

THE CLONING, EXPRESSION AND ACITIVITY OF
EIGHT PECTIN DEGRADING ENZYMES FROM A
THERMOPHILIC FUNGUS

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LIST OF ABBREVIATIONS

ANTS	8-Aminonaphthalene-1,3,6-trisulphonate
APTS	9-Aminopyrene-1,4,6-trisulphonate
CZE	capillary zone electrophoresis
GalA	galacturonic acid
Glc	glucose
HG	homogalacturonan
IFI	invasive fungal infections
kD	kilodalton
NMR	nuclear magnetic resonance
PG	polygalacturonase
RG	rhamnogalacturonan
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
RGases	rhamnogalacturonases
Rha	rhamnose
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
XGA	xylogalacturonan
XGase	xylogalacturonase

μl	micro liter
μg	micro grams
mg	milli grams
min	minute(s)
mL	milli liter
mM	milli molar

CHAPTER I

INTRODUCTION

1.1 *ASPERGILLUS*

1.1.1 BRIEF STATEMENT

Aspergillus is a genus including several hundred mold species, which could be found in various environments worldwide. Most recent data showed there are 250 known species of *Aspergillus*[1], all of which are classified within the Ascomycota [2]. Currently this number is expected to keep increasing. The name *Aspergillus* was given by Micheli in 1729. The microscopic spore-bearing structure reminded Micheli of aspergillum, a word referring to holy water sprinkler. In 1926 Thom and Church published the first major monograph on the Aspergilli. Since then, it became one of the well-known and frequently studied mold groups. Aspergilli are prevalent in the natural environment, and easy to cultivate on laboratory media. Some of their species are economically important. These traits attracted much attention onto researching them [3]. Originally, *Aspergillus* was considered as saprophytes, as it was firstly found in a great amount on decaying vegetation. However, later research showed it could also use other substrates as carbon resource, such as feces, animal tissues [4], and even low rank coal [5]. *Aspergillus* also has many industrial usages, as in producing chemicals and processing food.

Academically, one species, *A. nidulans* has become a model organism in understanding fundamental concepts in metabolic pathway regulation, the cell cycle, parasexual cycle, hyphal polarity and intron splicing [3].

1.1.2 NUTRITION PATTERN

Fungi are seen as heterotrophic. However, the biggest difference between them and other heterotrophic organisms is: others eat their food and then digest, while fungi do the opposite: first digest their food and then uptake it. In this particular process, they apply one special nutritional strategy. They degrade surrounding big organic molecules down into small ones by excreting acids and enzymes. Then they absorb these small molecules as their “food”. *Aspergillus* employs this type of life style, which we usually define as decomposition. This process plays an important role in natural cycling of carbon in which *Aspergillus* contributes to replenishment of the supply of carbon dioxide and other inorganic compounds [3]. The “menu” of *Aspergillus* lists starches, hemicelluloses, cellulose, pectins, fats, oils, chitin, and keratin. Decomposition efficiency reaches its highest if there is enough nitrogen, phosphorus and other essential inorganic nutrients. Meanwhile, they also offer food for other organisms living around them during this process [6]. Some species can even make use of substrates under extreme conditions, like low pH, low water content or high sugar concentration. *A. glaucus* could feed on salted dry fish [7], while some *Aspergillus* species which are moderately xerophilic could feed

on dried grains, nuts and spices, which contain little water [8]. What attracts public attention is, sometimes *Aspergillus* species also feed on human-made substrates, such as paper and textiles. This process is defined as biodeterioration [3]. One significant example was observed in 1969, *A. versicolor* was the main cause of damage to a Ghirlandaio fresco in the Ognissanti Church after the Florence flood in Italy: 74% of the isolate was it [9].

1.1.3 TAXONOMY

Traditional taxonomists distinguish *Aspergillus* species in different ways. The most common one is based on the color of spores. Spores of *A. niger* group are black. The *A. ochraceus* group is usually yellow and brown, while *A. fumigatus*, *A. nidulans*, and *A. flavus* are white and green (Fig. 1.1)[3]. Because the morphological and growth responses of Aspergilli are sensitive to different nutrients, another way to identify species is based on pure cultures grown on defined media. Early taxonomic micrographs employed a media, which is adapted by Dox from Czapek, usually called ‘Czapek–Dox medium’. This medium has sucrose as the carbon source and nitrate as the nitrogen source[10]. The morphology of *Aspergillus* is also very sensitive to subtle change within air exchange, light and volume of the medium [11]. A recent method is to grow *Aspergillus* on a variety of different media in order to identify species [12]. The modern technique of DNA sequencing helps in the taxonomy, while also resulting in some confusions [3].



Figure 1.1, Different *Aspergillus* species. Photo from Dr. David Midgley, University of Sydney, Australia

1.1.4 PROLIFERATION

The great majority of *Aspergillus* species are observed to employ a homothallic crossing system. However, some scientists hold an opinion that all species of *Aspergillus* have the capability of forming a sexual phase, which means heterothallism within *Aspergillus* probably is common while difficult to observe [13]. Their supporting evidence is, through the study on *Aspergillus* genome, some strictly anamorphic species are revealed to have more variability than expected. Additionally, the genes which are essential for ascocarp development, meiosis and other aspects of sexual reproduction are also found within these genomes [14, 15]. The finding of these genes led to a hypothesis that due to cryptic

heterothallism we have not seen teleomorph within some *Aspergillus* species such as *A. fumigatus*, which are anamorphs of ascomycetes [16, 17]. Some other teleomorphic species which are closely related to *Aspergillus*, for example, *Neosartorya fischeri*, have both MAT-1 and MAT-2 loci within the same genome, but apparently on different chromosomes [18]. In this aspect, *A. nidulans* has one significant characteristic: the location of MAT genes is on separate chromosomes [14]. Latest research supports that heterothallism is more common in *Aspergillus*. Samples of *A. fumigatus* were collected from 91 places in Dublin, Ireland. The ratio of MAT-1 to MAT-2 within these strains was 1:1 [19]. *A. parasiticus* from Georgia was also researched on opposite mating type[20].

Aspergillus disperse their spores by drifting on air currents [3]. When the spores reach a liquid or solid surface, they would deposit. If the environment is right, they would start germinating[21].

1.1.5 INDUSTRY USAGE

Aspergillus has wide use in industry. *A. niger* has a long history of producing citric acid [22, 23], which is an important ingredient in the food, pharmaceutical and cosmetic area. It is also used as a hardener in adhesive and for retarding the setting of concrete [24]. It has a nickname "bulk chemical", with an estimated production of 1.6 billion kg per year [25]. *A. niger* also is useful in producing gluconic acid, which has several purposes. It is an additive in cleaning metal, and also medicine ingredient in the therapy for calcium and iron deficient patients. Another species, *A. terreus*, is useful in producing itaconic acid,

which is a synthetic polymer [3]. Fermentation of *A. oryzae* conducted so as to get kojic acid and related products, which can be used in skin whitening and synthesis of flavor enhancers [26].

Aspergillus is also widely employed in food fermentation and enzyme production. For example, *A. oryzae* and *A. sojae*, also called koji mold [27], are suitable for growing on starch and other carbohydrate-rich substrates[28]. They secrete amylases to degrade rice starch, which later can be fermented to make rice wine[29]. They are also used in legume fermentation, whose products exist widely in daily life such as miso and soy sauce[30]. In modern commercial enzymology, two of the major producers for hydrolytic enzymes are *A. niger* and *A. oryzae* [31], because both of them have been highly engineered [32, 33].

1.1.6 EFFECT ON HUMAN/ANIMAL

Aspergillus spores are almost everywhere in our living environment. As a result of evolution, human and animals have got used to them. Thus, generally they do not lead to adverse health problems. However, sometimes *Aspergillus* is still able to cause human/animal diseases. There are three major pathways: 1) mycotoxins; 2) allergenic responses; 3) localized or systemic infections. The widely accepted idea is, allergies and asthma are caused by an active host immune response because of the presence of exogenous stimuli, such as fungal spores or hyphae [3]. However, the aetiology is not clear [34, 35].

High concentrations of *Aspergillus* spores in air are harmful to the human body in two ways. The first one is what was just mentioned, to over act an immune reaction. One well-known example is malt worker's lung. The workers inhale a big amount of *A. clavatus* and *A. fumigatus* spores from contaminated barley in manufacturing beer. These two *Aspergillus* species lead to an occupational mycosis [36]. The other way is to infect the human body. Around 40 out of 250 *Aspergillus* species have been reported as human pathogens [12], among which *A. fumigatus* is the most significant one [37, 38]. For example, invasive fungal infections (IFI) are the main reason for the sharp improvement in high mortality in the immunocompromized patient population [39]. Previous research showed that molds, especially *Aspergillus spp.* are the major causes [40, 41]. *A. fumigatus* is the most common pathogen in this genus, while it is also one of the most common kinds of the airborne saprophytic fungi [42]. Thus, humans and animals are constantly exposed to numerous conidia of *A. fumigatus*. Luckily, the innate immune mechanisms can normally remove conidia in the immunocompetent host [42].

The results of *Aspergillus* infection are all termed 'aspergillosis'. Aspergillosis is an all-encompassing name. It refers to all diseases caused by *Aspergillus* infection. Human aspergillosis is not contagious, but is attracting increased attention in modern medical care [37, 38].

Due to its caused biodeterioration to stored crops and a potential pathogen to field crop, especially at high moisture, *Aspergillus* firstly was seen as a serious problem to our economics [43]. The first time in which people were aware that fungi could not only rot plant material but also threaten food and feeds was the discovery of aflatoxin, which is one type of mycotoxin and was believed to associate with wide deaths of poultry, trout and other domesticated animals [44]. In fact, besides aflatoxin *Aspergillus* also has other kinds of mycotoxins such as ochratoxin, patulin and fumigillin [45, 46]. Aflatoxin mainly attack mammal's liver [3]. There are several factors which could affect the possibility of aflatoxicosis in human body, including limited food availability, environmental conditions that help mould grow on foodstuffs, and lack of regulatory systems for aflatoxin monitoring and control [47, 48]. It is difficult for us to avoid aflatoxin because some *Aspergillus* species such as *A. flavus* is able to grow widely at each stage of our food chain [49]. It is reported that aflatoxin can be found in all major cereal crops [3] and other crops including cassava, chilies, corn, cotton seed, millet, peanuts, rice, sorghum, sunflower seeds, tree nuts and a variety of spices intended for human or animal food use [50]. Metabolites of aflatoxin have been detected in eggs, milk products and meat from animals are fed contaminated grain [46, 51].

1.2 INTRODUCTION OF THREE MAIN *ASPERGILLUS* SPECIES

1.2.1 *ASPERGILLUS FUMIGATUS*

Aspergillus fumigatus is one of the most common saprophytic fungi. It is important in carbon and nitrogen cycling [52-54]. Naturally, it is living in soil and feeding on organic

debris like most other *Aspergillus* species[3]. Because of its airborne conidia which have a diameter of 2-3um, the small size of conidia makes them buoyant in air. So although *A. fumigatus* does not employ particular mechanism to release conidia into the air, environmental disturbances and strong air currents help in its dissemination [42]. The reason makes it considered as one of the most ubiquitous fungi species [55-57].The bad aspect is, it is easy for the conidia to get into lung alveoli [10]. In fact we inhale at least 700 *A. fumigatus* conidia daily [58-60]. That is one of the main reasons for *A. fumigatus* being a major pathogen of humans [42].

1.2.2 ASPERGILLUS NIVEUS

Besides *A. fumigatus*, there are some other *Aspergillus* species reported in IFI cases [61]. The first case has been reported that *A. niveus* was the allogeneic human leukocyte antigen of a 21 year-old woman. It followed allogeneic hematopoietic stem cell transplantation for Fanconi anemia [62]. It is an uncommon clinical species, which usually exists in the air and in the agricultural field [63, 64]. Genomically speaking, *A. niveus* is close to *A. terreus* [62]. Data from Dr. Patricia Canaan shows that *A. niveus* is also close to *A. fumigatus*. Although the whole genome has not been compared, some enzymes' DNA sequences have and show only several base pair differences (data show below).

1.2.3 ASPERGILLUS NIDULANS

Aspergillus nidulans is referred to as *Emericella nidulans* in its sexual form which is also called a teleomorph. It has been used as a model organism since 1976 because it is non-pathogenic on humans [65, 66]. In the *Aspergillus* genus, it was the first species to have its whole genome sequenced, within which automated gene prediction tools predicted 9,451 open reading frames (ORFs). Some of the predicted ORFs were functionally annotated [67].

1.3 PLANT CELL WALL

1.3.1 BRIEF STATEMENT

Plant cell wall is an extracellular matrix which is rigid and defines the shape and size of the cell [68]. It is the most striking difference between plant cells and animal cells.

Because of its rigidity, it has lost ability to develop nervous systems, immune systems, and most importantly, mobility. But it still possesses varied physical and chemical properties, plays an important role in many plant developmental processes, such as growth, proliferation, and fruit and vegetable ripening [69-71]. In the cell walls, some polysaccharides are able to bind heavy metals, which may accumulate heavy metals in naturally occurring biofilms. But this property is also used to remove heavy metals from polluted waste water in a biological approach. Wall polysaccharides have many commercial uses. Many types of plant-based food are composed mainly of cell walls. Cell walls are also main texture of gels and some kinds of stabilizer. Therefore, cell wall structure and organization attracts great interests of plant scientist, nutritionist, and food industry.

Plant cell wall is divided into two parts: primary cell wall and secondary cell wall. They are believed to have different structures and functions. Primary cell walls surround cells which are growing and expanding. Thus, the primary cell wall not only gives cell mechanically support but also can expand to let cells grow and proliferate [72]. Most of the apoplast in growing tissue is composed of primary cell wall and middle lamella. The main functions of primary cell wall includes: i) providing the structural and mechanical support to the cell; ii) defining and maintaining the cell shape and size; iii) resisting internal expanding pressure to cell; iv) controlling rate and direction of growth; v) determination for plant architecture and form; vi) regulation of material diffusion through the apoplast; vii) storage for carbohydrate - walls of seeds could be metabolized if necessary; viii) protection against pathogens, dehydration, and other environmental factors; ix) producing and transferring biologically active signal; and x) -interaction between cells. Besides, plant-derived foods contain much primary cell walls or the polysaccharide which is the major component of primary cell wall [72]. Usually, the main component of primary cell walls in plant tissues and cells is polysaccharides. Besides, there are some other components, such as proteins(e.g. enzymes), glycoproteins (e.g. hydroxyproline-rich extensins), phenolic esters (e.g. ferulic and coumaric acids),and ionically and covalently bound minerals (e.g. calcium and boron) [73]. These minor components play important roles in cell functions. For example, the protein expansins are supposed to regulate wall expansion [74]. Secondary cell wall is much stronger and thicker than primary cell wall. It is the place for accumulation of most polysaccharide of plants [72]. One of its main functions is to provide strength.

Both primary and secondary cell wall is mainly composed of cellulose and hemicellulose, with different portions. Secondary cell wall also contains a high level of lignin. Lignin forms a network around the hemicellulose and cellulose making the secondary cell wall hydrophobic.

1.3.2 CELLULOSE

Cellulose is major component of plant cell wall. In primary cell walls it composes 20- 30 % of the total dry weight while for secondary walls it accounts 40- 45% of the total dry weight [75]. This most common polysaccharide has a formula of $(C_6H_{10}O_5)_n$. It has no taste or no odor[76]. It is a straight chain polymer, without coiling or branching. It has β (1, 4)-D-glycosidic bonds. Different degrees of polymerization determine different properties of cellulose. Cellulose of wood pulp has chain length of 300-1700 units while cotton and bacteria cellulose have 800-10000 unites [77]. These chains form microfibrills by forming hydrogen bonds between multiple hydroxyl groups on the glucose from one chain and oxygen atoms on the same or on a neighbor chain. These microfibrills range from $5\ \mu\text{m} - 7\ \mu\text{m}$ in length and 30nm in width. The microfibrills have high tensile strength, which is necessary in cell walls. The microfibrils make a carbohydrate matrix, which can support plant cells by conferring rigidity to them. In vascular plants, rosette terminal complexes (RTCs), which contain the cellulose synthase enzymes, synthesize cellulose at the plasma membrane. The RTCs are hexameric protein structures [78]. There are at least three different cellulose synthases in the RTCs, all of which are

encoded by *CesA* genes, in an unknown stoichiometry. Different *CesA* genes are involved in primary and secondary cell wall biosynthesis [79].

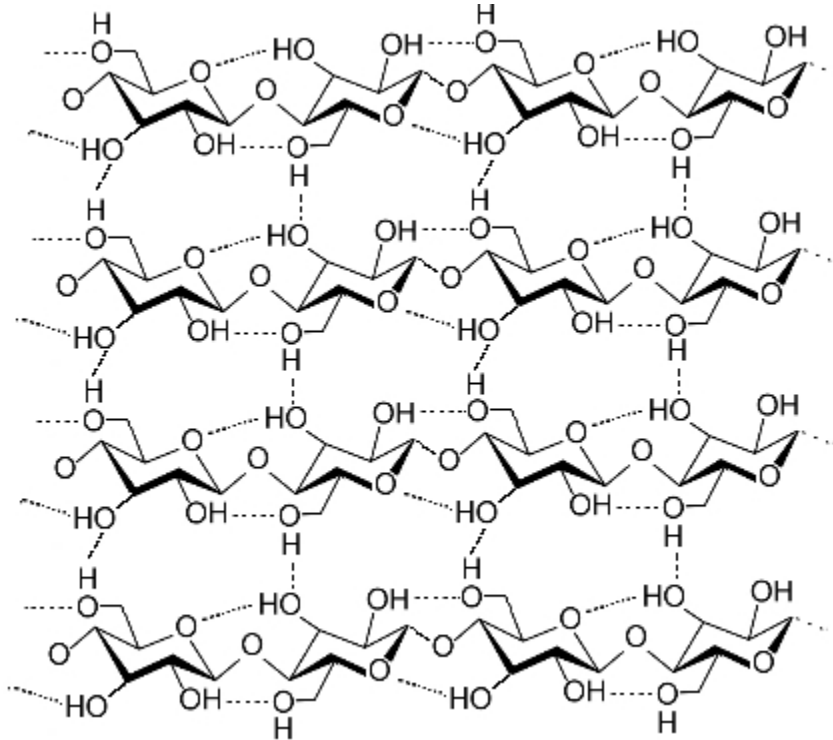


Figure 1.2 Cellulose strand. From Luca Laghi, University of Bologna, Italy.

1.3.3 HEMICELLULOSES

Hemicelluloses are defined as polysaccharides in cell walls, which water cannot dissolve while aqueous alkali can. Chemically, they are referred as polysaccharides that have a backbone of 1,4-linked β -D-pyranosyl residues in which O_4 is in the equatorial orientation[80]. Unlike cellulose having only glucose, they contain other sugars than glucose, such as xylose, mannose, galactose, and arabinose. Their backbones are β -1, 4-D pyranose residues. Sometimes they also have sidechains including L- arabinose units as

in arabinoxylan and the fucose in xyloglucan. The most common residues are xylose and mannose. There are several types of hemicelluloses, including xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan. The major types of hemicelluloses are glucuronoarabinoxylans and xyloglucan. They compose about 20% of the total dry weight of primary cell wall and about 20% - 35% of the total dry weight of secondary cell wall, respectively [75]. Hemicellulose exists more in secondary cell walls than in primary cell walls. Hemicellulose can be solubilized in alkaline solvents. And it could be hydrolyzed by dilute acid and hemicellulases [81]. An alternative term proposed for hemicellulose is cross-linking glycans.

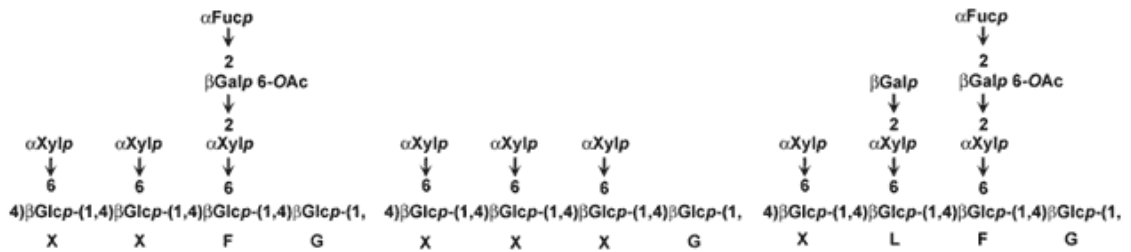


Figure 1.3 Xyloglucan. Complex Carbohydrate Research Center, the University of Georgia

1.3.4 PECTIN

Pectin is made of a complex set of polysaccharides which exist in most primary cell walls. Pectin is mainly composed of 1, 4-linked α -D-galactosyluronic acid residues with a few other sugars including arabinose, rhamnose, galactose and xylose. Based on this,

pectic polysaccharides are categorized into four different structural classes:

homogalacturonan (HG), rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II) and xylogalacturonan (XGA). Usually pectin is suggested to have two types of region: smooth region such as HG, and hairy region such as RG [82].

1.3.4.1 HOMO GALACTURONAN (HG)

HG is a linear chain composed of α -1, 4-D galactosyluronic acid residues. Though it is simple and smooth, it composes nearly 60% of the pectin in plant cell walls. Up to 80% of the carboxyl groups of the GalA residues are esterified with methanol while the O-2 and O-3 position of hydroxyl groups could be acetylated [83-85].

Homogalacturonan (HG)

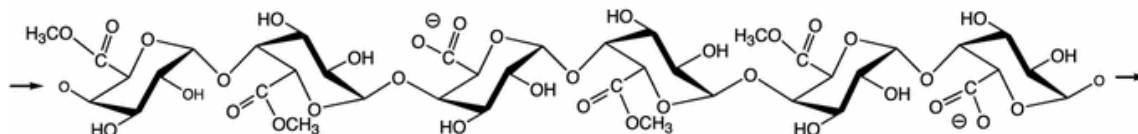


Figure 1.4 Homogalacturonan with methyl esterification[86]

1.3.4.2 RHAMNO GALACTURONAN I (RG I)

RG I composes 20-35% of pectic polysaccharides. This term refers to a group of closely related cell wall pectic polysaccharides that have a backbone composed of the repeating disaccharide (1, 2)- α -L-rhamnosyl-(1, 4)- α -D-galacturonic acid [87, 88]. Usually the O-2

and O-3 positions of the GalA residue on the backbone are acetylated [89], while various neutral sugars branches of mainly D-galactose and L-arabinose are attached to O-4 of rhamnose. The nature of the sidechains depends on the origin of the pectin [90], the stage of cell growth, and tissue development[91]. The function of RG is still little known and no RG has been fully characterized [92, 93].

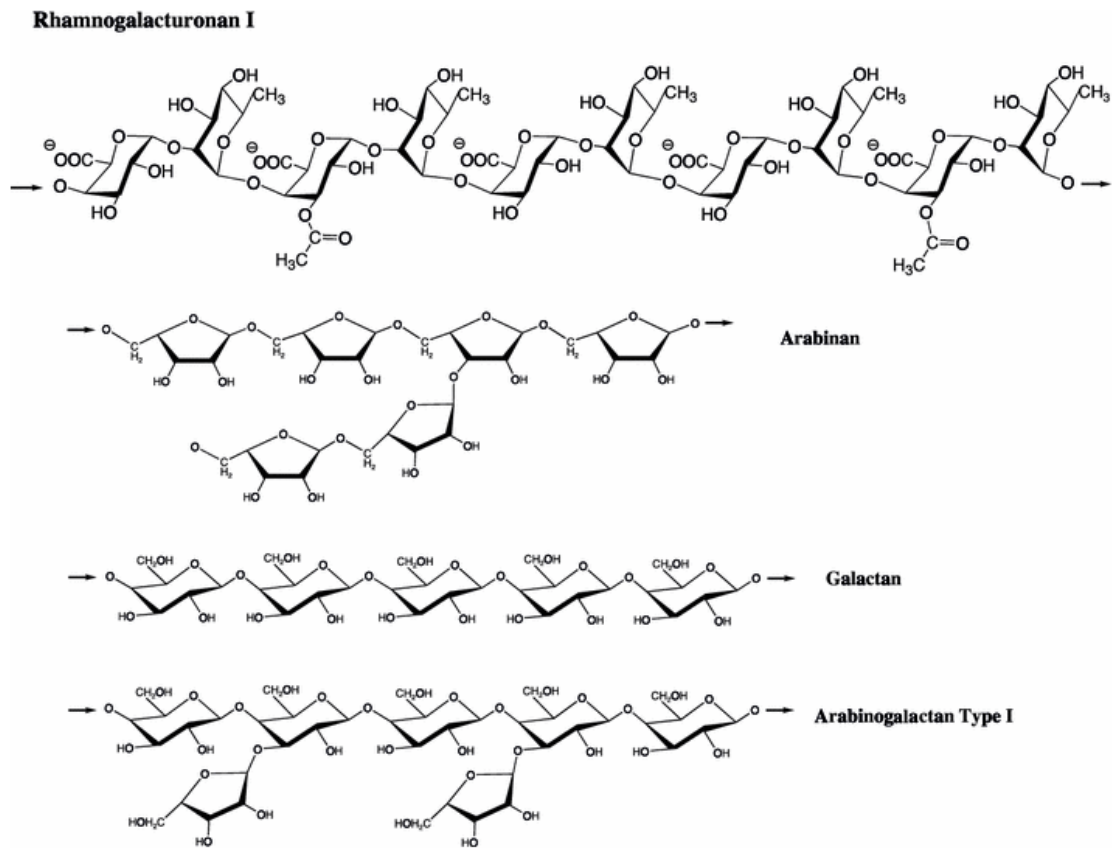


Figure 1.5 Three types of rhamnogalacturonan I side groups attached to the C4 position of the rhamnopyranosyl residue: (1, 5)- α -L-arabinans, (1, 4)- β -D-galactan, and arabinogalactan Type I[86]

1.3.4.3 RHAMNOGALACTURONAN II (RG II)

RG II composes about 5% of the pectic polysaccharides. It was identified in 1978. It is found to be conserved in all primary walls [94]. Though small molecular weight (usually 5-10Kd), it has a more complex structure when compared to all other pectic polysaccharides. Although RG II shares a similar name with RG I, they are different both structurally and functionally. The RG II has a backbone which is composed of at least 8 1, 4-linked α -D-GalpA residues. C-2 and C-3 of the backbone are attached to two types of structurally distinct disaccharides [94, 95], and two complex hepta-to nonasaccharides. There are several kinds of uncommon sugars which are linked to RG II, such as 2-keto-3-deoxy-D-lyxo-heptulosaric acid (Dha) [96], 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) [97], 2-O-methyl L-fucose, 2-O-methyl D-xylose, D-apiose [98], and 3-C-carboxy-5-deoxy-L-xylose (L-aceric acid) [99]. Sometimes two RG II molecules form self-associated dimers via boron-diester bonds through apiosyl residues of side chain via boron-diester bond. The dimer structure of RG II plays important roles in strengthening the cell wall, plant growth and development.[100-102]

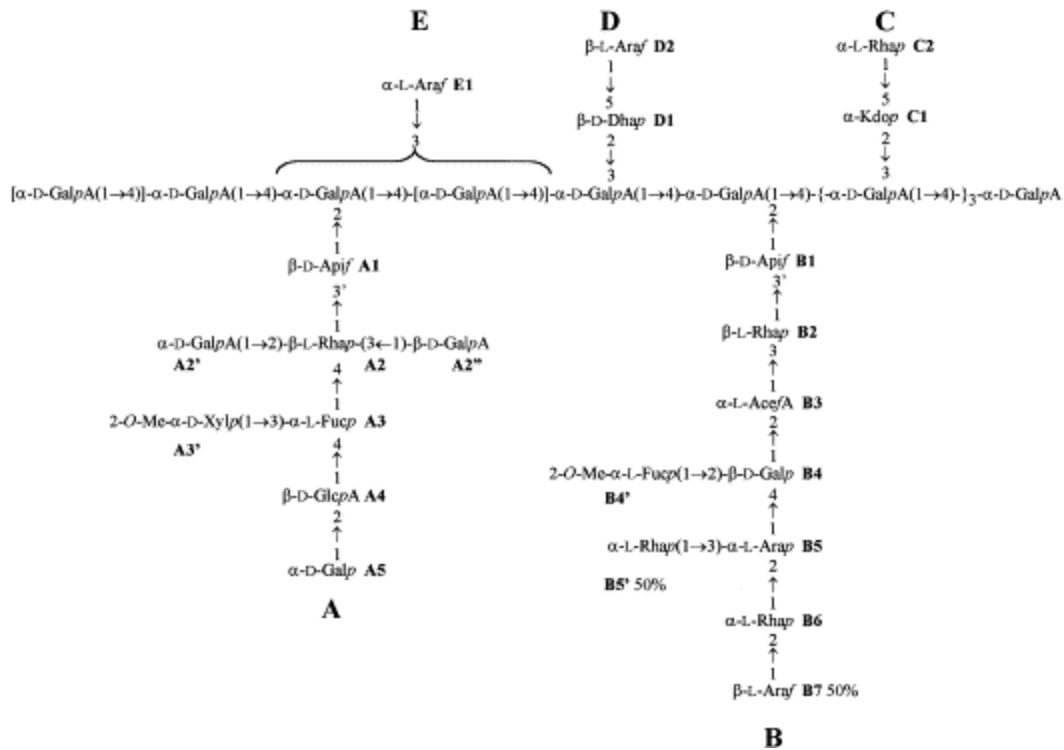


Figure 1.6 Schematic representation of the primary structure of RG-II monomer (mRG-II)[103].

1.3.4.4 XYLOGALACTURONAN (XGA)

XGA is one of less well-known types of pectic polysaccharide. XGA has a backbone which is a chain of α -(1, 4)-linked D-galacturonic acid. Often at the O-3 position it is substituted with β -D-xylose [104]. XGA was proposed to be a side chain of RG I in the “hairy” regions of pectin [105]. It is present in storage tissues or reproductive organs [106].

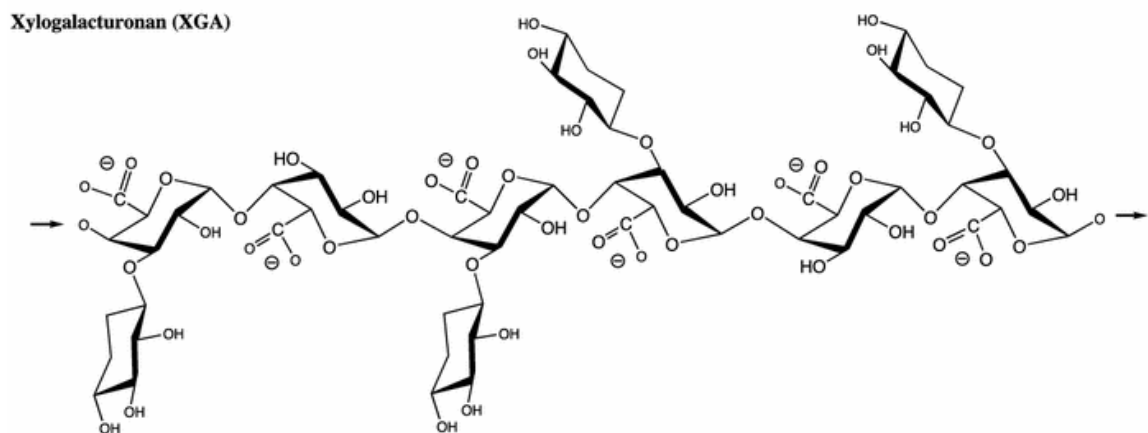


Figure 1.7 Xylogalacturonan are homogalacturonans substituted with α -D-xylopyranosyl residues at the C3 position of GalA units[86]

1.4 ENZYMES

1.4.1 POLYGALACTURONASE

When fungi encounter plants, the plant cell wall is the first line of defense. Fungi have evolved a pool of pectic enzymes or pectinases, including endo-polygalacturonases, exo-polygalacturonases (both of them are termed PGs for short), pectate lyases, and pectin lyases. [107-109]. These enzymes are thought to hydrolyze the cell wall in the first stage of pathogenesis. They degrade polygalacturonan in cell walls by hydrolyzing or trans eliminating the 1, 4 α -D galacturonic acid linkages in smooth region of pectin [110, 111]. Previous research showed that PGs are the first enzyme during this process[112]. After they play the pioneer role, other enzymes can digest the cell wall much more easily [113]. In correspondence, plants have evolved some protection mechanisms. One of them is polygalacturonase inhibitor proteins (PGIPs for short) [114].

In fact, PGs are not only from fungi, but are also encoded by a large gene families in plants. They do not only work negatively on plants. 35 years ago, PGs were first reported to function in disassembly of pectin critical to many plant developmental processes [107]. For example, PG's activity has been reported in organ abscission[115, 116], pod and anther dehiscence[117], pollen grain maturation and pollen tube growth[118, 119], cell expansion[120, 121], and ripening fruit [107].

1.4.2 RHAMNOGALACTURONASE

Pectin, which is a major component of cell wall, has a hairy region consisting of rhamnogalacturonan [82]. Previous research did not focus very much on the enzymes targeting this region [122]. Rhamnogalacturonases (RGases for short) are such enzymes. They are endo-acting enzymes. Two different RGases have been described with different mechanisms. RGase A functions by hydrolysing the glycosidic linkage α -D-GalUA(1, 2)- α -L-Rha [82], while RGase B works on the same substrate by cleaving the glycoside bond α -L-Rha(1, 4)- α -D-GalUA through β -elimination[123, 124]. Later research found the RGases B is a lyase while RGases A is a hydrolase [124]. Sometimes the RG is acetylated at the C2 or C3 position of GalUA [123, 125]. The acetylation could decrease the effectiveness of degradation by RGase A and B. So usually RGase works along with rhamnogalacturonan acetyl esterase [126].

1.4.3 XYLOGALACTURONASE

In the hairy region of pectin, there is often xylogalacturonan, which has a galacturonan backbone with frequent xylose substituents[104]. In 1996, one exo-polygalacturonase was reported to be able to remove a Xyl-GalA disaccharide from XGA [127]. In 2000, van der Vlugt-Bergmans, et al. reported on a xylogalacturonase (XGase for short) having 406 amino acids which digests xylogalacturonan using an endo mechanism [104].

CHAPTER II

MATERIALS AND METHODS

2.1 pEXPYR+

pExpyr+ was a gift from Dr. Rolf Prade, Department of Microbiology and Molecular Genetics, Oklahoma State University. This plasmid was constructed based on pFE, which originated from pBR322 [128]. The map is not available but the sequence is. It has an Ampicillin resistance gene for selection in *E. coli*, and a Zeocin resistance gene for screening in fungus. For a secure screen, it also has genes for producing uridine and uracil to complement to uridine⁻/uracil⁻ cell lines. The two cloning sites are: Not I upstream and Xba I downstream of insertion site. Right before the Not I site, there is a fragment encoding a signal peptide. Right after the Xba I site there are six CAT repeats encoding a His-tag which could be used in purification.

2.2 GENOMIC DNA EXTRACTION FROM *A.NIVEUS*

The genomic DNA extraction protocol is below:

- 1) Inoculate minimal media plates (containing appropriate supplements) with spores of *A. niveus* to create confluent growth;
- 2) Lyophilize mycelia overnight and homogenize tissue;
- 3) Add 600ul of genomic extraction solution. Heat at 68 °C for 10 min;
- 4) Centrifuge at 13000RPM for 5 min and transfer supernatant into a fresh tube;
- 5) Add 50ul 5M K-OAc, mix by inversion and place on ice for 10 min;

- 6) Repeat step 4 once;
- 7) Add 2.5 volume 95% ethanol of current mixture;
- 8) Centrifuge at 13000RPM for 5 min and discard the supernatant;
- 9) Add 1ml 70% ethanol to wash and centrifuge at 13000RPM for 2 min;
- 10) Repeat step 9 once;
- 11) Dry pellet well and add EB or H₂O.

The protocol for genomic extraction solution is below:

0.5M EDTA	1ml
10% SDS	1ml
H ₂ O	8ml
<hr/>	
Total	10ml

2.3 EXO-POLYGALACTURONASE AND ENDO-POLYGALACTURONASE EXPRESSION, COLLECTION AND PURIFICATION

Three exo-polygalacturonases and three endo-polygalacturonases were cloned from *Aspergillus niveus*. Because the genome sequence of *A. niveus* was not available when the project started, we designed the primers based on the corresponding sequences in *A. fumigatus*. The open reading frames of exo-polygalacturonases in *A. fumigatus* are AFUA_6G02980@7470, AFUA_7G06410@9053 and AFUA_8G02630@9409. And the open reading frames of endo-polygalacturonases are AFUA_1G17220@1581, AFUA_4G13920@5789 and AFUA_8G01970@9340. The signal peptide prediction was based on the protein sequences from *A. fumigatus*. It was performed on the SignalP

program (available online <http://www.cbs.dtu.dk/services/SignalP/>). The primer sequences were

GCGGCCGCTTCACTCGTTCGAGGAAC/TCTAGATGCGCAGTTAACATCCAG,
GCGGCCGCATGGCGACCACGGATCGA/TCTAGAACCCTCCTGTAGCACT,
GCGGCCGCATTGCAGTTGATGGCCTT/ TCTAGATGCCCTGTATTTTAGGCT,
GCGGCCGCTCTCCCGCGGCTGAGCCA/ TCTAGAGCATTGGGCAACGCTAGG,
GCGGCCGCGCCCCTGCTCCGTCTCGC/ TCTAGAGCAAGAAGCACCTGAGGG,
GCGGCCGCGCTGATCTCGAGACTCGC/ TCTAGAGCATGAGGCGCCAGCAGG,

correspondingly. Primers were inserted with Not I and Xba I. Predicted signal peptides were removed. The optimal temperatures for each pair of primers were determined by gradient PCR. The gradient PCR program is below:

- 1) 94 °C for 3 min;
- 2) 94 °C for 30 sec;
- 3) 55-65 °C for 30 sec;
- 4) 72 °C for 2 min;
- 5) repeat step 2 to 4 for 35 cycles;
- 6) 72 °C for 10 min.

The PCR reaction system was below:

H ₂ O	34.3ul
10X PCR buffer (Mg ²⁺ free)	5ul
25mM MgCl ₂	3ul
dNTP Mixture (2.5mM each)	4ul

DNA template (20-100 ng/ul)	1ul
Primer 1	1.25ul
Primer 2	1.25ul
Takara taq (5unit/ul)	0.2ul
<hr/>	
Total	50ul

The PCR polymerase was purchased from Takara (Lot No. K5401FA). After the optimal temperatures were determined, all the fragments were amplified by High Fidelity Taq (Invitrogen, 11304-011). All the fragments could be amplified at 59 °C. The system reaction protocol was the same as for the Takara polymerase. The PCR products were purified using a Gel Extraction Kit (Qiagen, Cat. No. 28706). Because the High Fidelity Taq could add a single overhang A at 3' the end of the PCR product, the purified fragments were ligated to pGem-T easy (Promega, Cat. No. A1360). The ligation reaction protocol is below:

Purified DNA fragment	3ul
pGem-T easy	1ul
Fast ligation buffer	5ul
Ligase	1ul
<hr/>	
Total	10ul

The ligation system was incubated at 4 °C for overnight. Then the ligation product was transformed into competent cells (Invitrogen, 18265-017). The transformation process is below:

- 1) Mix 50ul competent cell with 5ul ligation product;
- 2) Incubate on ice for 30 min;
- 3) Heat shock at 42 °C for 20 sec;
- 4) Move onto ice immediately, incubating for at least 2 min;
- 5) Add 950ul SOC;
- 6) Shake at 37 °C for 1 hour;
- 7) Spread onto solid media with 100ug/ml Amp, 8ul 1M IPTG and 10ul 40mM X-Gal;
- 8) Incubate at 37 °C overnight.

The white colonies were selected to perform colony PCR. The reaction protocol was almost the same as before, but the template was replaced by colonies. After the recombinants were confirmed, they were submitted for sequencing. The sequencing primers were the T7 promoter and the SP6 promoter. Once the target recombinants were found, the enzyme encoding DNA fragments were cut from pGem-T easy by Not I (Promega, Cat. No. R6431) and Xba I (Promega, Cat. No. R6181). Then they were ligated into pExpyr+. The ligation protocol is below:

Fragment	3ul
pExpyr+	1ul
Ligase buffer	2ul
Ligase	0.1ul
H2O	3.9ul

Total

10ul

Ligase was purchased from Invitrogen (Cat. No. 15224017). Ligation reaction was incubated at room temperature for at least 1 hour (optimal 4 °C for overnight). Then the ligation product was transformed into competent cells. The process was as described above. The recombinants were confirmed as described before.

The recombinants were transformed into *A. nidulans* protoplasts. The protoplast production process is below:

- 1) Grow *A. nidulans* on minimal medium with 1ug/ml pyridoxine and 100ug/l uracil/uridine at 37 °C for two nights;
- 2) Move spores into liquid medium and shake at 30 °C/250RPM overnight;
- 3) Harvest mycelium with a suction flask, funnel and filter paper;
- 4) Wash with Mycelium Wash Solution;
- 5) Wash with DSPS;
- 6) Transfer mycelium to a 50ml plastic centrifuge tube.
- 7) Add all enzymes (500mg Lysing Enzymes from *Trichoderma harzianum*, Sigma L1412-10G; 250mg Lysozyme from Chicken Egg White, Sigma L7651-1G) and 100mg BSA (Sigma A-4503), into the centrifuge tube with mycelium, and vortex to dissolve;
- 8) Shake at 30 °C/250RPM for 2 hours;

- 9) Use a suction flask with a sterile glass tube placed inside and a sterile funnel with miracloth (Calbiochem Cat. No 475855), transfer the protoplasts to the funnel and collect the filtrate in a 50ml centrifuge tube;
- 10) Spin at 7500 RPM for 10 min;
- 11) Dump the supernatant, and resuspend the pellet in 45ml STC50;
- 12) Repeat step 10 and 11 3 times to remove protoplasting enzymes;
- 13) Resuspend the final pellet in 1ml of STC50;
- 14) Keep in the refrigerator for at most 15 days.

The transformation process for *A. nidulans* is below:

- 1) Add 10ug DNA to a 15ml falcon tube;
- 2) Add STC50 to make the volume 50ul;
- 3) Add 150ul protoplasts, mix well by swirling and incubate at room temperature for 10 min;
- 4) Add 2ml PEG 4000, mix well by gently rolling on the bench and incubate at room temperature for 20 min;
- 5) Add 12ml STC50 and mix well by inverting the tube;
- 6) Spread 2ml of transformation product onto plate containing minimal medium with 1X pyridoxine and 1.2M Sorbitol;
- 7) Leave plates with agar side down and incubate at 37 °C for 1 day;
- 8) Invert plates and continue to incubate at 37 °C for another 2 days to see transformants.

The protocols for solution are below:

1) Mycelium Wash Solution (250ml)

0.6M MgSO₄ 36.972g

Autoclave and store at room temperature

2) Double Strength Protoplasting Solution (DSPS) (100ml)

1.1M KCl 8.2016g

0.1M Citric Acid 1.921g

1M KOH to pH 5.8

Sterilize by filtration and store at room temperature

3) STC50 Solution (250ml)-Must make fresh every 15 days

1.2M Sorbitol 54.65g

50mM CaCl₂ 1.85g

50mM Tris.HCl (pH 7.5) 12.5ml

Autoclave and store at room temperature

4) 60% PEG Solution (10ml) Must make fresh daily

PEG 4000 6g

STC50 5ml

Heat in a microwave oven for 15-30 sec.

Adjust final volume to 10ml.

Store at room temperature until use.

The *A. nidulans* colonies with a potential of expressing complete enzymes were confirmed by conidia PCR. The program of conidia PCR was almost the same as the previously described expect the first step. The first step was 94 °C for 15 min. The purpose was to break the conidia in order to release genomic DNA[129]. The ability to express active enzyme was confirmed by trial-expression. The medium protocol is below (in 100ml):

20x Nitrate Salt	5ml
1000X Pyridoxine	100ul
1000X Trace Elements	100ul
Glucose	1g
Maltose	5g
pH Adjust to 7.0	

Maltose was used here as inducer. Transformants were incubated in media at 37 °C for 48 hours. The activity of enzymes was confirmed using CZE and APTS labeled GalA₁₈. The reaction protocol is below:

Reaction buffer	20ul
Original medium	1ul
APTS-labeled substrate	0.2-0.5ul

The medium was concentrated 30 times by ultra-filtration (Milipore, Cat. No. 13722).

The protein mix was dissolved in buffer A. Then the protein mix was purified using 200ul Ni-NTA (Qiagen, Cat. No. 30210). The process is below:

- 1) Mix buffer A containing protein with Ni-NTA;
- 2) Shake at 4 °C for at least 4 hours, usually for overnight;
- 3) Pour protein-Ni-NTA into the column to make a bed;
- 4) Wash the bed with 10 column volumes of buffer A;
- 5) Elute with several 500ul aliquots of buffer B (each called one fraction).

Buffer A (also called washing buffer, 1 liter):

50 mM NaH₂PO₄ 6.90 g NaH₂PO₄ H₂O (MW 137.99 g/mol)

300 mM NaCl 17.54 g NaCl (MW 58.44 g/mol)

20 mM imidazole 1.36 g imidazole (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH.

Buffer B (also called elution buffer, 1 liter)

50 mM NaH₂PO₄ 6.90 g NaH₂PO₄ H₂O (MW 137.99 g/mol)

300 mM NaCl 17.54 g NaCl (MW 58.44 g/mol)

250 mM imidazole 17.00 g imidazole (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH.

SDS-PAGE analysis

SDS-PAGE protein gel was performed to analyze the expressed protein. The protocol for the resolving gel (10%, 5ml) is below:

H ₂ O	1.9ml
30% acryl-bisacrylamide	1.7ml
1.5M Tris (pH 8.8)	1.3ml
10% SDS	0.05ml
10% ammonium persulfate	0.05ml
TEMED	0.002ml

When the resolving gel has solidified, which usually takes 30 min, a 5% stacking gel was made. The protocol for the stacking gel (5%, 3ml) is below:

H ₂ O	1.34ml
30% acryl-bisacrylamide	0.5ml
0.5M Tris (pH 6.8)	1.14ml
10% SDS	0.03ml
10% ammonium persulfate	0.03ml
TEMED	0.003ml

Electrophoresis buffer (5X, 1 liter) protocol is below:

Tris	15.1g
Glycine	94g
SDS	5g

When being used, it should be diluted by 5 times.

The protein ladder was purchased from Invitrogen (Cat. No 10748010). The electrophoresis was performed at a voltage of 120V.

Western Blotting was performed to confirm the existence of His-tag. The process was below.

- 1) Separate protein on SDS-PAGE gel at 120V.
- 2) Transfer all protein on the gel onto nylon membrane at 30V for overnight (if it is PVDF membrane, the membrane must be put into methanol for later use).
- 3) Wash the membrane with Phosphate Buffered Saline with Tween-20 (PBST) for 5 min. Repeat this step three times.
- 4) Block the membrane with blocking buffer (3% BSA or 3% non-fat dry milk powder in 3% PBST) for 1 hour.
- 5) Wash the membrane with PBST for 5 min. Repeat this step three times.
- 6) Incubate the membrane with primary antibody (diluted 3000X in 3% BSA in PBST) for 1 hour. Here the primary antibody is mouse anti-His monoclonal antibody.

- 7) Wash the membrane with PBST for 5 min. Repeat this step three times.
- 8) Incubate the membrane with secondary antibody (diluted 5000X in 3% BSA in PBST) for 1 hour. Here the secondary antibody is rabbit anti-mouse monoclonal antibody. The secondary antibody is labeled by HRP (horseradish peroxidase).
- 9) Wash the membrane with PBST for 5 min. Repeat this step five times.
- 10) Incubate the membrane with GE ECL Western Blotting Detection Reagents (Cat. No. 61). After two-minute incubation, examine the membrane at Alpha FluoroChem.

The membrane transferring buffer is below (for 1 liter).

Tris	3.02g
Glycine	14.4g
Methanol	100ml

The PBST is below (for 1 liter).

NaCl	8g
KCl	0.2g
Na ₂ HPO ₄	1.44g
KH ₂ PO ₄	0.24g
Tween-20	2ml

2.4 RHAMNOGALACTURONASE EXPRESSION, COLLECTION AND PURIFICATION

One rhamnogalacturonase was cloned from *A. niveus*. Because the complete genome information of *A. niveus* was not available when the project started, we designed the primers based on the corresponding sequences of *A. fumigatus*. The open reading frame of rhamnogalacturonase in *A. fumigatus* is AFUA_5G10530@6817. The primer sequences were GCGGCCGCTCACTTTCCGGCTCTGTC/
TCTAGATATTGCGCTCCCGAAGTG. The expression protocol has been described above. The activity was confirmed using CZE and an APTS labeled (GalA-Rha)₈ rhamnogalacturonan fragment [130].

2.5 XYLOGALACTURONASE EXPRESSION, COLLECTION AND PURIFICATION

One Xylogalacturonase was cloned from *A. niveus*. Because the complete genome information of *A. niveus* was not available when the project started, we designed the primers based on the corresponding sequences of *A. fumigatus*. The open reading frame of xylogalacturonase in *A. fumigatus* is AFUA_8G06890@9731. The primer sequences were GCGGCCGCGCTCCCTCGCAGGTTGAG /
TCTAGAACCCGAAGCACCCGAGGT. The expression protocol has been described above. The activity was tested using partially hydrolyzed gum tragacanth for digestion, followed by being labeled with ANTS.

	Forward	Reverse
17220	GCGGCCGCTCTCCCGCGGCTGAGCCA	TCTAGAGCATTGGGCAACGCTAGG
13920	GCGGCCGCGCCCCTGCTCCGTCTCGC	TCTAGAGCAAGAAGCACCTGAGGG
10503	GCGGCCGCTCACTTTCCGGCTCTGTC	TCTAGATATTGCGCTCCCGAAGTG
02980	GCGGCCGCTTCACTCGTTCGAGGAAC	TCTAGATGCGCAGTTAACATCCAG
06410	GCGGCCGCATGGCGACCACGGATCGA	TCTAGAACCCTCCTGTAGCACT
01970	GCGGCCGCGCTGATCTCGAGACTCGC	TCTAGAGCATGAGGCGCCAGCAGG
02630	GCGGCCGCATTGCAGTTGATGGCCTT	TCTAGATGCCCTGTATTTTAGGCT
06890	GCGGCCGCGCTCCCTCGCAGGTTGAG	TCTAGAACCCGAAGCACCCGAGGT

Table 2.1 Primer sequences of all the enzymes

2.6 EMPTY VECTOR EXPRESSION, COLLECTION AND PURIFICATION

Empty vector was transformed into *A. nidulans* as a negative control. The expression protocol has been described above. The media was collected and concentrated 30 folds.

The activity was tested on each substrate.

2.7 ACTIVITY UNIT MEASUREMENT

Different substrates were used to measure the activity of the enzymes. For endo-galacturonases and exo-galacturonases, 1% pectic acid was used as substrate. For rhamnogalacturonase, 0.5% HMW (High Molecular Weight rhamnogalacturonan) was used as substrate. For xylogalacturonase, 0.5% partially hydrolyze gum tragacanth was

used as substrate. Substrate was dissolved in 0.1M NH₄Ac, pH 4.1 for polygalacturonases, or in 50mM NH₄Ac, pH 4.5 for rhamnogalacturonase and xylogalacturonase.

For each enzyme, the reaction system was: 100ul reaction buffer (0.1M NH₄Ac, pH 4.1 for polygalacturonases, or 50mM NH₄Ac, pH 4.5 for rhamnogalacturonase and xylogalacturonase), 100ul substrate and 50ul concentrated media. The mixture was incubated at 37 °C for 1 hour. Then 200ul DNS was added and the mixture was incubated at 100 °C for 5 min. Data was read on HP Diode Array Spectrophotometer (8345A), at 590nm. Assays were run in triplicate. In the following product characterization assay, for each enzyme the amount which could digest 0.5ug substrate in 1 hour at 37 °C was employed.

2.8 PRODUCT CHARACTERIZATION

For different enzymes, different substrates were used. 1% pectic acid was used for polygalacturonase; 0.5% HMW was used for rhamnogalacturonase; 0.5% partially hydrolyzed gum tragacanth was used for xylogalacturonase. The reaction system was composed of 10ul substrate (dissolved in 0.1M NH₄Ac, pH 4.1 for polygalacturonases, or in 50mM NH₄Ac, pH 4.5 for rhamnogalacturonase and xylogalacturonase) and the amount of enzyme described above. 2ul aliquots were collected at 5min, 1hour, 6hours, 12hours, 24hours and 48hours, respectively.

2.9 CAPILLARY ZONE ELECTROPHORESIS

The collected products were labeled by ANTS. The labeling protocol is mixing 2ul of product, 10ul of 23mM ANTS (3:17 v/v acetic acid: water) and 1ul of 1M NaBH₃CN (in Me₂SO). The mixture was incubated at 80 °C for 1 hour. Samples were examined using a custom built instrument that has a laser-induced fluorescence detector. A helium-cadmium laser excited the samples, with an intensified charge-coupled device (ICCD) camera for detection of the fluorescence. A fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used as the column for separating of oligosaccharides. 0.1 M NaH₂PO₄ buffer (pH2.5) was used as the running buffer during runs and rinsing buffer for the capillary between runs. We injected buffer and samples into the capillary through the cathodic end (negative electrode) by siphoning for 6 seconds. The electrophoresis was performed at 18 kV.

The APTS labeling and detecting method is below. Substrates were mixed with 8-aminopyrene-1,3,6-trisulfonic acid (APTS 100 mM in 15% acetic acid) along with 1M NaBH₃CN (in Me₂SO). The mixture was heated at 80 °C for 1 hour. The substrates were digested by different enzymes, and the digested products were analyzed using a BioFocus 2000 (Bio-Rad Laboratories) capillary electrophoresis system with the enhanced laser-induced fluorescence detector. Column used for separating oligosaccharide was a fused-silica capillary with 50µm internal diameter, the same as the one used in ANTS detection. The samples were injected by 4.5psi of helium pressure, instead of siphoning, at the cathodic end (negative electrode). The electrophoretic system was working at 15 kV/70-100 µA and 20 °C. The same buffer, 0.1M NaH₂PO₄ buffer (pH2.5) was used as a running buffer while the rinsing buffer was 1M NaOH. Digested labeled substrates were

excited at 488nm, and the emission was collected using a 520nm band pass filter.

CHPATER III

RESULT

3.1 PCR

The gradient PCR determined the suitable temperatures for the DNA segments of all enzymes. All the segments worked well between 55 °C-65 °C (Fig. 3.1 A, B). The lengths of all the DNA segments are between 1300bp-1800bp.

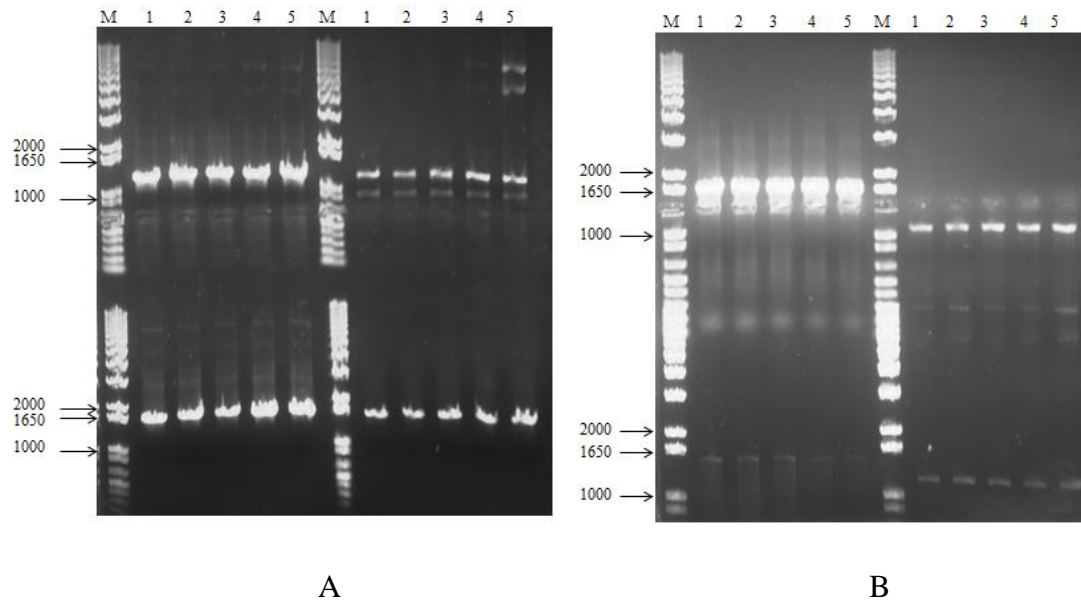


Figure 3.1 PCR results of all the eight enzyme segments. The temperatures for the five lanes are: 1-55.3 °C, 2-56.7 °C, 3-59.3 °C, 4-63.5 °C, and 5-64.8 °C (from left to right). A are 17220, 13920, 10503 and 02980; and B are 06410, 01970, 02630 and 06890. The figures were obtained on the 1% agarose gel.

3.2 TRANSFORMATION INTO *E. COLI*

There were still some blue colonies, usually more than white colonies (Fig. 3.2. Bigger image available upon request). All the white colonies were picked up to perform colony PCR. The PCR result showed that about 30% of the white colonies were recombinants (Fig. 3.3).

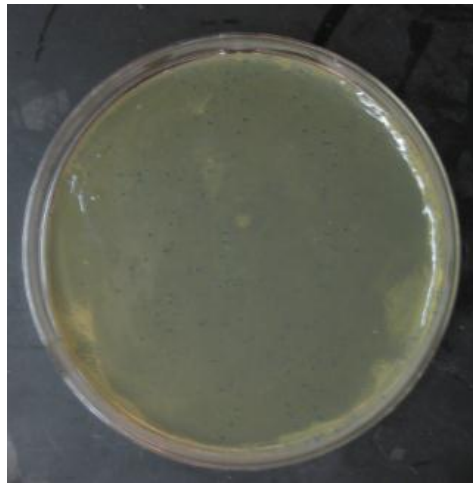


Figure 3.2 Blue/white colony screen. On this plate, there are about 50 blue colonies and about 15 white colonies.

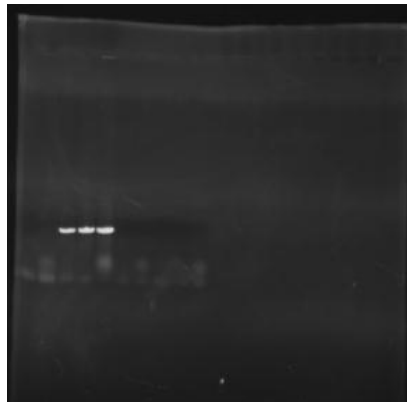


Figure 3.3 Colony PCR results of ten white colonies.

3.3 TRANSFORMATION INTO *ASPERGILLUS NIDULANS*

On each plate there were 4-20 colonies. The colony size varied. Most of the colonies were white in color while some were darker, yellow or brown in color (Fig. 3.4).The

darker ones did not grow not as well as the white ones. The recombinants were confirmed by spore-PCR. Spores from about 15% of the colonies contained the complete DNA segment (Fig. 3.5).

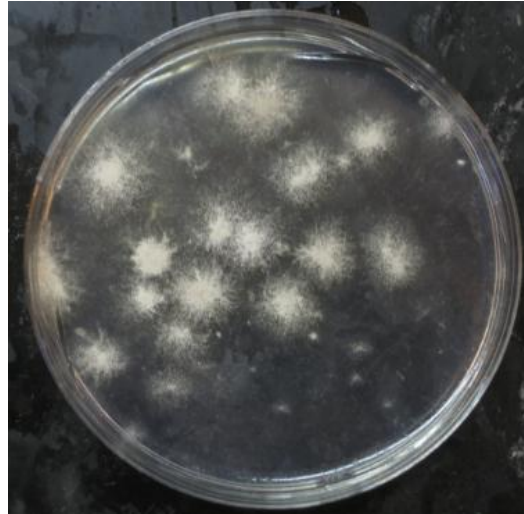


Figure 3.4 Transformed *Aspergillus nidulans* growing on minimum media with zeocin and glucose. This is 02980. This plate was incubated at 37 °C for two days.

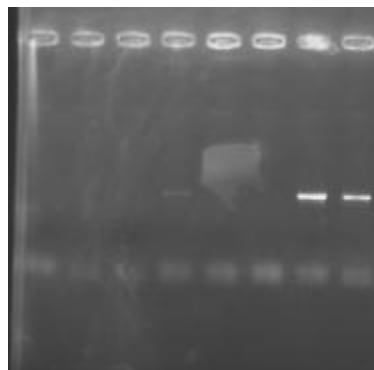


Figure 3.5 Spore PCR of seven colonies. The last one is positive control.

3.4 WESTERN BLOTTING

After being concentrated, the media was concentrated 50 folds again and applied onto an SDS-PAGE protein gel. The theoretical sizes of all expressed enzymes vary between 48kDa and 55kDa while the actual sizes are much bigger, varying between 60kDa and

115kDa (Fig. 3.6 A, B). Some of the lanes were not clear due to low concentration.

Western Blotting was performed to confirm the presence of the His-tag. Although all enzymes showed several bands on SDS-PAGE gel, only one of them displayed two clear signals in Western Blotting (Fig. 3.7).

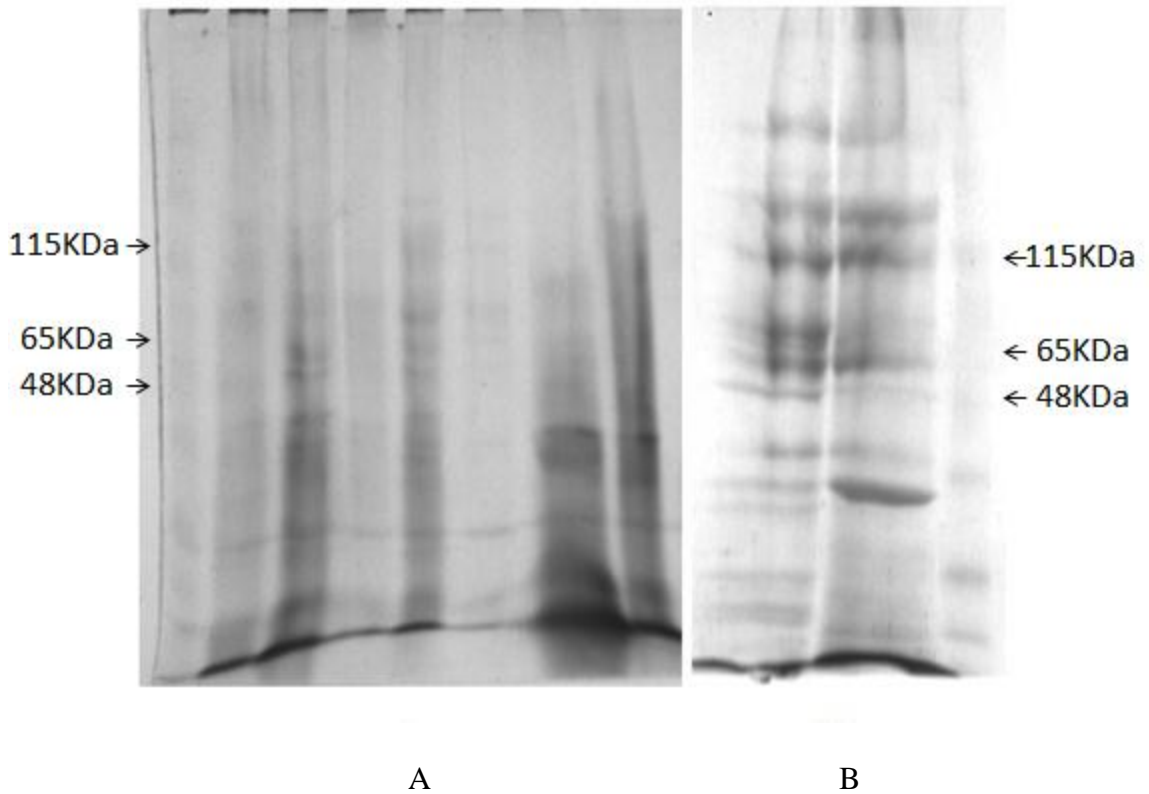


Fig 3.6 SDS-PAGE protein gel for all the enzymes. A, from left to right are: marker, 17220,13920,10503,02980,06410,01970,02630 and 06890. B, from left to right are: 13920, empty vector and marker.

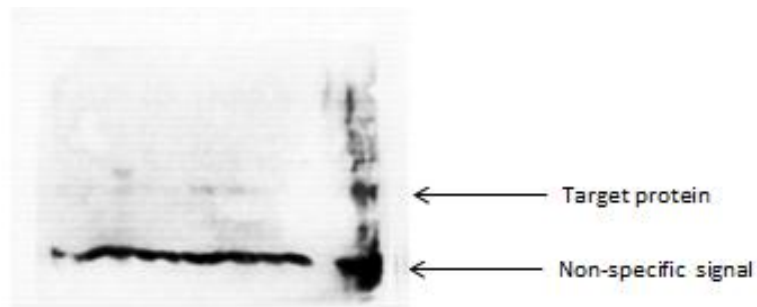


Figure 3.7 Western Blotting result for all the enzymes. From left to right are: 17220,13920,10503,02980,06410,01970,02630, 06890, negative control (empty vector), water and positive control.

3.5 PROTEIN PURIFICATION

The AFUA_4G13920@5789 and AFUA_5G10530@6817 transformants are shown as examples. Before applying to the nickel column, the concentrated media showed activity. After applying to the nickel column, the collected flow-through showed activity (Fig. 3.8 B, Fig. 3.9 B). However, the elution buffer fractions showed no activity (Fig. 3.8 C, Fig. 3.9 C), like negative control (Fig. 3.8 A, Fig. 3.9 A). The same result was observed for all expressed enzymes (data not all shown). Assays were run three times.

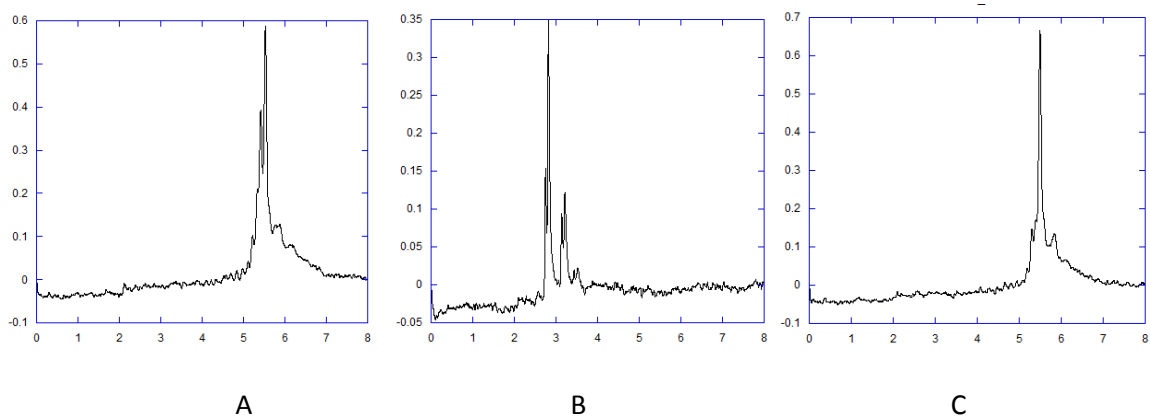


Figure 3.8 APTS labeled substrate for polygalacturonase. A is negative control without adding any enzyme. Empty vector media yielded the same result. B is concentrated media of exo-galacturonase (02980). Flow-through yielded the same result. C is collected elution buffer.

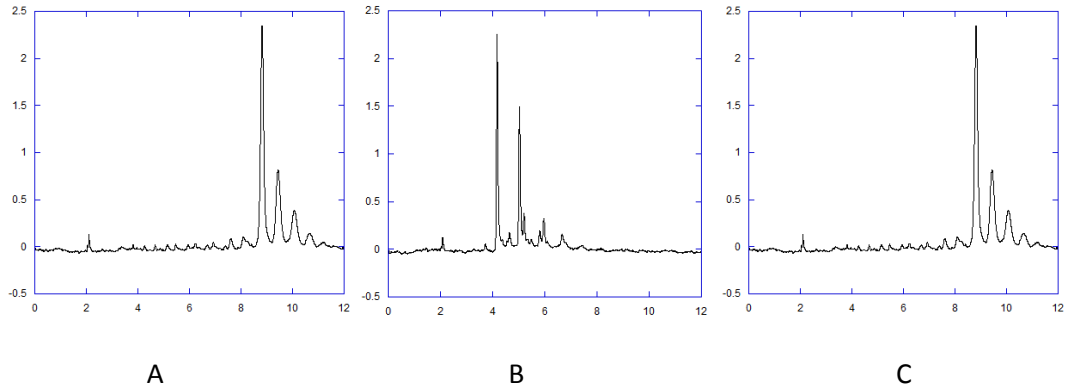


Figure 3.9 APTS labeled substrate for rhamnogalacturonase digestion. A is negative control, without adding any enzyme. Empty vector yielded the same result. B is concentrated media of rhamnogalacturonase (10530). Flow-through yielded the same result. C is collected elution buffer.

3.6 ENDO-POLYGALACTURONASES

The AFUA_1G17220@1581 is composed of 378 amino acids, with the first 19 ones forming the signal peptide. The AFUA_4G13920@5789 is composed of 368 amino acids, with the first 18 ones forming the signal peptide. The AFUA_8G01970@9340 is composed of 364 amino acids, with the first 23 ones forming the signal peptide. All of

them belong to the PLN 03003 superfamily and the Glycoside Hydrolase Family 28 multi-domains (data from blastp result, NCBI).

The DNA sequences of these three endo-polygalacturonases from *A. fumigatus* and *A. niveus* were compared. Based on the sequence of *A. fumigatus*, several differences were found in each enzyme of *A. niveus*. In 17220, *A. niveus* has four differences. Two of them are silent differences, counting from the beginning, 294 ACG->ACA, whose amino acid is still Thr; and 876 AAG->AAA, whose amino acid is still Lys. There are two missense differences, 52 CCT->TCT, which changes Pro into Ser, and 477 ATA->ATG, which changes Ile into Met. In 13920, *A. niveus* has three differences, all of which are silent differences. They are 132 GCC->GCA, whose amino acid is still Ala; 420 ATT->ATC, whose amino acid is still Ile; and 771 CAA->CAG, whose amino acid is still Gln. In 01970, *A. niveus* has three differences. Two of them are silent differences, 90 CAG->CAA, whose amino acid is still Gln; and 741 ACT->ACC, whose amino acid is still Thr. The last one is a missense difference, 94 ATC->GTC, which changes Ile into Val.

For 17220, the product characterization result is below (Fig. 3.16). From start to end, the products were mainly GalA₁-GalA₅, while some longer ones with fewer amounts. In order to avoid the possibility that after long digestion it would digest GalA₅ into shorter ones, we incubated the reaction system at 37 °C for longer time, 3 days, 4 days, 5 days and 7 days (Fig. 3.10). No product change was observed within GalA₁-GalA₄ besides the amount of them increased. However, the amount of GalA₅ decreased. And these products longer than 5 residues were digested at the end of 7 days. It was observed that for each

product there might be double peaks. The double peaks were caused by lactonization of products. Zhang et al. reported that the secondary peaks could be eliminated by adding an equal volume of 0.1M NaOH [131].

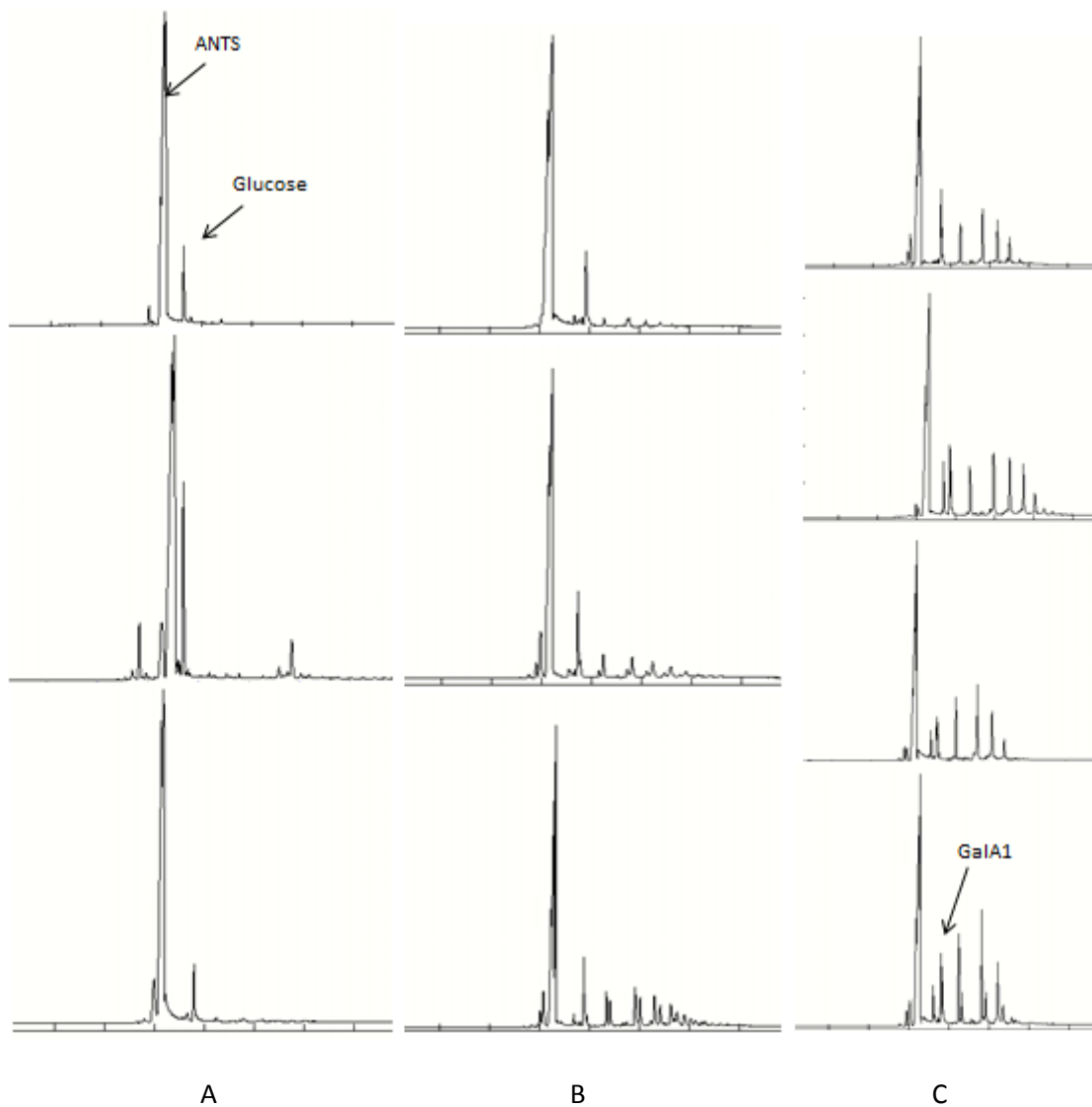


Figure 3.10 The 17220 product characterization result. From top to bottom, A are 5 min, 1 hour and 6 hours; B are 12 hours, 1 day and 2 days; and C are 3days, 4 days, 5 days and 7 days . X axis is time and Y axis is emission intensity.

For 13920, the product characterization result is below (Fig. 3.11). 13920 started working faster than other polygalacturonases. In the first 6 hours, the main product was GalA₁. After 6 hours, products of longer length (GalA₂-GalA₅) began to accumulate. Longer incubation was performed to make sure if 13920 could digest any product. At the end of 5th day, the final products were GalA₁ and GalA₂.

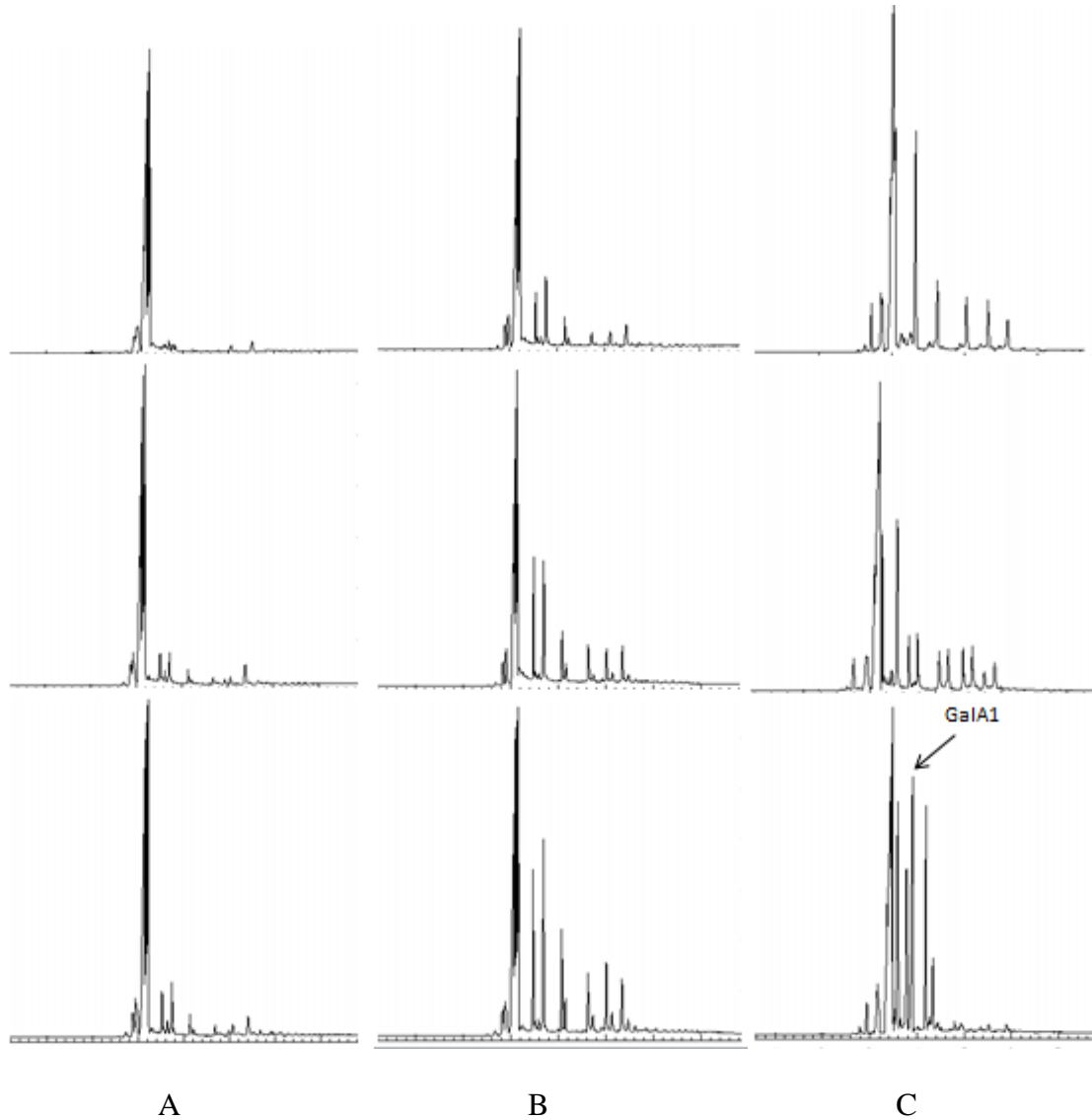


Figure 3.11 The 13920 product characterization result. From top to bottom, A are 5 min, 1 hour and 6 hours; B are 12 hours, 1 day and 2 days; and C are 3 days, 4 days and 5 days. X axis is time and Y axis is emission intensity.

For 01970, it worked in a very similar way as 17220. The product characterization result is below (Fig. 3.12). From start to 2 days, the products were always GalA₁-GalA₅. In order to avoid the possibility that after long digestion it would digest GalA₅ into shorter ones, we incubated the reaction system at 37 °C for longer time, 3 days and 5 days. No product change was observed within GalA₁-GalA₅ besides the amount of them increased. However, some longer products began to appear. It is proposed that probably the 01970 worked in a random style, which also generated longer products longer than GalA₅. With the development of time, the amount of longer products accumulated enough to show peaks.

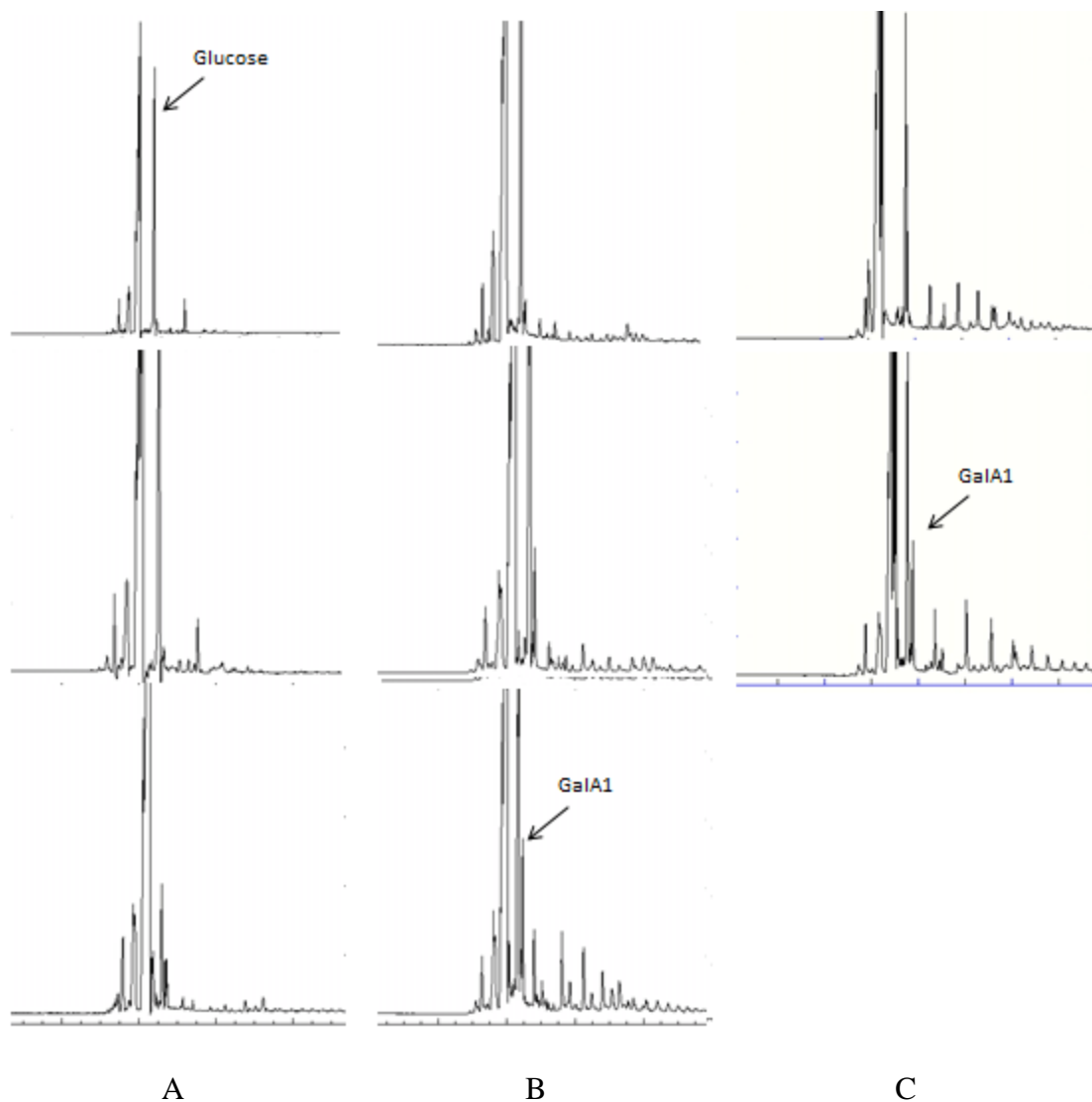


Figure 3.12 The 01970 product characterization result. From top to bottom, A are 5 min, 1 hour and 6 hours; B are 12 hours, 1 day and 2 days; and C are 3 days and 5 days. X axis is time and Y axis is emission intensity.

3.7 EXO-POLYGALACTURONASES

The AFUA_6G02980@7470 is composed of 442 amino acids, with the first 23 ones forming the signal peptide. The AFUA_7G06410@9053 is composed of 496 amino acids, without signal peptide. The AFUA_8G02630@9409 is composed of 364 amino acids, with the first 16 ones forming the signal peptide. All of them belong to the PLN03003 superfamily and the Glycoside Hydrolase Family 28 multi-domains (data from blastp result, NCBI).

In 02980, *A. niveus* has three differences, all of which are silent ones. Counting from the beginning, they are 387 AAC->AAT, whose amino acid is still Asn; 429 ATT->ATC, whose amino acid is still Ile; and 693 TAC->TAT, whose amino acid is still Tyr. In 06410, *A. niveus* has eight differences. There are two silent differences, 315 TCT->TCC, whose amino acid is still Ser; and 1398 GAT->GAC, whose amino acid is still Asp. There are five missense differences: 596 AAT->AGT, which changes Asn into Ser; 1184 GGC->GCC, which changes Gly into Ala; 1216 CCA->GCA, which changes Pro into Ala; 1313 AGC->AAC, which changes Ser into Asn; and 1448 GGG->GCG, which changes Gly into Ala. There is a triple base pair insertion: 51 ATA, which inserts an Ile. In 02630, *A. niveus* has eight differences. There are four silent differences: 168 GGC->GGT, whose amino acid is still Gly; 453 TCC->TCT, whose amino acid is still Ser; 765 GAT->GAC, whose amino acid is still Asp; and 771 GGT->GGC, whose amino acid is still Gly. There are four missense differences: 71 GCT->GTT, which changes Ala into Val; 185 CAT->CGT, which changes His into Arg; 210 AAT->AAG, which leads Asn into Lys; and 1328 AGG->AAG, which changes Arg into Lys.

For 02980, the product characterization result is below (Fig. 3.13). From start to end, the product was always GalA₁. Its digestion mechanism is exo-style, which means it cut one residue at one time from the end.

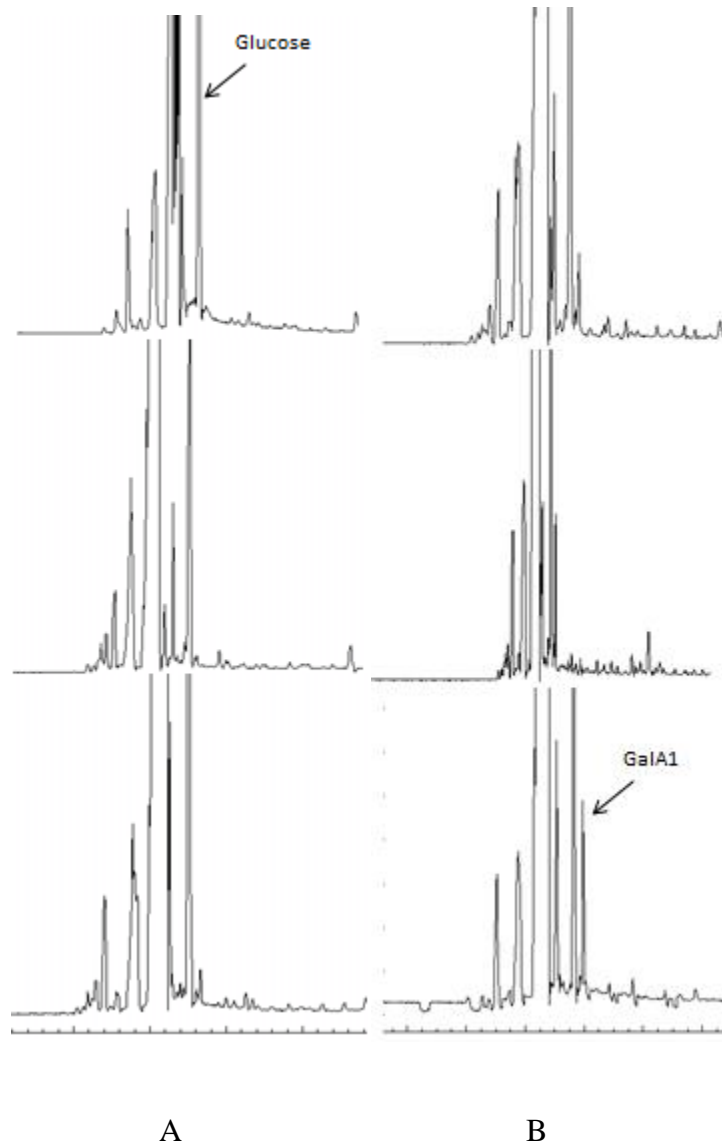


Figure 3.13 The 02980 product characterization result. From top to bottom, A are 5 min, 1 hour and 6 hours; and B are 12 hours, 1 day and 2 days. X axis is time and Y axis is emission intensity.

For 06410, it is noteworthy that 06410 could not work on long substrate such as GalA₁₈ (Fig. 3. 14). It was found in activity confirmation. In order to get a better understanding, GalA₃, GalA₄ and GalA₅ were used as substrates. No convincing evidence was found that it could digest them efficiently (Fig. 3.15). However, it still could work on pectic acid. The product characterization result is below (Fig. 3.16). From start to end, the product was always GalA₁. Its digestion mechanism is exo-style, which means it cut one residue at one time from the end. Since pectic acid is mainly composed of polygalacturonic acid with variant lengths, it is still unknown about the size of substrate which 06401 could work on.

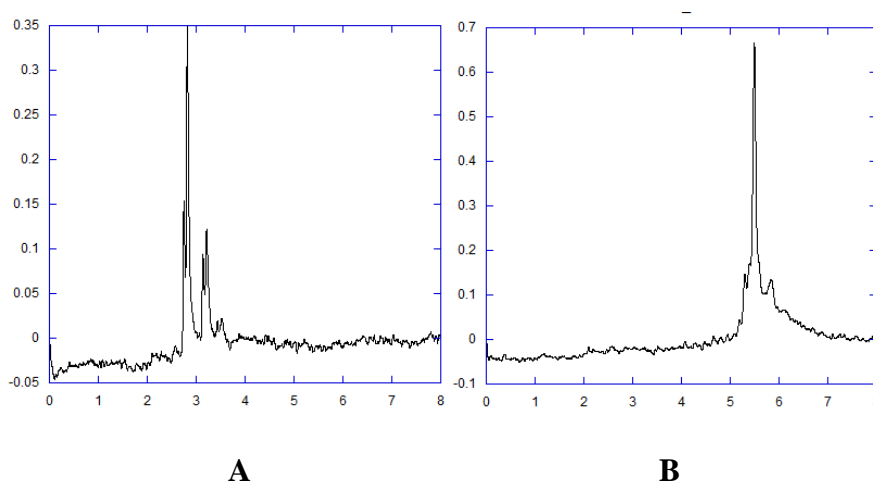


Figure 3.14 Digestion on APTS labeled GalA₁₈ result. A is 02980 and B is 06410.

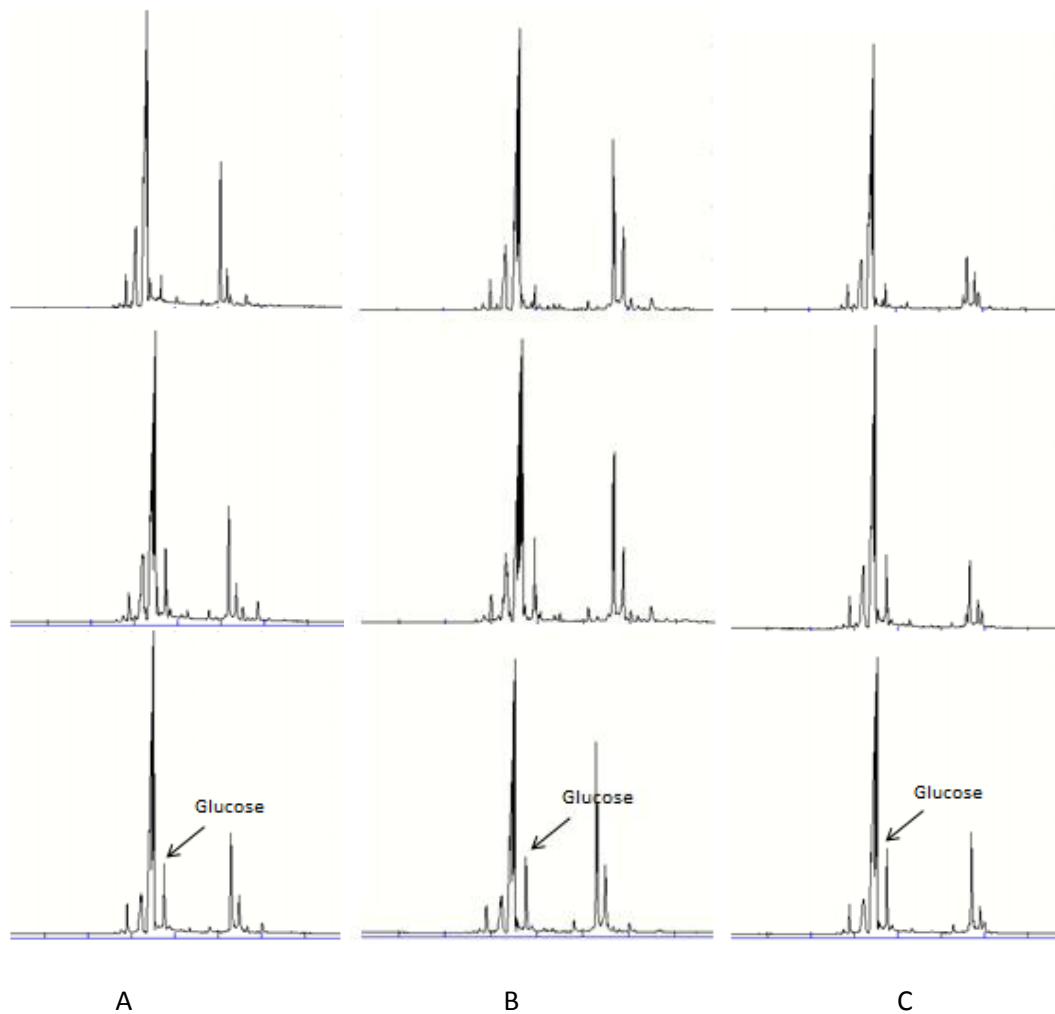


Figure 3.15 The 06410 product characterization result. From top to bottom are 5 min, 3 days and 5 days. A is GalA₃; B is GalA₄ and C is GalA₅. X axis is time and Y axis is emission intensity.

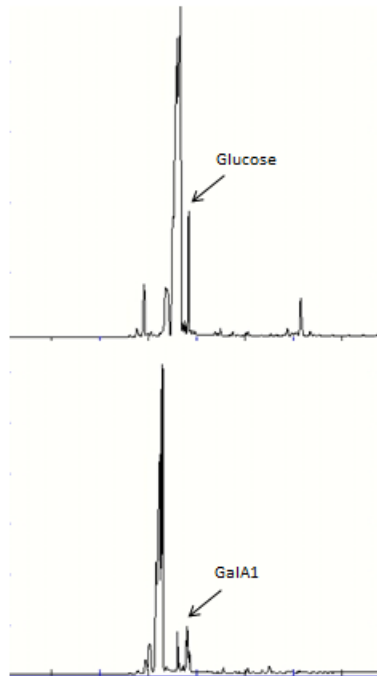


Figure 3.16 The 06410 product characterization result on pectic acid. Top is 5min and bottom is 2 days. X axis is time and Y axis is emission intensity.

For 02630, the product characterization result is below (Fig. 3.17). From start to end, the product was mainly GalA₁ while some less amount of GalA₂. Its digestion mechanism is exo-style, which means it usually cut one residue at one time from the end. Sometimes it cut two.

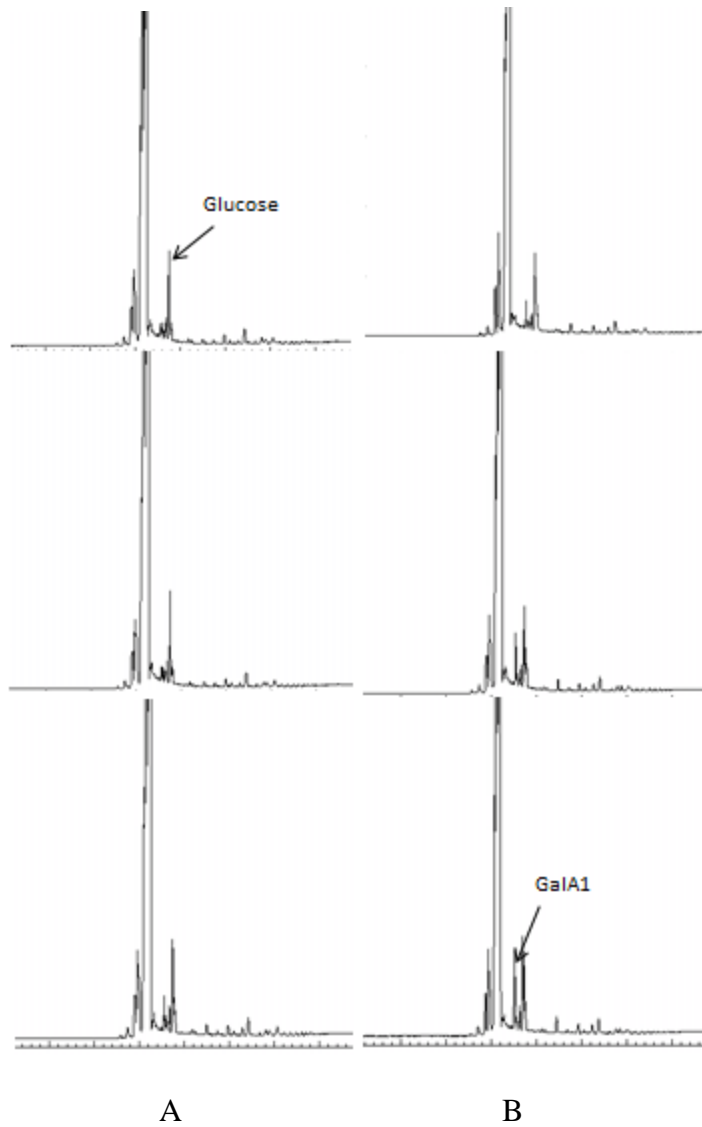


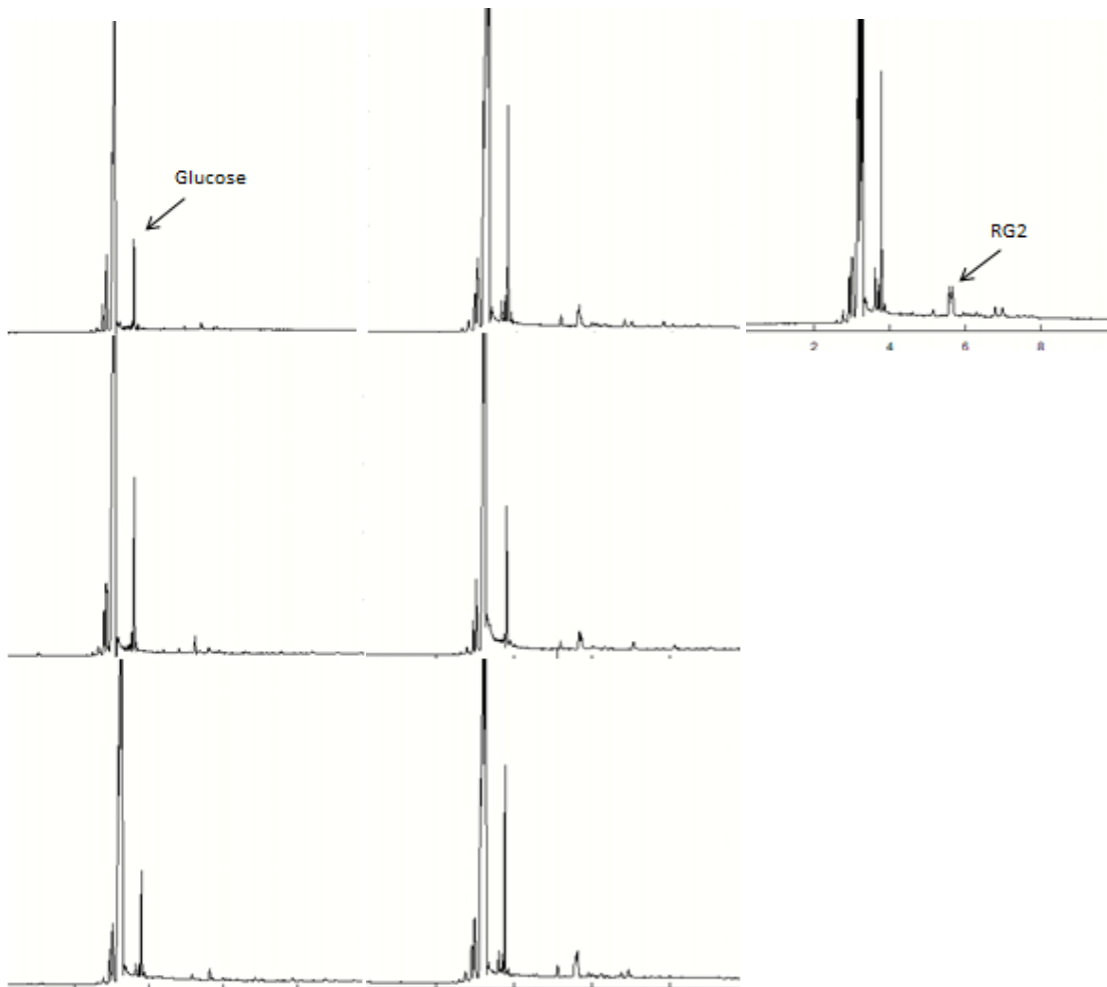
Figure 3.17 The 02630 product characterization result. From top to bottom, A are 5 min, 1 hour and 6 hours; and B are 12 hours, 1 day and 2 days. X axis is time and Y axis is emission intensity.

3.8 RHAMNOGALACTURONASE

The AFUA_5G10530@6817 is composed of 521 amino acids, with the first 21 ones forming the signal peptide. It belongs to the PLN03003 superfamily and the Pectate Lyase 3 multi-domains (data from blastp result, NCBI).

In 10503, *A. niveus* has four differences. There are three silent differences: 267 GGT->GGA, whose amino acid is still Gly; 477 TGC->TGT, whose amino acid is still Cys; and 837 TTG->TTA, whose amino acid is still Leu. There is one missense difference: 1190 TCC->TTC, which changes Ser into Phe.

For 10503, the product characterization result is below (Fig. 3.18). After 1 hour, RG₂ began to accumulate. After 2 days, the amount of RG₂ was very obvious, while RG₃ and RG₄ were a little. In order to confirm this, 5-day incubation was performed. The final products were RG₂, RG₃ and RG₄.



A

B

C

Figure 3.18 The 10503 product characterization result. From top to bottom, A are 5 min, 1 hour and 6 hours; B are 12 hours, 1 day and 2 days; and C is 5 days. X axis is time and Y axis is emission intensity.

3.9 XYLOGALACTURONASE

The AFUA_8G06890@9731 is composed of 409 amino acids, with the first 21 ones forming the signal peptide. It belongs to the PLN03003 superfamily and the Glycoside Hydrolase Family 28 multi-domains (data from blastp result, NCBI).

In 06890, *A. niveus* has six differences. There are three silent differences: 285 AAT->AAC, whose amino acid is still Asn; 837 TCG->TCA, whose amino acid is still Ser; and 924 TCC->TCA, whose amino acid is still Ser. There are three missense differences: 520 CGC->TGC, which changes Arg into Cys; 1070 GCT->GTT, which changes Ala into Val; and 1057 GTG->TTG, which changes Val into Leu.

For 06890, the product characterization result is below (Fig. 3.19). NMR was performed to analyze the product, but no useful information was obtained. The product details will be identified using NMR again soon.

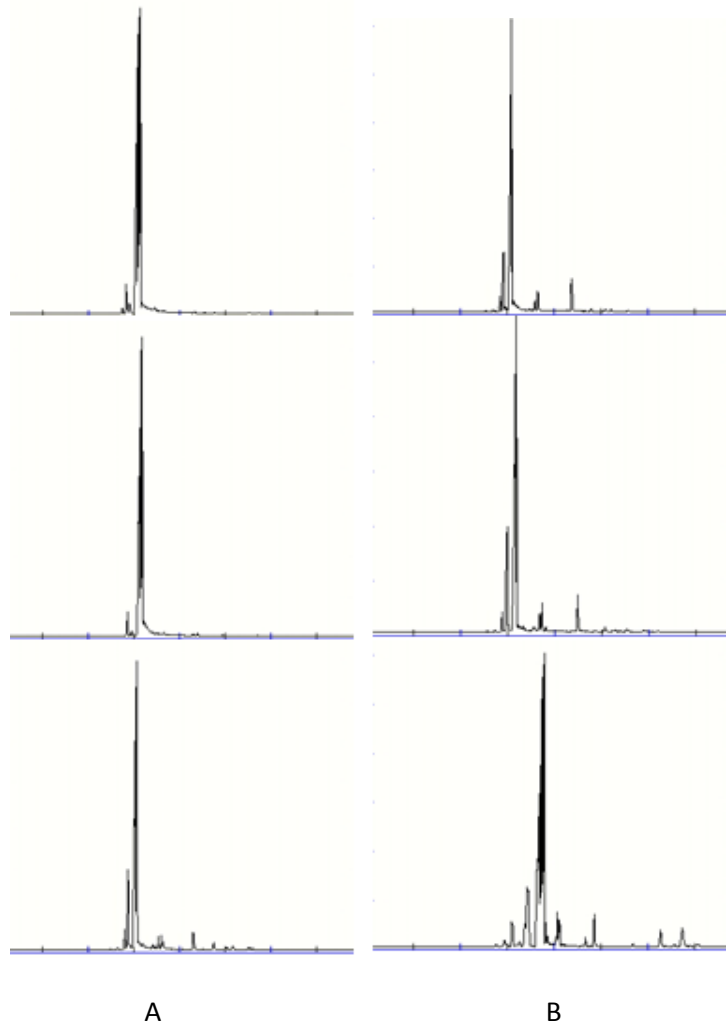


Figure 3.19 The 06890 product characterization result. From top to bottom, A are 5 min, 1 hour and 6 hours; and B are 12 hours, 1 day and 2 days. X axis is time and Y axis is emission intensity.

3.10 EMPTY VECTOR

The concentrated media from the empty-vector-colony showed no activity against any of the substrates (Fig. 3.20). There are some other negative controls as reference (Fig. 3.21).

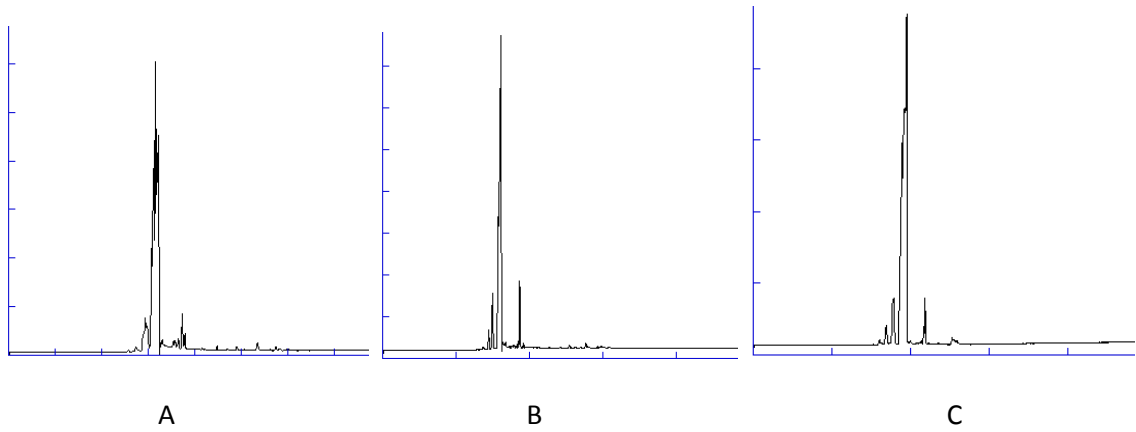


Figure 3.20 Empty-vector-colony 2-day digestion results. A is pectic acid, B is HMW and C is partially hydrolyzed gum tragacanth.

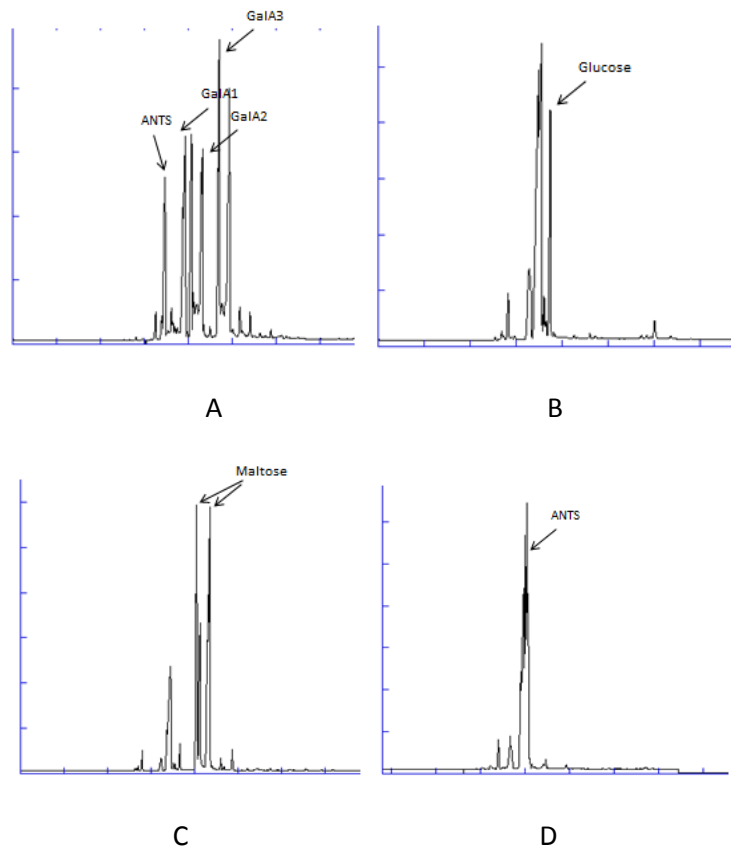


Figure 3.21 Negative controls. A is GalA₁-GalA₃, B is glucose; C is maltose and D is ANTS (with labeling anything).

CHAPTER IV

DISCUSSION

In the current world, energy is a big problem. The main energy source is fossil energy, including coal, oil and natural gas. These sources support 70% of current energy consumption [132]. They have many advantages. First, there is still a great amount of them left for us. Second, they are easy to transport and to store. Third, current technologies for using them are mature. Fourth, they are relatively safe. However, they also bring us many disadvantages. First, although they still exist in a great amount, the depletion rate is very high. It is predicted that in next decade they probably will run out [133]. Second, they are non-renewable. The process of forming fossil energy needs millions of years. Third, their usages cause heavy pollution to our environment. Each year, coal burning produces 3,700,000 tons of carbon dioxide, 10,000 tons of sulfur dioxide, 10,200 tons of nitrogen oxide, 720 tons of carbon monoxide, 500 tons of small airborne particles, 48 tons of mercury and 114 pounds of lead (data from Union of Concerned Scientists). Oil has a well-known potential hazard: oil spill. It refers to the release of oil into ocean or coastal water. It damages ecology greatly. Worse, it demands months or even years to clean up, and decades to recover [134]. There are, of course, other types of energy. Nuclear power is popular, and highly efficient. However, its safety is always a hot topic which leads to arguments. The Chernobyl disaster in 1986 affected a big area of Europe. Even now, the effect is still evident. Recently, the earthquake and tsunami that happened in Sendai, Japan damaged the nuclear power plant seriously. It is

reported that the nuclear fuel has already leaked out into the Pacific Ocean, impacting the round sea and countries including Korea, China, even the United States. Wind power is also a popular energy. Currently it accounts for 2.5% of worldwide electricity [135, 136]. Its biggest advantage is little environmental effect compared to other kinds of energy sources. But the availability limits its usage greatly. It is also a problem to solar energy. Solar energy availability is influenced by many factors such as daytime length, atmospheric effects, latitude and power transmission infrastructure.

Bioenergy is a promising substitute for current energy sources. In most cases, bioenergy could be considered as biofuel, which supported 1.8% of worldwide transport fuel [137]. Biofuel includes bioethanol, biodiesel, bioether, biogases and solid biofuels such as wood, sawdust and grass. The most common method for using solid biofuels is burning the raw biomass or densified products. The main component of solid biofuel is cell walls, which mostly compose of cellulose, hemicellulose and pectin. Fungi are successful organisms, which feed on a variety substrates. Some species take cell wall as carbon resource. They secrete enzymes to degrade cell walls, then absorb the products as their food. Based on this background, we cloned several enzymes from *A. niveus*. One is to degrade xylogalacturon; one is to degrade rhamnogalacturon; and six are to degrade polygalacturonan.

We obtained the *A. niveus* from Dr. Rolf Prade, while he received it from Brazil. The sequencing result showed it is very close to *A. fumigatus* with some insignificant

differences (data also shown above). However, it is white in color while *A. fumigatus* is green. Additionally, *A. niveus* is more pathogenic than *A. fumigatus*. Dr. Prade found *A. niveus* killed mice much faster. Therefore, it is difficult to conclude if the *A. niveus* is the true *A. niveus* or just *A. fumigatus* with some mutations.

All the eight enzymes from *A. niveus* have been compared by ClustalW2. The phylogenetic tree has been generated (Fig. 4.1). It is obvious that three endo-polygalacturonases are closely related to one another. Similarly, three exo-polygalacturonases are also closely related. However, to our surprise, rhamnogalacturonase is closer to endo-polygalacturonases than distance between endo-galacturonases and exo-polygalacturonases. Another point attracted our attention that AFUA_7G06410@9053 is apparently longer than other polygalacturonases (Fig. 4.2), while having no signal peptide. Probably it is an intracellular exo-polygalacturonase. We propose that the longer structure might account for the function of the 06410 to work on short substrates. The blastp result showed the non-homologous in 06410 is also non-homologous to other known conservative domain. However, the specific length of substrate for 06410 still remains unknown.

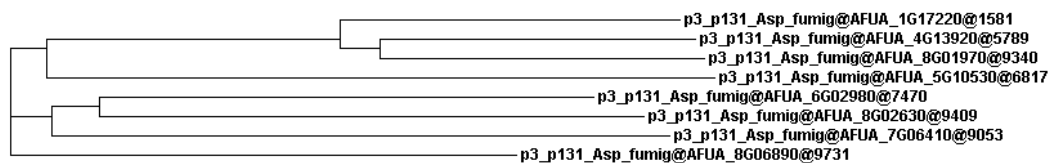


Figure 4.1 The phylogenetic tree concerning eight enzymes. Generated by ClustalW2, EMBL-EBI, www.ebi.ac.uk

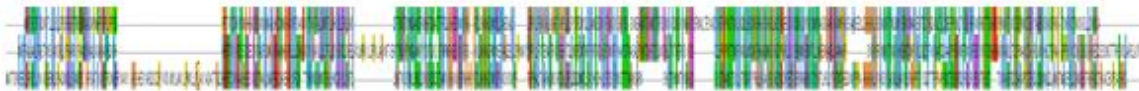


Figure 4.2 Amino acid sequence alignment among the three exo-polygalacturonases. From top to bottom: 02980, 01970 and 06410. Cyan is hydrophobic amino acid; green is polar uncharged amino acid; red is positive charged amino acid; purple is negative charged amino acid; lemon is proline; and scarlett is cysteine or glycine.

The six enzymes showed similar functions with a few differences. The final products of 17220 are believed to be GalA₁-GalA₄ while the ones of 13920 are GalA₁ and GalA₂. The 01970 gave mainly GalA₁-GalA₅ while a little amount of longer ones. Both the 02980 and 06410 produced single GalA. The 02980 worked on long chain while the 06410 could only work on some short substrate. We did not determine the exact length of optimal substrate for the 06410. Last, the final products of 02630 are GalA₁ and GalA₂. We proposed a model for all six of them. *A.niveus* secreted five of them into surrounding environment. The 02980 and the 02630 digest polygalacturonan in an exo-acting mechanism. They cut off one residue or two at one time from one end. The 17220, the 13920 and the 01970 digest polygalacturonan in an endo-acting mechanism. The 01970 degrades long substrate into shorter ones randomly, which are mainly composed of from one to five residues, but also having some longer ones. Then the 17220 degrades the ones which are longer than five residues into from one to five residue products. The 13920 continues to make the 17220-product into even shorter ones, usually only one or two

residues. Some of the products might be transported into cell by some membrane transportation protein(s). Then the 06410 degrades the short products in an exo-style, which makes substrate into single galacturonic acid.

In 1999 endo-polygalacturonase II from *A. niger* was crystallized. The active sites were scanned. Five residues are believed to be the active sites: Asp¹⁸⁰, Asp²⁰¹, and Asp²⁰², Arg²⁵⁶, and possibly Lys²⁵⁸ [138]. Our data shows that based on the endo- and exo-polygalacturonase sequences of *A. fumigatus*, *A. niveus* has several differences, in which some leads to missense mutations. However, these differences do not involve in any active sites of the known residues.

His-tagging is a popular method employed in protein purification. The expression plasmid, pExpyr+, has a DNA sequence encoding for a Hist-tag at the C terminal of the to-express protein. However, all the expressed enzymes did not show any evidence that they bind to the Ni-NTA resin. There are many possible explanations for this phenomenon. Previous research showed that the endo-polygalacturonase from *Colletotrichum lupine* has a heptamer structure, which hides the C terminal of each monomer (Fig. 4.3)[139]. There are some other structures of endo-polygalacturonase. The endo-polygalacturonase II from *A. niger* does not show an oligomeric structure (Fig. 4.4). From the structure, the C terminal is on the surface, not folded inside. However, there are two structures which may have brought steric hindrance to the C terminal. The first is a zinc ion at the Asp³³⁶, which is located near to the C terminal. The second is a

disulfide bridge between Cys³⁵³ and Cys³⁶², in which the latter one is the first amino acid at the C terminal [138]. These structural features might be responsible for the failure in purification. In 1997, Petersen et al. cloned and crystallized RGase A from *A. aculeatus*. The C-terminal is not hidden inside. Instead, it is exposed outside (Fig. 4.5). It was found that all 18 O-linked glycosylation sites are at the C-terminal tail. The glycosylation is believed to protect the tail from proteolytic degradation. Additionally, this C-terminal tail is closed to the core of this enzyme molecule [140]. These two properties could account for the RGase His-tag purification failure. And because not only RGase but also all other enzymes showed much bigger sizes than expected, we proposed glycosylation is the main reason accounting for size changes and failures in purification of all the enzymes. Besides, Western Blotting showed signals at the lower molecular weight positions, indicating the proteins at higher molecular weight positions could not bind to His-tag antibody. It is also evidence supporting that glycosylation stopped exposure of His-tag. There is a third possible explanation. Fig. 4.6 shows that the RGase B from *A. niger* and RGase from *A. niveus* has an extra domain compared to RGase from *A. aculeatus* and RGase A from *A. niger*. Fu et al. defined it as the domain H, which is rich in serine and threonine. This region is non-homologous to any pectinase [141]. It has been proposed that the domain H might get involved in specificity of enzymes and substrates binding [142]. Because the 10503 also has such a domain H, the serine and threonine might compete with His-tag to bind to Ni ion. That is the third possible explanation. In order to get more support for this proposal, the glycosylation prediction was performed on the RGase. The prediction result showed that at the C-terminal could be highly O-glycosylated (Fig. 4.7).

It is noteworthy that the DNA fragments of all enzymes were cloned directly from genomic DNA, instead of cDNA. As is well known, genomic DNA contains introns. Thus, the traditional way to express protein is to clone from cDNA, which does not have introns. This method could avoid possible splicing errors. However, at this time no obvious splicing errors are evident because all expressed enzymes showed activity and the His-tag was expressed correctly.



Figure 4.3 Crystal structure of endo-polygalacturonase from *Colletotrichum lupine* [139]

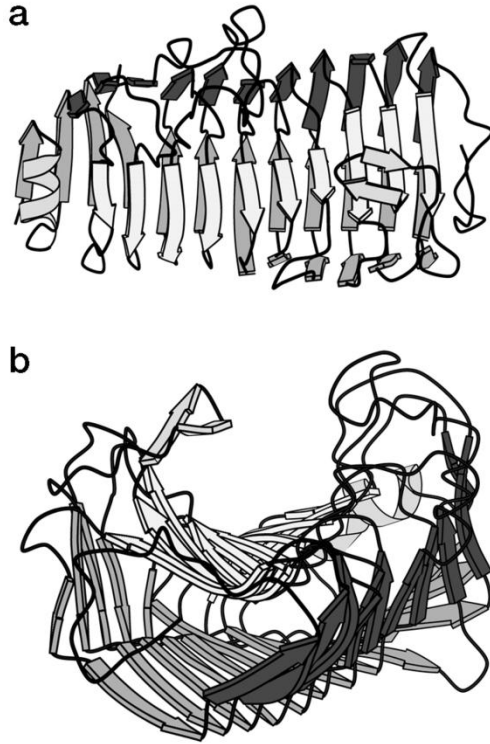


Figure 4.4 Crystal structure of endo-polygalacturonase II from *A. niger* [138]

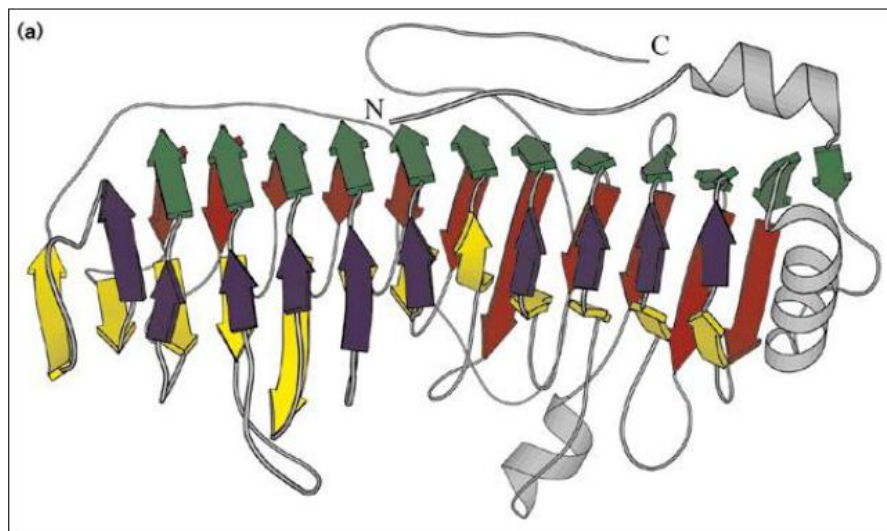
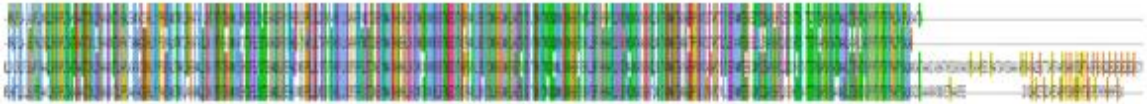
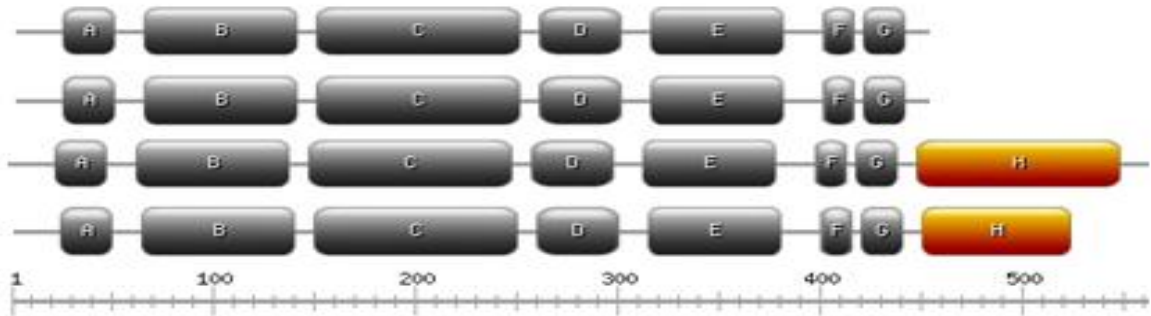


Figure 4.5 Crystal structure of RGase A from *A. aculeatus* [140]



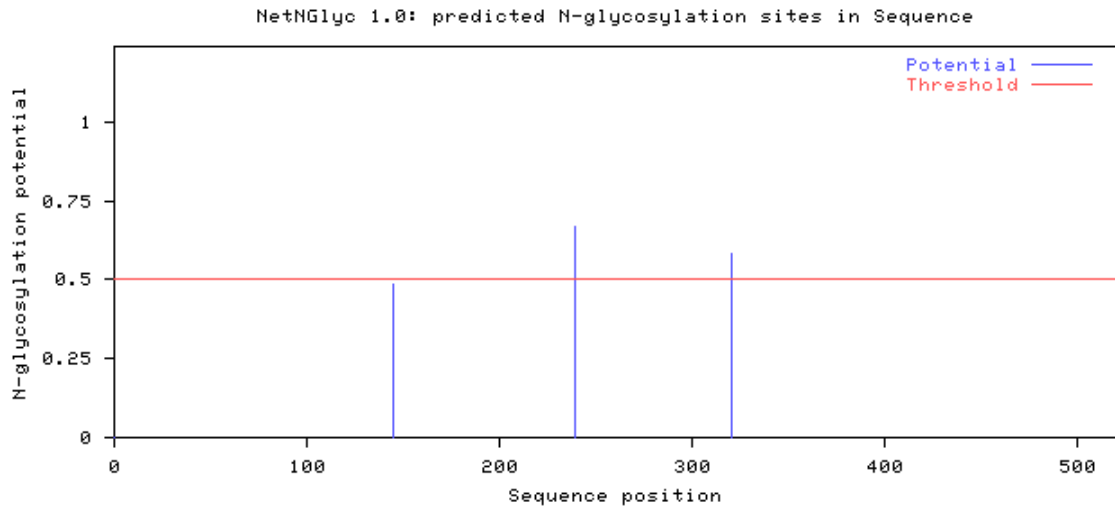
A



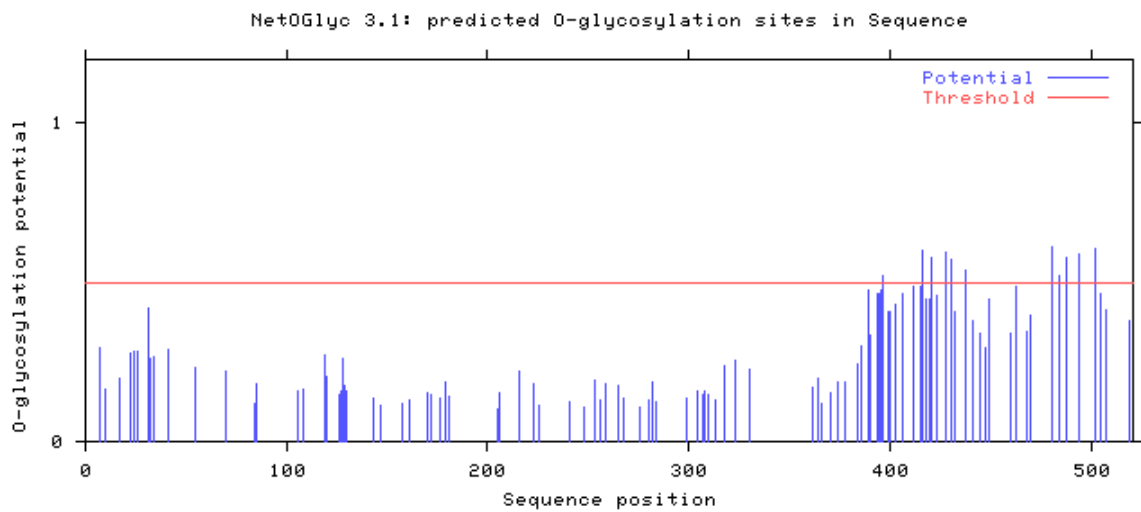
B

Figure 4.6 Sequence alignment among four RGases. From top to bottom: RGase A from *A. niger*, RGase from *A. aculeatus*, RGase B from *A. niger*, and RGase from *A. niveus*.

A is amino acid sequence alignment among four RGases. In A, cyan is hydrophobic amino acid; green is polar uncharged amino acid; red is positive charged amino acid; purple is negative charged amino acid; lemon is proline; and scarlett is cysteine or glycine. In B, domain A,B,D,F and G are RGase specific; domain C and E are pectinase specific; and domain H is non-homolous C-extension.



A



B

Figure 4.7 Glycosylation on RGase prediction results. A is N-glycosylation and B is O-glycosylation.

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Candidate for the Degree of Master of Science

Major Field: Biochemistry and Molecular Biology

Scope and Method of Study: One big part of biomass is in the form of plant cell walls. Fungi play an essential role in carbon and nitrogen recycling, in which one of the means is by degrading polysaccharides that human cannot utilize efficiently. To get a deeper insight into degradation of pectin, one of the major groups of plant polysaccharides six polygalacturonases, one rhamnogalacturonase, and one xylogalacturonase from a thermophilic fungus, *Aspergillus niveus* found widely in soil and decaying organic matter, were cloned and expressed in *Aspergillus nidulans* fused to a his tag. These enzymes break down different components of pectin. Their functions and activities are studied by cloning using *E. coli*, expression using *A. nidulans*, purification using Ni-NTA, and activity determination using various substrates.

Findings and Conclusions: Western Blotting detected that the His-tag was expressed correctly. However, purification using Ni-NTA failed, indicating the His-tag was not able to bind to Ni resin. SDS-PAGE protein gels showed the actual sizes of the proteins were larger than theoretical sizes. Expression and purification studies suggested that all eight enzymes expressed in *A. nidulans* were probably highly glycosylated. Pectic acid was used as substrate for the six polygalacturonases; rhamnogalacturonan from citrus pectin was used for the rhamnogalacturonase and partially hydrolyzed gum tragacanth was used for the xylogalacturonase. Only one exo-polygalacturonase does not have signal peptide. Among eight enzymes, three of six polygalacturonases are exo-polygalacturonases and the others are endo-polygalacturonases. For the exo-polygalacturonases, all of them cut off one or two GalA at a time. One has some limitation on length of substrate. The specific limitation remains unknown. For the endo-polygalacturonases, all of them work in endo-style. Final products from pectic acid for one of them were GalA₁-GalA₄. For another they were only GalA₁ and GalA₂ while for the third the main products were GalA₁-GalA₅. For the rhamnogalacturonase, the final products were RG₂ and RG₃. And for the xylogalacturonase, the information about final products is unavailable at present. NMR will be performed for their identification.

ADVISER'S APPROVAL: Andrew J. Mort
