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Purification Strategies for Microbial Pectinases

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Abstract

Microbial pectinases currently occupy a place of prominence among biocatalysts owing to their capability to catalyze a diversity of reactions in aqueous and non-aqueous media. Specific action of these enzymes has initiated remarkable attention among industrialists and researchers. Pectinases from a large number of fungal and a few bacterial sources have been purified to homogeneity. This has assisted in successful sequence and three dimensional structure determination leading to a better understanding of their unique structural function relationships during various hydrolytic and synthetic reactions. Since protein purification is normally done in a series of sequential steps involving a mixture of different techniques. This article presents a critical review of different approaches which have been employed for the purification of fungal and bacterial pectinases. This will be immense help for researchers while planning microbial pectinase purification.

Keywords: Bacterial, fungal, pectinases, purification

Introduction

Enzymes are well known delicate biocatalysts that accomplish a multitude of chemical reactions in the metabolism of almost all organisms' *viz.,* plants, animals, fungi, bacteria and viruses. From an industrial perspective, only a constrained number of enzymes are commercially exploited in the detergent, food, leather processing, pharmaceutical, diagnostics, and fine chemical industries (1-3). Pectinases constitute a diverse group of enzymes which catalyze the hydrolysis of pectin polymers (4). Pectin polymers are structural polysaccharides which forms an important component of middle lamella and primary cell wall of higher plants (Fig-1) (4).

Primarily, these are made up of á (1-4) linked D-galacturonic acid residues (5). Depending on their mode of catalysis, the enzymes hydrolyzing pectin are broadly known as pectinases (Table-1 and Fig-2), which include endo-polygalacturonase (E.C. 3.2.1.15), exo-polygalacturonase (E.C. 3.2.1.6.7), pectin lyase (E.C. 4.2.2.10) and pectin esterase (E.C. 3.1.1.11) (6-7).

Pectinases are alone account for about one quarter of the world's food enzyme production (8). It has been reported that microbial pectinases account for 25% of the global food enzymes sales (9). Pectinases are of great significance (Table-2), extensively used in the clarification of fruit juices, extraction of vegetable oil, treatment of pectin waste waters, degumming of plant fibers, pulp and papermaking, and for coffee and tea fermentation (10-16).

Most of the commercial production of pectinases is limited to some species of microorganisms *viz., Bacillus licheniformis* (17), *Bacillus cereus* (17), *Bacillus subtilis* (18), *Bacillus polymyxa* NCIM 2534 (19), *Lactobacillus plantarum sp.* (20), *Lactobacillus pentosus* SJ65 (20), *Bacillus sp.* (21-27), *Staphylococcus aureus* (17),

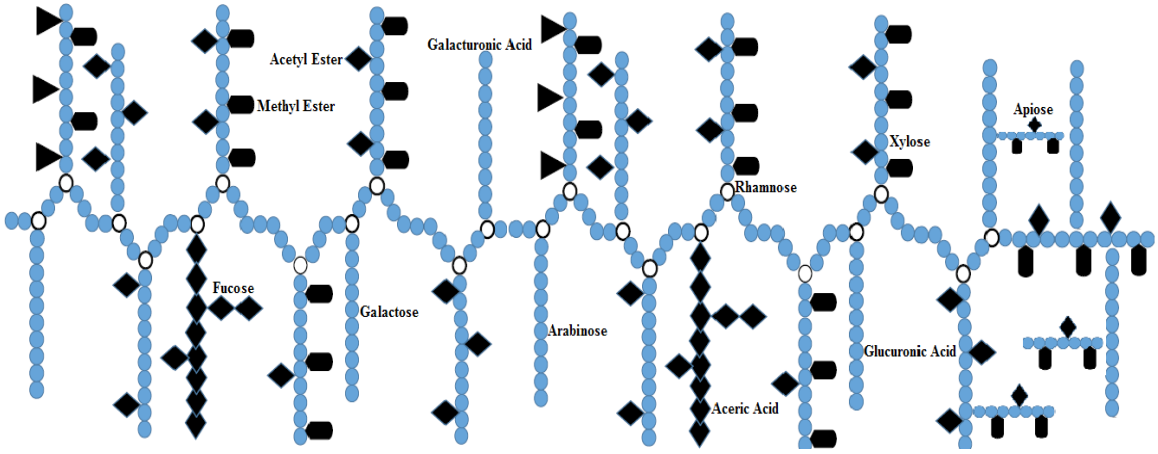

Fig. 1. Basic Structure of Pectin

Table-1. Pectolytic enzymes classification based to the mode of action

No	Enzyme	E.C. Number	Main Substrate	Mode of Action	Product
1	**Esterases**				
	Pectin methyl esterase	3.1.1.11	Pectin	Hydrolysis	Pectic acid + methanol
	Pectin acetyl esterase	3.1.1.6	Pectin	Hydrolysis	Pectic acid + methanol
2	**Depolymerases**				
	*Hydrolases*Proto pectinases		Proto pectin	Hydrolysis	Pectin
	Endo poly- Galacturonase	3.2.1.1.5	Pectic acid	Hydrolysis	OligoGalacturonates
	Exopolygalacturonase	3.2.1.6.7	Pectic acid	Hydrolysis	MonoGalacturonates
3	**Lyases**				
	Endo pectate lyase	4.2.2.2	Pectic acid	Trans elimination	Unsaturated Oligo Galacturonates
	Exo pectate lyase	4.2.2.9	Pectic acid	Trans elimination	Unsaturated Oligo Galacturonates
	Endo pectinlyase	4.2.2.10	Pectin	Trans elimination	Unsaturated methyl Oligo Galacturonates

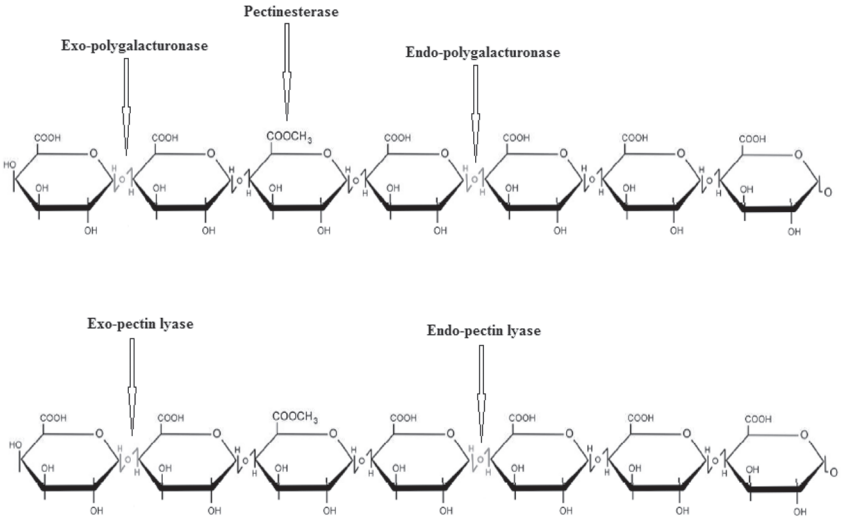

Fig. 2. Pectinases Mode of Catalysis

Erwinia sp. (27), *Leuconostoc lactis* VJ52 (20), *Pediococcus pentosaceous* (20), *Streptomyces sp.* (28), fungi *viz., Aspergillus niger* (27-44), *Aspergillus terreus* (45), *Aspergillus versicolor* (42), *Aspergillus flavus* (30, 31, 42), *Aspergillus awamori* (46), *Aspergillus ocharceous* (31), *Aspergillus japonicas* (30), *Aspergillus foetidus* (47), *Aspergillus aculeatus* (47), *Aspergillus candidus* (48), *Aspergillus carbonarius* (49-51), *Fusarium oxysporum* (27, 42), *Fusarium moniliforme* (52), *Gliocladium viride* (48), *Kluyveromyces* (27, 53), *Mucor racemous* (42), *Mucor hiemalis* (42), *Penicillium canescens* (27, 48), *Penicillium citrinum* (42), *Penicillium griseoroseum* (54), *Penicillium jenseni* (42), *Penicillium dierckxii* (55), *Penicillium occitanis* (56), *Penicillium frequentans* (57, 58), *Penicillum oxalicum* (59, 60), *Penicillium viridicatum* (61), *Rhizopus stolonifer* (27, 42, 48, 62), *Botrytis cinerea* (63), *Cladosporium cladosporioides* (64), *Chaetomium globosum* (30), *Lentinus edodes* (65), *Polyporus squamosus* (66), *Rhodotorulla sp.* (67), *Trichoderma viride* (42), *Thermoascus auriantacus* (68) and yeast *viz., Saccharomyces cerevisae* (16, 27).

Pectinases contain glycine rich consensus sequence (69). Knowledge of the three-dimensional structure of pectinase plays a significant role in designing and engineering pectinase for specific purposes. Most commercial applications do not require homogeneous pectinase preparations; a certain degree of purity, however, enables efficient and successful usage. In addition, purification of enzymes allows successful determination of their primary amino acid sequence and three-dimensional structure. The X-ray studies of pure pectinase enable the establishment of the structure-function relationships and contribute for a better understanding of the kinetic mechanisms of pectinase action on hydrolysis and synthesis (69). Further, purified pectinase preparations are needed in industries employing the enzymes for the bio catalytic production of fine chemicals and pharmaceuticals.

Purification of pectinolytic enzymes : The main constraints in traditional purification strategies include low yields and long time periods. Alternative new technologies such as membrane processes, and aqueous two-phase systems are

gradually coming to the forefront in the purification of pectinases (44, 45, 53, 60, 70). Industries today look for purification strategies that are inexpensive, rapid, high yielding and agreeable to large-scale operations. Purification protocols available in literature are important for consultation when attempting to purify any new preparation. Thus, a review article summarizing up-to-date literature on purification of pectinases serves as a ready reference for researchers engaged in the area of protein purification. The earlier research in this field highlight clearly the importance of designing optimal purification schemes for various microbial pectinases (25, 28, 63).

The present review enlists the various purification procedures applied by different workers. The extent of purification differs with the order of purification steps and this aspect is assessed through the different purification protocols pursued by various investigators and the attempts made in our laboratory for designing purification protocol for bacterial and fungal pectinases have also been discussed. On analyzing 112 articles on pectinases published from 1990 to 2015, some conclusions have been drawn about the percent of different methods used in the purification of these enzymes.

Pre-purification steps : The analysis of enzyme activity in the crude extract does not indicate either an isolated action or the presence of a multi enzyme system working in synergy on the substrate degradation. The characterization of purified enzymes is an important research line since it provides discrimination between the enzyme complex components about substrate degradation mechanism, optimum activity conditions and enzyme synthesis regulation. Most of the microbial pectinases are extracellular and the fermentation process is usually followed by the removal of cells from the culture broth, either by centrifugation or by filtration. The cell-free culture broth is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents. About 80% of the purification schemes attempted thus far have used a precipitation step, with 60% of these using ammonium sulphate and acetone followed by a combination of several chromatographic methods such as gel filtration and affinity chromatography.

Table-2. Application of Pectinases

S.No	Area	Application
1	Juice industry	Juice clarification
2	Textile industry	They are capable of depolymerizing the pectin breaking it into low molecular water soluble oligomers improving absorbency and whiteness of textile material and avoiding fiber damage
3	Pulp and paper industry	Effective in bio bleaching of mixed hard wood and bamboo Kraft pulp
4	Wine industry	Improve wine characteristics of colour and turbidity, improvement of chromaticity and stability of red wines
5	Biological applications	In protoplast fusion technology and plant pathology
6	Coffee and tea fermentation	Fermentation by breaking pectins present in tea Leaves
7	Oil extraction	By avoiding emulsification formation

Precipitation is usually used as a fairly crude separation step, often during the early stages of a purification procedure, and is followed by chromatographic separation. Increase in pectinases activity depends on the concentration of ammonium sulfate solution used (10, 71). Large quantities of material can be handled, and this step is less affected by interfering non-protein materials than chromatographic methods. In comparison to other techniques, which give lower yields (60-70%), precipitation methods often have high average yield (80%) (72). Salting out (ammonium sulfate precipitation) is useful for concentrating dilute solutions of proteins. It is also useful for fractionating a mixture of proteins, since large proteins tend to precipitate first while smaller ones will stay in solution. Rosenberg *et al* obtained 4.6 fold purification with 89.6% of polygalacturonase recovery when they used 70% ammonium sulfate saturation (73).

Hara *et al* obtained around 1.5 fold purification levels with 85% of enzyme recovery when they used 90% ammonium sulfate saturation to precipitate polygalacturonase from *Aspergillus niger* (74). Whereas 3 fold of purification and 68.3% of yield were attained at 70% ammonium sulfate saturation as reported by Darrieumerlou *et al* (75). Charlotte *et al* purified two isozymes of polygalacturonase from *Botrytis cinerea* by addition of 90% ammonium sulfate (63). Kashyap *et al* partially purified pectinase from *Bacillus sp.* DT7 by addition of 40-100% solid ammonium sulphate to the cell-free supernatant (26). Gyan *et al* purified pectinase from *Bacillus subtilis* by addition of 60% ammonium sulphate saturation (25). Das *et al* purified pectinase from *Streptomyces sp.* GHBA10 by addition of 80% ammonium sulphate precipitation (28). Mehraj *et al* purified polygalacturonase produced by *Aspergillus foetidus* MTCC 10367 by addition of 80% ammonium sulphate fractionation (76). Essam *et al* purified pectinase from *Thermomyces lanuginosus* by addition of 60% ammonium sulphate (77).

Chromatographic steps (Table-3)

In order to characterize and study the properties of microbial pectinases the enzymes must be purified. Important purification methods for the isolation of different pectinases are briefly summarized in this section. Most of the time, a single chromatographic step is not sufficient to get the required level of purity. Hence, a combination of chromatographic steps is required. Ion exchange chromatography is the most common chromatographic method; used in 67% of the purification schemes analyzed and in 29% of these procedures, it is used more than once (77).

The most frequently employed ion-exchangers are the diethylaminoethyl (DEAE) group in anion exchange and the carboxymethyl (CM) in cation exchange. Strong ion exchangers based on triethylaminoethyl groups and Sephacryl are becoming more popular in pectinase purification (74-84). Gel filtration is the second most frequently employed purification method, used in 60% of the purification schemes and more than once in 22% of them (81).

Pectinases from various sources of microorganisms have been purified to homogeneity. An exo-PG has been separated from mycelial extracts of *Aspergillus niger* by eluting from DEAE cellulose with 0.2 M sodium acetate buffer at pH 4.6. purification was efficient with 209 fold increase in specific activity with a recovery of 8.6% and the enzyme displayed its full activity only in the presence of Hg^{2-} ions (85). A second PG was isolated with 205 fold increase in specific activity with a recovery of 1%. These two PGs are differentiated by their optimum pH and PGII was not inhibited by chelating agents and did not require Hg^{2-} for activity (86). Benkova *et al* developed a purification statergy for the isolation of extracellular PG and PE (87). The enzyme was salted out with ammonium sulphate and precipitated with ethanol after gel filtration through Sephadex G-25. Repeated chromatography on DEAE-cellulose column

Table-3. Source, Substrate and Methods for Pectinase Production and Purification

S.N.	Name of the Organism	Type of Fermentation and Substrate Used	M.W in kDa	Purification Process	Ref.
1	*Aspergillus sp.*	Submerged and Solid State Fermentation Pectin, Glucose, Wheat bran extract, Corn meal, Sucrose, Soy bran, Sugar cane bagasse and Grape pomace	36-79	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	4, 54, 55, 58, 60, 80, 100
2	*Amycolata sp.*	Submerged Fermentation	30	Ultrafiltration, Ion exchange and Hydrophobic interaction chromatography.	105
3	*Bacillus sp.*	Submerged Fermentation and Solid State Fermentation Vegetable Waste	106	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	8, 17
4	*Botrytis cinerea*	Submerged Fermentation Vegetable Waste	52	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	63
5	*Erwinia carotovora*	Submerged Fermentation Pectin	42	Amonium sulphate saturation	64
6	*Kluyveromyces marxianus*	Submerged Fermentation Pectin	75	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	83
7	*Macrophomina phaseolina*	Submerged Fermentation Pectin	56	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	84
8	*Mucor sp.*	Submerged Fermentation and Solid State Fermentation Sugar beet pulp and Citrus pectin	66	Amonium sulphate saturation and Gel filtration	81, 82
9	*Penicillium sp.*	Submerged Fermentation and Solid State Fermentation Citrus pectin, Sugar beet pectin, Pectin, Orange, Sugar cane bagasse, Wheat bran bagasse and Wheat bran	31-45	Amonium Sulphate saturation, Gel filtration, Ion exchange and Affinity chromatography	54, 55, 56, 58, 60, 80, 103
10	*Rhizopus oryzae*	Submerged Fermentation Pectin	31	Gel filtration and Ion exchange chromatography	55
11	*Sclerotium sp.*	Submerged Fermentation Pectin	38	Gel filtration, Ammonium sulfate precipitation and Ion exchange chromatography	62
12	*Streptomyces sp.*	Submerged Fermentation Pectin	32	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	6
13	*Thermoascus aurantiacus* CBMAI-756	Solid State Fermentation Wheat bran and Orange bagasse	29.3	Gel filtration and Ion-exchange chromatography	104
14	*Trichoderma sp.*	Solid State Fermentation and Submerged Fermentation Sugar beet pulp	30-72	Amonium sulphate saturation, Gel filtration and Ion exchange chromatography	82

yielded a homogeneous preparation of enzyme. Exo-PG, Endo-PG and pectinesterase have been seperated from the culture filtrate of *Trichoderma reesei* by Sephadex chromatography (88).

Polygalacturonase from *Rhizopus stolonifer* has been purified up to 10 fold by ethanol precipitation followed by CM-Sepharose 6B ion exchange chromatography and gel filtration by Sephadex G-100 (89). PG and PL (pectinlyase) from *Aureobasidium pullulans* LV10 have been seperated by CMSepharose 6B followed by column chromatography (DEAE-cellulose column) and gel filtration on sephadex G-100 (89). PG and PL (pectinylase) have been seperated into PG I and PG II and PL I and PL II, respectively. Pectatelyase (PGL) was synthesized by *Amycolata* species and the extracellular crude enzyme has been purified to homogeneity by both cation and anion exchange columns and hydrophobic interaction chromatography (89). It has been observed that purification resulted in a 4 fold increase in specific activity with 37% recovery. Pectinases from *Clostridium acetobutylicum* ID 9136 a UV mutant has been purified by cation exchange chromatography on a Sepharose column by eluting with NaCl (90). Endo pectate lyase synthesized by *Bacillus macerans* has been purified by ammonium sulphate precipitation followed by DEAE Sephadex A-50 chromatography and CM Cellulofine chromatography (91). Similarly endo pectate lyase I/IV have been isolated from the culture filtrate of *Erwinia carotovora* by CM Sepharose CL 6B chromatography, Sephadex S-200 gel filtration and isoelectric focusing (92).

Kobayashi *et al* purified the first bacterial exo-PG from *Bacillus sp.* strain KSM-P443 to homogeneity (93). This enzyme releases exclusively mono-galacturonic acid from polygalacturonic acid (PGA), Di, Tri, Tetra and Penta galacturonic acids. They also determined the N-terminal sequence and concluded that no sequence matched with other pectinases reported to date. An extracellular endo PG produced by *Aspergillus awamori* IFO 4033 was purified homogeneity using cation exchange and size exclusion chromatographic columns (94). Sakamoto et al isolated propectinase N (PPN) and propectinase R (PPR) from the culture filtrate of *Bacillus subtilia* IFO3134 (95). These enzymes have been purified by hydrophobic interaction chromatography on butyl-toyopearl 650 M, cation exchange chromatography on CMtoyopearl 650 M and gel filtration on sepharose 12HR. These enzymes have been found to be stable over a wide range of pH and temperature. Endo pectate lyase produced by *Erwinia caratovara* FERM P-7576 has been selectively co-sedimented with an extracellularly produced lipopolysaccharide lipid complex (96). The cell free broth was precipitated and the enzyme separated by gel chromatography with a specific activity of 710 U/mg of protein. Co-sedimentation has been affected by pH and Ionic strength. Denis *et al* studied the effect of shear stress on purification of five isoenzymes of pectate lyase produced by *Erwinia chrysanthemi* 3937 in ultrafiltration equipment (97). Activity was not affected during 7 h of pumping and 36% activity was lost after 25000 passes.

New affinity matrices have been developed for the purification of pectinases, which possess better mechanical and chemical stability than those cross linked one with pectic acid (98). The culture filtrate was desalted on Sephadex G-25 column. The supports used were salinized controlled pore glass, silica gel salinized with 5-aminopropyl triethoxylsilane. All supports were activated with 3(3-dimethyl amino propyl) carbodiimide and best results were obtained with salinized controlled pore glass. Gupta et al developed an affinity precipitation technique for separation of selective proteins using heterobifunctional ligands (99). They used a soluble form of the ligand for affinity binding and then precipitation was induced for separating the protein complex. Alginate was used as successful ligand for pectinases. Wu *et al* studied the partitioning behavior of endo-PG and total protein from *Kluyveromyces marxianus* culture broth in polyethylene glycol (PEG) and PEG-potassium phosphate aqueous two phase system (ATPS)

(100). Both enzyme and total protein partitioned into the bottom phase in broth systems. Since, the enzyme protein content in the total protein of the fermentation broth was higher, they proposed that separating endo-PG was concentration rather than separation.

An efficient concentration was achieved using ATPS with polymer recycling and dialysis. Immobilized metal ion affinity polysulphone hollow fiber membranes with a high capacity for protein adsorption were tested for commercial pectic enzyme fractionation (101). The flow through (unbound) fraction has higher activity for pectinlyase and PE was retained on the column. Similarly Savary *et al* developed a rapid and simple method to separate PE from PG and other pectinases in potato enzyme preparations using perfusion chromatography (102). PE was eluted at low salt concentrations (80 mM NaCl) and PG at high salt concentrations (300 mM NaCl). The development of such columns looks promising for economical purification strategies for pectinases. A literature survey showed that extensive work has been carried out on the purification of PG, PE and PGL and less attention has been focused on PMG propectinase and oligogalacturonases. Charlotte *et al* purified crude extracts of polygalacturonase from *Botrytis cinerea* to homogeneity by applying Biologic duo flow system (Bio-Rad) liquid chromatography (63). Desalting of the crude protein was performed by gel filtration on HI Prep desalting column (Pharmacia) equilibrated with 50 mM Tris-HCl buffer and the desalted protein extract was loaded on an anion exchange column (Econo Pac High Q) (PG-I 6% and PG-II 3.7% recovery and eluted with 50 mM Tris-HCl buffer with a step gradient from 0 to 1 M NaCl. These fractions were applied to Size exclusion chromatography on SEC 250 column after this PG-I recovery 0.07 and PG-II 0.06% recovery.

Kashyap *et al* purified pectinase from *Bacillus sp.* DT7 by anion exchanger, DEAE-Sephacel equilibrated with 20 mM Tris HCl (pH 7.5) buffer (26). The proteins were eluted with a NaCl gradient (0.2 M). Using DEAE-Sephacel chromatography, 67.2 fold purification of the enzyme was achieved and its specific activity was found to be 730.3 U/mg of protein. Fractions showing pectinase activity were pooled, concentrated using a Centricon P-10 unit, and loaded on Sephadex G150 column. Protein was eluted with Tris-HCl buffer (pH 7.5). As pectinase activity was detected in fractions 21 ± 30, these fractions were pooled, concentrated and dialysed against 0.01 M Tris-HCl buffer (pH 8.0). This phase of purification yielded a 131.8 fold increase in the purification of pectinase and its specific activity was 1433 U/mg protein. Gyan *et al* purified pectinase from *Bacillus subtilis* by agarose ion-exchange column elution was carried out by linear gradient of NaCl 0.1 M-0.6 M (25). After this the specific activity was 4.5 U/mg and enzyme was 1.5 fold purified. Maximum yield observed about 80%.

Das *et al* purified pectinase from *Streptomyces sp.* GHBA10 by gel filtration chromatography (Sephadex G-200 column) equilibrated with 0.2 M phosphate buffer 7.5 pH (28). The activity of the crude and purified pectinase was evaluated as 250 U/l and 658 U/l, respectively. The specific activity of the crude and purified pectinase was recorded as 744 U/mg and 2610 U/mg, respectively. After column chromatography, 3.5-fold increase in the specific activity was noted. The crude Polygalacturonase produced by *Aspergillus foetidus* MTCC 10367 was subjected to gel filtration chromatography on a Sephadex G-100 column. Polygalacturonase activity for purified enzyme was found to be 40.1 U/ml, protein concentration was 0.5 mg/ml and specific activity was determined as 80.2 U/mg. A purification fold of 119.59 and % recovery of 71.55 was achieved (76). Pectinase enzyme was partially purified by Sephadex G-100 column chromatography. It showed an increased specific activity of *P. chrysogenum* pectinase to 145.84 IU/mg protein and enzyme recovery of 48.12%. Preparations of the enzyme obtained from culture filtrates of *P. italicum* were subjected to ion exchanger DEAE-cellulose and carboxymethyl cellulose chromatography at pH 8 and 6, respectively. The

preparations yielded the elution pH value of 8.6 and the elution volume of 15.1 ml and about three-fold higher specific activity (103).

The polygalacturonase from *Mucor circinelloides* ITCC 6025 was purified about 13.3 fold with a specific activity of 31.74 IU/mg giving a yield of 3.4% after Sephacryl S-100 gel-permeation chromatography which resulted in almost a single peak when absorbance was recorded at 280 nm (81). The crude enzyme solution obtained by *Thermoascus aurantiacus* CBMAI-756 culture on solid-state fermentation applied on Sephadex G-75 gel column showed only one peak of enzyme activity, which was detected between 160.0 ml and 256.2 ml. This step resulted in an increasing in the specific activity from 60.0 U/mg to 331.6 U/mg protein, in 5.2 fold enzyme purification and 58.8% yield. In the second step, 50 ml of enzymatic extract was applied on ion-exchange chromatography, using 20 mM acetate-NaOH buffer, at pH 4.0. Two protein peaks were observed from the elution volumes of 42.0 ml and 88.2 ml before the start of the salt gradient and three between 0.15 M and 0.7 M NaCl. Polygalacturonase was eluted at 0.9 M salt concentration. The specific activity increased from 331.6 U/mg to 5351.5 U/mg protein, with 89.2 fold enzyme purification and 14.2% yield (104). The crude pectate lyase from an *Amycolata sp.* was applied to a DEAE Sepharose column. Most of the pectate lyase did not bind to the anion-exchange resin and eluted from the column with the starting buffer. A small fraction of pectate lyase bound weakly to the resin and was eluted at a very low NaCl concentration (105).

Essam *et al* obtained *Thermomyces lanuginosus* pectinase specific activity up to (5.812 Umg^{-1}) by Sephadex G-200 Gel Filtration column chromatography (77). Saflastirilmasi *et al* purified pectinase from *Trichoderma viridi* by ion exchange column chromatography (sephadex G100), the enzyme purity increased 5.59 fold, with a specific activity of 97.2 U/mg (106). Mohsen *et al* purified polygalacturonase from *Asperigillus niger* U-86 by Sephadex G-75 column and obtained 9.5 fold of purification and 60.2 %. Recovery (107). Contreas *et al* purified 470 fold the PGI from a culture extract of *Aspergillus kawakii* with a recovery of 8.6 % of the initial activity in three steps: acetone precipitation, Sepharose Q and Sephacryl S-100 column chromatographies (108). The PG from *Thermoascus aurantiacus* was isolated with 21 fold increase in specific activity with a recovery of 24.6 % by Sephadex G-75 gel filtration followed by SP-Sepharose ion exchange chromatography (109).

Celestin et al purified 9.37 fold one pectinase produced by *Acrophialophora nainiana* which has exopolygalacturonase and pectin lyase activity, 60.6% of the enzyme was recovered after three steps: Sephacryl S-100 gel filtration, DEAE-Sepharose ion exchange and another gel filtration on Sephadex G-50 (110). Kashyap *et al* developed a purification strategy for the isolation of the pectin lyase from *Bacillus* sp. DT7 (26). The enzyme was precipitated with ammonium sulphate followed by DEAE-Sephacel and Sephadex G-150 column chromatographies. The pectin lyase produced by *Aspergillus flavus* was purified 58 fold with a recovery of 10.3% of the initial activity in three steps: ammonium sulphate fraction, DEAE-Cellulose ion exchange and Sephadex G-100 gel filtration (110). Semenova *et al* isolated five pectinases produced by *Aspergillus japonicus*, PGI, PGII, PEI, PEII and PL, by hydrophobic and ion exchange column chromatographies (111). The polygalacturonase from *Streptomyces lydicus* was purified with 57.1 fold increase in the specific activity and a yield of 54.9% after ultrafiltration followed by CM-Cellulose and Sephadex G-100 column chromatographies (112).

Conclusions

Most purification patterns for pectinases are based on multistep strategies. However, in recent years, new techniques have been developed that may be skillfully used, usually as a first step in purification processes leading to high recovery. A mixture of traditional chromatographic processes can indeed be expected to yield rapid and high recovery. As the tertiary structures of several pectinases are known today, one can also imagine

that the design of novel, highly specialized purification procedures based upon molecular recognition will be developed, allowing even easier and more efficient separation and recovery. An overview of various pectinase purification procedures shows that no conclusions can be drawn regarding an optimal sequence of chromatographic methods that maximizes recovery yields and purification fold. Based on the nature of the pectinase produced by the organism, one has to design the protocol for purification involving precipitation and chromatographic steps. However, it will also be guided by the purity of the enzyme required for its usage, which is important from the economic viewpoint. Very few examples of enzyme substrate affinity purification for pectinase exist. Affinity for cofactors and substrate analogues are other possibilities that have not been fully exploited in the purification of pectinases.

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