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Protease Enzymes: Present Status and Future Perspectives for Industrial Sector

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A B S T R A C T

Proteases or peptidases constitute the largest group of enzymes in bio industry with a long array of uses. They play an invincible role in industrial biotechnology, especially in detergent, food and pharmaceutical arena. Interest has been growing in microbial proteases which have eco friendly as well as commercial importance. This focused review encompasses an overview on proteases, mainly of microbial sources in a handy module. Its classification with evolutionary insight, major sources of proteases (animal, plant and microbial including fungal, bacterial), and their general properties are discussed. In addition to this, an overview on the applications of proteases in detergent, tannery, food, metal recovery and waste treatment industries is also addressed briefly.

Introduction

Enzymes are used in many environmental- friendly industrial purposes, as they are efficient, selective, accelerate and speed up reactions by forming transition state complexes with their substrate which reduces the activation energy of the reaction. With the advancement in biotechnology, especially in the area of genetics and protein engineering, have opened a new era of enzyme applications in many industrial processes and experiencing major research and development initiatives, resulting not only in the development of a number of new products but improvement in the process and performance of several existing processes also. Alkaline proteases (or Subtilisins, E.C. 3.4.21.14) are an important category of enzymes that hydrolyze protein peptide bonds under alkaline conditions. They are widely used in detergents, leather processing, silk processing, medicine, food, animal feed, environment protection, chemical processing and (as an alternative for protease K) in DNA isolation (Kwon *et al.,* 1994; Kumar and Takagi, 1999; Kirk *et al.,* 2002; Mei and Jiang, 2005; Mukherjee *et al.,* 2008). In 1994, the total market for industrial enzymes accounted for approximately $400 million, of which enzyme worth $112 million were used for detergent purposes (Hodgson, 1994).

Proteases encompass about 60% of the total enzyme market and stand amongst the most precious commercial enzymes. The global proteases market is

expected to uphold a CAGR of 6.4% during the forecast period of 2017 -2022. It is estimated to be worth around USD 3.29 billion by 2022.

Sources of Proteases

Proteases are essential constituents of all forms of life on the earth, including prokaryotes, fungi, plants and animals, because they are necessary for living organisms. Microorganisms account for a two-third share of commercial protease production in the world (Jisha *et al.,* 2013). Protease of bacteria, fungi and viruses are increasingly studied due to its importance and subsequent applications in industry and biotechnology. Commercial application of microbial protease is attractive due to the relative ease of large scale production as compared to proteases from plants and animals.

Plant Proteases

The use of plants as a source of proteases is governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Papain, bromelain, keratinases and ficin represent some of the well-known proteases of plant origin (Kumari *et al.,* 2012). Papain is a cysteine protease (EC 3.4.22.2) extracted from the latex of papaya (*Carica papaya*). The rough enzyme has broad specificity due to the mixture of several proteases. Bromelain (EC 3.4.22.32) is a crude extract from the pineapple (*Ananas comosus*) plant. Bromelain is present in all parts of the pineapple plant but the stem is the most common commercial source. Ficin is extracted from the latex of ficus and is a sulfhydryl proteinase with cysteine at the active site (EC 3.4.22.3). It preferentially cleaves at tyrosine and phenylalanine residues. Ficin has proven to be a versatile low-cost biocatalyst useful in peptide synthesis (Sekizaki *et al.,* 2008).

Animal Proteases

The mainly familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennins. These are ready in pure form in bulk quantities. However, their construction depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies (Kumari *et al.,* 2012).

Trypsin is a serine protease (EC 3.4.21.4) found in the digestive system and is responsible for the breakdown of food proteins. Trypsin has a optimum pH and temperature of about 8 and 37°C respectively and predominantly cleaves proteins at the carboxyl side of the lysine and arginine**.** Chymotrypsin is found in the pancreatic extract of animals (EC 3.4.21.1). The enzyme cleaves peptides at the carboxyl side of tyrosine, tryptophan and phenylalanine although over time it also hydrolyzes other amide bonds, particularly those with leucine-donated carboxyls**.** Pepsin is a digestive protease (EC 3.4.23.1) released by the chief cell in the stomach of almost all vertebrates that function to degrade food proteins into peptides. Pepsin is produced in its zymogenic form i.e pepsinogen, whose primary structure has additional 44 amino acids. This zymogen is activated by hydrochloric acid (HCl), which is released from the parietal cell in the stomach lining**.** Rennin is an aspartic acid protease (EC 3.4.23.4), produced as an inactive precursor, prorennin in stomachs of all nursing mammals but more specifically in the fourth stomach of cows. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in *k*-casein to generate insoluble para-*k*-casein and C-terminal glycopeptides (Ghafoor and Hasnain, 2010).

Microbial Proteases

Proteases are broadly distributed in microbial population viz. bacteria, actinomycetes, viruses, and fungi. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes and account for around two-thirds of commercial manufacture worldwide. Alkaline serine proteases (EC 3.4.21) are the most important group of commercial enzymes (Kumar and Takagi, 1999). In progress world demand for proteases has led to an

interest in microbial proteases because of their rapid growth, cost effectiveness and the ease with which they can be genetically modified to generate high yielding strains with more efficient enzymes with desirable properties required for their diverse applications.

The proteases available nowadays in the market are derived from microbial sources. This is due to their high output, limited cultivation space requirement, easy genetic manipulation, broad biochemical diversity and desirable characteristics that make them suitable for biotechnological applications (Singhal *et al.,* 2012). Proteases are ubiquitous and found in several microorganisms such as protozoa, bacteria, yeast and fungi. Microbial proteases can be cultured in large quantities in a relatively short time by established methods of fermentation and they also produce an abundant, regular supply of the desired product. Microorganisms account for a two-third share of commercial protease production in the world (Vishwanatha *et al.,* 2010).

Microbial proteases are among the most significant hydrolytic enzymes and have been studied extensively since the advent of enzymologist. Microorganisms involved a large array of proteases, which are intracellular and extracellular. Intracellular proteases are important for diverse cellular and metabolic processes, such as speculation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products. At the similar time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes (Gupta *et al.,* 2002).

Bacteria

Protease production is an inherent capacity of all microorganisms; and a large number of bacterial species are known to produce alkaline proteases of the serine-type, although very few are recognized as commercial producers. Microbial proteases account two third of the total worldwide enzyme sales as they possess almost all the characteristics desired for biotechnological applications (Genckal, 2004). Most of the commercial proteases are of bacterial origin (Prakasham *et al.,* 2006). Though proteases are produced by a variety of bacteria such as *Pseudomonas aeruginosa*, *Flavobacterium*, *Clostridium*, *Staphylococcus aureus*, *Achromobacter*, *Thermoactinomyces* and species belonging to *Streptomyce, Bacillus* is the major source which secretes a variety of soluble extracellular enzymes (Nirmal *et al.,* 2011).

Alkaline proteases from the bacterial source are broadly used in detergent formulations due to their activity and stability at high pH from 9.0 to 11.0 and temperature 50-60°C. Neutral proteases of bacterial origin are active at pH 5.0-8.0 and between 35-40°C. Compared to alkaline proteases, neutral proteases have lower thermo-tolerance. Protein hydrolysate produced by neutral proteases of microbial origin has less bitterness compared to the one using animal trypsin and hence finds application in food industry. Neutral proteases are also used in the brewing industry. Alkalophilic bacteria are also known to produce proteases. The primary report of alkaline protease by an alkalophilic *Bacillus* sp. strain 221 was published in 1971 by Horikoshi (1971). Bacteria are mainly important alkaline protease manufacturer with the genus *Bacillus* being the most prominent source because of their ability to produce a large amount of protease having significant proteolytic activity and stability at high pH and temperature. *Bacilli* genus alone contributes more than 70% due to ease in production and purification (Kumar and Vats, 2010; Hema and Shiny, 2012; Ash *et al.,* 2018). Among the various sources, soils samples are known to be the common habitats frequently investigated for isolation of proteases.

Fungi

Filamentous fungi can effectively produce various hydrolytic enzymes and one of the major groups of

secreted enzymes in fungi is the protease. Fungi are known to produce acid, neutral, alkaline and metalloproteases. Fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity (Yike, 2011). One of the first known representatives of proteases was proteinase K, an alkaline enzyme from *Engyodontium album* also known as *Tritirachium album* (Kotlova *et al.,* 2007). Several reports are available on production of proteases by fungi belonging to the genera *Aspergillus* (Hajji *et al.,* 2007; Vishwanatha *et al.,* 2010), *Penicillium* (Zhu *et al.,* 2009; Krishna *et al.,* 2009), *Rhizopus* (Kumar *et al.,* 2005), *Humicola* (Aleksieva *et al.,* 2003), *Thermomyces* (Jensen *et al.,* 2002).

Among fungi, the ability of many species of *Aspergillus* to produce proteases is well known. For example, *Aspergillus oryzae* produces acid, neutral and alkaline proteases. Endo and exoproteases from *A. oryzae* have been used to modify wheat gluten, an insoluble protein, by limited proteolysis thereby facilitating its handling and machining (Singhal *et al.,* 2012).

Viruses

Viral proteases are concerned in the processing of proteins that cause fatal diseases like AIDS and cancer. Mature enzymes encoded within the human immunodeficiency virus type 1 (HIV-1) genome protease (PR); reverse transcriptase (RT) and integrase (IN) are derived from proteolytic processing of a large polyprotein (Gag-Pol). The viral PR catalyzes Gag-Pol processing, which is active as a homodimer (Olivares *et al.,* 2007). The HIV-1 protease is a homodimeric enzyme composed of two identical subunits of 99 amino acids. Each subunit contains the conserved sequence Asp-Thr-Gly that provides the aspartyl group essential for catalysis.

Classification of Proteases

Proteases constitute a very huge and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima and stability profile.

Proteases can be classified in different ways. Through organism plant, animal and microbial, where animal and plant derived proteases are produced by extraction while microbial mainly fungal and bacterial derived proteases are produced by fermentation.

Proteases can be classified by their pH activity ranges *viz.* acid, neutral, alkaline, and by their peptide bond specificity endopeptidases, exopeptidases, or amino acid specific proteases. According to the most favorable pH classification, proteases may be classified by the optimal pH in which they are active like Acid proteases, Neutral proteases, Basic proteases or alkaline proteases (Kumari *et al.,* 2012).

According to the Committee of International Union of Biochemistry and Molecular Biology (1992), proteases are classified in subgroup 4 of group 3 (hydrolases). The exopeptidases are classified mainly on the basis of their actions. Only peptides with unsubstituted terminus are attacked with the exception of a very small number, grouped as omega peptidases (E.C. 3.4.19) one of the examples is Peptidyl–glycinamidase (3.4.19.2.), which can release certain modified terminal residues. Proteases or proteolytic enzymes are enzymes that break peptide bonds between amino acids of proteins. The process is called proteolytic cleavage, a common mechanism of activation or inactivation of enzymes. They use a molecule of water for this and are thus classified as hydrolases. There are currently four classes of proteases:

Serine proteases

Cysteine proteases

Aspartic proteases

Metalloproteases

Serine proteases

Serine proteases (E.C. 3.4.21) are characterized by serine at the active site. They are numerous and widespread among bacteria, fungi, and viruses. They are found in endopeptidases as well as exopeptidases. Three residues which form the catalytic triad are essential in the catalytic process *i.e* His (base), Asp (electrophile) and Ser (nucleophile). The first step in the catalysis is the formation of an acyl-enzyme middle between the substrate and the essential serine. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate and then the peptide bond is cleaved. During the second step or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme in Fig. 2.1. One of the example of serine protease is Chymotrypsin (E.C. 3.4.21.1), which also involves the configuration of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is an attacking nucleophile as a substitute of Ser residue. His residue provides a general base and accepts the OH group of the reactive Ser residue (Rao *et al.,* 1998). The occurrence of serine proteases has been reported in only a few fungi. Intracellular enzymes with properties similar to serine proteases have been reported in *Trichosporon species*, *Oidiodendron kalrai,* and *Nannitzla fulva.* Extracellular serine proteases have been observed in *Microsporium* species, Aspergillus *oryzae,* and *Sporotrichum pulverulentumi,* Most of these enzymes are active at pH 5.0-8.0.

Catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential Cys and His play the same role as Ser and His respectively as in serine proteases. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighboring imidazolium group of His. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps (Barrett *et al.,* 2012).

Cysteine proteases (E.C.3.4.22) are common in animals, eukaryotic microorganisms, and bacteria as well as in plants. In animals, they are sequestered lysosomal compartments in the cytoplasm, where they are thought to be involved in intracellular protein turnover. Cysteine proteinases are not secreted as intestinal digestive enzymes in higher animals but are found in midguts of several families of Hemiptera and Coleoptera where they appear to play important roles in the digestion of food proteins. These particular insects characteristically have mildly acidic pH in their mid guts near the pH optima of cysteine proteinases at pH 5.0. In plants the most common cysteine protease is Papain (E.C.3.4.22.2) present in papaya fruit plays a key role in digestive processes involving breaking down tough protein fibers (Vishwanatha *et al.,* 2010).

Aspartic proteases

Aspartic proteases (E.C.3.4.23) are characterized by maximum activity at low pH 3.0-4.0 and insensitivity to inhibitors of the other three groups of enzymes. They are widely distributed in fungi (pepsins E.C.3.4.23.1) and rarely found in bacteria or protozoa. Most aspartic proteases are sensitive to epoxy and diazo ketone compounds in the presence of copper cations. They are also inhibited by pepstatin or streptomyces pepsin inhibitor. Catalysis by aspartic proteases does not involve a covalent transitional through a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers: one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism, leads to the formation of a non-covalent neutral tetrahedral intermediate (Barrett *et al.,* 2012).

These enzymes are specific beside aromatic or bulky amino acid residues on both sides of the cleavage point. Catalytic behaviors involve two aspartic acid residues. The catalytic mechanism of the aspartic proteases requires the initial binding of a water

molecule at the active site before the nucleophilic attack on the substrate peptide bond. Most of the fungal aspartic proteases are unstable above neutral pH and are not found in cultures growing at neutral or alkaline pH (Nirmal *et al.,* 2011).

Metalloproteases

Metalloproteases (E.C.3.4.24) have pH optima between pH 5.0-9.0 and are sensitive to metal-chelating reagents, such as EDTA, but are unaffected by serine protease inhibitors or sulphydryl agents. Many of the EDTA- inhibited enzymes can be reactivated by ions, such as zinc, calcium, and cobalt. These are widespread, but only a few have been reported in fungi. Most of the bacterial (Sepia proteinase E.C.3.4.24.2) and fungal metalloproteases are zinc- containing enzymes, with one atom of zinc per molecule of enzyme. The zinc atom is essential for enzyme activity. Calcium is required to stabilize the protein structure the catalytic mechanism by metalloprotease leads to the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group (Barrett *et al.,* 2012). Alternatively, proteases may be classified by the optimal pH in which they are active:

Acid proteases

Acid proteases obtained by both bacteria and fungi. The enzymes have pH optima of 2.0-4.0. These proteases are used in medicine, in the digestion of soy protein for soy sauce making and to break down wheat gluten in the baking and food industry.

Neutral proteases

Neutral proteases secreted by both fungi and bacteria. They are comparatively unstable and ions such as Ca^{++}, Na^{+} and Cl^{-} must be added for more stability. The pH range of activity is moderately narrow and the enzymes are not very stable to enlarged temperatures. The neutral proteases are also quickly inactivated by alkaline proteases. Because of these limitations, they have restricted industrial application but do find some uses in leather and food industry for the manufacture of crackers, bread, and rice cake.

Alkaline proteases

All microorganisms follow a usual distribution pattern based on the pH dependence for their optimal growth, and the majority of these microorganisms are known to proliferate well at near neutral pH values. As the pH moves away from this neutral range the number of microorganism decreases. Alkaline proteases have an optimum pH greater than or equal to 9.0. They are most commonly used as detergent additives. They are more stable at high temperature and in the alkaline range 9.0 – 11.0 (Ash *et al.,* 2018). They are stable in association with chelating agents and perborates (Nadeem *et al.,* 2013).

Alkalophilic microorganisms constitute a diverse group that thrives in highly alkaline environments. They have been further categorized into two broad groups, namely alkalophiles and alkalotolerants. The term alkalophiles are used for those organisms that were capable of growth above pH 10.0, with an optimal growth around pH 9.0, and are unable to grow at pH 7.0 or less. On the other hand, alkalotolerant organisms are capable of growing at pH values 10.0 but have an optimal growth rate at pH 7.0.

The extreme alkalophiles have been further subdivided into two groups, namely facultative and necessitate alkalophiles. Facultative alkalophiles have optimal growth at pH 10.0 or above but can grow well at neutrality, while obligate alkalophiles fail to grow at pH 7.0. One of the most important and noteworthy features of many alkalophiles is their ability to modulate their environment. They can convert neutral medium to alkaline and high alkaline medium to optimize external pH for growth (Ray, 2012).

Extracellular Protease Production and Method

As the term extracellular applies, it is recognized as that enzyme which functions outside the cell in which it originates. Be it a fungi or bacteria, the source can be mesophiles acquired everywhere. Mesophilic bacteria and fungi are usually obtained from soil there is no temperature restraint to abide in the investigational work. As recognized the *Bacillus* sp. are well known for the production of extracellular protease. Most bacteria from the *Bacillus* species can be obtained from soil and are preferred to be used in the production of extracellular enzymes.

Basically, there are two methods of production of enzymes, one is submerged fermentation (SmF) and other is solid-state fermentation (SSF). In SmF, microorganisms grow in suspended form in liquid media supplemented with protein-rich sources like soya bean meal, wheat bran, casein corn step liquor, soya oil etc. Most of the times, the major production cost of enzymes is estimated to be due to the growth medium necessitating optimization of fermentation conditions for cost-efficient enzyme production. Most of the commercial proteases are generally produced by SmF.

There is no universal medium for protease production and media composition varies from organism to organism (Vishwanatha *et al.,* 2010; Nirmal *et al.,* 2011). In SSF, different agricultural residues are used as solid support like wheat bran, rice bran, soya bean cake, lupine or combination of substrates like sunflower meal and wheat bran. SSF processes are usually simpler and use wastes or agro industrial substrates such as defatted soybean cake, wheat and rice bran for enzyme production. The minimal amount of water allows the production of metabolites in a more concentrated form making the downstream processing less time consuming and less expensive. The product can be recovered in highly concentrated form as compared to those obtained by SmF. With regard to cost economics, SSF has been proved to be more efficient than SmF (Sumantha *et al.,* 2006). Peptidases normally employed to commercial levels in the textile and agro-industries are crude extracts (Kumar and Takagi, 1999). However, purification is required before deepening information on the operational features of any enzyme. There are no strict rules for purification of peptidases, but a general scheme for purification can be outlined: product recovery, isolation and purification, and eventual stabilization (Gupta *et al.,* 2002).

Purification of Proteases

Studies on the expenditure effective purification method are very much essential for the industrially important enzyme-like protease. To differentiate an alkaline protease and determine its potential viable application it is necessary to purify the enzyme thus produced. Salt precipitation and chromatographic separation ensure purification of the enzyme to some extent. Protein gel electrophoresis reveals the degree of purification and as a result, several other techniques are employed for purification of the same to a further extent. Patil and Jadhav (2017) isolated, purified and partially characterized thermostable serine alkaline protease enzyme from a *Bacillus subtilis* PE II. They have extracted proteases in bulk amount, checked its molecular weight is 15 kDa, optimal pH at 10.0, the optimum temperature is 60°C with casein as substrate. This enzyme is activated by metal ions, for example, Ca2+ Mg2+ and Mn2+ but its activity is powerfully inhibited by phenyl methyl sulphonylthoride (PMSF) and disopropyl thorophosphates (DFP).

Application of Proteases

Food and feed industry

Microbial proteases have been exploited in the food industries in many ways especially alkaline proteases in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks (Neklyudov *et al.,* 2000).

The alkaline elastase and thermophilic alkaline protease are used as meat tendering enzymes. During the preparation of bread, proteases are added to modify wheat gluten and milk proteins. Endo and exo proteinases from *Aspergillus oryzae* are used to modify wheat gluten by limited proteolysis resulting in the improvement of dough elasticity permitting easier machining and consequent increase in loaf volume, better grain, symmetry, and texture. Proteases are also used in biscuit, cracker, and cookie manufacturer. Proteases find huge potential in various foods and feed industrial applications such as in dairy industry (milk protein; casein and whey protein hydrolysis for use in cheese flavor development), baking industry (treatment of flour in the manufacture of baked goods and improvement of dough texture, flavor, and color in cookies, *etc.*), brewing industry, soy protein hydrolysis, soy sauce production, gelatin hydrolysis, meat protein recovery, fish protein hydrolysis and meat tenderization and improves digestibility of animal feeds (Jisha *et al.,* 2013). Proteolytic enzymes are used for processing strong gluten flours with high resistance and elasticity and low extensibility. The dough obtained from strong gluten flours cannot expand under pressure of the gas fermentation which shows that it has little capacity to retain the gas. Dough elasticity is improved at low doses of protease and it is reduced at higher doses.

Dairy industry

The major application of proteases in dairy industries is the manufacture of cheese. In cheese making, the primary function of proteases is to hydrolyze the specific peptide bond to generate *p*k-casein and macro peptides. The most significant property of acidic proteases is the ability to coagulate milk proteins (casein) to form curds from which cheese is prepared after the removal of whey. By virtue of this property, microbial acidic proteases have largely replaced the calf enzyme (rennet), facilitating the expansion of the cheese manufacture industry whose development was hurdled by animal rights issues. Alkaline protease was also used for the production of whey protein hydrolysate, using cheese whey in an industrial whey bioconversion process. The proteases produced by GRAS (generally regarded as safe) microbes such as *Bacillus subtilis*, *Mucor michei*, and *Endothia parasitica* have been used in cheese production. They are also involved in lactose reduction and flavor modification in dairy applications (Ray, 2012).

Leather industry

Leather manufacture is one of the highly polluting industries and generates solid wastes as well as liquid effluents. The major source of pollution is from dehairing step in the pre-tanning operations due to the use of hazardous chemicals like lime and sulphide. Leather processing involves a number of steps such as soaking, dehairing, bating, degreasing and tanning etc. Though proteases have been used for bathing for more than a century, their use for soaking and dehairing is more recent.

The purpose of soaking is to swell the hide and addition of a small amount of protease to soaking liquor were found to facilitate water uptake and reduce the time required for swelling. Since skin and hair consist of proteins, their selective removal with proteases is environmentally friendly. The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental pollution and safety hazards.

Thus, for environmental reasons, the bio-treatment of leather using an enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction, thus being eco-friendly. Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. Alkaline protease with elastolytic and keratinolytic activity has been used in leather processing industries. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced. Bating is traditionally an enzymatic process involving pancreatic proteases. However, recently, the use of microbial alkaline proteases has become popular. Alkaline proteases speed up the

process of dehairing, because the alkaline conditions enable the swelling of hair roots; and the subsequent attack of protease on the hair follicle protein allows easy removal of the hair (Ray, 2012; Jisha *et al.,* 2013).

Detergent industry

One of the major applications of microbial proteases is in detergent industry, which accounts for around 25% of the total worldwide sale of enzymes. Though the first protease to be used in commercial detergents was pancreatic trypsin way back in 1913, the first detergent containing bacterial protease was introduced only in 1956 under the trade name Bio-40. Novo Industry introduced Alcalase, an alkaline protease produced by *Bacillus licheniformis* (BIOTEX). All the proteases presently used in the market are serine proteases produced by *Bacillus* strains. Enzymes have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies.

Applications of detergent proteases have grown substantially and the largest application is in household laundry detergent formulations. The increased reliance of detergent manufacturers on enzyme technology is because of consumer recognizable cleaning benefits, the addition of completely new performance benefits, fabric restoration, and an increased performance/cost ratio, because of the availability of more efficient enzymes and the industry trend toward reduced pricing (Jisha *et al.,* 2013; Ash *et al.,* 2018).

In addition, enzyme suppliers and detergent manufacturers are actively pursuing the development of new enzyme activities that address the consumer expressed need for improved cleaning, fabric care, and antimicrobial benefits. Apart from their use in laundry detergents, proteases are also popular in the formulation of household dishwashing detergents and both industrial and institutional cleaning detergents (Ray, 2012). Some of the fungal proteases are also reported to be suitable for the detergent application (Hajji *et al.,* 2007).

Silk degumming

One of the least explored areas for the use of proteases is the silk industry and only a few patents have been filed describing the use of proteases for the degumming of silk. Sericin, which is about 25% of the total weight of raw silk, covers the periphery of the raw silk fibers, thus providing the rough texture of the silk fibers. This sericin is conventionally removed from the inner core of fibroin by conducting shrink-proofing and twist-setting for the silk yarns, using starch (Vaithanomsat *et al.,* 2008). The process is generally expensive and therefore an alternative method suggested is the use of enzyme preparations, such as protease, for degumming the silk prior to dyeing. The silk-degumming efficiency of an alkaline protease from *Bacillus* sp. RGR-14 was studied and results were analyzed gravimetrically (fiber weight reduction) and by scanning electron microscopy (SEM) of treated silk fiber. After 5 h of incubation of silk fiber with protease from *Bacillus* sp., the weight loss of silk fiber was 7.5%. Alkaline proteases remove serecin covering the silk fibroin and increase the luster without damaging the properties of the fiber (Gulrajani *et al.,* 2000).

Medical and Pharmaceutical industry

Alkaline proteases are also used for developing products of medical importance. Collagenases with alkaline protease activity are increasingly used for therapeutic applications in the preparation of slow-release dosage forms. A new semi-alkaline protease with high collagenolytic activity was produced by *Aspergillus niger* LCF9. The enzyme hydrolyzed various collagen types without amino acid release and liberated low molecular weight peptides of potential therapeutic use (Genckal, 2004).

Pharmaceutical and clinical applications of proteases include their use in digestive aids and treatment of

burns. Collagenases with alkaline protease activity are increasingly used for therapeutic application in the preparation of slow release dosage forms. Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery. These Waste films contain 1.5–2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Conventionally, this silver is recovered by burning the films, which causes undesirable environmental pollution. Furthermore, a base film made of polyester cannot be recovered using this method. Since the silver is bound to gelatin, it is possible to extract silver from the protein layer by proteolytic treatments. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled. A new semi-alkaline protease with high collagenolytic activity was produced by *Aspergillus niger* LCF9 which hydrolyzed various collagen types and liberated low molecular weight peptides of potential therapeutic use without the release of amino acids (Barrett *et al.,* 2012). Alkaline proteases are used for developing products of medical importance. The proteases are also used as potential bacteriocidal agents and for removal of protein contaminants from antibiotic preparation. An asparginase isolated from *E. coli* is used to eliminate aspargine from the bloodstream in the various forms of lymphocytic leukemia. An alkaline protease with fibrinolytic activity is used as a thrombolytic agent (Kumar and Takagi, 1999). These include elastolytic activity for the preparation of elaterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses, as a thrombolytic agent having fibrinolytic activity (Ray, 2012).

Chemical industry

Proteases have been used successfully for the synthesis of dipeptides and tripeptide, regioselective sugar esterification and dia-stereoselective hydrolysis of peptide esters. Enzymatic peptide synthesis offers several advantages over chemical methods, e.g. reactions can be performed stereospecifically and reactants do not require side-chain protection, increased the solubility of non-polar substrates, or shifting thermodynamic equilibria to favor synthesis over hydrolysis. Enzymatically synthesized small peptides (usually di or tripeptides) are being used successfully for human and animal nutrition and also as pharmaceuticals and agrochemicals (Ray, 2012).

Some relevant examples are the synthesis of the leading non-caloric sweetener aspartame, the lysine sweet peptide, kyotorphin, angiotensin, enkephalin and dynorphin and some nutritional dipeptides and tripeptides. Protease is also used for the production of biodegradable films, coatings, and glues from keratinous waste products like hair, feathers, skin, fur, animal hooves, horns etc. for compostable packaging, agricultural films or edible film applications (Singh *et al.,* 2011).

Management of industrial and household wastes

The global environment is gradually deteriorating because of the socio-economic activities of humankind such as processing industries. Many industrial processes cause adverse changes in the immediate environmental change and therefore being challenged by society. Of these, leather industries and the increased amount of feathers generated by commercial poultry processing may represent a pollution problem and needs adequate management. The proteases solubilize proteinaceous waste and thus help in lowering the biological oxygen demand of aquatic systems. Recently, the use of alkaline protease in the management of wastes from various food-processing industries and household activities opened up a new era in the use of proteases in waste management. Proteases can be effectively used for degradation of protein containing waste and help in clearing pipes and to remove clogs in blocked drainage pipes (Sawant and Nagendran, 2014).

In this regard, the use of alkaline protease in the management of wastes from various industries and household activities opened up a new era in the use of proteases in waste management. Proteases

solubilize proteinaceous waste and thus help lower the biological oxygen demand of aquatic systems. Alkaline protease from *B. subtilis was* used for the management of waste feathers from poultry slaughterhouses feed. Large quantities of waste are generated in the form of feather, hair, left over protein rich solids from the meat industry. For example, feathers constitute around 5% of the body weight of the animal and proteases with keratinolytic activity are being successfully used for hydrolysis of keratin-rich products like hair and feather to prepare animal feed and food, amino acids and peptides (Sharma *et al.,* 2017).

Other applications

Some of the other applications of proteases include their use in basic research, peptide synthesis, sizing of fabrics, optical resolution of amino acids, for dissociation of cells from monolayer animal cell culture etc. Their selective peptide bond cleavage is used in without the need for the tedious blocking and deblocking steps that are common in enantio- and region selective organic synthesis. Such high selectivity also affords efficient reactions with few by-products thereby making enzymes an environmentally friendly alternative to conventional chemical catalysts (Shine *et al.,* 2016).

Market dynamics

The primary factors that drive the global market for proteases are its wide use in the food industry. Protease enzymes are diverse ingredients. It helps in reducing manufacturing costs of food products and also to improve the digestibility of some foods, they are critical for the development of many of today's FMCG products. The main reason for the growth of this market is the multiple uses of proteases in diverse industries. Its eco-friendly due to its non toxic and non-pathogenic attributes is an added advantage that leads to a surge in the market. Presence of stringent regulations has pull down the proteases market to an extent. Growing product demand in various applications including paper, biofuels, rubber, photography, biological detergents, contact lens cleaners and molecular biology will promote industry expansion over the next eight years.

This review is mainly focused on the general aspects of proteases giving special emphasis on the industrial applications of the proteases. Proteases play a decisive role in detergent, pharmaceutical, leather, food and agricultural industries. Currently, the estimated value of the global sales of industrial enzymes is over 3 billion USD, of which proteases account for about 60% of the total sales. Microbial alkaline proteases already play a pivotal role in several industries, mainly in the detergents, leather processing, silver recovery, medical purposes, food processing, feeds, and chemical industries, as well as in waste treatment their potential is much greater and their applications in novel proteases are likely to increase in the near future. Advancement in biotechnology offers a constructive position for the development of proteases and will continue to facilitate their applications to provide a sustainable environment for improving the quality of human life.

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