CHARACTERISATION OF PECTATE LYASE PELQ1 FROM *Saccharobesus litoralis*

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CHARACTERISATION OF PECTATE LYASE PELQ1 FROM *Saccharobesus litoralis*

by

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LIST OF ABBREVIATION

A280	absorbance at 280 nm
bp	base pair
CBM	carbohydrate-binding module
CP	citrus pectin
DE	degree of esterification
DM	degree of methylation
DP	degree of polymerization
$GalA_1$	galacturonic acid with 1 DP unit
GalA	galacturonic acid
kDa	kilodalton
L	litre
M	molar
mg	milligram
min	minute
monoGalA	monomer of galacturonic acid
oligoGalA	oligomer of galacturonic acid
PCR	polymerase chain reaction
Pel	pectate lyase
PGA	polygalacturonic acid
pH	potential hydrogen
rpm	revolution per minute
sec	second
V	volt
v/v	volume per volume
w/v	weight per volume

PENCIRIAN PEKTAT LIASE PELQ1 DARIPADA *Saccharobesus litoralis*

ABSTRAK

Gen *pelQ1* daripada bakteria marin *Saccharobesus litoralis* CCB-QB4 mengekodkan enzim pektat liase yang mengandungi dua domain, iaitu domain polisakarida liase keluarga 1 (PL1) dan domain pengikat karbohidrat keluarga 13 (CBM13). CBM13 yang berfungsi sebagai pengikat substrat yang lazimnya hadir dalam pektat liase (Pel) daripada sumber laut dan bukan sumber daratan. Tambahan pula, kajian terhadap Pel marin adalah berkurangan kerana pektat adalah bukan sumber karbohidrat utama dalam laut. Oleh itu, kajian ini bertujuan untuk menjelaskan mekanisma penguraian PelQ1 dengan membandingkan aktiviti enzim yang bermodul lengkap (PelQ1-Full) dan enzim yang disingkirkan domain CBM13 (PelQ1-PL1). Kedua-dua enzim telah diklon, ditulenkan, dicirikan serta mekanisma penguraian telah dijelaskan. PelQ1-Full memotong secara eksolitik substrat asid poligalakturonik (PGA) untuk menghasilkan asid trigalakturonik tidak tepu sebagai produk majoriti, akan tetapi, ia memotong substrat pektin sitrus (CP) kepada asid digalakturonik tidak tepu sebagai produk majoriti apabila kepekatan enzim yang tinggi digunakan. PelQ1-Full berfungsi secara optimum dalam kondisi pH 9.0, 30°C, dan 0.6 mM $CaCl_2$. Perbandingan antara kedua-dua enzim ini menunjukkan nilai kecekapan (k_{cat}/K_M) yang lebih tinggi sedikit terhadap PGA daripada CP, manakala hasil TLC menunjukkan bahawa domain CBM13 berkemungkinan membantu pengikatan substrat. Model homogi AlphaFold menunjukkan bahawa domain PL1 mempunyai struktur β-heliks selari dan persamaan jujukan asid amino yang tinggi dengan PelC dari *Dickeya dadantii*. Domain CBM13 pula menunjukkan struktur β-lipatan trefoil yang serupa dengan domain CBM13 lain. Mekanisma penguraian PGA dan CP menunjukkan

bahawa domain PL1 dapat memuatkan asid heksagalakturonik tidak bermetilasi pada subtapak -3 hingga +3 dan menghuraikannya kepada asid trigalakturonik, namun ia mengikat pentagalakturonik dan tetragalakturonik dengan kurang kuat dan memotong substrat tersebut dengan lebih perlahan. Bagi pektin sitrus yang banyak bermetilasi, sekurang-kurangnya tiga unit asid galakturonik tidak bermetilasi yang berturutan adalah diperlukan untuk mengikat pada subtapak -1 hingga +2. Hal ini kemungkinan mengakibatkan mekanisma penguraian CP yang berbeza daripada mekanisma penguraian PGA.

CHARACTERISATION OF PECTATE LYASE PELQ1 FROM

Saccharobesus litoralis

ABSTRACT

The *pelQ1* gene from the marine bacterium *Saccharobesus litoralis* CCB-QB4 encodes a pectate lyase that consists of two domains, which are polysaccharide lyase family 1 (PL1) and carbohydrate-binding module family 13 (CBM13). CBM13 which is involved in substrate binding is often found in pectate lyases (Pels) from marine sources but not terrestrial sources. Moreover, the study of marine Pels is lacking since pectin is not one of the major sources of carbohydrates in the marine environment. Therefore, this study aimed to elucidate the cleaving mechanism of PelQ1 by comparing the activity of the full-length enzyme (PelQ1-Full) and the truncated enzyme with CBM13 removed (PelQ1-PL1). Both enzymes were cloned, purified, characterized, and their cleaving mechanisms were elucidated. PelQ1-Full exolytically cleaved the substrate polygalacturonic acid (PGA) to produce a majority of unsaturated trigalacturonic acid; however, it cleaved the citrus pectin (CP) into a majority of unsaturated digalacturonic acid when high enzyme concentrations were used. PelQ1-Full worked in an optimal condition of pH 9.0, 30°C, and 0.6 mM $CaCl_2$. Comparison between the two enzymes showed that there was a slightly higher catalytic efficiency (k_{cat}/K_M) towards PGA than CP, while TLC results showed that the CBM13 domain might help substrate binding. The AlphaFold homology model of the PL1 domain showed that it had a structure of parallel β-helix and a high sequence similarity with PelC of *Dickeya dadantii*. The CBM13 domain showed a structure of β-trefoil fold similar to other CBM13 domains. The cleaving mechanisms towards PGA and CP showed that the PL1 domain could accommodate unmethylated hexagalacturonic acid

at subsites -3 to +3 and cleave it into trigalacturonic acid; however, it bound to pentagalacturonic acid and tetragalacturonic acid less tightly and cleaved them more slowly. For citrus pectin that is highly methylated, at least three consecutive unmethylated galacturonic acid units were required to bind to subsites -1 to +2. This could have led to possibly a CP cleaving mechanism different from that for PGA.

CHAPTER 1

INTRODUCTION

1.1 Rational of research

Pectin is a group of complex heteropolysaccharides that can be found naturally in citrus fruit peel and pulp, and is composed of a backbone of galacturonic acid (GalA) that is attached to methyl ester and branches of neutral sugar side chains (Voragen et al., 2009). Due to its complex structure, pectin is rarely synthesized chemically and instead, it is cleaved enzymatically or chemically from its original form as it is more straightforward and less time-consuming (Nepogodiev et al., 2010). With its low cost and non-toxic properties, pectin has found its way into many applications. According to the market forecast (https://www.futuremarketinsights.com/reports/pectin-market), the global pectin market holds a share of US$ 890.9 million in 2023 and is estimated to value US$ 1.5 billion by 2033. Pectin is approved as a food additive (EU code E440) that functions as a gelling agent in jams and emulsifiers in low-fat dairy products (Cirimminna et al., 2016; Vanitha & Khan, 2020). The shorter form of pectin – pectin oligosaccharides (POS) – is also found to possess prebiotic potential and anti-cancer properties (Chung et al., 2017; Glinsky & Raz, 2009).

To cleave pectin, various pectinases are involved. One of them is pectate lyase (Pel, EC 4.2.2.2) that targets the α-1, 4-linked glycosidic acid to produce unsaturated galacturonate residues in the de-esterified pectin (also termed "pectate") (Marin-Rodriguez et al., 2002). Due to their secretion by a large number of phytopathogens that cause domestic crop loss, an extensive amount of terrestrial Pels have been studied compared to marine Pels (P. Wu et al., 2020). Marine Pels can be equally useful in the study of biofuel alternatives (Berlin et al., 2007), which requires pectinases in the process of cell disruption as a better option that consumes less energy (Zhang et al.,

2022). Pectinases are also found in marine Bacteroidetes which play an important role in marine carbon cycling (K. Tang et al., 2017).

In recent years, studies of marine Pels have yielded some interesting findings. These include the discovery of an alternative pectin-degradation pathway and substrate-binding domains in marine Pels (Hehemann et al., 2017; Hobbs et al., 2019). An increasing number of marine organisms have also been found to encode pectin polysaccharide utilization loci (PUL) (Hehemann et al., 2017; Hobbs et al., 2019; K. Tang et al., 2017), including those that target the pectin in marine diatoms and seagrasses (Hobbs et al., 2019). Zosterin, a type of marine pectin found in seagrasses of the Zosteraceae family, is composed of apiogalacturonan (AGU), a structure with D-apiose residues attached to the homogalacturonan backbone. The presence of marine pectin can be traced back to the immediate ancestor of land plants, Charophyte green algae, which are shown to have simpler pectin structures with abundant homogalacturonan-Ca^{2+} complex and only a small amount of rhamnogalacturonan-I (Domozych et al., 2014). Seagrasses are also found with a low methyl ester content of less than 10% which is relatively lower than citrus fruits.

Numerous studies on Pels have helped us build a substantial understanding of the cleaving mechanism of Pels towards the de-esterified galacturonic acid chain. To obtain an unsaturated product, the mechanism involves several aspects such as the anti β-elimination, endolytic or exolytic mechanism, production of dimer or trimer, and presence or absence of the CBM domain in Pels (Hehemann et al., 2017; Hobbs et al., 2019; Hugouvieux-Cotte-Pattat et al., 2014; Seyedarabi et al., 2010). However, the exact cleaving mechanism of polygalacturonic acid (PGA) and citrus pectin (CP) is still not well understood. Therefore, more studies regarding this can help understand the cleaving mechanism. It should be noted that the homogalacturonan backbone of

pectin is methylated in nature and removal of the methyl ester groups is needed to obtain PGA. Therefore, PGA does not exist in nature.

Saccharobesus litoralis strain CCB-QB4 is a novel bacterium isolated from the coastal region of Queensbay Penang. Whole genome sequencing found that it encodes various pectin catabolic genes similar to several marine bacteria (Hehemann et al., 2017; Hobbs et al., 2019; K. Tang et al., 2017), and one of the *pel* gene contains an extra CBM13 domain. Therefore, this project aimed to study the characteristics, cleaving mechanism, and structure of this marine Pel which we named PelQ1 along with the effect of CBM13 on its kinetic efficiency. Protein sequence alignment of PelQ1 revealed a high structural similarity with the terrestrial PelC of *Dickeya dadantii* (Lietzke et al., 1996), which the PL1 catalytic domain of PelQ1 might share a similar optimal working condition and structure with. As for the CBM13 domain, only proteins of low structural similarity were obtained. It was speculated to be involved in the binding of pectin. Protein crystallization of the full-length enzyme (PelQ1-Full) was also attempted to study the interaction of PelQ1's two domains and the substrate.

1.2 Objectives

1. To produce recombinant full-length PelQ1 (PelQ1-Full) and the PL1 domain of PelQ1 (PelQ1-PL1) for characterization.
2. To characterize PelQ1-Full in terms of its optimal working condition; and compare its kinetic efficiency with PelQ1-PL1 to study the function of CBM13.
3. To elucidate the cleaving mechanism of PelQ1-Full towards PGA and CP.
4. To study the structure of PelQ1-Full with protein crystallization.

CHAPTER 2

LITERATURE REVIEW

2.1 *Saccharobesus litoralis*

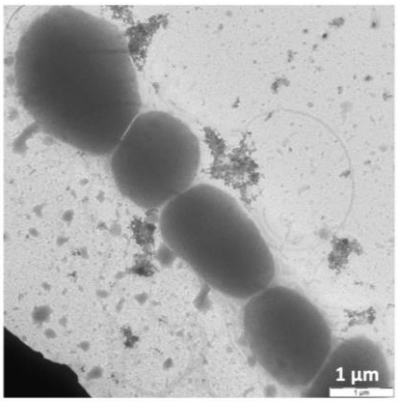

Figure 2.1 Cell morphology of *Saccharobesus litoralis*, taken from the webpage of Centre for Chemical Biology (https://ccb.usm.my/index.php?option=com_content&view=article&id=426&catid=1%205&Itemid=272).

Saccharobesus litoralis strain CCB-QB4 (Figure 2.1) was previously isolated from marine algal turf at the coastal site of Penang, Malaysia (5° 19′ 30′′ N; 100° 18′ 12′′ E) designated as *Catenovulum*-like bacterium that contains numerous genes that encode carbohydrate-active enzymes (CAZymes) (Lau et al., 2019).

In the following year, it was proposed to be a novel genus *Saccharobesus* and species *litoralis* which means “fat sugar-eating organism of the shore” (Table 2.1) (Amrina et al., 2021). This strain is unique compared to the four reference strains found in the literature; as *S. litoralis* can utilize all seven carbon sources (α-D-Glucose, D-Fructose, D-Mannitol, Turanose, D-Sorbitol, Cellobiose, and Dextrin) while none of the reference strain could utilize D-Mannitol and D-Sorbitol.

Table 2.1 Classification of *S litoralis* from domain to species level.

Classification	
Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Alteromonadales*
Family	*Alteromonadaceae*
Genus	*Saccharobesus*
Species	*litoralis*

S. litoralis is a gram-negative, rod-shaped bacterium that moves using flagellum and thrives under aerobic conditions. It grows optimally at 30°C, pH 7-8, at 2-4% (w/v) NaCl. This bacterium is capable of producing agarases like its closely related genus *Catenovulum* and *Algibacillus*. However, unlike *Algibacillus agarilyticus* RQJ05, it requires an absolute carbon source to grow (Meng et al., 2021).

2.1.1 *Saccharobesus litoralis* genes in pectin degradation and utilization

Among the *S. litoralis* genes for carbohydrate-active enzymes (CAZymes), many are involved in the metabolism of uronic acids such as alginate, ulvan, and pectin which are found in seaweed (Furusawa et al., 2021). Pectin is also found largely in terrestrial plants and has a more complex structure than seaweed.

S. litoralis may target one of the main regions of pectin – the homogalacturonan (HG) region which makes up the backbone of pectin and is the simplest and most abundant in pectin. According to the CAZy database, these genes include seven Pels. Four of the Pels have CBM domains that may bind to arabinan, arabinoxylan, pectin, and HG; and two Pels with additional carbohydrate esterase from family 8 (CE8) domains that may function to de-esterify the methyl group in the HG region. These enzymes, along with five of the glycoside hydrolases (GHs) were speculated to de-esterify and depolymerize the HG chain to produce unsaturated digalacturonate

products. These then undergo further linearization and isomerization catalyzed by the isomerase KduI and dehydrogenase KduD before entering the citric acid cycle for energy production.

PelQ1 (abbreviated as Pd1_PL1 in Table 2.2), which was the focus of this project, consists of an N-terminal catalytic domain from polysaccharide lyase family 1 (PL1), and a C-terminal carbohydrate-binding module (CBM13) (Figure 2.2).

Table 2.2 Genes of *S. litoralis* involved in pectin degradation (Furusawa et al., 2021).

Abbreviation	Function	CAZymes	GeneBank ID
Pd1_PL1	Pectate lyase	PL1, CBM13	WP_108605188.1
Pd2_PL1		PL1, CBM35, CBM77	WP_108605291.1
Pd3_PL1		PL1, CBM35	AWB69198
Pd4_PL1		PL1, CE8	WP_159084287.1
Pd5_PL1		PL1, CE8	WP_108605272.1
Pd6_PL3		PL3, CBM13	WP_108605187.1
Pd7_PL10		PL10	WP_108605295.1
Pd8_GH105	Unsaturated galacturonate hydrolase	GH105	WP_108605228.1
Pd9_GH105		GH105	WP_108605230.1
Pd10_GH105		GH105	WP_108605292.1

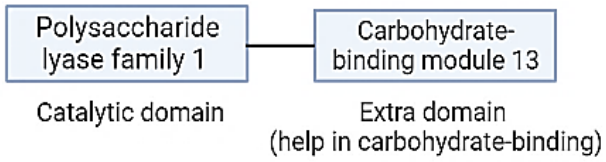

Figure 2.2 Structural domains of PelQ1. It contains two domains, the polysaccharide lyase family 1 (PL1) and carbohydrate-binding module family 13 (CBM13).

2.2 Pectin

Pectic substance is a general term used to describe all the compounds that are made up of galacturonic acid (GalA) residues. The GalA residues when present in a long chain are termed polygalacturonic acid (PGA). In nature, the GalA residues exist in different degrees of polymerization (DP), methyl content, acetyl content, and neutral sugar side chains (Voragen et al., 2009).

Depending on their gelling properties, pectic substances are referred to in four different terms – protopectin, pectinic acid, pectin, and pectic acid (Kertesz et al., 1944) as explained in Table 2.3. Pectin is used in a generic sense to refer to all water-soluble pectic substances (BeMiller, 2019).

Table 2.3 Nomenclature of pectic substances (Kertesz et al., 1944; Sista Kameshwar & Qin, 2018).

Nomenclature	Definition
Pectic substance	A group of complex colloidal carbohydrate derivatives that contains a high proportion of galacturonic acid unit.
Protopectin	**Water-insoluble** form that is found in the plant cell wall as precursor of pectic substance which upon degradation yield pectin or pectic acid.
Pectin	**Water-soluble** galacturonic acid with different methyl content and degree of neutralization. Under suitable condition, it can form gel with sugar and acid.
Pectinic acid	Galacturonic acid with high methyl ester content, the salt of pectinic acid is called ***pectinate***. Under suitable condition, it can form gel with sugar, acid, or for those with low methyl content, with metallic ion.
Pectic acid	Galacturonic acid that is almost free of methyl content, the salt of pectic acid is called ***pectate***.

2.2.1 Early discovery of pectic substances

Pectic substances were first extracted from tamarind by chemist Louis-Nicholas Vauquelin in 1790 (Vauquelin, 1790), which was later coined as "pectic acid" by Henri Braconnot in 1825 to refer to the simple galacturonic acid without any methyl ester. The term was derived from the Greek word "πήκτίζ" meaning to congeal or solidify (Muzzarelli et al., 2012).

2.2.2 Pectin nomenclature in early literature and current time

As more pectic substances with different physical properties were discovered, several attempts were made to unify the nomenclature for the pectic substances. One of the nomenclature systems that is frequently mentioned in the literature was set by the Committee for the Revision of the Nomenclature of Pectic Substances in 1944 (Kertesz et al., 1944) as listed in Table 2.3 of Section 2.2.

However, the set of nomenclature was not entirely agreed upon and modifications were made according to the researchers' understanding. Among them, Doesburg has pointed out that the definitions failed to address water-soluble pectin that is high in methyl ester but cannot gel without sugar and acid, as well as that is low in methyl ester and can gel without sugar but with metal (Doesburg, 1965).

Therefore, it is more useful to group the pectic substance into high-methoxyl pectin (DM>50%), low-methoxyl pectin (DM<50%), and pectate (with negligible methyl ester content) as seen in the commercial pectin available on the market (Doesburg, 1965; Hotchkiss, et al., 2002). Pectin is widely utilized in the food industry such as jam making, food emulsified and stabilizing agent (Gavahian et al., 2021).

2.2.3 Pectin source and function in plants

Pectin can be found in abundance within the primary cell, secondary wall, and middle lamella of terrestrial plants (Voragen et al., 2009) which play a significant role in controlling cell wall mechanics and regulating plant development (Y. Yang & Anderson, 2020). According to the literature, there is up to 35% of pectin found within the primary cell wall of dicotyledon (Fry, 1988) and commercial pectin is mostly acquired from citrus peel and apple pomace (Voragen et al., 2009).

Pectin is not the dominant carbon source in the marine environment (Hobbs et al., 2019). However, with researchers seeking alternatives to biofuel production, there is a growing interest in pectin from marine sources. Pectin-like components can be found in marine algae and seagrasses. Marine algae from Charophytes are found to be ancestral to land plants (Kranz et al., 1995; Manhart & Palmer, 1990). *Zygnematophyceae* is an example of Charophyte Green Algae (CGA) that is composed of cell wall made of homogalacturonan-calcium complex. It allows the algae to have high water-holding capacity, which in turn delays water loss (Herburger et al., 2019). Additionally, the marine seagrass zosterin is also found to consist of predominantly apiogalacturonan (AGU) (Ovodova et al., 1968).

2.2.4 Pectin structure

Pectin is a family of complex heteropolysaccharides that is primarily made up of galacturonic acid (GalA) residues on the pectin backbone, which is then further attached with acidic methyl esters and neutral sugar side chains (Voragen et al., 2009).

The basic unit of pectin – galacturonic acid (GalA) – is the oxidized form of galactose where the sixth carbon made up of hydroxymethyl group CH_2OH is converted to carboxyl group COOH (Figure 2.3). Pectin has a complex structure which can be divided into three main groups – homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) as shown in Figure 2.4.

Among them, homogalacturonan (HG) is present as the most abundant pectic component accounting for up to 60% of the pectin structure (Caffall & Mohnen, 2009; Voragen et al., 2009). It forms the backbone of the pectin structure by linking galacturonic acid (GalA) into 1, 4-linked-α-D-galacturonic acid. Side chains may be attached to the GalA residues and they can be methyl-esterified and/or partially O-acetylated at varying degrees (Ridley et al., 2001).

Rhamnogalacturonan I (RG-I) is made of a backbone of alternating α-1, 4-linked galacturonic acid (GalA) and α-1, 2-linked rhamnose (Rha) (I. R. Silva et al., 2016). Additionally, neutral sugar side chains, primarily β-D-galactopyranosyl and α-D-arabinofuranosyl residues branch out of Rha residues. These complex structures are associated with the function of plant cell walls. RG-I is present in 20 to 35% of the total pectin mass (I. R. Silva et al., 2016).

Rhamnogalacturonan II (RG-II) is a distinctive part of HG that is highly conserved in vascular plants (Voragen et al., 2009). It has a complex structure with a total of 13 different sugar residues forming 6 chains denoted as chain A to chain F. SG is present in 10% of total pectin mass in higher plant cell walls (Mancuso et al., 2021).

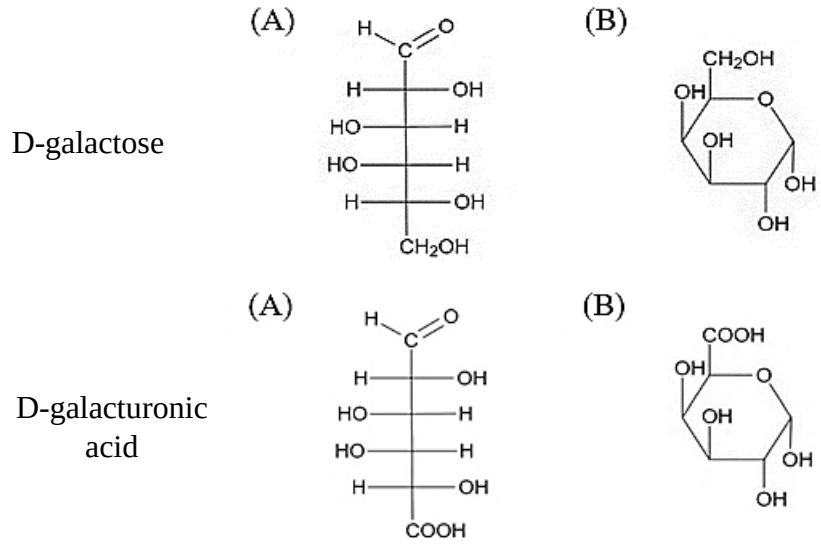

Figure 2.3 Fischer projection and Haworth projection of D-galactose and D-galacturonic acid. (A) Fischer projection and (B) Haworth projection. Drawn with ChemDraw (https://chemdrawdirect.perkinelmer.cloud/js/sample/index.html).

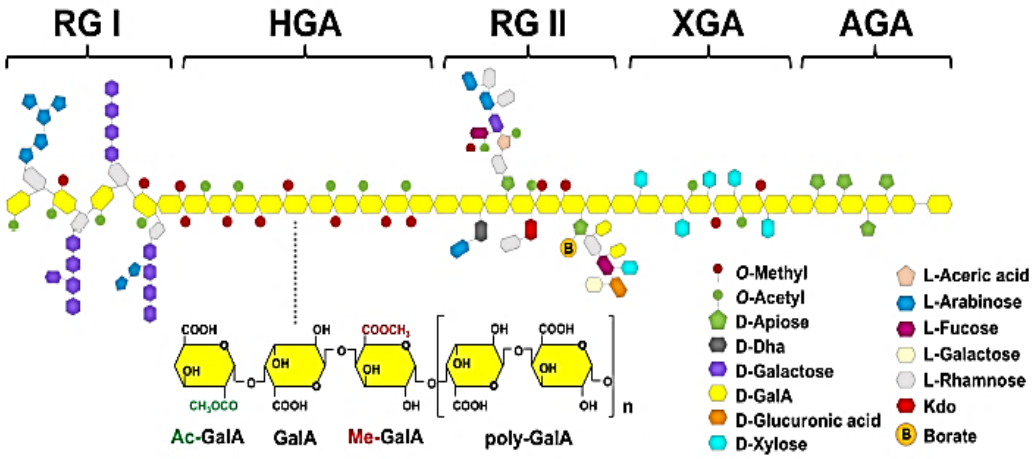

Figure 2.4 Pectin structure. Structural domains showed are HG, RG I, RG II, and minor structural domains XGA (xylogalacturonan) and AGA (arabinogalacturonan) (H.-C. Wu et al., 2018).

2.2.5 Pectin gelling ability and degree of methylesterification (DM)

Pectin gelation plays a significant role in intercellular adhesion and support in the junction zone, as well as a thickening and gelling agent in jam-making, and drug delivery (Cao et al., 2020). Pectin gelation occurs when negatively charged unmethylated GalA interacts with positively charged cations such as calcium ions (Celus et al., 2018). The percentage of methyl ester in pectin frequently correlates with the gelling ability of pectin (Voragen et al., 2009) because the neutral methylated carboxyl attached to GalA do not interact with positively-charged calcium ion (Cao et al., 2020). Lack of calcium-pectin interaction in low-methylated (LM) pectin will lead to a lesser ability to form gel and *vice versa*.

The "egg box model" (Figure 2.5) was originally proposed in 1973 to describe the gelation mechanism of alginate, and it has been used to explain gelation in pectin. In this model (Figure 2.5), two antiparallel chains of polygalacturonic acid interacts with calcium ion, forming dimer and eventually multimer (Cao et al., 2020; Grant et al., 1973). This allows the formation of stable pectin-calcium complex with shorter GalA chain (Braccini & Pérez, 2001).

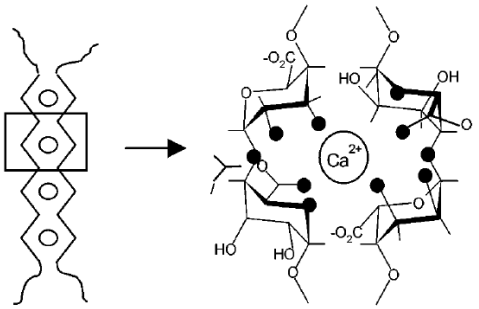

Figure 2.5 The egg box model. The model used to describe alginate and later pectin showing the interaction of alginate guluronate residue and calcium with reference (Braccini & Pérez, 2001).

2.3 Pectinase

2.3.1 Type of pectinase

Pectin can be degraded with a group of enzymes called pectinase. Pectinases are classified based on their mode of action as follows – (i) Pectinases that cleave GalA that is esterified (esterase) or non-esterified (depolymerase); (ii) Pectinases that carry out hydrolysis (hydrolase) or β-elimination (lyase); and (iii) Pectinases that target the inside of the chain (endo) or chain terminus (exo).

A variety of pectinases are available to break down the complex structure of pectin. Pectinases that target the homogalacturonan region are listed in Figure 2.6. However, pectinases that target the rhamnogalacturonan region are much lesser in number. During the degradation of RG-I structure, hydrolase and lyase initially break down the RG backbone, followed by accessory enzymes that degrade the RG side chain (I. R. Silva et al., 2016).

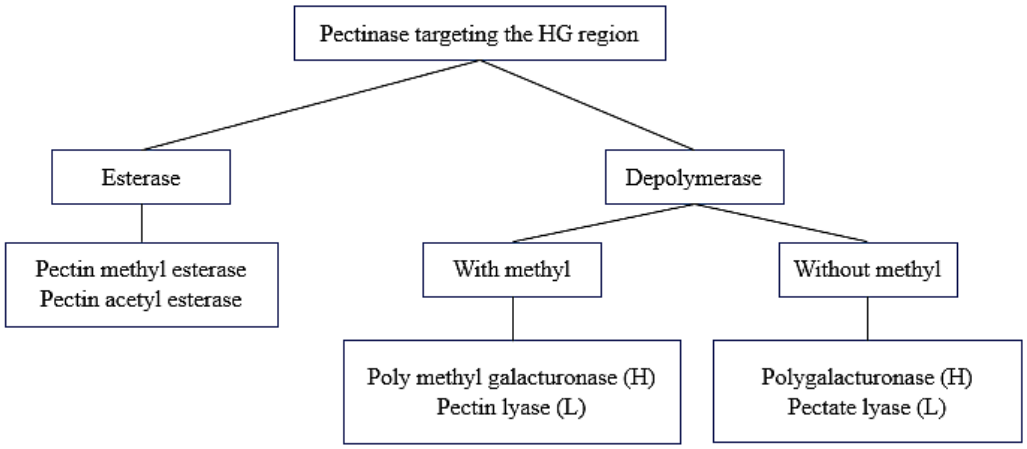

Figure 2.6 Pectinases targeting the homogalacturonan region of pectin. The enzymes are classified into esterase and depolymerase. Depolymerase further cleaves pectate with or without methyl ester with reference (Sharma et al., 2013). H: Hydrolase, L: Lyase.

2.3.2 Pectin degradation pathway

In terrestrial Pels, the degradation of homogalacturonan follows the canonical homogalacturonan metabolism pathway (Figure 2.7A) (Hobbs et al., 2019). The degradation occurs sequentially in the extracellular space, periplasm, and cytoplasm.

Degradation starts in the extracellular space, where extracellular enzymes such as carbohydrate esterases (CE) are secreted to de-esterify the HG backbone; simultaneously, depolymerization is also done by endo-cleaving polysaccharide lyases (PLs) and glycoside hydrolase family 28 (GH28). These oligomers are transported into the periplasm through porins of the KdgM family. In the periplasm, the oligomers are further broken down into shorter disaccharide and trisaccharide by PL and exo-GH28 and are then transported into the cytoplasm through ATP-dependent TogMNAB transporter. Once they are inside the cytoplasm, the short oligomers are cleaved into saturated and unsaturated monosaccharide by oligogalacturonate lyase PL22. Both saturated GalA and unsaturated GalA are converted into key metabolite 2-keto-3-deoxygluconate (KDG) by different enzymes. Saturated GalA is converted by UxaC, UxaB, and UxaA; while unsaturated GalA is converted by KdgF, KduI isomerase, and KduD reductase. According to (Abbott & Boraston, 2008), KDG is finally converted into pyruvate and 3-phosphoglyceroladehyde which enters the citric acid cycle for energy production.

The degradation for homogalacturonan in the marine organisms follows an alternative pathway in which it was only proposed recently (Figure 2.7B). It was proposed because marine bacteria *Pseudoalteromonas* sp. PS47 was found to encode an extra GH105 enzyme that was able to convert unsaturated GalUA into DKI within the periplasmic space and did not require the PL22-KdgF pair. The gene for GH105 was similarly encoded in the pectin polysaccharide utilization loci (PULs) of marine

bacteria *Pseudoalteromonas haloplanktis* ANT/505 and *Gramella Flava* JLT2011 (Hobbs et al., 2019).

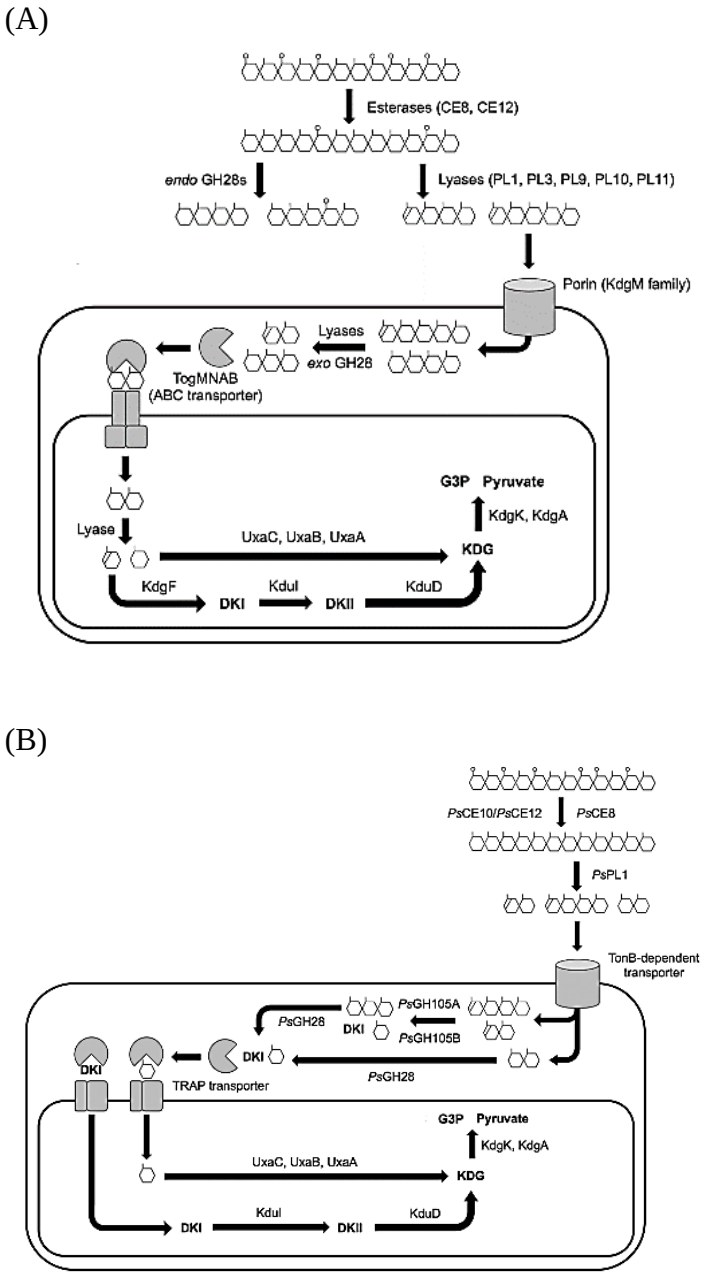

Figure 2.7 Comparison of homogalacturonan metabolism pathway between land and marine organisms. (A) Canonical homogalacturonan metabolism pathway. (B) Proposed homogalacturonan metabolism pathway of the marine *Pseudoalteromonas* sp. PS47 (Hobbs et al., 2019).

2.4 Pectate lyases (Pels)

Pectate lyases (Pels, EC 4.2.2.2), also known as pectate transeliminase are enzymes capable of cleaving de-esterified pectin (also termed “pectate”) that are made up of α-1, 4-linked galacturonic acid chain. The degradation generates 4, 5-unsaturated galacturonate at the non-reducing end (Marin-Rodriguez et al., 2002).

Pels are widely found to be produced by phytopathogens such as *Dickeya* and *Pectobacterium* (previously designated as genus *Erwinia*) capable of causing soft-rot disease (Charkowski, 2018). Soft-rot disease is a major phytobacterial disease that causes plant crops to become soft and watery (Figure 2.8), leading to an unpleasant smell that makes them unsuitable to be sold and causing devastating economic losses (UW-Madison Plant Pathology, 2021).

The soft rot enterobacteria have developed a remarkable ability to break down the tough plant cell wall by producing a wide range of plant cell wall degrading enzymes. For example, *Dickeya dadantii* is capable of producing at least 11 pectinases including eight endo-Pels (PelA, PelB, PelC, PelD, PelE, PelI, PelL, and PelZ) and three exo-Pels (PelN, PelX, PelW) (Hassan et al., 2013). In most cases, the pectinases are encoded in the pectin PULs (polysaccharide utilization loci) (Hobbs et al., 2019).

Figure 2.8 Bacterial soft rot disease in potato tuber (UW-Madison Plant Pathology, 2021).

2.4.1 Source of Pels

In addition to the soft rot phytopathogen, as mentioned in Section 2.4, many other organisms are also capable of producing Pels. Some notable examples are rhizospheric bacteria, soil bacteria, plant-pathogen nematode, and microbiome in ruminant and human gut (Hugouvieux-Cotte-Pattat et al., 2014).

Compared to the terrestrial environment, the Pels from marine sources are significantly lesser. However, recent research has focused on marine bacteria and has found that they also possess pectin-degrading genes. These bacteria include *Pseudoalteromonas haloplanktis* ANT/505 (Hehemann et al., 2017; Truong et al., 2001), *Pseudoalteromonas* sp. PS47 (Hobbs et al., 2019), *Gramella Flava* JLT2011 (K. Tang et al., 2017), and *Microbulbifer mangrove* DD-13T (Imran et al., 2017).

2.4.2 Classification of Pels

Pels can be classified into the polysaccharide lyase (PL) family, namely PL1, PL2, PL3, PL9, PL10. Each PL family has their respective structural topology and proton abstractor (Table 2.4) (Hugouvieux-Cotte-Pattat et al., 2014).

The PL1 family has the largest group of Pels found in plants, fungi, and bacteria (Hugouvieux-Cotte-Pattat et al., 2014). Some of the earliest Pels that were structurally studied including PelC (Yoder et al., 1993) and PelE (Lietzke et al., 1994) from *Dickeya dadantii*, and BsPel from *Bacillus subtilis* (Pickersgill et al., 1994) are from the PL1 family. Among the PL family, PL3 is unique as it is the only PL family having an animal representative – nematode. It is believed that the *pel* genes were perhaps acquired through horizontal gene transfer from bacterial genes (Chen et al., 2021).

The PL1 family has a parallel β-helix topology which is similar to that of PL3 and PL9. In this topology, amino acid chains are connected in the form of a β-strand

and folded into a right-handed superhelix (Hugouvieux-Cotte-Pattat et al., 2014). The structural difference between PL1 members is the size and conformation of loops protruding from the β-helix core where the catalytic site is located (Zheng et al., 2012).

Table 2.4 Polysaccharide lyase (PL) family, basic topology, and proton abstractor of Pels.

Polysaccharide Lyase (PL) family	Basic topology	Proton abstractor
PL1	β-helix	Arg
PL2	$(\alpha/\alpha)_7$ barrel	Arg
PL3	β-helix	Lys
PL9	β-helix	Lys
PL10	$(\alpha/\alpha)_3$ barrel	Arg

2.4.3 Degradation mechanism and biochemical properties of Pel

Pels degrade pectate using the anti β-elimination mechanism which is well-studied (Figure 2.9) (Seyedarabi et al., 2010). It can be separated into a three-step process namely (i) Abstraction of hydrogen at C5 by the catalytic arginine to generate an enol intermediate, which is followed by (ii) Electron shuffling from the carboxylate group (COO^-) towards the bond between C4-C5, leading to the formation of a double bond, and (iii) Elimination of the leaving group. During degradation, calcium is shown to be important for the acidification of the C5 proton before the proton is abstracted by arginine, and it mediates the protonation of the leaving group for cleavage to happen.

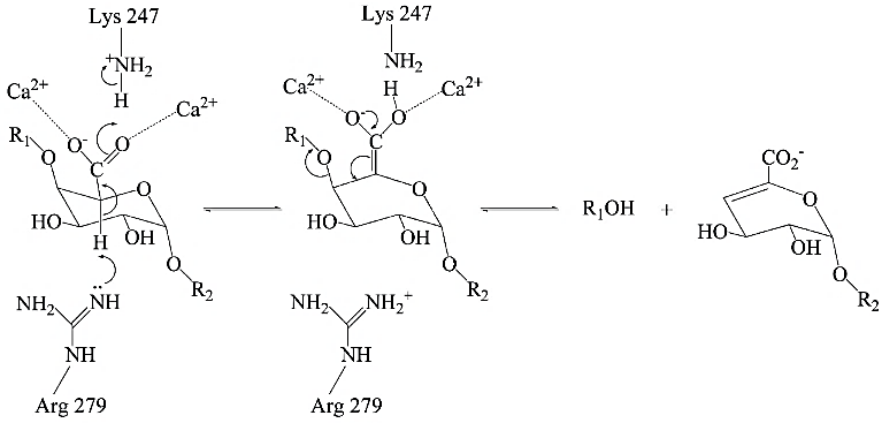

Figure 2.9 Anti β-elimination of Pel from *Bacillus subtilis* (BsPel) (Seyedarabi et al., 2010).

Most Pels require cations such as calcium for catalysis, but there are exceptions. For example, Bsp165PelA from *Bacillus* sp. N16-5 requires only a trace amount of calcium for catalysis (Zheng et al., 2012). Additionally, some Pels from PL2 such as YePL2A from *Yersinia enterocolitica* requires transition metal Co^{2+}, Mn^{2+} and Ni^{2+}, instead of Ca^{2+} (Abbott & Boraston, 2007).

Most Pels have an optimal activity at alkaline pH ranging from pH 8.0 to pH 11.5. However, a small number of Pels are acidic or neutral in their optimal pH range including – PeLI, II, III of *Aspergillus niger* MIUG 16 with pH 4.2 to 6.0 (Dinu et al., 2007), PelN of *Dickeya dadantii* with pH 7.4 (Hassan et al., 2013), and Pel-22 of *Bacillus pumilus* BS22 with pH 7.5 (Ouattara et al., 2010).

In a study by Wu et al. (2020), the optimal temperature for Pels was classified into three groups that work best at the range of equal or less than 30°C, 40°C to 60°C, and 60°C to 70°C. The first group, called cold-active Pels, have an optimal temperature of 30°C or below and are produced by microorganisms from marine or cold Antarctic environments. There are currently three cold-active Pels documented including two Pels from *Pseudoalteromonas haloplanktis* ANT/505 (Truong et al., 2001) and one from *Massilia eurypsychrophila* (Y. Tang et al., 2019). The second group is mesophilic Pels with the optimal temperature ranging from 40°C to 60°C. Most of the terrestrial Pels found are in this group. The third group is the thermo-active Pels range from 60°C to 70°C, with the highest temperature found to be 90°C from PelA of *Thermotoga maritima* (Kluskens et al., 2003) and Pel SWU of *Bacillus* sp. RN1 (Sukhumsiirchart et al., 2009).

2.4.4 Application of Pels and pectin oligosaccharides (POS)

Since the majority of Pels have an alkaline optima pH, they have practical applications in various industries such as textile, paper pulp, and plant fiber processing industry (Sharma et al., 2013). Pels are used in textile processing and cotton bioscouring to remove hydrophobic material to increase fiber wettability (P. Wu et al., 2020). For paper pulp processing, Pels are utilized for the retting of Mitsumata bast to proceed with Japanese paper making (Sharma et al., 2013). The use of Pels as an alternative to harsh chemicals in these industries has environmental benefits, along with the enzymatic approach that targets non-cellulosic material while maintaining the integrity of the raw material (Gang et al., 2010).

The degraded product of pectin, pectin oligosaccharide (POS) are non-digestible oligosaccharide (NDO) that gives numerous health benefits to the host by selectively stimulating the growth of *Bifidobacteria* and *Lactobacillus* and not pathogenic gut bacteria (Gullón et al., 2013; Holck et al., 2014). Gut microbes such as *Bacteroidetes thetaiotaomicron* can ferment POS (Luis et al., 2018), which produces short-chain fatty acids (SCFAs) that aid in the maintenance of intestinal barrier integrity, mucus production, and protection against inflammation, thus reducing the risk of colorectal cancer (Y. P. Silva et al., 2020).

CHAPTER 3

MATERIALS AND METHODS

Saccharobesus litoralis was first isolated from the algal turf at Queensbay, Penang, by a research team from the Centre for Chemical Biology (Amrina et al., 2021). The complete genome sequence revealed the existence of many CAZymes genes in *S. litoralis* including pectin-catabolic genes (Furusawa et al., 2021) which target pectin, an uronic acid that is abundant in terrestrial but not marine environments. One of the genes codes for the putative pectate lyase PelQ1, and this project aimed to better understand its characteristics and the function of its CBM13 domain.

3.1 In silico analysis

In order to predict the function and secondary structure elements of PelQ1, it was submitted to in silico analysis.

3.1.1 NCBI Standard Protein BLAST

The PelQ1 protein sequence (GenBank ID: WP_108605188) was subjected to NCBI Standard Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) for sequence alignment to search for its reference proteins to ensure that important structural features were included in the cloning sequences.

3.1.2 Check for signal peptide using SignalP

The protein sequence of PelQ1 was subjected to SignalP (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) to predict the presence of

signal peptide and cleavage site which were then excluded from the cloning process. This was to prevent the protein from getting transported out of the cytoplasmic space.

3.2 Cloning

3.2.1 High-nutrient Artificial Seawater Medium (H-ASWM) agar and broth

Broth and agar plates for H-ASWM were prepared and autoclave-sterilized according to the previous study (Furusawa et al., 2021). The compositions of the H-ASWM medium are listed in Table 3.1, and the ingredients were mixed in distilled water and adjusted to pH 7.6 using 1M NaOH before autoclaved. Different from H-ASWM agar, H-ASWM broth was supplemented with 1% glucose which could also be utilized by *S. litoralis* since it did not contain agar.

Table 3.1 Composition of H-ASWM (broth and agar). All components were mixed and autoclaved, with exception on 1% glucose which was filtered-sterilized and added into autoclaved medium.

H-ASWM broth	H-ASWM agar
2.4% (w/v) Artificial Sea Salt	
0.5% (w/v) Tryptone	
10 mM HEPES	
1% (v/v) glucose	1.5% (w/v) agar powder

3.2.2 Bacterial culture and genomic DNA extraction

S. litoralis was a gift from Dr. Go Furusawa and it was cultured according to the literature (Furusawa et al., 2021). The bacterium was streaked from a glycerol stock and onto H-ASWM agar plate, followed by incubation at 30°C for 16 to 20 hours to get the desired colony size for inoculation. Verification of contamination was done by observing the colony morphology which was pale-white, smooth, circular, and with pits that formed around the colonies due to the bacterium's ability to degrade agar, while a microscopic view showed rod-shaped cells with lateral flagella. One to two colonies were picked to ensure sufficient cells and were inoculated into 10 mL of H-ASWM broth supplemented with 1% (v/v) glucose, and incubated at 30°C for 16 hours with constant agitation of 200 rpm. A suitable volume of overnight bacterial culture was harvested and their genomic DNA was extracted using HiYield^{TM} Genomic DNA

Mini Kit according to the manual instruction (Blood/Bacteria/Cultured Cells) (Real Biotech Corporation, Taiwan).

3.2.3 DNA insert amplification with Polymerase Chain Reaction (PCR)

The homologous recombination method (Jacobus & Gross, 2015) was used in the cloning of recombinant genes. Primer pairs were designed to have gene sequences with overlapping regions of 40 bases that were complementary to the plasmid regions of pET-21a-d (+) (Novagen, Germany), as listed in Table 3.2.

The primers for PelQ1-Full and truncated PelQ1-PL1 were amplified using a high-fidelity polymerase KAPA HiFi HotStart ReadyMix PCR Kit (KAPA Biosystems, USA). The preparation of PCR reagents (Table 3.3) and cycling temperature (Table 3.4) were listed.

Table 3.2 List of customised primers. Underlined indicated the gene region and non-underlined indicated plasmid overlapping region. Start codon ATG was added.

Primer	Sequence (5'- 3')
$PelQ1_{Full}$ – Forward (T_m = 64.7°C)	AAATAATTTTGTTTAACTTTAAGAAGGAGAT ATACAT**ATG**AGCGGCTTTCACACGCAAAA
$PelQ1_{Full}$ - Reverse (T_m = 72.5°C)	CTTTGTTAGCAGCCGGATCTCAGTGGTGGTG GTGGTGGTGCTGGCGGGTTAATTCAAACA
$PelQ1_{PL1}$ – Forward (T_m = 64.7°C)	AAATAATTTTGTTTAACTTTAAGAAGGAGAT ATACAT**ATG**AGCGGCTTTCACACGCAAAA
$PelQ1_{PL1}$ – Reverse (T_m = 74°C)	CTTTGTTAGCAGCCGGATCTCAGTGGTGGTG GTGGTGGTGTCCCTTGTTAGCGCCCGCTG

Table 3.3 PCR components for PelQ1-Full and PelQ1-PL1.

Component	Final concentration
PCR-grade water	N/A
KAPA HiFi HotStart ReadyMix (2×)	1×
Forward Primer (10 μM)	0.3 μM
Reverse Primer (10 μM)	0.3 μM
Template DNA	10 – 100 ng