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PROTEASES - A SINGLE GROUP OF ENZYME BRIDGING INDUSTRIAL AND THERAPEUTIC DEMANDS

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ABSTRACT

Proteases are an expanding class of enzyme that holds great promise in the industry and therapeutics. The protease constitutes one of largest consumed enzyme for several industrial applications in last few decades. The application of protease is not restricted to only industrial level and in recent decades many of protease enzymes have been approved for therapeutics application. Till date, US FDA has approved 12 different proteases for therapeutic applications and many more are in clinical trial studies. The industries like detergent, food processing, leather, textile and paper industry have employed more than 80% of world protease production. These amazing enzymes not only improved industrial processes leading to high through output but also helped in cleaning the environment. Several industry including leather and textile industry are associated with massive amount of chemical consumption a major source of pollutant have been optimized with use of proteases with high efficiency and good quality of product. Further, the emerging scope of thermostable protease had led to industrial efficiency one step ahead. The thermostable proteases have been already implemented in biological research and therapeutics and current prospect is finding novel sources and producing these enzymes by recombinant DNA technology. The most important invention in protease was exploring a protease for management of vascular disorders. The available thrombolytic are serine protease clinically approved for management of several vascular disorders. However, many time industrial operations are carried out extreme low temperature and hence activity of catalyst always falls in controversy. The psychrophilic proteases have emerged in last decades to solve such complication and enable many industrial operation and therapeutics often run at lower temperatures. In this review, author has bought scope of protease in industry and therapeutics as well. The novel categories of protease such as thermophilic and psychrophilic are ideal choice for several applications. In this review we have given a complete overview on protease, thermophilic protease and psychrophilic protease for industrial and therapeutic application.

INTRODUCTION

In the present scenario enzymes have become an integral part of life and had shown their impact in many ways including house hold application, therapeutic application, research and industrial application (1). The application of these enzymes increasing exponentially which further emphasized in finding novel sources and technology to meet the commercial demands (2). The microbial world, a prime source for enzymes had served for many decades to conquer industrial and therapeutic targets (3). The commercial production of protease enzyme for industry and therapeutics has expanded enormously. However, the advancement of technology in biological sciences; molecular biology, DNA technology and enzyme engineering technology had made remarkable achievement in last two decades (4, 5). More than 70% total enzyme production is utilized by industrial processes alone and rest for therapeutic and research activities (6).

The enzymes not only offer a better industrial yield but also became more essential to minimize environmental pollution (7). The several industrial processes often run with massive amount chemical leading to environmental damage. Here, these amazing molecules had played viral role in optimizing conventional industrial processes into efficient one and same time protection of environment (8). The leather processing is classical example which is associated with huge amount of chemical consumption and leading to environmental pollution (9). The textile industry and other such as chemical industries are major threats for environment need to optimized using modern enzyme based industrial process (10). The proteases constitute a very large and complex group of enzymes which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles (11). The versatility in physiochemical properties of proteases had result this one group of enzyme for diverse application. The proteases are primarily of six major categories and each of class of protease is specific for their substrate (12). Further, each class of protease possesses several subcategories and subfamily defines reason for diverse substrate specificity. Each category of protease offers enzymatic reaction differently used commercially for industrial application and therapeutics (13). The entire biological world is enriched with such potential biomolecules including plant, animal and microbial world (14). The plants such as papaya are prime source for several proteases which have been used from ancient time for various applications. The animal based proteases are becoming more popular especially for therapeutic application as these source offer enzyme with less complication in biological system (15). However, the microbial world is still first choice for these commercial enzymes for both therapeutic and industrial operations. There are several reasons for choosing microbes as a potential source for protease and other enzymes, one,

microbes are easy to grow and manipulate than other sources (16). Second the diversity in microbial world which offers diverse protease for several applications. The modern commercial production of any class of enzyme including protease carried out by recombinant DNA technology and hence, single cell microbes are easy to manipulate at genetic level for commercial need (17, 18).

Classification of proteases

The protease is group of enzyme widely distributed in biological world including plant, animal and most important microbial world. This enzyme is associated with series of vital physiological functions in each organism such as blood coagulation mechanism in higher mammals, defense system in microbial world, digestive enzymes etc. (19). The proteases have been classified into six major groups based on their molecular mechanism and targeted substrate. Further, these six major groups were classified into many subgroups based on digestion patterns of enzyme (20). The classes in protease group are as given below-

i. Serine proteases
ii. Threonine proteases
iii. Cysteine proteases
iv. Aspartate proteases
v. Glutamic acid proteases
vi. Metalloproteases

There is another approach used for classification of protease based on their optimal pH at which these enzymes offer a maximum enzymatic activity. The acid protease where the maximum activity of protease reported in lower pH and acid protease ranges in their activity in pH 1-6 (21). Alkaline protease belongs to a group of protease where higher pH is essential for a protease to offer its proteolytic activity and activity ranges in between 8-14. On Other neutral protease offers their enzymatic activity at pH 7.0. These proteases offer their enzymatic activity at a particular pH due to pH act as an activation factor for these enzymes (22). For example trypsin a proteolytic enzyme in the gastrointestinal track produced in inactive form that is chymotrypsin and need a lower pH to remove extra amino acid from the active site of the pro-enzyme.

i. Acid protease
ii. Alkaline protease
iii. Neutral protease

1. Serine protease (EC 3.24.21)

Serine protease is one of the major classes of protease group that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the active site (23). Serine proteases are found ubiquitously in both eukaryotes and prokaryotes with diverse enzymatic activity. However, two prime categories of serine protease: chymotrypsin-like (trypsin-like) or subtilisin-like have been studied in detail (24). The serine proteases due to diverse enzymatic activity are vital for many physiological functions such as immune response, blood coagulation and digestion. These serine proteases are explored for therapeutic and industrial applications in the last few decades (24). The antiviral drug and thrombolytics are the classical example of serine protease application in modern medicine. Further, serine proteases have tremendous scope of industrial application and many serine proteases have been isolated and purified to meet commercial demand (25). The breakthrough was when Subtilisin was produced by recombinant DNA technology from microbial origin one of the major proteolytic enzymes implemented for industrial application (26).

2. Threonine proteases (EC 3.4.25)

The threonine proteases are a family of proteolytic enzymes possesses a threonine (Thr) residue within the active site. The threonine protease exhibited proteolytic activity by recruiting secondary alcohol at N-terminal to threonine for catalysis (27). Two major subfamily of threonine protease were reported are as proteasome and acyltransferases. The threonine proteases have a significant role in cellular physiology and very few have been employed in commercial application (28).

3. Cysteine proteases (EC 3.4.22)

The other industry and therapeutically important protease subgroup is Cysteine proteases which also known as thiol protease. The thiol protease exhibits its catalytic property with a shared common mechanism which involves a nuleophilic thiol in a catalytic triad or dyad (29). The Cysteine proteases are primarily present in all the fruits including papaya, pineapple, fig and kiwi fruit. Cysteine proteases are used as an ingredient in meat tenderizers. There are more than 14 subfamilies of cysteine proteases have been reported and many of them had played a significant role in building novel therapeutics against diseases caused by viruses (30). Many viruses like polio and hepatitis C have been successfully targeted by cysteine protease to inhibit their pathogenic island to overcome such diseases (31).

4. Aspartic proteases (EC 3.4.23)

The aspartic proteases are other physiologically important group of protease enzymes which use an aspartate residue for catalysis of their peptide substrates (32). The Aspartic proteases require a

low pH for their enzymatic activity and are distributed in the entire biological world including prokaryotic and eukaryotic. Among the most studied aspartic proteases is a eukaryotic aspartate protease include pepsins, cathepsins, and renins (33). These enzymes are essential for maintaining several vital biological functions and many of them have been employed for industrial process optimization (34).

5. Glutamic acid protease (EC 3.4.19)

The glutamic acid proteases are limited to filamentous fungi another class of protease available for several application. These proteases are having much scope in food processing industry (35). The glutamic acid protease also had shown its potential in developing several drugs such as antitumor and anticancer (36).

6. Metalloproteinase (MMPs) (EC 3.4.24)

In contrast to other proteases, a metalloproteinase or metalloprotease (MMPs) catalytic mechanism involves a metal. These proteases have great scope in management of neurological disorders as tools for drug delivery and cancer management (37).

Industrial applications

The protease constitute a group of industrially important enzyme dominate commercial market by more than 70% of available enzymes. The proteases are ubiquitously distributed in biological world but microbial world and alone bacillus produces more than 80% of proteases enzyme used for different commercial applications (38). Due to wide range of substrate affinity and diverse sources of proteases the applications of proteases are unique and widely applicable in different industries. The protease among the six major categories acid protease and alkali proteases have been used extensively in last three decades (39). Several industries like leather and textile (synthetic fabric and silk) revive themselves due to one group enzyme and that is proteases. Further, there is tremendous scope of protease in chicken and paper industry to produce commercial products in higher efficiency (40).

Leather processing

Leather is one of prime commodity for India to earn foreign currency. The commercial production of leather has been increased remarkably in last three decades and India is second largest exporter of leather both processed and unprocessed followed by China (41). Leather making is a tedious process and consume a lots of time and expenditures in term of chemicals water resources and manpower. The old conventional method of leather making is not practical now days as are not environmental friendly and lead to low grade leather (42). The hide essentially treated with several enzymes including protease for removal of hairs and other skin protein like collagen for

better texture and appearance. The processing of leather need incubation of hide with protease at higher temperature hence conventional proteases often fail to provide required output (43). Hence, thermostable protease is ideal and most convenient option for leather making and leather dehairing (44).

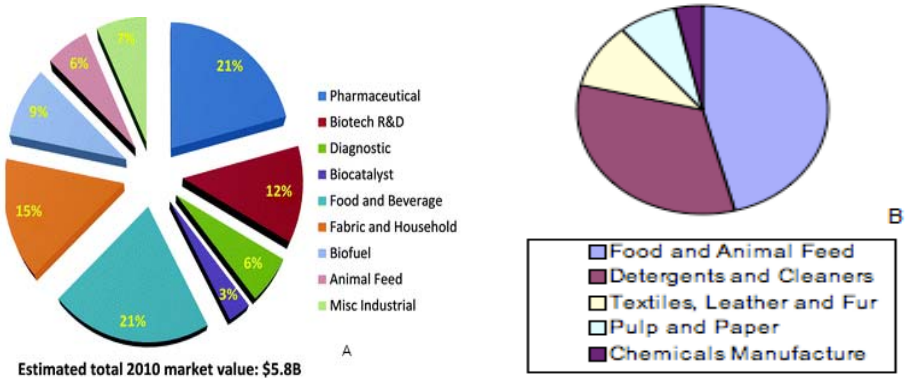

Figure 1. Commercial application of protease and other enzyme in therapeutics (A) and protease contribution in industry (B).

The protease based leather not only offer free from environmental pollution same time it provides better quality of leather compare from chemical based leather making. Several microbial species have been explored for hunt of thermostable protease for leather processing (45). The bacterial strains such as *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens and Bacillus. cereuses are* the few studies for commercial production of thermostable and conventional protease. Many fungi also explored as source for thermostable protease for leather making including *Aspergilous parasiticus, Aspergilous effuses, Aspergilous ochraceus and Penicillium griseofulvum* (46). The another advantage in using thermostable protease in leather making use of immobilized protease for repeated application cut down production cost (47).

Silk processing

Silk is another commodity for foreign currency and India stand in top ten nations producing quality silk. The silk production and processing are two different events need skills and time to achieve high quality product. The quality of silk depends on silk processing especially silk degumming an operation where several treatments requires for removal of wax and fat deposited on silk protein fiber called as fibroin (48). The conventional methods for silk processing and degumming are carried out with use of detergent basically synthetic origin often cause detects in silk protein fiber. Further, chemical treated silk neither possesses shining appearance nor appropriate mechanical properties (49). The grades of silk purely depend of skilled processing where a series of operation required giving glow and mechanical features. The use of enzyme in

silk processing especially silk degumming results in quality silk and lots of protease have been employed for such operation in last one decade. The conventional protease often used to achieve silk degumming and reaction often carried out at higher temperature which abolishes enzymatic activity. The current prospect of silk processing and production has acknowledged the potential of thermostable protease and many thermostable proteases have been isolated and implemented for silk processing (50).

Paper industry

Another significant application of proteases alone and in combination with lipase is paper manufacturing. These enzymes not only offer large scale production of paper from hydrolytic methods but also useful in recycling of used paper (51). Paper is made of up of cellulose routinely produced by plant by hydrolyzing their pulp and spreading along with appropriate shaping and sizing. Paper industry had grown exponentially and lead to crisis due to limitation of plant resource hence significant of protease and other enzyme became more vital in recycling paper for commercial demand (52). In the process of paper making protease play crucial role in removing protein part from plant tissue prior to subject for lipase treatment. Here, use of lipase is essential and protease is complimentary in paper making. The protease used in paper making is not limited to removal of protein content of plant tissue but also utilized for production of some useful product as secondary products (53).

Chicken industry

The demand for meat is growing rapidly and world production of poultry has been increasing steadily since the last five decades as prime food items (54). The chicken industry is one of major source of food supplying chain in modern world and had grown exponentially. The chicken processing involves removal of feather and other non-edible protein content by enzymatic mechanism at different temperatures (55). Further use of protease is not restricted to the chicken processing it also became an integral part of poultry feed for growing chickens. The use of protease as poultry feed not only offer high nutritional value but also environmental friendly. The different proteases are added to feed with the purpose of increasing dietary protein hydrolysis and thus enabling improved nitrogen utilization. The most important were the benefits related to reduce emissions of ammonia using protease as poultry feed, which help reduce both health risks and environmental impacts, such as acidification and eutrophication (56).

The vast quantities of chickens are being utilized every day in the society that produces a large amount of feathers waste in poultry industries. So far, feathers are known to have been chemically and physically prepared to be used as feather meal as well as digestible nutritional protein for

animal feed (57). Because of the complex, rigid and fibrous structure of keratin, poultry feather is a challenge to anaerobic digestion. It's poorly degradable under anaerobic conditions. However, application of appropriate pretreatments methods hydrolyzes feather and breaks down its tough structure to corresponding amino acids and small peptides (58). The use of biological catalyst i.e. enzyme is another alternate for biodegradation of feathers. The proteases from microbial world are competent to produce keratinase proteases which have keratinolytic activity and are capable to keratinolyse feather α- keratin (59). These microbial based keratinase proteases help the bacteria to obtain carbon, sulfur and energy for their growth and maintenance from the degradation of α - keratin. Several keratinases from different microorganisms such as *Bacillus sp. Bacillus licheniformis Burkholderia*, *Chryseobacterium*, *Pseudomonas*, *Microbacterium sp*., *Chryseobacterium sp., Streptomyces sp.* has been isolated and studied to date.

Detergent industry

The commercial use of proteases as detergent for household and industrial application have recognize and implemented in eighties' (60). Microbial proteases are classified into acidic, neutral, or alkaline groups, depends on the required conditions for their activity and on the characteristics of the active site group of the enzyme, i.e. metallo, aspartic, cysteine- or sulphydryl- or serine-type. The alkaline proteases which are active in a neutral to alkaline pH, especially serine-types, are the most important group of enzymes used in protein hydrolysis, waste treatment and many other industrial applications (61). The alkaline protease from *Bacillus subtilis* was used as main ingredient in household and industrial detergent. The most recognized protease as detergent is Subtilisins extracellular alkaline serine proteases, which offers catalysis of proteins and peptide amides. Further, a series of research finding lead to development of several other enzymes as detergent such as Savinase is one of these enzymes; Alcalase, Esperase and Maxatase are others (62).

To meet the commercial demand several microbial strain were explored as source of these potent enzyme and majority of these enzymes were produced from single microbial genus is Bacillus. The commercially available enzyme detergents are Maxatase and Alcalase come from *Bacillus licheniformis*, Esperase from an alkalophilic strain of a *Bacillus licheniformis*, and Savinase from an alkalophilic strain of *Bacillus amyloliquefaciens (63)*. The use of enzyme as detergent is beneficial in many aspects, one highly selective to substrate and required in small quantities. Further, chemical based detergents are main cause of environmental pollution and also associated with several negative effects on biological surface. The use of enzyme as detergent does not offers any negative impact on environment and are completely safe (64). Hence, unlike conventional

chemical based detergents there is no buildup of unrecovered enzymes or chemicals that must be removed from the system at the end of process. Although, biological detergent is a promising technology; it has some limitations and disadvantages, as well. Currently, the main disadvantage of using alkaline proteases is the high cost of the enzymes production. The immobilization of such potent molecules for repeated use surely cut down cost factor (65).

Enzyme	Category	Source
Subtilisins	Serine protease	*Bacillus subtilis*
Streptopain	Cysteine protease	*Streptococcus sp.*
Papain	cysteine protease	*Papaya sp.*
Asclepain	Serine protease	*Asclepias curassavica*
Clostripain	Alkaline protease	*clostridium histolyticum*
Alcalase	Alkaline protease	*Bacillus licheniformis*
Esperase	Alkaline protease	*Bacillus lentus*

Table 1. List of few different protease from biological origin for various industrial applications.

These proteases are approved by USFDA for commercial application and are in sale in different brand name.

Therapeutics applications

Therapeutic application of protease

Apart from industrial applications protease are essential for many vital functions and regulate physiology at the molecular level. The physiological importance of these proteases has turned it into therapeutic potential and many of the proteases (serine protease) have been approved for clinical application (66). The proteases have been employed in the management of vascular disorders, cancer management, as antimicrobial, cytotoxic, anti-inflammatory, antioxidant (67).

Serine protease as Thrombolytics

The serine proteases have been explored in the last few decades for their therapeutic potential and clinically approved for management of vascular disorders. The vascular disorders, both cardiac and cerebral are a major cause of death and physical deformities worldwide (68). The vascular disorders often caused by lack of blood supply to vital organs and primarily due to blockage in vascular pipeline. The blood coagulation is a complex process governed by several plasma proteins and inflammatory mediator to avoid loss of blood and plasma component during any damage or injury (69). The blood clot inside the vascular pipeline is a sign of failure of the blood coagulation system and results in myocardial infarction, thromboembolism, cerebral ischemia and pulmonary embolism (70). The counter these life threatening event another component of blood plasma get activated and lead to clot dissolution under healthy homeostasis. The plasminogen another plasma protein gets activated into plasmin and lead to clot dissolution (71, 72).

Enzyme	Category	Molecular Mechanism	Source
Tissue plasminogen activator (t-PA)	Serine protease	activating circulating plasminogen (inactive form) into plasmin (active)	Human plasma
Urokinase like plasminogen activator (u-PA)	Serine protease	activating circulating plasminogen (inactive) into plasmin (active)	Human Urine
Streptokinase (SK)	Serine protease	activating circulating plasminogen (inactive) into plasmin (active)	Streptococcus species
Staphylokinase (SAK)	Serine protease	activating circulating plasminogen (inactive) into plasmin (active)	Staphylococcus species
Earthworm fibrinolytic enzyme (EFE)	Serine protease	activating circulating plasminogen (inactive) into plasmin and direct on fibrin	Earthworm Species (*Lumbricus rubellus)*

Table- 2; The serine proteases extensively used as thrombolytic from different sources. The most of these serine proteases are approved by US FDA and are plasminogen activators.

Under pathological conditions and failure of coagulation system need infusion of external agent for activation of plasminogen into plasmin and further clot dissolution. These agents called as thrombolytics are primarily plasminogen activators (73). Interestingly, thrombolytics approved for clinical applications are serine protease form different sources and dissolved blood clots by activating plasminogen into plasmin. The first ever serine protease was used for management of vascular disorder was tissue plasminogen activator from human plasma a serine protease (74). Further, urokinase and microbial based thrombolytics such as streptokinase and staphylokinase are in clinical application with high precision and accuracy (75). Recently, earthworm fibrinolytic enzyme (EFE) has been explored for its therapeutic potential for vascular disorders and again a serine protease of molecular weight range 20-33kDa (76).

Serine protease as anticancer

Cancer/ tumors are the second largest cause of human death and physical disability worldwide after vascular disorders. The emergence of drug dependence and use of cytotoxic drug in the combating advance tumor lead to the problem even worse. Hence, there was need of alternate medicine/ novel drug molecule for management of such health devastating elements (77). Recently, several serine proteases have been identified and characterized for the cytotoxic and antitumor properties (78). The most of protease exhibited cytotoxic activity and antitumor activity are serine protease. In a study 1987, the bacterial protease was evaluated for cancer management in mice using intravenous administration for solid tumors (79). Several proteases from different

sources were isolated, including plant, animal and microbial origin for antitumor activity. In a study 2013, protease inhibitor was examined for antitumor activity and an anti-proliferative activity of *Lavatera cashmeriana* was reported towards Human Cancer Cells (80). Several plant based protease has shown tremendous scope in combating cancer.

A protease inhibitor from Mini-Black Soybean was analyzed and was reported for excellent antitumor and HIV-1 Reverse Transcriptase Inhibitory activity (81). Further, in 2012 a protease component from *Eleusine coracana* has shown tremendous scope in combating tumor evaluated on Human Chronic Myeloid Leukemia Cells. Further, earthworm protease was reported for excellent cytotoxic and antitumor activity (82). In a study 2013, a serine protease was purified and characterized for antitumor activity in different cancer cell lines against standard anticancer drugs. The protein purification, study and MTT assay revealed that a 15kDa serine protease from Indian earthworm *Pheretima posthuma* was able to inhibit cancer cell growth by maximum 70% in against tamoxifen and 5` Fluoro Uracil (83). Further, in 2007 a protease inhibitor component from buckwheat was studied for its antitumor potential. In this study a trypsin protease inhibitor has shown significant inhibition activity on myeloid leukemia K562 cell lines (84).

Serine protease as Anti-inflammatory

The inflammation is one most common biological response for any infection and tissue damage. The inflammation ranges from acute to chronic and may be lethal if not cured properly (85). The available treatments for inflammation are either use of Non-Steroidal anti-inflammatory drugs NSAIDs for acute cases of inflammation and or steroidal drugs along in combination with NSAIDs for chronic cases. The uses of the protease from different sources were examined for anti-inflammatory activities in last two decades. The most significant result was reported in the case of serratiopeptidase a protease from enterobacterium Serratia Sp. E-15 was able to combat inflammation, both acute and chronic (86). This was a breakthrough in finding novel therapeutics for combating inflammation, which was awaited to overcome the toxic effects caused by chemical based drugs. Further, the earthworm protease and plant extracts were evaluated for anti-inflammatory activity. Several species of earthworms and plant extract possessing protease/short peptides has shown specific inhibition of cyclooxygenase pathway (87). The use of protease for combating inflammation was much safe and specific as they are required in very little quantity and are highly specific in finding their substrate. The preliminary investigation suggested that the earthworm protease has shown its specificity to COX-I and COX-II and negligible affinity for lipoxygenase pathway (88).

Serine protease as Antimicrobial

The antimicrobial peptides (AMPs) are basically small molecular weight proteins with broad spectrum antimicrobial activity against bacteria, viruses, and fungi (89). The commercial demand of antimicrobial agents has been increased exponentially in the last two decades and different sources have been explored in isolation of such amazing biomolecules (90). These peptides possess a different molecular mechanism to kill the microbial population and which is due to different arrangements of amino acids in peptide backbone. More likely peptides/proteins having proteolytic activity are much significant for commercial application as antimicrobial peptides (91). The serine proteases one of a class of protease group have been studied in plant, animal and microbial world for exploring their capacity to kill microbes (92). In a study 2011, a protease inhibitor from *Coccinia grandis* plant leaves has shown tremendous potential in inhibiting microbial growth in a broader spectrum (93). These antimicrobial peptides are integral part of innate immunity and significantly found in lower animals, including nematodes and microbial world (94). The earthworm is enriched in such compounds and many studies have been carried out to investigate these amazing molecules. In the year 2012, Indian earthworm *Pheretima posthuma* was explored for an antimicrobial protease possess a broad spectrum of antimicrobial activity (95). The amphibian such as frog and toads and many reptiles are also being potential source of antimicrobial peptides. Mellitin strong AMPs from bee efficiently target both gram positive and gram negative pathogens. Further, the arthropods, the insect family produce several cationic and anionic AMPs (97). Magainin, an AMP from frog not only offers a broad range of microbial inhibition but also target at the genetic level against many viruses (98).

Antimicrobial Peptides	Source of AMP	Molecular Mechanism
Magainin	Frog	Permeabilizes bacterial membrane
A & β-Defensin	Mammals, analogues in insects and fungi	Many are strongly salt antagonized; cell membrane and intracellular targets, inhibits macromolecular synthesis
Mellitin	Bee	Membrane destabilizing
Polyphemusin	Horseshoe crab	Very potent, translocate into cells
Indolicidin	Bovine	Inhibits macromolecular synthesis, Ca2++ - calmodulin interaction
Cecropin A	Silk moth	Membrane destabilizing
Buforin II	Toad	Binding of nucleic acid

Table 3 The list of natural antimicrobial peptides from biological origin and their molecular mechanism

Apart from these therapeutic applications several other proteases have been employed in the treatment of life threatening diseases and disorders. The most important finding was HIV 1 protease target gene expression in HIV virus a potential medicine for AIDS (99). The protease is available for commercial application in the name of Saquinavir (Roche), ritonavir (Abbott), indinavir (Merck), nelfinavir (Pfizer/Agouron), amprenavir (Vertex/Glaxo Wellcome) FDA approved for therapeutic use (100). The Proteasome another important protease enzyme is commercially available to combat cancer more precisely lung, colon and breast cancer. From the recent investigations Hepatitis C virus NS3 protease has shown a significant scope in combating hepatitis, which is under clinical trial studies and very soon will be available for clinical application (101). Further, the caspases and ICE (caspase-1) are more useful in case of sepsis and autoimmune disorders like Rheumatoid arthritis, osteoarthritis (102). The angiotensin converting enzyme (ACE) and neutral endopeptidase are ideal for the maintaining blood pressure and minimizing risk of vascular disorders (103). The presenilin-1 β-secretase BACE and matrix metalloproteases (MMPs) will be future medicine for neurological disorders like Parkinson and Alzheimer disease (104). The proteases have shown their role in designing drug and gene delivery vehicles for cancer therapeutics and gene therapy (105).

New Generation proteases

There are several industrial processes and therapeutic application need a slight different environment for operation. These hearse operating conditions lead to loss of enzymatic activity of conventional protease and other enzymes too (106). The developments of thermal and chemical resistant enzymes are huge in demand and thermostable protease has a great scope in therapeutics and industry (107). Similarly, many industrial operations often carried out a lower temperature and need an enzyme operate at lower temperatures. More precisely, food processing and soft drink formulation need enzymes to work at lower temperature even at preservation conditions (108). The conventional proteases often recover from mesophiles biological sources are not ideal choice for such industrial and therapeutic applications and need alternate to fulfill need (109).

Thermostable protease

The optimal activity of conventional protease ranges 30-40^{0}C which is not appropriate for much industrial operation including leather and textile industry (110). The thermostable proteases are protease enzyme isolated from thermophilic microorganism of optimal activity in the range of 40-130^{0}C (111). The thermophilic microbes have evolved for such hearse condition and their protein have evolved to offer enzymatic activity in higher temperature. The most important invention in case of thermophilic enzyme was taq polymerase completely changes molecular biology (112).

The taq polymerase was isolated from a thermophilic bacterial *Thermus aquaticus* in 1965 by Thomas D. Brock became integral part of polymerase chain reaction. Thermostable proteases are the choice for many industrial processes and are free from inhibitory effect of various chemical and inhibitors (113).

The microbial world is one prime source of such amazing enzyme and has been explore in last few decades. The bacteria, fungi and several animals have been reported for ideal source of thermostable proteases. Among these source bacterial source are more commonly employed for purification and production by recombinant DNA technology (114). The one bacterial group bacillus alone possesses more than 70% of protease enzyme and thermophilic protease. Subtilisin is one classical example of thermostable protease commercially available for detergent industry both larger scale and house hold application (115). Several fungal strains have been identified and many thermostable proteases have been isolated and purified for commercial application (116). The thermostable proteases not only offer their enzymatic activity at higher temperature but also offer ease in storage for long time period. The immobilizations of an enzyme often reduce enzyme activity due to chemical treatment or interaction with immobilization support in case of conventional enzyme (117). The thermostable proteases are ideal for immobilization study leas to repeated use without loss of enzymatic activity (118).

Psychrophilic proteases

Apart from mesophilic and thermophilic proteases, the psychrophilic microorganisms have the largest distribution on earth and if one considers the extent of area where temperature remains permanently below 10°C including the deep-sea waters, mountains and Polar Regions (119). The climate of lower temperature essentially requires physiological and biochemical adaptations of microorganisms. These psychrophilic microorganism have evolved in conditions where an optimal temperature of 10°C or lower remains throughout their life period (120). To survive in such hearse condition organisms have evolved with cold adaptive proteins enables physiological functioning at lower temperatures. Further, in order for growth to occur in low temperature environments, cellular components from membranes and transport systems to intracellular solutes, protein and nucleic acids must adapt to the cold (121). The psychrophilic organisms are competent to produce proteins/enzymes that can function in cold environment and simply denature at high temperature. There proteins/enzymes undergo the conformational changes necessary for catalysis with a lower energy demand (122).

These organism and proteins/enzymes are having great potentials for food processing industry and much therapeutics where lower temperature is essential (123). Several research investigation

have been carried out to explored the potential of proteins and enzymes from such sources The psychrophilic microorganisms are potential source of industrial enzymes (eg. lipase and protease) and fundamental metabolic (eg. glycolytic pathway) (124). The prime application of psychrophilic proteases is in food industry where these enzyme process several biochemical reaction at lower temperature and offer enzymatic reaction even storage conditions enhances self-life of products (125). The extracellular lipases and proteases represent two of the most important groups of extracellular hydrolytic enzymes. Apart from proteases physchrophiles are enriched in glyceraldehyde 3-phosphate dehydrogenase (GAPDH), triose phosphate isomerase (TPI) and phosphofructokinase (PFK) (126).

SUMMARY

In current scenario it's difficult to imagine an industrial operations and therapeutics without enzymes. Several types of enzymes have been identified, characterized in last few decades and are being produced in large scale for commercial need. These amazing enzymes are not offering ease in process catalysis result in a higher yield with limited resources but also are environmental friendly (127). Several industries which have been in controversy for their pattern of operation leading to environmental pollution have been completely refined in clean and green industry. The classical example is leather processing and textile industry (silk and synthetic fabric processing) have been associated with used of massive amount of chemicals and environmental pollution (128). Use of enzyme in such operation results in good quality product and does not have any negative impact on environment. Similarly, detergent industry, paper industry and chicken industries have acknowledged potential of protease and opted for higher yield. Additionally, thermostable protease and psychrophilic protease lead to industrial revolution one step ahead by offering enzymatic catalysis in extreme higher and lower temperature and in presence of various chemicals (129). Apart from industrial applications, proteases have been used enormously as therapeutics. The most significant contribution of protease as therapeutics is external thrombolytics for management of vascular disorders including both cardiovascular and cerebrovascular (130). The tissue plasminogen activators (t-PA) and urokinase like plasminogen activators (u-PA) are serine protease from human plasma and urine respectively. Additionally, microbial based thrombolytics including streptokinase (SK), staphylokinase (SAK) and their recombinant variants are also serine protease have changed thrombolytic therapy (131).

In last decade, another external thrombolytic from earthworm origin again a group of serine protease have shown its potential for the management of vascular disorders. These proteases were also implemented for management of inflammation and earthworm serine protease and

serratiopeptidase are in clinical application from long time (132). The serine protease as anti-inflammatory agent offer selective inhibition of COX I and COX II pathways leading to minimal side effects often reported with NSAIDs and steroids conventionally used for various types of inflammation managements (133). The antimicrobial property of protease is a breakthrough to develop biological peptides and protease for various commercial applications. Several proteases from animal, plant and microbial origin have been studied for their antimicrobial potential. However, role of protease in management of cancer and tumor is most highlighted area of current research work worldwide (134). The protease from different sources have been evaluated for anti-tumor and anticancer propertied and have shown excellent results in protection against cancer. The protease from animal origin such as earthworm protease and proteases from other representatives of Annelida are most effective anticancer agent from animal origin (135). The development of antiviral drugs against adeno and retrovirus are becoming more common now days. HIV 1 protease is one classical example for developing drugs against HIV and other retroviruses (136). Still lots of refinements are required for developing more efficient protease for industrial and therapeutic applications. Moreover, more emphasis is needed in novel classes of proteases such as thermostable and psychrophilic protease to refine industrial processes and therapeutics for mankind (137).

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REFERENCES

1. Anwar A., and Saleemuddin M., “alkaline proteases: a review”, Bioresour Technol 1998; Vol. 64:175–183.
2. Mala B.R., Aparn M.T,, Mohini S.G., and Vasanti V. D., “Molecular and biotechnological aspects of microbial proteases.” Microbiology and Molecular Biology Reviews 1998; Vol. 62(3): 597-635.
3. Rao M. B., Tanksale A. M., Ghatge M. S., and Deshpande V.V., “Molecular and biotechnological aspects of microbial proteases”. Microbiol Mol Biol Rev 1998; Vol. 62:597–635.
4. Craik C. S., Page M. J., Madison E. L., “Proteases as therapeutics”, Biochem J. 2011 Vol. 1; 435(1):1-16. doi: 10.1042/BJ20100965.
5. Li Q., Yi L., Marek P., Iverson B. L., “Commercial proteases: present and future”, FEBS Lett. 2013; 17;587(8):1155-63. doi: 10.1016/j.febslet.2012.12.019.

6. Schallmey M., Singh A., and Ward O. P., "Developments in the use of Bacillus species for Industrial production". Can. J. Microbiol., 2004; Vol. 1; 1-17.
7. Ahuja S. K., Ferreira G. M., and Moreira A. R., "Utilization of enzymes for environmental applications". Crit Rev Biotechnol. 2004; Vol. 24(2-3):125-54.
8. Falch E. A., "Industrial enzymes--developments in production and application". Biotechnol Adv. 1991; Vol. 9(4):643-58.
9. Alane Beatriz Vermelho, Claudiu T. Supuran, and Jose M. Guisan "Microbial Enzyme: Applications in Industry and in Bioremediation", Enzyme Res. 2012; 980681. doi: 10.1155/2012/980681.
10. Van Beilen J. B., Li Z., "Enzyme technology: an overview". Curr Opin Biotechnol. 2002 Vol. 13(4):338-44.
11. Prashant T Sanatan, Purushottam R Lomate, Ashok P Giri, and Vandana K., "Characterization of a chemostable serine alkaline protease from Periplaneta Americana", BMC Biochem. 2013; Vol. 14: 32. doi: 10.1186/1471-2091-14-32.
12. Carlos López-Otín and Judith S. Bond, "Proteases: Multifunctional Enzymes in Life and Disease- Minireviews" J. Biol. Chem. 2008, Vol. 283:30433-30437. doi: 10.1074/jbc.R800035200.
13. Barett, A. J., "Proteolytic enzymes: serine and cysteine peptidases". Methods Enzymol. 1994; Vol. 244:1–15.
14. Barett, A. J., "Proteolytic enzymes: aspartic and metallopeptidases". Methods Enzymol. 1995; Vol. 248:183.
15. Dancer, B. N., and Mandelstam J., "Production and possible function of serine protease during sporulation of *Bacillus subtilis*. J. Bacteriol. 1975; Vol. 121: 406–410.
16. Gupta R., Beg Q. K., and Lorenz P., "Bacterial alkaline proteases: molecular approaches and industrial applications, Appl Microbiol Biotechnol, 2002 Vol. 59:15–32.
17. Jaeger K. E., Eggert T., Eipper A., and Reetz M. T., "Directed evolution and the creation of enantioselective biocatalysis". Appl Microbiol Biotechnol 2001; Vol. 55:519–530.
18. Jang J.S., Kang D, O., Chun M. J., Byun S. M., "Molecular cloning of a subtilisin J from *Bacillus stearothermophilus* and its expression in *Bacillus subtilis*". Biochem Biophys Res Comm 1992: Vol. 184:277–282.
19. Godfrey, T., and West S., "Industrial enzymology, 2nd ed., p. 3. 1996, Macmillan Publishers Inc., New York, N.Y.
20. Keil, B**.,** **"**Specificity of proteolysis". Springer-Verlag KG, Berlin, Germany, 1992.

21. Nelson G., Young T. W., "Extracellular acid and alkaline proteases from *Candida olea*". J Gen Microbiol. 1987 133(6):1461-9.
22. Yamada T., Ogrydziak D. M., "Extracellular acid proteases produced by *Saccharomycopsis lipolytica"*. J Bacteriol. 1983; 154(1):23-31.
23. Hedstrom L., "Serine protease mechanism and specificity. Chem. Rev. 2002: Vol. 102: 4501–4524.
24. Dodson G., and Wlodawer A., "Catalytic triads and their relatives". Trends Biochem. 1998; Vol. 23: 347–352.
25. Czapinska H. and Otlewski J. (1999) "Structural and energetic determinants of the S1-site specificity in serine proteases. Eur. J. Biochem. 260: 571–595
26. Hess G. P. "Chymotrypsin. Chemical properties and catalysis", The Enzymes, 1971; Vol. 3 (3), pp. 213–248, Boyer P. D. (ed.), Academic Press, New York.
27. Teaster T., Baird, J., William D., and Charles S. Craik "Conversion of trypsin to a functional threonine protease", Protein Sci. 2006; 15(6): 1229–1238. doi: 10.1110/ps.062179006.
28. Hedstrom L., Trypsin: a case study in the structural determinants of enzyme specificity, Biol Chem. 1996; 377(7-8):465-70.
29. Ishisaka R., Kanno T., Akiyama J., Yoshioka T., Utsumi K., Utsumi T., "Activation of caspase-3 by lysosomal cysteine proteases and its role in 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH)-induced apoptosis in HL-60 cells". J. Biochem. 2001; Vol. 129(1):35-41.
30. Ishisaka R., Utsumi K., Utsumi T., "Involvement of lysosomal cysteine proteases in hydrogen peroxide-induced apoptosis in HL-60 cells". Biosci Biotechnol Biochem. 2002; 66(9):1865-72.
31. Daisuke Kato, Kelly M Boatright, Alicia B Berger, Tamim Nazif, Galia Blum, Ciara Ryan, Kareem A H Chehade, Guy S Salvesen & Matthew Bogyo, Activity-based probes that target diverse cysteine protease families, Nature Chemical Biology, 2005; Vol. 1, 33-38 doi:10.1038/nchembio707.
32. Polgár L. "The mechanism of action of aspartic proteases involves 'push-pull' catalysis". FEBS Lett. 1987; 13; 219 (1):1-4.
33. Wu H., Downs D, Ghosh K, Ghosh AK, Staib P, Monod M, Tang J. "Candida albicans secreted aspartic proteases 4-6 induce apoptosis of epithelial cells by a novel Trojan horse mechanism", FASEB J. 2013; Vol. 27(6):2132-44. doi: 10.1096/fj.12-214353.

34. Naglik J. R., Challacombe S. J., Hube B., "Candida albicans secreted aspartyl proteinases in virulence and pathogenesis". Microbiol Mol. Biol. Rev. 2003; 67(3):400-428.
35. Glazer, A.G and Nikaido, H., "Microbial, Biotechnology: Fundamental of applied Microbiology", Freeman and Company, Washington: 1995; 256-259
36. Outrup, H., and COL Boye, "Microbial proteinases and biotechnology. In Fogarty, W.M. (ed), Microbial enzymes and biotechnology, 2nd Ed, London and New York, 1990, Elsevier Science Publisher.
37. Zucker S., Cao J., Chen W. T., "Critical appraisal of the use of matrix metalloproteinase inhibitors in cancer treatment", Oncogene. 2000; Vol. 27(56):6642-50.
38. Anwar, A., and Saleemuddin, M., "Alkaline proteases- A Review", Bioresource Technology, 1998, Vol. 6, (3), pp. 175-183.
39. Anwar, A. and Saleemuddin, M., "Alkaline protease from Spilosoma obliqua: potential applications in bioformulation". Biotechnology and Applied Biochemistry, 2000, Vol. 31(2), p. 85-89.
40. Qing Liu, Shujing Sun, Meizi Piao, and Ji Young Yang, "Purification and Characterization of a Protease Produced by a Planomicrobium sp. L-2 from Gut of Octopus vulgaris", Prev Nutr Food Sci. 2013; 18(4): 273–279. doi: 10.3746/pnf.2013.18.4.273.
41. Dayanandana, A., Kanagarajb, J., Lesley Sounderraja, Govindarajua, R., Suseela Rajkumara, G.,"Application of an alkaline protease in leather processing: an ecofriendly approach", Journal of Cleaner Production, 2003; Vol 11(5), Pages 533–536.
42. Kamini, N.R., Hemachander, C., Mala, J.G.S. and Puvanakrishnan, R. "Microbial enzyme technology as an alternative to conventional chemicals in leather industry. Curr.Sci.1999; Vol. 77:80-86.
43. Peper, K.W. and Wyatt, K G., "Enzymatic unhairing of heavy hides. J. Ind. Leather Technol. Asso, 1989; 36: 214-233.
44. Godfrey, T., Leather, In Industrial Enzymology, 2nd Ed., Edited by Godfrey, T., and S. West, Stocton Press, New York, 1996; pp. 285-291.
45. Gupta, R., Gupta, K., Sexena, R., Khan, S., "Bleach-stable, alkaline protease from Bacillus sp." Biotechnol. Lett. 1999; Vol. 21, 135–138.
46. Sutar I I, Vartak H G, Srinivasan M C & Siva Raman H, "Production of alkaline protease by immobilized mycelium of Conidiobolus", Enzyme Microb Technol, 1986; Vol. 8: 632-634.

47. Laxman R S, Sonawane A P, More S V, Rao B S, Rele M V, Jogdand V V, Deshpande V V & Rao M B "Optimization and scale up of production of alkaline protease from Conidiobolus coronatus", Proc Biochem, 2005; Vol. 40: 3152–3158.
48. Zheng, L., Du, Y., and Zhang J., "Degumming of ramie fibers by alkalophilic bacteria and their polysaccharide-degrading enzymes," Bioresource Technology, 2001; Vol. 78(1), pp. 89–94.
49. Bruhlmann, F., Leupin M., Erismann, K. H., and Fiechter, A., "Enzymatic degumming of ramie bast fibers," Journal of Biotechnology, 2000; Vol. 76(1), pp. 43–50.
50. Xiao L., Wang G. X., and Chen G, J., "The research advancement of the enzymatic degumming of ramie (*Boehmeria nivea*): a review," The Journal of Microbiology, 2004; Vol. 31(5), pp. 101–105, 2004.
51. Bajpai P., "Application of enzymes in the pulp and paper industry", Biotechnol Prog. 1999; Vol. 15(2):147-157.
52. Gutiérrez A, Del Río J C, Martínez A.T., "Microbial and enzymatic control of pitch in the pulp and paper industry". Appl. Microbiol Biotechnol. 2009; Vol. 82(6):1005-18. doi: 10.1007/s00253-009-1905-z.
53. Karn S. K., Kumar P, Pan X., "Extraction of lipase and protease and characterization of activated sludge from pulp and paper industry". Prep Biochem Biotechnol. 2013; Vol. 43(2):152-62. doi: 10.1080/10826068.2012.712589.
54. Oxenboll K. M., Pontoppidan K., and Fru-Nji F., "Use of a Protease in Poultry Feed Offers Promising Environmental Benefits", International Journal of Poultry Science, 2011; Vol. 10 (11): 842-848.
55. Kim J.M., Lim W. J., and Suh H. J., "Feather-degrading Bacillus species from poultry waste" Process Biochemistry, 2001; Vol. 37, 287–291.
56. Willams CM, Shih JCH., "Enumeration of some microbial groups in thermophilic poultry waste digesters and enrichment of a feather-degrading culture." J. Appl Bacteriol, 1989; Vol. 67:25–35.
57. Shama G, Berwick PG., "Production of keratinolytic enzymes in a rotating frame bioreactor." Biotechnol Tech, 1991; Vol. 5:359–362.
58. Sangali S, Brandelli A., "Isolation and characterization of a novel feather-degrading bacterial strain". Appl Biochem Biotechnol, 2000; Vol. 87:17–24.

59. Saurabh, S., Jasmine, I., Pritesh, G. and Rajendra Kumar, S. "Enhanced productivity of serine alkaline protease by Bacillus sp. using soybean as substrate", Malaysian Journal of Microbiology, 2007; Vol 3(1), pp. 1-6.
60. Rathindra Mohan Banika, Monika Prakashb, Laundry detergent compatibility of the alkaline protease from *Bacillus cereus* Microbiological Research, 2004;Vol. 159(2), 30 June 2004, Pages 135–140.
61. Banik R M, Prakash M., "Laundry detergent compatibility of the alkaline protease from Bacillus cereus". Microbiol Res. 2004; Vol. 159 (2):135-140.
62. Saeki K, Ozaki K, Kobayashi T, Ito S. "Detergent alkaline proteases: enzymatic properties, genes, and crystal structures". J. Biosci Bioeng. 2007; Vol. 103(6):501-508.
63. Ito S, Kobayashi T, Ara K, Ozaki K, Kawai S, Hatada Y. "Alkaline detergent enzymes from alkaliphiles: enzymatic properties, genetics, and structures", Extremophiles. 1998; Vol. 2(3):185-190.
64. Ito S. "Alkaline cellulases from alkaliphilic Bacillus: enzymatic properties, genetics, and application to detergents". Extremophiles. 1997; Vol. 1(2):61-66.
65. Ward, O.P "Proteolytic enzymes"M Moo-Young (Ed.), Comprehensive Biotechnology, Vol. 3Pergamon, Oxford (1985), pp. 789–818.
66. Maurer H. R., Bromelain: biochemistry, pharmacology and medical use. Cell Mol Life Sci. 2001; 58 (9):1234-45.
67. Grabs V, Nieman D. C., Haller B, Halle M, Scherr J. "The effects of oral hydrolytic enzymes and flavonoids on inflammatory markers and coagulation after marathon running: study protocol for a randomized, double-blind, placebo-controlled trial". BMC Sports Sci Med Rehabil. 2014; 22; 6(1):8. doi: 10.1186/2052-1847-6-8.
68. Mahendra K. Verma, Yogendra K. Verma, "Conventional thrombolytics need to refine at molecular level for safe and efficient management of cerebrovascular disorders- an overview"Int J Pharm Pharm Sci, 2013; Vol 5(l), pp 448-454.
69. Rijken DC, Lijnen HR. "New insights into the molecular mechanisms of the fibrinolytic system". J Thromb Haemost. 2009; Vol. 7(1):4–13.
70. Mahendra Kumar Verma, KK Pulicherla, "Lumbrokinase – A Potent and Stable Fibrin–Specific Plasminogen Activator" International Journal of Bio-Science and Bio-Technology, 2011; Vol. 3(2), pp 57-69.
71. Ambrus, C. M., Back, N., and Ambrus, J. L. "On the mechanism of thrombolysis by plasmin", Circ. Res., 1962; Vol. 10:161- 165.

72. Rouf S A, Moo-Young M, Chisti Y. "Tissue-type plasminogen activator: characteristics, applications and production technology".Biotechnol Adv. 1996; Vol. 14:239–266.
73. Nicholl S M, Roztocil E, Davies M G. "Plasminogen activator system and vascular disease", Curr Vasc Pharmacol. 2006; Vol. 4(2):101-116.
74. Collen D, Lijnen HR. "Tissue-type plasminogen activator: a historical perspective and personal account. J Thromb Haemost. 2004; 2(4):541-546.
75. Peng Y, Yang X, Zhang Y. "Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity in vivo". Appl Microbiol Biotechnol. 2005 Vol. 69(2):126-132.
76. Hu Y, Meng XL, Xu JP, Lu W, Wang J. "Cloning and expression of earthworm fibrinolytic enzyme PM(246) in Pichia pastoris". Protein Expr Purif. 2005; Vol. 43(1):18-25.
77. Kim R, Inoue H, Tanabe K, Toge T. "Effect of inhibitors of cysteine and serine proteases in anticancer drug-induced apoptosis in gastric cancer cells". Int J Oncol. 2001; Vol. 18(6):1227-1232.
78. Jedinak A, Maliar T. "Inhibitors of proteases as anticancer drugs". Neoplasma. 2005; Vol. 52(3):185-92.
79. Shimizu T, Pommier Y. "Camptothecin-induced apoptosis in p53-null human leukemia HL60 cells and their isolated nuclei: effects of the protease inhibitors Z-VAD-fmk and dichloroisocoumarin suggest an involvement of both caspases and serine proteases". Leukemia. 1997; Vol. 11(8):1238-1244.
80. Rakashanda S, Qazi A. K, Majeed R, Rafiq S, Dar I M, Masood A, Hamid A, Amin S. "Antiproliferative activity of Lavatera cashmeriana- protease inhibitors towards human cancer cells". Asian Pac J Cancer Prev. 2013; Vol. 14(6):3975-8.
81. Patick A.K., and K. E. Potts K. E., "Protease Inhibitors as Antiviral Agents", Clin Microbiol Rev.1998; Vol. 11(4): 614–627.
82. Saxena L, Iyer BK, Ananthanarayan L. "Purification of a bifunctional amylase/protease inhibitor from ragi (Eleusine coracana) by chromatography and its use as an affinity ligand. J Chromatogr B" Analyt Technol Biomed Life Sci. 2010 Vol. 1;878(19):1549-54. doi: 10.1016/j.jchromb.2010.04.009.
83. Mahendra Kumar Verma, Francies Xavier, Yogendra Kumar Verma, and Kota Sobha, Evaluation of cytotoxic and anti-tumor activity of partially purified serine protease isolate from the Indian earthworm Pheretima posthuma, Asian Pac J Trop Biomed. 2013; Vol. 3(11): 896–901. doi: 10.1016/S2221-1691(13)60175-6.

84. Park SS, Ohba H. "Suppressive activity of protease inhibitors from buckwheat seeds against human T-acute lymphoblastic leukemia cell lines". Appl Biochem Biotechnol. 2004; Vol. 117(2):65-74.
85. Tasaka K, Meshi T, Akagi M, Kakimoto M, Saito R, Okada I, Maki K. "Anti-inflammatory activity of a proteolytic enzyme, Prozime-10". Pharmacology. 1980; Vol. 21(1):43-52.
86. Shilpa P. Jadav, Nilesh H. Patel, Tarang G. Shah, Maganlal V. Gajera, Hiren R. Trivedi, and Bharat K. Shah, "Comparison of anti-inflammatory activity of serratiopeptidase and diclofenac in albino rats", J Pharmacol Pharmacother. 2010 Jul-Dec; 1(2): 116–117. doi: 10.4103/0976-500X.72362.
87. Mahendra Kumar Verma and Kota Sobha, "Antioxidant and Anti-Inflammatory Properties of Autolysed Extract of the Indian Earthworm *Pheretima posthuma* after Preliminary Purification - An *In Vitro* Study", RJPBCS 2013; Vol 4(4) pp 888-898.
88. Benedek B, Kopp B, Melzig MF. Achillea Millefolium L."Is the anti-inflammatory activity mediated by protease inhibition?" J Ethnopharmacol. 2007; Vol. 5; 113(2):312-317.
89. Izadpanah A, Gallo R L. "Antimicrobial peptides", J Am Acad Dermatol. 2005; Vol. 52(3):381-390.
90. Havard Jenssen, Pamela Hamill, and Robert E. W. Hancock, "Peptide Antimicrobial Agents", Clin Microbiol Rev. 2006; Vol. 19(3): 491–511. doi: 10.1128/CMR.00056-05.
91. Pinheiro da Silva F, Machado M C., "Antimicrobial peptides: clinical relevance and therapeutic implications". Peptides. 2012; Vol. 36(2):308-314. doi: 10.1016/j.peptides.2012.05.014.
92. Sitaram N, Nagaraj R. "The therapeutic potential of host-defense antimicrobial peptides". Curr Drug Targets. 2002; Vol. 3(3):259-267.
93. Satheesh L S, Murugan K. "Antimicrobial activity of protease inhibitor from leaves of Coccinia grandis (L.) Voigt". Indian J Exp Biol. 2011; Vol. 49(5):366-374.
94. Gallo RL, Murakami M, Ohtake T, Zaiou M., "Biology and clinical relevance of naturally occurring antimicrobial peptides". J Allergy Clin Immunol. 2002; Vol. 110(6):823-831.
95. Yogendra Kumar Verma, Mahendra Kumar Verma, "Earthworm- A Potential Source For Stable And Potent Antimicrobial Compounds- Isolation And Purification Study", International Journal of Pharmacy and Pharmaceutical Sciences, 2012; Vol. 4(4):540-543.
96. Adade CM, Oliveira IR, Pais JA, Souto-Padrón T., "Melittin peptide kills Trypanosoma cruzi parasites by inducing different cell death pathways". Toxicon. 2013; Vol. 69:227-39. doi: 10.1016/j.toxicon.2013.03.011.

97. Wei Chen, Liaofu Luo, "Classification of antimicrobial peptide using diversity measure with quadratic discriminant analysis", Journal of Microbiological Methods, 2009; Vol. 78(1), pp 94–96.
98. Almeida KC, Lima TB, Motta DO, Silva ON, Magalhães BS, Dias SC, Franco OL. "Investigating specific bacterial resistance to AMPs by using a magainin I-resistant Escherichia coli model". J Antibiot (Tokyo). 2014; 7. doi: 10.1038/ja.2014.48.
99. Debouck C. "The HIV-1 protease as a therapeutic target for AIDS". AIDS Res Hum Retroviruses. 1992; Vol. 8(2):153-164.
100. Urs Lüthi, Proteolytic Enzymes as Therapeutic Targets", EBR; ESBAtech, 2002: 1-5.
101. Ferenci P, Reddy K R. "Impact of HCV protease-inhibitor-based triple therapy for chronic HCV genotype 1 infection". Antivir Ther. 2011; Vol. 16(8):1187-201. doi: 10.3851/IMP1934.
102. Nakano S, Ikata T, Kinoshita I, Kanematsu J, Yasuoka S. "Characteristics of the protease activity in synovial fluid from patients with rheumatoid arthritis and osteoarthritis". Clin Exp Rheumatol. 1999; Vol. 17(2):161-170.
103. Scott BB, McGeehan GM, Harrison RK. "Development of inhibitors of the aspartyl protease renin for the treatment of hypertension". Curr Protein Pept Sci. 2006; Vol. 7(3):241-254.
104. Mantle D, Falkous G, Ishiura S, Perry RH, Perry EK. "Comparison of cathepsin protease activities in brain tissue from normal cases and cases with Alzheimer's disease, Lewy body dementia, Parkinson's disease and Huntington's disease". J Neurol Sci. 1995; Vol. 131(1):65-70.
105. Mahendra Kumar Verma. *et al*. "Current prospects of nano-designs in gene delivery- aiming new high for efficient and targeted gene therapy" *International Journal of Biopharmaceutics.* 2013; 4(3): 145-165.
106. Jun Ogawa, Sakayu Shimizu, "Microbial enzymes: new industrial applications from traditional screening methods", Trends in Biotechnology, 1999; Vol. 17, pp. 13–20.
107. Ogawa J, Shimizu S. "Industrial microbial enzymes: their discovery by screening and use in large-scale production of useful chemicals in Japan". Curr Opin Biotechnol. 2002; Vol. 13(4):367-375.
108. Periasamy Anbu, Subash C. B. Gopinath, Arzu Coleri Cihan, and Bidur Prasad Chaulagain "Microbial Enzymes and Their Applications in Industries and Medicine", Biomed Res Int. 2013; 204014, doi: 10.1155/2013/204014.
109. Underkofler, L. A., Barton, R.R. and Rennert, S.S., "Production of Microbial Enzymes and Their Applications", Appl Microbiol. 1958; Vol. 6(3): pp 212–221.

110. Vida Akhtar Kazemi, Parvin Nahid, Soheila Yaghmaei, Mohammad Amin Sabzevari, "Alkaline protease production by immobilized cells using *Bacillus licheniformis"*, Scientia Iranica, 2013; Vol. 20,(3), pp. 607–610.
111. Chenel J P, Tyagi RD, Surampalli RY. "Production of thermostable protease enzyme in wastewater sludge using thermophilic bacterial strains isolated from sludge". Water Sci Technol. 2008; Vol. 57(5):639-645. doi: 10.2166/wst.2008.004.
112. Nedumpully Govindan P, Monticelli L, Salonen E. "Mechanism of taq DNA polymerase inhibition by fullerene derivatives: insight from computer simulations". J Phys Chem B. 2012; Vol. 6; 116(35):10676-83. doi: 10.1021/jp3046577.
113. Ohta Y, Ogura Y, Wada A. "Thermostable protease from thermophilic bacteria-Thermostability, physiocochemical properties, and amino acid composition". J Biol Chem. 1966; Vol. 25; 241(24):pp 5919-5925.
114. Latiffi AA, Salleh AB, Rahman RN, Oslan SN, Basri M. "Secretory expression of thermostable alkaline protease from Bacillus stearothermophilus FI by using native signal peptide and α-factor secretion signal in Pichia pastoris". Genes Genet Syst. 2013; Vol. 88(2):85-91.
115. Jayakumar R, Jayashree S, Annapurna B, Seshadri S. "Characterization of thermostable serine alkaline protease from an alkaliphilic strain Bacillus pumilus MCAS8 and its applications". Appl Biochem Biotechnol. 2012; Vol. 168(7):1849-1866. doi: 10.1007/s12010-012-9902-6.
116. Li AN, Ding AY, Chen J, Liu SA, Zhang M, Li DC. "Purification and characterization of two thermostable proteases from the thermophilic fungus Chaetomium thermophilum", J Microbiol Biotechnol. 2007; Vol. 17(4):624-631.
117. Wilson SA, Young O A, Coolbear T, Daniel R M. "The use of proteases from extreme thermophiles for meat tenderization". Meat Sci. 1992; Vol. 32(1):93-103. doi: 10.1016/0309-1740(92)90019-Z.
118. Cowan D A, Daniel RM. "The properties of immobilized caldolysin, a thermostable protease from an extreme thermophile". Biotechnol Bioeng. 1982; Vol. 24(9):2053-2061.
119. Villeret, V., Chessa, J. P., Gerday, C., and Van Beeumen, "Preliminary crystal structure determination of the alkaline protease from the Antarctic psychrophile Pseudomonas aeruginosa", Protein Sci. Nov 1997; Vol. 6(11): 2462–2464. doi: 10.1002/pro.5560061121
120. Mikhailova AG, Likhareva VV, Khairullin RF, Lubenets NL, Rumsh LD, Demidyuk IV, Kostrov SV, "Psychrophilic trypsin-type protease from Serratia proteamaculans", Biochemistry (Mosc). 2006; Vol. 71(5):563-570.

121. Martinez R, Schwaneberg U, Roccatano D., "Temperature effects on structure and dynamics of the psychrophilic protease subtilisin S41 and its thermostable mutants in solution", Protein Eng Des Sel. 2011; Vol. 24(7):533-44. doi: 10.1093/protein/gzr014.
122. Zeng R, Zhang R, Zhao J, Lin N. "Cold-active serine alkaline protease from the psychrophilic bacterium Pseudomonas strain DY-A: enzyme purification and characterization". Extremophiles. 2003; Vol. 7(4):335-7.
123. Aghajari N, Van Petegem F, Villeret V, Chessa JP, Gerday C, Haser R, Van Beeumen J. "Crystal structures of a psychrophilic metalloprotease reveal new insights into catalysis by cold-adapted proteases. Proteins. 2003; Vol. 50(4):636-647.
124. Zhang SC, Sun M, Li T, Wang QH, Hao JH, Han Y, Hu XJ, Zhou M, Lin SX. "Structure analysis of a new psychrophilic marine protease", PLoS One. 2011; Vol., 6(11): e26939. doi: 10.1371/journal.pone.0026939.
125. Susana C Vazqueza, Silvia H Coriab, Walter P Mac Cormack, "Extracellular proteases from eight psychrotolerant antarctic strains", Microbiological Research, 2004; Vol. 159(2), Pages 157–166.
126. Adams M, Perler F B, Kelly RM, "Extremozymes: Expanding the limits of biocatalysis. Biotechnology 1995; Vol. 13: pp 662-668.
127. Salleh, A.B., Basri, M., Razak, C., "The effect of temperature on the protease from Bacillus stearothermophilus strain F1. Mal. J. Biochem. Mol. Biol 1997; Vol. 2, pp 37–41.
128. Banerjee, V., Saani, K., Azmi, W., Soni, R.,"Thermostable alkaline protease from Bacillus brevis and its characterization as a laundry additive. Proc. Biochem. 1999 Vol. 35, pp 213–219.
129. Chaloupka J. "Temperature as a factor regulating the synthesis of microbial enzymes". Microbiol Sci 1985; 2:86–90.
130. Coughlin SR. "Protease-activated receptors in hemostasis, thrombosis and vascular biology". J Thromb Haemost. 2005;Vol. 3(8):1800-1814.
131. Coughlin SR. "Protease-activated receptors in vascular biology". Thromb Haemost. 2001; Vol. 86(1):298-307.
132. Rajic A, Kweifio-Okai G, Macrides T, Sandeman RM, Chandler DS, Polya GM. Inhibition of serine proteases by anti-inflammatory triterpenoids. Planta Med. 2000; Vol. 66(3):206-210.
133. Shigetomi H, Onogi A, Kajiwara H, Yoshida S, Furukawa N, Haruta S, Tanase Y, Kanayama S, Noguchi T, Yamada Y, Oi H, Kobayashi H. "Anti-inflammatory actions of serine protease

inhibitors containing the Kunitz domain". Inflamm Res. 2010; Vol. 59(9):679-87. doi: 10.1007/s00011-010-0205-5.

134. Roessner A, Krüger S, Kido A. "Cellular proteases and invasion", Verh Dtsch Ges Pathol. 2000; Vol. 84: 69-76.
135. Jedinak A, Maliar T. "Inhibitors of proteases as anticancer drugs". Neoplasma. 2005;Vol. 52(3):pp 185-192.
136. Nakazawa H, Tsuneishi E, Ponnuvel KM, Furukawa S, Asaoka A, Tanaka H, Ishibashi J, Yamakawa M., "Antiviral activity of a serine protease from the digestive juice of Bombyx mori larvae against nucleopolyhedrovirus", Virology. 2004; Vol. 30; 321(1):154-162.
137. Qing Li, Li Yi, Peter Marek, Brent L. "Commercial proteases: Present and future", FEBS Letters, 2013; Vol. 587(8), pp 1155–1163.