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Biotechnological Significance and Applications of Alkaline Protease: A Review

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ABSTRACT

Because they catalyze reactions without changing themselves, enzymes are like catalysts in nature. Any enzyme that hydrolyzes the peptide bonds that connect amino acids in a polypeptide chain is considered a protease and initiates protein catabolism. They are useful for both synthetic and degradative purposes. Enzymes are most often found in microbes, and they are more active and stable than enzymes found in plants or animals. Because they multiply quickly, take up little room, and are amenable to genetic manipulation, microbes are a great choice for producing enzymes because we can easily change their features to suit our needs. Different proteases are known as endopeptidases or exopeptidases, depending on whether they work at or away from the termini. On the other hand, proteases are categorized as acidic, alkaline, or neutral depending on their ideal pH. Here we had a look at the many biotechnological uses of alkaline proteases, including in the food, tannery, brewery, silver recovery, and other industrial sectors as well as in medicine, ecology, and sustainability initiatives. The purification processes as well as the optimum condition of the protease was also discussed, in order to provide a comprehensive review about applications of alkaline proteases for sustainable development.

INTRODUCTION

Because they catalyze processes without changing themselves, enzymes are like catalysts in nature (Fig. 1). For thousands of years, people have used enzymes without fully comprehending their nature or function [1]. The enigma of enzymes has been unraveled and their amazing properties have been better exploited in an ever-increasing variety of applications thanks to the scientific community's efforts over the last several generations [2]. The majority of the world's enzymes come from microbes, and compared to enzymes made from plants or animals, they're far more stable and effective [3]. Due to their fast growth rate, small cell size, and simplicity of genetic manipulation, microbes are ideal sources of enzymes because of their versatility and usefulness in a wide range of applications [3,4]. Actually, almost 60% of all enzymes sold globally are microbial proteases [5]. Any enzyme that hydrolyzes the peptide bonds that connect amino acids in a polypeptide chain is considered a protease [6]. This process initiates protein catabolism. They are useful for both synthetic and degradative purposes. Proteolytic enzymes are highly valued for both their physiological functions and their practical uses in various industries [7]. Plants, animals, and microbes are only a few of the many diverse sources of proteases, which are physiologically essential for all forms of life [8].

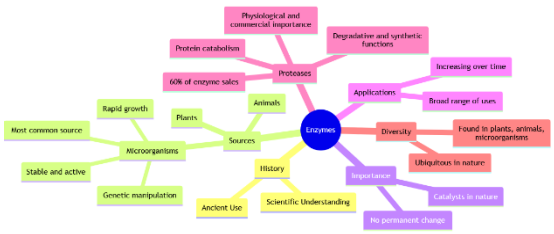

Fig. 1. A mindmap of the various uses of enzymes in the modern world (designed using Mermaid v10.6.1).

Sources of commercial proteolytic enzymes

Plant proteases

Several criteria, including the availability of area for cultivation and climatic conditions that promote optimal growth, govern the

utilization of plants as a source of proteases. It takes a long time for plants to produce proteases for commercial use. However, there are some famous plant proteases, such as papain, bromelain, keratinases, and Ficin. The enzymes used to coagulate milk in the cheesemaking process are plant proteases [9].

Animal proteases

Pancreatic trypsin, chymotrypsin, pepsin, and rennin are the proteases that originate from animals [10]. Animal protease production is contingent upon the accessibility of cattle for slaughter. Therefore, it is not practical from an industrial standpoint.

Microbial proteases

Because of their easy genetic modification and small cultivation area requirements, microorganisms are a promising source of proteases, as indicated before. A wide variety of proteases, both intracellular and extracellular, are generated by these organisms. Protein turnover, sporulation, differentiation, hormone and enzyme maturation, and cellular protein pool management are just a few of the many metabolic and cellular processes that rely on intracellular proteases. In order for cells to absorb and use hydrolytic products, extracellular proteases are critical for protein hydrolysis in cell-free settings [10]. Protein 3 degradation in industrial processes has also made commercial use of them [11].

Bacterial proteases

Soil, air, and water aren't the only places you may find bacteria; they're also present in animal cells. In instance, marine bacteria are abundant in all seas and can be found in a broad variety of habitats, from the ocean floor to the digestive tracts of fish. Underwater mountains, seamounts, deep-sea sediments, and even the top of an algae cell are just a few of the diverse ecosystems found in the ocean. For their survival in such diverse and frequently harsh environments, these microbes have evolved distinct and highly specialized strategies. Temperatures can vary greatly, from 33 degrees Celsius in tropical waters to as low as 5 degrees Celsius in the deep sea and polar regions, while salinity levels can range from very salty to barely brackish water. Bacteria found in the ocean have shown to be an excellent source of new biologically active compounds due to their extraordinary resilience in the face of extreme environmental stress. These compounds have the ability to revolutionize various industries, including health and food production, thanks to their unique qualities.

Fungal protease

Fungi, like bacteria, may produce complex enzymes from a broad range of sources. Aspergillus oryzae is one example; it makes proteases that are acidic, neutral, and alkaline. The pH range in which fungal proteases are active is typically larger, spanning 4 to 11, and they show a broad selectivity for substrates. But compared to bacterial enzymes, they aren't as fast and can't withstand as much heat. The solid-state fermentation process allows for the commercial production of fungal enzymes. For fungal acid proteases, the sweet spot is between 4 and 4.5, and they stay put anywhere from 2.5 to 6.0. Their high specificity for both pH and temperature make them ideal for use in cheesemaking. At pH 7.0, fungal neutral proteases are active, and chelating substances block them. They have the unique capacity to hydrolyze bonds between hydrophobic amino acids and are active peptidases 4. So, they can lessen the bitterness of hydrolyzed proteins in meals by enhancing the activity of proteases from other sources [13].

Viral proteases

The reproduction of viruses that cause dangerous diseases like cancer and AIDS relies heavily on viral proteases, which play a critical role in digesting viral proteins. These proteases are essential for the maturation of viral particles and enable the virus to replicate effectively. Many different types of viruses, including retroviruses, have proteases that can break down large proteins into smaller functional units, a process that is vital for their survival and propagation. Interestingly, no metalloproteases are involved in the replication process of viruses, which distinguishes them from other biological systems. It is also worth noting that all proteases encoded by viruses are endopeptidases, which specifically cleave peptide bonds within proteins, rather than at the ends.

Retroviral aspartic proteases, in particular, have been the subject of extensive research due to their crucial role in the lifecycle of retroviruses like HIV. The mutations in these proteases have been studied in-depth in terms of their purification, expression, and enzymatic characterization. Much of the research has focused on understanding their structure at the molecular level, as this knowledge is essential for the development of targeted inhibitors. The three-dimensional structures of these viral proteases, along with their interactions with synthetic inhibitors, have been a significant focus of scientific study. This research is crucial because the development of effective inhibitors is considered one of the most promising strategies to slow or halt the spread of the AIDS epidemic—a devastating disease that has impacted millions of people worldwide, causing significant social and health challenges.

Classification of proteases based on the site of action

Based on whether they operate at or away from the termini, proteases are classified as exopeptidases or endopeptidases, respectively.

Exopeptidases

Only the N- or C-termini of polypeptide chains are active exopeptidases. Some enzymes, known as aminopeptidases, release a single amino acid residue, while others, known as carboxypeptidases, release a single amino acid or a five-dipeptide. These enzymes act at either the free N-terminus or the free C-terminus of proteins, respectively. Certain exopeptidases are dipeptidase selective or remove terminal residues that are isopeptide bond replaced, connected, or cyclized. Most carboxypeptidases fall into one of three categories, distinguished by the types of amino acids present at the enzyme's active site:

Endopeptidases

Endopeptidases have a preference for neutralizing peptides in their interior, rather than at their C or N termini. Enzyme activity is reduced when amino or carboxyl groups are unbound. These endopeptidases can be ordered further, according to the reactive groups at the active site involving catalysis, into serine- (EC 3.4.21), cysteine- (EC 3.4.22), aspartic-proteinases (EC.3.4.23) and metalloendopeptidases or metalloproteinases (EC 3.4 24). Enzymes are categorized in the EC subgroup if their reaction mechanisms are not well understood. 3.4.1. 99. It is based on the action mechanism. There were six main categories of patients that were found.

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

Threonine and glutamic acid proteases were not fully characterized until the late 20th century, with threonine proteases being described in 1995 and glutamic acid proteases in 2004. These proteases function through a distinct mechanism to cleave peptide bonds. Specifically, they involve the creation of a 6-amino acid residue, which includes a threonine and either a cysteine or a water molecule (conditional on the class of protease, such as aspartic acid, metallo-, or glutamic acid proteases). This setup facilitates the nucleophilic attack on the peptide's carboxyl group. To make a nucleophile capable of attacking, a catalytic triad is often utilized. In this case, a histidine residue plays a key role in activating other residues such as serine, cysteine, or threonine, transforming them into nucleophiles. This mechanism is essential for the protease's function in breaking down peptides. The MEROPS database is an important resource for protease classification and provides an up-to-date, detailed categorization of proteases into their respective families. It continuously updates the classification to account for new findings and variations within protease mechanisms [15].

Serine proteases

Serine proteases represents one of the most widely distributed groups of proteolytic enzymes, found across a broad spectrum of organisms, including animals, microbes, and viruses. These enzymes are characterized by the presence of a serine residue at their active site, which plays a crucial role in their catalytic activity. Serine proteases are typically inhibited by compounds like diisopropyl fluorophosphates (DFP) and phenylmethyl sulfonyl fluoride (PMSF), which target their active sites. Additionally, many of these protease can be inhibited by thiol reagents, such as p-chloromercuric benzoate (pCMB), likely due to the proximity of a cysteine residue near the active site, though this residue does not directly participate in the catalytic mechanism of the enzyme.

Serine proteases tends to exhibit activity at neutral to alkaline pH levels, with an optimal pH range typically between 7 and 11. Their widespread present in viruses, bacteria, and eukaryotic organisms underscores their essential role in biological processes, highlighting their importance across different life forms. These proteases are categorized into four main classes: chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC), and *Escherichia coli d*-Ala–d-Ala Peptidase A (SE). The molecular masses of serine proteases generally range from 18 to 35 kDa, reflecting their diverse structures and functional roles in various biological processes.

Metalloproteases

Metalloproteases are considered to be the most diverse and complex group of proteolytic enzymes, showcasing an extensive range of catalytic mechanisms. These enzymes are distinguished by their requirement for divalent metal ions, such as zinc or manganese, which are essential for their catalytic activity. The metal ions are typically located in the enzyme's active site, where they assist in stabilizing the transition state during peptide bond cleavage. In fact, most fungal and bacterial metalloproteases are zinc-containing enzymes, making zinc a crucial cofactor for their function.

They are highly sensitive to metal chelators like EDTA, which binds to the metal ions, disrupting their ability to perform the necessary catalytic reactions. On the other hand, metalloproteases are not affected by sulfhydryl agents, which do not interact with the metal ions in the same way they interact with enzymes that contain cysteine in their active sites.

Metalloproteases are classify based on their substrate specificity and pH preference into four distinct categories. These categories includes (i) neutral proteases, which are most active at a neutral pH; (ii) alkaline proteases, which function best under basic conditions; (iii) Myxobacter I proteases, which are primarily produced by Myxobacteria and have unique substrate preferences; and (iv) Myxobacter II proteases, which are similar to Myxobacter I but differ slightly in terms of catalytic activity and substrate specificity. Each group plays a vital role in the biological systems where they are found, reflecting their functional diversity and adaptability in various environments.

Aspartic proteases

Aspartic acid proteases, also commonly referred to as acidic proteases, are a class of endopeptidases that rely on aspartic acid residues for their catalytic activity. These enzymes are widely distributed in fungi, but are comparatively rare in bacteria, making them more characteristic of fungal species. Aspartic proteases is known to be inhibited by a variety of compounds, including epoxy- and diazo-ketone compounds, particularly in the presence of copper ions. Additionally, they can be inhibited by pepstatin, a well-known inhibitor, or by a Streptomyces-derived pepsin inhibitor.

Most aspartic proteases exhibit maximal catalytic activity at an acidic pH range of 3 to 4, which aligns with their typical isoelectric points, usually found within the pH range of 3 to 4.5. The molecular weights of these enzymes typically fall within the range of 30 to 45 kDa, though variations may exist depending on the specific organism or type of aspartic protease. These proteases plays crucial roles in various biological processes, including protein turnover and regulation, and their activity is finely tuned to the acidic environments in which they function best.

Cysteine/ Thiol proteases

The activity of all cysteine proteases depends on the catalytic site, which typically consists of cysteine and histidine residues. These proteases are generally only active in the presence of reducing agents, which are essential to maintain their active form. Cysteine proteases are sensitive to various sulphydryl agents, including pCMB, iodoacetic acid, iodoacetamide, and heavy metals, which can inhibit their activity. However, they can be activated by reducing agents such as DTT, EDTA acid, potassium cyanide, or cysteine, which help to maintain the integrity of the catalytic site and ensure efficient function.

Cysteine proteases are classified based on their side chain specificity into four major groups: (i) papain-like proteases, (ii) trypsin-like proteases, which have a preference for cleavage at the arginine residue, (iii) proteases that are specific to glutamic acid, and (iv) a broad category known simply as "others," which includes proteases with more unique or less characterized specificities. These enzyme are generally most active in slightly acidic to neutral pH ranges, typically between 5 and 8, which corresponds to the conditions commonly found in cellular environments.

Based on optimal pH

Another classification of proteases is related to the optimal pH at which they are active:

i. Acid proteases
ii. Neutral proteases
iii. Alkaline proteases

Table 1. A summary of the various types of proteases.

Category	Description
Plant Proteases	- Governed by factors like land availability and climatic conditions. Commercial production takes a long time. Examples: Papain, bromelain, keratinases, Ficin. Used in cheese industry as milk-clotting enzymes.
Animal Proteases	- Examples: Pancreatic trypsin, chymotrypsin, pepsin, rennin. Production depends on livestock availability. Not feasible for industrial production due to dependency on livestock.
Microbial Proteases	- Limited space required for cultivation. Susceptible to genetic manipulation. Includes both intracellular (important for cellular processes) and extracellular proteases (vital for hydrolysis of proteins in cell-free environments). Commercially exploited in industrial processes.
Bacterial Proteases	- Ubiquitous in soil, air, water, and animal tissue. Marine bacteria have unique survival mechanisms, making them a promising source of novel biologically active substances [12.
Fungal Protease	- Produced by fungi like Aspergillus oryzae. Active over a wide pH range (pH 4 to 11). Lower reaction rate and heat tolerance than bacterial enzymes. Useful in cheese making due to narrow pH and temperature specificities [13].
Viral Proteases	- Involved in processing of viruses causing diseases like AIDS and cancer. Includes serine, aspartic, and cysteine peptidases. Research focused on their structure and interaction with inhibitors [19].
Classification of Proteases	- Exopeptidases: Act at the ends of polypeptide chains. Endopeptidases: Act in the inner regions of peptide chains.
Serine Proteases	- Characterized by a serine group at the active site. Generally active at neutral and alkaline pH (pH 7-11). Subdivided into chymotrypsin, subtilisin, carboxypeptidase C, and Escherichia coli d-Ala–D-Ala Peptidase A.
Metalloproteases	- Require divalent metal ions for activity. Mostly zinc-containing in fungi and bacteria. Sensitive to metal chelators like EDTA. Classified into neutral, alkaline, Myxobacter I, and Myxobacter II proteases.
Aspartic Proteases	- Depend on aspartic acid residues for catalytic activity. Found in fungi, rarely in bacteria. Inhibited by compounds like pepstatin. Active at pH 3–4.
Cysteine/Thiol Proteases	- Activity depends on catalytic site consisting of cysteine and histidine. Sensitive to sulphydryl agents. Activated by reducing agents. Classified into papain-like, trypsin-like, specific to glutamic acid, and others. Active at pH 5-8.

Biotechnological Applications of Proteases

The chemical, feed, leather, medicinal, food, meat tenderization, cheese, and waste treatment industries could all benefit significantly from proteases, which are powerful enzymes with considerable industrial potential, particularly as detergent additives. These enzymes, known for their stability and efficacy, can enhance a wide range of industrial processes by breaking down specific proteins, making tasks more efficient. Through enzyme-aided (partial) digestion, proteases facilitate the creation of applications or products with high added value, thus contributing to the optimization of various production methods. Laundry detergents, which target stains based on proteins, are among the primary consumers of proteases. These detergents rely on proteases to break down protein-based stains such as food, blood, and sweat, effectively improving cleaning performance [19].

For proteases to be useful as detergent additives, they must remain stable and active when mixed with other common detergent chemicals. These include fillers, surfactants, fabric softeners, bleach activators, builders, and bleach. The ability of proteases to maintain their activity in such complex mixtures is key to their success in the detergent industry. Another notable application of proteases is in textiles, where they are used to soften and enhance the luster of raw silk fibers. Proteases achieve this by breaking down the stiff and dull gum layers of sericin, a protein found in silk. This treatment not only gives the silk a new look but also imparts a special sheen to the fabric. Similarly, wool and silk can be treated with proteases to achieve a soft, lustrous finish, making these enzymes valuable in the textile and fashion industries [20].

As Detergent Additives

Out of the three types of enzymes used in detergents—proteases, amylases, and lipases—alkaline proteases account for sixty-five to sixty-five percent of the world's industrial enzyme sales. The development of subtilisins as typical detergent proteases has utilized all the tools of enzyme technology over the past 20 years, resulting in a steady flow of new and improved enzymes [21]. Proteases have been incorporated into detergents since 1913, when Roehm first used pancreatic extracts [22]. Since their introduction, the commercial and technological efficiency of bacterial enzymes has steadily increased, particularly following their widespread availability in the 1960s [23]. Initially, these enzymes were produced using Bacillus species, specifically *Bacillus amyloliquefaciens a*nd *Bacillus licheniformis.* These Bacillus species are well-known for producing alkaline proteases, which are the primary components of subtilisins [24].

The cleaning power of proteases in detergents is largely responsible for their widespread use as additives. These enzymes not only enhance washing efficiency, but they also facilitate lower wash temperatures and shorter agitation periods, typically following a pre-wash soaking phase. This makes detergents with proteases more energy-efficient and effective in stain removal. For detergents to work optimally, the enzymes, including proteases, must be highly active and stable across a wide range of temperatures and pH values. Furthermore, it is important that enzymes maintain low-level activity to avoid unnecessary degradation of fabric or other materials during washing cycles [26].

In Silver Recovery

Alkaline proteases is potentially exploited for silver recovery in the bioprocessing of X-ray films containing silver in the gelatin layer. The conventional practice of silver recovery using burning films pose a major environmental pollution problem, releasing harmful fumes and contributing to air pollution. Enzymatic hydrolysis, on the other hand, offer a sustainable alternative. The enzymatic hydrolysis of gelatin layers on X-ray films enables not only silver recovery but also recycling of the polyester film base, making it a eco-friendly process. This method significantly reduces the environmental impact compared to traditional burning methods, while also allowing for the valuable silver to be reclaimed [24-29].

Medical Uses

Many different areas of medicine and biopharmaceutics make use of proteases. In addition to removing clots and aiding wound healing, proteases can enhance the efficacy of certain medications. For example, one protease found in pineapple stems is commonly used to reduce inflammation, while another type of enzyme is employed to treat severe sepsis by breaking down harmful proteins involved in the infection process. Furthermore, collagenases, which exhibit alkaline protease activity, have shown medicinal applications due to their ability to degrade collagen and other extracellular matrix components, making them useful in various therapeutic contexts [28].

One specific alkaline protease with fibrinolytic activity is used as a thrombolytic agent to break down blood clots, thereby aiding in the treatment of conditions like thrombosis. It is widely recognized that proteases derived from *Bacillus s*pecies are generally safe for human use, making them an attractive option for therapeutic applications [29]. In the biopharmaceutical sector,

proteases are integral to products like enzymatic debridement agents and contact lens enzyme cleaners. In the case of enzymatic debridement, proteases help remove necrotic tissue from skin ulcerations in a delicate and selective manner, which accelerates wound healing by promoting the body's natural healing processes. This makes proteases essential in improving patient outcomes, particularly in managing chronic wounds and infections [31].

Food Industry

The food industry has utilized proteases for centuries. Rennet, a protease derived from the stomachs of unweaned calves, has traditionally been employed in the production of cheese. This enzyme helps coagulate milk proteins, aiding in the formation of curds. Additionally, the hydrolysis of proteins by alkaline proteases can produce various hydrolysates with distinct peptide profiles, with up to eleven different types depending on the source and conditions used [32]. These hydrolysates can be derived from both plant and animal proteins, offering a diverse range of applications in food production. One commercially available alkaline protease, commonly known as Alcalase, has a broad specificity, although it shows a slight preference for hydrophobic amino acids at the ends of peptide chains.

Neutral proteases are particularly important in the production of soy sauce and other soy-based products, as they help reduce bitterness by breaking down undesirable peptides. These proteases are also used in the brewing industry due to their resistance to natural plant proteinase inhibitors, making them reliable for improving product quality and consistency [33]. Proteases are also essential in the production of sausages, luncheon meats, and breads. In the meat industry, they can help recover proteins from animals (and fish) that would otherwise go to waste after slaughter. Furthermore, proteases are sometimes used in baking. By partially hydrolyzing the gluten in dough, proteases can help reduce preparation times, which can be particularly useful in commercial bakeries. However, to ensure that the enzyme does not affect the final product during baking, it is inactivated early in the process using a heat-labile fungal protease [30].

Tannery, Leather and Wool industries

Enzyme synthesis is heavily utilized in the leather industry, particularly in the extraction of hair from animal skins, where alkaline proteases play a critical role [34]. This method is considered less harsh and more environmentally friendly compared to traditional techniques that relied on sodium sulfide, which often involved toxic chemicals. Alkaline proteases with keratinolytic and elastolytic activities are integral to the biotreatment of leather, especially in processes like dehairing and bating, which help in softening skins and hides.

In an alkaline environment, root hairs can swell, and the proteins in hair follicles are readily digested by proteases, making hair removal much more efficient. This helps produce supple leather, which is ideal for crafting various leather goods and clothing. The enzymatic breakdown of elastin and keratin, along with the removal of hair remnants and swelling collagen, leads to a smoother, more pliable material. In addition, proteases have been used for shrinkproofing wool in the past, a process that enhances the wool's quality by providing a glossy sheen.

The protease papain, for example, works by partially hydrolyzing the scale tips of wool fibers, improving the texture and durability of the fabric [35].

For Waste Treatment

By dissolving proteins in waste materials, alkaline proteases offer promising biotechnological applications in both the food industry and residential waste management. These enzymes can efficiently break down proteins in various types of organic waste, contributing to the management and recycling of food scraps and other waste materials. For example, feather waste, which is typically discarded and difficult to decompose, has been treated with enzymes produced by *Bacillus subtilis [*36]. This process not only helps in waste reduction but also opens up possibilities for converting such waste into valuable products, such as amino acids and bioactive peptides, adding value to otherwise discarded materials.

Protein Engineering

With the increasing demand for alkaline proteases, substantial research has been conducted on DNA shuffling and cloning techniques to enhance the production and functionality of these enzymes. To complement gene-shuffling methods, preexisting structural knowledge is being employed, enabling more targeted and efficient enzyme design. Modern biotechnology has created a favorable environment for protease research and development, potentially contributing to more sustainable and eco-friendly living practices. The beneficial properties of proteases and their significant potential for improving a wide range of consumer and industrial products have been thoroughly explored by Ward (2022) [37].

An example of protease production is the enzyme derived from *Serratia marcescens,* which was successfully produced by cloning and expressing its gene in *Escherichia coli.* This process allowed the host cells to secrete the protease into the extracellular medium, making it more accessible for various applications [38]. Genetically modified microorganisms are now responsible for producing over half of the enzymes used in industrial processes, underscoring the critical role of biotechnology in advancing enzyme production and optimization [37].

Significance of microbial proteases

Alkaline proteases make up over 65% of the global enzyme market [39], with microbial proteases being highlighted as more advantageous compared to those derived from plants and animals [40]. As the industrial demand for enzymes, particularly alkaline proteases, continues to rise annually, marine microorganisms are drawing increasing attention for their potential biotechnological applications (**Table 2**). The chemical, food, pharmaceutical, leather, and detergent industries, in particular, could benefit from the extracellular proteases produced by various *Bacillus s*pecies, such as *Bacillus cereus, Bacillus sterothermophilus, Bacillus mojavensis, Bacillus megaterium,* and *Bacillus subtilis [*41]. For example, a thermostable alkaline protease was isolated, produced, and characterized from *Bacillus licheniformis M*IR 29 by Ferrero et al. [43]. Additional species of *Bacillus k*nown for producing alkaline proteases have also been reported by Christensen et al. [44]. In particular, a parametric analysis of *Bacillus firmus p*rotease production in batch and fed-batch cultures was conducted by Bohacz et al. [45].

Table 2. General application of alkaline proteases.

Application Area	Description	Key Points
As Detergent Additives	- Proteases, amylases, and lipases used in detergents. Alkaline proteases hold 60-65% of the global industrial enzyme market. Development of subtilisins as detergent proteases. Use dates back to 1913. Produced using *Bacillus* species. Improved washing efficiency, allows for lower wash temperatures and shorter agitation periods.	- High activity and stability over a broad range of pH and temperatures. Active at low levels.
In Silver Recovery	- Alkaline proteases exploited for silver recovery from X-ray films. Enzymatic hydrolysis of gelatin layers enables silver recovery and recycling of polyester film base.	- Eco-friendly process compared to conventional burning films.
Medical Uses	- Used to treat blood clots, clean wounds, and enhance antibiotic effectiveness. Types include anti-inflammatory agents and protein-degrading enzymes for severe sepsis. Collagenases with alkaline protease activity used therapeutically. Safe for humans, used as thrombolytic agents.	- Useful in biopharmaceutical products like contact-lens enzyme cleaners and enzymatic debridement.
Food Industry	- Used for centuries, e.g., rennet in cheese production. Alkaline proteases hydrolyze proteins to produce hydrolysates. Neutral proteases used in soy sauce production, brewing industry. Active role in meat processing, baking industry.	- Alcalase has broad specificity. Proteases recover proteins from animal and fish waste. Heat-labile fungal protease used in baking.
Tannery, Leather, and Wool Industries	- Significant enzyme consumption in leather industry. Alkaline proteases used for dehairing hides. Keratinolytic and elastolytic activities beneficial in leather processing. Used to 'shrinkproof' wool, giving it a silky lustre.	- Safer and more pleasant than traditional methods. Produces good, soft leather for various products.
For Waste Treatment	- Alkaline proteases used for waste management in food industry and household activities. Solubilizes proteins in waste. *Bacillus* subtilis enzymes used for waste feather treatment.	- Contributes to sustainable waste management.
Protein Engineering	- Research on DNA shuffling and cloning for alkaline proteases. Utilizes existing knowledge on structures and gene-shuffling techniques. Advances in biotechnology create a niche for protease development. Serratia marcescens protease gene cloned and expressed in E. coli.	- Over 50% of industrially important enzymes produced by genetically engineered microorganisms. Focus on sustainable environment and product improvement.

Halophilic bacteria are another promising source of alkaline proteases due to their remarkable adaptability to high-salinity environments [46]. After screening, Bacillus strains found in the Kakinada saltern pond were identified as having high levels of alkaline protease activity [47]. Enzymes produced by alkalophilic microbes are well-known for their proteolytic activity and excellent pH stability, as documented by Gessesse and Gashe [48]. A highly stable and detergent-compatible alkaline protease was isolated from *Bacillus licheniformis a*t Chalawa Dam, Kano, Nigeria [49].

The synthesis and characteristics of alkaline proteases from *Pseudomonas spp. h*ave been described by Chakraborti et al. [50]. Tang et al. [51] investigated a solvent-stable protease isolated from *Pseudomonas aeruginosa P*T 121, focusing on its biochemical properties and potential applications. Ramesh et al. [52] demonstrated that an alkaline-resistant *Streptomyces i*solate produced a thermostable alkaline protease. Cui et al. [53] purified a protease from *Pseudomonas aeruginosa P*D100 and explored its potential uses. A mutant strain of *Bacillus sp. a*lso produced an alkaline protease, which was purified and studied by Thakur et al. [54]. For environmentally conscious detergent applications, Rejisha et al. [55] described the process of producing, purifying, and characterizing a detergent-stable, halotolerant alkaline protease.

Additionally, the utilization of halophilic and alkaline proteases, isolated and optimized through bioprocessing from marine *Streptomyces,* has been explored as a cleaner for contact lenses [56,57]. The enzymatic properties of alkaline protease produced by alkalophilic *Bacillus sp. i*n an inexpensive medium were also studied [56]. Furthermore, a novel alkaline protease was produced by cultivating alkalophilic bacteria on chicken feathers [57,58]. One study found that using agro-wastes as a substrate to extract alkaline protease from *Serratia marcescens i*n soil was an efficient and cost-effective method [59]. Other studies have confirmed the cost-effectiveness of using agro-wastes as a substrate for alkaline protease production [56,58]. Overall, the production of purified proteases depends on several variables that need to be optimized for efficient enzyme synthesis.

Various protease purification methods have been documented, including electrophoresis [62], chromatography [61], and precipitation [60]. Studies have highlighted several proteases with unique properties, such as a thermostable alkaline protease derived from the alkalophilic *Bacillus megaterium-TK1,* and a serine alkaline protease from *Bacillus mojavensis,* which is both oxidation-stable and thiol-dependent. Additionally, a 56 kDa cold-active protease was isolated from *Serratia sp. TGS1 a*nd studied [57]. Muhammad et al. [58] described the extraction and characterization of extracellular alkaline proteases from a gene pool of *Bacillus s*trains. The synthesis and characteristics of alkaline protease in a *Bacillus cereus s*train, obtained from tannery waste, were also explored [59], while alkaline protease synthesis and purification were carried out with a *Bacillus s*train from Modj, Ethiopia [60].

Further research includes the purification and characterization of *Aspergillus flavus p*roteases produced via solid-state fermentation by Chimbekujwo et al. [64]. Khan et al. [65] partially described the genome and serine protease of a novel *Bacillus subtilis i*solate. The role of external proteases in hydrolyzing large proteins and intracellular proteases in regulating metabolism has been examined, with a new haloalkaliphilic *Bacillus 1*6 species found to contain an extracellular alkaline protease [61].

The solid-state fermentation method has been employed to produce thermostable alkaline proteases. For example, a new strain of *Pseudomonas w*as used to produce such a protease [62], and a similar study examined the use of protease produced from *Bacillus amyloliquefaciens f*or dehairing skins and hides [59]. Notably, *Serratia marcescens h*as been shown to outproduce *B. subtilis i*n terms of protease production, earning it recognition as an excellent protease producer [63, 64]. An efficient metalloprotease was identified in *S. marcescens S*3-R1 from the Korean ginseng rhizosphere [65], while soil samples from dairy waste yielded *S. marcescens s*trains with potential applications in detergent, leather, and silver recovery due to their ability to produce thermostable alkaline proteases active under high pH

conditions [66]. Additionally, a novel cysteine protease produced from dairy waste was studied [67].

The importance of alkaline proteases in various industries continues to grow, and recent studies have explored new sources and applications for these enzymes. For example, after isolating obligate alkaliphilic bacteria from Chalawa's estuary habitats, Joshi et al. [68] documented lipase, amylase, and protease activity under acidic conditions. Song et al. [42] reviewed microbial alkaline proteases and emphasized their resistance to highly acidic environments.

Aspergillus species, particularly *Aspergillus niger,* have become primary sources for bulk commercial enzyme synthesis, largely due to their ability to grow on inexpensive media and produce large quantities of enzymes [66]. These enzymes, especially proteases, are heavily utilized in the detergent industry, with studies like Wei et al. [67] investigating the compatibility of *A. niger a*lkaline proteases with commercial detergents. Other bacterial species such as *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus amyloliquefaciens h*ave also been widely used in various sectors, including feed production, brewing, and washing, due to their enzymatic versatility [25, 62, 70, 69]. A specific alkaline protease from *Bacillus licheniformis UV-9 h*as been used to evaluate various detergent formulations [72].

In leather processing, alkaline proteases have been explored as depilating agents, which help remove hair during tanning [73]. Similarly, proteases are employed in silk processing, where they break down the sericin gum, improving the texture and gloss of raw silk [74]. The pharmaceutical industry has also studied microbial proteases for potential applications in drug development and other biotechnological fields [42, 70]. Notably, Amira and Eida [71] demonstrated that *Bacillus subtilis s*ubsp. *subtilis a*lkaline protease can be used for silver recovery from used X-ray films.

Marine organisms, particularly eukaryotic marine protists like thraustochytrids from mangrove ecosystems, have garnered attention for their production of alkaline proteases with potential uses in detergent and leather industries [78]. Aruna et al. [72] and others have studied *Nocardiopsis alba f*or its thermostable, cellulolytic, alkaline protease, demonstrating the bacterium's biochemical characteristics and protease structure. Similarly, *Pseudoalteromonas atlantica w*as analyzed by Auta et al. [73] for the impact of physiological conditions on enzyme activity.

Fungi from deep-sea ecosystems are also promising sources of proteases, as demonstrated by research into species from the Central Indian Ocean Basin, which exhibit cold- and alkaline-tolerant protease activity [66]. *Aspergillus ustus,* for example, produced cold-tolerant alkaline proteases under ambient pressure at 30°C, unaffected by exposure to various commercial detergents [75]. The growing interest in marine and extreme-environment microorganisms underscores the vast biotechnological potential of alkaline proteases, highlighting their applications in a variety of industrial sectors. This work aims to provide a comprehensive overview of alkaline proteases, emphasizing their significance and broad range of uses in biotechnology.

Future challenges

The biotechnological applications of alkaline proteases have gained significant attention in recent years due to their versatility and potential for sustainable development. As the world transition towards a more eco-friendly and circular economy, the need for environmentally-friendly biocatalysts like alkaline proteases has become increasingly crucial [76]. These enzymes are not only efficient in industrial applications but also offer a promising solution to the growing demand for green technologies. One of the primary future challenge in this field is the development of cost-effective and scalable production methods for alkaline proteases. The use of alternative substrates, such as agro-industrial waste, can help achieve this goal by aligning with the principles of the circular economy. Furthermore, the use of green solvents, such as ionic liquids and deep eutectic solvents, for the extraction and purification of these enzymes can contribute to the overall sustainability of the process [77]. These environmentally-friendly solvents minimize the need for harmful chemicals, ensuring a safer and more sustainable production process.

Another challenge lies in the optimization of enzyme properties and activity through protein engineering and directed evolution techniques. By enhancing the stability, specificity, and catalytic efficiency of alkaline proteases, researchers can expand their applications in various industries, including food processing, detergent manufacturing, and leather production [76-78]. These advancements could lead to more efficient enzyme-based processes that are not only cost-effective but also reduce environmental impact.

To achieve real progress in sustainability, a more circular production from renewable biomass is necessary. The use of alternative substrates in bioprocesses, such as agroindustrial waste, complements circular economy principles. This shift towards renewable sources would drastically reduce reliance on fossil fuels and reduce the overall carbon footprint of industrial processes. Furthermore, to guarantee the development of green processes, other aspects must be observed, such as the use of biosolvents for the recovery and purification of biocompounds.

This is an essential step to ensuring that the entire biotechnological production process is sustainable and aligns with eco-friendly principles. The increased use of non-renewable energy sources over the decades has caused changes in the economy, society, and the environment. In this regard, serious environmental problems and future limitations of these sources indicate the need for environmentally friendly alternatives for energy sources. Technologies for the production of alternative energy from agroindustrial residues can help increase production without the threat of competition with food production. Moreover, utilizing agricultural byproducts for energy and enzyme production can mitigate waste and offer a sustainable alternative to traditional energy sources [79-82].

CONCLUSION

In conclusion, alkaline proteases have emerged as essential biocatalysts in numerous industrial applications, offering sustainable solutions across sectors like food processing, detergents, leather, and pharmaceuticals. The microbial production of alkaline proteases, particularly from species like *Bacillus a*nd *Aspergillus,* has revolutionized enzyme synthesis, making it more cost-effective and environmentally friendly. These enzymes are valued for their stability at high pH levels and temperatures, enabling their use in demanding conditions, such as in detergent formulations and leather depilation processes. Research has shown that diverse microbial sources, including marine and estuarine organisms, can produce alkaline proteases with unique properties. Marine bacteria such as *Pseudoalteromonas atlantica a*nd *Serratia marcescens,* as well

as fungal species like *Aspergillus ustus,* demonstrate cold- and alkaline-tolerant protease activities, making them particularly valuable in extreme environments. Furthermore, advancements in genetic engineering and enzyme production methods have significantly improved the yields and specificities of these proteases. The ability to modify microorganisms to enhance protease production has paved the way for new applications, such as silver recovery from X-ray films and efficient waste management. The versatility of alkaline proteases also extends to agricultural and biotechnological industries. The use of agricultural waste as a substrate for enzyme production not only reduces costs but also contributes to sustainable practices. With their wide range of uses, alkaline proteases have proven to be a pivotal component in advancing greener technologies. In future, continued research and development will likely lead to even more optimized production methods and novel applications. The growing demand for alkaline proteases and their broad industrial potential underscores their importance in shaping the future of biotechnology. Therefore, understanding and enhancing the production of alkaline proteases is critical to meeting the challenges of modern industry and environmental sustainability.

AI DECLARATION

We employed advanced AI-based tools during the initial phases of our research and manuscript preparation, specifically Mermaid, ChatGPT, ScholarAI, and Grammarly. These tools served as supplementary resources, assisting with data collection, analysis, and manuscript editing. However, they were not involved in data interpretation or formulating scientific conclusions. The final interpretations, conclusions, and scholarly work, including the structure and coherence of the arguments presented, are solely the responsibility of the authors.

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