Pectinases

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1. INTRODUCTION

Pectinases are a group of at least seven different enzymatic activities that contribute to the breakdown of pectin from a variety of plants. Pectin is a structural polysaccharide found in primary cell wall and middle lamella of fruits and vegetables. Pectin has a complex structure; the predominant structure consists of homopolymeric partially methylated poly-α-(1,4)-galacturonic acid. Sections of α-(1,2)-L-rhamnosyl- α-(1,4)-D-galacturonosyl containing branch-points with L-arabinose and D-galactose can be incorporated in the main polymeric chain. Pectin may also contain residues of D-glucuronic acid, D-apiose, D-xylose and L-fucose attached to poly- α-(1,4)-D-galacturonic acid sections (Pérez et al. 2003). As other enzymes, pectinases contained in natural ingredients have been used long time ago, e.g. the production of coffee and chocolate, where pectinases produced by wild microorganisms improved the fermentation step to remove grain mucilage. Pectinases are very useful to determine the structural characteristics of pectic substances and to prepare plant cell protoplasts for studies of genetic engineering (Shubakov & El'kina 2002). Due to its ability to degrade cell wall, pectinases have been used in juice and wine processing for the last 70 years. They are extensively used in food industry to increase juice yields, to accelerate juice clarification and to produce juice concentrates from grapes, berries, pears, apples, carrots, beets, green peppers and citrus fruits. Pectinases are also used to increase the colour of juices, promoting antioxidants formation and favour the extraction of colour, flavour components and fermentable sugars when added to grapes of musts during wine production. Removal of the inner wall of lotus seed, garlic, almond and peanut is also carried out by pectinases. Pectinase world-wide consumption is above 7 x 10^6 tons per year.

2. SOURCE AND TYPE OF PECTINASES

Although plants and microorganisms produce them, the most common source of commercial pectinases is the filamentous fungus *Aspergillus* sp. that produces a complex of pectinolytic enzymes, including the de-esterifying and chain-splitting enzymes. They are also obtained from tomatoes and oranges.

According to the reaction mechanisms, pectinases splitting homopolymeric partially methylated poly-α-(1,4) galacturonic acid (homogalacturonan) can be classified as: i) esterases, ii) hydrolytic depolymerases, and iii) eliminative depolymerases (Table 1). Pectinases degrading hairy regions (rhamnogalacturonan I) are not described in this chapter, but they have been described by Voragen et al. (2003) and include rhamnogalacturonan-hydrolases and lyases.

A. Pandey et al. (Eds.), *Enzyme Technology*

Table 1. Classification of pectinases from enzyme nomenclature

Name	EC number	Reaction type
Pectinesterase	3.1.1.11	Hydrolysis of carboxylic ester
Polygalacturonase	3.2.1.15	Hydrolysis of *O*-glycosyl bond
Galacturan 1,4-α-galacturonidase	3.2.1.67	Hydrolysis of *O*-glycosyl bond
Exopoly-α-galacturonosidase	3.2.1.82	Hydrolysis of *O*-glycosyl bond
Endo-pectatelyase	4.2.2.2	Elimination (C-O bond cleavage)
Exo-pectatelyase	4.2.2.9	Elimination (C-O bond cleavage)
Endo-Pectinlyase	4.2.2.10	Elimination (C-O bond cleavage)

2.1. Pectinesterase

Pectinesterase (E.C. 3.1.1.11) is an enzyme that catalyses the hydrolysis of methylated carboxylic ester groups in pectin into pectic acid and methanol. It is used in the treatment of certain foodstuffs and can be produced by a wide variety of plants and micro-organisms. Table 2 presents some of the micro-organisms producing pectinesterase.

Although pectin is the natural substrate of pectin esterase, methyl pectate (Versteeg 1979) and methylated oligogalacturonides (Shevchik et al. 1996) have also been used as substrates. Pectinesterase activity is stimulated by $(NH_4)_2SO_4$ (Versteeg 1979), Mg^{2+}, NaCl (Lim et al. 1983) and inhibited by Cu^{2+}, Hg^{2+} (Lim et al. 1983), D-galacturonate, polygalacturonate (Pitkänen. et al. 1992) and pectate (Markovic et al. 1985). Studies carried out on pectinesterase immobilisation revealed a 5-fold increase in K_m value after immobilisation (Markovic et al. 1985). The immobilised enzyme did not act on pectin with a high esterification degree and presents lower activity than the free enzyme. Table 3 presents the optimal values of pH and temperature of pectinesterases produced by different microorganisms. Plant pectinesterases release a methoxyl group adjacent to a free galacturonic acid and slide along the homogalacturonan to produce pectins with blocks of free carboxyl groups, whilst fungal pectinesterases saponify methyl esters in more or less random fashion (Ralet et al. 2002).

2.2. Polygalacturonase

Endo-polygalacturonases (E.C. 3.2.1.15) are important enzymes in fruit ripening and in fungal and bacterial attack on plants and are used in treatment of certain vegetables such as tubers, apples, etc. The enzymatic reaction involves random hydrolysis of *O*-glycosyl bonds in 1,4-α-D-galactosyluronic linkages in homogalacturonans. Most of the studies dealing with endo-polygalacturonase characteristics have been carried out with strains of *Aspergillus*, (particularly, *A. niger*), *Erwinia carotovora* and *Saccharomyces fragilis*. However, several plant pathogenic and saprophytic fungi and bacteria have also been studied. Table 4 lists some of the microorganisms reported as endo-polygalacturonase producers.

Homogalacturonan is the natural substrate of endo-polygalacturonases. However, a wide variety of oligogalacturonides are used as substrates, producing several products, depending on the nature of substrate. For example, molecules containing two or more monomers of galacturonate act as substrate for this enzyme. Soluble (homogalacturonan and methylated homogalacturonan) and insoluble (protopectin) substrates can be used as inducers for microbial endo-polygalacturonases. Hydrolysis of α-D-

Table 2. Microorganisms producing pectinesterases

Microorganism	References	Microorganism	References
Aspergillus carbonarius	Versteeg 1979	*Corynebacterium sp.*	Versteeg 1979
Aspergillus foetidus	Markovic & Machova 1985	*Erwinia chrysanthemi*	Heikinheimo et al. 1991, Pitkänen et al. 1992, Shevchik et al. 1996
Aspergillus japonicus	Versteeg 1979	*Fusarium oxysporum*	Versteeg 1979
Aspergillus kawachii	Contreras-Esquivel 2003	*Fusarium roseum*	Versteeg 1979
Aspergillus niger	Versteeg 1979		
	Lobarzewski et al. 1985	*Gibberella* sp	Versteeg 1979
Aspergillus oryzae	Ueda et al. 1982		
	Lim et al. 1983		
	Kitamoto et al. 1999	*Kluyveromyces fragilis*	Versteeg 1979
Aureobasidium pullulans	Manachini et al. 1988	*Penicillium chrysogenum*	Versteeg 1979
Botryosphaeria ribis	Versteeg 1979	*Pseudomonas solanacearum*	Versteeg 1979
Botrytis cinerea	Versteeg 1979	*Saccharomyces cerevisiae*	Gainvors et al. 1994
Chaetomium globosum	Versteeg 1979	*Torulopsis candida*	Versteeg 1979
Clostridium multifermentans	Versteeg 1979	*Trichoderma lignorum*	Versteeg 1979
Colletotrichum trifolii	Versteeg 1979	*Trichoderma reesei*	Markovic et al. 1985
Corticium rolfsii	Yoshihara et al. 1977	*Xanthomonas malvacearum*	Versteeg 1979

Table 3. Optimal values of pH and temperature for activity of microbial pectinesterases

Micro-organism	pH	Temperature (°C)	References
Aspergillus foetidus	4.8	52	Markovic et al. 1985
Aspergillus oryzae	8.2	50	Lim et al. 1983
Aureobasidium pullulans	4.5	50	Manachini et al. 1988
Erwinia chrysanthemi	7.5	40	Shevchik et al. 1996

galacturonosyl-(1,4)-*O*- α-D-galacturonate has been reported for the endo-polygalacturonases produced by *Botrytis cinerea* and *Aspergillus niger* (Deuel & Stutz 1958). Endo-polygalacturonase activity produced by *Rhizopus stolonifer* was stimulated by Co^{2+}, Fe^{3+}, Mg^{2+} (Manachini et al. 1987) and was inhibited by Ba^{2+}, Hg^{2+}, Mn^{2+} and Zn^{2+} (Manachini et al. 1987, Trescott & Tampion 1974). Other endo-polygalacturonases were also inhibited by Ag^{+}, $Ba^{2+}$and Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+} and Pb^{2+} (Sakai et al. 1982, 1984, Lim et al. 1980). Values of K_m of endo-polygalacturonases decrease as the number of monomers into the galacturonide oligomer increases. Endo-polygalacturonases produced by some bacteria, yeast and fungi has been in the range of $0.01mM<K_m<12.5mM$. The physiological significance of this variability is not yet understood.

Table 4. Microorganisms producing endo-polygalacturonases

Microorganism	References	Microorganism	References
Aspergillus foetidus	Deuel & Stutz 1958	*Galactomyces reesei*	Sakai & Yoshitake 1984
Aspergillus japonicus	Schejter & Marcus 1988 Ishii & Yokotsuka 1972	*Kluyveromyces marxianus*	Sakai et al. 1984
Agrobacterium vitis	Herlache et al. 1997	*Mucor pusillus*	Foda et al. 1984
Aspergillus aculeatus	Foda et al. 1984	*Neurospora crassa*	Deuel & Stutz 1958
Aspergillus alleaceus	Sreenath et al. 1986	*Penicillium capsulatum*	Gillespie & Coughlan 1989 Gillespie et al. 1990
Aspergillus aureus	Deuel & Stutz 1958	*Penicillium expansum*	Deuel & Stutz 1958
Aspergillus kawachii	Contreras-Esquivel 2003	*Penicillium frequentans*	De Fatima-Borin et al. 1996
Aspergillus niger	Cooke et al. 1976 Heinrichova & Rexová-Benková 1977 Heinrichova & Dzurova 1981 Raab 1992 van Santen et al. 1999 Parenicova et al. 2000	*Pseudomonas solanacearum*	Ofuya 1984
Aureobasidium pullulans	Sakai & Takoka 1985	*Rhizopus arrhizus*	Schejter & Marcus 1988
Bacillus mesentericus	Deuel & Stutz 1958	*Rhizopus stolonifer*	Manachini et al. 1987 Trescott & Tampion 1974
Bacillus sp.	Horikoshi 1972	*Rhizopus tritici*	Deuel & Stutz 1958
Botrytis cinerea	Deuel & Stutz 1958	*Saccharomyces cerevisiae*	Blanco et al. 1994 Blanco et al. 1998 Hirose et al. 1999 Gainvors et al. 2000
Clostridium felsineum	Deuel & Stutz 1958	*Saccharomyces fragilis*	Lim et al 1980
Corticium rolfsii	Tagawa and Kaji 1988	*Sclerotinia sclerotiorum*	Oliva et al. 1999 Fraissinet-Tachet et al. 1995
Erwinia carotovora	Ried & Collmer 1986 Saarilahti et al. 1990 Lei et al. 1992 Herlache et al. 1997 Palomaeki & Saarilahti 1997 Pickersgill et al. 1998 Pickersgill et al. 1999	*Trichoderma koningii*	Schejter & Marcus 1988 Fanelli et al. 1978 Tagawa & Kaji 1988
Erwinia chrysanthemi	Collmer et al. 1988 Ried & Collmer 1986	*Trichoderma reesei*	Markovic et al. 1985
Fusarium monoliforme	Deuel & Stutz 1958 Caprari et al. 1996	*Verticillium albo-atrum*	Schejter & Marcus 1988
Fusarium oxysporum	Strand et al. 1976 di Pietro & Roncero 1996	*Verticillium dahliae*	Deuel & Stutz 1958

Table 5. K_m values of some endopolygalacturonases.

Microorganism	Substrate	K_m (mM)	References
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	3.67	Heinrichova & Dzurova 1981
Galactomyces reessii	(1,4-α-D-galacturonaide)$_2$	3.98	Sakai & Yoshitake 1984
Trichosporun penicillatum	(1,4-α-D-galacturonaide)$_2$	4.26	Sakai et al 1982
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	12.5	Heinrichova & Rexova-Benkova 1977
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	1.82	Heinrichova & Dzurova 1981
Trichosporun penicillatum	(1,4-α-D-galacturonaide)$_2$	2.2	Sakai et al 1982
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	3.31	Heinrichova & Rexova-Benkova 1977
Galactomyces reessii	(1,4-α-D-galacturonaide)$_2$	0.71	Sakai & Yoshitake 1984
Trichosporun penicillatum	(1,4-α-D-galacturonaide)$_2$	0.87	Sakai et at 1982
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	0.89	Heinrichova & Dzurova 1981
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	1.85	Heinrichova & Rexova-Benkova 1977
Aspergillus niger	(1,4-α-D-galacturonaide)$_2$	0.7	Heinrichova & Dzurova 1981
Aspergillus niger	Pectate	0.01	Heinrichova & Rexona-Benkova 1977
Trichosporun penicillatum	Polygalacturonic acid	0.04	Sakai et al 1982
Aspergillus niger	Polygalacturonic acid	0.34	Heinrichova & Dzurova 1981

Depending on the type of microorganisms, endo-polygalacturonases have a wide range of optimal pH and temperature values (Table 6). Such variability may be useful for special conditions to be applied at industrial operations and may be the justification for looking at biodiversity as a source of special strains for such industrial applications.

Table 6. Optimal values of pH and temperature and molecular weight for endopolygalacturonases produced by some microorganisms

Microorganism	pH	Temperature (°C)	Molecular weight (kDa)	References
Aspergillus aculeatus	3.0	60		Foda et al. 1984
Aspergillus niger	5.0	40	46	Heinrichova & Rexová-Benková 1977
Clostridium thermosulfurogenes	5.5	75	320	Schink & Zeikus 1983
Erwinia carotovora	5.5	37		Lei et al. 1992 Saarilahti et al. 1990
Kluyveromyces marxianus	5.0	60	44	Hirose et al. 1999
Mucor pusillus	4.5	50		Foda et al. 1984
Penicillium capsulatum	4.7	52	51.6	Gillespie & Coughlan 1989 Gillespie et al. 1990
Saccharomyces cerevisiae	6.0	45	45	Hirose et al. 1999

Table 7. Optimal values of pH and temperature for activity of exo-polygalacturonases produced by some microorganisms

Microorganism	pH	Temperature (°C)	References
Fusarium oxysporum	5.6	60	Vásquez et al. 1993
Aspergillus niger	5.2	45	Heinrichova & Rexová-Benková 1976
Geotrichum lactis	5.0	40	Pardo et al. 1991 Pardo & Gacto 1992
Fusarium oxysporum	5.0	40	Martínez et al. 1991

Table 8. Bacteria and fungi producing endo-pectatelyase

Microorganism	References
Bacteria	
Bacillus subtilis	Nasser et al. 1990 Nasser et al. 1993 Sakamoto et al. 1994
Bacteroides thetaiotaomicron	McCarthy et al. 1985
Erwinia carotovora	Sugiura et al. 1984 Ried & Collmer 1986 Lei et al. 1987 Yoshida et al. 1991 McMillan et al. 1992 Heikinheimo et al. 1995
Pseudomonas solanacearum	Ofuya 1984
Streptomyces nitrosporeus	Sato & Kaji 1977,1980
Thermoanaerobacter italicus	Kozianowsky et al. 1997
Thermomonospora fusca	Stutzenberger 1987
Xanthomonas campestris	Nasuno & Starr 1967
Fungi	
Fusarium oxysporum	Huertas-González et al. 1999
Fusarium solani	Crawford & Kolattukudy 1987 Guo et al. 1995a,b Guo et al. 1996
Penicillium oxalicum	Ikotun 1984

2.3. Exo-polygalacturonases

Galacturan 1,4-α-galacturonidases (E.C. 3.2.1.67) are enzymes that degrade polygalacturonan by hydrolysis of the glycosidic bonds from the non-reducing ends yielding the corresponding 1,4- α-D-galacturonide and galacturonic acid. They are produced by a wide variety of plants and microorganisms. Strains of fungi such as *Aspergillus*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Penicillium* and *Trichoderma* have been used for biochemical characterisation of these enzymes. They are also produced by some

bacterial strains of the genus *Bacillus* and *Butyrivibrio*. Although the natural substrates of exo-polygalacturonases are pectic acid and pectin, these enzymes have a preference for oligogalacturonates and present activity with some arabinogalactans. The exo-polygalacturonase was inhibited by Ca^{2+} (Vásquez et al. 1993), glucose (Pardo et al. 1991, Pardo & Gacto 1992) and D-galactopyranuronic acid (Kester et al. 1996, 1999). When (1,4)- α-D-(galacturonide)$_n$ was used as substrate with n = 2 to 7, the exo-polygalacturonase activity of *Aspergillus tubingensis* showed preference for the high molecular weight molecules (K_m = 1.44 and 0.32 mM for n = 2 and n = 7, respectively). The optimum pH for activity of these enzymes is below 7 and has been 3.0 as minimum for polygalacturonase produced by the plant pathogen fungal *Colletotrichum capsici*. The specific activity of the exo-polygalacturonase produced by this fungus was near to 25 times higher than that produced by *Aspergillus niger* (Behere et al. 1993). Table 7 presents the optimal values of pH and temperature of exo-polygalacturonases produced by different microorganisms.

2.4. Exo-poly-α-galacturonosidases

Exo-polygalacturonosidases (E.C. 3.2.1.82) hydrolyse the pectic acid from the non-reducing end, releasing digalacturonates. These enzymes are produced by bacterial strains such as *Clostridium thermosaccharolyticum* (van Rijssel et al. 1993), *Erwinia carotovora* (Kegoya et al. 1984), *E. chrysanthemi* (Ried & Collmer, 1985), *Pseudomonas sp.* (Hatanaka & Imamura, 1974), *Ralstonia solanacearum* (Huang & Allen, 1997) and *Selenomonas ruminantum* (Heinrichova et al. 1989). Their natural substrate is pectin and has K_m values as high as 1159 mM measured with trigalacturonate for the enzyme produced by *Selenomonas ruminantum* (Heinrichova et al. 1989).

2.5. Endo-pectatelyases

Endo-pectatelyases (EC 4.2.2.2) do eliminative cleavages of pectate at the C-O bonds to give oligosaccharides with 4-deoxy- α-D-gluc-4-enuronosyl groups at their non-reducing ends resulting in formation of products with a double bond between C-4 and C-5. A wide variety of bacteria and some groups of plants and phytopathogenic fungi produce these enzymes. The enzyme production was constitutive in *Bacillus* sp. and *Fusarium solani*, but was induced by pectin or pectic acid in *E. anoideae* (Kamimiya et al. 1977). Its production by *B. subtilis* was repressed by glucose (Sakamoto et al. 1994). Table 8 presents some of the bacteria and fungi that produce this enzymatic activity.

Table 9. Molecular weight, pH and temperature optimal values for endo-pectatelyases produced by some microorganisms

Microorganism	K_m(mM)	pH	Temperature (°C)	References
Bacillus macerans	45000	9.0	60	Kamimiya et al. 1977
Bacillus subtilis	32000	8.0	60	Sakamoto et al. 1994
Colletotrichum gloesporioides	39000	8.9	35	Wattad et al. 1994
Erwinia anoideae	36000	9.0	35	Kamimiya et al. 1977
Streptomyces nitrosporeus	39000	9.3	5	Sato & Kaji 1977
Thermoanaerobacter itallicus	148000	9.0	80	Kozianowsky et al. 1997

Table 10. Microorganisms producingendo-pectinlyases

Micro-organism	References	Micro-organism	References
Bacteria		*Aspergillus niger*	Mayans et al. 1997 Kester & Visser 1994 Hanisch et al. 1978
Bacillus sp.	Jong-Chon et al. 1998	*Aspergillus oryzae*	Lim et al. 1983
Erwinia aroideae	Kamimiya et al. 1974	*Aspergillus sojae*	Ishii & Yokotsuka 1972
Erwinia carotovora	Itoh et al. 1982	*Colletotrichum lindemuthianum*	Wijesundera et al. 1984
Lachnospira multipara	Silley 1986	*Fusarium oxysporum*	Guevara et al. 1996
Pseudomonas fluorescens	Schlemmer et al. 1987	*Penicillium expansum*	Silva et al. 1993
Pseudomonas marginalis	Sone et al. 1988 Nikaidou et al. 1995	*Penicillium italicum*	Alana et al. 1991
Fungi		*Penicillium paxilli*	Szajer & Szajer 1982
Aspergillus japonicus	Ishii & Yokotsuka 1972 Dinnella et al. 1995	*Rhizoctonia solani*	Bugbee 1990

Oligogalacturonates, pectate, pectic acid, polypectate, polygalacturonic acid and pectin are used as substrates of endo-pectatelyase. The endo-pectatelyase activity required Ca^{2+} and was stimulated by Mg^{2+} (Sato & Kaji 1977, Brühlmann 1995), Mn^{2+} (Kobayashi et al. 1999, Miyazaki 1991, Guo et al. 1995a,b) and Sr^{2+} (Kobayashi et al. 1999, Brühlmann 1995); it was inhibited by several metal ions such as Mn^{2+}, Mg^{2+}, Cu^{2+} (Sugiura et al. 1984), Ba^{2+}, Cd^{2+} (Kamimiya et al. 1977, Sakamoto et al. 1994) and Ca^{2+} (Tardy et al. 1997). The K_m values of the enzyme produced by *Streptomyces nitrosporeus* for penta, tetra and trigalacturonic acids were 0.23, 0.19 and 1.5 mM, respectively (Sato & Kaji 1977). Endo-pectatelyase has maximal activities at alkaline pH values. Table 9 shows the molecular weight and the optimal pH and temperature values for endo-pectatelyase activities produced by different microorganisms.

2.6. Exo-pectatelyases

Exo-pectatelyases (E.C.4.2.2.9) do eliminative cleavages at the C-O bonds of 4-(4-deoxy- α-D-galact-4-enuronosyl)-D-galacturonate at the reducing end of unesterified pectin. These enzymes are produced by *Clostridium multifermentans* (Macmillan & Phaff 1966, Miller & Macmillan 1970, Macmillan & Vaughn 1964), *Erwinia chrysanthemi* (Shevchik et al. 1998, 1999a,b), *E. carotovora* (Ikeda et al. 1984, Kegoya et al. 1984), *Streptomyces nitrosporeus* (Sato & Kaji 1979) and *S. massasporeus* (Sato & Kaji 1980). Low molecular weight galacturonates are better substrate than polygalacturonate (Shevchik et al. 1999a). The exo-pectatelyase produced by *C. multifermentans* was inactive with pectin of high methoxyl content (Macmillan & Vaughn 1964). Production of exo-pectatelyase by *S. massasporeus* required Ca^{2+} and was stimulated by Mn^{2+} and Sr^{2+} (Sato & Kaji 1980). Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and Sr^{2+} stimulated exo-pectatelyase from *C. multifermentans* (Macmillan & Phaff 1966). Mn^{+2} also stimulated exo-pectatelyase production by *E. chrysanthemi* (Shevchik et al. 1999a). Ba^{2+}, Cu^{2+}, Hg^{2+} and Mg^{2+} inhibited the enzyme activity (Hatanaka & Ozawa 1972, 1973, Ikeda et al. 1984). The exo-pectatelyase produced by *E.*

Table 11. Effect of metal ions on pectinlyase activity

Microorganism	Stimulated by	Inhibited by	References
Aspergillus japonicus	Ca^{2+}		Ishii & Yokotsuka 1975
Aspergillus niger	Ca^{2+},Na^{+}		Kester & Visser 1994
Aspergillus oryzae	Ca^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}		Lim et al. 1983
Aspergillus sojae	Ca^{2+}, Co^{2+}, K^{+},	Hg^{2+} Mg^{2+}, Mn^{2+}, Na^{+}	Ishii & Yokotsuka1972
Bacillus sp.	Ca^{2+}	Co^{2+}, Fe^{2+},Hg^{2+}, Mn^{2+}, Zn^{2+}	Jong-Chon et al. 1998
Colletotrichum lindemuthianum	Ca^{2+}		Wijesundera et al. 1984
Erwinia areideae	Ba^{2+},Ca^{2+},Co^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}		Kamimiya et al. 1974
Fusarium oxysporum	Ca^{2+}		Guevara et al. 1996
Penicillium expansum		Ca^{2+}	Silva et al. 1993

Table 12. Characteristics of pectinlyases produced by different micro-organisms

Microorganism	pH	Temperature (°C)	Molecular weight (kDa)	References
Aspergillus japonicus	6.0	55	32	Ishii & Yokotsuka 1975
Aspergillus oryzae	8.5	5	34	Lim et al. 1983
Aspergillus sojae	7.0	-	32	Ishii & Yokotsuka 1972
Bacillus sp.	6.0	40	52	Jong-Chong et al. 1998
Colletotrichum lindemuthianum	9.3	-	27	Wijesundera et al. 1984
Erwinia aroideae	8.0	40	28	Kamimiya et al. 1974
Penicillium expansum	7.0	4	36.5	Silva et al. 1993
Pseudomonas marginalis	8.5	-	32	Sone et al. 1988

carotovora had K_m value as low as 1.3 μM with polygalacturonate (Ikeda et al. 1984). The pH and temperature optimal values for these enzymes are from 8.5 to 9.5 and 40 to 45°C, respectively. Molecular weights of 39 kDa and 54 kDa were found by gel filtration for the exo-pectatelyases produced by *S. nitrosporeus* and *S. massasporeus*, respectively (Sato & Kaji 1979, 1980).

2.7. Endo-pectinlyases

Endo-pectinlyases (E.C. 4.2.2.10) or pectinlyases give oligosaccharides with terminal 4-deoxy-6-methyl-α-D-galact-4-enuronosyl groups by eliminative cleavage of pectin. They are produced by some bacterial

and fungal strains (Table 10). Endo-pectinlyases produced by *A. japonicus* (Ishii & Yokotsuka 1975, Dinnella et al. 1995) and *A. niger* (Kester & Visser 1994, Mayans et al. 1997) have been studied and characterised.

Pectin, polygalacturonic acid, methoxylated pectic acid and polymethylpolygalacturonate methyl glycoside are used as substrate by the pectinlyases. The enzyme produced by *A. niger* was stimulated by Ca^{2+} and Na^{+} (Kester & Visser 1994) and was inhibited by acetate, formate, propionate, iso-butyrate and butyrate. The soluble enzyme had a pH optima of 5.2 (Hanisch et al. 1978) and a molecular weight around 35.5 kDa. Table 11 presents the stimulatory and inhibitory effect of some metal ions on pectinlyases produced by several microorganisms.

K_m values for the different pectinlyases have been generally estimated with citrus pectin as substrate, obtaining values of 3.2 mg/mL for *Pseudomonas fluorescens* (Schlemmer et al. 1987) and 15.0 mg/mL for *Penicillium italicum*. When pectin was used as substrate, K_m values of 1.36, 2.5 and 9.0 mg/mL were obtained with pectin lyases from *A. oryzae* (Lim et al. 1983), *Penicillium paxilli* (Szajer & Szajer 1982) and *P. expansum* (Silva et al. 1993), respectively. Optimal pH values from 5 to 9.8 have been reported. Table 12 presents some of the characteristics of pectinlyases produced by different microorganisms.

3. PRODUCTION OF PECTINASES

Pectinases are produced by a few microorganisms and strains of *A. niger*, *A. oryzae* and *A. aculeatus* are mainly used. Other species such as *Penicillium expansum* are also used for pectinase production at industrial scale. Pectinase production at large scale is carried out mainly by submerged culture (deep-tank process), although solid-state fermentation (SSF) is also used.

3.1 Submerged fermentation

Industrial fermentors for submerged fermentation (SmF) have volumes from 20 to several hundred cubic meters. Since most of the microorganisms used for pectinases production are aerobic, air must be supplied at rates from 0.1 to 2.0 vvm. Oxygen transfer from the gas phase to the liquid phase is enhanced by mechanical or airlift agitation of the culture medium and agitation helps to maintain homogeneous conditions of pH, temperature and dissolved oxygen in the broth. Pectinases production by a mutant strain of *A. niger* growing in a culture medium with sugar beet slices supplemented with malt extract and mineral salts was strongly influenced by the oxygen uptake rate (Zetelaki-Horvath & Vas 1981). While growth was stimulated at an oxygen uptake rate (OUR) of 100 mmol/Lh, pectinesterase, endo-polygalacturonase and pectinlyases production was stimulated at OUR values of 13, 49 and 60 mmol/Lh, respectively. However, the macerating activity, due to pectinesterase and endo-polygalacturonase activities, gave two maximal values at 12 and 14 mmol/Lh. On the other hand, some yeast strains such as *Kluyveromyces marxianus* produced nine isoenzymes forms of endo-polygalacturonases growing under anaerobic conditions with glucose as sole carbon source (Harsa et al. 1993). Most of the pectinases are inducible enzymes that require the presence of the inducer to be synthesised. Although pectin is the natural inducer for pectinases production, its elevated cost makes difficult its use at industrial level. A number of agricultural products containing pectin and other polysaccharides have been used for pectinase production. Depending on the raw material used as source of inducer and carbon, the culture

medium needs to be supplemented with minerals to improve the microbial growth and enzymes production. Ammonium sulphate is widely used as nitrogen source. Macro elements such as P, K, Mg are generally supplemented as KH_2PO_4 and $MgSO_4$ and micro elements such as Mo, Zn, Fe, Mn and Co are added as mineral salts at low concentrations (below 0.05%, w/w). A comparative study of polygalacturonases production by *A. niger* and *P. dierckxii* from different pectin sources showed that sugar beet pectin (at 10 g/L) was the most active inducer and ammonium sulphate the best source of nitrogen for polygalacturonase production by both the strains (Shubakov & El´kina 2002). The use of a mixture of sugar beet pulp and alkaline extracted sugar beet pulp instead of sugar beet pulp alone slightly increased the polygalacturonase production by *Trichoderma reesei* (Olsson et al. 2003).

As stated above although pectinases production by fungi has been mostly inducible, a constitutive exo-pectinase was produced by *Aspergillus* sp. CH-Y-1043 grown on glucose, sucrose, fructose, glycerol and galacturonic acid (Aguilar & Huitrón 1990). Pectinases production by *Aspergillus* sp. (Solís et al. 1990, Aguilar & Huitrón 1987) and *Neurospora crassa* (Polizeli et al. 1991) was induced by pectin and repressed by glucose and by the degradation products of pectin. In contrast, *Erwinia carotovora* (Tsumuyu, 1977) produced a pectatelyase that was induced by the breakdown products of pectic acid. The complexities of the regulatory mechanisms involved on pectinase production require permanent programs for the selection of catabolic resistant strains. However, technological aspects such as fed-batch cultures or solid- state fermentation can be used to minimise the catabolic repression by glucose or by the breakdown products of pectin (Solís-Pereira et al. 1993, Díaz-Godínez et al. 2001).

3.2. Solid-state fermentation

Solid-state fermentation (SSF) used for the production of polygalacturonases by *A. niger* with sugar cane bagasse as solid support showed that endo-polygalacturonase and exo-polygalacturonase productivities were 18.8 and 4.5 higher in SSF than in SmF (Solís-Pereira et al. 1993). Apparently, the regulatory phenomena such as induction-repression related to pectinases synthesis by *A. niger* are different in the two types of fermentation (Solís-Pereira et al. 1993). The use of sugar cane bagasse as a sole carbon source allowed higher production (6-fold) of pectinesterase and polygalacturonase by *A. niger* in SSF as compared with that obtained in submerged fermentation. Moreover, glucose addition improved pectinase production in SSF but it was decreased in submerged fermentation (Maldonado & Strasser 1998). Similar results were obtained with polyurethane (as inert support) and a culture medium containing pectin as carbon source, showing that protease production is lower in SSF than in SmF (Díaz-Godínez et al. 2001). However, Morita and Fujio (1999) compared specific polygalacturonase activities from *Rhizopus* sp. MKU 18 of a metal-ion-regulated liquid medium and a wheat bran solid medium. Their work suggested that some advantages can be found in producing polygalacturonase by metal-ion-regulated liquid medium. The use of washed sugar cane bagasse as support, impregnated with a defined culture medium showed that pectinases produced by SSF were more stable at pH and temperature than those produced by SmF (Acuña-Argüelles et al. 1995). Although pectinases production at low water activity (a_w) values was lower than that obtained at high a_w values, the specific activity increased up to 4.5 times in SSF (Acuña-Argüelles et al. 1994). Polygalacturonase production by thermophilic *Thermoascus aurantiacus* by SSF using a mixture of sugar cane bagasse and orange bagasse (1:1) at different

culture conditions (pH, substrate moisture and temperature) was highest at pH 5, substrate moisture 70% and 50°C (Martins et al. 2003).

4. PECTINASE ASSAYS

4.1. Pectinesterases

Pectinesterase activity can be estimated by automatic or manual titration of the H^+ produced by the reaction in a solution containing the enzyme and its substrate. To 10 mL of 0.5% (w/v) pectin in 0.1 M NaCl, 2 mL of the enzyme solution is added. The pH is adjusted to 4.5 with 0.1 M NaOH and the mixture is incubated for 65 min at 35 °C (Maldonado & Strasser de Saad 1998). Pectinesterase activity is measured by determining the carboxyl groups released by titration with 0.02 N NaOH. Pectinesterese activity from plant sources is usually determined at pH 7.5 (Rexová-Benková & Slezárik 1966), but fungal pectinesterases are assayed at pH 4.5. The activity is expressed as the number of milliequivalents of methyl ester groups cleaved by enzyme per min. Pectins with degrees of esterification above 65% have been used to determine pectinesterase activity. Pectinesterase activity can be determined in a pH-stat at pH 4.0 to 7.7 (Whitaker 1984). Free methanol can be also measured by gas chromatography and used to assay the enzyme activity (Versteeg 1979). Pectinesterase activity can also be estimated spectrophotometrically by monitoring the colour changes of the indicator dye added to the reaction mixture (Vilariño et al. 1993).

4.2. Hydrolytic depolymerases

Enzymatic activities such as endo- and exo-polygalacturonase and endo and exo-polymethylgalacturonases are usually assayed by the release of reducing groups or by the reduction of viscosity of a solution containing pectin (Solís et al. 1990), sodium pectate (Aguilar and Huitrón 1986) or polygalacturonic acid (Manachini et al. 1988) as substrate. The endo-PG activity of the filtrate is determined by adding 2 ml of filtrate to 8 ml of a 1% (w/v) apple pectin solution and measuring the viscosity in a viscometer. One endo-polygalacturonase activity unit (U) is defined as the quantity of enzyme, which caused a reduction in viscosity of 50% in 30 min of reaction under standard conditions (Dartora et al. 2002). To measure the release of reducing sugars, 0.3 mL of the enzymatic solution is added to a solution containing 1 mL of 0.9% of substrate and 0.7 mL of buffer; samples are incubated at 45°C for 30 min and the reducing groups determined by a colorimetric method (see below). One exo polygalacturonase activity unit is defined as the amount of enzyme that liberates 1 mmol of reducing group per minute (Solís-Pereira et al. 1993). The reaction mixture for the hydrolytic depolymerising enzymes contains from 0.2% (Miyairi et al. 1985) to 1.0% (Kumar and Lonsane 1988) of substrate and are carried out at temperatures from 30°C (Miyairi et al. 1985) to 50°C (Zetelaki 1976) and pH values from 3.8 (Zetelaki 1976) to 5.5 (Manachini et al. 1988). Different sources of pectin can be used as substrate for determination of these enzymatic activities. The Miller (1959) and Somogyi-Nelson (Somogyi 1952) reagents are used to measure released reducing groups. The exo-poly-α-D-galacturonosidase activity is assayed in a reaction mixture containing 0.5% D-galacturonan at pH 6.0 (Collmer et al. 1982). In order to inhibit eliminative activities, 2mM EDTA is added to the reaction mixture. The amount of released reducing groups is estimated by the arsenomolybdate method (Nelson 1944). The thiobarbituric acid assay (Ayers et al. 1966) is used to check if the pectolytic activity was due to transelimination action and/or to hydrolase activity. The occurrence of peaks at 510 and 550 nm is indicative of the presence of hydrolase and lyase activities respectively (Manachini et al. 1988).

4.3. Eliminative depolymerases

Pectinlyase and pectatelyase are assayed spectrophotometrically by monitoring the increase in absorbance at 235 nm of a solution containing from 0.25% to 1% of substrate. The reaction mixture containing 1mL of substrate and 0.5 mL of enzyme solutions is incubated at 30°C for 60 min. The reaction is stopped by adding 3.5 mL of 0.5 M HCl. One unit of eliminative depolymerase activity is defined as the amount of enzyme that releases 1 μmol of 4,5 unsaturated digalacturonic acid per minute. A molar extintion coefficient of 5550 M^{-1} cm^{-1} is used for this calculation (Manachini et al. 1988). The enzymatic reaction is carried out at pH values from 5.5 (Manachini et al. 1988) to 9.0 (Durrands & Cooper 1988) and temperatures from 30°C to 35°C. Since calcium is an activator of these enzymatic activities, 10^{-4} M $CaCl_2$ is added to the reaction mixture (Tsuyumu 1979). Before the spectrophotometrical determination, excess of the substrate and the enzyme can be precipitated, adding successively 0.6mL of a 9% solution of $ZnSO_4$, 9mL of H_2O and 0.6mL of a 0.5N solution of NaOH. The mixture is shaken vigorously and centrifuged for 15 min at 8000 rpm, then subjected to photometry at 235 nm. (Bravova et al. 1982). For pectatelyase determination, pectin with a low methoxyl content or polygalacturonate can be used as substrate, whilst high methylated pectin is used for pectinlyase determination. Molar extinction coefficients of 4600 M^{-1} cm^{-1} and 5500 M^{-1} cm^{-1} are used to calculate the pectatelyase and the pectinlyase activities, respectively. These enzymatic activities can be also colorimetrically assayed after reaction with thiobarobituric acid, being one unit of pectinlyase defined as the amount of enzyme which creates 1 nM of unsaturated product (4-dehydro-5-ketouronic acid) per min (Szajer & Szajer 1982).

5. PURIFICATION OF PECTINASES

Pectinases produced by different microorganisms have been purified (partially or to homogeneity). The first purification/concentration step involves precipitation with ammonium sulphate or with ethanol. Ultrafiltration is also employed to concentrate the enzymatic extract followed by different steps of gel filtration, affinity or ion exchange chromatography, which may produce a homogeneous preparation of the enzymes present in the extract.

An enzymatic preparation from *A. niger* containing five endo-polygalacturonases and one exo-polygalacturonase was purified by affinity chromatography, followed by chromatofocusing and gel permeation chromatography. The exo-polygalacturonases were purified to homogeneity and endo-polygalacturonase was partially purified (Kester & Visser, 1990). Three endo-polygalacturonases produced by *A. carbonarius* were isolated to apparent homogeneity by molecular sieve chromatography on Sephacryl S-200 followed by ion exchange chromatography on CM Sephadex and gel filtration on Sephadex G-50 (Devi & Rao, 1996). Endo-polygalacturonase I from *Stereum purpureum* was purified to homogeneity by ion exchange chromatography on CM-52 followed by gel filtration on Sephadex G-100 (Miyairi et al. 1985). An enzymatic extract produced by *Neurospora crassa* was precipitated by ethanol, followed by chromatography on Biogel P-60. The pooled fractions were applied to a DEAE-cellulose column and finally applied to a CM-cellulose column. Polygalacturonase was eluted as a single peak with a purification factor of 56.14. Pectatelyase and pectinlyase eluted in three fractions with purification factors from 5 to 21 (Polizeli et al. 1991). Cross flow filtration was used to concentrate the endo-polygalacturonase present in the filtrated culture broth of *Mucor flavus*. The dialysed concentrate was purified to homogeneity by two ion-exchange chromatography steps on CM-Sepharose CL-6B (Gadre et al. 2003). An exo-polygalacturonatelyase produced by *Bacillus sp.* was purified to homogeneity by salting-out with ammonium sulphate followed by gel filtration on Superose 12 HR10/39, hydrophobic

interaction chromatography on a Phenyl-Superose HR5/5 column and ion exchange chromatography on a Mono-Q- HR5/5 column. The final yield and purification factor were 20% and 45.4%, respectively (Singh et al. 1999). An aqueous two-phase system (polyethyleneglycol/potassium phosphate) was used to purify 4 different pectinolytic activities from a commercial enzyme preparation (Lima et al. 2002). The best purification factors were observed in the upper phase for the systems containing high molecular weight polyethyleneglycol without NaCl, obtaining a purification factor of 5.49, 16.28, 16.64 and 14.27 for exo-polygalacturonase, endo-polygalacturonase, pectinesterase and pectinlyase, respectively.

6. CONCLUSIONS

Microbial pectinases are complex group of polymer-splitting enzymes that breakdown homogalacturonan and rhamnogalacturonan pectin-regions. They are widely used as aid processing in food industries. However, the fields of new industrial and analytical applications are being extended in recent years making necessary to study more deeply into these enzymes. Although extensive studies have been carried out on the production of microbial pectinases under various conditions from different carbon sources employing different microbial species and fermentation systems, yet there is not yet a comprehensive set of models of pectinase regulation. Apparently much is yet to be understood on kinetic studies in fermentors for pectinase production in SSF as SSF could be potential tool for its commercial production with better economic feasibility. The regulatory phenomenon such as induction-repression or activation-inhibition could be different in submerged culture and SSF. Similary, it is necessary to carry out efforts to understand the importance of enzymes related to the degradation or modification of hairy regions and those enzymes considered as accessories.

7. PERSPECTIVES

From the description as above, it would be worth to peruse the production of pectinases related to the degradation of rhamnogalacturonan region and those considered as accessories. More work is needed on bioreactor kinetics to analyse the whole process: Since, high concentrations of carbon source inhibit enzyme synthesis in submerged fermentation, it could be worth to explore the production using fed batch fermentation with the corresponding optimisation studies. Continuous production process is yet another aspect to explore. Furthermore, the fact that pectinase production by SSF is not strongly repressed by carbon sources makes this field an attractive way to produce pectinases. It should also be stressed that literature on high cell density cultivation for pectinases production is not available. Further studies on design of level reactors for the production of pectinases can be attempted. An interesting opportunity is the production of pectinases by SSF of these enzymes using genetically modified strains suitable to SSF conditions.

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