



# Microbial proteases: ubiquitous enzymes with innumerable uses

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## Abstract

Proteases are ubiquitous enzymes, having significant physiological roles in both synthesis and degradation. The use of microbial proteases in food fermentation is an age-old process, which is today being successfully employed in other industries with the advent of 'omics' era and innovations in genetic and protein engineering approaches. Proteases have found application in industries besides food, like leather, textiles, detergent, waste management, agriculture, animal husbandry, cosmetics, and pharmaceuticals. With the rising demands and applications, researchers are exploring various approaches to discover, redesign, or artificially synthesize enzymes with better applicability in the industrial processes. These enzymes offer a sustainable and environmentally safer option, besides possessing economic and commercial value. Various bacterial and fungal proteases are already holding a commercially pivotal role in the industry. The current review summarizes the characteristics and types of proteases, microbial source, their current and prospective applications in various industries, and future challenges. Promoting these biocatalysts will prove significant in betterment of the modern world.

**Keywords** Purification · Protease engineering · Therapeutic · Nematicidal · Biofilm · Prion protein

## Introduction

Proteases represent a broad group of enzymes which break down or hydrolyze proteins or peptides. The proteases act on the peptide bonds joining the adjacent amino acid residues in a protein molecule and cleave them leading to formation of shorter peptides and amino acids (Razzaq et al. 2019). These hydrolytic enzymes are ubiquitous in nature and have been found in all living forms encompassing the eukaryotes like plants, animals, fungi, protists as well as the prokaryotic domains of bacteria and archaea. Even several viruses are also known to encode their own proteases (Bernardo et al. 2018).

As per the Enzyme Commission classification, proteases are placed in the class 3(hydrolases), sub-class 4 with each proteolytic enzyme assigned unique number as EC 3.4.x.x (Contesini et al. 2017). These enzymes have been categorized on the basis of various parameters like the site of action, the type of substrate, active pH range, mechanism of action involving particular amino acid present in the active site (Guleria et al. 2016a, b). Depending on the site of action, broadly these enzymes can be classified as endopeptidase and exopeptidase. The former tend to hydrolyze non-terminal peptide bonds, leading to formation of shorter peptides, while the latter act on peptide bonds located at the termini

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of the substrate. Also, depending on the termini on which a particular exopeptidase acts preferentially, exopeptidases are further categorized into aminopeptidases or carboxypeptidases depending on whether they act on the N-terminal or C-terminal respectively (Naveed et al. 2021). The exopeptidases release dipeptides or tripeptides or amino acids and correspondingly shortened peptides.

Another approach of protease categorization is based on the presence of specific amino acid residue(s) in the active site and mechanism of action. Based on this approach, the major classes of protease are serine proteases, cysteine protease, threonine proteases, glutamic proteases, asparagine proteases, aspartic proteases, mixed proteases, etc. (Contesini et al. 2017). The MEROPS database (<https://www.ebi.ac.uk/merops/>) is a resource providing comprehensive details about the different types of proteases. The enzymes are categorized on the primarily on the basis of their phylogenetic relationships and mechanism of action in this database (Rawlings et al. 2018). Similarly, the proteolytic enzymes have also been sorted into alkaline proteases, acidic proteases and neutral proteases on the basis of their optimal pH range (Tavano et al. 2018).

The proteases play a very significant role in the physiology and metabolism of all living organisms. Apart from the obvious role in the digestion of proteins and peptides, these enzymes also play significant role in the regulation of a vast array of physiological processes by controlling various stages involved in the protein synthesis, protein activation–inactivation, signaling, protein turnover as well as gene expression (Bond 2019). Protease and protease inhibitors account for more than 2% of the human genes. Not only do the proteases play important role in the normal functioning of the body and maintenance of homeostasis, but also these have significant role in infections, immunity, inflammation and disease development (Patel et al. 2018). For example, various common characteristic features of cancer like uncontrolled growth, angiogenesis, metastasis, immune evasion have been associated with various aberrant protease activities. On the other hand, proteases have also been found to play crucial role in tumor suppression (Dudani et al. 2018). Therefore, proteases are being utilized for prognostic, diagnostic as well as therapeutic purpose in the field of cancer management (Dudani et al. 2018). Owing to the crucial role of proteases in the life cycle of all the organisms including the infectious agents, the proteolytic enzymes offer lucrative avenue for drug discovery as is evident in case of protease inhibitors like ritonavir which is widely used for controlling Human Immunodeficiency Virus (HIV) infections (Tigabu et al. 2020). Recently, Jo et al. (2020) screened a flavinoid library for assessing the potential antiviral activities of the flavinoids which inhibited the 3C-like protease of coronaviruses and reported promising results in case of Herbactin, rhoifolin and pectolinarin. Apart from this, there are

many other applications of proteases in the field of medicine like control of cardiovascular diseases, digestive disorders, inflammatory diseases as well as promotion of tissue repair in cases of burns, fractures, accidental or surgical trauma, etc. (Kumar and Jain 2018; Bond 2019). Hence, both proteases and protease inhibitors play a crucial role in the field of medicine (Agbowuro et al. 2018).

The proteases also find a very significant role in various industries. The classical example of industrial application of proteases is for cleaning purposes like detergent additive, contact lens cleaning solution component, etc. (Salwan and Sharma 2019; Singh and Bajaj 2017; Lam et al. 2018). The textile industry makes use of proteases for a variety of purposes ranging from degumming of silk, biopolishing of wool in a very much sustainable and eco-friendly approach (Chatha et al. 2017; Mamo and Assefa 2018). Similarly, leather industry is also reducing the utilization of chemicals and laying more and more emphasis on the application of proteolytic enzymes for carrying out different steps of leather processing (Fang et al. 2017). A lot of keratinous wastes are generated by poultries, slaughter houses, etc., which are difficult to manage and cause a lot of problems like soil pollution, water pollution, aesthetic problems, clogging of drains, spreading of diseases, etc. (Kamarudin et al. 2017). Another proteinaceous waste of concern is collagen, which is generated by fish processing industries, sea-food processing industries as well as slaughter houses. Improper disposal of such wastes also pose a great pollution threat apart from the direct health risk to humans and animals (due to possible transmission of pathogenic microbes). Proteases, especially the keratinases and collagenases are also playing pivotal role in the arena of waste management and pollution control by breaking down these problematic components (Bhagwat and Dange 2018; Razzaq et al. 2019; Yusuf et al. 2019). The keratinases (proteases, which are capable of degrading keratin) have been used for degradation of keratin in the waste materials, thus helping in the waste management. Also, the keratinase treated residues may be used as animal feeds and nitrogenous fertilizers (Kumawat et al. 2018). Collagenase can be used for extraction of collagen from the fish and animal carcasses, which in turn will not only lead to lower waste generation, but also help in reclaiming collagen (Pal and Suresh 2016). The collagen can be used by various food, pharmaceutical and cosmetic industries.

Apart from these, proteases possess a plethora of applications in case of the food industries like manufacture of protein supplements, infant food, debittering of protein hydrolysates, meat tenderization, production of various types of fermented foods and beverages like cheese, beer, removal of haze from beverages, etc. (Banerjee and Ray 2017; dos Santos Aguilar and Sato 2018). The proteolytic enzymes also act as important tools in carrying out various molecular biology and genetic engineering experiments. The applications

of proteases in various fields are discussed in detail in the subsequent sections.

Proteases represent a wide array of enzymes capable of acting on a variety of proteinaceous substrates. Not only do these enzymes carry out degradative activity, but also these are also capable of synthetic activity. Proteases have been utilized for synthesis of peptides for application in diverse fields like food industry, medicine, agriculture, etc. Białkowska et al. (2017) have specifically reviewed the application of proteases from thermophilic, halophilic and psychrophilic organisms for peptide synthesis. Zanutto-Elgui et al. (2018) reported production of milk peptides with antimicrobial and antioxidant properties with the help of fungal proteases from *Aspergillus oryzae* and *A. flavipes*.

## Scope of the review

This review aims to provide state of the art information related to the proteases with emphasis on microbial origin proteases. First part of this review highlights the various categories of proteases, second showed various strategies of production and purification of the enzyme. The subsequent section presents a comprehensive overview about some of the pertinent applications of proteases. It is then followed by an in depth analysis about the concept of protein engineering and its application in the improvement of properties of proteases. The review entails the compilation and analysis of up to date information about the microbial proteases.

## Microbial proteases

Proteases are among one of the oldest enzymes to be discovered and studied, with several animal origin proteases like pepsin, trypsin and plant origin proteases like papain being discovered and characterized as early as the 1800s or the earlier decades of 1900s (Bond 2019). Subsequently, the microbial proteases also came into the focus and quickly became quite popular owing to several advantages associated with them (Guleria et al. 2014; Singh et al. 2016). Microorganisms, as source of proteases, offer several inherent advantages like rapid production rates, lower investment in terms of land and time requirements, not being subjected to climatic influences (Bhatia et al. 2021). Moreover, due to the diverse environments in which the microorganisms are found, it is quite easier to screen out the microbes producing enzymes with desirable characteristics (Putatunda et al. 2019). For example, if one is interested in thermostable proteases, it is highly probable to get such enzymes from thermophilic bacteria growing

in hot springs (Sahay et al. 2017). Moreover, due to shorter generation time and relatively simpler genetic make-up, the genetic manipulation of microorganisms is much easier (Ali et al. 2016). This in turn, further opens up vistas for designing 'tailor-made' enzymes with the desired features. The ethical issues confronted by the workers in dealing with animal origin proteases are also absent in case of microbial origin proteases (da Silva et al. 2016a, b, 2017). The capability of microbes to produce extracellular proteases further makes the production process much easier by simplifying the downstreaming (Razzaq et al. 2019). Not only this, several microbes have been grown on cheaper substrates or waste materials, with the aim of further making the process more economical (Hamza 2017; Limkar et al. 2019).

A lot of work has been done on various proteases of microbial origin, some of the recent ones are presented in Table 1. From the commercial point of view, bacterial origin proteases and fungal origin proteases have received a lot of attention and that is why many researchers have explored these enzymes. As per Gurumallesh et al. (2019), the microbial proteases form two-third of the total proteases used in the industries. The major contributors are the bacterial alkaline proteases and fungal acidic proteases, although the viral proteases are also important. Protease producing microorganisms have been isolated from a variety of environments like rhizosphere soil, slaughter house soil or floor washing, sewage, food waste, etc. (Jadhav et al. 2020; Hakim et al. 2018; Ash et al. 2018; Prajapati et al. 2017). These have also been from saline environments like the marine environments, sea sediments, hypersaline lakes, salted food, soda lakes, etc. (Maruthiah et al. 2016; Boughachiche et al. 2016; Ibrahim et al. 2019). Among the bacteria, the genus *Bacillus* has a very prominent place in terms of the commercial production of proteases, while the fungal sources are dominated by the genera *Aspergillus*, *Trichoderma*, *Penicillium*, etc. (Gurumallesh et al. 2019). Apart from these, many researchers have also explored the endophytic fungi for production of proteases for various purposes (Meshram et al. 2016; Mandal and Banerjee 2019). Meshram and Saxena (2016) reported proteolytic and fibrinolytic activity in an endophytic fungal isolate *Lasiodiopodia pseudotheobromae* obtained from the stem of the *Aegle marmelos*, and proposed its potential use for dissolution of blood clots.

Many different proteases have been identified and characterized in different viruses; however, from the commercial point of view, these have not gained much importance and mainly serve as targets of various antiviral drugs. Some of the viral origin proteases include the cysteine proteases, identified in Adenovirus, Alphavirus; serine protease reported in Hepatitis C Virus; aspartic protease found in HIV, etc. (Sharma and Gupta 2017).

**Table 1** Advantages and disadvantages of different techniques/methods employed for enzyme production and improvement of enzymatic yield

Sr. no.	Method/ technique	Advantages	Disadvantages	References
1. Fermentation	SSF	Cost effective Higher product recovery that too in concentrated form Superior volumetric productivity Inexpensive substrate requirements Low energy requirement Low capital investment Low waste water output Lower levels of catabolite repression	Restricted to the microorganisms that can grow under reduced environments No defined concentration of media components Usually slower because of diffusion barrier imposed Difficult to control process parameters Difficulties in scale-up	Doriya et al. (2016), Sharma et al. (2017), Contesini et al. (2018)
	SMF	Better process control Easy recovery of extracellular enzymes Better distribution of medium components Better heat and mass transfer can be achieved Better diffusion of microorganism Commercially available in large scale	Complex in operation Products are dilute and less stable Requires high moisture content High energy requirement Cost intensive High release of effluents	Doriya et al. (2016), Contesini et al. (2018)
2. Genetic engineering methods	Site-directed mutagenesis	Directed High efficiency Mutation library is small Simple methods for mutagenesis Mimic natural evolution	The structure–function relationships of the target Enzyme should be clear	Yang et al. (2013)
	Directed evolution		Random Mutation library is large Low efficiency	
	Saturation mutagenesis	Easy Laboratory evolution of enzymes with greater activity, stereoselectivity, and stability High efficiency	Mutation library is large Creating high-quality SM libraries is difficult	
	Truncation		The relationships between domains and properties should be clear	
	Iterative saturation mutagenesis	High-quality libraries More efficient and faster than others (e.g., saturation mutagenesis)	Mutation library is large	
	Fusion	The structure–function relationships of the target enzyme is not necessary	There is not a clear selection criterion of fusion sequence	

Table 1 (continued)

Sr. no.	Method/technique	Advantages	Disadvantages	References
3.	Metagenomics	<p>Direct way of identifying novel enzyme</p> <p>Selects clones with functional activities</p> <p>Does not need identification of homologies to genes of known functions</p> <p>Adds novel functional annotations and thereby contributes to nucleic acid and protein databases</p>	<p>Time consuming screening</p> <p>A huge number of clones are required to be screened</p> <p>High-throughput screening is crucial</p> <p>Low hit rate of positive clones</p> <p>Low throughput</p> <p>Requires quality DNA</p>	<p>Steele and Wolfgang (2005), Alma'abadi et al. (2015), Hosokawa et al. (2015)</p>
4.	Genome editing using CRISPR/Cas9	<p>Uses next-generation sequencing (NGS) technology</p> <p>Desired niches can be explored for microbial enzymes</p> <p>Simple &amp; efficient</p> <p>Easy to use</p> <p>Cost-effective compared to previous gene editing techniques [(e.g., Transcription activator-like effective nucleases (TALENs)]</p> <p>Can analyse the interaction of genes and relationship between genetic differences and expression (phenotype)</p> <p>High-yield multiplexing available</p> <p>Highest engineering feasibility</p>	<p>For enzymes identifications there is need for the detection of gene variants with conserved domain or motif of the known functions</p> <p>The methods does not necessarily finds the novel gene</p> <p>Off target effect may alter the gene functioning leading to genomic instability</p> <p>Require protospacer adjacent motif</p>	<p>Zhang et al. (2015), Omodamilola and Ibrahim (2018), Uddin et al. (2020)</p>

## Alkaline proteases

The bacterial proteases used for various commercial purposes generally belong to the alkaline proteases and neutral proteases categories. The alkaline proteases, especially the extracellular proteases, from the members of the genus *Bacillus*, which are usually the serine proteases have been in use since quite long time (Pathak and Rathod 2018). The enzymes from *Bacillus* have drawn a lot of attention and hence a lot of information is available with respect to their activity, size, structure, etc. (Contesini et al. 2017). Two of the most important types of alkaline proteases are subtilisin Carlsberg and subtilisin novo, which are again obtained from the genus *Bacillus* (Contesini et al. 2017). Moreover, the strains of *B. subtilis* and other related members of *Bacillus*, used for enzyme production for commercial purposes are regarded as GRAS (generally recognized as safe) (Zhang et al. 2019). So, even now more and more reports about alkaline protease from novel strains of *Bacillus* sp. as well as novel proteases from already discovered strains are being published from around the globe (Guleria et al. 2016a; Hakim et al. 2018; Dorra et al. 2018; Jadhav et al. 2020; Qamar et al. 2020; Far et al. 2020). Mothe and Sultanpuram (2016) carried out the purification and characterization of serine alkaline protease from a novel *B. caseinilyticus*. Hammami et al. (2020) have reported the characterization of a novel H<sub>2</sub>O<sub>2</sub>-tolerant alkaline protease from *B. invictae* AH1. Recently, Zhou et al. (2020) carried out genetic modification of *B. licheniformis* 2709 which lead to 62.19% enhancement in alkaline protease production along with reduction in the formation of mucopolysaccharides and other foaming agents.

Although *Bacillus* genus proteases are most widely characterized and used, there are reports of several other bacteria capable of producing alkaline proteases. Alkaline protease production by *Streptomyces* sp. isolated from saltpan of Algeria was reported by Boughachiche et al. (2016). A novel thermostable keratinolytic alkaline protease producing *Streptomyces* sp. Al-Dhabi-82 was reported by Al-Dhabi et al. (2020). Similarly, Masi et al. (2017) reported alkaline protease production from *Enterococcus hirae* and *Pseudomonas aeruginosa* isolated from dairy effluents. They further carried out formation of nanocomposite of the bacterial protease with super paramagnetic nanoparticles leading to protease stability enhancement. Not only the bacteria like *Bacillus*, *Pseudomonas*, etc. have the capability of producing alkaline proteases, but several fungi have also been reported to produce these enzymes (Sharma 2019). Meshram et al. (2016) reported that a metalloprotease (with 8.0 pH optima) produced by endophytic fungus *Xylaria curta* possessed appreciable fibrinolytic properties, and thus has the potential of being

used for therapeutic purposes. da Silva et al. (2018) have carried out thermodynamic investigations on the serine alkaline protease produced by the fungus *Aspergillus tamaritii* URM4634. Nadeem et al. (2019) reported significant alkaline protease production from *Chetomium globosum*.

The bacterial alkaline proteases have generally pH optima in the range of 8–12. Many of these enzymes also display higher thermostability and activity at elevated temperatures (Naveed et al. 2021). Being capable of showing activity in the alkaline pH range, the alkaline proteases have been very much in use as an additive to the detergents, for enhancement of the stain removal properties (Guleria et al. 2016a; Asha and Palaniswamy 2018). Similarly, leather processing is carried out at high pH, hence the alkaline proteases can act as suitable additives for the tanning industries (Hussain et al. 2017; Hakim et al. 2018).

## Acidic proteases

As the name itself indicates, the acidic proteases tend to show their activity in the acidic pH range, mostly between pH 3.8 and 5.6, with the optimum pH being 3–4 and isoelectric point between 3 and 4.5 and belong to the group of aspartic proteases (Machado et al. 2016; Razzaq et al. 2019). The microbial acidic proteases are broadly categorized into pepsin-like and rennin-like (Razzaq et al. 2019). Unlike the alkaline proteases, which are mostly produced by the bacteria, the fungi are the primary producers of acid proteases, with the members of the genera *Aspergillus*, *Penicillium*, *Endothia*, *Mucor*, etc. being the most commonly reported ones (Mammo and Assefa 2018).

There are several reports of novel acid proteases being produced by the molds as well as some yeasts (Mandujano-González et al. 2016). An aspartic protease of *Rhizomucor miehei*, which can serve as an alternative enzyme for milk clotting during cheese production, was characterized by da Silva et al. (2016a, b). Souza et al. (2017) characterized an acidic protease with optimum pH of 5.0 and optimum temperature of 55 °C, from *Aspergillus foetidus* isolated from soil sample. Similarly, da Silva et al. (2017) characterized an aspartic protease with promising milk clotting properties from *Phanerochaete chrysosporium*. Sun et al. (2018) cloned the aspartic protease gene from *Rhizomucor miehei* into *Pichia pastoris* and reported better meat tenderization as compared to papain (which is a plant origin protease, commonly used for meat tenderization). Deng et al. (2018) reported that recombinant aspartic protease from *Trichoderma harzianum* expressed in *Pichia pastoris* was effective against several pathogenic fungi like *Botrytis cinerea*, *Mucor circinelloides*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Rhizoctonia solani*, and *Candida albicans*. Song et al. (2020) carried out biochemical characterization of

two new *Aspergillus niger* aspartic proteases and reported both the enzymes had 50 °C as the optimum temperature, with the pH optima of PepAb and PepAc being 2.5 and 3.0 respectively.

A few bacteria have also been reported to produce acidic proteases. Proteases from *Enterococcus faecium* strain 1.15 and *Lactobacillus plantarum* strain 1.13, isolated from Bakasam (a type of fermented meat) by Putranto et al. (2017) and (2020a), displayed rennin-like activity. Similar activity was also shown by the protease produced by *L. paracasei* strain 2.12, which was isolated by Putranto et al. (2020b) from goat milk.

The acidic proteases play a very significant role in the food and beverage industry. Animal origin enzymes like rennin were originally used for milk clotting during cheese production, but these are now being replaced by microbial origin proteases (Mammo and Assefa 2018). Similarly, the acid proteases are also used for preparation of protein hydrolysates as well as in bakeries for improving the characteristics of dough (Singh and Bajaj 2017). The acid proteases are also important in removal of haze from juices and beer.

## Neutral proteases

Neutral proteases refer to those proteolytic enzymes which show their activity in either in neutral pH or slightly alkaline pH or slightly acidic pH, with the maximal activity within the pH range of 5–8 (Razzaq et al 2019). Like the alkaline proteases, the neutral proteases are also contributed by the genus *Bacillus* (Contesini et al. 2017; Razzaq et al. 2019). Many of the neutral proteases fall into the category of metalloprotease and have requirement of a divalent positively charged ion for their activity.

The neutral proteases have been utilized for various purposes including the brewing and baking industries (Razzaq et al. 2019). Aissaoui et al. (2016) characterized a neutral protease from *Trichoderma harzianum* and carried out hydrolysis of *Scorpaena notata* (fish) viscera. They reported anti-bacterial peptide production in the fish viscera hydrolysate. Ao et al. (2018) undertook the characterization of neutral protease from *Aspergillus oryzae* Y1 isolated from naturally fermented broad beans and reported it to be a 45 kD protein with 7.0 optimum pH and 55 °C optimum temperature. De Oliveira et al. (2020) reported about production of a relatively thermostable protease by the fungus *Moorella speciosa*, with 6.5 as the optimum pH. However, on immobilization on MAT540 microspheres, the optimum pH changed to 5.74. Deng et al. (2021) reported about the possible application of the recombinant neutral protease rNpI as fish feed additive to improve protein digestion and growth.

## Microbial protease production technology

### Microorganisms and source

The first step in the production of microbial proteases is the isolation and selection of candidate microorganisms. The microorganisms are isolated from a variety of sources/ecosystems followed by selection for the desired character. These sources/ecosystems have specific or adverse feature which reflects the characteristics of the enzyme to be produced; for example, dumping sites, hot springs, soda lake, soil samples from milk processing plants, poultry waste sites, meat waste contaminated soil, tannery waste, detergent industry, leather industry, and wood factory (Anandharaj et al. 2016; Kshetri et al. 2016; Suleiman et al. 2020; Sharma et al. 2017; Chu 2007; Kandasamy et al. 2016; Ramakodi et al. 2020). Other sources includes saltern Qingdao at China, Izmir Gulf at Turkey, composting soil, sewage sludge, vermicomposting pit soil, sea mud, marine sediments, southern ocean, and mangrove sediments (Baweja et al. 2017; Li et al. 2017; Sonune and Garode 2018; Bhatia et al. 2021). A variety of protease producers including bacteria, fungi, and actinomycetes have been isolated and identified. Some bacterial strains reported to produce protease are *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus lentus*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Bacillus circulans*, *Geobacillus* SBS-4S, *Bacillus alkaloophilus*, *Bacillus halodurans*, *Bacillus pumilus*, *Bacillus clausii*, *Bacillus licheniformis* and *Bacillus safensis* (Briki et al. 2016; Ahmad et al. 2020; Alves et al. 2016; Contesini et al. 2018). Fungus is the next major microbial group known for protease production. The fungi reported as protease producer are *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus clavatus* ES1, *Fusarium* sp. *Aspergillus niger* KIBGE-IB36, *Penicillium chrysogenum* X5, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus nidulans* HA-10, *Aspergillus oryzae*, *Penicillium chrysogenum*, *Mucor* sp., *Cephalosporium* sp., *Rhizopus* sp., *Trichoderma* and *Penicillium italicum* (Abu-Tahon et al. 2020; de Castro et al. 2015; Benmrud et al. 2018; Sattar et al. 2019; Naveed et al. 2021). *Aspergillus* and *Trichoderma* are two dominant strains exploited for industrial protease production. Other than bacteria and fungi, actinomycetes, a group of bacteria having morphology similar to fungi are also known to produce proteases. For example, *Streptomyces nogalator* produces extracellular protease found to be useful in depilation of goat skin (Mitra and Chakrabartty 2005). Another study by Bajaj and Sharma (2011) isolated alkalithermotolerant protease from *Streptomyces* sp. DP2, which is having thermal stability at 90 °C. For economic improvements, a number of microorganisms have been exploited for protease

production, but a quest for cost-effective industrial grade enzyme from microbial sources is still under consideration (Guleria et al. 2018; Razzaq et al. 2019).

## Fermentation

Present, microbial protease production is carried out using both solid-state fermentation (SSF) and submerged fermentation (SmF) (Contesini et al. 2018; Razzaq et al. 2019; dos Santos Aguilar and Sato 2018). The SmF accounts for approximately 90% production of all industrial enzymes, normally using wild type or genetically engineered microorganisms. In SmF, the microbial growth occurs in liquid medium with high free water availability and is more suited for bacteria. The process offers advantages such as ease of control of physicochemical variables, better distribution of medium components and recovery of extracellular enzyme. However, the enzymes produced are dilute and less stable. Generally, microbial protease production by SmF uses different substrates such as fructose, sucrose, lactose, yeast extract, peptone and complex industrial by product i.e., molasses, residual biomass, effluents, and corn steep liquor (Gimenes et al. 2019; Contesini et al. 2018; Anandharaj et al. 2016).

By contrast, in SSF, microbial growth takes place on a solid support in absence or near absence of free water with adequate moisture to support metabolic activity and growth of microorganisms (Soccol et al. 2017; dos Santos Aguilar and Sato 2018). The SSF is more suited for fungi due to their closeness between natural habitat and medium, and requirement of less moisture than bacteria i.e., 40–60% is enough for fungi (Singhania et al. 2009). In comparison to SmF, the SSF offers advantages of low cost, simplicity, higher yield, product stability, lower sterility requirements, and possibility of exploiting numerous agro-industrial wastes as substrate (Contesini et al. 2018). However, a limited number of microorganisms can grow under reduced moisture conditions, which is a limitation in SSF. Additionally, parameters like particle size of substrate and moisture content must need to be studied cautiously (Chiliveri et al. 2016; Contesini et al. 2018). A variety of low cost substrates including wheat bran, wheat straw, red gram husk, cow dung and sugarcane bagasse have been used for protease production in SSF (Kalaikumari et al. 2019; Razzaq et al. 2019). The typical fermentation duration of bacterial and fungal protease production processes are 2–4 days and 3–5 days, respectively (dos Santos Aguilar and Sato 2018).

The protease production is greatly influenced by physicochemical and nutritional factors, such as substrate, nitrogen, carbon, pH, temperature, moisture, aeration, agitation, dissolved oxygen and inorganic salts (Mukhtar and Haq 2013; Niyonzima and More 2013; Kasana et al. 2011; Gimenes et al. 2019). There is no defined culture condition

and medium for microbial protease production, and every microbial strain has its own particular nutritional and physicochemical requirements to reach maximum enzyme production (Kasana et al. 2011; Gimenes et al. 2019).

Numerous researchers have reported microbial protease production using SmF and SSF (Ida et al. 2017; Limkar et al. 2019; Zhao et al. 2019; Osmolovskiy et al. 2021; Matkawala et al. 2021). Sandhya et al. (2005) and da Silva et al. (2013) conducted a comparative study between SmF and SSF using *A. oryzae* and *A. fumigatus*. In SSF, the protease yield was enhanced up to 3.5 times in *A. oryzae* and 30 times in *A. fumigatus* using wheat bran as substrate. Ida et al. (2017) using *Aspergillus fischeri* and *Penicillium citrinum* performed SmF in a medium having crushed feathers (0.5%) as a nitrogen source and reported production of two collagenolytic serine peptidases. The results of enzymatic assay showed production of 460 U/mL at 72 h and 760 U/mL at 168 h with the fungi *A. fischeri* and *P. citrinum*, respectively. Protease production by bacteria was reported in many studies, for example, Anandharaj et al. (2016) reported alkaline metalloproteases using *B. alkalitelluris* TWI3, Rehman et al. (2017) reported metalloprotease using *B. subtilis* KT004404, de Oliveira et al. (2017) using *Bacillus* sp. CL33A, and Baweja et al. (2016a, b) reported alkaline protease using *B. pumilus* MP 27 in SmF. Another study described production of acidic proteases from *Pediococcus acidilactici* SH in SmF (Imdakim et al. 2015). Jensen et al. (2010) under SmF revealed a new bacterial glutamic peptidase and its heterologous expression using *B. subtilis* expression clone. In addition, a variety of bacterial strains from the genus *Bacillus* have been reported to exhibit considerable protease production in SSF, for example, Qureshi et al. (2016) reported 12,200.0 U/g protease activity using *Bacillus* sp. BBXS-2, Vijayaraghavan et al. (2014) reported 4813 U/g activity using *B. cereus* in SSF.

## Recent approaches to improve protease yield

To improve yield of protease enzyme from microbial sources, scientists have utilized different techniques including cloning and overexpression, fed batch and chemostat fermentation, and optimization of parameters (medium and growth conditions) using one factor at a time method and statistical approaches such as response surface methodology. The continuous efforts are being made to enhance the production of protease enzyme using traditional i.e., UV or chemicals as well as recent techniques i.e., genetic engineering, gene editing by clustered regularly interspaced short palindromic repeats (CRISPR), protein engineering and metagenomics (Kumar et al. 2012; Homaei et al. 2016; Rehman et al. 2017; Guleria et al. 2017; Thakur et al. 2020). The advantages and disadvantages of these techniques are summarized in Table 2. Scientist are using



**Table 2** Selected novel microbial proteases and their potential applications

Microorganism	Source of isolation	Type of protease	Potential application	References
<i>Alcaligenes faecalis</i> APCMST-MKW6	Marine sediment	Halophilic organic solvent tolerant protease	Deproteinization of shrimp shell waste	Maruthiah et al. (2016)
<i>B. subtilis</i>	Soil from tannery area	Alkaline protease	Animal hide dehairing, detergent additive	Hussain et al. (2017)
<i>Halobacillus</i> sp. CJ4	Hypersaline lake	Thermo-solvent stable protease	Peptide synthesis and detergent formulation	Daoud et al. (2017)
<i>Aspergillus foetidus</i>	Savannah soil	Acidic protease	Food industries	Souza et al. (2017)
<i>B. subtilis</i> AKAL7 and <i>Exiguobacterium indicum</i> AKAL11	Poultry waste mixed soil	Alkaline protease	Animal hide dehairing, removal of gelatin from X-ray film	Hakim et al. (2018)
<i>B. halotolerans</i> strain CT2	Tunisian potato	Alkaline protease	Detergent additive	Dorra et al. (2018)
<i>Bacillus cereus</i> FT 1	Organic matter mixed soil	Alkaline protease	Detergent additive	Asha and Palaniswamy (2018)
<i>B. tequilensis</i>	Soil	Fibrinolytic protease	Blood clot dissolution	Xin et al. (2018)
<i>B. safensis</i> strain RH12	Off-shore oil fields	Serine alkaline protease	Detergent additive	Rekik et al. (2019)
<i>Salipaludibacillus agaradhaerens</i> strain AK-R	Soda lake	Serine alkaline protease	Detergent additive	Ibrahim et al. (2019)
<i>Lactobacillus paracasei</i> 2.12	Goat milk	Rennin-like protease	Milk clotting activity	Putranto et al. (2020b)
<i>Streptomyces</i> sp. Al-Dhabi-82	Soil	Thermostable alkaline protease	Keratinous waste degradation	Al-Dhabi et al. (2020)
<i>B. atrophaeus</i> NIJ	Hydrocarbon-contaminated soil	Serine alkaline protease	Detergent additive	Rahem et al. (2021)
<i>B. stearothermophilus</i>	Olive oil mill sols	Thermostable alkaline protease	Detergent additive	Karray et al. (2021)

genetic engineering technology for improving quality, lowering cost and increasing yield of enzyme to capture global biotechnology market. Several bioengineered enzymes with higher stability are being produced using rDNA technology in detergent industry (Razzaq et al. 2019).

Protein engineering is another important technique for hyper-production of enzyme (Li et al. 2020). Normally, the enzyme activity and stability depends on the composition and position of the amino acids. Protein engineering alters structure of protease in a way that enhances the specificity, activity, and stability of the enzyme. Protease engineering has been extensively investigated for varied industrial purposes (Xu et al. 2015; Vojcic et al. 2015; Banerjee and Ray 2017). Presently, directed evolution and random design are the two main methods used for engineering proteases. Additionally, computer-aided protein-directed evolution (CAPDE) approach which involves a blend of directed evolution methods and computational designing have further strengthen the protein engineering techniques (Banerjee and Ray 2017; Verma et al. 2012; Thakur et al. 2020).

Database mining is another important thrust area in sequence-related segment and metagenomics is an ideal tool to process sequence-related information collected from the DNA of environmental microorganisms (Prayogo et al. 2020). The isolated sample is then screened for open reading

frames to determine genes that encodes for a novel enzyme (Gurumallesh et al. 2019). A variety of enzymes with novel bio-catalytic activity have been isolated and identified through metagenomics based screening. Waschowitz et al. (2009) identified a novel protease enzyme using metagenomics technology. Verma et al. (2021) conducted in silico mining of solid tannery waste metagenome and reported structural characterization of protease. Devi et al. (2016) identified and characterized gene Prt1A which encodes for alkaline serine protease from the metagenomic library of tannery sludge. The Prt1A protease showed optimal activity at temperature 55 °C and pH 11. Similarly, Pessoa et al. (2017) screened a metagenomic library from mangrove sediments and identified a protease producing clone. The enzyme was further purified with a yield of 77.27% and a specific activity of 8.5 U/μg. García-Moyano et al. (2021) through a two-step functional screening approach, identified a new marine metagenome-derived protease that can hydrolyze insoluble zein at temperatures up to 50 °C and pH 9–11. The enzyme was derived from a bacterial group who's potential to degrade zein was unknown.

Now a days, researchers are using most effective gene editing tool i.e., CRISPR with high efficiency in attaining gene modification (Martínez et al. 2017). The CRISPR system have two main components i.e., a Cas9 enzyme and

a single guide RNA (SgRNA) that selectively target genes to induce DNA breakage. The bacterial strain *B. subtilis* ATCC 6051 is actively involved in the production of industrial enzymes and have efficient protein secreting ability. However, insignificant transformation efficiency associated with this strain is a major hurdle faced by researchers. In such conditions, CRISPR/Cas9 was preferred by Zhang et al. (2016a, b). Similarly, García-Moyano et al. (2020) have engineered *B. subtilis* by adapting two CRISPR/Cas plasmids to the cloning technology and their tool allowed versatile editing at any chosen genomic position or at a fixed genomic locus. The engineered *B. subtilis* successfully produced protease enzyme. Though, there are numerous existing and forthcoming enzyme production techniques, the demand for enzyme in industries still persists (Gurumalles et al. 2019).

## Purification of proteases

Once the protease is produced the next step is to purify the enzyme, which is a complex process. Presently, numerous methods and techniques are available for enzyme purification. The selection of method and technique depends on the source of enzyme i.e., extracellular or intracellular (Razzaq et al. 2019). The enzyme purification is not a one-step process, rather it uses a blend of different methods and technologies. Economical techniques must be used during microbial protease purification with an aim to produce a cost effective and high-value quality product (Guleria et al. 2015; Banerjee and Ray 2017; Razzaq et al. 2019).

First, the culture is separated from the fermentation medium either by centrifugation or filtration or differential sedimentation followed by concentration of supernatant using ultrafiltration and precipitation through salts or organic solvents for recovery of protein from biologically crude mixture. In the salt precipitation, ammonium sulfate is commonly used for the purification of enzymes due to its simplicity, high solubility, non-denaturation, and non-generation of heat. However, ammonium sulfate is corrosive and requires monitoring during disposal (Gimenes et al. 2019). To avoid denaturation, the enzymatic solution needs to be cooled to 4–5 °C during salt precipitation (Abidi et al. 2014; Huang et al. 2017). Other precipitation techniques involve addition of organic solvents such as methanol, ethanol, isopropanol, and acetone. Ethanol, is a cheap resource which is produced globally and has satisfactory hydrophobicity as well as great aqueous solubility to minimize the occurrence of denaturation (Golunski et al. 2011). Precipitation by ethanol is a catchy technique due to its good physicochemical properties, complete water miscibility, low toxicity, high volatility, and low cost (Sun et al. 2010). However, ethanol is flammable. The ethanol precipitants are recovered by

flash evaporation, whereas acid precipitants and ammonium sulfate require dialysis to adjust the ionic strength (Gimenes et al. 2019). In addition, methods like temperature-sensitive hydrogel, activated charcoal, heat treatment of enzyme, lyophilization, and polyethylene glycol (PEG)-35000 are also used to concentrate alkaline proteases (Prabhavathy et al. 2013; Gimenes et al. 2019; Gupta et al. 2002; Razzaq et al. 2019).

Further purification of enzyme requires use of one or more chromatography techniques viz., ion exchange chromatography (IEC), gel filtration chromatography, affinity chromatography (AC), hydrophobic interaction chromatography (HIC), and dye ligand chromatography (Banerjee and Ray 2017; Gupta et al. 2002). The production of purified proteases has been attained successfully using gel filtration and ion exchange chromatography (CM-Sephadex, DEAE-Sephadex) from various microbial sources i.e., *B. circulans* and *B. cereus* AT (Kanmani et al. 2011; Sa et al. 2012). The aqueous two-phase systems (ATPS) and foam fractionation are other preferred methods that have been used on small scale and are expecting their commercial utilization (Gimenes et al. 2019).

## Microbial proteases and its industrial applications

Protease is one of the most widely used industrial enzymes having tremendous applications in various industries including detergent, leather, silver recovery, dairy, baking, beverages and pharmaceutical industries etc. New protease mediated industrial processes are continuously being accelerating. A small summary of protease application is being sketched in Fig. 1. Various applications of the protease are elaborated and discussed as below.

### Detergent industry

Protease enzyme has been used in detergents since late 1990s as one of the largest industry to use proteases. The use of proteases in the detergent industry accounts for 20% of the global sale of enzymes and accounting for 30–40% of global enzyme revenues, and is predicted to expand further, with a CAGR of about 15.5% between 2020 and 2025 (Razzaq et al. 2019). Protease digests food such as milk, egg, beef, fish, blood, and other bodily secretions stains and improves detergent washing quality with less soaking and agitation time. These enzymes used as detergent additives can be useful in very small amounts because of their ionic ability, over a large pH and temperature spectrum and have a longer shelf life along with improved detergent's efficacy (Singh et al. 2016). Serene



**Fig. 1** Applications of protease enzyme in various industries/sectors

proteases produced by *Bacillus* sp. and alkaline proteases by some fungal strains widely used in detergent formulations due to their ease of manufacturing, down streaming are gaining prominence in detergent industry (Tekin et al. 2020). Protease produced by *Bacillus licheniformis* RP1 has been identified as a strong detergent ingredient with stable action in a solution of 10% (w/v) commercial detergent (Fabs Perfect), implying its commercial use. Cocktails of enzymes such as protease, amylase, cellulase, and lipase are also used in many formulations to increase the washing effect for household purposes (Singh et al. 2016). Recently, rDNA technology was used to create bioengineered detergent proteases with improved stability and shelf life (Vojcic et al. 2015; Su et al. 2020). The substitution of a few unique amino acid residues for bleach and oxidation stability of proteases has been researched using protein engineering (Guleria et al. 2016a, b, c).

### Waste management

Proteases are used to process various forms of waste, including solid, liquid, and hazardous waste. Protease enzymes also assist in waste degradation by converting waste small simple molecules that may utilise by other organisms for metabolic activities and thus help lower the biological oxygen demand of aquatic systems. A formulation containing hydrolytic enzymes derived from *B. subtilis*, *B. amyloliquefaciens*, and *Streptomyces* sp. has been prepared and patented as Genex for depilation and cleaning of hairs from drains and clogged pipes (Naveed et al. 2021). Villa et al. (2013) reported the application potential of keratin hydrolysates produced from feathers for formulation of hair shampoos. A thermostable and pH-stable protease from *Micromonospora chaiyaphumensis* S103 demonstrated excellent potential for deproteinization of shrimp wastes for chitin processing (Mhamdi et al. 2017).

Alkaline protease has recently been used in the waste management of different food-processing businesses and home activities. Protein-based residues in food bioprocessing sectors such as milk and meat processing activities are a major contaminant (Majumdar et al. 2015). Various cleaning procedures using enzymatic alternatives minimize cleaning expenses and possible environmental dangers while also increasing equipment lifetime. A product on the market that contains proteolytic enzymes from *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Streptomyces* sp., as well as a disulfide reducing agent (thioglycolate), aids in the cleaning of blocked pipes (Naveed et al. 2021). The most advantageous element of employing enzymes in cleaning methods is that the enzymes themselves are biodegradable and will not destroy the environment after they are no longer required. In contrast to other remediation approaches, there is no accumulation of biomass or chemicals that must be removed; nonetheless, one disadvantage of employing proteases for bioremediation is the high expense of the enzymes themselves Villa et al. (2013).

### Leather industry

Proteases are used to break down non-collagenous skin constituents and remove non-fibrillary proteins. The application of enzymes for leather processing enhances the quality of leather and gives stronger and softer leather with less spots (Fang, et al. 2017). The use of enzymes in the manufacture of leather has improved leather quality while reducing environmental pollution. Increased application of alkaline protease at emerging leather industries is due to the elastolytic and keratinolytic activity (Moridshahi et al. 2020). These influential properties of alkaline protease are very effective in leather processing industries. The particular uses of protease are found to be relevant in the soaking, bating, and dehairing phase of preparing skin and hides (Zhou et al. 2018). Enzymatic unhairing does no surface damage, and high-quality leather can be recycled without substantial chemical reduction. Microbial alkaline proteases have become very popular in leather industries. Application of enzymes in leather processing may help developing eco-benign process that is less harmful to the environment. Considering the high risks associated with conventional leather processing, there is considerable emphasis on developing enzymatic approaches that are cleaner and safer (Kalaikumari et al. 2019; Ammasi et al. 2020). Such biobased processes would help reducing emissions. Thus, exploitation of enzymes for leather processing is beneficial in terms of improved process efficiency, highly specific enzyme-based catalysis, enhanced leather output and superior quality of leather.

### Photographic industry

Silver recovery from waste photofilms is a big business. The exposed nearly 2.0 billion radiographs are taken each year, including chest X-rays, mammograms and CT scans (Cavello et al. 2015). X-ray films have approximately 5–15 g of silver per kg of film. Around 18–20% of the world's silver needs are supplied by recycling photographic waste (Asgher et al. 2018). Since silver is linked to gelatin in the emulsion layer, it is possible to break the same and release the silver which could be used as source of secondary silver. Enzyme-based method may be developed that may not only recover silver efficiently with minimal impact on the environment (Al-Abdalall and Al-Khaldi 2016). Proteases have been reported to possess excellent gelatinolytic activity for successful recovery of silver from X-ray films. Proteases from *Purpureocillium lilacinum* (Cavello et al. 2015) have also shown good gelatin hydrolysing ability. Alkaline proteases produced by *B. subtilis*, *Streptomyces avermectinus*, and *Conidiobolus coronatus* have been successfully reported to recover silver from X-ray films, ensuring that the process is eco-friendlier over the use of chemicals. The enzyme-based silver extraction from X-ray films relies more on renewable energy resources than on fossil fuel and, thus, might offer an overall eco-safe process.

### Silk degumming

Silk fabric is composed of two fibroin filaments surrounded by a cementing layer of proteinaceous sericin and this gives a rough texture to silk but after its removal silk fibres turns very soft (More et al. 2017). Proteases which can cleave the peptide bonds of sericin without destroying the fibroin may have potential for application as degumming agents (Suwannaphan et al. 2017). Traditionally harsh chemicals used in degumming process that reduce the strength of silk, shorten shelf life of silk, not eco-friendly and even harmful to the environment. Enzymes like trypsin, papain and some other bacterial proteases are used for degumming process. Plant proteases like papain and bromelain (are the effective cocoon cooking enzymes that are commonly used for the processing of cocoons whereas some bacterial protease also has been found to be very effective in hydrolysing sericin (Singh et al. 2016). Joshi and Satyanarayana (2013) reported a recombinant alkaline serine protease from *B. lehensis* which enhanced the softness and shine of silk fibres. Thus, the enzyme-based technology could be used effectively for silk degumming in industries as an eco-friendly alternative (More et al. 2017). These enzymes not only increases the strength of the yarn but also prevent various environmental issues; therefore, proteases are greatly used for degumming of silk and projected as a replacement of the harsh and energy demanding chemicals for treatment process. Thus,

enzyme-based silk degumming is an eco-friendly approach that may potentially help reducing usage of hazardous chemicals and undesired emissions due to usage of chemicals and fossil fuels.

### Biopolishing of wool

Alkaline proteases are used for the manufacture of shrink-proof wool. Wool fibres are covered in overlapping scales pointing towards fibre tips, which could be hydrolysed by protease action. However, protease treatment in general damages the wool by causing excessive loss of strength, and lowering the anti-felting ability, which in turn may lead to additional damage to the fibre interior during wool processing. Therefore, specific proteases whose action is limited to cuticle scale of wool fibre are desired. Keratinase can be used in textile processing for efficient production of shrink-resistant fibre that has improved handling properties (Lateef et al. 2015). A keratinase from *Brevibacillus parabrevis* CGMCC10798 was purified and characterized for its excellent potential for wool processing (Zhang et al. 2016a, b). A novel recombinant keratinase expressed in *E. coli* BL21 (DE3) exhibited high specificity towards some substrates including wool. The protease has the potential for application in wool processing (Su et al. 2020). *Bacillus* sp. G51 exhibited the highest wool-keratinolytic activity. Thus, eco-friendly treatment approaches based on enzyme cock-tail (with protease combination) may help designing the organic wool processing (Srivastava et al. 2020).

### Biofilm removal

Biofilms are formed by extracellular polymeric substances (EPS) which are mainly consist mostly of polysaccharides, proteins and nucleic acids form structural matrix which help the bacteria to attach to the surface to facilitate the survival in the adverse conditions and environments (Lohse et al. 2020). Biofilms are detrimental to both human life and industrial processes due to their association with infection, pathogen contamination, biofouling, and slime formation. Harmful biofilms may cause grave economic losses due to reduced productivity, decreased product quality and loss of time and expenses for biofilm removal and impose an economic burden on companies besides contamination and biofouling. The most effective method for removing a biofilm is by the clean-in-place (CIP) method in combination with chemicals involving manual scrubbing of the affected area. But the method is impractical for larger structures where regions like joints, filters or gaskets are not easily accessible (Nahar et al. 2018). Microbial enzymes may be safer and more efficient alternatives to traditional chemical means of biofilm removal. Proteases are the most commonly used biofilm removal agents however, other hydrolases in

combination with proteases, help to eliminate existing biofilms and prevent bacteria from adhering to surfaces (Lohse et al. 2020).

### Food industry

The use of chemicals in the food industry is increasingly being recognized as hazardous and natural alternatives are being explored by food technologists around the globe. Use of eco-friendly microbial proteases has shown prominent potential in replacing chemical agents, while improving piquant properties of the food products. The potential of microbial proteases as biocatalysts has been employed since time immemorial in the fermentation of food items (Singh et al. 2016). It is also known that proteases of microbial origin are more stable than their counterparts from animals or plants, with better consistency and easier potential for optimization or process modification (Gurung et al. 2013). The microbial species mainly employed in production of proteases for food industry (Table 2) have been isolated from the traditional sources of fermented foods. The US Food and Drug Administration GRAS list may be referred to identify the *Bacillus* (*Bacillus subtilis*, *B. licheniformis*) and *Aspergillus* (*Aspergillus niger*, *A. oryzae*) species, whose products are labelled as 'generally safe'.

### Medical field

Proteases show promising therapeutic properties in medical field such as gauze, non-woven tissues, and ointment composition. Certain lytic enzyme deficiency syndromes are diagnosed to be aided by an oral administration of alkaline proteases (Joshi and Satyanarayana 2013). The use of this fibrinolytic enzyme suggests its future application as an anticancer drug and in thrombolytic therapy (Jaouadi et al. 2012). Slow-release dosage form preparation containing collagenases with alkaline proteases is extensively used in therapeutic applications. The hydrolysis of collagen by the enzyme liberates low molecular weight peptides without any amino acid release for therapeutic use (Suwannaphan et al. 2017). For the treatment of various diseases, such as burns, carbuncles, furuncles, and wounds, a preparation of elastase immobilized on bandage is used. Proteases possess distinct therapeutic properties which are helpful in developing various drugs against fatal diseases like anticancer, antimicrobial, anti-inflammatory infections and dissolving clots class with diverse other applications (Srilakshmi et al. 2015; Kumar and Jain 2018). Nowadays proteases are useful in treatment of cystic fibrosis, sepsis, digestive disorders, retinal disorder, and many more disease. Beside this they are also help in reducing the discomfort of breast engorgement in lactating women (Banerjee and Ray 2017). Asparaginase from *Escherichia coli* and collagenase also play a

major role in removing asparagine from the blood in forms of lymphocytic leukemia, burns and wounds, respectively (Muneer et al. 2020). Nattokinase from *Bacillus subtilis* is also helpful against cardiovascular disease (Lan et al. 2020). Microbial proteases also act as the therapeutic targets for protease inhibitor drugs in various disorders and conditions, including bacterial and viral diseases (Table 3).

### Prion protein degradation

Prion protein or Scrapie protein (PrP<sup>Sc</sup>) is a misfolded infectious form of protein and the causative agents of transmissible spongiform encephalopathies in a variety of mammals, including bovine spongiform encephalopathy in cattle. In humans, prions cause Creutzfeldt–Jakob disease, variant Creutzfeldt–Jakob disease, Gerstmann–Straussler–Scheinker syndrome, fatal familial insomnia and kuru (Silveira et al. 2005). All known prion diseases affect the structure of the brain or other neural tissue, and all are currently untreatable and universally fatal. Proteases may help degrading wrongly folded proteins. The protease (MSK103) was also effective against dried PrP<sup>Sc</sup>. Thermostable keratinase from *B. pumilus* KS12 has potential for degradation of Sup35NM (Rajput and Gupta 2013). It may be envisaged that potentially novel

proteases may be discovered that may help developing bio based therapeutics for prion diseases in humans and animals.

### Peptide synthesis

Enzymatic peptide synthesis has several advantages, including enantioselectivity, free racemization, ecologically benign reaction conditions, over chemical synthesis that requires expensive defensive groups, solvents, or reagents (Agyei et al. 2016; Antink et al. 2019). Protease from viral, fungal, plant and animal origins has received a great deal of interest in recent years for the synthesis of many small peptides, especially dipeptides and tripeptides utilizing either a thermodynamically or a kinetically controlled bioprocess. (Kumar and Bhalla 2005; Motyan et al. 2013; Yang et al. 2020). Organic solvent-tolerant alkaline proteases from the genera *Aspergillus*, *Bacillus*, and *Pseudomonas* have shown promise for peptide synthesis (Song et al. 2020). Protease-produced therapeutic bioactive peptides that serve as nutraceuticals, digestive aids, antioxidants, blood pressure regulators, and foe diet and medications (Nongonierna and FitzGerald 2015; Nandan and Nampoothiri 2020). Computer-aided drug design, in conjunction with genetic

**Table 3** The applications of proteases in the food processing industry (Razzaq et al. 2019; Singh et al. 2016; Sharma et al. 2017)

Application	Microbial strain	Protease	Characteristics
Brewing and cereal mashing	<i>Bacillus</i> and <i>Aspergillus</i> sp.	Neutral proteases	↑ Filterable extract volume ↑ Alpha-amino N <sub>2</sub> amount in wort
Producing cereal extract concentrates	<i>Bacillus</i> and <i>Aspergillus</i> sp.	Neutral proteases	↑ Filterable extract yield
Clarifying beer haze or chill haze	<i>Bacillus</i> sp.	Neutral zinc metalloprotease	Solubilizes residual proteoglycans
Cheese making	<i>Aspergillus</i> sp.	Calf chymosin	First recombinant FDA approved heterologous product
	<i>Brevibacterium aurantiacum</i>	Neutral proteases	Produce free amino acids for metabolism by breaking down casein
Baking	<i>Aspergillus niger</i>	Heat-labile proteases	Partial hydrolysis of gluten in preparation of flour dough
	<i>Sphingomonas capsulate</i> , <i>Lactobacillus</i> sp.	Neutral proteases	Enhancing flavor and nutritional value
Protein hydrolysates—additives to food and feed for humans or animals	<i>Bacillus</i> sp. <i>Bacillus licheniformis</i> <i>Aspergillus oryzae</i> and <i>Rhizopus niveus</i>	Alkaline serine proteases Neutral proteases Acidic proteolysis	Modification of proteins like soy, gelatin, whey or caseins; hydrolysates or tenderization of fish and meat; fortifying fruit juices; therapeutic dietary blood pressure regulators
Soy sauce production	<i>A. saitoi</i> <i>A. oryzae</i>	Pepsin-like acid protease Endoacting enzyme	Hydrolysis of soybean protein Produces free amino acids, optimum pH 4–4.5—enhances flavor of soy sauce
Digestive aids	<i>Aspergillus</i> sp.	Aspergillopeptidase A	Low pH optimum
Food processing	<i>Bacillus</i> sp.	Alkaline serine proteases, neutral proteases	Flavor enhancing
	<i>Aspergillus</i> sp.	Fungal proteases	

and protein engineering techniques, can also aid in the discovery of new peptidases.

### Nematicidal activity

Protease can also be used as biocontrol agents against pathogenic nematodes. Traditional chemical-based methods are not only toxic to humans and cause significant environmental pollution but also lead to the emergence of nematicide resistance (Singh and Bajaj 2017; Yang et al. 2019). Nematicidal microbial proteases, on the other hand, are natural biocontrol agents that offer several advantages like being non-toxic, environmental friendly, economically sustainable, besides improving soil characteristics and plant growth.

There are several reports of proteases from non-*Bacillus* spp. being used as biocontrol agents against nematodes. Alkaline protease from *B. lehensis* has been found to be useful as a biocontrol agent for plant pathogenic nematode *Meloidogyne incognita* (Joshi and Satyanarayana 2013). *Saccharomonospora viridis* isolated from soil sample secreted extra-cellular cuticle-degrading protease that had remarkable nematotoxic activity against *Panagrellus redivivus* (Darwesh et al. 2019). One of the major mechanisms for nematicidal capacities was observed to be the ability of bacteria to produce proteases and/or chitinases. Thus, development of bio-based strategies for controlling and managing the pests may contribute substantively towards environmental health as this would lead to reduced application of chemical-based pesticides (Table 4).

### Contact lens cleansing

Proteases are important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymatic debriders. It is well known that the deposits on contact lenses consist of proteins, lipids, and mucin as tear components. Protein deposits are natural deposits on contact lenses which are unavoidable as they are formed by the interaction of the protein in our natural tears and the contact lenses.

Generally, contact lens cleaning solutions have been prepared using plant (papain) and animal (pancreatin, trypsin and chemotrypsin) proteases but most of them impart an unpleasant odor to the cleansing bath or develop an odor after a few hours of use (Liu et al. 2018). Several microbial proteases from *Bacillus* species, *Streptomyces* sp., and *Aspergillus* sp. were reported to clean tear films and debris of contact lenses to overcome these drawbacks and to make cleaning composition odorless and safe, i.e., not producing an allergic response or causing irritation to the eyes, bacterial proteases are gaining importance (Singh and Bajaj 2017). Several reports are available on production of proteases from bacterial cultures with *Bacillus* sp. as the dominating organism. Protease from *Bacillus* sp.158 exhibited ability for cleaning of tear films and efficiently removed the protein deposits from contact lenses (Razzaq et al. 2019). The enzyme could effectively be used to remove protein deposits from contact lenses and, thus, help increasing the transmittance of lenses. Proteases from several *Bacillus* sp., viz. *B. subtilis*, *B. licheniformis*, *B. thermophiles* and *B. cereus*, exhibited excellent activity against artificial tear solution and, thus, could be of importance for contact lens cleansing (Singh et al. 2016; Contesini et al. 2018).

### Animal feed

Animal feeds supplemented with proteases enzymes can enhance the nutrient bioavailability of the food constituents or as an element in an animal diet formulation to help protein digestion in the gastrointestinal tract (Hejdysz et al. 2020). The influence of a supplemental dietary mixture of different proteases in granular form cysteine protease and a serine endopeptidase on the productivity variables in dairy cattle, using as a model lactating Holstein cows was studied by Park et al. (2020). The outcome of the experiment showed that supplemental protease enhances milk and meat production effectiveness and enhances parameters of nitrogen status (Yang et al. 2020). A novel serine protease, expressed in *Bacillus licheniformis* as feed protease, permitted a significant augmentation

**Table 4** Therapeutic applications of protease inhibitors and their target enzymes (Culp and Wright 2017; Kumar and Jain 2018)

Target enzyme	Therapeutic application of protease inhibitor
Bacterial methionine aminopeptidases	Broad-spectrum antibacterial drugs Regulation of angiogenesis and Cancer
Glutamyl aminopeptidase or Aminopeptidase A	Antihypertensive drugs, act through regulation of angiotensin
Alanyl aminopeptidase or Aminopeptidase N from <i>Lactobacillus delbrueckii</i>	Therapeutic and regulatory potential in diseases like cancer, rheumatoid arthritis, diabetic nephropathy, leukemia and inflammatory diseases Antiviral agents (corona virus) Antibacterial ( <i>E. coli</i> )
Dipeptidyl peptidase IV	Type 2 diabetes; Immunological disorders
X-prolyl dipeptidyl aminopeptidase	Antibacterial ( <i>Streptococcus gordonii</i> )

in the degree of the hydrolysis, solubilization, and digestibility of proteins, improving broiler performance by enhancing protein and energy digestibility (Naveed et al. 2021).

## Market potential of proteases

Changing cleaning habits, higher advancements in protein engineering technologies to produce high performing products, and rapid industrialization are driving forces behind the booming market growth for proteases, which is further fueled by rapid industrialization and growing environmental concern in food and beverages, livestock feed, soaps and detergents, pharmaceuticals, and other industries. The protease market is expected to reach USD 2.21 billion by 2021, with a CAGR of 6% from 2016 to 2021 (Protease Market 2021). The protease market is divided into three segments based on their sources: microorganisms, plants, and animals, with the microbial sector increasing at the quickest pace in terms of value due to the inability of plant and animal sources to fulfil global protease demand. Because of its high-temperature functional characteristics, the microbial protease market is expected to gain momentum due to its commercial viability to produce large revenues with extensive penetration across the textile manufacturing, detergent, food and beverage, and paper and pulp sectors (Gupta et al. 2013). For example, in 2019, DuPont Nutrition & Biosciences released OPTIMASE, an unique range of biobased enzymes for liquid detergents in medical equipment cleaning applications. Novozymes released Formea Prime, a new protease enzyme, in 2021 to enhance its Formea range of protein drink ingredients and to assist beverage formulators in overcoming the problems associated with utilizing whey protein hydrolysates. Similarly, Amunix Pharmaceuticals, Inc. raised \$117 million in a Series B fundraising round to advance AMX-818, a masked and protease-activated T-cell engager that targets HER2-expressing solid tumors, into the clinic. Some of the key participants are Advanced Enzyme Technologies, Specialty Enzymes and Biotechnologies (Advanced Enzymes USA), Biocatalysts (BRAIN AG), DuPont and DSM, Enzyme Technical Associations (ETA), and others are developing new and robust protease for various applications in food and beverages, livestock feed, soaps and detergent, pharmaceuticals, and other industries such as textile and leather, waste management, chemical industries, silver recovery, photography, and biofuel (Protease Market 2021).

## Protease engineering

Although there has been tremendous increase in our knowledge of proteases in terms of their sequence and evolutionary significant relationships, as well as technologies

to engineer recombinant proteins as per our convenience, still there is a lot to know when producing a protein with ideal applications in biotechnology industry. Naturally occurring proteases might not be optimal for industrial application but with progress in bioengineering technology, they can be specifically “edited” for desirable applications. Challenges being dealt by the geneticists and protein engineering scientists while designing desirable proteases are.

- Discovering or engineering novel proteases with industrial or pharmaceutical applications.
- Increasing the yield, output, or cost-effectiveness of the product of the enzyme.
- Improving the characteristics of the enzyme like increasing thermal stability or widening/altering substrate specificity, optimal pH, etc., or preventing inactivation by auto-proteolysis.

Exploring the data from available plethora of biodiversity is the invaluable resource based on which the proteases or microorganisms can be engineered for the industry. For instance, metagenomics data of the enzymes isolated from extremophiles, or ‘extremozymes’, may possess unique activities ideally required for commercial applications (Bilal et al. 2018; Bruno et al. 2019). The only limitation will be that maintaining these extremophiles in laboratory conditions might reduce their cost-effectiveness (Table 5).

The methods of protein engineering that are employed for engineering novel enzymes for industrial or pharmaceutical applications may be categorized into mainly two approaches—rational redesigning and directed evolution (Baweja et al. 2016a, b; Fig. 2). Rational redesigning approach is based on the method of ‘site-directed mutagenesis’ whereas directed evolution approach is based on natural unbiased evolution through ‘random mutagenesis’. Microbial hosts, such as *Escherichia coli* or *Bacillus* sp. have been ideal tools for such metabolic engineering approaches.

## Site-directed mutagenesis

Selected amino acid of a particular site is edited creating a mutated protein. It is preferred for those proteins whose 3-D structures and functional characteristics are well established. The evaluation of the mutant protein is quicker since the variants produced are few. Site-directed mutagenesis is done generally to impart the proteins with desirable properties like improved catalysis or activity, stability like thermostability, substrate specificity, solubility, expression, etc. while also being utilized for immobilization of enzymes for the targeted industrial application (Baweja et al. 2016a, b; Chen et al. 2018a, b).



**Table 5** Nematicidal properties of microbial proteases

Target nematode	Microbial source of nematicidal protease	Type(s) of microbial protease(s)	Mode of nematicidal action
<i>Meloidogyne</i> sp. (root-knot nematode)	Endophytic bacteria ( <i>Bacillus cereus</i> ) (Hu et al. 2020)	Collagenases Chitinases Serine proteases	Degradation of cuticular constituents (juveniles) and egg shells
<i>Meloidogyne</i> sp. (root-knot nematode)	Actinomycetes <i>Saccharomonospora viridis</i> strain Hw G550 (Darwesh et al. 2019)	Alkaline protease	↓ nematode reproduction, no. of females, eggs, galls, egg-masses, larvae, and developmental stages
<i>Meloidogyne incognita</i> (root-knot nematode)	Fungus <i>Lecanicillium psalliotae</i> (Yang et al. 2019)	Serine protease Ver112	Degradation of cuticular constituents and egg shells
<i>Radopholus similis</i> (burrowing nematode)	Genetically modified <i>Pseudomonas fluorescens</i> pf36 (p4MCS & p4Tn5) (Chen et al. 2018a, b)	Protease PASE4	Nematostatic (↓ growth) and nematicidal
<i>Meloidogyne incognita</i> (root-knot nematode)	Root bacteria in tomato plants <i>Enterobacter asburiae</i> HK169 (Oh et al. 2018)	Collagenases Chitinases Serine proteases	Nematicidal ↓ gall formation
<i>Meloidogyne ethiopica</i> and <i>Xiphinema index</i>	Seven Rhizobacteria strains (Abalalay et al. 2017)	Exoenzymes: Collagenases Chitinases Serine proteases Lipases	Nematicidal ↓ egg hatching
<i>Caenorhabditis elegans</i> (soil nematode)	Soil bacterium <i>Bacillus thuringiensis</i> (Peng et al. 2016)	Collagenase metalloproteases	Degradation of intestine of host nematode
<i>C. elegans</i> and <i>Meloidogyne incognita</i>	<i>Alcaligenes faecalis</i> ZD02 (Ju et al. 2016)	Extracellular serine protease	Nematostatic (↓ growth) and nematicidal

## Random mutagenesis

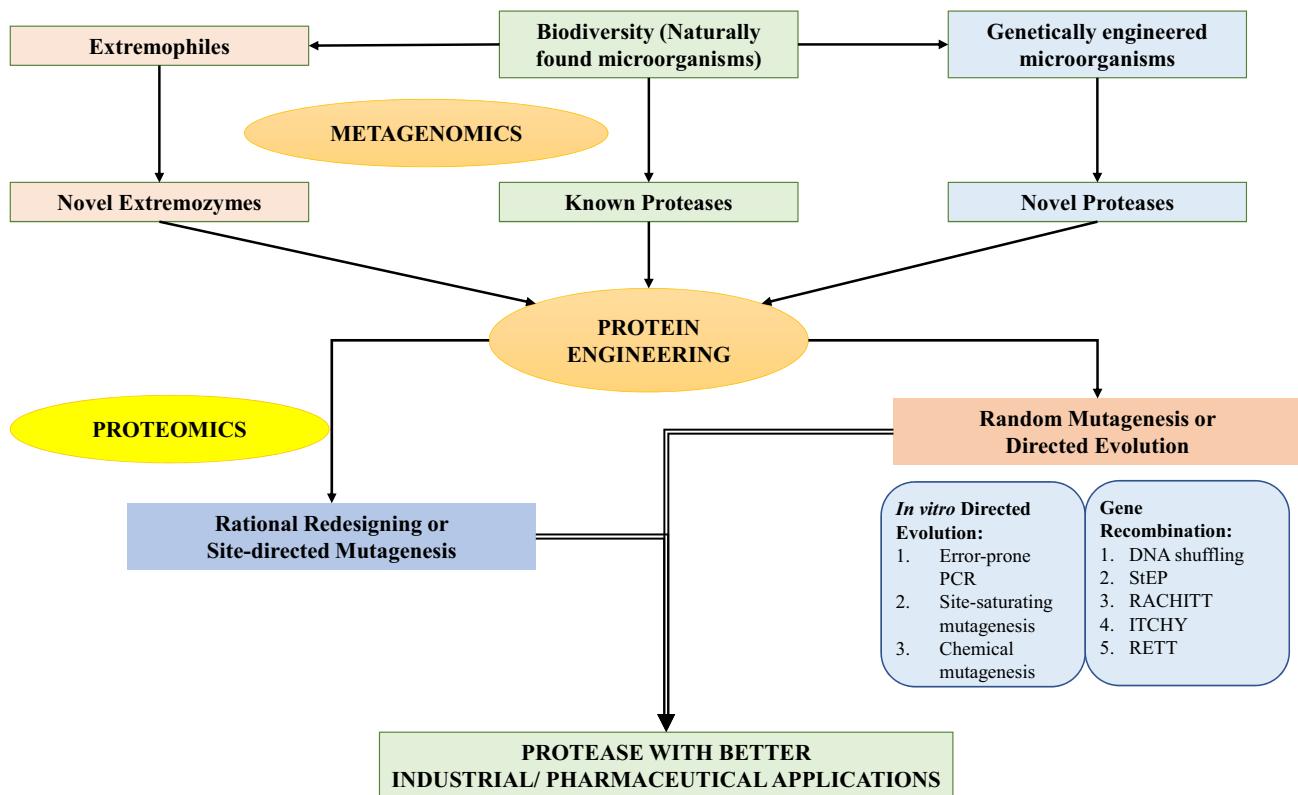
The technique is based on introducing random mutations in a gene. The mutated protein products are produced within weeks in the form a library of mutants and require high throughput screening strategies for selecting the desired variant. The quality of library decides the engineered protein formed. This technique may also be employed for those proteins whose structural and functional characterization is not fully known. This is an indiscriminate method of mutagenesis and involves various techniques summarized in Fig. 2. Error prone PCR is generally the technique employed for random mutagenesis. Examples of applications include enhanced high/low pH tolerance, thermostability, substrate or product specificity, and so on (Baweja et al. 2016a, b).

The selection of method is based on the desirable protein product and may involve more than one method, including combination of both site-directed and random mutagenesis techniques (Chen et al. 2018a, b; Weng et al. 2015).

## Future prospects of proteases

Proteases are a unique class of enzymes that bear both degradative and synthetic potential, along with the advantage over chemical processes of being biological in nature.

Hence, being a part of clean and green technology, the environmentally friendly applications of proteases are vast, holding promising potential for the future. Their therapeutic potential against controlling the spread of epidemics like malaria and AIDS and diseases like cancer is being researched. Last few decades have enhanced our knowledge to explore the applications of these enzymes in industries beyond the food technology, like textiles, leather, detergent, agriculture, and pharmaceutical industry. The ‘era of OMICS’ has further provided future directions of utilizing proteolytic potential of these enzymes in nanotechnology and site-specific or targeted drug delivery systems (Zhao et al. 2020), as well as for imaging technology in cancer diagnostics (Abousaway et al. 2021). Novel engineered proteases activated by small molecules hold the future of specifically controlling the activity of human proteases, and significantly changing the course of cell cycle and probably even cell death (Gerry and Schreiber 2020). These molecules present the potential strategies of targeting the otherwise unreachable targets inside the cells, promising great clinical applications in future. Engineering the proteases in accordance with their prerequisite application is the challenge for future.



**Fig. 2** Basic principles in protease engineering (Baweja et al. 2016a, b). *StEP* staggered extension process, *RACHITT* random chimeragenesis on transient templates, *ITCHY* iterative truncation for the creation of hybrid enzymes, *RETT* recombined extension on truncated templates

## Conclusion

Applications of proteases in many areas of industry are already well established, while they are being explored as promising tools in pharmaceuticals (therapeutic and diagnostics), waste management, production of bioenergy, agriculture and food industry. The bottlenecks in the production of novel and desirable microbial proteases can be narrowed down to their substrate specificity, stability, and production costs. New age technologies are increasingly being used to discover or engineer novel proteases overcoming these obstacles. While directed evolution is being utilized for optimizing available enzymes, rational redesigning approaches aim at producing new ones. Future efforts of replacing the questionable practices in industry with enzyme or protease-based methods will significantly benefit both environment and survival of human species.

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