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Revisiting microbial pectinases: An understanding between structure-functional relationship in the arena of genetic engineering

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ABSTRACT

In food and beverage sector, biotechnological applications of pectinase have been increasing day by day. However, subdued production volume and low purity of the pectinase in commercial scale still remain a challenge. As a result, researchers are continuously exploring the opportunity to express modern tools such as genetic engineering, metagenomic study, and metabolic engineering to exploit microbes as a promising source for pectinases. Although this enzyme can be found naturally in plants, microbial pectinases retained a high value preference due to its easy fermentation in different bioreactors and inimitable physicochemical attributes. Microbial pectinase has immense potential to contribute in different areas such as textile industries, pharma sector, paper, and pulp industry, environmental engineering, agricultural economics in addition to that food, and beverage industries. The assertion of gene manipulation for better production of pectinase by means of elementary molecular devices and conventional fermentation procedures has been correlated in this study to get a bird's eye view in the structure-functional relationship of the microbial pectinases.

1. INTRODUCTION

In fermentation technology, enzymes are exceptionally resourceful catalysts sometimes from microbial origin which can perform a variety of reactions both *in vivo* and *in vitro*. Pectinase is one of such enzymes responsible for degradation of pectic substances. Pectic materials can be identified as an assorted group comprises principally of D-galactopy- ranosyluronic acids connected to polygalacturonic acid alpha 1-4 glycosidic linkage with a trivial quantity of L-rhamnose linked with beta 1–2 glycosidic linkage [1]. Numerous adjacent chains entailing of L-arabinose, D-galactose, and β-D-xylose can also be found alongside those glycosidic bonds. Pectinases can be obtained naturally in plants or can be obtained from different microbes. Pectic substances are one of the key components of middle lamella of plant cell walls which makes around one third weight of the total biomass of the plant (dry basis). Pectinase which is naturally exist within the plant begins to digest that middle lamella which leads to unstiffening of fruits thus called ripening. Various microbes including bacteria, fungi, and yeast can produce pectinases which are fundamentally critical due to their involvement in plant-pathogenesis, plant-microbe interaction, and degradation of dead plant materials. The commercial application for microbial pectinases is gaining world-wide attention now a days due to their adaptability in wide range of environment, broad substrate specificity, and high enzyme activity [2]. However, due to limitations in wild strains genetic engineering opening a new era for microbial pectinase production for biotechnology industries including textile, food, and feed, and pharmaceutical. [3]. In addition, different types of bioreactors are being designed to the overcome unusual limitations for the enzyme production such as ass transfer, nutrient mixing, proper aeration, and many more. Novel strategies such as metagenomic study and metabolic engineering are being introduced to get the maximum benefit of cutting-edge biomolecular tools for biotechnological advancement of microbial pectinase production [4].

Various cultural conditions such as temperature, moisture content, production time, pH, nutrient mixing, and aeration affect can limit the production of pectinase. These elements are essential for promoting, stimulating, enhancing, and optimizing pectinase production [5]. Few studies suggested to keep pH to be maintained below 5.5 to prevent pectinase degradation and above 2.6 to avoid limitation in the production of pectinase. To ensure smooth pectinase production, a sufficient number of organic nitrogen components, such as those found in peptone and yeast extract, must be added to spawn significant enzymatic activity [6]. To obtain pectinase enzymes with the required specific activity, nutrient contents may be modified with KH_2PO_4, Na_2HPO_4, $FeSO_4.7H_2O$, $CaCl_2$, peptone, $MnSO_4.7H_2O$, H_3BO_3, and citrus pectin. [7] In the solid state and submerged fermentation method, filamentous fungi are mostly used while pellet form of cultures is used in downstream fermentation process because of the very low viscosity of fermentation medium. Usage of pellets makes the mass transfer of oxygen and nutrients much efficient and also separating the pallets

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from the fermentation medium is comparatively easier [8]. Increasing the agitation rate raises the oxygen pressure in the system but does not boost output as the structure of the enzyme changes at such high agitation rates. Lowering the aeration rate, on the other hand, may result in a large drop in pectinase production yields because oxygen availability is a critical limiting factor for both growth and pectinase production. Agitation and aeration are to a lesser complex method that can be operated cost effectively when it comes to industrial pectinase enzyme production.

The current review deals with brief analysis of newly found microbial sources for pectinase production and their genetic modifications for pectinase yield improvement. The unique structure of the enzyme and their role in different functionality has also been correlated to get a proper scenario on structure-functional relationship of microbial pectinases. In final part, a concise representation has been made to identify key strategies being adapted by researchers for partial purification of microbial pectinase. In overall, a successful attempt has been made to represent the occurrence and functionality of microbial pectinases which might be helpful to design strategies for enhanced microbial pectinase production for industrial applications worldwide.

2. STRUCTURE AND FUNCTIONAL RELATIONSHIP OF PECTINASE

2.1. Classification Based on the Mechanism of Action of Pectic Enzymes

Pectic enzymes are broadly grouped in to three types based on the mechanism of action, namely, pectinesterase (PE) (which are de esterifying enzymes), depolymerizing enzymes (hydrolases and lyases), and protopectinase. PE eliminates methyl residues from pectin forming pectic acid and protopectinase solubilizes insoluble protopectin to polymerized soluble pectin. Alpha 1,4-glycosidic bonds are cleaved by the depolymerizing enzymes [5,6]. Depolymerizing enzymes have two mechanisms to act on pectin substances. One is hydrolysis when water is introduced over the oxygen bridge, they promote the hydrolytic cleavage and the other one is transelimination lysis in which they use a trans-elimination reaction to break the glycosidic bond without the use of water molecules [7]. Depolymerases are classified into four main groups: Polygalacturonase, polygalacturonate lyase, polymethylgalacturonase, and polymethyl galacturonate lyase**.** The hydrolysis mechanisms of polygalacturonase and poly methyl galaacturonase break the components such as pectate and pectin. They are broken down by elimination enzymes polygalacturonate lyase and polymethylgalacturonate lyase [8].

Classification of Pectic Enzymes Based on Site of Action

There are two types based on the site of action, they are endopectinases and exopectinases. Endopectinases cleaves pectin's internal bonds randomly. These enzymes are primarily secreted by fungi and are used extensively in fruit juice and food industry. Endo acting pectate lyase from a strain of *Bacillus pumilus* was used in cotton bioscouring [9].

Exopectinasaes cleave the long chain polysaccharides which are at the exterior. Exopoloygalacturonase enzyme was produced from Bacillus species by solid-state fermentation (SSF). It was used for wastewater treatment and degumming [10] *Fusarium* species produce exoplygalacturonase which had high enzyme activities at optimum conditions [11]

2.3. Classification Based on Site of Production of Pectinases

There are two types of pectinases rooted on the site of production which are extracellular and intracellular pectinases. Extracellular pectinases are simple to extract from the culture media. In the manufacture of extracellular pectinases, the downstream process stages are quite straightforward. An extracellular pectate lyase was discovered in a mutant strain of Bacillus tequilensis isolated from river samples, and it was discovered that it may be used in a variety of bioscouring procedures [12]. Extracellular pectinases generated by new strains such as Chryseobacterium indologenes are used in the fruit processing industry [13] Intracellular pectinases are produced within the cell, created, retained, and function inside the cell. Many stages are involved in the downstream process to destroy the cell and isolate the enzyme. The polygalacturonic acids found in deteriorating plant materials might be degraded by an intracellular polygalacturonic acid transeliminase isolated from *Klebsiella* and *Yersinia* species [14]

2.4. Biochemical Basis of the Structure Function Relationship

Following the discovery of the three-dimensional structural aspect of pectinases, the molecular basis of enzymatic action was discovered. Every single amino-acids play a function in the active regions of the enzymes; however, the structure-function connection researches of the available pectinases could be taken as the prototypes for the related family members. The pectinase(PelC) gene from *Erwinia chrysanthemi* was cloned in *Escherichia coli* and [15] explained its crystal structure. Similar configurations were revealed in other pectinase family members, of the species *Bacillus subtilis* pel [16], PelA and PelE of *E. chrysanthemi* [17,18], pectin lyasases such as PLA and PLB from *Aspergillus niger* reported in [19], the polygalacturonases of species Erwinia from [20], endo-polygalacturonase one and two from *A. niger* [21,22], respectively, endopolygalacturonase enzyme from the *Fusarium* species mentioned in the [23]. A large right-handed cylinder is a formed by each structure which is made of a single β-strand domain. The structural rules established were compatible with the parallel beta helix domain fold with novelty because of the peculiar positioning of the existing three parallel beta strands in every turn of the helix, the prism-shaped cylinder found in the center, and the complete helical turns' (seven-nine) presence. The three parallel beta sheets, namely, the PB1, PB2, and lastly PB3 are made when the strands of the successive turns line-up. PB2 and the antiparallel beta sandwich formed by PB1 and PB2 are almost perpendicular. Even if the disintegration of pectin differs between esterases, lyases, and hydrolases, the sites of substrate binding which are determined by the sequences, structural similarities, and the site-directed mutagenesis studies are all the same. The structural changes in the loops are thought to be related to enzymatic and maceration features that are subtle. There is currently no organization in place to represent the PMG family.

2.4.1. Polygalacturonases or PG

According to [24], the polygalacturonase gene in the species of Aspergillus has a length of 1107–2495 nucleotides. Polygalacturonase I was isolated from *A. niger* and contains 367 aminoacids and 1101 nucleotides. The glycoside hydrolase family 28 includes all polygalacturonases (endo or exo) (GH28). The four highly conserved areas were discovered after analyzing the sequences of aminoacid of every polygalacturonases from diverse origin. The amino acids of the mentioned four motifs are NTD, RIK G/SHG, and G/QDD, Two of these four regions (G/QDD and G/SHG) are catalytic, whereas the others (G/QDD and G/SHG) are substrate-binding. In most circumstances, the gap (amino acids number) between the preserved motifs is kept constant. It is probable that the exact spacing between the highly

conserved motifs is critical for substrate stability [25]. The anomeric structure of the product is inverted by the inversion of glycosidases by endo PG. A proton is supplied to the glycosidic oxygen by a general catalyst which is an acid, and a base with catalytic property directs the attack on nucleophile of a molecule of a molecule of water on the carbon which is anomeric of the galacturonate moiety bound at the-1 subsite, which results in hydrolysis. The crystal or lattice structures of *Stereum purpureum* native, endo PG I, and ternary output complexes containing two molecules of galacturonate showed the substrate binding process, active site construction, and reaction process [26]. In site-directed mutagenesis tests, His195, Lys228, Arg226, and Tyr262 which are charged amino acids of *A. niger* endo PG II produced a tenfold or higher increased Km values. The importance of carboxyl group specificity in subsite 1 for effective substrate binding has thus been verified. In comparison, replacing Asp173 only resulted in a twofold gain in Km but a significant loss in Kcat [27]. Asp173 aminoacid is hypothesized to act as a basic acid catalyst, giving glycosidic oxygen a proton. The substrate's strong binding to the sub site +1 is due to electrostatic interactions between carboxyl groups and the base residues, as well as the precise recognition of the epimer galactose. Endo PGs can only degrade free polygalacturonate and not substrates esterified by methyl, which explains why they can only cleave free polygalacturonate [26].

2.4.2. Poly galacturonate lyase or PGL

In the PGL family of proteins, they are thought to have a common enzymatic mechanism and the enzyme uses a beta-elimination action to cleave pectates at random, resulting in the non-reducing end with a trimer product with galacturonosyl residue consisting of 4,5-unsaturated bond [28].

Three stages are involved in β-elimination reaction of pectinolytic cleavage), which are carboxyl group neutralization at the scissile glycosidic link, C5 proton removal plus the transfer of proton to the glycosidic oxygen. R218K PGL mutant which is catalytically inert, Pel C obtained from the strain *Erwini chrysanthemi*, and the cell wall fragment of a plant (penta Galp) make up the Michaelis complex. This is one of the PGL superfamily members' structures that discloses critical insights about the enzyme process [29]. Within a single strand of pectate, the oligosaccharide to the protein is connected by the Ca^{2+} ions the surrounding uronic acid moieties are also connected the same way. The Ca^{2+} locations found differ significantly from the inter-strand Ca^{2+} ions hypothesized to connect PGA helices [7].

A protein-liganded calcium ion coordinates the −1 and +1 subsite saccharide carboxyl groups, the arginine catalytic base placed near to the alpha carbon hydrogen, and a number of other invariant enzyme substrate interactions, along with mutagenesis statistics, all link to a prevalent polysaccharide anti beta elimination path for Pectate lyase and Poly galacturonate lyase**.** The lack of homologs within pectate lyase families shows that these catalytically identical enzymes originated independently, which might be due to their different natural roles [30].

2.4.3. Pectin lyase or PL

In the same way as PGLs are cleaved by enzymes, PLs are cleaved by the same β-elimination process. PL, on the other hand, is selective for very high methylated versions of the substrate and it is not a must for calcium ions to be present for their functions. According to crystal structures, both the apo forms of *A. niger* pectin lyase A and *A. niger* pectin lyase B adopt the parallel helix fold and are physically nearly identical [31].

The substrate attachment clefts and catalytic activity appears to differ significantly in some structures of PGL and PL although they have many structural similarities in them, which may represent changes in substrate specificities.

Variability in substrate specificity is caused by two factors. To begin with, the active part cleft loops of PL are substantially longer and contain more of an intricate structure, containing two beta-strands that form an antiparallel beta sheet. Second, the presumed active site of PL enzyme features an aromatic cleft composed of tryptophans (four) and tyrosines(three), which contributes to active site's architecture. Despite the fact that these enzymes have a similar structure and function, their strategies for identifying and binding substrates differ significantly [32].

2.4.4. Pectate Lyase or PeL

The lattice structures of pectate lyase C, pectate lyase E, and pectate lyase from the strain *B. subtilis* were found to have a parallel helix which is right-handed with a big loop area. This indicated that amino acids Asp184 and Arg 279 were conserved in the family of pectate and pectin lyase. The parallel helix on the other side consisted Val-Trp-Ile-Asp-His (Val, Ile substitutable) which were conserved regions [32]. Besides from the helical structure, *Cellvibrio japonicus* and *Yersinia enterocolitica* both have unusual configurations such as (α/α) toroid and (α/α) barrel structures [33]. Pectate lyase C has 353 amino acids and two disulfide linkages, according to an early investigation and calcium ions are needed for their action [34]. There are eight coils in the parallel beta helix which is right-handed extracted from *E. chrysanthemi*, each of which has three strands which are linked by three turns, according to the precise structure. Due to the staking of the coils, three parallel sheets are created, and the structure is supported by a large network of inter-strand hydrogen bonding.

There are three main amino acids which make up the parallel helix's core which are hydrophobic, aromatic, or polar amino acids. The above-mentioned amino acids are found to be in the interior and build lengthy ladders by forming amino acids from neighboring coils in a row. The amino acids on the outside part are arranged in a random pattern, forming loops of varied lengths that extend from the core. According to Vitali *et al*. [35], sequencing investigation of the 14 extracellular pectate lyases plus the 7 pectin lyases revealed 10 unique amino acids clustered around the active region, five of these are associated to catalysis. The catalytically important amino acids are Asp131, Asp-144, His145, Thr206, and Arg218, whereas the remaining five amino acids which are Gly6, Gly12, G13, Trp-142, and Pro-220 are unreactive. Furthermore, there are two distinct clustering of amino acids: Asp131, Arg218, and Pro220 are found in the active site's Ca^{2+} binding region, while the remaining seven amino acids are present on the contrary direction of the parallel helix.

2.4.5. Pectin methyl esterase or PME

Mayans *et al*. [36] mention that pectin methyl esterase is a carbohydrate esterase from the family 8. PME had been reported to have some of the conserved and also semi-conserved amino acids just like other pectinases in the multiple sequence alignment. PME is made up of helices that are right-handed and amino acids over 80% are located in the Ramachandran plot's most favorable region. The 1–4 links of D galacturonosyl units which are the ester bonds are hydrolyzed by pectin methyl esterase, which results in galacturonosyl polymer and methanol which are negatively charged. 30–50KD was the weight of greater number of microbials pectinases [19]. The active site and the cleft that the substrate joins in PME's are similar to that of PGL and PL, as a result, many aromatic amino acids surround the core region of the substrate binding cleft, in which Asp136 and Asp 157 are found

in the center of the active site. The function of Asp136 and Asp157 in PE action was shown by [37]. During the first cleavage phase during methanol releases, Asp157 attacks the carboxy methyl carbonyl carbon with a nucleophilic assault, while Asp136 may operate as a proton donor (acid). Later, Asp136 absorbs the hydrogen atom which is coming from the molecule of water. This breaks the bond between the enzyme and substrate which allows the enzyme to reactivate its active site.

3. LARGE-SCALE PRODUCTION OF MICROBIAL PECTINASE IN BIOREACTORS

Fermentation process needs to have a proper relationship with the microbe physiology and many of the fermentation factors; pH, temperature, aeration, moisture content, and the solid substrate used among others. Scaling up the SSF process and estimating biomass has become a big difficulty, prompting academics to work tirelessly to find answers. A variety of bioreactors have been developed that can help with scale-up fermentation process and, to some extent, online tracking of numerous factors. The modern bioreactors control the main difficulty faced in a traditional fermentation system which is heat and mass transfer [38,39]. Table 1 shows how various bioreactors are used along with specific microbes in pectinase production.

Figure 1 shows the commonly adopted different fermentation strategies for microbial pectinase production in general process.

4. PECTINASE PRODUCTION FROM WILD TYPE AND GENETICALLY MODIFIED ORGANISMS

4.1. Overview of Microbial Sources

Pectinase enzyme claims 10% from all manufacturing enzymes throughout the globe and out of all food enzymes, 25% of them are microbial pectinases. Pectinase enzymes can be obtained from higher plants and microorganisms both. However, microorganisms are mostly preferred in large scale production. Several microbes, for example, fungi, yeasts, bacteria, actinomycetes, and protozoan are involved in production of pectinases. Decaying plant tissue is the most common substrate for these microorganisms [33]. Production of microbial pectinase is more effectual due to usage of low-cost recyclable raw materials such as agroindustrial wastes, ease of production, and consist of certain physiochemical properties. Microbial pectinases are a main part of plant and microbe symbiosis, studies of diseases in plants caused by pathogenic microbes and dead organic material decomposition. There are several types of pectinolytic enzymes which have different molecular mass and kinetic properties (discussed in the previous section of this manuscript). Microbes which produce several pectinase enzymes are more efficient toward plant infection and degradation [54]. These pectinolytic enzymes are expressed either by one or several genes present in microbial genome. Production of the polygalacturonases is one of these examples. In *Fusarium moniliforme* (a pathogenic fungi), it is expressed by one gene but in *A. niger*, the same is encoded by a family of several genes [55]. In sixteen different growth conditions, *A. niger* reported the expression of 26 penctinolytic genes and this has been used for making a profile for each pectinolytic gene in the fungi [56]. Bacteria, fungi, or other genetically modified organisms capable of producing pectinases are called pectinolytic organism. Among then, pectinolytic bacteria are easily available, have short lifespan, environmentally friendly, and cheap source of production of pectinases [1,2]. Pectinolytic bacteria can withstand high pH and temperatures also [3,57]. Bacterial pectinases are slightly alkaline or neutral in pH. As a result, they are the better source for industrial applications. *Bacillus sp*., *Pseudomonas sp*., and actinomycetes are main sources of alkaline pectinases [3,58,59]. Bacterial enzymes are extracellular products so they can be easily

Table 1: An outline of various bioreactors used in pectinase production.

Type of bioreactor	Micro-organism used	Substrate	Principle	Yield	References
Pilot scale packed bed bioreactor	*Aspergillus niger*	Wheat bran, sugar cane bagass	SSF	1840 U/kg of dry solid per h	[40]
Pilot scale packed bed bioreactor	*Aspergillus niger*	Wheat bran, sugarcane bagass	SSF	22U/g	[41]
Packed bed bioreactor with recycled flow for continuous production	*Kluyveromyces marxidnus*	Spent grain	SSF	Volumetric productivity P (v)=0.98 U/ml/h	[42]
Pilot scale Packed bed bioreactor	*Aspergillus Oryzae*	Citrus pulp waste, sugarcane bagass	SSF	33 to 41 U/g	[43]
Column tray bioreactor	*Aspergillus niger*	Lemon peel pomace	SSF	Approx. 2181 U/l	[44]
Tray bioreactor	*Aspergillus sojae*	Bran of wheat	SSF	298 U/g substrate	[45]
Surface culture bioreactor	*Aspergillus niger*	Pectin	SSF	Exo-PGase and Endo-PGase 1.5 and 0.014 U/ml respectively.	[46]
Double surface bioreactor	*Aspergillus niger*	Wheat bran	SSF	45 U/g	[47]
Rotatory drum bioreactor	*Aspergillus niger*	Rice bran	SSF	4 U/g	[48]
Rotatory drum bioreactor	*Aspergillus niger*	Sugarcane bagass, orange pomace	SSF	ND	[49]
Stirred Tank bioreactor	*Aspergillus flavipes*	Orange peelo	Submerged fermentation	Exo 670.7 and endo 28.2 U/l/h batch fermentation	[50]
Packed bed and tray-type	*Aspergillus awamori*	Grape pomace and orange peel	SSF	4 IU/g	[51]
Flask reactor	*Bacillus sp. DT7*	Wheat bran, rice bran and apple pomace	SSF	8050 U/g	[52]
Flask type	*Aspergillus tubingensis*	Papaw peelings	SSF	246.83 U/g	[53]

obtained [60]. Bacillus and Cocci species account for approximately half of commercial enzyme production [61,62]. Bacterial strains are easy to modify so enzyme technologies are applied on these to produce high yield of pectinase enzymes through genetic modifications [63]. Thirty strains of *Bacillus polymyxa* were used for production of pectin and pectic acid as of the first *in vitro* fermentation studies that reported back in 1955 [64]. Some other examples of bacterial sources are *Pseudomonas solanacearum* (Schell *et al.* 1994), *Lactobacillus lactis* subsp. Cremoris [65]**,** *E. chrysanthemi* B341 [66], *Lachnospira pectinoschiza* [67], and *E. chrysanthemi* 3604 [68]. Pectinase from fungi is commonly used in fruit juices preparations because their pH value is also similar to many fruit juices which ranging from pH 3–5.5 [69,33]. In this sector, almost 90% enzyme is extracted using molds such as *Trichoderma harzianum*, *A. niger*, and *Rhizopus* into the culture medium [70]. However, rather than fungi, bacteria are used in industrial application, because fungi tend to secret several enzymes with pectinolytic enzymes which lead to turbidity and resulting high cost in production process. Some of examples major of pectinolytic fungi are *A. niger* [71,72], *Phytophthora infestans* [73], *Penicillium frequentans* [74], and *Penicillium occitanis* [75]. Other than that pectinase enzyme is also found in yeast such as *Rhodotorula* sp**.** [76], *Saccharomyces cerevisiae* [77]**,** and nematodes, protozoan, insects, and higher plants.

4.2. Arena of Genetic Engineering for Commercial Microbial Pectinase Production

First, commercial microbial pectinase was produced in 1930 by Kertesz for apple juice clarification. Commercialized production of microbial pectinase is being continuously improved through using low-cost raw materials as substrate, multisteps screening processes of microbes, implementation of novel technologies strain improvement, modified fermentation techniques, etc. [33,78]. For the production of commercially available pectinases involved these microorganisms such as Pectinex SP-L, Novo Nordisk Pectinex TM, Pectinex SP-L [79], and Pectinex 1XL at several industries like CCM International Ltd and Carolina Biological Supply Company [80]. The production can be either submerged or SSF [81]. SSF technique can be applied with *Aspergillus awamori* [63,82], *B. subtilis* [83], and *Penicillium viridicatum* [84] whereas submerged fermentation is mostly applied in production of xylano-pectinolytic enzymes from *B. pumilus* [85]. SSF technology is easier and simpler, requires lower space and low cost, and it also improves the conditions of higher filamentous fungi which grow on solid wet substrates and their spores become more stable, resistant to drying, and even after freeze drying, they have higher germination period of time compared to submerged fermentation [86].

Some high yielding natural isolates are *Xanthomonas*, *Pseudomonas*, *Erwinia*, *Streptomyces* sp. QG-11-3, *Thermotoga maritima*, *A. niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Rhizopus stolonifer*, *Mucor racemous*, *Mucor hiemalis*, *Penicillium jenseni*, *Penicillium citrinum,* and *Trichoderma viride* [87,88]

As the main target of strain improvement is to produce high yield of pectinase enzyme, to achieve this outcome can use methods such as recombination/gene transfer, genome shuffling, mutations, protoplast fusion, and other genetic engineering techniques [4] Recombinant technologies are often used to produce strains that have high expression of certain protein and it leads to high purity of enzyme which might be industrial point of interest. Various expression hosts such as *E. coli, S. cerevisiae, and Pichia pastoris* are used to produce highly efficient enzymes using their promoter control in recombinant technology. These enzymes tend to have more yield, tolerance to high temperature, and pH which can be useful in various applications. Those examples of recombinant sources produce higher yield of pectinases than wild type strains. In addition, other than physical-induced mutations such as UV-induced spontaneous mutations have been reported in *Penicillium griseoroseum* mutant strains which leads to 7.8-fold greater yield than the normal type [89]. Some recent findings showed that that Pel (BacPelA) gene from *Bacillus clausii* expressed in *E. coli* can increase the pectinase production up to 8378.2 U/ml [90] whereas endo polygalacturanase acid stable gene from *Penicillium oxalicum* and *Aspergillus aculeatus,* respectively, expressed in *P. pastoris* can increase the pectinase production up to 1828.7 U/ml [91] and 2408.7 U/ml [92], respectively. Similar trend was observed during expression gene encoding polygalcturonase from plasmid pAN52pgg2 in *P. griseoroseum* resulting 266-fold greater production of pectin lyase and 27-fold greater times of polygalacturonase than the normal strain [93].

To study about the mechanism and active site of pectate lyase, several sites directed mutagenesis experiments have been used in strains of *E. chrysanthemi* and *A. niger* [94]. In addition, studies related to mutagenesis reported substrate bindings to Ca^{2}+ ions as a complex and absolute requirement for pectate lyase [95]. Further studies showed that single mutations in *Xanthomonas campestris* have been also initiated to yield thermostable pectate lyase enzymes and directed studies used on producing 12 mutants of pectate lyase, which are more tolerant to the higher temperature than parental strains [96]. Site directed mutagenesis based on polygalactauronidase used to indicate sites of histidine and aspartic acid amino acids which are part of enzyme activity using recombinant stains of *S. cerevisiae* in *P. pastoris.*

Another couple of simple yet powerful new techniques are genome shuffling and protoplast fusion. For high pectinase production, accelerating the process of directed evolution by promoting the recombination among diverse mutants with improved genotypes for breeding is known as Genome shuffling [97]. In this experiment, Patnaik and his colleagues took natural strain *B. subtilis* ZGL 14 and treated them with UV and 60Co-γ irradiation and resulted mutated strains of *B. subtilis* UV12, UV10 and UV-S45 (resulted from UV treatment), and *B. subtilis* C-S50 and S9 (obtained from 60Co-γ irradiation) which has higher alkaline pectinase activity than the natural strain *B. subtilis* ZGL 14. Then, used these transformed colonies and natural strain of *B. subtilis* ZGL14 as parental strains for genome shuffling, at the end of the first round of genome shuffling, 106 colonies were selected and resulted in 30% of enhancement in production of alkaline pectinase production. After that 150 colonies were obtained at the end of the second round of genome shuffling and screened. Finally, it resulted in nine mutated strains. Among them, *B. subtilis* FS105 had the highest enzymatic yield of alkaline pectinase and it was 1.6 times higher than the natural stain *B. subtilis* ZGL14.

Aspergillus carbonarius is a fungus which is well known for production of high yield of pectinase but on the solid substrate of wheat bran, due to the weak colonization, it shows low pectinase production. To overcome this [4] carried out a strain improvement process to obtain high yield of pectinase production by interspecific fusion of protoplasts of *A. carbonarius* and *A. niger* resulted mutant strain which has capability of producing high yield of pectinase enzyme because due to the genetic recombination occurred during the protoplast fusion, the genetic character which reasons higher production of pectinase has acquired from the *A. niger.*

In the category of chemical mutagenic agents, a study by [98], *Aspergillus tamarii strain RMLC-10*, which was isolated from spoiled

chicku subjected into sodium azide, nitrous acid, EMS treatment, and UV treatment. Among these highest enhanced activity of polygalacturonase production was detected by *Aspergillus tamarii mutant strain SA-11* after subjecting conidial suspension to the 1% (w/v) sodium azide solution for different periods of time. In this case, sodium azide had directly affected the genetic composition by functioning on precursors in metabolic pathways of particular fungal strain and resulted enhanced production of polygalacturonase enzyme [99,100] found a successful way to enhance the activity of pectinase enzyme after subjecting *Penicillium notatum* strain *MH-61* to nitrous acid and EtBr (ethidium bromide) by following the previous experiment of [101]. According to his methodologies at first, centrifuged spore suspension of *P. notatum* strain MH-61 washed with phosphate buffer and then treated with sodium acetate. Second, different concentrations of sodium nitrate solutions were added, and after 10 min, phosphate buffer was added to stop the reaction. As nitrous acid has the ability to remove amino groups from Adenine, Cytosine, and Guanine, it leads to oxidative deamination of nitrogenous bases [102]. To increase pectinolytic activity of an enzyme, *P. notatum MH-61* was treated with (0.5 mg/ml) ethidium bromide resulting in nine mutant strains. The highest enzyme activity was detected from *P. notatum MH-EB9* mutant strain and it was enhanced by 1.84 folds as compared to the

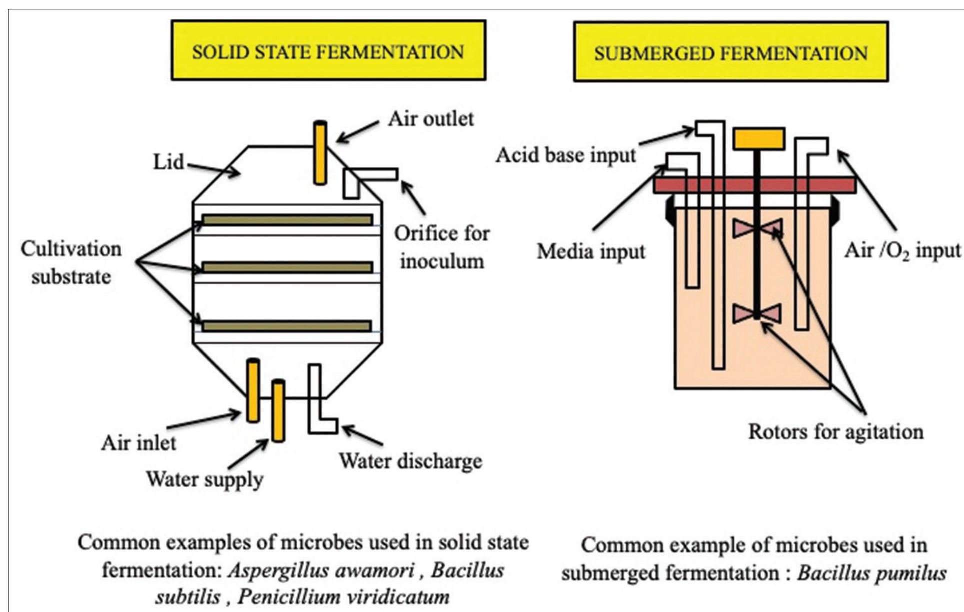

Figure 1: Different fermentation strategies for microbial pectinase production.

Table 2: Novel technologies for enhanced pectinase production.

Targeted host/sample for study	Genetic engineering approach	Observation/Findings	References
Rumen of diary-cow	Meta genomic analysis	Identification of pectinolytic microbes like *Ruminococcus, Bacteroides sp., Prevotella sp*. etc.	[105]
Sheep	Meta genomic analysis	Presence of pectinase producing genes of *Fibrobacter, Bacteroides, Butyrivibrio*, and *Prevotella*	[106]
Soil	Meta genomic analysis	Opening reading frame expressed in *E. coli* which is similar to *Bacillus licheniformis* which process under wide range of temperature and pH	[107]
Recombinant expression of pelB gene in *E. coli* from soil meta-genome	Meta genomic analysis	Better bioscouring agent for textile industry	[108]
Thermophilic compost meta-genome	Meta genomic analysis	Presence of pectin lyase, polygalacturonas, arabinofuranosidase and galactosidase which are pectin degrading enzymes	[109]
Mutant strains of *P.expansum* and *P.griseoroseum*	Metabolic engineering	Use of calcium ions and Polyethylene glycol resulted in recombinant RGE27 with 3-fold and 1.2 fold increase in polygalacturonase and pectin lyase	[110]
First 6 signal peptides in *Bacillus subtilis*	Metabolic engineering	poly galacturonidase production with comparative and screening analysis resulted in Efficient poly galacturonidase secretory expression with yield of 313.7 U/ml	[111,112]
Bacillus subtilis	Metabolic engineering	Use of Shine-Dalgarno sequence and strong P43 promoter resulted in increase of poly galacturonidase to 446.3 U/ml	[112,113]
Bacillus subtilis	Metabolic engineering	Use of fed batch fermenter techniques resulted in 17.6 U/ml/h productivity and 632.6 U/ml yield	[113,114]

wild strain's activity due to the planar structure of EtBr gets inserted between nitrogenous bases of DNA as it known as an intercalating agent [102]. In another similar experiment conducted by [103], *A. niger* was subjected to ethidium bromide (6 mg/ml) treatment for 60 min and resulted in 1.44 times enhanced yield of pectinase as compared to parent strain. The experiment of [102] shows contradictory results when comparing to the experiment of [103] The reason might be the *P. notatum* used in the experiment of [102] was more prone to mutagenesis by ethidium bromide even at low concentrations.

In the category of physical mutagenic agents, experiment which was carried out to study the effect of UV Light in strain improvement of *P. notatum* MH-61 by [100], the particular fungal strain was subjected to UV treatment for different intervals of time. At 60 min of UV exposure, the growth of fungal strain was completely inhibited. Repeatedly carried out the experiment for several rounds and resulted in five mutant strains. The mutant strain *Penicillium notatum* MHUV9 was detected for the highest activity of pectinase enzyme and it was i.e., 5.1 ± 0.10 U/ml/min. Munir and his colleagues were followed the procedure mentioned in Shakibaie et al., 2018 and resulted seven mutant variants of *Aspergillus tamarii RMLC strain*, after 1 mL of conidial suspension was subjected to UV light for different time of exposures [98]. Haq *et al*. [104] carried out an experiment to reveal the possible mechanisms and pathways that are reasonable to the enhanced activity of production of pectinase enzyme by *A. niger mutant strain EIMU2* and it was the result of mutation occurred in wild type *A. niger EIM-6* after exposing to the UV treatment. In this experiment, to achieve comparative proteomics of wild and mutant strains after extracting crude enzymes from two strains, they have respectively followed the steps of filtration, precipitation, dialyzation, and concentration. Then, performed Bradford method for quantitative assay and resulted highest concentration of enzyme from *A. niger mutant strain EIMU2* which achieved from UV mutagenesis of wild type *A. niger EIM-6* and it was 8.92 lg/lL. The different dilutions

Table 3: Purification strategies for microbial pectinase.

Microbial source of pectinase	Purification strategy	Specification	Result	References
Trichoderma viridi	Ammonium sulfate precipitation	Gradual precipitation from 30% up to 90% in 5% increments followed by dialysis using 0.01 M Tris-HCl buffer	97.2 U/mg specific activity	Irshad *et al*., 2014 [115]
Streptomyces chartreusis	Ammonium sulfate precipitation	Gradual precipitation followed by dialysis using 0.08% Sodium azide	42.09 U/mg specific activity	Patel *et al*., 2021 [116]
Bacillus subtilis	Ammonium sulfate precipitation	Gradual precipitation up to 65% followed by dialysis using phosphate buffer	217.44 U/mg specific activity	Takcı *et al*., 2016 [117]
Bacillus tequilensis	Ammonium sulfate precipitation	Gradual precipitation up to 70% followed by dialysis using 2% NaHCO3, 0.05% EDTA	1.5% (w/v) pectin concentration	Koshy *et al*., 2019 [118]
Penicillium cyclopium	Aqueous two-phase system	$(NH_4)_2SO_4$ has been used as the salt while polyethylene glycol 4000, polyethylene glycol 1000, polyethylene glycol 1500, polyethylene glycol 6000 polyethylene glycol 10 000 and fractionated dextran (Mr~500 000) used as the polymers	Purification factor of 1.28 for the endo-pectinase activity	Prodanović *et al*., 2008 [119]
Polyporus squamosus	Aqueous two-phase system	Polyethylene glycol 4000 (PEG) as the polymer against a crude dextran	Partition coefficient of 2.45 for endo pectinase	Antov, 2004 [120]
Aspergillus oryzae	Aqueous two-phase system	Micellar Triton X-114/sodium phosphate	20% concentrated crude extract (wt/wt).	Jaramillo *et al*., 2013 [121]
Aspergillus niger	Aqueous two-phase system	First alcohol and salt ATPS with different ratios such as 16/16, 18/20, 20/20 and 24/22 (by mass)	51.4%	Trentini *et al*., 2020 [122]
Aspergillus niger	Aqueous two-phase system	PEG - Potassium phosphate buffer with 16, 18, 20 and 28% (by mass)	4.8%	Trentini *et al*., 2020 [122]
Rhizopus oryzae	Sephadex size exclusion chromatography method	Sephadex G-25 column	NS	Hamdy, 2005 [123]
Hylocereus polyrhizus	Sephadex size exclusion chromatography method	Sepharacryl S-100 column	NS	Amid *et al*., 2014 [124]
Aspergillus niger	Sephadex size exclusion chromatography method	Sephadex G -100 column	NS	Mehmood *et al*., 2019 [125]
Schizophyllum commune	Gel method	SDS PAGE method	355 U/mg specific activity	Ahmed *et al*., 2016 [126]
Hylocereus polyrhizus	Sephadex size exclusion chromatography method	Sephadex G-75	NS	Khatri *et al*., 2015 [127]
Aspergillus niger	Affinity Precipitation	Microwave treated alginate incubated with Pectinex Ultra SPL and mixed with acetate buffer followed by sephadex G-25 separation	83% recovery	Mondal *et al*., 2004 [128]
Aspergillus niger	Affinity Precipitation	Magnetic latex beads for absorption of enzyme then desorbed using carbodiimide coupling	81% recovery	Tyagi *et al*., 1995 [129]

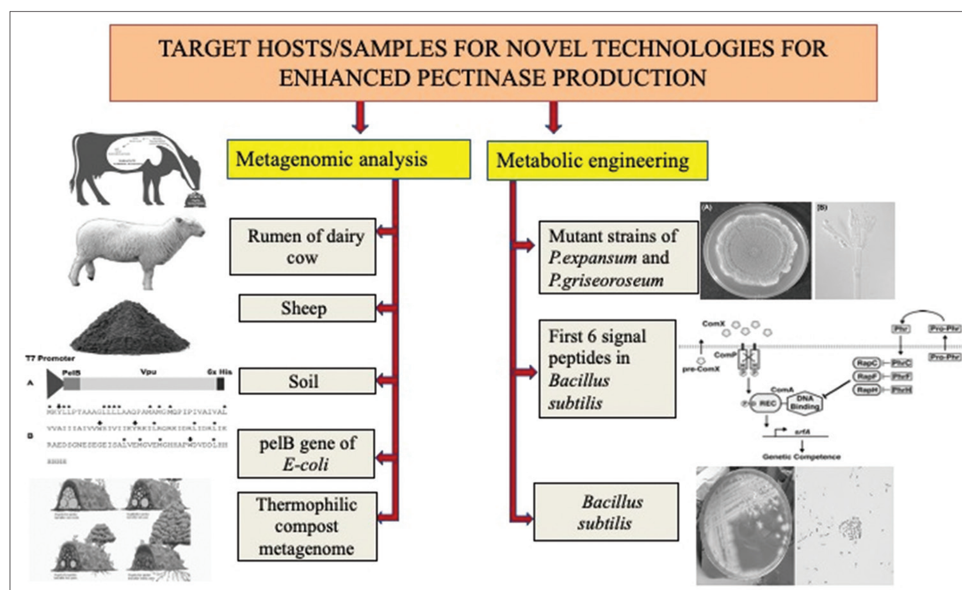

Figure 2: Novel strategies for enhanced pectinase production.

of cell suspensions of soil fungi *A. niger* were prepared and exposed to UV germicidal lamp (256 nm). Survived microbial colonies were isolated and screened to determine the improvement of the pectinase enzyme yield [103]. In the past decade, novel technologies considered with microbial pectinase production have been modified with cutting edge molecular biology tools to produce more efficient pectinolytic enzymes with specific characteristics and to produce more affordable sources of pectinases than the commercial fungal species. As a result, hyper active and thermos-tolerant pectinases which are made due to changes in primary structure are being produced by protein engineering and *in vitro* evolution techniques. Table 2 showed some of these novel technologies along with their observations where metagenomics and metabolic engineering approach has been introduces for enhance pectinase production. Table 3 suggests assorted references and their preliminary purification strategies for pectinases for specific microbes after fermentation. Figure 2 shows the commonly adopted different novel strategies for enhanced production strategies for microbial pectinase in general process.

5. CONCLUSION AND FUTURE PERSPECTIVE

The enzymes from biological origins are substituting conventional chemicals in food and pharma industries as they reduce the energy demand for substrate transformation and do not produce any greenhouse gas in the nexus of global warming to carbon neutrality. Microbial enzymes especially pectinase are being applied in various industries (textiles, leather, paper and pulp, research and development, pharmaceutical, agriculture, detergent, waste, biorefineries, photography, and food industries), thus making them very essential in several industrial production processes. Microbes are being preferred by the researchers as their survival capability in harsh conditions, high growth rate, low space requirement, independent to seasonal variations, and many more. To meet the ever-increasing demand, conventional optimization procedures are still in process and seem favorable for pectinase cocktail production. However, as discussed throughout the manuscript – the forthcoming enzyme biotechnology relying on genetic engineering centered optimization strategies. Although microbes are exceptionally suitable for valuable metabolite products, they typically make them for their personal benefits – thus, avoiding overproduction of the metabolites. In strain improvement, genetics have had a protracted narration of contributing diverse microbial products in the form of mutagenesis and screening/selection for high yield microbial strains and recombinant DNA technology. In addition, novel strategies like metagenomic study showing promising results in which expression vectors from existing diversified wild strain microbes can be protracted to screen metagenomes particularly in archaebacteria for high value pectinase production. Microbial pectinases can also be produced through unconventional metabolic engineering (e.g., proteomic study) practices to integrate novel properties thus leading to augmented impending marketable application. In this way, the expedition for novel tactics for enhanced microbial pectinases production shall indubitably be the utmost imperative field for the future study in terms of industrially important enzymes.

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

No conflicts, informed consent, or human or animal rights are applicable to this study.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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