STRAIN AND PROCESS IMPROVEMENT FOR POLYGALACTURONASE PRODUCTION BY *ASPERGILLUS CARBONARIUS*

THESIS

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in
Biotechnology

By
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DECLARATION

I hereby declare that this thesis entitled **"Strain and process improvement for polygalacturonase production by *Aspergillus carbonarius*"** submitted to the University of Mysore, for the award of Doctor of Philosophy in Biotechnology is the result of research work carried out by me in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, under the guidance of **Dr. S. Umesh Kumar**, during the period May 2001 to November 2004.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

Place: Mysore **K.S. Venkatesh**

Date: 10th November 2004 **Candidate**

Dr. S. Umesh Kumar
Head
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10th November 2004

CERTIFICATE

I hereby certify that this thesis entitled **"Strain and process improvement for polygalacturonase production by *Aspergillus carbonarius*"** submitted by **Mr. K.S. Venkatesh** for the award of Doctor of Philosophy in Biotechnology, to the University of Mysore, is the result of Research work carried out by him in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, under my guidance during the period May 2001 to November 2004.

S. Umesh Kumar
Guide

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PREFACE

PREFACE

The multitude of biosynthetic and catabolic pathways of Aspergillus has defined the competitive survival of the fungus in the natural ecosystem. The studies on the metabolic diversity characterized by colonization ability of various substrates resulted in the identification of Aspergillus species for several industrial applications. The understanding of the fungal genome using methods of molecular genetics and cellular manipulations defined Aspergillus as a cell factory for the production of human growth hormone, food enzymes, interferon etc. Despite the above advances, the phenomenon of protein secretion by the fungus in relation to its growth physiology is less understood. This is because the fungus grows on solid substrate so also in liquid cultures.

Of the carbohydrate degrading enzymes produced by the fungus, pectinolytic enzymes have several applications in food and beverage industries. For degrading a particular polymer substrate like pectin, the fungus is known to produce several forms of enzymes identical in catalytic activity but differing in kinetic properties. From this laboratory, polygalacturonases of *Aspergillus carbonarius* have been characterized and application for beverage industry described. However, fundamental questions on the enzyme production in relation to the biology of the fungus still remain unclear.

This thesis unravels the physiology of *A. carbonarius* in relation to multiple enzyme secretion with reference to polygalacturonase production.

INTRODUCTION

INTRODUCTION

Species of *Aspergillus* are the earliest domesticated filamentous fungi which due to versatility in metabolic activity are found almost everywhere on any conceivable type of substratum. The use of this fungus as starter cultures for food fermentation is known for many centuries (Cook and Campbell-Platt, 1994) and they form a very large proportion of all the moulds encountered in industrial work like, production of citric acid, lovastatins, and industrial enzymes. *Aspergillus* spp are known as major contaminants of environment, weeds of laboratory cultures, agents for food spoilage and causative organism for certain human, animal and bird diseases. Their enormous potential that exploited continuously has resulted in the sequencing of the genomes of *A. nidulans*, *A. fumigatus* and *A. flavus* (http://www.*Aspergillus*-genomics.org; http://www.*Aspergillus*.man.ac.uk). A survey of literature showed that the research developments described of *Aspergillus* have been towards perfecting the fungus as cell factories for the production of industrial and biological biomolecules (Punt *et al*., 2002).

The review of literature presented here focuses only of those aspects that are relevant to the present investigation.

ASPERGILLUS

Aspergilli play an important role in environment due to their saprophytic nature. *Aspergillus,* an anamorphic genus, produces asexual spores (conidia, conidiospores) on specialized structures characteristic of the genus, the “aspergillum”. The name *Aspergillus* coined by Micheli in 1729 originated from Latin word *aspergillum* (a container for distributing holy water) meaning, rough head (Thom and Church, 1926). Raper and Fennell (1965) described 132 species of the fungus subdivided into 18 groups based on their morphology.

Most of the anomorphs species are readily identified by cultivating on Czapek and malt extract agars. International Commission on *Penicillium* and *Aspergillus* (ICPA) recommended growth of the fungus on cornmeal agar, oatmeal agar and CBS malt agar media for 10-14 days at 25^0C for teleomorph identification (Samson, 1994). Misidentification of species reported was due to high degree of diversion and variation occurring in the genus *Aspergillus* due to cultural conditions. *Aspergillus* taxonomy has been refined by a multidisciplinary approach of biochemistry, molecular biology and serology that included cell wall composition, enzymes, electrophoretic protein profiles, nucleic acid composition etc (Samson, 1993). The genus until 1994 contained, 186 anamorphic species with 72 named teleomorphs (Samson, 1994).

MORPHOLOGY OF *ASPERGILLUS*

The genus *Aspergillus* is a large taxon among the Hyphomycetes. The characteristic conidial morphology unique to *Aspergillus* is the aseptate stipe terminating in a vesicle on which, the phialides and metulae are borne. The combination of colour, shape, size and arrangement of conidial heads form the basis for species identification. Wehmer classified species of *Aspergillus* into micro and macro *Aspergilli*, based on the size of the conidial heads (Thom and Raper, 1945). *A. fumigatus*, *A. nidulans* and *A. versicolor* were grouped under micro *Aspergilli* due to smaller heads that formed columnar masses. *A. niger*, *A. clavatus*, *A. ochraceus* and *A. wentii* were classified as macro *Aspergilli* due to their globose and large heads. *A. flavus* and *A. candidus* produced heads of different sizes and shapes in the same colony.

In *Aspergillus,* conidium formation starts with the differentiation of foot cells in the mycelium. Unbranched conidiophores arise from the foot cell, more or less perpendicular to its axis and enlarge towards the apex and terminate in a swelling called vesicle. The vesicle may be globose, hemispherical, elliptical or

mere thickening of the stalk. From whole surface or upper part of the vesicle, more or less cylindrical spore bearing structures called phialides or sterigmata with or without intermediate cells known as metulae, originate. *Aspergilli* with only phialides are referred to as uniseriate while, biseriates are those with both metulae and phialides. Based on the formation of foot cells and simultaneous production of phialides, the *Aspergilli* are differentiated from monoverticillate *Penicillia* that form phialides successively (Onions *et al*., 1981). Conidia produced successively from the tips of the phialides form unbranched chains of different shapes and colours. Based on conidial wall and morphology, species were differentiated by Iizuka (1955). Multinucleate vesicles initially provide one or more nuclei to primary sterigmata. In the case of biseriate structures, secondary sterigmata (phialides) receive nuclei from metulae (primary sterigmata). Sterigmata form spores, which in many species are uninucleate. In *A. glaucus*, a group up to 12 nuclei has been recorded. *A. niger* produces dark brown to black globose conidial heads from which chains of conidiospores radiate. Conidial heads splitting into columns is characteristic of *A. japonicus*.

ASPERGILLUS CARBONARIUS

A. carbonarius (Bainier) Thom was placed under the *A. niger* group since it formed black coloured colonies and globose radius conidial heads. Colonies on Czapek agar reach 2.5 – 3.5 cm in diameter in 10 days. The fungus produces lanose mycelium first and on maturation of conidia, surface of colonies turn black and granular, except for a 1 – 2 mm wide margin. Reverse of the colony is colourless. Prolonged incubation induces a light yellow to blackish colour in the middle of the colony. Some instances of exudates were described which initially are colourless and later become brown. Conidiophores of *A. carbonarius,* which measure 5 – 6 μm in length, are much bigger than that of *A. niger*. The conidial heads are usually 500-600 μm in diameter and are radially arranged. They formed spherical vesicles that measure 60 – 80 μm in diameter. The phialides are arranged in two layers, and like vesicle, they are brown in colour. Primary

phialides measure 25 – 50 x 8 – 11 μm in length. Secondary sterigmata are usually smaller and measure 8 – 15 μm x 6 – 8 μm in size. Conidia are globose, with hyaline spines in youth. Upon aging, dark distinct warts measuring 5.5 – 8 μm large arise (Fassatiova, 1986).

This mould is widely distributed on various agricultural products including grains. The species is particularly predominant in vegetable and fruit store houses (Fassatiova, 1986). The synonyms of *A. carbonarius* are *A. fonsecaeus, A. pulchellus, Sterigmatocystis carbonaria,* and *S. acini-uvae.*

VARIATIONS IN *ASPERGILLUS*

Due to the ability of *Aspergillus* species to secrete broad range of enzymes, they are the major agents that are responsible for the decomposition of biological materials in the environment. Their flexible, adaptive metabolism and natural variants occurring in the species due to growth conditions make the genus ubiquitous and cosmopolitan. Spontaneously occurring variants in the fungus can be identified as sectoral growth and isolation of hyphae from these atypical growth sectors from single spore cultivation generally result in unique colonies. *A. flavus* obtained from cotton seed and soil of desert valleys of Arizona showed a number of variations in morphology, enzymes and toxins produced. Abundant but small sclerotia (<400 μm in dia) and large quantities of aflatoxins production by soil isolates of *Aspergilli* have been described from desert valleys of Arizona. In contrast, isolates from cottonseed had fewer but larger sclerotia and produced less aflatoxin. Variations were reasoned to adaptation of the fungi to the ecological niche during co-evolution of species over a long period of time (Cotty *et al.*, 1990). Many species of the genus domesticated for many centuries for use in food fermentation differ from their wild counterparts both in morphological and physiological characters. These isolates

usually have larger spores and establish mycelium more rapidly on substrates than their wild counterparts (Wickow, 1984).

Variation in *Aspergilli* can be induced by mutation or recombination. Several stable mutants were obtained experimentally by growing *A. niger* on media containing poisonous substances. Colourless *A. niger* mut. *cinnamomeus* and brownish coloured colonies of *A. niger* mut. *schemannii* were isolated by Thom and Church (1926) using the above procedure. Wheldon (1940) used low voltage cathode rays to induce mutation in *A. niger* while Raper *et al*. (1945) induced mutation with ultraviolet radiation to obtain mutants. In a detailed study on mutation of *A. niger* by UV, Raper and his colleagues (1945) showed effects on physiology of the mutants like inability to grow on nitrate containing medium while showing normal growth and sporulation on ammonia as nitrogen source (Raper *et al*., 1945). Swansson (1952) reported increased rate of mutation in *A. terreus* by pretreatment of conidia with infrared and low levels of mustard gas. Stapleton and Hollaender (1952) stated that a reduction in water or oxygen content before UV irradiation reduced the mutation rate. Induced mutation is one of the standard procedures followed in most of the industrial process that use microorganisms. In this laboratory, UV induced mutation was used as a procedure to improve *A. niger* and *A. carbonarius* for the production of industrial enzymes like amylases and polygalacturonases (Eugene-Raj *et al*., 2003; Ravi-Kumar, 2004; Venkatesh *et al*., 2003).

CARBON METABOLISM IN *ASPERGILLUS*

A wide variety of organic compounds are used as carbon and energy source by *Aspergilli* (McCullough *et al*., 1977). Embden-Meyerhoff-Parnas pathway, pentose phosphate pathway and TCA cycles occur in *Aspergillus* (Blumenthal, 1965; Niederpruem, 1965). Carbon utilization is little affected by the nature of nitrogen source but mutants differ in carbon metabolism and growth

on nitrate or ammonia (Hankinson, 1974). Certain amino acids serve as a poor carbon source for *Aspergilli* even though they are used as nitrogen sources (Kinghorn and Pateman, 1977). Efficiency of carbohydrate utilization is influenced by its concentration in the medium, trace metals and growth pH (Perlamn, 1965). Of all the monosaccharides, glucose, fructose and mannose are readily utilized by the fungus but galactose is less metabolized (Berry, 1975). Xylose, arabinose and several other pentoses support the growth of the fungus as they are incorporated in to the pentose phosphate pathway (Dean, 1972). Glucose reportedly suppressed the uptake of galactose while, inhibition in the uptake of glucose in *A. nidulans* by acetone is also known (Romano and Kornberg, 1968).

The most extensively studied form of carbon regulation in *Aspergillus* is carbon repression, a phenomenon where in the easily metabolized carbon source temporarily prevent the uptake or use of other less favourable carbon sources (Ronne, 1995). Mediation of carbon regulation through carbon repression protein A (CREA) has been demonstrated in the fungus. CREA inhibits transcription of many target genes by binding to the specific sequences 5'-SYGGRG-3' in the promoter regions (Kulmburg *et al*., 1993). But how the CREA synthesis is triggered by signal from favoured carbons source like glucose is still unknown (Ruijter and Visser, 1997). The genes regulated by CREA are grouped into three systems such as: genes encoding enzymes involved in catabolism of less preferred carbon sources (cellulose, pectin, xylan and araban), genes encoding gluconeogenic and glyoxylate cycle enzymes and genes related to secondary metabolism like penicillin production (Ruijter and Visser, 1997).

Detailed understanding on the function of CREA protein was derived from studies on *cre*A mutants of *A. nidulans* (van der Veen *et al*., 1995). A characteristic property of mutants affected in CREA function is compact colony morphology and reduced sporulation on solid-state media (Hynes and Kelly,

1977). The *A. nidulans cre*A gene has been cloned and characterized (Dowzer and Kelly, 1991). The product of *cre*A is a DNA binding protein containing two zinc fingers whose binding to promoter prevents transcription of the target gene (Shroff *et al*., 1996). The CCAAT elements found in *Aspergillus* genes indicate possible involvement of CCAAT binding factors like the HAP2/HAP3/HAP4 complex of yeasts, in carbon repression (Ruijter and Visser, 1997).

NITROGEN METABOLISM IN *ASPERGILLUS*

Though *Aspergilli* can utilize a wide range of nitrogen compounds as soul source of nitrogen, certain compounds like ammonia, glutamine, and glutamate are preferentially used. When these primary sources are not available or available in low concentrations, many other sources like nitrate, nitrite, purines, amides, amino acids, peptides, and proteins are used (Pateman and Kinghorn, 1976). Urea is degraded to ammonia by urease in a one step reaction. Mutants of *A. nidulans* defective in urease production were shown not to grow on media containing urea as soul nitrogen source (Pateman and Kinghorn, 1976). When the fungus utilizes salts of ammonia, the pH of the medium drops rapidly and consequently the growth ceases. Incase of ammonium nitrate, the sharp drop in pH indicated preferential use of ammonia over nitrate as nitrogen source (Morgan and MacMillan, 1954).

In *A. nidulans*, assimilation of nitrate has been demonstrated *via* nitrite to ammonia catalyzed by nitrate reductase and nitrite reductase (Gross, 1969). Nitrate and nitrite induced the production of the two enzymes and the associated hydroxylamine reductase but ammonia repressed their expression (Pateman *et al*., 1967). It has been shown that nitrate and *nir*A, (a gene that regulates enzyme activity necessary for nitrate reduction) increased levels of enzymes involved in pentose phosphate pathway (PPP) since, reduction of nitrate to ammonia consumed NADPH provided by PPP (Hankinson and Cove, 1974).

Though formamide is used only as a source of nitrogen, *Aspergilli* utilize acetamide as both carbon and nitrogen source. The enzymes involved in their utilization (acetamidase, formamidase) are induced by amides while ammonia repressed them (Hynes, 1975). The nitrogen regulation was reported to dependent on the nature of the carbon source available to *Aspergillus* (Kinghorn and Pateman, 1977). Studies on mutants affected in regulation of ammonia utilization and its repressive effect resulted in the identification of ammonium repression gene, *are*A (Chang *et al*., 2000; Pateman and Kinghorn, 1977).

The genes that responded to available nitrogen sources reported to be governed in parallel by major (global) and minor (pathway specific) regulatory genes (Marzluf, 1997). Major positive acting regulatory gene *are*A that mediate global nitrogen repression and derepression has been characterized from *A. nidulans* (Kudla *et al*., 1990), *A. oryzae* (Christensen *et al*., 1998) and *A. parasiticus* (Chang *et al*., 2000). The gene product of *are*A has been shown to be a transcription factor, which structurally and functionally resemble GATA family of DNA binding proteins (Kudla *et al*., 1990; Merika and Orkin, 1993). The global regulator protein AREA functions in combination with one or more pathway-specific factors. Such factors are believed to achieve an active form upon binding to a specific inducer (Marzluf, 1997). Pathway-specific factors well studied in *Aspergillus* are AMDA (acetate), AMDR (acetamide), FACB (acetate), NIRA (nitrate), PRNA (proline) and UAY (urate) (Andrianopoulos and Hynes, 1990; Burger *et al*., 1991; Karz and Hynes, 1989; Lints *et al*., 1995; Suarez *et al*., 1995). Their expression has been reported to depend on derepression signal from globally acting AREA factor.

STRUCTURE OF PECTIN

Pectic substances are the most complex acidic high molecular weight heteropolysaccharides that occur in plant cells as cementing substances. Large portion of this polysaccharide is made of anhydrous polygalacturonic acid units. The carbonyl groups of polygalacturonic acid may be partly esterified by methyl groups or partly or totally neutralized by one or more bases (Fogarty and Kelly, 1983). In 1944, the Committee for the Revision of the Nomenclature of Pectic Substances (a subdivision of the American Chemical Society) defined complex substances as follows.

Protopectin: The water insoluble parent pectic substance, which occur in plants and from which pectic substances are produced.

Pectinic Acids: It is the term used to designate colloidal polygalacturonic acids containing more than a small portion of methyl ester groups. They form gels under suitable conditions with sugar and acid and if the methyl content is low, gel formation occur with certain ions.

Pectin or Pectins: Water soluble pectinic acids of varying methyl ester content and degree of neutralization which form gels with sugar and acid.

Pectic Acid: Pectic substances composed of colloidal polygalacturonic acid and essentially free of methyl ester groups. The salts of pectic acids are either normal or acid pectates.

Pectic substances are used as gel forming agents in industries. Hence, there are definitions based on application apart from composition based naming system. Pectins with high methoxy content (>50%) that gel only in the presence of a relatively high sugar and acid content are called ‘slow-setting pectins’ while ‘fast-setting pectins’ denote pectins with lower methoxyl content that form gels with less sugar and acid content (Sakai *et al*., 1993).

Pectin is a heteropolysaccharide formed by anhydrous galacturonic acid and non-uronides bound to the unbranched chain of α-1,4-glycosytic bonded galacturonic acid units. This complex polysaccharide can be clearly distinguished as two defined regions, the smooth and hairy regions (de Vries *et al*., 1982). The region consisting of a backbone of α-1,4 linked D-galacturonic acid residues which can be acetylated at *O*-2 or *O*-3 and methylated at *O*-6 is called "smooth" region or homogalacturonan backbone **(Fig. 1)** while the hairy region **(Fig. 2)** identified through structural studies on pectin from apple (Schols and Voragen, 1996), sugar beet (de Vries and Visser, 2001; Ishii and Matsunaga, 1996), grape (Vidal *et al*., 1999) and cultured sycamore cell walls (McNeil *et al*., 1984) revealed the three different structures, a xylogalacturonan consisting of *D*-xylose substituted galacturonan backbone, rhamnogalacturonan and arabinogalacturonan.

In hairy regions, the galacturonic acid backbone was reportedly interrupted by α-1,2-linked *L*-rhamnose residues. This part was referred to as homogalacturonan I. Long side chains consisting mainly of *L*-arabinose and *D*-galactose were attached to the rhamnose residues at *O*-4. In sugar beet and apple pectins, ferulic acid was found at *O*-2 of galactose residues as terminal residues to cause further complexities (de Vries and Visser, 2001). Galacturonic acid residues in rhamnogalacturonan have also been reported to be esterified at *O*-2 or *O*-3 positions (Schols and Voragen, 1994). Rhamnogalacturonan II is a polysaccharide made of ~30 monosaccharides units with a backbone of galacturonic acid residues substituted by four side chains. These side chains also contained uncommon sugars such as 2-*O*-methoxy-*L*-fucose and 3-deoxy-*D*-manno-2-octulosonic acid (Kester and Visser, 1990). Arabinogalactans I have a main chain of *D*-galactose units linked by $\beta(1\rightarrow4)$ glycosidic bonds with side chains made of $\alpha(1\rightarrow5)$ linked *L*-arabinose polymers, which is attached by $\alpha(1\rightarrow3)$ glycosidic bond to the galactose main chain (Whitaker, 1984). In contrast to arabinogalactans I, arabinogalactans II had a main chain of $\beta(1\rightarrow3)$

linked *D*-arabinose units interrupted by side chains composed of $\beta(1\rightarrow6)$ linked galactose units (Whitaker, 1984). The variations in pectin structures described above, were attributes of protective mechanism that the plants have evolved to protect against invading microorganisms.

ENZYMES INVOLVED IN PECTIN DEGRADATION

Pectic enzymes are widely used in fruit processing industries for the preparation of whole fruit juices. Due to their activity on plant tissues, pectic polymers that form the major constituent of middle lamella of higher plant primary cell walls are degraded. By this process, contents of the whole fruit are extracted. Other industrial applications of pectic enzymes include clarification of wines, production of baby foods, hydrolysis of cellulosic biomass and in the generation of protoplasts for cell culture research (Lang and Dornenburg, 2000).

Pectinases are a group of enzymes that act on pectic polymers (predominantly 1,4-linked α-D-galacturonic acid and their methoxylated derivatives). Pectin is said to be the most complex heteropolysaccharide so far described (Sakai *et al*., 1993). Enzymes degrading such a complex structure are not only numerous but also vary in their degrading mechanisms. Based on their catalytic action, pectin-degrading enzymes have been classified into two major groups, depolymerising enzymes and saponifying enzymes or esterases. The depolymerising enzymes are further classified according to (a) hydrolytic or trans-eliminative cleavage of glycosidic bonds by endo- or exo- mechanism of degrading process, (b) preference for pectic or pectin as substrate and, (c) ability to act on homogalacturonan part (smooth region) or hairy region (de Vries *et al*., 1982; Fogarty and Kelly, 1983).

Pectin Methylesterase

This enzyme is also referred to as pectin esterase, pectin pectyl hydrolase (EC 3.1.1.11), pectase, pectin methoxylase and pectin demethoxylase (Whitaker, 1984). Pectin esterases are produced by some bacteria, plants and fungi. For active pectin depolymerising enzyme formulations, pectin methylesterase with polygalacturonase and/or pectates lyase are necessary (Pilnik and Voragen, 1993). Pectin esterases have a high specificity for methyl ester of polygalacturonic acid. Enzymatic desterification of pectin proceeds in a linear fashion along the substrate and because of the release of pockets of free carbonyl groups in this manner, the resultant product becomes highly sensitive to calcium (Whitaker, 1984).

Polygalacturonases

These enzymes hydrolyze the α-1,4 glycosidic linkages in homopolygalacturonan backbone. There are four types of polygalacturonases based on their preference for the type of pectin like, poly[α(1→4)-*D*-methylgalacturonic acid] or pectinic acid, poly [α(1→4)-*D*-galacturonic] and based on the mechanism by which they hydrolyze the polymer, exo fashion releasing products in a successive manner or oligosaccharides in an endo manner.

Endopolygalacturonases (EC 3.2.1.15) catalyze random hydrolysis of α-1,4-glycosidic linkages in pectic acid. They have been characterized from higher plants, molds, yeasts and bacteria **(Table 1)**. Due to their endo-hydrolyzing activity, they cause a rapid drop in substrate viscosity and the rate and degree of hydrolysis decrease rapidly with increasing degree of methoxylation. Mono-, di-, and tri-galacturonan units accumulate as end products at the end of hydrolysis (Devi and Rao, 1996; Pilnik and Voragen, 1993; Schwan *et al*., 1997).

Endopolygalacturonases of *Aspergillus* constitute the major component in formulations used in food industry.

Exopolygalacturonases (EC 3.2.1.67) catalyze the hydrolysis of α-1,4-glycosidic linkage of pectic acid in a sequential manner from non-reducing end. Fungal exopolygalacturonases release galacturonic acid monomer as end products and they can degrade dimers as well. Bacterial exopolygalacturonases have a neutral pH optima and they produce di-galacturonic acid as a main end product (Kester and Visser, 1990; Whitaker, 1984). These enzymes do not bring about rapid reduction in viscosity. Reduction in viscosity caused by this enzyme has a linear relation with appearance of reducing groups from substrate (Keon and Waksman, 1990).

Polymethylgalacturonases directly hydrolyze α-*D*-1,4-glycosidic bond of highly methoxylated pectic acid without the involvement of esterases. Though endo- and exo- enzymes of this group have been described in literature (Sakai *et al*., 1993), Fogarty and Kelly (Fogarty and Kelly, 1983) questioned their existence since according to them the reports were based on impure preparations of polygalacturonase containing pectin esterase activity.

Pectic Lyases

This group of enzymes catalyze cleavage of α-1,4-glycosidic linkages by transelimination. Activity of this enzyme result in galacturonides with an unsaturated bond between C_4 and C_5 at the non-reducing end of the galacturonic acid formed. They are not produced by plants but reported mostly from bacteria. Like polygalacturonases, endo- and exo- acting enzymes have been reported in lyases as well (Nasuno and Starr, 1967). All the transeliminases acting on pectin require Ca^{2+} for their catalytic function and their pH optima range from 5 to 9. Polymethylgalacturonan lyases are the only enzymes demonstrated to break

methoxylated pectin without requiring esterase activity and they are found in fungi (Sone *et al*., 1988).

Enzymes that act on Hairy Region of the Pectin

Recent investigations have identified the enzymes that act on hairy region of pectic substances as important for plant cell wall degradation. These pectin hairy regions degrading enzymes act in synergy with enzymes that hydrolyze smooth chain. The enzymes in this category are rhamnogalacturonan hydrolases, rhamnogalacturonan lyases, acetyl esterases, xylogalacturonan hydrolases and arabinofuranosidases (Suykerbuyk *et al*., 1997; van der Vlugt-Bergmans *et al*., 2000; Vidal *et al*., 1999; Whitaker, 1990).

PECTINASES OF *ASPERGILLUS*

Several enzymes involved in the degradation of pectin have been purified and gene sequences obtained. Polygalacturonase I and II (PG I and PG II) were identified as major enzymes produced by *A. niger* (de Vries and Visser, 2001; Kester and Visser, 1990). Screening genomic DNA libraries of *A. niger* using probes designed based on the NH_2-terminal sequence of PG-I and PG-II resulted in isolation of five more genes. The products of these genes were also detected in *A. nidulans* transformants by western blot analysis using antibody raised against PG I. The sequence of one of the polygalacturonases (*PGC*) identified three introns in the genomic DNA. The product of the gene was 383 amino acids preproprotein that is cleaved after a pair of basic amino acids. In the upstream region of *A. niger* polygalacturonase gene, a conserved sequence CCAAT apparently represented a binding site for the regulatory protein (Bussink *et al*., 1992a).

Sequence analysis of *pga*A and *pga*B identified one intron in the former and two introns in the latter. Apparently, these enzymes were also synthesized as preproenzymes with the prosequences cleaved by a KEX2 like dibasic peptidase after the lysine and arginine residues (Bussink *et al*., 1990). The N-terminal amino acid sequence of several polygalacturonases of *Aspergillus* identified three different enzymes. However, polygalacturonase III produced by *A. niger* and *A. oryzae* were similar (Cho *et al*., 2001). Sequence analysis of polygalacturonase identified 27 amino acids characteristic of a secretory signal sequence in NH_2-terminal, which is cleaved off in the mature protein (Bussink *et al*., 1991a; Ruttkowski *et al*., 1990).

A similar processing of rhamnogalacturonan acetyl esterase (RGAE) from *A. aculeatus* was described. The *rha*1 cDNA was also found to encode a signal peptide of 17 amino acids. Absence of significant similarity of the amino acid sequence of this enzyme with other pectinases suggested the enzyme RGAE as a novel enzyme representing a new family of esterases (Kauppinen *et al*., 1995). Endo xylogalacturonan hydrolase (xghA) of *A. tubingensis* cDNA analysis revealed the presence of 18 amino acid signal sequence in the NH_2-terminus. Comparison of xghA amino acid sequence with sequences in European Molecular Biology Laboratory (EMBL) data library revealed homology to polygalacturonase sequences of prokaryotes, fungi and plants and *rhg*A and *rhg*B sequence of *A. aculeatus* and *A. niger* (van der Vlugt-Bergmans *et al*., 2000). The pectin methyl esterase protein of *A. aculeatus* had a molecular mass of 43 kDa as estimated on SDS-PAGE gels. The cDNA gene sequence revealed an apparent signal sequence of 17 amino acids with a typical signal cleave site between Ala-17 and Ala-18 (Christgau *et al*., 1996).

Strain Improvement

Though natural isolates of *Aspergillus* produce polygalacturonases, strain improvement to increase enzyme production is usually carried out by random mutagenesis. Random mutagenesis by UV or NTG and selection of mutants resistant to antimetabolites such as 2-deoxy-D-glucose (*dgr*) or resistant to glycerol catabolic repression were shown to overproduce enzyme since the mutants overcame the effective catabolic repression during submerged growth of fungus (Solis *et al*., 1990). Tubular fermentor based studies suggested that slow growing *dgr* mutants of *A. niger* produced higher activities of polygalacturonase (Leuchtenberger and Mayer, 1992). *Aspergillus* mutants that formed aggregates or pellets during submerged fermentation were described to produce higher quantities of enzyme compared to parent strain and overproduction was reasoned to their better fermentor behaviour such as less viscosity, better growth, higher aeration and efficient mixing of medium (Gibbs *et al*., 2000; Leuchtenberger and Mayer, 1992).

In the case of strain improvement for enzyme production in solid-state cultures, a specific protocol for the selection of strains well adapted to SSF has not been defined (Antier *et al*., 1993b). Documented heterogonous nature of solid state culture medium such as low water activity (a_w), osmotic gradient due to heterogenous distribution of solutes, matrix structure and adsorption characters of solid support limited selection protocols after mutation. Low a_w and high osmotic pressure can lead to modification in the phospholipid fatty acid saturation which cause the reduction in membrane permeability in fungi for protein secretion (Lesage *et al*., 1993). Accumulation of reducing sugars at low a_w values in solid-state medium probably due to membrane transport limitation has been postulated (Acuna-Arguelles *et al*., 1994). Mutants selected for tolerance to high osmotic pressure and low a_w showed better colonization of substrate with increase secretion of enzymes (Antier *et al*., 1993a). The use of

low a_w as a selective factor for 2-deoxy-D-glucose resistant (*dgr*) mutant resulted in higher polygalacturonase production in solid-state cultures (Solis *et al*., 1990).

Secretion of Pectinases by *Aspergillus*

The ability to secrete a large array of biopolymer degrading enzymes *Aspergillus* was postulated to evolutionary consequence gained by the species (van Gorcom *et al*., 1994). Though more than 100 different enzymes have been characterized from versatile species like *A. niger* (Oxenboll, 1994), the mechanism of gene induction for polygalacturonase production is still unknown since pectin is a high molecular mass polysaccharide. Induced secretion of polygalacturonases by galacturonic acid and other monomers of pectic substances have been reported. The basal level of pectic enzyme activities detected in medium containing no pectic substances suggested low molecular mass products released from pectin by enzyme activity acting as inducers for the synthesis of pectinases (Leone and van den Heuvel, 1987).

Regulation of Pectinase Production in *Aspergillus*

Production of lesser pectin esterase activity by *Aspergillus* when grown in a substrate made of deesterified lemon peel with alkali pretreatment compared to the esterase activity induced by untreated peel revealed the importance of inducer composition for the type of pectic enzyme produced by the fungus (Maldonado *et al*., 1989; Maldonado *et al*., 1986). Naturally occurring inducers such as lemon peel were reported to produce four times higher polygalacturonase activity than purified pectin from the same source (Larios *et al*., 1989). Components of medium that affect polygalacturonase production are nitrogen source and phosphates. Higher concentration of ammonium salts increased the enzyme productivity with lower concentration of phosphates. Among source of nitrogen, ammonium salts yielded higher pectinase activities

than nitrates. When *Aspergillus* was grown in nitrogen limiting media, a delay in the production of polygalacturonase was reported (Aguilar and Huitron, 1986).

Polygalacturonase production by filamentous fungi is repressed by readily available carbon source (Siessere and Said, 1989). Synthesis of the enzymes was induced by galacturonic acid or polygalacturonic acid in *A. niger* at transcription level (Maldonado *et al*., 1989). Glucose repressed the production of polygalacturonases at transcription level but addition of cAMP derepressed enzyme synthesis (Torakazu *et al*., 1975). A similar regulation was seen in the case of pectin esterase enzyme production by *Aspergillus* (Taragano *et al*., 1997). A number of reports showed that repression of polygalacturonase synthesis in *Aspergillus* during submerged growth by simple sugars was very effective and stronger than in SSF (Acuna-Arguelles *et al*., 1995; Solis-Pereira *et al*., 1996).

Aspergilli that produce pectic enzymes are known to grow over a wide pH range. In *A. kawachi*, it was demonstrated that the pH of the culture broth influenced the type of polygalacturonase produced by the fungus (Kojima *et al*., 1999). Thus, a role for pH regulatory protein (PacC) was identified in pectinase gene regulation. Generally, pH regulation is mediated by *pac* genes, *pac*C, *pad*A, *pal*B, *pal*C, *pal*F, *pal*H and *pal*I. Mutations that mimic acidity, alkalinity, and neutrality gene expressions have been obtained. This suggested direct involvement of a key transcription factor, which is a product of *pac*C, in the regulation of gene expression by ambient pH. The major arabinofuronosidase gene was reportedly regulated by PacC (Gielkens *et al*., 1999). Hence, regulation of pectinase gene expression apparently is controlled by pH as in the genes encoding several cell wall degrading enzymes like xylanases and cellulases (MacCabe *et al*., 2002; Stewart and Parry, 1981).

THE PROBLEM

THE PROBLEM

Pectinases are enzymes involved in the degradation of plant cell wall. Hence, these enzymes find application in food industries for the extraction of intracellular components from plant materials. In the beverage industry, they are used for the production of clarified fruit juices while in wine production, use of pectinases improve colour extraction from grapes. Pectinases along with other cell wall degrading enzymes are also used for oil extraction from pectin rich fruits like, the lemon and olive.

Polygalacturonase, the major constituent of pectinase formulations, is industrially produced from the fungus, *Aspergillus niger*. For industrial applications, polygalacturonases of *A. carbonarius* were also described from this laboratory (Devi and Rao, 1996). However, the fungus produced the enzyme only when grown in solid-state cultures made of wheat bran (Kavitha and Umesh-Kumar, 2000). This study revealed very little enzyme production by the fungus in shake-flask cultures. Differential regulation as the cause for decreased yields of polygalacturonase during shake-flask growth was postulated and to evidence the hypothesis, mutants of *A. carbonarius* were isolated to examine the phenomenon governing polygalacturonase secretion by *A. carbonarius*. The details of the experiments are described in the **First Part** of the thesis.

In this study, a mutant over producing the enzyme during shake-flask growth was isolated. Studies using the mutant showed that polygalacturonase secretion was induced by acidic pH of the culture broth and that multiple enzyme forms arise due to differential enzyme glycosylation. These results are described in the **Second Part** of the Thesis.

Since the isolation of polygalacturonase overproducing mutant of *A. carbonarius* suggested its application for industrial enzyme production by

submerged fermentation, cultural conditions were standardized to define an industrial process. The **Third Part** of the thesis describes these results so also the scaleup of fermentation to 120 L for enzyme production using the mutant strain of *A. carbonarius.*

MATERIALS AND METHODS

METHODS AND MATERIALS

MICROBIOLOGICAL METHODS

Media, glass and plastic ware were generally sterilized in an autoclave at 121^{0}C (15 lbs pressure) for 15 min. Inoculations were performed in a Laminar Flow hood. Other aseptic methods described for microbiological work were generally followed.

WATER

Glass distilled water was used for all media preparations. Ultra-pure water obtained from Milli Q filtration unit (Millipore) was used for buffer preparation, enzyme assays and protein determination.

CHEMICALS

Laboratory grade reagents and chemicals were used for media preparation. For biochemical studies, analytical or pure chemicals were used. They were procured from s.d. fiNE-CHEM Ltd., Mumbai, India; Hi-Media, Mumbai; Ranbaxy Laboratories Ltd., India, and Sigma Chemicals, St. Louis, USA. Bulk media components like wheat bran, corn flour, corn starch and corn steep liquor were purchased from local market. Peptone, yeast extract, and Agar-Agar were from Hi-Media, Mumbai, India.

Sodium alginate (medium viscosity), peptide-N-glycosidase F (PNGase F) of *Flavobacterium meningosepticum*, enzyme substrates like sodium polygalacturonate, casein (Hammarsten grade), 4-chloro-1-naphthol, standards for enzyme assays and protein estimation, galacturonic acid, tyrosine, and bovine serum albumin were procured from Sigma Chemicals, St. Louis, USA.

Detection reagents like Folin-Ciocalteau's phenol reagent, silver nitrate, Coomassie Blue G 250, R 250 and Amido black were obtained from Loba Chemie, India.

Acrylamide, N, N'-methylene-bis-acrylamide, ammonium per sulphate, N, N, N', N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulphate, dithiothreitol, and mercaptoethanol were from BDH Laboratories and Sigma Chemicals. Termamyl and Pectinex 3XL were from Novo Nordisk Laboratories, Denmark.

Serological reagents like goat anti-rabbit IgG tagged with biotin and avidin-peroxidase conjugate were also from Sigma Chemicals. Buffer salts like Tris, sodium acetate and the detergent Triton X-100 were from Sigma Chemicals. Nitrocellulose membranes were procured from Amersham Pharmacia Biotech, UK.

THE ORGANISM

The holotype of *Aspergillus carbonarius* (Bainier) Thom (Accession No. CFTRI 1047) used in this study was isolated in this laboratory from grapes (Devi and Rao, 1996; Sreekantaiah *et al*., 1975). The wild type and mutants used in this study were grown on Yeast extract-Peptone-Dextrose (YPD) agar slants for four to five days at 30^{0}C and maintained at 4^{0}C. Subculturing was done periodically every two months.

Yeast Extract-Peptone-Dextrose Agar

		$g\ L^{-1}$
Yeast Extract	-	10

Peptone	-	20
Dextrose	-	20
Agar-Agar	-	20
pH	-	5.5

INDUCTION OF MUTATION

Three-day-old spores of *A. carbonarius* from YPD slant culture were spread on petriplates containing pectin agar medium and mutation was induced (30-40% kill) by inverting the plates on a UV trans-illuminator (302 nm). The plates after mutation were incubated in the dark at 4^0C for 12 h to avoid light induced repair of the mutation induced. Following incubation at 42^0C for 24 h (Temperature Selection), the survivors were identified as growing colonies when incubated at 30^0C for 48 – 72 h. From the surviving colonies, fast growing colonies on pectin agar that showed bigger zone of clearance were screened for aerial growth of mycelia, resistance to 2-deoxy-D-glucose (0.01%) and pellet formation in shake-flask cultivation by picking mycelia to fresh pectin agar plate and inoculation in shake-flasks.

Inoculum for shake-flask cultivation was developed in corn flour broth using spores. After growth at 30^0C for 48 h on an orbital shaker (150 rpm), the inoculum was transferred to corn flour salt medium and incubated at 30^0C for 24 h on an orbital shaker, as above. Enzyme production was quantitated in the culture broths during the growth period. For protease production, the fungi, were grown in corn starch-peptone-yeast extract medium.

Pectin Agar

		g L^{-1}
Ammonium dihydrogen orthophosphate	-	0.9
Di-ammonium hydrogen phosphate	-	2.0

Magnesium sulphate heptahydrate	-	0.1
Potassium chloride	-	0.5
Citrus pectin (Sigma)	-	10.0
Agar-Agar	-	20.0
pH	-	5.5

Corn Flour Broth

		g L^{-1}
Corn flour	-	40
Peptone	-	10
Yeast extract	-	6
pH	-	5.5

Corn Flour Salt Medium

		g L^{-1}
Corn flour	-	25
$(NH_4)H_2PO_4$	-	3.13
$(NH_4)_2HPO_4$	-	3.13
pH	-	5.5

Corn starch-Peptone-Yeast Extract Medium

		g L^{-1}
Corn Starch	:	100
Peptone	:	10

Yeast Extract	:	5
pH	:	5.5

ENZYME ASSAYS

Schimadzu UV-visible recording spectrophotometer (UV-160A) was used for all spectrophotometric analyses.

Polygalacturonase Assay

Polygalacturonase activity was determined using 0.5% polygalacturonic acid (sodium salt) substrate prepared in 0.1 M sodium acetate buffer (pH 4.3). Reducing sugars were quantitated as galacturonic acid equivalents released due to enzyme activity as determined by spectrometric analysis by the Nelson-Somogyi method (Somogyi, 1952). Galacturonic acid standards were used as standards.

Polygalacturonic acid substrate (2 mL) equilibrated to 50°C in a water bath was treated to 100 µL of suitably diluted enzyme and the reaction was allowed to procede for 10 min. The reaction was stopped by drawing 100 µL and mixing with 1 mL freshly made alkaline copper sulphate solution. Nine hundred micro liter of water was added to this mixture and kept in boiling water bath for 20 min. After cooling, the colour was developed with 1 mL arsenol molebdate reagent and the volume was made to 10 mL with distilled water. Galacturonic acid formed was estimated by determining absorbance at 540 nm read against enzyme and substrate blanks. Activity corresponded to µmol galacturonic acid formed min^{-1} for 1 mL culture filtrate or one gram moldy bran.

Alkaline copper reagent

Solution A

Anhydrous sodium carbonate : 2.5 g

Sodium bicarbonate : 2.5 g

Sodium potassium tartarate : 2.5 g

Solution B

Copper sulphate pentahydrate : 15g

Concentrated sulphuric acid : 1-2 drops

These were dissolved in Milli Q water, volume made to 100 mL and stored at room temperature in a stoppered brown bottle. Alkaline copper reagent was prepared fresh for assay by mixing solutions A and B in the ratio of 25:1.

Arsenol molybdate reagent

Ammonium molybdate : 5 g

Concentrated sulfuric acid : 4.2 mL

Sodium arsenate : 0.6 g

The above were dissolved in Milli Q water and the volume made to 100 mL. The solution was incubated at 37^0C on an orbital shaker overnight. The reagent was stored at 4ºC in a stoppered brown bottle.

Acid Protease Assay

Protease activities in the culture filtrates were assayed by determining tyrosine equivalents due to protease action on casein. Released tyrosine equivalents were estimated using Folin-Ciocalteau Phenol reagent (Ichishima, 1970). The released tyrosine equivalents were measured by adding 2.5 mL of

0.4 M sodium carbonate solution and 0.5 mL Folin-Ciocalteau Phenol reagent (1:5 diluted with water) to 0.5 mL of sample and incubating at 30°C for 30 min. The colour intensity was measured at 660 nm against the blank. Tyrosine (Sigma, 0.02 mg/mL) was used as standard.

Casein (2%) prepared in 0.1 M sodium acetate-HCl buffer (pH 2.7) was used as substrate for protease activity. A uniform suspension of casein was made in 10 mL buffer by stirring the suspension for 15 min in a boiling water bath. After cooling to room temperature, the volume was made to 100 mL using 0.1 M sodium acetate-HCl buffer (pH 2.7).

Acid protease activity was determined using 2% casein (Hammarsten) prepared in 0.1 M sodium acetate-HCl buffer (pH 2.7) as substrate. The reaction was carried out at 30^{0}C for 30 min and stopped by the addition of 0.4 M trichloroacetic acid. The clear filtrate from the reaction mixture obtained after centrifugation (4000 g for 5 min) was analysed for tyrosine released using Folin-Ciocalteau Phenol reagent (Ichishima, 1970). The protease activity was quantitated as μmoles of tyrosine released from casein in one minute at 30^{0}C.

TOTAL PROTEIN DETERMINATION

Protein concentrations were routinely determined by the dye binding method using Coomassie Brilliant Blue G250 (Spector, 1978). The reagent for this assay consisted of Coomassie Blue G-250 prepared in 3% perchloric acid (optical density of the prepared reagent at 465 nm was adjusted between 1.3 – 1.5). Protein was quantitated by adding appropriately diluted sample to 1.5 mL of the reagent and making up the volume to 3.0 mL with milliQ water. Optical density was measured at 595 nm. Bovine serum albumin was used as standard. Specific activity of enzymes in liquid cultures corresponded to activity estimated for one milligram protein.

ELECTROPHORESIS

SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) was performed in 10% gels (Laemmli, 1970). The protein samples for loading the gel were prepared in sample buffer containing dithiothreitol (DTT). The proteins were visualized by silver staining (Morrisey, 1981). Molecular mass of the proteins was determined by running the following standards alongside the protein samples in SDS-PAGE. Molecular mass standards were obtained from Bangalore Genei, India.

Molecular Mass Standards	**kDa**
Phosphorylase b	97.4
Bovine Serum Albumin	66.0
Ovalbumin	43.0
Carbonic anhydrase	29.0
Soyabean Trypsin Inhibitor	20.1
Lysozyme	14.3

Based on the migration of standards, the molecular mass of the samples was determined using Bioprofil Image Analysis System (Vilber Lourmat, France).

ZYMOGRAM

SDS-PAGE under non reducing condition was performed in 10% acrylamide gels containing 0.67 mg/mL of sodium polygalacturonate (Sigma). After electrophoresis, the gels were washed with 0.01 M Tris-HCl buffer (pH 8.3) containing 20% isopropanol to remove SDS. Following two washes in 0.1 M sodium acetate buffer pH 4.3 (15 minutes each), the gels were incubated in the same buffer at 35^0C for 12 h for enzyme activity. For visualizing enzyme

activities, the gels were washed in 0.01M Tris-HCl buffer (pH 8.3) thrice (10 minutes each) to remove the digested fragments. The gels were stained with 0.1% toluidine blue in 0.01M Tris-HCl buffer (pH 8.3) for five minutes. Destaining was done by washing the gel for 6 h with intermittent change of the same buffer (McKeon, 1988) and the polygalacturonase activities were visualized as clear zones against a purple background.

POLYGALACTURONASE PURIFICATION

Polygalacturonase was purified by alginate affinity procedure (Gupta *et al*., 1993). The culture filtrate after shake-flask growth or extracts of moldy bran obtained from solid-state cultures was used as enzyme source. Culture filtrate was mixed with equal volume of 0.5% sodium alginate (Sigma Chemicals) prepared in MilliQ water of pH 4.3 (pH adjusted using dilute HCl). The mixture was stirred for 15 min, allowed to stand for 30 min at 30^0C and the alginate enzyme complex was precipitated by the slow addition of 0.02 M $CaCl_2$ solution pH 4.3 (volume equivalent to crude culture filtrate). The mixture was allowed to stand undisturbed for 15 min for uniform precipitate formation. Precipitate was separated by centrifugation (4000 g for 5 min) and washed with 0.02 M $CaCl_2$ solution (pH 4.3) four times. The enzyme from the alginate precipitate was eluted in a minimum volume of pH adjusted Milli Q water containing 0.5 M NaCl and 0.2 M $CaCl_2$ (pH 4.3). The eluted aliquot was dialyzed against pH adjusted MilliQ water (pH 4.3) and equilibrated by dialysis against 0.1M sodium acetate buffer (pH 4.3). Proteins in the dialyzed aliquots was concentrated using PEG-20000.

The 61 kDa enzyme was separated from the 42 kDa enzyme by electro elution from the gels after preparative SDS-PAGE. Protein bands on the preparative SDS-PAGE were visualized by immersing the gels in 1M KCl for 2 min without fixing (Walker *et al*., 1977). Protein at 61 kDa position (which was

seen as white regions against a pale white background) was cut into small pieces and taken in 0.1M sodium acetate buffer (pH 4.3). Electroelution was carried out at 100 mA for 10 min using the same buffer.

SEROLOGICAL METHODS

Antibodies

Antibodies against purified polygalacturonase (42 kDa enzyme) were raised in rabbits. Rabbits were immunized with 18 μg pure protein taken in saline (0.85% NaCl). Intra muscular injections of protein mixed with equal volumes of Freund's complete adjuvant (Sigma) were administered on days 0, 14, 28 and 42 (Shankar and Umesh-Kumar, 1994). Two days after the last injection, blood was collected from the marginal ear veins of the animal. Antiserum separated from the blood clot was stored at –15ºC with sodium azide as preservative.

Ring Test

Serially diluted antibody in Tris buffer saline (0.01 M Tris buffer, pH 8.0; NaCl 0.85%) was taken in Durham's tubes and an equal volume of antigen was layered on the antibody with out mixing. The tube was incubated at 37^0C and the reaction was visualized as white precipitate at the interface of antigen and antibody. Ring test was regularly used to check the antibody titer of bleeds.

Ouchterlony Immuno Double Diffusion

Two millimeter thick 1% agarose gels were prepared in 0.01M Tris buffer saline (pH 8.0; 0.85% NaCl) on glass slides. Wells were cut using cork borer for loading the reagents. The perpheral wells contained the antigen and central well the antibody. The slides were incubated at 37ºC in the humid chamber for 24-48 h for the reaction. Reactions were visualized as white precipitates between the

peripheral and central wells. To confirm reactions, slides were stained with Coomassie Blue R250 (Nagarajan and Umesh-Kumar, 1990).

Enzyme Inhibition Assay

The presence of antibody to the enzyme was confirmed by performing enzyme inhibition assay. Nitrocellulose membranes containing immobilized antibody were incubated for 30 min at 37^0C in a solution of enzyme containing a defined activity. The residual enzyme activity was estimated in the enzyme solution and decrease in enzyme activity defined antibody mediated absorption of the protein (Suresh *et al*., 1998).

Western Blotting

For Western blot analysis, protein samples after separation by SDS-PAGE on 10% gel were electro-blotted on to nitrocellulose membrane at 130 mA for 20 min. Membranes were blocked at 37^0C for one hour by shaking in 0.01M Tris-HCl buffer (pH 8.0) containing 10% skim milk. The proteins were identified indirectly using antibody raised to the pure polygalacturonase protein (1:500 dilution in 0.01M Tris-HCl buffer saline, 0.85% NaCl pH 8.0) and anti-rabbit goat IgG tagged with horseradish peroxidase obtained from Sigma (1:1000 dilution in 0.01M Tris-HCl buffer saline, 0.85% NaCl pH 8.0). Tris-HCl buffer (50 mM; pH 7.6) containing hydrogen peroxide (0.025%) and 4-chloro-1-napthol (0.4%) was used as substrate for the enzyme reaction.

DEGLYCOSYLATION

Purified enzyme proteins (42- and 61-kDa polygalacturonases) was denatured with 0.1% SDS by boiling for 5 min and deglysosylated at 37^0C for 48 h using peptide-N-glycosidase F (PNGase F) of *Flavobacterium meningosepticum* (Sigma, USA) taken in 50 mM Tris-HCl buffer (pH 8.0)

containing 1% Triton X-100 and 0.02% sodium azide. The reaction was stopped by adding electrophoresis sample buffer. The products of the reaction were analyzed after SDS-PAGE and Western blot reactions using antibody raised to 42 kDa polygalacturonase protein. Native enzyme was deglycosylated at 37^{0}C for 4 h using PNGase F in 0.25 mM Tris-HCl buffer (pH8.0). Enzyme activity was measured at pH 4.3 as above. Controls consisted of untreated protein and protein incubated in 0.25 mM Tris-HCl buffer (pH 8.0) containing heat inactivated PNGase F.

N-TERMINAL SEQUENCING

Purified proteins (42- and 61- kDa after SDS-PAGE in 10% gels) were electroblotted to PVDF (polyvinylidene difluoride) membranes (Biorad) using 0.1 M CAPS [3-(cyclohexylamino)-1-propane sulphonic acid] buffer at pH 11.0 (Dubey *et al*., 2000). Microsequencing was performed at Alta Bioscience, The University of Birmingham, UK using the membrane bound protein. The sequence identities were detected by BLAST search using the NCBI data base.

CULTIVATION OF FUNGUS

Shake-flask Cultivation

Spores from one-week-old slants were inoculated into 10 mL corn flour broth taken in 50 mL Erlenmeyer flasks and incubated at 30^{0}C for 48 h in an orbital shaker (150 rpm) for developing the inoculum.

The inoculum developed was transferred to 500 mL Erlenmeyer flasks containing 100 mL of corn flour salt medium. After 24 h growth at 30ºC in an orbital shaker (150 rpm), culture filtrates were obtained by filtration through Whatman No.1 filter paper. After centrifugation (6000 g for 5 min) the culture broths were used as enzyme.

In some experiments, glucose, xylose, galactose, mannose and pectin were used at 2% levels as carbon source replacing corn flour in the broth.

Solid-state Cultivation

For polygalacturonase production by solid-state fermentation, the fungus was grown on wheat bran medium consisting of the following components.

Wheat bran solid-state medium

Wheat Bran - 20g

NaCl - 47mg

Yeast Extract- 32 mg

2N HCl - 1.25 mL

Distilled Water- 6.75 mL

The components were mixed thoroughly in petriplates. The plates were autoclaved at 15 lbs psi (121^{0}C) for 45 min (Kavitha, 2001).

Spores from one-week-old slants were inoculated into 10 mL corn flour broth (Corn flour, 40 gL^{-1}; Peptone, 10 gL^{-1}; Yeast Extract, 6 gL^{-1}; pH 5.5) taken in 50 mL Erlenmeyer flasks. For developing the inoculum, the flasks were incubated at 30^{0}C for 48 h in an orbital shaker (150 rpm). The fungus grown as above, was mixed with 20g of sterile wheat bran medium aseptically.

For obtaining the enzyme after solid-state growth of the fungus, 10 g of the moldy bran was shaken with 100 mL 0.1M acetate buffer (pH 4.3) in an

orbital shaker (200 rpm) for 30 min. The extract obtained after filtration through Whatman No. 1 filter paper constituted the crude enzyme.

pH CONTROLLED CULTIVATION

Spores of the fungus was inoculated in 10 mL corn flour broth taken in 50 mL Erlenmeyer flasks and grown for 24 h on an orbital shaker. The primary inoculum was transferred to 100 mL corn flour salt medium buffered to pH 5.5 using 0.25 M citrate phosphate buffer. After the growth of the culture for 24 h, the fungal biomass was removed by aseptic filtration. The mycelia washed with sterile water were inoculated into corn flour salt medium buffered to pH 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 with 0.25 M citrate phosphate buffer. Flasks were incubated in orbital shaker (200 rpm) at 30ºC for growth. The culture filtrates after every 6 h growth was assayed for polygalacturonase activity. Simultaneously, the enzyme protein was analyzed by western blot reactions.

IDENTIFICATION OF THE ENZYME IN THE FUNGUS

The culture filtrates proteins, intracellular proteins and proteins of the periplasmic space were obtained as follows: Intracellular proteins were extracted from the fungal mycelium by grinding 24 h old mycelium with glass beads (106 microns and finer; Sigma) in a pestle and mortar; the aliquots obtained by freezing and thawing the fungal mycelia in a minimum amount of 0.1 M acetate buffer (pH 4.3) constituted proteins of the fungal periplasmic space (Patel, 1985); the culture filtrates were used directly as source of extracellular proteins.

The presence of the enzyme in the samples was analyzed by performing western blot reactions after SDS-PAGE. Antibodies developed against the 42 kDa polygalacturonase were used for probing the western blots.

SCALEUP OF FERMENTATION

Laboratory Scale Fermentor Trials

Spores from one-week-old slants were inoculated into 10 mL of corn flour broth medium taken in 50 mL Erlenmeyer flasks. The flasks were incubated at 30ºC for 48 h in orbital shaker (200 rpm) for inoculum development. The primary inoculum was scaled up to 100 mL by transferring the content of the 50 mL flasks into 90 mL fresh medium prepared in 500 mL flasks. After incubating the flasks at 30ºC for 18 – 24 h on an orbital shaker (150 rpm), the content were transferred to 900 mL medium and grown as above.

Nine liter of corn flour salt medium was prepared in 15 L Bioengineering Fermentor and initial pH was adjusted to pH 5.5. Medium was sterilized at 121ºC (15 lbs pressure) for 15 min. After cooling the medium to 30ºC, the fermentor was inoculated with 1 L inoculum developed as described above. Conditions maintained during fermentation were:

Temperature	:	$30^{0}C$
pH	:	Maintained at 4.0 using 2 M $(NH_4)_3PO_4$ solution
Agitation	:	600 - 800 rpm
DO	:	60% Saturation
Vessel Pressure	:	0.5 bar
Air flow rate	:	0.75 – 1.0 vvm

In some experiments, pH of the culture medium was not maintained. Culture samples were collected periodically and assayed for enzyme activity. pH of the culture broth was adjusted to 4.0 using 2 M NaOH before harvesting the culture for downstream processing.

Fed-Batch Fermentation Trials

One liter inoculum developed as described in laboratory fermentor trials was used to inoculate 10 L corn flour salt medium (initial pH 5.5) prepared in 15 L Bioengineering Fermentor. Fermentation conditions were maintained as mentioned above but the pH of the culture medium was not maintained. When pH of the culture broth dropped to 3.0, 2 L culture broth was removed aseptically from the fermentor and 2 L fresh corn flour salt medium (pH 3.0) was added. Fermentation was allowed for 4 – 6 h and pH of culture broth was adjusted to pH 4.0 using 2 M NaOH. The culture broth was harvested for down stream processing. Periodical samples collected during fermentation were analyzed for enzyme activity.

Pilot-plant Fermentation Trials

Submerged fermentation was carried out using Sortorius fermentor (200 L working volume) using corn flour salt medium (initial pH 5.5).

Inoculum Development

One week old spores of mutant strain of *A. carbonarius* was inoculated into 50 mL Erlenmeyer flasks containing 10 mL of corn flour broth medium. They were incubated at 30ºC for 48 h in orbital shaker. The 48 h old culture broth was transferred to 500 mL Erlenmeyer flasks containing 100 mL of corn flour salt medium and incubated as above for 24 h (pre-inoculum). One liter of inoculum was transferred into inoculum raising fermentor (Bioengineering Fermentor, working volume 15 L) containing 9 L corn flour salt medium. Fermentor conditions maintained were:

Temperature	:	28ºC
Agitator speed	:	350 – 400 rpm
Aeration	:	10 - 15 L min^{-1}

Culture pH was allowed to decline during growth of the fungus. After 18 – 24 h of growth, the culture was transferred to production fermentor.

Fermentation

The medium components (corn flour salt medium) for 125 L was mixed in deionized water and pH of the medium was adjusted to 5.5 using 2 M HCl followed by sterilization at 121.1ºC for 20 min. Inoculum from 10 L fermentor, measuring 9.5 L was pumped to fermentor aseptically.

Fermentation Conditions

Temperature	:	30^0C
PH	:	Not adjusted
Agitation	:	350 – 400 rpm
DO	:	85% Saturation
Vessel Pressure	:	0.5 bar
Air flow rate	:	130 – 150 L/min

When the culture broth pH dropped to 2.9, fermentation was terminated for down stream processing after adjusting the broth pH to 4.0 using 2 M NaOH. Periodical samples collected during fermentation was analyzed for polygalacturonase activity.

DOWNSTREAM PROCESSING

The downstream processing involved separation of fungal biomass from broth by filtration using plate and frame filters followed by centrifugation. The

filtered broth was concentrated using Miniton Filter Plates fitted with Miniton-S filter sheets (polysulphone membranes on polyolefin support) with 10000 Da molecular mass cut off (Millipore Bedford, USA). Samples collected periodically were analyzed for polygalacturonase activities during concentration.

STABILITY OF ENZYME

Temperature

The enzyme in 0.1 M sodium acetate buffer (pH 4.3) was incubated in water bath at 27, 30, 40 and 50ºC for 30 and 60 min. The residual enzyme activity was determined after comparing the untreated controls.

pH

The enzyme taken in 0.1 M sodium acetate buffer at pH 2.5, 3.0, 3.5, 4.0 and 4.5 was stored for 6 h at room temperature (~27ºC) and the activity was assayed at pH 4.3.

In another experiment, stability of purified 42- and 61-kDa polygalacturonases at pH 8.0 was studied after deglycosylating the enzyme proteins with PNGase. The enzyme taken in 50 mM Tris-HCl buffer (pH 8.0) was incubated at different temperatures and the residual activity was assayed at pH 4.3.

Storage

Crude culture filtrate (pH 4.0) obtained after shake-flask growth of the mutant strain was stored at 4ºC and room temperature after adding 1000 ppm of sodium benzoate. Activity was assayed after every ten days. Controls consisted of the crude culture filtrate stored as above, but without added sodium benzoate. The activity variation between the initial assay (control) and in the stored samples was tabulated.

EXTRACTION OF FRUIT JUICE

Ripe banana and apple procured from market were washed, peeled and cut into small pieces. The fruit was pulped using a food processor. Pectinase formulation or enzyme concentrate obtained after downstream processing were added at the level of 0.001 to 0.01% to fruit pulp and incubated for maceration at 30^0C and 50^0C for 4 h. Juice from the digest was separated by centrifugation. The juice was analyzed for specific gravity, degree Brix and pH.

EXPERIMENTAL RESULTS

PART I

ASPERGILLUS CARBONARIUS STRAIN IMPROVEMENT FOR POLYGALACTURONASE PRODUCTION

1. *ASPERGILLUS CARBONARIUS* STRAIN IMPROVEMENT FOR POLYGALACTURONASE PRODUCTION

Synergistic action of many enzymes is a requirement for degrading the complex plant cell wall polysaccharides. Synergy has been reported for many enzymes from *Aspergillus* (de Vries and Visser, 2001) and in the case of pectin degradation, synergy has been shown by the strong influence of pectin methyl esterase on the activity of polygalacturonase (Christgau *et al*., 1996). Similar reports are also available on pectin hairy region degrading enzymes (Kauppinen *et al*., 1995; Kroon and Williamson, 1996) and main chain degrading enzymes because, hairy regions restrict the activity of the common polygalacturonases produced by microorganisms (de Vries *et al*., 2000).

Pectinases are industrially produced by *Aspergillus niger*, a GRAS (Generally Regarded As Safe) fungus (de Vries and Visser, 2001; Pariza and Foster, 1983; Taylor and Richardson, 1979). Pectin degrading commercial enzyme formulations consist of polygalacturonases as the major component (Kester and Visser, 1990). Several endopolygalacturonases produced by *Aspergillus* hydrolyze pectin within smooth region. Multiple polygalacturonases of identical catalytic activity but varying in kinetic and molecular mass have been reported (de Vries and Visser, 2001). Of the seven polygalacturonases characterised from *A. niger*, endopolygalacturonase II, according to reports, is the major and highly active enzyme of commercial formulations used in food processing industries (Benen *et al*., 1999). The functionality of endopolygalacturonase II in fruit processing is due to its ability to degrade the pectin polymer present in the middle lamella of the cell causing release of the intracellular content (Sakai *et al*., 1993). In *A. niger,* molecular mass of endopolygalacturonases range from 35 to 80 kDa (de Vries and Visser, 2001). The three polygalacturonases of molecular mass 43, 71 and 83 kDa

characterized of *A. kawachii* differed in their *N*-terminal amino acid sequences. But the *N*-terminal sequence of the 71 kDa enzyme was reported to be similar to *A. niger* and *A. oryzae* polygalacturonases (Kojima *et al*., 1999). The high specific activities reported of the 42 kDa enzyme from *A. carbonarius* (Devi and Rao, 1996) apparently suggested this protein as unique to the fungus. The variation occurring in polygalacturonases of *Aspergillus* has also been useful to classify *Aspergillus* for taxonomic purposes (Kuster van Someren *et al*., 1990). Other pectinolytic enzymes described are pectin lyases (van Houdenhoven, 1975), pectin methyl esterase (Christgau *et al*., 1996), rhamnogalacturonan hydrolases (Mutter *et al*., 1994), rhamnogalacturonan lyase (Kofod *et al*., 1994), rhamnogalacturonase acetylesterase (Kauppinen *et al*., 1995), xylogalacturonan hydrolases and arabinofuranosidases (Whitaker, 1990).

Polygalacturonases are difficult to classify strictly as endo- or exo-enzymes in the traditional sense (Cooper *et al*., 1978). Polygalacturonase from *Colletotrichum lindemuthianum* exhibited bimodal pH optima and at pH 4.2 the enzyme acted like an exoenzyme whereas at pH 5.4, it showed a typical endopolygalacturonase activity (Keon and Waksman, 1990). Based on gene sequence of endo- and exo- polygalacturonases and rhamnogalacturonases of *Aspergillus*, they have been grouped under glycosidase family 28, which has an inverting mechanism of hydrolysis (de Vries and Visser, 2001).

Polygalacturonase production in *Aspergillus* has been shown to be induced by pectin and products released due to degradation of pectin (Bussink *et al*., 1992b; Suykerbuyk *et al*., 1996). However, constitutive secretion of certain polygalacturonases has also been described (Mikhailova *et al*., 1995; Parenicova *et al*., 2000a). Differential regulation of polygalacturonase expression during solid-state and submerged cultivation of *Aspergillus* has been reported by Acuna-Arguelles *et al.* (1995), Minjares-Carranco *et al.* (1997) and Iwashita *et al.* (2002).

Many studies on structural features of polygalacturonases have shown that endopolygalacturonase II of *A. niger* folds into right-handed parallel β-helical structure comprising of 10 complete turns with the β-helix formed by four parallel β-sheets (van Santen *et al*., 1999). The catalytic cleft, according to the authors, is made of a loop formation due to two turns between four β-helical structures. Four disulfur bridges found in endopolygalacturonases were reported to be conserved in all *A. niger* polygalacturonases (van Santen *et al*., 1999). Single *N*-glycosylation with a heterogenous carbohydrate was reported in endopolygalacturonase II of *A. niger* (van Santen *et al*., 1999). X-ray crystallography studies of polygalacturonase purified from *A. aculeatus* revealed that the enzyme was highly glycosylated with one *N*- and ten *O*-glycosylation and all of them were located near the *N*-terminus (Cho *et al*., 2001). Enzymatic deglycosylation of these enzymes resulted in their complete inactivation (Stratilova *et al*., 1998). Polygalacturonases of various microorganisms were shown to have several conserved domains, particularly a highly conserved region in *C*-terminal (Ruttkowski *et al*., 1991; Stratilova *et al*., 1993). Common structural features also seemed to exist among polygalacturonase from ascomycete fungi.

In spite of the above reports, variations in enzyme properties and the need for production of multiple endopolygalacturonases by *Aspergillus* have not been described in literature. Since *A. carbonarius* produces multiple polygalacturonases, studies were carried out on the fungus to explain the phenomenon of multiple polygalacturonase production.

1.1. POLYGALACTURONASES OF *A. CARBONARIUS*

Aspergillus carbonarius (CFTRI 1047) was isolated in this laboratory for extracellular polygalacturonase and a solid-state fermentation process was described for the enzymes production (Sreekantaiah *et al*., 1975). Characterization studies of *A. carbonarius* enzymes showed that the organism produced three polygalacturonases of molecular mass 61-, 42- and 47- kDa (PG-I, PG-II and PG-III) and that the 42 kDa enzyme was unique due to its high specific activities to polygalacturonate substrate (Devi and Rao, 1996). Since it was found that the colonization ability of the fungus on wheat bran substrate was affected due to poor amyloglucosidase production, strain construction by interspecies protoplasmic fusion of *A. niger* and *A. carbonarius* resulted in a fusant with higher polygalacturonase activity due to improved amylase secretion (Kavitha and Umesh-Kumar, 2000). The extracts obtained from solid-state cultures of the fusant strain showed the 61 kDa enzyme as the major polygalacturonase produced (Kavitha, 2001). Since the 42 kDa enzyme is the highly active enzyme unique to *A. carbonarius* (Devi and Rao, 1996), strain improvement was proposed in the present study for obtaining a strain of *A. carbonarius* that produced the highly active polygalacturonase during shake-flask growth. As a prerequisite, enzyme profile of the wild type *A. carbonarius* was first studied to identify the polygalacturonases produced by the fungus.

Spores of *A. carbonarius* grown in corn flour broth medium for 48 h were used to inoculate wheat bran solid-state medium taken in petriplates (Kavitha and Umesh-Kumar, 2000). After 60 h growth, the enzymes secreted in the bran culture were extracted using 10 volumes of 0.1M sodium acetate buffer (pH 4.3). Total polygalacturonase activity was determined by Nelson-Somogyi (1952) method and the enzyme extract was used for zymogram and protein analysis by SDS-PAGE to identify the form of the enzyme. The details of the procedure are described in Materials and Methods.

Zymogram analysis of the crude extract of the wild type *A. carbonarius* grown by solid-state fermentation showed the occurrence of only two polygalacturonase proteins of molecular mass 61- and 42-kDa **(Fig. 3)**. Of these, one of the proteins appeared highly active as visualized by large clearance zone in the zymogram reaction **(Fig. 3, lane C)**. Migrational variations of proteins were seen in the zymogram reactions due to binding of the enzyme with the polygalacturonan substrate incorporated in the polyacrilamide gel.

The enzyme zymogram reaction showed that only two polygalacturonases were produced by *A. carbonarius* when grown in solid-state and one of them appeared to be the highly active 42 kDa enzyme.

1.2. POLYGALACTURONASE PRODUCTION BY *A. CARBONARIUS* IN SHAKE-FLASK CULTURES

Preliminary experiments showed that *A. carbonarius* when grown in shake-flask cultures did not produce polygalacturonase. Since literature have shown that polygalacturonase secretion is induced in *Aspergillus* by pectic substances during submerged growth (Maldonado *et al*., 1989; Maldonado *et al*., 1986), in the present investigation, *A. carbonarius* was grown in media containing pectin and other sugars to induce enzyme production during shake-flask growth. In this study, the fungus was grown in media made of 2% carbon source such as corn flour, pectin, xylose, galactose, mannose and glucose. All the media contained $(NH_4)_2HPO_4$ 3.13 gL-1; $(NH_4)H_2PO_4$ 3.13 gL^{-1} as the nitrogen source. After 48 h growth, polygalacturonase activity in culture filtrate was assayed. The details of the procedure are described in Materials and Methods.

Mycelial mat formation occurred during shake-flask growth in the media. Activity determination for polygalacturonases in the culture broth showed very little enzyme production during shake-flask growth despite induction with pectin **(Table 2)**. Growth of the fungus resulted in the reduction of the culture pH after 48h.

The results of this study showed that polygalacturonase production by *A. carbonarius* in shake-flasks was not inducible even by amending the medium with pectic substances as evidenced by the similar enzyme activities in all the media containing different carbon sources.

1.3. *ASPERGILLUS CARBONARIUS* STRAIN IMPROVEMENT FOR POLYGALACTURONASE PRODUCTION IN SHAKE-FLASK CULTURE

In the earlier experiment it was shown that *A. carbonarius* produced very little polygalacturonase when grown as shake-flask cultures. The secretion of low quantities of the enzyme suggested operation of a stringent regulation in the fungal cell that affected enzyme production during shake flask growth. The earlier observation that in solid-state more enzyme was secreted by *A. carbonarius* (Kavitha and Umesh-Kumar, 2000) reasoned absence of such a regulation probably due to cultural conditions affecting enzyme production as shown in *A. niger* earlier (Acuna-Arguelles *et al*., 1995; Iwashita, 2002; Minjares-Carranco *et al*., 1997). The observation that enzyme production was not induced by pectic substrate during shake-flask growth (Experiment 1.2) suggested secretional difficulties and/or physiological differences in the fungus occurring during growth in shake-flasks. In this regard, permeability barrier of cell walls to high molecular mass proteins (Gordon *et al*., 2000a; Gordon *et al*., 2000b; Wosten *et al*., 1991) gains importance since, reports from this laboratory on amylase secretion identified its processing by a protease activity for secretion (Dubey *et al*., 2000; Ravi-Kumar *et al*., 2004). In the light of the above observation, a mutation protocol was developed to isolate mutants of *A. carbonarius* affected in secretory pathway (Ravi-Kumar *et al*., 2004) in order to understand the physiology of the fungus in relation to polygalacturonase secretion during shake-flask growth.

Mutation was induced in *A. carbonarius* CFTRI 1047 by exposing the wild type strain to UV irradiation. The mutants were analyzed for polygalacturonase secretion to study the mutation effect in relation to the enzyme production. The

details of the experimental protocol for induced mutation are described in Materials and Methods.

Selection of mutants for temperature tolerance at 42°C was the primary step in the mutation protocol **(Fig. 4)**. Those mutants that appeared early were considered fast growing mutants and further selection on 2-deoxy-D-glucose resulted in strains that secreted higher quantities of polygalacturonase when grown in shake-flask cultures **(Table 3)**. Eighty six mutants of *A. carbonarius* were isolated in this study after the first mutation step. Screening of their culture filtrates for extracellular polygalacturonase, found all the mutants to secrete higher quantities of the enzyme, when compared to the 9 U mL^{-1} (36 U mg^{-1}) enzyme produced by the parent strain. Of the mutants, those that appeared early (60 h incubation) on plates as colonies visible to the naked eye, secreted more enzyme (75 U mL^{-1}; 800 U mg^{-1}) than the slow growers (20-40 U mL^{-1}) which appeared after 72 h incubation of the plates. The observation that fast growing mutants produced more enzyme prompted us to attempt further mutational improvement of the selected mutant, for fast growing colonies. Screening of the 11 mutants that appeared in 50 h after the second mutation round, resulted in the identification of a colony that produced 130 U mL^{-1} (1200 U mg^{-1}) enzyme. Mutation of this culture evolved fast growing colonies (44 h) and isolation of a mutant secreting 180 U mL^{-1} polygalacturonase (1800 U mg^{-1}). When this strain was mutated, a single colony that appeared in 36 h was found to secrete 300 U mL^{-1} enzyme in the culture broth with a specific activity as high as 3500 U mg^{-1}.

A significant observation in this study was that the mutants isolated for polygalacturonase overproduction **(Table 3)** also showed a concomitant increase in the secreted extracellular acid protease **(Table 3)**. The mutant when grown on pectin agar and gelatin agar plates resulted in shriveled compressed colonies **(Fig. 5)**. In liquid cultures, the mutants assumed pellet morphology **(Fig. 6)**.

Isolation of the mutant overproducing both polygalacturonase and acid protease during shake-flask growth suggested a common mechanism regulating the secretion of the two enzymes under the cultivation conditions.

Significant observations of the study were:

a. Pellet growth of the mutants in liquid cultures and increased polygalacturonase production during shake flask growth.

b. Compressed growth of the colonies of the mutants on agar plates.

The above observations in relation to the biology of the fungus are discussed in the 'Discussion' section.

1.4. POLYGALACTURONASE PRODUCTION BY THE MUTANTS OF *A. CARBONARIUS* DURING SOLID-STATE GROWTH

The strain improvement experiments resulted in the isolation of mutant strains that overproduced polygalacturonase in shake-flask cultures **(Fig. 5)**. Since differential regulation of polygalacturonase production in *Aspergillus* based on cultivation condition has been reported (Acuna-Arguelles *et al*., 1995; Iwashita, 2002; Minjares-Carranco *et al*., 1997), the effect of mutation on polygalacturonase secretion during solid-state growth was studied with the mutants isolated.

The mutants were grown on wheat bran medium for 60 h and the enzyme secreted in the fermented bran was extracted using 0.1M sodium acetate buffer (pH 4.3) for assaying the enzyme activities. The details are described in Materials and Methods.

Of the many mutants screened, those mutants that showed reduced sporulation produced more enzyme as evidenced by higher activities determined. However, the activity differences were not significant. The mutant selected in this study (UV-10046) for polygalacturonase overproduction during shake-flask growth showed no appreciable variation in enzyme production in solid-states when compared to the parent **(Table 4)**. All the cultures produced ~400 Units of enzyme per gram dry bran. When the acidified wheat bran medium was amended with 2% glucose, the parent culture of *A. carbonarius* and also its mutant UV-10 showed 60% reduction in enzyme production. In the other mutants, enzyme production was unaffected during growth in solid-state cultures under glucose-amended conditions since ~400 Units of enzyme for one-gram dry bran was estimated in these cultures.

The above results suggested that glucose regulation of enzyme production during solid-state growth does occur and in some of the mutants, such a regulation was absent.

1.5. EFFECT OF PECTIN AND SIMPLE SUGARS ON POLYGALACTURONASE PRODUCTION DURING SHAKE-FLASK OF THE MUTANT

Many studies in fungi have shown that pectinase production is affected due to glucose catabolite repression (Galliotou-Panayotou *et al*., 1997; Solis-Pereira *et al*., 1993; Torakazu *et al*., 1975). An earlier experiment identified such a regulation in *A. carbonarius* since the growth of the fungus on wheat bran solid-state substrate amended with 2 % glucose resulted in reduced yields of polygalacturonase enzyme (Experiment 1.4). These results and the observation that polygalacturonase production in the UV-10046 mutant was not regulated by glucose suggested, experiments to study the phenomenon of enzyme induction in the mutant isolated for polygalacturonase over production during shake-flask growth.

Enzyme production by the fungus during shake-flask growth was studied after growing the fungus in the media made of corn flour or 2% pectic substances or monomers of the pectin heteropolysaccharide like polygalacturonic acid, galacturonic acid, xylose, mannose, galactose and glucose, and ammonium salts were used as nitrogen source for growth. and in media that contained corn flour replaced with . After 24 h growth, the extracellular polygalacturonase produced was quantitated as activity units. The details of the procedure are described in Materials and Methods.

Growth of the mutant in all the media resulted in the acidification of the culture broth and production of polygalacturonase **(Table 5)**. Even though some variation was found in the activities of the enzyme assayed from media amended with different carbon sources, the differences in enzyme production were not appreciable. Interestingly, polygalacturonase production was best observed in

the medium containing mannose or glucose since high specific activities of polygalacturonase were determined in the culture filtrates **(Table 5)**.

The results of this study suggested that the mutant strain of *A. carbonarius* isolated for polygalacturonase overproduction, was affected in general secretion and enzyme production was independent of glucose (mannose) regulation. Since glucose repression was absent in the mutant, constitutive enzyme production occurred even during growth in glucose medium.

1.6. KINETICS OF POLYGALACTURONASE PRODUCTION BY *A. CARBONARIUS* MUTANT DURING SHAKE-FLASK GROWTH

Isolation of a mutant strain of *A. carbonarius* (UV-10046), which constitutively overproduced polygalacturonase during growth in shake-flask cultures in this study, is new to literature. Hence, kinetics of enzyme overproduction by the mutant strain in shake-flask cultures was studied in corn flour salt medium.

The mutant was grown in corn flour salt medium and enzyme assays were carried out with the culture filtrate at regular intervals of time. pH of the culture broth were also recorded. The details are described in Materials and Methods.

Rapid acidification of the medium was observed during growth of the mutant strain in corn flour medium. Though polygalacturonase production was associated with growth, extreme acidity of the culture pH after 12 h caused an upsurge in the polygalacturonase production as identified by high activity determinations at acidic pH **(Fig. 7)**.

The results of this study suggested induction of enzyme production by acidic culture pH.

1.7. IDENTIFICATION OF POLYGALACTURONASES PRODUCED BY THE MUTANT STRAIN DURING SHAKE-FLASK GROWTH

Though *A. carbonarius* was reported to produce three polygalacturonases during solid state growth, only two enzymes were identified in this study **(Fig. 3)**. Since mutation of the fungus resulted in the isolation of a polygalacturonase overproducing strain and its culture filtrate showed high specific activities of the enzyme (Experiment 1.6), SDS-PAGE analysis was carried out with the culture filtrate protein to identify the type of polygalacturonase produced by the mutant.

The mutant strain of *A. carbonarius* (UV-10046) was grown in corn flour salt medium for 24 h and the culture filtrate obtained after filtration and centrifugation was used for SDS-PAGE analysis. Zymogram reactions were also carried out to identify the type of polygalacturonase present in the culture broth. The details of the procedure are described in the Materials and Methods.

The profile of secreted proteins as evidenced by silver staining analysis of the SDS-PAGE gels **(Fig. 8, lane B)** identified production of only a few proteins in the culture broth by the mutant of *A. carbonarius* (UV-10046). Of all the proteins secreted, overproduction of 42 kDa protein was evidenced by the protein quantity showing high intensity.

Zymogram analysis **(Fig. 8, lane C)** showed a highly reactive polygalacturonase protein. The intensity of the reactive zone corresponded with the quantity of the 42 kDa protein identified in the silver nitrate stained gel. This suggested overproduction of the 42 kDa enzyme by the mutant upon shake-flask growth in corn flour salt medium.

Selective production of the highly active 42 kDa polygalacturonase by the mutant strain of *A. carbonarius* when grown in shake-flask cultures is discussed in the later section of the thesis.

2. SECRETIONAL REGULATION OF POLYGALACTURONASE DURING SHAKE-FLASK GROWTH OF *A. CARBONARIUS*

Genetic complexity of the fungal pectinolytic system in relation to regulation of individual genes (Bussink *et al*., 1992a) suggested induction of polygalacturonases in *Aspergillus* by sugar beet pectin, apple pectin, polygalacturonic acid, and a combination of rhamnose and galacturonic acid (Bussink *et al*., 1992b; Kojima *et al*., 1999; Solis-Pereira *et al*., 1993). Esquivel and Voget (2004) speculated environmental conditions as the cause for the secretion of different polygalacturonases by *A. kawachii*. Polygalacturonase genes of *S. sclerotiorum* were differentially expressed during growth on polygalacturonic acid (Parenicova *et al*., 2000a). Though this indicated different physiological functions for each of the polygalacturonases, the role for individual polygalacturonase in the degradation of plant cell wall was not explained. A study on *A. niger* polygalacturonase secretion during solid-state and submerged fermentations showed an influence of carbon sources on enzyme production (Solis-Pereira *et al*., 1996; Solis-Pereira *et al*., 1993). In solid-state, production of the enzyme was not reduced due to the presence of readily metabolizable sugars. Conversely, increase in their concentration resulted in higher enzyme yields. However, in submerged fermentation polygalacturonase activities estimated in culture broths showed reduced enzyme yields when readily metabolizable sugars were used and this could not be reversed by induction with pectin (Solis-Pereira *et al*., 1993). The ratio of the components of pectinolytic enzymes produced by *Aspergillus* was also found to depend on the type of inducer used (Bodie *et al*., 1994). In *A. niger*, studies suggested that the physiology of polygalacturonase secretion and regulation were different during solid-state and submerged growth (Minjares-Carranco *et al*., 1997). Though a number of reports evidenced regulation of polygalacturonase production by sugars, detailed information on carbon regulation of polygalacturonase at molecular level is still unavailable.

Readily metabolizable sugars like glucose were shown to repress polygalacturonase production in *Aspergillus* (Bussink *et al*., 1991b; Fogarty and Kelly, 1983; Kester *et al*., 1996; Solis-Pereira *et al*., 1993). Certain reports on *A. niger* polygalacturonase demonstrated *pga*A and *pga*B specific mRNA increase in the mycelia of *A. niger* grown in sucrose containing medium and mRNA level increase on addition of fresh sucrose following incubation (Parenicova *et al*., 2000a). This result and no difference detected on growth and polygalacturonase production with galacturonic acid or pectin suggested constitutive expression of pgaA and pgaB polygalacturonases in *A. niger*. Coregulation of the genes during transcription (Bussink *et al*., 1992a) by CREA and HAP mediated carbon repression factors (Ruijter and Visser, 1997) probably reasoned the need for conserved sequence in polygalacturonase genes.

Growth of *Aspergillus* is known to cause acidification of culture medium (Visser *et al*., 1994) and ammonium salt as nitrogen source caused rapid acidification of the medium (Morgan and MacMillan, 1954). pH regulation of genes of filamentous fungi (Denison, 2000) encoding cell wall degrading enzymes including pectin has not been studied in detail (de Vries and Visser, 2001). *A. kawachii* produced two polygalacturonases with different *N*-terminal sequences in liquid cultures (Kojima *et al*., 1999). Of these, both the molecular forms were secreted at pH 2.0 while, only one of the enzyme was identified at pH 5.0. This indicated, pH of the culture medium influencing production of polygalacturonases. In a later study, the variation was related to mRNA levels of the corresponding polygalacturonase (Nagai *et al*., 2000). Polygalacturonase gene cloned from *A. kawachii* showed the occurrence of consensus nucleotide sequence for PacC binding sites due to which regulation and activation by ambient pH was proposed (Nagai *et al*., 2000). This major regulatory factor in *Aspergillus* is a zinc finger protein (PacC) with a dual function, inducing alkaline-expressed genes and repressing acid-expressed genes at alkaline pH (Tilburn *et*

al., 1995). Activated PacC binds to the specific sequence, GCCARG of the promoter regions in the target genes. Proteolytic modification occurring at *C*-terminal of PacC in alkaline condition was shown to activate the protein and this process has been reported to be mediated by *pal* signaling pathway (Mingot *et al*., 2001; Mingot *et al*., 1999; Penalva and Arst Jr, 2002).

Purification and characterization of polygalacturonases from several fungi have revealed a large variation in their physical and chemical properties (Barthe *et al*., 1981; Keon and Waksman, 1990; Kester and Visser, 1990). In spite of these variations, amino acid compositions of homogenous polygalacturonases showed similarity suggesting that the basic structure of the protein is maintained in different fungi (Cervone *et al*., 1986). Keon and Waksman (1990) also reported high similarities in the *N*-terminal amino acid sequences among polygalacturonases of three *Ascomycetes* fungi, *A. niger*, *Sclerotinia sclerotiorum,* and *Collectotrichum lindemuthianum.* This suggested a common structural feature with variation in external surface, identified by absence of antibody cross reactivity, within the polygalacturonases.

Secretion of multiple forms of polygalacturonase that differ in molecular size, pH optima, and specific activity have been the general observation from most of the *Aspergillus* species (Stratilova *et al*., 1993). Though polygalacturonases of *A. niger* showed variation in physical and kinetic properties, they have been reported to have identical mode of hydrolytic activity (inverting glycanase) by single displacement mechanism of glycosidic linkage (Biely *et al*., 1996). Molecular mass of *A. niger* polygalacturonases reportedly varied from 35 to 85 kDa with a narrow range in K_M values (de Vries and Visser, 2001). Physicochemical properties of three polygalacturonases (endo-IIIA, endo-IIIB, endo-IV) characterized of commercial formulations derived from *A. niger* were similar to endopolygalacturonase I of *A. niger*. The specific activity of seven enzymes described from *A. niger* ranged from 25 to 4000 U mg^{-1} (Benen

et al., 1999; Parenicova *et al*., 2000a; Parenicova *et al*., 2000b). These enzymes also showed antigenic similarity (Kester and Visser, 1990). The four polygalacturonases characterized from *A. awamori* showed variation in properties according to the ambient pH of the medium from which the enzyme was harvested (Yoshikawa *et al*., 1995). From this laboratory, three molecular forms of polygalacturonases were described from *A. carbonarius* (Devi and Rao, 1996).

A. flavus produced three distinct polygalacturonases in cotton bolls and also in culture filtrates. In a plant pathogenic strain of *A. flavus,* production of polygalacturonases was related to virulence (Clevaland and Cotty, 1991). An endopolygalacturonase of *A. parasiticus* identical to that of *A. flavus* has also been identified (Cary *et al*., 1995). The deduced amino acid sequence of endopolygalacturonase of *A. parasiticus* encoded by *pec*A gene contained 363 amino acids. The sequence established a close similarity to other known fungal polygalacturonases. The deduced amino acid sequence showed a high sequence homology to PGA-I, PGA-II and PGA-C of *A. niger* and PGN-1 of *Cochliobolus carbonum* (Cary *et al*., 1995). The characteristic variation found in *N*-terminal amino acid sequence of *A. niger* polygalacturonases was explained to amino acid insertion or deletion that normally occurred in phytopathogenic fungi (Bussink *et al*., 1992a).

In spite of the above variation in molecular mass and kinetic properties of the enzyme, the primary protein sequence suggested a basic structure consisting of approximately 360 amino acids with 27 amino acids sequence at N-terminal functioning as the signal peptide (Bussink *et al*., 1991a; Bussink *et al*., 1991b; Bussink *et al*., 1990; Ruttkowski *et al*., 1991). The degree of nucleotide sequence similarity reported amongst polygalacturonases (Nagai *et al*., 2000) was 95.1% with PG and PGII of *A. niger* RH 5344 and *A. tubingensis* NW 756 and 93.1% and 60.1% with PGII and PGI respectively of *A. niger* N 400.

Occurrence of multiple molecular forms of polygalacturonases in fungi was reasoned to proteins encoded by several genes, although in the maize pathogen *C. carbonum*, only one polygalacturonase gene was found (Durrands and Cooper, 1988; Scott-Craig *et al*., 1990). Bussink *et al.* (1992a) identified a family of diverged genes (*pga*A, *pga*B, *pga*C, *pga*D and *pga*E) encoding polygalacturonases in *A. niger*. The deduced amino acid sequence of the gene products suggested that 36.5% amino acids were conserved. They also proposed the presence of at least four other genes encoding polygalacturonases. The study hypothesized events that predated speciation in some filamentous fungi (Bussink *et al*., 1992a). Gene duplication and subsequent divergence of the duplicated genes apparently caused the high sequence homology and common structural features in *A. niger* polygalacturonase genes.

Despite the above reports, studies on polygalacturonases of *A. carbonarius* has been confined to characterization of the enzymes (Devi and Rao, 1996), their production in solid-state cultures (Ghildyal *et al*., 1981), polygalacturonase recovery from fermented wheat bran (Singh *et al*., 1999) and strain improvement by protoplast fusion (Kavitha and Umesh-Kumar, 2000).

PART II

SECRETIONAL REGULATION OF POLYGALACTURONASE DURING SHAKE-FLASK GROWTH OF *A. CARBONARIUS*

2.1. PURIFICATION OF POLYGALACTURONASE SECRETED BY THE MUTANT STRAIN OF *A. CARBONARIUS*

Though three polygalacturonases of *A. carbonarius* were characterized (Devi and Rao, 1996), the mutant strain isolated in the present study (UV-10046), when grown in shake-flasks secreted only the 42 kDa enzyme as the major protein **(Fig. 8)**. Thus, there appeared a stringent regulation governing polygalacturonase secretion in *A. carbonarius*. In order to study the regulation mechanism, the enzyme was purified from the culture broths of the mutant after shake-flask cultivation.

The enzyme from the culture filtrate was purified to homogeneity by affinity chromatography using alginate. For antibody production, the pure 42 kDa polygalacturonase protein mixed with equal volumes of Freund's complete adjuvant was injected to rabbits. Serum containing antibodies was separated from blood collected by marginal ear vein bleeding of the animals. The details of the procedure are described in Materials and Methods.

Purification of the enzyme by alginate affinity chromatography resulted in 87% enzyme recovery with six-fold purification in a single step. Silver staining the SDS-PAGE gels showed only one protein which confirmed the homogeneity of the purified enzyme preparation **(Fig. 9)**. Highest purity of the enzyme was further confirmed by a single reactive precipitate in the Outerlony immuno double diffusion assay using the homologous antibodies **(Fig. 10)**. Western blot reaction **(Fig. 9)** also showed the occurrence of only one reactive protein in the purified enzyme protein. The pure enzyme and the antibodies against the same were used for the experiments described in the later sections of the thesis.

2.2. PROPERTIES OF PURIFIED POLYGALACTURONASE OF *A. CARBONARIUS*

The polygalacturonase purified by alginate affinity (Exp. 2.1) was used for the experiment.

pH

The enzyme was found to be active in the acidic pH range between 4.0 and 4.5 with optimum activity at pH 4.3 **(Fig. 11)**. Incubating the enzyme for 48 h at pH 4.0 showed a reduction in activity by 7% since only 93% of the residual activity was assayed after. Stability studies showed that the enzyme was stable for 6 h at pH 4.3. The enzyme was less stable at pH 3.0 and 5.0 since 40 and 70% loss in activity was recoded after storing for 6 h at those pH.

Temperature

In order to study the effect of temperature on enzyme, activity assays were carried out at pH 4.3, the optimum pH. The results showed optimum activity of the purified enzyme between 40-50^{0}C **(Fig. 12)**. Reduced enzyme stability of the enzyme was evidenced by 93% loss of activity assayed at 60^{0}C. In order to confirm stability of the enzyme at 50ºC, activity assays were performed with the enzyme incubated at 25, 30, 40 and 50^{0}C for 30 and 60 min.

A loss of 50% activity was determined in the enzyme incubated at 40ºC for 30 min. Though the enzyme showed maximum activity at 50ºC, it was very unstable at this temperature since a loss of ~90% in activity was revealed after 30 min incubation **(Table 6)**.

K_M

The K_M value of the 42 kDa polygalacturonase was determined by assaying the activities of 0.15μg purified enzyme with different concentrations of polygalacturonate substrate. K_M of the enzyme on polygalacturonate substrate was found to be 2.5% **(Fig. 13)**.

Other Activities

Preliminary experiments showed that the purified enzyme was active on methoxylated pectin. It also hydrolyzed apple pulp to release juice.

Thus, the polygalacturonase purified was found to be an enzyme active at acidic pH with high affinity to polygalacturonate substrate.

2.3. REACTIONS OF THE ANTIBODY RAISED TO 42 KDA POLYGALACTURONASE

Even though antigenic similarity has been demonstrated among polygalacturonases of *A. niger* (Kester and Visser, 1990), it was earlier shown in this laboratory that, a particular polygalacturonase of *A. carbonarius* was unique to the fungus (Kavitha, 2001). Since the highly active 42 kDa polygalacturonase occurred only in *A. carbonarius*, homology of this protein with other polygalacturonases was studied by antibody reactivity.

For this experiment, the polygalacturonases produced by *A. carbonarius* in solid-state culture and those present in the commercial preparation of *A. niger* were used as antigen. The samples separated by SDS-PAGE were probed for polygalacturonase in the western blot reactions with the antibody raised to the purified 42 kDa polygalacturonase of *A. carbonarius*. The details are described in Materials and Methods.

When the extract obtained after solid-state cultivation of *A. carbonarius* was used for the western blot reactions, the antibody identified two polygalacturonase proteins corresponding to molecular mass 42- and 61-kDa **(Fig. 14)**. Antibody cross-reaction was also seen with a number of proteins of molecular mass ranging from 37- to 70-kDa of *A. niger* enzyme preparation **(Fig. 14)**.

The reactivity of the antibody to several polygalacturonase proteins of *A. niger* and 61 kDa enzyme of *A. carbonarius* suggested serological homology of the enzyme proteins produced by the two species of *Aspergillus*.

2.4. pH INDUCED POLYGALACTURONASE SECRETION DURING SHAKE-FLASK GROWTH OF THE MUTANT STRAIN OF *A. CARBONARIUS*

It is a well known observation that growth of *A. niger* in shake-flasks causes acidification of the medium (Visser *et al*., 1994). Though this observation suggested pH induced gene regulation of protein synthesis and secretion (Denison, 2000; Penalva and Arst Jr, 2002), studies on ambient pH affecting polygalacturonase production by *Aspergillus* has not been reported in literature. Growth of mutant strain of *A. carbonarius* (UV-10046) in shake-flask cultures caused acidification of the culture medium and the results of an earlier study (Experiment 1.6) showed an upsurge in polygalacturonase activity when the pH of the culture broth was acidic. This suggested pH induced polygalacturonase secretion in *A. carbonarius*. To evidence this observation, polygalacturonase production by the mutant strain during growth was studied.

The mutant strain UV-10046 was grown in liquid culture made of corn flour salt medium. Samples of the culture broth of the growing fungus were analyzed for polygalacturonase proteins by SDS-PAGE and western blot reactions using antibodies raised to 42 kDa polygalacturonase. For some of the studies, mycelia of the mutant strain grown in corn flour salt medium as above were inoculated to fresh media buffered to pH 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 with 0.25 M citrate phosphate buffer. Periodical samples of the culture broth drawn every six hours of growth were analyzed for polygalacturonase proteins using antibodies developed to 42 kDa polygalacturonase in western blot reactions. The detail of the procedure is described in Materials and Methods.

Reactions of the antibody identified the production of a small concentration of a 61 kDa polygalacturonase and the 42 kDa enzyme by the

mutant strain of the fungus during shake-flask growth. Concomitant acidification of the culture during growth caused secretion of higher quantities of the 42 kDa polygalacturonase as evidenced by the reaction intensity of the protein in the western blot reactions **(Fig. 15)**. When the fungus was cultivated under pH controlled conditions using buffered media, more enzyme production was observed at pH 3.0 **(Fig. 16)**. In another experiment, fungus grown at pH 5.0 was found to produce very little enzyme compared to that obtained when the fungus was grown at pH 3.0 **(Fig. 17)**.

The results evidenced that polygalacturonase secretion is induced by acidic culture pH. In the above reactions, appearance of a protein with molecular mass lower to the 42 kDa **(Figs. 16, 17)** was also seen. The protein occurred in low concentrations as faint reactive bands **(Figs. 16, 17)**. The results are discussed in the later section of the thesis.

2.5 ACID PROTEASE SECRETION BY *A. CARBONARIUS* MUTANTS DURING SHAKE-FLASK GROWTH

Filamentous fungi secrete multiple polygalacturonases identical in catalytic activity but varying in physical and kinetic properties (Cotton *et al*., 2003; de Vries and Visser, 2001). Studies on the genetics of *A. niger* polygalacturonase explained secretion of smaller enzymes by the fungus due to post translational modification by cleavage of the signal peptides of prepro-protein (Bussink *et al*., 1990; Fraissinet-Tachet *et al*., 1995; Ruttkowski *et al*., 1991) apparently by proteases (Bussink *et al*., 1990). However, the proteases involved have not been described. In the light of the earlier reports that protease processing occur in *A. niger* for multiple amylase secretion (Dubey *et al*., 2000; Ravi-Kumar *et al*., 2004), studies were carried out to identify the possible involvement of protease for multiple polygalacturonase secretion by *A. carbonarius*. Since acidic pH of the culture broth induced polygalacturonase secretion during shake-flask growth of the mutant (Experiment 2.4), acid protease activity was assayed in the culture broth of the mutants. The details are described in Materials and Methods.

The assay for protease activity in the culture broth of the mutant showed a time dependent increase which suggested a relationship between decreasing culture pH and acid protease production and polygalacturonase secretion **(Fig. 18)**. Increased polygalacturonase production at pH 3.0 could be related to over three fold increase in protease activity at pH 3.0. At this pH, western blot analysis identified the presence of a 40.5 kDa protein apart from the 42 kDa polygalacturonase **(Fig. 18)**. The above results suggested a role for protease in the secretion of multiple polygalacturonase by the fungus. These results are discussed in the later part of the thesis.

2.6. POLYGALACTURONASE SECRETION BY THE MUTANT STRAIN OF *A. CARBONARIUS* DURING SHAKE-FLASK GROWTH

Reduced porosity of cell walls to high molecular mass proteins has been reported to affect protein secretion in *Aspergillus* (Gouka *et al*., 1997; Wessels, 1993). In the case of multiple amylase secretion by *A. niger,* though a single high molecular mass protein was identified in the cell, occurrence of lower enzyme forms in the culture filtrates was reasoned to protease processing, probably by a carboxy peptidase-like activity (Ravi-Kumar *et al*., 2004), in the cellular periplasmic space (Dubey *et al*., 2000). Higher acid protease secretion identified along with polygalacturonase production when the mutant strain of the fungus was grown in shake-flasks, suggested probable protease processing of polygalacturonase for secretion. For this study, intracellular, periplasmic and extracellular (secreted) proteins were extracted from the mutant strain of *A. carbonarius* to identify the type of enzyme present in these three fractions.

After shake-flask cultivation of the mutant (UV-10046) strain of *A. carbonarius* in corn flour salt medium, the mycelia were used for the extraction of intracellular and periplasmic proteins (Materials and Methods). Culture filtrates were used directly as extracellular proteins. The polygalacturonases in the above preparation were identified after SDS-PAGE by western blot analysis (Materials and Methods).

No polygalacturonase was detected in the intracellular fractions **(Fig. 19)**. Only the 42 kDa polygalacturonase was identified in the mycelial periplasm and culture filtrates **(Fig. 19)**.

The above results suggested that all the polygalacturonases produced by the cell were efficiently secreted without being processed by protease.

2.7. PROTEASE INHIBITORS AND POLYGALACTURONASE SECRETION DURING SHAKE-FLASK GROWTH OF THE MUTANT

Though protease processing of polygalacturonase in *A. carbonarius* could not be identified (Experiment 2.6), the results were further confirmed by analyzing the polygalacturonase produced after growing the fungus in a medium containing protease inhibitors.

The mutant UV-10046 strain of *A. carbonarius* was grown in corn flour salt medium containing protease inhibitor cocktail tablets (Boehringer Mannheim GmbH, Germany). The concentration of protease inhibitors used ranged from 10 – 100 mg/100 mL medium. Protease inhibitor tablet was dissolved in distilled water and filtered sterile for addition to the autoclaved medium. Culture filtrates after 24 h growth were analyzed for polygalacturonase produced by performing western blot reactions. The details of the procedure are described in Materials and Methods.

The presence of protease inhibitors in the medium did not show any apparent effect on the growth of the fungus. Western blot analysis of the culture filtrates after 24 h growth showed the secretion of only the 42 kDa polygalacturonase in all concentrations of protease inhibitor tried **(Fig. 20)**. A reduction in the molecular mass of the enzyme was found in the culture filtrates of the fungus grown in media containing over 40 mg/100 mL protease inhibitors.

The above results further supported the earlier observation that proteases have no role in polygalacturonase protein processing in *A. carbonarius*.

2.8. DEGLYCOSYLATION OF POLYGALACTURONASES

Production of multiple forms of polygalacturonases reported from solid-state grown *A. carbonarius* (Devi and Rao, 1996) is intriguing in the light of the observation that the mutant strain isolated in this study produced only the 42 kDa enzyme as the major protein during shake-flask growth. Since, protease processing of the enzyme could not be identified, antibody reactivity to a lower molecular mass polygalacturonase protein (~40.5 kDa) detected in the culture filtrates of the mutant strain, suggested a probable role for glycosylation in the production of higher molecular mass enzyme. In order to test this hypothesis, the polygalacturonase proteins were enzymatically deglycosylated using PNGase F to record changes in their molecular mass in SDS-PAGE gel.

The 61 kDa polygalacturonase purified from the solid-state grown *A. carbonarius* and the purified 42 kDa polygalacturonase were deglycosylated using the enzyme PNGase-F. Homogeneity of the purified proteins was checked by SDS-PAGE. The 61- and 42-kDa enzymes purified for this study showed a specific activity of 930 Umg^{-1} and 6350 Umg^{-1} with polygalacturonan substrate respectively. The procedural details for enzymatic deglycosylation of the protein are described in Materials and Methods.

The results showed that upon deglycosylation, the 61 kDa protein attained a molecular mass of 42 kDa and extended incubation with PNGase F reduced its molecular mass to 40.5 kDa **(Fig. 21)**. Deglycosylation of 42 kDa protein also caused a reduction in the molecular mass to 40.5 kDa **(Fig. 21)**.

The significance of the results in relation to molecular mass and other properties of polygalacturonase is discussed in the later sections of the thesis.

2.9. STABILITY OF DEGLYCOSYLATED ENZYMES

The secreted proteins of fungi are usually glycosylated (Conesa *et al*., 2001; Gouka *et al*., 1997) and in the case of polygalacturonases, it has been shown that solid-state cultures produce enzymes containing more glycans compared to the less glycosylated forms secreted in liquid cultures (Acuna-Arguelles *et al*., 1995). Glycosylation is said to impart structural and functional stability to the proteins as shown in the case of increase thermal stability of polygalacturonases (Lang and Looman, 1995). Since deglycosylation causes enzyme inactivation (Stratilova *et al*., 1998), studies were carried out to examine stability of deglycosylated polygalacturonase of *A. carbonarius* to pH.

For this experiment, native enzymes were deglycosylated at 37^0C using PNGase F prepared in 25 mM Tris-HCl buffer (pH 8.0). Enzyme activity was measured at pH 4.3, as described in Materials and Methods. Controls consisted of untreated proteins and proteins incubated in Tris-HCl buffer (pH 8.0) containing heat inactivated PNGase F.

The deglycosylation of polygalacturonases at pH 8.0 caused over 90% loss in activity even though the activity assays were performed at pH 4.3. Incubation of the deglycosylated enzymes at pH 4.3 did not result in enzyme renaturation. However, the native 61- and 42-kDa enzymes were more stable at pH 8.0 because controls incubated at that pH, when assayed, showed only 40 and 70% activity losses respectively.

The above results suggested reduced stability of the deglycosylated enzymes at pH 8.0.

2.10. *N*-TERMINAL SEQUENCE OF POLYGALACTURONASES

The results of the above experiments suggested differential glycosylation as the cause for the secretion of multiple forms of polygalacturonase by *A. carbonarius*. To confirm these results, *N*-terminal amino acid sequence of the proteins were obtained by microsequencing of the protein electroblotted to PVDF membranes. Microsequencing was performed at Alta Bioscience, The University of Birmingham, UK using the membrane bound proteins.

The *N*-terminal amino acid sequences of the 42 kDa proteins as determined by microsequencing was GS(C)TF **(Annexure 1)**. In the case of 61 kDa polygalacturonase, only an indicative sequence XVTXXF that did not show homology to any polygalacturonases was deciphered after microsequencing **(Annexure 2)**.

The above results are discussed in the later sections of the thesis.

PART III

POLYGALACTURONASE PRODUCTION BY *A. CARBONARIUS* MUTANT AND FERMENTATION SCALEUP

3. POLYGALACTURONASE PRODUCTION BY *A. CARBONARIUS* MUTANT AND FERMENTATION SCALEUP

Numerous phytopathogenic fungi e.g. *Colletotrichum lindenuthianum, Trichoderma coningii, Rhizhoctonia fragariae, Sclerotinia sclerotiorum, S. fructigena* and saprophytic molds such as *Aspergillus, Mucor* and *Rhizopus* are capable of synthesizing different pectinases (Leuchtenberger and Mayer, 1992). Of the different species of saprophytic molds, *A. niger* is the favoured organism for pectinase production (Aguilar and Huitron, 1986; Galliotou-Panayotou *et al*., 1997) due to the GRAS status of the fungus (Pariza and Foster, 1983). *A. niger* also has a number of characters like good fermentation capabilities and high level protein secretion (de Vries and Visser, 2001) that make it an ideal organism for widespread enzyme production. The reviews on microbial pectinases are those of Whitaker (1984); Sakai *et al.* (1993); Benen and Visser (1996); Lang and Dornenburg (2000); Venkatesh and Umesh-Kumar (2004).

Industrially, polygalacturonases of *Aspergillus* species are produced by solid-state (SSF) or submerged fermentation (SmF) methods. However, several literature reports have regarded solid-state fermentation (SSF) as a more suitable method for obtaining higher enzyme yields (Maldonado *et al*., 1986; Minjares-Carranco *et al*., 1997; Pilnik and Rombouts, 1981; Solis-Pereira *et al*., 1993; Ward, 1985).

Solid-state Fermentation

Production of enzymes by SSF was introduced at industrial scale mainly in Oriental countries (Pandey, 1992) where agro industrial residues and biomass are abundant as cheap raw material (Castilho *et al*., 2000). Solid-state cultivation conditions resemble the natural growth habitat of molds and hence it

was speculated that the fungi grow faster to colonize the substrate and secrete large quantities of enzymes (Iwashita, 2002). According to the available literature, physiology of filamentous fungi in relation to enzyme production in SSF is different since the molecular mechanisms that regulate gene expression are influenced by conidiophore formation and invasive growth of fungus (Iwashita, 2002; Minjares-Carranco *et al*., 1997). In fungi, many polygalacturonases are inducible enzymes (Kilara, 1982; Siessere and Said, 1989) but its production is less affected by carbon repression in SSF (Solis-Pereira *et al*., 1993). The enzymes produced by the fungus grown in SSF have higher K_M values with stability to higher temperature and broad range of pH (Acuna-Arguelles *et al*., 1995) and these differences were explained to differential glycosylation occurring in secreted proteins of fungi growing in SSF which gives more stability to the enzyme in terms of pH and temperature (Willick and Seligy, 1985).

The factors that influence pectinase production by SSF are the ability of the strain to colonize the substrate and medium composition. In general, media consist of a carbohydrate solid support with minerals and corn steep liquor, yeast extract and/or peptone as nitrogen source (Kilara, 1982). Though wheat bran is the mostly used solid-state substrate, cassava fibrous waste residue (Budiatman and Lonesane, 1987), citrus pulp waste (Siessere and Said, 1989), sugar cane bagasse (Acuna-Arguelles *et al*., 1995), coffee pulp (Antier *et al*., 1993a) and orange peel (Ismail, 1996) have also been reported to be substrates for pectinase production. Strong acidic condition (pH 2-3) of medium has been reported to influence polygalacturonase secretion but initial pH around 4.5 to 5.5 has been a requirement for substrate colonization by fungi (Cavalitto *et al*., 1996; Galliotou-Panayotou *et al*., 1997; Hugouvieux *et al*., 1997). Temperature during fermentation is maintained using cold sterile moist air (R_H 80%) in the range of 28 – 35^{0}C in chambers called koji rooms. In case of rotating cylinders, flow of water on outer surface is used to control the temperature (Fogarty and Kelly, 1983).

Submerged Fermentation (SmF)

In SmF process for polygalacturonase production, the liquid medium consists of a number of components. Reported carbohydrates sources used in this process include glucose, molasses, corn syrup, starch, starch hydrolysate, milled cereal products, etc. Nitrogen for fungal growth is derived from ammonium salts, corn steep liquor, distillers' solubles, yeast extract, gelatin and casein. Mineral supplementation is provided in the form of inorganic salts (Fogarty and Kelly, 1983). Pectin sources used for enzyme induction during SmF growth of fungi have been beet pulp, citrus peel and apple pomace (Kilara, 1982). Polygalacturonase productivity in *Aspergillus* species is reported as a function of temperature, composition of medium and pH. Growth of *A. niger* and assaying the enzyme produced suggested faster growth at 29ºC and enzyme production at 37ºC. Requirement of complex medium such as lemon peel for polygalacturonase production (Bailey, 1990; Larios *et al*., 1989) and higher enzyme activities in acidic pH of the production medium (Aguilar and Huitron, 1990; Aguilar *et al*., 1991; Galliotou-Panayotou *et al*., 1997) suggested a stress physiology.

Downstream Processing

Pectinase product recovery from SmF process is simpler because the enzyme is secreted in to the culture broth. The downstream processing involves separation of biomass by filtration or centrifugation followed by concentration. In the case of enzyme produced by SSF, extraction using water by a counter current extraction system is followed. In some cases, the fermented mass is dried at low temperature for storage and extraction performed when needed (Castilho *et al*., 1999; Castilho *et al*., 2000). The enzyme solution obtained is also partially purified or concentrated by ultrafiltration (Kilara, 1982).

Since production of polygalacturonase by the mutant strain of *A. carbonarius* is new to literature, scaleup of fermentation was studied.

3.1. MEDIA FOR POLYGALACTURONASE PRODUCTION IN SHAKE-FLASK CULTURES

For successful commercial production of polygalacturonase, balanced medium made of cheaper raw materials is crucial. Hence, several media components were explored for optimizing enzyme production in shake-flask cultures using the mutant strain of *A. carbonarius* (UV-10046).

The fungus was grown in media of varying compositions as described in **Table 6**. Culture filtrates obtained after 24 h growth was assayed for polygalacturonase activity. The details of the procedure are described in Materials and Methods.

Growth of the fungus and polygalacturonase production were recorded in all the media used in this study. Use of corn flour as carbon source resulted in maximum enzyme yields **(Table 7)**. A highest enzyme yield of 348 UmL^{-1} was obtained only in the medium made of corn flour and ammonium salts **(Table 7)**. A specific activity of 3500 U mg-1 suggested that the enzyme produced was the major protein in the culture filtrate.

Based on the above experiments, the corn flour salt medium was used for scaleup studies.

3.2. LABORATORY SCALE FERMENTOR STUDIES

Following the encouraging results from shake-flask trials for production of polygalacturonase using the mutant strain of *A. carbonarius*, scaleup studies were undertaken using the corn flour medium.

Ten liters of the medium made of corn flour 40 gL^{-1}; $(NH_4)H_2PO_4$ 5 gL^{-1}; $(NH_4)_2HPO_4$ 5 gL^{-1} was prepared and the initial pH was adjusted to 5.5. Fermentation was carried out in a 15 L Bioengineering^R fermentor **(Fig. 22)**. To avoid foaming during fermentation, 0.5 mL of polypropylene glycol (antifoam) was added to the medium. One liter inoculum raised as described in Materials and Methods was used as the starter culture. Conditions maintained during fermentation were

Temperature	:	30^0C
pH	:	Maintained at 4.0 using 2M $(NH_4)_3PO_4$ solution
Agitation	:	600 - 800 rpm
DO	:	60% Saturation
Vessel Pressure	:	0.5 bar
Air flow rate	:	1 – 1.5 vvm

Assay for enzyme activity at regular time intervals (4 h) showed highest enzyme activity in the culture broths at 40th h of fermentation. At this period of time, 304 UmL^{-1} polygalacturonase activity was determined. The specific activity of the enzyme in this sample was 1550 Umg^{-1} protein. The enzyme activity was found to reduce with increasing fermentation period after 40 h.

Despite enzyme production, problems such as increased viscosity of the medium affecting down stream processing and longer fermentation periods were encountered in this trial. Due to increased viscosity of the fermented broth, plate and frame was not effective for filtration.

In order to over come the process difficulties described above, fermentation trials were carried out using a medium that contained 25 $gL^{-}1$ corn flour; 3.13 gL^{-1} $(NH_4)H_2PO_4$ and 3.13 gL^{-1} $(NH_4)_2HPO_4$. In the first 10 L trial, the conditions described above were maintained. In this trial, a polygalacturonase activity of 292 U mL^{-1} culture filtrate was obtained after 24 h fermentation with a specific activity of 2147 U mg^{-1} protein.

In this fermentation trial also viscosity problems affected down stream processing of the fermented culture broth.

In order to reduce the problems associated with viscosity development during corn flour fermentation by the fungus, a third 10 L fermentation trial was attempted without maintaining the pH of the medium at pH 4.0. In this fermentation trial, growth of the fungus acidified the culture broth and when the pH reduced to ~3.0, highest activities of the enzyme (340 UmL^{-1}) with a specific activity of 3300 Umg^{-1} protein was obtained in 24 h period **(Fig. 23)**. Absence of viscosity in the culture broth was evidenced by easy down stream processing by plate and frame filtration.

3.3. POLYGALACTURONASE PRODUCTION BY FED-BATCH FERMENTATION

The observation that the fungus rapidly acidified the culture broth together with a steep increase in enzyme production when the culture broth attained a pH ~ 3.0 (Experiment 3.2) suggested, development of a fed batch fermentation protocol for obtaining improved yields of enzyme using the mutant strain of *A. carbonarius*.

In this experiment, the starter culture of the mutant developed as described in Materials and Methods was inoculated to 15 L Bioengineering fermentor that contained 10 L medium made of corn flour 25 gL^{-1}; $(NH_4)H_2PO_4$ 3.13 gL^{-1}; $(NH_4)_2HPO_4$ 3.13 gL^{-1} (initial pH adjusted to 5.5). During the growth of the fungus the pH was not maintained and the drop of the medium pH was recorded. Fresh corn flour salt medium of pH 3.0 was fed to the fermentor after the culture attained a pH of 3.0 (20 h). Fermentation was continued for 4 h and the culture filtrate when assayed for polygalacturonase activity showed a 167% increase in polygalacturonase production in 4 h **(Fig. 24)**. This corresponded to an activity of 367.5 UmL^{-1} culture filtrate.

Thus, fed batch fermentation was standardized as a process for polygalacturonase production using the mutant strain of *A. carbonarius*.

3.4. PILOT PLANT STUDIES

For pilot plant studies a batch fermentation was carried out in a 200 L Sortorius fermentor. The medium used for this experiment contained 25 gL^{-1} corn flour; 3.13 gL^{-1} $(NH_4)H_2PO_4$ and 3.13 gL^{-1} $(NH_4)_2HPO_4$ (pH adjusted to 5.5). Inoculum for the experiment was prepared from shake-flasks as shown in **Fig. 25**.

Fermentation was carried out for 24 h maintaining the following conditions:

Temperature	:	30^0C
Agitation	:	350 – 400 rpm
DO	:	85% saturation
Air flow rate	:	130 – 150 L/min
pH	:	Not maintained

When the pH of the broth dropped to 2.9, fermentation was terminated after estimating the enzyme activity.

Highest polygalacturonase activity of 340 UmL^{-1} with a specific activity of 2500 U mg^{-1} protein was assayed after 24 h of growth of the fungus in the 200 L fermentor. The fungus was found to grow as pellets **(Fig. 26)** during fermentation.

Thus, the batch process was scaled up for polygalacturonase production using the mutant strain of *A. carbonarius*.

3.5. DOWN STREAM PROCESSING

At the end of fermentation, the pH of the culture broth was adjusted to pH 4.0 using 0.1 M NaOH solution. Since the mutant strain formed pellets during growth in fermentor, biomass separation from culture broth was done by filtration using plate and frame filter without any filter aid. The culture filtrate free of mycelia obtained was concentrated using Minitan Filter Plates and Miniton-S filter sheets as described in Materials and Methods.

Recovery of polygalacturonase enzyme after fermentation is described in **Table 7**. A recovery of 78% after 30-fold enzyme concentration (concentrated sample 2) suggested a simple system for enzyme concentration **(Table 8)**. Increase in specific activity was due to removal of contaminating low molecular mass proteins from the culture filtrates during membrane concentration since a 10,000 Da molecular mass cut off membrane was used.

Hence, the enzyme could be concentrated by membrane filtration.

3.6. STUDIES ON ENZYME STABILITY

Commercial applications of enzymes involve enzyme formulations (Kilara, 1982) for synergetic action (de Vries *et al*., 2000). For formulations, single enzyme produced is mixed according to the requirement for application. Hence, stability of enzyme produced by microbial fermentation is a crucial factor that define a commercial technology. Since polygalacturonase production described in this study is only one of the components of pectic enzymes, its stability during storage was studied in order to define commercial applications of the enzyme.

Culture filtrate samples of the mutant strains of *A. carbonarius* obtained after down stream processing of the broth obtained by fermentation of corn flour salt medium were stored at 4ºC and 28±2ºC with 1000 ppm sodium benzoate as preservative. Controls constituted samples stored under the above conditions without the added preservative.

The samples stored with and without preservative at 28±2ºC lost ~40% enzyme activity in 40 days. However, both the control and preservative added samples stored at 4^0C showed very little loss in enzyme activity until 150 days, the period of the experiment **(Fig. 27)**. Polygalacturonase protein of the culture filtrate and fermented wheat bran extract when stored under conditions described above showed some degradation **(Fig. 28)**.

The results on the stability of the enzyme when stored at 4ºC described above further supported the commercial application of the enzyme produced by fermentation. Though a small degradation of the enzyme protein was observed during storage, the loss appeared to be negligible.

3.7. STUDIES ON THE APPLICATION OF POLYGALACTURONASE PRODUCED BY THE MUTANT STRAIN OF *A. CARBONARIUS*

Polygalacturonases are extensively used in fruit and vegetable processing industry to enhance recovery of products like juice, flavour, colour and oils. Activity of pectin hydrolyzing enzymes also cause viscosity reduction of pulp by neutralizing the electrostatic charges of particles in suspension. This aids in agglomeration and sedimentation of the insoluble solids in the juice thus facilitating the filtration process for obtaining higher juice concentration without gel formation (Faigh, 1995). A typical pectinase preparation contains pectin esterase, polygalacturonase, and polygalacturonan lyase with additional activities of arabinase, rhamnogalacturonases, cellulase and hemicellulases. Starch degrading enzymes may also be added at the clarification stage (Faigh, 1995). Low temperature processing of fruit juices retains better flavour and colour. This necessitates demand for enzymes that work efficiently at low temperatures. In the light of the above, studies on the application of the polygalacturonase produced by the mutant strain of *A. carbonarius* by submerged fermentation were carried out.

Polygalacturonase activity profile on commercial citrus pectin was assayed at different temperatures ranging from 10 to 60°C. The results showed that the enzyme was active from 20 to 50°C with highest activity at 40 and 50ºC. Loss in activity at 60°C suggested low stability of the enzyme at this temperature **(Table 9)**. Application of polygalacturonase produced by the mutant strain of *A. carbonarius* enhanced juice yield from macerated apple. These results compared well with the performance of a commercial enzyme under identical conditions **(Table 10)**. The enzymes of the mutant and that from the commercial sample (4800 UmL^{-1}) were added to 450 g of fruit pulp respectively. The

samples were incubated at 30°C and 50°C for 2 – 4 h. The juice formed was filtered and tested for specific gravity, degree brix and pH. The results showed that sugar levels were not significantly different in the two samples **(Table 10)**. The pH of the juice produced was also comparable to the pH of the juice obtained with the commercial samples **(Table 10)**.

These results suggested that the mutant strain is a promising candidate for commercial production of polygalacturonase by submerged fermentation.

DISCUSSION

DISCUSSION

Studies on polygalacturonases of *Aspergillus carbonarius* described that the fungus is a good source of polygalacturonase enzyme for industrial applications. Since the fungus was originally isolated from spoilt grapes (Sreekantaiah *et al*., 1975), its ability to produce particular polygalacturonase that efficiently degraded plant pectin explained the ecological adaptation so also the colonization ability of the fungus on solid substrates. Perhaps this specialization was taken advantage of when a solid-state fermentation process was described for industrial enzyme production using the fungus (Ghildyal *et al*., 1981). However, the process of enzyme production was not viable due to cost economics affected by low enzyme yields and heavy sporulation characteristic of the species making the fermentation process less attractive. Strain improvement procedure attempted in this laboratory to improve the efficiency of the solid state process resulted in the isolation of a hybrid strain obtained by inter specific protoplast fusion of glucoamylase producing *A*. *niger* and polygalacturonase producing *A. carbonarius.* Selection for amylase over producing fusant for higher colonizing ability during growth on wheat bran solid substrate made of starch (Kavitha and Umesh-Kumar, 2000), improved polygalacturonase productivity and reduced growth-associated sporulation (Kavitha, 2001). But, industrial adaptability of the strain was still not effective since such a strain improvement procedure did not define a culture for production of the highly active 42 kDa polygalacturonase that the fungus is known for (Devi and Rao, 1996).

Polygalacturonases of *A. carbonarius*

Though the production of a highly active 42 kDa polygalacturonase by *A. carbonarius* (Devi and Rao, 1996) has several industrial significance, the biology of the fungus in relation to multiple polygalacturonase secretion or the need for the secretion of multiple enzymes by *Aspergillii* and other phytopathogenic fungi

(de Vries and Visser, 2001) are still unclear. *A. carbonarius* when grown on solid-state, produced two polygalacturonases of molecular mass 61- and 42-kDa **(Fig. 3)**. The 47 kDa protein, which is the third form of the enzyme (Devi and Rao, 1996), was not identified in this study. Apparently the solid-state cultivation of the fungus is affected by uncontrolled factors like the quality of substrate and fermentation parameters and it is highly likely that cultural conditions used in this study allowed the secretion of the only two enzymes by the fungus.

Even though *A. carbonarius* secreted polygalacturonases in solid-state cultures, an important observation in this study was on the production of very little enzyme when the fungus was grown in shake-flask cultures **(Table 2)**. Differences in cellular regulation of enzyme secretion during shake flask and solid-state growth of *Aspergillus* is known to literature (Antier *et al*., 1993b; Iwashita, 2002) and it appears that protein production in shake flasks are stringently governed by such regulations while it is absent or non functional in the cells of the fungus growing on solid state substrate.

Mutants of A. *carbonarius*

Mutant analysis that suggested the importance of secretional regulation in enzyme production during shake-flask growth of fungi so also identified a mutational protocol for application as a procedure for industrial strain development. Mutants of the fungus affected in the protein secretion pathway were easily isolated in this study by selection for temperature tolerance after mutagenesis using ultraviolet rays. As was described earlier in relation to amylase secretion in *A. niger* (Ravi-Kumar, 2004), mutants of *A. carbonarius* were found to over produce proteases when grown in shake flask cultures. Overcoming secretional regulations in *A. carbonarius* apparently resulted in faster growth rates of the mutants since such mutants could efficiently hydrolyze and utilize polymeric carbon and nitrogen substrates in the growth medium due

to higher quantities of secreted enzymes. The significant differences in growth physiology of the selected mutants **(Fig. 5, 6; Experiment 1.3)** in comparison to the wild type showed that many determinants govern cellular regulation for protein excretion in fungi and multiple mutation steps are needed to isolate industrially important mutants. In their study, Reynaga-Pena and Bartnicki-Garcia (1997) derived CREA mutants (carbon repression protein A) of *A. niger* by selecting survivors after treatment with a UV dose that killed over 90% cells. Since industrial strain development requires selection of several useful characters, harsh mutagen dosage like the above was avoided by multiple mutagenesis step and selecting mutants using UV dosage that resulted in only 40-50% kill.

The mutant of *A. carbonarius* (UV 10056) selected for the overproduction of polygalacturonase in shake flask cultures was found to secrete only a single enzyme, unlike the parent **(Fig. 8)**. The enzyme purified from the culture broths showed a molecular mass of 42 kDa by SDS-PAGE **(Fig. 9)** and was highly active with a specific activity of 6350 units mg^{-1} protein on polygalacturonate substrate. The pure enzyme was optimally active at pH 4.3 and at 50° C **(Fig. 11, 12)**. The enzyme had a low affinity to polygalacturonic acid substrate as evidenced by high K_M **(Fig. 13)**. Thus, a mutant strain of *A. carbonarius* that selectively overproduced the highly active enzyme was isolated in this study. The probable reasons for the selective production of this protein has been discussed below.

Multiple polygalacturonase production by A. *carbonarius* is due to differential glycosylation of the enzyme protein

A. carbonarius has been reported to secrete copious quantities of polygalacturonase in solid state cultures (Devi and Rao, 1996; Kavitha and Umesh-Kumar, 2000). In the present study, it was found that the fungus

produced only very little enzyme when grown in shake-flasks **(Table 2)**. The enzyme production in shake-flasks could not be induced by pectin, polygalacturonic acid or pectic substances **(Table 2)**. Protein secretion in *Aspergillus* occurs through hyphal apical cells during shake flask cultivation (Khalaj *et al*., 2001) and Iwashita's report (2002) that solid state growth of the fungus induces more hyphal branching suggested a mutation approach for the isolation of branching mutants of the fungus (Reynaga-Pena and Bartnicki-Garcia, 1997) for polygalacturonase production in shake flasks.

Branching mutants show pellet growth in liquid medium (Bocking *et al*., 1999) and the 86 mutants of the parent strain of *A. carbonarius* affected in cytoskeletal organization for induced branching isolated in the present study, also grew as pellets in shake flasks. Screening of culture filtrates for extracellular polygalacturonase, found all the mutants to secrete higher quantities of the enzyme, when compared to the 9 U mL^{-1} (36 U mg^{-1}) enzyme produced by the parent strain. Of the mutants, those that appeared early (60 h incubation) on plates as colonies visible to the naked eye, secreted more enzyme (75 U mL^{-1}; 800 U mg^{-1}) than the slow growers (20-40 U mL^{-1}) which appeared after 72 h incubation of the plates. The observation that the fast growing mutants produced more enzyme prompted us to attempt further mutational improvement of the selected mutant, for fast growing colonies. Screening of the 11 mutants that appeared in 50 h, resulted in the identification of a colony that produced 130 U mL^{-1} (1200 U mg^{-1}) enzyme. Mutation of this culture evolved fast growing colonies (44 h) and isolation of a mutant secreting 180 U mL^{-1} polygalacturonase (1600 U mg^{-1}). When this strain was mutated, a single colony that appeared in 36 h was found to secrete 300 U mL^{-1} enzyme in the culture broth with a specific activity as high as 3500 U mg^{-1} **(Table 3)**. The variation in the quantities of enzyme secreted by the mutants can only be approximated to the number of hyphal tips due to induced branching since, enzyme secretion is known to occur through hyphal tips.

Highly branching mutants affected in CREA (carbon repression protein A) have been reported to have a compressed colony morphology (Hynes and Kelly, 1977; Reynaga-Pena and Bartnicki-Garcia, 1997). The mutant isolated for polygalacturonase over production described above, also exhibited compressed colony morphology, when grown on agar plates **(Fig. 5)**. This particular colony morphology, characteristic of the mutant suggested that, constitutive enzyme secretion occurred during shake flask growth of the mutant in the medium containing starch (corn flour) due to affected carbon repression.

Antibodies raised to the 42 kDa enzyme purified from the mutant culture filtrates, reacted with the 61 kDa polygalacturonase of *A. carbonarius* secreted in solid-state **(Fig. 14)**. Antibody cross reactivity was also seen with a commercial enzyme preparation of *A. niger* containing high molecular mass enzymes **(Fig. 14)**. These reactions showed homology of the fungal polygalacturonases and probable occurrence of the 61 kDa polygalacturonase in *A. carbonarius* as a glycosylated form of the 42 kDa enzyme. In order to ascertain the effect of glycosylation on the protein molecular mass, the 61- and 42-kDa polygalacturonases were deglycosylated using the enzyme PNGase F. Upon deglycosylation, the 61 kDa protein was converted to 42 kDa protein and extended incubation with PNGase F, reduced its molecular mass to 40.5 kDa **(Fig. 21)**. Deglycosylation of 42 kDa protein also caused a reduction in its molecular mass to 40.5 kDa **(Fig. 21)**. Antibody reactivity to deglycosylated proteins confirmed that the cross reactivity described above was not due to antibodies recognizing glycans in the glycosylated proteins. Western blot analysis of the culture broth proteins of the mutant for the type of enzymes produced during growth showed increased secretion of 42 kDa polygalacturonase during acidification of the culture broth and occurrence of a 40.5 kDa protein at pH 3.0 **(Figs. 16-18)**. The culture broths of the mycelia grown at pH 5.5 and incubated at pH 3.0 when analyzed for enzymes also

showed similar results **(Fig. 17)**. The above data evidenced induction of polygalacturonase production by acidic pH in *A. carbonarius* and secretion of the underglycosylated 42 kDa polygalacturonase and unglycosylated 40.5 kDa proteins during shake flask growth due to an impaired glycosylation at acidic pH.

Efforts to study the kinetic properties of the deglycosylated native enzyme protein were not successful since, protein deglycosylation using PNGase F was possible only at pH 8.0 and at this pH, over 90 % activity loss occurred in the deglycosylated enzymes. Incubation of the deglycosylated enzymes at the assay pH (pH 4.3) did not result in enzyme renaturation. This could be due to the glycans conferring more pH stability to the proteins because, in the native 61- and 42-kDa enzymes (controls), only 40 and 70 % activity losses were recorded at pH 8.0. When *N*-terminal amino acid sequences of the two proteins were determined by microsequencing, the clear sequence GS(C)TF **(Annexure 1)** of the 42 kDa enzyme of *A. carbonarius* was identical to the *N*-terminal sequences reported for the polygalacturonases of *A. tubingensis* (Nagai *et al*., 2000), *A. niger* (Ruttkowski *et al*., 1991), *A. oryzae* (Kojima *et al*., 1999) and *Sclerotinia sclerotiorum* (Fraissinet-Tachet *et al*., 1995). Only an indicative sequence XVTXXF **(Annexure 2)**, which did not show homology to any polygalacturonases, was deciphered in the 61 kDa enzyme, despite repeated attempts.

Even though amino acid differences seemed to occur at the *N*-terminus of the two polygalacturonases of *A. carbonarius*, the variation can be due to amino acid insertion or deletions that are known of polygalacturonases produced by phytopathogenic fungi (Bussink *et al*., 1992a). This is explained by antigenic similarity of the polygalacturonases, the deglycosylated 40.5 kDa form corresponding to the molecular mass approximately deduced of the 360 amino acids reported of polygalacturonases (Keon and Waksman, 1990; Nagai *et al*., 2000) and sequence identity of the 42 kDa *A. carbonarius* enzyme with

polygalacturonases of other fungi. The data obtained throws light on the adaptation of the fungus to environmental changes such as growth substrate and pH and changes in the posttranslational modifications of polygalacturonases under different conditions.

Complex regulation of polygalacturonase secretion during shake flask growth of *A. carbonarius*

Studies on amylase secretion in *A. niger* from this laboratory showed that proteases play an important role in the post translational modification of proteins for secretion in *A. niger* (Dubey *et al*., 2000; Ravi-Kumar *et al*., 2004). Such a processing that occurred in the cellular periplasmic space was found necessary for overcoming secretional difficulties associated with reduced porosity of *Aspergillus* cell walls to proteins of molecular mass above 45 kDa (Dubey *et al*., 2000). Even though protein glycosylation was identified as the cause for multiple polygalacturonase secretion by *A. carbonarius*, higher extra-cellular acid protease production concomitant with over production of polygalacturonases in the mutants **(Table 3, Fig. 8)** suggested a role for the acid proteases in polygalacturonase processing. However, the results on polygalacturonase overproduction even under protease-inhibited conditions **(Fig. 20)** showed that higher secretion of acid proteases was incidental due to mutation affecting the secretory pathway with no relevance for polygalacturonase processing.

The mutant isolated for polygalacturonase overproduction in shake-flask, when grown in solid-state culture, behaved identical to the wild type *A. carbonarius* in the proteins and enzymes it secreted **(Table 4)**. Despite the above, the fungi showed variation in the polygalacturonase enzyme yields when grown in a medium amended with 2% glucose **(Table 4)**. Glucose amendment reduced enzyme production by ~60% in the case of wild type while in the mutant,

the enzyme yields were unaffected. Shake flask growth of the mutant in glucose medium (corn flour replaced with 2% glucose) also showed no variation in the production of the enzyme **(Table 5)**. From the results above and the observation that very little enzyme is produced by the parent strain in shake flasks in spite of the growing culture acidifying the culture broth, it was inferred that, relief from glucose repression is important for induced enzyme secretion by pH. The mutant responded to acidic pH environment for enzyme over production during shake flask growth, because it was affected in glucose regulation.

Mutational analysis of *A. carbonarius* in relation to polygalacturonase secretion identified a complex regulatory mechanism involved in fungal gene expression. The results also explained the need for strains not affected in protein secretion for gene expression studies. Glucose repression overriding the effects of pH regulation identified by mutant analysis reasoned insufficient literature on fungal gene regulation by pH (Penalva and Arst Jr, 2002) although, it is a general observation that *A. niger* rapidly acidified the medium as it grows (Visser *et al*., 1994).

Submerged fermentation process for polygalacturonase production by the mutant strain of *A. carbonarius*

In this study, batch and fed-batch fermentation protocols for polygalacturonase production by submerged fermentation were developed. In the batch process, increased hyphal branching of the mutant was taken advantage of for obtaining increased yields of the enzyme in culture broths. The increased branching that induced pellet growth of the mutant fungus aided in easy downstream processing to separate the biomass from the fermented broth **(Experiment 3.5)**. Since the mutation relieved the mutant strain of glucose

catabolite repression, a cheap medium containing corn flour could be used to produce the enzyme by fermentation.

In conclusion it can be stated that by inducing hyphal branching by UV mutagenesis, wild type *A. carbonarius* that produces very little polygalacturonase in shake flasks can be made to excrete more enzyme. Temperature selection of UV mutants of the fungus induced faster growth rates in the fungus thereby, reducing the fermentation period for enzyme production in shake flask cultures. Mutational analysis described in this study also identified glucose catabolite repression overriding pH regulation for polygalacturonase production by the fungus. This is in contrast to the observation on pH regulation overriding glucose regulation in the case of isopenicllin-N-synthase gene in *A. nidulans* (Espeso *et al*., 1993). The pH induced polygalacturonase production by the mutant strain of *A. carbonarius* in shake-flask cultures resulted in a fed batch fermentation process for increased enzyme production by submerged fermentation process. The enzyme produced by such a process had the characteristics for industrial applications since it was stable at 4^oC for over 150 days and liquefied banana and apple to yield clear juices. The observation that differential protein glycosylation resulted in several forms of polygalacturonases with differences in kinetic properties showed the ecological adaptability of the fungus.

SUMMARY

SUMMARY

This thesis describes identification of the primary polygalacturonase protein secreted by *Aspergillus carbonarius*, its glycosylation resulting in higher molecular enzyme forms and the fungal growth physiology in relation to enzyme production.

Wild type *A. carbonarius* produced two polygalacturonases of molecular mass 61- and 42-kDa when grown in solid-state culture made of wheat bran. In submerged cultures, the fungus produced only very little enzyme. Polygalacturonase production by *A. carbonarius* in shake-flask could not be induced by pectin or pectin derivatives as carbon source.

Mutants of the fungus isolated after UV-treatment and temperature selection secreted polygalacturonase when grown in shake-flasks. These mutants grew as pellets due to increased hyphal branching during shake-flask cultivation. One of the mutants (UV-10046) isolated for fast growth rates, overproduced (300 U ml^{-1}) polygalacturonase in shake-flask cultures. This mutant exhibited compressed colony morphology on agar plates. In solid-state cultures made of wheat bran, the mutant strains behaved like the parent in the quantity of enzyme secreted. In the wheat bran medium containing 2 % glucose, enzyme secretion was affected in the parent but not in the mutant UV-10046. No appreciable difference in polygalacturonase production was observed when the mutant strain (UV-10046) was grown in shake-flask containing pectin and other simple sugars as carbon source.

Kinetics of polygalacturonase production by the mutant of *A. carbonarius* (UV-10046) during shake-flask growth showed that the enzyme production was

induced by acidic culture pH. Overproduction of the 42 kDa polygalacturonase by the mutant was observed by SDS-PAGE analysis of the culture filtrate.

The 42 kDa polygalacturonase purified by alginate affinity precipitation demonstrated optimum activity at temperature 40-50ºC and pH 4.3. However, the enzyme was stable only at temperatures below 30ºC. A high specific activity of 6350 U mg^{-1} was estimated with polygalacturonate substrate using the purified enzyme. Low affinity of the enzyme to the polygalacturonic acid substrate was evidenced by high K_M (2.5 %). Antibodies raised to the purified enzyme cross reacted with the 61 kDa polygalacturonase produced by the fungus in solid-state cultures and several high molecular mass enzyme forms that were present in the commercial samples of *A. niger*.

Studies on enzyme production during growth of the fungus in the shake-flask revealed pH induced enzyme secretion. pH controlled cultivation confirmed induced enzyme synthesis at pH 3.0. Basal level enzyme secretion at pH 5.0 was also observed. Rapid acidification of the culture broth and induced enzyme secretion at pH 3.0 resulted in the occurrence of a protein of molecular mass 40.5 kDa in the culture filtrates. The evolution of the lower molecular mass enzyme protein was not due to proteolytic hydrolysis by the acid proteases present in the culture filtrates since, the 40.5 kDa protein was also identified when the culture was grown in presence of protease inhibitors. The above results suggested differential glycosylation as the cause for the secretion of multiple enzyme forms by the fungus. In order to evidence differential glycosylation of the proteins, the 61- and 42-kDa enzymes were deglycosylated using PNGase F. The molecular mass of the deglycosylated protein was estimated by SDS-PAGE and western blot analysis of the products. Upon deglycosylation, the 61- and 42-kDa enzyme resolved as 40.5 kDa proteins in SDS-PAGE gels. The glycans were found to have a role in enzyme conformation for stability to pH.

The *N*-terminal of the 42 kDa protein had a sequence GS(C)TF. Only an indicative *N*-terminal sequence XVTXXF was deciphered in the 61 kDa polygalacturonase.

Highest yields of the enzyme in shake-flasks using the mutant UV-10046 were obtained when the fungus was grown in a medium containing corn flour. The conditions standardized resulted in the up scaling the batch fermentation process to 125 L for enzyme production.

pH induced enzyme secretion during shake-flask growth of the fungus also resulted in the description of a fed-batch fermentation protocol for increasing enzyme yield during submerged fermentation process.

Growth of the fungus as pellets in the fermentor aided in easy separation of the fungal mycelia from culture filtrate after fermentation using plate and frame filter. The raw culture filtrate could be concentrated 30 fold by membrane filtration. Stability of the enzyme during storage at 4ºC and ability of the enzyme preparation to degrade apple and banana fruit pulps to yield juice, described a process for industrial polygalacturonase production using the mutant strain of *A. carbonarius*.

The results are discussed in relevance to literature pertinent to this investigation.

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FIGURES

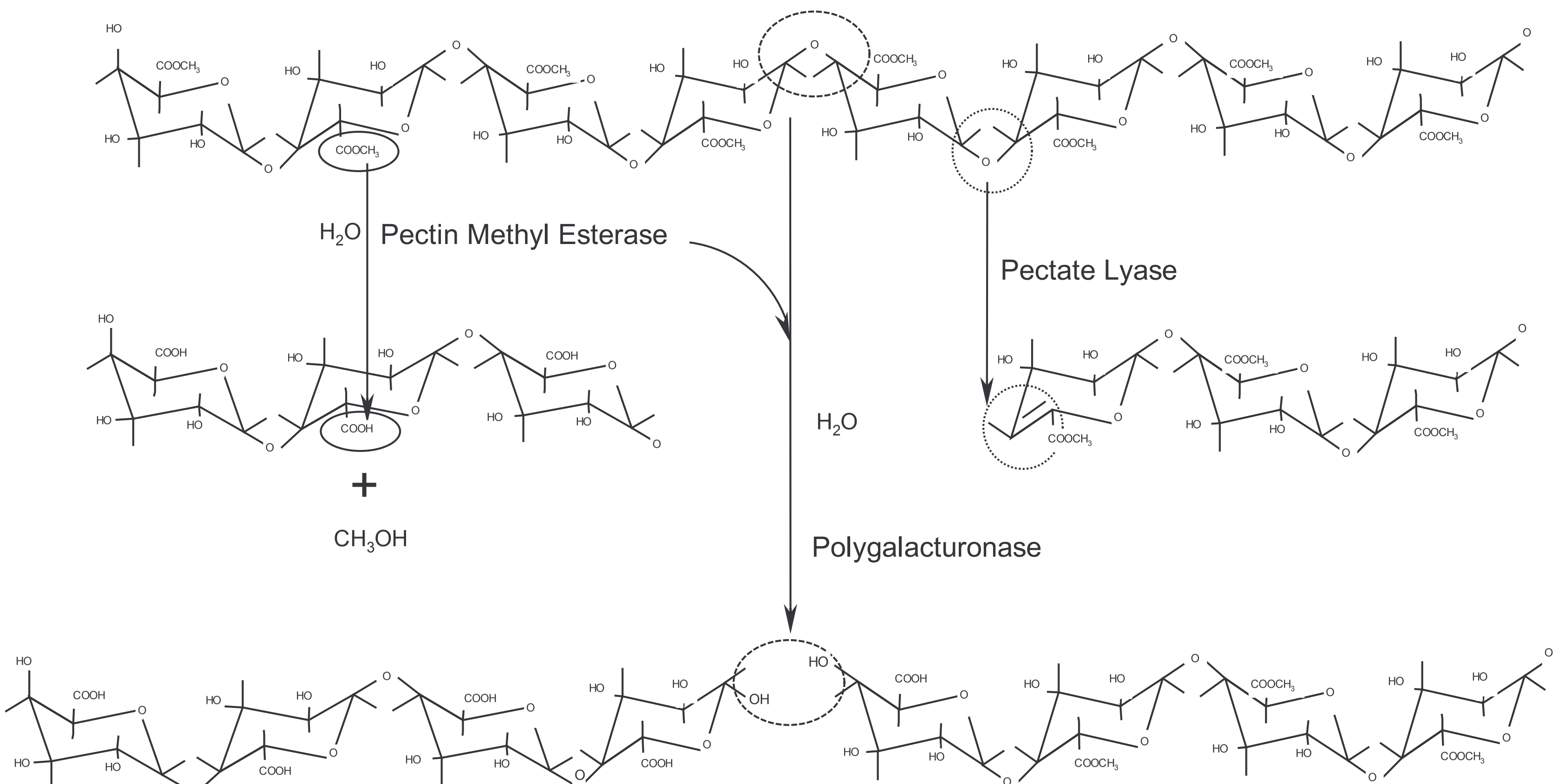


Figure 1. Pectin main chain structure and enzymes involved in degradation

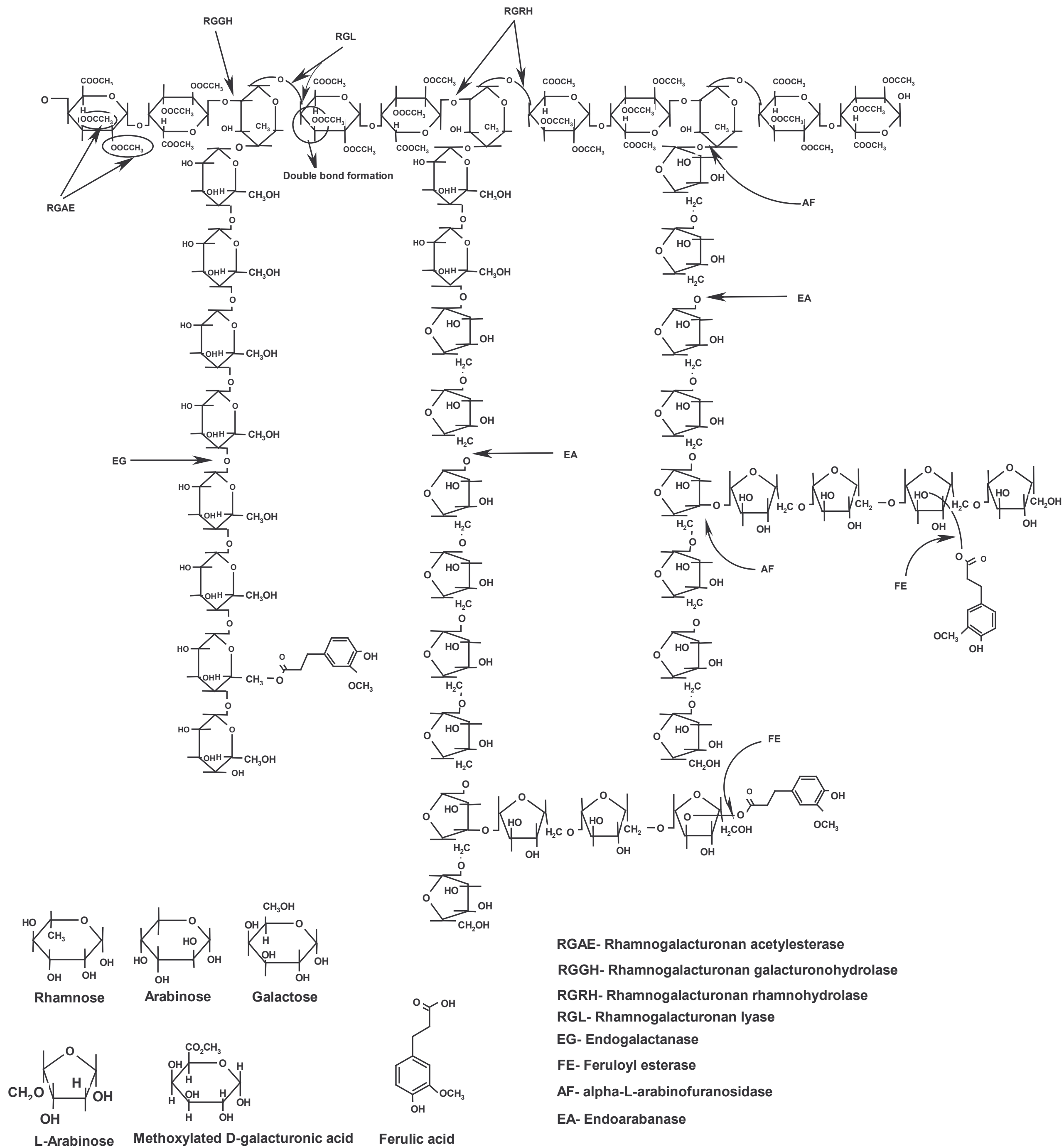


Figure 2. Structure of hairy region of pectin and pectic enzymes involved in the degradation.

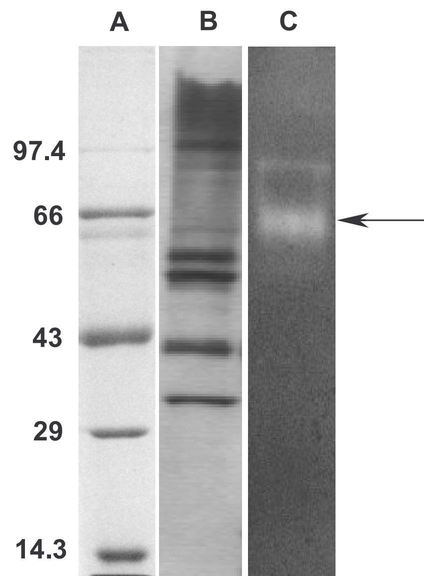


Figure 3. Polygalacturonases of *A. carbonarius* produced during growth in solid state cultures.

A. Molecular mass standards

B. Silver stained crude proteins (5 µg) in the extracts of moldy wheat bran after 60 h growth

C. Zymogram reaction identifying the high active polygalacturonase in B (arrow)

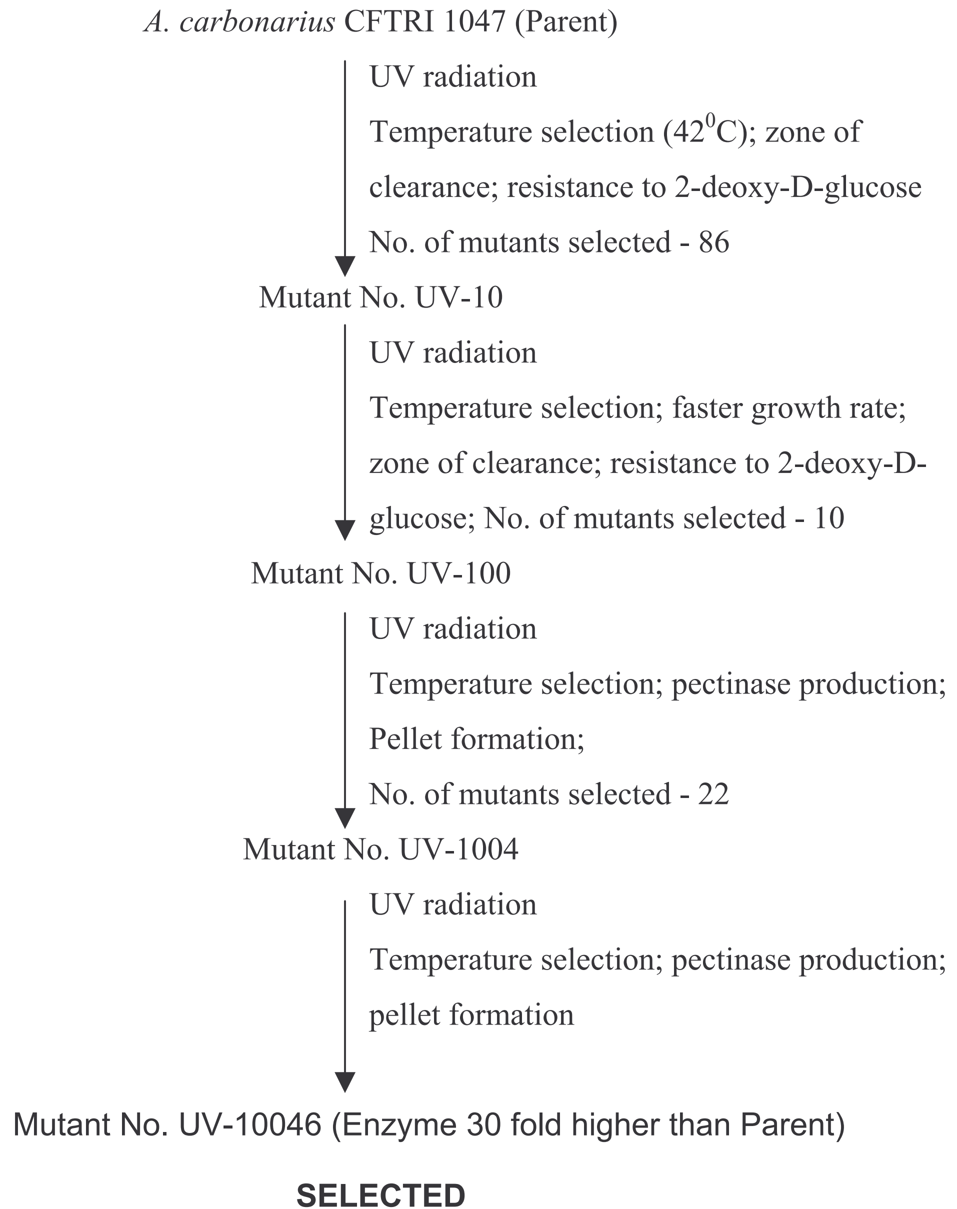
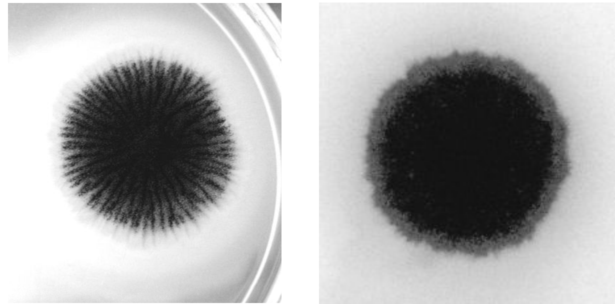
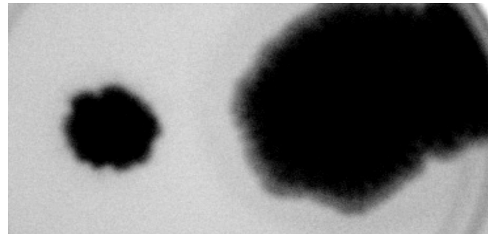


Figure 4. Mutational protocol for *A. carbonarius* CFTRI 1047 strain improvement for polygalacturonase overproduction in shake-flasks



Parent **Mutant UV-10046**

Pectin Agar Medium



Mutant UV-10046 Parent

Gelatin agar medium

Figure 5. Growth of the parent and mutant strains of *A. carbonarius* on agar plates

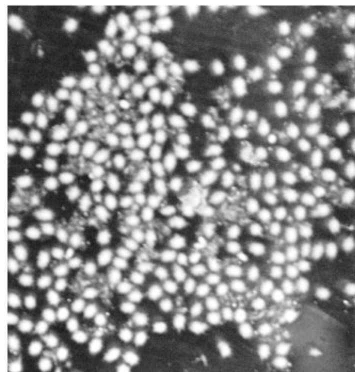


Figure 6. Pellet growth of the mutant strain (UV-10046) of *A. carbonarius* during shake-flask cultivation in corn flour medium

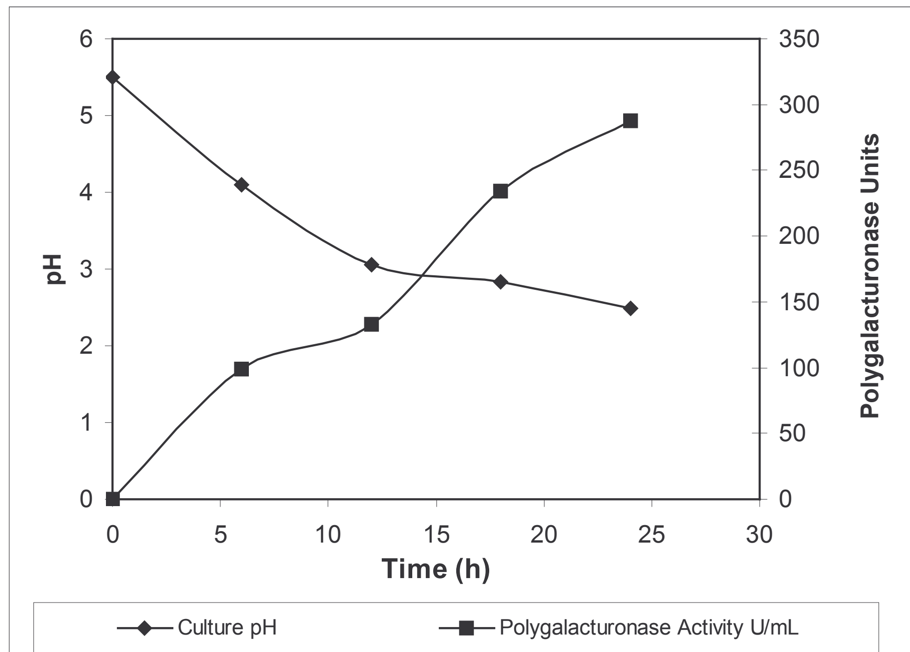


Figure 7. Polygalacturonase production by UV-10046 mutant of *A. carbonarius* in relation to pH during shake-flask growth in corn flour medium

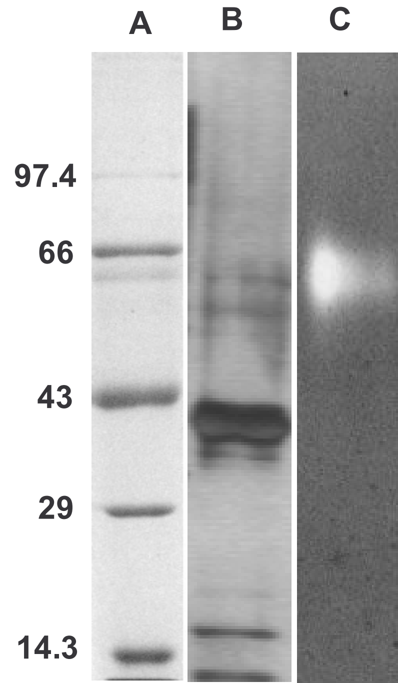


Figure 8. Overproduction of 42 kDa polygalacturonase by the mutant strain of *A. carbonarius* in shake-flask cultures.

A. Molecular mass standards

B. Silver stained culture filtrate proteins of the mutant strain of *A. carbonarius* (5 μg)

C. Zymograms identifying polygalacturonases in B (Shifts in protein mobility due to incorporated polygalacturonate substrate in the gels)

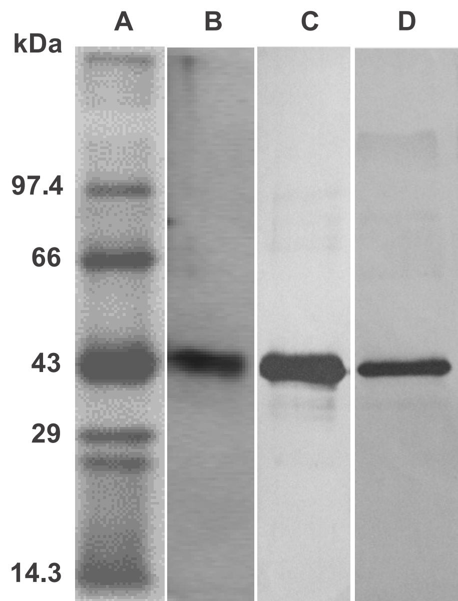


Figure 9. Antibody cross reactivity to polygalacturonases of the mutant strain (UV-10046) of *A. carbonarius*.

Antibodies raised to the 42 kDa polygalacturonase were used to probe the western blots containing SDS-PAGE separated proteins.

A. Silver stained molecular mass standards

B. Silver stained SDS-PAGE gel showing the pure 42 kDa polygalacturonase protein (2µg).

C. Homologous antibody reaction of B (1µg)

D. Antibody reactivity to the specific polygalacturonase present in the crude culture filtrate (1µg)

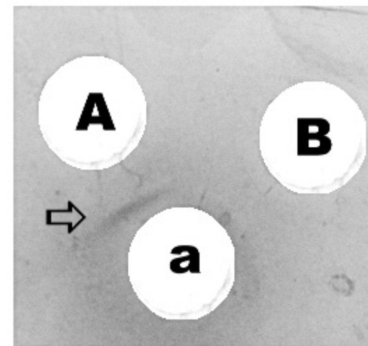


Figure 10. Outerlony immuno double diffusion reaction

A. Purified 42 kDa polygalacturonase and (a) homologous antibody

B. Culture filtrate proteins

Arrow: The precipitation reaction

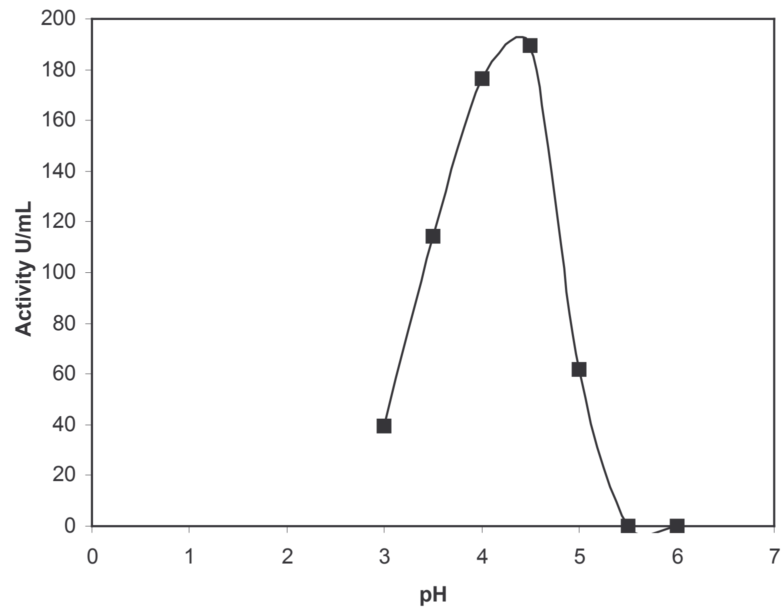


Figure 11. Effect of pH on the activity of polygalacturonase purified from the mutant strain of *A. carbonarius*

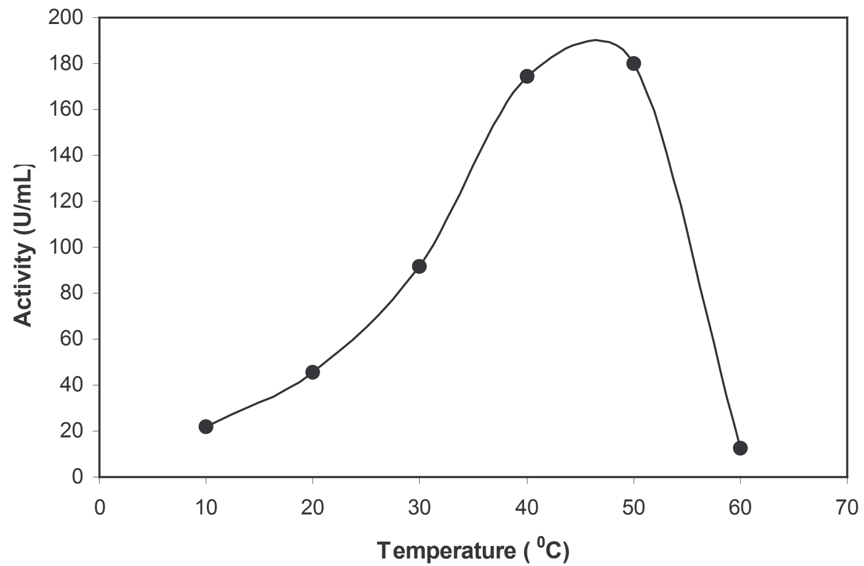
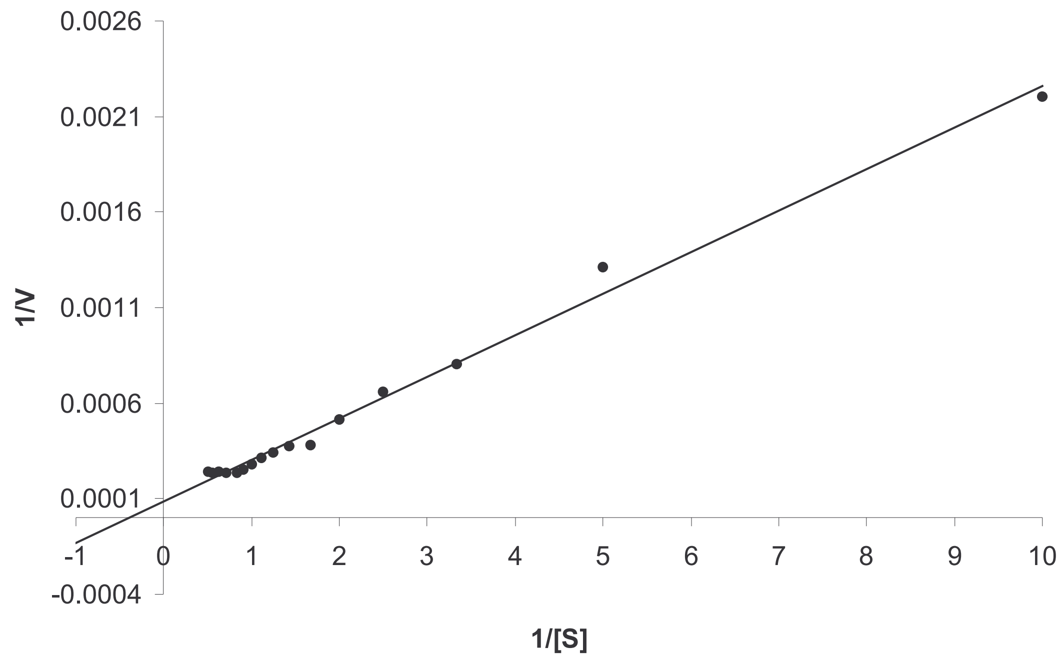


Figure 12. Activity of purified polygalacturonase from the mutant strain of *A. carbonarius* at different temperatures



$K_M = 2.5\%$

Figure 13. Substrate affinity of the 42 kDa polygalacturonase purified from mutant strain of *A. carbonarius* on polygalacturonic acid

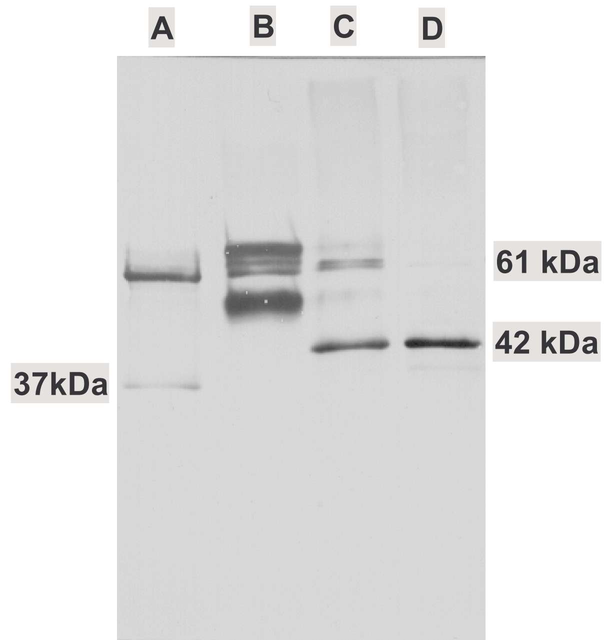


Figure 14. Reactivity of the antibody to polygalacturonases of *A. niger* and *A. carbonarius*.

A & B. Commercial pectinase samples of *A. niger* (A: 0.5 μg protein, B: 1.0 μg)

C. Crude proteins of the solid-state grown *A. carbonarius* mutant (1.5 μg protein)

D. Purified 42 kDa polygalacturonase of the mutant strain of *A. carbonarius* (homologous reaction; 0.5 μg protein)

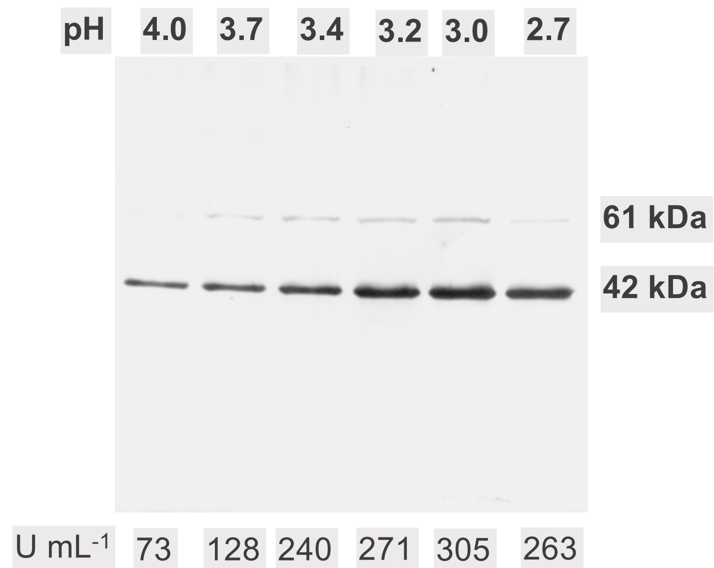


Figure 15. Polygalacturonase production in relation to culture broth pH during shake-flask growth of the mutant strain of *A. carbonarius*.

The fungus was grown in corn flour salt medium and culture filtrates after different periods of growth were analyzed by western blot analysis for polygalacturonase protein (Identical concentration of one μg protein was used in all lanes). The figures at the bottom of the gel refer to polygalacturonase activities mL^{-1} culture filtrate ($U\ mL^{-1}$). Culture pH is shown above the blot.

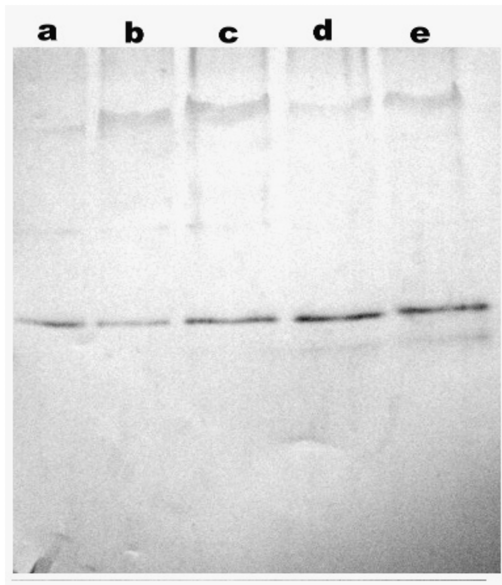


Figure 16. Effect of pH on polygalacturonase production by *A. carbonarius*.

The fungus was grown at pH 5.0 (a), 4.5 (b), 4.0 (c), 3.5 (d) and 3.0 (e). in a buffered medium. Identical protein concentrations (1 µg) were used in all lanes. Polygalacturonase was identified in the western blot reaction by the intensity of the reaction with homologous antibody.

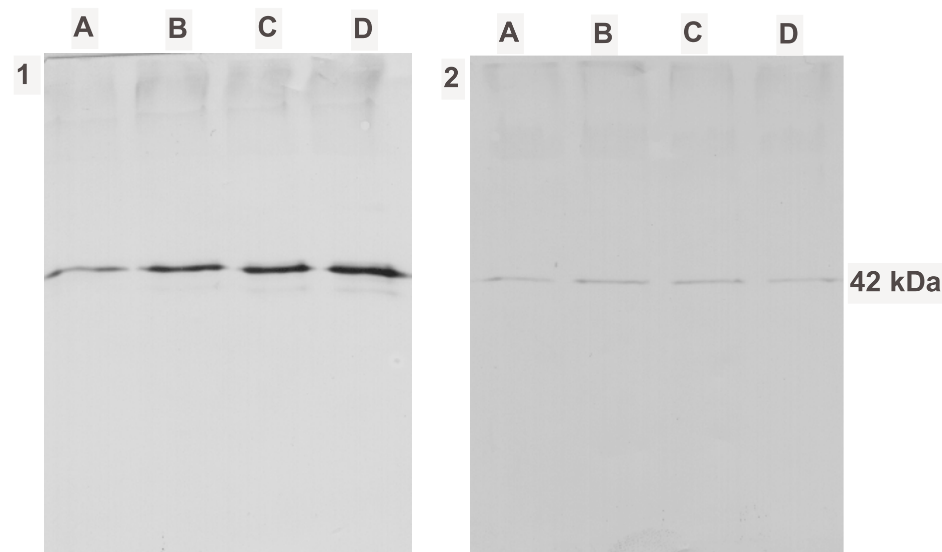


Figure 17. pH induced expression of the high active polygalacturonase in the mutant strain of *A. carbonarius*.

Mycelia grown at pH 5.5 in buffered medium for 24 h was transferred to media buffered to pH 3.0 (1) and pH 5.0 (2). Culture filtrates (1.5 µg protein, equal in all lanes) after 6 h (A), 12 h (B), 18 h (C) and 24 h (D) were used for western blot analysis.

Probe: Homologous antibody raised to 42 kDa polygalacturonase.

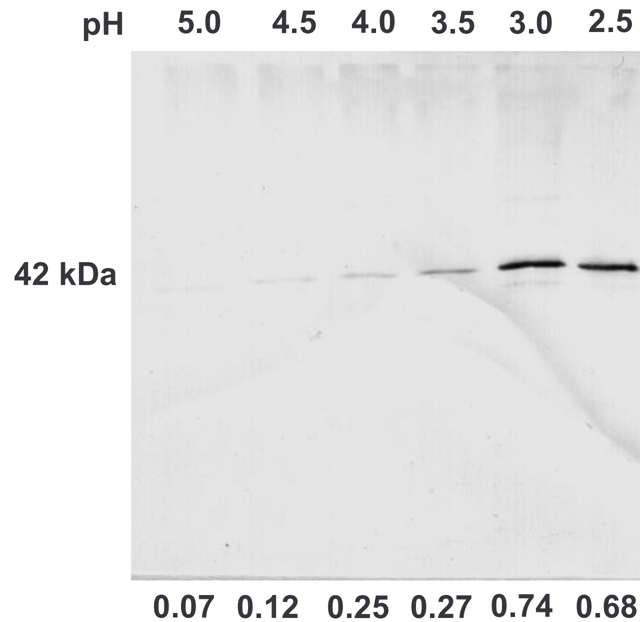


Figure. 18. Effect of culture pH on protease and polygalacturonase production during shake-flask growth of the mutant strain of *A. carbonarius*.

The mutant strain of the fungus was grown in the medium buffered to the required pH for 24 h and polygalacturonase was identified by western blot reactions. The values at the bottom of the gel correspond to the specific activity (U mg^{-1}) of acid proteases at the respective pH.

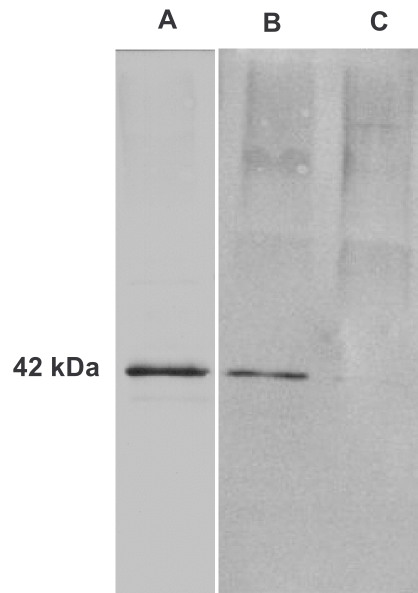


Figure 19. Western blot reactions identifying polygalacturonase in the extracts of mycelia of the mutant strain of *A. carbonarius* grown in shake flasks (1.5 μg protein was loaded in each lane).

A. Culture filtrate proteins

B. Periplasmic proteins

C. Intracellular proteins

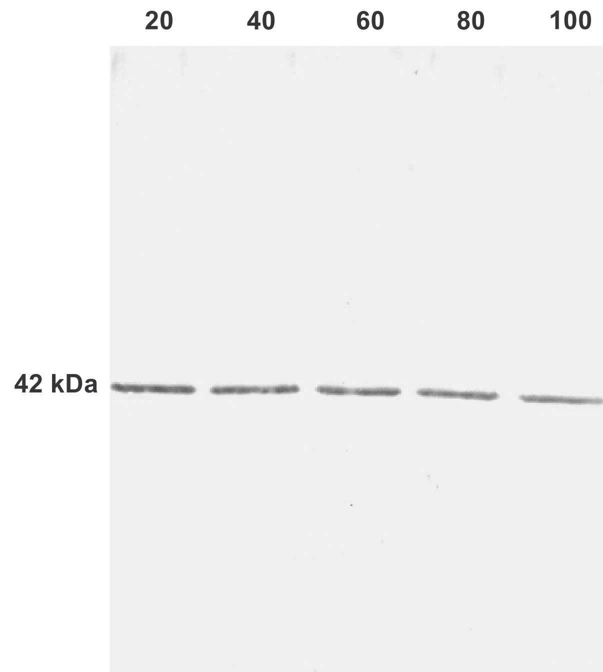


Figure 20. Western blot reaction identifying polygalacturonase in culture filtrates of shake-flask grown mutant strain of *A. carbonarius.* The fungus was grown in corn flour salt medium containing protease inhibitors. The figures on each lane refer to the concentration (mg/100 mL) of protease inhibitor.

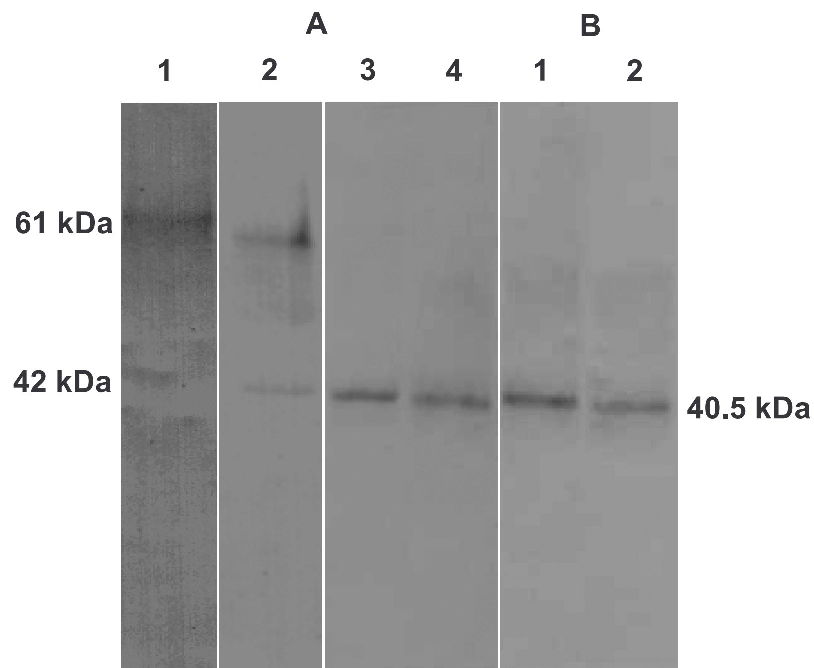


Figure 21. Western blot reactions identifying the deglycosylated polygalacturonases of *A. carbonarius*.

The 61- and 42- kDa polygalacturonases were treated to PNGase F and the products analyzed by western blot reactions after protein separation by SDS-PAGE.

A. Products formed from 61 kDa enzyme (1) and after 5 h (2), 10 h (3), and 15 h (4) digestion.

B. Products formed from 42 kDa enzyme (1) and after 5 h (2) digestion.

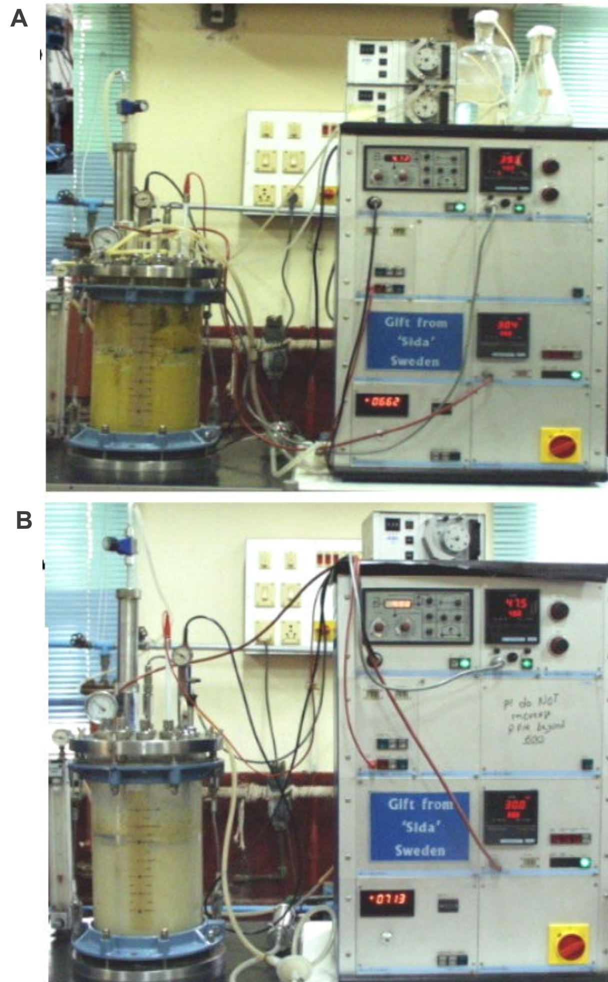


Figure 22. The Bioengineering fermentor (15 L) used for lab-scale fermentation for polygalacturonase production

A. Fermentation with pH of the medium maintaining at pH 4.0

B. Fermentation without pH maintenance

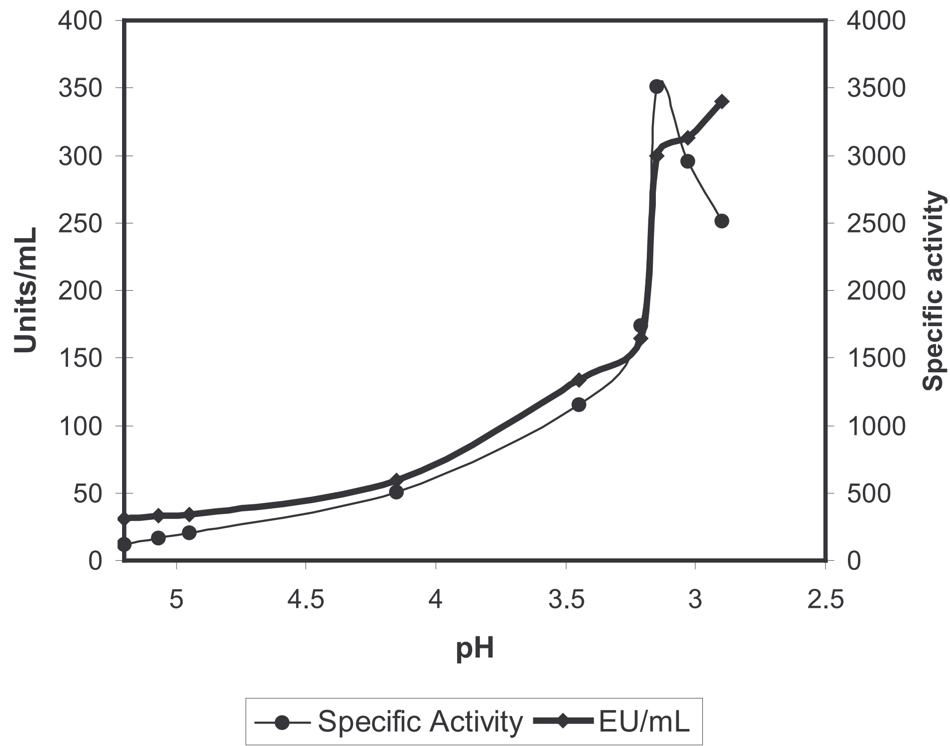


Figure 23. Polygalacturonase production during growth of the mutant strain of *A. carbonarius* in 10 L fermentor

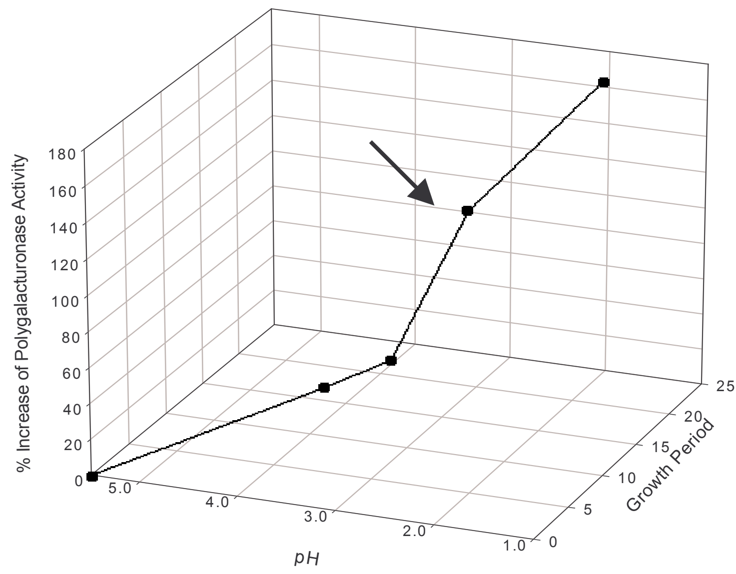


Figure 24. Polygalacturonase yield during fed batch fermentation using the mutant strain of *A. carbonarius* (Arrow: addition of fresh medium at pH 3.0)

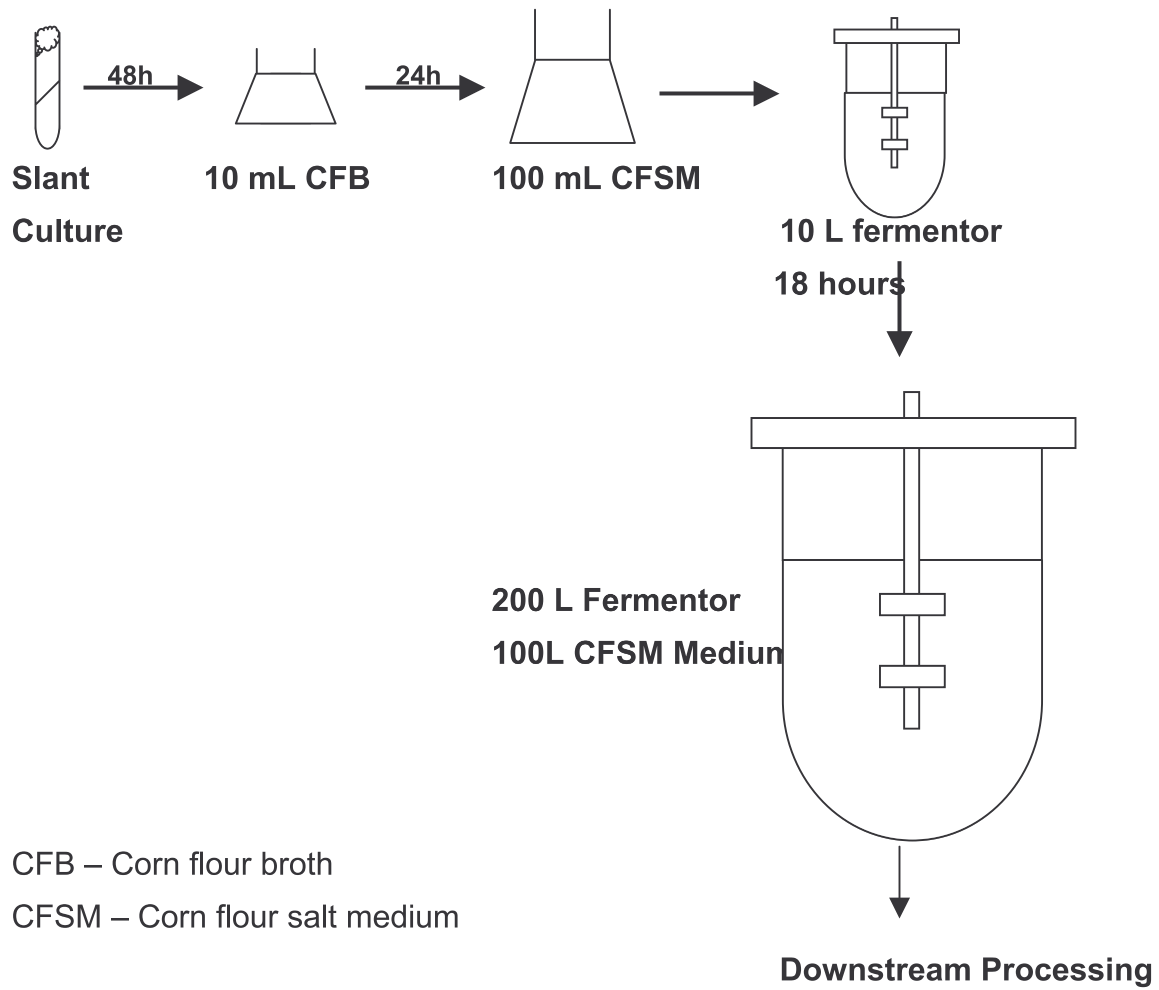


Figure 25. Flow chart for scale up of fermentation for polygalacturonase production using the mutant strain of *A. carbonarius*

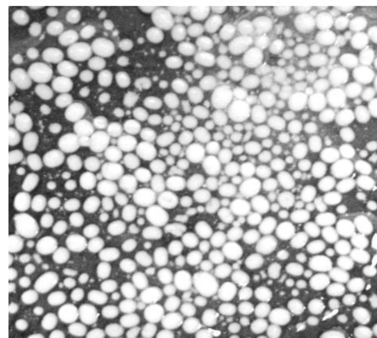


Figure 26. Pellet formation by the mutant strain of *A. carbonarius* in 200 L fermentor.

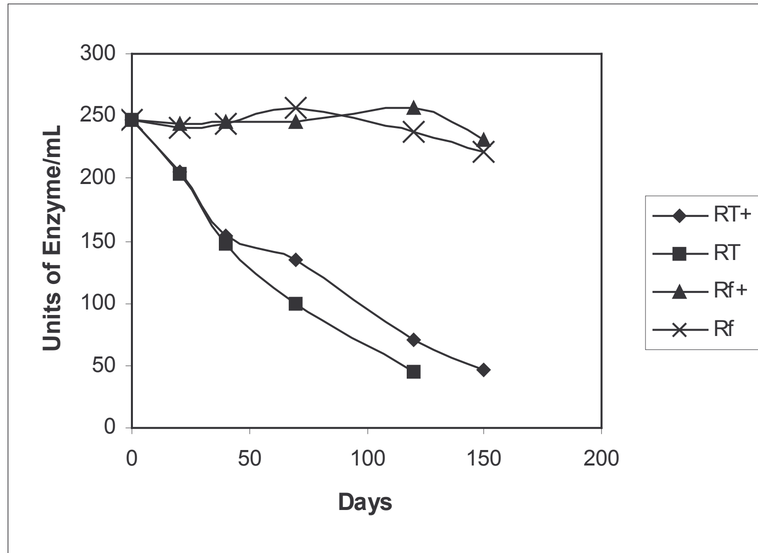


Figure 27. Stability of polygalacturonase obtained from shake-flask grown mutant strain of *A. carbonarius* during storage. (RT: Room temperature; Rf: Refrigeration; RT+: Room temperature + preservative in sample; Rf+ : Refrigeration + preservative in sample)

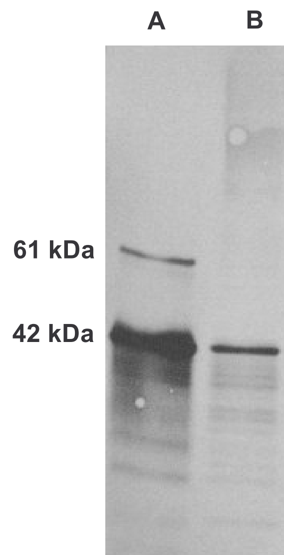


Figure 28. Western blot analysis of polygalacturonase samples from the mutant strain of *A. carbonarius* after storage for 150 days

A. Extract from fermented wheat bran B. Culture filtrate from SmF

TABLES

Table 1. Characteristics of pectic enzymes produced by microorganisms

Enzyme	Organisms	Molecular Weight (kDa)	pH Optima	Reference
Endopolygalacturonase	*Colletotrichum lindemuthianum*	38	4.2 & 5.4	(Keon and Waksman, 1990)
Endopolygalacturonase	*Rhizopus stolonifer*	60	4.6 - 4.8	(Trescott and Tampion, 1974)
Endopolygalacturonase (PGIII) Exopolygalacturonase (PGI) Exopolygalacturonase (PGII))	*A. niger*		5.0 5.0 5.0	(Behere *et al*., 1993)
Endopolygalacturonase (Endo-I) Endopolygalacturonase (Endo-II) Endopolygalacturonase (Endo-IIIA) Endopolygalacturonase (Endo-IIIB) Endopolygalacturonase (Endo-IV)	*A. niger*	55 38 57 57 59	4.9 4.8 4.3 4.5 4.8	(Kester and Visser, 1990)
Endopolygalacturonase (endoPG)	*A. niger*	35.5	4.5	(Lang and Looman, 1995)
Endopolygalacturonase (endoPG)	*C. lindemuthianum*	42	5.2	(Hugouvieux *et al*., 1997)
Endopolygalacturonase (PGA) Endopolygalacturonase (PGB) Endopolygalacturonase (PGD)	*A. niger*	35 35 51	4.0 5.0 4.2	(Parenicova *et al*., 2000a) (Parenicova *et al*., 2000a) (Parenicova *et al*., 2000b)

Endopolygalacturonase (PGI) Endopolygalacturonase (PGA) Endopolygalacturonase (PGB)	*A. oryzae*	 41 39	4.0 5.0 5.0	(Ueda *et al*., 1982) (Kitamoto *et al*., 1998) (Kitamoto *et al*., 1998)
Endopolygalacturonase E_1 Endopolygalacturonase E_2	*A. niger*	35 80	4.1 3.8	(Cooke *et al*., 1976)
Endopolygalacturonase I Endopolygalacturonase II Endopolygalacturonase III	*Kluveromyces fragilis*	46 50 30	4.0-5.0 4.0-5.0 4.0-5.0	(Inque *et al*., 1984)
Endopolygalacturonase I (PG I) Endopolygalacturonase II (PG II) Endopolygalacturonase III (PGIII)	*A. carbonarius*	61 42 47	4.0 4.1 4.3	(Devi and Rao, 1996)
Endopolygalacturonase II	*A. niger*	35	4.5	(van Santen *et al*., 1999)
Endopolygalacturonase Pgl1P	*Saccharomyces cerevisiae*	42	4.5	(Gainvors *et al*., 2000)
Endopolygalacturonases	*K. marxianus*	45, 42, 39, 36	5.0	(Schwan *et al*., 1997)
Endoxylogalacturonan hydrolase	*A. tubingensis*		5.0	(van der Vlugt-Bergmans *et al*., 2000)
Exopolygalacturonase	*A. aculeatus*	42		(Beldman *et al*., 1996)
Exopolygalacturonase (exo-PG I) Exopolygalacturonase (exo-PG II)	*A. niger*	66 63	3.8 4.5	(Hara *et al*., 1984)
Exopolygalacturonase ($exoPG_1$) Exopolygalacturonase ($exoPG_2$)	*A. alliaceus*	40 40	3.5 6.0	(Mikhailova *et al*., 1995)

Pectate lyase (PEL A)	*A. nidulans*	40		(Dean and Timberlake, 1989)
Pectates lyase (Ply A)	*A. niger*	43	7.5-8.5	(Benen *et al*., 2000)
Pectin lyase	A. joponicus	32	5.2	(Ishii and Yokotsuka, 1975)
Pectin lyase (PL I) Pectin lyase (PL II) Pectin lyase (PL B)	*A. niger*	37.5 37.5 40	 8.5-9.0	(van Houdenhoven, 1975) (van Houdenhoven, 1975) (Kester and Visser, 1994)
Pectin methyl esterase (PME1)	*A. aculeatus*	43	4.6	(Christgau *et al*., 1996)
Rhamnogalacturonan hydrolases RhgA Rgase A Rhamnogalacturonan rhamnohydrolase (RG-RH) Rhamnogalacturonan galacturonohydrolase RG-GH	*A. aculeatus*	51 59 84 66	3.0-4.0 3.5 4 4	(Schols *et al*., 1990) (Kofod *et al*., 1994) (Mutter *et al*., 1994b) (Mutter *et al*., 1994a)
Rhamnogalacturonan Hydrolase A (RHG-A), Rhamnogalacturonan Hydrolase B (RHG-B)	*A. niger*	72, 70	3.6, 4.1	(Suykerbuyk *et al*., 1997)
Rhamnogalacturonan lyase (Rgase B)	*A. aculeatus*	55	6.0	(Kofod *et al*., 1994)
Rhamnogalacturonase acetylesterase (RGAE)	*A. aculeatus*	26.7	6.0	(Kauppinen *et al*., 1995)

Table 2. Effect of carbon sources on polygalacturonase production by *A. carbonarius* in shake-flasks.

Carbon Source	pH		Polygalacturonase Activity (UmL^{-1})	Specific Activity ($mgmL^{-1}$)
	Initial*	Final**		
Corn flour	5.5	2.4	9	36
Glucose	5.5	2.3	8	63
Galactose	5.5	2.3	11	64
Xylose	5.5	2.4	8	50
Mannose	5.5	2.3	11	96
Pectin	5.5	2.8	19	106

The medium contained 2% carbon source and 0.313% $(NH_4)_2HPO_4$ and $(NH_4)H_2PO_4$

* pH adjusted with 0.01M HCl/0.01M NaOH

** After 48 h growth

Table 3. Polygalacturonase and protease produced by mutants of *A. carbonarius* in the culture broth.

Mutation Steps	No. of mutants analyzed after each mutation step	Range of	
		Polygalacturonase (U mL^{-1})	Protease (U mg^{-1})
Parent	-	9 (36)	(0.20)
1	86	20-75 (100-800)	(0.20-50)
2	11	75-130 (800-1200)	(0.50-0.60)
3	22	130-180 (1200-1600)	(0.70)
4	1	300 (3500)	(0.78)

Figures in parentheses are the range of specific activities (U mg^{-1})

Table 4. Polygalacturonase production by *A. carbonarius* in solid-state cultures

Strain	Polygalacturonase Activity (U g^{-1} dry bran)	
	Wheat Bran Medium	**Wheat Bran medium amended with 2% Glucose**
Parent	400	240
Mutant UV-10	391	262
Mutant UV-100	396	373
Mutant UV-1004	420	400
Mutant UV-10046	422	420

Table 5. Effect of carbon sources on polygalacturonase production by *A. carbonarius* mutant strain UV-10046 grown in shake flasks

Carbon source	pH		Polygalacturonase ($U\ mL^{-1}$)	Specific Activity ($U\ mg^{-1}$)
	Initial*	Final**		
Corn flour	5.5	2.48	288	2362
Glucose	5.5	2.49	260	2723
Galactose	5.5	2.49	266	2236
Xylose	5.5	2.57	250	2420
Mannose	5.5	2.39	290	2865
Pectin	5.5	2.70	270	2666

The medium contained 2% carbon source and 0.313% $(NH_4)_2HPO_4$ and $(NH_4)H_2PO_4$

* pH adjusted with 0.01M HCl/0.01M NaOH

** After 24 h growth

Table 6. Temperature stability of 42 kDa polygalacturonase enzyme of the mutant strain of *A. carbonarius*

Temperature (ºC)	Treatment (min)	Residual Activity (%)
27	30	100
27	60	100
30	30	100
30	60	100
40	30	55.73
40	60	12.87
50	30	11.05

Table 7. Optimization of media components for polygalacturonase production by the mutant strain of *A. carbonarius* in submerged cultures (Initial pH was 5.5), 24 h

Media Components	Medium No. (Ingredient Concentration of gL^{-1})																	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	***16***	**17**	**18**
Corn flour	40	40	100*	40			40	40	40	40	40	40	40	40	20	***25***	30	40
Corn starch*					40	40												
Pectin		2		1			1	1	1									
Peptone																		10
Yeast extract																		6
Corn steep liquor	10	10	27	10	10	20			5									
$(NH_4)_2HPO_4$							5	10	5	15	5	20	10	10	2.5	***3.13***	3.75	
$(NH_4)H_2PO_4$											5		10		2.5	***3.13***	3.75	
Gelatin																		
Polygalacturonase Activity ($U\ mL^{-1}$)	155	178	40	102	94	78	153	179	77	318	322	218	178	233	255	***348***	270	329
Specific Activity of polygalacturonase ($U\ mg^{-1}$)	1600	2325	106	1606	775	370	1550	2161	2011	1158	1202	646	636	735	1508	***3500***	2290	2012

* Hydrolyzed with Termamyl at 100ºC for 15 min and used

Table 8. Enzyme recovery during downstream processing

Sample Description	Volume (L)	Polygalacturonase		Specific Activity (U/mL)	% Recovery
		U mL^{-1}	Total Units		
Culture Broth at the end of fermentation (pH 2.9)	50	350	1,75,00,000	2500	100
After biomass separation	46	347	1,59,62,000	3420	91.2
Concentrated Sample 1	3.067	4815	1,47,67,605	4720	84.4
Concentrated Sample 2	1.536	8920	1,37,01,120	4140	78.3

Table 9. Culture filtrate polygalacturonase activity profile of the mutant strain of *A. carbonarius* on citrus pectin.

Assay Temperature	Enzyme Activity (UmL^{-1})	Specific Activity (Umg^{-1})
10°C	19	203
20°C	40	423
30°C	86	917
40°C	168	1793
50°C	204	2177
60°C	26	277

Table 10. Apple juice treatment and juice analysis by enzyme concentrate obtained from mutant strain of *A. carbonarius* and a commercial formulation.

Enzyme Sample	Temperature and Duration (h)	Specific Gravity	Degree Brix	pH	% Juice Yield
Enzyme concentrate from mutant	30°C, 4	1.0675	14	4.25	72.25
	50°C, 2	1.0556	11.4	4.20	76.3
Commercial formulation	30°C, 4	1.06912	14.2	4.00	75.5
	50°C, 2	1.0071	12	3.85	81.1