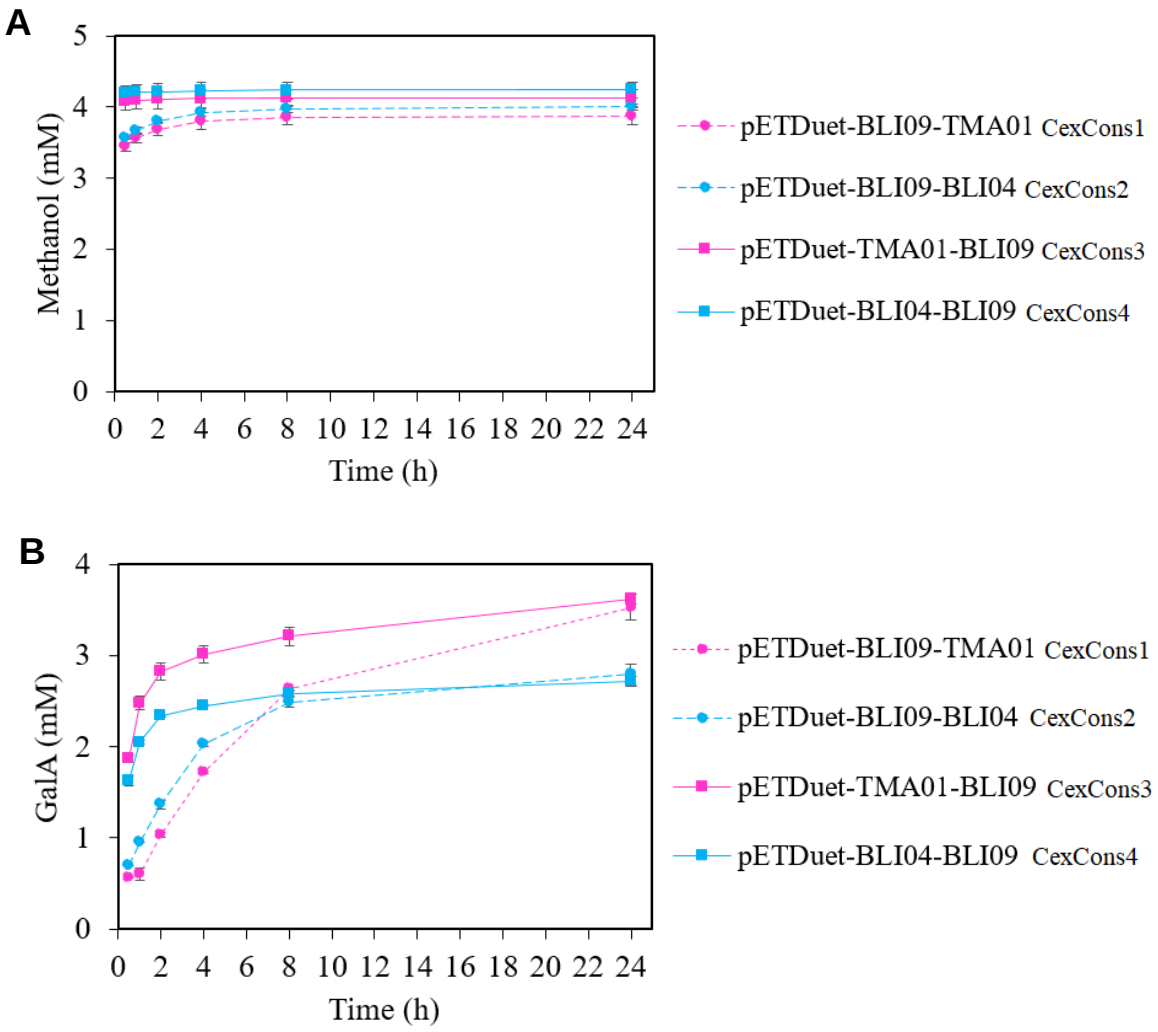

Figure 4.6. Synergistic activity between BLI09 PME paired either with TMA01or BLI04 exo-PGs as co-expressed enzymes using apple pectin. (A) Methanol and (B) GalA quantification. The reactions were carried out at 50 °C using 0.5% (w/v) substrate in 20 mM phosphate buffer pH 7; and with 0.5 and 2 U mL^{-1} of TMA01 and BLI04, respectively. The activity of BLI09 was calculated in the volumes containing the U mL^{-1} of exo-PGs mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.8.1; and methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). CexCons: co-expression construct.

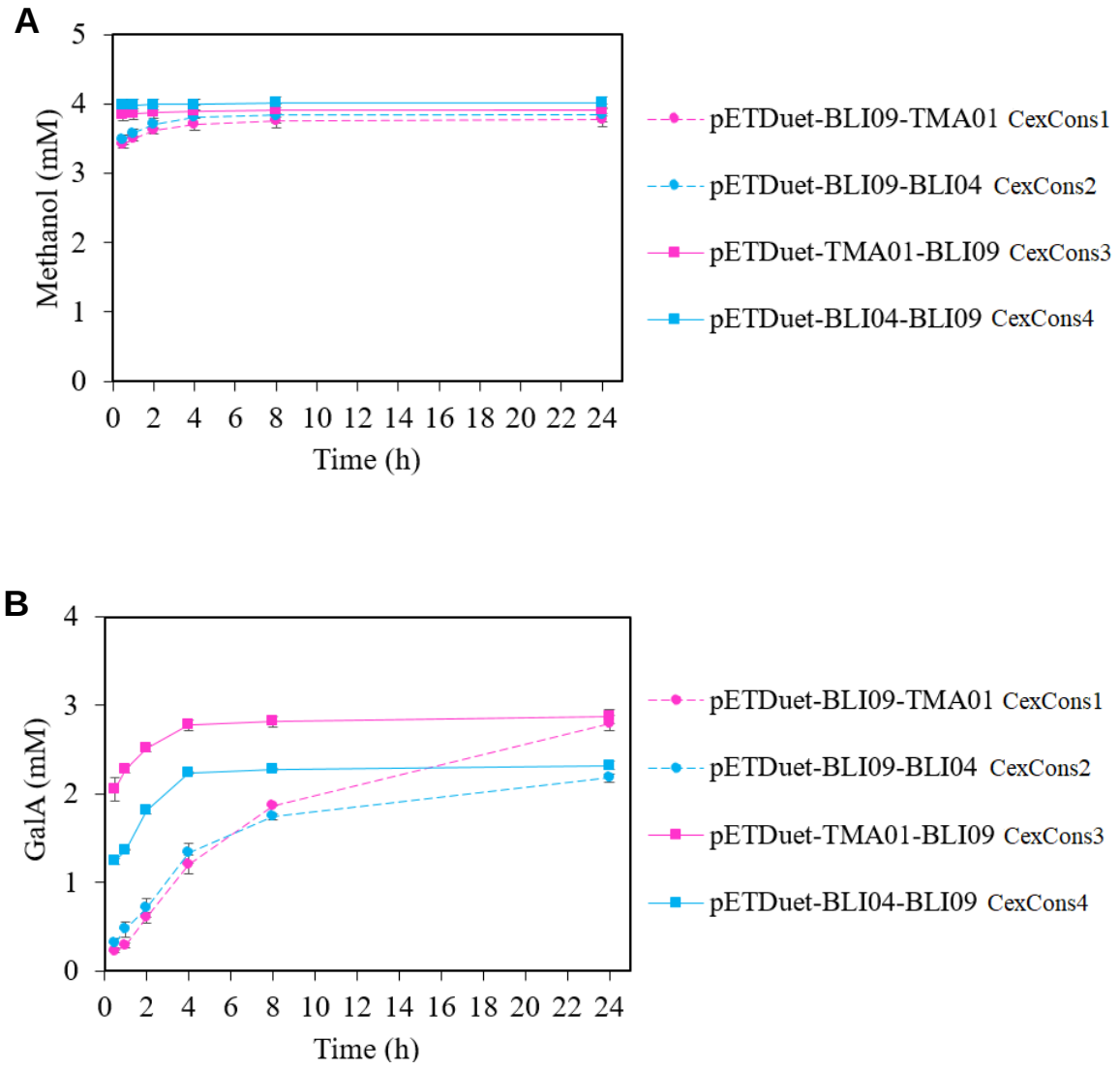

Figure 4.7. Synergistic activity between BLI09 PME paired either with TMA01or BLI04 exo-PGs as co-expressed enzymes using citrus pectin. (A) Methanol and (B) GalA quantification. The reactions were carried out at 50 °C using 0.5% (w/v) substrate in 20 mM phosphate buffer pH 7; and with 0.5 and 2 U mL^{-1} of TMA01 and BLI04, respectively. The activity of BLI09 was calculated in the volumes containing the U mL^{-1} of exo-PGs mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.8.1; and methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). CexCons: co-expression construct.

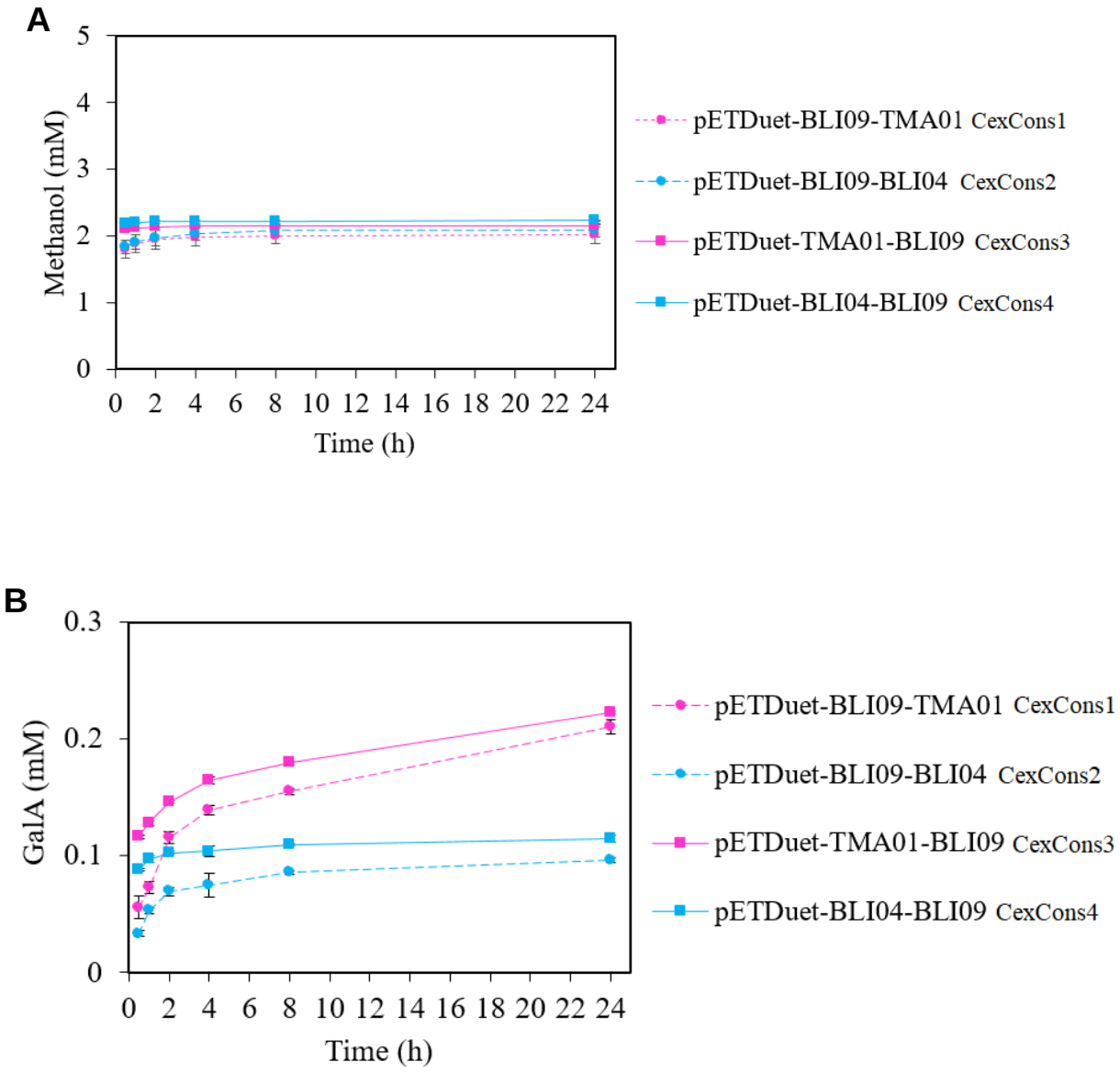

Figure 4.8. Synergistic activity between BLI09 PME paired either with TMA01or BLI04 exo-PGs as co-expressed enzymes using sugar beet pectin. (A) Methanol and (B) GalA quantification. The reactions were carried out at 50 °C using 0.5% (w/v) substrate in 20 mM phosphate buffer pH 7; and with 0.5 and 2 U mL^{-1} of TMA01 and BLI04, respectively. The activity of BLI09 was calculated in the volumes containing the U mL^{-1} of exo-PGs mentioned before Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.8.1; and methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). CexCons: co-expression construct.

Comparing the synergistic action of co-expressed pectinases with that using individual expressed enzymes, methanol released was around 3 mM after 4 h of reaction for apple and citrus pectin using individually expressed enzymes, while it was 4 mM using the co-expression constructs 3 and 4. In the case of sugar beet pectin, 2 mM methanol was released using the individually expressed enzymes as well as all the co-expression constructs. Regarding GalA, 2-2.5 mM were released after 4 h of reaction from the synergistic reactions using individually expressed enzymes, while 2.5-3 mM were quantified using the co-expression constructs 3 and 4. These findings showed that co-expression constructs 3 and 4 were more efficient for GalA release with respect to individual expression, although the same exo-PGs activity was used in both cases. These results could be justified by the very high activity of BLI09 in these constructs (40.8 and 86 U mL^{-1}) with respect to the individually expressed enzyme (9 U mL^{-1}). In sugar beet pectin, low amounts of GalA were released using individually expressed enzymes and co-expressed enzymes (less than 0.25 mM). In the case of individually expressed enzymes, purified enzymes were used for the synergistic reactions. Whereas in the case of co-expression constructs, clarified lysates were used.

Our results demonstrated that co-expression constructs containing a PME and exo-PG expressed in a single host allowed to obtain GalA reducing the enzymes production cost. In addition, clarified lysates were efficiently used representing a reduction in the purification costs of the enzymes. In the case of sugar beet pectin, although this substrate was demethylated using both the co-expression constructs and individually expressed enzymes individually, low concentrations of GalA were released. It could be due to the presence of a high percent of acetyl groups in this substrate (20%) in comparison with apple and citrus pectin (~ 1.5%). The presence of acetyl groups hinders the exo-PGs activity, thus the addition of a PAE in the synergistic reactions could improve GalA release. In Section 4.3.3, the results about cloning and expression of a PAE from *Bacillus licheniformis* are presented.

4.3.3 Cloning and expression of a PAE from *Bacillus licheniformis*

A PAE (EC 3.1.1.6, UniProtKB A0A415J918) from *Bacillus licheniformis* named as PAE21 was cloned in pET29a (+): *SacB-BsaI* (Dobrijevic *et al.*, 2020) giving as a result the pET29a_SacB_BsaI-PAE21 plasmid successfully expressed in *E. coli* BL21(DE3). The primers used are described in Table S 1 (Appendix 4), Section 2.3 in Chapter 2. No signal peptide was detected in PAE21, and the enzyme was cloned with a C-terminal His6-tag. The gene of 663 bp encodes a protein of 220 aa. Figure 4.9 shows the expression of PAE21 with a molecular weight of ~25 kDa. This enzyme has an optimum pH of 8 keeping around 90% of its activity at pH 7 and exhibits maximum activity at 50 °C. In addition, it presents good thermal stability up to 50 °C (Remoroza *et al.*, 2014)

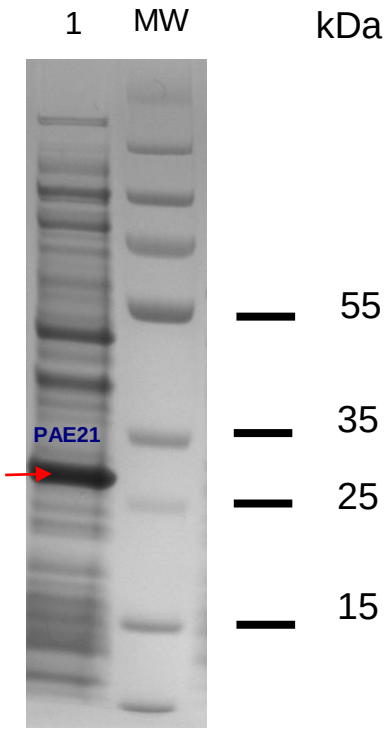

Figure 4.9. SDS-PAGE showing the expression of PAE21 from *Bacillus licheniformis* DSM 13 in *E. coli* BL21(DE3). Lane 1: clarified lysate, fifteen micrograms of protein were loaded. MW, molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa). Molecular weight of PAE21 is ~25 kDa. SDS-PAGE analysis was carried out as described in Section 2.9.2 in Chapter 2. PAE: pectin acetylesterase.

4.3.4 Synergistic action between an exo-PGs and PME as co-expressed enzymes along with a PAE and AF

As described in Section 4.3.2, low GalA concentrations were released by co-expression constructs using sugar beet pectin. It was hypothesised that the addition of a PAE into the reactions will deacetylate sugar beet pectin improving GalA release. Thus, the PAE21 from *Bacillus licheniformis* was successfully cloned and expressed. The capacity of this enzyme to deacetylate sugar beet pectin was tested using different activities at pH 7, 50 °C and 300 rpm until 24. It was found that 0.022 U mL^{-1} of PAE21 released 0.4 mM acetic acid after 4 h of reaction reaching a plateau at this time. Hence, this activity was used for the synergistic reactions.

Likewise, as sugar beet pectin contains highly branched arabinans formed by Ara in the RG-I of its structure, a thermophilic AF from *Geobacillus thermoglucosidasius* was also added into the synergistic reactions. AF catalyses the removal of Ara residues from arabinan side chains in RG-I (Cárdenas-Fernández *et al.*, 2017). It is expected that the addition of this AF depolymerises the RG-I and leaves the GalA backbone more accessible to the action of enzymes such as PAEs, PMEs and exo-PGs leading to a GalA release improvement (Figure 4.10). This enzyme has an optimum pH of 7 and exhibits maximum activity at 80 °C keeping around 80% of its activity at 50 °C. In addition, it presents good stability up to 50 °C (Cárdenas-Fernández *et al.*, 2018). Before carrying out the synergistic reactions, different activities of AF were assayed to test its capacity to remove Ara residues from sugar beet pectin. The maximum quantified amount of released Ara was 0.2 mM using 0.1 U mL^{-1} of AF. Hence, this activity was used for the synergistic reactions.

Synergistic reactions between co-expression constructs 3 and 4 along either with PAE21 or PAE21 + AF were carried out at pH 7 at 50 °C and 300 rpm until 24 h. These conditions of pH and temperature were chosen to ensure the activity of all the enzymes involved in the synergistic reactions. Methanol release was 2 mM (Figure 4.11 A) similar to the concentration released using only the co-expressed enzymes (Figure 4.8 A). Thus, the addition of either PAE21 or PAE21 + AF into the synergistic reactions did not affect BLI09

activity. While acetic acid release was 0.6 mM after 4 h of reaction, reaching a plateau at this point (Figure 4.11 B). This value was higher (1.5-fold) than that obtained using only PAE21 in sugar beet pectin (0.4 mM after 4 h of reaction). This result may be explained by the presence of BLI09 in the synergistic reactions, which demethylates pectin enhancing PAE21 activity. It has been reported that PAEs exhibit higher activity in low methylated substrates (Remoroza *et al.*, 2014). Also, it was noticed that the addition of AF did not affect PAE21 activity.

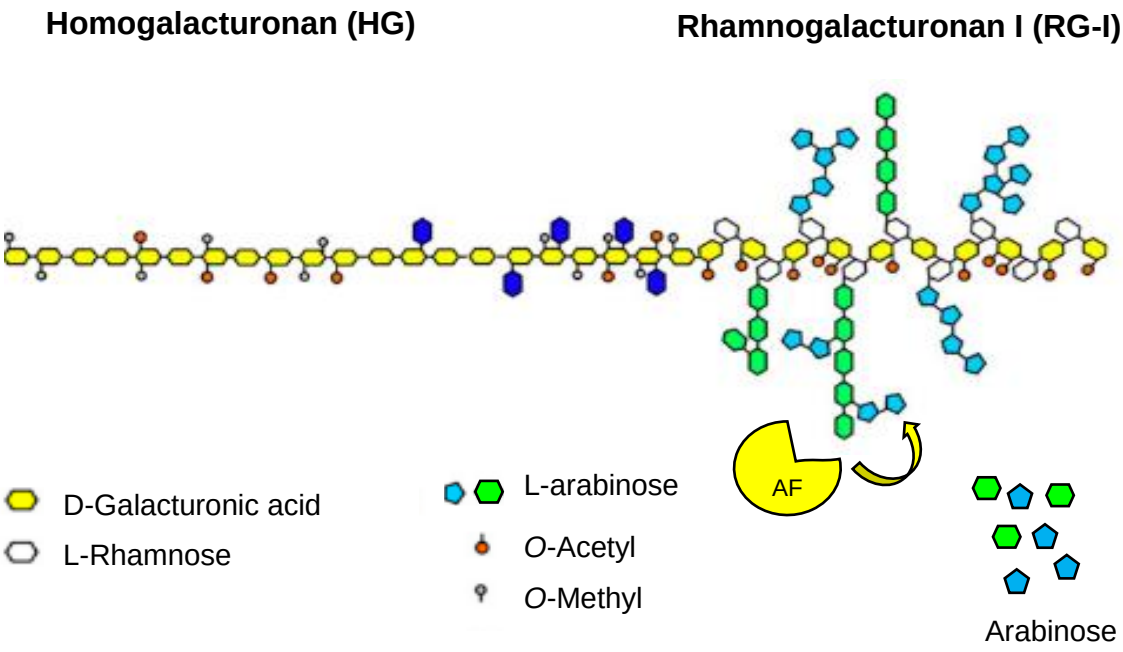

Figure 4.10. AF activity on the RG-I backbone catalysing the removal of Ara residues from arabinan side chains.

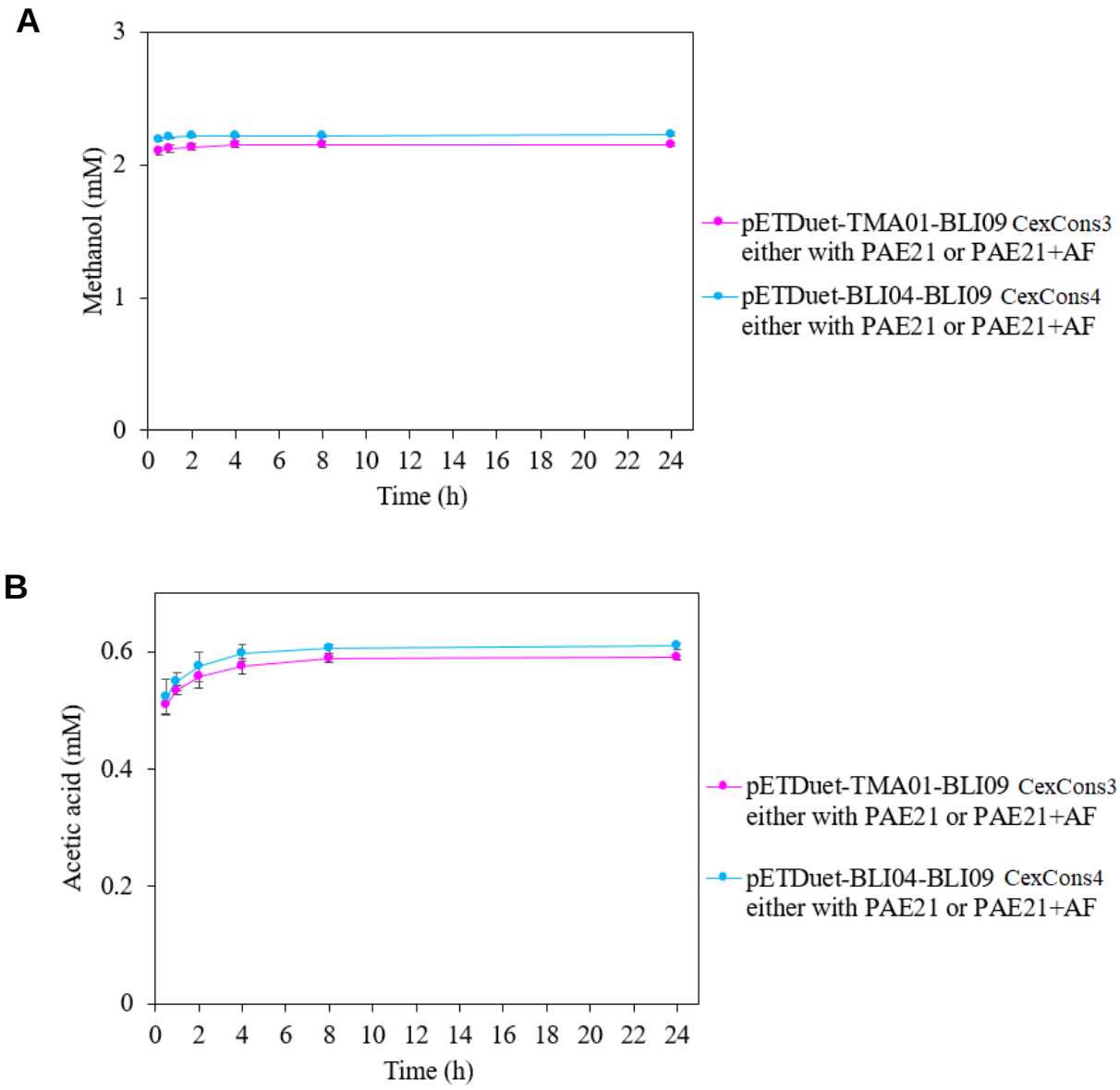

Figure 4.11. Synergistic activity between pectinases from co-expression constructs 3 and 4 along either with PAE21 or PAE21 + AF using sugar beet pectin. (A) Methanol and (B) acetic acid quantification. The reactions were carried out at 50 °C using 0.5% (w/v) substrate in 100 mM phosphate buffer pH 7; and with 0.5 and 2 U mL^{-1} of TMA01 and BLI04, respectively. The activity of BLI09 was calculated in the volumes containing the U mL^{-1} of exo-PGs mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.8.2; and methanol and acetic acid were quantified following the procedures described in Sections 2.9.4 and 2.9.6, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). CexCons: co-expression construct. PAE: pectin acetylesterase, AF: arabinofuranosidase.

Regarding Ara, the maximum released amount was around 0.2 mM (Figure 4.12 A). In sugar beet pectin from biomass, it has been determined that the percentage of Ara as a monomer is almost the double (23%) respect to GalA (14%) (Cárdenas-Fernández *et al.*, 2017). Hence, it was expected that higher Ara concentrations than GalA would be released. However, these low values may be explained due to the commercial sugar beet pectin used in this experiment has been chemically treated to have a predominant GalA content (65%). Despite sugar beet pectin was both demethylated and deacetylated and Ara was released, low GalA concentrations were still released. Maximum concentrations of around 0.6 (8%) and 0.8 mM (10%) GalA were quantified using co-expression constructs 3 and 4 along either with PAE21 or PAE21 + AF, respectively (Figure 4.12 B). Thus, the addition of the AF and PAE21 did not considerably enhance the amount of GalA released. A possible explanation might be due to PAE21 mechanism of action and the position of acetyl groups in GalA residues. It has been reported that PAE21 deacetylate sugar beet pectin at *O-3* positions in non-methylated GalA residues (Remoroza *et al.*, 2014). Based on this information, it is suggested that even if *O-3* positions are deacetylated, *O-2* positions will remain acetylated. This *O-2* acetylation may hinder exo-PGs activity and therefore GalA release. Another possible explanation might be the distribution of acetyl groups in the substrate associated to the deacetylation pattern of PAEs. A blockwise distribution of acetyl groups in HG from commercial sugar beet pectin has been reported (Ralet, Crépeau and Bonnin, 2008). PAEs, similar to PMEs, have a random or blockwise deacetylation pattern. Therefore, if acetyl groups in sugar beet pectin are blockwise distributed and assuming that PAE21 deacetylates in a random manner, blocks of deacetylated pectin will not be produced. Thus, the presence of acetyl groups will obstruct exo-PGs activity and GalA release (Figure 4.13). Finally, the low GalA release might be explained by the presence of exo-PGs inhibitors in the commercial sugar beet pectin used as substrate in this experiment.

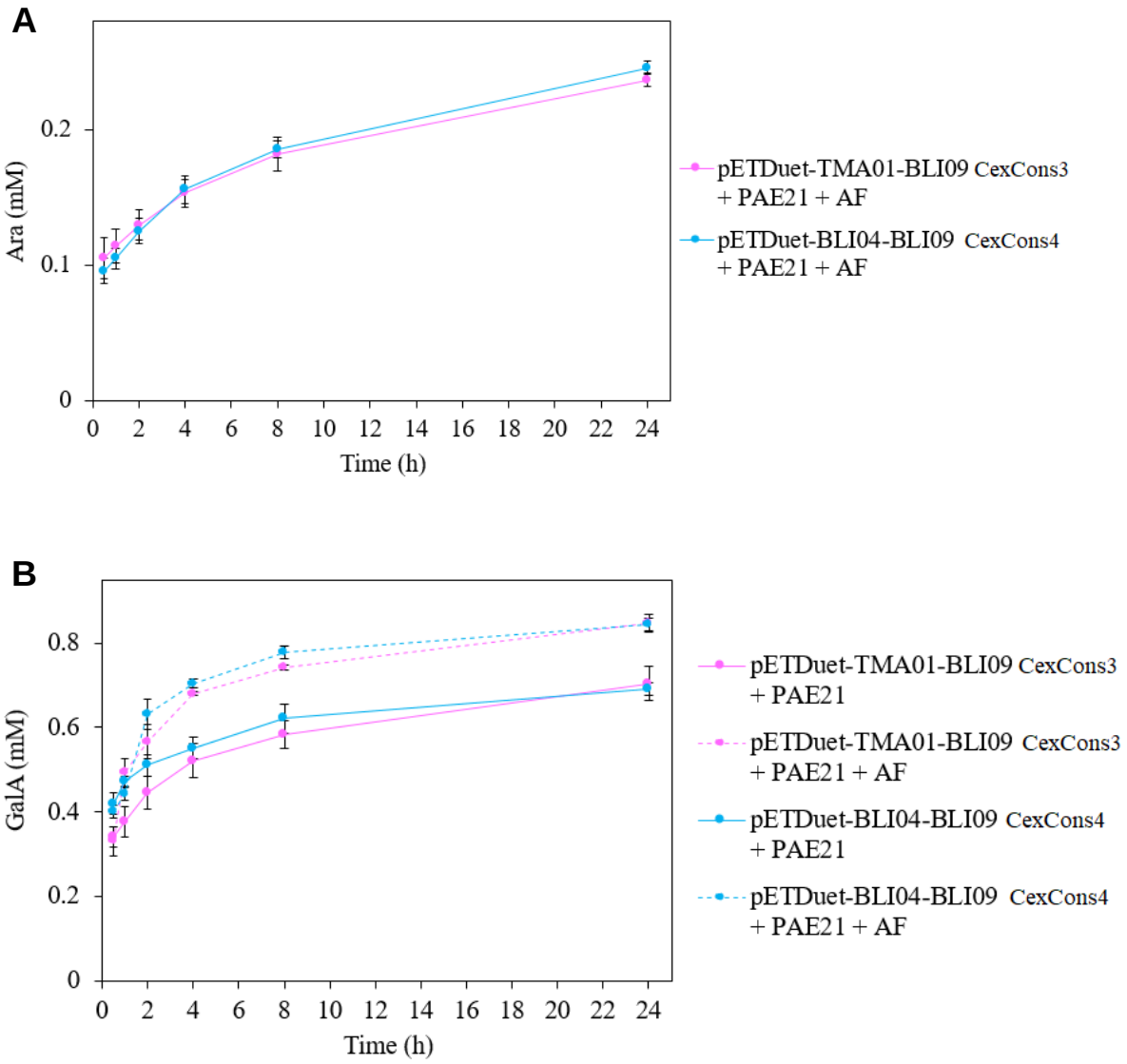

Figure 4.12. Synergistic activity between pectinases from co-expression constructs 3 and 4 along either with PAE21 or PAE21 + AF using sugar beet pectin. (A) Ara and (B) GalA quantification. The reactions were carried out at 50 °C using 0.5% (w/v) substrate in 100 mM phosphate buffer pH 7; and with 0.5 and 2 U mL^{-1} of TMA01 and BLI04, respectively. The activity of BLI09 was calculated in the volumes containing the U mL^{-1} of exo-PGs mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.8.2; and Ara and GalA were quantified following the procedures described in Sections 2.9.5. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). CexCons: co-expression construct. PAE: pectin acetylesterase, AF: arabinofuranosidase, GalA: galacturonic acid, Ara: arabinose.

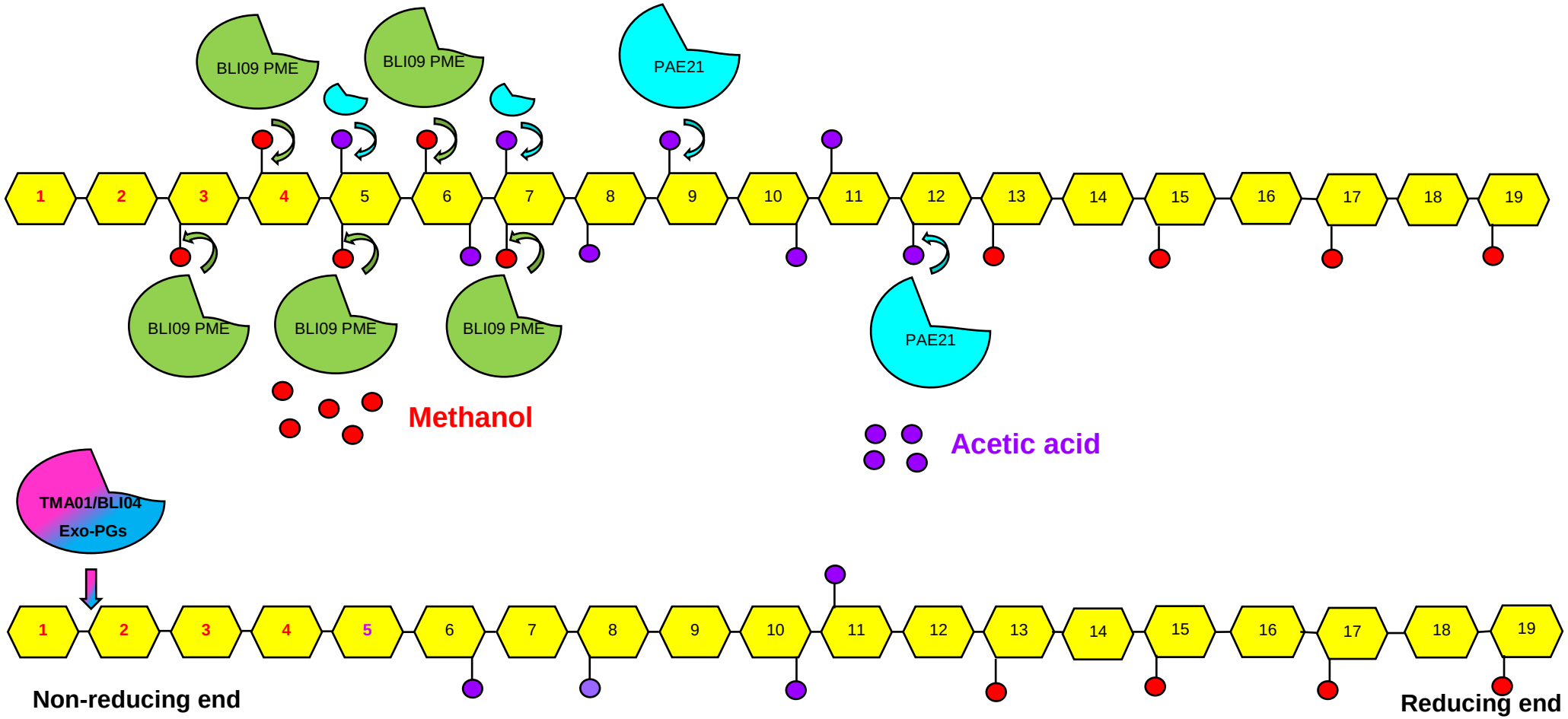

Figure 4.13. Synergistic action between BLI09 PME either with TMA01 or BLI09 exo-PGs as co-expressed enzymes along with PAE21 on the HG backbone in sugar beet pectin. It is illustrated the blockwise demethylation pattern of BLI09 producing fragments of non-methylated pectin, the blockwise distribution of acetyl groups in sugar beet pectin and the deacetylation of PAE21 at *O-3* positions of non-methylated GalA suggesting a random deacetylation pattern. In addition, it is presented the exo-PGs action in the non-reducing end of demethylated and deacetylated pectin leading to monomeric GalA release. No major increase in GalA release was observed with the addition of PAE21 into the synergistic reactions. GalA monomers highlighted in red (from 1 to 4) can be released by BLI09 paired either with TMA01 or BLI04. While the additional GalA monomer highlighted in purple (number 5) can be released with the addition of PAE21. (⬡) GalA, (🍒) methyl groups and (🍒) acetyl groups. PME: pectin methylesterase, PAE: pectin acetylesterase, exo-PG: exo-polygalacturonase, HG: homogalacturonan and GalA: galacturonic acid.

4.3.5 Product inhibition assays

Product inhibition assays using methanol, GalA and acetic acid were tested in pectinases from co-expression constructs 3 and 4 since they were the most efficient to GalA release. These assays were carried out using several concentrations of each inhibitor and 0.5% (w/v) apple pectin as substrate.

4.3.5.1 Methanol and galacturonic acid effect on PMEs and exo-PGs

Figures 4.13 and 4.14 present the effect of methanol on pectinases of co-expression constructs 3 and 4, respectively. Methanol had an inhibitory effect on BLI09 from around 6 mM causing almost a complete inhibition from 14 mM (Figures 4.13 A and 4.14 A). Respecting TMA01 and BLI04, a slight inhibitory effect of methanol was observed from 7 mM (Figures 4.13 B and 4.14 B). These findings indicate that BLI09, TMA01 and BLI04 were not inhibited by this product during the synergistic reactions using the co-expression constructs 3 and 4 where 4 mM methanol was released.

Figures 4.15 and 4.16 show the effect of GalA on pectinases of co-expression constructs 3 and 4, respectively. Concentrations higher than 7 mM inhibited BLI09 (Figures 4.15 A and 4.16 A) indicating that this enzyme was not inhibited by the 3 mM GalA released during the synergistic reactions. GalA concentrations higher than 3 and 2.5 mM GalA inhibited TMA01 and BLI04, respectively (Figures 4.15 B and 4.16 B). Thus, product inhibition might explain why only GalA below 3 and 2.5 mM were achieved in the synergistic reactions with TMA01 and BLI04, respectively. Competitive inhibition by GalA has been reported for some exo-PGs (Kester *et al.*, 1996; Baciu and Jördening, 2004; Bélafi-Bakó *et al.*, 2007). In competitive inhibition, the inhibitor binds to the active site of the enzyme by competing with the substrate. The inhibition effect can be reduced by increasing substrate concentration (Baciu and Jördening, 2004). This type of inhibition could be tackled through a simultaneous reaction and product recovery. Although synergistic action between PMEs and exo-PGs is a promising strategy to release monomeric GalA from pectin, factors such as product inhibition limit the efficiency of the process.

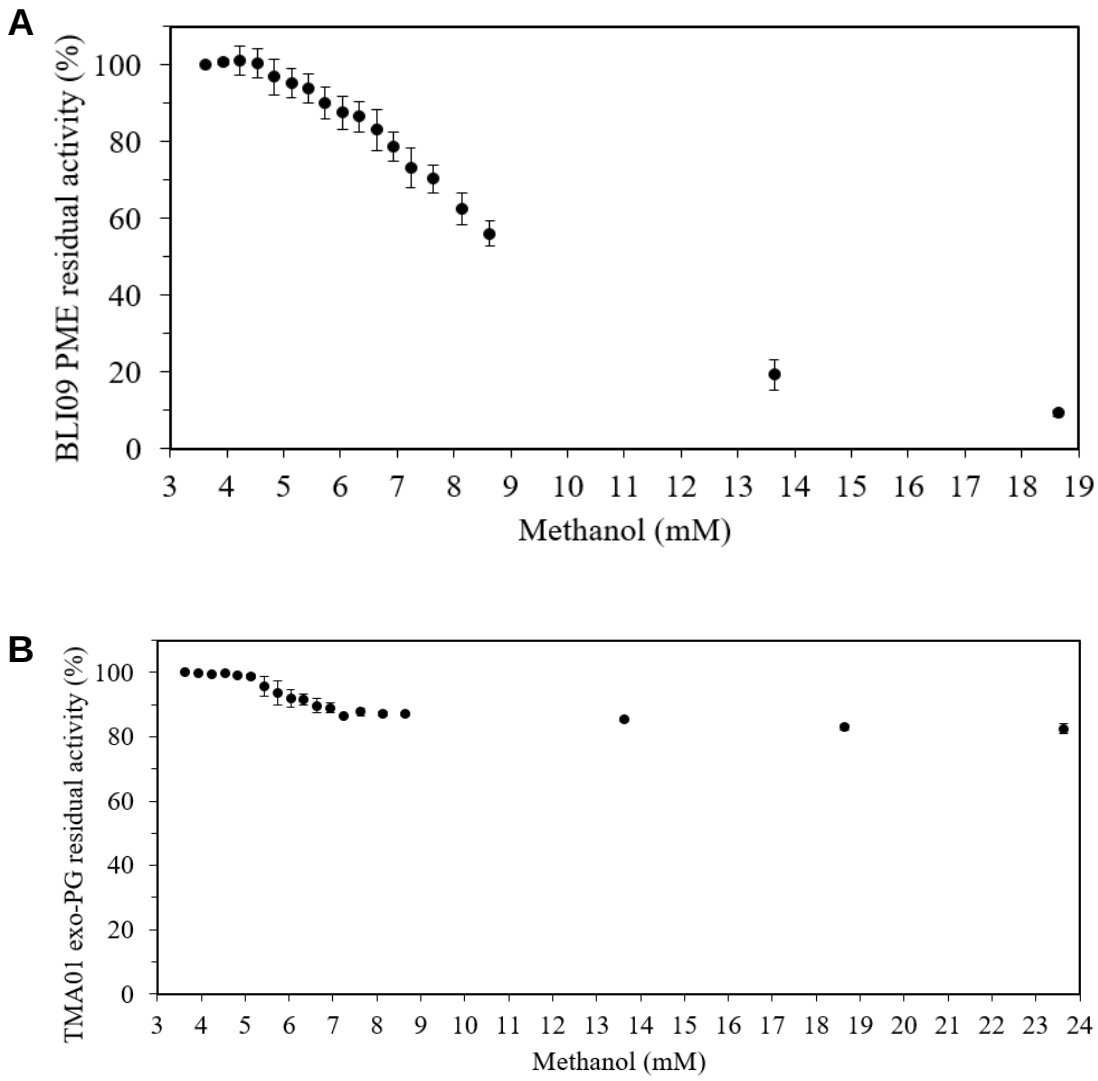

Figure 4.14. Product inhibition effect of methanol on pectinases of co-expression construct 3. (A) Methanol effect on BLI09 and (B) TMA01. The reactions were carried out at 50 °C and 300 rpm for 30 min using 0.5% (w/v) apple pectin in 20 mM phosphate buffer pH 7; and with 0.5 U mL^{-1} of TMA01. The activity of BLI09 was calculated in the volume containing the U mL^{-1} of TMA01 mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.7.7.1. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). PME: pectin methylesterase, exo-PG: exo-polygalacturonase.

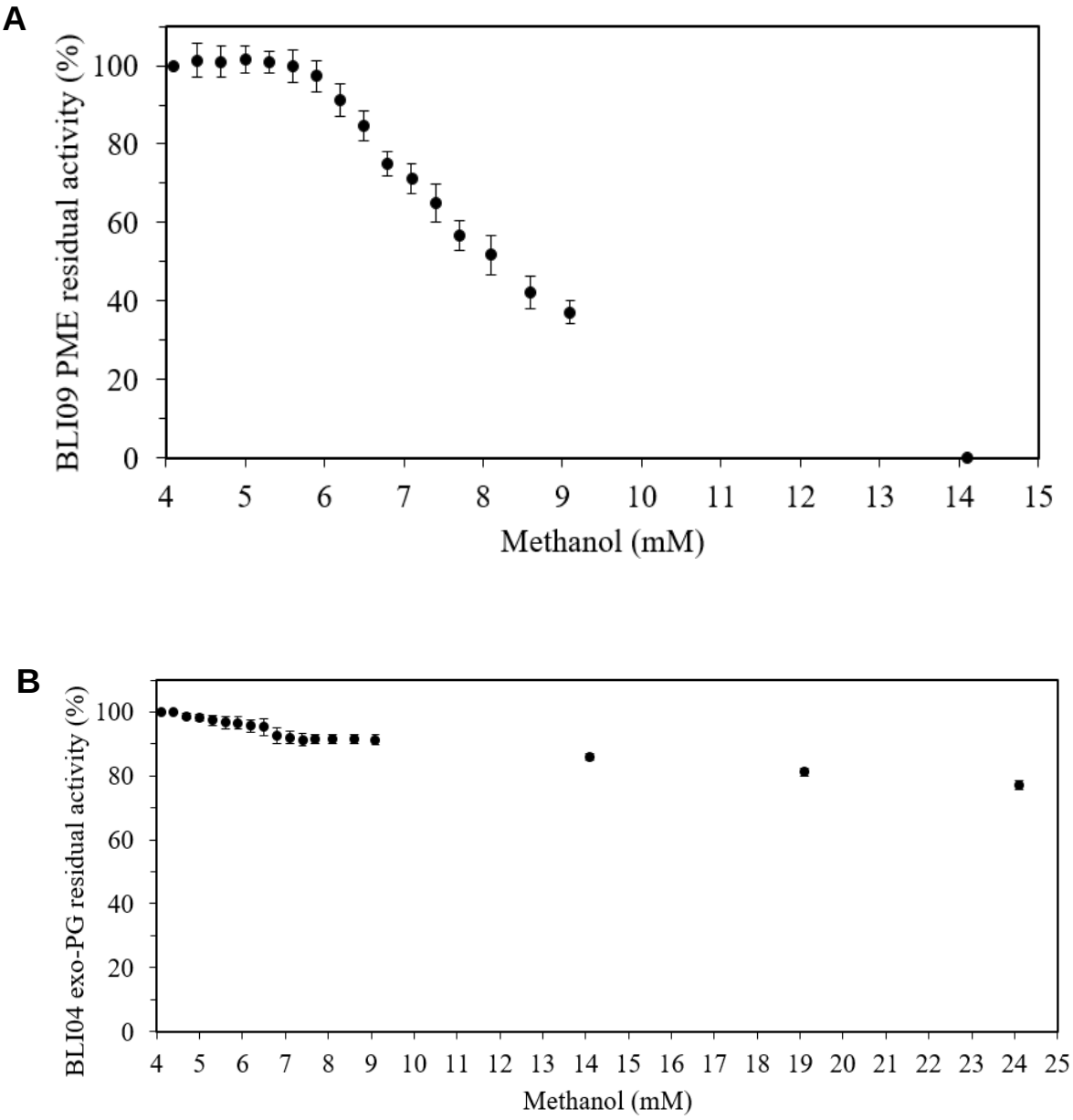

Figure 4.15. Product inhibition effect of methanol on pectinases of co-expression construct 4. (A) Methanol effect on BLI09 and (B) BLI04. The reactions were carried out at 50 °C and 300 rpm for 30 min using 0.5% (w/v) apple pectin in 20 mM phosphate buffer pH 7; and with 2 U mL^{-1} of BLI04. The activity of BLI09 was calculated in the volume containing the U mL^{-1} of BLI04 mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.7.7.1. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). PME: pectin methylesterase, exo-PG: exo-polygalacturonase.

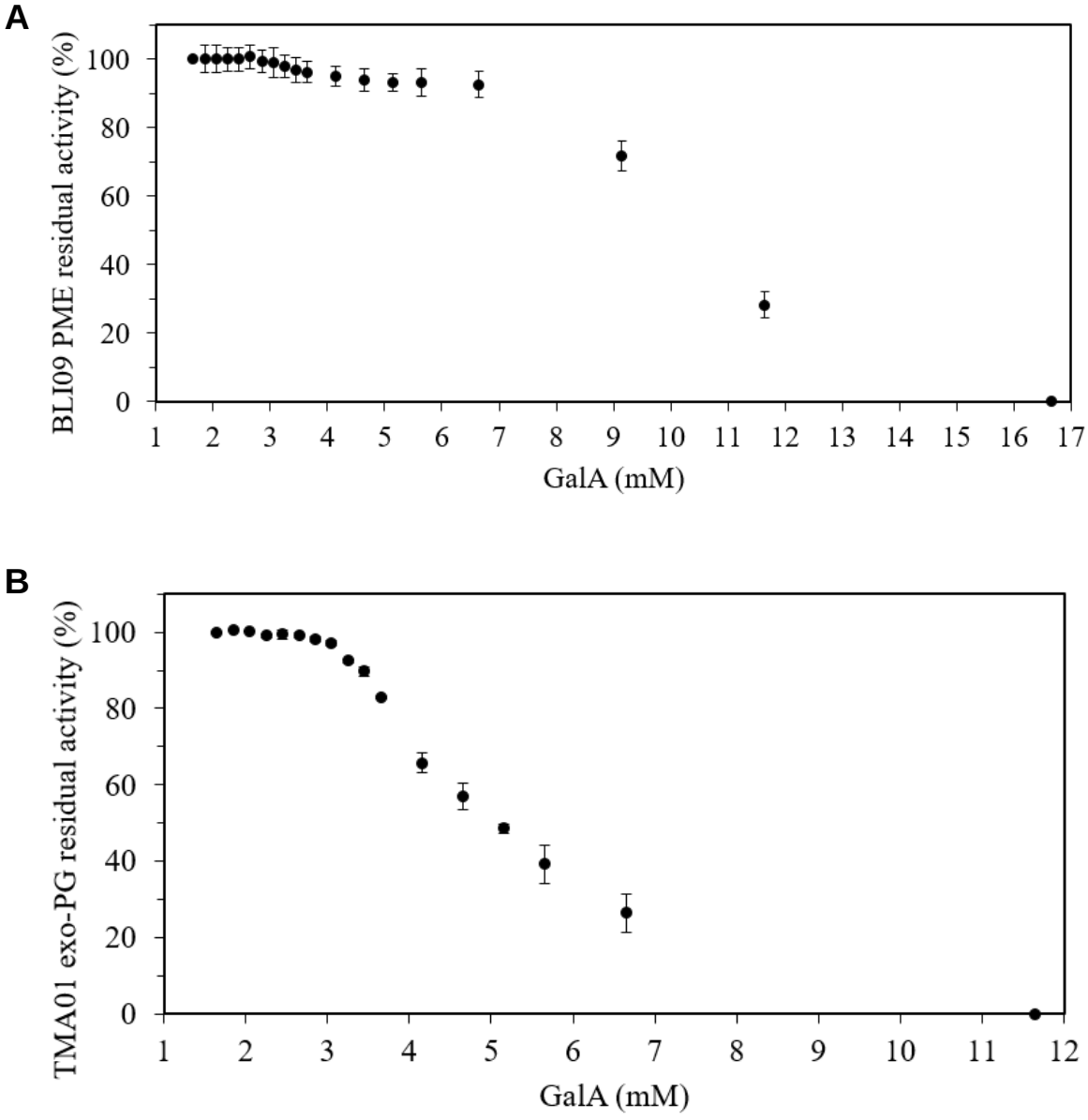

Figure 4.16. Product inhibition effect of GalA on pectinases of co-expression construct 3. (A) GalA effect on BLI09 and (B) TMA01. The reactions were carried out at 50 °C and 300 rpm for 30 min using 0.5% (w/v) apple pectin in 20 mM phosphate buffer pH 7; and with 0.5 U mL^{-1} of TMA01. The activity of BLI09 was calculated in the volume containing the U mL^{-1} of TMA01 mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.7.7.1. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). PME: pectin methylesterase, exo-PG: exo-polygalacturonase, GalA: galacturonic acid.

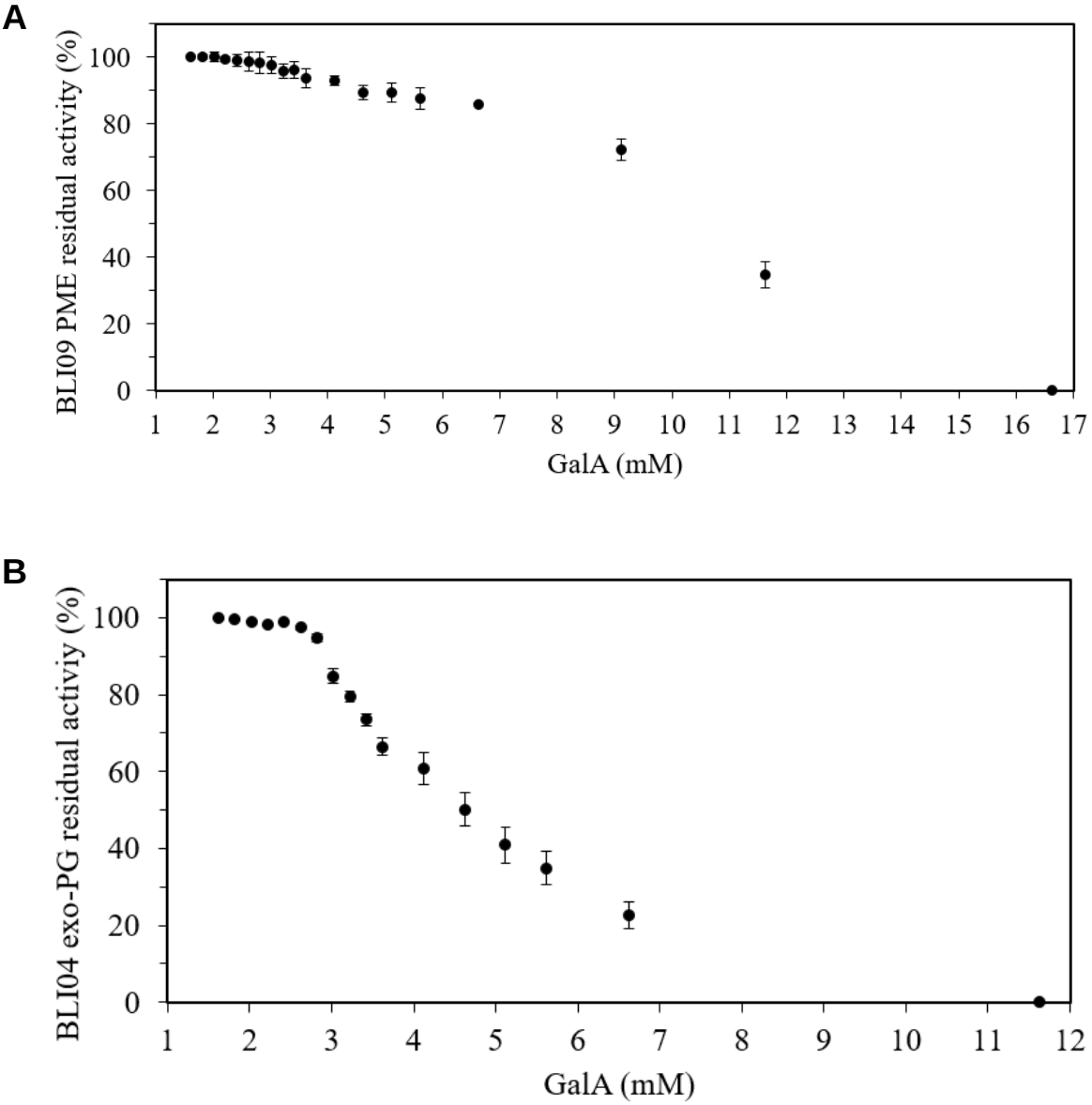

Figure 4.17. Product inhibition effect of GalA on pectinases of co-expression construct 4. (A) GalA effect on BLI09 and (B) TMA01. The reactions were carried out at 50 °C and 300 rpm for 30 min using 0.5% (w/v) apple pectin in 20 mM phosphate buffer pH 7; and with 2 U mL^{-1} of BLI04. The activity of BLI09 was calculated in the volume containing the U mL^{-1} of BLI04 mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.7.7.1. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). PME: pectin methylesterase, exo-PG: exo-polygalacturonase, GalA: galacturonic acid.

4.3.5.2 Acetic acid effect on PMEs and exo-PGs

Low GalA concentrations were released in the synergistic reaction between co-expressed pectinases along with PAE21 using sugar beet pectin (Figure 4.11 B in Section 4.3.4), which might be due to a potential inhibition of the enzymes by the acetic acid released during the reactions. Figures 4.17 and 4.18 present the

effect of acetic acid on pectinases of co-expression constructs 3 and 4, respectively. Our results showed that BLI09, TMA01 and BLI04 maintained more than 90% of its activity up to 10 mM acetic acid concentration. These findings indicated that the released acetic acid during the synergistic reactions (0.6 mM) did not inhibit BLI09, TMA01 and BLI04.

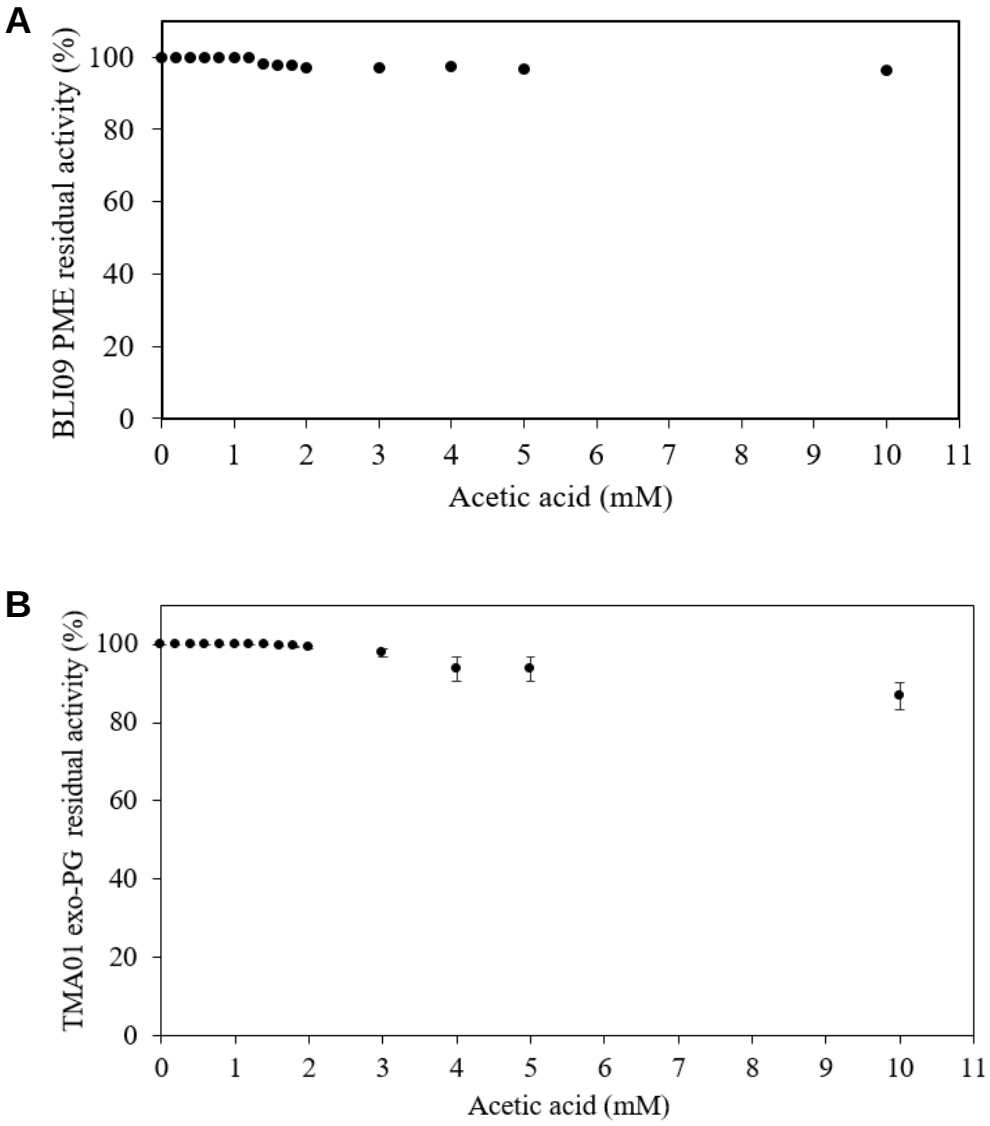

Figure 4.18. Product inhibition effect of acetic acid on pectinases of co-expression construct 3. (A) Acetic acid effect on BLI09 and (B) TMA01. The reactions were carried out at 50 °C and 300 rpm for 30 min using 0.5% (w/v) apple pectin in 20 mM phosphate buffer pH 7; and with 0.5 U mL^{-1} of TMA01. The activity of BLI09 was calculated in the volume containing the U mL^{-1} of TMA01 mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.7.7.2. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). PME: pectin methylesterase, exo-PG: exo-polygalacturonase.

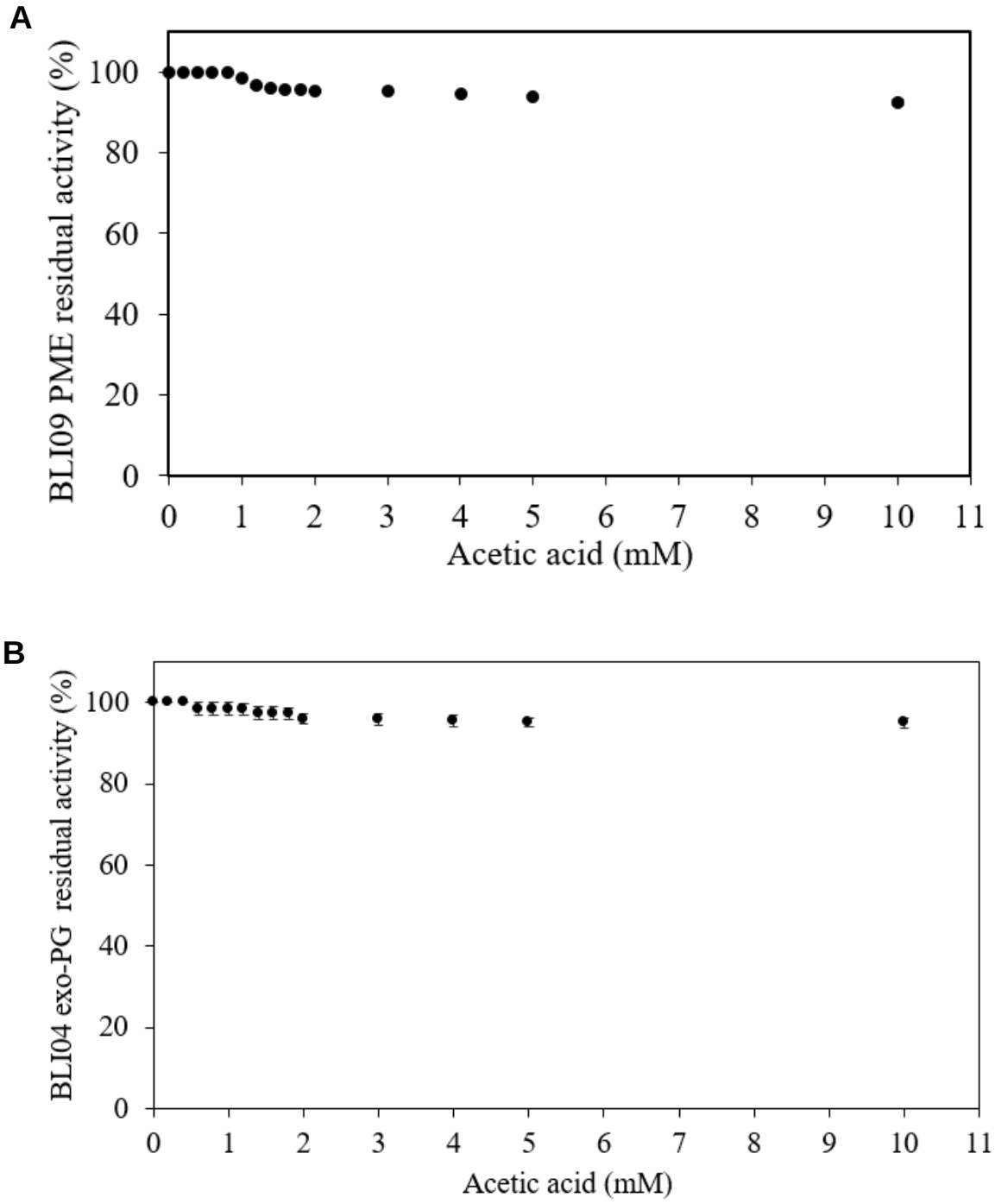

Figure 4.19. Product inhibition effect of acetic acid on pectinases of co-expression systems 2. (A) Acetic acid effect on BLI09 and (B) BLI04. The reactions were carried out at 50 °C and 300 rpm for 30 min using 0.5% (w/v) apple pectin in 20 mM phosphate buffer pH 7; and with 2 U mL^{-1} of BLI04. The activity of BLI09 was calculated in the volume containing the U mL^{-1} of BLI04 mentioned before (Table 2.4, Section 2.8.1 in Chapter 2). The assay was performed as described in Section 2.7.7.2. Methanol and GalA were quantified following the procedures described in Sections 2.9.4 and 2.9.5, respectively. All these sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). PME: pectin methylesterase, exo-PG: exo-polygalacturonase.

4.4 Conclusions

The aim of this chapter was to co-express a thermophilic PME (BLI09) and exo-PG (TMA01 or BLI04) in a single plasmid and host for a cost-effective pectin bioconversion into GalA. In addition, it was to evaluate the addition of PAE21 and AF into the reactions to improve GalA release from sugar beet pectin. Four co-expression plasmids containing a PME and exo-PG were constructed. In co-expression constructs 1 and 2 (pETDuet-BLI09-TMA01 and pETDuet-BLI09-BLI04, respectively), BLI09 was cloned in MCS-1 and TMA01 and BLI04 in MCS-2. In co-expression constructs 3 and 4 (pETDuet-TMA01-BLI09 and pETDuet-BLI04-BLI09, respectively), the cloning order of the genes was inverted with respect to co-expression constructs 1 and 2. In all the co-expression constructs, each gene was under the control of its own T7 promoter and both shared one T7 terminator. Regarding the pectinases expression, TMA01 and BLI04 were well expressed in all the co-expression constructs. The expression results indicated that for exo-PGs expression, the cloning order of the genes and the presence of a T7 terminator behind were not essential. On the other hand, BLI09 expression was enhanced and more successfully in co-expression constructs 3 and 4 where the enzyme was cloned downstream exo-PGs in MCS-2 and with the presence of a T7 terminator behind. Strategies such as the addition of a T7 terminator behind BLI09 to improve its expression in co-expression constructs 1 and 2 were not assayed due to both enzymes were successfully expressed in co-expression constructs 3 and 4. Comparing the purified pectinases of the co-expression constructs, enzymatic activity and protein concentration of BLI09 were 8 and 9.5-fold higher in co-expression constructs 3 and 4 than in co-expression constructs 1 and 2, respectively with the highest values in co-expression construct 3 (2556 U mL^{-1} and 0.142 mg mL^{-1}). Meanwhile, similar enzymatic activity and protein concentration values were found for TMA01 and BLI04 in all co-expression systems (23 - 36 U mL^{-1} and 0.182 - 0.228 mg mL^{-1}). Specific activities for the BLI09, TMA01 and BLI04 were similar in all the co-expression constructs (17 800, 166 and 118 U mg^{-1}, respectively). Similarly, comparing the co-expressed enzymes with those individually expressed, lower enzymatic activity and protein concentrations were determined for BLI09 in co-expression constructs 3 and 4 (3.5 and 7.5-fold

lower, respectively) with respect to individual expression. For TMA01 and BLI04, enzymatic activity and protein concentration in co-expressed enzymes were similar to individual expression. Specific activity for the three pectinases in the co-expressed enzymes was similar to individual expression.

From all the synergistic reactions with the co-expression constructs using apple, citrus and sugar beet pectin; the most efficient for GalA release were co-expression constructs 3 and 4 which allowed to release 4 mM methanol as well as 3 and 2.5 mM GalA, respectively from apple and citrus pectin. These values were slightly higher than using individually expressed enzymes (3 mM methanol as well as 2.5 and 2 mM GalA in the reactions between BLI09 paired either with TMA01 or BLI04, respectively). From sugar beet pectin, despite it was demethylated only around 0.2 mM GalA was released by all co-expression constructs as well as by individually expressed enzymes. To improve GalA release from this substrate, PAE21 and AF were also added to the previous synergistic reactions. As a result of it, sugar pectin was demethylated (2 mM methanol), deacetylated (0.6 mM acetic acid) and Ara was released (~0.2 mM), but still low concentrations of GalA were quantified (0.6-0.8 mM). In summary, AF did not enhance GalA released from sugar beet pectin, since low concentrations of Ara were quantified which could be explained by its low percent in this commercial substrate. In addition, PAE21 did not improve GalA release and possible explanations could be related to PAEs mechanism of action and the distribution of acetyl groups in sugar beet pectin. PAE21 deacetylate pectin at *O-3* positions in non-methylated GalA and in a random deacetylation pattern. In addition, acetyl groups are in a blockwise distribution in sugar beet pectin. All of it might cause that some acetyl groups such as *O-2* positions remain in the pectin molecule, obstructing the exo-PGs activity and thus releasing low GalA concentrations. Another possible explanation might be the presence of inhibitors on this commercial substrate.

Product inhibition assays testing the effect of methanol on pectinases of co-expression constructs 3 and 4, showed that BLI09, TMA01 and BLI04 were not inhibited by this product during the synergistic reactions. Regarding GalA, BLI09 was not inhibited by this product. However, TMA01 and BLI04 were inhibited by GalA at concentrations higher than 3 mM and 2.5 mM, respectively, the same

maximum concentrations released because of the synergistic reactions using these enzymes. These results indicated that GalA release was limited by the product inhibition effect of this product on TMA01 and BLI04 which can be tackled through *in situ* the product recovery during the reactions. Product inhibition assays using acetic acid revealed that BLI09, TMA01 and BLI04 were not inhibited by this compound even at high concentrations (10 mM). It demonstrated that the acetic acid concentration released in the synergistic reactions with PAE21 (0.6 mM) was not related to the low GalA released from sugar beet pectin.

This chapter allowed co-expressing pectinases which act synergistically in a single host to obtain GalA from pectin-rich substrates. It makes possible the production of the two pectinases in a single culture reducing the production cost of the enzymes. Likewise, the results of pectin bioconversion into GalA using the clarified lysates of the co-expression constructs demonstrated to be comparable with those using the purified individually expressed enzymes. It contributes reducing the cost of enzymes purification. Finally, the study of the synergistic action between different kinds of pectinases contribute to a better understanding of pectin composition in the different substrates as well as of the mechanisms of action of pectinases. All this knowledge provides further insights for the applicability of pectinases and for improving pectin rich-biomass bioconversion into valuable compounds.

CHAPTER 5 THERMOPHILIC PECTATE LYASES AND THEIR SYNERGISTIC ACTION WITH PECTINESTERASES FOR IMPROVING PECTIN DEPOLYMERISATION

5.1 Introduction

Pectin depolymerisation is fundamental in a number of industrial processes playing an important role for improving quality and yield. In addition, small oligogalacturonates with potential bioactive properties could be produced as a result of pectin degradation (Yuan *et al.*, 2019). Pectin is a complex heteropolysaccharide formed by several substructures including HG, RG-I and RG-II, being HG the most abundant. HG is a backbone of a α-1,4-GalA that can be methylated or acetylated and accounts ~ 65% of the molecule (Chiliveri and Linga, 2014; Hugouvieux-Cotte-Pattat, Condemine and Shevchik, 2014; Zeuner *et al.*, 2020).

The most important pectinases act on the HG backbone among which are PMEs, PAEs, PGs and PGLs. PMEs and PAEs de-esterify pectin demethylating and deacetylating it, releasing methanol and acetic acid, respectively (Remoroza *et al.*, 2014, 2015). Meanwhile, PGs and PGLs catalyse the cleavage of the (1→4)-α-D-glycosidic bonds on the non-esterified HG. Regarding PGLs, these enzymes act through a transelimination mechanism releasing unsaturated products with a double bond between C4 and C5 of the residue situated at their non-reducing ends (Hamouda *et al.*, 2020; Li, Foucat and Bonnin, 2021; Sheladiya *et al.*, 2022). PGLs can exhibit and endo and exo activity, endo-PGLs act on a random manner giving unsaturated oligogalacturonates, while exo-PGLs catalyse the cleavage of unsaturated disaccharides (pectate disaccharide-lyases) or trisaccharides (pectate trisaccharide-lyases) from the reducing ends of pectin as the major products (Atanasova *et al.*, 2018).

Most of the commercial pectinases are from mesophilic bacteria and fungi, which are active at restricted conditions of pH and temperature. High temperatures improve processes in which pectin is involved since this substrate is more soluble at these conditions. There are scarce reports about thermophilic

pectinases, thus these enzymes represent an important contribution due to their potential applications (Li *et al.*, 2014; Su *et al.*, 2015; Atanasova *et al.*, 2018; Hamouda *et al.*, 2020). Moreover, the synergistic activity between thermophilic PGLs and esterases such as PMEs and PAEs constitutes a promising eco-friendly and more efficient alternative for pectin depolymerisation. This enzymatic pectin depolymerisation is attractive and useful in several fields including textile industry, paper making, coffee and tea fermentation, pectin wastewater treatment, juice industry, wine making, biorefineries and to produce oligosaccharides with potential bioactive properties (Chiliveri, Koti and Linga, 2016; Sharma, Patel and Sugandha, 2017; Espejo, 2021).

5.2 Aims and objectives

The aim of this chapter was to carry out functional characterisation of thermophilic PGLs as well as explore their synergistic activity with PMEs and a PAE for improving esterified pectin depolymerisation. The key objectives of the chapter are outlined below:

1. To purify and functionally characterise three thermophilic PGLs.
2. To assess the synergistic action between PGLs and PMEs individually expressed using apple, citrus and sugar beet pectin.
3. To assess the synergistic action between PGLs, PMEs and a PAE individually expressed to improve sugar beet pectin depolymerisation.
4. To evaluate product inhibition of acetic acid on PGLs individually expressed.

5.3 Results and discussion

5.3.1 Purification of individually cloned and expressed thermophilic PGLs

PGLs were cloned and expressed individually including a C-terminal His_6-tag. For characterisation purposes, they were fully purified by affinity chromatography by using a His_6-Tag Ni-affinity resin as specified in Section 2.6.1 in Chapter 2. All the studied enzymes were expressed in their soluble form.

TMA14 is a tetramer of ~ 151.2 kDa formed by monomers of ~ 40.6 kDa (Kluskens *et al.*, 2003). The single band of ~ 40 kDa observed in Figure 5.1 A corresponds to the size of TMA14 monomers. Figures 5.1 B and 5.1 C confirm the molecular weights reported by UniProtKB for TFU19 (53.6 kDa) and TFU20 (49.9 kDa), respectively. No enzymatic activity was detected in loading and washing fractions. After the purification process, around 90% of the three PGLs was recovered.

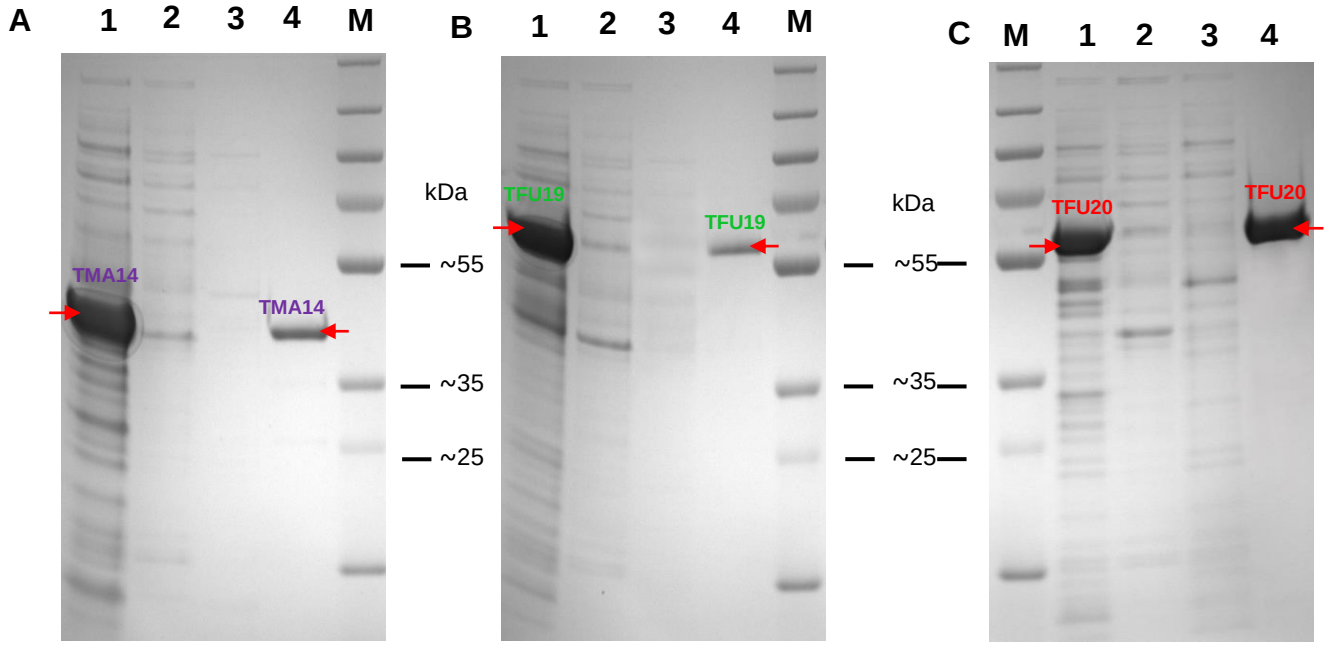

Figure 5.1. SDS-PAGE analysis of affinity chromatography purification from (A) TMA14, (B) TFU10 and (C) TFU20 PGLs. Lanes: 1, clarified cell lysate; 2, loading; 3, washing and 4, elution. Fifteen micrograms of protein were loaded per lane. M represents molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa). Molecular weights of TMA14, TFU19 and TFU20 are 40.6, 53.6 and 49.9 kDa, respectively. Enzymes purification and SDS-PAGE analysis were carried out as described in Sections 2.6.1 and 2.9.2, respectively in Chapter 2.

5.3.2 Functional characterization of thermophilic PGLs

5.3.2.1 Influence of Ca^{2+} and other ions on the activity of PGLs

PGLs activity depends on Ca^{+2} and not activity is detected in absence of this ion (Atanasova *et al.*, 2018; Hamouda *et al.*, 2020). Figure 5.2 presents the influence of different Ca^{2+} concentrations (from 0.25 to 1 mM) on PGLs activity (~0.25 U mL^{-1}). It was observed that the higher the Ca^{+2} concentration, the higher the PGLs activity. However, the enzymes exhibited only 50% of their activity with 0.25 mM Ca^{2+} and more than 90% with Ca^{+2} concentrations

between 0.6 and 1 mM. But no significant increase on the activity was observed between this range of concentrations (Tables S 5, S 6 and S 7, Appendix 9). In addition, high Ca^{2+} concentrations tend to bind to blocks of non-methylated pectin forming gels which could be an issue specially in synergistic reactions between PGLs and PMEs. Due to these reasons, 0.6 mM Ca^{2+} (for 0.25 U mL^{-1} of enzyme) was chosen as the optimum concentration for PGLs activity and this concentration was used for all the PGLs assays.

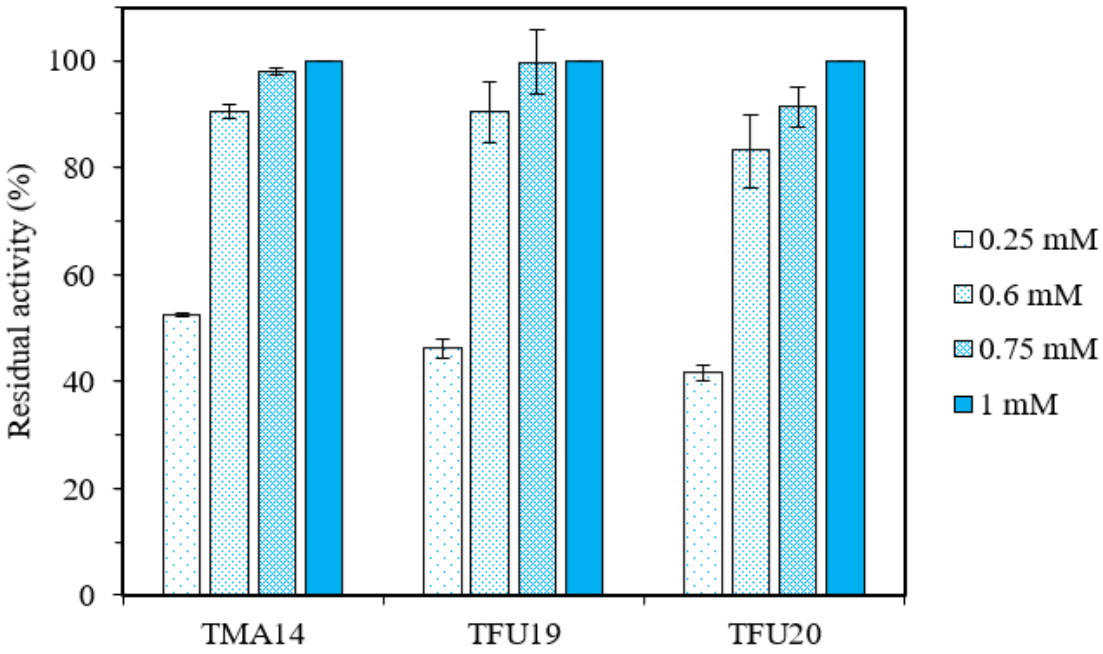

Figure 5.2. Influence of Ca^{2+} on the activity of TMA14, TFU19 and TFU20 PGLs. The enzymes were pre-incubated with the ion at room temperature for 15 min. The PGL activity was determined using 0.5% (w/v) polyGalA at pH 8 and 50 °C. Not PGL activity was observed in absence of Ca^{2+}. The residual activity was expressed as a percentage of the maximum activity. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.2 and 2.9.3.3 in Chapter 2. PolyGalA: polygalacturonic acid.

After determining the optimum Ca^{2+} concentration for PGLs activity, the influence of other ions such as Mg^{2+}, Mn^{2+} and Zn^{2+} was tested at 1 mM in presence of 0.6 mM Ca^{2+} (Figure 5.3). TMA14, TFU19 and TFU20 were completely inhibited by Zn^{+2}, while Mg^{2+} produced a slight increase on TFU20 activity (1.3-fold) and did not affect the activity of the other PGLs. Meanwhile, Mn^{2+} produced a significant improvement on the activity of TMA14 (1.3-fold) (Table S 8, Appendix 9) and on the activity of TFU20 (2.4-fold) (Table S 9, Appendix 9). However, this ion inhibited the activity of TFU19 by around 40%.

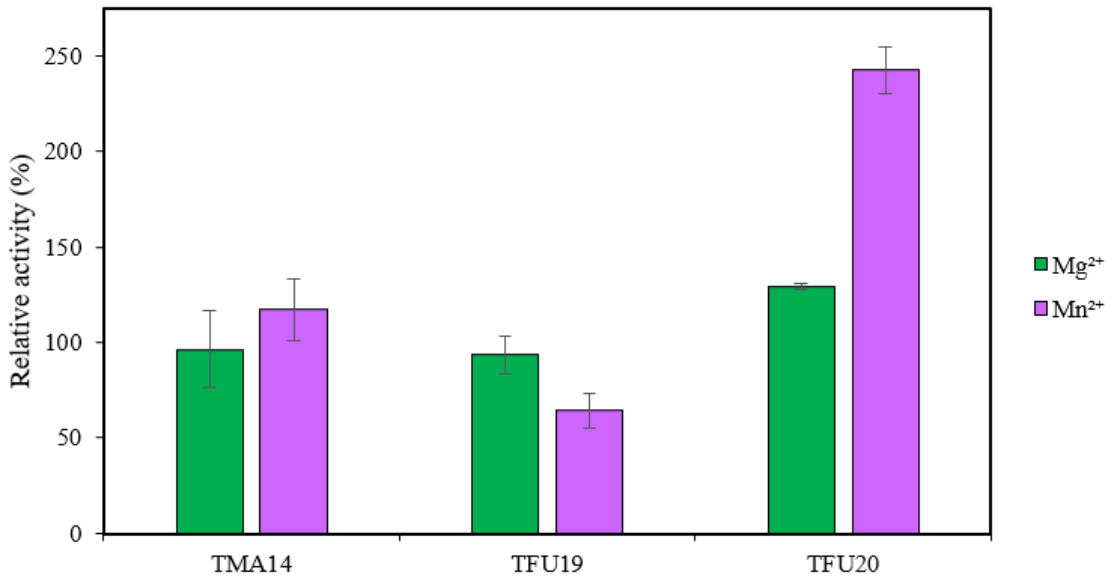

Figure 5.3. Influence of ions on the activity on TMA14, TFU19 and TFU20 PGLs at 1 mM. The enzymes were pre-incubated with the ions at room temperature for 15 min in presence of 0.6 mM Ca^{2+}. The PGL activity was determined using 0.5% (w/v) polyGalA at pH 8 and 50 °C. Zn^{2+} inhibited completely the activity of the enzymes. The relative activity was expressed as the percentage of activity compared with a control in presence of only 0.6 mM Ca^{2+}. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.2 and 2.9.3.3 in Chapter 2. PolyGalA: polygalacturonic acid.

Due to 1 mM Mg^{2+} increased the activity of TFU20, additional concentrations such as 0.25, 0.5 and 0.75 mM were assayed to determine if a further rise could be achieved (Figure 5.4). However, no major increase was observed and due to 1 mM is a high concentration and increased TFU20 activity in only 30%, this ion was not considered for adding in the subsequent reactions of this enzyme. Similarly, due to 1mM Mn^{2+} improved the activity of TMA14 and TFU20, concentrations of 0.25, 0.5 and 0.75 mM were also tested (Figure 5.5). It was found that the optimum Mn^{2+} concentration for TMA14 was 0.25 mM (for 0.25 U mL^{-1} of enzyme), increasing its activity by 1.3-fold. While the optimum concentration of this ion for TFU20 was 0.5 mM (for 0.25 U mL^{-1} of enzyme) increasing its activity by 2.5-fold. These optimum Mn^{2+} concentrations for TMA14 and TFU20 were added in all the subsequent reactions of these enzymes. In previous studies, the reported PGLs have been inhibited by most of the divalent cations, although Mn^{2+} and Mg^{2+} have increased the activity of some of them (Hamouda *et al.*, 2020; Zheng *et al.*, 2020; Zheng, Guo, *et al.*, 2021; Sheladiya *et al.*, 2022).

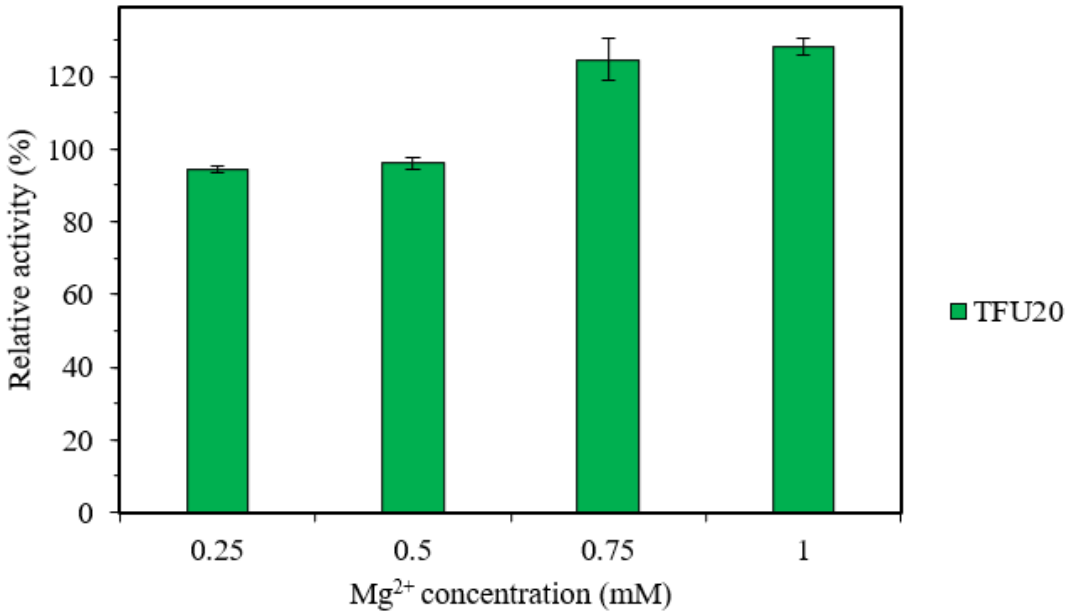

Figure 5.4. Influence of Mg^{2+} on the activity on TFU20 PGL. The enzyme was pre-incubated with the ion at room temperature for 15 min in presence of 0.6 mM Ca^{2+}. The PGL activity was determined using 0.5% (w/v) polyGalA at pH 8 and 50 °C. The relative activity was expressed as the percentage of activity compared with a control in presence of only 0.6 mM Ca^{2+}. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.2 and 2.9.3.3 in Chapter 2. PolyGalA: polygalacturonic acid.

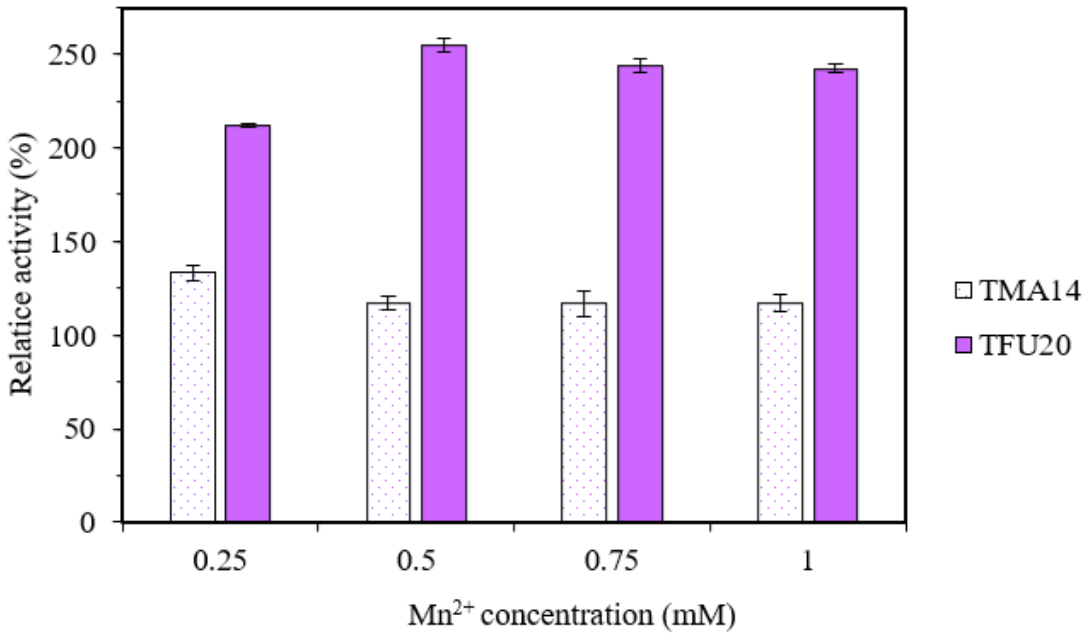

Figure 5.5. Influence of Mn^{2+} on the activity on TMA14 and TFU20 PGLs. The enzymes were pre-incubated with the ion at room temperature for 15 min in presence of 0.6 mM Ca^{2+}. The PGL activity was determined using 0.5% (w/v) polyGalA at pH 8 and 50 °C. The relative activity was expressed as the percentage of activity compared with a control in presence of only 0.6 mM Ca^{2+}. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.2 and 2.9.3.3 in Chapter 2. PolyGalA: polygalacturonic acid.

5.3.2.2 Influence of pH on the activity of pectate lyases

TMA14 was active at mild acidic, neutral, and alkaline conditions in a range of pH between 6 and 12, exhibiting more than 50% of its maximum activity at neutral and alkaline conditions between pH 7 and 11. The optimum pH for this enzyme was 10 (Figure 5.6 A). TFU19 was active in a range of pH between 7 and 12. However, it presented only 7% of its maximum activity at pH 7 being more active at alkaline conditions. The optimum pH for this enzyme was also 10 and kept 80% of its maximum activity at pH 9 and 11 (Figure 5.6 B). TFU20 presented activity at mid acidic, neutral and mild alkaline conditions in a range of pH between 6 and 10 being more active at these last two conditions. The optimum pH for this enzyme was 7, retaining around 70% of its maximum activity at pH 8 (Figure 5.6 C). According to the reported literature, most PGLs have shown greater activity at alkaline conditions at pH values above 8 and only a few of them are neutral or acidic (Atanasova *et al.*, 2018; Zheng, Xu, *et al.*, 2021). In this study, TMA14 and TFU19 were more active at alkaline conditions and TFU20 at neutral conditions. Despite TFU20 is produced by *Thermobifida fusca* same as TFU19, both exhibited different pH activity profiles. Alkaline thermophilic PGLs are attractive in textile industry (for retting and degumming vegetal fibres), paper making, coffee and tea fermentation, and pectin wastewater treatment. While neutral and acidic PGLs are used mainly in juice industry and wine making (Chiliveri and Linga, 2014; Chiliveri, Koti and Linga, 2016; Zheng *et al.*, 2020; Subash and Muthiah, 2021). Pectinases active in a wide range of pH could be useful for the production of oligogalacturonates with bioactive properties known as POS (Yuan *et al.*, 2019; Zheng, Guo, *et al.*, 2021; Zheng, Xu, *et al.*, 2021).

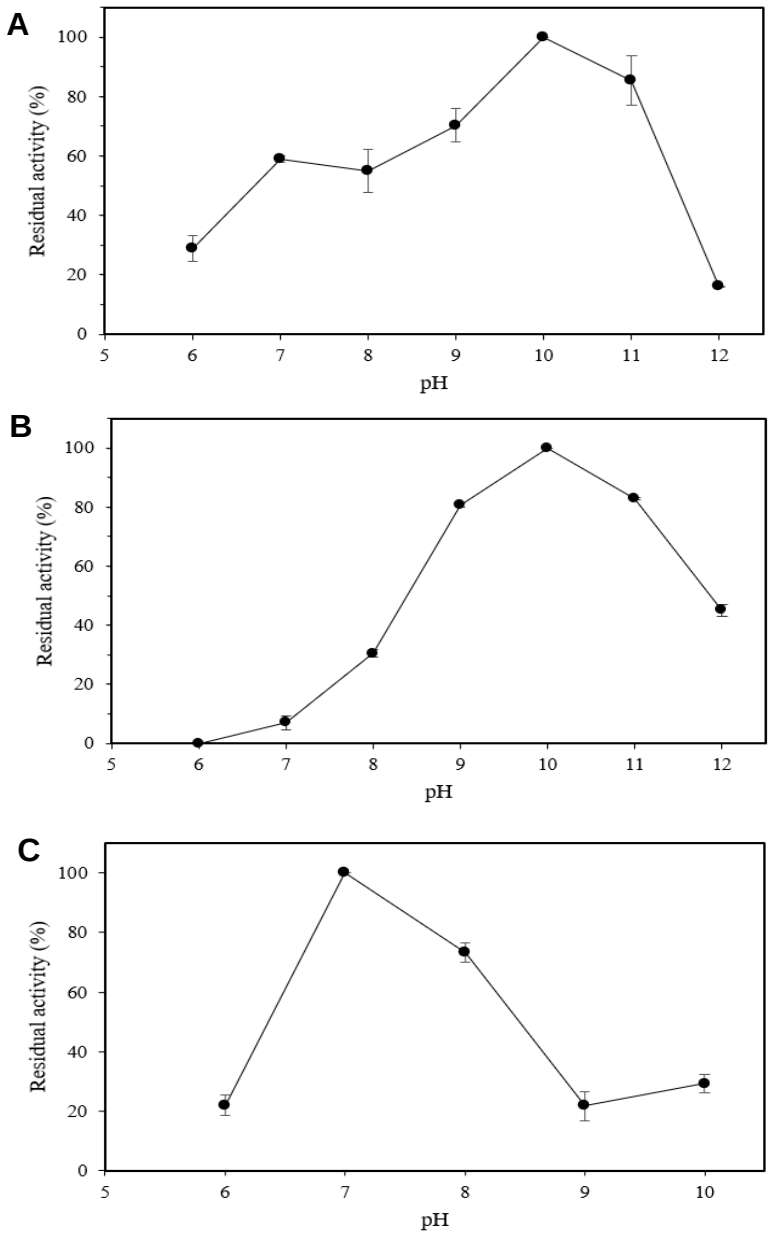

Figure 5.6. Influence of pH on the activity of purified (A) TMA14, (B) TFU19 and (C) TFU20 PGLs (~ 0.25 U mL^{-1}). The experiment was carried out using 0.5% (w/v) polyGalA and the reactions were incubated at 50 °C for 15 min. The relative activity was expressed as a percentage of the maximum activity. Error bars represent one standard deviation from the mean (n = 2). The assay was performed as described in Sections 2.7.1 and 2.9.3.3 in Chapter 2. PolyGalA: polygalacturonic acid.

5.3.2.3 Influence of temperature on the activity of pectate lyases

TMA14 was active in a temperature range between 40 and 100 °C. The optimum temperature of this enzyme was 80 °C, showing more than 50% of its maximum activity at 70, 90 and 100 °C. (Figure 5.7 A). TFU19 exhibited maximum activity between 60 and 80 °C, presenting more than 80% of it at 40

and 50 °C (Figure 5.7 B). TFU20 was active between 40 and 70 °C, being 50 °C its optimum temperature. This enzyme showed 80 and 60% of its maximum activity at 40 and 60 °C, respectively (Figure 5.7 C).

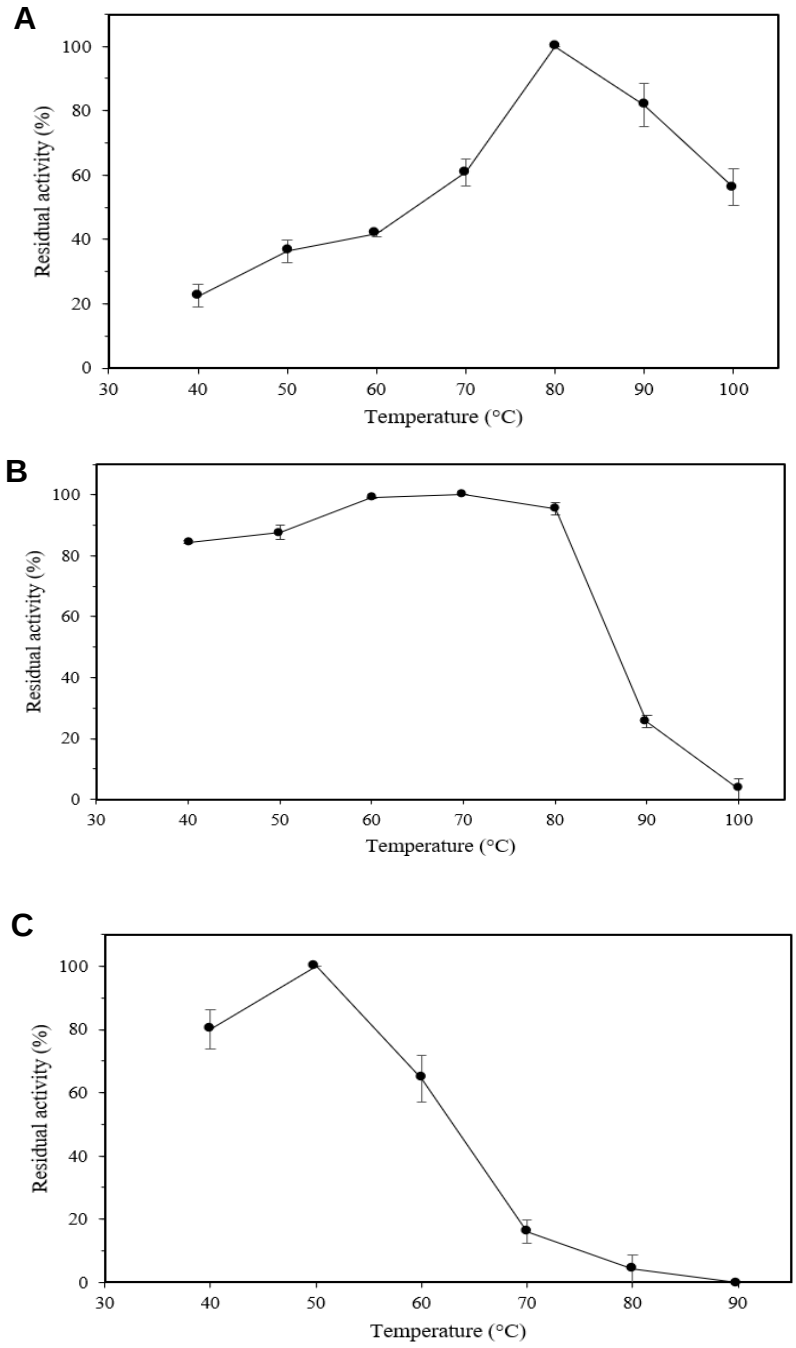

Figure 5.7. Influence of temperature on the activity of purified A) TMA14, (B) TFU19 and (C) TFU20 PGLs (~ 0.25 U mL^{-1}). The experiment was carried out using 0.5% (w/v) polyGalA at pH 8 and the reactions were incubated for 15 min. The relative activity was expressed as a percentage of the maximum activity. Error bars represent one standard deviation from the mean (n = 2). The assay was performed as described in Sections 2.7.3 and 2.9.3.3 in Chapter 2. PolyGalA: polygalacturonic acid.

5.3.2.4 Thermal stability of the pectate lyases

Thermal stability of TMA14, TFU19 and TFU20 PGLs was tested in absence and in presence of 0.6 mM Ca^{2+}. From Figure 5.8 A is observed that TMA14 presented great stability up to 70 °C in absence of Ca^{2+}, remaining more than 80% of its activity after 24 h of incubation. Thermal stability at 80 °C and above was also assayed in presence of 0.6 mM Ca^{2+}. The presence of this ion improved the stability of the enzyme at 80 and 90 °C keeping 97 and 55% of its activity, respectively after 8 h of incubation. While in absence of Ca^{2+}, at 80 °C the enzyme remained 55% of its activity after 8 h of incubation and at 90 °C it lost its activity after 30 min incubation. At 100 °C even in presence of Ca^{2+}, the enzyme remained only 20% of its activity after 30 min of incubation. Figure 5.8 B presents thermal stability results of TFU19. The enzyme was very stable up to 50 °C in absence of Ca^{2+}, maintaining more than 70% of its activity after 24 h of incubation. Thermal stability at 60 and 70 °C was also performed in presence of Ca^{2+}. At 60 °C, no difference was observed in the stability profile of the enzyme with or without Ca^{2+}. In both cases, the enzyme showed stability keeping more that 60% of its activity after 8 h of incubation. At 70 °C, the presence of Ca^{2+} improved the stability of the enzyme after 2 h of incubation, since it remained 63% of its activity in presence of the ion and only 12% in absence of it. Figure 5.8 C provides thermal stability results of TFU20. From this figure we can see that the enzyme exhibited good stability up to 50 °C in absence of Ca^{2+} retaining more than 85% of its activity after 24 h of incubation. Thermal stability at 60 and 70 °C was carried out also in presence of Ca^{2+} and the thermal stability profile of the enzyme was similar either in absence or in presence of this ion. At 60 and 70 °C, the enzyme maintained 60 and 50% of its activity, respectively after 2 h incubation. Most of the commercial PGLs are from mesophilic microorganisms showing restricted activity at elevated temperatures. Several industrial processes that involve pectin depolymerisation are more efficient at high temperatures such as retting and degumming in textile industry, paper making and juice extraction, thus thermophilic enzymes are desirable for these processes (Zhou *et al.*, 2015; Cheng *et al.*, 2020).

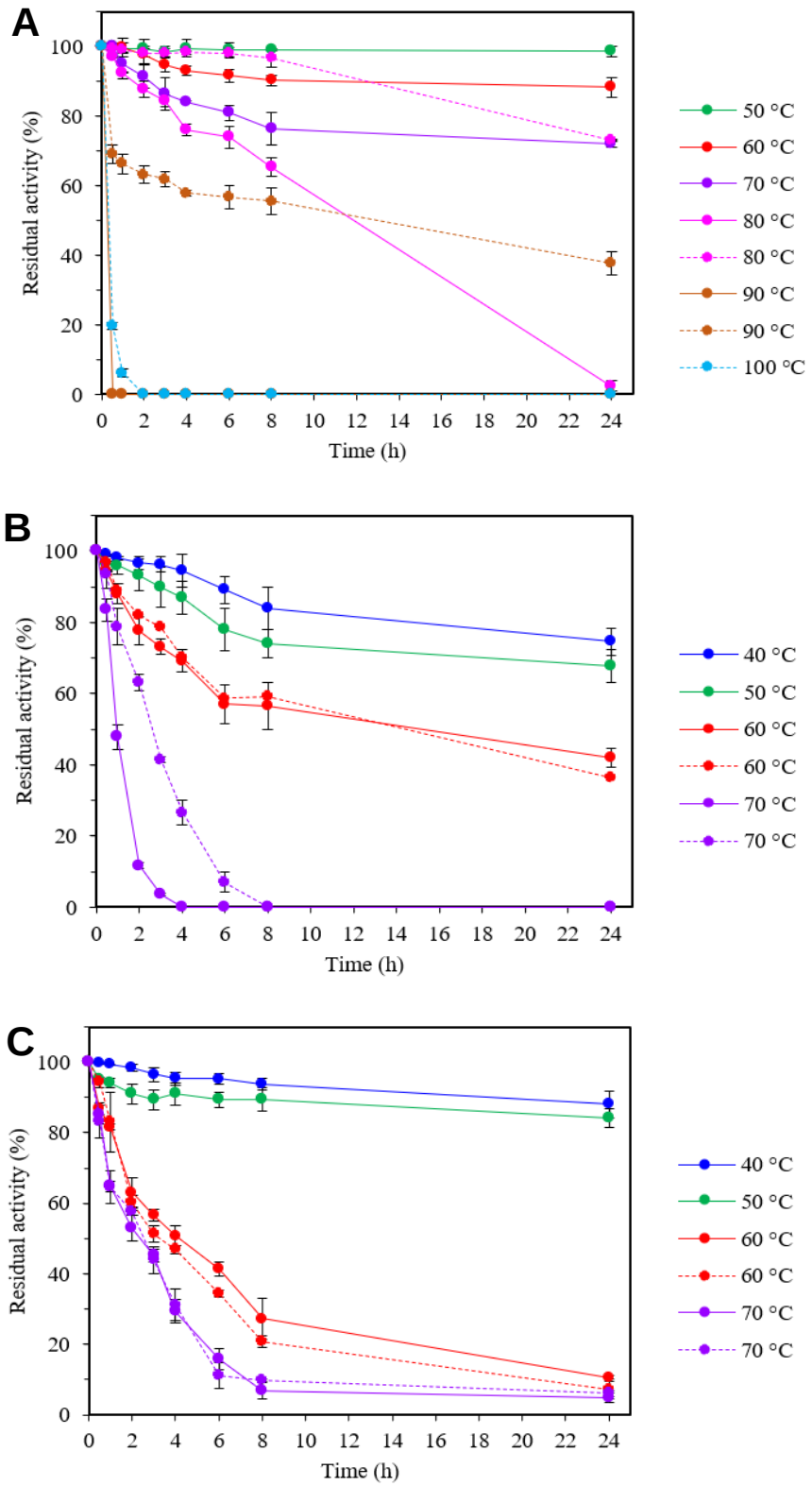

Figure 5.8. Thermal stability of purified (A) TMA14, (B) TFU19 and (C) TFU20 PGLs (~1.5 U mL^{-1}), (—) in absence of Ca^{2+} and (…) in presence of 0.6 mM Ca^{2+}. The enzymes were pre-incubated with the ion at room temperature for 15 min. After the indicated time of incubation, the PGL activity was determined using 0.5% (w/v) polyGalA at pH 8 and 50 °C. The residual activity was expressed as the percentage of the starting activity. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.4 and 2.9.3.3 in Chapter 2. PolyGalA: polygalacturonic acid.

5.3.3 Kinetic characterization of thermophilic PGLs

Kinetic data of TMA14, TFU19 and TFU20 PGLs were successfully fitted to the Michaelis-Menten equation (Equation 2.4, Section 2.7.5 in Chapter 2) and no substrate inhibition was observed (Figure 5.9). *kcat* values were calculated using the Equation 2.3 (Section 2.7.5 in Chapter 2). The protein concentration and molecular weight used for TMA14 were 19.78 µg mL^{-1} and 40.61 kDa, respectively; for TMA14 33.94 µg mL^{-1} and 53.62 kDa, respectively; and for TFU20 20.47µg mL^{-1} and 49.89 kDa, respectively. The regression coefficients (R^2) of Equations 2.3 and 2.4 were 0.99. The kinetic parameters values of the three PGLs are summarised in Table 5.1. The *Vmax* of TMA14 was 82.77 µmol min^{-1} mg^{-1}; while the *Km* and *kcat* were 45.11 µM and 56.02 s^{-1}, respectively. A higher *Km* (60 µM) has been reported for this enzyme using polyGalA (Kluskens *et al.*, 2003). Likewise, the *Vmax* of TFU19 was 26.84 µmol min^{-1} mg^{-1}; while the *Km* and *kcat* were 38.05 µM and 23.98 s^{-1}, respectively. Finally, the *Vmax* of TFU20 was 48.3 µmol min^{-1} mg^{-1}; while the *Km* and *kcat* were 19.5 µM and 40.16 s^{-1}, respectively. Comparison between the kinetic parameters of the three PGLs showed that the *Vmax* and *kcat* of TMA14 were higher than those from TFU19 and TFU20. However, the *Km* of TMA14 was also higher, which means that this enzyme exhibited lower affinity towards polyGalA. Both TFU19 and TFU20 are from *Thermobifida fusca*, however TFU20 showed better kinetic parameters. Thus, both *Vmax* and *kcat* were 1.8-fold higher as well as *Km* was 1.9-fold lower in TFU20 respect to TFU19.

Kinetic information of thermophilic PGLs previously reported (Table 5.1) shows that some of them have exhibited a Michaelis-Menten kinetics (Su *et al.*, 2015; Zheng *et al.*, 2020) similar to the enzymes from this study. Additionally, it is observed that a PGL from *Paenibacillus* sp. presented the highest *Vmax* and *kcat* values, but also the highest *Km* indicating its lower affinity towards the substrate (Li *et al.*, 2014). PGLs from *Caldicellulosiruptor bescii* and *Caldicellulosiruptor kronotskyensis* also presented high *Vmax* and *kcat* values being their *Km* values similar to those from the enzymes of this study (Su *et al.*, 2015; Hamouda *et al.*, 2020). TFU20 along with PGLs from *Caldicellulosiruptor* presented the lowest *Km* values showing more affinity towards the polyGalA.

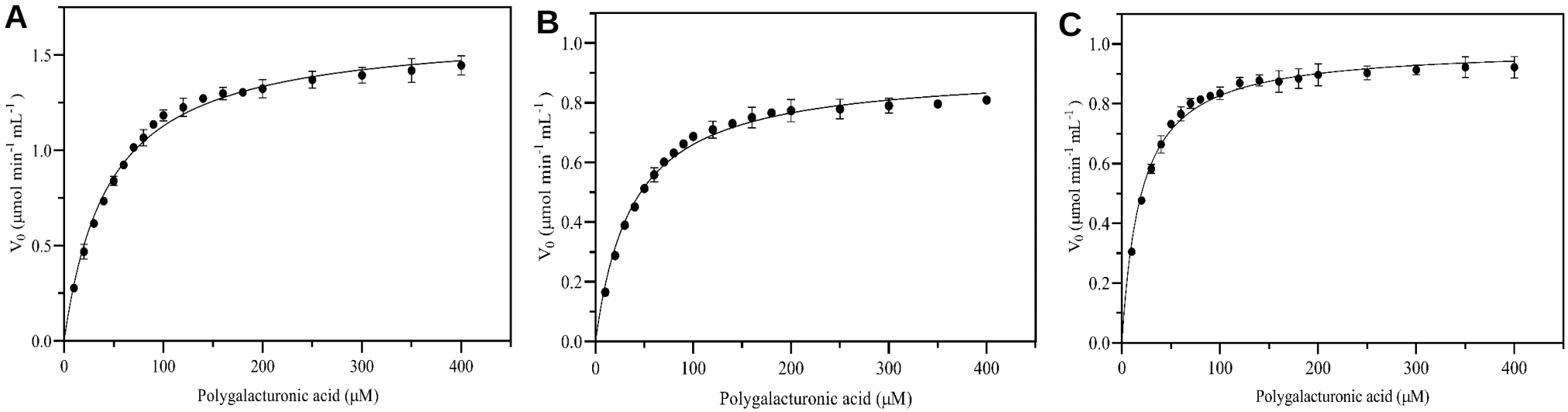

Figure 5.9. Michaelis-Menten kinetics of (A) TMA14, (B) TFU19 and (C) TFU20 PGLs using polyGalA as substrate. The enzymes velocity was measured at optimum conditions of pH, temperature and ions concentration. TMA14 and TFU19: pH 10 and 80 °C and TFU20: pH 7 and 50 °C; 0.25 and 0.5 mM Mn^{2+} were used for TMA14 and TFU20, respectively. Kinetic data were analysed by non-linear regression and successfully fitted to Equation 2.4 (Section 2.7.5 in Chapter 2) using GraphPad Prism 8. The parameters are detailed in Table 5.1. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Section 2.7.5 in Chapter 2. PolyGalA: polygalacturonic acid.

Table 5.1. Kinetic parameters of TM14, TFU19 and TFU20 PGLs and comparative kinetic information with other thermophilic PGLs using polyGalA as substrate. Kinetic parameters of TMA14, TFU19 and TFU20 were calculated using Equations 2.3 and 2.4 (Section 2.7.5 in Chapter 2).

Bacteria	**Enzyme**	***Vmax*** **(μmol min^{-1} mg^{-1})**	***Km*** **(μM)**	***kcat*** **(s^{-1})**	***kcat/Km*** **(s^{-1} $μM^{-1}$)**	**Reference**
Thermotoga maritima	TMA14[a]▪	82.77 ± 1.01	45.11 ± 1.90	56.02 ± 0.31	1.24 ± 0.16	This study
Thermobifida fusca	TFU19[a]▪	26.84 ± 0.29	38.05 ± 1.64	23.98 ± 0.13	0.63 ± 0.08	This study
Thermobifida fusca	TFU20[b]▪	48.3 ± 0.39	19.5 ± 0.88	40.16 ± 0.20	2.06 ± 0.23	This study
Thermotoga maritima	PelA[a]	ND	60	ND	ND	Kluskens *et al.* (2003)
Bacillus sp. strain N16-5	BspPelA (EA)[b]	ND	46.40 ± 5.33	3932 ± 136	84.74 ± 5.30	Zhou *et al.* (2015)
Caldicellulosiruptor bescii	CbPL3[c]	1446 ± 162.4	42.67 ± 8	2132.7	49.98	Hamouda *et al.* (2020)
Caldicellulosiruptor bescii	CbPL9[c]	57.3 ± 4	21.07 ± 2.77	127	6.03	Hamouda *et al.* (2020)
Paenibacillus sp.	PelN[d]	4120 x 10^3	92.8 ± 9.33	3290 x 10^3	35.45 x 10^3	Li *et al.* (2014)
Caldicellulosiruptor kronotskyensis	Pel-863[e]▪	172.8 ± 5.6	16 ± 1.33	ND	ND	Su *et al.* (2015)
Bacillus sp. RN1	BspPel[a]▪	ND	58.4	116.1	1.98	Zheng *et al.* (2020)

Reactions were performed at [a]80 °C, [b]50°C, [c]65 °C, [d]45 °C and [e]70 °C. ▪Michaelis-Menten kinetics, ND: not determined. Errors represent one standard deviation about the mean (n = 2).

5.3.4 Substrate specificity of thermophilic PGLs

5.3.4.1 Substrate specificity determined by reducing sugars quantification

The substrate specificity reactions of the three PGLs were determined at their optimum conditions of activity which are detailed in Table 5.2. Non-esterified substrates such as polyGalA as well as substrates with different degrees of esterification (methylation and acetylation) such as apple, citrus and sugar beet pectin were used (Table 2.2, Section 2.7.6 in Chapter 2). Since PGLs prefer non-esterified substrates, their activity with polyGalA was considered as 100%. From Figure 5.10, it is observed that the three PGLs exhibited activity towards esterified substrates such as apple, citrus and sugar beet pectin being TFU19 the most active followed by TMA14 and finally by TFU20. TMA14 and TFU19 exhibited similar activities towards apple and citrus pectin which are high methylated (~ 55%) and sugar beet pectin which is high acetylated (20%). Meanwhile, TFU20 was more active towards apple and citrus pectin presenting lower activity towards sugar beet pectin.

Table 5.2. Optimum conditions of activity of TMA14, TFU19 and TFU20 PGLs.

Optimum condition	**PGLs**		
	***TMA14**	***TFU19**	***TFU20**
pH	10	10	7
Temperature (°C)	80	80	50
Mn^{2+} (mM)	0.25	-	0.5

*All the enzymatic reactions were carried out including 0.6 mM Ca^{2+} for 0.25 U mL of enzyme.

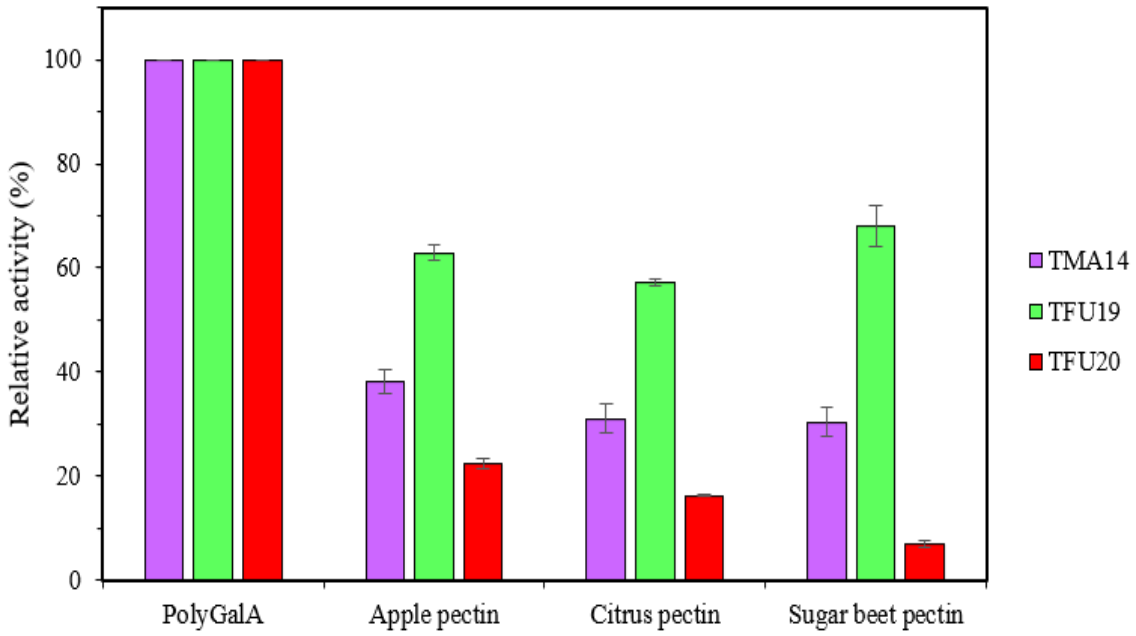

Figure 5.10. Effect of the degree of pectin esterification on the activity of purified TMA14, TFU19 and TFU20 PGLs. The PGL activity was performed using ~ 0.25 U mL^{-1} of each enzyme at optimum condition of activity (Table 5.1) with an incubation time of 15 min. The relative activity was expressed as the percentage of the activity towards polyGalA which was considered as 100%. Error bars represent one standard deviation from the mean (n = 2). The experiment was carried out as described in Sections 2.7.6 and 2.9.3.3 in Chapter 2. PGL: pectate lyase, PolyGalA: polygalacturonic acid.

5.3.4.2 Substrate specificity determined by gel filtration chromatography (GFC)

The substrate specificity reactions were incubated up to 4 h and samples were taken periodically. These samples were used to determine the molecular weight distribution of the produced oligogalacturonates using GFC. All the results presented correspond to 30 min reaction, since same depolymerisation profiles were obtained at the other indicated times.

Figures 5.11, 5.12, 5.13 and 5.14 present the depolymerisation profiles of polyGalA as well as of apple, citrus and sugar beet pectin, respectively by the three PGLs action. Figures A show the gels filtration chromatograms, and Figures B provide the molecular weight distribution of the produced oligogalacturonates calculated based on the predominant peaks. At optimum conditions of TMA14 and TFU19 (pH 10 and 80 °C), polyGalA, apple pectin and citrus pectin presented two main peaks of around 200 and 650 kDa, while sugar beet pectin presented an additional small peak of around 120 kDa. Both TMA14 and TFU19 depolymerised polyGalA in three peaks of around 70, 100 and 120 kDa. While all apple, citrus and sugar beet pectin were depolymerised by TMA14 and TFU19 in three (70, 120 and 200 kDa) and two (70 and 120 kDa) peaks, respectively but keeping the main peak of 650 kDa corresponding to the substrates. On the other hand, at optimum conditions of TFU20 (pH 7 and 50 °C), polyGalA as well as apple, citrus and sugar beet pectin presented two peaks of around 20 kDa and 650 kDa. TFU20 depolymerised polyGalA in peaks of around 20 and 120 kDa. While apple, citrus and sugar beet pectin were depolymerised by this enzyme in peaks of around 20 and 200 kDa but keeping the main peak of 650 kDa corresponding to the substrates.

The results mentioned above demonstrated that the three PGLs from this study were able to depolymerise the esterified apple, citrus and sugar beet pectin at optimum conditions of activity. Similar depolymerisation profiles were observed for these three substrates despite the high percent of acetyl groups in sugar beet pectin. TMA14 released oligogalacturonates predominantly of around 70, 100 and 200 kDa; TFU19 of 70 and 120 kDa; and TFU20 of 200 kDa. However, the main peak of 650 kDa of these substrates was degraded by the three PGLs only in the non-esterified polyGalA keeping detectable in the esterified substrates. These results confirmed the preference of PGLs by non-esterified substrates and evidenced the need of a synergistic action between them with PMEs and PAEs to improve esterified pectin depolymerisation.

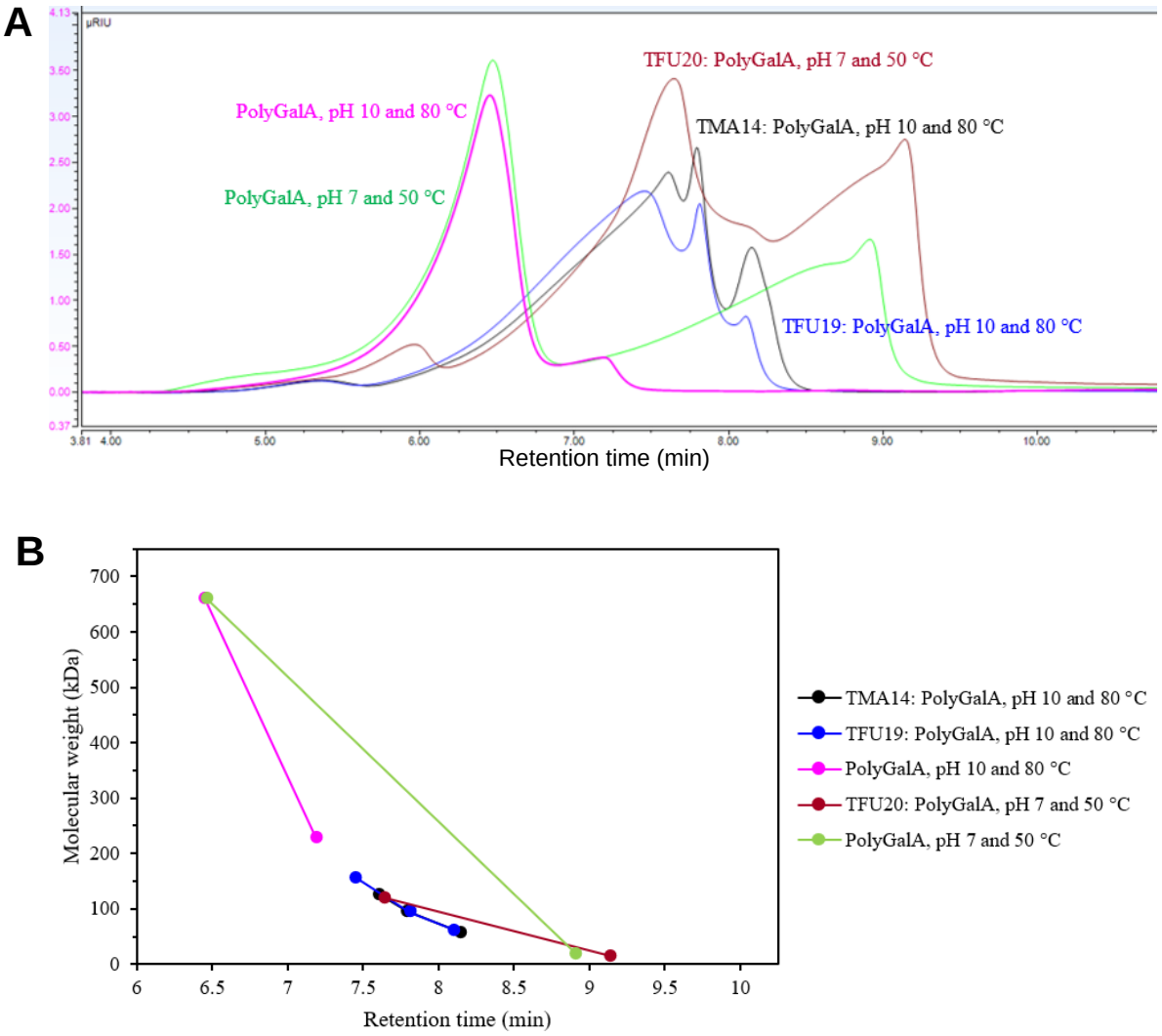

Figure 5.11. PolyGalA depolymerisation by PGLs action at optimum conditions of activity (Table 5.2) (A) Gel filtration chromatograms and (B) molecular weight distribution of the produced oligogalacturonates. The reactions were carried out using 0.5% (w/v) polyGalA and 0.25 U mL^{-1} of each enzyme. These results correspond to an incubation time of 30 min. The experiment was performed as described in Section 2.7.6 and 2.9.7 in Chapter 2. PolyGalA: polygalacturonic acid.

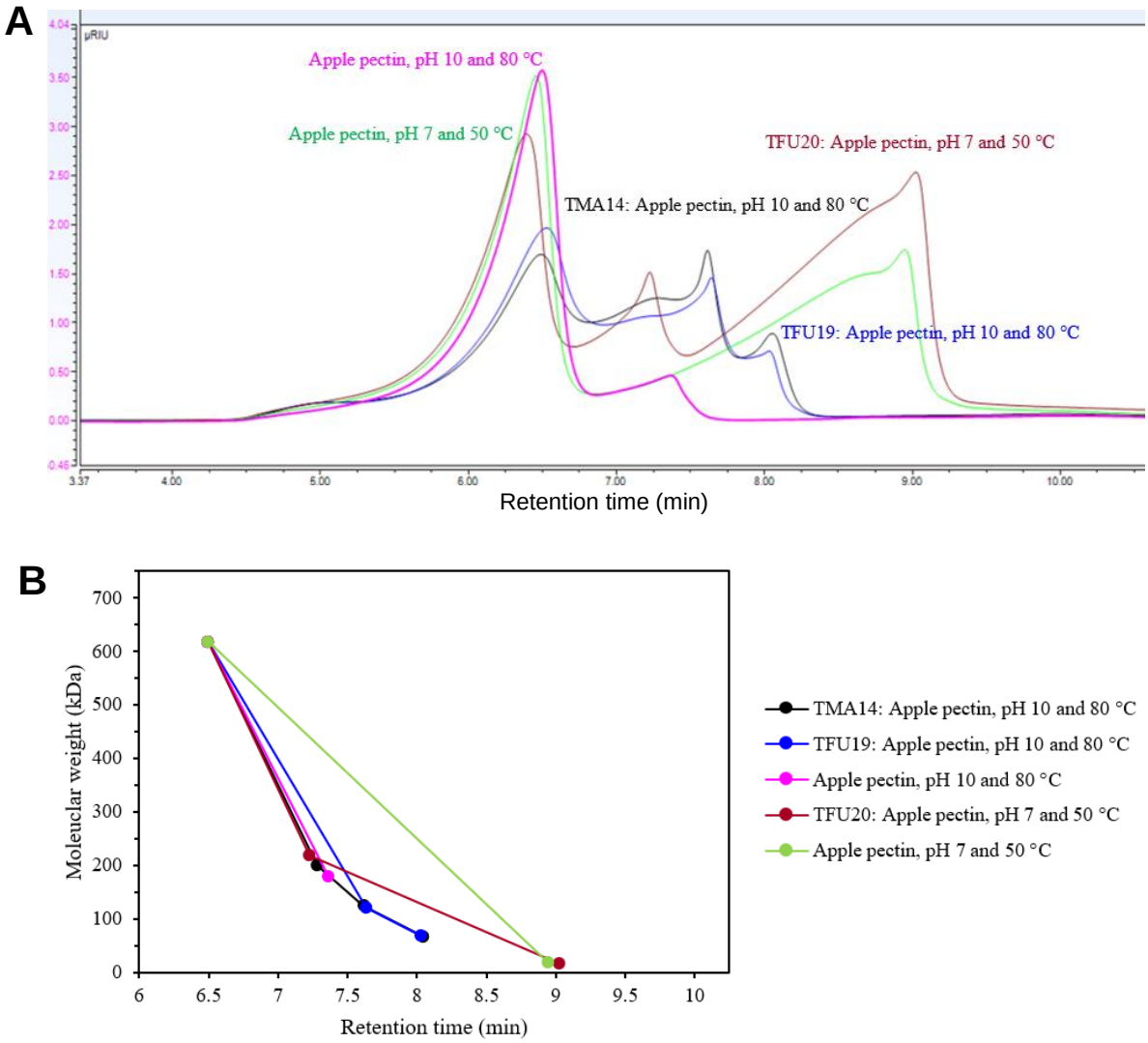

Figure 5.12. Apple pectin depolymerisation by PGLs action at optimum conditions of activity (Table 5.2) (A) Gel filtration chromatograms and (B) molecular weight distribution of the produced oligogalacturonates. The reactions were carried out using 0.5% (w/v) apple pectin and 0.25 U mL^{-1} of each enzyme. These results correspond to an incubation time of 30 min. The experiment was performed as described in Section 2.7.6 and 2.9.7 in Chapter 2.

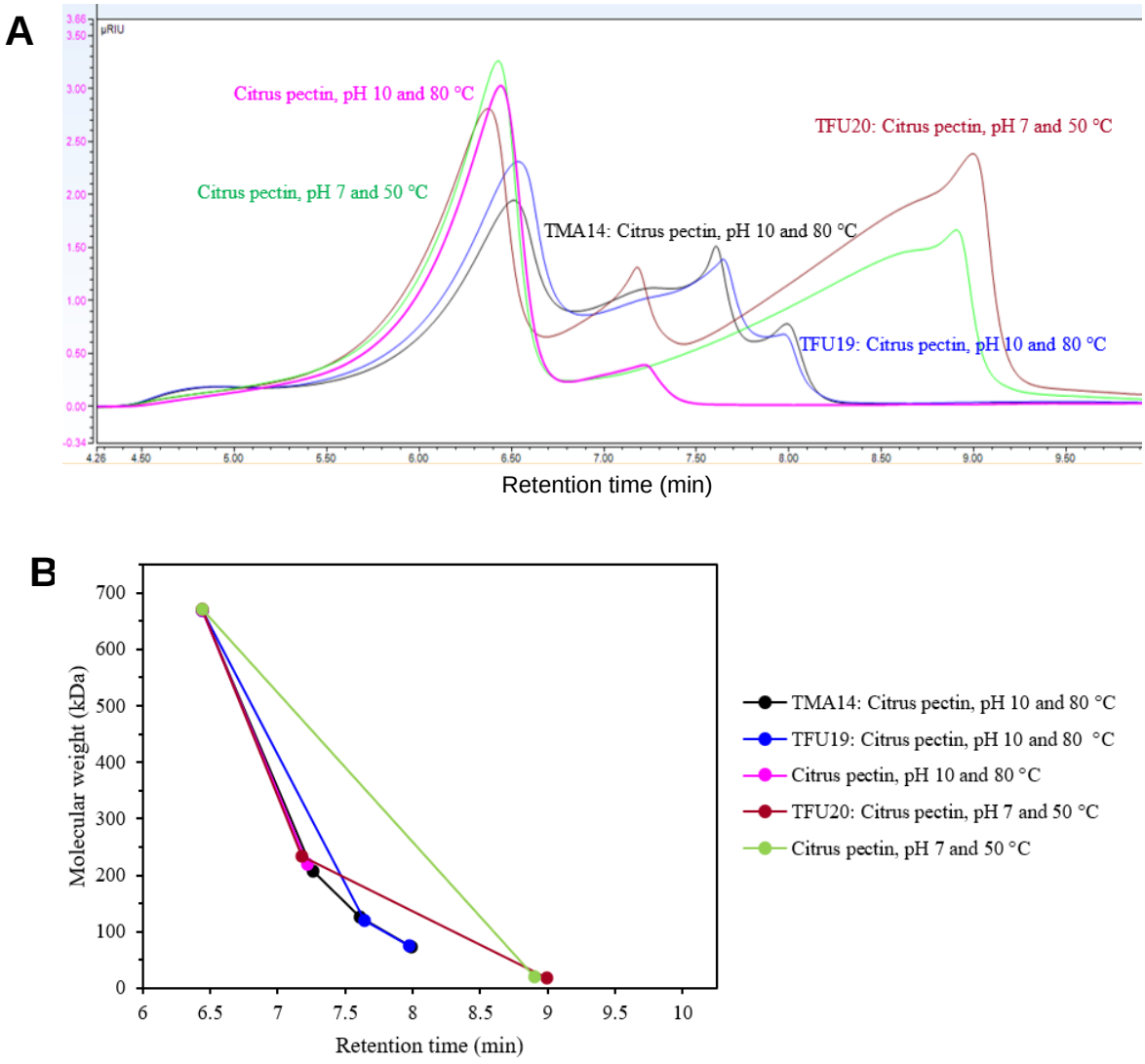

Figure 5.13. Citrus pectin depolymerisation by PGLs action at optimum conditions of activity (Table 5.2) (A) Gel filtration chromatograms and (B) molecular weight distribution of the produced oligogalacturonates. The reactions were carried out using 0.5% (w/v) citrus pectin and 0.25 U mL^{-1} of each enzyme. These results correspond to an incubation time of 30 min. The experiment was performed as described in Section 2.7.6 and 2.9.7 in Chapter 2.

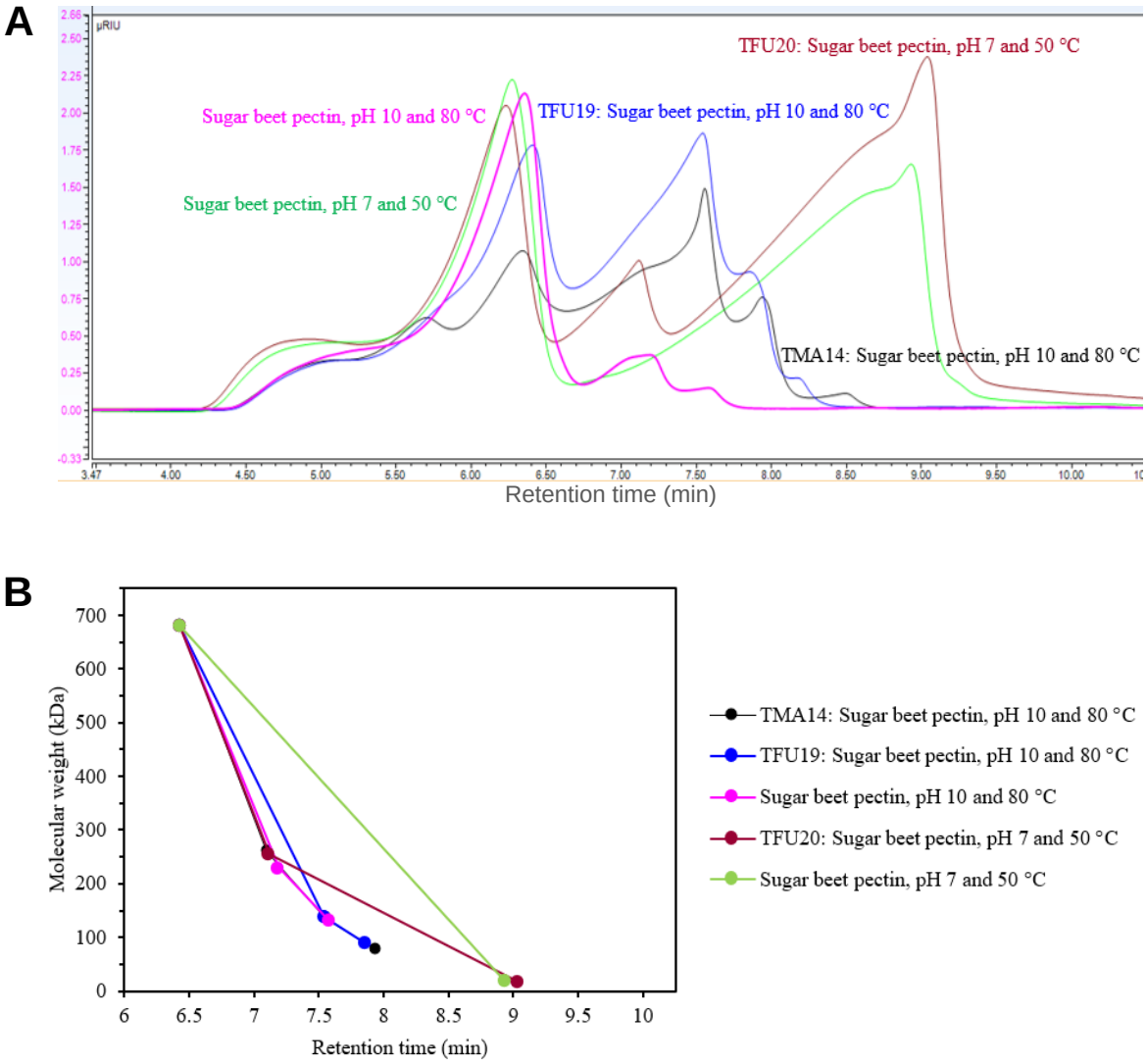

Figure 5.14. Sugar beet pectin depolymerisation by PGLs action at optimum conditions of activity (Table 5.2) (A) Gel filtration chromatograms and (B) molecular weight distribution of the produced oligogalacturonates. The reactions were carried out using 0.5% (w/v) sugar beet pectin and 0.25 U mL^{-1} of each enzyme. These results correspond to an incubation time of 30 min. The experiment was performed as described in Section 2.7.6 and 2.9.7 in Chapter 2.

5.3.5 Synergistic action between PGLs and PMEs

5.3.5.1 Synergistic activity determined by reducing sugars quantification

As shown in Section 5.3.4, PGLs prefer non-esterified substrates and the presence of methyl and acetyl groups affect their activity and as consequence pectin depolymerisation. Hence, synergistic activity between PGLs and PMEs to improve esterified pectin depolymerisation was evaluated. Six reactions were

set up between BLI09 or SAM10 PMEs paired either with TMA14, TFU19 and TFU20 PGLs using apple pectin as substrate. For these reactions, 9 and 18 U mL^{-1} of BLI09 and SAM10, respectively, were used since these amounts were the optimum to achieve this substrate maximum demethylation (Section 3.3.8 in Chapter 3). Different activities of PGLs were tested until reaching a plateau for reducing sugar quantification during the time of reaction. Thereby, 0.25, 0.5 and 0.1 U mL^{-1} of TMA14, TFU19 and TFU20 were used. One of the major limitations when several enzymes take part in one process is the different optimal conditions for individual enzymes (Thite and Nerurkar, 2018). Compatible operational conditions were set up for the synergistic reactions even if the optimum activity of the individual enzymes was compromised. All the synergistic reactions were carried out at pH 7 and 300 rpm. Regarding temperature, the synergistic reactions with BLI09 PME were incubated at 50 °C up to 24 h, while the reactions with SAM10 PME at 40 °C (unstable at 50 °C) for 1 h for demethylation and then increased to 50 °C for depolymerisation up to 24 h. Samples were taken periodically for methanol and reducing sugars quantification. Similar results were obtained in the synergistic reactions using either BLI09 (Figure 5.15) or SAM10 (Figure 5.16). Methanol was released since the first hours of reaction reaching around 3.5 mM (Figure 5.15 A and 5.16 A). Regarding reducing sugars, around 6 and 7 mM were quantified in the synergistic reactions with TMA14 and TFU20, respectively after 4 h of reaction. But, with TFU19 only around 2.5 mM reducing sugars were quantified after 4 h of reaction (Figures 5.15 B and 5.16 B). These results can be explained because the operational conditions at which the synergistic reactions were carried out (pH 7 and 50 °C) favoured the activity of TFU20 (optimum activity at pH 7 and 50 °C). Meanwhile, TMA14 (optimum activity at pH 10 and 80 °C), presented 60 and 40% of its maximum activity at pH 7 and 50 °C, respectively. Regarding TFU19 (optimum activity at pH 10 and 80 °C), its optimum activity was severely compromised at the conditions of the synergistic reactions because despite this enzyme showed 80% of its maximum activity at 50 °C, it presented less than 10% of it at pH 7 and below. On the other hand, less than 2 mM reducing sugars were quantified using only PGLs. In summary, the most successful synergistic reactions for pectin depolymerisation at these reaction

conditions were between BLI09 or SAM10 PME paired either with TMA14 or TFU20 PGLs.

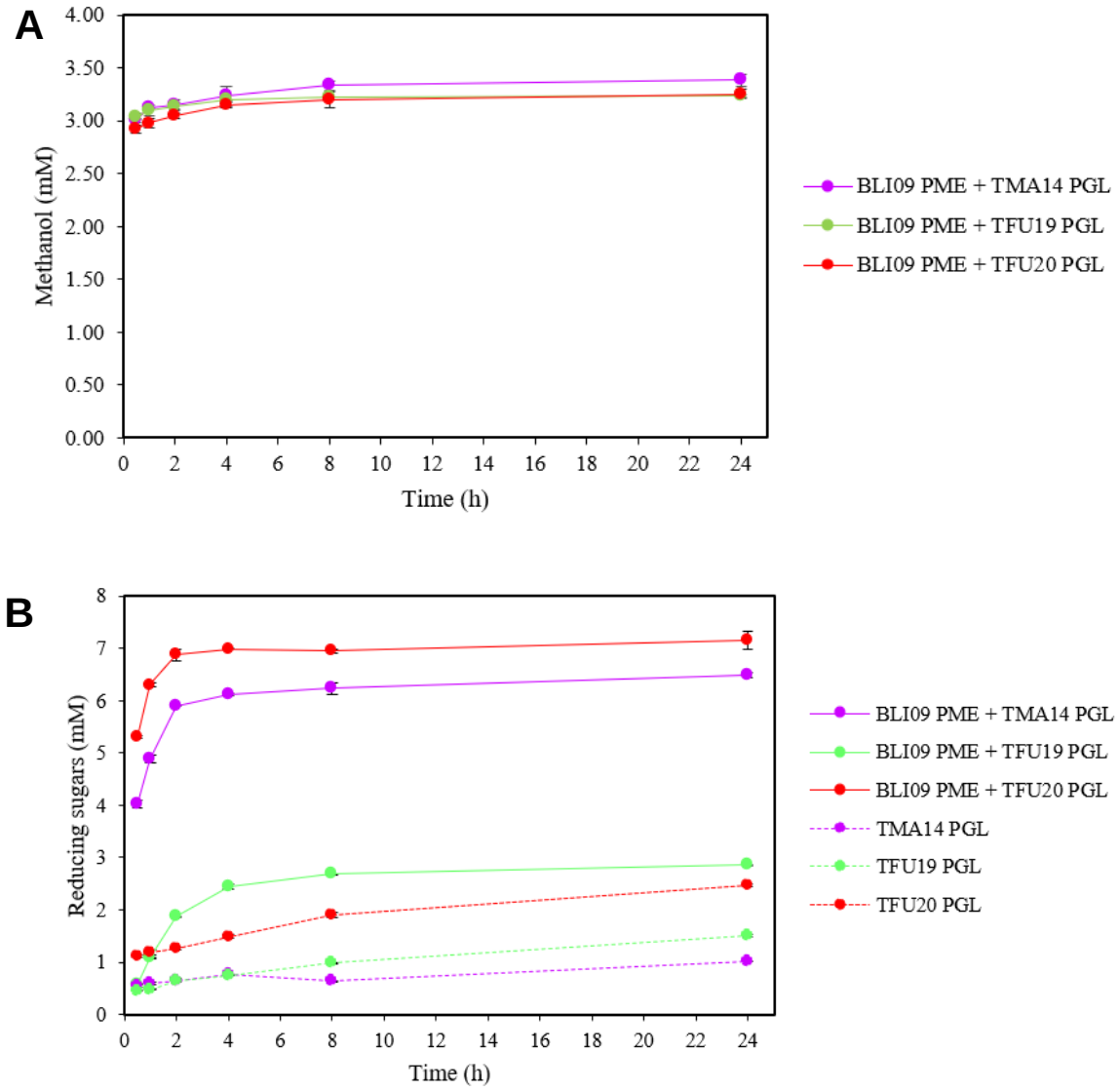

Figure 5.15. Synergistic activity between BLI09 PME paired either with TMA14, TFU19 or TFU20 PLGs using apple pectin. (A) Methanol and (B) reducing sugars quantification. The reactions were carried out using 0.5% of substrate in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm up to 24 h. These results were obtained using 9 U mL^{-1} of BLI09 as well as 0.25, 0.5 and 0.1 U mL^{-1} of TMA14, TFU19 and TFU20, respectively. The assay was performed as described in Section 2.8.3 and methanol was quantified following the procedure described in Sections 2.9.4. These sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2).

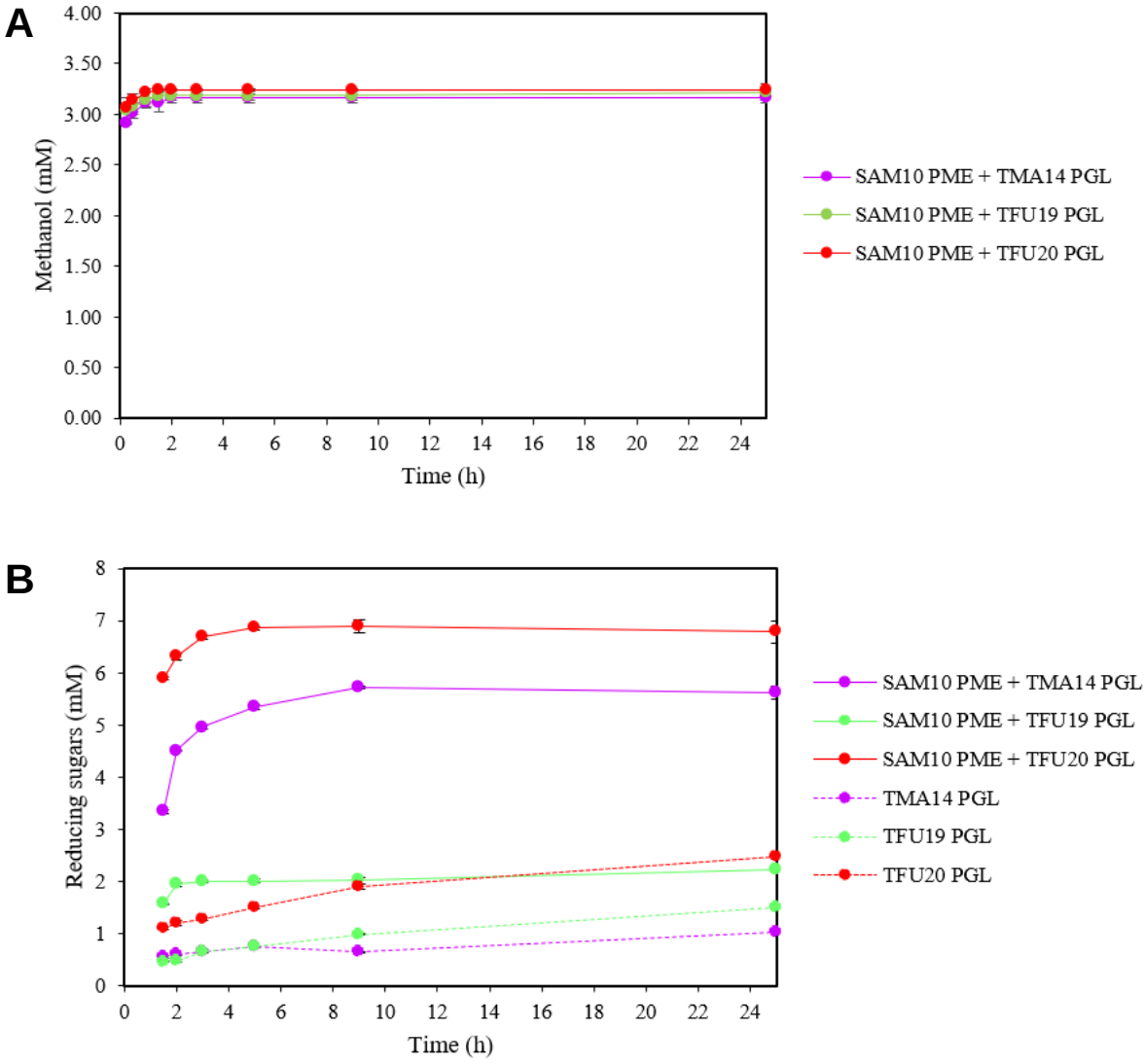

Figure 5.16. Synergistic activity between SAM10 PME paired either with TMA14, TFU19 or TFU20 PLGs using apple pectin. (A) Methanol and (B) reducing sugars quantification. The reactions were carried out using 0.5% of substrate in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm up to 24 h. These results were obtained using 18 U mL^{-1} of SAM10 as well as 0.25, 0.5 and 0.1 U mL^{-1} of TMA14, TFU19 and TFU20, respectively. The assay was performed as described in Section 2.8.3 and methanol was quantified following the procedure described in Sections 2.9.4. These sections are in Chapter 2. Error bars represent one standard deviation from the mean (n = 2).

5.3.5.2 Synergistic activity determined by GFC

According to the results obtained in Section 5.3.5.1, the most successful synergistic reactions for apple pectin depolymerisation were between BLI09 or SAM10 PME paired either with TMA14 or TFU20 PGLs. Hence, four synergistic reactions were set up between these pectinases using apple, citrus and sugar beet pectin, and the molecular weight distribution of the produced oligogalacturonates was analysed by GFC.

For these reactions, two enzymatic activities were assayed for each PGL based on the results of reducing sugars quantification previously described. Thereby, 0.25 and 1 U mL^{-1} were used for TMA14 and 0.1 and 0.5 U mL^{-1} for TFU20. In all the synergistic reactions, methanol release by both PMEs was 3.5 mM in apple and citrus pectin and around 2 mM in sugar beet pectin (data not shown). Figures 5.17 and 5.18 show the synergistic activity between BLI09 or SAM10 PMEs with TMA14 PGL using apple pectin, Figures 5.19 and 5.20 using citrus pectin and Figures 5.21 and 5.22 using sugar beet pectin. Figures A1 and B1 present the gel filtration chromatograms while Figures A2 and B2 show the molecular weight distributions of the produced oligogalacturonates. From Figures 5.17, 5.19 and 5.21, it was observed that all apple, citrus and sugar beet pectin presented two main peaks of 20 and 650 kDa, and similar depolymerisation profiles were obtained for these three substrates by the synergistic action between BLI09 or SAM10 PMEs with TMA14 PGL. Thus, using 0.25 U mL^{-1} of TMA14 peaks of 15 and 100 kDa were produced in the synergistic reactions with BLI09, while peaks of 10 and 30 kDa in the reactions with SAM10. Meanwhile, using 1 U mL^{-1} of TMA14 peaks of 8 and 25 kDa were produced in the synergistic reaction with BLI09, while peaks of 4 and 10 kDa in the reactions with SAM10. In both cases, using only TMA14, peaks between 10 and 240 kDa were produced but keeping the main peak of 650 kDa corresponding to the substrates. Enzymatic activity of 1 U mL^{-1} was the optimum for the synergistic reactions with TMA14 since same depolymerisation profiles were obtained using higher activities.

The comparison of the synergistic activity between BLI09 or SAM10 using 0.25 and 1 U mL^{-1} of TMA14 in apple, citrus and sugar beet pectin is better shown in Figures 5.18 A, 5.20 A and 5.22 A, respectively. In addition, control reactions using only TMA14 with polyGalA were tested and the molecular weight distribution of the produced oligogalacturonates was analysed by GFC (Figures 5.18 B, 5.20 B and 5.22 B). From these results, it was observed that polyGalA, similar to apple, citrus and sugar beet pectin, presented two main peaks of around 20 and 650 kDa which were depolymerised by 0.25 U mL^{-1} of TMA14 in three peaks of 15, 40 and 100 kDa. While 1 U mL^{-1} of this enzyme, depolymerised polyGalA in two peaks of 8 and 25 kDa. These results showed that similar depolymerisation profiles were obtained using 0.25 and 1 U mL^{-1} of TMA14 in its synergistic reactions with BLI09 in the three substrates and in its action with polyGalA. Meanwhile, smaller oligogalacturonates were obtained using 0.25 and 1 U mL^{-1} of TMA14 in its synergistic reactions with SAM10 in the three substrates than in its action with polyGalA. These findings demonstrated that the synergistic activity between PMEs with TMA14 was efficient for esterified pectin depolymerisation. The smallest oligogalacturonates obtained in the synergistic reactions with SAM10 could be explained by the random demethylation pattern of this enzyme, respect to blockwise demethylation of BLI09, which might favour the exo-PGL activity of TMA14 (EC 4.2.2.22) for releasing unsaturated trigalacturonates from the reducing end of pectin as major products (Figure 5.23).

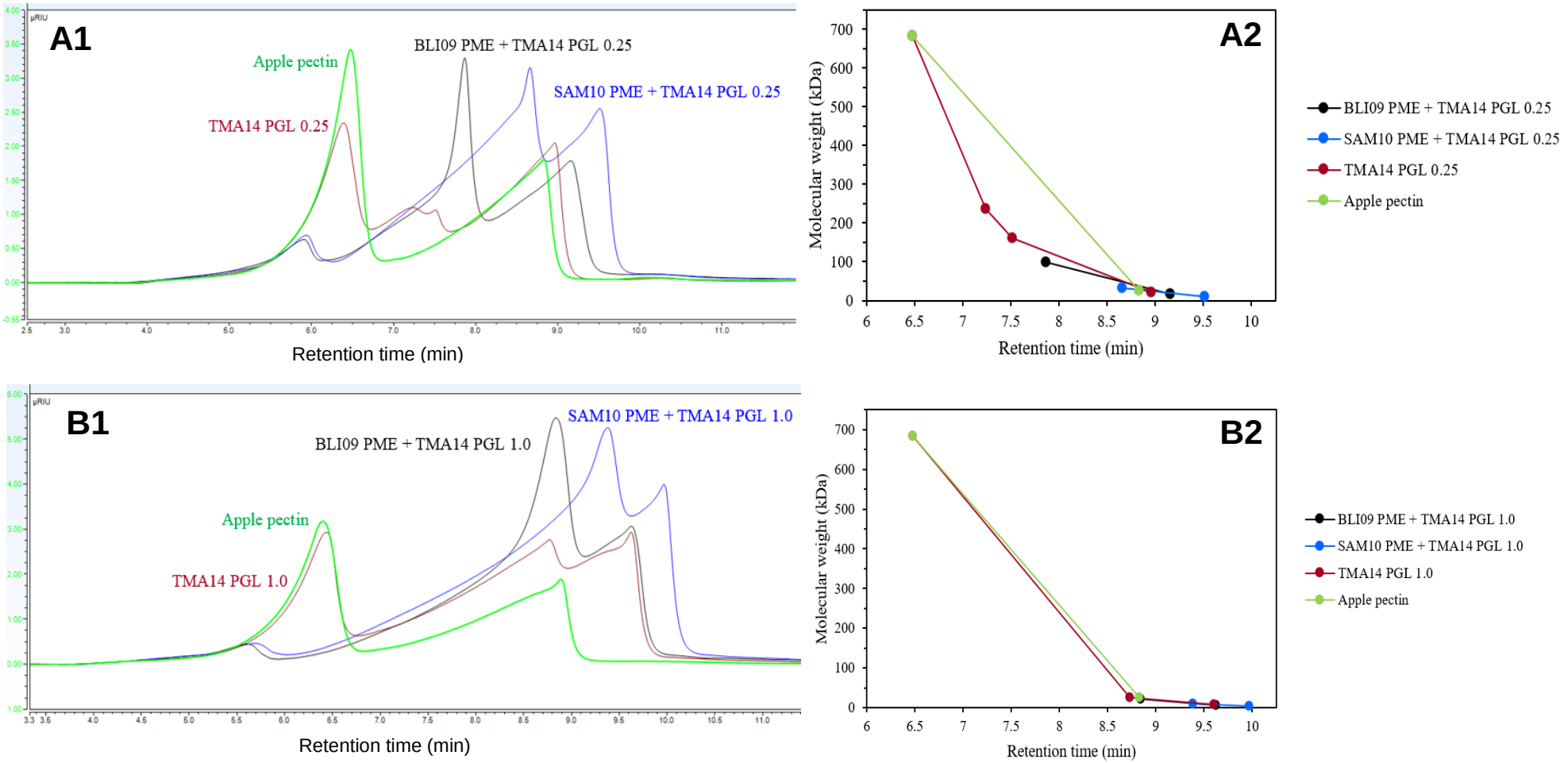

Figure 5.17. Synergistic activity between BLI09 or SAM10 PMEs with TMA14 PGL using apple pectin. (A1) Gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates after 2 h of reaction using 0.25 U mL^{-1} of TMA14. (B1) Gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates after 30 min of reaction using 1 U mL^{-1} of TMA14. In both cases, the activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. The reactions were carried out using 0.5% (w/v) apple pectin in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

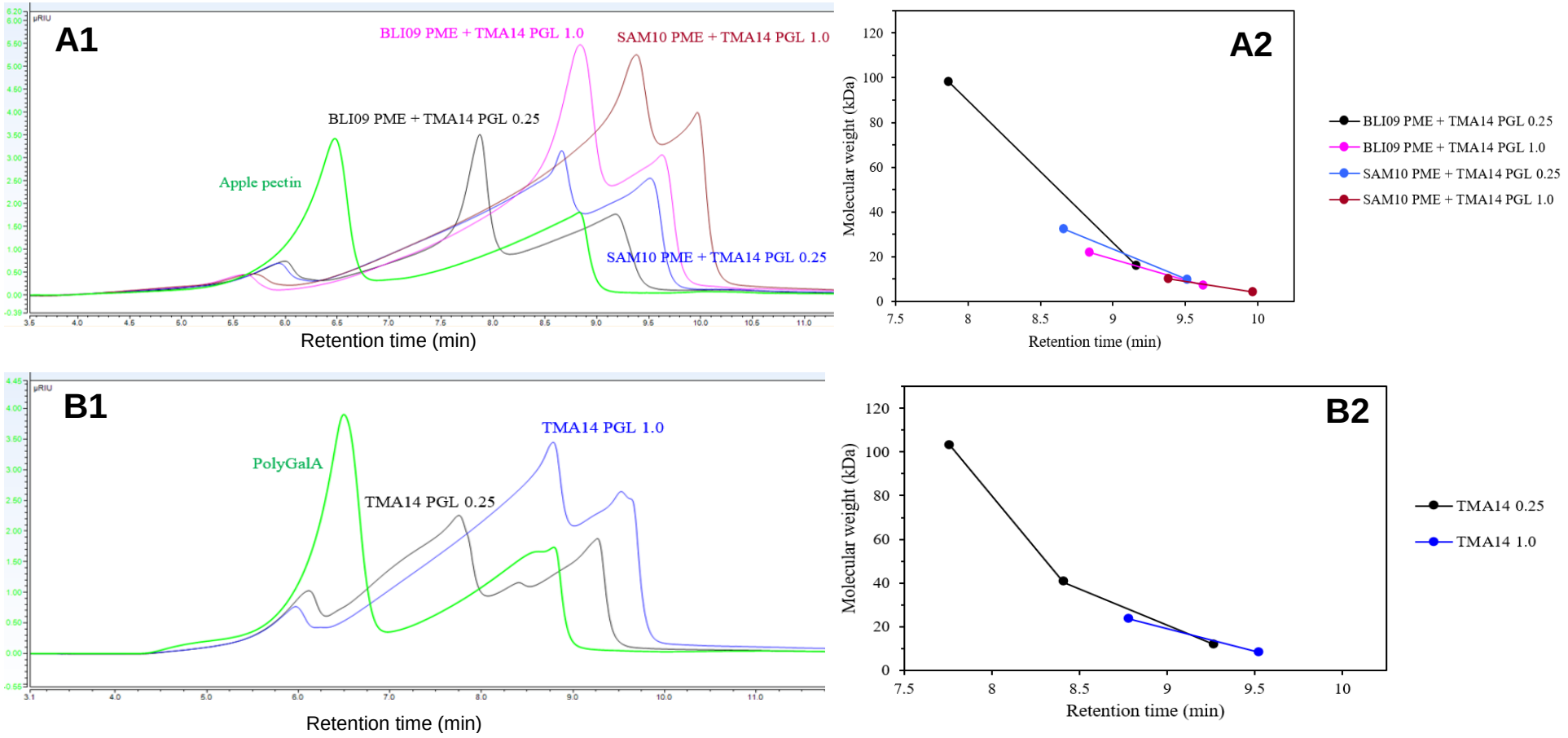

Figure 5.18. Comparison between the synergistic activity of the BLI09 or SAM10 PMEs with TMA14 PGL using 0.25 and 1 U mL^{-1} of this enzyme in apple pectin: (A1) gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates. The activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. In addition, control reactions of polyGalA depolymerisation only by TMA14 are shown: (B1) gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates. These results correspond to 2 h and 30 min of reaction for 0.25 and 1 U mL^{-1} of TMA14, respectively. The reactions were carried out using 0.5% (w/v) apple pectin and 0.5% (w/v) polyGalA in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

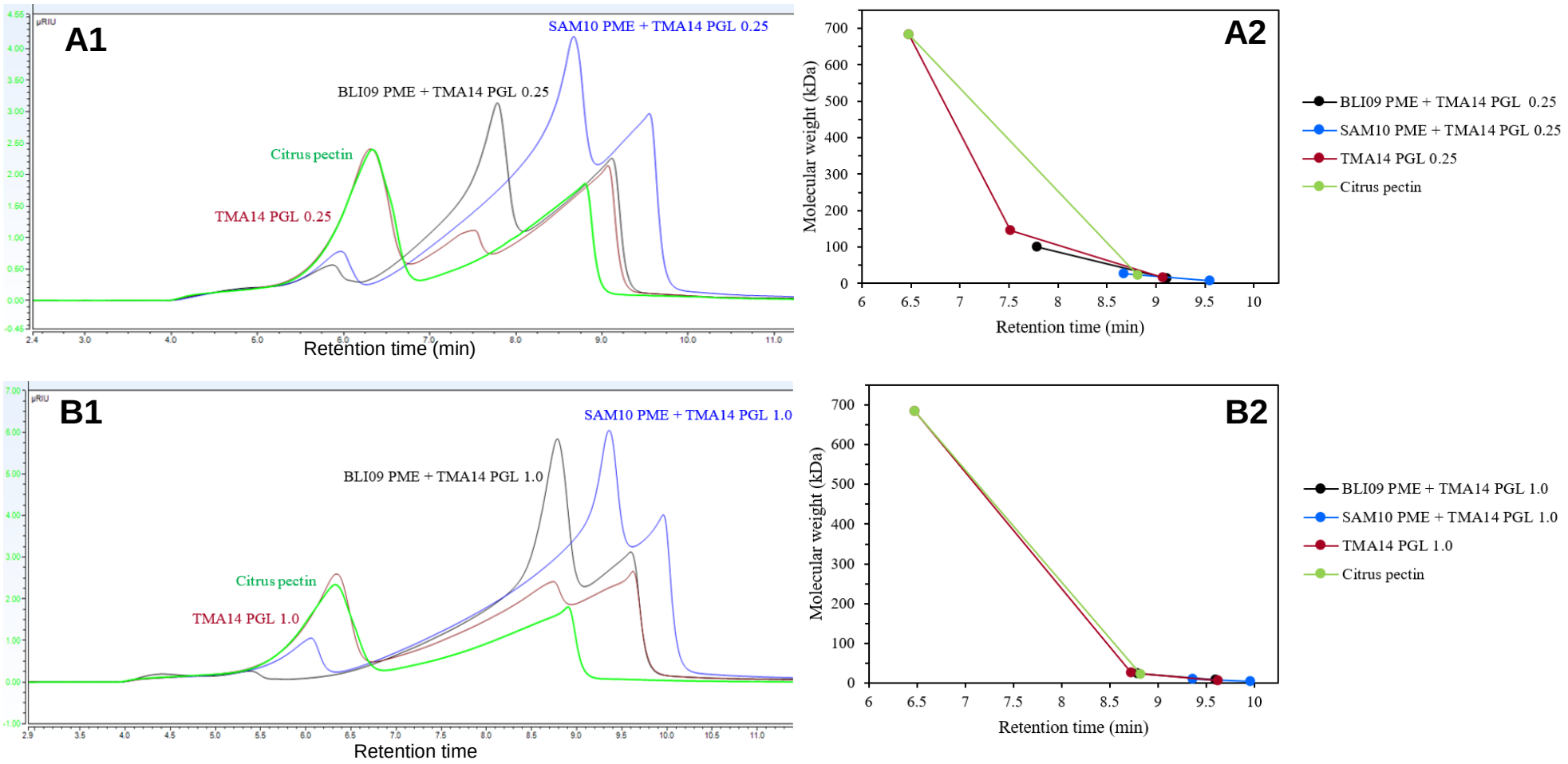

Figure 5.19. Synergistic activity between BLI09 or SAM10 PMEs with TMA14 PGL using citrus pectin. (A1) Gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates after 2 h of reaction using 0.25 U mL^{-1} of TMA14. (B1) Gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates after 30 min of reaction using 1 U mL^{-1} of TMA14. In both cases, the activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. The reactions were carried out using 0.5% (w/v) citrus pectin in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

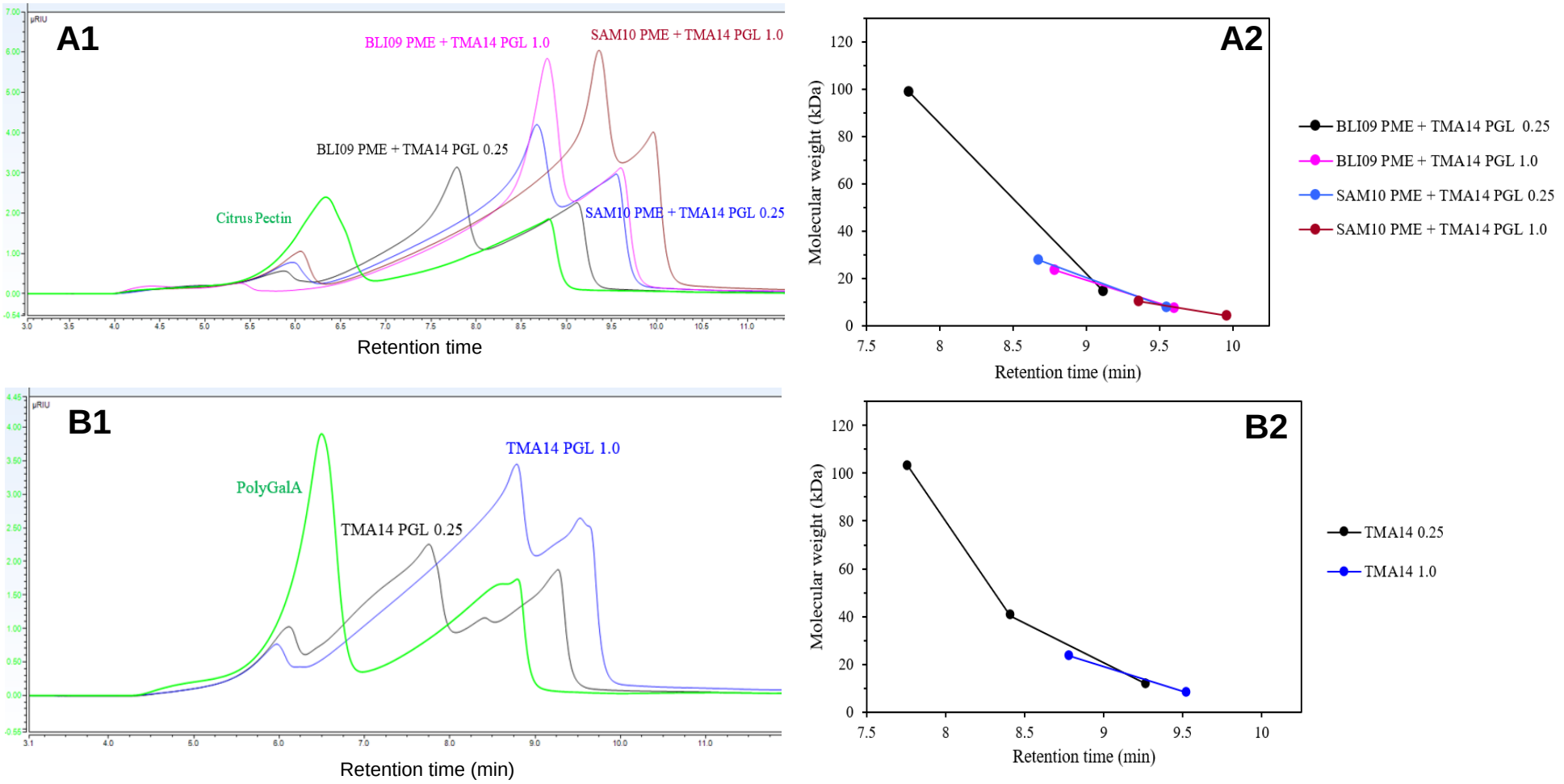

Figure 5.20. Comparison between the synergistic activity of BLI09 and SAM10 PMEs with TMA14 PLG using 0.25 and 1 U mL^{-1} of this enzyme in citrus pectin: (A1) gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates. The activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. In addition, control reactions of polyGalA depolymerisation only by TMA14 are shown: (B1) gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates. These results correspond to 2 h and 30 min of reaction for 0.25 and 1 U mL^{-1} of TMA14, respectively. The reactions were carried out using 0.5% (w/v) citrus pectin and 0.5% (w/v) polyGalA in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

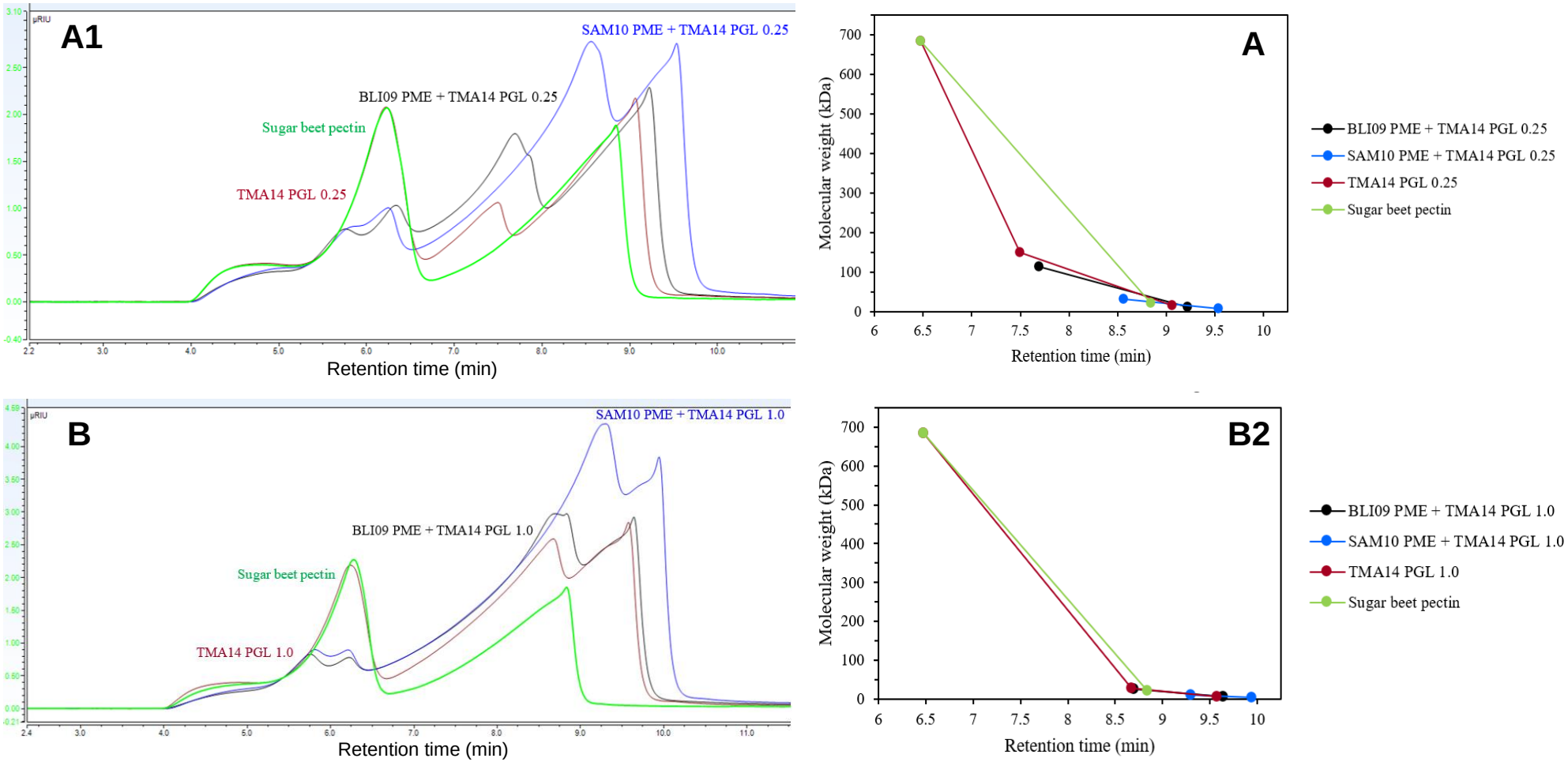

Figure 5.21. Synergistic activity between BLI09 or SAM10 PMEs with TMA14 PGL using sugar beet pectin. (A1) Gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates after 2 h of reaction using 0.25 U mL^{-1} of TMA14. (B1) Gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates after 30 min of reaction using 1 U mL^{-1} of TMA14. In both cases, the activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. The reactions were carried out using 0.5% (w/v) sugar beet pectin in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

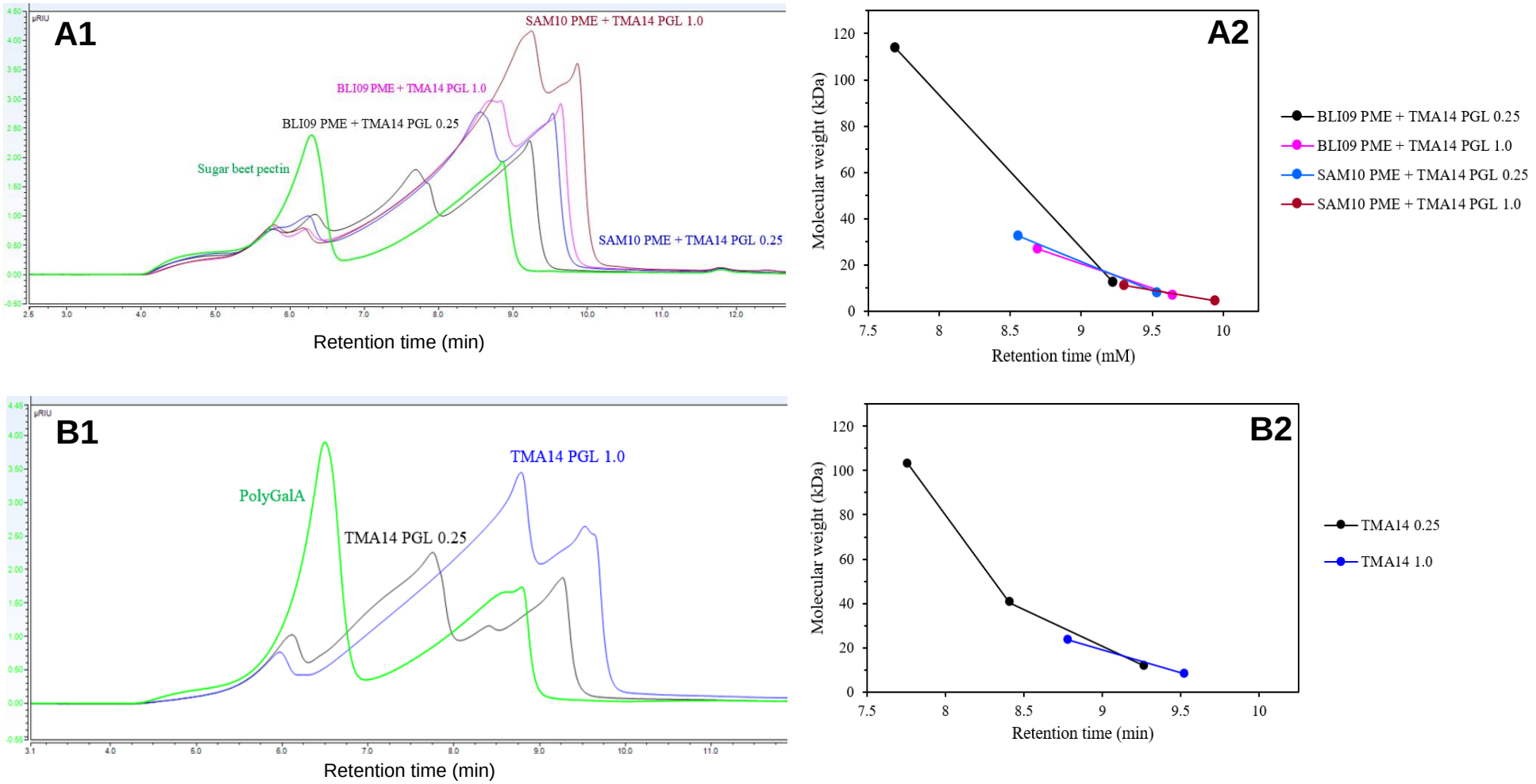

Figure 5.22. Comparison between the synergistic activity of BLI09 and SAM10 PMEs with TMA14 PGL using 0.25 and 1 U mL^{-1} of this enzyme in sugar beet pectin: (A1) gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates. The activity of BLI09 and SAM10 were 9 and 18 U mL^{-1}, respectively. In addition, control reactions of polyGalA depolymerisation only by TMA14 are shown: (B1) gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates. These results correspond to 2 h and 30 min of reaction for 0.25 and 1 U mL^{-1} of TMA14, respectively. The reactions were carried out using 0.5% (w/v) sugar beet pectin and 0.5% (w/v) polyGalA in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

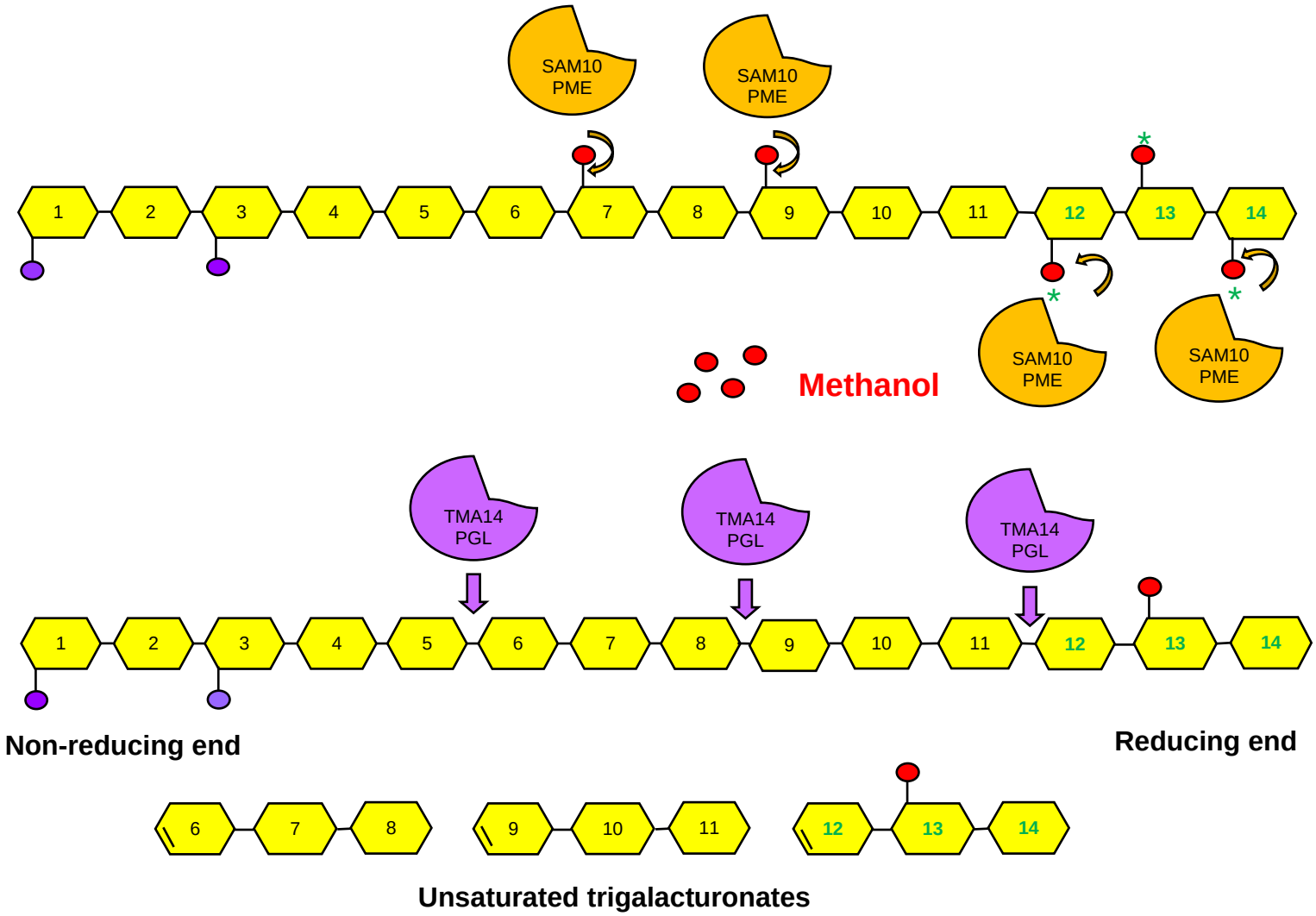

Figure 5.23. Schematic representation of consecutive SAM10 PME and TMA14 PGL activities on HG backbone of pectin. SAM10 PME demethylates pectin in a random manner, then TMA14 PGL acts on the reducing end of non-methylated pectin releasing unsaturated trigalacturonates as major products. Meanwhile, (*) are methanol groups that would be released by BLI09 PME in a blockwise demethylation pattern allowing to release only the trigalacturonate formed by 12, 13 and 14 GalA residues. (⬡) GalA, (🍒) methyl groups and (🍒) acetyl groups. HG: homogalacturonan, PME: pectin methylesterase, PGL: pectate lyase and GalA: galacturonic acid.

Figures 5.24 and 5.25 show the synergistic activity between BLI09 or SAM10 PMEs with TFU20 PGL using apple pectin, Figures 5.26 and 5.27 using citrus pectin and Figures 5.28 and 5.29 using sugar beet pectin. From Figures 5.24, 5.26 and 5.28, it was observed that all apple, citrus and sugar beet pectin presented two main peaks of 20 and 650 kDa, and similar depolymerisation profiles were obtained for these three substrates by the synergistic action between BLI09 or SAM10 PMEs with TFU20 PGL. Thus, using 0.1 U mL^{-1} of TFU20 peaks between 15 and 170 kDa were produced in the synergistic reactions with BLI09, while peaks of around 9 and 30 kDa in the reactions with SAM10. Meanwhile, using 0.5 U mL^{-1} of TFU20 peaks of around 15 and 70 kDa were produced in the synergistic reactions with BLI09, while peaks of around 8 and 25 kDa were produced in the reactions with SAM10. In both cases, using only TFU20, peaks between 20 and 370 kDa were produced but keeping the main peak of 650 kDa corresponding to the substrates. Enzyme activity of 0.5 U mL^{-1} was the optimum for the synergistic reactions with TFU20 since same depolymerisation profiles were obtained using higher activities.

The comparison of the synergistic activity between BLI09 and SAM10 using 0.1 and 0.5 U mL^{-1} of TFU20 in apple, citrus and sugar beet pectin is better shown in Figures 5.25 A, 5.27 A and 5.29 A, respectively. In addition, control reactions using only TFU20 with polyGalA were tested and the molecular weight distribution of the produced oligogalacturonates was analysed by GFC (Figures 5.25 B, 5.27 B and 5.29 B). From these results, it was observed that polyGalA presented two main peaks of around 20 and 650 kDa which were depolymerised by 0.1 U mL^{-1} of TFU20 in two peaks of 15 and 140 kDa. While 0.5 U mL^{-1} of this enzyme, depolymerised polyGalA in two peaks of 15 and 85

kDa. These findings showed that similar depolymerisation profiles were obtained using 0.1 and 0.5 U mL^{-1} of TFU20 in its synergistic reactions with BLI09 in the three substrates and in its action with polyGalA. Meanwhile, smaller oligogalacturonates were obtained using 0.1 and 0.5 U mL^{-1} of TFU20 in its synergistic reactions with SAM10 in the three substrates than in its action with polyGalA. These findings demonstrated that the synergistic activity between PMEs with TFU20 was efficient for esterified pectin depolymerisation. The smallest oligogalacturonates obtained in the synergistic reactions with SAM10 could be explained by the random demethylation pattern of this enzyme which might favour the endo-PGL activity of TFU20 (EC 4.2.2.2) (Figure 5.30).

In summary, smaller fragments were observed using 1 U mL^{-1} of TMA14 and 0.5 U mL^{-1} of TFU20 in their synergistic reactions with SAM10 (4-10 and 8-25 kDa, respectively). Comparing the synergistic reactions between both PMEs with TMA14 and those between both PMEs with TFU20, similar depolymerisation profiles were obtained using 0.25 and 0.1 U mL^{-1} of TMA14 and TFU20, respectively in the three substrates. However, using 1 and 0.5 U mL^{-1} of TMA14 and TFU20, respectively smaller fragments were produced in the synergistic reactions with TMA14. These findings can be explained due to TMA14 is an exo-PGL and releases unsaturated trigalacturonates as major products, although di and tetragalacturonates can also be released. On the other hand, TFU20 is an endo-PGs and releases oligogalacturonates of variable size.

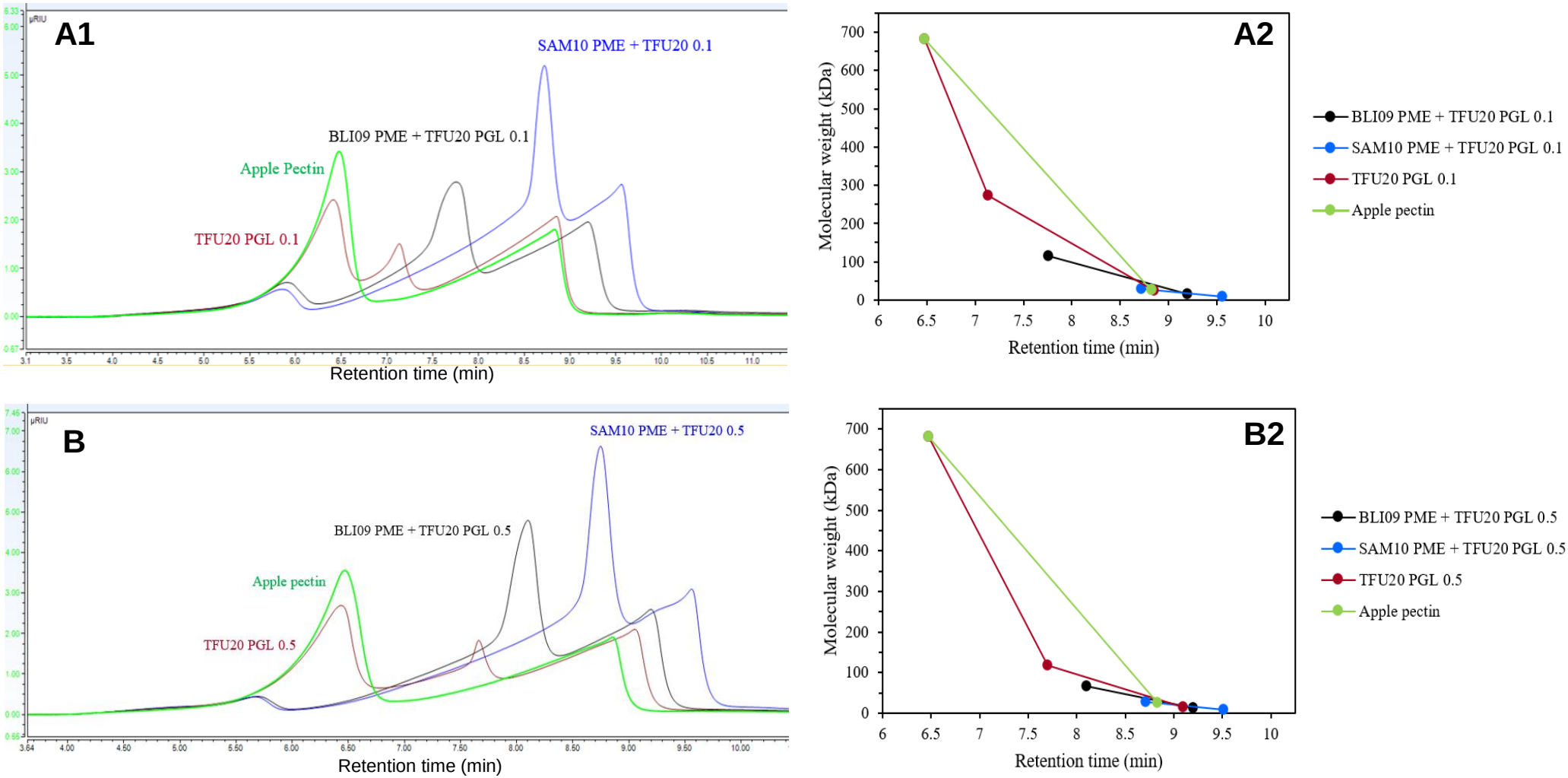

Figure 5.24. Synergistic activity between BLI09 or SAM10 PMEs with TFU20 PGL using apple pectin. (A1) Gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates after 2 h of reaction using 0.1 U mL^{-1} of TFU20. (B1) Gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates after 30 min of reaction using 0.5 U mL^{-1} of TFU20. In both cases, the activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. The reactions were carried out using 0.5% (w/v) apple pectin in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm up to 24 h. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

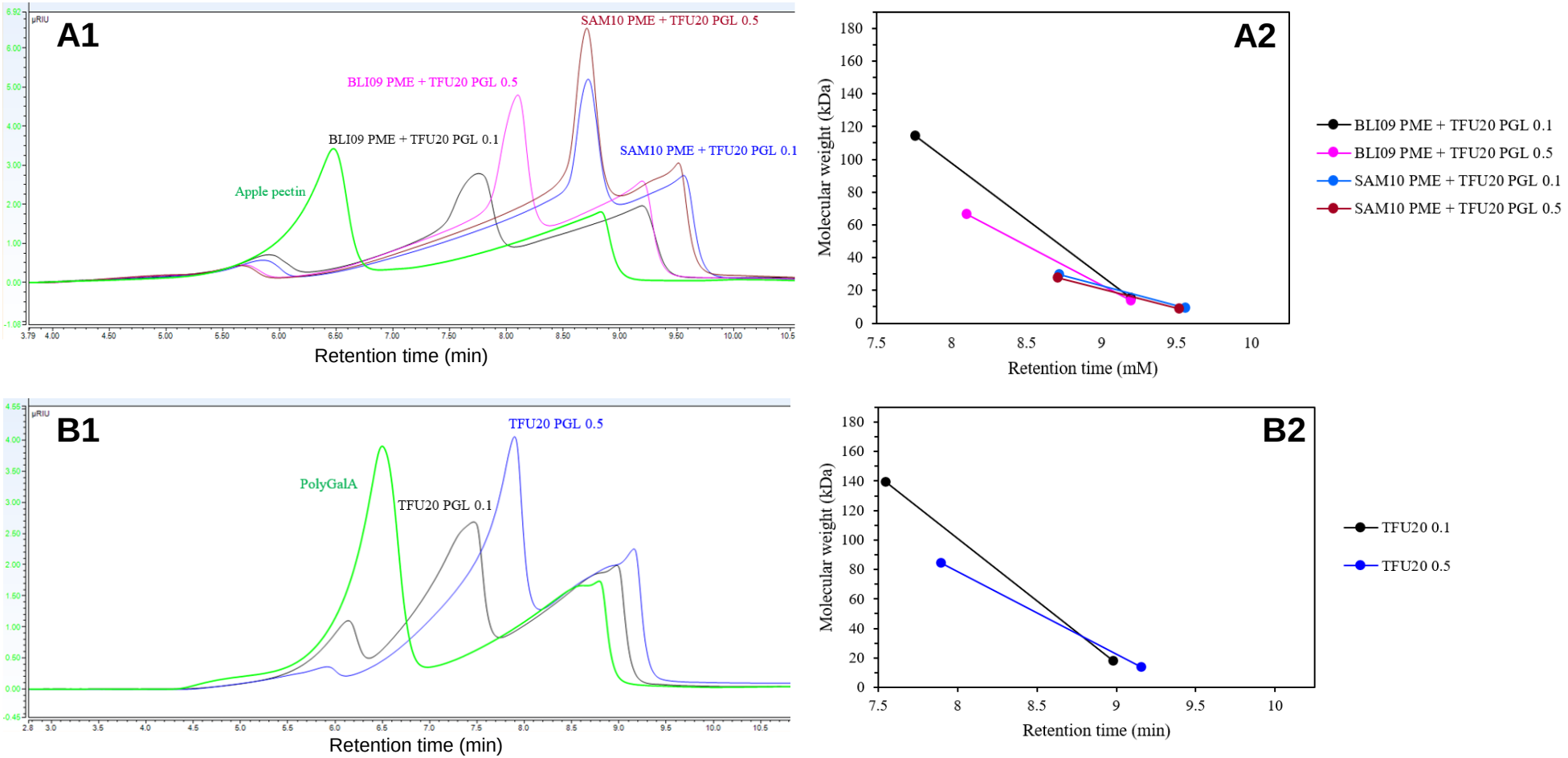

Figure 5.25. Comparison between the synergistic activity of BLI09 and SAM10 PMEs with TFU20 PGL using 0.1 and 0.5 U mL^{-1} of this enzyme in apple pectin: (A1) gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates. The activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. In addition, control reactions of polyGalA depolymerisation only by TFU20 are shown: (B1) gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates. These results correspond to 2 h and 30 min of reaction for 0.1 and 0.5 U mL^{-1} of TMA14, respectively. The reactions were carried out using 0.5% (w/v) apple pectin and 0.5% (w/v) polyGalA in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

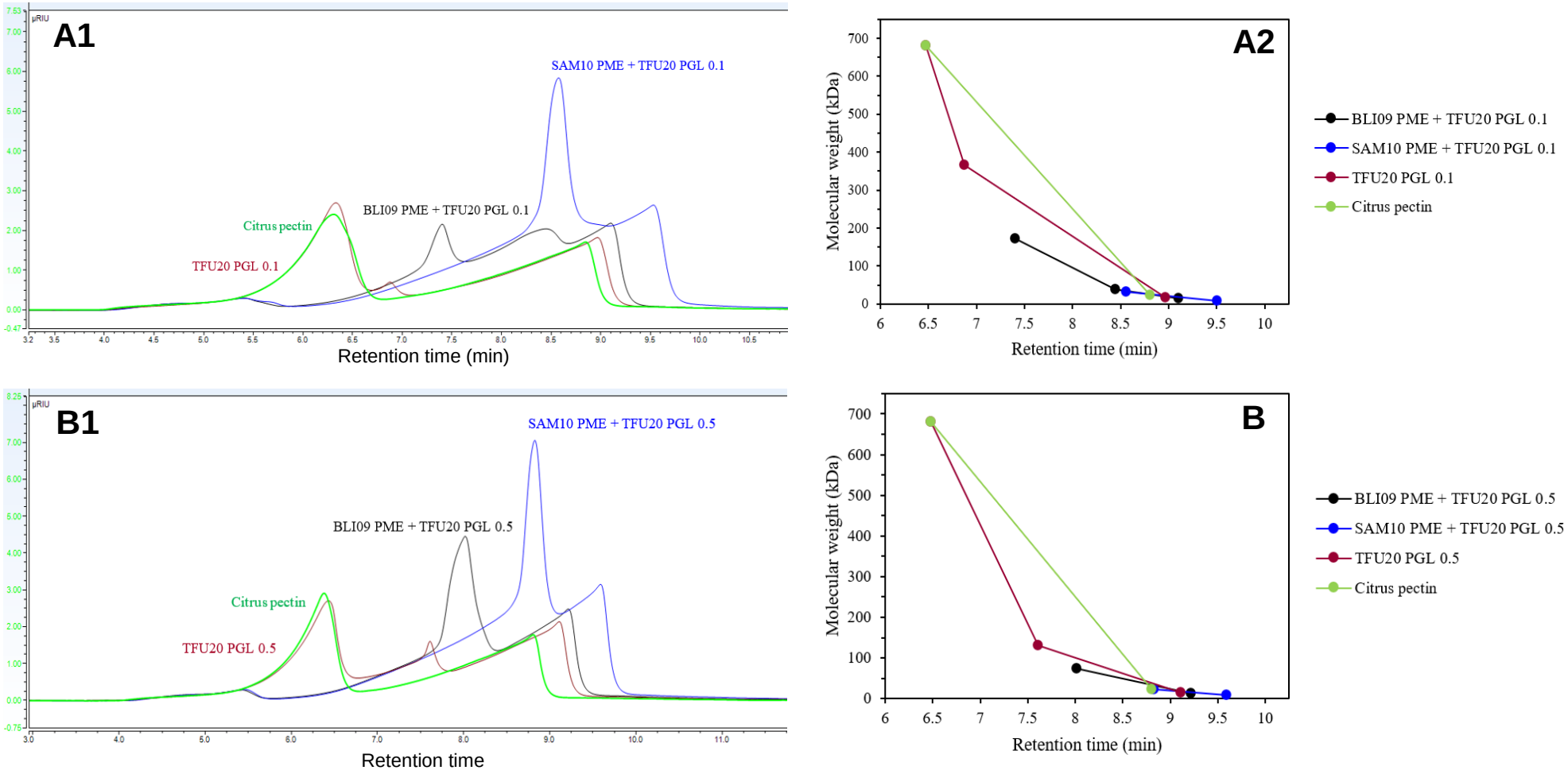

Figure 5.26. Synergistic activity between BLI09 or SAM10 PMEs with TFU20 PGL using citrus pectin. (A1) Gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates after 2 h of reaction using 0.1 U mL^{-1} of TFU20. (B1) Gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates after 30 min of reaction using 0.5 U mL^{-1} of TFU20. In both cases, the activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. The reactions were carried out using 0.5% (w/v) citrus pectin in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm up to 24 h. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

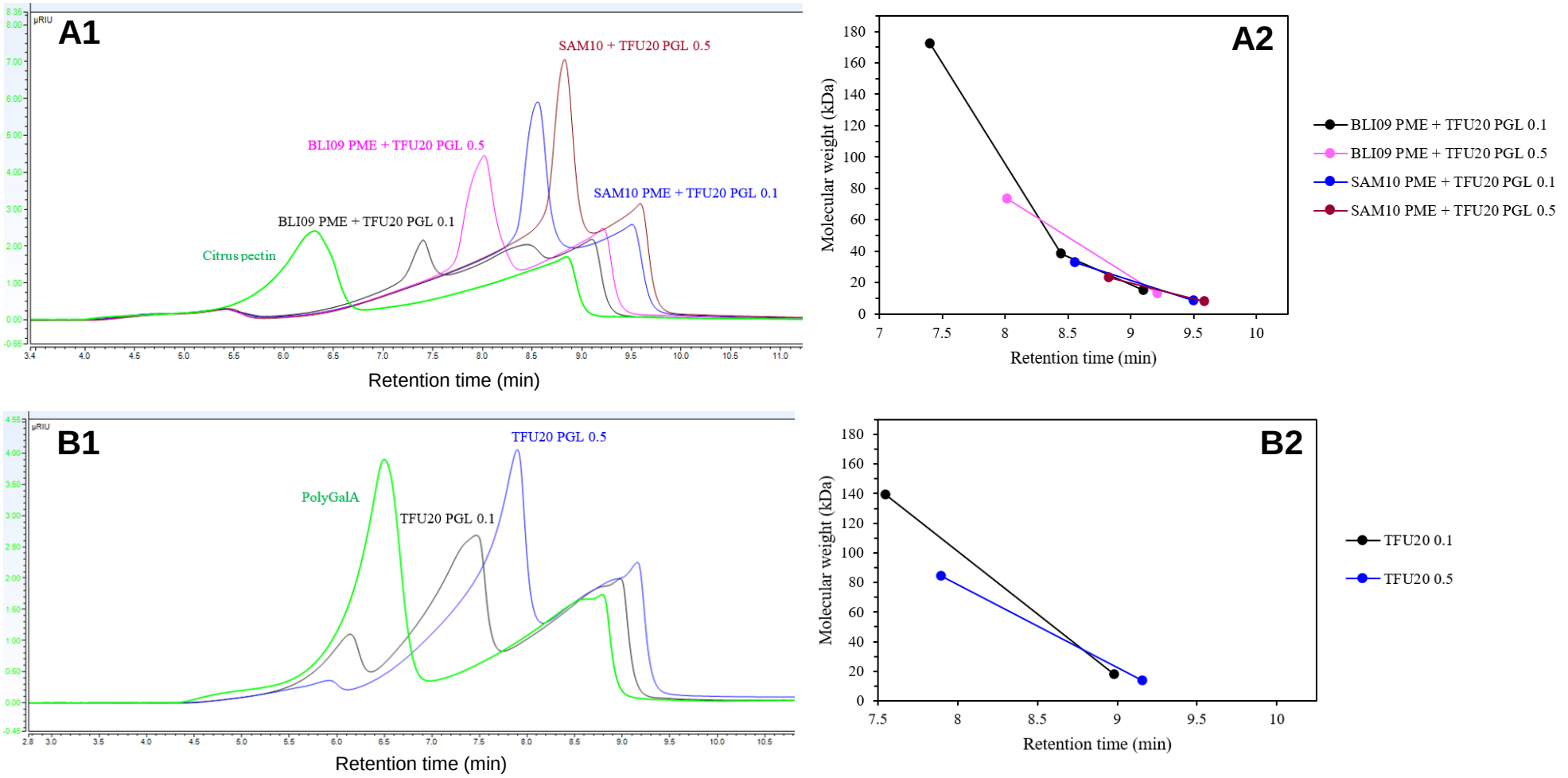

Figure 5.27. Comparison between the synergistic activity of BLI09 and SAM10 PMEs with TFU20 PGL using 0.1 and 0.5 U mL^{-1} of this enzyme in citrus pectin: (A1) gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates. The activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. In addition, control reactions of polyGalA depolymerisation only by TFU20 are shown: (B1) gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates. These results correspond to 2 h and 30 min of reaction for 0.1 and 0.5 U mL^{-1} of TMA14, respectively. The reactions were carried out using 0.5% (w/v) citrus pectin and 0.5% (w/v) polyGalA in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

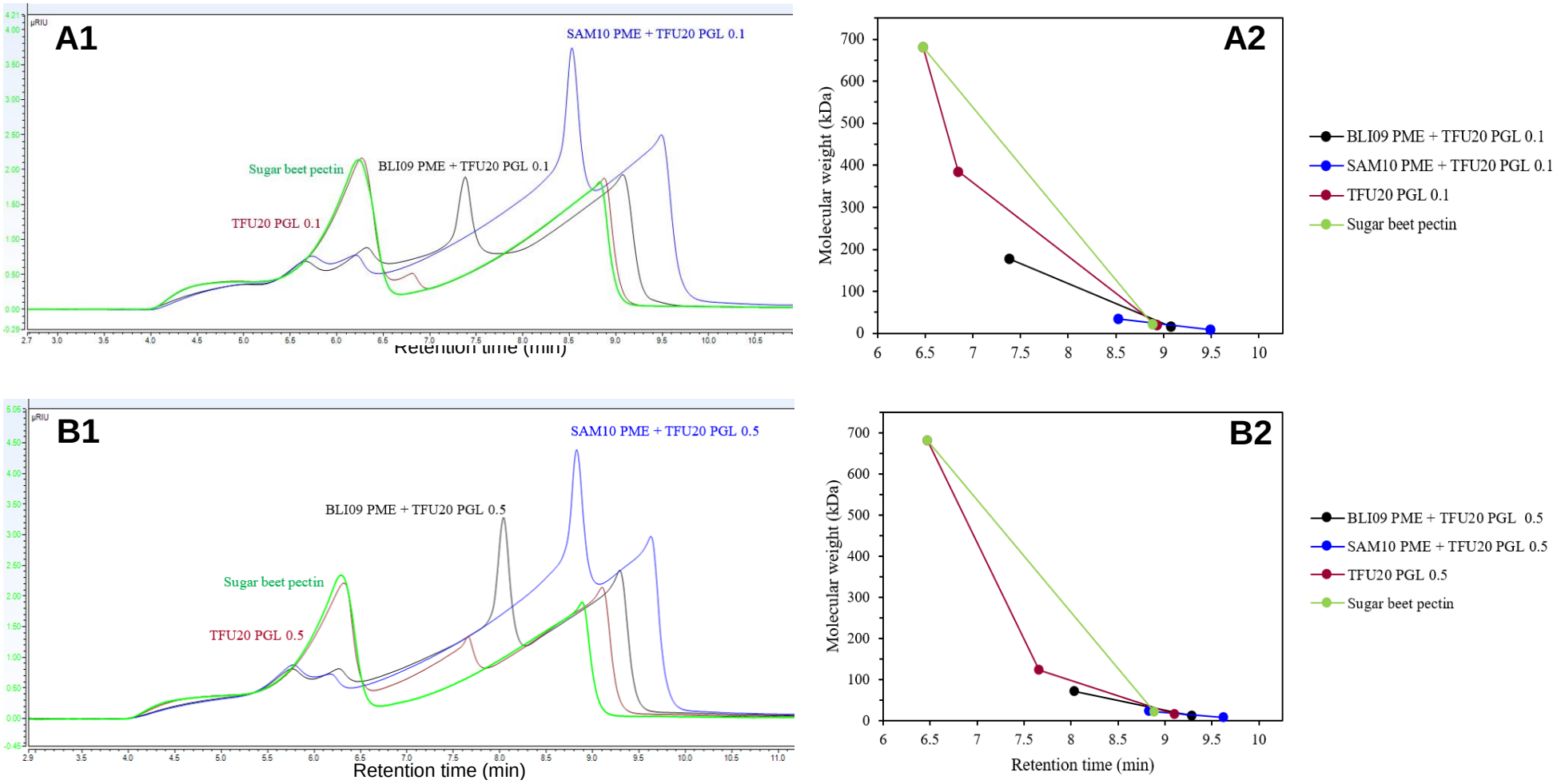

Figure 5.28. Synergistic activity between BLI09 or SAM10 PMEs with TFU20 PGL using sugar beet pectin. (A1) Gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates after 2 h of reaction using 0.1 U mL^{-1} of TFU20. (B1) Gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates after 30 min of reaction using 0.5 U mL^{-1} of TFU20. In both cases, the activity of BLI09 and SAM10 was 9 and 18 U mL^{-1}, respectively. The reactions were carried out using 0.5% (w/v) sugar beet pectin in 20 mM phosphate buffer pH 7 at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

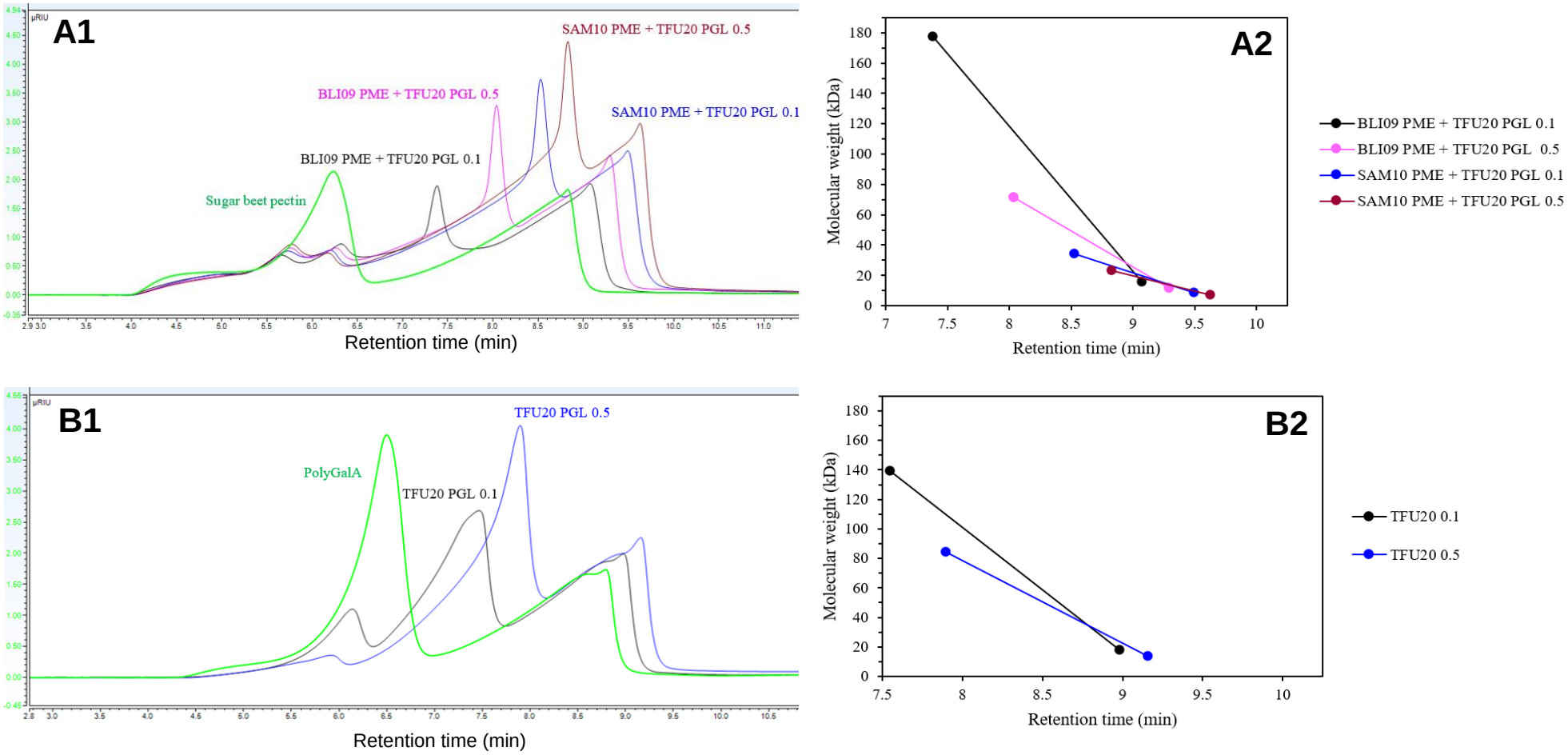

Figure 5.29. Comparison between the synergistic activity of BLI09 and SAM10 PMEs with TFU20 PGL using 0.1 and 0.5 U mL^{-1} of this enzyme in sugar beet pectin: (A1) gel filtration chromatograms and (A2) molecular weight distribution of the produced oligogalacturonates. The activity of BLI09 and SAM10 was 9 and 18 U mL^{-}, respectively. In addition, control reactions of polyGalA depolymerisation only by TFU20 are shown: (B1) gel filtration chromatograms and (B2) molecular weight distribution of the produced oligogalacturonates. These results correspond to 2 h and 30 min of reaction for 0.1 and 0.5 U mL^{-1} of TMA14, respectively. The reactions were carried out using 0.5% (w/v) sugar beet pectin and 0.5% (w/v) polyGalA in 20 mM phosphate buffer pH 7at 50 °C and 300 rpm. The assay was performed as described in Section 2.8.3.2 and 2.9.7 in Chapter 2.

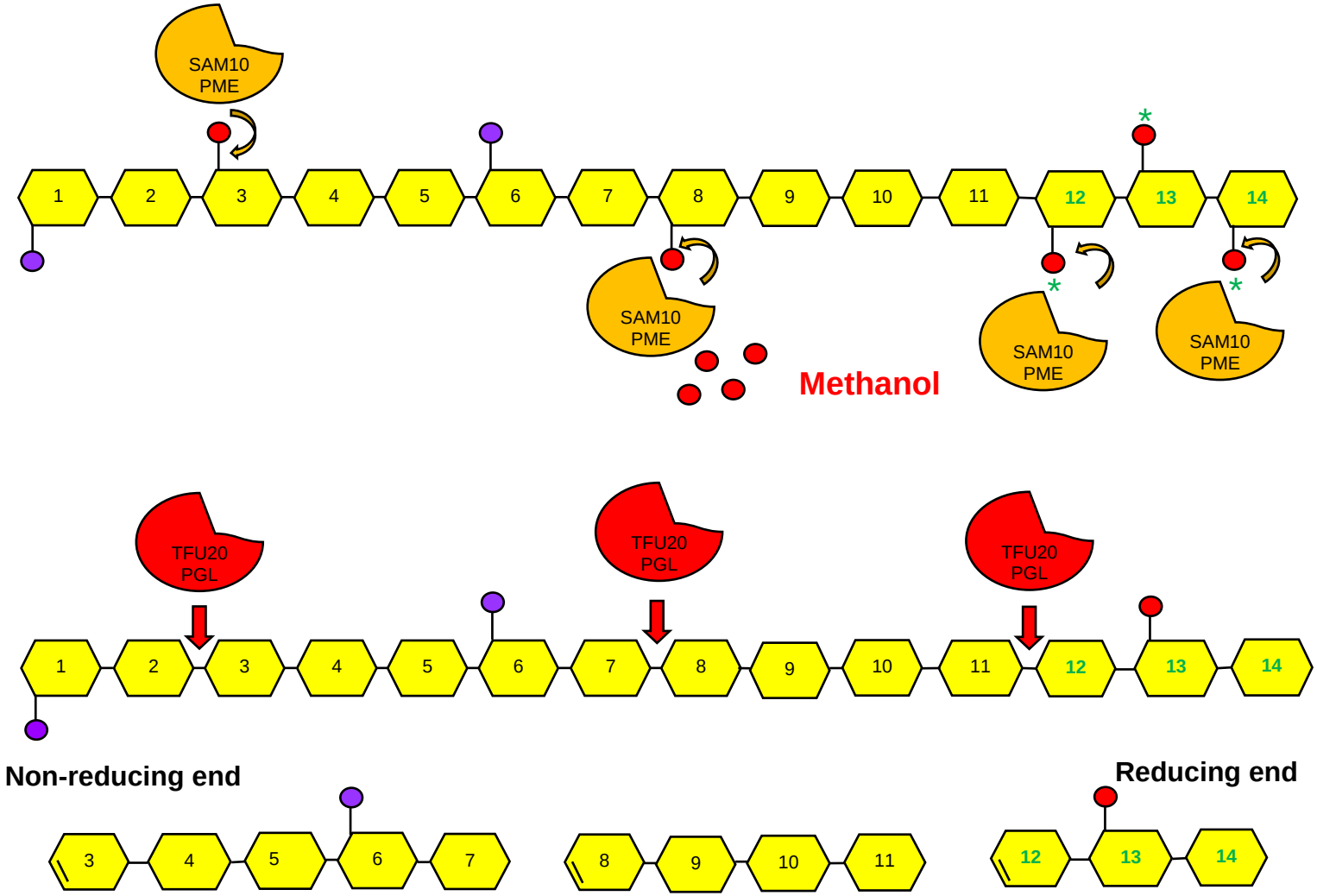

Figure 5.30. Schematic representation of consecutive SAM10 PME and TFU20 PGL activities on HG backbone of pectin. SAM10 PME demethylates pectin in a random manner, then TFU20 PGL acts on non-methylated pectin releasing unsaturated olygalacturonates. Meanwhile, (*) are methanol groups that would be released by BLI09 PME in a blockwise demethylation pattern allowing to release only the oligogalacturonate formed by 12, 13 and 14 GalA residues. (⬡) GalA, (🍒) methyl groups and (🍒) acetyl groups. HG: homogalacturonan, PME: pectin methylesterase, PGL: pectate lyase and GalA: galacturonic acid.

5.3.6 Synergistic action between PGLs and PMEs along with a PAE individually expressed

Synergistic action between PGLs, PMEs and a PAE was assayed in sugar beet pectin since this substrate is 20% acetylated. It was hypothesised that the addition of a PAE into the synergistic reactions will improve sugar beet pectin depolymerisation by removing acetyl groups. Synergistic reactions between BLI09 or SAM10 PMEs paired either with TMA14 or TFU20 PGLs along with a PAE were set up. Reactions without the addition of the PAE were used as controls.

For these reactions, 9 and 18 U mL^{-1} of BLI09 and SAM10 PMEs, respectively were used as well as 1 and 0.5 U mL^{-1} of TMA14 and TFU20 PGLs. While 0.022 U mL^{-1} of PAE21 were used. The reactions were carried out at compatible operational conditions for all the enzymes (pH 7 and 50°C). Methanol and acetic acid were released at concentrations of 2 and 0.6 mM, respectively. However, as we can see from Figure 5.31, the presence of PAE21 in the synergistic reactions did not improve sugar beet pectin depolymerisation since same depolymerisation profiles were obtained in presence and in absence of this enzyme. These findings could be related to the distribution of acetyl groups in sugar beet pectin as well as to the mechanism of action of PAE21. According to the literature, acetyl groups are distributed in a blockwise manner in sugar beet pectin (Ralet, Crépeau and Bonnin, 2008), and PAE21 deacetylates sugar beet pectin at *O-3* positions in non-methylated GalA residues acting in a random way (Remoroza *et al.*, 2014). These factors might have caused that PAE21 does not play a key role in the synergistic action with PGLs and PMEs for pectin depolymerisation (Figure 5.32).

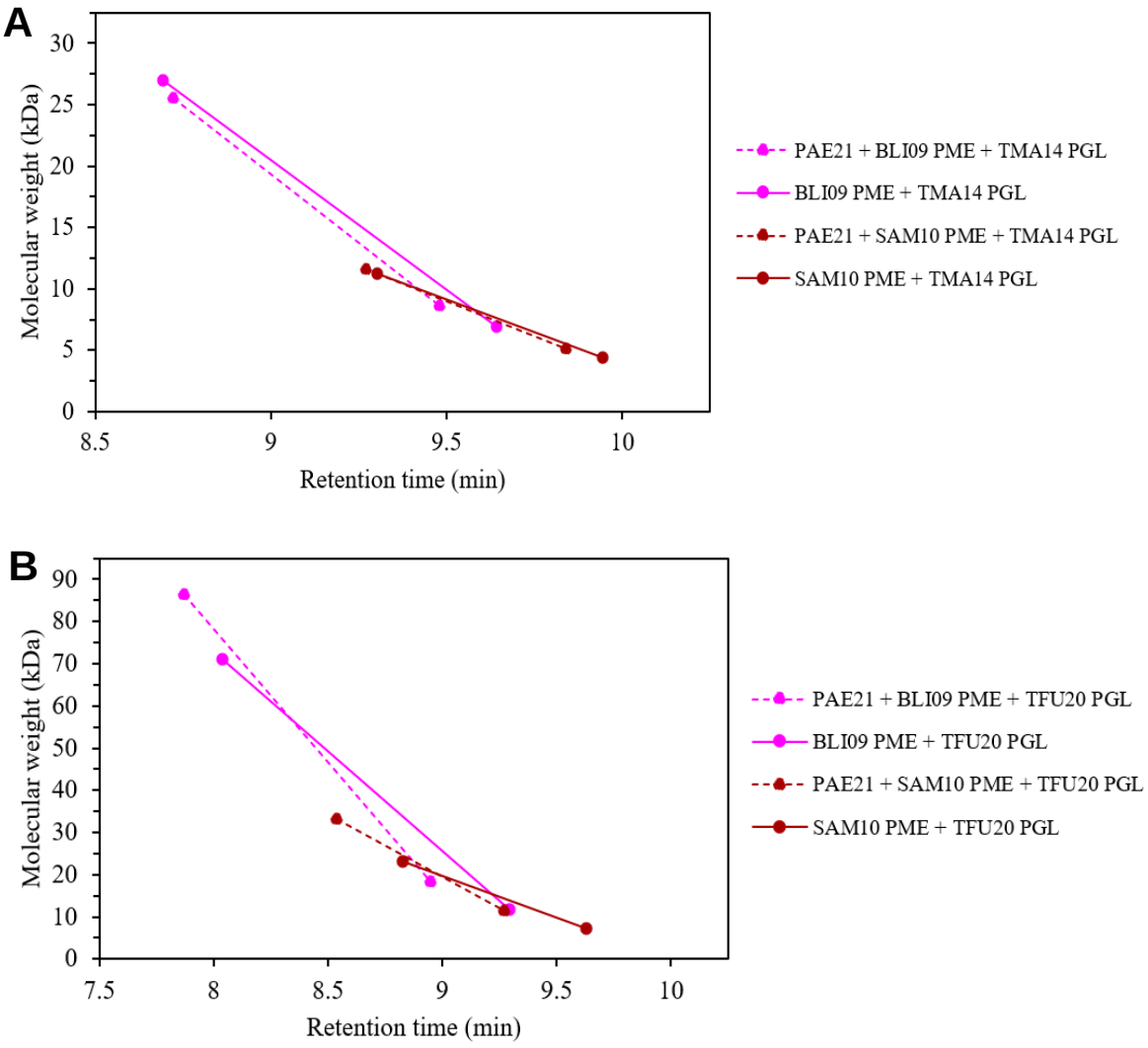

Figure 5.31. Molecular weight distribution of the produced oligogalacturonates by the synergistic activity between PMEs, PGLs and a PAE using sugar beet pectin. (A) Synergistic action between BLI09 or SAM10 PMEs with TMA14 PGL with and without the addition of PAE21. (B) Synergistic action between BLI09 or SAM10 PMEs with TFU20 PGL with and without the addition of PAE21. The reactions were carried out using 0.5% substrate in 100 mM phosphate buffer pH 7 at 50 °C and 300 rpm for 30 min. For the reactions, 9 and 18 U mL^{-1} of BLI09 and SAM10, respectively were used. Likewise, 1 and 0.5 U mL^{-1} of TMA14 and TFU20, respectively were used. The assay was performed as described in Sections 2.8.3.2 and 2.9.7 in Chapter 2. PAE: pectin acetylesterase.

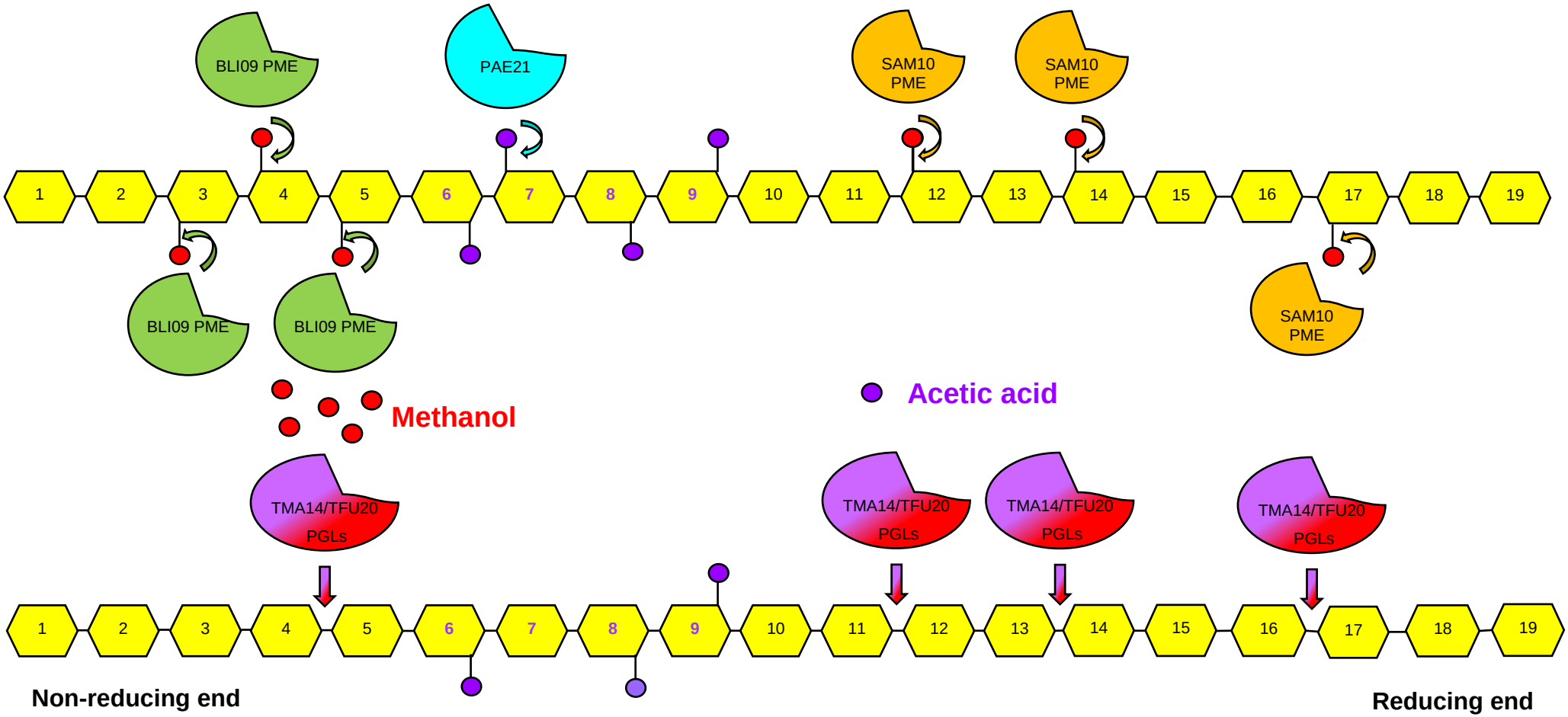

Figure 5.32. Synergistic action between PMEs and PGLs along with PAE21 on the HG backbone in sugar beet pectin. It is illustrated the blockwise and random demethylation patterns of BLI09 and SAM10 PMEs, respectively; the blockwise distribution of acetyl groups in sugar beet pectin and the deacetylation of PAE21 at *O-3* positions of non-methylated GalA suggesting a random deacetylation pattern. In addition, it is illustrated the subsequent action of the PGLs leading to the unsaturated oligogalacturonates release. Despite some acetyl groups were removed, no major increase in oligogalacturonates release was observed with the addition of PAE21 into the synergistic reactions. Glycosidic bonds between the GalA residues highlighted in purple (from 6 to 9) might not be breakdown by the PGLs because there are remaining acetyl groups that have not been removed due to their *O-2* positions and the PAE21 mechanism of action. (⬡) GalA, (⊤) methyl groups and (⊤) acetyl groups. PME: pectin methylesterase, PGL: pectate lyase, PAE: pectin acetylesterase, HG: homogalacturonan and GalA: galacturonic acid.

5.3.7 Effect of acetic acid on PGLs

As described in Section 5.3.6, the presence of a PAE21 in the synergistic reactions between PMEs and PGLs did not improve sugar beet pectin depolymerisation. It was hypothesised that one of the factors might be PGLs inhibition by the acetic acid released during the reactions. From Figure 5.33, it is observed that TMA14 and TFU20 PGLs remained more than 80 and 90% of its activity, respectively up to 1.5 mM acetic acid. These findings indicated that the acetic acid release in the synergistic reactions using sugar pectin (0.6 mM) did not inhibit TMA14 and TFU20.

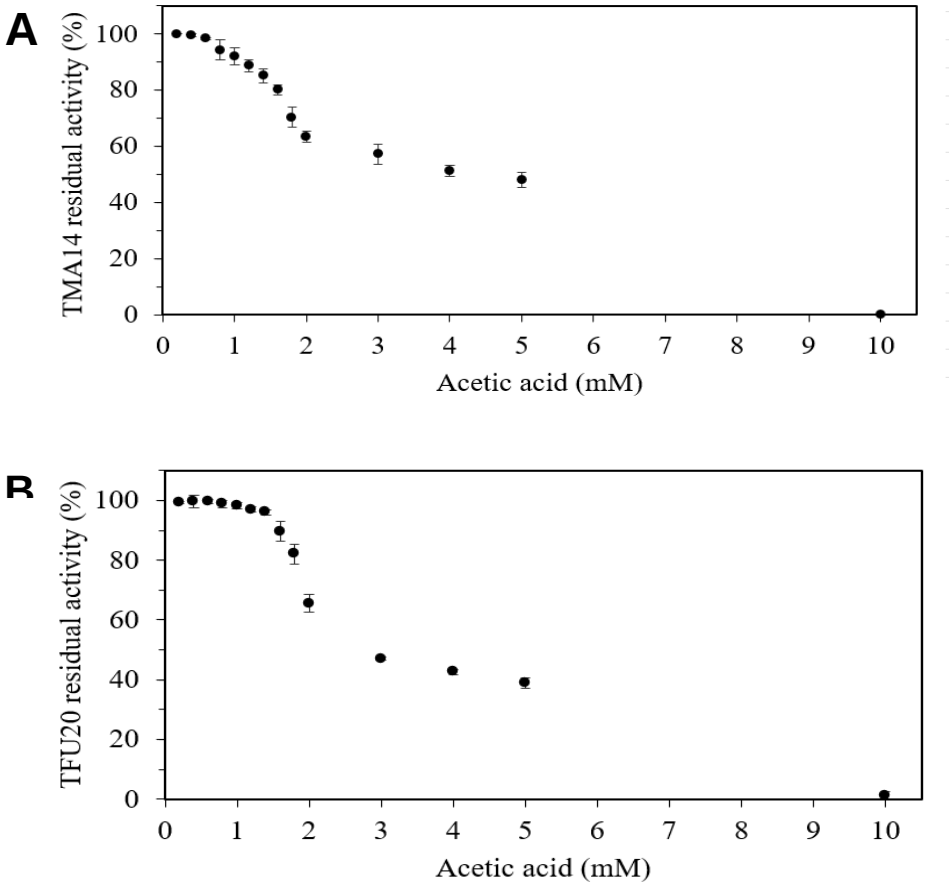

Figure 5.33. Effect of acetic acid on (A) TMA14 and (B) TFU20 PGLs. The reactions were carried out at 50 °C for 15 min using 0.5% polyGalA in 20 mM Tris-HCl pH 8; and with 0.25 U mL^{-1} of each enzyme. The assay was performed as described in Section 2.7.7.3 in Chapter 2. Error bars represent one standard deviation from the mean (n = 2). PGL: pectate lyase.

5.4 Conclusions

The aim of this chapter was to carry out functional characterization of three thermophilic PGLs (TMA14, TFU19 and TFU20) as well as explore their

synergistic action with other pectinases such as PMEs and a PAE for improving esterified pectin depolymerisation. The functional characterization assays showed that the activity of these enzymes was dependent on Ca^{2+} finding 0.6 mM as the optimum concentration. Other ions such as 0.25 and 0.5 mM Mn^{2+} improved the activity of TMA14 (1.3-fold) and TFU20 (2.5-fold), respectively. TMA14 and TFU19 exhibited optimum activity at pH 10 and 80 °C, while TFU20 at pH 7 and 50 °C. Concerning thermal stability, TMA14 exhibited great stability up to 70 °C and in the presence of Ca^{2+} up to 90 °C for up to 24 h. TFU19 and TFU20 presented high stability up to 60 and 50 °C, respectively and Ca^{2+} did not improve their stability at higher temperatures. The three PGLs showed Michaelis-Menten kinetics and no substrate inhibition was observed. Substrate specificity assays demonstrated that the three PGLs were able to depolymerise esterified substrates at their optimum conditions of activity. The PGLs from this study were active in a wide range of pH and temperature and presented good stability at high temperatures. In addition, they were able to degrade different esterified substrates. All these characteristics make them applicable in different industrial processes.

GFC assays showed that TMA14 and TFU19, at their optimum condition of activity (pH 10 an 80 °C), were able to depolymerise polyGalA as well as of apple, citrus and sugar beet pectin in peaks from 70 to 200 kDa. While TFU20, at its optimum conditions of activity (pH 7 and 50 °C), was also able to depolymerise polyGalA as well as of apple, citrus and sugar beet pectin in peaks of 120 and 200 kDa. However, the main peak of all these substrates (650 kDa) was completely depolymerised by the three PGLs only in the non-esterified polyGalA and was still detectable in the esterified apple, citrus and sugar beet pectin. These findings confirmed the preference of PGLs by non-esterified substrates and evidenced the need of a synergistic action with PMEs and PAEs to improve esterified pectin depolymerisation.

Hence, six synergistic reactions between the three PGLs (TMA14, TFU19 and TFU20) and the two PMEs (BLI09 and SAM10) were set up at compatible operational conditions for both pectinases (pH 7 and 50 °C). Apple pectin was used as substrate and the produced oligogalacturonates were quantified as reducing sugars. Concentrations of 6 and 7 mM of reducing sugars were

determined in the synergistic reactions between the PMEs with TMA14 and TFU20, respectively. However, in the reactions with TFU19 only 2.5 mM were quantified, which can be explained because the optimal activity of this pectinase was severely compromised at the operational conditions of the synergistic reactions. The most successful synergistic reactions for apple pectin depolymerisation were between BLI09 or SAM10 paired either with TMA14 or TFU20.

Thus, four synergistic reactions between these pectinases were assayed using apple, citrus and sugar beet pectin and the molecular weight distribution of the produced oligogalacturonates was analysed by GFC. The three substrates presented two main peaks of 20 and 650 kDa and exhibited similar depolymerisation profiles by the synergistic action between the pectinases. Using 0.25 U mL^{-1} of TMA14, oligogalacturonates ≤ than 100 and 30 kDa were released after 2 h of reaction in the synergistic reactions with BLI09 or SAM10, respectively. While using 1 U mL^{-1} of TMA14, oligogalacturonates ≤ than 25 and 10 kDa were released after 30 min of reaction in the synergistic reactions with BLI09 or SAM10, respectively. Regarding TFU20, using 0.1 U mL^{-1} of this enzyme, oligogalacturonates ≤ than 170 and 30 kDa were released after 2 h of reaction in the synergistic reactions with BLI09 or SAM10, respectively. While using 0.5 U mL^{-1} of TFU20, oligogalacturonates ≤ than 70 and 25 kDa were released after 30 min of reaction in the synergistic reactions with BLI09 or SAM10, respectively. In addition, it was found that the synergistic reactions between TMA14 or TFU20 with BLI09 using the three esterified substrates released oligogalacturonates of comparable size that those released in control reaction by TMA14 or TFU20 in the non-esterified polyGalA. Meanwhile, the synergistic reactions between TMA14 or TFU20 with SAM10 in these esterified substrates released smaller olygalacturonates than those produced in control reactions by TMA14 or TFU20 in polyGalA. These findings demonstrated the efficiency of the synergistic reactions between PMEs and PGLs for improving esterified pectin depolymerisation. The smaller oligogalacturonates released in all the synergistic reactions with SAM10 could be explained by the random demethylation pattern of this enzyme which might favour TMA14 and TFU20 activities. Furthermore, smaller oligogalacturonates were released in the

synergistic reactions between the PMEs with TMA14 in comparison with TFU20, because of the exo activity of TMA14 which releases mainly trigalacturonates, while TFU20 has an endo activity releasing oligogalacturonates. Overall, the smallest oligogalacturonates were generated in the synergistic reactions between SAM10 either with 1 U mL^{-1} of TMA14 (4 – 10 kDa) or 0.5 U mL^{-1} of TFU20 (8 – 25 kDa). Previous studies have reported that these low-molecular weight POS (3 - 30 kDa) have exhibited different bioactive properties (Yuan *et al.*, 2019; Zheng, Guo, *et al.*, 2021; Zheng, Xu, *et al.*, 2021).

Finally, it was demonstrated that the addition of PAE21 in the synergistic reactions did not enhance sugar beet pectin depolymerisation since similar depolymerisation profiles were observed with or without the addition of this enzyme. These results could be explained by acetyl groups distribution in sugar beet pectin as well as by PAE mechanism of action. To confirm that the acetic acid produced in the synergistic reactions was not inhibiting PGLs action, product inhibition assays using this compound were carried out. The results showed that the concentration of acetic acid produced (0.6 mM) did not produce PGLs inhibition.

In summary, the work from this chapter studied novel thermophilic pectate lyases providing information about their operational conditions, stability, kinetic parameters and substrate specificity. The functional characterisation of enzymes is fundamental to set up compatible operational conditions for those such as pectinases which act in a synergistic way. In addition, the synergistic activity between thermophilic PMEs and PGLs improved esterified pectin depolymerisation. The PGLs from this study as well as their synergistic activity could be useful in several fields of industry improving yield and quality through an eco-friendly bioprocess. Finally, this study contributes to expand the application of thermophilic pectinases in different fields of industry as well as to valorise pectin-rich biomass.

CHAPTER 6 GENERAL CONCLUSIONS AND FUTURE WORK

6.1 General conclusions

Millions of tonnes of pectin-rich biomass are mainly produced by food and sugar industries as well as from agricultural activities. In some cases, this biomass is sold as undervalued products and in other cases is causing pollution problems. Biocatalysis using pectinases for pectin-rich biomass recycling, is a promising alternative to valorise it with economic and environmental advantages. Thereby, the discovery and study of thermophilic pectinases is gaining increasing importance due to their applications in industrial biotechnology. This work was focused on identifying novel thermophilic pectinases and studying their characteristics of activity to be used in synergistic reactions for pectin bioconversion and depolymerisation. The results of this research have demonstrated the applicability of these enzymes to obtain bio-based compounds such as GalA and oligogalacturonates as well as for improving industrial processes in which pectin depolymerisation is fundamental. The key findings and their relevance are summarised in the following sections.

6.1.1 Identification and characterisation of novel thermophilic pectinases

The primary objective of this research was to identify putative thermophilic pectinases for cloning and expression as well as carry out their functional characterisation. Seven thermophilic pectinases were identified through a genome mining approach which were successfully cloned and expressed. From these enzymes: two were PMEs, one from *Bacillus licheniformis* (BLI09) and one from *Streptomyces ambofaciens* (SAM10); two exo-PGs, one from *Thermotoga maritima* (TMA01) and *Bacillus licheniformis* (BLI04); and three PGLs, one from *Thermotoga maritima* (TMA14) and two from *Thermobifida fusca* (TFU19 and TFU20). The identification of novel thermophilic pectinases belonging to different types contributes to expand the knowledge about these enzymes since they have a number of advantages with respect to their mesophilic counterparts. Subsequently, they were fully functionally characterised and the determination of the influence of ions, pH and temperature on their activity was carried out. In addition, thermal stability was

studied and the kinetic parameters for each enzyme were determined. Mn^{2+} significantly increased the activity of exo-PGs and PGLs, while did not affect the activity of PMEs. In general, exo-PGs and PGLs were active in a wide range of pH (7-11), while PMEs mainly between pH 6 and 7. Regarding temperature, exo-PGs showed good activity between 50 and 90 °C, PMEs between 40 and 60 °C and PGLs between 50 and 80 °C (TFU20 between 40 and 60 °C). All the enzymes exhibited thermal stability at 50 °C for up to 24 h. Characterisation studies are fundamental since it allows to determine the conditions of activity of each enzyme as well as to set up accurate operational conditions in a bioprocess in which more than one enzyme is involved. Substrate specificity assays showed that exo-PGLs and PGLs preferred non-esterified substrates evidencing the need of a synergistic action with PMEs for improving pectin bioconversion and depolymerisation.

6.1.2 Synergistic action between thermophilic pectinases for pectin bioconversion into bio-based chemicals

Following identification and characterisation of thermophilic pectinases, the synergistic activity between exo-PGs and PMEs for pectin bioconversion into GalA was explored. The highest GalA concentrations were released by the synergistic reactions between BLI09 PME either with TMA01 (2.5 mM) or BLI04 (2 mM) exo-PGs using apple and citrus pectin as substrates. In these synergistic reactions, the HG backbone was blockwise demethylated by BLI09 PME, subsequently the exo-PGs acted on the blocks of non-methylated pectin releasing the GalA. The synergistic reactions between SAM10 PME either with TMA01 or BLI04 exo-PGs released low GalA concentrations, probably due to the mechanism of action of SAM10. This enzyme might demethylate pectin in a random manner, thus the blocks of non-methylated pectin where exo-PGs act would not be generated. In all the synergistic reactions using sugar beet pectin as substrate, low GalA concentrations were released. It could be explained by the high percentage of acetyl groups in the HG backbone of this substrate, which hinders the exo-PGs activity. The findings demonstrated the capacity of thermophilic pectinases to act in a synergistic way to achieve pectin bioconversion into bio-based chemicals such as GalA. GalA is a valuable bio-

based chemical precursor of other compounds such as L-galactonic acid; L-ascorbic acid; mucic acid; 2,5-furandicarboxylic acid; adipic acid; as well as nylon and other biopolymers (Taguchi, Oishi and Iida, 2007; Wiebe et al., 2010; Lavilla et al., 2011; Kuivanen et al., 2014). In addition, this work further provides novel insights into the potential of these enzymes for the valorisation of pectin-rich biomass feedstocks within a biorefinery context.

6.1.3 Co-expression of thermophilic pectinases for a cost-effective pectin bioconversion into bio-based chemicals

Since the synergistic action between thermophilic PMEs and exo-PGs demonstrated to be efficient for pectin bioconversion into GalA, the following objective was to co-express them in a single plasmid and host for a cost-effective process. The enzymes from the most successful synergistic reactions which were between BLI09 PME paired either with TMA01 or BLI04 exo-PGs were chosen for co-expression in pETDuet-1. When the co-expression systems were constructed, the effect of the gene cloning order in the expression of the enzymes was evaluated. The results showed that both the PME and the exo-PG were well expressed only when the PME was cloned in MCS-2 downstream any of the exo-PGs and with the presence of a T7 terminator behind. The findings revealed that the gene cloning order and the presence of a T7 terminator was crucial specially for BLI09 good expression. The synergistic activity of the co-expressed enzymes allowed the release of 3 and 2.5 mM GalA from the reactions between BLI09 either with TMA01 or BLI04, respectively using apple and citrus pectin as substrates. Through the co-expression of two kinds of pectinases in a single plasmid and their production in a single host, a cost-effective pectin bioconversion can be achieved. Co-expression allows to reduce enzymes production cost since multiple enzymes are produced in a single culture. In addition, in this case clarified lysates were used giving comparable results to the individually purified enzymes. It also contributes to reduce the enzymes purification costs. However, product inhibition assays revealed that both TMA01 and BLI04 exo-PGs were inhibited by 3 and 2.5 mM GalA, respectively indicating that it was the reason because these maximum concentrations were produced during the synergistic reactions. In the

synergistic reactions using sugar beet pectin as substrate, a PAE was added into the synergistic reactions in order to deacetylate the HG backbone and improve GalA release. Despite PAE addition, still low GalA concentrations were still obtained, probably related to the mechanism of action of this enzyme and the position of the acetyl groups in the HG backbone. Overall, co-expression of thermophilic pectinases in a single host and the use of the clarified lysates allowed pectin bio-conversion into GalA which contribute to reduce the production and purification costs of the enzymes. However, product inhibition should be tackled using strategies such as *in situ* product recovery.

6.1.4 Synergistic action between thermophilic pectinases for pectin depolymerisation

Finally, the synergistic activity between thermophilic PMEs and PGLs for improving esterified pectin depolymerisation was explored. The synergistic reactions between BLI09 or SAM10 PMEs with TMA14 PGL as well as between BLI09 and SAM10 PMEs with TFU20 PGL were able to degrade the main peak of 650 kDa observed in apple, citrus and sugar beet pectin. Similar depolymerisation profiles were obtained using the three substrates mentioned above. The synergistic reactions between TMA014 with BLI09 or SAM10 depolymerised pectin in peaks ≤ than 25 and 10 kDa, respectively. While the synergistic reactions between TFU20 with BLI09 or SAM10, depolymerised pectin in peaks ≤ than 70 and 25 kDa, respectively. All these results were obtained after 30 min of reaction. The most efficient pectin degradation was achieved by the reactions between TMA14 or TFU20 with SAM10 were oligogalacturonates ≤ than 10 and 25 kDa were produced, respectively. It is important to mention that using only the PGLs, pectin was slightly degraded but its main peak of 650 kDa was still detectable. These findings demonstrated that the synergistic activity between thermophilic pectinases improved considerably and efficiently esterified pectin depolymerisation releasing unsaturated oligogalacturonates. Pectin depolymerisation is key in industrial processes such as in juice extraction and clarification in juice industry, retting and degumming of natural fibres in textile industry, fermentation of grapes juice in wine making and biopulping in paper making (Irshad *et al.*, 2014; Cheng *et al.*, 2020; Espejo,

2021; Subash and Muthiah, 2021). In addition, the smaller oligogalacturonates obtained could be used as functional compounds due to their potential bioactive properties (Combo *et al.*, 2013; Prandi *et al.*, 2018). This work provides further insights about the wide application of thermophilic pectinases in industry for improving yield and quality with economic and environmental advantages.

6.2 Future work

Future work includes extending some of the reported results in this thesis as well as to further explore their applicability. The main aspects related to future work are summarised below:

- As discussed in Section 3.3.3, from the 31 thermophilic pectinases identified through a genome mining approach of our documented bacterial collection, seven of them were successfully cloned and functionally characterised. The remaining 24 were not studied since the producing bacteria were not available or nonspecific PCR products were obtained during the genes amplification. The study of these enzymes could give the possibility of discovering more pectinases with special and valuable characteristics to be applied in industrial biotechnology, for example the ability to degrade native pectins from a range of different plants.

- The *in situ* removal of GalA from the synergistic reactions between PMEs and exo-PGs should be performed. It will solve the product inhibition issues of this compound on exo-PGs and will improve pectin bioconversion giving higher yield of GalA.

- The inclusion of an endo-PG in the synergistic activity between PMEs and exo-PGs could improve GalA release. It is due to the fact that endo-PGs catalyse the random cleavage of HG in pectin and have variable tolerance to methyl and acetyl groups. The endo-PGs activity gives, as a result, a number of oligogalacturonates in which PMEs and exo-PGs could act, releasing GalA.

- For improving GalA release from sugar beet pectin, besides the inclusion of an endo-PG in the synergistic reaction between PMEs and exo-PGs, other PAEs could be studied. These PAEs could be assayed to assess their capacity to deacetylate pectin in a blockwise pattern to allow access for the exo-PGs to act. In addition, natural sources of sugar beet pectin could be used instead of commercial substrates which have a number of inhibitors.

- It would be important to carry out a comparative economic analysis regarding the use of individually produced and co-expressed enzymes for pectin bioconversion into GalA. It will provide more detailed information about the advantages of using the clarified lysates of co-expressed enzymes produced in a single host in comparison with using purified enzymes produced in separate cultures.

- Finally, the synergistic reactions between PMEs and PGLs could be assayed by simulating industrial processes at laboratory scale. It will demonstrate the feasibility of using these enzymes for pectin depolymerisation in industry.

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APPENDICES

APPENDIX 1: Mechanisms of reaction of pectinolytic enzymes

alpha-D-galactopyranuronosyl-(1-4)-alpha-D-galactopyranuronosyl-(1-4)-alpha-D-galactopyranuronosyl-(1-4)-alpha-D-galactopyranuronosyl-(1-4)-alpha-D-galactopyranuronic acid + H2O = alpha-D-galactopyranuronosyl-(1-4)-alpha-D-galactopyranuronosyl-(1-4)-alpha-D-galactopyranuronic acid + alpha-D-galactopyranuronosyl-(1-4)-alpha-D-galactopyranuronosyl-(1-4)-alpha-D-galactopyranuronic acid

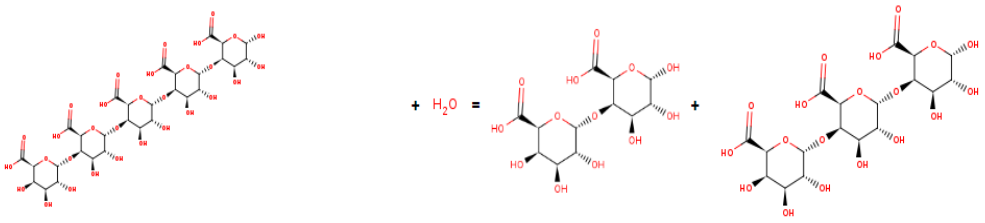

Figure S 1. Mechanism of reaction of endo-PGs (EC 3.2.1.15). BRENDA (https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.15).

[(1->4)-alpha-D-galacturonide]n + H2O = [(1->4)-alpha-D-galacturonide]n-1 + D-galacturonate

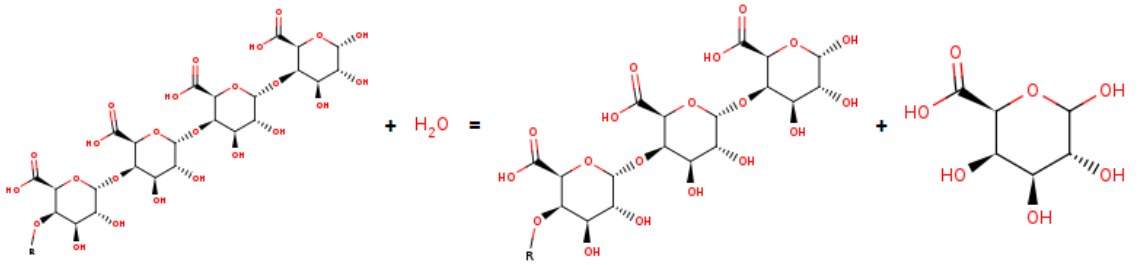

Figure S 2. Mechanism of reaction of exo-PGs (EC 3.2.1.67). BRENDA (https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.67).

(GalAalpha(1-4))n + H2O = (GalAalpha(1-4))n-2 + 4-O-alpha-D-galactopyranuronosyl-alpha-D-galactopyranuronic acid

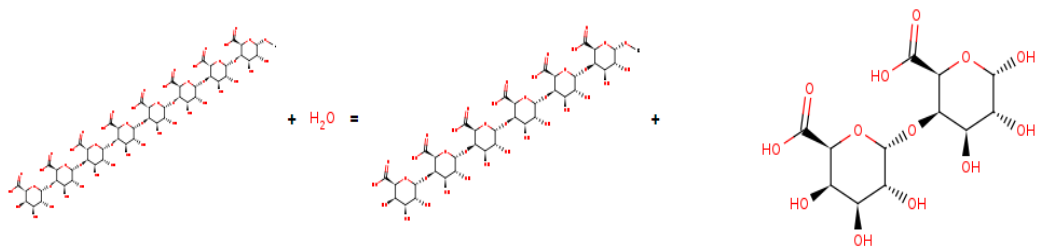

Figure S 3. Mechanism of reaction of exo-PGs (EC 3.2.1.82). BRENDA (https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.82).

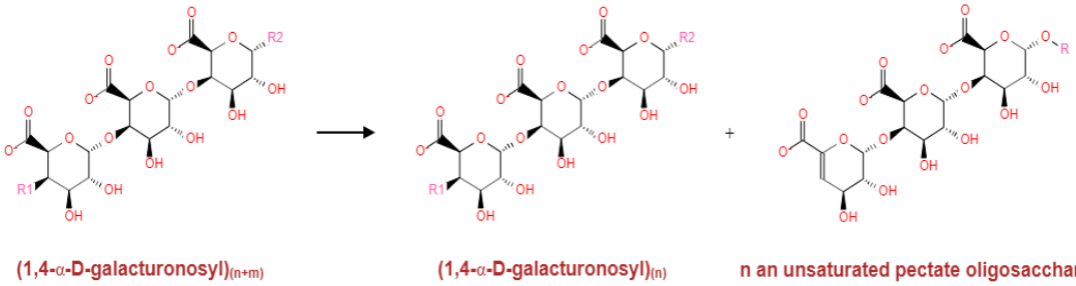

Figure S 4. Mechanism of reaction of endo-PGLs (EC 4.2.2.2). MetaCyc (https://biocyc.org/META/NEW-IMAGE?type=REACTION&object=4.2.2.2-RXN).

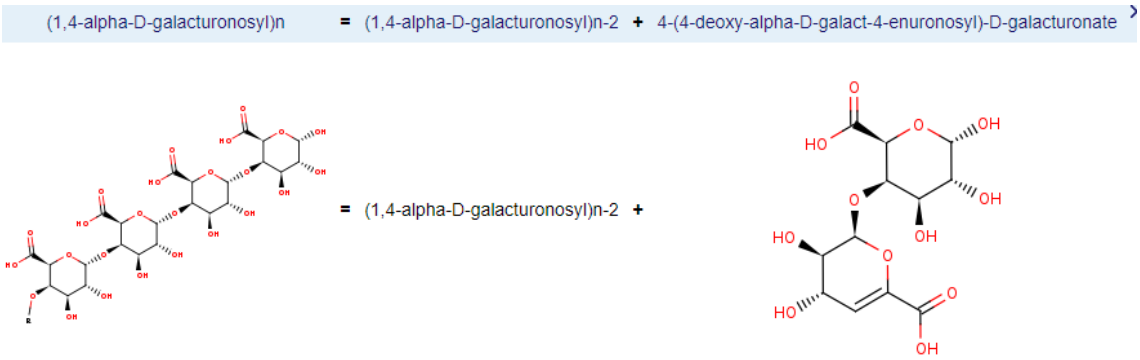

Figure S 5. Mechanism of reaction of exo-PGLs (EC 4.2.2.9) (pectate disaccharide-lyase). BRENDA (https://www.brenda-enzymes.org/enzyme.php?ecno=4.2.2.9).

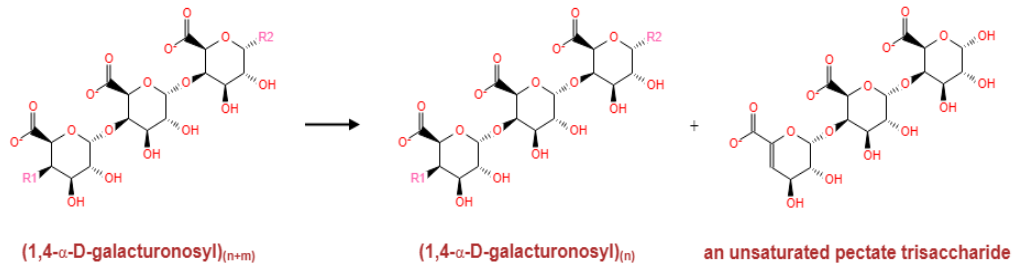

Figure S 6. Mechanism of reaction of exo-PGLs (EC 4.2.2.22) (pectate trisaccharide-lyase). MetaCyc (https://biocyc.org/META/NEWIMAGE?type=REACTION&object=4.2.2.22-RXN).

[6-O-methyl-alpha-D-GalpA]n = [6-O-methyl-alpha-D-GalpA]m + 4-deoxy-6-O-methyl-alpha-D-galact-4-enuronosy-[6-O-methyl-alpha-D-GalpA]n-m-1

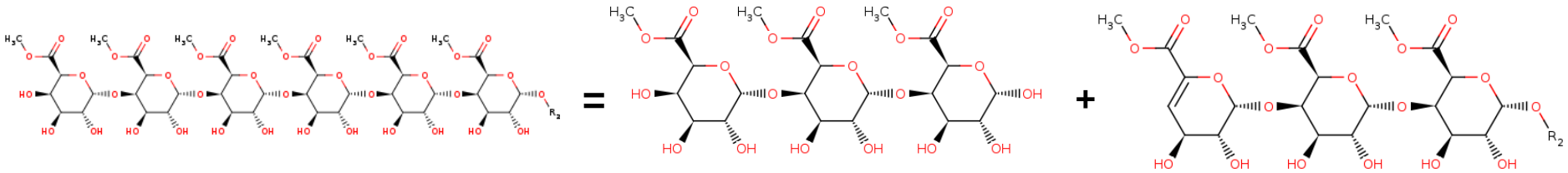

Figure S 7. Mechanism of reaction of endo-PLs (EC 4.2.2.10). BRENDA (https://www.brenda enzymes.org/enzyme.php?ecno=4.2.2.10#SYNONYM)

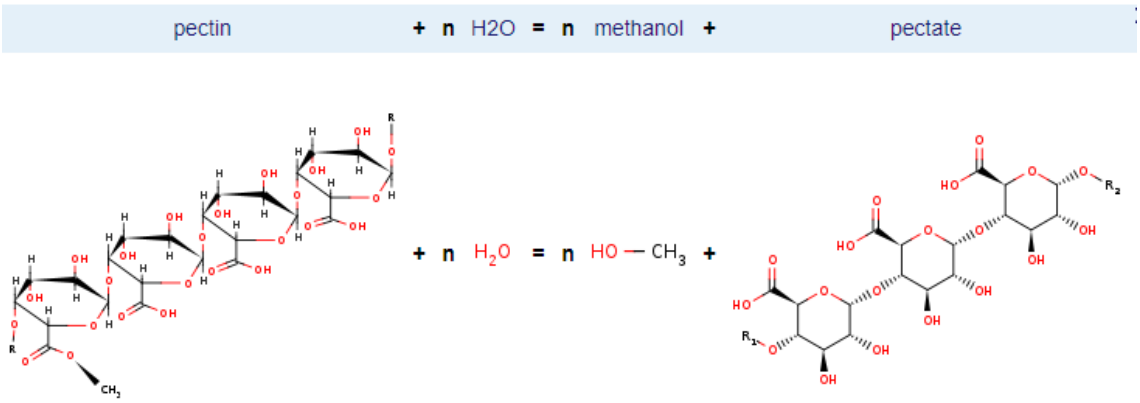

Figure S 8. Mechanism of reaction of PMEs (EC 3.1.1.11). BRENDA (https://www.brenda-enzymes.org/enzyme.php?ecno=3.1.1.11

APPENDIX 2: DNA sequences of genes encoding pectinases

DNA sequences correspond to cloned genes and nucleotides in bold correspond to the signal peptide.

> pTMA01

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> pBLI04

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> pBLI09

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> pSAM10

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> pTMA14

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> pTFU19

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> **pTFU20**

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GGCGGCGCTGCGGCCGACCCCGAACACGTCTACGTTGTCACGACCTACGCCGAACTGCG
CGAGGCACTGGCCGGAGGCCGCACCAACGACACACCCAAGATCGTCTTCCTCAAGGGGC
GCATCGACGCCAACACCGACGAGCACGGCAACCAGCTCACCTGCGACGACTACGCCGAC
CCGGAATACGACTTCGACGCCTACCTCGCTACCTACGACCCCGAAGTCTGGGGCTGGGA
CCAGGAACCGTCCGGCCCCCTCGAAGAAGCGCGGGAACGCTCCTACCGCAACCAGCGCG
ACCAGGTCGTCATCGAAGTCGGCTCCAACACGACCCTCATCGGCCTGGGCGACGACGCC
ACACTCGTCGGCGCCCAGGTCATGGTGGACAGCGTCGACAACGTGATCATCCGCAACAT
CATCTTCGAAACCGCCCAGGACTGCTTCCCCCAGTGGGATCCCACGGACGGCCCCGAAG
GGAACTGGAACTCCGAATTCGACGGAGTCTCGGTCCGCCGCTCCACCCACGTGTGGATC
GACCACAACGAGTTCAGCGACGGCGCAGTACTCGACCGTGACCTGCCCGAATACTTCGG
CCGCGAATTCCAGGTCCACGACGGGCTCCTGGACATCACCCACGGGGCCGACCTGGTCA
CCGTCTCCTACAACGTGCTGCGCGACCACGACAAGACCATGCTCATCGGCAGCACCGAC
TCGCCAACCTACGACGTGGGCAAACTGCGGGTCACCCTGCACCACAACCGCTGGGAGAA
CGTGCTGCAGCGCGCTCCCCGGGTCCGCTACGGGCAGGTCCACGTGTACAACAACCACT
ACGTGATCCCTGCCACGCCCGAGGGCGAGAAGACCTACGAGTACTCGTGGGGGGTCGGC
GTGGAATCCGCGCTGTACGCGGAGAACAACTACTTCGACATCGACCCGTCCGTCGACTT
CTCCCAGGTCGTCGCCCACTGGAAGGGCACCCAGATGTACGAGAAGGGAAGCTACGCCA
ACGGCCGCTCCCGGCACCACCAGGTGAGCTTCCTCGACGAATACAACGCGGTCCACAGC
CCGACCATCGAGAACAAGCAGACCTGGAGCCCTCCGCTCCACGGCCGGATCGACCCCAC
CCAGTCGGTGCCCGCCAAGGTGCAGAAAGCCGGGGTGGGCCACATCCTCTGA

APPENDIX 3: Amino acid sequences of pectinases

Protein sequences correspond to cloned genes and amino acids in **bold** correspond to the signal peptide.

TMA01

MIMEELAKKIEEEILNHVREPQIPDREVNLLDFGARGDGRTDCSESFKRAIEEL
SKQGGGRLIVPEGVFLTGPIHLKSNIELHVKGTIKFIPDPERYLPVVLTRFEGI
ELYNYSPLVYALDCENVAITGSGVLDGSADNEHWWPWKGKKDFGWKEGLPNQQE
DVKKLKEMAERGTPVEERVFGKGHYLRPSFVQFYRCRNVLVEGVKIINSPMWCV
HPVLSENVIIRNIEISSTGPNNDGIDPESCKYMLIEKCRFDTGDDSVVIKSGRD
ADGRRIGVPSEYILVRDNLVISQASHGGLVIGSEMSGGVRNVVARNNVYMNVER
ALRLKTNSRRGGYMENIFFIDNVAVNVSEEVIRINLRYDNEEGEYLPVVRSVFV
KNLKATGGKYAVRIEGLENDYVKDILISDTIIEGAKISVLLEFGQLGMENVIMN
GSRFEKLYIEGKALLK

BLI04

MSLQKIKEEIVKKLKVPVFPNRSFDVTSFGADENGKNDSTEAIQKAIDQAHQAG
GGRVTVPEGVFLSGALRLKSNVDLHIAKGAVIKFSQNPEDYLPVVLTRFEGVEL
YNYSPLIYAYEADNIAITGKGTLDGQGDDEHWWPWKRGTNGQPSQEKDRNALFE
MAERGIPVTERQFGKGHYLRPNFIQPYRCKHILIQGVTVLNSPMWQVHPVLCEN
VTVDGIKVIGHGPNTDGVNPESCKNVVIKGCHFDNGDDCIAVKSGRNADGRRIN
IPSENIVIEHNEMKDGHGGVTIGSEISGGVKNVIAEGNLMDSPNLDRALRIKTN
SVRGGVLENIYFHKNTVKSLKREVIAIDMEYEEGDAGDFKPVVRTVDVKQLKSM
GGQYGIRVLAYDHSPVTGLKVADSEIDGVDVPMELKHVKDPVFSNLYINGKRYD
SHKA

BLI09

MVNREAAAIRLTVSKDGDGEFQTVQEAIDALPEYSREQKVIFIKKGVYKEVVHI
PATKPFVKLIGENRYETVITYDNYAGKEKEGGGKYGTTGSSSVFIYADHVEAEN
LTFENSFDRTKVDTTDTQAVAVYAKGNRMTFKYVRFIGRQDTLFVNDGTQYFYQ
CYIEGDVDFIFGGARAVFEECQIHSADRGSATNNGYVTAASTHIAKPFGLLITN
CRVTSDAADGTVYLGRPWHPGGDPDAIASVLYHRCDLGAHIKPEGWTDMSGFSA
ADARLYEYGNTGPGAISHEARRQLADHEAEKWTIENVLDGWNPKVES

SAM10

MHRPAPSRRGFLLTTAGAGAALALTAAPAVAGTRPRPRPFGRYGSPAARRTPRT
LYVDPHGRGDFTSVQAAVNAAPGDGWTLVLAPGTYRETVSVDTDRTGATWIGAS
QDPRDVVIVFDNAAGTPKPDGSGTYGTSGSATTTVRADGFTARWITFANDWLRA
GHPGWTGTQAVALKAMGDRTAFHHCRFLGHQDTLYADSRDLGHFARQYYAHCHV
EGDVDFVFGRATAVFEGCHFRTLLRTDLSAAPHGFVFAPSTAGANPRGYLVTNG
RVSSEAPDGHYKLARPWVPSSDVTARPMLTVRETRLGAGIDAVAPYADMRDTYP
WQEQRFREYRNTGPGAAVTVPENRPQLTRAEAASHTREAYLGDWRPYARHR

TMA14

MLMRFSRVVSLVLLLVFTAVLTGAVKASLNDKPVGFASVPTADLPEGTVGGLGG
EIVFVRTAEELEKYTTAEGKYVIVVDGTIVFEPKREIKVLSDKTIVGINDAKIV
GGGLVIKDAQNVIIRNIHFEGFYMEDDPRGKKYDFDYINVENSHHIWIDHCTFV
NGNDGAVDIKKYSNYITVSWCKFVDHDKVSLVGSSDKEDPEQAGQAYKVTYHHN
YFKNCIQRMPRIRFGMAHVFNNFYSMGLRTGVSGNVFPIYGVASAMGAKVHVEG
NYFMGYGAVMAEAGIAFLPTRIMGPVEGYLTLGEGDAKNEFYYCKEPEVRPVEE
GKPALDPREYYDYTLDPVQDVPKIVVDGAGAGKLVFEELNTAQ

TFU19

MGRSITRRLASTLATAAVVTAGLTLPVSPASAQTGSATGYAALNGGTTGGAGGQ
VVRATTGTEIHQALCQRPSTSTPIIIQVEGTINHGNTSKVSGPGCDTASDKIEL
KKISNVTIVGVGNGALFDQIGIHIRESRNIIIQNVHIRNVKKSGSPTSNGGDAI
GMEKDVRNVWVDHVTLEASGGESEGYDGLFDLKDNTQYVTLSYSILRNSGRGGL
IGSSESDLSNGYITFHHNLYENIDSRTPLLRGGVAHMYNNHYVRLNNSGINSRA
GARAKVENNYFQNSKDVLGTFYTNERGYWEVRGNIFDNVTWSSQGNENYPAGPN
PQSTTSVSIPYSYRLDDAGCVPQIVSQTAGANKGNRVSDGNCRAEDPGNPDPGN
PDPGNPDPGTPPGGTNLSIGADADGSSKASGTSYKNVIDGDMSTYWSPSGSTGR
ISVKWGSNVTVSAIHIREAAGAVGNIGSWRVVNNDTGAVLATGTGAGVITFTPT
TLRKINFEILSSNGTPRVAEFETYAG

TFU20

MRRAATLGVALALPLTLAAPSSALAQPHHHAGVSPKAEQVAREVLAPNDGWAAY
DGGTTGGAAADPEHVYVVTTYAELREALAGGRTNDTPKIVFLKGRIDANTDEHG
NQLTCDDYADPEYDFDAYLATYDPEVWGWDQEPSGPLEEARERSYRNQRDQVVI
EVGSNTTLIGLGDDATLVGAQVMVDSVDNVIIRNIIFETAQDCFPQWDPTDGPE
GNWNSEFDGVSVRRSTHVWIDHNEFSDGAVLDRDLPEYFGREFQVHDGLLDITH
GADLVTVSYNVLRDHDKTMLIGSTDSPTYDVGKLRVTLHHNRWENVLQRAPRVR
YGQVHVYNNHYVIPATPEGEKTYEYSWGVGVESALYAENNYFDIDPSVDFSQVV
AHWKGTQMYEKGSYANGRSRHHQVSFLDEYNAVHSPTIENKQTWSPPLHGRIDP
TQSVPAKVQKAGVGHIL

APPENDIX 4: Primers

Table S 1. Primers used in the PCR amplification of genes encoding putative thermostable pectinases.

Bacteria	Enzyme code	Primer nucleotide sequence	Tm (°C)	Restriction site	Plasmid
Thermotoga maritima DSM 3109	TMA01	F: 5'-AAA**CATATG**ATCATGGAAGAACTGGCAAAAAAGATTG-3'	70.1	*Nde*I	pET-29a-TMA01
		R: 5'-AAAA**CTCGAG**TTTCAGCAGAGCTTTACCTTCGATGTAAAGC-3'	72.2	*Xho*I	
Bacillus licheniformis DSM 13	BLI04	F: 5'-AAA**GCTCTTC**GATGAGTCTGCAGAAAATAAAAGAAGAGA-3'	60.9	*Sap*I	pET29a_SacB_SapI-BLI04
		R: 5'-AAA**GCTCTTC**GGTGGGCTTTGTGTGAGTCATAGC-3'	60.4	*Sap*I	
Bacillus licheniformis DSM 13	BLI09	F: 5'-AAA**GCTCTTC**GATGGTTAATCGGGAGGCAGC-3'	61.3	*Sap*I	pET29a_SacB_SapI-BLI09
		R: 5'-AAA**GCTCTTC**GGTGTGATTCAACTTTAGGATTCCAG-3'	60.1	*Sap*I	
Streptomyces ambofaciens DSM 40053	SAM10	F: 5'-AAA**GCTCTTC**GATGGGCACCCGCCCCCGCCCG-3'	73.0	*Sap*I	pET29a_SacB_SapI-SAM10
		R: 5'-AAA**GCTCTTC**GGTGGCGGTGCCGGGCATACGGCCGC-5'	73.0	*Sap*I	
Thermotoga maritima DSM 3109	TMA14	F: 5'-AAA**GCTCTTC**GATGTCTCTCAATGACAAACCTGTGGGATTTGC-3'	62.0	*Sap*I	pET29a_SacB_SapI-TMA14
		R: 5'-AAA**GCTCTTC**GGTGCTGAGCCGTATTTAGTTCTTCAAACACCAG-3'	62.0	*Sap*I	
Thermobifida fusca DSM 43792	TFU19	F: 5'-AAA**GCTCTTC**GATGCAGACCGGCAGCGCCACCGGCTACG-3	73.0	*Sap*I	pET29a_SacB_SapI-TFU19
		R: 5'-AAA**GCTCTTC**GGTGGCCGGCGTAGGTTTCGAACTCGGCCACCCG-3'	73.0	*Sap*I	
Thermobifida fusca DSM 43792	TFU20	F: 5'-AAA**GCTCTTC**GATGCAGCCGCACCACCACGCCGGGGTC-3'	73.0	*Sap*I	pET29a_SacB_SapI-TFU20
		R: 5'-AAA**GCTCTTC**GGTGGAGGATGTGGCCCACCCCGGCTTTCTGCAC-3'	72.0	*Sap*I	
Bacillus licheniformis DSM 13	PAE21	F: 5'-AAA**GGTCTC**TTATGATGCGAGACATTCAGTTGTTTTTGGC-3'	62.0	*Bsa*I	pET29a_SacB_BsaI-PAE21
		R: 5'-AAA**GGTCTC**GGGTGTAAAGGAATTCCCGCTTCTTTTGCTTCCTG-3'	61.0	*Bsa*I	

Nucleotides in **bold** correspond to the restriction site of the enzyme.

Table S 2. Primers used in the PCR amplification of thermophilic pectinases for the construction of co-expression plasmids containing a PME and exo-PG in pETDuet-1.

Co-expression construct		Enzyme	Primer nucleotide sequence	Tm (°C)	Restriction Enzyme
1	pETDuet-BLI09-TMA01	BLI09 PME (MCS1)	F: 5'-AAA**GGATCC**GATGGTTAATCGGGAGGCAGCTGCTATA-3'	62	*BamH*I
			R: 5'-AAA**GCGGCCGC**CTATGATTCAACTTTAGGATTCCAGCCGTCC-3'	62	*Not*I
		TMA01 exo-PG (MCS2)	F: 5'-AAA**CATATG**ATCATGGAAGAACTGGCAAAAAAGATTG-3'	60	*Nde*I
			R: 5'-AAAA**CTCGAG**TTTCAGCAGAGCTTTACCTTCGATGTAAAGC-3'	62	*Xho*I
2	pETDuet-BLI09-BLI04	BLI09 PME (MCS1)	F: 5'-AAA**GGATCC**GATGGTTAATCGGGAGGCAGCTGCTATA-3'	62	*BamH*I
			R: 5'-AAA**GCGGCCGC**CTATGATTCAACTTTAGGATTCCAGCCGTCC-3'	62	*Not*I
		BLI04 exo-PG (MCS2)	F: 5'-AAA**CATATG**AGTCTGCAGAAAATAAAAGAAGAGATTGTAAAGA-3'	60	*Nde*I
			R: 5'- AAAA**CTCGAG**GGCTTTGTGTGAGTCATAGCGTTTTC-3'	60	*Xho*I
3	pETDuet-TMA01-BLI09	TMA01 exo-PG (MCS1)	F: 5'-AAA**GGATCC**GATGATCATGGAAGAACTGGCAAAAAAGA-3'	59	*BamH*I
			R: 5'-AA**GCGGCCGC**TTATTTCAGCAGAGCTTTACCTTCGATGTAAAGC-3'	62	*Not*I
		BLI09 PME (MCS2)	F: 5'-AAA**CATATG**GTTAATCGGGAGGCAGCTGCTATACGG-3'	66	*Nde*I
			R: 5'-AAAA**CTCGAG**TGATTCAACTTTAGGATTCCAGCCGTCCAATACG-3'	64	*Xho*I
4	pETDuet-BLI04-BLI09	BLI04 exo-PG (MCS1)	F: 5'-AAA**GGATCC**GATGAGTCTGCAGAAAATAAAAGAAGAGATTGT-3'	59	*BamH*I
			R: 5'-AA**GCGGCCGC**TTAGGCTTTGTGTGAGTCATAGCGTTTTC-3'	60	*Not*I
		BLI09 PME (MCS2)	F: 5'-AAA**CATATG**GTTAATCGGGAGGCAGCTGCTATACGG-3'	66	*Nde*I
			R: 5'-AAAA**CTCGAG**TGATTCAACTTTAGGATTCCAGCCGTCCAATACG-3'	64	*Xho*I

Nucleotides in **bold** correspond to the restriction site of the enzyme. PME: pectin methylesterase, exo-PG: exo-polygalacturonase, MCS: multiple cloning site.

APPENDIX 5: Recombinant plasmids containing thermophilic pectinases

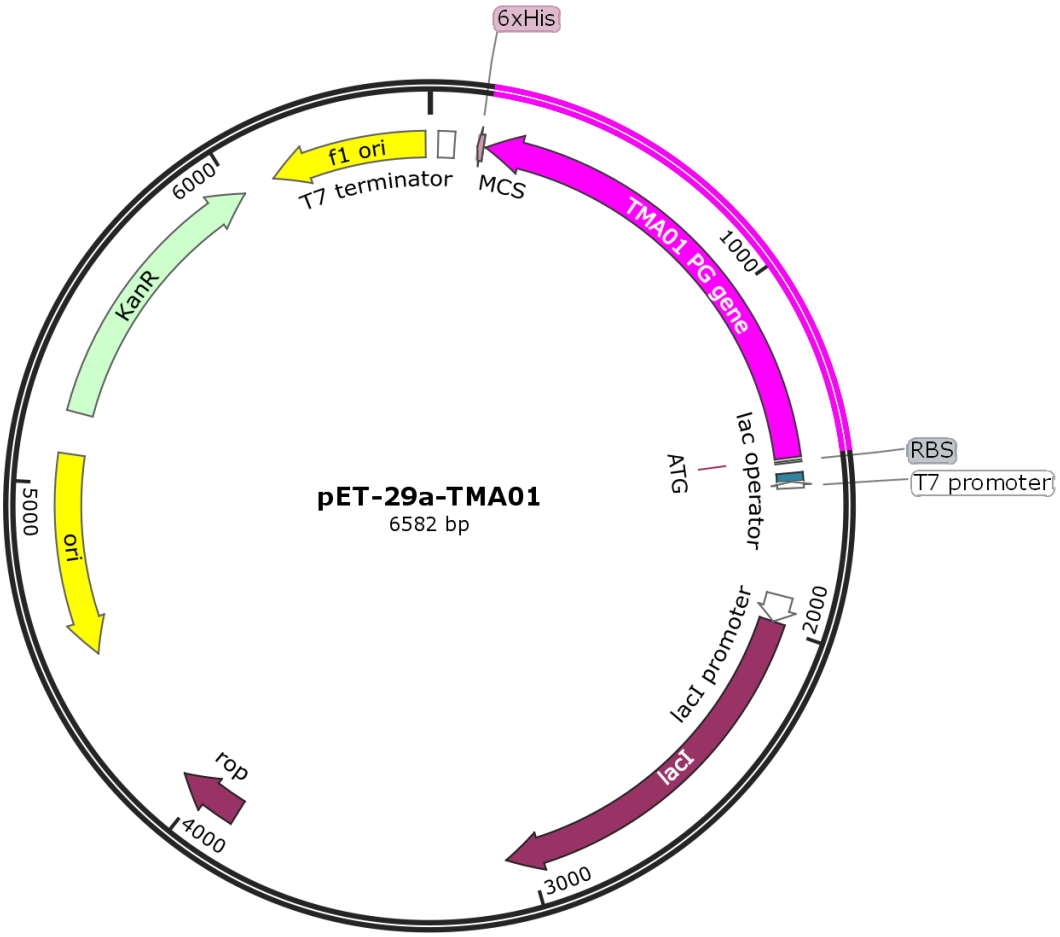

Figure S 9. Recombinant plasmid pET-29a-TMA01 containing the TMA01 exo-PG from *Thermotoga maritima* DSM 3109.

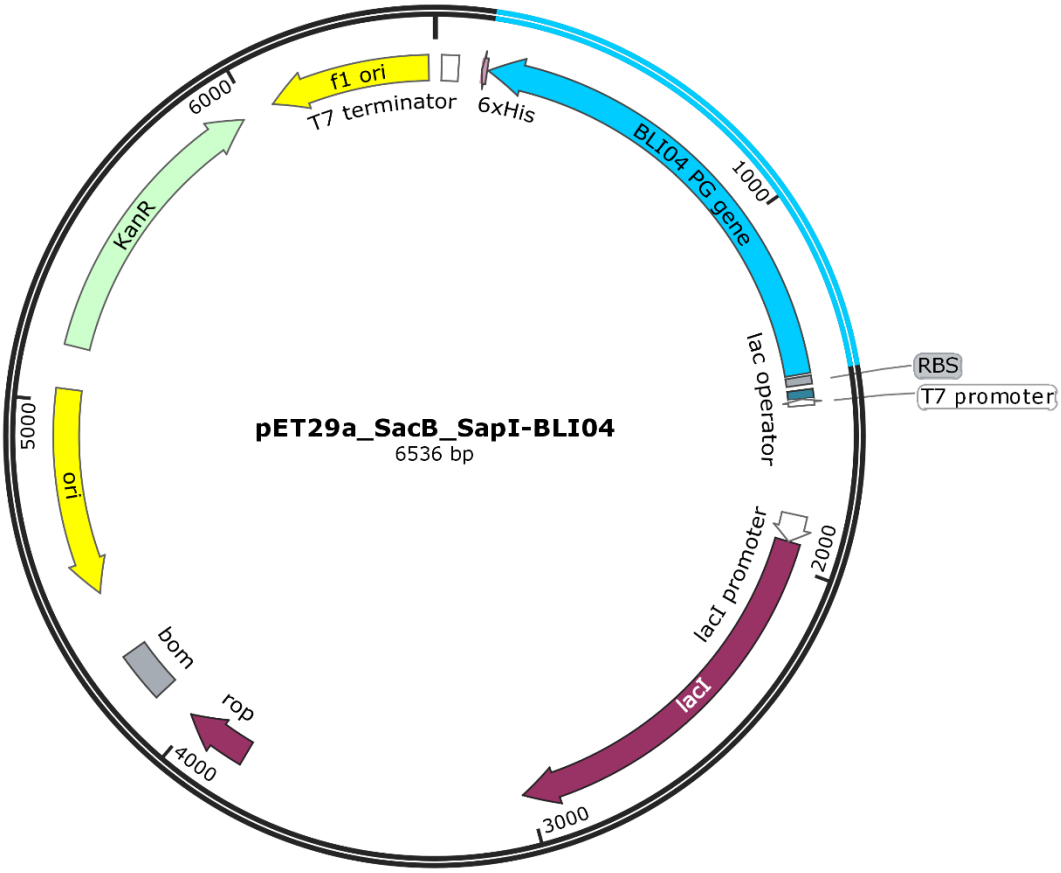

Figure S 10. Recombinant plasmid pET29a_SacB_SapI-BLI04 containing the BLI04 exo-PG from *Bacillus licheniformis* DSM 13.

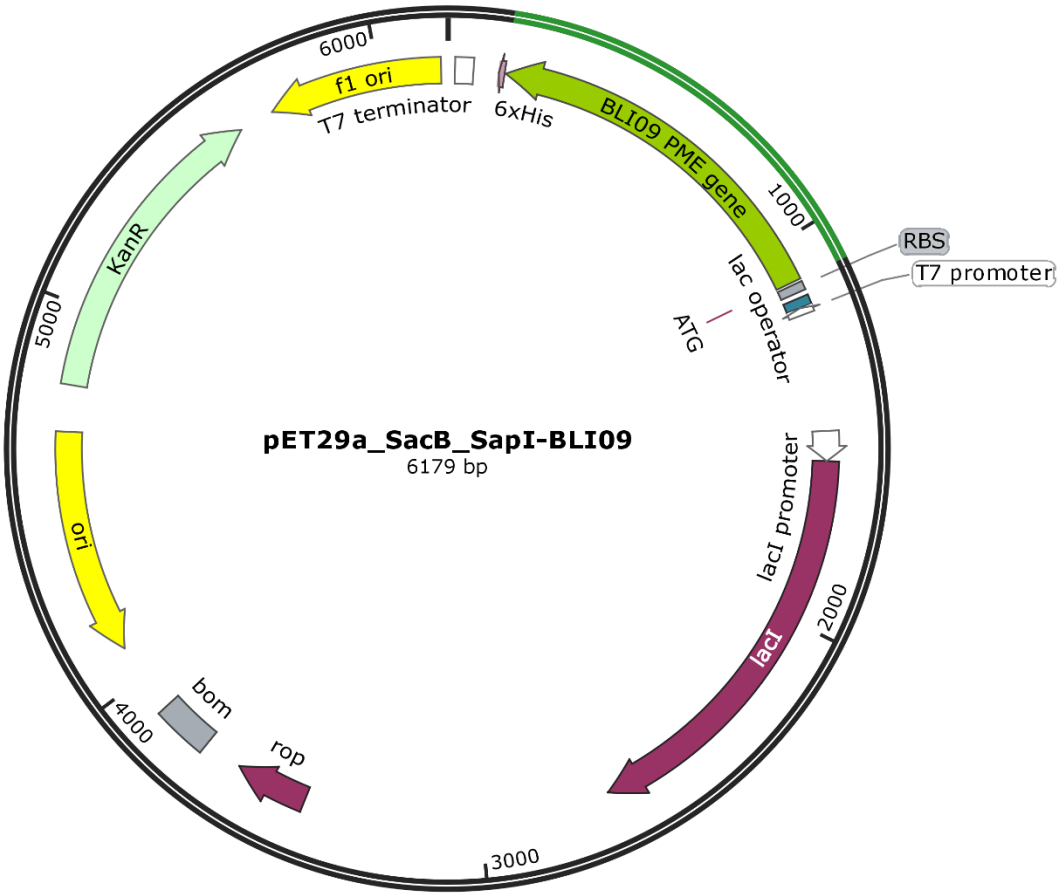

Figure S 11. Recombinant plasmid pET29a_SacB_SapI-BLI09 containing the BLI09 PME from *Bacillus licheniformis* DSM 13.

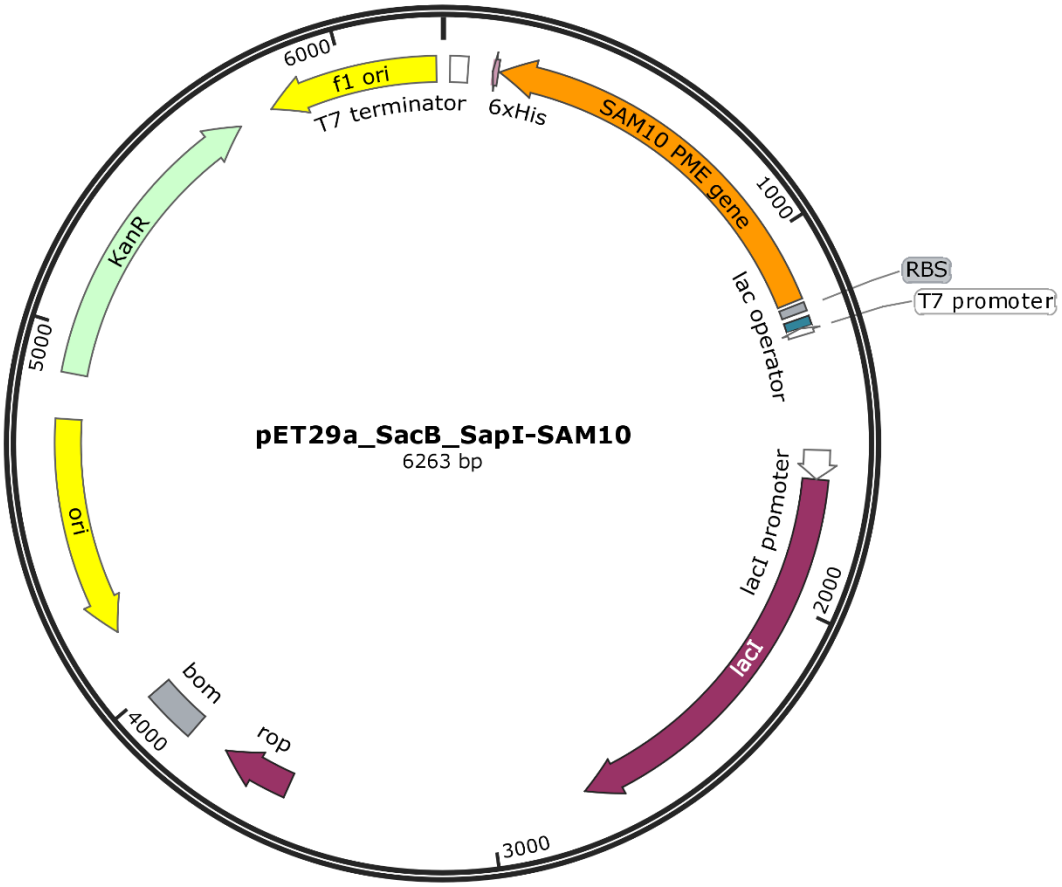

Figure S 12. Recombinant plasmid pET29a_SacB_SapI-SAM10 containing the SAM10 PME from *Streptomyces ambofaciens* DSM 40053.

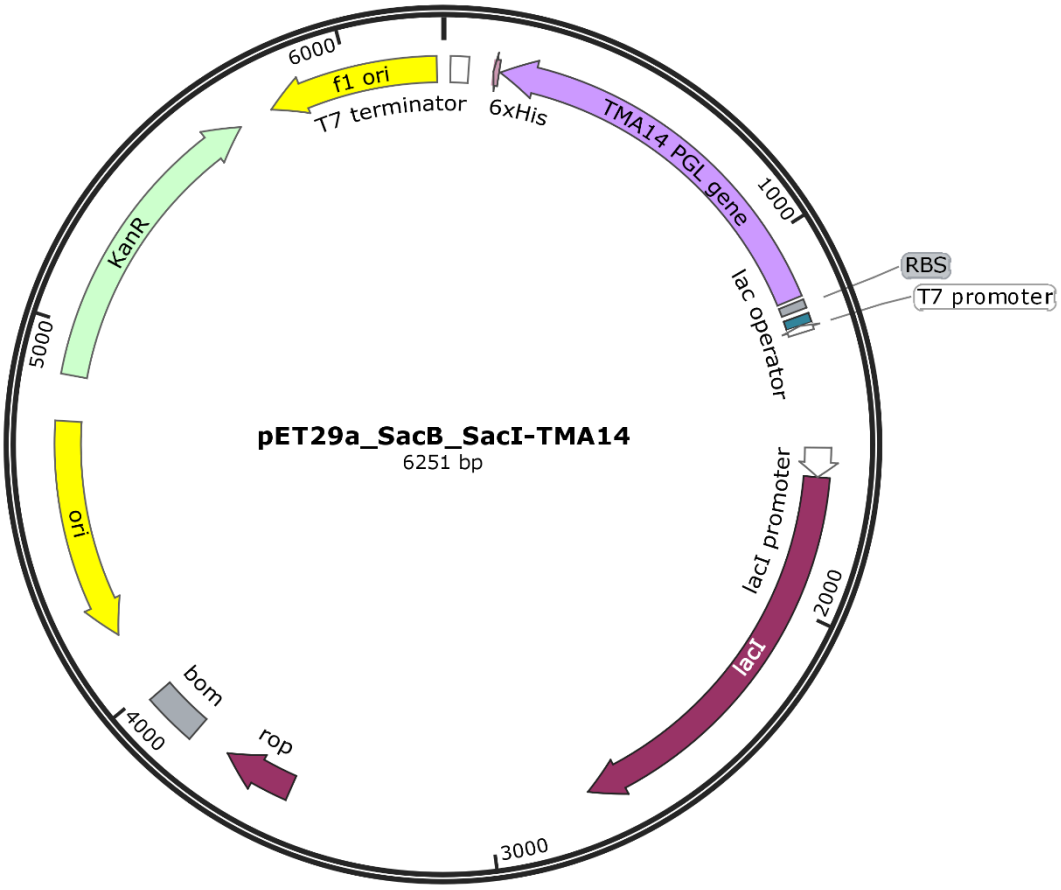

Figure S 13. Recombinant plasmid pET29a_SacB_SapI-TMA14 containing the TMA14 PGL from *Thermotoga maritima* DSM 3109.

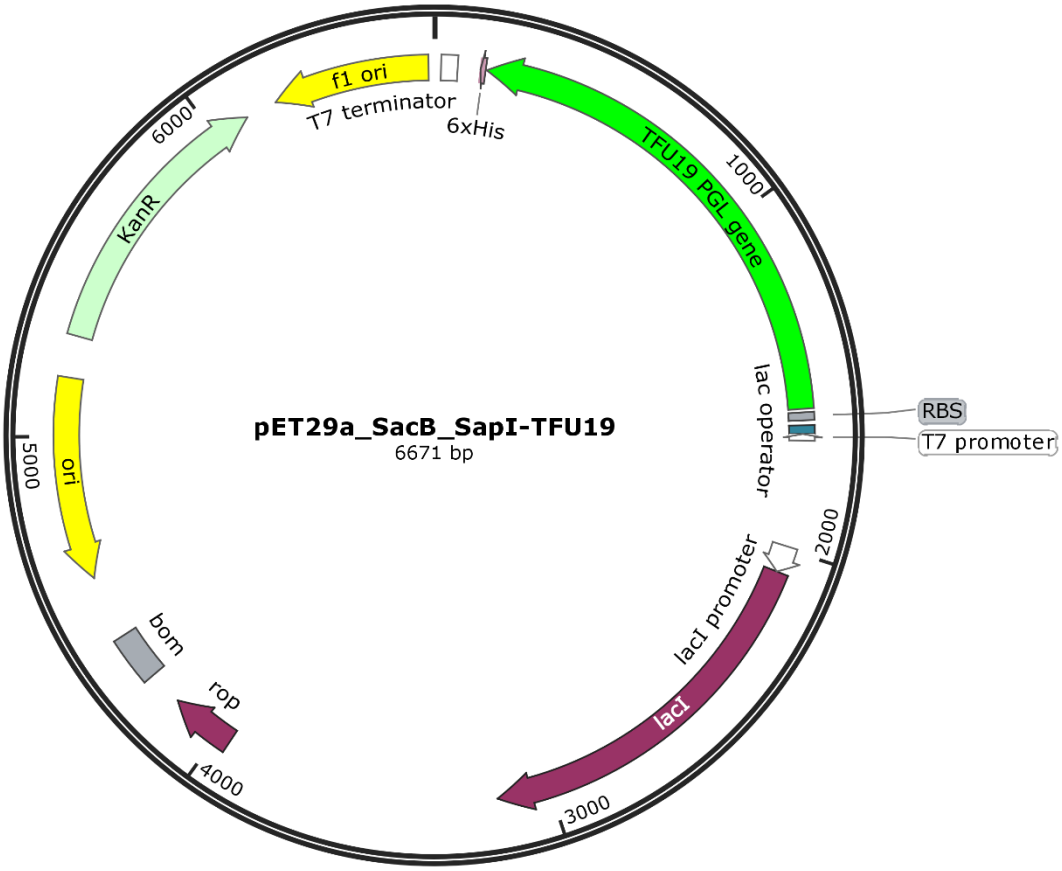

Figure S 14. Recombinant plasmid pET29a_SacB_SapI-TFU19 containing the TFU19 PGL from *Thermobifida fusca* DSM 43792.

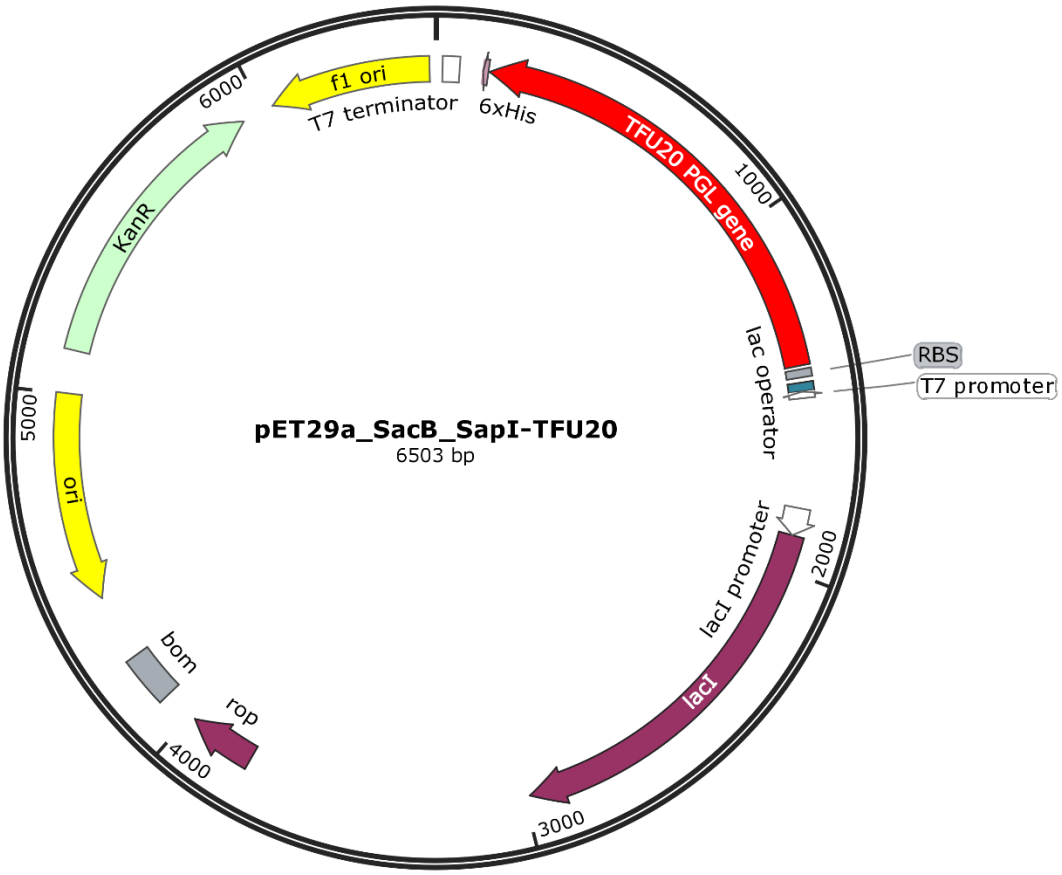

Figure S 15. Recombinant plasmid pET29a_SacB_SapI-TFU20 containing the TFU20 PGL from *Thermobifida fusca* DSM 43792.

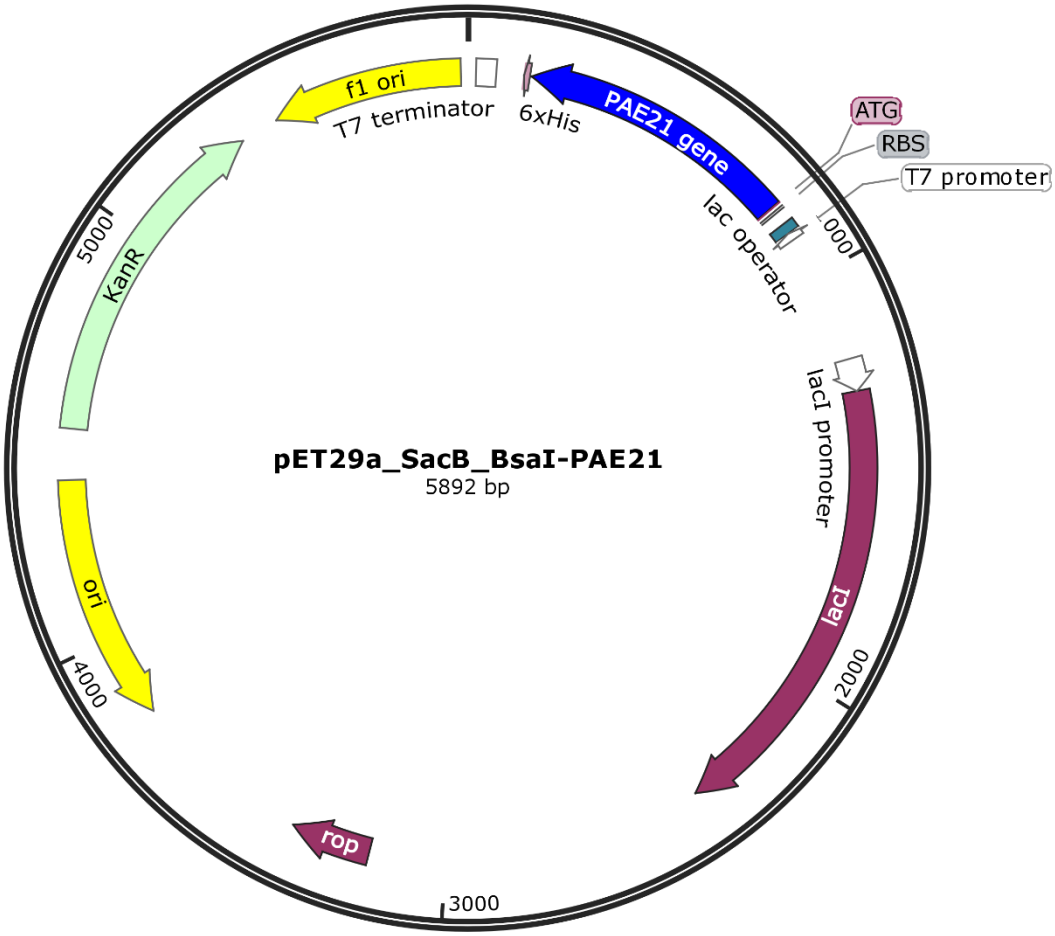

Figure S 16. Recombinant plasmid pET29a_SacB_BsaI-PAE21 containing the PAE21 from *Bacillus licheniformis* DSM 13.

APPENDIX 6: Calibration curves

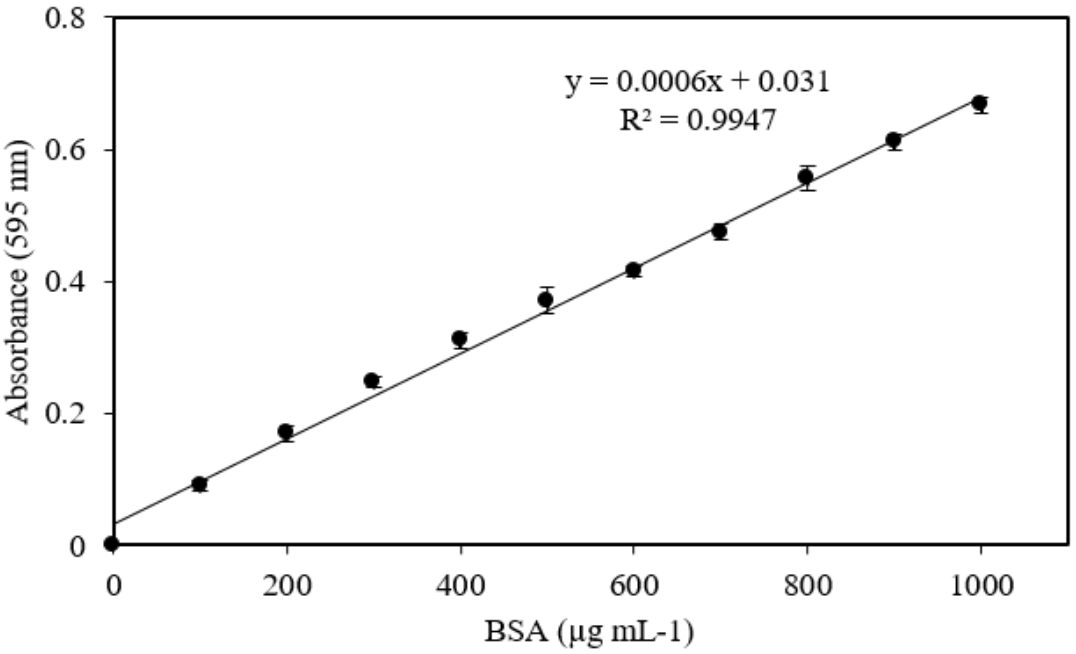

Figure S 17. Calibration curve for protein assay detailed in Section 2.8.1. Error bars represent one standard deviation from the mean (n = 2).

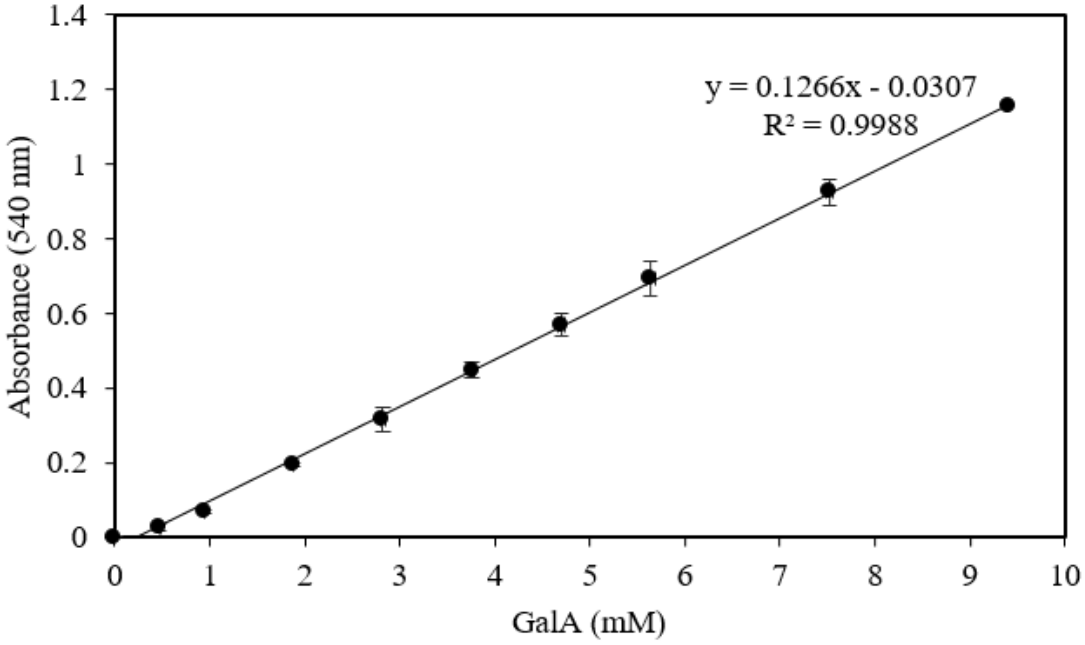

Figure S 18. Calibration curve for PG activity described in Section 2.8.3.1. Error bars represent one standard deviation from the mean (n = 2).

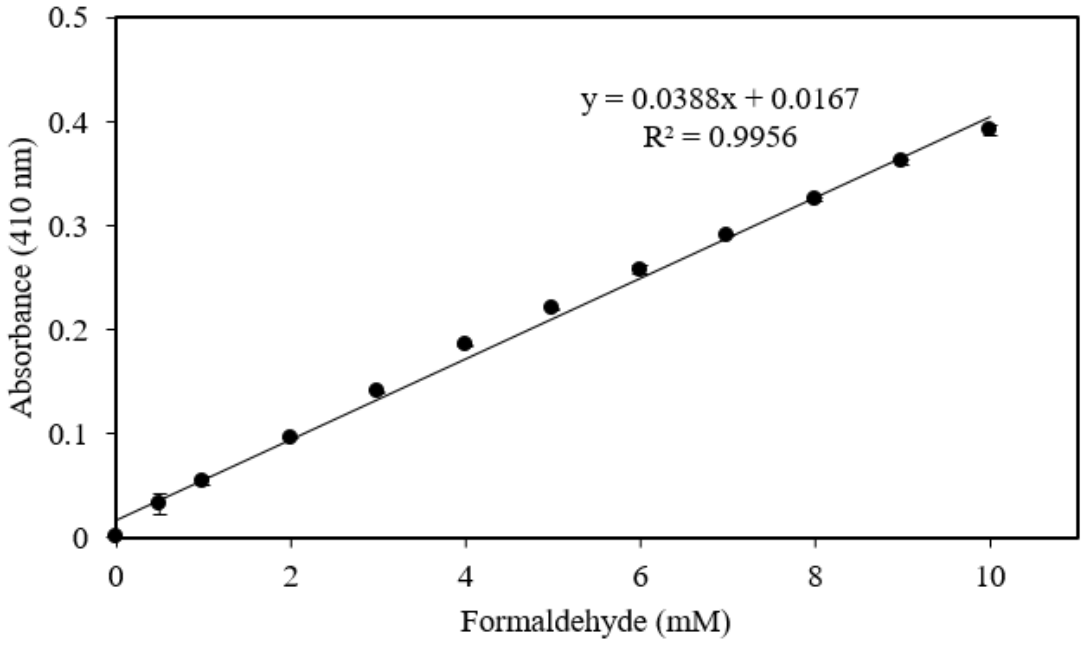

Figure S 19. Calibration curve for PME activity based on methanol quantification using AO and Fluoral-P as detailed in Section 2.8.3.2. Error bars represent one standard deviation from the mean (n = 2).

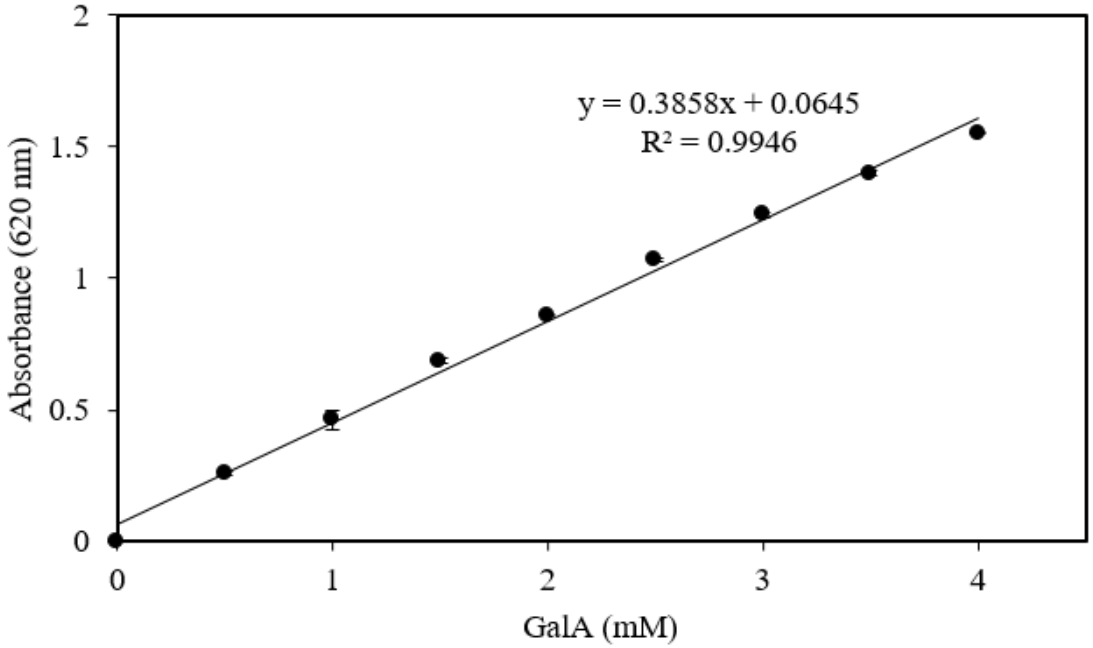

Figure S 20. Calibration curve for PME activity based on the use of pH indicator as detailed in Section 2.8.3.2. Error bars represent one standard deviation from the mean (n = 2).

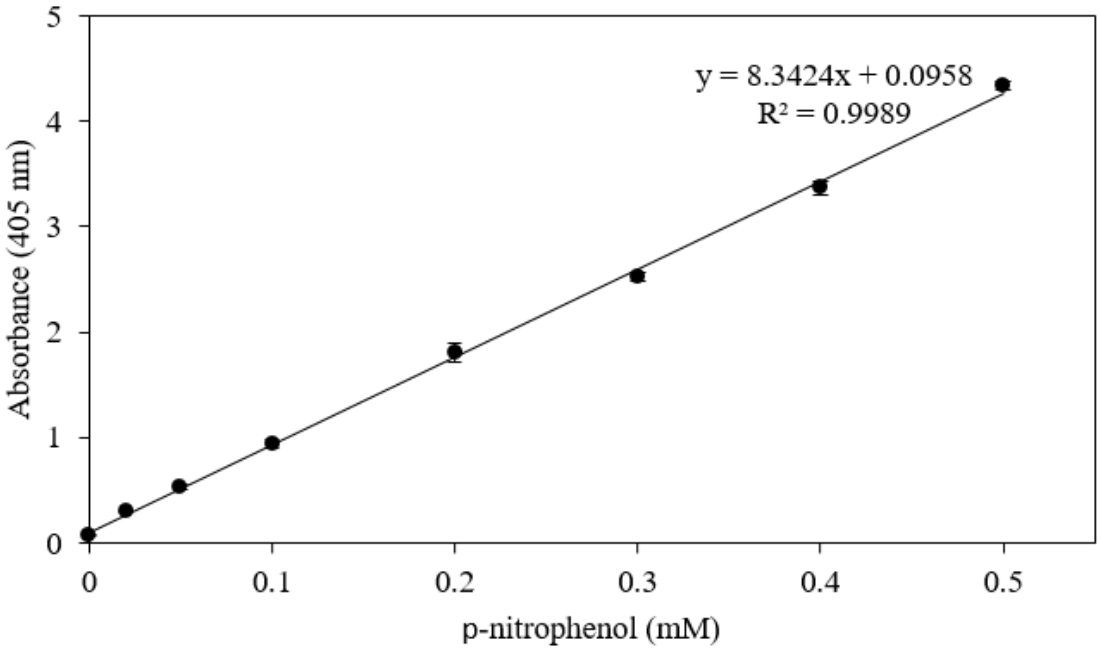

Figure S 21. Calibration curve for PAE activity based on p-nitrophenol quantification as detailed in Section 2.8.3.4. Error bars represent one standard deviation from the mean (n = 2).

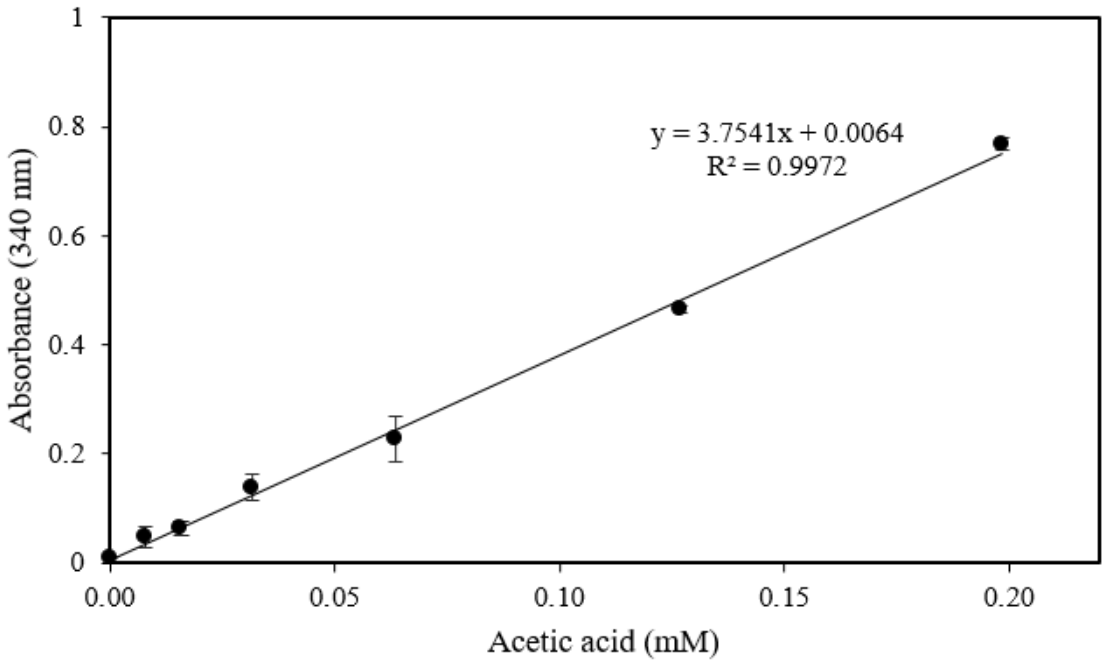

Figure S 22. Calibration curve for acetic acid quantification as detailed in Section 2.8.6. Error bars represent one standard deviation from the mean (n = 2).

APPENDIX 7: Schematic representation of co-expression plasmids construction

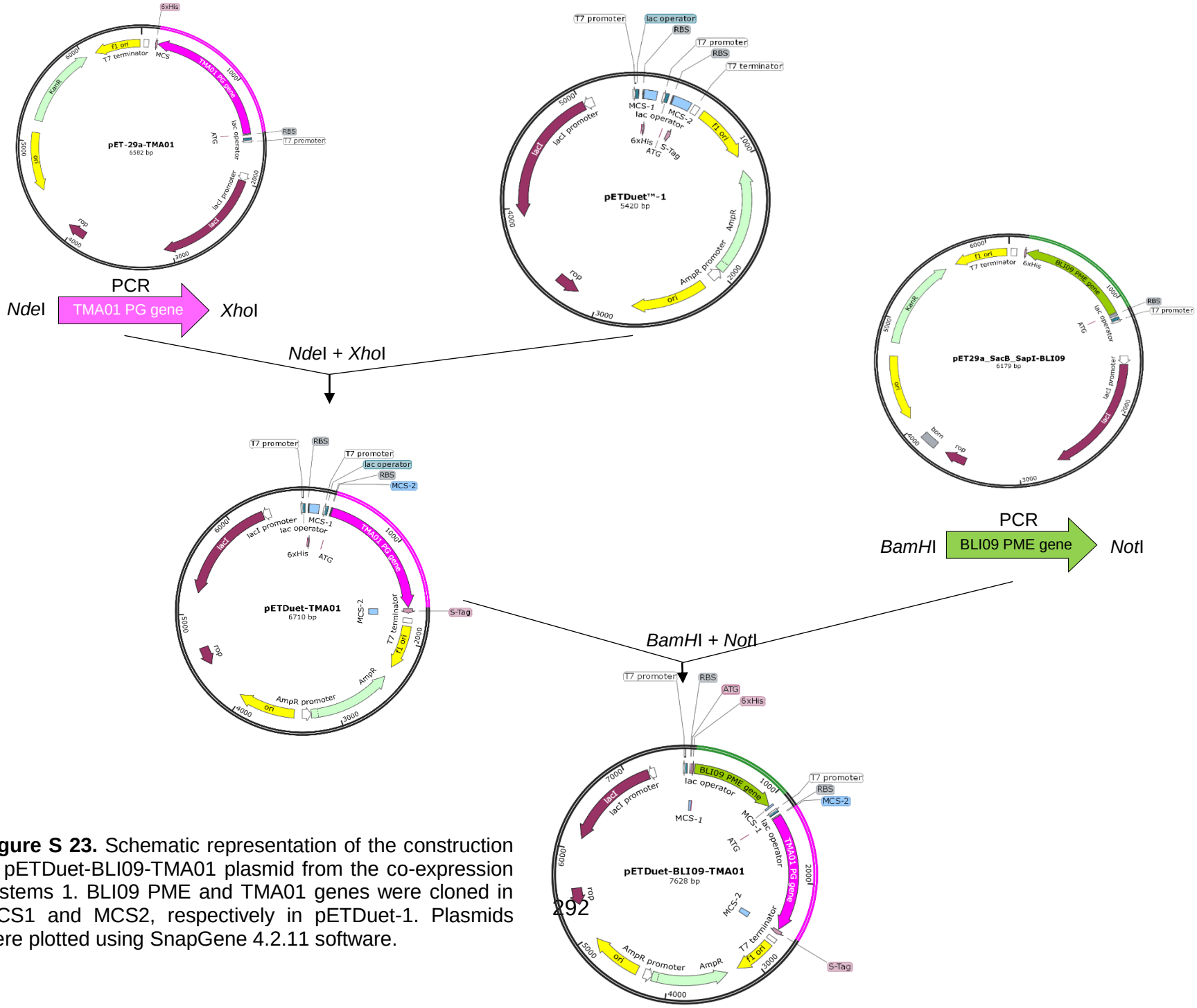

Figure S 23. Schematic representation of the construction of pETDuet-BLI09-TMA01 plasmid from the co-expression systems 1. BLI09 PME and TMA01 genes were cloned in MCS1 and MCS2, respectively in pETDuet-1. Plasmids were plotted using SnapGene 4.2.11 software.

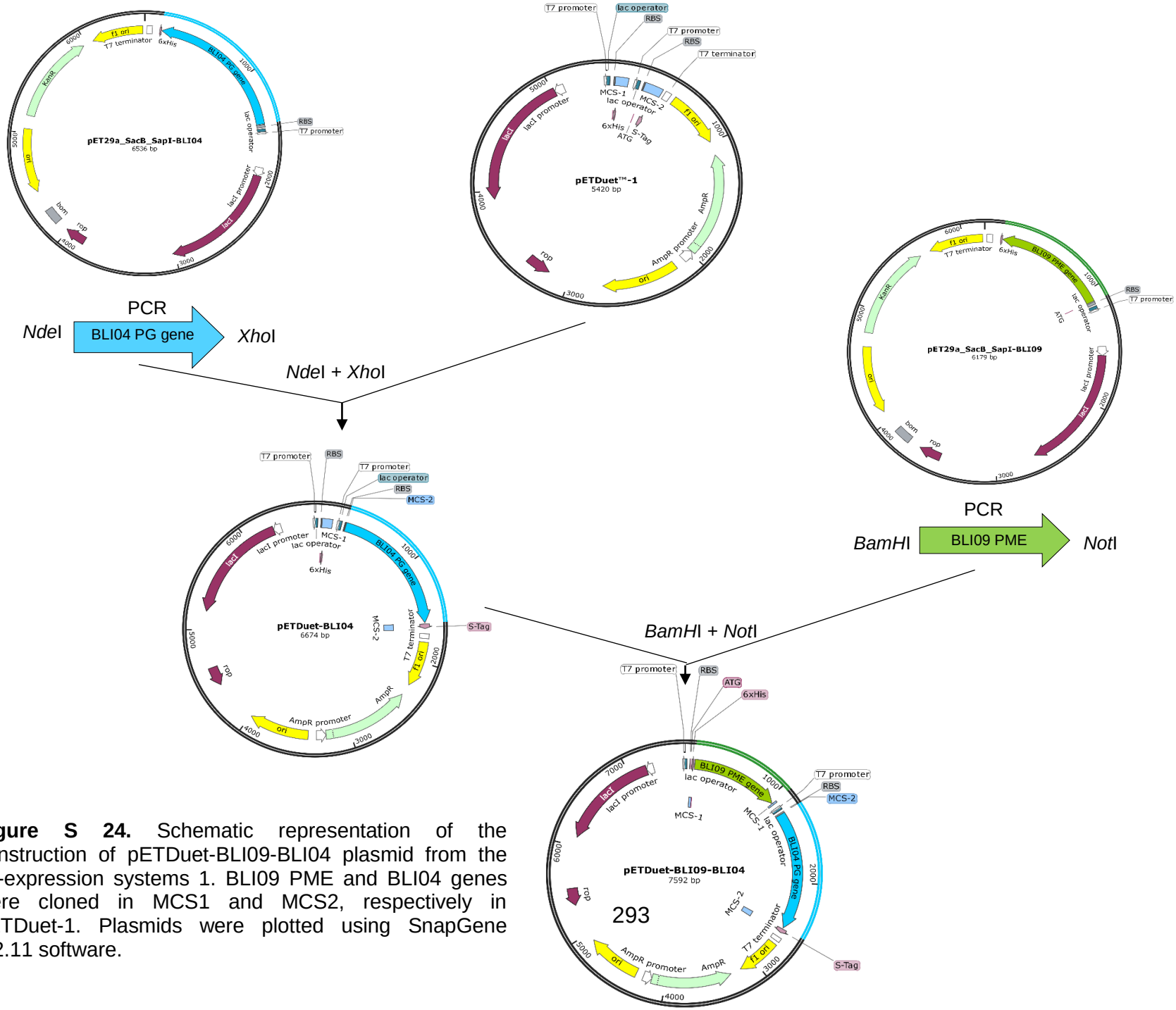

Figure S 24. Schematic representation of the construction of pETDuet-BLI09-BLI04 plasmid from the co-expression systems 1. BLI09 PME and BLI04 genes were cloned in MCS1 and MCS2, respectively in pETDuet-1. Plasmids were plotted using SnapGene 4.2.11 software.

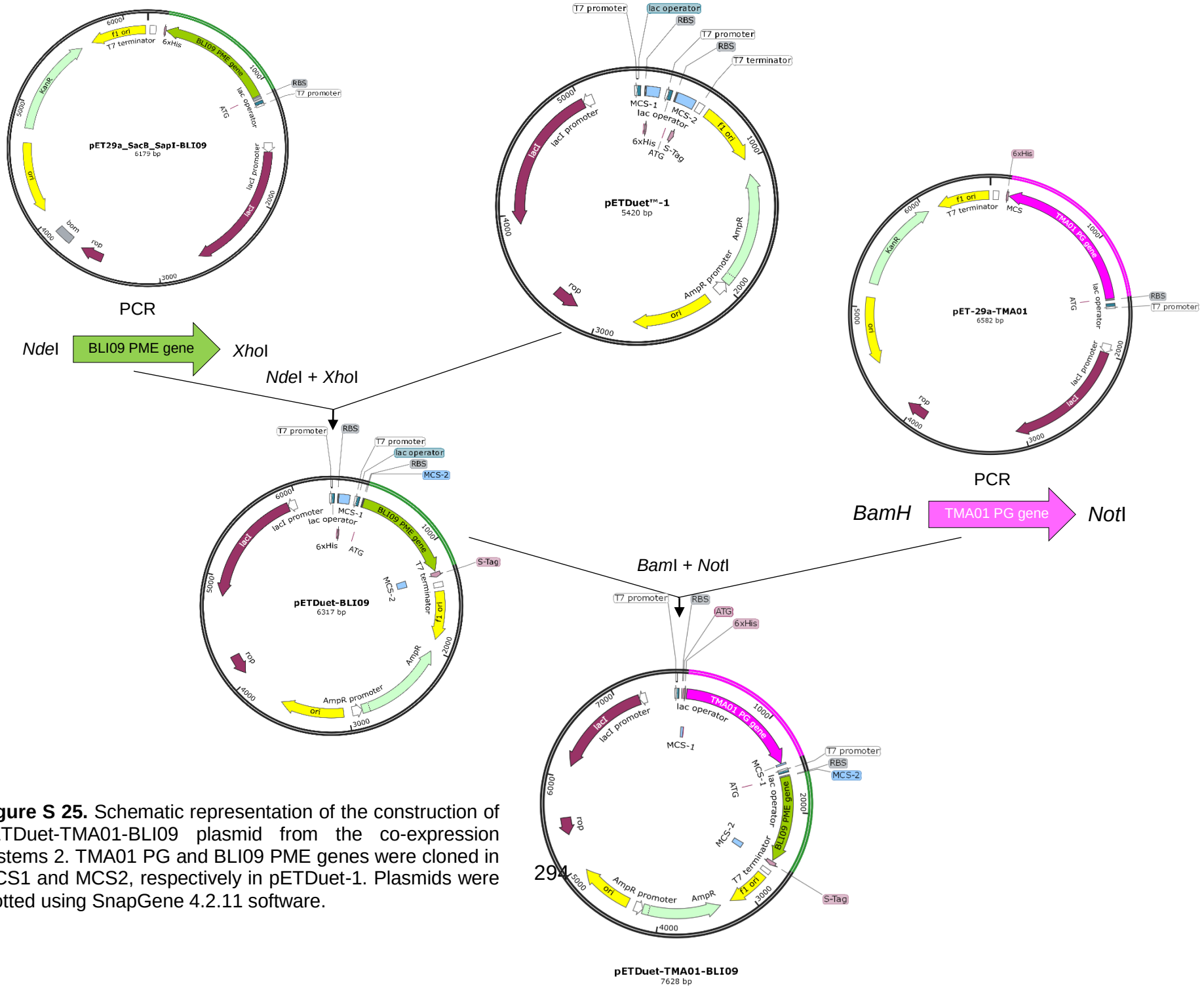

Figure S 25. Schematic representation of the construction of pETDuet-TMA01-BLI09 plasmid from the co-expression systems 2. TMA01 PG and BLI09 PME genes were cloned in MCS1 and MCS2, respectively in pETDuet-1. Plasmids were plotted using SnapGene 4.2.11 software.

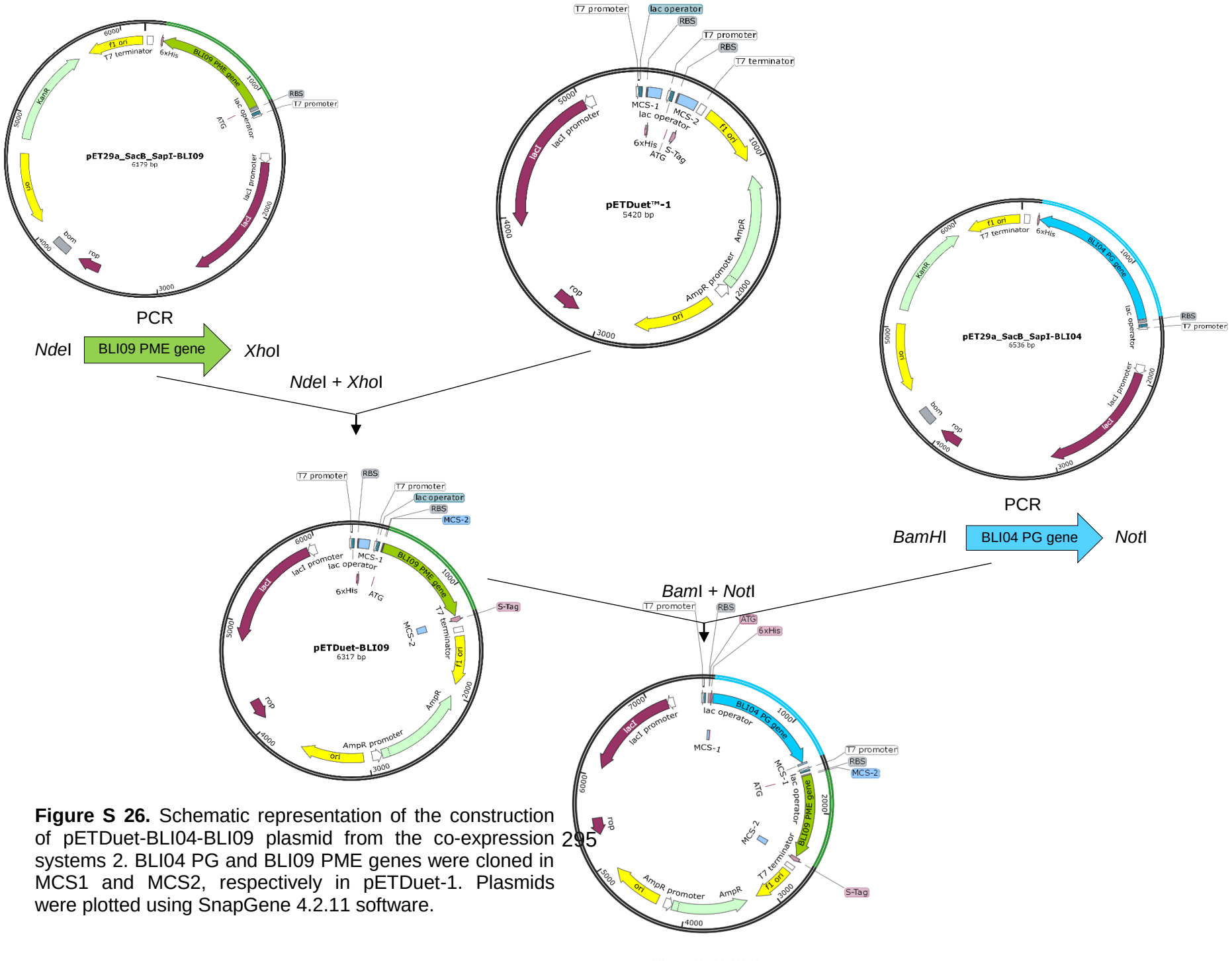

Figure S 26. Schematic representation of the construction of pETDuet-BLI04-BLI09 plasmid from the co-expression systems 2. BLI04 PG and BLI09 PME genes were cloned in MCS1 and MCS2, respectively in pETDuet-1. Plasmids were plotted using SnapGene 4.2.11 software.

APPENDIX 8: Analytical chromatograms

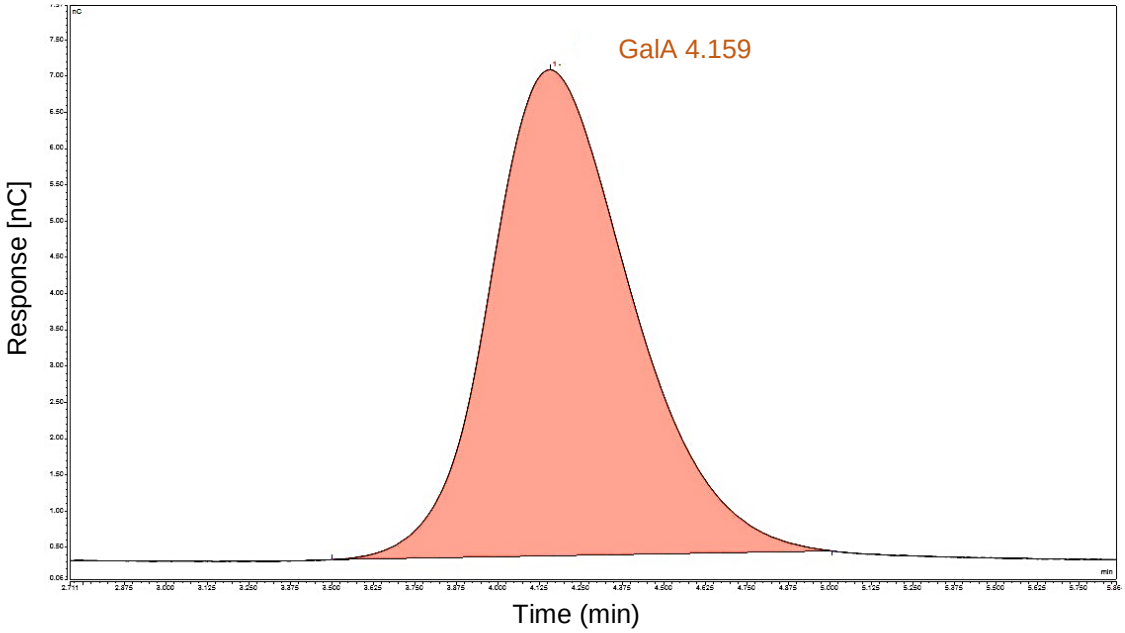

Figure S 27. Analytical chromatogram of synergistic reactions showing GalA release because of the pectic substrates hydrolysis.

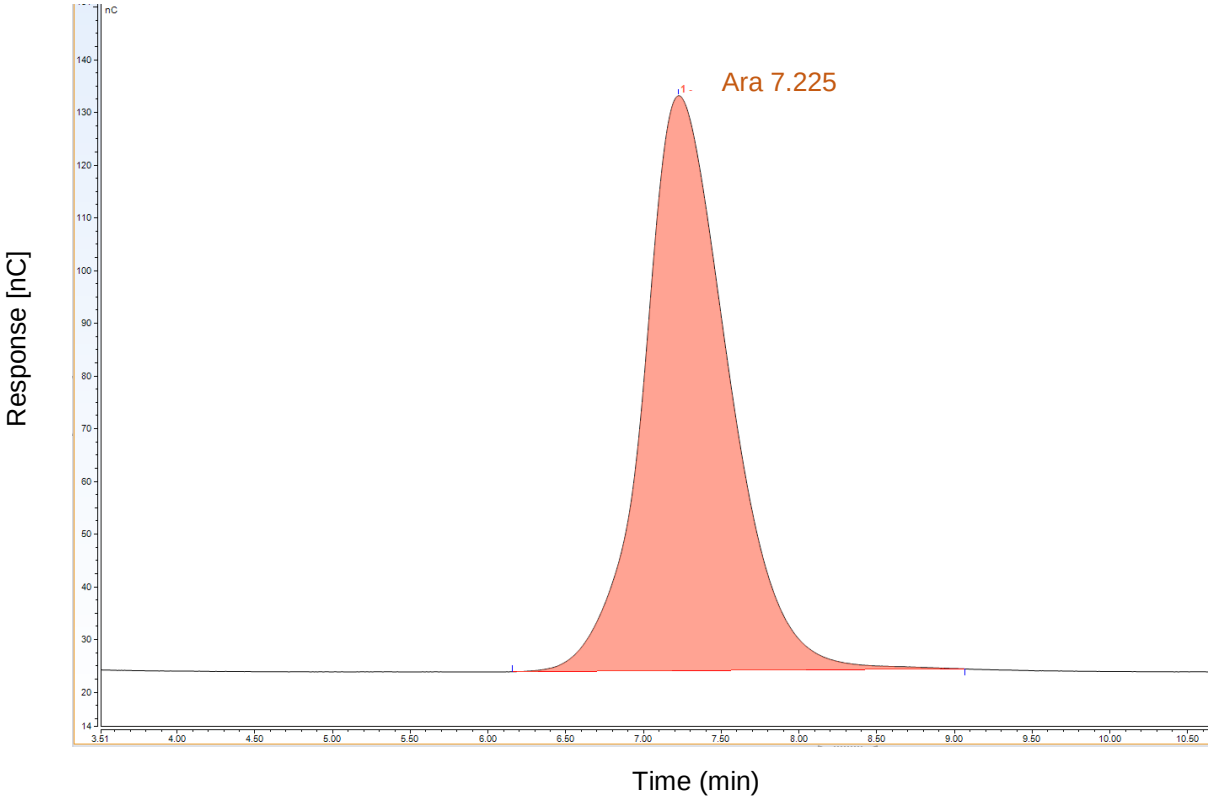

Figure S 28. Analytical chromatogram of synergistic reactions showing Ara release because of the pectic substrates hydrolysis.

APPENDIX 9: Statistics

Table S 3. Student's t-test for the effect of 1 mM Mn^{2+} on TMA01 exo-PG activity.

	Mn^{2+}	C
Mean	0.470	0.309
Variance	0.000	0.000
Observations	4.000	4.000
Pooled variance	0.000	
Hypothesised Mean Difference	0.000	
df	6.000	
t stat	14.014	
P(T<=t) one-tail	0.000	
t critical (one-tail)	1.943	
P(T<=t) two-tail	0.000	
t critical (two-tail)	2.447	

C: control without the presence of any ion. $p = 0.05$.

Table S 4. Student's t-test for the effect of 1 mM Mn^{2+} on BLI04 exo-PG activity.

	Mn^{2+}	C
Mean	0.339	0.266
Variance	0.000	0.000
Observations	4.000	4.000
Pooled variance	0.000	
Hypothesised Mean Difference	0.000	
df	6.000	
t stat	13.552	
P(T<=t) one-tail	0.000	
t critical (one-tail)	1.943	
P(T<=t) two-tail	0.000	
t critical (two-tail)	2.447	

C: control without the presence of any ion. $p = 0.05$.

Table S 5. One-way ANOVA for the effect of 0.6, 0.75 and 1 mM Ca^{+2} on TMA14 PGL activity.

Source of variation	SS	df	MS	F	P-value	F crit
Between Groups	3.45E-05	2	1.72E-05	4.306	0.131	9.552
Within Groups	1.2E-05	3	4E-06			
Total	4.65E-05	5				

p = 0.05

Table S 6. One-way ANOVA for the effect of 0.6, 0.75 and 1 mM Ca^{+2} on TFU19 PGL activity.

Source of variation	SS	df	MS	F	P-value	F crit
Between Groups	0.000705	2	0.0003	3.622	0.158	9.552
Within Groups	0.000292	3	9.73E-05			
Total	0.000997	5				

p = 0.05

Table S 7. One-way ANOVA for the effect of 0.6, 0.75 and 1 mM Ca^{+2} on TFU20 PGL activity.

Source of variation	SS	df	MS	F	P-value	F crit
Between Groups	0.004747	2	0.0023	2.165	0.261	9.552
Within Groups	0.003288	3	0.0010			
Total	0.008035	5				

p = 0.05

Table S 8. Student's t-test for the effect of 1 mM Mn^{2+} on TMA14 PGL activity.

	Mn^{2+}	C
Mean	0.182	0.133
Variance	0.000	0.000
Observations	4.000	4.000
Pooled variance	0.000	
Hypothesised Mean Difference	0.000	
df	6.000	
t stat	13.626	
P(T<=t) one-tail	0.000	
t critical (one-tail)	1.943	
P(T<=t) two-tail	0.000	
t critical (two-tail)	2.446	

C: control without the presence of any ion. $p = 0.05$.

Table S 9. Student's t-test for the effect of 1 mM Mn^{2+} on TFU20 PGL activity.

	Mn^{2+}	C
Mean	0.664	0.133
Variance	0.000	0.000
Observations	4.000	4.000
Pooled variance	0.000	
Hypothesised Mean Difference	0.000	
df	6.000	
t stat	36.843	
P(T<=t) one-tail	0.000	
t critical (one-tail)	1.943	
P(T<=t) two-tail	0.000	
t critical (two-tail)	2.446	

C: control without the presence of any ion. $p = 0.05$.

APPENDIX 10: Presentations and publications

Flores-Fernández, C. N., Cárdenas-Fernández, M., Lye, G. J. and Ward, J. M. (2022) 'Co-expression of thermostable pectinases for cost-effective pectin bioconversion', Biochemical and Molecular Engineering XXII: The dawn of a new era, Engineering Conferences International (ECI), Cancun, Mexico, June 26-30.

Flores-Fernández, C. N., Cárdenas-Fernández, M., Lye, G. J. and Ward, J. M. (2022) 'Thermostable pectinases for pectin bioconversion into D-galacturonic acid: enzymes characterisation and their synergistic action', 18th International Conference on Renewable Resources and Biorefineries (RRB 2022), Bruges, Belgium, June 1-3.

Flores-Fernández, C. N., Cárdenas-Fernández, M., Lye, G. J. and Ward, J. M. (2022) 'Synergistic action of thermophilic pectinases for pectin bioconversion into D-galacturonic acid', Enzyme and Microbial Technology, 160 (June). doi: 10.1016/j.enzmictec.2022.110071.

Flores-Fernández, C. N., Cárdenas-Fernández, M., Lye, G. J., & Ward, J. M. (2023). 'Co-expression of thermophilic pectinases in a single host for cost-effective pectin bioconversion into D-galacturonic acid'. Frontiers in Catalysis, 3 (February). doi:10.3389/fctls.2023.1112154.