Chapter 2
Mass Multiplication, Production Cost Analysis and Marketing of Protease

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Abstract Proteases are widely distributed enzymes that play an important role in both synthesis and breakdown. The catalytic characteristics of proteases have allowed their use in a variety of industrial processes, including detergents, leather, textiles, medicine, feed, and trash. Researchers are exploring numerous techniques to discover, redesign, or artificially manufacture enzymes with improved applicability in industrial processes in response to the expanding demands and applications. Proteases have been successfully used as chemical substitutes and environmentally benign indications for nature and the environment. The most common protease producers are *Bacillus* sp. and *Aspergillus* sp., which are produced via submerged and solid-state fermentation, respectively. Thermostable and solvent-tolerant proteases are important for biotechnological and industrial applications because of their resistance to denaturing agents and chemicals. The current chapter highlights the microbial sources, mass production, existing and future uses of microbial proteases in various sectors, and the estimated costs to assist new entrepreneurs.

Keywords Proteases · Detergent industry · Leather industry · Textiles industry · Entrepreneur

2.1 Introduction

Enzymes are proteins produced by living organisms that catalyse chemical reactions in highly efficient and environmentally favourable ways. All enzymes, which are classified into six types, are essential for survival, and their malfunction causes disease (Homaei 2015). Proteases or proteolytic enzymes can break down peptide bonds in proteins. Proteases are classified as hydrolases in class 3 and peptide hydrolases or peptidases in subclass 3.4, according to the International Union of

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Biochemistry and Molecular Biology Nomenclature Committee. Proteases, often known as proteolytic enzymes, are the most basic and versatile enzymes involved in the functions of living organisms (Beg et al. 2003). Proteases are required for optimal cellular metabolism, particularly the mitochondrial processes, in biological contexts (Quiros et al. 2015). It also regulates the size, structure, and composition of essential proteins through numerous biochemical processes (Troncoso et al. 2022).

In terms of economics, new protease research is expanding because it accounts for 60% of all commercialized enzymes in the world. Proteases are now used in a variety of industries, including the leather and detergent industries, food technology, silver recovery, feed, chemical and waste treatment, and pharmaceutical manufacturing (Homaei et al. 2016). They are important in a variety of physiologic processes, including development, apoptosis, regulatory mechanisms, infection, fecundation, allergic responses, blood clotting, tumour growth, and bone remodelling, as well as in therapeutic targets (anti-inflammation, digestion, and wound healing) (Barzkar et al. 2018).

The classification of proteases remains difficult owing to the diversity of their methods of action and architecture. Protease enzymes are classified into two types based on the peptide bond cleavage site and their functions: exopeptidases and endopeptidases. The International Union of Biochemistry recognizes four mechanistic classes, each with six families of proteases: serine carboxy proteases (EC 3.4.16), serine proteases (EC 3.4.21), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23), metallocarboxy proteases (EC 3.4.17), and metalloprotease I (EC 3.4.24), depending on the specific catalytic amino acid (or metal) involved in proteolysis (Jornitz et al. 2011). However, three novel systems have recently been discovered: the threonine-based proteasome system, glutamate-glutamine system of equolisin, and sedolisin serine-glutamate-aspartate system (Mamo and Assefa 2018). Aspartic proteases (EC 3.4.23) are peptidases with diverse properties. It contains two aspartic acid residues (Asp32 and Asp215) within its active site, which are important for catalysis. Most aspartic proteases have isoelectric points in the pH range of 3–4.5, with optimal enzyme activity at low pH (pH 3–4). The inability of plant and animal proteases to meet the current global enzyme demands has increased interest in microbial proteases. Microbial proteases were chosen over plant proteases because they possess the majority of the properties required for biotechnological applications.

Microbial enzymes play a wide range of biochemical, physiological, and regulatory roles. Microbial proteases have played an important role in the manufacturing of traditional fermented foods for generations, and the industrial enzyme industry, which is dominated by microbial protease products, delivers biocatalysts to a wide range of industries (dos Santos Aguilar and Sato 2018). Peptide bond cleavage in industry can be performed enzymatically or chemically. Chemical, alkaline, or acidic hydrolysis is more difficult to manage and produces hydrolysates containing altered amino acids (Tavano 2013). Proteases can catalyse specific and selective protein changes. Proteins with restricted proteolytic activity may have a wide range of applications. For example, using particular hydrolytic conditions, hydrolysed products with diverse functional qualities can be created and used in a variety of sectors.

These functional features are critical because they determine the main aspects of a finished product and define its use.

2.2 Mass Microbial Protease Production Technology

2.2.1 Microorganisms and Source

The inability of plant and animal proteases to meet the current global demands for the enzyme has prompted interest in microbial proteases. Isolation and selection of potential microbes is the first step in the synthesis of microbial proteases. Microbial protease enzymes are favoured over plant and animal proteases because of the desirable features for biotechnological applications. Microorganisms are isolated from various sources and ecosystems and are then selected for appropriate characteristics (Patel and Dudhagara 2020). For example, hot springs, dumping sites, soda lakes, soil samples from milk processing plants, meat waste contaminated soil, detergent industry, leather industry, poultry waste sites, wood factory, and tannery waste have specific or adverse features that affect the characteristics of the enzyme to be produced (Solanki et al. 2021). Protease producers from bacteria, fungi, and actinomycetes have been isolated and identified. Some bacterial species that have been reported to produce proteases include *Bacillus subtilis*, *B. amyloliquefaciens*, *B. halodurans*, *B. licheniformis*, *B. lentus*, *B. circulans*, *B. safensis*, *B. pumilus*, *B. pseudofirmus, B. clausili*, *B. alkaloophilus*, *B. lehensis*, *B. stearothermophilus*, *E. coli*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Pyrococcus furiosus*, *Thermus aquaticus*, *T. thermophilus*, *T. stature*, *Geobacillus* SBS-4S, *Streptomyces nogalator*, and *S. avermectus* (Mamo and Assefa 2018; Razzaq et al. 2019; Solanki et al. 2021). Among them, *Bacillus* sp. has been extensively studied for protease production on a large scale and is used in a variety of industries, including leather, detergents, pharmaceuticals, and textiles. The fungi reported as protease producers are *Aspergillus niger*, *A. sterreus*, *A. favus*, *A. oryzae*, *A. fumigates*, *A. nidulans* HA-10, *A. clavatus* ES1, *A. saitoi*, *Botrytis cinerea*, *Fusarium* sp. *Penicillium chrysogenium*, *P. italicum*, *Conidiobolus coronatus*, *Cryphonectria* (Endothia) *parasitica*, *Mucor* sp., *Cephalosporium* sp., *Rhizopus* sp., and *Trichoderma* (Naveed et al. 2021). *Aspergillus* and *Trichoderma* are two major strains used for industrial protease production. However, thermophilic and halophilic proteases are gaining popularity in biotechnological applications because of their thermal stability and ability to preserve their activity under high stress from organic solvents.

2.2.2 Bioprocesses for Protease Production

A variety of approaches are being used for enzyme production from a dominant microbial source for economic improvement, but the search for high-quality

enzymes from bacteria for industrial usage continues. Low protease titres have always been a major concern, so several researchers are attempting to improve production titres through a variety of approaches, such as using better bioprocess technologies, using cheaper or crude raw materials as substrates for enzyme production, and bioengineering the microorganisms (Razzaq et al. 2019). Microbial growth substrates account for nearly two-fourths of total manufacturing costs (Singh et al. 2015). Researchers worldwide have been working on bioprocess optimization strategies to boost protease production and specific activities. For cost-effective generation of microbial proteases, both solid substrate and submerged fermentation (SmF) are used. Solid-state fermentation (SSF) has regained popularity in the last few decades owing to high titres of enzyme synthesis using fungal cultures. Protease secretion was most strongly influenced by lignocellulosic substrates. In SSF, the readily available substrate wheat bran is shown to be more promising for protease synthesis, whereas other inexpensive substrate sources such as cow dung, agro-industrial waste, groundnuts, and wheat bran can be effective in the manufacture of proteases (Hamza 2017). Furthermore, easily available substrate sources such as molasses from sugar industry waste, dairy sludge, and effluents have the potential for value-added product enzyme synthesis, while also helping to reduce environmental pollution (Corral et al. 2018; Razzaq et al. 2019). Some substrates considerably increased proteolytic enzyme synthesis without the addition of specific inducers to the growth medium. Nonetheless, the benefits of improved monitoring and management are still connected to concealed culture. Protease production in cultures is growth dependent and is regulated by a variety of factors such as substrate type, medium pH, and nutrient availability. Large-scale production of proteases necessitates an understanding and effective control of the producer's growth and enzyme production capabilities (dos Santos Aguilar and Sato 2018). Microbial proteases include induction and repression mechanisms (Zhang et al. 2020), which must be considered in the process design and media formulation for protease synthesis. Genetic modification, however, provides researchers with a new way to manipulate the microbial genome using various biotechnological tools to improve the yield of proteases with desired properties. Cloning and overexpression, strain screening, fed-batch, and chemostat fermentation are all methods that scientists have used to boost protease yield for industrial use. Genetic engineering with the goals of enzyme hyperproduction, cost-effectiveness, and quality aids scientists in capturing the global biotechnology market.

2.2.3 Solid-State Fermentation (SSF)

Solid-state fermentation refers to fermentation that occurs in the absence or near the absence of free water. SSF for the synthesis of industrial enzymes is rapidly gaining popularity as a cost-effective method because microorganisms, particularly fungal cultures, produce comparatively high metabolite titres under fermentation conditions that are comparable to those found in nature (Singhania et al. 2009; Singhania 2011).

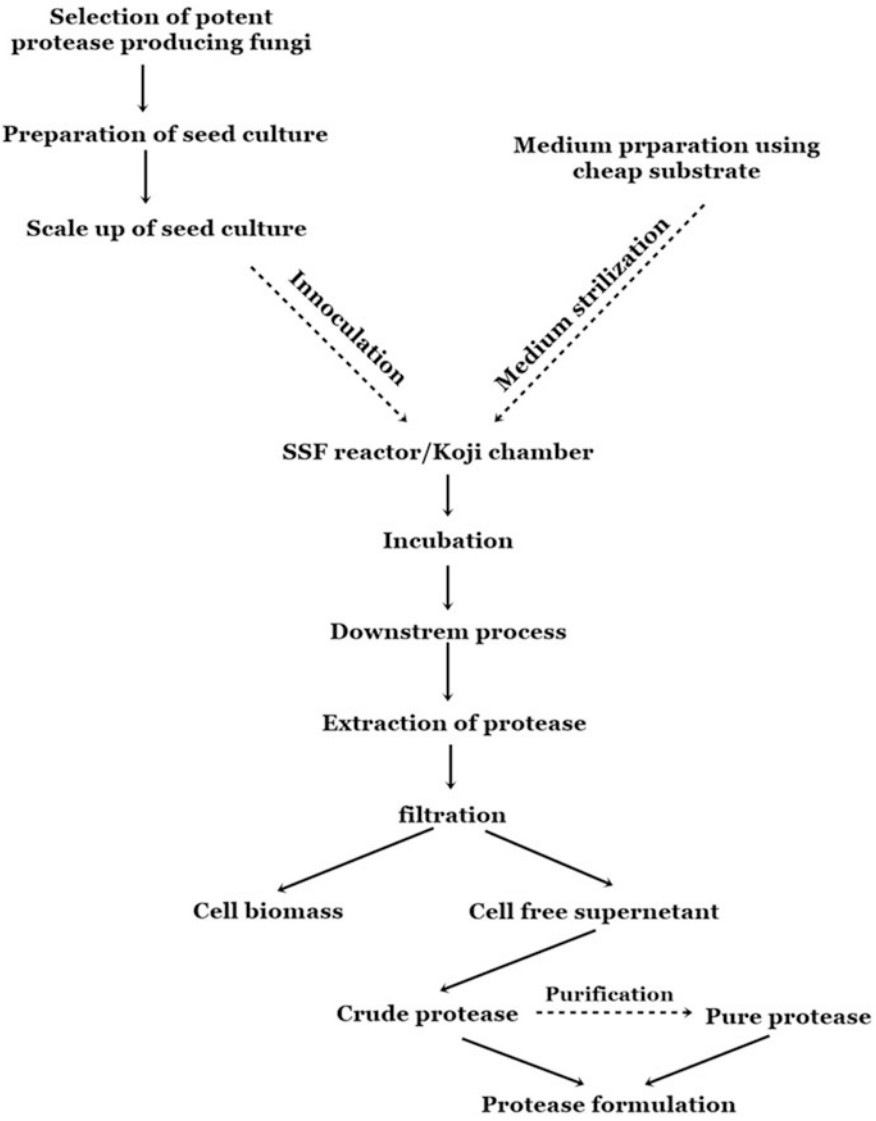

Fig. 2.1 Flowchart of protease production by SSF

Fungi such as *A. oryzae*, *M. miehei*, *C. parasitica,* etc. have been used to produce proteases utilizing SSF, in which a basal mineral salt medium was used to wet the substrate. Figure 2.1 shows the general steps of the SSF protease production process. For economic reasons, the Koji chamber can be utilized for large-scale production (Singhania 2011), but maintaining sterility is problematic. Agro-industrial waste can be used as a substrate. Inocula for protease synthesis can be created in a stirred tank reactor and sprayed onto a sterile medium in a shallow tray. Spores or mycelia can be

used as an inoculum. Temperature and humidity are controlled inside the koji chamber, and incubation is allowed for 7 days or as prescribed. A suitable buffer or distilled water with an adequate tTeen percentage is used as the extraction liquid. To remove biomass and cell detritus, the medium is homogenized with extraction liquid and centrifuged. The extracellular cellulase in the supernatant can be concentrated by acetone precipitation or salting out, or it can be used directly as a crude enzyme.

Substrate composition, pH, temperature, moisture content, and aeration are all critical parameters in protease production via SSF. The organism and substrate used may affect the working conditions. Fungi, for example, prefer an acidic pH and low moisture content (35–70%) compared to bacteria (70–90%) and grow best at 25–30 °C, whereas bacteria prefer a neutral pH and high moisture content and grow best at 35–37 °C. Owing to the low cost of input and the possibility of using naturally available sources, solid substrate fermentation may be a better technique for commercial protease production. Another key component is pH, which influences microbe development and, as a result, protease production. The pH of a solid substrate is difficult to monitor, but the pH of the basal medium, which usually contains nitrogen sources with buffering capabilities, can be altered. Heat transport is always limited because it is poor in the solid layer and overheating occurs in the substrate particle. This makes it difficult for spores to germinate, mycelia to proliferate, and enzymes to accumulate and secrete. Temperature management in a solid-state fermenter environment is very simple, but the temperature adjustment within the solid substrate layer is more complicated. Controlling the moisture content of the medium is also important for protease formation, which is a necessary component for microorganism development. The void space and gas-phase volume within the solid substrate are reduced when the water content is high, which increases the mass transfer resistance of oxygen and carbon dioxide, as well as the risk of contamination, whereas a low water content is unfavourable for spore germination and substrate swelling.

2.2.4 Submerged Fermentation

Fermentation in the presence of excess water is known as submerged fermentation. Owing to superior monitoring and handling, almost all large-scale enzyme production facilities use the proven technology of SmF. *B. subtilis*, *B. subtilis* var. *natto*, and *B. amyloliquefaciens* have been reported to produce proteases. As previously stated, the nature of the substrate, medium pH, nutrient availability, inducer supplementation, fermentation temperature, and other factors have a significant impact on protease synthesis in cultures (SmF). Increased production in the fermenter can be achieved through a gradient feed of a suitable substrate and maintaining ideal process conditions. Large-scale protease production requires an understanding and proper control of the producer's growth and enzyme production capabilities.

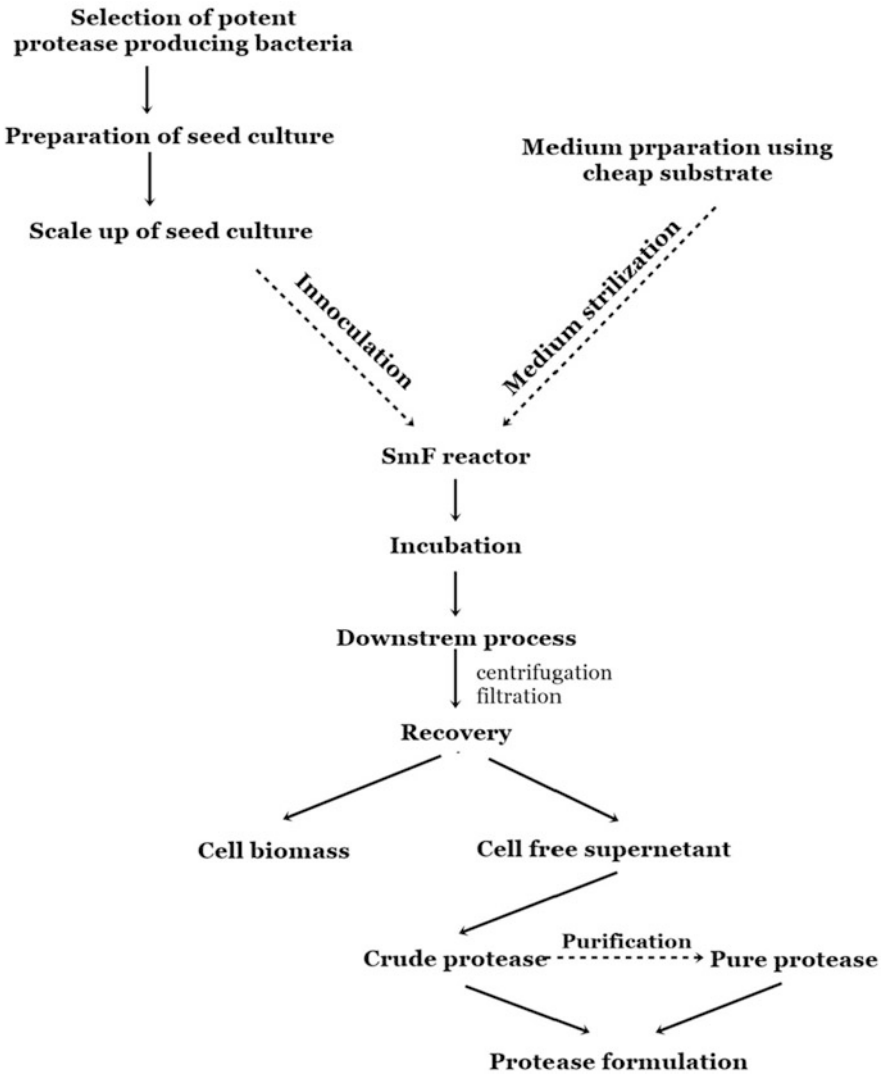

Fig. 2.2 Flowchart of protease production by SmF

Large bioreactors are available for submerged fermentation, and they also allow for easy control of many operating conditions such as pH, temperature, aeration, etc. Figure 2.2 depicts the general procedures involved in the manufacture of protease by submerged fermentation. SmF is the most widely used technology for the industrial production of primary and secondary metabolites. Because all the parameters required for modelling can be monitored in submerged fermentation, SmF has been used in the majority of modelling studies for metabolite synthesis (Singhania 2011).

2.3 Analysis of the Protease Production Cost

Here, we estimate the cost of generating proteases from bacteria and fungi, which will be useful for new entrepreneurs (Table 2.1).

2.4 Application of Protease

Proteases are the most important hydrolytic enzymes, where alkaline proteases are the most important enzymes in the enzyme market (Mahajan et al. 2015). Microbial acid proteases are primarily used in the detergent, food, leather, and pharmaceutical industries, whereas only a few alkaline protease products are marketed effectively (Fig. 2.3). Microbial proteases have a wide range of uses in the following industries.

2.5 Detergent Industry

Proteases are commonly used in the detergent industry on a commercial basis. Various detergent formulations containing proteases as essential components or ingredients have been used to clean domestic laundry, dentures, or contact lenses.

Table 2.1 Budget proposal for microbial protease production in small-scale operation

Cost item	Requirement	Amount (Rs)
Fixed capital cost estimation		
Total plant cost	Building construction	
	Electrical	
Equipment purchase	Laminar air flow	40,000
	Autoclave	20,000
	Fermenter (200 L)	5,60,000
	Incubator cum orbital shaker	45,000
	Centrifuge	20,000
Total cost		
Recurring cost estimation		
Raw materials	Wheat bran	7/kg; 105/month
Consumables	Glass wares and plastic wares	10,000[a]
Utilities	Media and chemicals	10,000[a]
Microbial strain	Fungal or bacterial strain from MTCC	2440/strain
Manpower	Microbiologist	25,000/month
	Labour	10,000/month
Transportation and waste treatment		
Total cost		

[a]Based on rate of utilization

Fig. 2.3 Application of microbial protease in various fields

Proteinaceous stains are particularly difficult to remove with regular detergents; nevertheless, such stains can be removed by utilizing microbial proteases. Furthermore, the addition of protease to detergent formulations increases the cleaning of proteinaceous stains and provides unique benefits that are not available with traditional detergent technologies. Enzymes are increasingly used in detergent formulations in industrialized countries, with enzymes found in more than 50% of all detergents (Hamza 2017). Most of these enzymes are produced by various bacterial species. *Bacillus* sp. have a wide range of applications in the textile and detergent industries. *B. cereus* BM1-produced protease has been reported as a good detergent ingredient, with stable action in a solution of 10% (w/v) commercial detergent, implying commercial use (Barberis et al. 2008).

2.6 Food Industry

Proteases are used in the food industry to modify proteins and increase the palatability and storage stability of protein sources. Proteases are added to milk cheese to hydrolyse kappa casein and prevent coagulation by stabilizing micelle formation. Because of its high specificity for casein, particularly the Phe105-Met106 link of k-casein, which is the first step in cheese manufacturing chymosin is the favoured protease in the cheese-making industry. Higher photolytic rates and shorter ripening times in cheddar-type cheeses were aided by microbial rennet from *B. amyloliquefaciens*. Proteases from *Mucor miehei* and *B. subtilis* have gradually replaced chymosin in cheese production. Alkaline proteases have been used to prepare protein hydrolysates with high nutritional value and well-defined peptide profiles. It also aids in tenderization of meat, particularly beef (Gupta et al. 2002). In the baking industry, endo- and exoproteases from *A. oryzae* have been used to change wheat gluten by restricted proteolysis, which reduces mixing time, improves dough texture, and boosts loaf volume. Wheat gluten solubility is substantially enhanced by the breakdown of wheat gluten by an acid protease from *A. usamii* under optimal conditions. The emulsifying activity index (EAI), water, and oil-holding capacity of wheat gluten increase dramatically after enzyme hydrolysis (Deng et al. 2016). Furthermore, after hydrolysis, the functional characteristics of the wheat gluten were enhanced.

2.7 Leather Industry

Proteases are enzymes utilized to degrade non-collagenous skin constituents and eliminate non-fibrillary proteins. The use of enzymes in leather processing improves the quality of leather, making it stronger and softer with fewer spots (Solanki et al. 2021). The elastolytic and keratinolytic actions of alkaline proteases have increased their use in the growing leather industry. Alkaline proteases are extremely useful in the leather processing industry. Proteases have been found to be useful in the soaking, bating, and dehairing stages of skin and hide preparation. Protease eliminates undesirable colours and aids in the creation of clean hides while lowering environmental pollution (Brandelli et al. 2010).

2.8 Medical Field

In medicine, various formulas including alkaline proteases produced by *B. subtilis*, such as gauze, non-woven tissues, and ointment compositions, exhibit promising therapeutic characteristics (Awad et al. 2013). The diagnosis of certain lytic enzyme-deficient diseases is aided by the oral introduction of alkaline proteases (Joshi and

Satyanarayana 2013). Fibrin breakdown has been found to be accomplished by alkaline fibrinolytic proteases. The utilization of fibrinolytic enzymes suggests that they could be used as an anticancer medicine or in thrombolytic therapy in the future (Jaouadi et al. 2012). The preparation of elastoterase immobilized on a bandage is used to treat various diseases such as burns, carbuncles, furuncles, and wounds (Palanivel et al. 2013).

2.9 Chemical Industry

Many alkaline proteases-producing microbes such as *B. pseudofirus* SVB1, *P. aeruginosa* PseA, and *A. flavus* have demonstrated significant success in peptide synthesis due to their durability in organic solvents (Razzaq et al. 2019). Several *Bacillus* and *Streptomyces* species that produce alkaline proteases in the water systems are active candidates for peptide and chemical synthesis (Yadav et al. 2015).

2.10 Miscellaneous Applications

Protease is also used in waste management, photographic industry, silk degumming, and beer and wine industries (Mamo and Assefa 2018; Razzaq et al. 2019). Poultry feathers with a very hard keratin structure account for 5% of body weight and are a great source of proteins for feed and food. Keratinolytic processes can convert poultry manure into feed and food. Alkaline proteases produced by *B. subtilis*, *Conidiobolus coronatus*, and *S. avermectnus* have been reported to successfully recover silver from X-ray films, ensuring that the process is more environmentally friendly than the use of chemicals. In bench-scale fermentation conducted at 200 °C, the addition of acid proteinase from *Saccharomycopsis fibuligera* 1570 and *Torulopsis magnoliae* 1536, as well as brewer's yeast, to brewer's wort demonstrated that the final bottled beer was resistant to haze formation. Acid protease enzymes can degrade the turbidity complexes formed by proteins in fruit juices and alcoholic beverages.

2.11 Market Trend of Protease

Proteases are one of the three major categories of industrial enzymes, accounting for more than 60% of the global enzyme sales. Microbes account for two-thirds of all commercial proteases sold worldwide. During this forecast period, the worldwide protease market is expected to develop at a CAGR of 5.8% (2022–2027). Since the late 1990s, proteases have been widely used in detergents. Protease use in the detergent industry accounts for 20% of the total enzyme sales. Proteases with the

names alcalase (*B. licheniformis*), Savinase (*Bacillus* sp.), Esperase (*B. lentus*), and Durazym (Protein designed, version of Savinase®) were introduced by Novoenzyme, Denmark, and used in detergents, degumming, and the textile industry. Denmark's Novo Industry is one of the world's leading protease producers, accounting for 40% of the global protease market. It produces three different types of proteases, Aquaderm, NUE, and Pyrase, which are used for soaking, dehairing, and bating, respectively (Sandhya et al. 2005). Fungal aspartic proteases (Aps) have been used as milk-clotting enzymes in the dairy sector for approximately 30 years because of the global scarcity of calf chymosin. Aspartic protease enzymes derived from *M. miehei*, *M. pusillus*, and *C. parasitica*, and sold under the brand names Rennilase®, Fromase®, Novoren®, Marzyme®, Hannilase®, Marzyme®, and Suparen® are commonly used in the manufacture of cheese (Mamo and Assefa 2018). The use of proline-specific endoproteinases (e.g., Brewers Clarex®, DSM, France) that target the degradation of haze-active proteins (e.g., hordeins) minimizes the production of storage haze in the finished beer. Similarly, proteases with the tread names proleather (*Bacillus* sp.), protease Savinase (*B. licheniformis*), and Biofeed protease (*B. licheniformis*) were utilized in the food and feed industry.

2.12 Conclusion

Microbial proteases are key hydrolytic enzymes that have been widely used since the beginning of enzymology. It has a wide range of uses in the detergent industry, bioremediation, food processing, and leather processing, and has been widely commercialized by a variety of companies. Owing to the use of low-cost basic materials and genetic manipulation, their use and manufacturing are rapidly increasing. New technology adoption is required to enhance cleaner production in all industries, particularly the leather and treatment industries, by replacing most existing chemical techniques with less expensive and more environment-friendly alternatives, particularly proteases.

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