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REVIEW PAPER

Potential application spectrum of microbial proteases for clean and green industrial production

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Abstract Enzymes are the cornerstones of metabolism and constitute the fundamental basis for existence of life. However, recently enzymes are being implicated in diverse industrial processes because of their specific and fast action for efficient bioconversion of substrate to product, and their capability to save raw materials, energy and chemicals for various manufacturing processes. Enzymes are considered as environment-friendly (green) chemicals that may potentially help replacing completely or reducing the usage of hazardous chemicals for industrial processes, thus promising sustainable production and manufacturing. Among various industrial enzymes microbial proteases dominate the world enzyme market due to their multi-faceted application potential in varied bioindustries like food, pharmaceutical, textile, photographic, leather and detergent. Promising applications of proteases in agricultural sector for instance may include biocontrol of pests, degumming of silk, selective delignification of hemp and wool processing. However, for successful industrial applications the proteases must be robust enough to suit the process conditions which are generally hostile. Proteases intended for industrial applications must have activity and stability over wide range of temperature and pH extremes for prolonged time periods and even in the presence of various potential enzyme inhibitors. Of various microbial proteases those from *Bacillus* spp. have got special significance because the latter are known for their ability to produce sturdy enzymes that might have suitability for industrial process conditions. The current article presents an interpretive summary of the recent developments on application potential of proteases for various industries.

Keywords Microbial enzymes · Protease · *Bacillus* · Applications · Eco-friendly · Pollution

1 Introduction

Green chemistry also called sustainable chemistry aims at utilizing preferably renewable raw materials, avoiding toxic and hazardous chemicals, thereby producing minimum wastes during commercial production and manufacturing (Sheldon et al. 2007; Sharma and Bajaj 2017). Furthermore, considering the ever-growing world energy demand, industrialization, rapidly depleting fossil-fuel reserves, environmental health issues, there is emphasis on development of sustainable industrial technologies that are environmentally safe and involve minimum emissions (Jegannathan and Nielsen 2013; Vaid and Bajaj 2017). As a result there is a paradigm shift from traditional concepts of chemical-based production and manufacturing to eco-benign processes that are equally efficient and economic (Sarrouh et al. 2012; Nargotra et al. 2016). Application of enzymes for industrial processes may contribute significantly towards development of environmentally benign processes (Bajaj and Jamwal 2013; Mhamdi et al. 2017). Enzyme-based technologies promise efficient utilization of raw materials, generation of minimal or no wastes, and shun the usage of toxic chemicals (Singh and Bajaj 2016, 2017).

Enzymes are the fundamental molecules that govern various metabolic processes in living systems, and

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constitute the very foundation for the existence of life. However, recently, enzymes have become the focus of intense research by process engineers and production chemists mainly due to recognition of their application potential in various industries. Enzyme-based industrial processes are much more environmentally safer than the traditional chemical ones and involve less emissions (Sheldon et al. 2007). But considering that enzymes are the biological molecules which are largely designed (by nature) to function under ambient conditions in the living systems, may not suit well for relatively hostile industrial process conditions (Bajaj et al. 2014). Therefore, discovery of sturdy enzymes that are capable of functioning under industrial processes has gained considerable research impetus in recent years (Sharma and Bajaj 2017). There is continuous and intense research focus on targeting process-apt robust enzymes either by exploiting the enormous natural microbial diversity or by bioengineering (Singh and Bajaj 2015; Guleria et al. 2016). Microbial enzymes may potentially be utilized for numerous industrial applications and may help replacing toxic and hazardous chemical catalysts (Nigam et al. 2012). The continual exploration of application potential of enzymes has expanded the enzyme industrial market at annual growth rate of 7.6% (David et al. 2009).

Proteases are one of the most important groups of industrial enzymes that have been studied extensively during recent years (Sarrouh et al. 2012). Proteases (EC 3.4) are a group enzymes which occur in all live forms, and execute a large variety of complex physiological and metabolic functions in living systems like cell division, signal transduction, digestion, blood pressure regulation, apoptosis and several others (Theron and Divol 2014). Proteases represent approximately 1–5% of the total gene content (Qazi et al. 2008). Proteases constitute 60% of the global industrial market due to their huge application potential in diverse industries (Fig. 1). Proteases are used in pharmaceutical industry (Kumar et al. 2015), food industry for peptide synthesis (Kumar and Bhalla 2005), leather industry for dehairing (Pillai et al. 2011; Singh and Bajaj 2017), photographic industry for silver recovery (Joshi and Satyanarayana 2013), detergent industry as an additive for detergent formulation (Giri et al. 2011) and in processing of keratin residues (Harde et al. 2011). In addition, the proteases are also used for other applications such as contact lens cleaning (Pawar et al. 2009), biofilm removal (Leslie 2011), isolation of nucleic acid (Motyan et al. 2013), pest control (Joshi and Satyanarayana 2013), degumming of silk (Mahmoodi et al. 2010) and selective delignification of hemp (Khan 2013).

The proteases intended for biotechnological processes must be robust enough and have the capability of kinetic and structural adaptations under extreme industrial microenvironments, e.g. extremes of temperatures, pH and presence of inhibitors (Singh et al. 2014). Such proteases may be targeted from microbial sources, especially the extremophiles, i.e. microbes which are inhabitants of extreme ecological niches (Białkowska et al. 2016). Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Rani et al. 2012). Microbes are the goldmines not only for proteases but also for other industrial products. Microorganisms represent the most preferred enzyme sources due to several unique advantages (Bajaj and Wani 2011; Sharma and Bajaj 2017).

Exploration of microbial diversity for targeting novel process-suitable proteases, has been an ongoing practice (Singh and Bajaj 2015, 2016). Among various microbial groups, bacteria due to their enormous diversity and other special merits have become interesting source for industrial enzymes including proteases (Bajaj and Sharma 2011). For industrial enzymes, strains of *Bacillus* spp. have gained much importance and account for 35% of total microbial enzyme sale (Jayakumar et al. 2012). This is because of the ability of *Bacillus* spp. to produce enzymes that suit well the process conditions in industries (Priya et al. 2014; Rehman et al. 2017). Enzymes from *Bacillus* spp. have poly-extremotolerance, i.e. capability of functioning under adverse process conditions like extreme of temperature, pH, presence of solvents, detergents and other potential enzyme inhibitors (Joshi and Satyanarayana 2013). Furthermore, *Bacillus* spp. may represent a model system for the heterologous expression of genes (Sadeghi et al. 2009). For biotechnological processes involving protein production, several species of *Bacillus,* viz. *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* and others, have become the most popular due to their excellent fermentation properties, high product yields and the complete lack of toxic by-products (Dijl and Hecker 2013; Singh and Bajaj 2016; Guleria et al. 2016). The enzymes being marketed by Novo Industry, Denmark, are produced from *Bacillus* spp. (Georgieva et al. 2006; Jisha et al. 2013). There are several reports of thermostable and wide range pH stable proteases from *Bacillus* spp. which have excellent compatibility for industrial processes (Baweja et al. 2016; Guleria et al. 2016). The genus *Bacillus* is represented by Gram-positive, spore-forming, rod-shape bacteria that are obligate aerobes or facultative anaerobes (Dijl and Hecker 2013).

In view of the grave threat posed by the polluting chemical-based industries, development of sustainable and environmentally friendly technologies is being emphasized. Proteases are the lead enzymes from industrial application view point. The current article presents a recent research survey of robust proteases especially from *Bacillus* spp. that are apt for industrial process conditions.

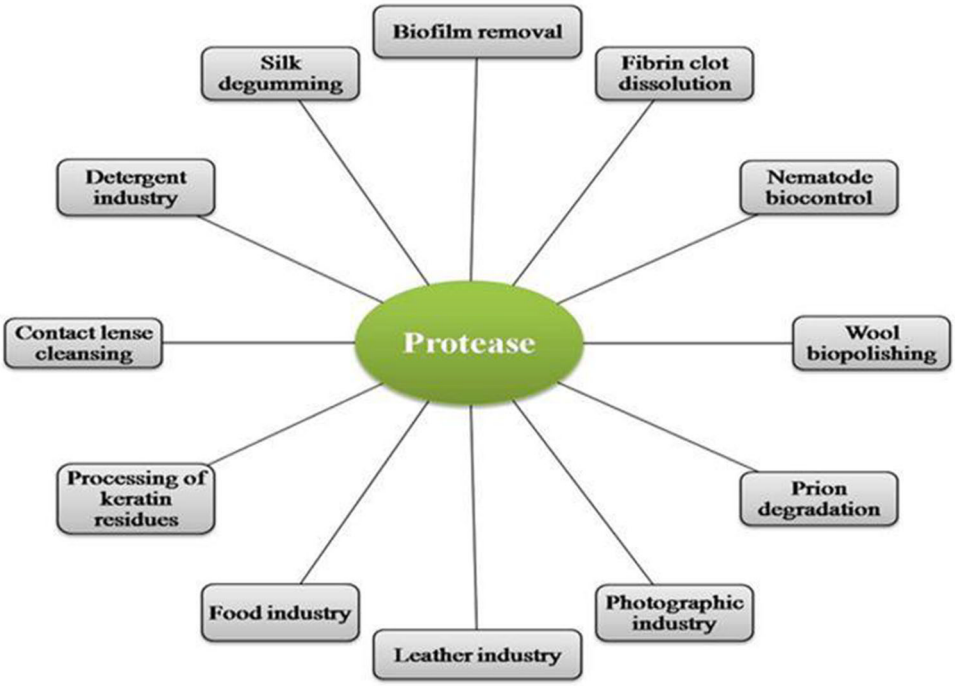

Fig. 1 Applications of proteases in different industrial sectors

2 Proteases in detergent industry

Detergent enzymes are eco-friendly solution that is being used to improve the cleaning efficiency of conventional detergents (Kumar et al. 2008; Khajuria et al. 2015). The industrial and economic importance of alkaline proteases, especially those from *Bacillus* spp. in the detergent industry, was realized during 1960s (Kazan et al. 2005). The use of enzymes in detergent formulations is now common in developed countries. Most of the currently marketed detergents contain enzymes (Kumar et al. 2008). The use of proteases in the laundry detergents constitutes a big market share and accounts for approximately 25% of the total worldwide sale of enzymes (Tanksale et al. 2001).

Today, the enzymes are the staple ingredients of powder as well as liquid detergents, stain removers, laundry pre-spotters, automatic dishwashing detergents and other industrial and medical cleaning products. Detergent industry is one of the largest industries where enzymes usage is being practised. The enzymes enhance the efficacy of detergents for removing proteins from cloths soiled with sweat, grass, milk, egg, blood, etc. (Ida et al. 2016). Proteases are the most extensively used enzymes in detergent formulations; however, amylases and lipases are also being added. Proteases work very efficiently as scissors to cut off the stains from cloths (Shankar and Laxman 2015). Contrary to enzyme detergents, the non-enzymatic detergents are inefficient in removing proteins from cloths as organic stains adhere strongly to textile fibres, and become permanent due to oxidation and denaturation caused during bleaching and drying operations. Performance of protease in laundry detergents has been reported to be enhanced when applied in combination with lipase, amylase and cellulase (Khan 2013).

The ideal detergent protease should possess broad substrate specificity to facilitate removal of a variety of stains due to blood, food and other body secretions. Activity and stability at high pH and temperature (Giri et al. 2011; Padmapriya et al. 2012) and compatibility with other components of detergents like chelating and oxidizing agents (Nascimento and Martins 2006; Tekin et al. 2012) are among the major pre-requisites for the proteases intended for application in detergents (Giri et al. 2011). Proteases from several *Bacillus* spp. are well characterized for usage as detergent additives, e.g. *B. subtilis*, *B. cohnii*, *B. clausii*, *B. licheniformis* and *B. brevis* (Guleria et al. 2016). *Alcalase* was the first detergent protease developed by Novozyme during 1960s. Since then several other enzymes have been developed by Novozymes for removing protein stains, viz. *Esperase*, *Savinase* and *Everlase*. All of these are well suited to detergent formulations particularly at alkaline pH (Sumantha et al. 2006). Protease from *Bacillus pumilus* MP 27 was considered as a good candidate for detergent industry due to its broad substrate range, activity over high and low temperatures and pH, and compatibility with surfactants and commercial detergents (Baweja et al. 2016). *Bacillus subtilis* KT004404, an isolate from hydrothermal vents, produced a metalloprotease that has high resistance towards anionic surfactants, solvents and detergents. Additionally, the protease had good

destaining properties which reflected its potential for application in detergent industries (Rehman et al. 2017). Extremely pH stable and thermostable proteases have been reported from *Micromonospora chaiyaphumensis* S103 (Mhamdi et al. 2017) and from *Aeromonas caviae* (Datta et al. 2016). Additionally, the proteases exhibited tolerance towards several organic solvents. Such proteases may have prospective for application as ideal additives for detergent formulations.

Keratinolytic proteases (keratinases) have the ability to bind and hydrolyse solid substrates like hair (Singh et al. 2014). Keratinolytic potential is an important property sought in detergent enzymes that may help removing protein substrates from solid surfaces. Keratinolytic proteases are attractive additives for detergents, especially for cleaning hard surfaces (Brandelli et al. 2010). Furthermore, keratinases may also help in the removal of keratinous soils particularly from collars of shirts, on which most proteases fail to act (Gessesse et al. 2003).

Application of enzymes in detergents not only enhances cleansing efficacy, especially for rigid biomaterials, but also allows the washing to be accomplished at relatively low temperatures, thus saving energy and making the process economic (Rehman et al. 2017). Supplementation of enzymes in detergents helps in reducing quantities of other hazardous chemical-based detergent components like soaps, oxidizing agents, chelating agents and surfactants, thus making detergents more eco-friendly. But there may be some limitations of enzymes usage in detergents, i.e. enzymes may be expensive to produce, may exhibit some allergic reactions, may get denatured at elevated temperatures and pH extremes and may digest some fabrics (wool). However, these limitations can be readily overcome by selecting the most apt detergent-compatible enzymes from the vast diversity of microbial proteases (Singh et al. 2014).

3 Proteases in leather industry

Vicious exploitation of natural resources, reckless usage of hazardous chemicals and consequential enormous environmental pollution have led to the concept of cleaner production. Tanneries represent one such industry that contributes heavily towards environmental degradation, and require serious attempts for mitigating its impact on the environment. Application of enzymes in leather processing may help developing eco-benign process that is less harmful to the environment (Pillai et al. 2011).

The studies on application of enzymes in leather processing and production were commenced in 1910. Since then a significant research has been undertaken, and currently several enzymes are being used at commercial level for leather processing. Leather processing involves several steps, viz. curing, soaking, liming, dehairing, bating, pickling, degreasing and tanning. The wastes or the effluents generated during these steps not only cause enormous environmental pollution and pose brutal threat to ecosystem, but may be severely health hazardous due to the presence of high concentrations of sulphide and chromium (Nilegaonkar et al. 2007). In leather processing, the primary objective is to remove hair and open up the fibre structure suitably to get the desired properties in final finished leather.

The conventional method of dehairing or depilation involves the usage of lime and sodium sulphide as it is more efficient and cheaper than other currently available technologies (Pillai et al. 2011). Chromium salts are the most commonly used tanning agents. But this process is highly polluting and health hazardous due to the presence of residues of these chemicals in tannery waste. The process poses a serious health threat to tannery workers (Nilegaonkar et al. 2007). The tannery effluent has high total dissolved solids (TDS) that contribute increased pollution load, i.e. BOD and COD in the wastewater.

The use of enzymes for leather dehairing process involves proteolytic cleavage of cementing substances which holds the hair to the skin so that the hair can be removed without destruction. Enzymatic dehairing may involve application of several enzymes like proteases, amylases and lipases. Moreover, the application of enzymes for leather processing enhances the quality of leather and gives stronger and softer leather with less spots (Madhavi et al. 2011). Enzymatic approach for leather processing is currently being explored as an eco-friendly option, especially for obviating or minimizing the conventional sodium sulphide-based processing (Sundararajan et al. 2011).

Different strategies have been used for the cost-effective production of dehairing proteases that may be used for developing greener and cleaner leather processing regime. Alkaline protease produced by *B. subtilis* strain VV under solid-state fermentation (SSF) on cow dung substrate, exhibited promising dehairing potential (Vijayaraghavan et al. 2012). Production of alkaline protease that has potential for application in leather processing was increased by mutagenesis of *B. licheniformis* N-2 using UV irradiation and other chemical mutagens (Nadeem et al. 2010). Leather industry waste was used as substrate (under SSF) for the production of alkaline protease from *B. cereus* 1173900 that has suitability for leather processing application (Ravindran et al. 2011).

Keratinolytic proteases with no collagenolytic activity and mild elastolytic activity are preferably used for dehairing process. Keratinase selectively breaks keratin tissue of the follicle, thereby pulling out intact hairs without affecting the tensile strength of leather (Macedo

et al. 2008). A metallokeratinase produced by *Acinetobacter* sp. R-1 had high keratinase activity and low collagenase activity. The keratinase showed reasonably good depilation on goat skin (Zhang et al. 2016b). Additionally, the ability of keratinase to modify the wool surface showed its application potential in textile industry.

Alkaline proteases from *Bacillus* spp. are the suitable candidates for efficient dehairing of hides in leather industry. There are several reports wherein dehairing potential of *Bacillus* proteases has been characterized, e.g. *B. altitudinis* GVC11 (Kumar et al. 2011), *B. cereus* MCM B-326 (Nilegaonkar et al. 2007), *B. cereus* VITSN04 (Sundararajan et al., 2011), *B. halodurans* JB 99 (Shrinivas et al. 2012), *B. pumilus* MCAS8 (Jayakumar et al. 2012), *B. subtilis* (Sathiya 2013) and *B. subtilis* KT004404 (Rehman et al. 2017). A thermostable alkaline protease from *Bacillus amyloliquefaciens* SP1 exhibited excellent dehairing (goat skin) potential (Guleria et al. 2016).

Considering the high risks associated with conventional leather processing, there is considerable emphasis on developing enzymatic approaches that are cleaner and safer. 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.

4 Proteases for processing of keratin wastes

Keratin-containing wastes are abundant in nature and have high protein content. However, accumulation of keratin wastes generally leads to environmental pollution as well as wastage of precious protein reserves (Mukherjee et al. 2011). Keratin wastes could be transformed into protein hydrolysate that may have application as amino acid-rich animal feed supplement. Moreover, the protein hydrolysate can be used for the production of various essential amino acids such as lysine. Villa et al. (2013) reported the application potential of keratin hydrolysates produced from feathers for formulation of hair shampoos.

Keratin in its native state structure is highly stable due to the presence of tightly packed helices and sheets with large number of disulphide bonds and is not easily degraded by common proteolytic enzymes like trypsin, papain and pepsin (Daroit et al. 2011). Composition and molecular configuration of keratin, its constituent amino acids, disulphide bonds and cross-linkages are responsible for its hardness and insolubility (Parradoa et al. 2014).

Keratin occurs in nature mainly in the form of hair, horn, nails and cornified tissue (Gupta and Nayak 2015). Feathers represent a rich protein source and contain about 90% of proteins and can be used for the production of protein-rich hydrolysate. Worldwide 24 billion chickens are killed annually, and around 8.5 billion tonnes of poultry feathers is produced. The poultry feathers are mostly discarded, and this not only adds to environmental pollution but also causes wastage of precious protein-rich reserve (Agrahari and Wadhwa 2010). Furthermore, incineration of the huge amounts of keratin solid waste may have several ecological disadvantages (Deydier et al. 2005).

Conventional methods of feather processing are cost and energy intensive, involve harsh process conditions like high pressure and temperature and cause loss of nutritional value (Grazziotin et al. 2006). Therefore, development of an eco-friendly enzyme-based technology for processing keratin waste is the need of the hour. Keratinases have been reported from several bacterial spp. (Singh et al. 2014). *Bacillus subtilis* K-5 utilized a wide range of keratinous wastes, viz. diverse feather types, nails, hair and scales, for growth and keratinase production. Keratinase exhibited activity and stability over a broad pH (5–10) and temperature range (50–90 °C) and showed multifarious application spectrum for blood stain removal from fabric, gelatin hydrolysis from waste X-ray films and dehairing of animal hide (Singh et al. 2014). A thermostable and wide range pH stable protease from *Micromonospora chaiyaphumensis* S103 showed excellent potential for deproteinization of shrimp wastes for production of chitin (Mhamdi et al. 2017).

Bacterial keratinases, mostly from *Bacillus* spp., have been used to convert hard-to-degrade keratin into protein substitutes (Kainoor and Naik 2010; Harde et al. 2011). Feather meal is nitrogen-rich, inexpensive and readily available source which may serve as potential substitute to guano (Brandelli et al. 2010). The non-conventional sources like wastes from agriculture, poultry, meat and fish industry have been exploited to meet the demand of low-cost protein foods. Studies have shown that keratin hydrolysates may serve as good organic fertilizers (Gousterova et al. 2005) and as amino acid supplements for health foods and pet foods (Gupta and Ramnani 2006). Two keratinases produced by *B. licheniformis* PWD-1, i.e. *Versazyme* and *Cibenza DP100,* have shown excellent results in broiler performance and growth of piglets (Odetallah et al. 2005; Wang et al. 2011).

Thus, the enzymatic hydrolysates produced by bioprocessing of keratin wastes may have potential for applications in food, feed and cosmetic industries. The process not only promises eco-friendly and sustainable approach for valorization of '*wastes to wealth',* but opens new avenues for development of potentially novel biotechnological processes that are good for environmental health.

5 Fibrinolytic proteases as thrombolytic agents

Fibrin is an insoluble protein derived from its soluble precursor, fibrinogen, which is involved in blood clotting. Fibrin plays a vital role in health and healing; however, formation of inappropriate clot especially under certain pathophysiological conditions in the body is a major risk factor for heart disorders (Bajaj et al. 2013, 2014). Homoeostasis of formation and dissolution of fibrin maintain appropriate viscosity in the vascular system. A shift in balance towards fibrin overproduction leads to unwanted clotting resulting in cardiac complications like acute myocardial infarction, ischaemic heart diseases, valvular heart diseases, peripheral vascular diseases, arrhythmias, high blood pressure and stroke (Mine et al. 2005).

Various thrombolytic agents have been used for the removal of clots, such as plasminogen activators, like tissue-type plasminogen activator and urokinase, which trigger the conversion of plasminogen into active plasmin (Hwang et al. 2007). Lumbrokinase from earthworm, fibrolase from snake venom and other plasmin-like proteins directly degrade fibrin, thereby dissolving thrombi rapidly and completely (Jayalakshmi et al. 2012). Although plasminogen activators and urokinase are still widely used in thrombolytic therapy, their expensive prices and undesirable side effects have prompted researchers to target novel, cheaper and safer resources (Agrebi et al. 2010; Deepak et al. 2010).

Microbial fibrinolytic proteases have been reported from several microorganisms including *Bacillus* spp. (Kim et al. 2009; Jo et al. 2011a). Production of fibrinolytic protease from a mutant strain of *B. cereus* GD55 was optimized using apple pomace as substrate (Raju and Goli 2014). *Bacillus* spp. are well known for production of potent fibrin-degrading enzymes (Wang et al. 2009; Bajaj et al. 2013) as shown in Table 1. Bafibrinase, a non-toxic, non-haemorrhagic fibrinolytic serine protease isolated from *Bacillus* sp., exhibited in vivo anticoagulant activity and thrombolytic potency (Mukherjee et al. 2012). Process optimization techniques have been used for enhancing production of fibrinolytic proteases (Ashipala and He 2008; Agrebi et al. 2009; Mahajan et al. 2012).

Bacillus amyloliquefaciens UFPEDA 485 produced fibrinolytic protease that exhibited long-term stability, and activity at physiological conditions, and could be of therapeutic importance for human and veterinary applications (de Souza et al. 2016). *Streptomyces* sp. CC5 produced a novel fibrinolytic protease that has high substrate specificity, strong thrombolytic activity, no toxicity and no prolonged bleeding time. Carrageenan-induced mouse tail (thrombosis model) was used for demonstrating excellent thrombolytic activity. Thus, the proteases may have potential for application in antithrombotic drug development (Sun et al. 2016).

A marine *Streptomyces violaceus* VITYGM produced an extracellular thrombolytic protease that had efficient blood clot lysis activity (Mohanasrinivasan et al. 2016). This novel actinoprotease may have the potential for developing drug for the treatment of cardiovascular diseases. A fibrinolytic enzyme was purified (36-fold) from *Cordyceps militaris*, a medicinal mushroom. The enzyme was characterized for several properties and analysed for amino acid sequence. The fibrinolytic enzyme was capable of degrading α-, β- and γ-chains of fibrinogen and activating plasminogen into plasmin. The enzyme can act as an anticoagulant and prevent clot formation by degrading fibrinogen. Thus, fibrin-degrading proteases may have great potential for prevention and treatment of thrombolytic diseases (Liu et al. 2017).

Keeping in view the fast emergence of cardiovascular problems including thrombosis, there is increased demand for chemotherapeutic thrombolytic agents. Most of the chemotherapeutics available are extremely expensive and have a range of side effects on the patients; therefore, there is a huge research impetus on investigating novel fibrinolytic agents that are safe, economic and effective for treatment.

6 Proteases for biofilm removal

Biofilms also termed as 'city of microbes' are a structure formed by extracellular polymeric substances (EPS) and the bacteria enclosed within it (Leslie 2011). The EPS consist mostly of polysaccharides, proteins and nucleic acids (Leroy et al. 2008). These substances form structural matrix which help the bacteria to attach to the surface. EPS also facilitate survival in the adverse conditions and environments (Kostakioti et al. 2013). Biofilm may be formed anywhere as long as the nutrients and a surface for adhesion are available. Biofilm is formed frequently in industrial systems associated with wastewater management, food processing, brewing, pulp and paper manufacturing and dairies.

Harmful biofilms may cause grave economic losses due to reduced productivity, decreased product quality and loss of time and expenses for biofilm removal (Leslie 2011). The durable microbial colonies in the biofilms impose an economic burden on companies as these may cause contamination by biofouling, i.e. the accumulation of microorganisms in aqueous environments. Bacterial adhesion is the initial step in colonization and is followed by increasing cell numbers and area of adherence, finally resulting in biofilm formation (Fig. 2). Biofilms are

Table 1 Fibrinolytic proteases from various *Bacillus* spp.

Organism	Source	Molecular weight, optimal pH and temperature	Comments	References
B. subtilis Natto B-12	Natto	29 kDa, pH 8.0 and 40 °C	Enzyme activated by Zn^{2+}	Wang et al. (2009)
B. subtilis LD-8547	Douchi	30 kDa, pH 8.0 and 50 °C	PMSF inhibited activity	Wang et al. (2008)
B. subtilis DC33	Traditional food in China	30 kDa, pH 8.0 and 60 °C	Soybean trypsin inhibitor inhibited activity	Wang et al. (2006)
B. subtilis A26	Marine water	28 kDa, pH 9.0 and 60 °C	Activity totally lost with PMSF	Agrebi et al. (2009)
B. sphaericus	Vector Control Research Centre, Puducherry	18.6 kDa	Fibrinolytic activity was similar to that of streptokinase	Balaraman and Prabakaran (2007)
B. subtilis ICTF-1	Marine niches	28 kDa, pH 9.0 and 50 °C	Activated by Ca^{2+} and inhibited by Zn^{2+}, Fe^{2+}, Hg^{2+} and PMSF	Mahajan et al. (2012)
B. subtilis DC-2	Douchi	–	CCD used to optimize enzyme production	Ashipala and He (2008)
B. amyloliquefaciens CH86-1	Cheonggukjang	27 kDa	Gene *apr* E86-1 was expressed in *B. subtilis*	Lee et al. (2010)
B. amyloliquefaciens MJ5-41	Meju	27 kDa, pH 7.0 and 45 °C	Gene *apr* E5-41 was expressed in *B. subtilis*	Jo et al. (2011b)
B. subtilis EAG-2	Ornamental plant nursery	27 kDa, pH 8.5 and 65 °C	Activity reduced by PMSF	Ghafoor and Hasnain (2010)
B. amyloliquefaciens CH51	Cheonggukjang	27 kDa, pH 6.0 and 45 °C	Tryptic soy broth was best for enzyme production	Kim et al. (2009)
B. licheniformis KJ-31	Jeot-gal	37 kDa, pH 9.0 and 40 °C	Activity inhibited by PMSF	Hwang et al. (2007)
B. subtilis GBRC1	University of Madras, Chennai	24.6–33.0 kDa, pH 7.0–12 and 50 °C	Inhibited by PMSF	Jayalakshmi et al. (2012)
B. subtilis K42	Soybean flour	20.5 kDa, pH 9.4 and 40 °C	Organic solvent stable	Hassanein et al. (2011)
B. subtilis A1	Soil	28 kDa, pH 6.0–10 and 50 °C	PMSF, DIFP and TPCK reduced enzyme activity	Yeo et al. (2011)
B. amyloliquefaciens An6	Soil	30 kDa, pH 9.0 and 60 °C	*Mirabilis jalapa* tuber powder used as complex media	Agrebi et al. (2010)
B. subtilis TP-6	Tempeh	29 kDa, pH 7.0 and 50 °C	Enzyme was stable towards organic solvents	Kim et al. (2006)
B. subtilis TKU007	Soil	28–30 kDa, pH 8.0 and 40 °C	Shrimp shell wastes used as sole C/N source	Wang et al. (2011)
B. subtilis	*Bacillus* genetic stock centre, Ohio, USA	–	CCRD was used to optimize medium	Deepak et al. (2008)
B. cereus NS-2	Soil	pH 9.0 and 40 °C	Fe^{2+} favours enzyme activity. Inhibited by Pb^{2+} and Hg^{2+}	Bajaj et al. (2013)

detrimental to both human life and industrial processes due to their association with infection, pathogen contamination, biofouling and slime formation. But sometimes biofilms are desirable and beneficial for example, in probiotics-gut adhesion (Gupta and Bajaj 2017), environmental technologies and bioprocesses (Hori and Matsumoto 2010).

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 (Jessen and Lammert 2003). But the method is impractical for larger structures where regions like joints, filters or gaskets are not easily accessible. 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 like *Savinase* and *Everlase*; however, other hydrolases have also been

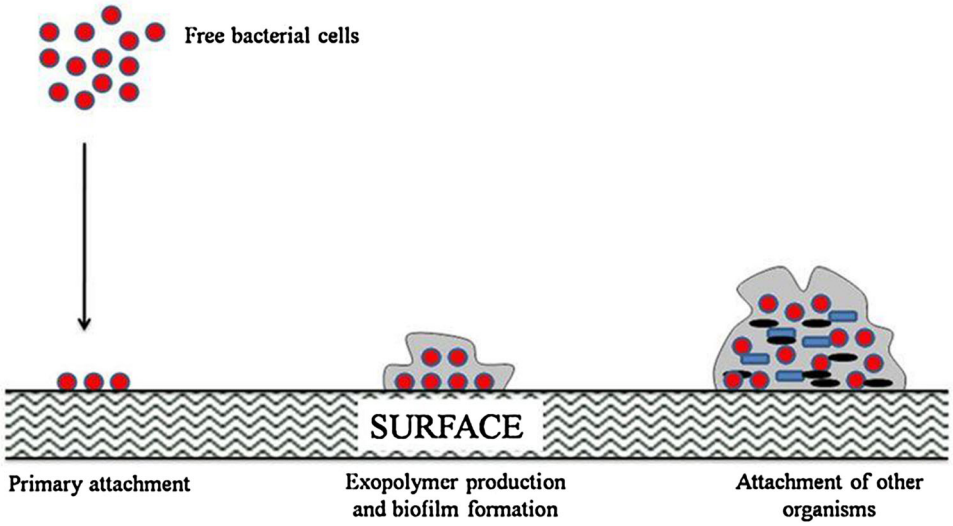

Fig. 2 Steps involved in biofilm formation

exploited (Molobela et al. 2010). Amylases, when used in combination with proteases, help to eliminate existing biofilms and prevent bacteria from adhering to surfaces (Deinhammer and Andersen 2011).

Considering the extreme conditions of industrial processes robust proteases that function optimally under such conditions are desired. Pernisine, a protease produced by *Aeropyrum pernix,* is optimally active at 90 °C and could be used as potential biofilm-removing agent (Molobela et al. 2010). Oulahal-Lagsir et al. (2003) examined the combined treatment which involved the application of ultrasound and enzyme preparations for removal of an *E. coli* model biofilm, made with milk on stainless steel sheets. Orgaz et al. (2006) demonstrated the use of enzymes from three different fungal sources, i.e. *Aspergillus niger*, *Trichoderma viride* and *Penicillium* spp., for their potential ability to remove *Pseudomonas fluorescens* biofilm.

7 Proteases for silk degumming

Silk, also known as the 'queen of fabrics', is a composite material with two fibroin filaments surrounded by a cementing layer of sericin (Mahmoodi et al. 2010; More et al. 2013). Silk processing from cocoons to the final finished clothing and articles involves several steps which include reeling, weaving, degumming, dyeing or printing and finishing (Zahn 1993). Degumming is the process where sericin, i.e. the silk gum gluing the fibroin filaments, is totally removed in order to obtain silk with desirable properties (Fig. 3). Degumming of silk is traditionally carried out with soap or alkali. These methods face major limitations like degummed silk obtained is not of uniform quality, big loss of strength of silk, short shelf life of silk and highly hygroscopic nature of silk. Additionally, the usage of chemicals in the traditional methods causes environmental pollution and makes the process environmentally unsafe. Therefore, there is great impetus on developing enzyme-based silk-degumming process that is low energy demanding, uses least chemicals, maintains high strength of silk fibre and is environmentally healthy (Nakpathom et al. 2009).

Proteolytic enzymes, which can cleave the peptide bonds of sericin without destroying the fibroin, may have potential for application as degumming agents. Proteases are being projected as a replacement of the harsh and energy demanding chemicals for treatment process. Plant proteases like papain (Nakpathom et al. 2009) and bromelain (Devi 2012) are the effective cocoon cooking enzymes that are commonly used for the processing of cocoons. A bacterial enzyme *Alkalase* marketed by Novo has been found to be very effective in hydrolysing sericin. The combination of lipase and protease has been reported to be an effective approach for dewaxing and degumming. Furthermore, this enzyme cocktail provides positive effects on wettability of silk fibres (Freddi et al. 2003). Thus, the enzyme-based technology could be used effectively for silk degumming in industries as an eco-friendly alternative.

The production of silk-degumming protease from *B. subtilis* C4 was optimized to get an enhanced protease yield (Romsomsa et al. 2010). Joshi and Satyanarayana (2013) reported a recombinant alkaline serine protease from a novel bacterium *B. lehensis* which enhanced the softness and shine of silk fibres. The protease could possibly be employed as an ecologically benign alternative to traditional harsh chemicals used in degumming processes. Furthermore, the nitrogen-rich discharges from enzyme-based silk-degumming process may potentially be utilized as a nutrient substrate for microbial growth and protease

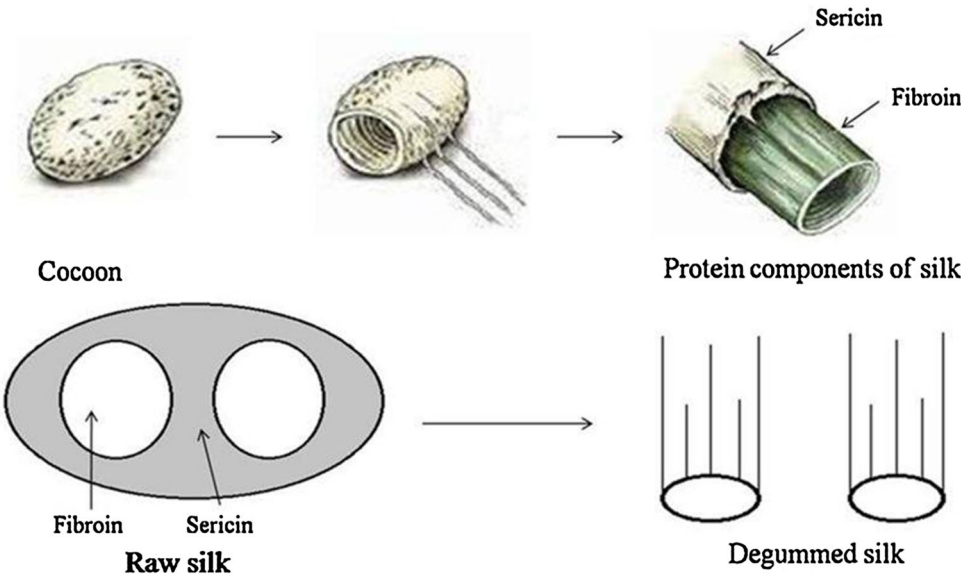

Fig. 3 Structure of silk fibre and degumming of silk

production. This degumming discharge has been used as substrate for protease production from *B. licheniformis* and *Aspergillus flavus* (Vaithanomsat et al. 2008). Moreover, the degumming waste liquor that is rich in sericin content is being used as a raw material for the production of sericin powder. The sericin powder may have application in cosmetic industry as a moisturizer, for hair-care products and as a natural textile finish. 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.

8 Proteases in photographic industry

The exposed X-ray films have approximately 5–15 g of silver per kg of film (Marinkovic et al. 2006). Silver recovery from waste photofilms is a big business. Around 18–20% of the world's silver needs are supplied by recycling photographic waste (Shankar et al. 2010). Nearly 2.0 billion radiographs are taken each year, including chest X-rays, mammograms and CT scans (Cavello et al. 2013). 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 (Fig. 4). The conventional method for silver recovery involves burning the films directly. This method is the most primitive one and generates undesirable foul smell and causes enormous environmental pollution by producing undesired emissions. The chemical method for silver recovery from X-ray films involves usage of acid and alkali, and thus, it is quite harsh and environmentally hazardous (Marinkovic et al. 2006; Ekpunobi et al. 2013). Furthermore, the polyester film on which emulsion of silver and gelatin is coated cannot be recovered by these methods.

Enzyme-based method may be developed that may not only recover silver efficiently but might have minimal impact on the environment (Nakiboglu et al. 2003). Proteases have been reported to possess excellent gelatinolytic activity for successful recovery of silver from X-ray films. Several *Bacillus* spp. proteases, viz. *B. lehensis* (Joshi and Satyanarayana 2013), *B. subtilis* (Nakiboglu et al. 2001; Kumaran et al. 2013), *B. cereus* (Bajaj et al. 2013) and *B. licheniformis* (Pathak and Deshmukh 2012), *B. subtilis* K-5 (Singh et al. 2014) and *B. licheniformis* K-3 (Singh and Bajaj 2017), have been demonstrated to possess good gelatinolytic activity for efficient silver recovery from X-ray films. Proteases from other sources like *Aspergillus versicolor* (Choudhary 2013) and *Purpureocillium lilacinum* (Cavello et al. 2013) have also shown good gelatin hydrolysing ability. High temperature and slightly alkaline conditions favour stripping off of the gelatin layer. Thus, thermostable alkaline proteases from *Bacillus* spp. are well suited for the process (Nakiboglu et al. 2001). Nutritionally enriched medium was used for the production of proteolytic enzyme that was capable of efficiently hydrolysing X-ray-bound gelatin (Kumaran et al. 2013).

Considering the eco-unfriendly nature of chemical-based methods of silver extraction from X-ray films, a greener approach that is based on application of enzymes is gaining attention. 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.

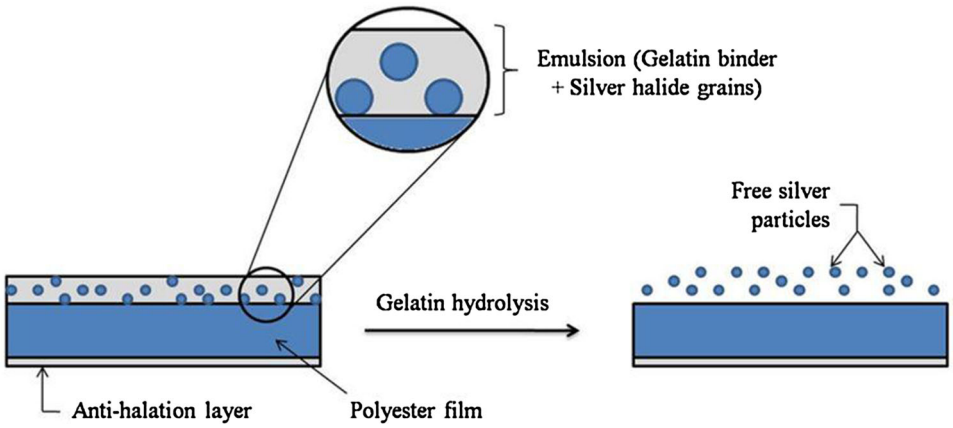

Fig. 4 Gelatin hydrolysis for the release of silver from X-ray film

9 Proteases in food industry

Proteases are used for a wide range of food processing applications, e.g. in dairy, bakery, fish and seafood processing, animal protein processing, meat tenderization, plant protein processing and generation of bioactive peptides. Major aim for application of enzymes in food processing is to enhance the nutritional and functional properties of foods such as improved digestibility, modifications of sensory quality, improvement of antioxidant capability and reduction of allergenic compounds (Tavano 2013). However, the choice of enzyme and the desired degree of hydrolysis must be realized by taking into account the taste, solubility and specific application properties of the hydrolysate product.

Microbial proteases have been exploited in the food industries in variety of ways. Primarily proteases have been used in the food industries for production of protein hydrolysates of high nutritional value. The protein hydrolysates can be used for blood pressure regulation, for infant food formulations, for specific therapeutic dietary products and for the fortification of fruit juices and soft drinks (Ray 2012). However, the bitter taste of protein hydrolysates is a major barrier in their wide range utilization. Intensity of bitterness is proportional to the presence of hydrophobic amino acids in the hydrolysates (Sumantha et al. 2006). Exopeptidases like leucine aminopeptidase and those that can cleave hydrophobic amino acids and proline are valuable in debittering protein hydrolysates (Sumantha et al. 2006).

Major application of proteases in dairy industry is the manufacture of cheese. The proteases produced by GRAS (generally recognized-as-safe) microbes help in hydrolysis of specific peptide bond to generate *p*-k-casein and macropeptides. Whey is an abundant liquid by-product of cheese-making process. Proteases convert whey into protein hydrolysate during whey bioconversion process. Proteases are used for chill-proofing of beer. The fresh beer produced may have haziness mainly due to the presence of complexes of proteins and tannins, which may be cleared by application of proteases. A metalloneutral protease from *B. amyloliquefaciens* SYB-001 had the ability to release more water-soluble proteins and may be suitable for brewing industry (Wang et al. 2013).

Application of exogenous proteases aids in the production of tender meat (Mageswari et al. 2017). Tenderness is one of the most desirable characteristics of meat. Some of cuts from the big carcases may not be considered as prime quality meat cuts due to their toughness. However, the application of proteases may help transforming such meat pieces into quality cuts just like the prime ones by tenderization. The two most often used meat-tenderizing enzymes are papain and bromelain. To a lesser extent, ficin, derived from fig tree latex, is also used (Payne 2009). Microbial proteases (bacterial and fungal) have also been explored for meat tenderization application (Qihe et al. 2006; Ha et al. 2013). Microbial proteases are also useful in baking process. Flour consists of gluten (glutenin and gliadin), starch, non-starch polysaccharides, lipids and trace amount of minerals. Protease-mediated weakening of gluten results in improved dough formation and enhanced dough rise mainly due to breakdown of complex network of glutenin and gliadin (Bajaj and Manhas 2012).

Application of proteases may help enhancing digestibility, solubility of proteins in foods and also upgrading the organoleptic properties of foods. Protease may be used for enzymatic synthesis of aspartame, a non-calorific artificial sweetener. The waste generated by food industry could be used as an inexpensive substitute for protease production by thermophilic strain *B. caldolyticus* DSM 405 (Jamrath et al. 2012).

The application of enzymes in various food processing units would not only substantially enhance the nutritional and functional attributes of foods but certainly help developing eco-friendly and sustainable bioprocesses that involve less usage of chemicals.

10 Proteases for prion degradation

A prion in the scrapie form (PrP^{Sc}) is a misfolded infectious form of protein. They are causative agents of transmissible spongiform encephalopathies in a variety of mammals, including bovine spongiform encephalopathy in cattle (Prusiner 1998). The name PrP^{Sc} is because of their discovery first in scrapie-affected sheep. PrP^{Sc} has high resistance to proteolytic digestion. Actually, the core of PrP^{Sc}, i.e. carboxy proximal, can withstand proteolysis even at very high level of proteinase-K. The presence of proteinase-K-resistant prion protein is considered as a definitive diagnostic test for prion diseases in humans and other species (Leske et al. 2017). Recently, however, absence of protease resistance in PrP^{Sc} has been observed, and a mechanism for protease-sensitive prion infectivity has been proposed (Leske et al. 2017).

In humans, prions cause Creutzfeldt–Jakob disease, variant Creutzfeldt–Jakob disease, Gerstmann–Straussler–Scheinker syndrome, fatal familial insomnia and kuru. All known prion diseases affect the structure of the brain or other neural tissue, and all are currently untreatable and universally fatal (Prusiner, 1998). Proteases may help degrading wrongly folded proteins. Yoshioka et al. (2007) identified a protease-producing *Bacillus* strain that was capable of degrading scrapie PrP^{Sc}. The protease (MSK 103) was also effective against dried PrP^{Sc}. 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 biobased therapeutics for prion diseases in humans and animals.

11 Proteases for 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. Wool is a special kind of keratin that has a high concentration of cysteine cross-links in the exocuticle of wool fibre. Proteases may have application potential for production of shrink-proof wool in an environmentally friendly process. However, protease treatment in general damages the wool by causing excessive loss of strength, and lowering the antifelting ability, which in turn may lead to additional damage to the fibre interior during wool processing (Wang et al. 2011). Therefore, specific proteases whose action is limited to cuticle scale of wool fibre are desired (Shen et al. 2007). Keratinase can be used in textile processing and may potentially replace the conventional physicochemical methods that are environmentally unhealthy. Thus, enzyme-based bioprocesses can be developed for efficient production of shrink-resistant fibre that have improved handling properties (Tahara et al. 2003).

A keratinase from *Brevibacillus parabrevis* CGMCC 10798 was purified and characterized for its excellent potential for wool processing (Zhang et al. 2016a). 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. 2017). Several strains of bacteria like *Bacillus*, *Exiguobacterium*, *Deinococcus* and *Micrococcus* isolated from Patagonian Merino wool, were reported to produce wool-degrading enzymes. *Bacillus* sp. G51 exhibited the highest wool-keratinolytic activity. LC-MS/MS analysis showed that two serine proteases of peptidase family S8 and a metalloprotease associated with Bacillolysin were responsible for hydrolysing keratin disulphide bonds. The enzyme substantially reduced the wool felting tendency without much weight loss. Thus, eco-friendly treatment approaches based on enzyme cocktail (with protease combination) may help designing the organic wool processing (Iglesias et al. 2017).

12 Nematicidal activity of protease

Nematoda is a diverse animal phylum inhabiting a broad range of environments. Depending on the species, a nematode may be beneficial or detrimental to plant health causing huge economic losses (Abad et al. 2008). Strategies followed against pathogenic nematodes include use of chemical nematicides and different biocontrol agents like fungi and bacteria (Tian et al. 2007). Traditional chemical-based method not only causes a significant environmental pollution but also leads to the emergence of nematicide resistance (Yang et al. 2013). The rhizobacteria have extensively been studied as biocontrol agents for the plant-parasitic nematodes. Among these bacteria, numerous *Bacillus* spp. strains have been reported to express activities that suppress the pests and pathogens, including nematodes (Radnedge et al. 2003). There are several reports of proteases from non-*Bacillus* spp. being used as biocontrol agents against nematodes (Siddiqui et al. 2005; Ward et al. 2012). Lian et al. (2007) demonstrated nematicidal activity of an extracellular cuticle-degrading protease Apr219 from *Bacillus* sp. strain RH219 isolated from rhizosphere.

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). *Bacillus* sp. B16 isolated from soil sample secreted extracellular cuticle-degrading protease that had remarkable

nematotoxic activity against *Panagrellus redivivus* (Qiuhong et al. 2006). A total of 120 bacterial strains of 30 species of the Bacillaceae and Paenibacillaceae were examined for nematicidal activities. Nine species, viz. *Bacillus thuringiensis*, *B. cereus*, *B. subtilis*, *B. pumilus*, *B. firmus*, *B. toyonensis*, *Lysinibacillus sphaericus*, *Brevibacillus laterosporus* and *B. brevis,* exhibited excellent nematicidal potential. Genome analysis was used for identification of potential virulence factors. One of the major mechanisms for nematicidal capacities was observed to be the ability of bacteria to produce proteases and/or chitinases (Zheng et al. 2016). Thus, development of biobased strategies for controlling and managing the pests may contribute substantively towards environmental health as this would lead to reduced application of chemical-based pesticides.

13 Proteases for contact lens cleansing

The tear film is a complex fluid composed mainly of water, lipids, proteins, sugars, mucin and carbohydrates. Films formed over the lenses provide a surface for the adhesion of opportunistic pathogens like *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Dutta et al. 2012). Adhesion and colonization by microorganisms, particularly bacteria, on contact lenses have been implicated in several adverse events including microbial keratitis (Willcox and Holden 2001), contact lens-related acute red eye (Szczotka-Flynn et al. 2010), contact lens peripheral ulcer (Wu et al. 2003) and infiltrative keratitis (Szczotka-Flynn et al. 2010). Therefore, cleansing of contact lenses is utmost important. Proteases have been used for preparing contact lens cleaning solutions. Mainly plant and animal proteases have been used for preparing contact lens cleaning solutions. Recently, some microbial proteases have been shown to be promising agents for contact lens cleaning. Protease from *Bacillus* sp. 158 exhibited ability for cleaning of tear films and debris of contact lenses (Pawar et al. 2009). The neutral protease isolated from *Bacillus* sp. 158 efficiently removed the protein deposits from contact lenses. The partially purified protease exhibited optimum pH and temperature for activity at pH 7.0 and 30 °C, respectively.

The enzyme could effectively be used to remove protein deposits from contact lenses and, thus, help increasing the transmittance of lenses (Pawar et al. 2009). Clear-Lens Pro, currently used in contact lens cleaning formulations, marketed by Novozymes, Denmark, is of microbial origin. This preparation is used to remove protein-based deposits and protein films from contact lenses. The protease used in this preparation is from *Bacillus* sp. which hydrolyses the protein in the deposits and films (Sumantha et al. 2006). Proteases from several *Bacillus* spp., viz. *B. subtilis*, *B. licheniformis*, *B. thermophilus* and *B. cereus*, exhibited excellent activity against artificial tear solution and, thus, could be of importance for contact lens cleansing. All the proteases had maximum activity at 40 °C and pH 8 (Ismail et al. 2014). Considering enormous campaign of bioeconomy, i.e. biobased products, processes and services, it is of course interesting to design and develop contact lens cleansing formulations using enzymes.

14 Future prospective of proteases

Though proteases are extensively applied enzymes in several sectors of industrial biotechnology, further research is required for exploring the full application potential of proteases. Several processes like peptide synthesis and sequencing, digestion of unwanted proteins, cell culturing and tissue dissociation, preparation of recombinant antibody fragments, study of structure–function relationships, removal of affinity tags and proteolytic digestion of proteins, require immense research impetus. Moreover, with the application of recombinant DNA technology and protein engineering microbes can be manipulated to enhance the production of specific high priority industrial enzymes. Extremophilic organisms could be exploited for production of process-suitable novel enzymes. Furthermore, molecular intricacies of mechanisms involved for application of proteases in diverse processes need investigation. Environment assessment tools like life cycle assessment, carbon footprint, environmental impact assessment, global warming, acidification, eutrophication and photochemical ozone formation could be employed for determining the impact of cleaner enzymatic processes in place of conventional processes.

15 Conclusions

Enzyme-driven industrial processes are the most appropriate alternatives to tedious, expensive and polluting traditional methods. Microbial proteases, especially from *Bacillus* spp., have enormously been exploited and constituted the backbone for several industries. The *Bacillus* spp. have the potential capability to produce industrially suitable enzymes which possess poly-extremotolerance, i.e. ability to withstand extremes of pH, temperatures, presence of organic solvents and a variety of other enzyme inhibitors. Thus, enzymes from *Bacillus* spp. meet the industrial process criteria. It is pertinent to refer *Bacillus* spp. as 'microbial factories' for industrial enzymes. Application of enzymes in detergents promises eco-friendly industrial processes which involve reduced usage of chemicals such

as soaps, surfactants, bleach, oxidizing and chelating agents in the detergent formulation. Addition of enzymes in detergents augments their washing efficacy, especially for dirt/dust of biological origin. Furthermore, application of enzymes helps washing to be executed at lower temperatures, thus saving energy and environment. Application of enzymes in leather processing regime makes it more efficient, eco-benign and safer, i.e. free of sulphide and chromium usage. Enzymatic bioprocessing of keratin wastes envisages eco-friendly and sustainable valorization of *wastes to wealth* and offers huge potential for food, feed and cosmetic industries. Application of proteases in leather and textile industry not only improves the process economy but also improves product quality and makes the processes eco-benign and more efficient. Protease application in silk and wool industries promises high-quality silk/wool and a safer and eco-friendly process. Application of proteases helps developing green process for silver extraction from X-ray films, thus mitigating the enormous environmental pollution due to conventional process. Microbial proteases may potentially be developed as specific therapeutics for prion diseases in humans and animals. Microbial proteases are being investigated for development of potential thrombolytic agents considering the high cost and undesirable side effects of the available chemotherapeutics. Proteases may be developed as potential biocontrol agents that may help mitigating environmental pollution due to chemical-based pesticides. Thus, successful commercialization of proteases for several biotechnological processes in industries paves the way for development of clean, green and sustainable processes. Furthermore, recent advancements in the areas of molecular biology and protein engineering must be exploited to develop novel/tailor-made enzymes with greater efficacies under prevailing industrial process microenvironments.

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