FROM NATURE TO INDUSTRY: HARNESSING THE POWER OF PECTINASE TOWARDS POTENTIAL BIOTECHNOLOGICAL APPLICATIONS

Hijab Zahra, Sikander Ali*, Ali Raza Nawab, M. Usman Ahmad and M. Nauman Aftab

Institute of Industrial Biotechnology (IIB), GC University Lahore, Pakistan
**Corresponding author (dr.sikanderali@gcu.edu.pk)*

ABSTARCT

Pectinase or pectinolytic enzymes perform the hydrolyzation of pectic substances. Pectinases are among the most prevalent enzymes in a variety of species, including fungi, bacteria, and plants. Not just, protopectinase, pectin esterases, lyases and polygalacturonases are present in abundance but this also makes them the most researched pectinolytic enzymes. Protopectinase stimulates the process of protopectin activation. Polygalacturonases are present in abundance of all other pectinolytic enzymes and with the addition of water they hydrolyze a polygalacturonic acid chain. Lyases are those enzymes which strengthen the separation of galacturonic acid polymer dissolution. Pectinesterases are known to release pectin and methanol by the process of removal of methyl-ester interaction of the pectin spine. Pectinolytic enzymes holds vital importance in the modern biotechnological period and that is based on their extensive usage for multiple processes including the reduced fruit juice extraction and specification, extraction process of cotton and plant, treating the contaminated water, extraction process of vegetable oil, fermentation process of tea and coffee, paper extracts, alcoholic beverages, poultry supplements and in food industries. The current revision primarily focuses on the following aspects including the structure and types of pectic substances, the separation of pectinolytic enzymes, their experimental techniques, biological and physicochemical properties and close view of their commercial applications.

Keywords: Pectinase, pectinolytic enzymes, pectinase production, biotechnological applications, pectic substances, food sector applications

INTRODUCTION

Enzymes are renowned biological molecules which drive different biochemical reactions. Pectinases are the enzymes that perform the process of degradation of pectic substances following the stages of de-polymerization and esterification. Pectinase has its vital role in the preparation of commercial enzymes which are used for the clarification of fruit juices (Roy *et al.,* 2018). In plants, pectic substance are present and their hydrolyzation process is done by heterogeneous group of enzymes i.e., pectinases. Along with the microbial organisms pectinases are widely distributed in higher plants. For plants, they hold a particular importance as they perform a major role in the extension of cell wall and soften the plant tissues for the stages of maturation and storage. With their action the ecological balance is maintained through the process of recycling and decomposition of waste plant materials. Pectinolytic enzymes have significant manifestations in the process of rotting following the plant pathogenicity and deterioration of fruit and vegetables. Based on their high demand in market, the microbial pectinase has been stated to hold the 25 per cent of global food enzymes revenue. Strikingly, all commercial level preparations for pectinase enzymes are achieved using fungal sources (Jayani *et al.,* 2005).

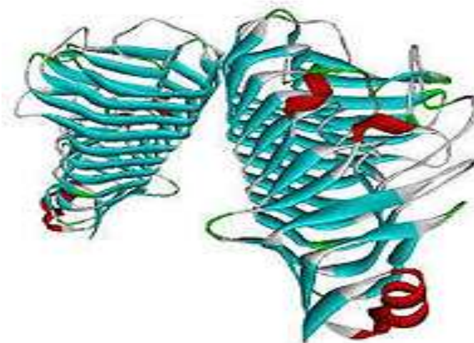


Fig. 1. 3D Pictorial Representation of Pectinase (chares Subash and Muthiah, 2021).

THE SUBSTRATE

Pectic Substances

Pectic substances are complex molecules which are the amalgam of different glycosidic macromolecule cells extensively present in higher plants. They are found in the main cell wall and are prime components of the lamellae in the middle, which is the adhesive layer, attached to the outside of the cells created between the walls of adjacent small cells. Putting it this way, the pectic substances are primarily involved in assuring the integrity and orderliness of plant tissue (Pedrolli *et al.,* 2009).

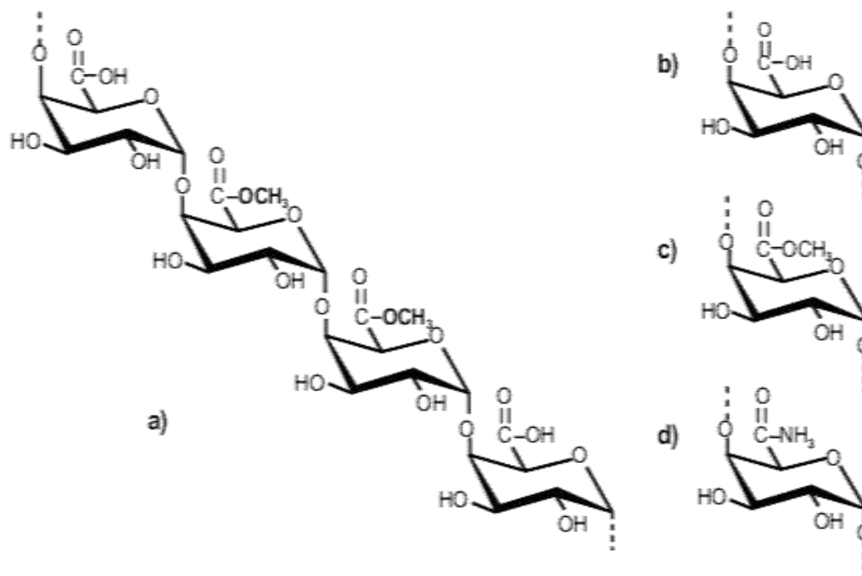


Fig. 2 Structures of Pectic Substances (Oumer, 2017)

Pectic substance is a common term that is applied for compounds having the pectinolytic enzymes as make-up substances. They have a high molecular weight, are poorly charged, with acidic nature, and have glycosidic macromolecules which are found in Kingdom Plantae. Pectic materials make up the "0.5-4.0%" portion of the new plant material (Jayani *et al.,* 2005).

Table 1. Composition of Pectin in Different Fruits and Vegetables (Jayani *et al*., 2005)

Fruit/ vegetable	Tissue	Pectic substance
Apple	Fresh	0.5-1.6
Sugar beet pulp	Dry matter	10.0-30.0
Banana	Fresh	0.7-1.2
Tomatoes	Dry matter	2.4-4.6
Peaches	Fresh	0.1-0.9
Potatoes	Dry matter	1.8-3.3
Strawberries	Fresh	0.6-0.7
Orange Pulp	Dry matter	12.4-28.0
Cherries	Fresh	0.2-0.5
Carrots	Dry matter	6.9-18.6
Peas	Fresh	0.9-1.4

ORIGIN AND OCCURRENCE

The pectinase enzyme is necessary for the proliferation of cells, fruit maturity, abscission senescence, and disease, among other functions of the cell wall. On commercial scale, the pectinase enzyme comes in handy when there is need to safeguard and enhance the procedures employed in the extraction and clarity of fruit juices, as well as the stiffness and texture of used fruits and vegetables. Pectinase enzyme has abundant presence in plants, in pathogenic bacteria and fungi. Including the organisms as "*Rhodotorula sp.*, *Phytophthora infestans*, *Erwinia chrysanthemi B341*, *Saccharomyces cerevisiae*, *Lachnospira pectinoschiza*, *Pseudomonas solanacearum*, *Aspergillus niger*, *Lactobacillus lactis* sub sp. *Cremoris*, *Penicillium frequencyans*, *E. chrysanthemi 3604*,

Penicillium occitanis and *A. japonicas"*. Many reports justify the emergence of PE in plants, namely, "*Carica papaya*, *Lycopersicum esculentum*, *Prunus malus*, *Vitis vinifera*, Citrus sp., *Pouteria sapota* and *Malpighia glabra"* (Sharma *et al.,* 2012).

CLASSIFICATION OF PECTIC SUBSTANCES

Pectinase enzymes can be classified in the four major types which are discussed as follows:

The first pectic substance, Protopectin is water-soluble and is found in hard tissues. Protopectin performs the restricted hydrolysis and as a result pectin or pectic acid are produced (Sakai, 1993). Pectic acid is the pectic substance which is a soluble polymer of galacturonans having a negligible amount of methoxyl groups. Normal or pectic acid salts are named as pectates (Barrett and Northcote, 1965). Pectinic acid is a polygalacturonan series which contains methylated galacturonate units in the range of >0 and <75%. Ordinary sat or Pectinic acid is called pectinates. The last type of pectic substances, Pectin (Polymethyl galacturonate) is a polymeric substance. These have at least, "75%" of carboxyl groups in which the galacturonate units are certified with methanol. Due to this feature it strengthens the cell wall (Jayani *et al.,* 2005).

Depending upon the action pattern the recent enzymes are broken down, such as the breakage is into endo - if the action pattern is random and the breakage is external if the action pattern is at the end (Shet *et al.,* 2018).

SOURCES OF PECTINASE ENZYME

Pectinase can be obtained from the following two main sources:

a) Plant Source

b) Microbial Source

Plant Source

Pectic enzymes can be found in high ratio in plants and are studied for the presence, especially in fruits where they are produced during the process of fruit ripening. The pectin absorbs the lamella in the middle of the plant cell wall and then the high salinity nature is required to separate them. For the said purpose, 10% NaCl is applied. Cold conditions are required for the complete removal and cleaning process because the plant enzymes have thermo-labile nature. The microbial enzymes have more benefits as compared to plant enzymes, including energy efficiency, short-term purification, cost-effectiveness, results reproduction, and industrial use (Nighojkar *et al.,* 2019).

Microbial Source

Aspergillus niger is the major source for pectinase. This enzyme has usage in the production of a wide variety of foods and to prepare easy digestion. The fungal species of pectinase are industrialized by the species *Aspergillus* and *Penicillium* as well (Amin *et al.,* 2019). The fungal pectin enzymes are used for the following major reasons:

1. for accelerating the clarification and screening standards
2. for the removal of pectin from the fruit container prior to suspending the gel into jam
3. For preventing the formation of undesirable jelly in fruits and vegetables extracted with purées
4. To obtain orange oil
5. for stabilizing the cloud in fruit juices (Uraz and Ozer, 2014).

ENZYME ENGINEERING AND OPTIMIZATION

a) Strategies for Enzyme Engineering

Rational Design: Based on structural and functional data, rational design entails making precise alterations to the amino acid sequence of pectinases. Site-directed mutagenesis, which introduces specific mutations to improve or alter enzyme characteristics, can do this. To increase the substrate specificity, thermal stability, pH tolerance, or other desirable properties of pectinases, rational design techniques can be used (Zhou and Wang, 2019).

Directed Evolution: An effective strategy for engineering pectinases is directed evolution. It entails creating genetic variety using techniques like PCR that is prone to mistake or DNA shuffling, then putting the variations through screening or selection procedures to find better enzyme versions. Pectinases with better characteristics, such as higher activity, improved stability, or changed substrate selectivity, can be produced by this repeated process of mutagenesis, screening, and selection (Anand *et al*., 2020).

Protein Engineering Tools: Pectinase engineering may be carried out using a variety of protein engineering tools and methods. These include protein engineering algorithms for creating combinatorial libraries, high-throughput

screening techniques for quickly assessing enzyme variants, and computational modeling and simulation approaches to predict the impact of amino acid substitutions on enzyme structure and function (Wen *et al*., 2009).

Metagenomic Approaches: Metagenomics is the process of extracting and analyzing genetic material from samples of the environment. It gives users access to a huge variety of genes and enzymes, including newly discovered pectinases with special qualities. Researchers may find and isolate pectinase genes from diverse settings using metagenomic approaches, which enable the identification of enzymes with enhanced properties or novel capabilities (Rebello *et al*., 2017).

Fusion and Chimeric Proteins: By merging several domains or functional units from various enzymes, fusion and chimeric proteins can be produced. By using this method, pectinases with improved characteristics or increased substrate specificities can be produced. Pectinase domains can be combined with additional catalytic or binding domains to create new enzymes with enhanced functionality (Jadaun *et al*., 2020).
These pectinase engineering techniques present methods to improve enzyme characteristics, broaden substrate specificity, increase stability, and produce novel functions. Pectinases can be customized for certain industrial purposes by researchers by combining various strategies and cutting-edge instruments.

b) Optimization of Pectinase Production
To maximize the enzyme yield, pectinase synthesis must be optimized by carefully adjusting the medium composition and fermentation conditions. The following techniques are for optimizing fermentation parameters and medium composition:

Media Composition Optimization

Carbon and Nitrogen Sources: Carbon and nitrogen sources must be carefully chosen and optimized in order for pectinase to be produced. Peptone, yeast extract, or ammonium salts are examples of nitrogen sources, whereas pectin, citrus peel, and glucose are examples of carbon sources. Through statistical experimental designs or response surface methodology (RSM), the concentrations and ratios of these sources may be optimized (Uzuner and Cekmecelioglu, 2015).

Trace Elements and Minerals: Adding minerals and trace elements may boost pectinase synthesis. Magnesium, potassium, iron, manganese, and other elements are some of them. Through optimization trials, their ideal concentrations can be found (Taragano *et al*., 1999).

Inducers and Enzyme Enhancers: Enzyme enhancers like calcium ions and inducers like pectin or certain carbohydrates may both boost the synthesis of pectinase. To maximize enzyme production, the concentration and time of their addition can be modified (Enshasy *et al*., 2018).

Fermentation Parameters Optimization
pH and Temperature: Temperature and pH: The pH and temperature factors have a big impact on pectinase synthesis. The best circumstances that encourage more enzyme synthesis are found by assessing a variety of pH and temperature values when optimizing these parameters. For optimum enzyme production, pH must be adjusted using buffers, and fermentation temperature must be maintained (Uzuner and Cekmecelioglu, 2015).

Agitation and Aeration: In the fermentation process, proper agitation and aeration guarantee an appropriate supply of oxygen and avoid the development of oxygen gradients, which can impact the synthesis of enzymes. Pectinase production can be improved by optimizing agitation speed, impeller design, and aeration rate (Enshasy *et al*., 2018).

Fermentation Time: Pectinase production is influenced by the length of the fermentation process. Determining the ideal fermentation period that permits the highest enzyme output is crucial. Monitoring the growth kinetics and enzyme activity over time can be used to assess this (Taragano *et al*., 1999).
It is feasible to increase pectinase synthesis and get larger enzyme yields in a productive and economical way by methodically optimizing the medium composition and fermentation parameters.

PRODUCTION AND EXTRACTION

The production of pectinolytic enzyme is achieved by the two main processes of fermentation including, solid state fermentation processes (SSF) and sub-merged fermentation processes (SmF) with the aid of various bacteria. Pectic enzymes are produced by bacteria, yeast, and fungi in both SmF and SSF settings. Bacterial enzymes have mainly alkaline and curable nature; on the other hand the fungi mainly produce acidic pectinases. SSF is considered to be more suitable for molds as compared to bacteria. One of the pioneers involved in pectinase synthesis using SSF said that pectinase production using *Aspergillus niger* was around "11 times greater" utilizing the SSF approach than the SmF method. Addition to this, the production of endo-polygalacturonases by *Paecilomyces clavisporus* was seen to be "28 times higher" per gram of solid substrate when the SSF method was applied as compared to 1ml of culture during the SMF. The process of SSF occurs when there is absence of water or near or no fluid is present. The exposure of secondary polygalacturonases (PG II) is thought to cause an increased production of polygalacturonases in the SSF, compared with that in SmF. PG II in SSF is chemically different from that produced in the SmF.

Industrial waste acts as a solid substrate to produce fermentative metabolite using molds. Molds can increase their production while using the agro waste similar to when present in their natural habitat. This characteristic makes molds very useful and efficient for the SSF processes. The fungus is very familiar to the SSF processes because the fungal hypha can grow in the agro residue, then penetrate the middle particle spaces, and develop the solid colonies. On the other hand, during the SmF, both nutrients and microorganisms are totally immersed in water which facilitates the breakdown of bacteria. Nevertheless, the SSF processes can be effectively managed and kept in control using the pectinolytic enzymes with slow production rates using the bacterial cultures. Comparisons of the SSF and SmF procedures for pectinase synthesis over a shorter period of time revealed that the SSF process produced more of the enzyme (Nighojkar *et al.,* 2019).

Submerged Fermentation

Submerged fermentation is an advanced system with applications in industrial scale for production of several types of small metabolites. SmF enzyme production methods are usually performed on dynamic reactor tanks under aerobic conditions using batches or batch systems (Sharma *et al.,* 2019). Submerged fermentation requires ample amount of water, consistent improvement and thus results in a lot of impurities. Solid state fermentation applications are found to be restricted to processes where unicellular devices are used. The main obstacles faced include the slow process control, very strong fermentation conditions and often unsatisfactory reproduction of results and complex product purification (Shet *et al.,* 2018).

PURIFICATION

Microbial pectinases must be in a well purified form to fully study and understand the properties they have and for their self-efficacy studies. Pectinases can be obtained from a wide array of microbial sources that have been further purified (Haile and Ayele, 2022). The *Aspergillus niger* genus produces exo-Polygalacturonases which are then separated by eluting process from DEAE cellulose using a buffer of 0.2M sodium acetate at pH 4.6 with a 209-fold increase in specific activity for having an 8.6% recovery. Second, the separation of pectinase is done by a 205-fold increase in specific activity with a 1% acquisition (Shrestha *et al.,* 2021).
Extracellular excretion of exo-polygalacturonase and polygalacturonase cells from "*Aspergillus foetidus* EGEK145" can be performed and revealed as 54 and 31 kDa, respectively. Over the past couple of years, significant improvements have been made in the purification process of pectinases to homosexuality using diversified purification methods. Following the advent of new matrix compounds with abilities of Pectinase separation, there if essential need for the clear and advanced purification methods such as immunochemical techniques. Similarly, to investigate the effect of pH between enzyme inactivity circular dichroism studies have been implied along with studying the modification of structural modification. The analytical use of the MALDI TOF fragment ion was used to identify and predict the exact weight of polygalacturonase from "*Fusarium graminearum"* (Shet *et al.,* 2018).

ENZYME CHARACTERIZATION METHODS

Molecular Weight Determination

A crucial component of pectinase characterization is molecular weight evaluation since it offers important information about their composition, structure, and possible uses. For determining molecular weight, a number of techniques can be used, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry.

One approach for determining the molecular weight of pectinases is gel filtration chromatography. Using this method, proteins are classified according to their molecular weight and size. Use of a column filled with permeable beads is involved. Pectinases are among the smaller molecules that may penetrate the column's pores and take longer

to move through it, leading to a prolonged retention period. On the other hand, larger molecules are eluted earlier because they do not penetrate the pores. The estimated molecular weight of pectinases can be calculated by comparing their elution volumes to those of recognized molecular weight standards.

Another popular method for figuring out the molecular weight of pectinases is SDS-PAGE. This technique coats denatured proteins with sodium dodecyl sulphate (SDS), a detergent that gives them a negative charge. The denatured proteins are subsequently sorted by electrophoresis via a polyacrylamide gel matrix according to their molecular weight. Larger proteins move more slowly through the gel than smaller proteins do. The molecular weight of the separated proteins may be determined by comparing the migration distances of the proteins to those of known molecular weight markers. The separated proteins can be seen using staining methods (Banu *et al*., 2010).

The molecular weight of pectinases may be precisely determined with the use of mass spectrometry. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) is a popular mass spectrometry technique. The pectinases are ionized and subjected to mass spectrometry in this method. The distribution of peptide fragments is shown by the ensuing mass spectra, which makes it possible to calculate the molecular weight of the pectinases.
These techniques, which include mass spectrometry, SDS-PAGE, and gel filtration chromatography, enable scientists to estimate the molecular weight of pectinases. Understanding their structure-function link and investigating their possible uses in diverse sectors require this information.

Isoelectric Focusing

A method used often to characterize pectinases is isoelectric focusing (IEF), notably to establish their isoelectric point (pI). The pH at which a protein has no net electrical charge is known as the pI, and it is a useful indicator of the charge characteristics and probable pH stability range of pectinases.

An electric field is spread across a gel matrix containing a pH gradient during the isoelectric focusing process. Small ampholyte molecules with a variety of pKa values are incorporated into the polyacrylamide or agarose gel matrix. A pH gradient is created inside the gel matrix by the ampholytes. A protein sample, such as pectinases, migrates to its pI location where it has no net charge when it is added to the gel. Based on their pI values, the proteins are divided, with basic proteins going towards the cathode and acidic proteins flowing towards the anode.
Following the separating procedure, the pectinases may be seen using a variety of staining methods, including silver or Coomassie Brilliant Blue. The isoelectric points of the pectinases may be calculated by comparing their migration sites with well-known pI markers.

IEF offers insightful information on the charge characteristics and pH stability range of pectinases. It helps to comprehend how they behave in various pH environments and how they may be used in a variety of sectors, such as biotechnology, textiles, and food processing.

CLASSIFICATION OF PECTINASE ENZYMES

Pectinases are the important class of enzymes that degrade pectic substrates by the processes of de-esterification reaction (done by esterases) or by the de-polymerization reactions (done by hydrolases and esterases) (Singh *et al.,* 2019).

Their classification depends upon the following parameters;
a) If the Oligo-D-galacturonate, the pectin or the pectic acid would be one of the ideal substrates for degradation
b) Whether the enzymes act by the process of hydrolysis or the trans-elimination
c) After the action of the enzymes, the cleavage would be endwise or random (Shet *et al.,* 2018).
The pectinases enzymes can be categorized into three main types; (Paloma and Saarilahti, 1997)

Proto-pectinases: These enzymes form much polymerized soluble pectin by the degradation of the insoluble protopectin.

Esterases: They cause the elimination of the methoxy esters and catalyze de-esterification of the pectin substrate.

Depolymerases: They bring about the hydrolysis of α (1→4) glycosidic bond present in the D-galacturonic acid portion of pectic substances (Jayani *et al.,* 2005).
Depolymerases perform their action on the pectic substances by two altered processes; the first one is hydrolysis, and the other one is the trans-elimination (Tapre and Jain, 2014).

The Depolymerases can be further divided into four main classes, and this division depends upon the suitable enzyme for the degradation of substrate, process of cleavage and the breakdown of glycosidic bonds. Polygalacturonase and poly-methyl-galacturonase causes the hydrolysis of the pectate and the pectin substrate. And

the polygalacturonate lyase and polymethylgalacturonate lyase causes the trans-elimination of the pectate and the pectin (Sharma *et al.,* 2012).
The recent classification of the enzymes is as follow;
1. Protopectinases
2. Polygalacturonases
3. Lyases
4. Pectin esterases

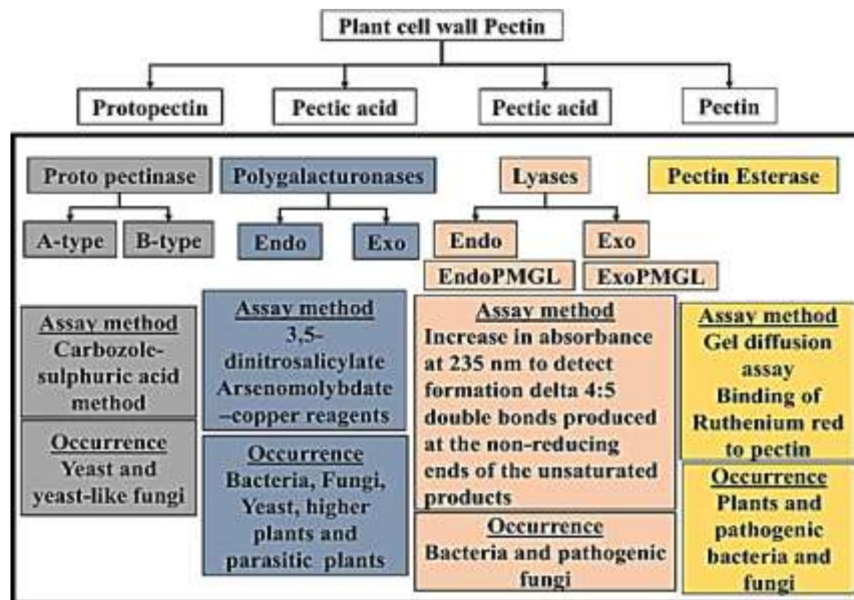
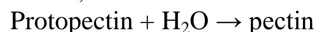


Fig. 3 Classification of Pectinase Enzymes (Sista Kameshwar and Qin, 2018).

1. Protopectinases

These enzymes split the glycosidic linkages favorably on the polygalacturonic acid making the unsaturated product by the process of the trans-elimination reaction. PGL requires the Ca^{2+} ions for functioning. Their action can be repressed by the chelating agents like EDTA (Oumer, 2017).
Protopectinases catalysis their reactions as follows;



$$\text{Protopectin} + H_2O \rightarrow \text{pectin}$$

Classification

Protopectinases or PPase can be further divided into two categories, on the basis of their reaction process. The "A-type PPases" reacts with the polygalacturonic acid i.e. the inner region of the protopectin. While the "B-type PPases" reacts mainly on outer site of polysaccharide chains which link polygalacturonic acid chain and the constituents of the cell wall (Jayani *et al.,* 2005).

Physiochemical Properties

All A-type Protopectinases have the same molecular mass of about 30 kDa. All of these three enzymes have the optimum pH of 5. All the A-type PPase are basic in nature except the PPase-F which is acidic in nature. The A-type PPase have vigorous role in the process of hydrolysis of the polygalacturonic acid. Type B PPases can easily be isolated from *B. subtilis* and *Trametes sunginea that* are termed as the PPaseC and the PPase-T. The molecular weight of PPase-C is 30 kDa and that of PPase-T is 55 kDa. The isoelectric points are 9.0 and 8.1 respectively. Such enzymes are profusely present in the agro-products such as; orange, apple, carrot, sugar beet, lemon, hassaku orange, burdock, radish etc. where particularly they act on the protopectin (Satapathy *et al.,* 2020).

Assay Methods

Calculating the amount of pectic material released from protopectin using the carbazole-sulphuric acid technique allows one to assess the activity of the protopectinase enzyme. The concentration of pectin is calculated from the standard curve as D-galacturonic acid. The enzyme that produces pectic material equal to 1mMol of D-

galacturonic acid per milliliter of the reaction mix under study is considered to have one unit of protopectinase activity (Jayani *et al.,* 2005).

2. Polygalacturonase

It causes the hydrolysis of "α-1, 4-glycosidic bonds" of the pectic acid. And the methodical name of the polygalacturonases is "Poly 1, 4-α-D-galacturonide or glycanohydrolase".

Classification

The poly-galacturonase can be classified into two categories i-e Endo-PG or Exo-PG. The Endo-Polygalacturonase cause the unsystematic hydrolysis of the α-1,4-glycosidic bonds (Yang *et al.,* 2018). The Exo-Polygalacturonase cause the hydrolysis of the α-1,4-glycosidic bonds successively on the pectic acid (Kubra *et al.,* 2018).

Physiochemical Properties

The endo PGs frequently occurs in diverse forms and their molecular masses ranges from 30–80 kDa and their isoelectric points range from 3.8-7.6. Many of the endo PGs have the acidic pH as their optimum pH ranging from 2.5–6.0. The values of their optimum temperature range from 30–50˚C. The exo-polygalacturonases are extensively produced in "*A. niger"*, "*Erwinia sp*." and in apples, carrots, citrus and peaches. The values of their molecular masses range from 30-50 kDa and their isoelectric points have the values range from 4.0-6.0. One of the important properties of the endo PGs is that they are the inverting glycosidases and causes the inversion of the anomeric arrangement of the products produced during the reaction (Sharma *et al.,* 2012).

Assay Methods

Polygalacturonase activity is measured by calculating, during the reaction the following parameters:

- The rate at which the amount of reducing groups gets increased
- The rate at which the substrate's solution viscosity gets decreased

The number of the reducing sugars can be calculated by the colorimetric techniques like "3, 5- dinitrosalicylate reagent or DNS technique" and the "arsenomolybdate–copper reagent technique". The enzyme that releases 1µMol-1 min-1 galacturonic acid under normal conditions is considered to have one unit of enzyme activity (Jayani *et al.,* 2005).

3. Lyases

Lyases are the class of enzymes that are also termed as the trans-eliminases because of their role in the splitting of the pectate polymers or the pectinate polymers in a trans-eliminative manner. They work by releasing a hydrogen atom from the fifth carbon and breaking the glycosidic bond at the fourth carbon atom to create an unsaturated product (Jayani *et al.,* 2005).

Classification

The lyases are grouped into two major types depending on the substrate on which they act. These types are the "polygalacturonate lyase" and "polymethylgalacturonate lyase". Though, these enzymes are sub-classed into five types, which are "endo-polygalacturonate lyase, exo-polygalacturonate lyase, endo-polymethylgalacturonate lyase, oligo-D-galactosiduronate lyase and exo-polymethylgalacturonate" (Patidar *et al.,* 2018).

Polygalacturonate lyases are also called as the pectate lyases they are categorized into two types on the basis of their pattern of action, i.e., the "endo-polygalacturonate lyases"; they act on their substrate in a random manner. The "exo-polygalacturonate lyases" act on their substrate from the non-reducing end of the pectic acid. The polymethylgalacturonate lyases are also called as the pectin lyases (Uluisik and Seymour, 2020). These enzymes are also divided into two categories i.e. the "endo-polymethylgalacturonate lyases", that breaks the α-1,4- glycosidic linkages of the pectin randomly and give rise to unsaturated products i.e. methyloligogalacturonates. The other type is the "exo-polymethylgalacturonate lyases"; they break the pectin substrate in a trans-eliminative manner and also form an unsaturated product i.e. Methyl-monogalacturonates. The process of trans-elimination that occurs at the terminal end of the unsaturated digalacturonate is mostly mediated by the enzyme Oligo-D-galactosiduronate lyases. It is primarily formed by the "pectate lyases" that convert them into the unsaturated monogalacturonates (Yadav *et al.,* 2008).

Physiochemical Properties

The pectin lyases can be efficiently formed by the microorganism. The isolated pectin lyases from the microorganisms have the different properties from each other. The subclass polygalacturonate lyases entirely need Ca^{2+} ions for their function and activation. Yet, the ions of “Co^{2+}, Mn^{2+}, and Ni^{2+}” are also required for the functioning and activation of the intracellular and cytoplasmic lyases. The polymethylgalacturonate lyases do not need the metallic ions for their functioning and activation. In short the two enzyme types are effective for performing their action in the pH ranging from 7.5–10.0 which is alkaline. The optimum temperature ranges for the working of both the enzymes ranges from 40-50˚C. The molecular masses of these enzymes ranges from “22-90 kDa”, while the molecular masses of the polymethylgalacturonate lyases ranges from “89-90 kDa” in the “*Aureobasidium pullulans* LV-10” and the “*Pichia pinus”*, correspondingly. The isoelectric points of these enzymes range from 5.2-10.7 (Satapathy *et al.,* 2020).

Assay Methods

The best method for determining how well lyases work is to determine the increase in absorbance at 235 nm wavelength brought on by the formation of D 4:5 double bonds at the non-reducing terminals of the products (Jayani *et al.,* 2005).

Table 2. Extensive Classification of Pectinase

Enzyme	Action Mechanism	Action Pattern	Primary Substrate	Product	References
Esterase					
1. Pectin methyl esterase	Hydrolysis	Random	Pectin	Pectic acid + methanol	(Dahiya and Singh, 2022)
Depolymerizing enzymes					
a. Hydrolases					
1. Protopectinases	Hydrolysis	Random	Protopectin	Pectin	(Li *et al.*, 2023), (Paloma and Saarilahti, 1997)
2. Endopolygalacturonase	Hydrolysis	Random	Pectic acid	Oligogalacturonates	
3. Exopolygalacturonase	Hydrolysis	Terminal	Pectic acid	Monogalacturonates	
4. Exopolygalacturonan-digalacturonohydrolase	Hydrolysis	Penultimate bonds	Pectic acid	Digalacturonates	
5. Oligogalacturonate hydrolase	Hydrolysis	Terminal	Trigalacturonate	Monogalacturonates	
6. Endopolymethyl-galacturonases	Hydrolysis	Random	Highly esterified pectin	Oligomethyl-Galacturonates	
b. Lyases					
1. Endopolygalacturonse lyase	Trans-elimination	Random	Pectic acid	Unsaturated oligogalacturonates	(Zheng *et al.*, 2023), (Uluisik and Seymour, 2020)
2. Exopolygalacturonase lyase	Trans-elimination	Penultimate bond	Pectic acid	Unsaturated digalacturonates	
3. Oligo-D-galactosiduronate lyase	Trans-elimination	Terminal	Unsaturated digalacturonates	Unsaturated monogalacturonates	
4. Endopolymethyl-D-galactosiduronate lyase	Trans-elimination	Random	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methyl-oligogalacturonates	
5. Exopolymethyl-D-galactosiduronate lyase	Trans-elimination	Terminal	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methyl-monogalacturonates	

4. Pectin Esterases

Pectinesterases are the class of enzymes that form the polygalacturonic acid and results in the separation of the methoxyl and the acetyl deposits from pectin. Pectinesterases that we isolate from fungi have multiple mechanisms, they remove the methyl group in random manner, while the pectin esterases that we isolate from plants have the single chain processes, and they act by aiming the non-reducing side or the free carboxyl group and then proceed in a linear way (Satapathy *et al.,* 2010).

Classification

Pectinesterases are categorized into two groups based on of the functional group they target. The two types are the "pectin methyl esterase or pectin esterase" and the "pectin acetyl esterase". Pectin methyl esterase performs by a single-chain process by changing the pectin to pectate. They do this by splitting methyl ester group of pectin and releasing the methanol. In this whole mechanism the length of the chains of the pectin is not shortened. Inconsistently, the pectin acetyl esterase forms the product acetate and pectic acid by catalyzing the process of hydrolysis of the acetyl ester residue of the pectin substrate (Pedrolli *et al.,* 2009).

Physiochemical Properties

The molecular mass of enzymes isolated from the microbial and the plant sources differs ranging from 30-50 kDa. The standard pH for working differs ranging from 4.0-7.0 except for the enzymes extracted from "Erwinia" whose pH occurs in alkaline state. Many enzymes have standard temperature range occurring from 40–60˚C and isoelectric points differing from 4.0-8.0. The industrial enzymes can be employed to preserve texture and inflexibility of the processed fruits. They are also utilized for the clarification of the fruit juices (Sharma *et al.,* 2012).

Assay Methods

PE working is freely determined by the process of the gel diffusion assay. The unit used to indicate the activity is calculated in Nano or Pico katals. These values can be determined from the standard curve showing the enzyme activity verses the stained zone diameter. Another method for determining the activity of the PE is by using a pH stat. Change in the pH occurs by the ionization of the carboxyl group and the product liberates a proton (Jayani *et al.,* 2005).

FACTORS AFFECTING PECTINASE PRODUCTION

Following factors affect the production and activity of pectinase enzyme:

Effect of Temperature

The highest temperature at which pectinase can be produced is "30 °C". The fermentation process proceeds slowly at 25 °C and the enzyme production is only approximately 50% of what it is after 48 hours at 30 °C. Growth typically occurs at "35 °C" (Hours *et al.,* 1988).

Effect of pH

The pectinase enzyme has high activity under low acidity, as evidenced by the observation of high enzyme activity at pH 5.8. According to the majority of research, pectinolytic enzymes have strong enzymatic activity in the pH range of 4.0 to 7.0. Even though the enzyme performed quite well at a pH of 5.8, a very high value at pH 8.0 was found. Both enzyme stability and activity may contribute to this. It has been discovered that fungal pectinases are stable across the pH spectrum, from acidic to alkaline (4.0–8.0), however their function and stability can peak at distinct pH levels (Sudeep *et al.,* 2020).

NUTRITIONAL FACTORS

The following nutritional factors affect the production and activity of pectinase enzyme.

Nitrogen Source

There has been substantial research on the impact of both inorganic and organic nitrogen sources on pectinase synthesis. On pectinase production, ammonium phosphate and ammonium sulphate have both had favorable effects. Ammonium sulphate improves the production of "*P. chrysogenum"* pectinase. In contrast, ammonium salt was also found to stimulate the production of pectinolytic enzyme in "*Aspergillus alliaceus"*. "Yeast, peptone and ammonium chloride" have been found to enhance pectinase production by up to "24%" and to increase pectinase production of glycine, urea and ammonium nitrate. Soybean diet (4%) showed significant "exo-pectinase activity of 5128 IU g^{-1}" and "endo-pectinase activity of 793 IU g^{-1}" (Samreen *et al.,* 2019).

Carbon Source

Wheat bran supports higher production of pectinase (589 U g^{-1}), on the other hand, pure pectin provides higher polygalacturonase production (642 U g^{-1}). Glucose (4-6%) increases pectinase production in a concentrated state and 6-8% sucrose provides a better pectinase yield in a solid state. *Solanum tuberosum* peels were an excellent source of carbon for producing polygalacturonase by *Bacillus firmus* (Samreen *et al.,* 2019).

APPLICATIONS

Pectinase enzyme holds vital applications in almost every field. The following are the major applications of pectinase enzyme.

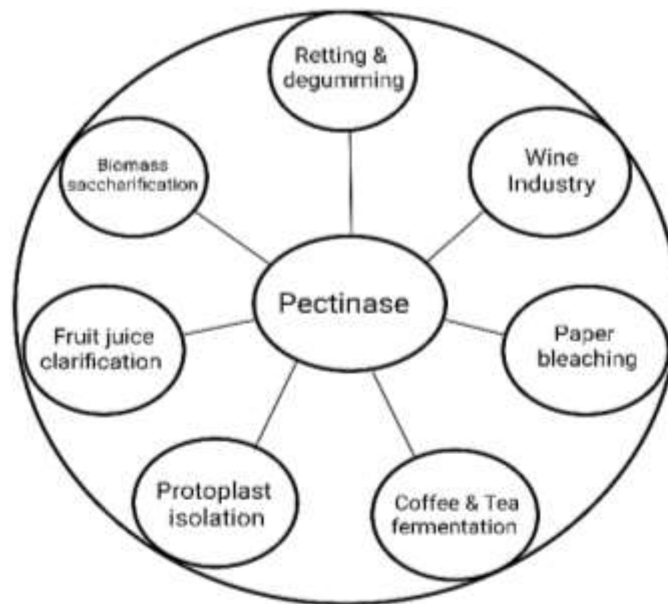


Fig. 4 Schematic representation of various applications of pectinases (Rebello *et al*., 2017).

a) Biotechnological Applications

Treatment of Waste Water

The vegetable food processing industry produces pectin, which contains wastewater as a by-product. The recycling of these pollutants containing the pectinolytic enzyme facilitates the removal of material and makes it ready for decomposition by effective sewage treatment (Hoondal *et al.,* 2002).

Pulp and Paper Industry

Enzymes are now being used by pulp and paper mills to fix issues with their production processes. Pectinase made by "*Erwinia carotovora* sp. and *Bacillus* sp." has been utilized to revive Mitsumata bast because of its potent performance. Pectinase decreases galacturonic acid polymers throughout the paper-making process and lessens the demand for cationic pectin solutions and peroxide bleaching filtrate (Shet *et al.,* 2018).

Processing of textile and cotton fibers bio-scouring

In place of the harmful soda traditionally employed for this purpose, pectinases have been utilized in conjunction with "amylases, lipases, cellulases, and hemi-cellulases" to remove sizing agents from cotton in a safe and environmentally acceptable manner. A unique method called "bio separation" may be used to remove non-cellulosic contaminants from a fiber that contains certain enzymes (Jayani *et al.,* 2005).

Red Wine Chromaticity and Stability

Prior to wine fermentation, pectinolytic enzymes are added to macerated fruit to improve the wine's appearance (humidity and color) in comparison to untreated wine. Enzymatic red wines have shown better chromatic properties than control wines. These wines have also shown great resilience compared to control (Revilla and Ganzalez-san, 2003).

Extraction of oil

Utilizing pectinases, citrus oils can be extracted, including lemon oil. They eliminate pectin's emulsifying abilities, which prevent oil from accumulating in citrus peel pieces (Jayani *et al.,* 2005).

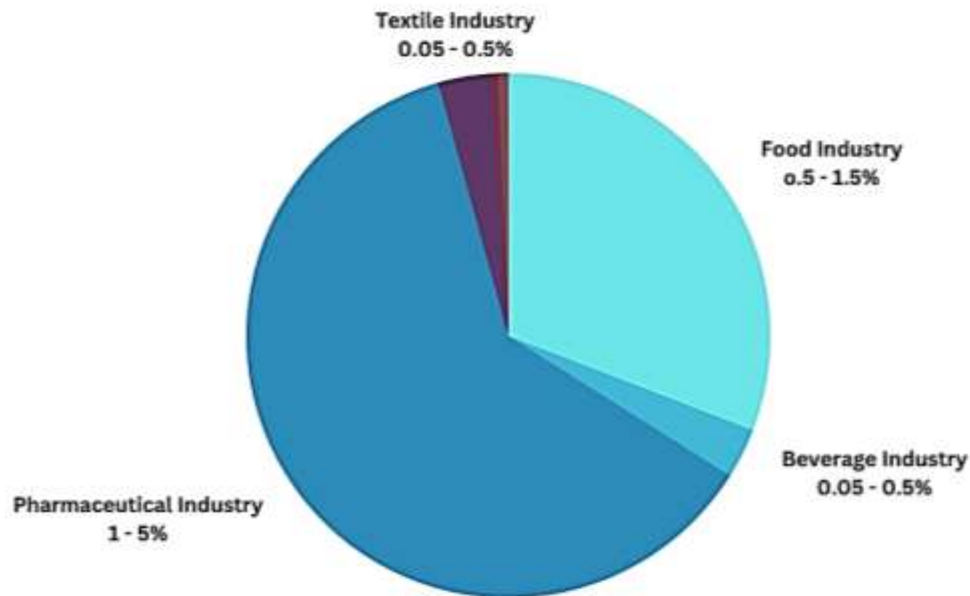


Fig. 5 Percentage of pectinase used in various sectors (Haile and Ayele, 2022)

b) Biomedical and Pharmaceutical Applications

The biological and pharmaceutical uses of pectinases show their adaptability and promise for creating cutting-edge methods for drug administration, tissue engineering, bioactive component extraction, and gastrointestinal health enhancement.

Drug Delivery Systems

Drug delivery methods are being developed using pectinases. Pectinases may alter pectin, a naturally occurring polysaccharide found in a variety of fruits, to create pectin derivatives with certain features. These modified pectins can be used as drug delivery systems for targeted and controlled release. Pectinases make it easier to modify pectin such that desirable drugs release kinetics, improved drug stability, and increased bioavailability may all be achieved.

Wound Healing and Tissue Engineering

Pectinases have demonstrated potential in applications for tissue engineering and wound healing. Pectin-based biomaterials, such as pectin hydrogels and scaffolds, can have their structural characteristics changed by pectinases to improve their capacity for tissue regeneration. Pectin-based materials' porosity, mechanical strength, and gelation qualities are all controlled by pectinases and are essential for fostering cell adhesion, proliferation, and tissue regeneration (Pereira *et al*., 2018).

Extraction of Bioactive Compounds

The extraction of bioactive substances from plant sources involves pectinases. Pectinases aid in the release of bioactive substances such phenolic compounds, flavonoids, and antioxidants by dissolving the pectinaceous components of plant tissues. Due to their antioxidant, anti-inflammatory, and anticancer qualities, these bioactive molecules may find use in medicine. The effective recovery of bioactive chemicals from plant sources is made possible by pectinase-assisted extraction.

Gastrointestinal Health

The possibility of pectinases enhancing gastrointestinal health has been researched. Pectin, which is a component of dietary fiber, can be difficult for humans to digest. Pectin may be enzymatically broken down by pectinases, producing soluble fiber and prebiotic oligosaccharides. These prebiotic substances enhance gut health and digestion by encouraging the development of good gut bacteria (Khan *et al*., 2013).

c) Applications in Food Sector

Pectinase enzyme has following applications in the food sector:

Tea and coffee fermentation

The walls of the tea cells contain pectinase enzymes of fungal origin, which break down pectin during the fermentation process. However, too much of these enzymes might harm the tea leaves; therefore a specific concentration must be maintained. By degrading the pectin in fast tea granules, they also have anti-inflammatory properties. A coating of mucilage from the coffee beans is removed by pectinase during the fermenting process. In order to facilitate fermentation, pectinase preparations are sprayed over cocoa beans. Filtered fermentation filtrate is a cheap method with the same purpose. But the enzymatic preparations of pectinase work better because the enzymes accelerate the fermentation process (Kubra *et al.,* 2018).

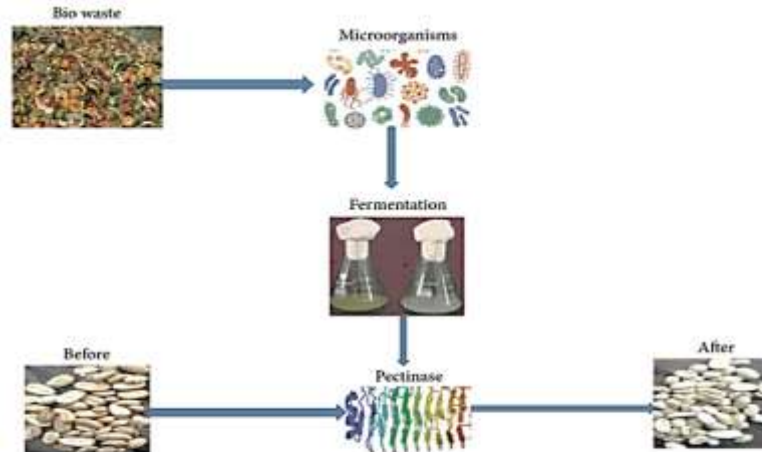


Fig. 6 Removal of mucilage from coffee beans via pectinase (Haile and Ayele, 2022)

Extraction of fruit juice

Pectinase is most frequently used in the production of fruit juice and clarifying. The viscosity of fruit juice and loss of weight are both influenced by pectin. To determine the origin of fruit drinks, pectinase and amylase are combined (Patel *et al.,* 2022). We shorten the filtering duration by as much as 50%. The volume of fruit juice from apples, bananas, and grapes has also been found to rise after pectinase treatment of fruit pulps. "Cellulases, arabinases, and xylanases", which have been used to improve the efficiency of fruit juice extraction, are combined to create pectinases (Sharma *et al.,* 2017). Commercially, pectinase absorption softens the orange peel so it can be removed. In the future, this method could become more popular in place of manually cutting canning pieces (Khan *et al.,* 2013). There is fourfold as much solid fruit produced when free stone peaches are combined with calcium and pectin methyl esterase. This can be used to prepare cucumbers if over-softening during boiling and storage is a possibility (Baker and Wicker, 1996).

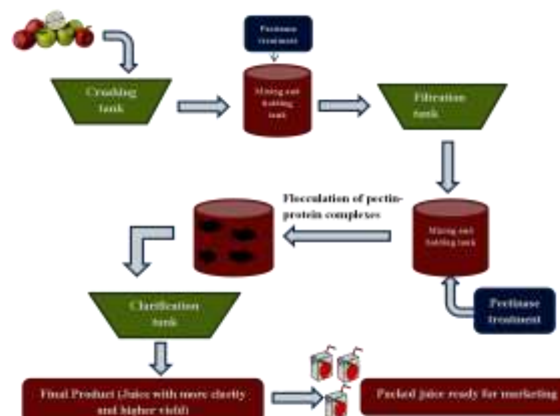


Fig. 7 Application of Pectinase in Fruit Juice Production (Verma *et al*., 2018)

Animal feed

The "enzyme cocktail", which is used to produce animal feed, contains pectinase. It also releases nutrients by hydrolyzing non-perishable fibers or by releasing nutrients that have been occluded by these fibers, lowering feces, and increasing the absorption of nutrients (Jayani *et al.,* 2005).

Pickling

Machine installation is the process by which the vacuum is pressed to drive the resin into a laminate. Pectinase has a vacuum absorption capacity and is therefore widely used to soften fruits and vegetables. Therefore, in the pickling business, where lubrication happens during boiling and storage, pectin methyl esterase and calcium are utilized (Kubra *et al.,* 2018).

Jams and jellies preparation

By de-methylating high methoxylated pectins, pectin esterase changes them into low methoxylated pectins. A calcium-based gelation-based component is present in pectin esterase, which reduces the amount of sugar needed to produce the gel. The production of gels, jellies, sauces, compotes, and soups uses this enzyme (Grassin and Fauquembergue, 1996).

d) Applications in Biorefineries

Biorefineries are establishments with the goal of converting biomass into various useful products through biochemical and thermochemical processes. Pectinases play an important role in these facilities. Following are some significant uses of pectinases in biorefineries:

Biomass Pretreatment

In biorefineries, pectinases are essential for the processing of biomass resources. Pectin is a complex polymer that is frequently found in biomass feedstock like agricultural leftovers and energy crops. Pectin can prevent enzymes from accessing cellulose and hemicellulose. In order to make cellulases and hemicellulases more accessible for enzymatic hydrolysis later on, pectinases are utilized to break down and remove pectin from biomass (Deb *et al*., 2022). The process of converting biomass is more effective overall thanks to this pretreatment phase.

Cellulosic Ethanol Production

A biofuel made from lignocellulosic biomass called cellulosic ethanol is produced using pectinases. In order to liberate fermentable sugars like glucose, which bacteria may use to ferment into ethanol, pectinases aid in the breakdown of the pectinaceous components of biomass (Amin *et al*., 2019). Pectinases increase the output of fermentable sugars and boost the process' efficiency during the saccharification stage in the synthesis of cellulosic ethanol.

Biogas Production

When producing biogas, where organic waste is anaerobically digested to produce methane-rich biogas, pectinases are used. Pectin, which is included in a variety of organic wastes, can help build dense layers and impede the digestive process. Pectinases aid in the degradation of pectin and accelerate the breakdown of organic waste, increasing the production of biogas and enhancing the effectiveness of digestion (Escamilla- Alvarado *et al*., 2021).

Value-Added Products

In biorefineries, pectinases can be used to extract and alter pectin from biomass to produce products with added value. Pectin's gelling, stabilizing, and emulsifying qualities make it useful in a wide range of food, pharmaceutical, and cosmetic sectors. Pectinases make it easier to extract and modify pectin, which makes it possible to create pectin-based products with improved functionality (Costa *et al*., 2022).

ENVIRONMENTAL IMPACT, CHALLENGES AND FUTURE PERSPECTIVES

1. Environmental Benefits of Pectinase-based Processes

Environmental benefits associated with pectinase-based processes include less waste production, improved resource efficiency, and reduced consumption of energy. These are described as follows:

Reduced Waste Generation

Pectin, a complex polymer present in plant cell walls, is efficiently broken down by pectinases. Pectinases can significantly reduce the production of waste in a variety of industrial settings. Pectinases are used in the food sector, for instance, to clarify fruit juice and remove pectin from leftover fruit. With this enzymatic method, pectin may be broken down more effectively, producing less waste (Garg *et al*., 2016).

Increased Resource Efficiency

By maximizing the extraction and use of important chemicals, pectinase-based techniques improve resource efficiency. Pectinases are employed to dissolve plant cell walls during the extraction of plant-based medicines, releasing bioactive substances that may then be processed. This enzymatic method increases the production and utilization of active chemicals while minimizing resource waste (Chauhan and Sharma, 2014).

Lower Energy Consumption

The benefit of pectinase-based techniques over conventional ones is that they use less energy. Enzymes operate in a kinder environment, needing less heat and less abrasive substances. As a result, the process uses less energy overall and has less of an impact on the environment. Pectinases have been used in a number of sectors, including pulp and paper, textiles, and food processing, where their enzymatic action takes the place of energy-intensive procedures (John *et al*., 2020).

Reduced Water Usage

Pectinases can help industrial operations use less water. Pectinases are used in the textile industry to degum natural fibers like flax and silk. The effective elimination of pectinaceous contaminants made possible by the enzymatic treatment lessens the requirement for thorough water washing and lowers water usage in general.

Eco-friendly Alternative to Chemical Processes

Chemical treatments can be replaced with pectinase-based procedures that are environmentally friendly. The use of strong chemicals, which may be harmful to both the environment and human health, is a common practice in traditional approaches. Industries can lessen or eliminate the requirement for such chemicals by using pectinases, leading to a cleaner and more environmentally friendly production process (John *et al*., 2020).

Contribution to Waste Valorization and Biorefineries

The development of biorefineries and waste valorization depend heavily on pectinase-based technologies. Pectinases may enzymatically hydrolyze pectin-rich agricultural leftovers and byproducts, such as citrus peels and apple pomace, to produce useful sugars and other bioactive substances. By allowing waste products to be used as feedstock, this strategy encourages a circular economy and lessens the load on landfills (Garg *et al*., 2016).

Thus it can be said that pectinase-based processes provide additional environmental advantages, making them more sustainable and advantageous for a variety of industries.

2. Challenges in Pectinase Research and Potential Future Directions

Challenges in Pectinase Research

a. **Substrate Specificity and Diversity:** Complex polysaccharides called pectins have various types of pectin backbones, side chains, and levels of methylation. The development of pectinases with broad substrate specificity to efficiently breakdown different forms of pectin is complicated by this structural diversity (Haile and Ayele, 2022). Researchers must investigate and create pectinases that can effectively target various pectin structures.

b. **Industrial Applicability:** Pectinases need to be modified to fulfill certain industrial demands. This covers ideal pH and temperature, suitability for the circumstances of the process, and stability during storage. It is still challenging to create pectinases with the best qualities for a variety of uses, including food processing, textile production, and biorefineries (Pedrolli *et al*., 2009). To increase pectinase's industrial usefulness, researchers should concentrate on optimizing its characteristics.

c**. Enzyme Stability and Shelf-life**: In severe industrial settings or during storage, pectinases are prone to denaturation and activity loss. Enzyme stability has to be improved if their shelf life and operational reliability are to be increased. To improve pectinase stability, methods including protein engineering, immobilization, and formulation techniques might be investigated (Ottone *et al*., 2020).

Potential Future Directions in Pectinase Research

a**. Exploration of Novel Sources and Biodiversity:** Microorganisms and plants that might be used as potential sources of pectinases still have a large undiscovered biodiversity. Researchers can find new enzymes with distinctive characteristics and wide substrate specificities by examining and isolating pectinases from various biological niches (Bibi *et al*., 2018). In order to access the genetic potential of uncultivable microbes, metagenomic methods can also be used.

b. **Enzyme Engineering and Directed Evolution:** Pectinase performance can be enhanced through enzyme engineering methods such protein engineering, site-directed mutagenesis, and directed evolution. Researchers can improve the catalytic effectiveness, substrate selectivity, stability, and other desired properties of pectinases by changing certain amino acid residues (Sharma *et al*., 2021). Pectinases may also be produced via directed evolution techniques like DNA shuffling and PCR, which are prone to errors.

c. **Integration with Other Enzymes or Microorganisms:** Pectinases can be more effective and have a wider range of uses if the synergistic interactions they have with other enzymes or microbes are investigated. The efficiency of pectin breakdown and product generation can be increased by co-immobilizing pectinases with other pertinent enzymes or by utilizing consortia of microorganisms, resulting in more environmentally friendly and long-lasting processes (Peng and de Vries, 2021).
Thus, researchers can get past the constraints in pectinase research and pave the way for improved enzyme performance, expanded applications, and more sustainable processes by tackling the issues through the investigation of novel sources, enzyme engineering, and integration with other biological systems.

CONCLUDING REMARKS

Most of the studies that have been done so far have been available focuses on testing, segregation, production, purification, formulation and utilizing pectinolytic enzymes for enhancing the yield of fruit juice and clarity. More reports are accessible about the applications pectinases in some industries, the cell reading features of pectinase and enzyme engineering. Understanding fluid enzyme regulation at the cellular level and the mode of action of various pectinolytic enzymes in pectic substances should be the focus of future research on pectic enzymes.

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