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REVIEW ARTICLE

A REVIEW ON MICROBIAL PRODUCTION OF AMYLASE AND PECTINASE FROM AGRICULTURAL WASTE: BIOTECHNOLOGY AND SCOPE

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ABSTRACT

Waste management is one of the biggest challenges in the modern world. Untreated wastes are the potential source for greenhouse gas emission, water and soil pollution. Millions of tonnes of fruits and vegetable waste (FVW) and agricultural waste (AW) are generated every year. FVW and AW are organic in nature and are rich source of polysaccharide components. Transformation of FVW and AW to value added products becomes the current need. Polysaccharides present in FVW and AW serve as nutrients for microbes and thus industrially important enzymes such as amylase and pectinase can be produced through microbial bio-processing of organic waste. Amylases are involved in various applications like starch coating, deinking, biopolishing, desizing, modification of synthetic fibers, stain cleaning etc. Pectinases are involved in various applications like fruit juice clarification, coffee and tea fermentation, waste water treatment, bio-scouring of plant fibres etc. Legitimate administration and management of waste can also ensure a cleaner environment and in future, a zero-waste economy. This review gives insights into how FVW and AW can be successfully harnessed as raw material for the microbial production of industrially important enzymes such as pectinase and amylase. This review also provides information on types of pectinases and amylases; their application and their novel improvement strategies.

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INTRODUCTION

Solid wastes represent the non-liquid class of waste which arise from animal and human activities and are considered unwanted (Ramachandra *et al*, 2018). Solid waste management is one of the biggest challenges in the modern world. Wastes can be classified into residential, industrial, medical, commercial, agricultural and municipal service wastes (Sharma et al, 2019). The amount of municipal solid waste (MSW) generated worldwide was estimated to be around 2 billion tons per year as of 2011 (Amoo and Fagbenle, 2013). It is estimated that the amount of MSW generation would increase to 9.5 billion tons per year by the year 2050 (FAO, 2009). India is the second most populous country in the world and its municipal waste generation depends on the consumption of goods by the population and their remaining residuals (Sharma *et al*, 2019). Sharholy et al (2008) mentioned that about 90 million tonnes (MT) of solid waste are generated annually in India. This increase can be due to rapid urbanization and changes in standard of living in urban cities which further led to waste mismanagement. Waste mismanagement include dumping in open spaces, disposal without treatment making it unfit for energy or resource recovery (Das *et al*, 1998).

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Untreated wastes are potential source of pollution and other socio-economic problems. Major portion of about 70-75% of MSW are organic in nature, of which food waste and fruits and vegetable waste (FVW) make up its primary constituents. With proper treatment, organic waste can become a source of raw material for several industries (Ramachandra *et al*, 2018; Chatterjee&Mazumder, 2018). In India, 50 million tons of FVW are discarded which has a worth of US$ 483.9 million (Sridevi and Ramanujam, 2012). Apart from MSW, agricultural wastes (AW) are generated as a consequence of various agricultural activities which comprises of garbage from the farms, poultry, slaughterhouses, manure, harvest wastes, fertilizer run-offs, plant-based wastes, etc. Milhau and Fallot (2013) reported that about 81.4 MT of agricultural wastes are generated every year in India. Because of the size of FVW and AW generated, transformation of such waste to value added products becomes essential. Polysaccharide components (like cellulose, pectin and starch) present in FVW and AW can be utilized by means of bioconversion for the production of enzymes. Several studies on development of enzymes were through microbial processing (Laufenberg et al, 2003). Microbial bio-processing of organic waste has been proven as a potential tool for production of enzymes (Panda *et al*, 2016). Thus, this review focuses on the microbial processing of FVW and agricultural waste into two commercially important enzymes: pectinase and amylase.

Starch: Due to increased population, there is a huge demand for agricultural production and food industries which in turn resulted in million tons of waste. Such waste contains residues obtained from agricultural activities during cultivation and processing of plants and from food industries like unused parts of roots, leaves, straw, cobs, pulps, seeds, peels, husks, etc which is known as agricultural wastes. Polysaccharides are the main chemical component present in agricultural wastes. The improper disposal of such waste creates a critical environmental issue and economic problem to agricultural and food industries. Polysaccharides contains useful chemicals that can be used as a raw material for other production chains. This provides a strategy to minimize waste as well as to produce sustainable energy (Donato *et al*., 2014). Starch is one of the major polysaccharides in plants. It is made up of two types of glucose polymers namely, amylose and amylopectin. In general, starches contain 20 – 30 % of amylose and 70 – 80 % amylopectin (Svihus*et al*., 2005). Amylose, a low molecular weight polymer that contains linear chains linked by α-1, 4 glycosidic bonds. Amylopectin, a high molecular weight polymer that contains a highly branched chains linked by α-1, 6 glycosidic bonds (Smith and Martin, 1993), (Mua and Jackson, 1997). The build-up of starch in agro-waste possess a threat to environment and hence it is necessary to dispose and manage the waste. Starch hydrolysis helps in both reduction of waste and production of industrially important enzyme, amylase that has potential applications in many industries.

Table 1. Sources of starch from agro-wastes and starch weight (%)

Waste source	Starch weight (%)	References
Cassava bagasse	50	Das and Singh, 2004
Corn bran	11.2	Rose *et al*., 2010
Corn fiber	15-20	Rose *et al*., 2010
Potato	67.5	Sanchez-Vazquez *et al*., 2013
Kiwifruit	40	Li and Zhu, 2018
Pineapple stem	11	Nakthong*et al*., 2017
Mango kernel	58	Kaur *et al*., 2004
Litchi seeds	53	Guo *et al*., 2018
Longan kernel	59	Guo *et al*., 2018
Loquat kernel	71	Guo *et al*., 2018
Annatto seeds	18-20	Zabot*et al*., 2018
Jackfruit seeds	60-80	Madrigal-Aldana *et al*., 2011
Avocado	27.5-29.8	Lacerda*et al*., 2014
Banana peel	30	Li *et al*., 2018

Starch content varies from different sources. The growth of microbes and production of enzyme depends on percentage of starch present in agro-waste. The sources of starch and its percentage from some of the agro-wastes is shown in Table 1.

Amylases: Amylase, a hydrolase enzyme that acts on starch molecules to yield products such as glucose and maltose units. Based on their mode of action, amylases are categorized into four groups: a) α-amylase (1, 4- α -D-glucan glucanohydrolase), an endoenzyme that acts on α-1-4 glycosidic bond, b) β-amylase (1, 4- α -D-glucan maltohydrolase), an exoenzyme that acts on alternate α-1-4 glycosidic bonds from the non-reducing ends to form maltose (two glucose units), c) glucoamylase (GA) (1, 4- α -D-glucan glucanohydrolase) also called as γ-amylase, an exoenzyme that acts on both α-1-4 and α-1-6 glycosidic bonds that yields single glucose units from non-reducing end, d) debranching enzymes, that acts on α-1-6 glycosidic bonds of starch (Reddy *et al*., 2005). In 1831, starch was hydrolysed by saliva which was reported to contain an enzyme "ptyalin", an amylase by Erhard Friedrich Leuchs (Ajay and Farhath, 2010). In 1833, French chemists AnselmePayen and Jean-François Persoz isolated amylase from barley malt that are heat labile (Banks and Greenwood, 1975). Amylases are obtained from several sources such as plants, animals and microorganisms (Sowmya *et al*., 2015) (Sidkey*et al*., 2010). Though amylases are obtained from various sources, microbial amylases are focussed as they are more stable comparatively, safer usage, economically bulk production capacity, microbes can be manipulated to produce enzymes of required characteristics and thus they have huge applications in industrial sector (Gupta et al., 2003; Tanyildizi*et al*., 2005). α-amylase is the most widely studied since it is a faster-acting enzyme and it has more potential applications in starch industry as well as in bakery that it shares maximum sales in market. Global alpha amylase enzyme is expected to grow from USD 278.23 Million in 2018 to USD 352.78 Million by 2026, of 3.01 % at a CAGR (Compound Annual Growth Rate).

Structure of α-Amylase

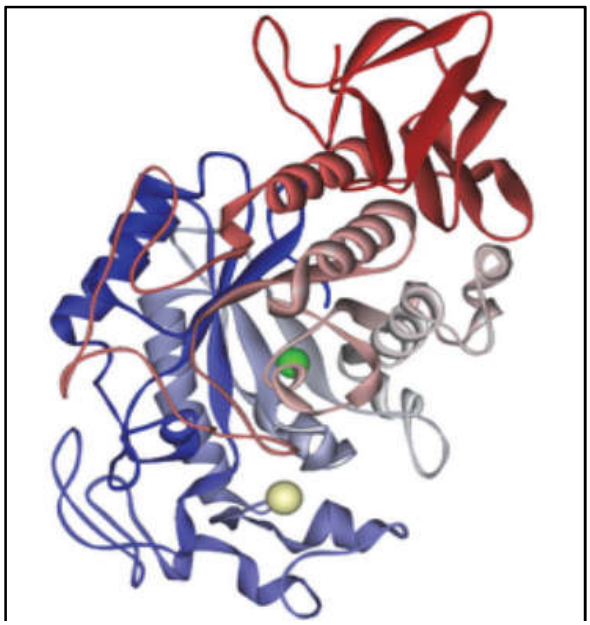

Figure 1. Three-dimensional structure of α-amylase (RCSB PDB accession code 1MSD; calcium binding regions are indicated)

Most of the α-amylase enzymes belongs to GH13 family, along with GH57, GH119 and eventually GH126 families (Ficko-blean*et al*., 2011). X-ray studies of bacterial and mammalian amylases have shown that all α-amylases consists of three domains namely, A, B and C. Domain A which is a central $(\alpha/\beta)_8$ TIM barrel that forms the core of the molecule. Domains B and C are located approximately at opposite sides of this TIM barrel (Nielsen and Borchert, 2000). α-amylases are said to be metalloenzymes which require metal (calcium) ions to maintain their activity, stability and structural conformation (Saboury, 2002) (Figure 1).

Production Methods of α-Amylase: There are mainly two methods for production of amylase namely, submerged fermentation and solid state fermentation, but latter is preferred due to advantages such as higher concentration of enzyme is produced, comparatively low production cost, generation of less effluent, recycling of waste materials and can be used as substrate, high volumetric productivity and

substrate is used slowly and constantly thereby reducing the continuous supply to the process (Couto and Sanroman, 2006) (Kunammemi *et al*., 2005). Due to increased demand and huge applications of amylase, it is necessary to reduce the production cost by utilizing different agro-waste as a low-cost carbon source and nitrogen source (Pandey *et al*., 2000). A summary list of amylase produced from different agro-substrate using various organisms is listed in Table 2.

Table 2. Production of amylase from different agro-substrates using various microorganisms, parameters such as temp and pH and respective enzyme activity is presented

Agro-substrate	Microorganism	Method	Temp (ºC)	pH	Enzyme activity	References
Coconut oil cake	*Aspergillus oryzae*	SSF	30	7	3388 $Ugds^{-1}$	Ramachandran *et al*., 2004
wheat bran	*Bacillus* sp. PS-7	SSF	37	5.9	4,64,000 Ug^{-1} dry bacterial bran	Sodhi *et al*., 2005
	*Aspergillus oryzae*IIB-6		30	5	7800 $Ugds^{-1}$	Fathima *et al*., 2014
Potato peel	*Bacillus subtilis* DM-03	SSF	50	8	532±5 Ug^{-1}	Mukherjee *et al*., 2009
Rice bran	*Streptomyces* sp. MSC702	SMF	50	7	373.89 $IUml^{-1}$	Singh *et al*., 2012
Soyabean meal	*Aspergillus.oryzae S2*	SSF	60	5.6	22118.34 Ug^{-1} dry substrate	Sahnoun *et al*., 2014
Brewery waste	*Bacillus subtilis* UO-01	SMF	36.8	6.6	9.35 $EUml^{-1}$	Blanco *et al*., 2016
Rape seed cake, feather	*Bacillus subtilis* PF1	SMF	70	6	16.39±4.95 μgml^{-1}	Bhange*et al*., 2016
Maize bran	*Bacillus coagulans*	SSF	50	7	22956 Ug^{-1}	Babu and Satyanarayana, 1995
Rice husk	*Bacillus subtilis*	SSF	37	7	21760 Ug^{-1}	Baysal*et al*., 2003
Mustard oil cake	*Bacillus coagulans*	SSF	50	7	5953 Ug^{-1}	Babu and Satyanarayana, 1995
Corn bran	*Bacillus* sp. PS-7	SSF	37	5.9	97600 Ug^{-1}	Sodhi *et al*., 2005
Gram bran	*Bacillus coagulans*	SSF	50	7	8984 Ug^{-1}	Babu and Satyanarayana, 1995
Bread waste	*Rhizopus oryzae*	SSF	30	5.5	100 Ug^{-1}	Benabda*et al*., 2019
Loquat kernel	*Penicillium expansum* MT-1	SSF	30	6	1012 Ug^{-1}	Erdal and taskin, 2010
Banana waste	*Bacillus subtilis*	SSF	35	7	7.63 $IUml^{-1}$ min^{-1}	Unakal*et al*., 2012
Cassava waste	*Bacillus sp.*	SSF	36	7	866 Uml^{-1}	Selvam *et al*., 2016
Date waste	*Aspergillus niger*	SSF	30	5.5	285.6 Uml^{-1}	Acoureneet *al*., 2014
Orange waste	*Streptomyces sp.*	SMF	30	7	8.26 Uml^{-1}	Mahmoud and Mounaimen, 2015
Papaya waste	*Aspergillus niger*	SSF	30	6	25.73 $IUml^{-1}$	Sharanappa*et al*., 2011

Improved Strategies for Amylase Stability and Production: The huge applications of amylases in industries demands more amylase production and its stability to act in all robust conditions. The main barriers for amylase stability are temperature, pH, Ca^{2+} independency and oxidant stability (Jiang *et al*., 2015) (Chi *et al*., 2010). Thermal stability of enzyme can be increased by inserting thermostable amylase producing gene from thermophile into mesophile. A thermostable amylase producing gene from *Thermococcus* sp. CL1 was cloned in *E. coli* and highest enzyme activity was found at 85 ºC for α-1, 4 glycosidic linkages and 98 ºC to cleave α-1, 6 glycosidic linkages (Jeon *et al*., 2014). Enzyme immobilization is another strategy to improve enzyme stability wherein *Laceyellasacchari*TSI-2 was immobilized on DEAE cellulose with Glutaraldehyde cross-linking which increased temperature, pH, solvents and surfactant stabilities (Shukla and Singh, 2016). Chemical modification which also proves to be powerful technique to improve stability of enzyme. Commonly applied chemical modification processes are acylation and acetylation. *Saccharomycopsisfibuligera*R64 α-amylase was chemically modified by acid anhydrides, dimethyl adipimidate (DMA) and polyethylene glycol (PEG) which increased enzyme stability against thermal, chelator (calcium ion) and proteolytic inactivation, respectively (Ismaya *et al*., 2013).

pH stability can be improved by protein stability engineering techniques such as helix capping, cavity filling and removal of deamidating residues (Nielsen and Borchert, 2000). Histidine residues of *B. subtilis* α-amylase was replaced with aspartic acid residues by site directed mutagenesis which increased stability and catalytic activity under acidic condition (Yang *et al*., 2013).

Since, α-amylase is metalloenzyme which is dependent on Ca^{2+}, it is necessary to have a Ca^{2+} independent or low Ca^{2+} requiring amylase enzyme. Methionine of was replaced by serine that significantly increased the oxidative stability of alkaline amylase of *Alkalimonasamylolytica* (Yang *et al*., 2012). Stability of amylase can also be improved by addition of stabilizing agents. Thermostability of *A. oryzae* α-amylase was enhanced by addition of two heavy atoms, gadolinium (Gd) and samarium (Sm) ions (Sugahara*et al*., 2013). In industrial enzymology, large scale production of proteins is a major hurdle and hence extracellular enzymes can be produced by heterologous expression which provides numerous advantages. Codon optimization strategy can be employed to increase the amylase production. α-amylase and glucoamylase gene variants which were designed using codon optimization strategy was expressed in *Saccharomyces cerevisiae* to enhance protein production and enzyme activity (Sakwa*et al*., 2018). Media modification for recombinant enzyme an also increase the enzyme production. *B. aryabhattai* β-amylase using recombinant *B. choshinensis* was produced where the production of enzyme was 41.7-fold higher than that obtained with the initial medium (Duan *et al*., 2019). Systematic engineering at transport and transcription levels enhances amylase production and its activity. Alkaline α-amylase activity of *B. subtilis* 168^{M} $P_{\Delta\text{-}27T}$ was increased by 250.6-fold, compared with *B. subtilis* 168^{M} A1 (Yang *et al*., 2020).

Applications of Amylases: Amylases has huge applications in industrial processes such as food, brewing, chemicals, paper, pharmaceutical and textile. α-amylase was the first enzyme to be commercialized and marketed. The first industrial production of α-amylase from *A. oryzae* known as "Taka diastase" which was used as a digestive aid was demonstrated by Dr. J. Takamine (Sivaramakrishnan*et al*., 2006). Some of its applications are discussed in brief in this review.

Food industry: Enzymes such as malt and fungal α-amylases are used in bread making from decades. α-amylase supplementation in flour enhances the rate of fermentation by increasing sugar in dough which improves taste, aroma, porosity of bread and it also enhances the bread volume and

crumb texture (Lundkvist *et al*., 2007). Chocolate syrup can be produced by treating the cocoa slurries with amylases which prevents syrup becoming thick and can increase the stability, flow properties at room temperature. This stabilized cocoa flavoured syrups can be added to conventional non-acid mixes for use in the production quiescently frozen chocolate flavored confectioneries (El-Aassar *et al*., 1992).

Glucose and fermentation industries: During sugar manufacturing, starch present in sugarcane is a major hurdle which can be eliminated or reduced by amylases. Since, amylases hydrolyse starch by breaking glycosidic bonds which yields glucose and dextrins, they are used for production of glucose and water-soluble dextrin in many industries (Kwak *et al*., 1998).Amylases helps in production of biofuels such as ethyl alcohol. They convert starch into fermentable sugars followed by action of ethanol fermenting microorganism such as *Saccharomyces cerevisiae* on those sugars which produces ethanol. Mould amylases are used in alcohol production and brewing industries due to advantages such as even action of enzymes in mashes, enhanced rate of saccharification, alcohol yield, yeast growth and economically significant (Maria *et al*., 2011).

Paper industry: Amylases is used to partially degrade starch in paper industry. The starch coating paper makes the surface sufficiently strong and smooth which improves the writing quality of paper and erasebility. Since starch is too viscous to paper sizing, they are partially hydrolysed by amylases rather than using expensive chemically modified starches (Okolo *et al*., 1995).

Textile and detergent industries: In textile industries, amylases along with other hydrolase enzymes are used to increase the stiffness of the products, biopolishing and bioscouring of fabric, antifelting of fool, cotton softening, denim finishing, desizing, wool finishing and modification of synthetic fibers (Chen *et al*., 2013) In detergent industries, amylases increase the detergents ability to remove tough stains such as starchy food deposits which makes the detergent eco-friendly. Amylases obtained from *Bacillus* and *Aspergillus* are used generally (Mitidieri *et al*., 2006).

Pectic Polysacharides: Pectic substances are high molecular weight, branched, heteropolysaccharides with negative charges present in the middle lamellar region of plant cell wall. It is water soluble and its molecular weight ranges from 60 – 130000 $gmol^{-1}$. It has a main backbone that contains long and linear chains of α-1,4–D-galacturonic acid (GalA) units with saccharides like D-xylose and D-apiose. An estimated 80% of carboxyl groups of galacturonic acid are esterified with methyl groups (Voragen *et al*, 2009; Khan *et al*, 2013). It is because of the presence of these components that there is development in texture in fruits and vegetables during its growth and maturation (Alkorta *et al*, 1998; Caffall and Mohnen, 2009). Protopectin, pectins and pectic acids make up the major components of pectic substances. Pectins are methyl esterified components, protopectins are water insoluble component present in intact tissues of unripe fruits and demethylated pectins are otherwise called as pectic acid or pectate or polygalacturonic acid (Samantha, 2019). The dicotyledonous plants contain 35% of pectin whereas grasses contain 2–10% of pectin in their primary cell walls (Voragen *et al*, 2009).

The various pectic polysaccharides like homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII) are found in plant cell wall.HG makes up about 60–65% of the total pectin (Munarin*et al*, 2012;Willats*et al*, 2001). HG is a linear homopolymer with α-(1-4) linked GalA residues. It is about 100nm long with an estimated length of about 200 galacturonic acid units. The D-galacturonic acid residues can be subjected to acetyl esterification at position C-2 or C-3 and methyl esterification at C-6 position (Jayani, 2005).XGA is similar in form to HG with 1→3 linked β-D-xylose present as single unit side chains at O-3 position of GalA residues (Zandleven*et al*, 2006). XGA can be abundantly found in reproductive tissues but can also be found in other tissues like Arabidopsis leaves (Zandleven*et al*, 2007). RG-I makes up about 20-35% of pectin, composed of a system of repeating groups of α-(1→2) linked L- rhamnosyl and α-(1→4) linked D- galacturonosyl residues (Harris and Smith,2006). Mostly GalA residues of RG-I are not methyl esterified (Kravtchenko et al, 1992). RG-II makes up about 10% of pectin and are highly conserved and complex structures found in plants (Munarin*et al*, 2012). It carries side chains of four different and complex sugar residues like apiose, aceric acid, 2-O-methyl fucose, 2-O-methyl xylose, 3-deoxy-D-manno-2-octulosonic acid (KDO), 3-deoxy-D-lyxo-2-heptulosonic acid (DHA) that are attached to nine GalA residues along with HG residues (Munarin*et al*, 2012, Liu *et al*, 2004). Normal growth of plant can be affected even with minor mutations in RG-II structure (Munarin*et al*, 2012). During ripening, the pectin backbone or side chains are broken down by fruit enzymes, resulting in a more soluble molecule (Kashyap *et al*., 2001).

Pectin Content from Agro-Wastes: Fruit by products that arise due to agro-industrial activities are a potential source of pectin. Pectin extracted from citrus peels, apple pomace and of late sugar beet pulp are used for making commercial form of pectin (Pacheco *et al,* 2019). Pectin can also be extracted from other agricultural wastes but its yield and physiochemical properties are determined by various extraction process. The most common agro-waste for pectin is orange peels which contains an appreciable amount of pectin content and are used as inducer for pectinase production (Nighojkar*et al*, 2006). Table 3provides details of the approximate pectin content present in certain fruit by-products. This data clearlyshows watermelon rinds with 13-30% pectin is higher than apple pomace which is used for commercial pectin production. This alsoillustrates the need for utilization of agro industrial waste as a substrate for the production of pectinase enzyme.

Table 3. Pectin content from fruit by products (Data modified from Picot-Allain *et al*, 2020)

Fruit by-product	% pectin by-product
Mango peel	10-15
Banana peel	4-6
Watermelon rinds	13-30
Passion fruit peel	15-20
Dragon fruit peel	17
Apple pomace	18-19

Pectinase: Enzymes (bio-catalysts) are proteins responsible for efficient catalytic activity and possess definite mode of action, stereospecific binding, eco-friendly nature and low energy expenditure (Bhardwaj *et al*., 2017). Pectinases are enzymes that belong to the family of polysaccharidases that

contribute to the breakdown of pectic polymers present in the plant cell wall and are also known as pectolytic or pectic enzymes (Prathyusha & Suneetha, 2011). The market size for pectinase in the year 2019 is 30 million $ and it is estimated to reach 35.5 million $ by the year 2024 (Global Pectinase Market Research Report, 2019).

Classification of pectinolytic enzymes: Division of pectinases based on its mode of action on pectic substances is given in Figure2.Pectinases are divided into two groups: (i) esterases and (ii) depolymerases based on its mode of action on pectin. Esterases are restricted to pectin, whereas, depolymerases can act even on pectic acid and oligo-galacturonates.

(i) ***Esterases:*** Esterases are of two types- (i) pectin methylesterase (PME) (EC.3.1.1.11) and (ii) pectin acetylesterase (PAE) (EC. 3.1.1.6). The common name for PME is pectin esterase and it performs random cleavage of methanol from the esterified carboxyl groups yielding pectic acid and methanol. Acetyl esterase is the common name for PAE and it performs hydrolysis of acetyl esters present in HG region of pectin.

(ii) ***Depolymerases:*** Depolymerases are classified based on their mode of action on pectin and pectic acid and oligo-d-galactosiduronates. Each group is divided into hydrolases (hydrolysis) and lyases (transelimination), which is further subdivided into endo and exo-enzymes based on their mode of action.

Depolymerases acting on pectin are (i) Polymethylgalacturonase (PMG) which performs the hydrolytic cleavage of α-1,4-glycosidic linkage in pectin and (ii) polymethylgalacturonate lyase (PMGL) which cleaves α-1,4-glycosidic linkage in pectin through trans-elimination reaction at non-reducing end yielding unsaturated methyl galacturonates. PMG is subdivided into two pectin hydrolases (i) endo-PMG that performs random hydrolytic cleavage of highly esterified pectin to form oligo-galacturonates and (ii) exo-PMG on the other hand performs terminal hydrolytic cleavage of pectin from the non-reducing end to form mono-galacturonates. PMGL is subdivided into two pectin lyases (i) endo-PMGL (EC. 4.2.2.10) that performs random cleavage by trans-elimination reaction in pectin to form unsaturated methyl oligo-galacturonates and (ii) exo-PMGL that performs terminal cleavage by trans-elimination reaction in pectin to form unsaturated methyl mono-galacturonates.

Depolymerases acting on pectic acid are**(i)** Polygalacturonase (PG) which performs the hydrolytic cleavage of α-1,4-glycosidic linkage in pectic acid and (ii) polygalacturonate Lyase (PGL) which cleaves α-1,4-glycosidic linkage in pectic acid through trans-elimination reaction yielding unsaturated galacturonates. PG is subdivided into three pectate hydrolases (i) endo-PG (EC. 3.2.1.15) that performs random hydrolytic cleavage on polygalacturonic acid to produce oligo-galacturonides, (ii) exo- PG-1 (EC. 3.2.1. 67) that performs terminal hydrolytic cleavage on polygalacturonic acid to form mono-galacturonate and (iii) exo-PG-2 (EC. 3.2.1.82) that

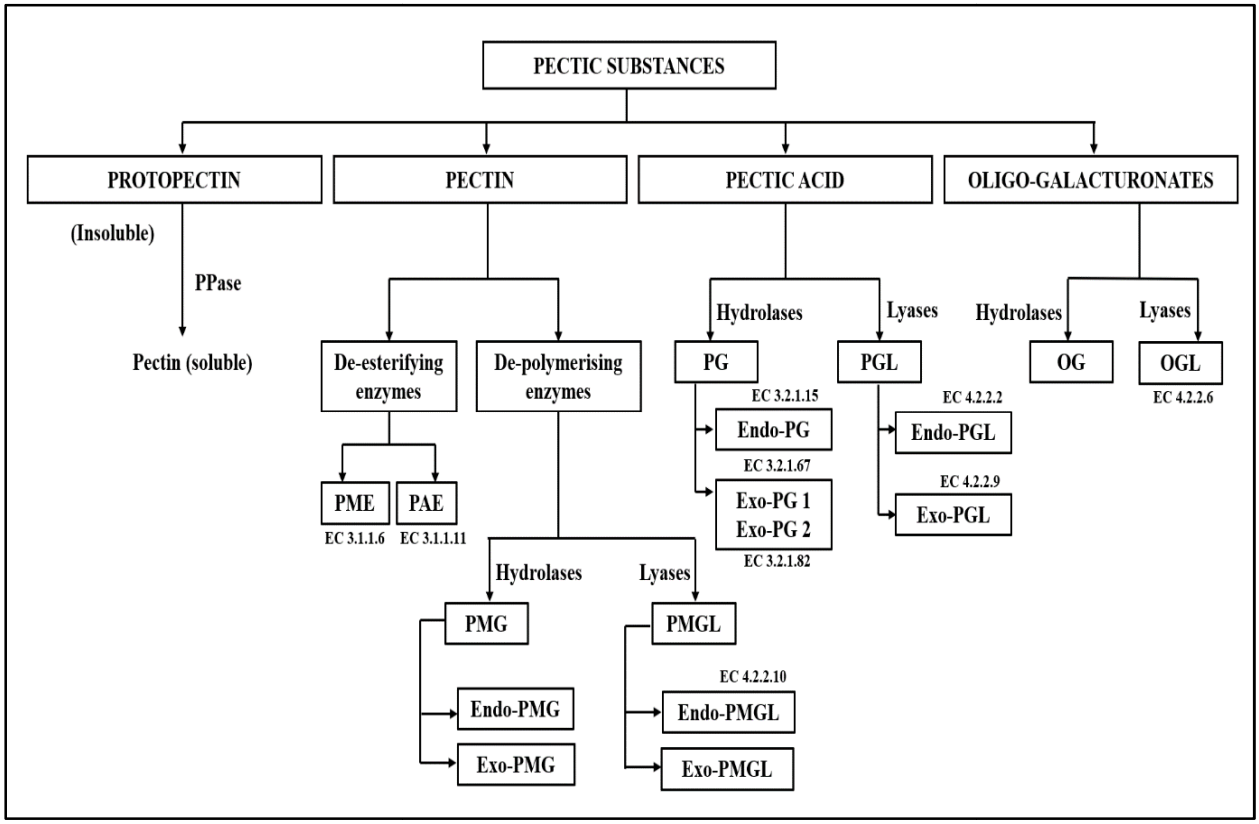

Figure 2. Division of pectinolytic enzymes acting on pectic subtances (data modified from Alkorta*et al*, 1998; Jayani*et al*, 2005; Kavuthodi and Sebastian, 2018). PMG: Polymethylgalacturonase, PMGL: Polymethylgalacturonate lyase, PGL: Polygalacturonate lyase, PG: Polygalacturonase, PME: Pectin methylesterase, PAE: Pectin acetyl esterase, OGL: Oligogalacturonate lyase, OG: Oligogalacturonase

performs penultimate hydrolytic cleavage on polygalacturonic acid to form di-galacturonate. PGL is subdivided into two pectate lyases (i) endo-PGL (EC. 4.2.2.2) performs random clevage by transelimination process on polygalacturonic acid to form unsaturated oligo-galacturonates and (ii) exo-PGL (EC. 4.2.2.9) performs cleavage of penultimate bonds from non-reducing end to form unsaturated di-galacturonates.

Depolymerases acting on oligo-galacturonates are (i)Oligogalacturonase (OG) that hydrolyse oligo-galacturonate to mono-galacturonate and (ii) Oligogalacturonate lyase (OGL) (EC. 4.2.2.6) that causes cleavage of unsaturated oligo-galacturonates by a transelimination process to form unsaturated mono-galacturonate.

Protopectinase (PPase) are another group of the pectic enzyme which is capable of converting insoluble native protopectin into soluble pectins.

Pectinolytic Microorganisms: Among the several organisms that produce pectic enzymes, microbial pectinases are the most important and widely studied for its role in degradation of plant material, plant pathogenesis and plant-microbe interaction (Lang and Domenburg, 2000). Although there are numerous reports available today on microbial pectinases, the first report on pectin degradation by the bacterium *Erwinia* sp. was produced by Elyrod (1942). Later, many reports on several bacterial, yeast and fungal species became available. Most widely studied bacterial species for pectinase production are *Bacillus* and *Erwinia*. Other bacterial genera reported for pectinolytic properties are species of *Streptomyces* (Ramirez-Tapias *et al.*, 2015), *Pseudomonas* (Sohail& Latif, 2016), *Lactobacillus* (Karam &Belarbi, 1995) etc. In case of fungi, *A. niger* is most commonly used for industrial production of pectinase. Other species of *Aspergillus* that were also reported to produce pectinase includes *A. oryzae A. fumigatus, A. terreus, A. soje, A. awamori* etc(Pedrolli*et al.*, 2009; Garg *et al.*, 2016). Further, species of *Penicillium, Fusarium, Mucor, Neurospora crassa*, *Sclerotinia sclerotiorum*were also reported to be involved in pectinase production (Pedrolli*et al.*,2009; Pedrolli& Carmona, 2014; Garg *et al.*, 2016). Only a few yeast species received attention for pectinase production and the recently reported ones are *Saccharomyces sp., Cryptococcus sp., Aureobasidium pullulans, Rhodotoruladairenensis, Kluyveromycesmarxianus, Geotrichumklebahnii, Wickerhanomycesanomalus*etc. (Alimardani Theuil *et al*., 2011; Merin *et al*., 2015; Hassan& Ali, 2016; Naumov*et al*., 2016).

Utilization of Agro-wastes for the Production of Pectinase: Submerged fermentation (SmF) and solid-state fermentation (SSF) are the two immensely used fermentation processes for the production of pectinase enzyme. SmF and SSF are applicable for bacteria, yeast and fungal species. Majority of the bacterial and fungal pectinase are alkaline and acidic pectinases respectively (Favela-Torres *et al*, 2006). In SSF process, microbes grow on the solid substrate in the absence of any liquid and fungi has the tendency to adapt to this solid substrate because of their hyphae. Therefore, fungi can even grow on cheap solid substrates like the agro wastes. *Aspergillusniger* reported 11 times higher pectinase production in SSF than in SmF. In SmF process, microbes are cultured along with nutrients in liquid broth and are most suitable for bacteria than fungi (Solis-Pereira *et al*, 1993; Graminha *et al*., 2008). The comparison between SmF and SSF process for the pectinase production by *A. niger* suggested higher production through SSF process (Patil and Dayanand, 2006). Agro-wastes are an economical and eco-friendly source of solid substrate and can be utilised for high enzyme production. Selection of microbes for a particular type of waste and optimising fermentation conditions like temperature and pH are essential for high enzyme production. List of reported microorganisms that can utilize agro-wastes for the production of pectinase along with its fermentation conditions are given in Table 4.

Apart from the agro-industrial wastes mentioned in Table 4, reports are also furnished on rice husk, papaya peel, mango peel and strawberry pomace. *Aspergillus tubingensis*produced PG by utilizing rice husk along with pectin media through SSF(Tai *et al*, 2014). *A. niger*produced PME using papaya peel which contains about 45-51% of esterified pectin (Van Alebeek et al, 2003). Dried papaya peels were used for the production of PG using *Aspergillus niger* AN07 (Patidar *et al*, 2017). Kumar *et al*, 2012 reported pectinase production by *A. foetidus* using mango peel through SSF. Chinese mushroom *Lentinus edodes* grew on strawberry pomace and produced maximum PG of 29.4 U/g (Zheng and Shetty, 2000).Some of the recent studies that provide insights into pectinase production using agro-wastes are by *B. safensis*M35 and *B. altitudinis* J208 that produced 411.58 U and 728.74 U of pectinase respectively using wheat bran and citrus peel in the production media (Thite*et al*, 2020), *A. nomius* MR103 that produced 4.83 IU/mg of PG through SSF when orange peels were used as substrate (Ketipally*et al*, 2019) and *B. subtilis* BKDS1 that produced pectinase through SmF using pineapple stem in production media (Kavuthodi and Sebastian, 2018). *Schizophyllum commune* used sweet lime peels to produce 480.45 U/ml of PL through SSF at pH-6 and temperature 35°C (Mehmood *et al*, 2019).

Application of Microbial Pectinases: Pectinase, a versatile industrial enzyme discovers its utilization in fruit juice extraction, preparation of jams and jellies, bio-scouring of cotton fibres in textile processing, degumming/ retting of plant bast fibres, wine processing, tea and coffee processing, waste water treatment, paper and pulp industry, animal feed industry, saccharification of agricultural substrates, purification of plant viruses and recycling of waste paper (Samanta, 2019). Acidic pectinases mainly produced by fungi are widely used in fruit juice clarification. Alkaline pectinases mainly produced by bacteria are used in waste water treatment, textile industry, paper pulp industry etc. Pectinases are of high demand in food processing industries compared to the other enzymes. Before processing, fruit pulp is turbid and viscous due to the presence of pectic polysaccharides. But the consumers prefer less viscous, clear and highly nutritive fruit juice for consumption. Several reports suggest that pectinase have an ability to diminish the viscosity and color of fruit juice and clarifies fruit juice by enzymatic degradation of pectic polysaccharides present in fruit pulp (Junwei*et al*., 2000; Chaudhri and Suneetha, 2012; Makky and Yusoff, 2015). It is applicable to clarify apple, orange, banana, pomegranate, mango and pineapple juice and date syrup. In the wine-making process, during the crushing of fruits, addition of pectic enzymes increases the volume of free run juice and reduces the pressing time. It also aids in juice filtration and clarification (Nighojkar*et al*, 2019).

Table 4. Utilisation of agro-waste for the production of pectinase (data modified from Garg *et al*, 2016).

Microorganism	Agro-waste substrate	Fermentation	T	pH	Enzyme activity
*Aspergillus niger*3T5B8	Wheat bran	SSF	32	-	PGA- 30.75 U/ml
*Penicillium veridicatum*RFC3	Orange bagasse, Wheat bran	SSF	30	-	PG- 55 Ug^{-1} PL- 3540 Ug^{-1}
Bacillus sp. DT7	Wheat bran	SSF	37	-	8050 Ug^{-1}
Aspergillus fumigates	Wheat bran	SSF	50	-	PG- 1270 Ug^{-1}
Aspergillus niger	Sunflower head	SSF	30	5	Endo pectinase - 19.8 Ug^{-1} Exo pectinase-45.9 Ug^{-1}
Aspergillus fumigatus MTCC 870	wheat flour	SmF	30	5	15.46 U/ml
Aspergillus heteromorphus	Orange peel	SmF	30	4.5	PME- 543units
Thermomucorindicae-seudaticae	wheat bran, orange bagasse	SSF	45	-	Exo PG- 120 U/ml
Fomes sclerodermeus	soy and wheat bran	SSF	28	-	PG- 26 Ug^{-1}
*Aspergillus sojae*M3	orange peel	SSF	22	-	PG >35 Ug^{-1}
Pseudozyma sp. SPJ	citrus peel	SSF	32	7	15092 Ug^{-1}
Aspergillus sojae	wheat bran, orange bagasse	SSF	37	6	PG-535.4 Ug^{-1}
Trichoderma viridi	orange peel	SSF	30	5.5	325 U/ml

Pectinolytic enzymes can also improve the chromaticity and stability of red wines (Revilla and González San José, 2003). Dyeing performance can be enhanced by using bio-scouring method instead of chemical method that uses chemical agents leading to toxicity. Bio-scouring ensures an eco-friendly means of removing non-cellulosic contaminants from the fibres by enzymatic treatment. It can increase the quality of the cotton in a safe manner and can also reduce the treatment time for bio-scouring using pectinase and lipase (Wang *et al*, 2007; Jayani*et al*, 2005; Klug-Santner*et al*, 2006; Kalantzi*et al*, 2010). Pectinases application extends to waste water treatment where food industry waste effluents that carries pectic polysaccharides are pre-treated with pectinases in order to reduce the cost and save time (Jayani*et al*, 2005). In paper and pulp industry, formation of sheet is an important process which gets affected due to the presence of pectins in the pulp as it causes yellowness of the paper (Garg *et al*, 2016). Bio-bleaching process can reduce cationic polymers by using pectinases in an eco-friendly manner. Finally, saccharification of agro-wastes by pectinases are used in the production of bio-ethanol (Biz *et al*, 2014).

Improvement Strategies: Several researchers reported novel strategies to improve the production, yield, properties and catalytic efficiency of pectinase. Some of the novel strategies are co-culturing of microorganisms, immobilization technique and genetic engineering techniques (Panda *et al*, 2016). Enzyme immobilization technique is defined as confining an enzyme within an insoluble matrix to prevent the enzyme from degradation and to make the enzyme stay at the targeted site for long time. This technique is of great value in food processing industries. Co- culturing of microorganisms can be defined as synergetic utilization of metabolic pathways of different specified microbial strains under aseptic condition to degrade the substrate and increase the production of pectinase. Genetic engineering strategies overcome the drawbacks like enzyme quality, productivity and substrate/ product inhibition. Recombinant DNA technology, protoplast fusion, developing mutant strains etc are few of the genetic engineering techniques used for pecinase production. Thermo alkaline pectate lyase gene (BacPelA) from an alkaliphilic *B. clausii* was cloned and overexpressed in *E.coli*. The recombinant mature BacPelA exhibited promising bioscouring properties of ramie fibres with total activity of 8378.2 Uml^{-1} (A235) and productivity of 239.4 $Uml^{-1}h^{-1}$ by high cell density cultivation in fed-batch fermentation using *E.coli*as host (Zhou *et al*, 2017).

Similarly, BliPelA gene from *B. licheniformis* was cloned and overexpressed in *E.coli*yielded 4.5 and 4.3 g/l^{-1} in 1l and 20l fed batch fermenters respectively. In another study, endo-PG from *Aspergillus aculeatus* was produced in *Pichia pastoris.* The recombinant enzyme demonstrated maximal activity of 2408.70 Uml^{-1} in the culture supernatant by high cell density batch fermentation, equivalent to 4.8 times greater yield than from shake-flask culture (Abdulrachman*et al,* 2017). An acid stable, endo-PG gene (*PoxaEn*PG28A) from *Penicillium oxalicum* was produced in *P. pastoris* and its recombinant expression yielded 1828.7 Uml^{-1}. *PoxaEn*PG28A proved its significance in fruit industries by improving the yields of fruit juices from banana, plantain, papaya, pitaya and mango (Cheng *et al,* 2017). The use of a constitutive promoter instead of the indigenous promoter of *Penicillium* greatly enhanced the production of pectinase. Attempts in the generation of catabolite repression resistant mutant strains of *Penicillium griseoroseum* by UV induction resulted in an increased pectinase production of 7.8-fold than the wild strain (Lima *et al*, 2017). Recombinant RGE27 obtained by protoplast fusion between complementary auxotrophic and morphological mutant strains of *P. griseoroseum* and *P. expansum* produced a threefold increase in PG and 1.2-fold increase in PL than the parental strain (Varavallo*et al*, 2007).Co-culturing of *B. subtilis* and *B. pumilus* achieved 11.25 IU/mL pectinase activity with apple pomace as the carbon source. Co-culturing of *Bacillus* species, achieved two-fold increase in pectinase production (Kuvvet *et al*, 2019). PG, immobilized in calcium alginate microspheres demonstrated reduction in turbidity in apple juice and also showed good thermal and freezing ability (Deng *et al*, 2019). *B. subtilis* FS105 obtained after genome shuffling exhibited an alkaline pectinase activity of 499U/ml and the molecular mechanism responsible for this activity was elucidated by aligning *rps*L gene sequences from original and fused strain. Data suggests that it was the substitution of amino acid sequences in ribosomal proteins that enhanced the biosynthesis of alkaline pectinase (Yu *et al*, 2019).

Production of enzyme is regulated by transcription factors, that can act as activator or repressor. Single point mutation (W361R) in GaaR (pectinolytic transcription factor) in *A. niger* showed constitutive production of pectinase. The endogenous gaaR gene was deleted and replaced with a DNA construct carrying a W361R point mutation (Alazi*et al*, 2019). Recent study using CRISPR/Cas9 genome editing system, to efficiently modify the functionality of transcriptional regulators in *A. niger* by generating on-site mutation in the native copy of the corresponding genes in the genome was

reported by Kun*et al*, 2020. The GaaR W361R mutant showed enhanced release of D-galacturonic acid from sugar beet pulp. This study revealed the use of CRISPR/Cas9 to generate such overproduction strains significantly reduced time and efforts compared to traditional approaches.

Conclusion

As FVW and AW are rich in polysaccharides, advances in microbial biotechnology have built an avenue towards the successful application of FVW and AW into value added products like enzymes. Pectinase and amylase, two industrially important enzymes that are on demand ceaselessly are produced through microbial bio-processing. Potency of the microbial strain can be improved with genetic engineering techniques like protoplast fusion, recombinant DNA technology, etc. Over expression of target gene in microbes can fulfil various purposes like enhancement of production, quality and catalytic activity of amylase and pectinase. More research is required to discover strains producing pectinase and amylase and their combination for more applications which could drastically reduce the cost of production. Thus, we can conclude from this review that commercial enzymes can be produced from organic waste and with proper management, greener environment in near future can be anticipated.

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