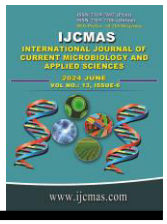
Int.J.Curr.Microbiol.App.Sci (2024) *13*(06): 1-23

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 13 Number 6 (2024)**
Journal homepage: http://www.ijcmas.com



Review Article https://doi.org/10.20546/ijcmas.2024.1306.001

Microbial Proteases: Sources, Significance and Industrial Applications

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Keywords

Bioremediation, classification, enzymes

Article Info

Received:
20 April 2024
Accepted:
22 May 2024
Available Online:
10 June 2024

A B S T R A C T

Microbial enzymes are the preferred source to plants or animals for enzyme integration into biotechnological processes that enhanced sustainable development due to its cost- effective, ease of operation, re-use advantages and consistent production. Enzymes are biocatalysts, they accelerate a chemical reaction. They are used in industries such as biofuel, cleaning/detergents, food, pharmaceuticals, textiles, bioremediation and many more. The present review attempts to provide descriptive information on the recent development in enzyme technology for industrial applications as well as sustainable development.

Introduction

Enzymes

Enzymes are biological catalysts that are proteins in nature and help to speed up the rate of metabolism and chemical reactions and can be found in all living organisms. In chemistry, enzymes have emerged as preferred tools and are being used increasingly in industrial processes due to their capacity to perform reactions with high specificity and efficiency (Nigam, 2013; Kumar and Sharma, 2016; Rekik *et al.,* 2019).

Among over 3000 enzymes that have been identified, only about 5% are exploited industrially (Robinson, 2015). The industrial application of enzymes has substantially reduced the demands for energy in many industries and the wastes generated from the application of enzymes in industries are biodegradable and non-toxic wastes that are friendly to the environment. Also, the use of industrial enzymes is more cost-effective and the possibility of integration of genetically engineered microbes to produce more stable and improved enzymes at an industrial scale can be achieved (Gurung *et al.,* 2013).

The rise in the demand for industrial enzymes is largely attributed to the increasing demand for enzymes as an alternative for both traditional and synthetic chemicals in many industrial processes because of the eco-friendly nature of enzymes application in industries unlike the use of chemicals which generates so much greenhouse gases.

The use of enzymes as an alternative for chemicals in industrial processes prevents the release of approximately 700 million kg of CO_2 into the atmosphere per year. Consequently, the industrial enzymes market has been growing steadily for the past 6 decades from a net worth of about USD 0.31 billion in 1960 to a net worth of USD 6 billion in 2020 (Figure 1) (Robinson, 2015).

Proteases

A protease is an enzyme that catalyzes proteolysis, breaking down proteins into smaller polypeptides or single amino acids, and spurring the formation of new protein products. They do this by cleaving the peptide bonds within proteins by hydrolysis, a reaction where water breaks bonds. Proteases are an important group of industrial enzymes and reported for almost 60% of the whole enzyme marketplace and also account for 40% of the entire global enzyme sale (Muthulakshmi *et al.,* 2011; Wahab and Ahmed, 2017; Sharma *et al.,* 2019). Proteases consist of not only a single enzyme but also contain a consortium of enzymes that includes (proteinases, peptidases, and amidases). Proteinases are involved in the hydrolysis of whole protein molecules releasing peptones and amino acids. Peptidases cause peptones hydrolysis and liberate amino acids, whereas the amidases catalyze the hydrolysis of amino acids and release ammonia (Munawar *et al.,* 2014; Maitig *et al.,* 2018). Proteases have a molecular weight of 18 to 90 kDa (Muthulakshmi *et al.,* 2011). Different types of proteases have different properties like catalytic activity, the specificity of the substrate, pH, temperature, active site-specificity, and stability profiles (Kumar and Vats, 2010).

Proteases are fundamental industrial enzymes having wide applications in chemical and biochemical processes because they can break peptide bonds present in proteins (Rohan, 2014). Proteases are present among all living organisms and vital for the process of cell differentiation, the period of growth, food proteins digestion, cell division, protein turnover, and cascades involved in blood clotting, apoptosis, signal transduction, and numerous infection-causing organisms' life-cycle including the retroviruses replication (Muthulakshmi *et al.,* 2011; Souza *et al.,* 2015). It is interesting to know that protease is the largest family of enzymes that accounts for 2% of the human genome.

Due to structural and functional diversity, proteases have a range of intracellular protein recycling as well as a cascade of nutrient digestion and amplification of the immune system (Dettmer *et al.,* 2013). Proteases contribute 60% of the total enzyme market because of their numerous applications in many industries like in detergent industry, leather industry, food industry, processing of meat, cheese making, paper and pulp, recovery of silver from photographic films, and bioremediation processes. These enzymes can also be used as a therapeutic treatment for inflammation and harmful lesions. Enzymes used in different industries like detergent, textile, and paper and pulp encompass the leading segment through 52% of market stock among the leading share of the enzymes market place is retained by alkaline proteases (Muthulakshmi *et al.,* 2011; Rohan, 2014; Fazilat, 2016; Wahab *et al.,* 2017).

Currently, the worth of the universal sale of industrialized enzymes has been estimated above 3 billion US$ (Sawant and Nagendran, 2014). The worldwide protease enzyme market is growing at a compound annual growth rate (CAGR) of 6.1% by 2024. The overall protease enzyme market income is expected to be valued as 3 billion US Dollars by 2024 (Proteases Regional Outlook, 2020). Now a day, most of the enzymes used in industries are hydrolytic and used for the degradation of different natural materials. Because of the wide use in dairy and detergent industries, proteases remain the dominant type of enzyme (Kirk *et al.,* 2002). Moreover, they are eco-friendly and typically tend to decrease the level of toxicity in the atmosphere after their usage in industries (Jegannathan and Nelsen, 2013).

Proteases constitute the largest product segment in the global industrial market for enzymes because they are extensively used in detergent and food industries (Acrofan, 2021; FOC Group, 2022). Additionally, with the development of science and technology, the use of protease enzymes in several bioremediation processes and leather treatments is increasing (Research and Markets, 2021).

Moreover, protease enzymes are being extensively used in the production of medicines, as protease enzymes treat multiple diseases, such as lung, heart, eye, digestive tract, and skin ulcer diseases as well as soreness (Shrivastava *et al.,* 2019). Thus, the demand for protease enzymes should continue to increase in the future.

The main sources of proteases are animals (e.g., calf stomach), plants (e.g., pineapple, fig, and papaya), microbes (e.g., *Bacillus* spp., *Pseudomonas* spp.) (Jisha *et al.,* 2013; Sun *et al.,* 2016; Chitte and Chaphalkar, 2017).

The production of enzymes from animal and plant sources, however, has been limited due to ethical issues, environmental reasons, and low-efficiency production processes. Commercially, microbial enzymes are popular due to their scientific and economic advantages as well

as their broad biochemical diversity (Jisha *et al.,* 2013). Proteases are a universal entity that is found everywhere, namely, in plants, animals, and microbes. The peptide bond present in the polypeptide chain of amino acids is hydrolyzed by means of proteases (Barrett and McDonald, 1986). Proteases are degradative enzymes and show specificity and selectivity in protein modification. A large number of enzymes from bacteria, fungi and plants are involved in biodegradation of toxic organic pollutants. Bioremediation is a cost effective and nature friendly biotechnology that is powered by microbial enzymes (Karigar and Rao, 2011). The research activity in this area would contribute towards developing advanced bioprocess technology to reduce the toxicity of the pollutants and also to obtain novel useful substances. In the industrial sector, *Bacillus* sp. is the most active and dynamic extracellular alkaline protease producer. Of the three largest groups of industrial enzymes, proteases are one of them, and their global market is drastically increasing annually. Of the 60% of enzymes marketed worldwide, proteases account for 20% (Rao *et al.,* 2009; Singhal *et al.,* 2012). Proteases are an integral component of existing life on earth, such as animals, plants, and microbes. By a process of fermentation, proteases can be isolated and purified in a relatively shorter period of time, exhibiting high substrate specificity and catalytic activity (Kumar and Takagi, 1999; Rifaat *et al.,* 2007; Singhal *et al.,* 2012). It is estimated that proteases account for 1–5% of the genome of infectious organisms and 2% of the human genome. According to researchers, proteases control the activation, synthesis, and turnover of proteins to regulate physiological processes (Rawlings *et al.,* 2004). Different physiological processes, such as formation, birth, aging, and even death is regulated by proteases (Chou *et al.,* 1997, 2003; Chou and Howe, 2002; Chou, 2006). Proteases are vital in the imitation and spread of infectious diseases, and because of their significant role in the life cycle, they are imperative for drug discovery. In more than 50 human proteases, a single amino acid mutation may lead to a hereditary disease. Proteases are involved in normal and pathophysiological processes or conditions. This involvement of proteases may lead them to produce a therapeutic agent against deadly diseases, such as cancer and AIDS (Rawlings *et al.,* 2004).

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

As per the Enzyme Commission classification, proteases are placed in the class 3(hydrolases), sub-class 4 with each proteolytic enzyme assigned unique number as EC 3.4.x.x (Contesini *et al.,* 2018). These enzymes have been categorized on the basis of various parameters like the site of action, the type of substrate, active pH range, mechanism of action involving particular amino acid present in the active site (Guleria *et al.,* 2014).

Depending on the site of action, broadly these enzymes can be classified as endopeptidase and exopeptidase. The former tends to hydrolyze non-terminal peptide bonds, leading to formation of shorter peptides, while the latter act on peptide bonds located at the termini of the substrate. Also, depending on the termini on which a particular exopeptidase acts preferentially, exopeptidases are further categorized into aminopeptidases or carboxypeptidases depending on whether they act on the N-terminal or C-terminal respectively (Naveed *et al.,* 2021). The exopeptidases release dipeptides or tripeptides or amino acids and correspondingly shortened peptides. Another approach of protease categorization is based on the presence of specific amino acid residue(s) in the active site and mechanism of action. Based on this approach, the major classes of protease are serine proteases, cysteine protease, threonine proteases, glutamic proteases, asparagine's proteases, aspartic proteases, mixed proteases, etc. (Contesini *et al.,* 2018).

The enzymes are categorized on the primarily on the basis of their phylogenetic relationships and mechanism of action in this database. Similarly, the proteolytic enzymes have also been sorted into alkaline proteases, acidic proteases and neutral proteases on the basis of their optimal pH range (Tavano *et al.,* 2018).

The proteases play a very significant role in the physiology and metabolism of all living organisms. Apart from the obvious role in the digestion of proteins and peptides, these enzymes also play significant role in the regulation of a vast array of physiological processes by controlling various stages involved in the protein synthesis, protein activation–inactivation, signaling,

protein turnover as well as gene expression (Bond, 2019).

Protease and protease inhibitors account for more than 2% of the human genes. Not only do the proteases play important role in the normal functioning of the body and maintenance of homeostasis, but also these have significant role in infections, immunity, inflammation and disease development (Patel *et al.,* 2018). For example, various common characteristic features of cancer like uncontrolled growth, angiogenesis, metastasis, immune evasion have been associated with various aberrant protease activities. On the other hand, proteases have also been found to play crucial role in tumor suppression (Dudani *et al.,* 2018). Therefore, proteases are being utilized for prognostic, diagnostic as well as therapeutic purpose in the Feld of cancer management (Dudani *et al.,* 2018). Owing to the crucial role of proteases in the life cycle of all the organisms including the infectious agents, the proteolytic enzymes offer lucrative avenue for drug discovery as is evident in case of protease inhibitors like ritonavir which is widely used for controlling Human Immunodeficiency Virus (HIV) infections (Tigabu *et al.,* 2020).

Recently, Jo *et al.,* (2020) screened a flavonoid library for assessing the potential antiviral activities of the flavonoids which inhibited the 3C-like protease of coronaviruses and reported promising results in case of Herbacetin, rhoifolin and pectolinarin. Apart from this, there a many other applications of proteases in the Feld of medicine like control of cardiovascular diseases, digestive disorders, inflammatory diseases as well as promotion of tissue repair in cases of burns, fractures, accidental or surgical trauma, etc. (Kumar and Jain 2018; Bond, 2019). Hence, both proteases and protease inhibitors play a crucial role in the Feld of medicine (Agbowuro *et al.,* 2018).

The proteases also find a very significant role in various industries. The classical example of industrial application of proteases is for cleaning purposes like detergent additive, contact lens cleaning solution component, etc (Salwan and Sharma, 2019; Lam *et al.,* 2018).

The textile industry makes use of proteases for a variety of purposes ranging from degumming of silk, biopolishing of wool in a very much sustainable and eco-friendly approach (Chatha *et al.,* 2017; Mamo and Assefa, 2018). Similarly, leather industry is also reducing the utilization of chemicals and laying more and more emphasis on the application of proteolytic enzymes for carrying out different steps of leather processing (Fang *et al.,* 2017). A lot of keratinous wastes are generated by poultries, slaughter houses, etc., which are difficult to manage and cause a lot of problems like soil pollution, water pollution, aesthetic problems, clogging of drains, spreading of diseases, etc (Kamarudin *et al.,* 2017). Another proteinaceous waste of concern is collagen, which is generated by fish processing industries, sea-food processing industries as well as slaughter houses. Improper disposal of such wastes also poses a great pollution threat apart from the direct health risk to humans and animals (due to possible transmission of pathogenic microbes).

Proteases, especially the keratinases and collagenases are also playing pivotal role in the arena of waste management and pollution control by breaking down these problematic components (Bhagwat and Dange, 2018; Razzaq *et al.,* 2019; Yusuf *et al.,* 2019). The keratinases (proteases, which are capable of degrading keratin) have been used for degradation of keratin in the waste materials, thus helping in the waste management. Also, the keratinase treated residues may be used as animal feeds and nitrogenous fertilizers (Kumawat *et al.,* 2018). Collagenase can be used for extraction of collagen from the fish and animal carcasses, which in turn will not only lead to lower waste generation, but also help in reclaiming collagen (Pal and Suresh, 2016). The collagen can be used by various food, pharmaceutical and cosmetic industries.

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

Proteases represent a wide array of enzymes capable of acting on a variety of proteinaceous substrates. Not only do these enzymes carry out degradative activity, but also these are also capable of synthetic activity. Proteases have been utilized for synthesis of peptides for application in diverse fields like food industry, medicine, agriculture, etc Białkowska *et al.,* (2017) have specifically reviewed the application of proteases from thermophilic, halophilic and psychrophlic organisms for

peptide synthesis. Zanutto-Elgui *et al.,* (2018) reported production of milk peptides with antimicrobial and antioxidant properties with the help of fungal proteases from *Aspergillus oryzae* and *A. favipes.*

Sources of Proteases

In living organisms, proteases play a vital role so their production takes place from different sources like plants, animals, and microbes (Muthulakshmi *et al.,* 2011; Rohan, 2014). The proteases produced from different sources have been represented in Figure 2.

Plants

Plants are used as a source of proteases that depends upon numerous factors like the accessibility of land intended for cultivation and the suitability of climate circumstances for growth. Furthermore, the production of proteases is a time taking process from plants. Some of the well-known proteases such as papain, bromelain, and keratinases are produced from plant sources (Sawant and Nagendran, 2014). Papain is produced from Papaya fruit (*Carica papaya*). It has the properties of milk clotting and protein-digesting with a wide pH range. Bromelain is a plant protease that is obtained from the leaf, juice, stem, and peel of pineapples. It can efficiently control the growth of tumor cells (Chanallia *et al.,* 2011). Keratinases are produced from botanical groups of plants, which are used for dehairing. Wool and hair digestion are significant for the formation of crucial amino acid-like lysine used for the avoidance of blockage of sewage systems (Rao *et al.,* 2001). In Pakistan, cysteine protease has been purified and characterized by maize leaves.

Animals

Trypsin, chymotrypsin, pepsin, and rennin are the most well-known proteases, which are originated from animals. The digestive enzyme, trypsin is found in the intestine and responsible for the food protein hydrolysis. Chymotrypsin is found in the excretory products of the pancreas (animals). Purified chymotrypsin is a costly enzyme, and is used in the analytical and diagnostic applications. Protease (pepsin) such as rennet is produced as an inactive precursor in the stomach of all mammals. By the action of pepsin enzyme, it is converted into its activated form. In the dairy industry, it is used widely for the formation of stable curd with high-quality favor. Pepsin enzyme has an acidic nature that is present in the stomach of approximately all vertebrates. As early as 1913, the use of pepsin was only in detergents, which are now replaced using a combination of microbial proteases (metal) and serine, which become resistant to degradation through alkaline conditions, detergents and elevated temperatures (Adinarayana and Ellaiah, 2003).

Microbes

Proteases that are available nowadays in the market are obtained from microorganisms. This is due to several reasons such as high rate of production, the limited requirement of cultivation space, wide biochemical diversity, easy genetic manipulation, and attractive characteristics that make them appropriate for biotechnological applications (Singhal *et al.,* 2012; Tavano *et al.,* 2018). However, due to the incapability of animal and plant proteases to fulfil the recent demands of the world industrial sector, scientists found an alternative solution in the form of microbial sources (Fazilat, 2016). Microbial-derived proteases account for about 40% of entire global enzymes sale (Freitas *et al.,* 2011). Microorganisms are responsible for the production of both intracellular and extracellular proteases. Intracellular proteases are vital for numerous metabolic end-products of cellular processes like differentiation, turnover of protein, sporulation, processing of hormones and proteins, removal of unnecessary protein, while the extracellular proteases are important for the consumption and hydrolysis of proteinaceous nutrients. Extracellular proteases have a significant role and multiple applications in different industries.

Microbial proteases have wide ranging applications in several fields, including baking, brewing, detergents, leather making, pharmaceuticals, meat tenderizing, cosmetics, medical diagnosis and so on (Christensen *et al.,* 2022; Akram *et al.,* 2023). In addition, with the rapid development of new fields, applications of microbial proteases are expanding to new areas, such feed industries (Bernardeau *et al.,* 2022; Cupi *et al.,* 2022), hydrolysis applications to prepare active peptides (Christensen *et al.,* 2022), and environmental protection applications, such as waste treatment and reuse (Ariaeenejad *et al.,* 2022; Asitok *et al.,* 2022; Zhai *et al.,* 2022). The proteases available in the market are of microbial origin because of their high yield, less time consumption, less space requirement, lofty genetic manipulation, and cost-effectiveness, which have made them suitable for biotechnological application in the market (Nisha and Divakaran, 2014). These microbial

proteases are preferred to plant and animal proteases because of the presence of all desired characteristics for industrial applications (Palsaniya *et al.,* 2012; Sathishkumar *et al.,* 2015). Proteases are isolated from different microbes like fungus, bacteria, and yeast. These applications illustrate the diversity and importance of proteases. The applications of proteases and their respective microbial sources by examining acid protease, neutral protease and alkaline proteases and their classification were discussed and briefly summarized in Figure 3.

Fungal

Proteases isolated from fungus are the curiosity of researchers due to their wide range of substrate specificity, stability under the unfavorable conditions, high diversity, and mycelium separation by the process of simple filtration. Fungal proteases are used in the modification of food proteins (Veloorvalappil *et al.,* 2013). Proteases produced from fungal sources have superior advantages over the proteases produced from bacterial sources and they are generally recognized as genetically regard as safe (GRAS) strains (Freitas *et al.,* 2011). Protease producing fungi are *Aspergillus niger, Aspergillus clavatus ES1, Aspergillus flavus, Aspergillus fumigates, Aspergillus malleus, Aspergillus nidulans HA-10, Aspergillus sojae and sydomi, Aspergillus terreus, Aspergillus oryzae, Cephalosporium, Fusarium species, Rhizopus* and *Penicillium italicum* (Charles *et al.,* 2008; Oyeleke *et al.,* 2010; Novelli *et al.,* 2016; Sharma, 2019). Among these fungal strains, *Aspergillus* is the famous group for protease production and microbial strains such as *Myxococcus*, *Neurospora*, *Penicillium*, *Ophiostoma* and *Rhizopus* are also frequent protease producers (Sharma *et al.,* 2015).

Bacterial

Bacterial proteases, which are alkaline in nature, have economic significance in different industries such as leather, food, laundry, silk, and detergent industry due to their higher catalytic activities and production capacities. Some of the protease producing bacteria are *Bacillus subtilis* (Romsomsa *et al.,* 2010; Santos *et al.,* 2018). *Bacillus alkaloophilus, Bacillus amyloliquefaciens, Bacillus clausii, Bacillus halodurans, Bacillus lentus and Bacillus licheniformis*, *Bacillus pumilus* (Al-Qodah *et al.,* 2013), *Bacillus circulans* (Jaswal *et al.,* 2008), and *Bacillus safensis* (Rekik *et al.,* 2019).

Protease in Food Processing Industry

Proteases are enzymes which catalyze the hydrolysis of peptide bonds present in proteins and polypeptides. They are widely used in detergent and pharmaceutical, followed by food industries. They represent 60 % of industrial enzymes on the market (Singh *et al.,* 2016).

The global demand for protease enzyme market has been growing at a compound annual growth rate (CAGR) of 5.3 % during the period 2014-2019. Their demand is expected to increase much further as they find applications in leather processing as well as bioremediation processes. Proteases can be classified based on their origin, catalytic activity and nature of the reactive group in the catalytic site.

The major sources of protease enzymes are animals, plant and microorganisms (both bacterial and fungal). Proteases are divided into two groups: exopeptidases and endopeptidases, based on the site of action on polypeptide chains (Rao *et al.,* 1998). The exopeptidases act on the ends of polypeptide chains and endopeptidases act randomly in the inner regions of polypeptide chains. The endopeptidases are further classified into six groups, based on the catalytic residue present in the active site: serine, aspartic, cysteine, metallo, glutamic acid and threonine protease (Li *et al.,* 2013).

Plant proteases such as bromelain, ficin and papain are widely used in food industry for various applications such as brewing, tenderization of meat, coagulation of milk and as a digestive aid (Patel *et al.,* 2013).

In addition, proteases are also used to improve the flavour, nutritional value, solubility and digestibility of food proteins as well as to modify their functional properties including coagulation and emulsification (Aruna *et al.,* 2014). Proteases are widely used in baking industry for the production of bread, baked foods, crackers and waffles. These enzymes are used to reduce the mixing time, decrease dough consistency and uniformity, regulate the gluten strength in bread and to improve the texture and flavour (Miguel *et al.,* 2013). The acid protease from *Aspergillus usamii* has been successfully employed for the improvement of functional properties of wheat gluten. The addition of protease could release sufficient peptides and amino acid levels in the wort to get a proper fermentation. Acidic fungal proteases are used in improving fermentation of beer as they are efficient even at low pH by balancing the amino

acid profile of beer. Another major application of proteases is associated with dairy industry (Damhus *et al.,* 2013).

Naturally occurring proteases contribute significantly to the flavour characteristics of cheese. They are used for the acceleration of cheese ripening, to modify the functional properties and reduce the allergenic properties of milk products (Damhus *et al.,* 2013). In cheese making, proteases are also used to hydrolyze the specific peptide bond to generate paracasein and macropeptides.

During cheese production from milk, proteases are added to hydrolyze kappa casein to prevent coagulation by stabilizing micelle formation. In the baking industry, for quicker preparation of dough, its gluten is partially hydrolyzed by a heat-labile fungal protease because of its early inactivation in subsequent baking.

Protein hydrolysate preparation with high nutritional value has been accomplished by the addition of microbial alkaline proteases. The bioactive peptides play an important role in various pharmaceutical drug formations and as potential molecules under stressed environmental conditions (Figure 4).

This preparation of hydrolysate is vital in infant food formulation and fortification of soft drinks and juices (Ray, 2012; Singhal *et al.,* 2012; Mótyán *et al.,* 2013; Singh *et al.,* 2016). The mackerel hydrolysates helped in the hydrolysis of protein molecules into free amino acids including carosine, anserine, and other small peptides through the use of proteases.

The hydrolysis of proteins into amino acids caused the formation of antioxidants that inhibit autoxidation of linoleic acid and the scavenging effects for α, α-diphenyl-β-picrylhydrazyl free radicals (Wu *et al.,* 2003; Li *et al.,* 2008). It was found that the long peptides with 1,400 Da molecular weight were stronger antioxidants as compared with smaller peptides with molecular weights of 200 to 900 Da (Foegeding *et al.,* 2002; Tavano, 2013). It has been found that the formation of extensive protein hydrolysates through sequential actions of exoproteases and endopeptidases coupled with the release and development of the post-hydrolysis processes was considered as the most efficient way to produce protein hydrolysates that showed well-defined characteristics during protein hydrolysis (Chalamaiah *et al.,* 2012; Power *et al.,* 2013).

The bioactive peptide produced from the hydrolysis of various food proteins plays an important role as antioxidants in cell (Nalinanon *et al.,* 2011; Kittiphattanabawon *et al.,* 2012). The protein hydrolysates showed excellent solubility, because of which the antioxidant activities of protein hydroxylates were enhanced (Chi *et al.,* 2015).

The bioactive peptides show anticalmodulin, anticancer, and hypocholesterolemia properties, and there are also multifunctional properties of the food-protein-derived peptides (Phoenix *et al.,* 2012; Nicolia *et al.,* 2014; Nongonierma and Fitz Gerald, 2015; Agyei *et al.,* 2016). In the food industry, proteases are utilized for modification, palatability, and storage life of all available sources of proteins.

High nutritional value preparations of protein hydrolysates are achieved by the use of alkaline proteases. In meat tenderization, alkaline proteases of microbial origin are of immense importance (Sumantha *et al.,* 2006).

Protein Modification

Microbial proteases are used to modify proteins. Protease-limited enzymatic hydrolysis of soybean protein can improve its solubility, emulsification, foaming and digestibility. Hydrolysis of peanut protein concentrates with *Aspergillus oryzae* crude protease extract resulted in their higher water- and oil-binding capacity as well as improved solubility, foam stability, and foaming capacity. When soybean protein isolate (SPI) was treated with alkaline protease accompanied by high-speed shearing homogenization, it significantly improved the emulsion stability of the SPI hydrolysates. As a result, the foaming properties of SPI were improved significantly (Hao *et al.,* 2022).

Microbial Fermentation

Proteases can hydrolyze the protein substrate in the fermentation medium into small peptides, making it easier for microorganisms to quickly absorb and utilize these substrates, improving fermentation efficiency.

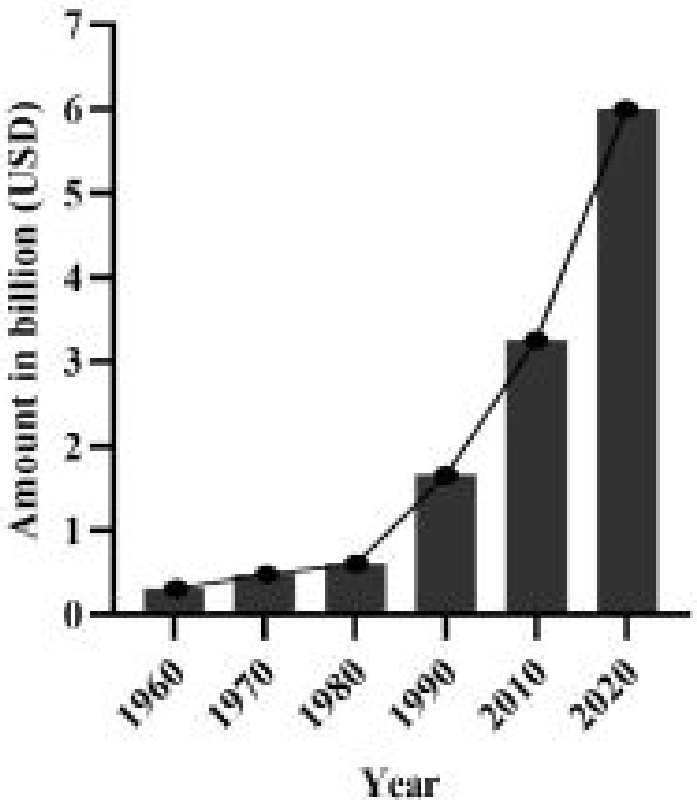
Other studies have found that during synergistic fermentation of bean dregs and soybean meal, adding multiple strains and protease promotes strain growth, organic acid secretion and amylase secretion and reduces sugar metabolism. Producing ethanol by microbial fermentation will cause hydrolysis by endogenous proteases and as a result will generate amino acids and peptides.

Table.1 A comparison among different types of proteases based on Optimal pH

Type of protease	pH range	Use of proteases	Sources	References
Alkaline	9 - 11	Detergent and leather industry	Mostly produced by bacterial species, such as *A. salinivibrio* sp. strain AF-2004, marine shipworms.	Miyaji *et al.,* 2006; Dodia *et al.,* 2008; Patil and Chaudhari, 2009; Soroor *et al.,* 2009; Simkhada *et al.,* 2010a; Vadlamani and Parcha, 2011
Acidic	3.8–5.6	Soy sauce, protein hydrolysate, digestive aids and in production of seasoning material, clearing beer and fruit juice.	Mostly produced by fungal species, such as *A. niger, A. oryzae, A. awamori*.	Sielecki *et al.,* 1991; Steele *et al.,* 1992; Zhang *et al.,* 2019; Pushpam *et al.,* 2011
Neutral	5–8	Food industry, brewing industry	Genus *Bacillus*	Sodek and Hofmann, 1970

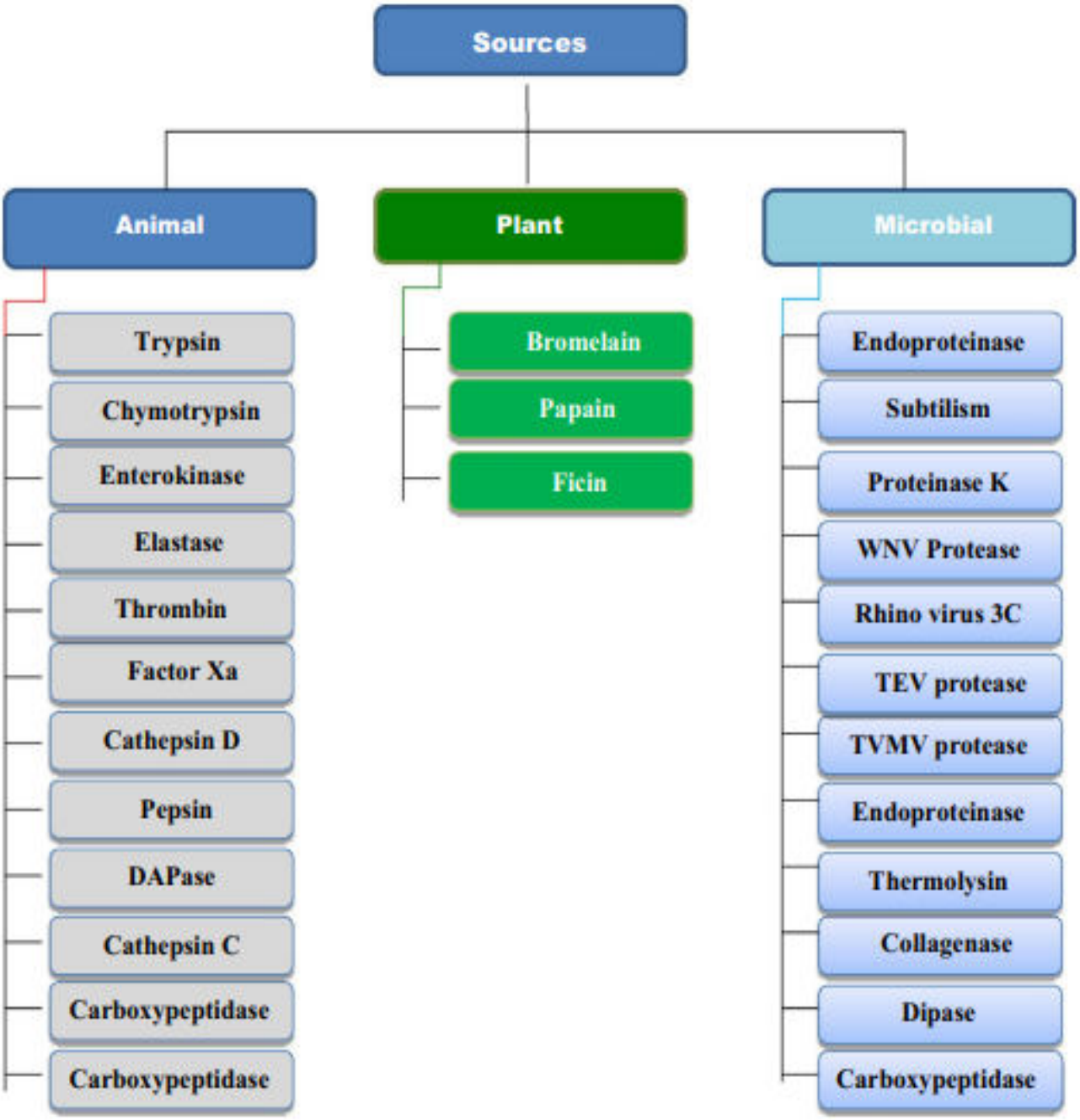
Source: (Razzaq *et al.,* 2019)

Figure.1 Graphical representation of the net worth of industrial enzymes market from 1960 to 2020.



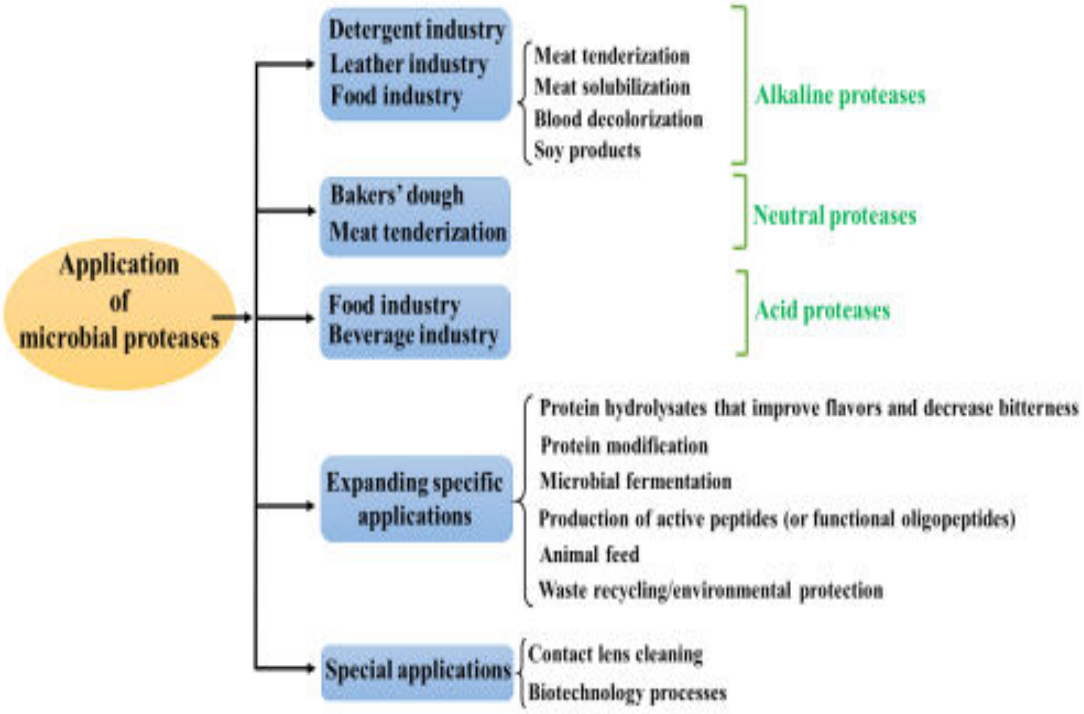
Source: (Robinson, 2015).

Figure.2 Proteases produced from different sources



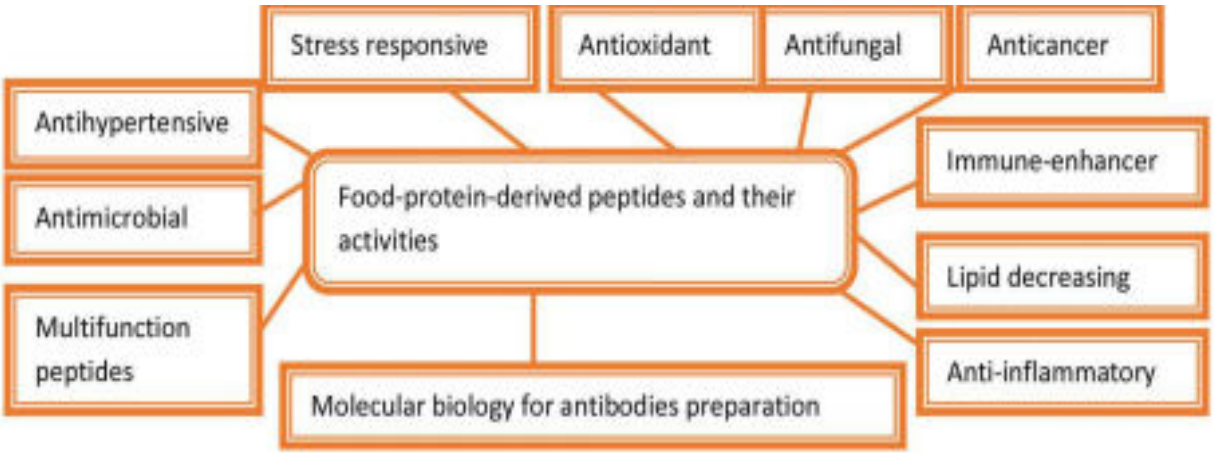
Source: (Muhammad, 2020)

Figure.3 The applications of microbial proteases



Source: (Song *et al.,* 2023)

Figure.4 Food protein-derived peptides and their roles



Source: (Razzaq *et al.,* 2019)

Amino acids and peptides can support the growth of microorganisms, which subsequently increases ethanol production. To improve ethanol yield and reduce fermentation time, exogenous proteases can be used to hydrolyze protein sources available in the raw materials in feedstock used for ethanol production (Heng *et al.,* 2022).

Animal Feed

Processing feed ingredients and applying exogenous proteases are the primary uses of exogenous proteases in animal feed. These proteases can be used to maintain high performance and reduce dietary protein levels.

Enzymatic hydrolysis is the best method when processing animal byproducts or plant-source feedstuffs.

Interesting activities from peptides from plant or animal sources include antihypertensive, antimicrobial, antioxidant, and immunomodulatory activities.

The environment also benefits from proteases by improving the utilization of protein materials and reducing nitrogen and ammonia excretions (Philipps-Wiemann, 2018; Hejdysz *et al.,* 2020).

Proteases are used in the following applications:

Livestock Feed

Adding *Bacillus licheniformis* to nursery diets that contain a low protein level can significantly improve nutrient digestibility, growth performance, and intestinal morphology of weaned pigs. Keratinolytic proteases can also use low-energy consumption to convert poultry feathers to a nutritionally upgraded protein-rich feedstuff for livestock from a potent pollutant.

Poultry Feed

Protease supplementation can improve the growth performance of broilers. Protease can be supplemented under low-protein conditions to achieve a breeding effect that is similar to a positive control (antibiotic). Proteases can alter the bacterial diversity in the cecum, which has a positive effect on broilers (Wang *et al.,* 2023).

Aqua feed

To improve the juiciness, flavor, tenderness, healthiness, and antioxidant capacity of grass carp meat, soy protein hydrolyzed by proteases has been added to a low-protein diet (Song *et al.,* 2020).

Beverage Industry

Acid proteases can degrade proteins in fruit juices and certain alcoholic beverages that cause turbidity, including black currant (Landbo *et al.,* 2006); cherry (Pinelo *et al.,* 2010); pomegranate (Cerreti *et al.,* 2017); and apple, orange, grape, and kiwi fruit juices. By adding acid proteases, the immediate turbidity is significantly reduced.

Adding proline-specific proteases from *Aspergillus niger* (Lopez and Edens, 2005) or *Aspergillus oryzae* (Kang *et al.,* 2014) when brewing beer can prevent chill-haze formation. This result indicates that proline-rich proteins perform hydrolysis due to a peptide fraction that cannot interact with polyphenols.

Protein haze is also a problem that occurs during the production of white wine. Early research has found that by using acid proteases in wine, protein haze formation can be reduced without damaging wine quality (Marangon *et al.,* 2012; Theron *et al.,* 2018). Apart from preventing protein haze, acid proteases also increase the α-amino nitrogen concentration necessary for microbial growth and generate better flavor during beer brewing (Lei *et al.,* 2013; Serna-Saldivar and Rubio-Flores, 2017).

Classification of Protease

The diversity and specificity of these indigenous enzymes owe their broad characterization. Proteases can be classified based on 2 factors:

1. Based on the active site of proteases, they are classified as exopeptidases and endopeptidases.
2. Based on Optimal pH in which they are active

Based on Active Site

Exopeptidases

Exopeptidases catalyze the splitting of distinctive protein peptide bonds adjacent to the carboxyl or amino terminals embodied in the substrate. Depending upon their precision regarding the site of action either C or N terminal, they are additionally characterized as two major classes carboxypeptidases or aminopeptidases (Sawant and Nagendran, 2014).

Aminopeptidases

Aminopeptidases are biased towards the free N-terminal of the polypeptide chain releasing a tripeptide, dipeptide, and single amino acid residue. After the recognition, they endeavor to remove the N-terminal methionine that might be present in proteins, which are heterologous in their expression but not present in many natural (mature) proteins.

Aminopeptidases occur in a wide range of microbial strains including fungi and bacteria. Overall, aminopeptidases behave as intracellular enzymes, however, one report highlighted that aminopeptidases originated from *Aspergillus oryzae* are extracellular enzymes (Rao *et al.,* 2001).

Carboxypeptidases

The carboxypeptidases exhibit their catalytic activities at a free C-terminal of the polymer of amino acids with a release of dipeptide and single amino acids. They are not primarily treated as endopeptidases because they leave the amino acid at a target protein. Rather, they can be labored for the removal of tags added at the C-terminal of the target protein. Amongst metallocarboxyprotease, type A carboxypeptidase primarily removes the branch or aromatic side chain comprising enzyme. While type, B is brought into action for basic amino acids (Motyan *et al.,* 2013).

Endopeptidases

Endopeptidases cleave the peptide bond at a distant site of the substrate (Sawant and Nagendran, 2014; Siroya *et al.,* 2020). Influenced by the presence of a particular functional group present on active site, endopeptidases are further cataloged as serine, aspartic, cysteine, and metalloproteases, which are summarized as follows.

Serine Proteases

Serine proteases enjoy wide diversity in nature occurring not only in the whole kingdom of cellular organisms but also present in many viral genomes. From the known proteolytic enzymes, one-third of them are serine proteases. These are generally endopeptidases in which the cleavage of the bond takes place in the central portion of the chain. Some of them are exopeptidases, which detach amino acids from the ending of the polypeptide sequence. Their name originates from the Ser residue, which is nucleophilic and resides at the active site of the enzyme. An acyl-enzyme intermediate is formed by the attack of the serine residue at the carbonyl end of the upcoming substrate peptide bond. Its nucleophilic activity is due to the triad complex of 'Asp', 'His' and 'Ser' residues (Page and Di, 2008; Thakur *et al.,* 2018).

Cysteine/Thiol Proteases

The active site of this class of proteases is comprised of cysteine residues. They have natural origin both in prokaryotic and eukaryotic organisms. Optimum pH for their proteolytic activity ranges from 6 to 8 and the optimum temperature is 50–70 °C. Hydrogen cyanide portrays the consequential role in the activation of this enzyme, owing to the regeneration of the SH group. Proteases can be inhibited by oxidizing agents and display sensitivity to the sulfhydryl agents e.g. p-CMB, however, they remain unchanged by metal-chelating agents (Muhammad, 2011).

Metalloproteases

Metalloproteases are mostly zinc-containing endopeptidases. Innumerable metal ions, for instance, cobalt, calcium, and zinc are involved in their reactivation, present in fungi. Most of the fungal and bacterial metalloproteases possess zinc containing enzymes. Zinc is required for their enzymatic activity and calcium is essential for the stability of protein structure. These enzymes have optimum pH ranging from 5 to 9, and are sensitive to metal chelating agents such as EDTA but are insensitive to cysteine inhibitors or sulfhydryl (Ellaiah *et al.,* 2002).

Aspartic Proteases

A comparatively nominal class of endopeptidases is aspartic proteases. Structurally, these proteases are bi-lobed having a central catalytic site, which is composed of a pair of aspartates. These proteases function on acidic pH and have shown their occurrence in animals, plants, and microorganisms.

A range of microbes secretes them as their virulence secretions that can also be mutualistic in breaking the proteins yielding the nitrogen in urea and thus illustrating the duality in their nature. Aspartic proteases are predominantly inclined towards the hydrophobic amino acids adjacent to the dipeptides bond (Vashishta *et al.,* 2007; Theron and Divol, 2014; Souza *et al.,* 2017).

The former two endoproteases employ residues residing in the active site having a nucleophilic attribute for proteolysis. While the latter two operate those residues that are significant in activating the molecules of water to undergo the nucleophilic attacks (Turk *et al.,* 2012). Some diverse proteases do not lie in the above-mentioned classification, for example, ATP-dependent proteases, which require ATP for their activation (Rao *et al.,* 2001).

Based on Active Optimal pH

Alkaline Proteases

The genus *Bacillus* is vital for commercially important alkaline protease (EC.3.4.21-24.99), which is active at alkaline pH ranging between 9 and 11 (Kocher and Mishra, 2009; Singhal *et al.,* 2012). These alkaline protease producers are distributed in water, soil, and highly alkaline conditions. From a variety of sources, such as detergent contamination, dried fish, sand soil, and slaughterhouses, segregation of alkaline proteases has been stated (Adinarayana *et al.,* 2003). The detergent industry consumes alkaline proteases most abundantly, which are serine proteases with an alkaline pH range (Gupta *et al.,* 2002). These alkaline serine proteases, which are easily inactivated by phenyl methane sulfonyl fluoride (PMSF), account for one-third of the share of the enzyme market (Page and Di Cera, 2008).

Alkaline proteases are unique in their activity and maintain a constant alkaline pH while being exploited for different formulations in pharmaceutical, food, and other related industries (Joo *et al.,* 2004; Dias *et al.,* 2008). A broad range of applications of these alkaline proteases are getting more attention from researchers with the hope of discovering new strains with unique properties and substantial activity. It is reported that for dehairing of animal skin and hides, *Bacillus* sp. provide the desired hydrolytic, elastolysis, and keratinolytic properties (Bhaskar *et al.,* 2007; Deng *et al.,* 2010; Shankar *et al.,* 2011). These *Bacillus* strains have the genus *Bacillus* is vital for commercially important alkaline protease (EC.3.4.21-24.99), which is active at alkaline pH ranging between 9 and 11 (Kocher and Mishra, 2009; Singhal *et al.,* 2012).

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Although alkaline proteases are produced by multiple sources (Ellaiah *et al.,* 2002; Prakasham *et al.,* 2005), with the increasing demand of protease in the market, and for cost-effectiveness, only those strains that show greater yield with hyperactivity will be accepted in the current biotechnological advancement (Kumar *et al.,* 2012).

Two essential types of alkaline proteases, such as subtilisin Carlsberg and subtilisin novo are obtained from *Bacillus* sp., which can be used as an industrial enzyme to produce zein hydrolysates (Miyaji *et al.,* 2006). In halophilic sources, different microbial sp. secreting serine alkaline proteases are also reported (Dodia *et al.,* 2008). The entomopathogenic bacterium *Photoradars* sp. strain EK1 (PhPrtPI) containing Cabvalkaline protease is categorized as a metalloprotease. Owing to its broad-spectrum specificity with different proteins and peptides, it is suggested that PhPrtPI provides nutrients to the nematodes by degradation of insect tissues (Soroor *et al.,* 2009). A Salinivibrio sp. strain, AF-2004, produces metallotype protease with a reasonable thermal tolerance and a broad range of pH (5.0–10.0). It is a highly recommended strain due to its thermal and halophilic properties (Amoozegar *et al.,* 2007). Another strain, *Bacillus clausii*, is also recommended for use at a commercial scale for the production of alkaline protease with the use of peptone, Cu, and fructose as the sole source of energy. The optimum pH and temperature recommended is 8–9 and 37–40◦C, respectively (Vadlamani and Parcha, 2011).

A strain of *Bacillus* sp., MPTK 712, isolated from dairy slush producing alkaline protease exhibits a symbiotic relationship with marine shipworms (Kumar *et al.,* 2012). Very rare microbes, such as *Kurthiaspiroforme* are also capable of producing alkaline protease (Amoozegar *et al.,* 2007). Some alkaline serine proteases recognized by goat skin metagenomics library shows homology to peptidases (Vadlamani and Parcha, 2011) and *Cryptococcusaureus* shows good bioactivity with optimum temperature (45–50◦C) and pH (9–10) (Kumar *et al.,* 2012). Different mushrooms producing alkaline protease are also reported (Li *et al.,* 2009; Pushpam *et al.,* 2011).

Acidic Proteases

Acid proteases are stable and active between pH 3.8 and 5.6 and are frequently used in soy sauce, protein hydrolysate, and digestive aids and in the production of seasoning material. The optimum pH of acidic proteases is 3–4 and the isoelectric point range is between 3 and 4.5 with a molecular weight of 30–45 kDa (Zheng *et al.,* 2011; Ravikumar *et al.,* 2012; Machado *et al.,* 2016). Furthermore, acid proteases are also exploited for use in clearing beer and fruit juice, improving texture of flour paste, and tenderizing the fibril muscle

In comparison with alkaline proteases, these extracellular acid proteases are mostly produced by fungal species, such as *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus awamori*, *Aspergillus fumigatus*, and *Aspergillus saitoi*. Most of the fungal extracellular acid proteases are known as *aspergillaopepsins*. Aspartic proteases are acid proteases consisting of 380–420 long chains of amino acid residues constituting the active site for catalytic activity. These acidic proteases are endopeptidases and grouped into three families: pepsin (A1), retropepsin (A2), and enzymes from Para retroviruses (A3). These three families are placed in clan AA. It is found that A1 and A2 are closely related to each other while members of the A3 family show some relatedness to families A1 and A2. An active site cleft of the members of the pepsin family is located between lobes of a bilobal structure (Pushpam *et al.,* 2011).

A great specificity of acidic proteases is exhibited against aromatic amino acid residues located on both sides of the peptide bond. These aromatic amino acid residues with peptide bonds are similar to pepsin but less stringent in action. Broadly, acidic proteases are divided into two groups: (i) pepsin-like enzymes and (ii) rennin-like enzymes produced by *Penicillium*, *Aspergillus*, *Rhizopus*, *Endothia*, and *Mucor*.

Neutral Proteases

Neutral proteases are defined as, such as they are active at a neutral or weakly acidic or weakly alkaline pH. Mostly neutral proteases belong to the genus *Bacillus* and with a relatively low thermotolerance ranging from pH 5 to 8 (Table 1). They generate less bitterness in hydrolysis of food proteins due to a medium rate of reaction; therefore, they are considered more valuable in the food industry. Neutrase is incorporated in the brewing industry due to its insensitivity to plant proteinase inhibitors. On the basis of high affinity toward hydrophobic amino acids, neutral proteases are identified and characterized. During production of food hydrolysate, it is slightly advantageous to control the reactivity of neutral proteases due to low thermotolerance (Zheng *et al.,* 2011).

Proteases play a pivotal role in the food processing industry, serving as indispensable enzymes with multifaceted applications. Their significance lies in their ability to catalyze the hydrolysis of proteins, contributing to various aspects of food production and enhancement, facilitating processes such as meat tenderization, dairy product manufacturing, and the production of protein-rich ingredients. Additionally, these enzymes contribute to the extraction of valuable bioactive peptides with potential health benefits. As the food industry continues to evolve to meet consumer demands for sustainable, healthier, and minimally processed foods, the role of proteases becomes increasingly prominent.

Statements and Declarations

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript. This is a self-funded research by the listed Authors.

Competing Interests

The authors have no competing interest to disclose because this study was self-sponsored by all the listed authors as a collaborative study. The authors did not receive support from any organization for the conduct of the study and the submitted manuscript.

Author Contributions

All authors contributed to the study conception and design as well as preparation, data collection and analysis. All authors contributed equally to all the previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical Consent

Ethical approval is not required for this type of work in Nigeria, since we did not test any material on human and animal subjects.

Consent to Participate

The consent to participate in this study was given by each author

Consent to Publish

The consent of each author was obtained prior to the commencement of the publishing process.

Data Availability

The datasets generated during and/or analyzed during the

current study are available from the corresponding author on reasonable request.

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How to cite this article:

Olusola Abayomi Ojo-Omoniyi, Dauphin Dighitoghi Moro and Oyinkansola Bridget Afolabi. 2024. Microbial Proteases: Sources, Significance and Industrial Applications. *Int.J.Curr.Microbiol.App.Sci.* 13(6): 1-23.
doi: https://doi.org/10.20546/ijcmas.2024.1306.001