Chapter

Proteolytic Enzymes Derived from a Macro Fungus and Their Industrial Application

Nagendra Kumar Chandrawanshi, Deepali Koreti, Anjali Kosre and Ashish Kumar

Abstract

Proteolytic enzymes are well known for catalyzing hydrolytic reactions. These enzymes fall under the group of large and complex, also known as proteases. Proteolytic enzymes mainly derived from microbial origin are favored because they have a short generation time, ease of genetic manipulation of microorganisms, and the availability of diverse species in nature. Macro fungi are significant and played an excellent role in degrading lignocellulosic compounds, such as mushrooms. They efficiently degrade cellulose and produce extracellular enzymes such as xylanases, cellulases, and ligninolytic enzymes. Furthermore, proteases play a significant role in fungi physiology, such as metalloproteinase, subtilases, aspartate, etc. Many worldwide researchers have reported the mycelial secretion of proteases from basidiomycetes. Thus, many protease extraction methods have been developed from the various categories of mushroom species, i.e., Pleurotusostreatus, Phanerochaetechrysosporium, Schizophyllum commune, Chondrostereumpurpureum, and Hypsizygusmarmoreus, etc. Furthermore, there is a high demand in the industry for specific proteolytic enzymatic activity. Numerous species of mushrooms have not been explored to date for the optimization and production of enzymes. Therefore, further detailed studies are required to expose the production mechanisms and application of proficient proteolytic enzymes from mushrooms. The present chapter will deliberately deal with proteolytic enzymes downstream processing and their various industrial applications.

Keywords: proteolytic enzymes, Basidiomycetes, macro fungi, mushroom, industrial application

1. Introduction

Enzymes are natural catalysts that evolve or require various biological processes and are utilized in various industrial applications. Scientists have recently focused on detecting new enzymes with various properties and best-suited commercial purposes [1, 2]. There are many advantages associated with industrial enzymes, such as reaction specificity, low energy needs, biodegradable sources such as plants, animals, and...
Hydrolases

microbes used for enzymes production and isolation. Proteases are the best studied and utilized in a group of enzymes that have the best substrate specificity. Total enzymes are produced at the industrial level, of which one-third are hydrolyses, and 65% are proteases. Proteases are hydrolytic enzymes that catalyze the interruption of the polymerization of protein. It evolves in the metabolic processes of biological activities in almost all organisms [3–6].

Different microbial sources have produced different proteases than plants and animals; microbial enzymes are more labor-intensive and best suitable for industrial applications [3, 7, 8]. Approximately two-thirds of commercial protease is produced from microbial origin in the world [6]. Microbial proteases production has advantages: short generation time, high growth rate, high yield, genetic modification is possible, cost-effective, and easy availability. These properties made microbial protease the best choice for biotechnological and industrial applications [9, 10].

Much research has been conducted to isolate and purify proteases from microbial sources and wieldy applied in industrial sectors [1, 11]. Bacteria are the most prominent microbes used for industrial-level protease production. Some groups of basidiomycetes also reported having proteases, and they provide the way for further study, fungal protease is neutral, acidic, or alkaline protease according to the species of fungi [12]. Fungi proteases have easy cell separation techniques, and a study revealed that micromycetes proteases have specific characteristics. Several fungal species include Aspergillus species, Fusarium graminarum, Chrysosporium keratinophilum, Penicillium chrysogenum, Pgriseofulvin, Scedosporium apiospermum, and Trametes cingulata. Of all these species, Penicillium and Aspergillus are the most widely studied [12–14].

Basidiomycetes are important wood-degrading fungi in biological communities, and some genera of this group have been used as a food source. They are well studied for their extracellular enzymes production properties, such as xylanases, cellulases, and ligninolytic enzymes [15]. Proteases play essential roles in the biochemical process in fungi and the essential completion of the life cycle [16]. Mushrooms are the known Basidiomycetes in the fungi group. They have been used as food products for centuries as well as reported for their biological activity, among which, species of Pleurotus are globally known and valued as good food source and ease of cultivation. Mushroom bioactive compounds such as protein, vitamins, and enzymes, etc., are also examined for their biological activity such as antitumor, anti-inflammatory antidiabetic, antiviral, antioxidant, hypocholesterolemic, antitumor, immunomodulatory, and hepatoprotective actions [17, 18]. They are a good source of vitamins, protein, minerals, and very low fat content. They contain a variety of bioactive compounds, including protease, and there are more than 20 proteases that have been isolated [17, 19, 20].

Thus, the isolation of new proteases from different mushroom species is a novel area of research that needs much exploration. There is still much progress required for the study of proteases from edible mushrooms and has great future opportunities in the area of genome, proteome, and metabolome of mushroom proteases. Also, open new research relates to exploring downstream processing and economic aspects of mushroom proteases.

2. Classification of proteolytic enzymes or proteases

Proteolytic enzymes significantly participate in the metabolism of organisms such as plants, animals, bacteria, fungi, and viruses. Proteases are not explored and are
essential in enzymology because of their substantial physiological significance and broad application in research activities [4]. Since proteolytic enzymes are requisite in providing nitrogen to xylotrophs under natural growth conditions (on living and dead wood), the absence of sufficient systematic information on secreted proteases of higher xylotrophic fungi is unnoticeable yet in biology. Research studies have been conducted to isolate and characterize proteolytic enzymes from the cultured mycelium and fruit bodies of basidiomycetes. Highly diverse types of structures and mechanisms of action, so proteases are not set aside with the rules of enzyme nomenclature [14]. So, the classification of these enzymes is often difficult. The enzyme that enters through the plasma membrane inside the cell is usually called an extracellular enzyme [6]. It must be classified into two categories according to their ability to cleave the peptide bonds as exopeptidases and cleave specific sites of peptide bonds as endopeptidases. They are industrially essential enzymes [21]. The diversity and specificity of these native enzymes are based on their broad characterization and isolation (Table 1). Based on active site present on proteases, they are classified as follows:

2.1 Exopeptidases

Exopeptidases are an enzyme that cleaves at the end site and requires free terminal groups close to the bond. It catalyzes the breakdown of specific peptide bonds after the carboxyl or amino terminals in the protein. Based on their efficiency in identifying the active site as either C or N terminal, they are further divided as carboxypeptidases or amino peptidases [36].

2.1.1 Aminopeptidases

Amino peptidases are the class of proteases enzymes that precisely cut at the N-terminal of the amino acid polypeptide chain, breaking it into dimer fragments or a single amino acid residue. After the recognition, they further remove the present methionine N-terminal of the polypeptide chain, which may differ in their expression. It is found in various microbial strains, including basidiomycetes fungi, molds, and bacteria, etc. Overall, amino peptidases work as intracellular enzymes; however, as per a report studied, amino peptidases that originated from Aspergillus oryzae fungal species are extracellular enzymes [3, 4].

2.1.2 Carboxypeptidases

This enzyme performs its catalytic reaction on the C-terminal of the amino acid chain, breaking peptide bonds into monomers form. These are not predominantly recognized as endopeptidases because they leave few amino acid molecules at the target site of the protein. Instead, it can be employed to eliminate the additional tags at the carboxyl-terminal of the target protein. Among specific peptidases, metallocarboxy protease, type A carboxypeptidase, is known primarily for removing amino acid of the aromatic side chain while type B acts on essential amino acids [37].

2.2 Endopeptidases

Endopeptidases act at specific site of the peptide bond of the substrate [36]. It cleaves the internal peptide bonds of proteins influenced by the existing functional group present on the active site of the peptide chain. It is further classified as follows:
<table>
<thead>
<tr>
<th>S N</th>
<th>Mushroom</th>
<th>Enzyme/protease</th>
<th>Cultivation condition</th>
<th>Method of purification/isolation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Pleurotus ostreatus</em></td>
<td>Laccase isoenzymes</td>
<td>Liquid culture</td>
<td>Polyacrylamide gel electrophoresis</td>
<td>[22]</td>
</tr>
<tr>
<td>2.</td>
<td><em>P. eryngii</em></td>
<td>Pleureryn</td>
<td>—</td>
<td>Ion exchange chromatography</td>
<td>[18]</td>
</tr>
<tr>
<td>3.</td>
<td><em>P. eryngii</em></td>
<td>Eryngeolysin</td>
<td>Fruiting body</td>
<td>Ion exchange chromatography</td>
<td>[23]</td>
</tr>
<tr>
<td>4.</td>
<td><em>P. citrinopileatus</em></td>
<td>Fruiting bodies</td>
<td>Ion exchange chromatography</td>
<td></td>
<td>[16]</td>
</tr>
<tr>
<td>5.</td>
<td><em>P. ostreatus</em></td>
<td>Fibrinolytic protease</td>
<td>Mycelia culture</td>
<td>SDS-PAGE</td>
<td>[24]</td>
</tr>
<tr>
<td>7.</td>
<td><em>P. ostreatus</em></td>
<td>Metal-dependent proteinases</td>
<td>Fruiting body</td>
<td>Ion exchange chromatography</td>
<td>[26]</td>
</tr>
<tr>
<td>8.</td>
<td><em>P. nebrodensis</em></td>
<td>Nebrodeolysin</td>
<td>Fruiting body</td>
<td>Ion exchange and gel filtration chromatography</td>
<td>[27]</td>
</tr>
<tr>
<td>9.</td>
<td><em>P. eryngii</em></td>
<td>Hemolysin</td>
<td>Fruiting body</td>
<td>SDS-PAGE</td>
<td>[28]</td>
</tr>
<tr>
<td>10.</td>
<td><em>P. eryngii</em></td>
<td>Fibrinolysis</td>
<td>solid-state conditions</td>
<td>SDS-PAGE</td>
<td>[29]</td>
</tr>
<tr>
<td>12.</td>
<td><em>Termitomycesalbuminosus</em></td>
<td>Alkaline protease</td>
<td>—</td>
<td>Ion exchange chromatography</td>
<td>[31]</td>
</tr>
<tr>
<td>13.</td>
<td><em>P. ostreatus</em></td>
<td>Fibrinolytic enzyme</td>
<td>Submerged culture fermentation</td>
<td>Ammonium sulfate precipitation, hydrophobic interaction, and gel filtration chromatographies</td>
<td>[32]</td>
</tr>
<tr>
<td>14.</td>
<td><em>Lentinus citrinus</em></td>
<td>Alkaline protease</td>
<td>Solid state fermentation</td>
<td>—</td>
<td>[33]</td>
</tr>
<tr>
<td>15.</td>
<td><em>P. sajor-caju</em></td>
<td>Signal Peptide Peptidase</td>
<td>Liquid culture</td>
<td>Ammonium sulphate precipitation, Ion-exchange chromatography, and HPLC</td>
<td>[34]</td>
</tr>
</tbody>
</table>

**Table 1.**
Enzymes production from the edible mushroom.
2.2.1 Serine proteases

These classes of proteases are broadly found in nature and present in cellular organisms. Along with all the identified proteolytic enzymes, a significant part is of serine proteases. It generally performs the cleavage action on the bond present in the central part of the amino acid chain. However, few Serine proteases act as exopeptidases by detaching the amino acids from the end terminal of the polypeptide chain. Its name derives from the Ser residue present in the peptide chain, which is nucleophilic and placed in the active site of the chain of amino acids. An intermediate substrate is formed by using the serine residues inform of acyl-enzyme at the C end terminal of the newly structured peptide bond [38].

2.2.2 Cysteine/thiol proteases

This enzyme contains cysteine residues at their active site present both in prokaryotes and eukaryotes microbes. It shows proteolytic activity at the 6–8 pH range with 50–70°C optimum temperature. Hydrogen cyanide is the key component that activates this enzyme, resulting in which SH group is formed in a polypeptide chain. Oxidizing agents can inhibit this kind of proteases and show sensitive action to the sulfhydryl agents, for example, p-CMB [39].

2.2.3 Metalloproteases

Metalloproteases are generally zinc-containing enzymes. In fungi or basidiomycetes, several metal ions such as calcium, cobalt, and zinc are involved in their reactivation. Zinc-containing enzymes and calcium are essential for proteineous activity and structural stability of protein at optimum pH 5–9. These are sensitive to an agent that causes chelation of metal, such as ethylen diamine tetracetic acid (EDTA), but are insensitive to cysteine inhibitors [40].

2.2.4 Aspartic proteases

It is a comparatively small class of endopeptidases that includes aspartic proteases. These proteases are composed of a pair of aspartates bilobed structures, including a leading catalytic site. It functions optimally on acidic pH and is present in nature. This enzyme is secreted by various microorganisms such as bacteria and fungus, as their virulence secretions. Also, it can perform the mutualistic function in the breakdown of proteins yielding nitrogen from urea. These kinds of proteases are primarily biased toward the hydrophobic amino acids nearer to the dipeptides bond. As compared with the other two endoproteases, it utilizes residues present in the active site showing nucleophilic attribute for proteolysis [41].

3. Proteolytic enzymes from mushroom species

As proteolytic enzymes are indispensable in supplying nitrogen to xylotrophs under natural growth conditions (on living and dead wood), the absence of sufficient systematic information on secreted proteases of higher xylotrophic fungi is not much explored [42]. The protein structure contains nitrogen, which is probably the reason for the secretion of extracellular proteolytic enzymes basidiomycetes or mushroom.
Hydrolases

The species belong to orders of basidial fungi, *Polyporales*, *Boletales*, and *Agaricales*, which are reported to secret proteolytic enzymes. Proteases secreted by mushrooms typically have a low molecular mass ranging from 26 to 50 kDa having isoelectric point’s up to 3.5–8.8. Acidic pH is usually optimal for these enzymes, ranging from 2.0 to 5.0. Proteinases isolated from *Hypsizygis (H.) marmoreus*, *P. ostreatus*, and *F. velutipes* are exceptional, as their optimal pH falls under the neutral range. Amino peptidases in the mushroom are usually intracellular enzymes; few reports have studied detecting extracellular aminopeptidases, such as *Trametella trogii*. Separately from endopeptidase activity, aminopeptidase, carboxypeptidase, and dipeptidyl aminopeptidase activities were revealed [43]. It is known that there is a high demand for industrial application of proteolytic enzymes with suitable specific properties and must be stable at various temperatures and pH. However, this chapter suggests that the studies of proteases from basidiomycetes fungus or mushroom recommend that further detailed studies are required to explore proteases’ mechanisms and physiological effects.

4. Role of proteolytic enzymes in mushroom

Proteases perform complex physiological functions, including protein catabolism; blood clotting, cell growth and migration, morphogenesis, and development [4]. Mushrooms or basidiomycetes fungi are heterotrophic organisms. They can utilize both organic and inorganic nitrogen sources as nutrition. An under natural conditions, they usually secrete various extracellular enzymes to decompose natural organic materials such as ligninolytic enzymes. Protease from mushrooms involves endopeptidases, and exopeptidases act one after another as the former produces many free C and N terminal ends and latter act on the peptide fragments, thus forming the decomposed protein. This broad specificity is a significant property of the fungal secreted proteases and other proteolytic enzymes employed to break down proteins. An investigation reported on fungus *T. trogii* utilizes these enzymes to efficiently break down various peptides in the substrate [43]. Proteases secreted in mushrooms participate in the active regulation of other synthesized enzymes, resulting in regulating some physiological processes in mushrooms species such as *P. ostreatus*, *P. chrysosporium*, etc. The activity of ligninolytic enzymes is regulated via their specific activation or inactivation by the extracellular proteases secreted them [22] and perhaps has the ability to degrade the proteins controlling heat shock response, DNA repair pathway programmed cell death [4]. The metalloprotease plays a significant role in the fruiting body formation in *P. ostreatus*. Previous studies demonstrated that mRNA content is noticeably higher in the primordial stages of fruit body formation than in the vegetative mycelium stage [44]. So the extracellular occurrence of the fungal proteolytic enzymes may help the fungus grow on the host by utilizing its nutritional contents, may act as a pathogenic agent for the host. Under favorable conditions, *P. pulmonarius* grows only on dead decaying wood showing as ubtilisin-like proteolytic activity using proteases [45].

5. Methods used for proteolytic enzymes recovery and production

Enzymes recovery and production from mushrooms were directly influenced by the substrate type, composition, and recovery methods. Various research studies
showed that solid-state and submerged fermentation mushrooms had been significantly used for enzyme production [46]. In solid-state cultivation, various agro wastes are utilized and produce fruiting bodies containing various metabolites primarily used for food sources. After harvesting, its by-products, spent mushroom substrate (SMS) that contains plenty of extracellular enzymes can be utilized as animal feeding and for enzymes recovery and production. It reported that laccase (EC 1.10.3.2) was the most prominent and common in *Pleurotus sajor-caju* [47], *P. ostreatus*, *L. edodes*, *Flammulina velutipes* and *Hericium erinaceum* [48], *A. bisporus* [49]. Another enzyme, lignin peroxidase productivity, was found to be the SMS of *P. sajor-caju* [47]. Researchers can significantly explore the production of lipases, pectinases, and phytases. Table 1 lists all the enzymes found in mushrooms and recovery methods. Convinced enzymes extraction and purification methods were widely applied, for example, dialysis, ultrafiltration, anion-exchange chromatography, and gel [50–52]. It is noteworthy that most of the investigations were carried out only for the fruiting body or mycelium of mushroom, not the SMS; therefore, it is an open possibility for the new finding for enzymes from SMS. Recently works were reported for enzyme recovery from SMS. Mayolo-Deloisa et al. [49] evaluated the use of aqueous two-phase systems to recover laccase from the residual compost of *A. bisporus* mushroom. They observed that valorizations of residual material give a 95% yield and have the potential for value-added products with commercial application [49]. Ko et al. [48] determined the production of amylase, cellulase, glucosidase, laccase, and xylanase from the SMS obtained from four edible mushrooms: *P. ostreatus*, *L. edodes*, *F. velutipes* and *H. erinaceum*, and evaluated its potential application using enzymes from SMS as industrial enzymes. It has been reported that a solvent such as water is used for enzymes recover from SMS with good activity; this fact is essential for an industrial application and related environmental concerns. However, the extraction of enzymes from submerged culture supernatant is more straightforward than from SMS because centrifugation is needed, and the obtained supernatant can be used as the crude enzyme. Some physical conditions are essential for optimizing scale-up, such as pH, temperature, extraction medium, incubation time, inoculums density, carbon and nitrogen source, and the impotent parameters for enzyme production.

### 6. Applications and future prospects

Novel investigation techniques revealed highly specific and selective protein modifications performed by proteases, including activating the zymogenic enzyme forms by limited proteolysis, forming hormones and other physiologically active peptides from precursor proteins, thrombus lysis, or the processing and transport of secreted proteins through the membrane (Figure 1). The vital role of proteolytic enzymes in metabolic and regulatory processes explains their occurrence in all living organisms [53].

#### 6.1 In the detergent industry

Proteases were used as a detergent centuries ago as the “Burnus” brand, along with sodium carbonate and pancreatic extract mixed in it [54]. Several industries, such as chemical, pharmaceutical, food processing, detergents, and leather processing, utilize the catalytic properties of proteases. Its application in the bioremediation of pollutants has also been reported. Several factors such as optimum substrate specificity,
temperature, optimum pH, chemical stability, and catalytic activity may vary because of a diverse group and also can affect the production of proteases [21].

6.2 Cell-free enzyme preparation

Immense interest has been grown in proteases due to their thermal ability in a wide range of temperatures. It is also used as detergents in the cell separation process for the production of cell-free enzyme preparations. In these perspective, fungal enzymes have applications as these are extracellularly secreted [55, 56].

6.3 In the pharmaceutical and food industries

Some proteases are also found to produce due to the infection process caused by foreign invaders such as bacteria, fungus, and viruses. A variety of steps regulate the mechanism of proteolytic enzyme reactions, including substrate specificity, ATP-directed protein degradation, restricted access to the active site, highly specific protein modifications. It can activate zymogenic forms of enzymes by restricted proteolysis activity [57]. Including these protease enzymes that cause diseases to host cells has become a good option for developing therapeutic agents for the diseases such as cancer, hepatitis, malaria, and candidiasis. It has also been reported to demonstrate potent immunomodulatory activity [4].

6.4 Leather industry

The leather industry involves various steps to obtain processed leather, for example, soaking, liming, hair removal, bating, deliming, and degreasing. These steps are applied using poisonous chemicals such as salt, lime, solvents, and sodium
sulfide, resulting in pollution. The exclusion of non-collagenous particles is required in leather processing, which decides the softness and durability of leather products [58, 59]. It can be controlled by applying enzymes such as proteases in the place of chemicals [60].

7. Conclusion

Most of the industrial proteases used are of microbial origin, especially of bacteria. These enzymes are preferentially selected because of their desired characteristics and lower cost. The bioengineering manufacture of microbial proteases is favored as they have short generation periods, high yield, ease of genetic desired modification, and diverse species available. Future opportunities are high in cutting-edge research from the pharmaceutical perspective of the protease gene. By the help via recombinant DNA technology, respective genes must have been cloned and sequenced to determine the function of enzymes that cause changes in the attributes of protease enzymes and enhance enzyme production for their commercial usage. In industries, proteases contribute to the high value-added products development, and the same way biological catalysts offer advantages over the use of chemical catalysts for numerous reasons, such as high catalytic activity, high specificity, and their availability in economically viable quantities.

Conversely, cost associated with the production of proteases from mushrooms or basidiomycetes is the major obstacle to their application in industries and pharmaceuticals. For that reason, further research studies should have been implemented to discover novel low-cost proteases from mushrooms and their application in commercial and industrial sectors. So, a great extent of the study of proteases from the mushroom requires further investigations.

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Conflict of interest

The authors declare no conflict of interest.
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