Cell Wall Degrading Enzymes of Fruit Spoilage Fungi (Review Article)

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Abstract: Fruits are an important part of the healthful lifestyle. Numerous cell wall degrading enzymes can be secreted by pathogenic fungi to breach and use the fruit cell walls as nutrient sources that reduce post-harvest life and finally lead to develop inedible, undesirable quality and soft rot spoilage. Plant cell wall polysaccharides can be divided into three groups, cellulose, hemicellulose, and pectin. Fungi in particular produce an abundance of extracellular pectinases and hemicellulases that are important factors for fungal spoilage. Organic acids such as benzoic and citric acids have been found to be antifungal agents.
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1. Introduction

Fruits play a vital role in human nutrition by supplying the necessary growth factors such as vitamins and essential minerals in human daily diet, and that can help to keep a good and normal health. Fruits are widely distributed in nature. One of the limiting factors that influence the fruits economic value is the relatively short shelf-life period caused by pathogens attacked. It is estimated that about 20–25% of the harvested fruits are decayed by pathogens during post-harvest handling even in developed countries (Droby, 2006; Zhu, 2006). In developing countries, post-harvest losses are often more severe due to inadequate storage and transportation facilities. Fungal fruits infection may occur during the growing season, harvesting, handling, transport, and post-harvest storage and marketing conditions, or after purchasing by the consumer. Fruits contain high levels of sugars and nutrients element, and their low pH values makes them particularly desirable to fungal decayed (Singh and Sharma, 2007).

The primary cell wall of fruit is composed of approximately 10% proteins and 90% polysaccharides, which can be divided into three groups: cellulose, hemicellulose and pectin (Nathalie, 2006). Numerous cell wall degrading enzymes can be secreted by pathogens to breach and use the plant cell walls as nutrient sources that reduce post-harvest life and finally lead to develop inedible, undesirable quality and soft rot spoilage. A remarkable array of polysaccharide degrading enzymes including xylanases, polygalacturonases, cellulases and α-amylases (Raviyan *et al.,* 2005; Netsanet *et al.,* 2009).

2. Plant cell wall polysaccharides

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They make up 90% of the plant cell wall and can be divided into three groups: cellulose, hemicellulose, and pectin (Nathalie, 2006). The plant cell wall is a resilient and structurally heterogeneous barrier composed of complex polysaccharides and diverse proteins (O'Neill and York, 2003). In addition to providing structural support and also a physical barrier, cell walls are an important line of defense against pathogens such as insects and pests. The main components of primary cell walls are members of two polysaccharide networks, one consisting of cellulose and hemicellulose, and the other consisting of pectic polysaccharides. In addition, primary cell walls often contain structural proteins such as the hydroxyproline-rich glycoprotein extension. Primary cell wall also, consists of cellulose fibrils tethered by hemicelluloses, principally xyloglucans in the majority of seed plant species. This load-bearing cellulose / xyloglucan network is embedded in an amorphous matrix of pectins and glycoproteins (Carpita and Gibeaut, 1993; Brett and Waldron, 1996).

2.1. Hemicelluloses (xylans)

Xylan constitutes the major component of hemicellulose; a complex of polymeric carbohydrates including xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose and D-mannose) and arabinogalactan (heteropolymer of D-galactose and arabinose) (Shallom and Shoham, 2003). This, together with cellulose (1,4-β-glucan) and lignin (a complex polyphenolic compound) make up the major polymeric constituents of plant cell walls (Kulkarni *et al.,* 1999). Within the cell wall structure, all three constituents interact via covalent and non-covalent linkages, with the xylan being found at the interface between the lignin and cellulose where it is believed to be important for fiber cohesion and plant cell wall integrity (Beg *et al*., 2001).

Xylan is found in large quantities in hardwoods from angiosperms (15–30% of the cell wall content) and softwoods from gymnosperms (7–10%), as well as in annual plants (<30%) (Singh *et al.,* 2003). It is typically located in the secondary cell wall of plants, but is also found in the primary cell wall, in particular in monocots. A complex, highly branched heteropolysaccharide, it varies in structure between different plant species, and the homopolymeric backbone chain of 1,4-linked β-D-xylopyranosyl units can be substituted to varying degrees with glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or p-coumaroyl side-chain groups (Azadi *et al.,* 2000). Wood xylan exists as O-acetyl-4-O-methylglucuronoxylan in hardwoods and as arabino-4-O-methylglucuron- oxylan in softwoods, while xylans in grasses and annual plants are typically arabinoxylans (Kulkarni *et al.,* 1999). The degree of polymerisation in xylans is also variable, with, for example, hardwood and softwood xylans generally consisting of 150–200 and 70–130 β-xylopyranose residues, respectively (Kulkarni *et al.,* 1999).

2.2. Pectins

Pectins constitute an important part of the primary plant cell wall and chemically pectic substances are essentially branched heteropolysaccharides containing between a few hundred and about one thousand building blocks per molecule with a back bone consisting of galacturonic acid residues part of which are methyl-esterified (Alkorta *et al.,* 1997). The most biochemical definition of pectin is a complex heteropolysaccharides composed mainly of D-galacturonic acid residues joined by α-1, 4-linkages that form homogalacturonan chains. This backbone structure ("smooth regions") alternates with branched regions ("hairy regions") which contain rhamnose, arabinane and arabinogalactane as side chains. Galacturonic acid units in both regions are partially methyl-esterified and acetylated (Lang and Dornenburg, 2000). The smooth region or homogalacturonan (HGA) part is a linear homopolymer of α -1,4 -linked – D- galacturonic acid (GalA) and is thought to contain some 100-200 GalA residues (Thibault *et al.,* 1993). HGA is an abundant and widespread domain of pectin and appears to be synthesized in the Golgi apparatus and deposited in the cell wall in a form that has 70-80% of GalA residues which are part methylated and can be acetylated (Mohnen, 1999).

HGA is involvement in calcium-mediated gel formation. The formation of gels is probably and between cell layers and a cross the middle lamella although, in many cases, factors in addition to calcium cross-links are responsible for maintaining the integrity of the pectic network (Jarvis, 1992). The capacity of HGA to participate in gel formation and to contribute to cell wall stiffening is regulated largely by the action of pectin methyl esterases " PMEs" remove methyl ester groups from HGA resulting in stretches of acidic residues that can associated with other HGA chains by calcium cross- links. The de-estrification of HGA appears to be a complex regulated process that does not occur uniformly throughout tissues or cell walls (Liberman *et al.,* 1999). The hairy regions known as rhamnogalacturonan (RG) I and II are characterized by stretches of 1, 2 α - L- rhamnose-1, 4-α-D-galacturonic acid dimmers to the rhamnose residues, L-arabinose and D-galactose can be attached. RG-I is abundant and heterogeneous and generally thought to be glycosidically attached to HGA (Willats *et al.,* 2001). Beside RG-I there RG-II is not structurally related to RG-I but is a branched pectic domain containing an HGA backbone. RG-II is a very minor component of plant cell walls and has an extremely complex structure (Voragen *et al.,* 1995).

2.3. Celluloses

Celluloses are the major organic compounds of cell wall polysaccharides and consists of a linear polymer of β-1,4-linked D-glucose residues. The cellulose polymers are found in plants as microfibrils (Nishiyama, 2009), and their main function is to ensure the rigidity of the plant cell wall. Cellulose is an outstanding commodity due to its abundance and distinctive structural properties. For example, its tension resistance is comparable to that of steel (Eckardt, 2003). However, cellulose is synthesized by cellulose synthase enzymes (CESAs) and is regarded as a major sink for atmospheric carbon in plants because it is the main component of the plant cell wall (Delmer and Haigler, 2002). Many plant cell walls are made up of three layers: middle lamellae, primary cell wall, and secondary cell wall. All the layers present in the cell wall have two phases: microfibrilar and matrix (Brett and Waldron, 1990). The microfibrilar phase, a crystalline phase, is composed of microfibrils of cellulose and the matrix phase, a non-crystalline phase, is composed of a variety of polysaccharides (pectins and hemicelluloses), proteins, and phenolic compounds (lignin, ferulic acid, coumaric acid, and others) (Brett and Waldron, 1990). The content of cellulose, expressed in dry weight, in the primary cell wall of poplars is 20-30%, whereas in the secondary cell wall it is 40-50% (Mellerowicz *et al*., 2001). The cellulose substrate contains various representation of lignin, which influences the resistance of substrate. The structure of cellulose consists of parallel glucan chains and is stabilized by hydrogen bonds (Mackulak *et al.,* 2010). The main structural unit of cellulose is D-glucose (Sulak and Smogrovicova, 2008). Kojima *et al.*(1999) reported that cellulose consists of linear β-1,4-linked D-glucopyranose chains that are condensed

by hydrogen bonds into crystalline structures called microfibrils. These microfibrils consist of up to 250 glucose chains and are linked by hemicelluloses (Carpita and Gibeaut, 1993). Cellulose is a linear homopolymer made up of β(1-4)-linked glucose residues and the UDP-glucose molecule acts as substrate for cellulose biosynthesis. An anhydroglucose, one glucose residue, is a monomer of cellulose. The dimer, two glucose residues β(1-4)-linked, called cellobiose is the structural repetitive unit of the cellulose chain. The degree of polymerization is determined by the number of monomers which compose each cellulose chain (Delmer, 1999). Two different ending groups are found in each cellulose chain edge. At one end of each of the chains, a non-reducing group is present where a closed ring structure is found. A reducing group with both an aliphatic structure and a carbonyl group is found at the other end of the chains. The cellulose chain is thus a polarized molecule. The β-1-4 linkage between glucose residues, in contrast to the α-1-4 linkage as occurs in starch, confers cellulose with unique structural features.

3. Fungal enzymes caused fruit spoilage

Many fruits and vegetables present nearly ideal conditions for the survival and growth of many types of microorganisms. Their structure is comprised mainly of the polysaccharides cellulose, hemicellulose, and pectin. The principal storage polymer is starch. Spoilage microorganisms exploit the host using extracellularlytic enzymes that degrade these polymers to release water and the plant's other intracellular constituents for use as nutrients for their growth. Fungi in particular produce an abundance of extracellular pectinases and hemicellulases that are important factors for fungal spoilage (Miedes and Ester, 2004; Al-Hindi *et al.,* 2011).

Pathogens secrete a remarkable array of polysaccharide degrading enzymes, including exo- and endo-polygalacturonases, pectin methylesterases, pectin lyases and pectate lyases, acetyl esterases, xylanases and a variety of endoglucanases that cleave cellulose, xyloglucan and other glucans (Gordon *et al.,* 2002; Verlent *et al.,* 2004; Raviyan *et al.,* 2005; Netsanet *et al.,* 2009). Extracellular hydrolytic enzymes are produced by plant-pathogenic fungi, so enable them to penetrate and infect the host tissue. These enzymes are collectively called cell wall-degrading enzymes (Kikot *et al.,* 2009). The increasing availability of genome-wide analyses of gene expression, product and function has enabled a survey of the cell wall degrading enzymes involved in the interaction between pathogens and host (Di Pietro, 2003; de Vries, 2005). Most cell wall degrading enzymes exist in large multigene families exhibiting diverse patterns of expression, suggesting functional specialization (Coutinho, 2003). The filamentous *Aspergillus niger* is a saprophytic fungus well known for its production and secretion of a variety of hydrolytic enzymes contributing to its ability to degrade plant polysaccharides such as cellulose, hemicellulose, pectin, starch and inulin (De Vries and Visser, 2001). Generally the enzymes that cause spoilage may be divided into two types; endo-enzymes which exist within the microorganism and exo-enzymes which are released by the microorganism. Pathogens secrete numerous cell wall degrading enzymes to breach the plant cell wall and use it as a source of nutrients.

3.1. Xylanases

The complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes. Endo- 1,4-β-D-xylanases (EC 3.2.1.8) randomly cleave the xylan backbone, β-D-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose while removal of the side groups is catalysed by a-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73) and p-coumaric acid esterases (EC 3.1.1.-) (Elegir *et al.,* 1994; Belancic *et al.,* 1995; Sunna and Antranikian, 1997; Teixeira *et al.,* 2010).

The xylanases have been reported mainly from bacteria (Gilbert and Hazlewood, 1993; Sunna and Antranikian, 1997), fungi (Sunna and Antranikian, 1997; Mohamed and Al-Hindi, 2012), actinomycetes (Beg *et al.,* 2000), and yeast (Liu *et al.,* 1999). There are different types of xylanases varying in substrate specificities, primary sequences, folds and physicochemical properties (Colina *et al.,* 2003). Among microbial sources, filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase activities are much higher than those found in yeasts and bacteria (Sunna and Antranikian, 1997; Krisana *et al.,* 2005). Filamentous fungi are capable of producing high levels of extra cellular enzymes and they can be cultivated very easily (Kar *et al.,* 2006).

3.2. Pectinases

Pectinases are a group of enzymes involved in degradation of pectin, that includes various enzymes classified into various classes and subclasses depending on the substrate specificity and mode of action, for example, methyl deesterases, hydrolases, and lyases. Primarily, these enzymes are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants (Murad and Azzaz, 2011). Pectinase production has been reported from bacteria including actinomycetes (Beg *et al.,* 2000), yeast (Reid and Ricard, 2000) and fungi (Huang and Mahoney, 1999; Mohamed *et al.,* 2003, 2006; Chellapandi, 2010; Al-Najada *et al.,* 2012). According to the cleavage site, pectinases are divided into three groups: (i) hydrolases

consisting of polygalacturonase, PGase (EC 3.2.1.15); (ii) lyase/trans-eliminases comprising pectin lyase, PNL (EC 4.2.2.10), and pectate lyase, PL (EC 4.2.2.2); (iii) pectin esterase, PE (EC 3.1.1.11) (Visser *et al.,* 2004; Yadav *et al.,* 2009). The fungus produces these enzymes to break down the middle lamella in plants and extract nutrients from the plant tissue. Pectin esterase catalyzes the deesterification of methylester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol (Cosgrove 1997). The resulting pectin is then acted upon by PGase and PL (Prade *et al.,* 1999). Production, biochemical characterization, and applications of PNL have been reviewed extensively (Yadav *et al.,* 2009). Pectin esterases are found in plants, plant pathogenic bacteria, and fungi (Jayani et al., 2005), while PGases are widely distributed among fungi, bacteria, and many yeasts (Murad and Azzaz, 2011). They are also found in higher plants and some plant parasitic nematodes (Yadav *et al.,* 2009). Polygalacturonases are important pathogenicity factors for other fungi, such as *Aspergillus flavus*, *Alternaria citri* and *Claviceps purpurea*, and for bacteria such as *Agrobacterium tumefaciens* and *Ralstonia solanacearum* (Di Matteo, 2006). The degradation of pectin chains by PGase loosens the primary cell wall allowing the cellulose-hemicellulose network to become more accessible for digestion and leads to the release of cell wall pectin-derived oligosaccharides, the oligogalacturonides (OGs), which in turn act as elicitors of a variety of plant defenses (Prade *et al.,* 1999). Majority of fungal PGases are directly involved in plant pathogenesis and they are termed as “virulent factors” of fungal pathogens. Pectinases are the first enzymes to be secreted by fungal pathogens when they attack plant cell walls (Idnurm and Howlett, 2001; Tomassini *et al.,* 2009).

3.3. Cellulases

Cellulases are a complex enzyme which consisting of endoglucanase, cellobiose hydrolase and β-glucosidase, hydrolyze the long chains of cellulose resulting in the liberation of cellobiose and finally glucose. The enzyme *β*-glucosidase has many distinct biological roles and exists in all living kingdoms, from simple bacteria to highly complex mammals. For example, in fungi saponin hydrolysing enzymes, such as avenacinases, which are a subset of *β*-glucosidases, have been identified as essential molecular tools for the pathogenicity of phytopathogenic fungi (Bouarab *et al.,* 2002). There are two well studied mechanisms that are used by cellulolytic microorganisms to degrade the cellulose present in plant cell walls. The well studied mechanisms use cellulases to hydrolyze the *β*-1,4 linkages present in cellulose. However, most aerobic microorganisms secrete a set of cellulases outside the cell (free cellulase mechanism) while most anaerobic microorganisms produce large multi enzyme complexes on their outer surface (cellulosomal mechanism) (Wilson, 2009). Many studied aerobic microorganisms use the free cellulase mechanism to digest cellulose (Wilson, 2008; Al-Hindi *et al.,* 2011). Brown rot fungi appear to use a different oxidative mechanism for degrading cellulose (Martinez *et al.,* 2009). Organisms using the free cellulase mechanism, secrete a set of individual cellulases, most of which contain a carbohydratebinding module (CBM) joined by a flexible linker to the catalytic domain although additional domains are present in some enzymes (Wilson, 2004). It is postulated that cellulose is decomposed through the synergistic actions of enzymes such as endo-*β*-l,4-glucanase, exo-*β*-1,4-glucanase and *β*-glucosidase. As to fungi, cellulolytic enzymes have been purified and

4. Fruit spoilage fungi

Fruits contain high levels of sugars and other nutrients, and they possess an ideal water activity for microbial growth; their low pH makes them particularly susceptible to fungal spoilage (Tournas and Katsoudas, 2005). Fruits are usually quite acid in the range pH 1.8- 2.2 (passion fruit, lemons) to 4.5-5.0 (tomatoes, figs) and are quite resistant to invasion by bacteria (Splittstoesser, 1978). Microbial spoilage of fruit and fruit products is usually caused by fungi due to low pH values of fruit and fruit products. On the other hand vegetables are susceptible to bacterial invasion due to vegetables pH values are near neutral. The more acid fruits and vegetables are liable to be spoiled by acidophilic organisms. While a slightly acid medium generally is considered to be optimum for fungi (Smith, 1979). Most molds grow well at ordinary temperature (mesophilic). The optimal temperature for most molds is around 25°C to 30°C, but some grow well at 35°C to 37°C or above, e.g., *Aspergillus* spp., and some grow at higher temperatures (thermophilic). A number of molds grow well at temperatures of refrigeration, (psychrotrophic) and some can grow slowly at temperatures below freezing (psychrophilic) (Frazier and Westhoff, 1988). Fungi are the most diverse organisms in the world. It is estimated that there are approximately 1.5 million species of fungi (Hawksworth, 2001). Molds are fungi that cover surfaces as fluffy mycelia and usually produce masses of asexual, or sometimes sexual, spores.

Molds are overwhelmingly present in postharvest diseases of fruits and vegetables. These pathogens are commonly members of the class *Ascomycetes* and the associated fungi *Imperfecti*. Mold spoilage of especially fresh fruit, is caused by species of *Penicillium, Phytophthora, Alternaria, Botrytis, Fusarium, Cladosporium, Phoma, Trichoderma, Aspergillus, Alternaria, Rhizopus, Aureobasidium, and Colletotrichum*. The symptoms include visible growth,

rots and discoloration, such as blue mold rot, gray mold rot, botrytis rot, and brown rot (Barth *et al*., 2009). Mold populations have been reported in various types of fresh-cut fruits and vegetables (Tournas, 2005). Banana (*Musa sapientum*) fruit peel is an organic waste that is highly rich in carbohydrate content and other basic nutrients that could support microbial growth. However, *Fusarium equiseti* was found to be associated with the ripening of bananas and also caused rot during storage (Odebode and Sanusi, 1996). In addition, Kazempour and Kamran (2005) reported that severe root rot of mulberry trees were associated with *F. solani*, *F. oxysporum*, *L. theobromae*, *R. solani* and *R. necatrix*. Sutthisa *et al.* (2010) found that *F. solani*, *F. oxysporum*, *F. phaseoli*, *F. culmorum*, *F. moniliforme*, *F. graminearum*, *F.scirpi*, *F. anthophilum*, *F. dlamini*, *F. dimerum* and *F. beomiforme* were associated with mulberry root rot disease. Al-Hindi *et al.* (2011) isolated and identified some fruit spoilage fungi and screening their plant cell wall degrading enzymes.

Although, thirty-one fungal apple diseases have been described in Korea. Lee *et al*. (1993) investigated apple and pear diseases from 1988 to 1992, and Uhm (1998) carried out a survey for apple diseases in Gyeongbuk province for 3 years from 1992 to 1994. Among the 17 fruit diseases found in this survey, white rot and bitter rot were major diseases of apple, causing economically important losses in apple-producing areas in Korea. Mango is the worst sufferer based on the percentage loss over the marketable period (Mandal and Dasgupta 1981). The pathogens that caused post harvest spoilage in mango were isolated and their extent of loss on mango was assessed by systematic survey at Coimbatore from field to consumer level. Mango fruits were predominantly affected by *Colletotrichum gloeosporioides* (anthracnose), *Botryodiplodia theobromae* (Stem end rot), mixed infections of both anthracnose and stem end rot, *Aspergillus niger* and *Rhizopus arrhizus* at different stages of marketing and storage (Prabakar *et al*., 2005).

5. Effect of organic acids on fruit spoilage fungi

Benzoic acid has been found to be antifungal agent (Pundir and Jain, 2010). Benzoic acid was found to be most effective against *Aspergillus niger, A. flavus, A. fumigatus* (Doughari *et al.,* 2007) and *A. awamori* and *A. Phoenicis* (Mohamed and Al-Hindi, 2012). Benzoates are used to inhibit the growth of molds, yeasts, and bacteria in acidic food products and antimicrobial such as benzoic acid is generally recognised as safe (GRAS) (Branen *et al.,* 1990). Benzoic acid and sodium benzoate are used primarily as antifungal agents (Eklund, 1985). The inhibitory concentration of benzoic acid against most yeast ranges from 20 to 700 µg/ml, while for molds it is 20 to 2,000 µg/ml (Chipley *et al.,* 2005). *Penicillium spinulosum* are highly resistant to chemical preservatives such as sorbic and benzoic acids, and can tolerate both high acid and lower water activity environments (De Boer *et al.,* 1995).

Citric acid was also found to be inhibitor against *Scopulariopsis* sp. (Doyle *et al.,* 2002) and *Fusarium oxysporum* and *A. tubingensis* (Al-Najada *et al.,* 2012). Evaluation of inhibitory effects of citric acid on the growth inhibition of some important pathogenic fungi *in vitro Trichophyton mentagrophytes* var. *mentagrophytes*, *Candida albicans*, *Aspergillus fumigatus*, and *Malassezia furfur* was studied. The results demonstrated that citric acid had fungistatic and fungicidal activities against all pathogenic fungi tested, and its effect on filamentous fungi was higher than that on yeasts (Shokri, 2011). Benzoic and citric acids were evaluated on the growth of *Geotricum candidum* (sour rot), *Penicillium digitatum* (green mould), and *P. italicum* (blue mould) and complete inhibition was observed in the linear growth of all tested fungi when exposed to benzoic and citric acids at concentrations of 4% and 2%, respectively (El-Mougy *et al.,* 2008).

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