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A REVIEW OF *BACILLUS SPECIES* ALKALINE PROTEASE PRODUCTION AND INDUSTRIAL APPLICATIONS

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ABSTRACT

Protein peptide linkages can be hydrolyzed by protease enzymes. They are present in every living thing and essential to many biological processes and the life cycle of many diseases, bacterial proteases are important medicinal ingredients. Enzyme delivery methods and new technology for rationally engineering proteases will considerably enhance the potential therapeutic applications of enzymes. Proteases are widely used agents in several pharmaceutical business industries. Proteases are present in every living thing. Because of their role in the life cycle of different pathogenic organisms, they could be targeted for the development of therapeutic drugs to treat serious illnesses such as AIDS, cancer, malaria, and bacterial infections. The uses of microbial proteases, including L-asparaginase, L-glutaminase, serrapeptase, serrazime, and L-asparaginase, are the primary emphasis of this review. These enzymes are being utilized to treat cancer, respiratory illnesses, cardiovascular disease, and inflammation. In microbial fermentations, Bacillus species remain the predominant bacterial workhorses. The primary microorganism involved in fermentation is *Bacillus subtilis* and other related species. Some of the most significant commercial enzyme manufacturers are *Bacillus* strains that can synthesize and secrete high amounts of extracellular enzymes (20–25 g/L). To summarize the medicinal uses of *Basillus* protease enzymes in the biological sciences, this paper will discuss the biotechnological features of these enzymes.

Keywords: Bacterial proteases, pharmaceutical applications, pathogenic, Protease.

INTRODUCTION

Proteases are essential enzymes involved in many biochemical processes, such as the metabolism and breakdown of proteins (Angmo et.al. 2016). Because of their usefulness in catalyzing the hydrolysis of peptide bonds in proteins, they are found in many different industries, including

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food, pharmaceuticals, and detergents. Production of alkaline protease may originate from isolates of proteolytic *Bacillus*. Because these isolates have demonstrated significant proteolytic activity at alkaline pH, they are appropriate for use in industrial settings where alkaline-stabilizing enzymes are needed. Endospore-forming aerobic, gram-positive, saprophytic microorganisms are categorized within the *Bacillus* genus.

They produce hydrolytic enzymes including mannanase, glucanase, and protease regardless of the culture medium employed, and they grow well in a basic medium. (M. Perez and R. Piad, 2007) One of the most significant classes of industrial enzymes, microbial proteases meets the needs of around 60% of the global enzyme market. It is possible to manufacture a large number of industrial enzymes with significant economic value by cultivating bacteria like Bacillus species. Every year more than 300 tons of enzymes primarily proteases are produced from *Bacillus* species. The function of microbial proteases in biotechnological processes is significant. (Rao and others, 1998) Using submerged fermentation, alkaline proteases were generated from three distinct Bacillus isolates. At pH 9, the isolates' maximal protease activity was seen. Many studies have been conducted on the synthesis of alkaline protease from isolates of the proteolytic Bacillus bacteria. (Gupta and Associates, 2002) the identification and improvement of Bacillus strains isolated from soil samples that produce alkaline proteases. They discovered that the Bacillus isolates could withstand high temperatures and showed strong protease activity at alkaline pH, which qualified them for use in industrial settings. Yu et al. conducted a second investigation to investigate the synthesis of alkaline protease from *Bacillus licheniformis*. To increase the synthesis of alkaline protease, they adjusted the fermentation parameters, including pH, temperature, and substrate concentration. They also looked into the enzyme's characterization and purification. The extraction method affected the protein's purity, which might range from 7% to 90%. Nonetheless, the separated protein's surface activity, solubility, and denaturation temperature were all greatly impacted by the extraction technique selected.

Proteases are extracellular enzymes that are classified as hydrolases (i.e., they break down proteins by adding water to peptide links) and generally have catalytic properties. Proteases primarily rely heavily on their active sites, substrate types, and modes of action. They contribute significantly to both commercial and physiochemical goals (65% of the enzyme market, for example). Apart from that, because of their unique characteristics, such as their extreme pH

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profiles, proteases are in high demand in a variety of industries, including the detergent, food, and beverage industries for cheese-making, beer clarifying, and beverage manufacturing. (Kirti Rani et.al. 2012)

These enzymes are divided into groups according to a number of criteria, including the kind of substrate, the site of action, the active pH range, and the mechanism of action, which involves a specific amino acid that is present in the active site (Guleria et al. 2016). These enzymes can be generically categorized as endopeptidase or exopeptidase, depending on the site of action. While the latter operate on peptide bonds at the substrate's termini, the former have a tendency to hydrolyze non-terminal peptide bonds, resulting in the creation of shorter peptides.

Additionally, exopeptidases are further classified as amino-peptidases or carboxy-peptidases based on the termini on which they preferentially operate, i.e., whether they act on the N- or C-terminal (Naveed et al. 2021).

DIVISION OF MICROBIAL PROTEASES

Proteases are classified by the Enzyme Commission (EC) as belonging to sub-group 4 (hydrolyzing peptide bonds) and group 3 (hydrolases). Peptides can hydrolyze internal peptide bonds (endopeptidases) or N- or C-terminal peptide bonds (exopeptidases), which has led to the division of proteases into two major categories. In contrast to endopeptidases, which are more significant in the industrial sector, exopeptidases are also employed in certain commercial applications. Separately, exopeptidases are classified as carboxypeptidases, which break the C-terminal peptide bond, and aminopeptidases, which cleave the N-terminal peptide structure. Proteases have also been categorized into different groups based on several other characteristics, including the presence of charged moieties at sites about susceptible bonds, their pH optima (as acidic, neutral, or alkaline), their specificity for particular substrates (collagenase, keratinase, elastase), or their homology to other proteases that have already been studied, including trypsin, pepsin, and others (trypsin-like, pepsin-like). Serine proteases were categorized by Morihara (1999) as trypsin-like proteinases, Staphylococcal proteinases, Myxobacter lytic proteinases, and alkaline proteinases. Endo proteases were categorized into four classes by Hartley (2000) according to their active location and inhibitor sensitivity.

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A. Serine Proteases

A serine group exists in the active site of serine protease enzymes. They are widespread and plentiful in bacteria, viruses, and eukaryotes, indicating the critical role they play in the life of living things. By acylating the active serine residue, organic phosphate esters inactivate serine proteases. With an optimal pH range of 7 to 11, serine proteases are typically active at neutral and alkaline pH values.

B. Cysteine/Thiol Proteases

Prokaryotes and eukaryotes both have cysteine proteases. Their pH ideal range is between 6 and 8, and they contain cysteine at their active site. Reducing substances like hydrogen cyanide can activate them, while oxidizing agents can inhibit them. They are not impacted by DFP or metal-chelating agents, however they are vulnerable to sulfhydryl (-SH) agents like p-CMB. The renewal of the SH group is what causes the activation by reducing agents. Their ideal temperature range is 50–70°C.

C. Aspartic Proteases

Aspartic proteases are generally distributed in animal, yeast, and mould cells but rarely found in bacteria. They are usually known as acidic proteases having aspartic acid remains at their active sites. They show specificity towards aromatic or bulky amino acids residues on both sides of the peptide bond and have optimum pH 3-4. The aspartic proteases are inhibited by pepstatin. Microbial aspartic proteases are further separated into two groups- (a) Pepsin-like proteases and (b) Rennin-like protease

D. Metallo-proteases

The most varied class of proteases that are catalytic are called metallo-proteases. Their need for a divalent ion to function is what distinguishes them. The catalytic mechanism of phosphate esters involves a metal ion, and these enzymes are sensitive to chelating agents but insensitive to sulfhydryl agents. This class of enzymes includes alkaline and neutral proteases from various microbial origins.

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CURRENT STUDIES ON ALKALINE PROTEASES

Raghunath T. et.al. (2002) reported in the control of living things' defense mechanisms, protease is a key component. With particular attention to their proteolytic capabilities, this review provides a thorough and detailed treatment of plant, animal, and microbial enzymes. This review's extensive list of biological sources of proteases amply illustrates the significance of microbial and plant proteases for practical and industrial applications. Proteases may find use in the food, dairy, detergent, leather, alcoholic beverage, brewing, meat, pharmaceutical, and picture industries.

As reported by Maghsoodi et.al. (2013) Alkaline protease has been used as an industrial catalyst more often in recent years. The protease enzyme is essential for many important industrial and commercial processes, including medical diagnosis and the food and textile sectors. A continuous fermentation system may be employed in the cell immobilization approach, which limits the free movement of microorganisms during the process. Using a variety of carriers, including chitosan, corn cob, and corn tassel, this technology has been applied in the current work to produce alkaline protease. The enzyme activity was 78.3 U/ml 72 hours before immobilization. Corn cob was determined to be the optimal carrier due to its high enzyme activity and immobilization capacity of 65%.

Schallmey, M. et.al. (2013) reported that the predominant bacterial workhorses in microbial fermentations are still Bacillus species. A few *Bacillus* species are on the Food and Drug Administration's GRAS (generally recognized as safe) list. *Bacillus subtilis* is the primary microbe involved in the continuous manufacture of the soya-based traditional fermentation. Some *Bacillus* strains are among the most significant commercial enzyme producers due to their ability to manufacture and secrete substantial amounts of extracellular enzymes (20–25 g/L). A range of new commercial enzyme products with the desired temperature, pH activity, and stability properties have been developed to address a variety of specific applications due to the genus's thermophiles and various species' capacities to ferment in the acidic, neutral, and alkaline pH ranges. These products have been developed by utilizing modern cloning and protein engineering techniques in conjunction with classical mutation and/or selection procedures. At

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first, it seemed that the host proteases were impeding efforts to synthesize and secrete large amounts of foreign recombinant proteins in *Bacillus* hosts.

According to another study by Murthy et.al. (2015), potato pulp powder is a significant byproduct that the potato processing industry produces in large quantities all over the world. Pollution of the environment is caused by the dumping of these wastes. The biotransformation of potato pulp powder by solid state fermentation for the synthesis of acid protease, an important food enzyme, is investigated with consideration to the use of agricultural byproducts as economic and environmental benefits. They investigated the biotransformation of potato pulp powder for the synthesis of acid protease, a food enzyme, as an economic and environmental solution. *A. oryzae* RIB 40 was found to be a promising choice due to its high production of acid protease. UV mutagenesis increased the parent strain's productivity, and the solid state fermentation of *A. oryzae* (F6) produced an 11-fold increase in protease activity. Glycine was released by the acid protease, improving food palatability.

Bacillus species proteases are most commonly categorized and utilised; there are reports of some other bacteria proficient of producing alkaline proteases. Production of Alkaline protease by *Streptomyces sp*. inaccessible from saltpan of Algeria was described by Boughachiche et.al. (2016).

In an attempt to create dehairing protease, Shakilanishi et.al. (2017) attempted to extract collagen from Chrome shavings. One of the main proteinous solid wastes of the leather industry, chrome shavings (chromium complexed collagenous waste scrapings), constitute a pollution risk. The recovery and repurposing of the waste's protein components can stop them from ending up in landfills. In order to produce dehairing protease at a lower cost than using protein wastes obtained from agriculture, the collagen hydrolysate made from chrome shavings was evaluated in the current study. Goat skin processing produced the chrome shavings used in the investigation. The designed medium (pH 8.0) containing 12 g/L of collagen hydrolysate from chrome shavings, 15 g/L of molasses, 3 g/L of K_2HPO_4, 2 g/L of NaCl, and 0.04 g/L of $CaCl_2$ was found to yield the maximum enzyme production of 203 ± 0.07 U/mL by *Bacillus cereus* VITS $N0_4$. The protease was stabilized and its activity was prolonged by collagen hydrolysate, as

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demonstrated by fluorescence spectral analysis. Using 22% (w/v) of 2-propanol and 14% (w/v) of K_2HPO_4 aqueous two-phase solution, the protease was purified with a yield of 88.1%.

The purity and activity of the enzyme were validated by native-polyacrylamide gel electrophoresis. In general, the categories of alkaline proteases and neutral proteases comprise the bacterial proteases that are utilized for diverse commercial applications. Alkaline proteases have been used for a considerable amount of time, particularly the extracellular proteases from the *Bacillus* genus, which are typically serine proteases (Pathak and Rathod 2018).

Nikolai Samburov et.al. (2018) state that Enzymes that are highly sought after and found in a wide variety of plants, animals, and microorganisms are known as proteases. They are crucial to biotechnological and industrial applications, which provide up lucrative business potential. It's needed to identify new and developing ways that could lower the expense. Therefore, the goal of this review is to provide a thorough overview of the physiological and biological activities of these enzymes, as well as an introduction to the various varieties. Lastly, the study will cover recent research on the potential of agro-industry and other low-cost sources and techniques for producing proteases.

Sudha et.al. (2018) showed that by using 16s rRNA gene sequencing, a new, powerful terrestrial bacterium that produced proteases was discovered and named *Exiguobacterium profundam* sp. MMI. Optimizing the culture conditions led to an increase in protease output. For the highest protease yield, physical and nutritional parameters like temperature, pH, and duration of incubation were tuned in addition to supplies of carbon and nitrogen. Research on the effects of various sources of carbon and nitrogen has shown that peptone and glucose increase the production of enzymes. For the synthesis of enzymes, 35°C and pH 9 were the ideal values, respectively. Using conventional protein markers, the protease enzyme from *Exiguobacterium profundam* sp. MMI MG951843.1 was electrophoresed on 10% (w/v) SDS-PAGE after being largely purified by ammonium sulfate precipitation. Seven bands can be seen in the electrophoretic profile of the partly purified enzyme extract, and the value of PM was 48.3 KDa, derived from the curve calibration markers to the known weight. 37.2, 28.2, 26.5, 24.5, 20.4, and 16.5 kDa. Protease is present in the sample as shown by the band at 48.3 kDa. A zymogram was used to measure the partly purified protease enzyme's caseinolytic activity.

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Bacillus subtilis strains and other similar members that are utilized to produce commercial enzymes are considered GRAS (generally recognized as safe). (Zhang et.al. 2019)

Zhou et.al. (2020) reported genetically modified *B. licheniformis* 2709, resulting in a 62.19% increase in alkaline protease production and a decrease in the synthesis of muco polysaccharides and other foaming factors.

The pH range of 8–12 is often the optimal range for bacterial alkaline proteases. According to Naveed et.al. (2021), many of these enzymes also exhibit increased thermostability and activity at higher temperatures.

PRODUCTION OF MICROBIAL PROTEASES

The majority of alkaline and neutral commercial proteases are made by bacteria in the *Bacillus* genus. The pH range in which bacterial neutral proteases are active is limited to pH 5-8, and they exhibit minimal heat tolerance. Neutral proteases are useful in the food industry because, compared to animal proteases, they produce less bitterness in hydrolyzed food proteins due to their intermediate pace of reaction. Neutrase is a neutral plant protease that is helpful in the brewing business because it is not affected by inhibitors.

The strong affinity that bacterial neutral proteases have for pairs of hydrophobic amino acids is one of their defining features. They can control their reactivity during the generation of food hydrolysates with a low degree of hydrolysis thanks to their low thermo tolerance. Serine proteases are unaffected by chelating chemicals however, some neutral proteases are part of the metalloproteases category and need divalent metal ions to function. The broad substrate specificity and high activity at alkaline pH 10, are characteristics of bacterial alkaline proteases. It is ideal for them to be at about 60°C. Bacterial alkaline proteases are useful in the detergent business because of these characteristics. (Mukesh Chander &Punit Puri2019)

Alkaline proteases can exhibit distinct properties when they are free and in an immobilized condition. Alkaline proteases can be easily repurposed and recovered after being immobilized for industrial usage. (Kumar, C.G. & Takagi, H. 1999)

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Fermentation in both submerged and solid states can provide proteolytic enzymes. Solid state fermentation is the best technique for fungal development since it mimics the fungi's natural environment. Sold-state fermentation (SSF) is favored over submerged fermentation (SMF) due to a number of factors, including its ease of use, low cost, high enzyme yields and concentrations, and utilization of widely accessible and reasonably priced agricultural leftovers as substrates (Chutmanop et al., 2008).

Many different types of alkalophilic microbes can produce extremely alkaline conditions that proteases are resistant to. The screening and selection of potential organisms for industrial production is made possible through the discussion of various isolation techniques. To boost the producer strain's efficiency to a commercial level, mutagenesis and recombinant DNA technology can be used for strain improvement. The several dietary and environmental factors that influence alkaline protease production are outlined. Numerous researchers also covered the characteristics and purification of these proteases, emphasizing the usage of alkaline proteases in a variety of industrial applications (Ganesh and Hiroshi, 1999).

The top *Bacillus subtilis* strains that produce proteases were examined by Geethanjali and Anitha (2011). Their research showed, for a 48-hour fermentation period, the pH, temperature, carbon, and nitrogen sources in the *Bacillus subtilis* production medium were adjusted. that using carbon as glucose and nitrogen as peptone, protease production may be optimized at pH - 9.0 and temperature 40 ˚C.

Some cultivation parameters were studied by Ruann Janser Soares and Helia Harumi (2013) and the results showed that the optimum fermentation medium was composed of wheat bran, 2.0 % (w/w) peptone and 2.0 % (w/w) yeast extract, and the conditions for maximum protease production were an initial moisture content of 50.0 %, an inoculums level of 107 spores g -1 and an incubation at 23 °C. Based on experimental design, the biochemical characterization revealed that the enzyme was stable between pH 4.5 and 6.0 and most active between pH 5.5 and 5.0, suggesting that it was an acid protease. 55 to 60°C was the ideal temperature range for activity, whereas 35 to 45°C was the stable range for the enzyme. Based on the findings, wheat bran has a lot of promise as a matrix to assist *Aspergillus oryzae's* generation of proteases during Solid State Fermentation (SSF).

APPLICATION OF ALKALINE PROTEASE

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From an industrial perspective, proteases are among the most significant types of enzymes, accounting for a significant portion of the global enzyme market. The most common application for proteases is as active components in detergents. In addition, they find extensive application in the leather sector, medical diagnostics, X-ray film recovery of silver, silk degumming, and food and feed industries, among other fields. Owing to their extensive utilization in industrial operations, numerous businesses commenced producing them on a commercial scale.

Chemical Industry

Applications employing biocatalysts in non-aqueous medium for peptide synthesis significantly value a high stability in the presence of organic solvents. Because of their stability in organic solvents, alkaline proteases from *Aspergillus flavus, Bacillus pseudofirmus* SVB1, and *Pseudomonas aeruginosa* PseA have demonstrated encouraging results for the possibility of peptide synthesis (Sen, 2011). Alkaline proteases from *Streptomyces sp*. strain AB1 and *B. pumilus* strain CBS exhibit significant organic tolerance and could be excellent choices for peptide synthesis in low-water settings. They have also been documented for organic synthesis in addition to peptide synthesis. The precursor di peptide of RGDS (Arg-Gly-Asp-Ser), Bz-Arg-Gly-NH (N-benzoyl argininyl glycinamide), is synthesized using Alcalase, an industrial alkaline protease, and is catalysed in water/organic co-solvent systems (Yang et.al.2006). According to Wang et.al. (2011), alkaline protease from *Bacillus licheniformis* is used in the synthesis of 2H-1-benzopyran-2-one derivatives. The polymerizable vinyl guaifenesin ester's region-specific synthesis has been examined using an alkaline protease derived from *Bacillus subtilis*.

Detergent industry

Modern industrial and domestic detergents have considerably benefited from the invention and improvement of alkaline proteases. They work well in the modern industrial and institutional cleaning environments of moderate temperature and pH levels. The industry uses a variety of enzymes, including cellulases, proteases, lipases, and analyzers (Ito et al., 1998). Because alkaline proteases may hydrolyze and remove proteinaceous stains such as blood, eggs, gravy,

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milk, etc. in high pH settings, they are widely used as detergent additives (Saeki et al., 2007). When included in detergent formulations, proteases and other enzymes should have high activity and stability throughout a wide pH and temperature range. The enzymes ought to work at low concentrations (0.4–0.8%). One of the most challenging design problems for biotechnologists is making the protease work with a wide range of commercial detergents and not be affected by common detergent ingredients like builders, surfactants, bleaching agents, bleach activators, fillers, fabric softeners, and other formulation aids. In the presence of specific stabilizers like CaCl2 and glycine, alkaline proteases from *Bacillus cereus*, *Bacillus pumilus* strain CBS, *Streptomyces sp.* strain AB1, *Bacillus licheniformis, Aspergillus flavus, Aspergillus niger, Bacillus brevis,* and *Bacillus subtilis* AG-1 have recently shown excellent detergent compatibility. Subtilisins have been enhanced in terms of their thermal stability and resistance to cheaters in order to withstand the extremes of high alkalinity and cheaters concentration in detergents (Bryan, 2000). Several oxidative stable serine proteases (OSPs) that are appropriate for usage in detergents have been identified from alkaliphilic Bacillus strains in order to prevent the loss of activity.

Photographic Industry

Silver has been successfully recovered from X-beam films using soluble proteases provided by *B. subtilis, Streptomyces avermectnus,* and *Conidiobolus coronatus*. This ensures that the cycle is more environmentally friendly than using synthetic compounds (Yang, et al., 2000). Its latent capacity has also been explained by silver recovery through the efficient use of thermally stable freak basic protease supplied by *Bacillus sp*. B21-2 (Dhawan and Kaur, 2007; Araujo et al., 2008).

Silver recovery

Among its many uses in jewelry, X-ray films, electronics, silverware, and photographic films, silver is an essential industrial metal. Silver may be recovered and repurposed because it is not destroyed during photography. 1-2 percent (w/w) silver is present in the gelatin layers of X-ray and photographic films. When used X-ray films are bioprocessed to recover silver, alkaline protease is a key component. The X-ray film's gelatin layer containing silver is removed by the

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enzyme. X-ray films were burned as part of the traditional silver recovery process, which harmed the environment. (Shankar S. et.al. 2011)

Waste treatment

In nature, fibrous proteins such as hair, nails, feathers, and horns are abundant waste materials. Waste materials can be transformed into valuable biomass by using the alkaline protease enzyme found in microbes. Protease solubilizes proteinaceous wastes, lowering the biological oxygen demand (BOD) in aquatic systems. Alkaline protease is a key player in waste management because of its ability to break down trash from homes and food processing companies. Chicken feathers could be broken down by the alkaline protease enzyme found in *Serratia sp.* HPC 1383. The observations revealed that it is possible to turn the vast amounts of chicken feathers produced by the poultry industry into extremely digestible animal feed. (Khardenavis AA et. al. 2009)

Food industry

The high nutritional value protein hydrolysate has been prepared using alkaline proteases. Protein hydrolysates come in a variety of forms and can be made from dietary proteins. In addition to being utilized in infant food formulations, certain therapeutic dietary items, and the fortification of fruit juices and soft beverages, protein hydrolysate is also crucial in the regulation of blood pressure. (Neklyudov AD et.al. 2000)

Brewing industry

The brewing industry uses proteinases to digest proteins and prevent the clouding of beer after chilling. It's crucial to remember that natural proteases are active when grains—typically barley—malt. These enzymes contribute to the clarity of the brew and are more nutritionally significant since they are both a substrate for the growth of yeast and an ingredient in the final product. The homogeneous protein distribution is not desired because it makes the foamy beer taste "empty" and loses its flavor. On the other hand, an imbalance in the distribution of proteins makes filtering more difficult and makes beer cloudy in storage. (Sanders et.al. 2018)

Pharmaceutical applications of Alkaline protease

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Alkaline proteases have a wide range of applications in medicine, from basic molecular activities to therapeutic uses for the entire organism, including inflammation and hemostasis. In the pharmaceutical sector, alkaline proteases are widely employed in the manufacturing of medications, including ointments for wound debridement. These days, alkaline proteases are widely employed, with Serratio peptidase being the most efficient type. It is possible to treat inflammation with alkaline protease. Furthermore, the anti-inflammatory properties of a class of serine protease from Indian Earthworms have been investigated. When a serine protease from Indian earthworms was tested in 2014 against breast cancer cell lines, the results demonstrated the enormous potential of proteases in the creation of anti-cancer treatments. In the future, enzymatic therapy may be a more effective drug than conventional approaches for treating cancer and other illnesses. (Saranraj P and Naidu M.A.,2014)

CONCLUSIONS

Modern biotechnology techniques have come a long way from lab curiosity to commercialization in a comparatively short amount of time. Recent developments in biotechnology and microbiology have created a supportive atmosphere for proteases' advancement and will continue to support their uses to provide a sustainable environment for human improvement. Alkaline protease has enormous promise in a variety of industries, including the food, detergent, leather, and pharmaceutical sectors. Currently, it takes an hour to investigate novel microorganisms for the synthesis of enzymes, which should have the adaptability to meet commercial demands.

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