INDUCTION OF PECTIC ENZYMES IN

ASPERGILLUS NIDULANS

By

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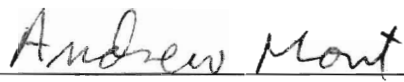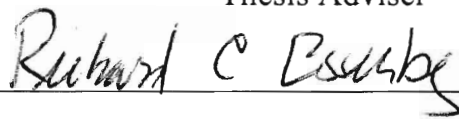
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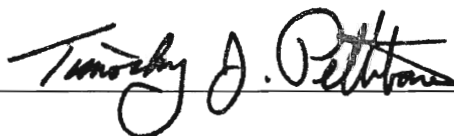INDUCTION OF PECTIC ENZYMES IN

ASPERGILLUS NIDULANS

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LIST OF ABBREVIATIONS

APTS	9-aminopyrene-1,4,6-trisulfonate
Ara	Arabinose
CZE	capillary zone electrophoresis
Endo-Ara	endo-arabinanase
Endo-PG	Endo-polygalacturonase
Endo-PL	Endo- Pectate Lyase
Exo-Ara	Exo-arabinase
Exo-PG	Exo-polygalacturonase
Exo-PL	Exo-Pectate Lyase
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic Acid
Glc	Glucose
GLC	gas liquid chromatography
HG	homogalacturonan
HPLC	high performance liquid chromatography
LIF	laser induced fluorescence
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
Man	Mannose
PG	polygalacturonase
PL	Pectate Lyase
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
Rha	Rhamnose
SCB	sodium cyanoborohydride
XGA	xylogalacturonan
Xyl	Xylose

CHAPTER 1

GENERAL INTRODUCTION

The fungi are an important group of organisms whose significance to humanity has been recognized for only a little more than a century, although some have been successfully domesticated for thousands of years without the realization of their existence. Being microorganisms, they do their work both for and against the interests of humanity in hiding, and become readily apparent only during their reproductive phases. The fungi are important as primary agents of decay in the cycling of carbon, nitrogen, and other nutrients in the biosphere and in the deterioration of useful materials and products. They cause serious diseases in plants and animals, including people, not only by their direct attack and invasion, but also indirectly through the secretion of toxins. Their abilities to synthesize many strange and wonderful compounds significant to us is not limited to deleterious activities, but includes the production of many materials important to the food, drug, and chemical industries. Although the control of diverse activities of fungi in preventing their deleterious activities and promoting their useful ones has been managed to some extend empirically, greater success can be expected from an informed basis of understanding their physiology and life cycles(1). One of the barriers against phytopathogenic fungi is the plant polysaccharide-rich cell wall. The vast majority of the fungi need to break these barriers to gain access to the plant tissue, and to

achieve this purpose they secrete a number of enzymes capable degrading the wall polymers. When fungi are grown on plant cell wall material *in vitro*, pectic enzymes are invariably the first enzymes to be secreted, followed by hemicellulases and cellulases (2).

The action of pectic enzymes and in particular polygalcturonases on cell walls appears to be a prerequisite for wall degradation by other enzymes. Only after pectic enzymes have acted on their substrates, the cellulose-xyloglucan framework, which is normally embedded in the pectin matrix, becomes accessible and inducers for cellulases and hemicellulases can be released. Purified pectic enzymes capable of cleaving the α-1,4-glycosidic bonds of homogalacturonan in an "endo" manner cause plant tissue maceration (cell separation). These enzymes also cause injury and death of unplasmolysed plant cells. It has been proposed that cell death results from a physical weakening of the wall caused by degradation of pectic polysaccharides, such that the wall can no longer resist the pressure exerted by the protoplast (3). The pectic polysaccharides are probably the most complex class of wall polysaccharides. Although much research has been directed to reveal the structure of pectins as they occur in the plant cell wall and pectins used as food ingredients, our understanding of structure-function relationships is rather limited. An important step to recognize individual subunits with a complex polysaccharide (i.e. pectin) can be made by using purified and well characterized enzymes.

In order to understand; (I) the architecture of pectin. (II) The many functions of pectin and pectin products of degradation that play important roles in many physiological processes in plants. (III) The fungal mechanism of cell wall degradation, we have to have enough information about pectic enzymes and other novel enzymes. In this study we

grew *A.nidulants* on pectin as a sole source of carbon to define the pectic enzymes secreted by this fungus and detect their activities. In contrast with frequently used chemical degradation methods, which usually have a poor selectivity, these pectic enzymes act in a defined way (4), so we detected the activities of some pectic enzymes by the action of each enzyme on reducing-end-labeled oligomer that give rise to different initial fluorescent products. Thus, they all can be distinguished from each other.

CHAPTER 2

LITERATURE REVIEW

PECTIN

Pectin is a family of complex polysaccharides present in all plant primary cell walls and middle lamellae. The complicated structure of the pectic polysaccharides, and the retention by plants of the large number of genes required to synthesize pectin, suggests that pectins have multiple function in plant growth and development (5). Pectins function as a 'glue' that holds the other cell wall polysaccharides, like cellulose and hemicellulose (i.e. xyloglucan or glucuronarabinoxylan), and proteins, such as hydroxyproline-rich glycoprotein extensin, together.

In plants, pectins are present in all stages of development and the composition depends not only on the species but also on tissue, stage of growth and maturity, and growth conditions. Pectins are heterogeneous with respect to both chemical structure and molecular weight (6). The cell secondary wall is not always present and is usually involved in providing structural support. Therefore, the cellulose content is increased and pectin diminished in secondary walls. Structural analysis of the pectic polysaccharides isolated from dicotyledonous and monocotyledonous plants, gymnosperms, and some lower plants had led to the identification of four polysaccharides found in all pectins: homogalacturonan (HGA), Rhamnogalacturonans I (RG-I), and rhamnogalacturonan II

(RG-II) (7). The pectic xylogalacturonans and apiogalacturonans are only present in some plants, and thus are not classified as typical pectic polysaccharides.

HOMOGALACTURONAN

Homogalacturonan (fig1) is a linear homopolymer of 1, 4-linked α-D-galacturonic acid, which is partially methyl-esterified at the C-6 carboxyl and may also contain other unidentified esters; the length of the HGA in pectin remains a matter of debate. Homogalacturonan isolated from some plants (e.g.. sugar beet and potato) is O-acetylated at C-3(5). Portions of the HGA from some plants such as apple, cotton and watermelon contain β-D-xylose linked to C-3 of GalA.(8). Such regions of xylosylated HGA are referred to as xylogalacturonan.

RHAMNOGALACTURONAN I

RG-I is a family of branched pectic polysaccharides that accounts for 7-14% of the primary wall. RG-I contains a backbone of up to 100 repeats of disaccharide [$\rightarrow$4)-α-D-GalρA-(1$\rightarrow$2)-α-L-Rhaρ-(1$\rightarrow$] (9). The galacturonic acid may be O-acetylated at C-3 or C-2 (fig2). The average molecular weight of RG-I from sycmore has been estimated to be between10^5-10^6 Da. Between 20-80% of the rhamnosyl residues are substituted at C-4, and occasionally at C-3, with oligosaccharide side chains composed mostly of arabinosyl and/or galactosyl residues. The number and type of different side chains in RG-I have not

Methyl esters

O-acetyl
esters

3- and 3,6-linked
β-D-galactan

4- and 4,6-linked
β-D-galactan

β-D-Gal*p*

4-GalA(1-2)-Rha-1
backbone

α-L-Araf

β-D-Glc*p*A

3 and 3,5-linked α-L-arabinan
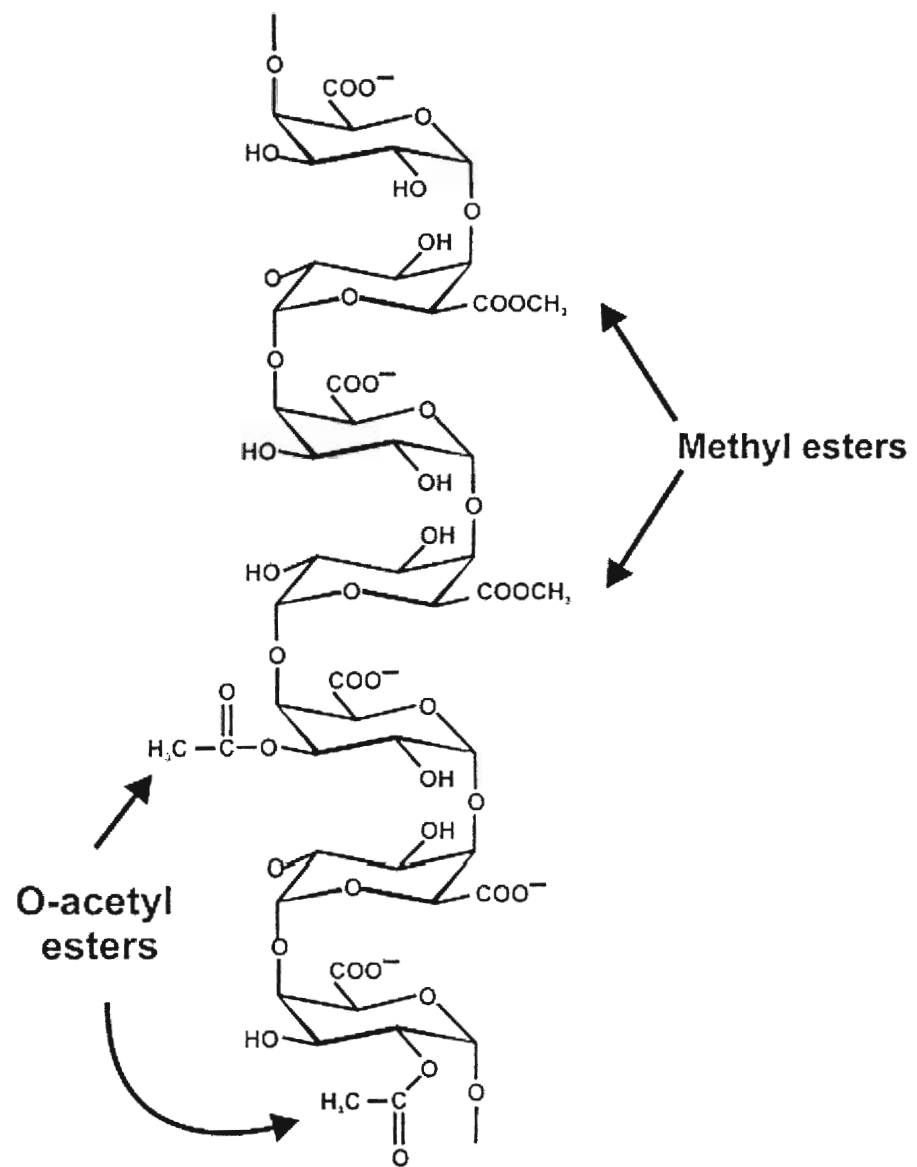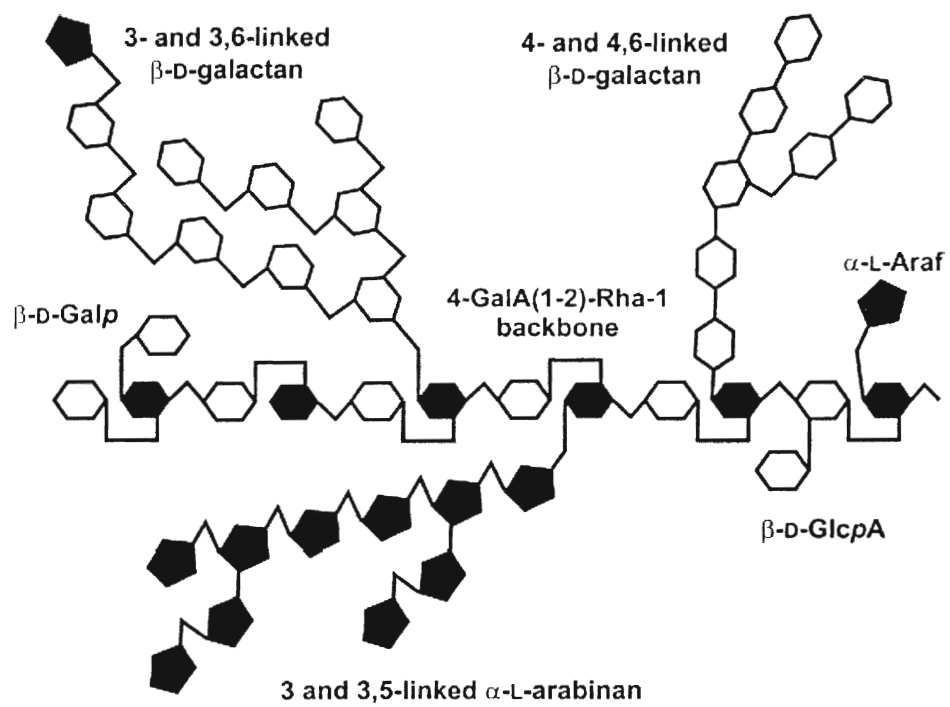
been determined and it is not known how much the structure of RG-I varies in different species (8).

RHAMNOGALACTURONAN-II

RG-II is not structurally related to RG-I since its backbone is composed of 1,4-linked α-D-Gal$p$A residues rather than the repeating disaccharide [$\rightarrow$4)-α-D-Gal$p$A-(1$\rightarrow$2)-α-L-Rha$p$-(1$\rightarrow$] (10). A nonsaccharide (side chain B) and an octasaccharide (side chain A) are attached to C-2 of some of the backbone GalA residues and two structurally different disaccharides (side chains C and D) are attached to C-3 of the backbone (fig3). The locations on the backbone of the side chains with respect to one another have not been established with certainty (5).

APPLICATIONS OF PECTIN

Pectins are used in many industries because they have broad range of applications such as a gelling agent, thickener, texturizer, emulsifier, and fat or sugar replacer in low-calories food, and as a component of many medicines. The main sources of commercial pectin are apple pomace and citrus peels (6). Pectins have been shown to possess a variety of pharmacological activities such as immunostimulating activity, anti-metastasis activity, anti-ulcer activity, anti-nephritis activity, and a cholesterol decreasing effect. Pectins are applicable also as a vaccine for typhoid fever (11).

A

βGalpA   α-L-Galp
3   2
αGalpA → 2 βRhap 4 ← αFucp 4 ← βGlcpA
                        3
3'
βApif   2-O-Me αXylp
2

?

?
3
Kdo
5
αRhap   C

D
βAraf
5
Dha
3

4)-αGalpA-(1,4)-αGalpA-(1,4)-αGalpA-(1,4)-αGalpA-(1,4)-αGalpA-(1,4)-αGalpA-(1,4)-αGalA~OH

2
βApif
3'
βRhap   B
3

OAc
2-O-Me Fucp → 2 βGalp → 2 αAcef ← OAc
4
βRhap → 3 αArap 2 ← αRhap 2 ← βAraf

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The degree of polymerization (DP) and degree of methylesterification (DE) determine the conditions of use. The DP of the pectin molecule varies from a few hundred to 1000 saccharide units. Based on the percentage of methylesterified galacturonic acid residues, pectins are classified as low (25-50%) and high (50-80%) methylesterified pectins. The low methylesterified (LM) pectin is obtained from the high methylesterified (HM) pectin usually by a treatment at mild acidic or alkaline conditions. The degree of methylation determines the gelling properties of pectin. In LM-pectin a gel is formed in the presence of Ca^{2+} over a wide pH range, pH=1.0 to 7.0 or higher. The HM-pectin requires quit a narrow pH range, around 3.0, and the presence of a cosolute (sucrose), at concentrations exceeding 55% by weight (12). About 80% of the world production of HM-pectin is used in the manufacturing of jams and jellies. LM-pectin is used for instance in a combination with carrageenan to produce sugar-free jams for diabetics and in fruit preparations for yogurt.

PECTINASES

The enzymes that hydrolyze pectic substances are known as pectic enzymes, pectinases, or pectinolytic enzymes. The production of pectic enzymes has been widely reported and thoroughly studied in bacteria and filamentous fungi because they play an essential role in phytopathogenesis. In addition, fungal pectinases are important in the food industry, especially in the extraction and clarification of fruit juices. An assumed natural role in plants includes fruit maturation, growth, abscission, and pollen development. Pectic enzymes are classified in two main groups, namely pectinesterases

(PE) which able to de-esterify pectin by removal of methoxyl residues, and depolymerases which readily split the main chain. The depolymerising enzymes are divided into polygalacturonases (PG), enzymes that cleave the glycosidic bonds by hydrolysis, and lyases (PL), which break the glycosidic bond by β-elimination. In addition, the latter two types of enzymes are classified on the basis of whether they exhibit a preferential hydrolytic power against pectin, pectic acid or oligogalacturonate as the substrate and whether the pattern of action is random (endo-) or terminal (exo-) (13).

Pectin methylesterases remove methyl groups rendering pectin polymers accessible to the depolymerizing enzymes such as lyases and polygalacturonases. Two different pectin methylesterases have been identified from *E. chrysanthemi*. The first was identified as an extracellular pectin methylesterase (pmeA) while the second, pmeB, was identified as an outer membrane lipoprotein. PmeB has a great activity on small pectic oligomers which can easily diffuse into the periplasm (14).

Based on sequence similarities the glycoside hydrolases that degrade pectin have been classified into the family 28. At present, this family consists of a few enzymes, such as (i) endopolygalacturonase(PG; EC 3.2.1.15) catalyzing random hydrolytic cleavage of α-1,4 glycosidic bonds in pectate and other galacturonans; (ii) exopolygalacturonase (EPG; EC 3.2.1.67) catalyzing the hydrolytic cleavage of one galacturonic acid residue from the non-reducing end of galacturonan; (iii) exo-poly-α-galacturonosidase (EPGD; EC 3.2.1.82) catalyzing the hydrolytic cleavage of two galacturonic acid residues from the non-reducing end of galacturonan; (iv) rhamnogalacturonase (RG; EC 3.2.1.-) catalyzing the hydrolytic cleavage of α-1,2 glycosidic bonds between D-galacturonic acid and L-rhamnose; and (v) endo-xylogalacturonan hydrolase (XGH; EC 3.2.1.-) catalyzing

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random hydrolytic cleavage of the glycosidic bond between D-galacturonic acid and L-xylose (15).

Pectin is composed of alternating homogalacturonan-smooth and rhamnogalacturonan-hairy regions. The smooth region is a polymer of α-1,4-linked D-galacturonic acid units, partially esterified, which are split by PGs, EPGs, EPGDs, pectin lyases, pectate lyases and de-esterified by pectin methylesterases. The hairy region consists of three different subunits, as identified in apples (16) (i) subunit I is xylogalacturonan, a galacturonan backbone heavily substituted with xylose and degraded by XGH; (ii) subunit II is a short section of rhamnogalacturonan backbone with many arabinan, galactan or arabinogalactan side-chains that can be degraded by arabinases and galactanases; and (iii) subunit III is rhamnogalacturonan consisting of alternating rhamnose and galacturonic acid residues which can be degraded by RGs.

Pectin, pectate, and rhamnogalacturonan lyases cleave the pectin backbone by β-elimination, which results in the formation of a Δ4,5-unsaturated nonreducing end. Pectin lyases prefer substrates with a high degree of methylesterification, whereas pectate lyases prefer those with a low degree of esterification. A clearer distinction between these two types of enzymes can be made based on the absolute requirement of Ca^{2+} ions for catalysis by pectate lyases versus the lack of Ca^{2+} ions for catalysis by pectin lyases(17).Until recently, it was not clear whether calcium is a part of the enzyme or involved in substrate binding. Recent x-ray studies on a pectate lyase from *Bacillus subtlis* complexed with calcium, shows that calcium interacts with an arginine residue that is conserved across all pectin and pectate lyases (14).

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ASPERGILLUS NIDULANS AS A MODEL ORGANISM

A. nidulans is a filamentous, homothallic fungus belonging to the family of ascomycetes, which is one of the most ubiquitous worldwide. The scientific name is derived from two Latin words; *aspergillum* which is a device used to sprinkle holy water (as this resembles the asexual reproductive structure of the organism called a conidiophore) and *nidulans* which means nest-like (which refers to the sexual structure called a cleistothesium). Although its correct taxonomic name is *Emericella nidullans* it is more widely known as *Aspergillus nidulans* (18).
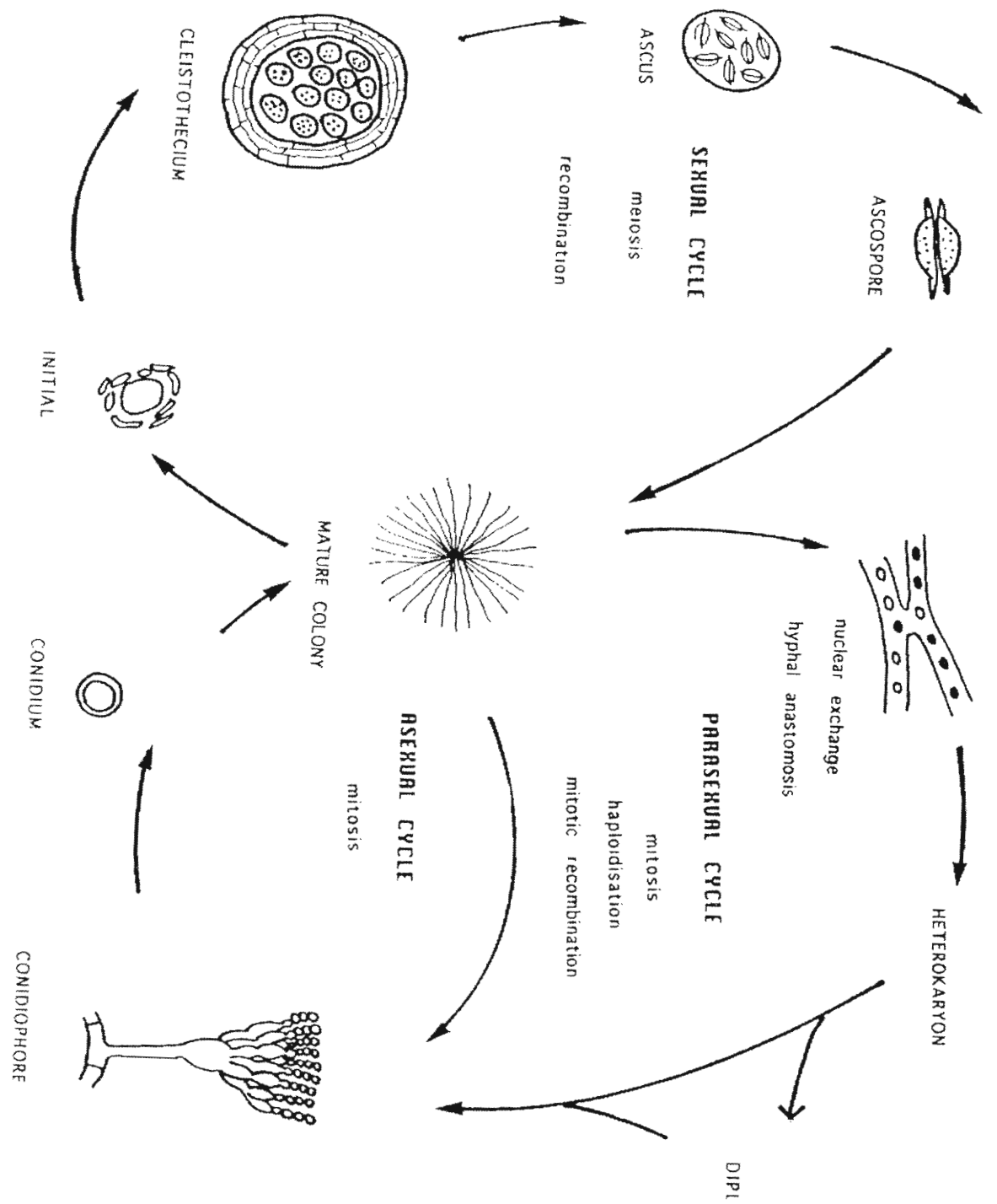
The Aspergilli can cause infections in man and animals; they are probably the most important contaminants of man-made and naturally occurring organic material. They are also able to produce mycotoxins during growth as contaminants of food. In their "non-destructive behavior" the metabolic abilities of these fungi are exploited for the production of extracellular enzymes. In the late 1940's, researchers were looking for an organism that would have characteristics suitable for detailed genetic analysis. Their search leaded them to the common soil fungus *Aspergillus nidulans*. Unlike a lot of other organisms considered at the time, *Aspergillus nidulans* fit all of their criteria. The advantages that *A.nidulans* offers include rapid growth on defined media, compact colonial morphology, uninucleate conidia (asexual spores), homothallism, meiotic (sexual) genetics, haploid nature and the ability to construct diploids and perform mitotic (parasexual) analysis. The relative ease of handling Aspergillus *nidulans* as a laboratory organism facilitates the investigation of physiology, genetics and molecular biology of a

lower, though multicellular, eukaryote and thus the organism became a useful tool for fundamental research (19).

A. nidulans, as mentioned before, has considerable advantages as a laboratory model organism. Above all, the three different life-cycles, i.e. vegetative-asexual, sexual and parasexual, which allow the isolation and characterization of mutants using powerful selection techniques. Conidia are uninucleated and this allows direct screening of mutants after replica-plating on different media. Since transformation procedures have been established for *A. nidulans* in the early 1980s, molecular genetic manipulation and reverse genetics have become standard techniques and today all modern tools to study the regulation of gene expression or developmental processes can be used in *A. nidulans*.

LIFE CYCLE

The life cycle includes alternation of generations, but it is the haploid state which is normally handled for the purposes of re-culturing and mutagenesis. In normal circumstances, the only diploid phase is transitory during sexual reproduction (figure.4) (2o). The outstanding characteristics of *A. nidulans* can be summarized in three main things: conidial heads typically columnar, usually dark yellow-green in color, but occasionally bluish green; conidiphores more or less brown pigmented, typically smooth, but occasionally showing surface concretions, usually sinuous; and ascospores in orange-red to blue-violet shades, usually showing definite equatorial crests (19).

CLEISTOTHECIUM

ASCUS

SEXUAL CYCLE

meiosis

recombination

ASCOSPORE

INITIAL

MATURE COLONY

PARASEXUAL CYCLE

nuclear exchange

hyphal anastomosis

mitosis

haploidisation

mitotic recombination

HETEROKARYON

CONIDIUM

ASEXUAL CYCLE

mitosis

CONIDIOPHORE

DIPL

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FUNGAL MEDIA AND GROWTH

Given water, an organic carbon source, inorganic sources of nitrogen, sulphur and phosphorus together with traces of metals, *A. nidulans* will grow well. Several recipes for minimal media of this type are known (App.1), for both solid and liquid culture conditions. Complex or complete media are normally used for everyday culturing and strain storage. There is a tremendous range of carbon sources utilized by *A. nidulans*, including hexoses, pentoses, polyols, alcohols, di-and poly-saccharides, and amino acids. Nitrogen sources used include ammonium or nitrate salts, purines, and amino acids. Many of these were discussed by Kinghorn and Pateman in the 1977 *Aspergillus* book. Although sulphate is the usual sulphur source, cystine or methionine is also used. Phosphorous is usually supplied as an inorganic phosphate (20).

For biochemical analyses, it is relatively easy to grow a large volume of mycelium in liquid culture in shaken flasks. By adjustment of pH, conidial inoculum size, medium constituents, temperature of incubation, it is possible to tip the balance in favor of pellet growth or a more homogeneous mycelial form. However, both are easily harvested either by filtration or centrifugation and can be dried for further analysis. There are many ways for measuring growth. But the more accurate method of assaying growth of fungus is assessment of dry –weight gain. This method is a destructive one and large numbers of cultures must be used successively in order to obtain data about growth rates over a period of time (21).

FUNGAL DEGRADATION OF POLYMERS AND OLIGOMERS

When aspergilli are in direct contact with their nutrients in the environment, smaller molecules (such as simple sugars and amino acids) can be directly absorbed by the cell. Large insoluble polymers such as cellulose, starch, and pectin must undergo a preliminary digestion before they can be used. Aspergilli can secret a broad range of enzymes that can degrade these complex polymers. The products that are formed reflect the composition of the polymers and include monomeric sugars like glucose, fructose, and mannose, and aromatic compounds such as ferulic acid and vanillic acid (20). The aspergilli also possess extracellular oxidases, such as glucose oxidase that converts glucose into D-gluconate.

The regulation of expression and secretion of polymer degrading enzymes is not completely clear yet, but the general concept is that low constitutive levels of particular enzymes are secreted and released in the medium or are bound to the conidial surface. These enzymes then produce 'signal molecules' when certain polymers are present. These molecules are taken by the cell, where they may be converted into or act directly as an inducer of synthesis of a subset of enzymes required to rapidly break down the polymer (21). Further, hydrolysis of polysaccharides is usually controlled by carbon repression, which inhibits synthesis of enzymes involved in polymer degradation when a more easily metabolizable carbon source is present (17).

PECTIN DEGRADATION AND EXPRESSION OF PECTINOLYTIC GENES

Pectin can be degraded by aspergilli using a number of different enzymes. Methanol is released from pectin by pectin esterase, yielding pectate and methanol. Pectate lyase degrades low esterified pectin, whereas highly esterified pectin can be degraded by pectin lyase. Polygalacturonases can degrade both low esterified pectin and pectate. A number of enzymes are involved in the degradation of the hairy region of pectin of which rhamnogalacturonase hydrolyses the rhamnogalacturonan backbone (20). The production of pectin main–chain-cleaving enzymes (mentioned above) and the expression of genes encoding these enzymes have been detected on apple pectin (22), and polygalacturonic acid (23). However, some pectinolytic enzymes have also been reported to be produced constitutively. Many differences in expression patterns are observed for the different polygalacturonase encoding genes. Constitutively expressed polygalacturonase encoding genes have been reported from *A. flavus*, *A. parasiticus* and *A. niger*. (16).

Three other polygalacturonase- encoding genes from *A. niger* were induced in the presence of sugar beet pulp, and promoter deletion analysis of one of these genes identified an important region responsible for high-level gene expression (5'-TYATTGGTGGA-3'). Recently, additional evidence has been obtained for the role of galacturonic acid as general inducer for pectinolytic genes in *A. niger*. Several genes encoding pectin main-chain-cleaving enzymes (pelA, plyA, pgaX, and rglA) and a gene encoding pectin methylesterase (pmeA) are expressed in the presence of galacturonic acid (16). Genes encoding arabinofuranosidases, endoarabinase, endogalacturonase, and β-galactosidase, all of which act on the pectin side chains, are also expressed on

galacturonic acid. However there are a number of factors, i.e. carbon source, pH of the medium, cAMP, catabolite repression and aeration that can be involved in fungal pectinase expression. A repressor, KDGR, controls the pectinase genes as well as the genes involved in pectin catabolism. The expression of several genes is also controlled by the PECS-PECM and PECT regulatory proteins, and by some global regulatory proteins such as the cAMP receptor protein (crp) (6).

ARABINAN DEGRADATION

Arabinans have a backbone that consists of α-1,5 linked α-L-arabinofuranosyl residues, to which other α-L-arabinofuranosyl residues are attached by α-1,2 or α-1,3 bonds . Endo –1, 5-α-l-arabinase hydrolyses glycosidic bonds in the arabinan backbone, α-L-arabinofuranosidase hydrolyses the terminal non-reducing α-L-arabinose substituents (24). Arabinan degrading enzymes have been purified from *A.niger*. Induction studies of the endo-arabinase and the two exo-α-L-arabinofuranosidases A and B showed that only complex substrates like sugar beet pulp induce the activities of all three enzymes, whereas L-arabitol and L-arabinose only induce α-l-arabinofruanosidase B. Ramon et al (1993) have purified two arabinan degrading enzymes from *A. nidulans* which have endo–arabinase and α-L-arabinofuranosidase activity. The enzymes have a much higher temperature optimum than the corresponding *A. niger* enzymes. All three genes, which encode the arabinan degrading enzymes activities in *A. niger* have been cloned and overexpresed (25).

INDUSTRIAL APPLICATIONS OF PECTINASES

Pectinases are of major importance in the beverage industry due to their ability to improve pressing and clarification of concentrated fruit juices. Pectinolytic enzymes and pectin methylesterase are used in the production of carrot puree. They are also used in debarking. Removal of the bark is the first step in all processing of wood and is traditionally a very energy-consuming process. Pectinolytic enzymes can help by the reduction in the amount of the required energy (16). Other applications for pectinolytic enzymes include increasing the yield of lemon peel oils (26). The enzymatic release of monosaccharides, the building blocks of the pectin polymer, is very important because these monosaccharides have different food and nonfood applications. Galacturonic acid can be enzymatically converted into L-ascorbic acid or can be used to produce surface-active agents by esterification with various fatty acids. Arabinose is a precursor of L-fructose and L-glucose, which can be used as sweeteners, and it can also be transformed to 5-deoxy-L-arabinose, a compound that has anti-Parkinson properties (16).

CHAPTER III

MATERIALS AND METHODS

STRAIN, MEDIA, AND GROWTH CONDITIONS

Aspergillus nidulants spores where provided by Dr. R. Prade (Microbiology Department, Oklahoma State University). To increase the total number of spores, a few spores were placed on agar-minimal medium (Petri dish) (see table), and inoculated upside down at 37°C for 2-3 days; the inoculation of spores resulted in approximately circular areas of mycelium (colonies), which have thousands of spores. Harvesting the spores was done by adding 2 ml water + 2 ml of 0.1% Tween 80 to every Petri dish and scraping the surface of the colony very gently with a sterile spatula. Spore counting was done using a hemacytometer.

For time course experiments and enzyme activity studies, a minimal medium culture containing 1% (W/V) carbon source was inoculated to a density of 1×10^8 conidia/ ml and shaken at 200 rpm, at 37°C. 20 ml samples were withdrawn using sterile pipette tips at intervals over 48 h and filtered through pre-weighed glass fiber filter paper. The growth of the spores depended on the glucose as a sole carbon source for the first 18 h of inoculation and immediately after that the fungal mass was filtered and washed with sterile water and then transferred to another minimal medium where the pectin was the

carbon source for another 24h. The filter papers were dried over night on a freeze drier and the dry weight of mycelia was determined. The filtrates were kept frozen at-20°C in 20 ml vials for further analysis of enzyme activities. The pHs of the filtrates were measured prior to being frozen.

HPLC GEL FILTRATION CHROMATOGRAPHY

Pectin polysaccharides were fractioned on Toyo pearl-Gel filtration column of HW 40(S) from Supelco Inc. Beads were packed in a stainless steel column (10mm × 500mm); the fractionation range was 100-10,000. Using a Dionex reagent pump system for elution, the column was equilibrated with 50 mM ammonium acetate, pH 5.2, and flow rate of 1.0 ml/min. the sugars were monitored by a refractive index detector (SHODEX R1-71). The fractions were collected every minute on a Gilson fraction collector. Certain fractions were collected from the above column, pooled and freeze dried.

GLC: GAS LIQUID CHROMATOGRAPHY

Sugar compositions were determined by GLC analysis of the trimethylsilyl methyl glycosides. Methanolysis and derivatization were performed using the protocol of Chaplin (1982) (27) as modified by Komalavilas and Mort (1989) (28). About 50 µl of sample was placed in a 4 ml glass vial with a Teflon-lined screw cap lid. 100 nmoles of inositol was added as an internal standard and dried in a speed vacuum. 200 µl of 1.5 M

methanolic HCl and 50 μl of methyl acetate were added to each vial. The vial was sealed tightly and placed in a heating block at 80 °C for at least 3 hours. After cooling to room temperature, a few drops of t-butanol were added to each vial and the sample was dried under a stream of N_2. 50 μl of trimethylsilylating reagent, which was prepared fresh in an exhaust hood by mixing 1 part of Tri-Sil with 3 parts of dry pyridine, was added to the sample and allowed to react for 15 minutes at room temperature. The derivatized sample was then evaporated gently under a stream of N_2 and redissolved in 100 μl of isooctane. The trimethylsilyl sugar derivatives were separated on a fused silica capillary column (30 m x 0.25 mm i.d., Durabond-1 liquid phase; J & W Scientific Inc., Rancho Cordova, CA) installed in a Varian (Sunnyvale, CA) 3300 gas chromatography equipped with an on column injector.

One μl of isooctane and 1 μl of sample was injected at 105 °C. The program was held at 105 °C for 1 minute. Then the temperature was raised to 160 °C at a rate of 10 °C/min and held for 4 min, then raised to 220 °C at a rate of 2 °C/min, finally raised to 240 °C at a rate of 10 °C/min and held for 10 min. Peaks were integrated on a Varian 4290 integrator. The amount of each monosaccharide was calculated by using an 'in-house' program based on the areas obtained relative to the internal standard area.

SUBSTRATE DERIVATIZATION

Labeling of GalA oligomers (7 & 11 residues) to be used as enzyme substrates was performed by a slight modification of procedures of Evanglista (29). Approximately 1 mg of the substrate was heated at 80°C for one hour in a mixture of 2 μL of 0.1 M APTS (in

25% acetic acid) and 20 μL of 1 M sodium cyanoborohydride (in DMSO) (5766). APTS derivatized samples were desalted on an HW40 (s) gel filtration column (15cm × 0.9cm) eluted with 50 mM NH4 AC 75%, acetonitrile 25%,at pH = 5.2 and monitored by a fluorescence detector (EX wavelength = 488, Em wavelength = 520). The labeled sample was dried on a speed vacuum and dissolved in sterile water to make a final concentration of 1 nmole/μL.

MALDI-TOF-MS

Oligosaccharides (polymers of 7 Gal A residues) were dissolved in 10μL water. 2μL of DOWEX-50W-50X8-200 beads in the ammonium form were added to the same volume of the oligosaccharide sample and let sit for at least 15 min to achieve complete desalting. 0.5 μL of the matrix TONC (TO/NC: 4/1), where TO = 100 mg 2,4,6-trihydroxy acetophenone/ml in methanol and NC = 15mg nitrocellulose/ ml in acetone/2propanol (50:50), was spotted on a 100 well plate, and allowed to dry and then 0.5 μL ammonium citrate (20 mM sol in H_2O) and 0.5 μL of the sample were added right on the matrix spot and let dry in air for a few minutes. Spectra were obtained on a Perseptive Biosystems Voyager matrix-assisted laser desorption time-of-flight mass spectrometer in the negative ion mode (Oklahoma State University, Department of Biochemistry, core facility). The identity of the different oligomers (substrates) was determined by using MALDI-TOF-MS.

ENZYME REACTIONS

Polygalacturonase assay

Endo and exo-polygalacturonase activity was assayed in 20 µL of 50 mM sodium acetate buffer, pH 4.00, containing 0.4 µL of culture filtrate and 10 µg of APTS-labeled oligomer of GalA (7 residues). The reaction mixture was incubated in a water bath for 25 minutes at 40°C, after that the enzyme was inactivated by boiling the reaction mixture for 15 minutes at 100°C.

Pectate lyase assay

Pectate lyase activity was assayed in 20 µL of 50 mM Tris-HCl buffer, pH 8.5, containing 2 µL of culture filtrate and 3 µg of APTS-labeled oligomer of GalA (11 residues) and 4 µL of 0.5 mM $CaCl_2$. The reaction mixture was incubated in water bath for 3 hours at 40°C, after which the enzyme was inactivated by heating the reaction mixture for 15 minutes at 100°C.

Arabinanase assay

Arabinanase activity was assayed in 25 µL of 50 mM sodium acetate buffer, pH 5.3, containing 1 µL of the culture filtrate and 2 µg of Arabinan (7 residues) as substrate. The reaction mixture was incubated in water bath for 25 minutes at 40°C, after that boiling the reaction mixture for 15 minutes at 100°C inactivated the enzyme.

Rhamnogalacturonase assay

Rhamnogalacturonase activity was assayed in 25 μL of 50 mM Tris-HCl buffer, pH 9.0 and 4.00, containing 2 μL of the culture filtrate (culture filtrate was first incubated for 30 minutes with 10 μL ANTS) and 2μg of GR10 (10 galacturonic acid residues + 10 rhamnose residues) as substrate. The reaction mixture was incubated in water bath for different times (from 30 minutes up to 18 hours) and at different temperatures (30°C, 35°C, and 40°C).

Methylesterase assay

Methylesterase activity was assayed in 20 μL of 50 mM Tris-HCl buffer, pH 7.0, containing 1 μL of the culture filtrate and 1μL of Gal.A4M (methylated oligomer of 4 GalA residues) as substrate. The reaction mixture was incubated in water bath for different times and different temperatures.

CAPILLARY ZONE ELECTROPHORESIS CZE

A fully automated system (BioFocus 2000, Bio-Rad) with LIF-detector, a high-voltage power supply capable of controlling voltages up to 30 kV and currents up to 300 μA with software-reversible polarity, and PC management of all functions including data and on-line integration was used for CZE analysis.

A fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm ID (31cm long) was used as the separation column for oligosaccharides. The capillary was placed in a temperature-controlled cartridge at 20°C, a potential of 15kV/70-100 μA, and

0.1 M sodium phosphate, pH 2.5, as running buffer, were used. Methods were programmed so that the capillary was rinsed with 1 M NaOH followed by running buffer before the injection and a dip-cycle was included after injection to prevent carryover between samples. Samples were pressure-injected at 4.5-5 psi*sec. APTS-labeled oligomers were excited at 488 nm and emission was collected through a 520 nm band pass filter.

CHAPTER IV

RESULTS AND DISCUSSION

GROWTH OF *A. NIDULANS* ON PECTIN

We investigated the ability of *A. nidulans* to grow on pectin as a sole carbon source. First the fungal spores were inoculated in a minimal medium culture that contains 1% glucose as sole carbon source for 18 hours and then the fungal mycelium and spores were filtered, washed, and transferred to 1% pectin minimal medium culture for 24 hours. The increase in the culture optical density and dry weight of the fungi indicated that *A. nidulans* was capable of utilizing pectin as carbon source, after a lag of ~ 4 hours. Mycelial dry weight increased in a sigmodial fashion, where the stationary phase appears after 18 hours of growth on pectin (fig5).

UTILIZATION OF PECTIN

Several gel filtration profiles of culture filtrates, which were withdrawn at 2-hour intervals, are shown in figure 6. From the chromatograms and figure 7, we can say that the pectin area (weight) decreased. Clearly the decrease

in the pectin amount is due to the fungi, which utilized the pectin as carbon source to survive. Pectin is a polymer and is expected to be included in the void volume. In the early hours of the fungal growth on pectin, a large amount of pectin was observed, however after 24 hours of the fungal growth the pectin peak was very small in comparison with previous peaks of the pectin (the area of pectin peak after 2 h / area of the same peak after 24 h was 1/ 7). The peaks of pectin were identified by comparison with the pectin peak at 0 h (control).

CARBOHYDRATE COMPOSITION OF PECTIN PEAKS

To know whether there were changes in sugar composition of the pectin during the growth of fungi, the sugar contents of pectin at 12 different time points were determined. Figure 8 shows GalA consumption. However, GalA was consumed more than other sugars and the reason for that could be that GalA is the most abundant component in pectin. The decrease in the amount of GalA, Rha, and Ara was linear after a lag of ~4h (fig 9). The Galactose amount was almost stable during whole incubation time.

PRODUCTION OF POLYGALACTURONASES

The time course of production of extracellular PG by *A.nidulans* grown in minimal medium containing pectin as a sole carbon source is shown in figure10. When fungi were grown on glucose as a sole carbon source, very low PG activity was detected. However, it is known that pectolytic enzyme production is subjected to catabolite repression, and in

the presence of glucose and any other carbon source the fungi utilize glucose first and after that they utilize the other carbon source (30). So it is clear that pectins or polygalacturonic acids are specific inducer of pectolytic enzymes.

The activity of PG enzymes in the filtrate increased dramatically after 4 hours and continued to increase until 24 hours. The pH of the culture filtrate increased from 6.5 to 8.5 during the course of the experiment (fig11). The optimum pH for PG was reported to be 4 (30). Figure 12 shows several electropherograms of the labeled polygalacturonate and the products of enzyme digestion. From the electropherograms we can say that exo-PG activity is much higher than endo-PG activity. Because exo-PG hydrolyses the pectate molecules in a uniform manner, beginning at a non-reducing end of the polymer, this enzyme cuts only one residue at the time from the non-reducing end. The substrate we started with (control) is 7 GalA residues, the first and largest amount of the products was oligomer of 6 GalA in the first 6 hours and so on until 16 hours. Endo-PG activity would only produce dimmer, trimer, and tetramer of GalA from the 7 GalA substrate (31). It is unlikely that the exo-enzyme degrades short oligomer (i.e. degrading trimer to dimer) in the presence of longer oligomer. Enzyme activity was calculated from the peak area ratio. The peak area ratio was calculated by dividing the sum of the area of the peaks representing the products of a particular enzyme activity by the sum of all peak areas. A Correction percentage was subtracted from both product area and total area; this correction percentage reflects the impurity of the substrate and it is the sum of areas of the peaks which are not products or substrate divided by the total area of the peaks; these peaks can be seen in the control which was the substrate before any enzymatic digestion. Each substrate has different correction percentage from the other.

The electropherograms in figure16 shows patterns that allow us to distinguish between the endo and exo enzymes. First of all the activity of the arabinases was very low until 20 hours of the incubation time, and a long lag phase was needed for the fungi to produce more enzymes to degrade the arabinan. The optimum pH for arabinase has been reported to be ~5.3. The substrate was an oligomer of 7 arabinose residues (17). The electropherograms of arabinase products after 20 h, 22 h, and 24 h showed a major product consisting of 5 Ara residues, which indicates the action of the exo-arabinase that cleaves two residues from the 7 residue oligomer. This may mean that the active site of the exo-enzyme needs at least 6 residues towards the non-reducing end from the position at which the hydrolysis occurs. In other words this enzyme may recognize 6 residues. We can't distinguish clearly between the two different exo-enzymes here, because of the methods we use which enable us of distinguishing between exo and endo enzymes only. But the other peaks in the electropherograms are probably products of endo 1, 5-α-L-arabinase and arabinofuranosidase.

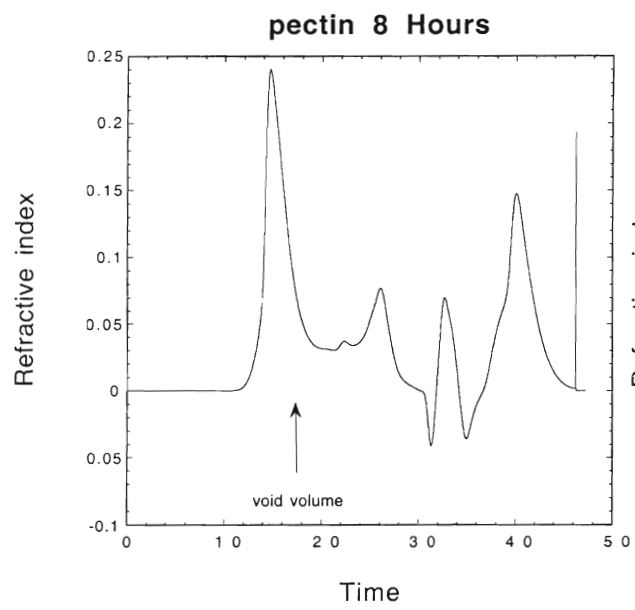PRODUCTION OF METHYLESTERASES AND RHAMNOGALACTURONASES

We tried to assay methylesterase activity in culture filtrates using a tetramer of GalA with a methyl ester group on the third residue from the non-reducing end as substrate. No activity was detected in the filtrates (fig17). That could be because we did not use the appropriate substrate. There must be methylesterase in the culture medium, because we know that the pectin, which was consumed by the fungi, was 70% methylesterified. There is no way for the fungi to consume that pectin before releasing the methyl residues from
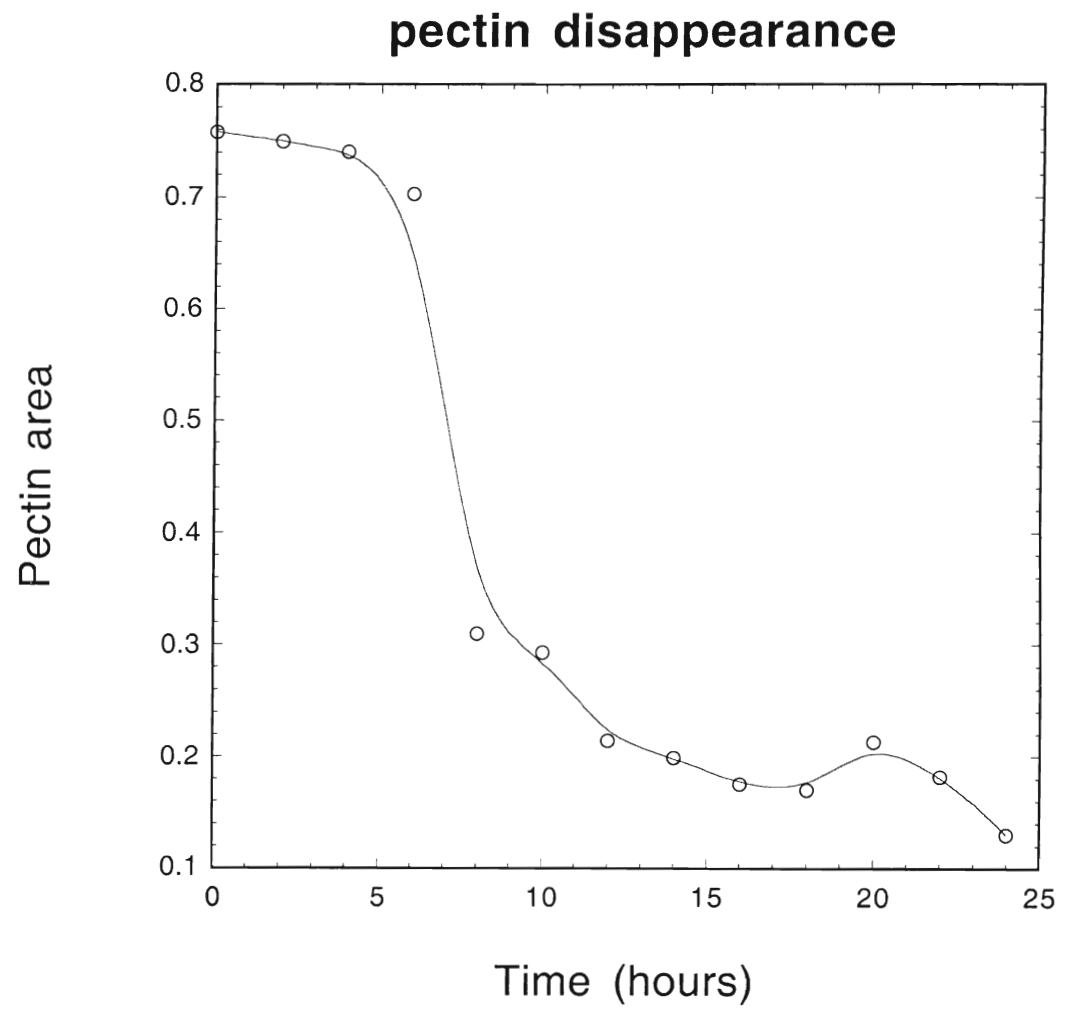
the backbone of the pectin. In other words, the ability of polygalacturonases and pectate lyases to degrade the pectin main chain depends on the activity of pectin methylesterase. Several methylesterases have been purified from *Aspergillus* spp (34, 35).
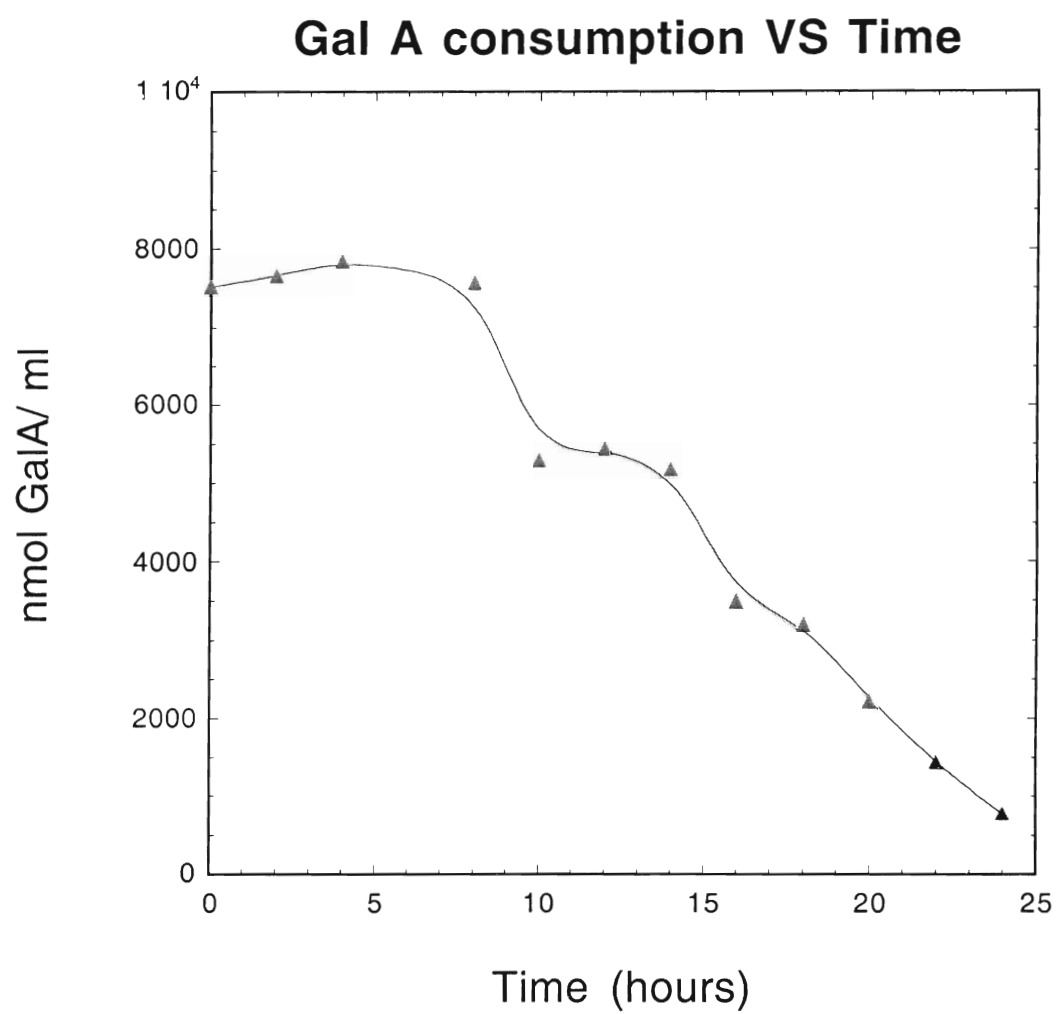
We could also not detect any rhamnogalacturonase activity in the culture filtrate (fig18), which may indicate that *A. nidulans* does not secret that enzyme. However rhamnogalacturonases have been reported in other fungi only a few times (36).
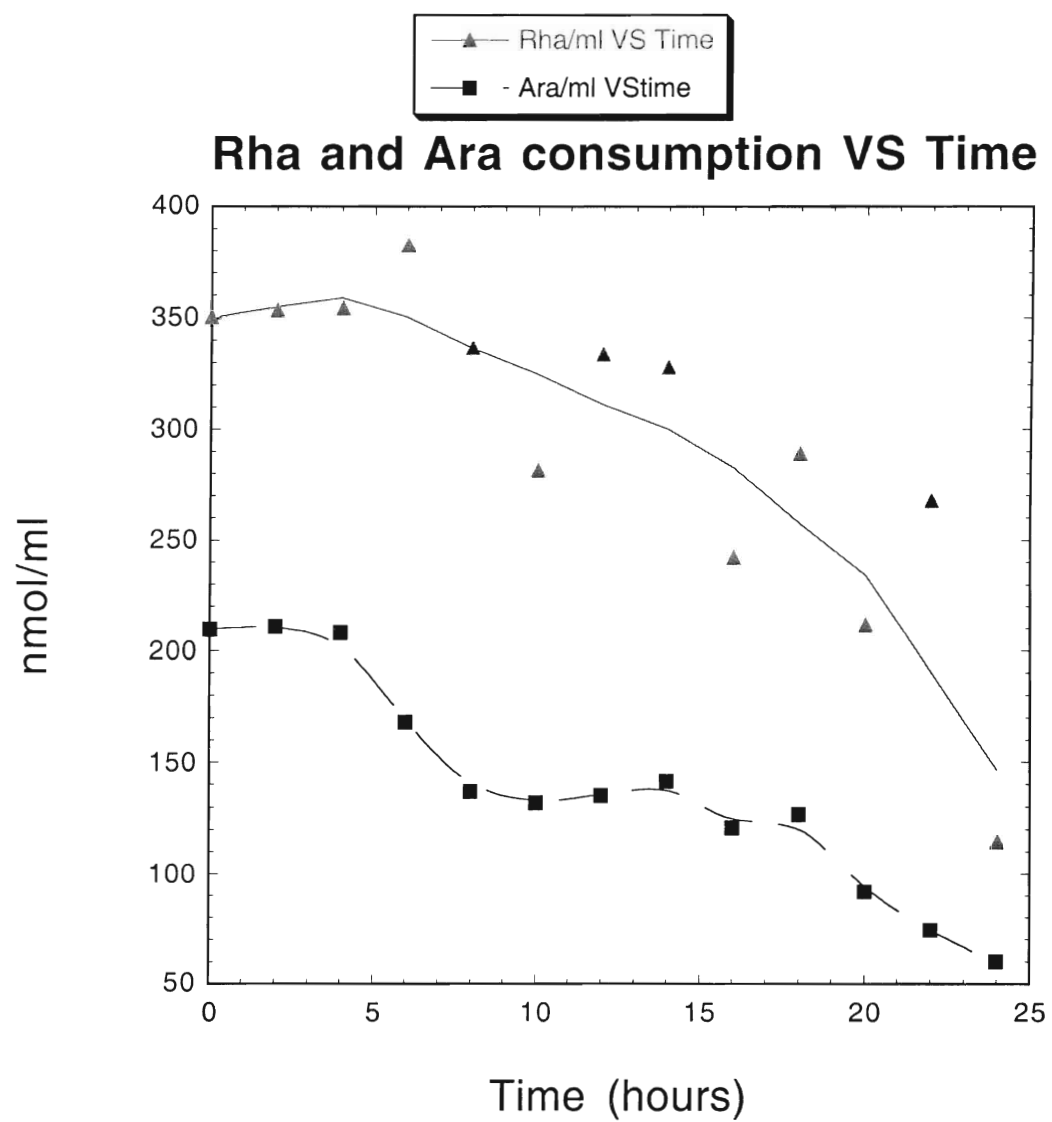
Fungal enzymes appear to be very sensitive to their ionic environment, and are usually closely regulated by pH. Indeed fungal enzymes present pH optima between 4 and 9. However, fungi such as *A. nidulans* can grow over a wide pH range, from 3 to 9.
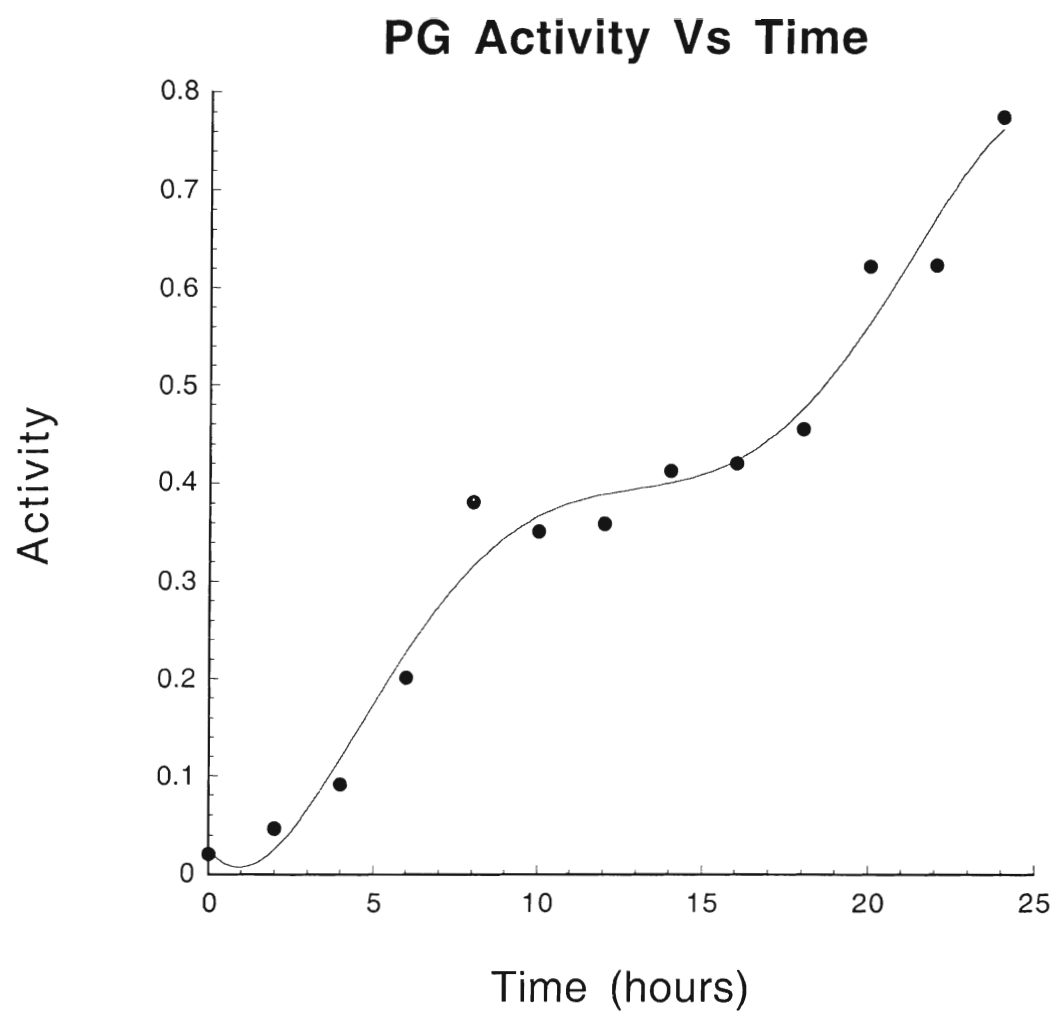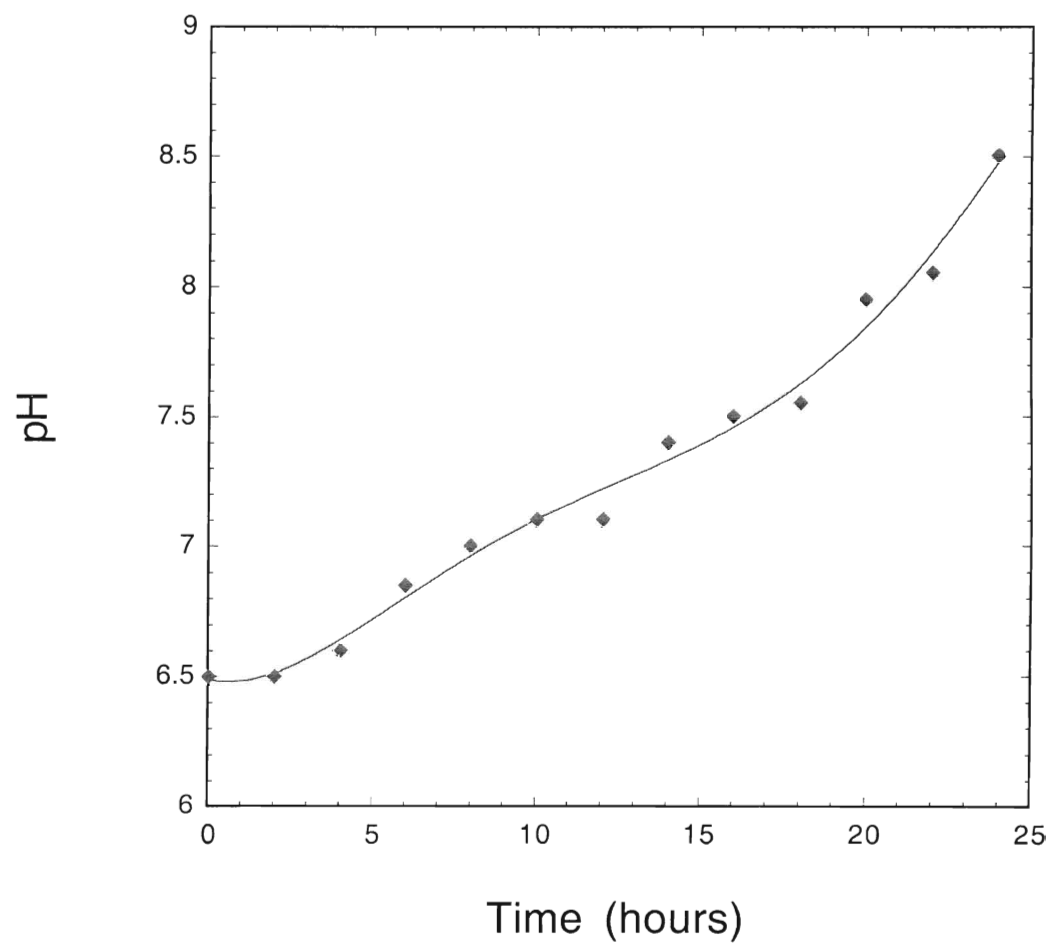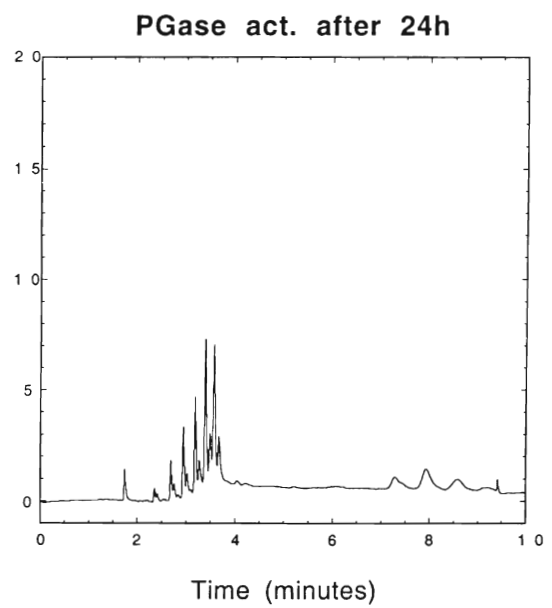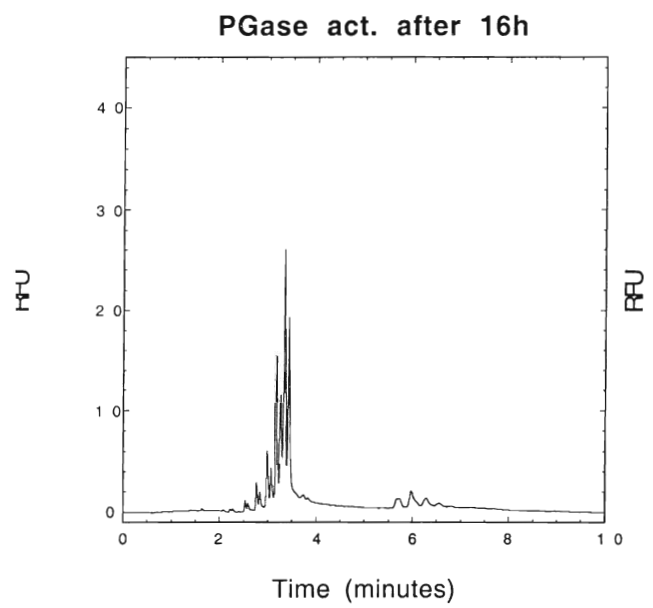
Dry Weight VS Time

pectin 8 Hours

Refractive index vs Time

void volume

pectin 16 Hours

Refractive index vs Time

void volume

pectin 24 Hours

Refractve index vs Time

void volume

pectin 0 Hours

Refractive index vs Time

void volume

35

pectin disappearance

Gal A consumption VS Time

Rha and Ara consumption VS Time

Legend:
- Rha/ml VS Time
- Ara/ml VStime

X-axis: Time (hours)
Y-axis: nmol/ml

38

PG Activity Vs Time

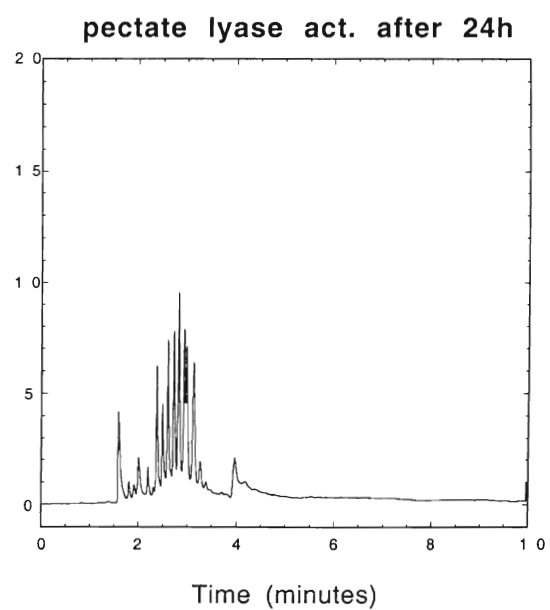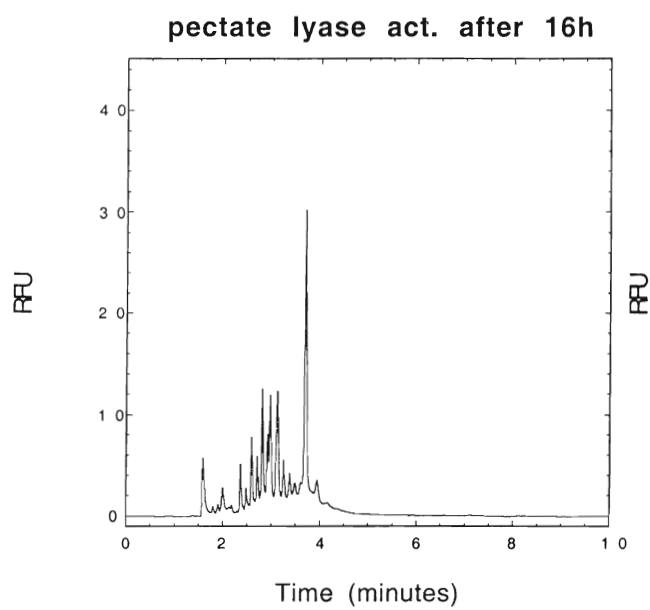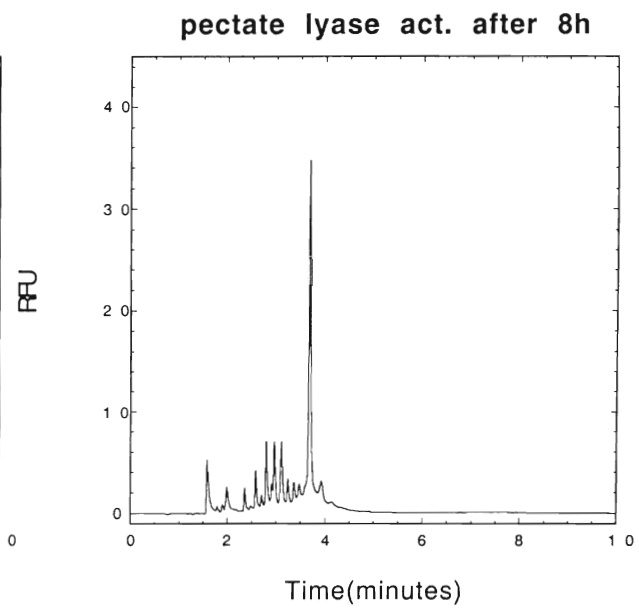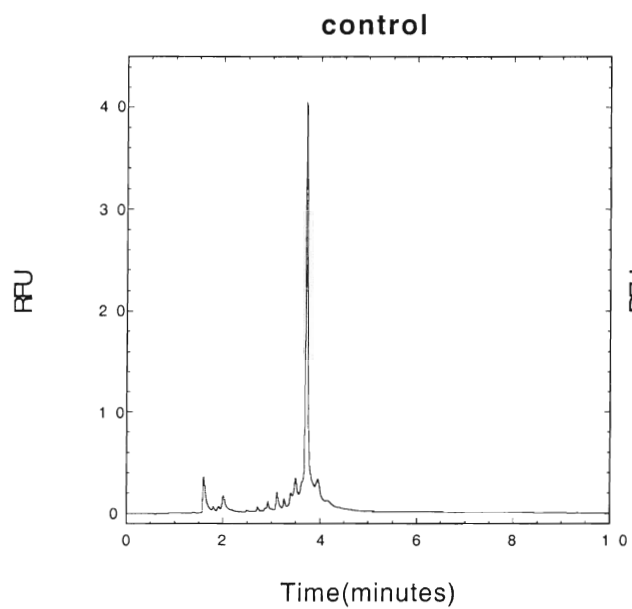# pH of the culture Medium VS Time

control

PGase act. after 8 h

PGase act. after 16h

PGase act. after 24h

Pectate Lyaes Activity VS Time

control

pectate lyase act. after 8h

pectate lyase act. after 16h
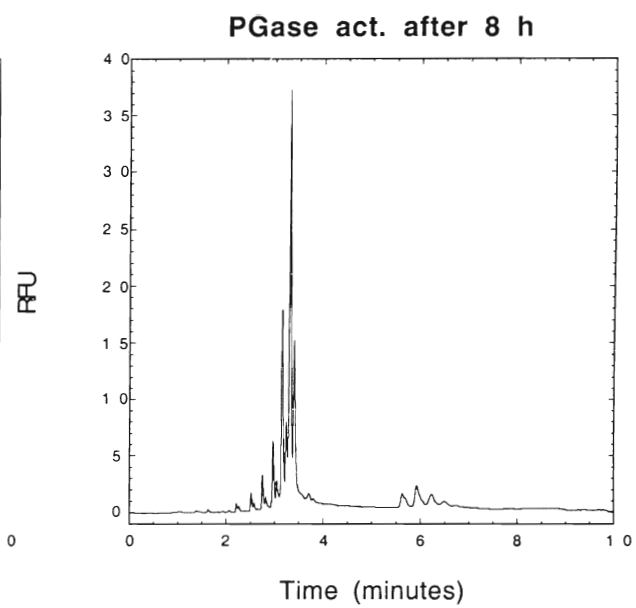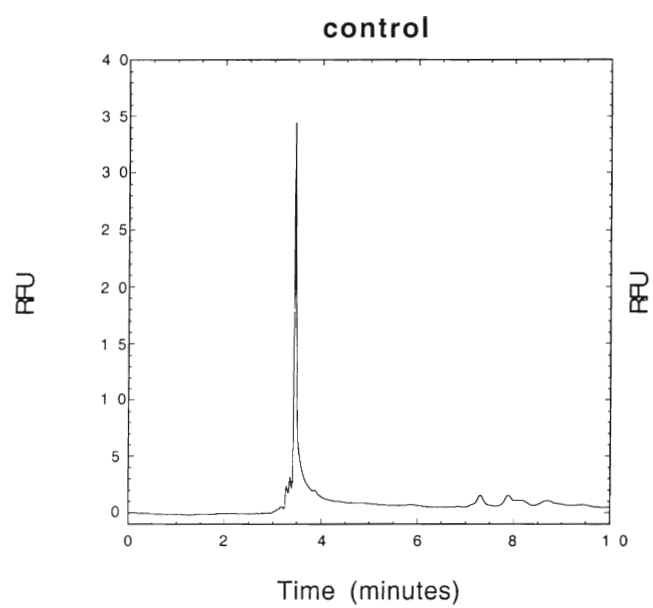
pectate lyase act. after 24h
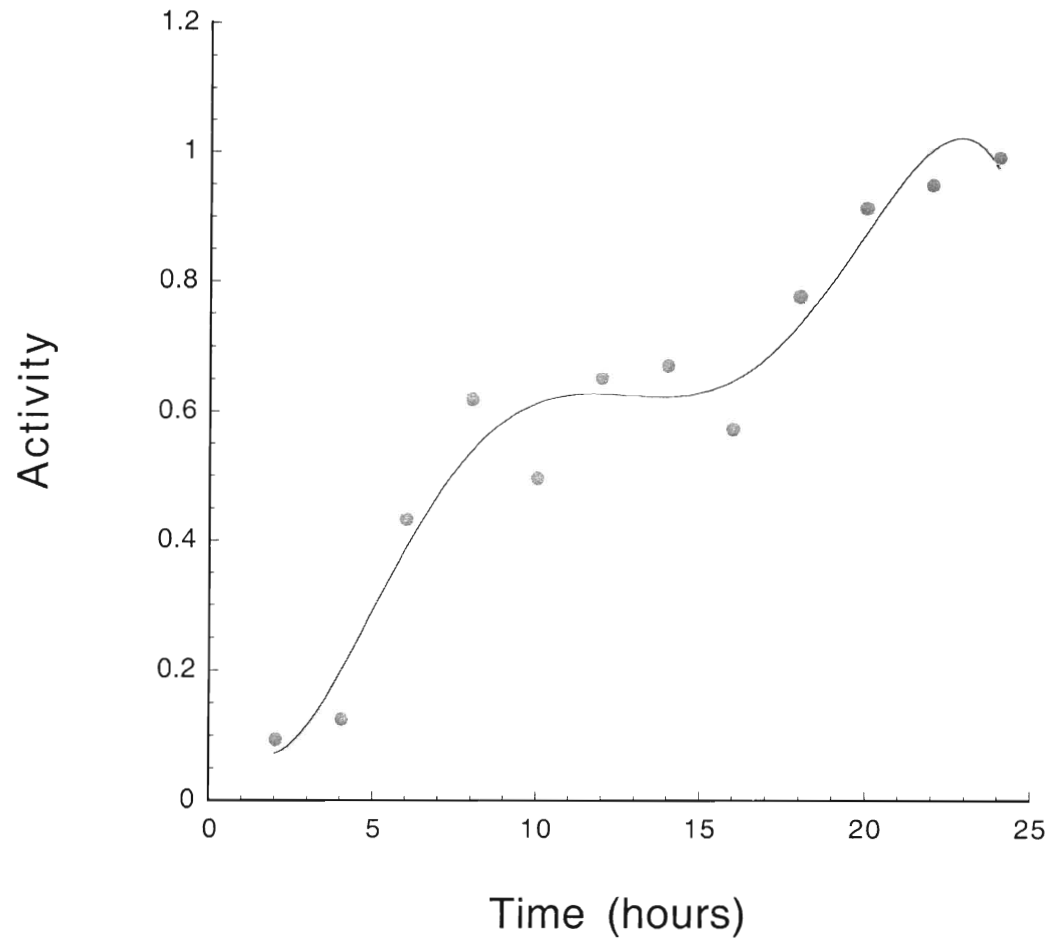
Arabinase   Activity. VS Time

control

arabinase act. after 14 h

arabinase act. after 22h

arabinase act. after 24h

methylesterase (control)

methylesterase Act. after 24h

Rhamnogalacturonase (control)
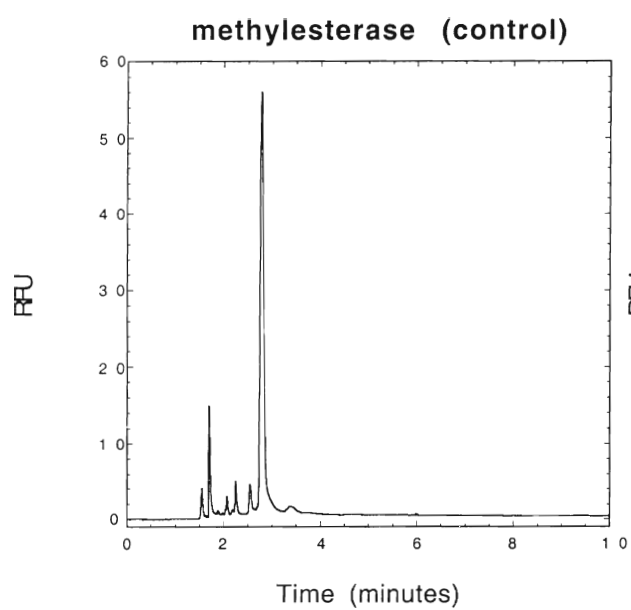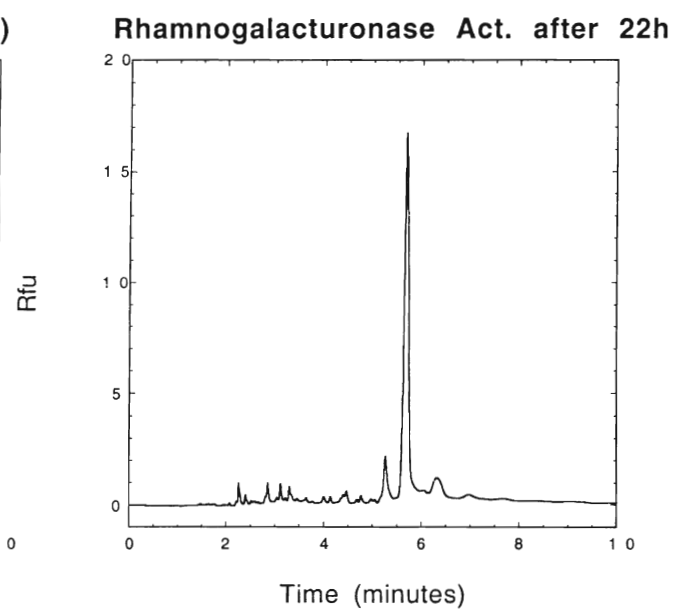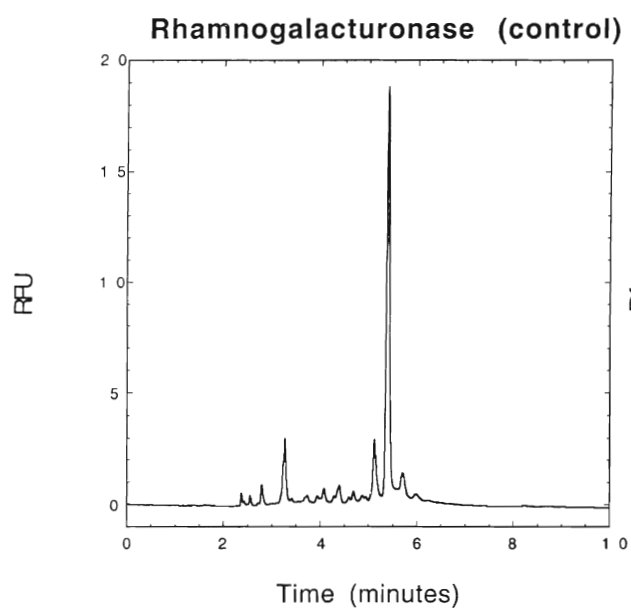
Rhamnogalacturonase Act. after 22h
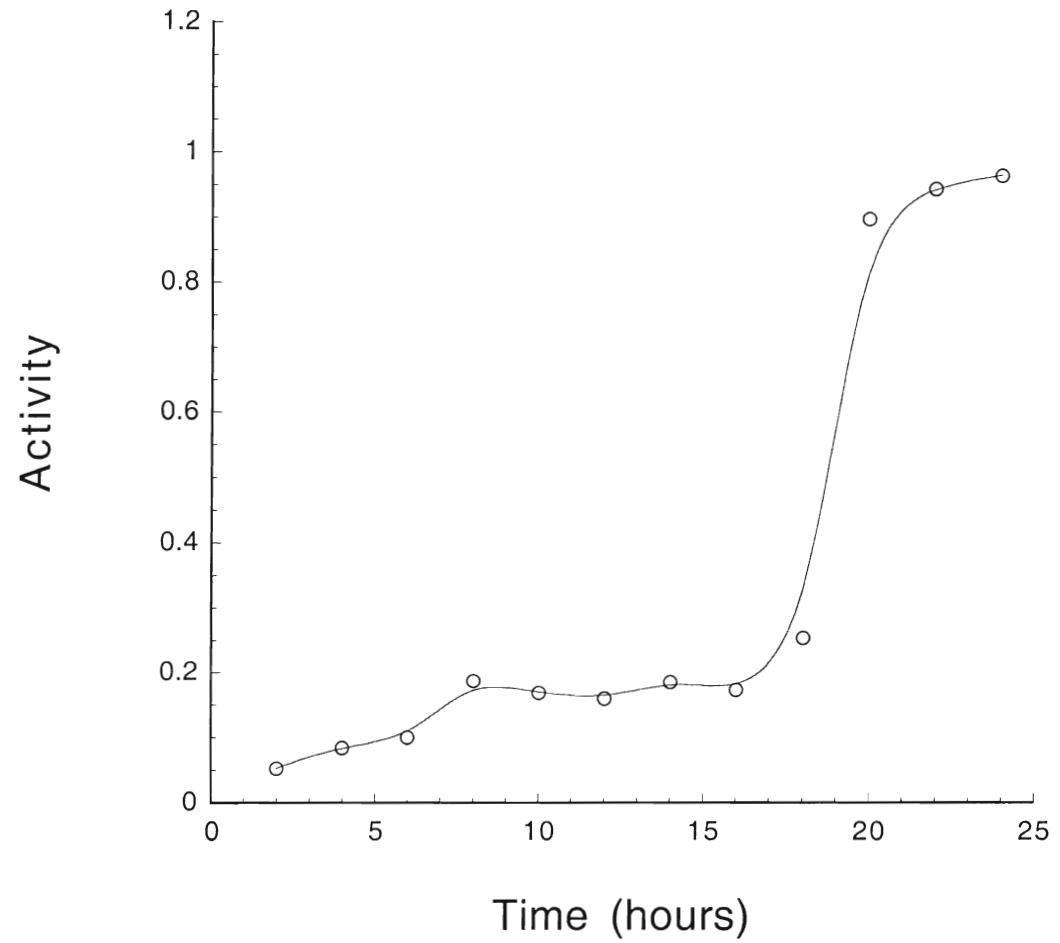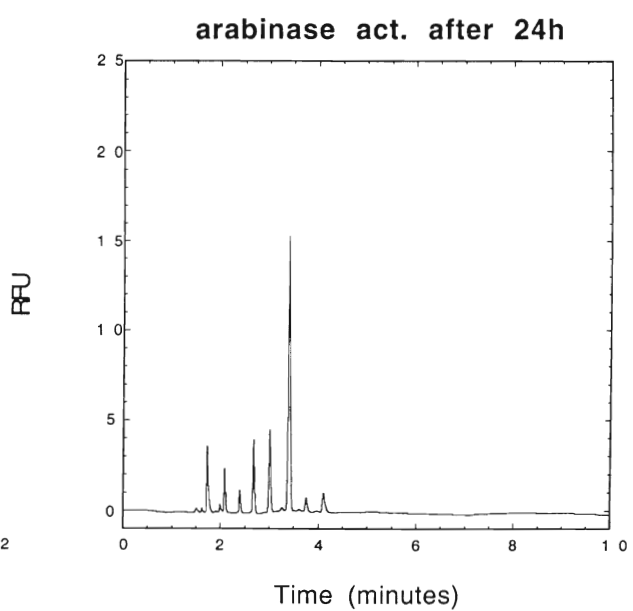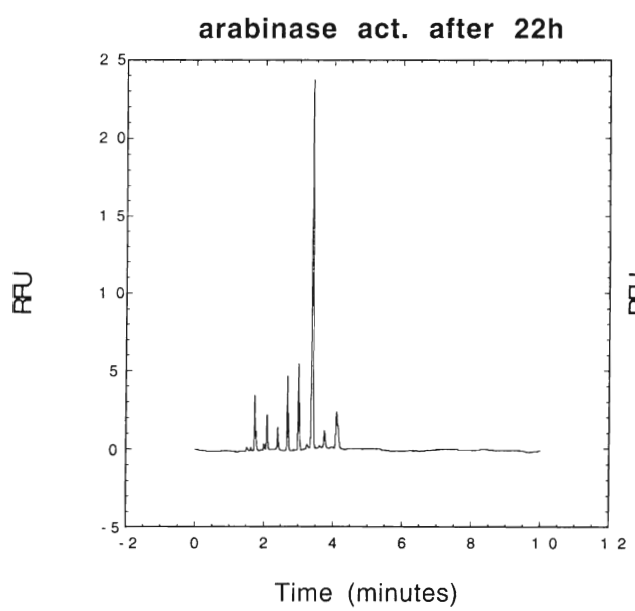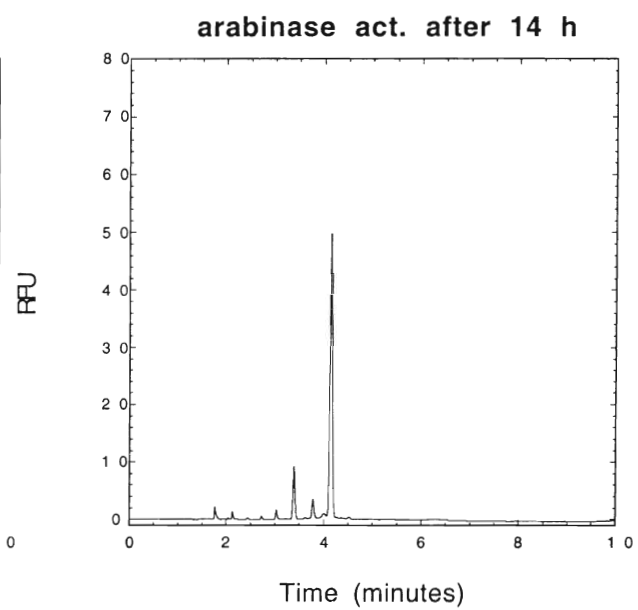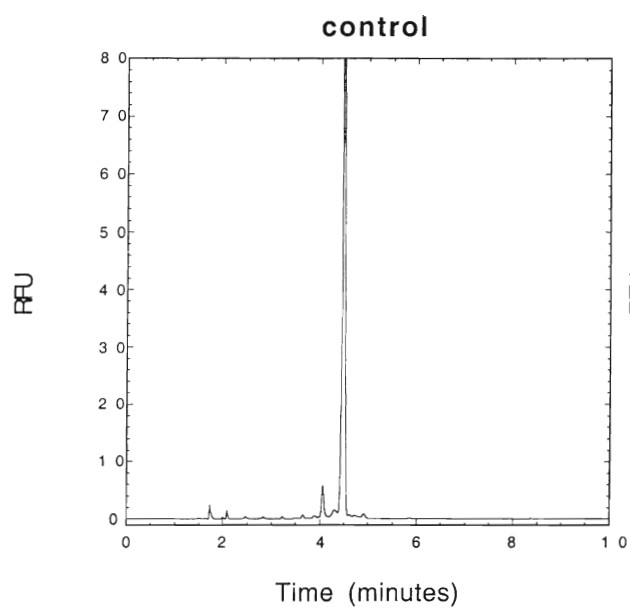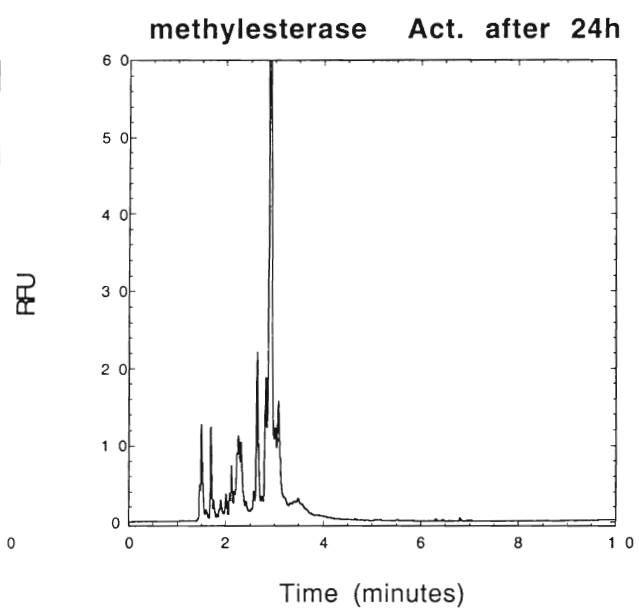
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ASPERGILLUS SOLID MINIMAL MEDIUM (as used at Cambridge, from Cove 1966)

Glucose	10.0 g
Salt solution	20.0 ml*
Difcobacto agar	14.0 g
Distilled water up to	1000 ml
10M sodium hydroxide to pH	6.5

* Salt solution

KCl	26.0 g
$MgSO4.7H_2O$	26.0 g
KH_2PO_4	76.0 g
Trace elements	75.0 ml**
Chloroform	1.25 ml as preservative
Water up to	1000 ml

** Trace element solution

$Na_2B_4O_7. 10 H_2O$	40.0 mg
$CuSO_4. 5H_2O$	400.0 mg
$FePO_4 H_2O$	800.0 mg (ferric)
$MnSO_4, 4H_2O$	800.0 mg
$Na_2MoO_4.2H_2O$	800.0 mg
$ZnSO_4.7H_2O$	8.0 g

The above medium requires a nitrogen source usually added when plates are poured (see below).

Aspergillus liquid minimal medium for conidiation (from Martinelli 1976).

Final concentration in liquid medium

Carbon source	10.0 g usually glucose
$MgSO_4.7H_2O$	0.25 g
$NaNO_3$	6.0 g
K_2HPO_4(0.1M)	26.5 ml
NaH_2PO_4(0.1M)	73.5 ml
Trace elements	10.0 ml *
Water up to	1000 ml

*trace elements

$CaCl_2$	140.0 mg
$MnCl_2.4H_2O$	39.0 mg
$ZnSO_4$	100.0 mg
$CuCl_2.2H_2O$	5.0 mg
$Na_2MoO_4.2H_2O$	0.5 mg
$CoCl_2.6H_2O$	0.15 mg
$FeCl_3$	100.0 mg
Citric acid	372.0 mg as chelator
Water up to	1000 ml

Many other nitrogen sources could be substituted for $NaNO_3$.

Common additions to any minimal medium

Nitrogen sources final concentration:

NaNO3	0.85 g/l
NH4 tartrate	0.46 g/l
Urea	0.303 g/l
Allantoin	1.0 g/l (added as solid)

Amino acids can used as nitrogen sources usually at 5 mM, whereas others are usually used at 10 mM.

Carbon sources final concentration

Glucose	1.0 g/l
Galactose	1.0 g/l
Ethanol	10.0 ml/l

Vitamins final concentration

Biotin	0.01 g/l
Nicotinic acid	1.0 mg/l
Riboflavin	2.5 mg/l

Amino acids final concentration

Arginine	0.526 g/l
Histidine	0.524 g/l
Proline	0.576 g/l

APPENDIX II

Classification of peptic enzymes

PEs (EC 3.1.1.11 de-esterify pectin by the removal of methoxyl residues. Depolymerases split the main backbone
1. By hydrolysis of α-(1,4) linkages 1.1. PGs, acting on pectate Exo-PG (EC 3.2.1.67) Endo-PG (EC 3.2.1.15) 1.2. Polymethylgalacturonases (PMG) (EC 3.2.1.15), actin on pectin 2. By β-elimination 2.1. PL, acting on pectate Exo-PL (EC 4.2.2.9) Endo-PL (EC 4.2.2.2) 2.2. Pectin methyl-lyase (PML) (EC 4.2.2.10), acting on pectin.

VITA $\sqrt{J}$

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