THE APPLICATION OF PECTINOLYTIC ENZYMES IN THE CONVERSION OF LIGNOCELLULOSIC BIOMASS TO FUEL ETHANOL

by

EMILY DECRESCENZO HENRIKSEN

(Under the Direction of Joy Doran Peterson)

ABSTRACT

The majority of ethanol is currently produced from corn; however, limited supply will require ethanol production from other sources of biomass that are rich in lignocellulose. The complexity of lignocellulose has necessitated the development of many different processes for the production of fuel ethanol from substrates containing lignocellulose, which can include physiochemical pretreatment to allow enzyme access, enzymatic saccharification to reduce substrates to fermentable sugars, and fermentation of those sugars by microorganisms. For the process to become economically feasible, inexpensive enzymes able to convert lignocellulose to fermentable sugars and ethanologenic microorganisms capable of fermenting those sugars are required. Ultimately, the most cost effective and efficient means of lignocellulose degradation will be achieved by consolidated bioprocessing, where a single microorganism is capable of producing hydrolytic enzymes and fermenting hexose and pentose sugars to ethanol at high yields. To achieve this end, ethanologen *Escherichia coli* KO11 was sequentially engineered to produce the *Klebsiella oxytoca* cellobiase and phosphotransferase genes (*casAB*) as well as a pectate lyase (*pelE*) and oligogalacturonide lyase (*ogl*) from *Erwinia chrysanthemi*, yielding strains LY40A (*casAB*), JP07C (*casAB*; *pelE*), and JP08C (*casAB; pelE; ogl*), respectively. *E. coli* JP08C produced significantly more ethanol than its parent strain, demonstrating the efficacy of the strategy. For future improvement of these technologies, new hydrolytic enzymes were sought by examining isolates from the hindgut of the aquatic, lignocellulose-degrading crane fly, *Tipula abdominalis*. A pectate lyase (*pelA*) from one isolate, *Paenibacillus amylolyticus* C27, was found while screening a genomic library in *E. coli* for pectinase activity. With its unusual activity on highly methylated pectin, PelA is useful for saccharification of sugar beet pulp, which is rich in this form of pectin. Moreover, while characterizing *P. amylolyticus* C27, production of polymyxin E_1 and E_2 was discovered, representing a novel source of these antibiotics.

INDEX WORDS: bioethanol, lignocellulose, pectin, pectate lyase, fermentation

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CHAPTER 1

INTRODUCTION

1.1 ABSTRACT

The majority of ethanol is currently produced from corn; however, limited supply will force ethanol production from other sources of biomass that are rich in lignocellulose. Lignocellulose is composed of cellulose, hemicellulose, and lignin, and can contain significant amounts of pectin. This complexity has necessitated the development of many different processes for the production of fuel ethanol from substrates containing lignocellulose, which can include physiochemical pretreatment to allow enzyme access, enzymatic saccharification to reduce substrates to fermentable sugars, and fermentation of those sugars by microorganisms. For the entire process to become economically feasible, inexpensive enzymes able to convert lignocellulose to fermentable sugars and ethanologenic microorganisms capable of fermenting those sugars are required. Ultimately, the most cost effective and efficient means of lignocellulose degradation will be achieved by consolidated bioprocessing, where a single microorganism is capable of producing hydrolytic enzymes and fermenting hexose and pentose sugars to ethanol at high yields. By examining microcosms whose functions are to degrade lignocellulose, novel enzymes and microorganisms may be discovered to meet these goals.

1.2 PURPOSE

Sixty percent of the United States crude oil is imported. The US Energy Policy Act of 2005 (http://www.ferc.gov) requires that 7.5 billion gallons of renewable fuels be incorporated into gasoline during or within the next 6 years. Ethanol is the most prevalent renewable fuel as US production exceeded 6 billion gallons in 2007 (Peterson & Ingram, 2008). Currently, the majority of ethanol is produced from corn; however, limited supply will force ethanol production

from other sources of biomass, of which the US produces 1 billion tons annually—enough to produce 80 billion gallons of renewable fuel (Figure 1.1) (Gray *et al.*, 2006).

Unlike corn, where the major component is starch, other sources of biomass are composed of 40-50% cellulose, 25-35% hemicellulose, and 15-20% lignin, with some containing significant amounts of pectin as well (Gray *et al.*, 2006). The highly complex structure of these materials has necessitated the development of many different processes for the production of fuel ethanol from substrates containing lignocellulose, which can include thermochemical pretreatment to allow enzyme access, enzymatic saccharification to reduce substrates to fermentable sugars, and finally fermentation of those sugars by microorganisms. Low-cost thermochemical pretreatments have been developed that allow enzymatic access; however, for the entire process to become economically feasible, inexpensive enzymes able to convert lignocellulose to fermentable sugars and ethanologenic microorganisms capable of fermenting those sugars are required (Eggeman & Elander, 2005). This chapter will provide an overview of relevant prior research and outline the goals to be met in order to facilitate industrial-scale bioconversion of lignocellulose to fuel ethanol.

1.3 LIGNOCELLULOSIC BIOMASS

Presently, the majority of bioethanol is derived from corn. However, with competing functions as food and feedstock, the continued use of corn as the sole source of bioethanol is not feasible. To circumvent problems associated with corn, carbon sources without competitive uses have been investigated. Abundant, sustainable, and renewable, lignocellulosic biomass provides the most plausible answer to this dilemma.

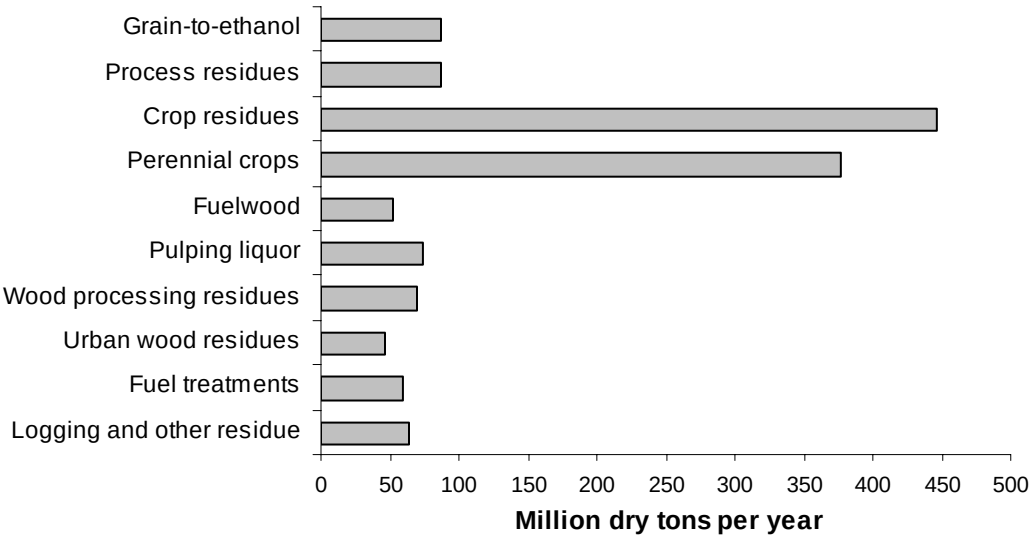

Figure 1.1 Potential forest and agricultural resources for the production of biofuels (Perlack *et al.*, 2005).

Investigation of current sources of lignocellulose revealed over 1 billon tons of biomass readily available for conversion to biofuels (Figure 1.1) (Perlack *et al.*, 2005). These sources range from dedicated crops and fuelwood to crop residues and industrial by-products. With current technology, the 1.3 billion tons of lignocellulose could be converted into enough bioethanol to supplant 30% of the United States' current energy usage for transportation. However, with a complicated and heterogeneous structure, different sources of lignocellulosic biomass will require different technologies to achieve higher efficiency.

Overall Structure of Lignocellulose. Lignocellulose is the main component of plant cell walls and provides a structural and protective barrier for the plant cells (Figure 1.2). Within the lignocellulose matrix, cellulose is the predominant homopolymer in the form of microfibril bundles and interacts with hemicelluloses, which are very chemically diverse and composed of

hexose and pentose sugars. Lignin crosslinks to these polysaccharides and is similarly heterogeneous, consisting of hydroxyphenylpropanoid units. Many sources of lignocellulose also contain pectin, which interacts with the cellulose, hemicellulose, and lignin, and is composed of galacturonic acid and other hexose and pentose pectic polysaccharides (Chang, 2007; Pauly & Keegstra, 2008).

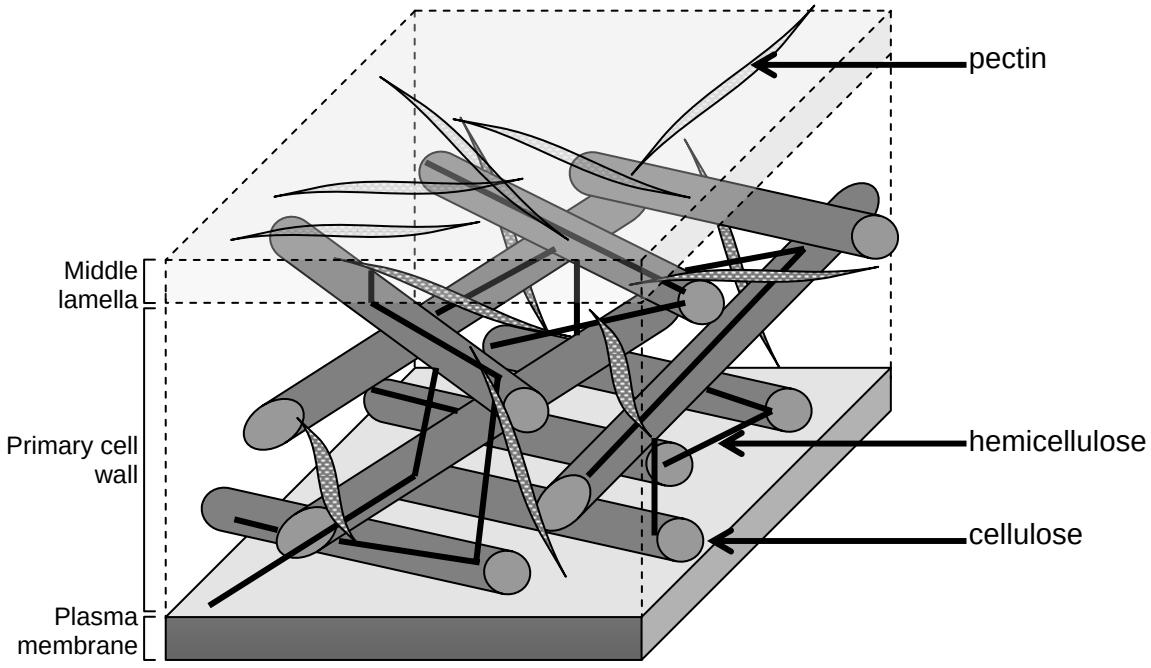

Figure 1.2 Basic structure of the plant cell wall, depicting cellulose, hemicellulose, and pectin. Lignin is omitted for clarity.

Cellulose. The most abundant biopolymer on earth and the main component of most plant cell walls, cellulose is composed of unbranched β-1,4-linked glucose monomers, with successive residues inverted 180º, forming repeating units of cellobiose (Figure 1.3) (Taylor, 2008). Primary plant cell walls are believed to have a degree of polymerization (DP) around 8000 monomers, while secondary plant cell walls have shown a DP of 14,000 to 15,000 (Brett, 2000).

Crystallization of cellulose results from extensive hydrogen bonding between parallel chains and forms insoluble microfibrils that contain approximately 36 glucan chains (Taylor, 2008).

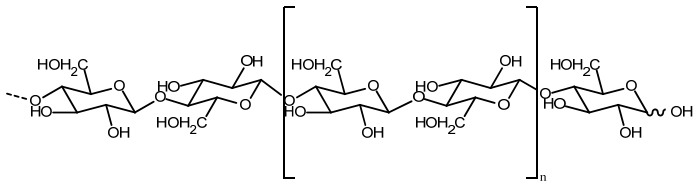

Figure 1.3 Structure of cellulose (Peterson *et al.*, 2008).

Hemicellulose. Hemicellulose is composed of heterogeneous branched and linear polysaccharides and it forms hydrogen bonds to cellulose microfibrils and is covalently bound to lignin (Chang, 2007; Shallom & Shoham, 2003). Xylan (Figure 1.4 A) is the predominant form of hemicellulose and is composed of D-xylopyranosyl linked by β-1,4-glycosidic bonds; the xylan backbone can be modified with 4-*O*-methyl-D-glucuronic acid, acetic acid, uronic acids, L-arabinofuranose, and phenolic compounds (*p*-coumaric and ferulic acids) bound to xylose subunits. β-mannans are another type of hemicellulose and can be comprised of repeating β-1,4-linked mannose or alternating mannose and glucose residues; galactomannan (Figure 1.4 B) contains α-1,6-linked galactose side chains attached to the mannose backbone (Shallom & Shoham, 2003).

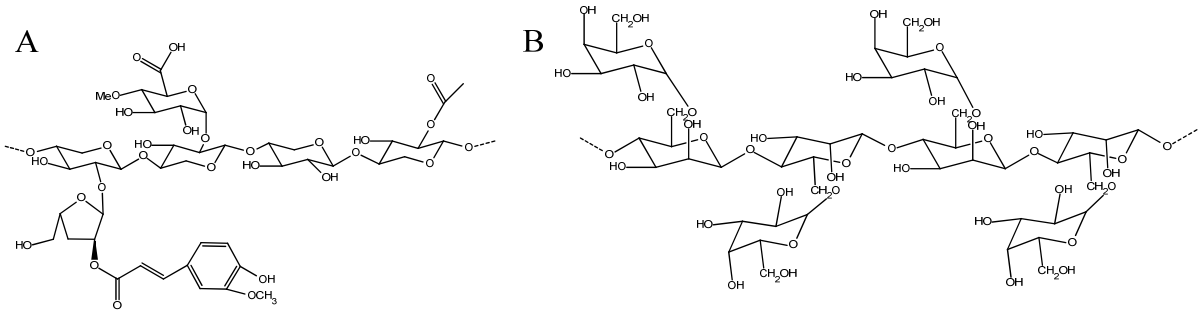

Figure 1.4 Composite structures of hemicellulose: **A)** xylan and **B)** galactomannan (Peterson *et al.*, 2008).

Lignin. Lignin is convalently bound to hemicellulose within plant cell walls and is the most recalcitrant of the biopolymers due to heterogeneity, aromaticity, and carbon-carbon crosslinking (Figure 1.6). Coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol are the monomers of lignin, and they form β- aryl ether, pheylcoumaran, resinol, biphenyl, and biphenyl ether motifs through crosslinking within the structure (Chang, 2007).

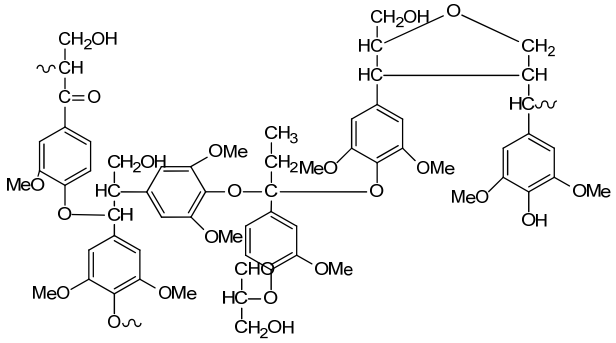

Figure 1.5 Composite structure of lignin (Chang, 2007).

Pectin. The lignocellulose complex of cellulose, hemicellulose, and lignin is associated with polysaccharides, glycoproteins, and proteoglycans, as well as other compounds and ions in the plant cell wall. The predominant macromolecule within this matrix is pectin (Figure 1.6) (Willats *et al.*, 2001). The pectin backbone can consist of a homopolymer of α-1,4-D-galacturonic acid

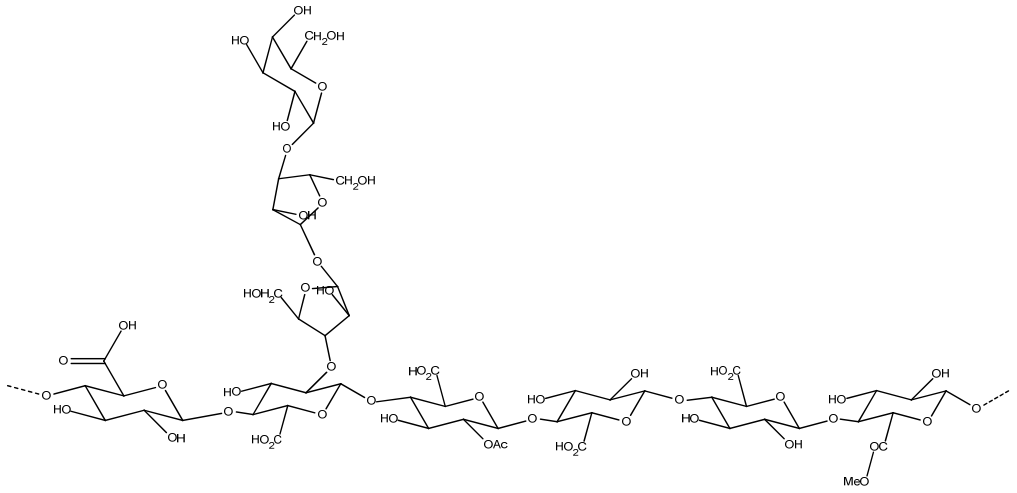

Figure 1.6 Composite structure of pectin. A homogalacturonan backbone is depicted to the right of the branch point, while a rhamnogalactuornan-I backbone is shown to the left. Typical sites of methylation and acetylation are depicted (Turner *et al.*, 2007).

(homogalacturonan) or repeats of the disaccharide α-1,2-L-rhamnose-α-1,4-D-galacturonic acid (rhamnogalacturonan-I). Typically, 70% to 80% of galacturonic acid residues are methylated at the C-6 carbon, and they can also be acetylated at the C-3 or C-2 carbons. Homogalacturonan can be substituted at the C-3 carbon with xylose and at the C-3 or C-2 carbon with apiose. Rhamnogalacturonan-I is often substituted at the C-4 carbon of rhamnose with galactose, arabinose, or galactan sugars. A third, branched pectic type, rhamnogalacturonan-II is widespread in pectin structures and consists of a homogalacturonan backbone with four

hetereopolymeric side chains that contain eleven different sugars, including apiose and aceric acid (Ridley *et al.*, 2001; Willats *et al.*, 2001).

1.4 PRETREATMENT AND SACCHARIFICATION OF LIGNOCELLULOSE

The complex structure of lignocellulose necessitates pretreatment of biomass prior to fermentation. Unlike corn and other substrates comprised mainly of starch, mechanical and/or thermochemical methods are used to breakdown the lignocellulose structure to allow access to enzymes, which are utilized to convert polymers to monomeric sugars for fermentation.

Physical and chemical pretreatments. Mechanical processes such as grinding, milling, or chipping are often employed to decrease the particle size of biomass. These methods increase the surface area of the lignocellulose and allow greater access for downstream pretreatments (Galbe & Zacchi, 2007). Another method involves gamma ray irradiation of cellulose, which decreases crystallinity by breaking the glycosidic bonds (Takacs *et al.*, 2000).

Alkaline, acid, and solvent-acid catalyst treatments are three commonly used chemical methods for breakdown of lignocellulose (Galbe & Zacchi, 2007). Treatment with base, like NaOH, causes swelling of the biomass, leading to increased surface area, decreased crystallinity and polymerization, and solubilization of large amounts of lignin and some hemicellulose. Alkaline treatment is very effective for agricultural residues and crops that contain lignin, but is generally less useful for materials that have low lignin contents.

Dilute acid pretreatment with sulfuric acid, performed between 140 to 200ºC, can solubilize hemicellulose to monomeric sugars. Some studies suggest this method releases toxic substances and inhibits fermentation; however, many procedures have been developed to relieve

this inhibition, including extraction with ether, alkali or sulfite treatment, and pre-incubation with the fungus *Trichoderma reesei*, which can degrade many fermentation inhibitors (Dien *et al.*, 2006; Llyod & Wyman, 2005; Mosier, 2005; Palmqvist & Hahn-Hagerdal, 2000; Wyman *et al.*, 2005).

A third type of method for chemical pretreatment is commonly called organosolv process—the use of a solvent (aqueous or organic) like ethanol or glycol with an inorganic acid catalyst like sulfuric or hydrochloric acid. As with dilute acid hydrolysis, lignin and hemicellulose are hydrolyzed by the acid, but they are then dissolved and recovered in the solvent phase. As these solvents are fermentation inhibitors, biomass must be thoroughly washed prior to fermentation (Galbe & Zacchi, 2007).

Physiochemical pretreatment. Physiochemical pretreatments combine mechanical and chemical methods of pretreatment. These methods include steam, hydrothermolysis (or liquid hot-water, LHW), and ammonia fiber explosion (AFEX).

Steam is one of the most common physiochemical pretreatment methods. Using high-pressure steam at 160 to 240ºC for seconds to minutes, hemicellulose is hydrolyzed, likely due to released organic acids in the lignocellulosic biomass (Brownell *et al.*, 1985; Knappert *et al.*, 1980); the method can be improved by the addition of an acid catalyst like sulfuric acid. After steam pretreatment, pentose and hexose sugars from the hemicellulose are solubilized while the cellulose remains solid, though more accessible to enzymatic degradation (Galbe & Zacchi, 2007).

LHW is very similar to steam pretreatment, but uses water at lower temperatures and lower biomass solids; an acid catalyst can be used to increase breakdown. As LHW uses more

water than steam, the released sugars tend to have a higher DP compared to those released from stream pretreatment and are in lower concentration, thus necessitating more energy input in later steps of the overall process (Bouchard *et al.*, 1991; Galbe & Zacchi, 2007).

A third physiochemical pretreatment is AFEX, which treats biomass with ammonia at temperatures below 100ºC and pressures above 3 MPa. While few monomeric sugars are released from the biomass, the lignocellulose structure is broken down to a form more accessible to enzymatic degradation (Galbe & Zacchi, 2007). A significant benefit of AFEX is that it does not appear to release many fermentation inhibitors (Sun & Cheng, 2002).

Enzymatic saccharification. The use of enzymes to saccharify lignocellulosic biomass is typically performed after other physical and/or chemical methods of pretreatment and can be accomplished prior to or in conjunction with fermentation. Pretreatment breaks down biomass to allow access to the enzymes, which can then hydrolyse the remaining cellulose, hemicellulose, and pectin polymers. Most enzymatic saccharifications are performed with commercially available cell-free extracts of fungal cultures, designed to provide predominantly cellulase, xylanase, or pectinase hydrolysis of the lignocellulose. The fungal enzymes typically have optima of 45ºC and pH 4.5, which can differ significantly from optimal fermentation conditions, especially when the ethanologen is a bacterium. The functional classes of enzymes that breakdown cellulose, hemicellulose, and pectin will be individually discussed; these classes have enzymes of both fungal and bacterial origin.

Cellulases

Cellulose degradation can occur via free, secreted enzymes or by enzyme complexes attached to the surface of microorganisms (a cellulosome) (Chang, 2007; Demain *et al.*, 2005; Newcomb & Wu, 2006). While anaerobic organisms typically possess cellulosomes, aerobic bacteria and fungi typically employ free enzymes. The degradation of cellulose is achieved through the action of three types of enzymes: endo-glucanases, cellobiohydrolases (or exo-glucanases), and β-glucosidases (Figure 1.7). Endo- and exo-glucanases cleave within or at the end of the glucan chain, respectively, and are classified based on both their structural fold and catalytic mechanism

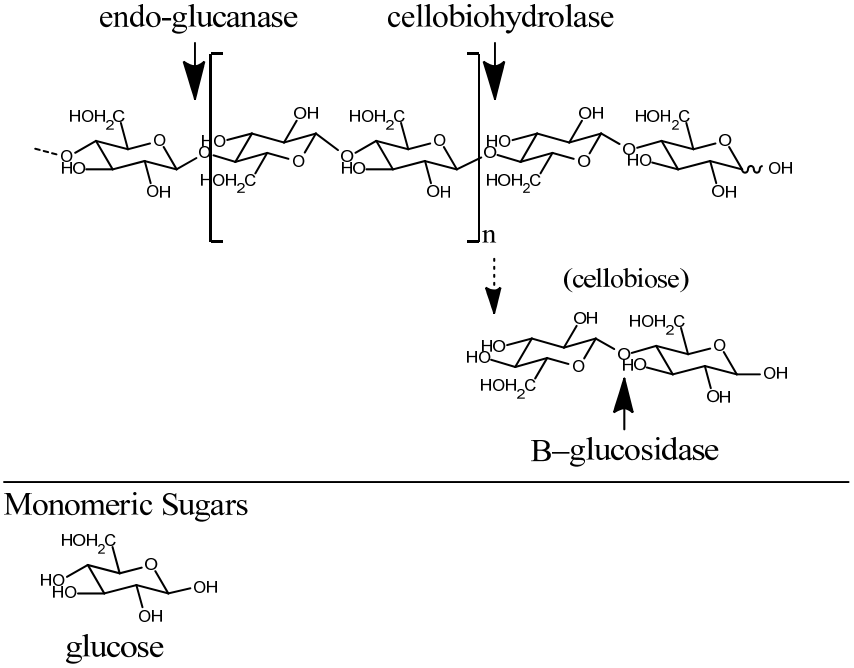

Figure 1.7 Cellulases. Sites of enzymatic cleavage are indicated by arrows. Resultant monomeric sugars are listed below the structure (Turner *et al.*, 2007).

(Henrissat & Davies, 1997). Hydrolysis of cellulose by glucanases is catalyzed by two carboxyl groups in the active site and can either invert or retain configuration of the anomeric carbon. Enzymes that retain chirality utilize a double-displacement mechanism with a covalent enzyme-

substrate intermediate while enzymes that invert chirality operate by a single-step concerted mechanism (Bayer *et al.*, 1998). β-glucosidases cleave cellobiose to monomeric glucose and are essential for overall cellulose degradation to glucose; accumulated cellobiose and/or glucose inhibit the activity of glucanases (Bayer *et al.*, 1998).

Hemicellulases

Hemicellulases are either glycoside hydrolases (GHs) or carbohydrate esterases (CEs), and are classified into families based on their activity and homology of primary sequence. GH enzymes are responsible for the hydrolysis of glycosidic bonds, while ester linked acetate and ferulic acids side chains are cleaved by CE enzymes. As the structure of hemicellulose is very heterogeneous, a wide array of enzymes is necessary for hydrolysis (Shallom & Shoham, 2003). Additionally, many hemicellulases have carbohydrate-binding modules in addition to catalytic domains; as much of the hemicellulose structure can be insoluble, the carbohydrate-binding modules are important for targeting of the enzymes to the polymers (Bourne & Henrissat, 2001).

Xylan is one major type of hemicellulose (Figure 1.8). Xylanases cleave the β-1,4 glycosidic bonds of the xylose backbone, while xylosidases hydrolyze resultant oligomers to monomeric xylose. Ferulic acid esterases and acetyl-xylan esterases cleave the ester bonds of ferulic acid and acetate side chains, respectively. Arabinofuranosidases hydrolyze arabinofuranosyl side chains from the xylose backbone and can have varying specificity as to the location of the arabinofuranosyl group. Finally, glucuronidases are responsible for the cleavage of glucuronic acid side chain α-1,2-glycosidic bonds (Shallom & Shoham, 2003).

A second form of hemicellulose is substituted β-mannan, such as galactomannan (Figure 1.9). Much like xylanases, β-mannanases are responsible for cleaving the mannose backbone to

oligomers, which are then hydrolyzed to monomeric mannose by mannosidases. Side chain moieties, like galactose, are cleaved by respective GHs, and, in this case, by α-galactosidases (Shallom & Shoham, 2003).

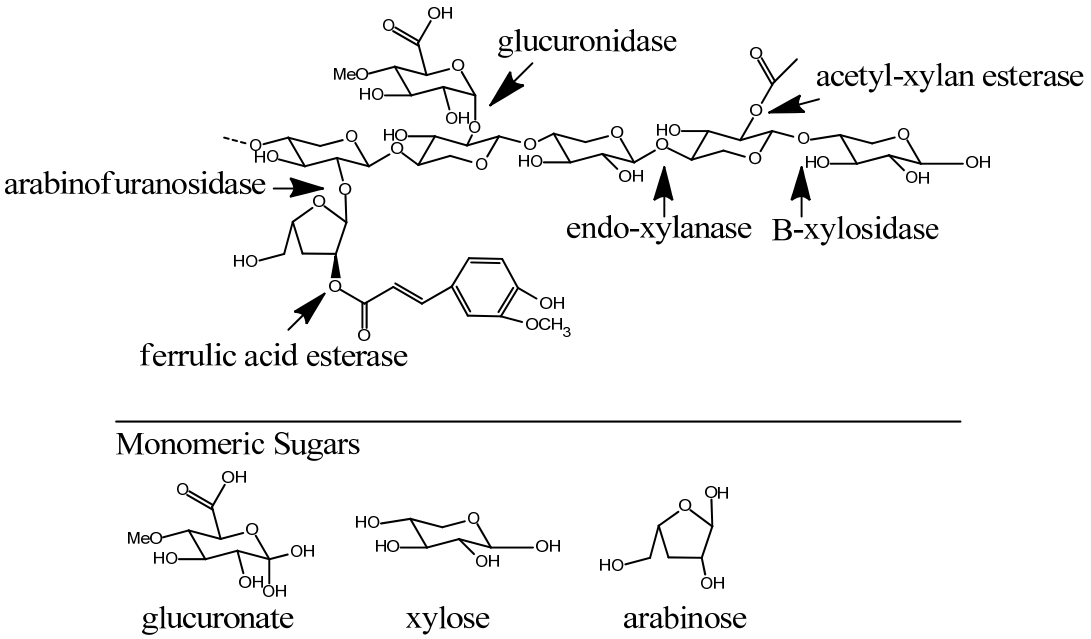

Figure 1.8 Xylanases. Sites of enzymatic cleavage are indicated by arrows. Resultant monomeric sugars are listed below the structure (Turner *et al.*, 2007).

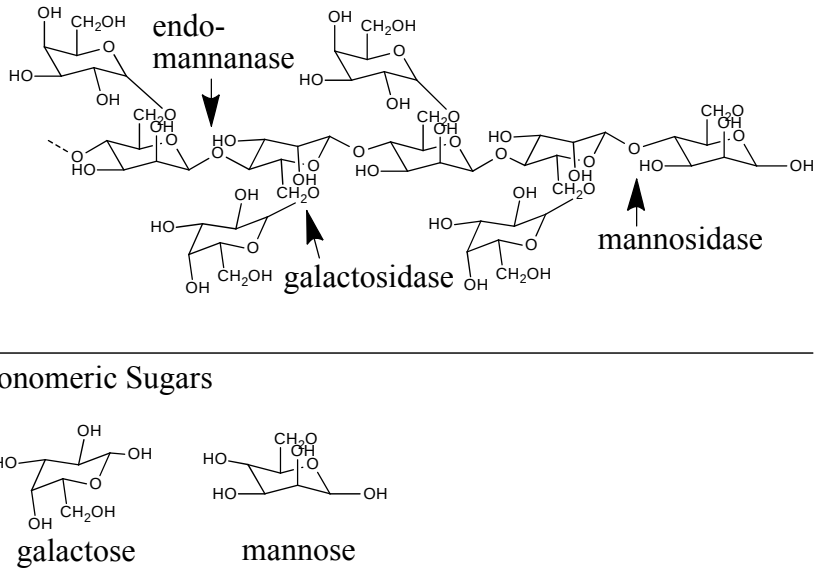

Figure 1.9 Galactomannanases. Sites of enzymatic cleavage are indicated by arrows. Resultant monomeric sugars are listed below the structure (Turner *et al.*, 2007).

Pectinases

Pectinases can be divided into three general activity groups: protopectinases, which act on insoluble pectic polymers; esterases, which de-esterify methyl and acetyl moieties from pectin; and depolymerases, which either cleave or hydrolyze glycosidic bonds with polygalacturonic acid polymers (Figure 1.10) (Jayani *et al.*, 2005). Protopectinases are usually unnecessary for degradation of lignocellulose if physical and/or chemical pretreatment methods have been employed prior to enzymatic saccharification.

Pectin methylesterases are well described in bacteria and fungi and are responsible for the hydrolysis of the ester linkages from the polygalacturonic acid backbone (Whitaker, 1984). Pectin acetylesterases, which act in the same manner as pectin methylesterases to remove acetyl groups, have been described in plants and fungi (Breton *et al.*, 1996; Searle-van Leeuwen *et al.*, 1996; Williamson, 1991); however, this type of enzyme has been found in only one bacterium,

Erwinia chrysanthemi 3937 (Shevchik & Hugouvieux-Cotte-Pattat, 1997; Shevchik & Hugouvieux-Cotte-Pattat, 2003). Pectin esterases are particularly important because many depolymerases cannot act upon methylated or acetylated pectin.

Pectin depolymerases act upon the polygalacturonate backbone and belong to one of two families: polygalacturonases or lyases. Polygalacturonases are responsible for the hydrolytic cleavage of the polygalacturonate chain, while lyases cleave by β-elimination giving a Δ 4,5-unsaturated product (Jayani *et al.*, 2005; Sakai *et al.*, 1993). For pectin polymers with a rhamnogalacturonan-I backbone, other hydrolases are also necessary; rhamnosidases hydrolyze rhamnose from the backbone, and arabinofuranosidases and galactosidases cleave arabinose and galactose, respectively, from substituted rhamnose subunits (Jayani *et al.*, 2005).

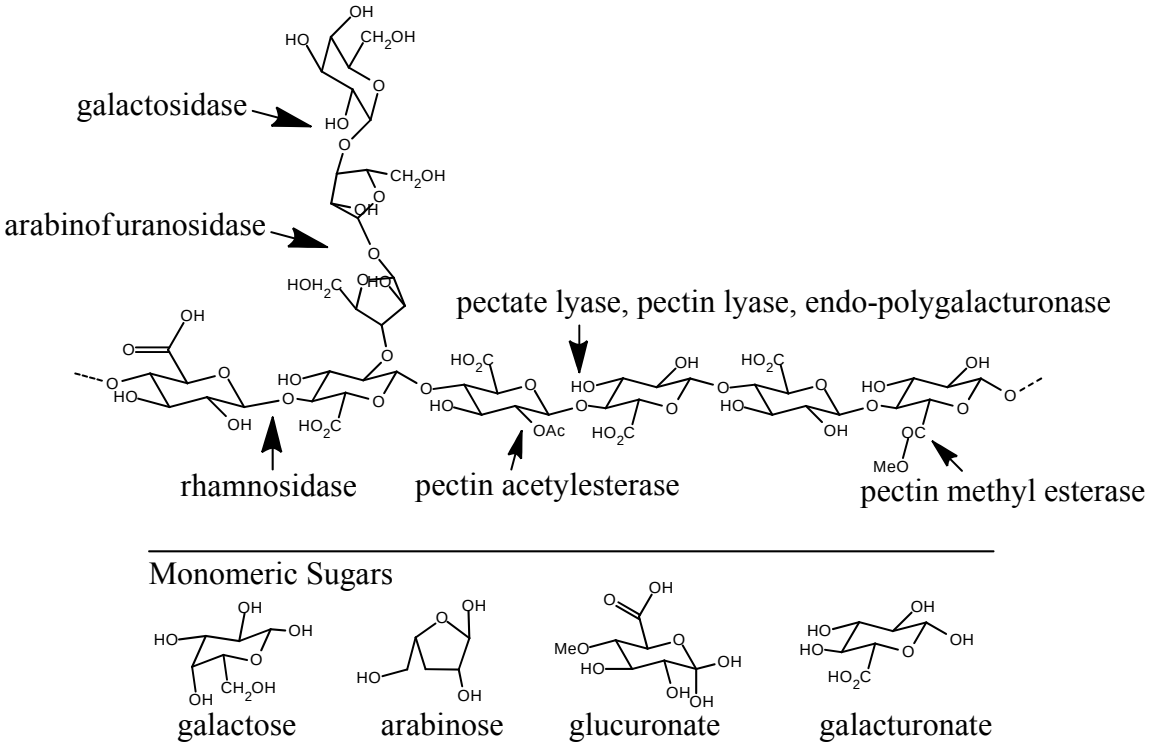

Figure 1.10 Pectinases. Sites of enzymatic cleavage are indicated by arrows. Resultant monomeric sugars are listed below the structure (Turner *et al.*, 2007).

1.5 FERMENTATION OF LIGNOCELLULOSE

After pretreatment of lignocellulosic biomass by physical and/or chemical methods and following or concurrent with enzymatic saccharification, fermentation is performed to produce ethanol. Fermentation conditions, including media, pH, temperature, and duration, are determined by the microorganism used. *Saccharomyces cerevisiae* has long been the organism of choice for industrial corn ethanol fermentations; however, with lignocellulosic biomass, there is no clear preeminent ethanologen. The following sections will discuss what is required of an ethanologen for viable lignocellulosic biomass fermentations to ethanol, as well as the advancements that have been made toward these goals.

Ethanologen requirements. When choosing a microorganism to use for fermentation of lignocellulose to ethanol, many considerations are necessary, including: ethanol yield, tolerance, and productivity; ability to ferment hexose and pentose sugars; robust growth and minimal growth requirements; resistance to inhibitors released during pretreatment; and facile heterologous gene expression and DNA manipulation (Dien *et al.*, 2003; van Zyl *et al.*, 2007). No ethanologen currently possesses all of these desired traits; however, many alterations have been made to ethanologens to design a strain with as many of these traits as possible.

Saccharomyces cerevisiae. *S. cerevisiae*, also known as Baker's yeast, has long been the workhorse for industrial bioethanol production from starchy biomass. This yeast has high ethanol productivity; tolerance to high concentrations of ethanol, saccharides, and inhibitors; and growth at low oxygen levels (Piskur *et al.*, 2006; van Zyl *et al.*, 2007). However, native *S. cerevisiae* strains are only capable of hexose utilization, while the main components of hemicellulose are

the pentose sugars xylose and arabinose; additionally, *S. cerevisiae* cannot utilize cellobiose, the major end product of cellulose degradation.

Current research with *S. cerevisiae* has focused on improving sugar utilization. Xylose isomerase from a *Piromyces* sp. was successfully engineered into *S. cerevisiae* allowing xylose fermentation (Kuyper *et al.*, 2003), as was the xylose metabolic pathway from *P. stipitis* (Kotter & Ciriacy, 1993) and the xylose isomerase from the bacterium *Thermus thermophilus (*Ho *et al.,* 1998; Ho *et al.,* 1999; Walfridsson, 1996). Likewise, arabinose fermentation has been enabled by the addition of arabinose-metabolizing genes from both fungal and bacterial sources (Richard *et al.*, 2003; Sedlak & Ho, 2001). The β-glucosidase from *Saccharomyces fibuligera* has been expressed in *S. cerevisiae* in high levels for cellobiose fermentation (van Rooyen *et al.*, 2005). Dual-fermenting strains for xylose and arabinose (Karhumaa *et al.*, 2006) as well as xylose and cellobiose (Katahira *et al.*, 2006) have also been constructed. Despite these advances in carbohydrate utilization, *S. cerevisiae* preferentially uses glucose before other sugars when co-metabolism is necessary; additionally, xylose transport into the cell competes with glucose, demonstrating specific transporters are necessary (Sedlak & Ho, 2004; van Zyl *et al.*, 2007). *S. cerevisiae* is also incapable of fermenting the uronic acid products of pectin, which are major carbohydrate sources from pectin-rich lignocellulosic biomass.

Zymomonas mobilis. The Gram-type negative bacterium, *Zymomonas mobilis*, is a particularly appealing ethanologen as it possesses a homoethanol fermentation pathway and high ethanol tolerance. In fact, *Z. mobilis* ethanol productivity and yield surpass those of *S. cerevisiae* (Rogers *et al.*, 1982), mostly due to its unique physiology. In *Zymomonas,* glucose is metabolized anaerobically through the Enter-Doudoroff pathway, yielding half as much ATP as that produced

by the glycolytic or Embden-Meyeroff-Parnas pathway. With lower ATP yields, the glucose flux is increased, fermentation enzymes are expressed constitutively, and less biomass with more fermentation products are produced (Dien *et al.*, 2003).

Unfortunately, *Z. mobilis* has a very limited carbohydrate utilization range and only ferments glucose, fructose, and sucrose. To improve sugar fermentation, strains of *Z. mobilis* have been engineered to ferment xylose, arabinose, and mannose. *Escherichia coli* xylose isomerase, xylulose kinase, transketolase, and transaldolase were engineered into *Z. mobilis* for xylose fermentation, and the strain co-fermented xylose and glucose even though the native glucose permease was utilized for import of xylose (Parker *et al.*, 1995; Zhang, 1995). An arabinose-fermenting *Z. mobilis* strain was created in the same way; five genes from *E. coli*—L-arabinose isomerase, L-ribulose kinase, L-ribulose-5-phosphate-4-epimerase, transketolase, and transaldolase—were engineered into *Z. mobilis* (Deanda *et al.*, 1996). However, the rate of arabinose fermentation was lower than that of the xylose-fermenting strain, possibly because import of arabinose through the native glucose permease was limiting (Parker *et al.*, 1995). These genes for xylose and arabinose fermentation were chromsomally integrated to produce *Z. mobilis* AX101; the strain will ferment 100% of glucose and xylose and approximately 75% of arabinose in culture with an ethanol yield of ~0.44 g ethanol g sugar^{-1} (Lawford & Rousseau, 2002). A major limitation of *Z. mobilis* AX101 and other strains is a low tolerance to inhibitors, especially acetic acid. Previous work with *Z. mobilis* 39767, the parental strain of *Z. mobilis* AX101, has demonstrated the ability to adapt strains to tolerate inhibitors (Lawford *et al.*, 1999), a necessity in the future development of this bacterium for use in lignocellulosic fermentations.

Escherichia coli. Unlike *S. cerevisiae* and *Z. mobilis*, *E. coli* produces mixed acids along with ethanol as fermentation products; it does, however, have the widest-ranging substrate utilization of any of these microorganisms, with the ability to ferment a number of pentose and hexose sugars, including uronic acid products of pectin degradation (Beall *et al.*, 1991; Doran *et al.*, 2000a; Grohmann *et al.*, 1994). In addition, it has a well-studied genetic system, prior industrial use, and minimal growth requirements; therefore, *E. coli* has many advantages for bioethanol production. However, there are several drawbacks, including a narrow growth pH range, mixed fermentation products, and public perception of danger due to pubilicity surrounding pathogenic strains of *E. coli*.

Native *E. coli* requires two NADH to make ethanol from pyruvate via pyruvate formate lyase (Figure 1.12), but only produces one NADH per pyruvate generated; to compensate for reducing power, succinic and acetic acids are also produced. The *Z. mobilis* pathway using pyruvate decarboxylase consumes a single NADH in converting pyruvate to ethanol. This pathway, including the alcohol dehydrogenase and pyruvate decarboxylase genes of *Z. mobilis*, was engineered into *E. coli* to produce ethanol, giving strain *E. coli* KO11 (Figure 1.11) (Ingram *et al.*, 1987; Ohta, 1991).

E. coli KO11 was evaluated for ethanol production from glucose and xylose as well as several biomass sources like *Pinus* wood, corn stover, sugar beet pulp, and sugarcane bagasse (Asghari *et al.*, 1996; Barbosa *et al.*, 1992; Doran *et al.*, 2000) and long-term adaptation of the strain increased ethanol tolerance by 10%, shortened fermentation duration, and increased inhibitor tolerance, giving strain LY01 (Yomano *et al.*, 1998). Another *E. coli* strain, SZ110, a

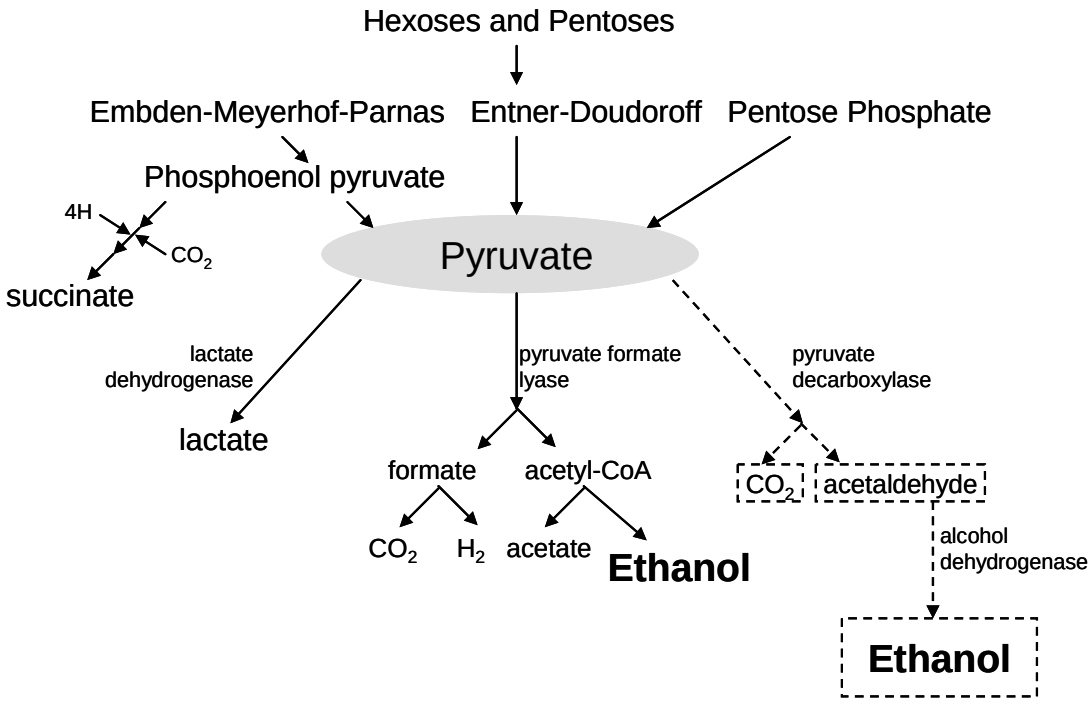

Figure 1.11 Ethanol production in *E. coli*. Native pathways are denoted by solid lines and the engineered pathway of *Z. mobilis* is denoted by dashed lines (Peterson *et al.*, 2008).

lactic acid producing derivative of KO11, was re-engineered to produce ethanol in minimal salts media, giving strain LY168 (Yomano *et al.*, 2007). *E. coli* LY168 has deletions in the lactate dehydrogenase, acetate kinase, and pyruvate formate lyase genes; the *Z. mobilis* pyruvate decarboxylase and alcohol dehydrogenase genes were inserted randomly (in LY168, the genes were located within *rrlE*, a 23S ribosomal RNA subunit) and *Pseudomonas putida* short-chain esterase was chromosomally integrated to reduce ethyl-acetate formation (Peterson & Ingram, 2008). With a theoretical yield of 0.51 g ethanol per g xylose, this strain produced 0.5 g ethanol g $xylose^{-1}$ in a minimal salts media with betaine (Yomano *et al.*, 2008).

A second approach to create a homoethanol pathway in *E. coli* has been recently described by two separate groups (Kim *et al.*, 2007; Zhou *et al.*, 2008). *E. coli* AH242, a K-12

derivative strain, is incapable of anaerobic growth due to mutations in *ldhA* and *pflB* that prevent NADH/NAD^+ cycling; Kim *et al.* (2007) chemically mutated this strain and selected derivatives capable of anaerobic growth—*E. coli* SE2378. The mutation was mapped to the pyruvate dehydrogenase (PDH) operon, which is usually expressed during oxidative growth; PDH activity was essential for anaerobic growth in SE2378 for redox balance. *E. coli* SE2378 fermented a mixture of glucose and xylose to ethanol with 82% yield (Kim *et al.*, 2007). Zhou *et al.* (2008) took a very similar approach. All competing fermentative genes were deleted from *E. coli* B, creating strain SZ420. This included lactate dehydrogenase, fumarate reductase, acetate kinase, and pyruvate formate lyase; anaerobic expression of the pyruvate dehydrogenase complex was enabled by engineering in a native transcriptional promoter expressed under anaerobic conditions. When grown on xylose and glucose, fermentation to ethanol was achieved with 90% yield (Zhou *et al.*, 2008). Both *E. coli* SE2378 and *E. coli* SZ420 demonstrate the ability to engineer a homoethanol pathway in *E. coli* without the addition of foreign genes. Further adaptation should improve yield and optimize this route of metabolic engineering, producing a nonrecombinant ethanologen.

1.6 PROCESS STRATEGIES

Many different strategies have been developed to optimize the conversion of lignocellulose to ethanol. Separate hydrolysis followed by fermentation (SHF) describes lignocellulose fermentation performed as discreet steps: mechanical and/or chemical pretreatment, enzymatic saccharification, fermentation, and, finally, distillation of ethanol. However, to lower the cost and increase efficiency, some processes have combined two or more of these steps.

Simultaneous saccharification and fermentation (SSF). SSF combines enzyme saccharification and fermentation into a single step, whereby the sugar stream created by biomass degradation is immediately consumed by the fermenting organism (Doran Peterson *et al.*, 2008). Patented in 1976 (Gauss *et al.*, 1976), the use of SSF has many benefits, including relief of enzyme inhibition by consumption of glucose and cellobiose; decreased likelihood of contamination as sugars are immediately utilized; and lower investure costs as fewer vessels are necessary (Olofsson *et al.*, 2008; Takagi *et al.*, 1977).

In SSF, mechanical and/or physiochemical pretreatments are still necessary. Enzymes, typically from a fungal source like *Trichoderma reesei* (Esterbauer *et al.*, 1991; Lynd *et al.*, 2002), are added along with the fermenting organism. The yeast, *S. cerevisiae*, has proven more suitable for SSF as its pH and temperature optima are closer to those of the enzymes, which generally have optima around pH 4.8 and 45ºC (Mandels & Sternberg, 1976). While choosing temperature and pH conditions that suit both the enzymes for saccharification and the ethanologen for fermentation are necessary to achieve the highest efficiency and lowest cost, some flexibility exists (Ingram & Doran, 1994).

Partial saccharification and co-fermentation (PSCF). PSCF combines aspects of SHF and SSF. In this process, enzyme saccharification is begun prior to fermentation under optimal conditions and allowed to proceed for a discreet period of time, usually several hours. After that time, the pH and temperature are altered to provide optimal conditions for the ethanologen, which is added to start fermentation. During fermentation, enzymes are not inhibited, but operate at reduced efficiency. As with SSF, using an ethanologen with pH and temperature optima close

to those of the enzymes utilized increases the efficiency of the process; however, PSCF is more efficient than SSF with bacterial ethanologens like *E. coli* (Doran Peterson *et al.*, 2008).

Consolidated bioprocessing (CBP). In CBP, a single microorganism would be capable of producing lignocellulose-degrading enzymes and fermenting hexose and pentose sugars to ethanol. Thus, the breakdown of the substrate and its fermentation to ethanol would occur in a single step without exogenous enzymes for saccharification. However, CBP has not been fully implemented to date. Two strategies are being employed to meet the goals of CBP: the native lignocellulolytic strategy to improve ethanol production in strains with high enzyme production and the recombinant lignocellulolytic strategy to improve enzyme production in strains with high ethanol production (Lynd *et al.*, 2005; Lynd *et al.*, 2008).

The native lignocellulolytic strategy has mostly focused on two *Clostridium* species, *C. thermocellum* and *C. cellulolyticum.* Introduction of the *Z. mobilis* pyruvate decarboxylase and alcohol dehydrogenase in *C. cellulolyticum* increased carbon flux and altered the fermentation production profile by decreasing lactate by 48% and increasing acetate and ethanol by 93% and 53%, respectively (Guedon *et al.*, 2002). *C. thermocellum* will produce ethanol to approximately 26 g L^{-1} and tolerate titers of 60 g L^{-1} (Lynd, 1996; Lynd *et al.*, 2002), indicating the potential of such cellulolytic organisms for ethanol production applications.

The recombinant lignocellulolytic strategy has focused on Gram-type negative ethanologens and the yeast *S. cerevisiae*. In addition to the engineering of carbohydrate utilization genes in some of these strains, heterologous expression of cellulases has been achieved in both *E. coli* and *Klebsiella oxytoca* using the *Erwinia chrysanthemi* endoglucanases CelZ and CelY and the *K. oxytoca casAB* operon for cellobiose utilization in *E. coli* (Lai *et al.*,

1997b; Moniruzzaman *et al.*, 1997b; Zhou *et al.*, 1999; Zhou & Ingram, 1999). Expression of cellulases and hemicellulases in *S. cerevisiae* has been accomplished using a variety of fungal and bacterial sources (van Zyl *et al.*, 2007).

CBP has the ability to increase the efficiency and lower the cost of lignocellulose fermentations to ethanol. However, organisms capable of producing the hydrolytic enzymes to degrade lignocellulosic biomass and then ferment the resultant monomeric sugars to ethanol must be developed.

1.7 DISCOVERY OF NEW ENZYMES

To engineer an ethanologen capable of CBP, hydrolytic enzymes with high specific activity and expression levels are necessary for heterologous expression. Moreover, in traditional multi-step fermentations, the enzymes used for lignocellulose degradation (cellulases, hemicellulases, and pectinases) represent a major cost of fuel ethanol production (Eggeman & Elander, 2005). Although the major commercial enzyme producers state that they have decreased the costs of cellulases, there is no low-cost source available to provide all the enzymes needed for effective conversion of lignocellulose to fermentable sugars. The investigation of environments harboring bacteria that are capable of degrading lignocellulose could provide a source of hydrolytic enzymes for both these applications.

Tipula abdominalis. A macroinvertebrate ubiquitous in stream ecosystems, *T. abdominalis* larvae degrade lignocellulosic leaf matter and thus play a central role in carbon cycling within the environment. The *T. abdominalis* larvae have a pronounced hindgut paunch where microorganisms are found within the lumen and directly attached to the gut wall (Klug &

Kotarski, 1980). Evidence suggests *T. abdominalis* larvae are incapable of tissue-level synthesis of lignocellulolytic enzymes; therefore, it is believed that the microbiota within the hindgut is responsible for the digestion of leaf lignocellulose (Lawson & Klug, 1989; Sinsabaugh *et al.*, 1985). Examination of the consortium has demonstrated high amounts of microbial diversity contained within the hindgut (Cook *et al.*, 2007). Culture-dependent methods have led to the description of 59 bacterial isolates, which were screened for lignocellulose degrading enzymes (Cook *et al.*, 2007). Five isolates, including *Paenibacillus amylolyticus* C27 and four other organisms with identical ribotypes, were able to degrade all nine model plant polymers examined (Cook *et al.*, 2007).

Paenibacillus amylolyticus. *Paenibacillus amylolyticus*, isolated from the hindgut of *T. abdominalis* (Cook *et al.*, 2007), is a Gram-type positive, spore-forming, facultative anaerobe with wide-ranging enzymatic capabilities (Ash *et al.*, 1993; Shida *et al.*, 1997). *P. amylolyticus* is believed to play a significant role in the *T. abdominalis* hindgut community. This organism has been isolated from four larvae in two states (Michigan and Georgia), both aerobically and anaerobically. *P. amylolyticus* C27 does not produce ethanol as a major fermentation product, and is thus natively ineffectual as the fermentative organism. However, as it has the exoenzymes with activities desired for a wide range of biomass conversions to fermentable products, *P. amylolyticus* C27 is an attractive candidate for biotechnology applications as a source of novel enzymes to engineer into ethanologens for CBP.

1.8 OBJECTIVES

Working towards the goal of consolidated bioprocessing, this study aims to engineer the ethanologen, *E. coli* KO11, to express cellulase and pectinase encoding genes. As pectin-rich biomass is prevalent in the United States, with sources such as sugar beet pulp and citrus peel, *E. coli* is first choice ethanologen for its ability to ferment the uronic acid products of pectin degradation (Doran *et al.*, 2000a; Doran Peterson, 2003).

In Chapter 2, known enzymes, including the *casAB* operon, which encodes a cellobiohydrolase and phosphotransferase system from *K. oxytoca* and *pelE,* which encodes a pectate lyase from *E. chrysanthemi*, were chromosomally integrated into *E. coli* KO11, to create strains LY40A and JP07C. Chapter 3 describes a novel pectate lyase, isolated from *P. amylolyticus* C27, and its applicability in fuel ethanol fermentations. In Chapter 4, a serendipitous discovery from *P. amylolyticus* C27 is described: the production of the antimicrobials polymyxin E_1 and E_2; while these antibiotics have been described previously, C27 represents a novel source for production. The Appendix describes the analysis of a second pectate lyase gene from *P. amylolyticus* with very low similarity to any other known pectate lyase.

CHAPTER 2

CHROMOSOMAL INTEGRATION OF THE *KLEBSIELLA OXYTOCA casAB* GENES FOR CELLOBIASE ACTIVITY AND THE *ERWINIA CHRYSANTHEMI pelE* AND *ogl* GENES FOR PECTATE LYASE AND OLIGOGALACTURONIDE LYASE ACTIVITY IN *ESCHERICHIA COLI* KO11[1]

[1] Henriksen, Emily DeCrescenzo, Lorraine P. Yomano, Brian C. Gardner, Lonnie O. Ingram, and Joy Doran Peterson. 2008. Chromosomal Integration of the *Klebsiella oxytoca casAB* Genes for Cellobiase Activity and the *Erwinia chrysanthemi pelE* and *ogl* Genes for Pectate Lyase and Oligogalacturonide Lyase Activity in *Escherichia coli* KO11. To be submitted to Applied and Environmental Microbiology.

2.1 ABSTRACT

Ethanologen *Escherichia coli* KO11 was sequentially engineered to produce the *Klebsiella oxytoca* cellobiase and phosphotransferase genes (*casAB*) as well as a pectate lyase (*pelE*) from *Erwinia chrysanthemi*, yielding strains LY40A (*casAB*) and JP07 (*casAB*; *pelE*), respectively. To obtain effective secretion of PelE, the Sec-independent pathway *out* genes from *E. chrysanthemi* on the cosmid pCPP2006 were provided to strain JP07 to construct strain JP07C. *E. coli* strains LY40A, JP07, and JP07C possessed significant cellobiase activity in cell lysates, while only strain JP07C demonstrated extracellular pectate lyase activity. Fermentation with sugar beet pulp at very low fungal enzyme loads during saccharification revealed significantly higher ethanol production for LY40A and JP07C compared to KO11. While JP07C ethanol yields were not considerably higher than LY40A, investigation of oligogalacturonide polymerization showed an increased breakdown of biomass to small chain (degree of polymerization $\leq$ 6) oligogalacturonides. Further engineering of *E. coli* JP07C to express Ogl, an oligogalacturonide lyase also from *E. chrysanthemi*, achieved even further breakdown of polygalacturonate to monomeric sugars and lead to higher ethanol yields.

2.2 INTRODUCTION

Sixty percent of the United States crude oil is imported. The US Energy Policy Act of 2005 (http://www.ferc.gov) will require that 7.5 billion gallons of renewable fuels be incorporated into gasoline in the next 6 years. Ethanol is the most prevalent renewable fuel with the US producing more than 6 billion gallons in 2007 (Peterson & Ingram, 2008). Currently, the majority of ethanol is produced from corn; however, limited supply will force ethanol production from other sources of biomass, of which the US produces 1 billion tons annually—enough to

produce 80 billion gallons of renewable fuel (Gray *et al.*, 2006). Moreover, use of waste biomass for fuel production positively affects greenhouse gases and carbon debt without causing land-use change (Fargione *et al.*, 2008; Searchinger *et al.*, 2008).

Unlike corn, where the major component is starch, other sources of biomass are composed of 40-50% cellulose, 25-35% hemicellulose, and 15-20% lignin (Gray *et al.*, 2006), and, in some, such as sugar beet pulp and citrus peel, pectin can also compose a significant portion of the lignocellulose structure. This highly complex structure has necessitated the development of many processes for the production of fuel ethanol from substrates containing lignocellulose, which can include thermochemical and/or mechanical pretreatment to allow enzymatic access, enzymatic degradation to reduce substrates to fermentable sugars, and finally fermentation of those sugars by microorganisms. Biomass from residues, like sugar beet pulp, does not require thermochemical or mechanical pretreatments because they are already partially processed; however, for the entire process to become economically feasible, optimization of enzymatic degradation of lignocellulose to fermentable sugars is required as is the development of ethanologens capable of fermenting those sugars. (Eggeman & Elander, 2005).

Most ethanol fermentations in the U.S. today use the yeast *Saccharomyces cerevisiae* to convert starch glucose into ethanol and CO_2; however, lignocellulosic biomass contains many hexose and pentose sugars that *S. cerevisiae* is unable to ferment (Peterson & Ingram, 2008). Thus, *Escherichia coli*, which is capable of using these hexoses and pentoses, was engineered as a biocatalyst for ethanol production by integration of the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) genes from *Zymomonas mobilis* into the chromosome of *E. coli* to generate strain K011 (Ohta *et al.*, 1991).

In this study we report improvement of *E. coli* K011 for partial saccharification and co-fermentation of lignocellulosic biomass, working towards a strain capable of achieving consolidated bioprocessing.

2.3 MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 2.1. *E. coli* strains were grown at 37ºC in Luria-Bertani (LB) medium supplemented with 2% wt/vol glucose for ethanologenic strains. When indicated, antibiotics were used at the following concentrations unless otherwise stated: chloramphenicol (Cm), 40 mg/L; ampicillin (Ap), 50 mg/L; kanamycin (Kn), 40 mg/L; erythromycin (Em), 150 mg/L; and spectinomycin (Spc), 50 mg/L. For enzyme assays, ethanologenic *E. coli* were grown in minimal media (MM) [0.02 M $(NH_4)_2SO_4$, 0.01 M $HOC(COOH)(CH_2COOH)_2$, 8 mM Na_2PO_4, 2 mM $MgSO_4 \cdot 7H_2O$, 1 mM KCl, 30 nM $FeSO_4 \cdot 7H_2O$] with 0.5% wt/vol glucose and either 0.5% wt/vol polygalacturonic acid or cellobiose. All chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Ipswich, Massachusetts). DNA sequencing reactions were performed at the Sequencing and Synthesis Facility at the University of Georgia (Athens, Georgia).

Genetic procedures and recombinant techniques. Standard methods were employed to construct plasmids and transfer DNA (Sambrook *et al.*, 1989). PCR was performed using either Platinum Taq (Invitrogen, Carlsbad, CA) or Phusion™ High-Fidelity DNA Polymerase Kit (New England BioLabs, Ipswich, Massachusetts), following the manufacturer's recommendations for reaction programs.

Chromosomal insertion of *K. oxytoca casAB* genes in *E. coli* KO11. The *casA*' gene from *K. oxytoca* was PCR amplified from pLOI1998 (Lai *et al.*, 1997) using primers LPY1 and LPY2 with a BglII restriction site engineered in before the ribosomal binding site and at the 5' end of *casA*. The *casA*' PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA), generating pLOI2085. The vector pBluescript II SK+ (Stratagene, La Jolla, CA) was digested with EcoRV and a BglII linker was ligated in, and then the plasmid was digested with HindIII. Digested pBluescript II SK+ was ligated to the BglII to AscI fragment (within *casA*) from pLOI2085 and the AscI to HindIII fragment (*casA* 3'-end, *casB*) from pLOI1998, creating pLOI2086 (*casAB*). The *lacY* and *lacA* genes were amplified from *E. coli* K011 via PCR using the *E. coli* ORFmer primer pairs and cloned into the vector pCR2.1 generating plasmids pLOI2053 (*lacY*) and pLOI2704 (*lacA*). Plasmid pLOI2086 was digested with HindIII and XhoI and treated with Klenow; pLOI2704 was digested with BamHI and XhoI and treated with Klenow; and the *lacA* gene was ligated into pLOI2086, resulting in pLOI2705 (*casAB:lacA*). Plasmids pLOI2705 and pLOI2053 were both digested with SacI and XbaI and the *lacY* gene was ligated into pLOI2705, resulting in plasmid pLOI2706 (*lacY:casAB:lacA*). Plasmid pLOI2706 was digested with KpnI and SacI and treated with T4 DNA polymerase; the resulting *lacY:casAB:lacA* fragment was ligated into the temperature conditional vector pST76-K (Posfai *et al.*, 1997) that had been digested with ClaI and SacI and treated with Klenow, resulting in plasmid pLOI2707.

To obtain a strong surrogate promoter, random *Z. mobilis* genomic Sau3A DNA fragments were ligated into the BglII site of pLOI2707 to generate a library that was screened for large colony size and dark red color on MacConkey agar plates with 2% wt/vol cellobiose. 4,000 colonies were screened and 300 colonies were retested to confirm high activity. A plasmid from one transformant that displayed the above screening characteristics was designated pLOI2708

Table 2.1. *E. coli* strains and plasmids used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics	Source or reference
Escherichia coli		
KO11	*pdc*$^+$*adhB*$^+$; Cm^r	(Ohta *et al.*, 1991)
LY40A	KO11 with *casAB*	This study
JP07	LY40A with *pelE*; Cm^r Ap^r	This study
JP07C	JP07 with pCPP2006; Cm^r Ap^r Spc^r	This study
JP08C	JP07C with pTOGL; Cm^r Ap^r Spc^r, Kn^r	This study
Plasmids		
pCPP2006	*out genes*; Spc^r	(He *et al.*, 1991)
pDMA160	pEVS107 derivative, mini-Tn7; mob; Em^r Kn^r	E.V. Stabb
pEDH24	pDMA160 derivative, consensus promoter	This study
pEDH25	pEDH24 derivative, *pelE*; Ap^r	This study
pEVS104	pRK2013 derivative; conjugal *tra* and *trb* genes	(Stabb & Ruby, 2002)
pLOI1998	*casR' AB*	(Lai *et al.*, 1997)
pLOI2053	pCR2.1 derivative, *lacY*$^+$	This study
pLOI2085	pCR2.1 derivative, *casA'*	This study
pLOI2086	pBluescript II SK+ derivative, *casAB*$^+$	This study
pLOI2090	*pelE*; Ap^r	This study
pLOI2704	pCR2.1 derivative, *lacA*$^+$	This study
pLOI2705	pLOI2085 derivative, *casAB*$^+$ *lacA*$^+$	This study
pLOI2706	pLOI2705 derivative, *lacY*$^+$ *casAB*$^+$ *lacA*$^+$	This study
pLOI2707	pST76-K derivative, *lacY*$^+$ *casAB*$^+$ *lacA*$^+$	This study
pLOI2708	pLOI2707 derivative, *lacY*$^+$ *casAB*$^+$ *lacA*$^+$, *Zm* promoter	This study
pPEL748	*pelE*	(Keen & Tamaki, 1986)
pST76-K	Ts; Cm^r	(Posfai *et al.*, 1997)
pTOGL	pCR2.1 derivative, *ogl*	This study
pUXBF13	R6K ori; *tns* genes; Ap^r	(Bao *et al.*, 1991)
Oligonucleotides		
LPY1	5′-GAGATCTTAAGGAAAAACAGCATGGA-3′	This study
LPY2	5′-ATAGCCGGCGTCCAGAAT-3′	This study
ORFmer-lacYF	5′-TTGCTCTTCCATGTACTATTTAAAAAACACAAAC -3′	Sigma Genosys
ORFmer-lacYR	5′-TTGCTCTTCGTTAAGCGACTTCATTCACCTGAC-3′	Sigma Genosys
ORFmer-lacAF	5′-TTGCTCTTCCATGCCAATGACCGAAGAATAAGAG-3′	Sigma Genosys
ORFmer-lacAR	5′-TTGCTCTTCGTTAAACTGACGATTCAACTTTATA-3′	Sigma Genosys
LacZ	5′-GGTGAAGTGCCTCTGGATGT-3′	This study
CasA	5′-CGCCTACCCGAGTGAGAATA-3′	This study
CasB	5′-GCAAAGCGGAAGTCTACCAG-3′	This study
CynX	5′-ATGCCTTCGGTGATTAAACG-3′	This study
Promoter	5′-CTAGTTGACATGATAGAAGCACTCTACTATATT-3′ 3′-AACTGTACTATCTTCGTGAGATGATATAACTAG-5′	E.V. Stabb
EDH160	5′-TGCTCAACGGGAATCCTGCTCT-3′	This study
EDH2090F	5′-GCGCATGGGCCCCACACAGGAAACAGCTATGACC-3′	This study
EDH2090R	5′-GCATGCGGGCCCGTTACCAATGCTTAATCAGTGAGG-3′	This study
EDHPelB	5′-TCAGCACGAACACGAACCGTCTTA-3′	This study
EDHPelE	5′-TGTGCTGCAAGGCGATTAAGTTGG-3′	This study
OglF	5′-GCGCACAGCTGTTGACATGATAGAAGCACTCTACTATATTCTAG AAGCGCTAAGGATTTACGGATGGC-3′	This study
OglR	5′- GAGCGCGATCGCATATGGGCACGGTTGCAGGTACAGTTATT-3′	This study

(insert size approximately 1000 bases). *E. coli* KO11 pKD46 (Datsenko & Wanner, 2000) was transformed via electroporation with the ApaI to SacI (*lacY-Zm* surrogate promoter-*casA-casB-lacA*) fragment from pLOI2708 to allow homologous recombination. Cells were grown in SOC medium with 5% wt/vol glucose at 37°C for one hour, then pelleted and resuspended in M9 minimal media with 5% cellobiose and 40 mg/L chloramphenicol. Cultures were transferred daily for 5 days, and dilutions were plated on MacConkey agar plates with 2% wt/vol cellobiose and 40 mg/L chloramphenicol. Colonies were screened for red color on MacConkey agar containing 2% wt/vol cellobiose and large colony size on LB plates with 2% wt/vol glucose and 600 mg/L chloramphenicol. The strain generated was named *E. coli* LY40A and integration was verified using custom primers (LacZ, CasA, CasB, and CynX) for the *lacZ* and *cynX* genes that flank the genomic insertion site.

Chromosomal insertion of *E. chrysanthemi pelE* gene in *E. coli* LY40A. A double-stranded *E. coli* consensus promoter sequence was constructed by heating two complementary single-stranded DNA oligos with overhangs at 98ºC for 10 min (E.V. Stabb). The oligos were cooled to room temperature, cloned into the AvrII site of pDMA160 to make pEDH24, and transformed into *E. coli* BW23474 using a standard heat shock protocol (Sambrook *et al.*, 1989). Directionality of the promoter was confirmed via DNA sequencing using the EDH160 primer. The BsrBI fragment carrying the *pelE* gene from pPEL748 (Keen & Tamaki, 1986) was inserted into the SmaI-PstI (Klenow treated) site of pUC18 to generate pLOI2090. The *pelE* and *bla* genes were amplified from pLOI2090 via PCR using primers EDH2090F and EDH2090R with engineered ApaI sites and cloned into pEDH24 at the ApaI site. Subsequent clones were investigated for directionality of the *pelE-bla* fragment, and the plasmid with *pelE-bla* in the correct orientation to the consensus promoter was named pEDH25. A triparental mating of

E. coli LY40A with *E. coli* BW23474 pUXBF13 (Bao *et al.*, 1991), *E. coli* BW23473 pEVS104 (Stabb & Ruby, 2002), and *E. coli* BW23474 pEDH25, was performed to insert the mini-Tn*7* transposon with *pelE* and *bla* into the chromosome, yielding strain *E. coli* JP07. Strain verification was accomplished by sequence analyses using primers EDHPelB and EDHPelE. Cosmid pCPP2006 (He *et al.*, 1991) was transformed into *E. coli* JP07 using standard heat shock protocol (Sambrook *et al.*, 1989), giving strain *E. coli* JP07C.

Construction of *E. coli* JP08C. To construct pTOGL, the oligogalacturonide lyase gene, *ogl*, was PCR amplified from *Erwinia chrysanthemi* 3937 using primers OglF and OglR and cloned into pCR2.1 using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). OglF contained the consensus *E. coli* promoter sequence employed in previous experiments. pTOGL was then transformed into JP07C via heat shock (Sambrook *et al.*, 1989), giving strain *E. coli* JP08C.

Cellobiase assay. Assays for cellobiase activity were performed essentially as described previously (Moniruzzaman *et al.*, 1997). Briefly, ethanologenic *E. coli* were grown in LB with 2% wt/vol cellobiose for 24 hours at 37ºC with shaking. Cells were harvested via centrifugation at 10,000 g for 10 minutes and lysed by sonication in 50 mM phosphate buffer, pH 7.2. Lysates were assayed for 15 minutes in 50 mM phosphate buffer with 2 mM *p*-nitrophenyl-β-D-1,4-glucopyranoside (PNPG). The reaction was terminated by the addition of 1 M Na_2CO_3 and ρ-nitrophenol content was measured at 410 nm. Units are defined as μmol product formed per minute per mL. Protein assays were performed on the supernatant by the Bradford method (Bradford, 1976), and enzyme activity reported as specific activity in U/mg protein. Data represents the mean of three separate experiments.

Pectate lyase assay. Assays for pectate lyase activity were performed as described previously (Collmer *et al.*, 1988). Briefly, ethanologenic *E. coli* were grown in MM with 0.5% wt/vol

glucose and 0.5% wt/vol polygalacturonic acid for 48 hours at 37ºC with shaking. Culture supernatant was harvested via centrifugation at 10,000 g for 10 minutes. Supernatant was assayed by rapidly mixing with substrate [60mM Tris-HCl, pH 7.2, 0.6 mM $CaCl_2$, 0.24% wt/vol polygalacturonic acid], both previously equilibrated to 37ºC, and monitoring the formation of 4,5-unsaturated products at 232 nm for 5 min with a linear rate of reaction for at least 30 s. Units are defined as μmol product formed per min per mL. Protein assays were performed on the supernatant by the Bradford method (Bradford, 1976), and enzyme activity reported as specific activity in U/mg protein. Data represents at least four separate experiments.

Sugar beet fermentations and analysis of ethanol production and reduced sugars. Fermentations were performed essentially as described previously (Doran *et al.*, 2000). Sugar beet pulp dry weight was calculated using a Denver Instrument IR 35 Moisture Analyzer (Denver, CO). In a blender, 10 g dry wt sugar beet pulp, 100 mL of 2X LB liquid media, and water to a final volume of 200 mL were blended at full speed for 10 s and then autoclaved in a 500 mL fleaker; blending was necessary to reduce particle size as very low fungal enzymes loads were used. The fleakers were placed in a water bath at 45°C and mixed with magnetic stirrers. The pH was adjusted to 4.5 using a Jenco 3671 pH controller (San Diego, CA). Spezyme CP (Genencorp; Copenhagen, Denmark) and pectinase from *Aspergillus niger* (Novozymes; Franklinton, NC) were added to the fleaker at concentrations of 0.5 FPU/g dry wt and 4 PGU/g dry wt, respectively. After 24 h, the pH was increased to 6.8 and the temperature was decreased to 35°C and maintained throughout the fermentation. Appropriate antibiotics were added to each fleaker, and they were inoculated to an OD_{550} 1.0 with *E. coli* strains KO11, LY40A, JP07C, or JP08C. Fermentations were run for 72 h with samples collected every 24 h.

To quantify ethanol production, gas chromatography (GC) was performed (Doran *et al.*, 2008); fermentation supernatant samples were filtered with a 0.22 μm filter prior to analysis. Ethanol concentrations were normalized to zero to account for ethanol added from antibiotic stocks. Reducing sugar analysis was performed using the dinitrosalicylic acid assay method (Miller, 1959).

Examination of oligogalacturonides. To quantify oligogalacturonides with a degree of polymerization (dp) less than 6, fermentation supernatant was diluted 1:3 in water and ethanol was added to a final concentration of 11% (vol/vol). The solution was incubated with agitation for 16 h at 4°C and then centrifuged at 7500 g for 15 min. This supernatant was diluted and analyzed at 235 nm (Spiro *et al.*, 1993). The absorbance of fermentation supernatant preparation of *E. coli* KO11 at 72 hours was used as the baseline. Data represents the average of two experiments.

2.4 RESULTS AND DISCUSSION

Construction of *E. coli* LY40A. Previous research identified cellobiose phosphoenolpyruvate-dependent phosphotransferase genes (*casAB*) from *K. oxytoca* that allowed rapid growth of *E. coli* DH5α with cellobiose as the sole carbon source (Lai *et al.*, 1997). However, when a plasmid containing *casAB* was transferred to *E. coli* K011, expression was poor; mutational studies of this plasmid in K011 suggested the native promoter was more tightly controlled in this strain (Moniruzzaman *et al.*, 1997). To create a stable, cellobiose-fermenting strain of *E. coli* K011, the *casAB* genes were inserted into the chromosome with a strong surrogate promoter.

For chromosomal insertion, the *casAB* genes were amplified from pLOI1998 (Lai *et al.*, 1997) and ultimately engineered into pLOI2707, a temperature conditional vector, with *lacY* and

lacA flanking the *casAB* genes. *Z. mobilis* genomic DNA was randomly inserted upstream of *casAB* on pLOI2707 to create a library; these clones were screened for large colony size and dark red color on MacConkey agar plates with 2% wt/vol cellobiose to find a strong surrogate promoter for cellobiose utilization. One plasmid, designated pLOI2708, which contained an insert of approximately 1 kb with a promoter, was chosen for further study. After electroporating *E. coli* K011 with pLOI2708 and selecting for *casAB* recombinants, cells were screened for red colony color on MacConkey agar containing 2% wt/vol cellobiose and LB agar containing 2% wt/vol glucose and 600 mg/L chloramphenicol to select for high expression of *casAB* and *Z. mobilis pdc* and *adhB*, respectively. The strain generated was named *E. coli* LY40A. Enzyme assays with ρ-nitrophenyl-β-D-1,4-glucopyranoside verified the absence and presence of cellobiase activity in K011 and LY40A, respectively (Table 2.2).

While chromosomal insertion of *casAB* improves *E. coli* K011 by enabling breakdown of cellobiose without supplemental cellobiase, the complexity of lignocellulosic substrates necessitates many other types of enzymes for breakdown. Further engineering of *E. coli* LY40A with additional types of enzymes should therefore enable decreased use of exogenous enzymes.

Table 2.2. Cellobiase and extracellular pectate lyase specific activity for *E. coli* KO11 and derivative strains (standard deviation; n=3)

E. coli	**Specific Activity (IU/mg protein)**	
Strain	**Cellobiase**	**Pectate Lyase**
KO11	0	0
LY40A	15.0 ± 0.4	0
JP07	15.8 ± 1.0	0.2 ± 0.3
JP07C	15.3 ± 1.1	18.9 ± 1.2
JP08C	5.4 ± 0.3	49.3 ± 0.9

Construction of *E. coli* JP07 and JP07C. In lignocellulosic substrates, pectin interacts with lignin, hemicellulose, and cellulose, and degradation of pectin is necessary to allow the disintegration of other components. Therefore, a pectate lyase, which cleaves the polygalacturonate repeating chains of pectin, was engineered into *E. coli* LY40A with a surrogate promoter.

For chromosomal integration, a mini Tn*7* system was used, which inserts as a single copy in the neutral *att* site in the *E. coli* chromosome (Bao *et al.*, 1991). *pelE* and *bla* were PCR amplified from pLOI2090 and cloned into pDMA160 with an *E. coli* consensus promoter, resulting in plasmid pEDH25. The plasmid was sequenced to verify promoter directionality and *pelE* sequence; a 61 bp deletion occurred between the promoter and *pelE*, but did not affect expression (data not shown). pEDH25 was conjugated into *E. coli* LY40A, and *pelE* transposed into the *att* site resulting in strain *E. coli* JP07.

Previous studies with *E. chrysanthemi* pectate lyases showed that a Sec-independent pathway, encoded by the *out* genes, was necessary for secretion of these enzymes (He *et al.*, 1991). A cosmid with a 40 kb fragment of the *E. chrysanthemi* genome containing the *out* genes, pCPP2006, was electroporated into strain *E. coli* JP07 to give strain *E. coli* JP07C (He *et al.*, 1991).

Enzyme assays with ρ-nitrophenyl-β-D-1,4-glucopyranoside were performed to ensure that cellobiase activity in *E. coli* JP07 and JP07C was not affected by the addition of *pelE* (Table 2.2). Subsequently, assays were performed with polygalacturonic acid to demonstrate extracellular pectate lyase activity (Table 2.2). *E. coli* KO11 and LY40A demonstrated no activity, while JP07 varied greatly, reaching, at the most, 0.5 U/mg protein; the occasional presence of activity could be attributed to cell lysis. *E. coli* JP07C, however, exhibited 18.9

U/mg protein of extracellular pectate lyase activity, demonstrating the functionality of the *out* genes secretion system.

Comparison of *E. coli* K011, LY40A, and JP07C. To demonstrate use of these engineered *E. coli* strains, sugar beet pulp fermentations were performed with very low fungal enzyme loads during pretreatment; low ethanol yields were obtained as expected (Figure 2.1A). Both *E. coli* LY40A and JP07C had significantly higher ethanol yields than *E. coli* KO11. Examination of reducing sugars demonstrates *E. coli* KO11's low yield: the high amount (140-185 μg/mL) of reducing sugars present throughout the fermentation corresponds to multimeric substrates the strain is unable to metabolize. Comparison with *E. coli* LY40A and JP07C suggests a major component of the *E. coli* KO11 reducing sugars is cellobiose, illustrating the significance of the addition of *casAB* to the strain.

Ethanol yields for *E. coli* JP07C were not significantly higher than those for LY40A. However, the concentration of reducing sugars for *E. coli* JP07C continually increased after 24 h while that of LY40A did not (Figure 2.1A). If PelE produced by *E. coli* JP07C is cleaving large polygalacturonate chains without releasing large amounts of monomeric sugars, the reducing sugar concentration would increase while ethanol production would not. To test this hypothesis, oligogalacturonides with a degree of polymerization (dp) greater than six were precipitated from the fermentation samples and the remaining oligogalacturonides with a dp of six or less were measured by absorbance at 235 nm. As seen in Figure 2.1B, the absorbance of *E. coli* JP07C is significantly higher than that of KO11 or LY40A throughout the fermentation, and, after fermentation of sugars released from the fungal enzymes, continues to increase from 24 to 72 h; this difference in absorbance corresponds to an increase of short chain oligogalcturonides throughout fermentation, demonstrating the enzymatic breakdown of polygalacturonate.

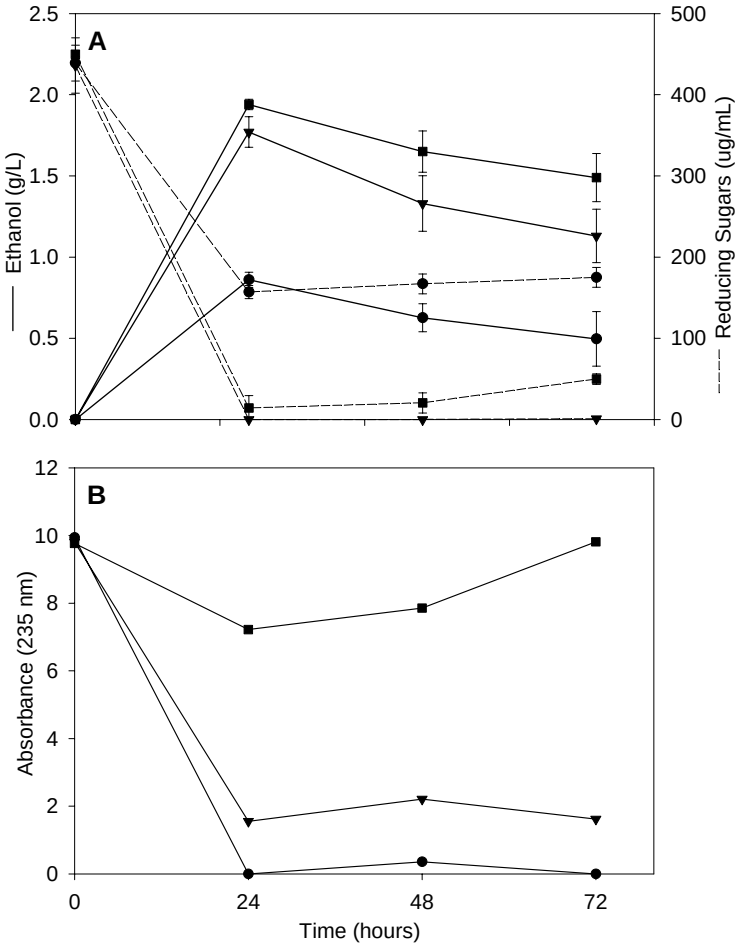

Figure 2.1. (A) Ethanol production and reducing sugars from sugar beet pulp fermentation for *E. coli* KO11, LY40A, and JP07C (standard error, n=3; solid lines indicate ethanol concentration and dashed lines represent reducing sugar concentrations) **(B)** Absorbance at 235 nm of oligogalacturonides with dp ≤ 6 from the above sugar beet pulp fermentation (data represents average of two experiments); ● K011; ▼ LY40A; ■ JP07C

Comparison of JP07C and JP08C. The oligogalacturonide lyase of *Erwinia chrysanthemi* 3937 (Collmer & Bateman, 1981) was transformed into *E. coli* JP07C to give strain JP08C, where *ogl* is maintained on plasmid pTOGL. Enzyme assays with JP08C demonstrated a large increase in the production of 4,5-unsaturated products, indicating ogligogalacturonide activity in addition to pectate lyase activity (Table 2.2). Sugar beet pulp fermentations were performed to determine if

this Ogl activity leads to higher ethanol yields than that of predecessor strains. The combination of *pelE* and *ogl* significantly increased ethanol production when compared to LY40A (Figure 2.2). Examination of reducing sugar concentrations for JP08C shows that while they are decreased in comparison to JP07C, they do continue to increase slightly throughout the fermentation; this suggests that polygalacturonic acid chains are being released from the sugar beet pulp, but are not being cleaved into di- and tri-galacturonides subject to oligogalacturonide lyase activity. As sugar beet pulp is highly methyl esterified (60%), the activities of both PelE and Ogl might be partially inhibited, and further addition of a pectin methylesterase could increase the activity of these two enzymes (Sun & Hughes, 1998).

Engineering these ethanologenic *E. coli* strains to produce lignocellulose degrading enzymes during fermentation can allow partial saccharification and co-fermentation, which enables decreased use of exogenous fungal enzymes in biomass saccharification steps, reducing the cost of the entire process. The addition of *casAB* for cellobiose utilization significantly impacts ethanol production from lignocellulosic biomass and eliminates the need for fungal cellobiases. While the addition of *pelE* did not display the same effect, secretion of pectate lyase did considerably increase degradation of polygalacturonate, and further engineering of *E. coli* JP07C to produce an oligogalaturonate lyase, *ogl*, allowed breakdown of polygalacturonate to monomeric sugars during fermentation and increased ethanol yield. Engineering of *E. coli* JP08C demonstrates the possibility of creating a strain of *E. coli* for consolidated bioprocessing, thereby eliminating the need for exogenous enzymes altogether (Lynd *et al.*, 2008). Further work to integrate cellulases, hemicellulases, and other pectinases will advance this goal of a single microorganism capable of both degradation and fermentation of lignocellulosic biomass.

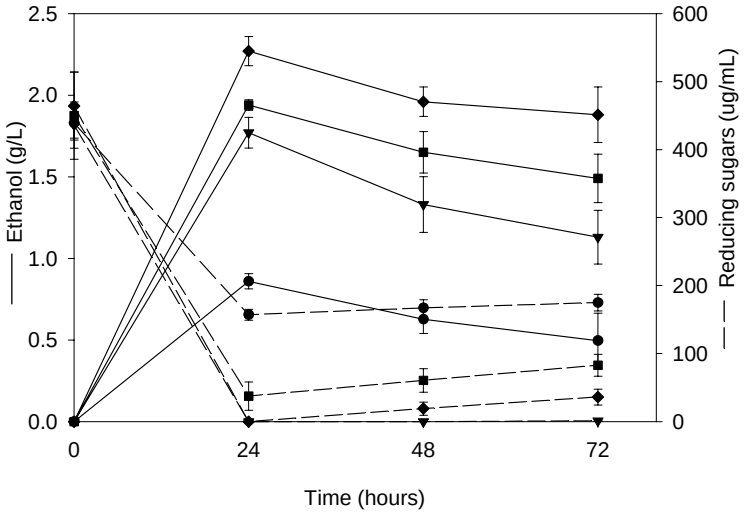

Figure 2.2. Ethanol production and reducing sugars from sugar beet pulp fermentation for *E. coli* KO11, LY40A, JP07C, and JP08C (standard error, n=3; solid lines indicate ethanol concentration and dashed lines represent reducing sugar concentrations); ● K011; ▼ LY40A; ■ JP07C; ♦ JP08C

2.5 ACKNOWLEDGEMENTS

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CHAPTER 3

CHARACTERIZATION OF A NOVEL PECTATE LYASE FROM *PAENIBACILLUS AMYLOLYTICUS* C27 WITH ACTIVITY ON HIGHLY METHYLATED PECTIN AND ITS DEGRADATION OF PECTIN-RICH LIGNOCELLULOSIC BIOMASS FOR FUEL ETHANOL PRODUCTION[1]

[1] Henriksen, Emily DeCrescenzo, Whitney E. Boland, and Joy Doran Peterson. 2008. Characterization of a novel pectate lyase from *Paenibacillus amylolyticus* C27 with activity on highly methylated pectin and its degradation of pectin-rich lignocellulosic biomass for fuel ethanol production. To be submitted to the Journal of Biotechnology.

3.1 ABSTRACT

A pectate lyase from *Paenibacillus amylolyticus* C27 was identified while screening a genomic library in *E. coli* for pectinase activity. The gene discovered, *pelA*, encodes a 222 amino acid protein. PelA demonstrated highest activity on polygalacturonic acid, but retained 60% and 56% of maximum activity on 8.5% and 90% methylated pectin, respectively. $CaCl_2$ was required for activity, and optima were pH 10.5, 45ºC, and 1.5 mM $CaCl_2$. PelA has high identity (95%) to PelA from *P. barcinonensis*, and is a subclass of the pectate lyase family 3 from saprophytic, non-pathogenic bacteria. The application of PelA in lignocellulose fermentations was examined by degradation of pectin-rich sugar beet pulp through quantification of small chain oligogalacturonides. Moreover, when *pelA* was expressed in ethanologen *E. coli* LY40A to give strain *E. coli* JP27, ethanol production nearly doubled, demonstrating the application of PelA in lignocellulose conversion to fuel ethanol.

3.2 INTRODUCTION

In the plant cell wall, the lignocellulose complex of cellulose, hemicellulose, and lignin is associated with polysaccharides, glycoproteins, and proteoglycans, as well as other compounds and ions. The predominant macromolecule within this matrix is pectin (Willats et al., 2001). The pectin backbone can consist of a homopolymer of α-1,4-D-galacturonic acid (homogalacturonan) or repeats of the disaccharide α-1,2-L-rhamnose-α-1,4-D-galacturonic acid (rhamnogalacturonan-I), and, typically, 70% to 80% of galacturonic acid residues are methylated. Homogalacturonan can be substituted with xylose or apiose, while rhamnogalacturonan-I is often substituted with galactose, arabinose, or galactan (Ridley *et al.*, 2001; Willats *et al.*, 2001).

The degradation of pectin requires both methylesterases and depolymerases. Pectin methylesterases are responsible for the hydrolysis of methylester linkages at random from the polygalacturonic acid backbone (Whitaker, 1984). Pectin depolymerases act upon the polygalacturonate backbone and belong to one of two families: polygalacturonases or lyases. Polygalacturonases are responsible for the hydrolytic cleavage of the polygalacturonate chain, while lyases cleave by β-elimination giving a Δ 4,5-unsaturated product (Jayani *et al.*, 2005; Sakai *et al.*, 1993). There are two types of lyases: pectate lyases, which cleave unesterified polygalacturonate, or pectate, and pectin lyases, which cleave methyl esterified pectin.

Pectate lyases are considered important virulence factors among phytopathogenic microorganisms (Barras *et al.*, 1994; Herron *et al.*, 2000), but have also been described in saprophytic microorganisms and thermophilic bacteria (Berensmeier *et al.*, 2004; Hatada *et al.*, 2000; Kluskens *et al.*, 2003; Nasser *et al.*, 1993). Pectate lyases have been classified into families based on amino acid similarity, which in turn suggests structural features (Coutinho & Henrissat, 1999).

Pectinases have many applications, including uses in the food and textile industries (Hoondal *et al.*, 2002; Kashyap *et al.*, 2001). Additionally, pectinases are important for the degradation of lignocellulose, where pectin can comprise a significant portion of the lignocellulose structure in many sources of biomass, including sugar beet pulp and citrus peel (Doran *et al.*, 2000; Doran Peterson, 2003). Sugar beet pulp is mainly composed of 24% cellulose, ~25% hemicellulose, and 24% pectin, with low amounts of lignin (<8%) (Doran *et al.*, 2000). As an agricultural residue, its fermentation to ethanol for fuel is renewable and sustainable.

Paenibacillus amylolyticus C27, isolated from the hindgut of the aquatic crane fly, *Tipula abdominalis*, possesses a wide range of lignocellulose-degrading enzymes, including pectinases (Cook et al., 2007). In this study, a pectate lyase from *P. amylolyticus* C27 is described. Like other pectate lyases from *Paenibacillus* and *Bacillus* spp., this enzyme displays unusual activity, combining traits of pectate and pectin lyases (Berensmeier *et al.*, 2004; Hatada *et al.*, 2000; Soriano *et al.*, 2000; Soriano *et al.*, 2006).

3.3 MATERIALS AND METHODS

Bacterial strains and plasmids. *P. amylolyticus* C27 was isolated from the hindgut of *Tipula abdominalis* (Cook *et al.*, 2007) and grown as described previously (Henriksen *et al.*, 2007) in either tryptic soy broth or Davis minimal media. Strains, plasmids, and oligonucleotides used for cloning are listed in Table 3.1. *Escherichia coli* strains were grown at 37ºC in Luria Bertani (LB) broth with 40 mg L^{-1} chloramphenicol (Cm), 50 mg L^{-1} ampicillin (Ap), or 40 mg L^{-1} kanamycin (Km), where indicated.

Library construction and enzymatic screening. Genomic DNA from *P. amylolyticus* C27 was prepared using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). After partial digestion with Sau3AI and agarose gel extraction of 2-5 kb fragments, *P. amylolyticus* C27 genomic fragments were ligated into BamHI digested pUC19, transformed into *E. coli* DH5α by heat shock (Sambrook *et al.*, 1989), and grown on LB agar with 50 mg/L ampicillin, 1 mg/L X-Gal, and 2.5 mg/L IPTG. Insert-containing transformants were screened for pectinase activity on polygalacturonase medium (Starr *et al.*, 1977). After growth, plates were flooded with 2N HCl and pectinase-producing colonies were identified by the appearance of clearing surrounding colonies.

Pectinase identification and subcloning. Plasmid pEDH13C2 from a pectinase-producing clone (13C2) was first sequenced with primer M13F followed by primer Seq2 at the Sequencing and Synthesis Facility at the University of Georgia. Primers PLAscF and PLAscR were used to

Table 3.1. Cloning strains and plasmids used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics	Source or reference
Escherichia coli		
DH5α	F⁻endA1glnV44thi-1recA1relA1gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	(Meselson & Yuan, 1968)
LY40A	*pdc*⁺*adhB*⁺*casAB* ⁺; Cm^r	(Henriksen *et al*., 2008)
JP27	LY40A with pEDH27; Cm^r Ap^r	This study
JP27O	LY40A with pEDH29; Cm^r Ap^r	This study
Plasmids		
pUC19	*lacZα*⁺; Ap^r	Invitrogen (Carlsbad, CA)
pEDH13C2	pUC19 derivative; *P. amylolyticus* DNA fragment with *pelA*	This study
pEDH27	pUC19 derivative; *pelA*⁺	This study
Oligonucleotides		
Seq2	5′-ACACTGAACGAAATGCTCCAAACC-3′	This study
PLAscF	5′-GTACAGGGCCCGGATCCTTGACATGATAGAAGCACTCTACT ATATTCTAGTGCTTCTACGGTTCTGTGGGACAA -3′	This study
PLAscR	5′-CGATCAAGCTTGGGCCCGAGCGGCCGCCTCGAGTCCACATG GTTTGGAGCATTTCG-3′	This study

subclone the pectinase gene, *pelA*, from pEDH13C2 into pUC19 using BamHI and HindIII sites, which were engineered into the forward and reverse primer, respectively, giving plasmid pEDH27. The forward primer also contained a consensus *E. coli* promoter (E.V. Stabb).

Enzyme assays. Pectate lyase assays were performed essentially as described (Collmer *et al.*, 1988; Soriano *et al.*, 2000) with *E. coli* DH5α pEDH27 cell extracts prepared by sonication. The standard enzyme assay mixture contained 0.2% (w/v) polygalacturonic acid (PGA, Sigma) or pectin (8.5% esterified citrus pectin purchased from MP Biomedicals (Irvine, CA) or 90% esterified citrus pectin purchased from Sigma (St. Louis, MO)) in a final volume of 1 mL 50 mM

glycine buffer pH 10.5 with 1.5 mM $CaCl_2$; the assay mixture and enzyme preparation were equilibrated to 45ºC and monitored for the formation of Δ-4,5-unsaturated products at 235 nm for 1 to 3 min. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol 4,5-unsaturated product per minute under the assay conditions described. Specific activity is reported as U/mg protein and the Bradford method was used to determine protein concentration of enzyme preparations (Bradford, 1976).

The pH optimum was determined at 40ºC with 1 mM $CaCl_2$ using the following buffers over the stated pH ranges: 50 mM sodium citrate, pH 3.0-4.0; 50 mM sodium acetate, pH 4.0-6.0; 50 mM sodium phosphate, pH 6.0-8.0; 50 mM Tris-HCl, pH 8.0-9.0; and 50 mM glycine, pH 9.0-12.0. The temperature optimum was determined at pH 10.5 in a range of 25-55ºC, and the $CaCl_2$ concentration optimum was determined at pH 10.5 and 45ºC in a range of 0-2.5 mM.

Degradation of sugar beet pulp and examination of oligogalacturonides. Precultures of *E. coli* carrying plasmid pEDH27 were grown in LB with 50 mg L^{-1} ampicillin overnight with shaking at 37ºC and inoculated into LB with 5% dry wt L^{-1} sugar beet pulp to OD_{550} 0.5. Sugar beet pulp cultures were grown at 37ºC with shaking and samples were removed every 24 h.

To quantify oligogalacturonides with a degree of polymerization (dp) less than 6, fermentation supernatant was diluted 1:3 in water and ethanol was added to a final concentration of 11% (vol/vol). The solution was incubated with agitation for 16 h at 4°C and then centrifuged at 7500 g for 15 min. This supernatant was diluted and analyzed at 235 nm (Spiro *et al.*, 1993) and compared to the absorbance of fermentation supernatant preparation immediately after inoculation.

Construction of *E. coli* JP27. Plasmid pEDH27 was transformed by heat shock (Sambrook *et al.*, 1989) into *E. coli* LY40A to construct strain JP27.

Sugar beet fermentations and analysis of ethanol production and reduced sugars. Fermentations were performed essentially as described previously (Doran *et al.*, 2000). Sugar beet pulp dry weight was calculated using a Denver Instrument IR 35 Moisture Analyzer (Denver, CO). In a blender, 10 g dry wt sugar beet pulp, 100 mL of 2X LB liquid media, and water to a final volume of 200 mL were blended at full speed for 10 s and then autoclaved in a 500 mL fleaker; blending was necessary to reduce particle size as very low fungal enzymes loads were used. The fleakers were placed in a water bath at 45°C and mixed with magnetic stirrers. The pH was adjusted to 4.5 using a Jenco 3671 pH controller (San Diego, CA). Spezyme CP (Genencor; Copenhagen, Denmark) and pectinase from *Aspergillus niger* (Novozymes; Franklinton, NC) were added to the fleaker at concentrations of 0.5 FPU/g dry wt and 4 PGU/g dry wt, respectively. After 24 h, the pH was increased to 6.8 and the temperature was decreased to 35°C and maintained throughout the fermentation. Appropriate antibiotics were added to each fleaker, and they were inoculated to an OD_{550} 1.0 with *E. coli* strains LY40A or JP27. Fermentations were run with samples collected every 24 h until completion.

To quantify ethanol production, gas chromatography (GC) was performed (Doran *et al.*, 2008); fermentation supernatant samples were filtered with a 0.22 µm filter prior to analysis. Ethanol concentrations were normalized to zero to account for ethanol added from antibiotic stocks. Reducing sugar analysis was performed using the dinitrosalicylic acid assay method (Miller, 1959).

3.4 RESULTS AND DISCUSSION

Cloning and identification of the pectate lyase. A library containing 2- to 5-kb chromosomal fragments of *P. amylolyticus* C27 was constructed in *E. coli* DH5α. A pectinase-positive clone,

13C2, was identified after screening approximately 4,000 clones, and sequencing of the plasmid carried in this clone, pEDH13C2, showed an insert of 2 kb. A single ORF of 669 bp was identified and named *pelA*. A putative ribosomal-binding (GAGGA) site is located eight nucleotides upstream of the ATG start codon; also upstream of *pelA* is a putative promoter with -10 (TTGTAA) and -35 (TTCTGT) elements. The deduced protein sequence of the ORF is 222 amino acids. The protein has an N-terminal region with features of a *Bacillus* signal peptide, and the most likely cleavage site is between amino acids 26 and 27 (Nielsen *et al.*, 1997).

PelA was compared to known proteins by performing a protein-protein BLAST (blastp) using the NCBI database (Altschul *et al.*, 1997). Homology was found to pectate lyases within family III (PL3), but not any other class. PelA was 95% identical to PelA from *P. barcinonensis* (Soriano *et al.*, 2000), but also showed high identity to other *Bacillus* sp. pectate lyases: 78% to *Bacillus* sp. KSM-P15 pectate lyase (Hatada *et al.*, 2000), 55% to *B. subtilis* PelC (Soriano *et al.*, 2006), 54% to *B. licheniformis* YvpA , and 53% to *Bacillus* sp. P-2850 pectate lyase. PelA has lower identity to phytopathogens *Fusarium solani* PelB (31%) (Guo *et al.*, 1995), *Erwinia chrysanthemi* PelI (15%) (Shevchik *et al.*, 1997), and *E. carotovora* Pel3 (12%) (Liu *et al.*, 1994). All of these enzymes have an arginine residue (Arg-157 in C27 PelA), which is believed to extract a proton during the β-elimination mechanism of the reaction (Akita *et al.*, 2001). Three of four signature blocks of conserved residues for PL3 enzymes (Shevchik *et al*., 1997) are found in PelA, but, like *P. barcinonensis* PelA, *Bacillus* sp. KSM-P15 PL, *B. subtilis* PelC, *B. licheniformis* YvpA, and *Bacillus* sp. P-2850 PL enzymes, the fourth block of residues is not conserved; it is replaced by another domain, not found in other pectate lyases (Figure 3.1) (Soriano *et al.*, 2006). Additionally, these enzymes have high homology to each other and lower cysteine content than other family PL3 pectate lyases. PelA appears to belong to a subgroup of

family PL3 enzymes from saprophytic bacteria (Soriano *et al.,* 2006) which includes *P. barcinonensis* PelA, *Bacillus* sp. KSM-P15 PL, *B. subtilis* PelC, *B. licheniformis* YvpA, and *Bacillus* sp. P-2850 PL.

Characterization of *P. amylolyticus* C27 PelA. SDS-PAGE analysis of *E. coli* DH5α carrying plasmid pEDH27 cell extract showed a band of approximately 23 kDa (the predicted size of PelA) not present in the extract of *E. coli* DH5α pUC19 (data not shown). These extracts exhibited pectate lyase activity on polygalacturonic acid (PGA), but did not show polygalacturonase, xylanase, or cellulase activity using dinitrosalycylic acid assays. PelA was active within a pH range of 7.5 to 11.5, with optimal activity at pH 10.5 (Figure 3.2A). The temperature optimum was 45ºC, but PelA retained at least 50% of its activity within a range of 25 to 50ºC (Figure 3.2B). $CaCl_2$ was necessary for activity, as it is for all known pectate lyases (Jurnak *et al.*, 1996), with maximum activity at 1.5 mM (Figure 3.2C). The activity of PelA on citrus pectin was also investigated. Assays with 8.5% and 90% methylesterified citrus pectin demonstrated activity at 60% and 56% of the maximum activity on PGA, respectively (Figure 3.2D).

The high activity of PelA on both PGA and pectins with low and high levels of methylation is unusual, but was also observed for PelA from *P. barcinonensis* and PelC from *B. subtilis* (Soriano *et al.*, 2000; Soriano *et al.*, 2006). Other family PL3 enzymes, like PelB and PelC from *E. chrysanthemi*, are active on PGA, but have highest activity on pectin with low levels of methylation with no activity on highly methylated pectin (Tardy *et al.*, 1997). Conversely, PelI from *E. chrysanthemi* and PelB from *E. carotovora* have highest activity on 45% and 68% methylated pectin, respectively, and low or no activity on PGA (Heikinheimo *et al.*, 1995; Shevchik *et al.*, 1998). Thus, the *P. amylolyticus* C27 PelA, *P. barcinonensis* PelA,

and *B. subtilis* PelC substrate utilization range, with activity on PGA as well as pectin with any degree of methylation, are unique among the pectate lyases described to date.

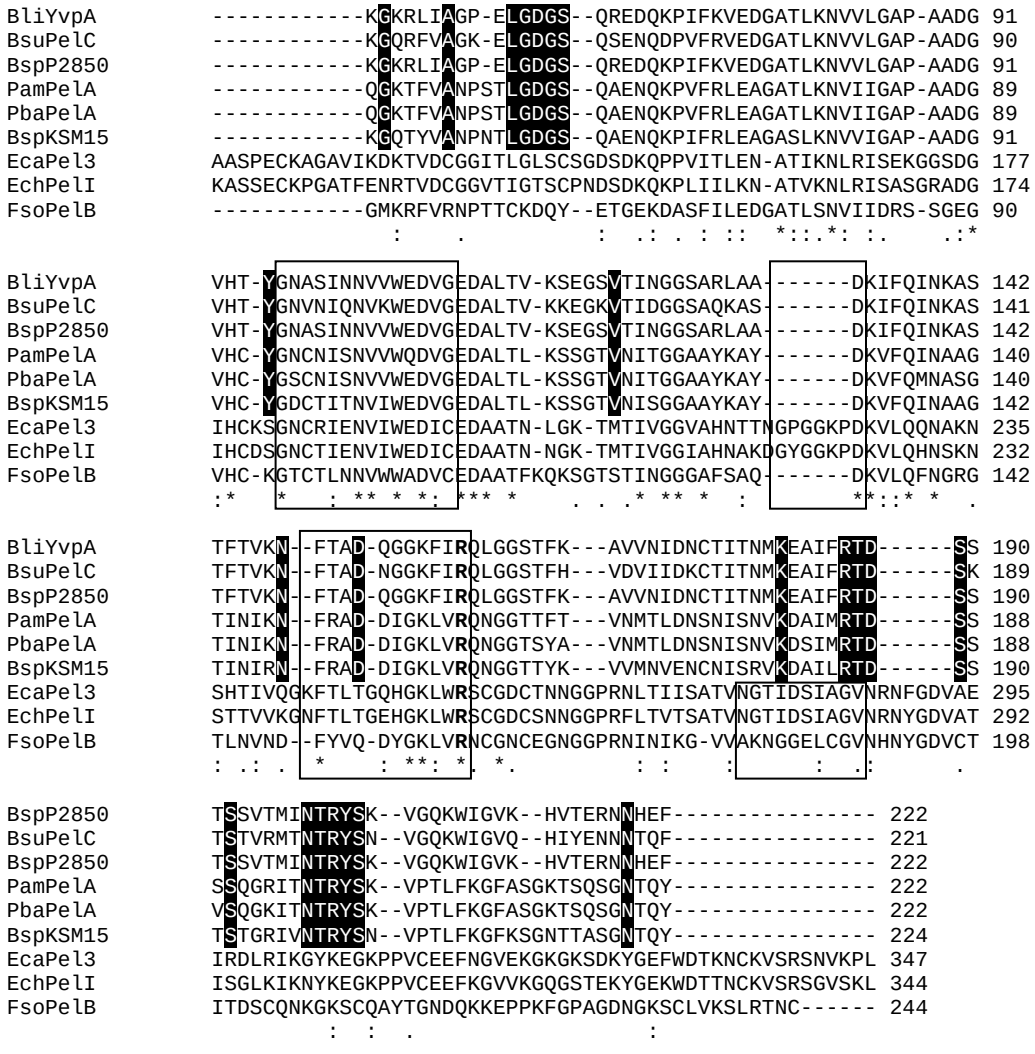

Figure 3.1. Amino acid alignment of pectate lyase class 3 enzymes (CLUSTAL W). Numbering begins at the N-termini of the proteins. Gaps are indicated by dashes. In the final line, identical amino acids are indicated by asterisks and conserved and semi-conserved residues by colons and dots, respectively. Family PL3 conserved residues are boxed. BliYvpA, *B. licheniformis* protein from gene *yvpA*; BsuPelC, *B. subtilis* pectate lyase C; BspP2850, *Bacillus* sp. P-2850 pectate lyase; PamPelA, *P. amylolyticus* pectate lyase A; PbaPelA, *P. barcinonensis* pectate lyase A; BspKSM15, *Bacillus* sp. KSM-P15 pectate lyase; EcaPel3, *E. carotovora* pectate lyase 3; EchPelI, *E. chrysanthemi* pectate lyase I; and FsoPelB, *F. solani* pectate lyase B.

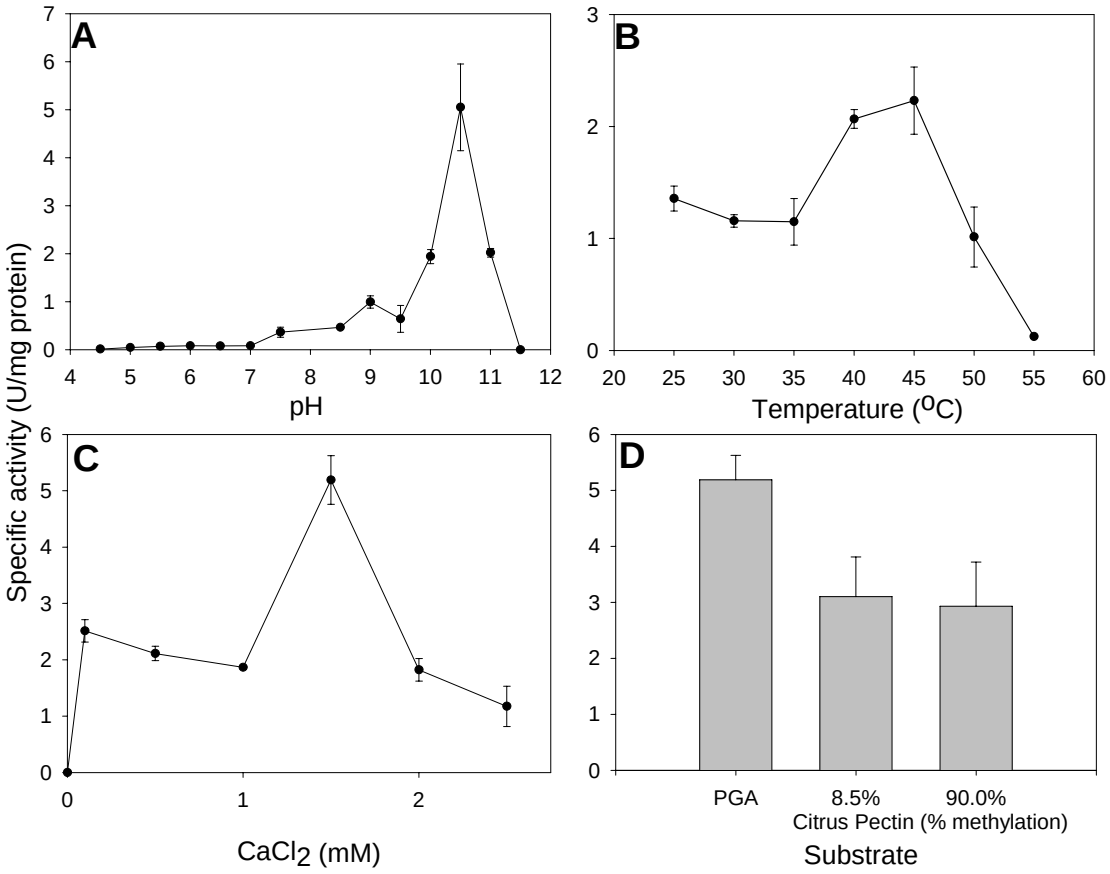

Figure 3.2. PelA optima for pH (A), temperature (B), and $CaCl_2$ (C), and activity on different pectic substrates (D).

While highly similar, *P. amylolyticus* C27 PelA, *P. barcinonensis* PelA (Soriano *et al.*, 2000), and *B. subtilis* PelC (Soriano *et al.*, 2006) do have distinct differences in activity optima and substrate preference. While the optima for C27 PelA is pH 10.5, both the *P. barcinonensis* PelA and PelC from *B. subtilis* have highest activity at pH 10 when assayed using the same method. The temperature optima differ for all three enzymes: for C27 PelA, it is 45ºC, *P. barcinonensis* PelA, 50ºC, and *B. subtilis* PelC, 65ºC. Likewise, activity on pectic substances

differs; the *B. subtilis* PelC shows highest activity on 22% methylated pectin, *P. barcinonensis* PelA on PGA or 22% methylated pectin, and C27 PelA on PGA.

Degradation of sugar beet pulp. To examine potential applications of the C27 PelA for saccharification in lignocellulose fermentations to fuel ethanol, its ability to degrade pectin in sugar beet pulp was examined. *E. coli* DH5α carrying plasmid pEDH27 was grown in LB with 5% dry wt L^{-1} sugar beet pulp and samples were taken to measure short chain oligogalacturonides that would be produced from pectate lyase activity. As shown in Figure 3.3, the amount oligogalacturonides with a degree of polymerization < 7 dramatically increased over 72 h for the strain expressing PelA, while *E. coli* DH5α pUC19 did not increase. As sugar beet pulp pectin is typically 60% methylated (Sun & Hughes, 1998), the ability of PelA to act on methylated pectin is desirable; the majority of decribed pectate lyases cannot significantly degrade pectin without added pectin methylesterase activity.

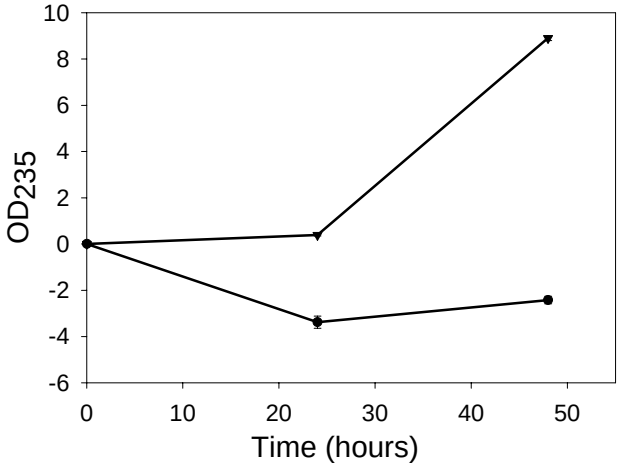

Figure 3.3. Comparison of oligogalacturonides with a dp < 7 after growth on sugar beet pulp for *E. coli* DH5α with pUC19 (●) or p13C2 (▼).

Sugar beet pulp fermentations with *E. coli* JP27. To better assess the applicability of PelA in fuel ethanol production processes, pEDH27 carrying *pelA* was added to ethanologen *E. coli* LY40A, a KO11 derivative (Henriksen *et al.*, 2008). *E. coli* LY40A and JP27 were grown in sugar beet pulp using very low fungal enzymes during saccharification; low ethanol yields were observed as expected. *E. coli* LY40A achieved a maximum ethanol yield of 1.79 g L^{-1} ethanol by 24 h (Figure 3.4). *E. coli* JP27, however, reached a maximum of 3.17 g L^{-1} ethanol by 120 h, after displaying a lag between 24 h and 48 h. Examination of reducing sugars shows that sugars liberated by fungal enzyme saccharification are consumed within 24 h for both strains. *E. coli* LY40A reaches its maximum ethanol production at 24 h because it is incapable of further lignocellulose degradation. *E. coli* JP27 also consumes the sugars released by fungal enzyme degradation by 24 h; however, after a lag (which was also observed with *E. coli* DH5α pEDH27, Figure 3.3) a small increase in the reducing sugar concentration with a concomitant increase in ethanol production is seen, demonstrating the degradation of pectin to smaller oligogalacturonides and release of fermentable sugars by PelA.

PelA is useful for many applications, including the degradation of pectin-rich biomass in partial saccharification and co-fermentation processes. Because PelA has activity on PGA and pectins with various degrees of methylation which are common in many different sources of biomass, its activity is more desirable than pectate lyases that are hindered by methylation. As demonstrated by *E. coli* JP27, nearly twice as much ethanol is produced with the addition of *pelA*. Optimimization of fermentation conditions with *E. coli* JP27 will lower cost by decreasing the amount of fungal enzymes necessary in saccharification pre-incubations.

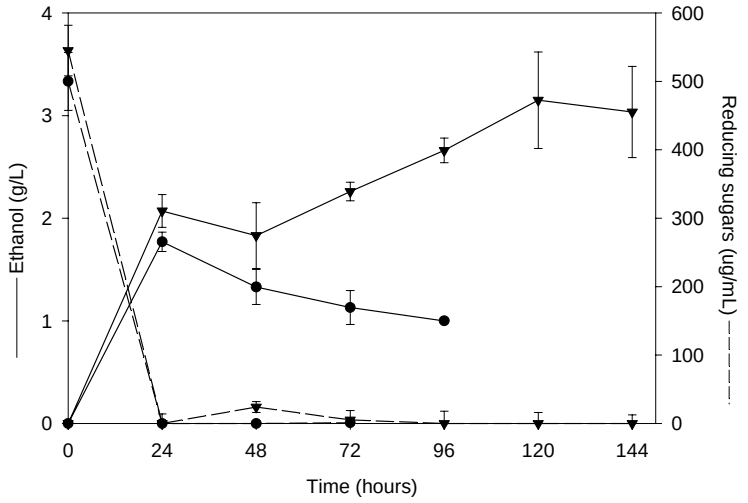

Figure 3.4. Ethanol production and reducing sugars from sugar beet pulp fermentation for *E. coli* LY40A (●) and JP27 (▼) (standard error, n=3; solid lines indicate ethanol concentration and dashed line represent reducing sugar concentrations).

3.5 ACKNOWLEDGEMENTS

We thank Dr. Debra Mohnen for assistance with oligogalacturonide quantification.

CHAPTER 4

POLYXYMIN E PRODUCTION BY *PAENIBACILLUS AMYLOLYTICUS* C27[1]

[1] Henriksen, Emily DeCrescenzo, Dennis R. Phillips, and Joy Doran Peterson. 2007. Production of Polymyxin E by *Paenibacillus amylolyticus* C27. Lett. Appl. Microbiol. 45:491-496. Reprinted here with permission of the publisher.

4.1 ABSTRACT

Aims. To investigate antibiotic production by bacteria isolated from the hindgut of *Tipula abdominalis*, the aquatic crane fly.

Methods and Results. A group of five isolates with 99.1% 16S rRNA sequence similarity to *Paenibacillus amylolyticus* were identified as antibacterial producers using the cross-streak method against both Gram positive and Gram negative bacteria. For one isolate, *P. amylolyticus* C27, biochemical tests were performed to confirm 16S rRNA identification and the antibacterials were purified using chromatographic methods. Post-source decay (PSD) mass spectroscopy (MS) was used to identify the antimicrobials, which were found to be polymyxin E_1 and E_2. Investigation of the remaining four isolates using PSD MS revealed they all produce polymyxin E_1 and E_2 as well.

Conclusions, Significance and Impact of the Study. Although variants of the polymyxin antibiotics are known to be produced by several species within the *Paenibacillus* genus, this first investigation of antibacterial production by bacteria isolated from the hindgut of *Tipula abdominalis* describes a novel source for polymyxin E production as well as the first report of antibiotic production by *P. amylolyticus*.

4.2 INTRODUCTION

As bacterial antibiotic resistance continues to increase (Theuretzbacher & Toney, 2006), habitats rich in microbial diversity merit investigation for novel antimicrobials.

Insect-microbe symbioses are ecological niches that harbor diverse microbial consortia, have high levels of species competition, and contain potentially novel organisms. Many species of insects have been shown to harbor diverse gut consortia (Breznak & Brune, 1994; Brune,

2005; Buchner, 1965; Dillon & Dillon, 2004; Kane & Pierce, 1994; Moran, 2001; Tanada & Kaya, 1993); however, insect-associated microbial consortia have been studied for less than 1% of described insect species (Kane & Mueller, 2002). Within a symbiotic environment such as an insect gut, antimicrobials may have dual purpose: associated microorganisms can produce antimicrobials to compete with other microorganisms in the consortia and to prevent colonization by insect pathogens (Dillon & Dillon, 2004).

The aquatic crane fly, *Tipula abdominalis*, is ubiquitous in stream ecosystems; the larval hindgut hosts a diverse consortia of microorganisms found within the lumen and directly attached to the gut wall (Klug & Kotarski, 1980). Fifty-nine bacterial isolates have been described and screened for antibacterial production (Cook *et al.* 2008). One group of isolates, with highest 16S rRNA sequence similarity to *Paenibacillus amylolyticus*, secreted antibacterials with broad spectrum activity against both Gram positive and Gram negative bacteria.

4.3 MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *Paenibacillus amylolyticus* C27, C25, C26, C28, and C30 were isolated from the hindgut extract of a *Tipula abdominalis* larva and grown aerobically at 28°C on tryptic soy agar (TSA) and in tryptic soy both (TSB) or Davis minimal media broth (MM) (Atlas, 1993). *Escherichia coli* ATCC 11303 was grown aerobically at 37°C on Luria-Bertani agar or in broth. *Enterococcus faecalis* ATCC 19433, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 29629, *Shigella flexneri* ATCC 9199, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, and *Streptococcus pyogenes* ATCC 19615 were grown aerobically at 37°C on TSA.

Antibacterial Screening. Antibacterial production was assessed via the cross-streak method (Abbott, 1958) with the primary streak of a *T. abdominalis* bacterial isolate on TSA for 24-48 hours incubation at 28ºC. Secondary streaks were incubated for 48 hours at 28ºC. Test organisms included: *E. coli* ATCC 11303, *S. aureus* ATCC 6538, and *B. subtilis* ATCC 6633.

Identification of *P. amylolyticus* C27. 16S rRNA gene sequencing and analysis was performed by Midi Labs, Inc. (http://www.midilabs.com) and sequence data have been submitted to GenBank databases under accession numbers AY504451-6, for isolates C25, C26, C27, C28, and C30, respectively. Biochemical assays were performed with C27 to confirm identification using standard laboratory procedures (Table 4.1) (Reva *et al.*, 2001; Shida *et al.*, 1997).

Assay for Antibacterial Activity. Antibacterial activity of the *Paenibacillus* isolates was assessed qualitatively by spread plating *E. coli* onto TSA and adding up to 50 µL of culture supernatant or antibiotic with incubation at 37°C for 12 to 16 hours. A zone of inhibition was interpreted as a positive result.

Purification of Antibacterial. *P. amylolyticus* C27 was grown aerobically on TSA at 28°C for 16 hours, and a 50 mL preculture of MM was inoculated at 28°C with shaking (250 rpm) for 16 h. 10 mL of preculture was used to inoculate 500 mL of MM at 28°C with stirring for 96 h. Cells were removed by centrifugation (10000 x g, 25 min) and filtration (Millipore ExpressPlus 0.22 µm vacuum filter). Purification of the antimicrobial was similar to that described for Polymyxin M (Martin *et al.*, 2003). Following isopropanol elution off an Amberlite XAD-16 column, two steps of reverse phase HPLC were performed using a C_{18} column (Supelco, 0.46 x 25 cm, 5 µm). All identifiable peaks were assayed for activity to determine elution time. A 16% to 50% v/v isopropanol in water (0.1% v/v TFA) gradient over 45 minutes at a flow rate of 0.5 mL min^{-1} was followed by a 50% to 90% v/v methanol in water (0.1% v/v TFA) gradient over 50

min at a flow rate of 0.5 mL min^{-1}. The antibacterials eluted at R_t= 33 and 38 min, as a small broad peak and sharp peak, respectively. Using this method, approximately 3 mg of combined antimicrobials were purified per L of culture.

Minimum inhibitory concentrations. Both *P. amylolyticus* C27 peaks with antibacterial activity from HPLC purification of 500 mL minimal media culture were combined. For analysis of polymyxin B (Sigma-Aldrich, St. Louis, MI), polymyxin E (Sigma-Aldrich, St. Louis, MI), and the *P. amylolyticus* C27 antibacterials, MIC determinations were performed according to the National Committee for Clinical Laboratory Standards (Table 4.2) (NCCLS, 2003). MIC analysis was performed in triplicate and results did not vary.

Amino acid analysis. The major active peak (R_t=38 min) was evaporated and resuspended in dH_2O at a concentration of 1 mg mL^{-1}. 10 μL was run on a Beckman amino acid analyzer at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. 10 nmol L^{-1} diaminobutyric acid was used as a standard.

Mass Spectrometric Analysis. Matrix-assisted laser desorption/ionization (MALDI) and Post Source Decay (PSD) analysis was performed on both *P. amylolyticus* C27 peaks (R_t= 33 and 38 min) from HPLC purification as well as polymyxin E (Sigma) and MM culture supernatant for the remaining five *Paenibacillus amylolyticus* isolates. The matrix used for MALDI analysis was a saturated solution of alpha-cyano-4-hydroxy cinnamic acid in 1:1:0.01 ratio of acetonitrile:water:trifluoroacetic acid. Sample was mixed with matrix solution in a 1:1 ratio on the target plate and allowed to dry at room temperature. A Bruker Daltonics Inc, Autoflex was used to acquire MALDI and PSD spectra. Ions of interest for PSD analysis were selected using a ± 10 m/z isolation window.

Fatty Acid Analysis. A mixed sample of both *P. amylolyticus* antimicrobials was lysed and methylated using 1 mol L^{-1} methanolic-HCl at 80°C for 18 hours, cooled on an ice bath, and flushed with dry nitrogen. Half-saturated sodium-chloride was added and the solution was extracted with chloroform. The chloroform layer was partitioned with water, anhydrous sodium sulphate was added to remove trace water, and the chloroform layer was dried. Sample was dissolved 1:1 in methanol:water and mixed with sinapinnic acid for MALDI analysis to detect the fatty acid methyl ester.

4.4 RESULTS

Antibacterial activity screening and identification of *P. amylolyticus* C27. Fifty-nine isolates from the hindgut of *T. abdominalis* were screened for antibacterial activity using the cross-streak method (data not shown) (Abbott, 1958). Five isolates inhibited growth of both *E. coli* and *S. aureus*. Partial 16S rDNA sequencing and BLAST homology searches in the NCBI nucleotide database revealed the antibacterial producing organisms had 99.1% identity to *Paenibacillus amylolyticus*, which is a Gram positive, spore-forming, facultative anaerobe (Ash *et al.*, 1993; Shida *et al.*, 1997); this organism has been isolated from four *T. abdominalis* insects in different states from both the hindgut lumen and wall. One strain, designated C27, was chosen for further analyses and biochemical tests were performed to confirm the species identity with comparison to other *Paenibacillus* species (Table 4.1) (Reva *et al.*, 2001; Shida *et al.*, 1997).

Assay for Antibacterial Activity. Antibacterial screens from *P. amylolyticus* C27 whole culture supernatant revealed activity against Gram negative species; cross-streak screenings with live cells exhibited inhibition of *S. aureus*. From minimal media, two compounds with an identical spectrum of antibacterial activity against Gram negative bacteria were isolated. MIC assays were

Table 4.1. Phenotypic characteristics of *P. amylolyticus* C27 and closely related organisms. Species: 1, *P. amylolyticus* C27; 2, *P. amylolyticus* (Nakamura, 1984; Shida *et al.*, 1997) ; 3, *P. pabuli* (Heyndrickx *et al.*, 1996; Nakamura, 1984; Shida *et al.*, 1997); 4, *P. illinoisensis* (Berge *et al.*, 2002; Shida *et al.*, 1997); 5, *P. polymyxa* (Claus & Berkeley, 1986; Heyndrickx *et al.*, 1996); 6, *P. popilliae* (Claus & Berkeley, 1986). v, variable, 10-89% of strains positive; w, weak; NT, not tested

Characteristic	*1*	*2*	*3*	*4*	*5*	*6*
Anaerobic growth	+	+	+	+	+	+
Catalase	+	+	+	+	+	-
Oxidase	-	-	-	-	-	-
Nitrate reduction	+	+	-	-	v	-
Acid from:						
Arabinose	+	+	+	+	+	-
Glucose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	-
Xylose	+	+	+	+	+	-
Gas from carbohydrates	-	-	-	-	+	-
Production of:						
Acetylmethylcarbinol	-	-	-	-	+	-
Indole	-	-	-	-	-	-
Hydrolysis of:						
Caesin	w	w	v	w	+	-
Starch	+	+	+	-	+	-
Use of citrate	-	-	-	-	-	-
Growth in:						
2% NaCl	+	+	+	+	+	-
5% NaCl	-	-	-	-	-	NT
0.001% lysozyme	+	-	-	v	v	+
Growth at:						
50ºC	-	-	-	+	-	-
pH 5.6	+	+	+	+	+	-

performed on purified antimicrobials and the spectrum of activity for the *P. amylolyticus* compounds was identical to polymyxin E and similar to polymyxin B (Table 4.2). All three compounds had low MIC values against the Gram negative organisms tested. MIC values were higher for Gram positive organisms, with polymyxin B more effective than both polymyxin E and *P. amylolyticus* C27 antibacterials.

Structure Determination. Initial MALDI analysis of the two active peaks from the final HPLC purification, R_t= 33 and 38 min, revealed molecular weights of 1155 and 1169 Daltons,

Table 4.2 Minimum inhibitory concentrations for Polymyxins B and E and the *P. amylolyticus* C27 antimicrobials. MICs were performed in triplicate, and identical values were obtained each time.

Test Organisms	*Polymyxin B (mg L^{-1})*	*Polymyxin E (mg L^{-1})*	*P. amylolyticus C27 (mg L^{-1})*
Gram Positive			
Enterococcus faecalis ATCC 19433	>128	>128	>128
Staphylococcus aureus ATCC 6538	32	128	128
Streptococcus pyogenes ATCC 19615	32	>128	>128
Gram Negative			
Escherichia coli ATCC 11303	2	2	2
Pseudomonas aeruginosa ATCC 27853	4	4	4
Salmonella typhimurium ATCC 29629	4	1	1
Salmonella typhimurium ATCC 29629	4	2	2

respectively. Amino acid analysis revealed the peptides are composed of leucine (Leu), threonine (Thr), with lysine and/or 2,4-diaminobutyric acid (Dab), which co-elute. Based upon the molecular weight, amino acid composition, and spectrum of activity, the compounds were suspected to be polymyxin E_1 and E_2 (1169 and 1155 Daltons, respectively) (Suzuki *et al.*, 1960; Suzuki *et al.*, 1963a; Suzuki *et al.*, 1963b; Suzuki & Fujikawa, 1964; Suzuki *et al.*, 1965). To confirm the antibacterials' identifications, Post source decay (PSD) mass spectrometry was employed on *m/z* 1170 (MH^+) and 1156 (MH^+) ions for both commercial polymyxin E (data not shown) and the *P. amylolyticus* antibacterials (Figure 4.1, A and B); identical fragmentation patterns were obtained. And, while only accurate to one *m/z*, fragmentation patterns are similar to those obtained by previous mass spectrometric studies (Govaerts *et al.*, 2002; Govaerts *et al.*, 2003).

To confirm the fatty acid moiety of the *P. amylolyticus* compounds, fatty acid methyl esters were examined using MALDI-TOF ion scanning. Using this method, the 6-methyloctanoic acid of the more abundant polymyxin E_1 was detected.

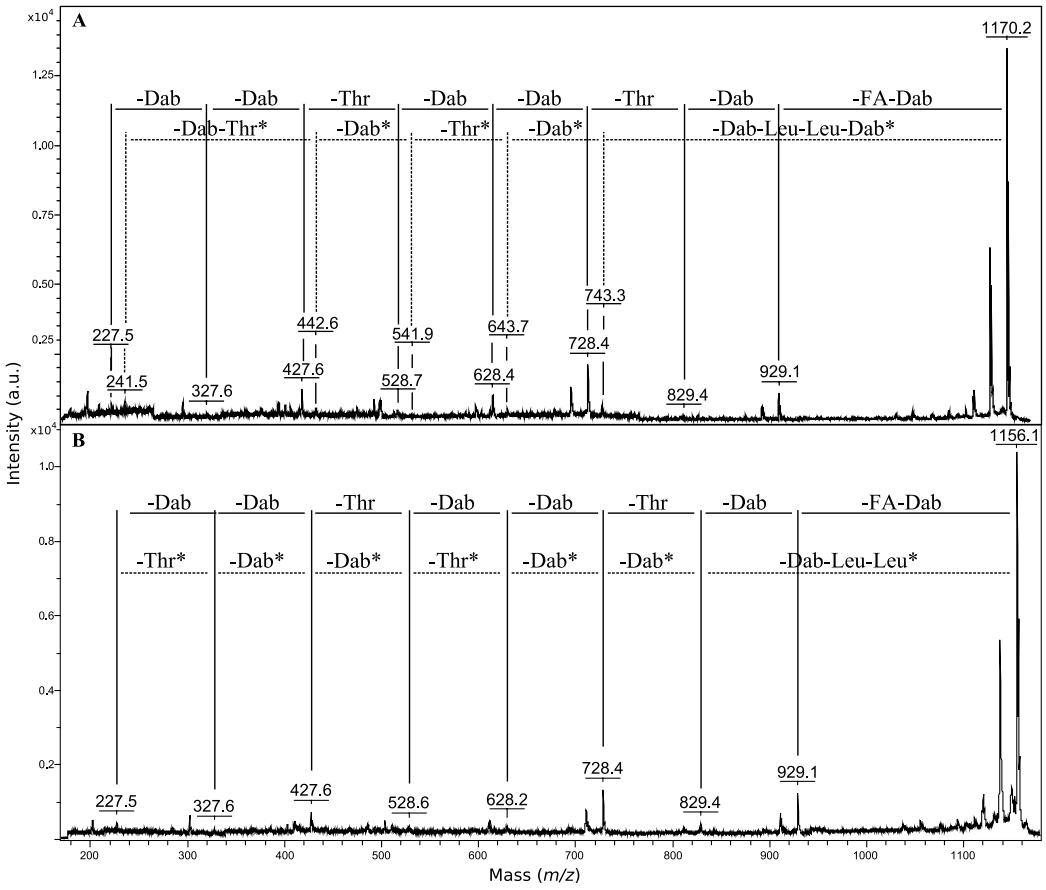

Figure 4.1. Post-source decay of *P. amylolyticus* C27 antimicrobials. For the antimicrobial R_t=38 minutes (1170 *m/z* [MH^+]) (A) the first fragmentation series is denoted by solid lines and begins when the terminal fatty acid moiety (6-methyloctanoyl) and Dab are lost, resulting in *m/z* 929.8 ion; fragmentation then continues sequentially. The second series, denoted by dotted lines, begins with fragmentation on the cyclic peptide portion of the molecule with loss of Dab-Leu-Leu-Dab, followed by sequential fragmentation. For the antimicrobial R_t=33 minutes (1156 *m/z* [MH^+]) (B), the first and second fragmentation series, which are denoted by solid and dotted lines, respectively, are difficult to distinguish as the expected *m/z* ions for both series in within one *m/z*; therefore, the *m/z* ions obtained in the PSD spectra can correlate to species in both the first and second series. Fragmentation occurs in the same manner as the first antimicrobial. An asterisk denotes *m/z* ions of the second series.

Screening of *P. amyolyticus* isolates for polymyxin E production. *P. amyloyticus* isolates C25, C26**,** C28, and C30 were examined for production of polymyxins E_1 and E_2 using MALDI analysis and qualitative antibacterial assays. Presence of a peak at 1170 (MH^+) and/or 1156 (MH^+) *m/z* in the MALDI spectrum and antibacterial activity identical to polymyxin E indicated

the presence of the antimicrobial(s). All of the isolates were positive for the production of both polymyxin E_1 and E_2 (data not shown).

4.5 DISCUSSION

Many species within the genus *Paenibacillus* produce variants of the peptide antimicrobial polymyxin, whose general structure consists of a decapeptide with a terminal fatty acid moiety (Martin *et al.*, 2003; Storm *et al.*, 1977). Polymyxins differ in amino acid and fatty acid composition and are formed by condensation reactions in the cytoplasm, directed by peptide synthases (Marahiel *et al.*, 1997). Although toxicity limited its medical applications during the past 50 years, the emergence of drug-resistant pathogens has caused a resurgence of clinical use (Li *et al.*, 2006; Markou *et al.*, 2003).

P. amylolyticus isolate C27 produces polymyxins E_1 and E_2 (colistin A and B), representing a novel source for production of these antimicrobials (Suzuki *et al.*, 1960; Suzuki *et al.*, 1963a; Suzuki *et al.*, 1963b; Suzuki & Fujikawa, 1964; Suzuki *et al.*, 1965) as well as the first description of antibiotic production for this *Paenibacillus* species. Using a three-step purification protocol, polymyxin E_1 and E_2 were separated and identified through comparison to commercial polymyxin E (composed of polymyxins E_1 and E_2). Fragmentation patterns observed for the compounds were nearly identical to previous studies, within the mass accuracy of PSD (Govaerts *et al.*, 2002; Govaerts *et al.*, 2003)). In Figure 4.2, A and B, a proposed mechanism of fragmentation for polymyxin E is outlined, but others are possible.

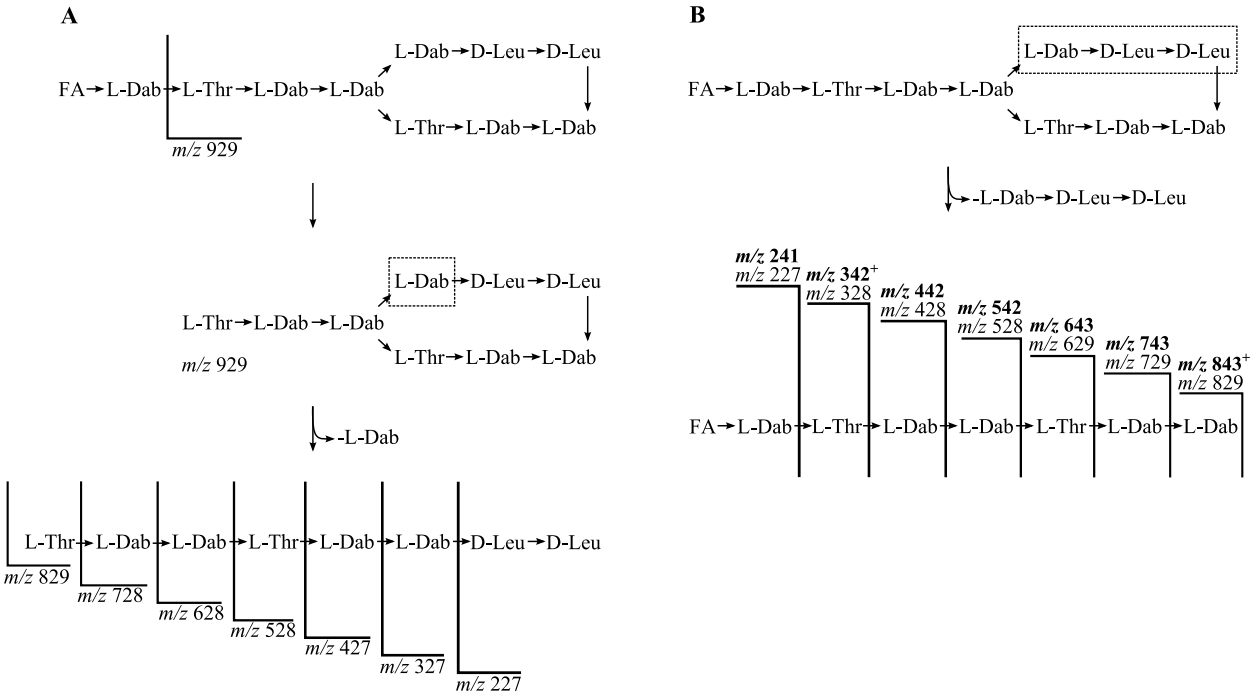

Figure 4.2. Proposed mechanism of MALDI fragmentation for polymyxin E_1 and E_2. A shows the primary route of fragmentation for the first series, as observed by PSD spectra (Figure 4.1, A and B), and B shows the primary route of fragmentation for the second series, as observed by PSD (Figure 4.1, A and B), with *m/z* values in bold and plain font for polymyxin E_1 and E_2, respectively. $^{+}$Fragments were not observed in PSD experiments.

Although fatty acid methyl ester (FAME) analysis only detected 6-methyloctanoic acid from *P. amylolyticus* polymyxin E_1, the PSD spectra for both polymyxin E_1 and E_2 exhibit fragments characteristic of their respective fatty acid moieties. MICs were identical for commercial and *P. amylolyticus* antibiotics, although assays with live cells suggest *P. amylolyticus* C27 may produce other antibacterial compounds with activity against Gram positive bacteria.

Bacterial symbionts of insects that produce polymyxin antibiotics in culture have been isolated previously. A yeast isolated from eggs of the brown planthopper *Nilaparvata lugens* was found to produce polymyxin M_1 (Jigami *et al.*, 1986), and a *Bacillus polymyxa* strain isolated from the oriental stinkbug *Plautia stali* produced polymyxin E_1 (Kenny *et al.*, 1989). However,

to the author's knowledge, this is the first investigation of antibacterial production by microorganisms isolated from the hindgut of *Tipula abdominalis*. While production of the antibiotic has not been demonstrated *in situ*, polymyxin E could have a role in colonization resistance and species competition as established in other insect species.

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CHAPTER 5

CONCLUSIONS

The need for renewable and sustainable energy has become increasingly apparent. Bioethanol is the most prevalent alternative fuel and provides a viable alternative to fossil fuels as it functions within existing infrastructure. To bring bioethanol production to the industrial scale necessary to displace fossil fuel usage, inexpensive enzymes able to convert lignocellulose to fermentable sugars and ethanologenic microorganisms capable of fermenting those sugars are required. The development of improved partial saccharification and co-fermentation (PSCF) as well as consolidated bioprocessing methodologies (CBP) will help to provide economical and efficient solutions.

Engineering ethanologenic *E. coli* strains to produce lignocellulose degrading enzymes during fermentation allows partial saccharification and co-fermentation, thus enabling decreased use of exogenous fungal enzymes in biomass saccharification steps, reducing the cost of the entire process. The addition of the *K. oxytoca casAB* operon into *E. coli* KO11 (strain LY40A) for cellobiose utilization significantly impacted ethanol production from lignocellulosic biomass and eliminateed the need for fungal cellobiases (Chapter 2). While the addition of the *E. chrysanthemi pelE* to LY40A (strain JP07C) did not increase ethanol production, secretion of pectate lyase did considerably increase degradation of polygalacturonate, and further engineering of *E. coli* JP07C to express an oligogalaturonate lyase, *ogl* (strain JP08C), allowed breakdown of polygalacturonate to monomeric sugars during fermentation and increased ethanol yield. Engineering of *E. coli* JP08C demonstrates the possibility of creating a strain of *E. coli* for consolidated bioprocessing, thereby eliminating the need of exogenous enzymes altogether.

While *E. coli* JP08C was engineered using known enzymes, the investigation of environments harboring bacteria that are capable of degrading lignocellulose could provide a source of novel hydrolytic enzymes for PSCF and CBP applications. Examination of the hindgut

microbial consortium of *Tipula abdominalis* found many isolates capable of lignocellulose degradation, and one group of five isolates, including *Paenibacillus amylolyticus* C27 and four other organisms with identical ribotypes, were able to degrade all nine model plant polymers screened. A library containing 2 to 5 kb chromosomal fragments of *P. amylolyticus* C27 was constructed in *E. coli* DH5α. Three pectinase-positive clones, 13C2, 19F6, and 23B3, were identified after screening approximately 4,000 clones (Chapter 3 and Appendix). The insert of clone 13C2 contained a pectate lyase, PelA, with the unusual characteristic of activity on both polygalacturonic acid and highly methylated pectin. The applicability of PelA for use in lignocellulose fermentations was investigated using sugar beet pulp, which is ~25% pectin with 60% methyl esterification. When *E. coli* pEDH27 (carrying *pelA* in pUC19) was grown in sugar beet pulp, a high amount of small chain oligogalacturonides were released, demonstrating saccharification of the biomass. Next, *pelA* was inserted into *E. coli* LY40A, giving strain JP27; when compared to *E. coli* LY40A fermentation on sugar beet pulp, JP27 produced nearly twice as much ethanol due to its breakdown of pectin. With its activity on highly methylated pectin, PelA will prove beneficial in PSCF and future CBP applications.

Examination of *P. amylolyticus* C27 also revealed another useful product: the production of the antibiotics polymyxin E_1 and E_2. *P. amylolyticus* C27 represents a novel source of the antibiotics, demonstrating the ability to uncover many useful compounds as well as enzymes from the investigation of microbiota in undescribed environments like the *T. abdominalis* hindgut.

Through continued exploration of lignocellulose-degrading habitats and engineering of ethanologenic microorganisms, tools will be developed to establish a sustabinable, renewable, and efficient bioethanol industry. By discovering and cloning new degradative enzymes with

high expression levels and specific activities, a microorganism capable of producing ethanol from many lignocellulosic biomass sources can be created. With a wide-substrate utilization range and manipulatible genetic system, *E. coli* is a promising ethanologen for PSCF and CBP processes.

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APPENDIX

ANALYSIS OF A NOVEL PECTATE LYASE FROM

PAENIBACILLUS AMYOLYTICUS C27

A genomic library of *Paenibacillus amylolyticus* C27 was constructed in *E. coli* DH5α and screened for pectinase activity. Clone 13C2, which contained *pelA*, was described in Chapter 3. Two other clones, 19F6 and 23B3, also exhibited pectinase activity and were further analyzed.

Sequencing of plasmid inserts isolated from 19F6 and 23B3 was performed at the Sequencing and Synthesis Facility at the University of Georgia with primer M13F, followed by primers 5'GGGTTCACCTTATTATCCGGACCT-3' and 5'-CAACAGCCATCGCTTCCAAAG GTT-3' for 23B3 and 5'-ACCGCAGCATCGCTTATGTAGGTA-3', 5'-AGTATCACTGTTGC CACGGAACCA-3', and 5'-TGGTGAGTCCATTAAAGCCGTCCA-3' for the plasmid in 19F6. Sequential sequencing reactions were examined for overlaps of at least 25 bp and assembled. Open reading frames of 1242 bp and 1029 bp were found in the insert from 19F6 and an open reading frame of 1242 bp was found in the insert from 23B3 (Figure A.1). Examination indicated that the sequence of the 1242 bp ORF was identical in both clones. A truncated portion of the 1029 bp ORF in 19F6 was also present in 23B3.

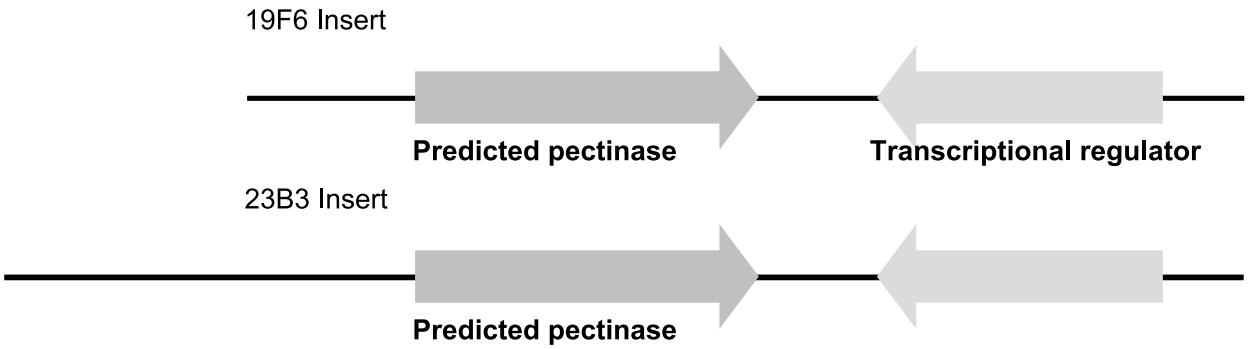

Figure A.1. Aligned inserts from clones 19F6 and 23B3. The predicted pectinase is an ORF of 1242 bp and the predicted transcriptional regulator an ORF of 1029 bp, which is truncated in 23B3.

The nucleotide sequence of the 1029 bp ORF was compared to sequences in the nonredundant and metagenomic NCBI databases by performing a BLAST (blastn) search (Altschul *et al.*, 1997). This ORF contained homology to *Bacillus* genes encoding transcriptional regulators, having highest identity (64%) to *B. stearothermophilus repA*, which is clustered with a β-mannanase, esterase, and α-galactosidase.

The nucleotide sequence of the 1242 bp ORF was compared to sequences in the nonredundant and metagenomic NCBI databases by performing a BLAST search (blastn) (Altschul *et al.*, 1997); however, no sequence of any significant similarity was found. The predicted ORF of 1242 bp encodes a protein of 414 amino acids that was compared to known proteins by performing a protein-protein BLAST (blastp) using the NCBI database (Altschul *et al.*, 1997). Homology was found to pectate lyases within family I (PL1) over the full length of the ORF. Highest homology (36.4% similarity, 24.1% identity by Smith-Waterman local alignment) was to a *Thermotoga maritima* pectate lyase. Pectate lyase assays of crude cell extract confirmed activity of *E. coli* clones 19F6 and 23B3 (181 U mg protein^{-1}), and the gene was denominated *pelB*, as it is the second pectate lyase identified in *P. amylolyticus* C27.

To determine relatedness to PL1 family enzymes, a BLAST search of PelB against a local database constructed from all PL1 family sequences listed in CAZy (Coutinho & Henrissat, 1999) was performed. A maximum likelihood tree was constructed from an alignment of all PL1 family sequences with an E value of less than 1e-15 (Figure A.2). Pectate lyases in family PL1 are poorly conserved on the sequence level, but share tertiary structure in the form of a parallel β-helix fold. While relatively distant from known enzymes, PelB appears to be a member of the PL1 family of enzymes.

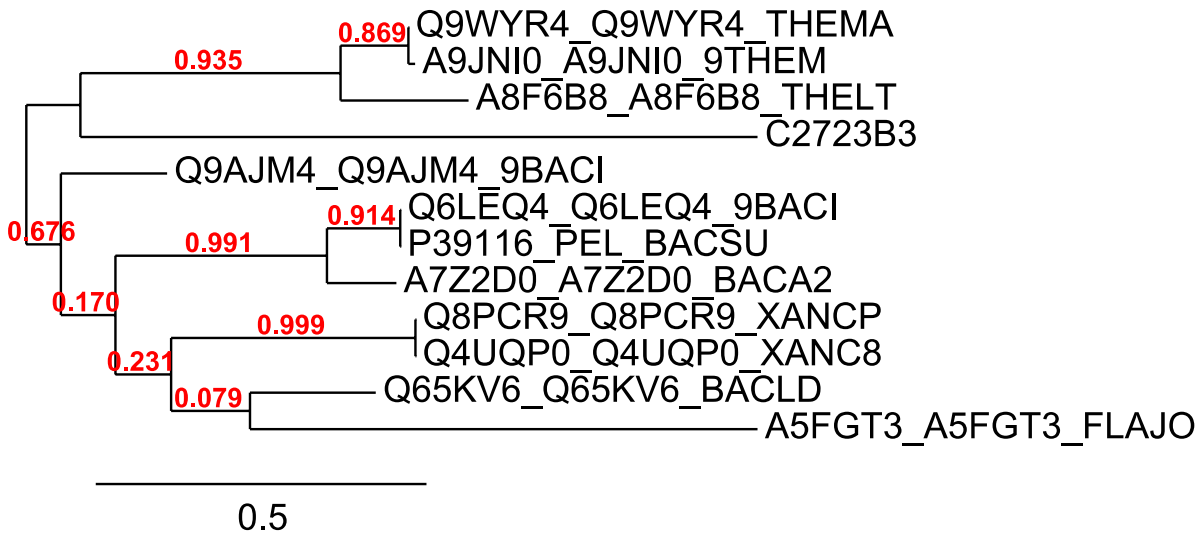

Figure A.2. Maximum likelihood tree of PL1 family enzymes with highest homology to *P. amylolyticus* C27 PelB. Bootstrap values are shown for each node. Protein sequences include (given UniProtKB/TrEMBL number) : [Q9WYR4], *Thermotoga maritima* PelA ; [A9JNI0], *Thermotoga* sp. RQ2 PL ; [A8F6B8], *Thermotoga lettingae* PL ; [Q9AJM4], *Bacillus* sp. TS-47 Pl47 ; [Q6LEQ4], *Bacillus* sp. PelK ; [P39116], *Bacillus subtilis* 168 PelC ; [A7Z2D0], *Bacillus amyloliquefaciens* PL ; [Q8PCR9], *Xanathamonas campestris* PL ; [Q4UQP0], *Xanthamonas campestris* 8004 PL ; [Q65KV6], *Bacillus licheniformis* DSM 13 PelII ; and [A5FGT3], *Flavobacterium johnsoniae* PL.

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