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Bacterial and Fungal Proteolytic Enzymes: Production, Catalysis and Potential Applications

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Abstract Submerged and solid-state bioprocesses have been extensively explored worldwide and employed in a number of important studies dealing with microbial cultivation for the production of enzymes. The development of these production technologies has facilitated the generation of new enzyme-based products with applications in pharmaceuticals, food, bioactive peptides, and basic research studies, among others. The applicability of microorganisms in biotechnology is potentiated because of their various advantages, including large-scale production, short time of cultivation, and ease of handling. Currently, several studies are being conducted to search for new microbial peptidases with peculiar biochemical properties for industrial applications. Bioprospecting, being an important prerequisite for research and biotechnological development, is based on exploring the microbial diversity for enzyme production. Limited information is available on the production of specific proteolytic enzymes from bacterial and fungal species, especially on the subgroups threonine and glutamic peptidases, and the seventh catalytic type, nonhydrolytic asparagine peptide lyase. This gap in information motivated the present study about these unique biocatalysts. In this study, the biochemical and biotechnological aspects of the seven catalytic types of proteolytic enzymes, namely aspartyl, cysteine, serine, metallo, glutamic, and threonine peptidase, and asparagine peptide lyase, are summarized, with an emphasis on new studies, production, catalysis, and application of these enzymes.

Keywords Bacteria · Bioprocess · Biotechnology · Fungi · Lyases · Proteolytic enzymes

Introduction

The biotechnology industry is expanding worldwide. In this context, the production of enzymes is crucial and has enabled large economic benefits. On a global scale, the market for enzymes has

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raised billions of dollars annually, and research work in the area has benefited from an increasing number of patents and published scientific articles. In current research, irrespective of the generated product (metabolite), the preference of the use of bacteria and fungi as production sources is attributed to the ease of handling, species diversity, capacity to degrade complex polymers (e.g., lignin and keratin), and high performance in the production process [1–4].

The biotechnological use of bacteria to obtain products of economic interest, in particular enzymes, has been explored for a long time. Among different bacterial species, the genus *Bacillus* has been extensively prospected in industrial bioprocess [5]. Other bacterial genera such as *Streptomyces* and *Pseudomonas* are also much employed as enzyme production sources.

Apart from the application of bacterial species in bioprocessing, fungal species are also explored in enzyme technology.

In 1991, Hawksworth [6] estimated that the number of fungal species in the biosphere was about 1.5 million. In a recent report by Chambergo and Valencia [7], the number of fungal species is estimated to be around 5.1 million. However, only 100,000 species have been cataloged. According to both the estimates, the fungal kingdom is recognized to possess a wide biodiversity. It is also predicted that more than 250 fungal species have been used to produce compounds of biotechnological interest.

Investigating the microbial diversity, recently, new catalytic types of proteolytic enzymes have been described, but with little discussion about these enzymes, such as the subgroups threonine peptidase, glutamic peptidase, and particularly the nonhydrolytic asparagine peptide lyase enzyme. This gap motivated the present review, which aims to provide an updated summary of bacterial and fungal proteolytic enzymes, with an emphasis on the subgroups aspartyl, cysteine, serine, metallo, glutamic, and threonine peptidase, and asparagine peptide lyase' production, specificity, and potential applications.

Proteolytic Enzymes: Mode of Action and Catalytic Specificity

The distribution of amino acids on primary structure of proteins is a fundamental feature that defines their structural, functional, and biological properties. The position and cooperativity of conserved residues is a common arrangement for proteolytic catalysis and a model for classification of these enzymes in *family* and *clans*. On evolutionary history of proteolytic enzymes, the conserved amino acid sequence is fundamental to the enzyme operativity [8], and the diad or triad catalytic is a significant example of a conserved structure essentially important for catalysis. On catalytic apparatus of peptidases, the positions of the amino acids represent the evolutionary history of these enzymes [9].

Proteases or peptidases are long known as enzymes that catalyze the rupture of peptide bonds into protein and peptide [9, 10]. They can be grouped according to (1) the source of production: animal, plant, and microbial; (2) details of the catalyzed reaction: (a) position of the cleavage site—endopeptidases and exopeptidases (amino and carboxypeptidase) and (b) the length of the substrate chain; (3) mechanism of action: main residue of the active site and nucleophile agent; and (4) homology and molecular structure [4, 8–10].

These enzymes comprise a notable group of biocatalysts with a broad scope of application, according to their biochemical properties and specificity to the substrate. In this context, specificity is an important parameter to be determined, which is essential to improve the understanding of the mechanism of action of the enzymes and evaluate their potential applications [4, 10].

In 1967, Schechter and Berger [11] proposed the nomenclature of the catalytic site of peptidases, dividing it into subsite series, where each subsite accommodates a single and

consecutive amino acid residue from substrate. The nonprimed side (P_n-P_4-P_3-P_2-P_1) is the amino terminal direction, and the primed side (P'_1-P'_2-P'_3-P'_4-P'_n) is the carboxyterminal direction of the substrate, while the corresponding subsites on peptidase are S_n-S_4-S_3-S_2-S_1-S'_1-S'_2-S'_3-S'_4-S'_n [12].

Probing the catalytic specificity of peptidases is important to elucidate the functional substrate recognition and to prospect the potential for virulence (pathogenicity) or application of the biocatalyst. Merheb-Dini et al. [13] investigated the functional properties of a metallopeptidase from the thermophilic fungus *Thermoascus aurantiacus*. The peptidase exhibited preference for arginine at position P_1 on substrate.

In other studies, Silva et al. [4] related a serine peptidase from *Aspergillus fumigatus* with preference for leucine at S'_3 and nonpolar amino acids at S_3. Neto et al. [14] reported a neutral peptidase from *Myceliophthora thermophila* with a specificity for alanine at S'_2, and Biaggio et al. [15] reported a serine peptidase from *Aspergillus terreus* with a nonpreference for basic (His and Arg) and acidic (Asp and Glu) amino acids at S_2. In general, these enzymes exhibited nonspecificity for the amino acids evaluated in various catalytic subsites, indicating a perspective for application assay as additives in detergent formulation, synthesis of bioactive peptides, and improvement of animal feed digestibility.

On the contrary, Silva et al. [16] described an aspartic peptidase secreted by *Rhizomucor miehei*, which exhibited high specificity on cleavage of the peptide bond between phenylalanine and methionine amino acids. Using the clotting sequence of *k*-casein, Abz-LSFMAIQ-EDDnp, the enzyme exhibited a low value for K_M (0.036 ± 0.003 μM) and the highest obtained value of catalytic efficiency (4722 ± 100 mM^{-1} s^{-1}) among the various substrates evaluated in the study. Because of its high catalytic performance, this peptidase may serve as an alternative enzyme in cheese manufacturing.

Analyses of catalytic specificity of intracellular peptidases strongly contribute to elucidate their cellular functions. The study of the autotransporter serine peptidase from *Fusobacterium nucleatum* revealed a specificity to cleave after Thr, Gly, Ala, and Leu residues at the P_1 position [17]. This serine peptidase is capable of damaging host tissue and inactivating immune effectors. It was found in *F. nucleatum*, a recognized pathogen associated with periodontal disease. The specificity study of peptidases is crucial to understand the enzymes that are known as key virulence factors and can help in the treatment choice of diseases [18].

Investigating the specificity, the fluorescence resonance energy transfer (FRET) peptides comprise the most versatile class of synthetic substrates currently available for proteolytic enzymes. This substrate class is useful for a wide application in enzymology, because of its simplicity, speed, and accuracy in determining the reaction constants [19].

Recently, a research has demonstrated that not all proteolytic enzymes are peptidase (E.C. 3.4). This discovery revealed a new proteolytic enzyme, asparagine peptide lyase (E.C. 4.3.2), in which cleavage of the peptide bond occurs by the action of amidine lyase [12]. Thus, seven catalytic types of proteolytic enzymes have been recognized to date: aspartyl, cysteine, serine, metallo, threonine, and glutamic peptidase, and asparagine peptide lyase, which is not a hydrolytic group (Table 1).

Aspartic Peptidase

Aspartic or aspartyl peptidases are acidic endopeptidase, whose catalytic apparatus has two residues of aspartic acid. It shows optimum activity at pH 3–6, isoelectric points in the range of

Table 1 The seven catalytic types of proteolytic enzymes

Catalytic type	Nucleophilic agent	Major production sources	Enzyme class	Recent scientific reports
Aspartic peptidase	Water molecule activated by two aspartates	Animal, plants, bacteria, fungi, and viruses	Hydrolase E.C. 3.4.	Silva et al. [16], Yegin et al. [20], Kumar et al. [21], Hsiao et al. [96], Aoki et al. [101]
Cysteine peptidase	Cysteine residue (Cys)	Animal, plants, bacteria, fungi, and viruses	Hydrolase E.C. 3.4.	Datta [25], Cabaleiro et al. [26], Dubey et al. [27], Rozs et al. [28]
Serine peptidase	Serine residue (Ser)	Animal, plants, bacteria, fungi, and viruses	Hydrolase E.C. 3.4.	Silva et al. [3, 4, 29], Biaggio et al. [15], Iqbal et al. [32], Joshi and Satyanarayana [33], Lv et al. [34], Prakash et al. [35]
Metallopeptidase	Water molecule activated by metallic ions	Animal, plants, bacteria, and fungi	Hydrolase E.C. 3.4.	Merheb et al. [13], Silva et al. [29], Neto et al. [37], Jashni et al. [38], Ruf et al. [39], Wang et al. [40]
Threonine peptidase	Threonine residue (Thr)	Bacteria, archaea, and fungi	Hydrolase E.C. 3.4.	Ward et al. [24], Kisselev et al. [43], Baird et al. [45]
Glutamic peptidase	Water molecule associated with glutamate	Bacteria and fungi	Hydrolase E.C. 3.4.	Kataoka et al. [46], Pillai et al. [47], Yabuki et al. [48], Jensen et al. [49], Kondo et al. [102]
Asparagine peptide lyase	Asparagine residue (Asn)	Bacteria, archaea, fungi, and viruses	Lyase E.C. 4.3.2	Rawlings [44]

3–4.5, and usual inhibition by pepstatin A—a hexapeptide produced by *Streptomyces*—and in the presence of copper(II) ion [20].

This peptidase group is used in various industrial sectors such as casein hydrolysates, peptide synthesis, and reduction of turbidity by degradation of the protein complex from fruit juice [20].

Besides, in cheese production, aspartic peptidases are much required as a substitute for chymosin from newborn ruminants. The extraction processing of chymosin from ruminants collides with ethical problems, and the search by microbial enzymes constitutes an attractive alternative with low cost and reduced production time [16, 21].

Endothia parasitica and species of *Penicillium*, *Aspergillus*, *Rhizopus*, *Mucor*, and *Rhizomucor* are examples of fungi used as production sources of aspartic peptidase. *R. miehei* is a thermophilic fungus with appreciable capacity for the production of acidic peptidase and potential for industrial application [16, 20].

Only few reports about bacterial acidic peptidases have been described. Some studies with aspartic peptidases are related to *Escherichia coli* and *Haemophilus influenzae* [22].

Cysteine Peptidase

Cysteine or thiol peptidases are endopeptidases and exopeptidases, whose catalytic action depends on a cysteine residue in the active site. The catalytic dyad His and Cys is present in all thiol peptidases. In papain-like cysteine peptidase, the catalytic apparatus generally consists of His, Cys, and Asp [23].

In general, these enzymes show maximal activity at pH 4.5–7 and molecular mass approximately 20–35 kDa. Because of its high tendency to oxidation of the thiol group, many cysteine peptidases need a reducing agent in its reaction medium. Cysteine peptidase shows inhibition by iodoacetic acid, *N*-ethylmaleimide, and E-64, among others [23].

Cysteine peptidases are present in all organisms. Many of these peptidases are found in viruses, bacteria, fungi, protists (cruzipain—*Trypanosoma cruzi*), and plants (papain). Mammals have two main groups of cysteine peptidases: calpains (type I and II) and lysosomal cathepsins [24]. Only little information about the secretion of fungal cysteine peptidase is known. Some examples of fungal thiol peptidases were reported by Datta [25] and Cabaleiro et al. [26]. In bacteria, cysteine peptidases are found in several species: *Porphyromonas* and *Lactococcus* genera [27], clostripain from *Clostridium histolyticum*, gingipain from *Porphyromonas gingivalis*, thiol keratinases from *Bacillus licheniformis* [28], etc.

Serine Peptidase

Serine peptidases comprise the most abundant group of peptidases. These enzymes are characterized by the presence of one serine residue at the active site, which is crucial for their catalytic performance. The catalytic triad consists of His, Ser, and Asp, and these enzymes can be an endopeptidase and exopeptidase. In general, serine peptidases show maximal activity at pH 6–11 and isoelectric points in the range of 4–6. Some alkaline serine peptidases from fungi, i.e., produced by *Aspergillus* sp. and *Neurospora Conidiobolu*, show optimum activity around pH 10 and high isoelectric points (8–9) [24].

Alkaline serine peptidases have different applications: in leather treatment, improvement of the flavor of cheese, and detergent industry [4]. This peptidase group exhibits inhibition by phenylmethylsulfonyl fluoride (PMSF), trypsin inhibitor, antipain, chymostatin, and others [4, 24].

Several studies have showed the production of serine peptidase from bacteria and fungi, for example, Silva et al. [3] with *Penicillium corylophilum* and *Penicillium waksmanii*, Silva et al. [29] and Silva et al. [4] with *A. fumigatus*, Graminho et al. [30] with *P. waksmanii*, Biaggio et al. [15] with *A. terreus*, Ida et al. [31] with *Aspergillus fischeri* and *Penicillium citrinum*, a thermostable peptidase from *Geobacillus stearothermophilus* [32], alkaline serine peptidase from *Bacillus lehensis* [33], subtilisin from *B. licheniformis* and *Bacillus amyloliquefaciens* [24], and keratinases from *Chryseobacterium* L99 sp. nov. [34] and *Bacillus halodurans* PPKS-2 [35].

Metallopeptidase

Metallopeptidases are endopeptidases and exopeptidases that are ion-dependent, usually zinc, for its catalytic performance. Other metallic ions, including cobalt, manganese, nickel, copper, and iron, have also been described for catalysis of these enzymes. The active site consists primarily of His-Glu-Xaa-Xaa-His, where "Xaa" is the same amino acid (repetition of the same amino acid). The histidine residue is involved in zinc ion accommodation, and glutamic acid residue directly related to catalytic function [24].

These peptidases exhibit a wide catalytic variety for proteolysis, such as collagenase and elastase (bacteria and fungi), hemorrhagic toxin of snake venoms, and bacterial thermolysin [24]. Metallopeptidases exhibit inhibition by chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and ethyleneglycoltetraacetic acid (EGTA), whose catalysis is influenced by the removal of divalent ions [13, 29, 37].

The production of metallopeptidase from fungi has been reported in several studies (e.g., Fernandez et al. [36] and Silva et al. [29] with *A. fumigatus*, Neto et al. [37] with *Eupenicillium javanicum*, Merheb-Dini et al. [13] with the fungus *T. aurantiacus*, and Jashni et al. [38] with *Fusarium oxysporum* f. sp. *lycopersici.*

Bacterial metallopeptidases have been reported by Ruf et al. [39] with neutral metallopeptidase from *Paenibacillus polymyxa*, aminopeptidases from *B. stearothermophilus* [40], and keratinases from *Chryseobacterium* sp. Kr6 [41]. Other metallopeptidases are also produced by *B. stearothermophilus* (thermolysin), *Achromobacter iophagus*, *Clostridium hystolyticum*, *Pseudomonas aeruginosa*, *Arthrobacter crystallopoietes* [24], etc.

Threonine Peptidase

Threonine peptidase was discovered in 1995, and currently, only little information about this enzyme group is available. It was found in complex subunits of the proteasome from *Saccharomyces cerevisiae* and constitutes the fifth proteolytic enzyme group, whose N-terminal threonine residue at the active site is necessary for its catalytic machinery [24, 42].

The complex proteasome is an important pathway for degrading of proteins in the cell [43]. In this intracellular complex, the hydroxyl group of threonine residue of this enzyme acts like the nucleophile agent, and in studies of proteolytic inhibition, a resistance by conventional peptidase inhibitors was noted. Threonine peptidase has also been found in proteasome from bacteria and archaea [24, 44].

However, some reports have revealed the replacement of threonine by serine residue located in the active site of archaebacterial proteasome (i.e., *Thermoplasma acidophilum*) [24, 43]. Thus, the nucleophile function of serine residue in archeabacterial proteasome and the conservation of the threonine nucleophile agent from bacteria to fungi have been unclear to date.

The nucleophile catalytic function of hydroxyl group of threonine residue located at the active site of proteolytic enzymes was also demonstrated by Baird et al. [45] in a study promoting the conversion of trypsin serine peptidase to a functional threonine peptidase.

Glutamic Peptidase

Initially identified as a carboxyl peptidase insensitive to pepstatin A, this is the sixth catalytic type of proteolytic enzymes. It was found primarily in *Scytalidium lignicolum* and grouped as an aspartic peptidase whose proteolytic activity did not present inhibition by pepstatin A [24]. Since 1972, the acidic peptidases insensitive to pepstatin A have been reported in bacterial and fungal species. However, by crystallographic studies, these bacterial peptidases revealed similarity with subtilisin structure. Their catalytic triad has been identified to be formed by serine, glutamic acid, and aspartic acid, and thus, they were classified into serine peptidase group [46, 47].

Subsequently, the acidic peptidase from *S. lignicolum* (Scytalidoglutâmico peptidase) and *Aspergillus niger* var. *microsporus* (Aspergiloglutâmico peptidase) revealed the presence of a catalytic dyad composed of glutamic acid and glutamine and a three-dimensional structure different from other peptidases known thus far. Currently, these enzymes are regarded as a different peptidase group, glutamic peptidases, having been precisely identified in 2004 [24, 44, 48].

Regarding its biochemical characteristics, the glutamic peptidase found in *A. niger* var. *microsporus* showed a maximum activity at pH 2 and pH 1.1, using casein and hemoglobin as substrate, respectively. Currently, peptidases with a similar molecular structure to Scytalido and *Aspergillus* glutamic peptidases have been described especially in fungi, such as *A. niger*, *Cryphonectria parasitica*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Talaromyces emersonii* [47, 48]. The production of bacterial glutamic peptidase from *Alicyclobacillus* sp. DSM 15716 was reported in 2010 [49].

Asparagine Peptide Lyase

Asparagine peptide lyase has been recently reported by Rawlings et al. [44] as the seventh catalytic type of proteolytic enzymes. This discovery offered a new concept about proteolytic enzymes, indicating that not all the rupture of peptide bonds is catalyzed by hydrolytic enzymes.

Different from all peptidases described to date, the asparagine peptide lyase is not a hydrolytic proteolytic enzyme, whose catalysis depends on an asparagine residue and the peptide bonds are cleaved by amidine lyase action. Homologs of this enzyme have been found in viruses, archaea, bacteria, and fungi. Asparagine peptide lyases have been identified in autotransporter proteins (Tsh protein precursor of *E. coli*), viral coat proteins, and intein-containing proteins (i.e., ATPase catalytic subunit A of *S. cerevisiae*, DNA helicase of *Synechocystis* sp.). All these asparagine peptide lyases perform only self-cleavages, with cleavage occurring on the C-terminal side to the active site asparagine residue.

Proteolytic Enzymes and Biotechnological Potential

Proteolytic Enzymes and Synthesis of Bioactive Peptides

The use of enzymatic hydrolysis to produce bioactive peptides from protein substrate is attractive for biotechnological studies, and currently, it constitutes the most common way to

generate these peptides. Certain characteristics like the short time for hydrolysis and enzymatic activity in mild and controlled conditions render proteolytic enzymes a valuable advantage over other methods for the synthesis of bioactive peptides [50].

In general, the peptides with biological functions are encrypted in a whole protein, and thus, the proteolysis is required to release them. Using a parent protein, enzymatic hydrolysis is used to release various peptides, in vitro and in vivo studies which have indicated a numerous collection of physiological functions, including antithrombotic, antioxidant, opioid activities, cytomodulatory and immunomodulatory effects, antimicrobial properties, inhibitory activity of angiotensin-converting enzyme I (ACE), antiobesity, and antigenotoxic activity [51, 52].

Bacterial and fungal peptidases such as the commercial enzymes Alcalase®, Neutrase®, and Flavourzyme® and enzymes prospected from microbial species are widely reported for the synthesis of bioactive peptides [52, 53]. For example, Coda et al. [54] selected *Lactobacillus* species to produce antioxidant peptides during sourdough fermentation of cereal flours. In this study, using mouse fibroblasts artificially subjected to oxidative stress, the authors reported the antioxidant activity of all the purified fractions, demonstrating the capacity of sourdough lactic acid bacteria to release bioactive peptides derived from enzymatic hydrolysis of native cereal proteins.

In a study with a peptidase from *Bacillus* sp. P45, Hidalgo et al. [50] evaluated the antioxidant effect of peptides obtained by proteolysis of bovine caseinate hydrolysates. Corrêa et al. [55] also described the antioxidant, antihypertensive, and antimicrobial effects of peptides derived from proteolysis of ovine caseinate by the peptidase secreted by *Bacillus* sp. P7. The bioactive peptides induced inhibition of the microbial growth to *Bacillus cereus*, *Corynebacterium fimi*, *A. fumigatus*, and *Penicillium expansum*.

The use of commercial fungal enzyme from *Aspergillus oryzae* (Flavourzyme®) was described by Cheong et al. [56] for the production of anticancer peptide from proteolysis of *Crassostrea gigas* proteins; the peptides were purified, and the results showed a promising effect to induce toxicity in cancer cells. An antioxidant activity of hydrolysates from soy protein using proteolytic enzymes secreted by *A. oryzae* LBA 01, Flavourzyme®, and Alcalase® was also reported in research using microbial enzymes [53]. In this research, the authors reported a 7.0-fold increase in the antioxidant potential of the hydrolysates after proteolysis, by which Flavourzyme® was the highest effective commercial peptidase for hydrolysis of soy protein.

Microbial peptidases from *Aspergillus sojae*, *A. oryzae*, and *B. licheniformis* were also used to generate bioactive peptides from whey protein with inhibitory activity to ACE. In this study, Moraes et al. [57] reported that several hydrolysates exhibited inhibitory activity >80%, particularly in hydrolysates derived from proteolytic activity of enzymes secreted by *B. licheniformis.*

A new alternative for the synthesis of peptides is devoted to predict the possible scissile point in the parent protein. Once the amino acid sequence of the protein substrate and the specificity of the enzyme used are known, in silico studies for digestion of protein can be performed [58, 59]. The prediction of peptides derived from enzymatic hydrolysis under a whole protein is an emergent proposal in specific cases, in which the biochemical properties of the substrate and enzyme are widely known [59].

Purification is generally conducted by chromatographic techniques such as reversed-phase, ion exchange, size exclusion, and ultrafiltration chromatography. Furthermore, amino acid sequence of the peptides can be identified by mass spectrometry, and for a future prospect, the chemical synthesis of peptides with biological activity is also possible [52, 58].

Table 2 shows some microbial enzymes used to produce bioactive peptides.

Table 2 The use of bacterial and fungal peptidases in the synthesis of bioactive peptides

Microbial source	Biological activity	Protein substrate	Reference
Lactobacillus strains	Antioxidant activity	Sourdough from cereal flour	Coda et al. [54]
Bacillus sp. P7	Antioxidant, antihypertensive, and antimicrobial activities	Ovine milk	Corrêa et al. [55]
Bacillus sp. P45	Antioxidant activity	Bovine sodium caseinate powder	Hidalgo et al. [50]
Chryseobacterium sp. kr6	Antioxidant activity	Soy protein	Oliveira et al. [103]
Chryseobacterium sp. kr6	Antioxidant, angiotensin-converting enzyme inhibitory (ACEI), and dipeptidyl peptidase-IV inhibitory activities	Chicken feather	Fontoura et al. [104]
Lactobacillus helveticus	Antioxidant, ACEI and immunomodulatory activities	Milk proteins	Elfahri et al. [105]
A. oryzae	Anticancer activity	Protein from *Crassostrea gigas*	Cheong et al. [56]
A. oryzae LBA 01	Antioxidant activity	Soy protein	De Castro and Sato [53]
A. sojae *A. oryzae* *B. licheniformis*	ACEI	Whey protein	Moraes et al. [57]
Torulaspora delbruekii KL66A *Galactomyces geotrichum* KL20B *Pichia kudriavzevii* KL84A *Kluyveromyces marxianus* KL26A	ACEI	Cow milk	Chavez-López et al. [106]

Improvement of Plant Biomass Degradation and Regulation of Lignocellulolytic Activity

The synergistic action of peptidases with lignocellulolytic enzymes is an important factor for the degradation of plant biomass and improvement of feed digestibility of poorly processed plant fibers in ruminant diet. Peptidases are capable of splitting peptide bonds into proteins from forage material in order to use them as nitrogen source [60]. This cooperative action of hydrolytic and oxidative enzymes favors the completion of plant biomass degradation [25].

Some reports related the secretion of fungal peptidases and the improvement of nutritional value in feed crops. In a study with *Pleurotus ostreatus*, Ramirez-Bribiesca et al. [61] reported an increase in protein content after 15 days of cultivation using corn straw substrate. The nutritional content of wheat straw residue was also improved by treatment with fungus *Ganoderma* spp. [62].

On the contrary, the proteolysis of enzyme repertoire secreted by bacteria and fungi is a challenge to biotechnological processes. The significant loss of enzymatic activity during cultivation in solid-state bioprocess (SSB) and submerged bioprocess (SmB) is an obstacle for enzyme technology, especially to large-scale production of the biocatalysts at low cost. In the last years, numerous studies have reported about peptidases involved in the regulation of lignocellulolytic activity. For example, Datta [25] and Cabaleiro et al. [26] reported the secretion of different peptidases by the white-rot fungus *Phanerochaete chrysosporium* and their influence on the degradation of ligninases. In a similar study, an enhanced lignolytic activity was observed after inhibition of the proteolysis by PMSF [63].

Other Commercial Applications

The use of proteolytic enzymes is observed in several segments, especially in food, detergents, leather, bioremediation, and basic research, including molecular biology (proteinase K) and proteomic techniques (trypsin) [2, 10].

Microbial peptidases comprise the main group of enzymes used industrially, accounting for about two thirds of the total marketed enzymes. In the pharmaceutical industry, they emphasize application examples as the use of collagenases in medical practices for the removal of stains and keloids and the treatment of burns and ulcers. The peptidase action on collagen, keratin, and elastin allows the use of these enzymes as an essential component in exfoliating creams and ointments [64].

In the food industry, peptidases can be used for the coagulation of milk by specific hydrolysis of peptide bond in *k*-casein (Phe^{105}-Met^{106}). In cheese production, the use of microbial peptidase is required as substitute for chymosin from newborn ruminants, because of ethical problems and decrease in the global supply of calf rennet as well as increase in the demand for cheese making [16].

Furthermore, according to Sinha et al. [65], whey protein resulting from cheese production has high nutritional value and can be used as a food supplement, being rich in essential amino acids, such as cysteine, a recognized amino acid with important antioxidant function. Hydrolysis of these proteins is carried out by acidic and alkaline peptidases, which provide biologically active peptides with potential to use as nutritional source in cases of child intolerance to milk proteins.

Hydrolysis of soybean protein has been carried out by alkaline and neutral peptidases. The proteolysis increases the protein solubility and can be used to improve the taste of the product.

The protein hydrolysates, in general, have a bitter taste because of the release of peptides containing hydrophobic amino acids at their ends (N and C terminus) and/or proline residue at the center of the molecule. For removal of the bitter taste, some endopeptidases and exopeptidases have been studied as valuable tools for specific cleavage of hydrophobic amino acids [66].

In baking, proteolytic enzymes can also be used as additives in the preparation of dough, such as peptidases from *A. oryzae*, whose action on gluten from wheat flour affects the elasticity and texture and contributes to the reduction of dough mixing time and production costs [10].

The use of peptidase as additives in detergent composition accounts for the largest commercial use of these enzymes. This application is due to activity and stability at alkaline pH and the formulation of detergents, as well as its action in a wide variety of protein substrates [10, 31, 67]. Currently, subtilisin from *Bacillus* species has been explored for application in detergent formulation [2].

In tanneries, the peptidases are used as an alternative way to replace the hazardous chemical additives. The use of proteolytic enzymes ensures the reduction of environmental pollution and improvement in the quality of the leather, and their use is based on the selective hydrolysis of noncollagenous and nonfibrous proteins, such as albumin and globulin [68].

Adding value to residual organic matter, keratinolytic peptidase could be used for the degradation of fibrous animal protein (keratin) into feathers, horns, hair, and nails available as natural waste. This enzymatic action would provide useful biomass to use as supplement for animal nutrition, among other applications [10, 41].

Microorganism and Peptidase Production

Two different systems, SmB and SSB, are used in microbial processes. In SmB, the variations in the composition of the culture medium, pH, and carbon and nitrogen sources affect the yield and diversity in peptidase expression [29, 69]. SmB has various advantages such as ease of control of physical and chemical variables (pH, temperature, and oxygenation of the culture medium) and better distribution of medium components [29].

SSB has been generally reported as a system in which enzyme titers are significantly higher than the submerged techniques. This advantage for enzyme production is probably due to fermentation conditions being closer of the natural fungal growth [29, 70]. In this bioprocess, the medium composition is also an important determinant of diversity and amount of enzyme production. Additionally, the advantages associated with SSB include lower energy requirements, generation of less wastewater, and possibility of using agro-industrial residues as fermentation substrates such as orange bagasse, sugarcane bagasse, cottonseed, rice bran, rice straw, and wheat bran, among others [3, 71].

Several researchers have reported the production of fungal peptidases from SmB and SSB. In the SSB system, the use of wheat bran as a substrate for peptidase production enhanced the yield up to 3.5 times in *A. oryzae* [72] and 30 times in *A. fumigatus* [29] than in the SmB system. Using the agro-industrial residues, Silva et al. [3] reported a better peptidase production using wheat bran than synthetic substrate (casein and albumin) with the fungus *P. corylophilum*. Merheb-Dini et al. [13] also reported the use of wheat bran for metallopeptidase production from *T. aurantiacus.*

Under SmB, Ida et al. [31] reported the secretion of two collagenolytic serine peptidases from *A. fischeri* and *P. citrinum* in fermentative medium containing 0.5% crushed feathers as a source of nitrogen. This experimental research demonstrated the great potential of animal

residue, chicken feather, for peptidase production. In caseinolytic assay, the results showed a peak production of 460 U/mL at 72 h with the fungus *A. fischeri* and a peak production of 760 U/mL at 168 h with *P. citrinum.*

Production of proteolytic enzymes from bacteria is also reported in several studies, for example, Kaur et al. [73] and Prakasham et al. [74] in SSB using *Bacillus* sp., Mahanta et al. [75] in SSB using *P. aeruginosa* PseA, and Wu et al. [76] with *Bacillus subtilis* natto under SmB cultivation. Imdakim et al. [77] also reported an acidic peptidase production from *Pediococcus acidilactici* SH in SmB, and in a study with the thermoacidophilic bacterium *Alicyclobacillus* sp. DSM 15716, Jensen et al. [49] described the production of a new bacterial glutamic peptidase and its heterologous expression using *B. subtilis* expression clone under SmB conditions.

In enzyme technology, biomolecule separation is a crucial step for a better understanding of the biochemical properties of enzymes [69]. Since the behavior of these biomolecules in the purification process is unpredictable, each enzyme requires a specific protocol. Purification processes are used to obtain the homogeneous peptidase. Therefore, alternate or sequential chromatographic procedures are adopted according to the chemical characteristics of the analyte.

Chromatographic methods, such as size exclusion, ion exchange, hydrophobic interaction, affinity, and reversed-phase chromatography, are often employed at the laboratory study [4, 5, 35, 78–80]. Preliminarily, precipitation and concentration techniques, such as the concentration of enzyme extract by tangential filtration, organic solvents [4, 80], or salt gradient [35, 78, 79, 81], are also adopted.

Alternatively, the heterologous expression of enzymes constitutes a valuable tool for high performance in production and purification [33, 49]. It also ensures the better conditions for crystallographic structure elucidation, protein engineering studies, application assays, and patent-product implementations using generally regarded as safe (GRAS) expression system.

In enzyme technology, the improvement of the enzymatic stability has been extensively explored. For peptidase application, the protein structural stability is a crucial biochemical property favoring its feasibility for commercial use. Enzymes are susceptible to denaturation by different physicochemical agents, including deamination, proteolysis, oxidation, inhibition, and structural changes due to pH and temperature [82–86].

For increasing thermal stability, techniques such as spray drying and enzyme immobilization have been adopted in enzyme technology [82–85].

Drying techniques commonly use adjuvants as stabilizing agents to minimize structural changes of protein and consequently loss of enzymatic activity. This technique is devoted to improve the enzyme stability, which provides advantages of long-term storage of the product under dehydrated condition and feasibility to use. Neto et al. [82] reported a successful spray drying methodology for metallopeptidases secreted by *E. javanicum* under SSB. In the drying process, using maltodextrin as adjuvant, the microencapsulated enzyme showed yields up to 66.12% and maintenance of enzymatic activity above 80% for 180 days at 4 and 25 °C.

In another study, a collagenolytic enzyme from *M. thermophila* was microencapsulated by spray drying process using maltodextrin in the concentration 1:1 and 1:2 (*w*/*w* adjuvant/enzyme). The results revealed a yield that ranged from 38.65 to 63.75% and maintenance of collagenolytic activity about 100% for 180 days at 30 °C [83].

Peptidase immobilization is also an alternative technique described in several studies to enhance the stability, activity, and reusability of soluble enzymes and simplify product

separation [84, 85]. In biotechnology, the main methodologies explore the physical adsorption and covalent attachment to polymers and sol–gels. These methodologies are used to obtain enzyme stabilization and reusability of immobilized enzyme [87, 88].

In a study of peptidase immobilization, covalent immobilization of the enzyme from *Bacillus* sp. was performed onto hollow core–mesoporous shell silica (HCMSS) nanospheres. The result showed higher immobilization yield by covalent conjugation (75.6%) than the physical adsorption (19.3–47.3%). In the evaluation of thermal stability, the immobilized enzyme retained around 63% of proteolytic activity after incubation at 60 °C for 1 h, while the free enzyme underwent a complete inactivation under the same thermal condition [84].

Figure 1 shows a summary of operations for microbial peptidases prospecting.

Thermophilic Proteolytic Enzymes

Under high-temperature conditions, enzymes from thermophilic microorganisms are attractive actors for industrial applications [89, 90]. Thermostable peptidases from bacteria and fungi are extensively prospected owing to their thermoactivity and increased solubility and, consequently, accessibility to the substrate at high temperatures. Furthermore, under these thermal conditions, the risk to microbial contamination is reduced [91].

Studies to characterize enzyme stability at different temperature and time incubation have revealed that thermophilic bacteria comprise some species with higher thermoactivity and stability than fungal species [91–94].

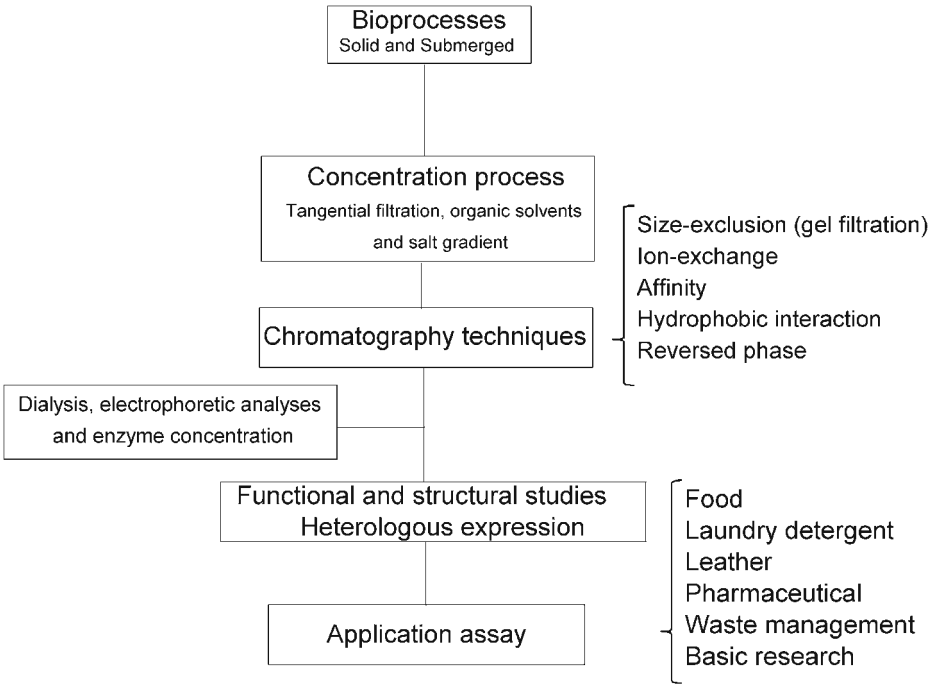

Fig. 1 Summary of the proteolytic enzyme processing: bioprocesses, biomolecule separation techniques, biochemical properties studies, and application assay

Recent reports about thermophilic peptidases have showed the biochemical and biotechnological potential of these microbial enzymes. Zanphorlin et al. [95] reported an experimental research with peptidase from thermophilic fungus *Myceliophthora* sp. The authors evaluated the purification and specificity of a neutral peptidase obtained under SSB. The enzyme exhibited an optimum hydrolysis at 40–45 °C using a synthetic substrate with amino acid sequence KLRSFK.

In another study, Silva et al. [16] reported an aspartic peptidase with relevant potential to milk clotting. The investigators prospected the peptidase from thermophilic fungus *R. miehei* under SmB at 45 °C using casein as the principal peptidase inductor. The enzyme exhibited maximal activity at 55 °C and residual activity of 70% at 45 °C for after 60-min incubation. In similar study, Hsiao et al. [96] performed the purification and characterization of an aspartic peptidase from *Rhizopus oryzae* with maximal activity at 75 °C.

Merheb-Dini et al. [97] also described the production of a milk-clotting peptidase from *Thermomucor indicae-seudaticae* N31 under SSB cultured at 45 °C for 8 days. In subsequent studies, application assays were performed; the authors described a high coagulant activity using milk powder dissolved in 0.01 M $CaCl_2$ and a low proteolytic action toward casein substrate. The milk-clotting and proteolytic activity was found to be 160.3 ± 11.8 and 2.1 ± 0.1 U/mL, respectively. Posteriorly, Merheb-Dini et al. [98] reported the use of this peptidase as coagulant in the preparation of Prato cheese. In comparison to the commercial coagulant, the enzyme exhibited a similar performance for Prato cheese manufacturing.

Subsequently, a new investigation using this peptidase from *T. indicae-seudaticae* N31 evaluated the manufacture of a high-cooked starter-free cheese variety, whose physicochemical and functional properties were similar to those of the commercial coagulants [99].

With thermophilic species more tolerant at high temperature, some bacteria have produced proteolytic enzymes with recognized thermal stability, for example, *Bacillus* sp., which was found to sustain temperatures up to 90 °C. The molecular analysis of 16 rRNA gene revealed a 99% similarity with *B. amyloliquefaciens* [92]; Toplak et al. [93] also reported a proteolytic enzyme secreted by *Coprothermobacter proteolyticus* having activity at temperatures of up to 80 °C, and Wilson and Remigio [94] exhibited an alkaline serine peptidase produced by a thermophilic bacterium (EP1001) with maximal activity at 75 °C.

Thermophilic bacteria inhabit at elevated temperature above 60 °C. Because of this, their laboratory cultivation is very challenging. Consequently, numerous efforts have been made to improve the cultivation and storage of thermophilic bacteria [92].

Complementarily, the use of protein engineering techniques has contributed for the improvement of thermal stability in peptidase; this is especially true for the subtilisin from *Bacillus* species employed in detergent formulation [100].

Conclusion

The microbial diversity has been highly explored by mankind, and from such studies, a variety of different peptidases with peculiar biochemical properties have been reported. Currently, seven different catalytic types of proteolytic enzymes are known. Bacteria and fungi serve as the potential production sources of these enzymes. By exploring the bacterial and fungal biodiversity, the enzyme technology has enabled a vast repertoire of peptidase applications,

leading to an increase in the number of scientific publications, especially related to microbial biochemistry. These studies have facilited the generation of new products with applications in pharmaceuticals, food, synthesis of bioactive peptides, basic research studies, leather treatment, and detergent industry, among others. Thus, for the future, continuous scientific investments are required for microbial enzymes prospecting for constant biotechnological advances and sustainable industrial development.

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