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Latest Overview of Proteases: A Review

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ABSTRACT: Proteolytic enzymes are found in all kinds of organisms i.e. viruses to animals. These peptidases are included in vast group of the enzymes used in the bio industry with wide applications. They play vital part in the industrial biotechnology specifically in food, detergent and the pharmaceutical industries. Microbial proteases which are environmental friendly are interestingly used for their commercial importance. This review displays an overview on proteases chiefly from sources of micro-organisms i.e. bacteria and fungi and their common properties are also discussed briefly. Proteases assume an essential part in detergent, pharmaceutical, leather, food industry, agricultural ventures. Microbial proteases assume an imperative part in various ventures, above all in the leather preparing, silver recuperation, medical purposes, food preparing, feeds, chemical enterprises, waste treatment.

Keywords: Enzymes; Protease; Source; Production and Application.

INTRODUCTION: Enzymes are macromolecular biological catalysts that accelerate chemical reactions. The molecules upon which enzymes may act are called substrates, and the enzyme converts the substrate into different molecules known as product. The first enzyme to be discovered was amylase[1] in which was originally isolated from barley and catalysis the conversion of starch into sugars. Enzymes have been utilized for thousands of year in microbial processes. Microbes & their enzymes have been applied for preparations of wines, beer, cheese and other milk products. The role of enzyme in fermentation process has been known for less than two hundred years. Versatility of protein molecules present in their diverse functioning including catalytic capabilities and structural organization. Enzymes are considered biocatalyst as they are involved in producing these molecules. Both fungal and bacterial species produce these molecules.[2] Protease also known as proteinase or peptidase is an enzyme group which is involved in proteolysis. Proteolysis is the hydrolysis of peptide bonds. Peptide bonds help in formation of polypeptide chain by linking amino acids together. Proteases have industrial uses and include more than 70% of the industrial enzyme.[3] Fungi and bacteria are the major source of production of this enzyme. Proteases are eco-friendly as the sources of production are generally non-pathogenic and non-toxic.[4]

Sources of Proteolytic Enzymes: Proteolytic enzymes are produced by all life forms such as plants, animals, and microorganisms. Enzymes from all these sources have been used during some point or other in the course of human history. However, to meet the huge demand of industries, microorganisms have been the mainstay of the source of proteolytic enzymes. A high yield of proteolytic enzymes can be obtained by culturing microorganisms, which grow in a short time and require less space compared to plant or animal sources.

Figure 1: Commercial available protease products.

Proteolytic Enzymes from Plant Sources: Proteolytic enzymes in plants are involved in key physiological process like photo inhibition, photomorphogenesis in seeds, and senescence.[5] Cysteine proteases derived from plant sources, such as papain and bromelain from papaya and pineapple, respectively, have been used as meat tenderizers as well in baking and production of protein hydrolysates.[6] The number of plant proteases used in industry is not very high, since 2005 proteolytic enzymes from plant sources have come into special focus for their therapeutic applications, as

many of them are active over a wide range of temperature and pH.[7] A protease isolated from the latex of *Synadenium grantii* with human fibrino (geno) lytic activity was reported.[8] Another cysteine protease, Pergularain from *Pergularia extensa* latex, has been studied, and it acts similar to thrombin in releasing fibrino peptides A and B from fibrinogen and thus has potential therapeutic use.[9] Plant cysteine proteases have been recognized to have antihelminthic activity and roles in mammalian wound healing, immune modulation, and treatment of digestive disorders.[10] Fastuosain, from *Bromelia fastuosa* and bromelain from *Ananas comosus* with antitumor properties have been reported.[11]

Proteolytic Enzymes from Animals: Pancreatic trypsin, chymotrypsin, pepsin, and chymosin (rennet) are the most important industrial proteases of animal origin. Chymotrypsin from pancreatic juice is used for diagnostic purposes. These enzymes were being obtained from slaughtered cattle; however, to meet the demands from industry, recombinant versions are being produced and tested for efficacy. Trypsin is a serine protease and is used in preparing medium for growing bacteria for research and industrial purpose.[12] The performance of animal derived trypsin compared to recombinant trypsin for use in clinical applications was similar.[13] Chymotrypsin is obtained from animal pancreatic extract and thus is an expensive enzyme. It has diagnostic and other analytical applications. A 2012 US patent (Patent No. US20120135460 A1) explains the production of recombinant porcinechymotrypsin and its use in food applications. Rennet is a pepsin-like protease, which is produced in the stomach of nursing mammals. Rennet from calf had a huge demand in the dairy industry for cheese production that is now being met by fungal enzymes.[14] The quality of the cheese made with recombinant chymosin has been shown to be comparable with that of cheese made with calf rennet.[15]

Table1: Sources of protease.

Enzyme	Source
Endopeptidases	
Trypsin	Animal
Collagenase	Microbial
WNV Protease	Microbial
Papain	Plant
Pepsin	Animal
Endoproteinase	Microbial
Thrombin	Animal
Thermolysin	Microbial
Ficin	Plant

Proteolytic Enzymes from Microbial Sources: Most microbial proteases are extracellular and thus are directly secreted into the fermentation broth, which makes the downstream processing easier to obtain the pure enzymes in bulk quantities compared with the proteases obtained from plants and animal sources. The most widely used microbial proteolytic enzymes come from bacterial and fungal sources. Proteolytic enzyme-producing bacteria as well as fungi have been isolated from various sources like mangrove sediments, seawater, offshore oil fields, poultry compost, and industrial effluents.[16-20] Bacterial proteolytic enzymes are generally active in neutral oralkaline pH. Members of the *Bacillus* and *Streptomyces* genera are the most commonly employed at the industrial level. Fungi, on the other hand, are versatile in enzyme production and produce acidic, neutral, and alkaline proteases. Bacterial neutral proteases are active in the pH range 5-8 and have low thermostability compared to the bacterial alkaline proteases. Neutrase is a neutral proteolytic enzyme used in the food industry for protein hydrolysate production.[21] Alkaline proteases from bacteria show optimal activityat alkaline pH and are quite thermostable, for example, proteases produced by *Bacillus licheniformis* and *Staphylothermus marinus.*[22] The bacterial proteases compared to fungal proteases possess higher thermostability and also higher reaction rate, except for enzyme produced by the thermophilic fungus *Malbranchea pulchella.*[12, 23]

Alkaline proteases have the largest market share as they are produced by the detergent and leather industries, which require these enzymes in huge amounts.[23, 24] Bacteria from the Bacillus genus have been a predominant source of alkaline proteases. Almost all proteases used in detergent formulations are subtilisins produced by *Bacillus* spp. new subtilisin-related recombinant proteolytic enzyme from a fungus, *Malbranchea sp.*, and produced by *Trichoderma reesei* has been introduced in detergent formulations.[23] Proteolytic enzymes from *Bacillus* and *Aspergillus* have replaced animal feces as a source of proteases in the leather-processing industry. Because of high demands for rennet in the dairy industry alternate sources for chymosin have been explored. Proteases from *Mucor michei*, *Bacillus subtilis*, and *Endothia parasitica* are now used for cheese production by the dairy industry. A proteolytic enzyme from *Aspergillus oryzae* is used in the modification of wheat gluten.[12] An extracellular solvent-stable alkaline metallo protease from *Pseudomonas aeruginosa* has been characterized as having the potential for synthesis of enzymatic peptides.[24] Leucine amino peptidases are being used in the production of protein hydrolysates. *Streptomyces* amino peptidases have garnered interest for protein hydrolysate production in the food industry because of their stability, high enzyme activity, and broad substrate specificity.[25]

Table 2: Fungal and bacterial sources of proteases.

Fungal Sources	Bacterial Sources
Aspergillus candidus	*Pseudomonas aerogenosa*
Aspergillus flavus	*Microbacterium sp.*
Aspergillus fumigates	*Lactobacillus helveticus*
Aspergillus niger	*Streptomyces microflavus*
Aspergillus oryzae	*Streptomyces vectus*
Rhizopus oligosporus	*Pseudomonas maltophilia*

Production of Proteolytic Enzymes: Enzyme-mediated processes are economically comparable to chemical process nowadays. Therefore, the reduction in the cost of enzyme production is a positive stimulus for the commercialization of enzyme-based processes. Proteolytic enzymes are one of the most important groups of industrial enzymes and account for nearly 60% of total enzyme sales.[12, 27]

Proteolytic enzymes can be produced by plants, animals, and microorganisms. The inability of the plant and animal proteolytic enzymes to meet current world demands has led to an increased interest in microbial sources. The relative ease of genetic manipulation and biodiversity of microorganisms make them a highly used source of proteolytic enzymes. Microbial proteases account for 40% of the total worldwide enzyme sales.[27] Microbial proteolytic enzyme production depends on the microorganism, medium composition, physicochemical properties, and the method of production. It is estimated that around 30-40% of the cost of production of industrial enzymes can be attributed to the cost of the growth medium.[28]

Selection of the microorganism is important to obtain the desired product. The microorganism should be able to secrete large amounts of proteolytic enzymes and give adequate yields in a short time period. The production of proteolytic enzymes is also affected by the medium components such as carbon and nitrogen sources and supplementation of mineral salts. Easily metabolizable carbon sources such as maltose, starch, molasses, wheat bran, and coffee pulp and coffee husk enhance the production of proteolytic enzymes.[29-31] Corn steep liquor, soybean meal, fish meal, and yeast extract enhance the production of proteolytic enzymes, whereas free amino acids decrease the production.[32] Proteolytic enzyme production is also affected by the physicochemical properties such as pH, temperature, moisture content, incubation period, inoculation size, and aeration.[33, 34] Proteolytic enzyme production is enhanced by vitamins such as biotin and growth promoters such as 1-naphthylacetic acid.[35] Microbial proteolytic enzymes are produced by fermentation methods. The success of fermentation depends upon the usage of low-cost raw materials, enzyme productivity, and ease of product recovery from the fermentation broth.

Solid-State Fermentation: Solid-state fermentation (SSF) utilizes a solid substrate in the free form and the absence of liquid for the growth of microorganisms. Water is either adsorbed on a solid supportor complexed with a solid matrix for the growth of the microorganisms. SSF is considered more natural than other types of fermentation, because it has conditions similar to those under which most microorganisms grow in nature. SSF has simple fermentation equipment and results in high volumetric productivity, relatively high concentration of product, less effluent generation, and relatively easy downstream processing.[36] Higher production of proteolytic enzyme can be achieved by using the best combination of medium components, such as carbon and nitrogen sources, metal ions, and surfactants, as well as by using optimized physicochemical properties such as pH, temperature, agitation, aeration, and inoculum size. Agro-industrial waste products such as wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, sawdust, corncobs, tea waste, aspen pulp, sugar beet pulp, peanut meal, groundnut oilcake, and mustard oil cake are generally used as the substrates for the SSF process for the production of proteolytic enzymes.[37, 38]

Submerged Fermentation: Submerged fermentation (SmF) uses a liquid fermentation medium with soluble nutrients. The substrate is dissolved or suspended in water, which is not a limiting factor. This fermentation process can be performed in shake flasks, a bench-scale fermenter, or an industrial-scale fermenter. In this type of fermentation, microorganisms are able to utilize a variety of carbon sources such as maltose, corn starch, etc. An industrial by product of the sugar industry, i.e., molasses, has been utilized extensively as a raw material for the carbon source. In addition, glucose is used as the main carbon source for the production of extracellular proteolytic enzymes in SmF with *Mucor mucedo*.[39] Higher proteolytic enzyme production was achieved in medium supplemented with peptone, followed by beef extract, casein, yeast extract, tryptone, and $NaNO_3$, with soybean meal as the organic nitrogen source.[40] Scale-up studies of proteolytic enzyme production from *Serratia marcescens* grown on fresh whey were performed.[41] Peanut meal was the best nitrogen source for proteolytic enzyme production compared to casein, peptone, and skim-milk powder.[42]

Immobilized Cell Technology: Immobilization of microbial cells has become popular in the field of proteolytic enzyme production. Immobilized cell

technology is often used to improve the bioprocess to gelatin enhanced yield of proteolytic enzymes as well as their long-term use and stability. Several natural and synthetic polymer matrices such as alginate, carrageenan, cellulose, agar, agarose, gelatin collagen, and polyacrylamide have been reported for immobilization of various microbes. Calcium alginate and polyacrylamide were found to be the best matrices for production of proteolytic enzymes.[43-46] Gelatin and radiated gelatin also manifested better results compared with calcium alginate.[47-49]

Purification and Characterization of Proteolytic Enzymes: Proteolytic enzyme purification processes may vary from single-step purification to multistep procedures. The enzyme purification strategy is based on information about the level of purity required, source of enzyme, properties of the target enzymes, and accompanying impurities. It is also important to determine the scale of purification.

The first step toward obtaining pure enzyme is the isolation from the source, which is generally microbial cells, a plant, or an animal tissue. Most of the industrial proteases are now obtained from microbial sources. The extracellular proteases are easy to separate from the cells by filtration or centrifugation.[18, 50] The retentate obtained after filtration or the culture supernatant obtained after centrifugation is concentrated by ultrafiltration.[51, 52] Various properties of enzymes like solubility, charge, size, and binding affinity have been exploited at various stages of purification. Ammonium sulfate at high concentrations has been widely used to purify active forms of proteases by reducing the solubility, resulting in precipitation. Different proteins precipitate at different concentrations of salt; therefore, by making a concentration gradient of ammonium sulfate several fractions containing different proteins can be obtained.[52] A detergent-stable alkaline protease from *Streptomyces koyangensis* has been purified by using this method.[18] Purification has also been done by ethanol-induced precipitation.[51] A thermo-stable alkaline protease produced by *Aspergillus niger* has been purified by this method.[53] The precipitated protease is recovered by centrifugation followed by dialysis to remove the salt or solvent. Aqueous two-phase systems (ATPSs) have also been used for purification of proteases. ATPSs comprise two water soluble polymers or one polymer and salt.[54] Sodium polyacrylate and polyethylene glycol-based ATPS has been used for purification of proteases expressed by *Penicillium restrictum.*[55] A modified organic solvent/salt-based ATPS using alcohol and salt combinations was used for purification of serine proteases from mango.[54] This method has several advantages including low cost, low toxicity, and easy recovery of alcohol compared to polymer-based ATPS. Precipitated proteases are recovered by centrifugation followed by dialysis to remove the salt. Intracellular proteases are isolated from the host cells by rupturing the cells by using a French press.[56] The cell extract is subjected to centrifugation to obtain a clarified cell-free extract. The second step of purification, to remove most of the impurities from the isolated enzyme, is a chromatography technique applied based on charge, size, or binding affinity. Gel filtration efficiently separates the molecules based on size. The dialyzed enzyme obtained after precipitation is loaded onto a pre-equilibrated gel-filtration column. During elution small proteins are retarded to a greater extent compared to the larger proteins, which elute in the initial fractions. The fractions that contain the protease are pooled and concentrated. A fungal proteolytic enzyme present in the culture filtrate was precipitated by using ethanol and purified by gel filtration using Sephadex G-100.[20] The ion-exchange chromatography technique utilizes the charge on the protein to bind to the column matrix. The proteins are eluted by varying the pH, which may change the surface charge depending on the pH, or by increasing the ionic strength of the elution buffer to enhance the affinity of proteins toward the mobile phase.[18, 56] The dialyzed filtrate obtained from filtration of the fermentation broth or the cell-free extract can be directly loaded onto an ion-exchange chromatography column. An alkaline protease was purified by a cation-exchange Mono-S Sepharose column by using a concentration gradient (0e500 mM) of NaCl.[18]

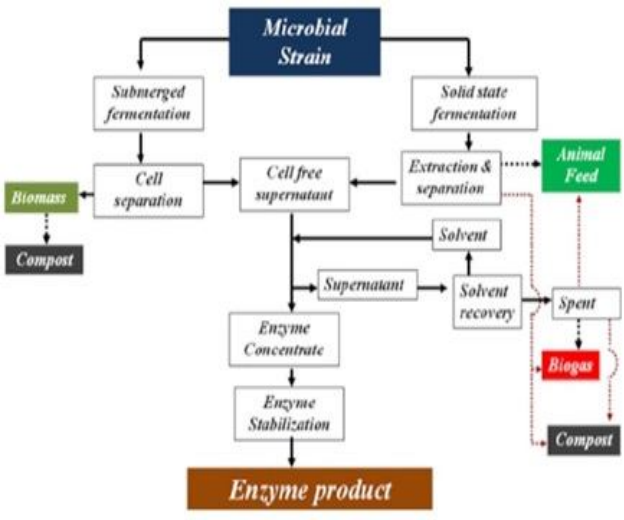

Figure 2: Flowchart of enzyme extraction.

Commercial applications of proteases: These are important enzyme group having different commercial applications involved in the food processing, in detergent industry, leather making and for therapeutic applications. In food industry, beverages, and milk in-

dustry, bakery and grains processing use huge quantity of protease and additional enzymes from various sources containing fungi. Ethanol fermentation, making of detergent for the biological applications have enhanced in the last few decades.

Table 3: Applications of proteases in different sectors.

Proteases	Industry	Application
Papain	Beverages	Haze removal and chill proofing
Neutral protease	Baking	Conditioning of dough
Fungal proteases and chymosin	Dairy	Calf rennet replacement, EMC Production, processing of whey protein
Subtilisn, alkaline protease	Detergents	Detergents for removal of stain of protein
Trypsin and other proteases	Leather	Leather bating, dehairing from skin
Several proteases	Food processing	Protein rich material modification
Trypsin	Medicine	Removal of dead tissues, dissolution of the blood clot

Protease in food processing: Different enzymes are required in food processing industries for making of nutritional and good quality food products. Proteases particularly play important role for this purpose. Production of candies and milk products require full processing that is catalyzed by sequence of the enzymes comprising of proteases. Processing of substrate is essential for both hard and soft drinks in beverage industry for good flavor and enhancement of shelf life of the product. Accurate oxidation of the raw material (berry, ripen seeds and leaves) is necessary for processing of coffee, cocoa powder and tea to form new items. In this proteases play significant role.[57]

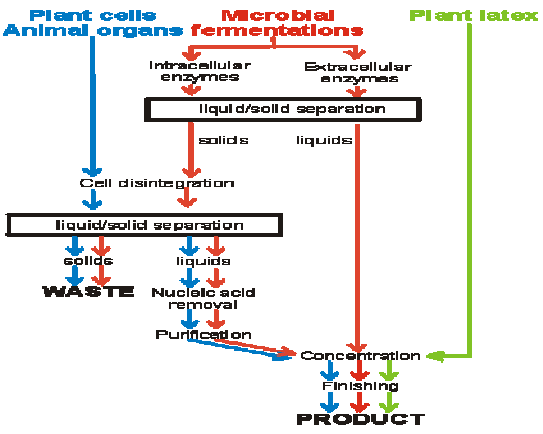

Figure 3: Procedure to enzyme extraction.

Proteases in Detergent Industries: Discovery of the thermostable protease improved effectiveness of the biological detergent. In current days, both the domestic and industrial detergents are using these enzymes highly. Use of proteases and amylases in laundry detergents is very common. Most of the powdered bleach additives contain enzymes which help in breakdown of the stains which are quite hard to remove by conventional surfactants alone. In detergents, protease help in hydrolyzing large sized protein molecules which are connected with the hard stains.[58]

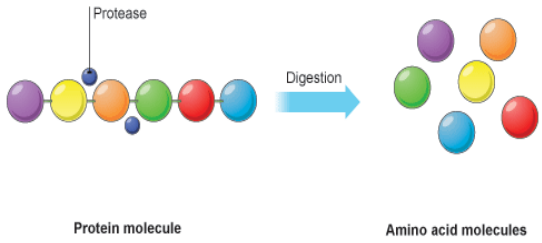

Figure 4: Enzyme action on protein.

Microbial proteases in leather Industry: Leather and detergent industries need use of polluting and toxic chemicals to break down proteins. In leather industries, chemicals are utilized in dehairing, soaking, degreasing and bating to remove the proteins linked with collagen.[59] Processed leather is obtained by going through these steps: soaking, bating, liming, deliming, degreasing, dehairing, and pickling.[60] All these steps are carried out by toxic chemicals such as sodium sulphide, solvents, lime and salts etc. so. It also adds to the environment pollution. Collagen is major leather forming protein which is present in skins and hides in connection with several globular proteins, like mucoid, albumin, globulin and fibrous proteins as reticulin, elastin and keratin.[58]

Plate1: Proteolytic activity on skim milk agar.

Silver recovery: Alkaline proteases have also applications in bioprocessing of utilized X-ray films for the silver recovery. Utilized X-ray films consist of about 1.5 to 2% silver. The conventional exercises of the

silver recovery by burning the films cause chief pollution problems of environment. Thus, the enzyme hydrolysis of the gelatin layers on X-ray films enable not only the silver, but the polyester film base to recycle. The alkaline protease from Bacillus coagulans PB-77 and Bacillus sp. B21-2 decomposed gelatinous coating over utilized X-Ray films.[61]

Waste treatment: Alkaline protease offer great applications for organization of waste from different industries of food Processing and the domestic activities. These have ability to make proteins soluble into the wastes via several step processes to recover the concentrates of liquids or the dry solids of great nutritional assessment for the fish or the livestock.[62] Enzymes may work on the precise unmanageable pollutants to exclude them via transformation or precipitation to the products. Alkaline proteinases from *Bacillus subtilis* are used in waste feather processing from the poultry slaughter houses.[63]

Textile Industry: Most significant commercial application of the proteases is textile industry in which enzyme treatment gives finishing elegant texture.[64] A thermostable protease is used in removing gum and impurities present in the core protein fiber specially in silk used world widely as most precious fiber. Degumming of silk is a growing industry; it utilizes proteases in large amount and forms fine quality of silk. Synthetic fabric is also treated with protease for the elegant and smooth finished product. Fungal proteases are most important enzymes in textile industry since a decade and it is increasing greatly. Indian sericulture has grownup double in the last one decade and the consumption of protease also enhances in various folds.[65]

Chemical industry: Enzymes present in organic solvents have their applications of biocatalysts in chemistry. The disadvantage of this method is reduced activity of enzymes under anhydrous conditions. Thus, we can find ways to activate enzymes in organic solvents due to practical importance.[61]

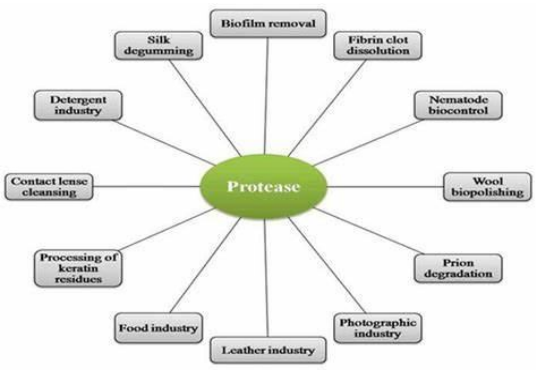

Figure 5: Application of protease in different sectors.

Future Scope: Proteases are unique class of the enzymes as they are of great physiological and commercial importance. They have both synthetic and degradative properties. Since, proteases are physiologically essential, they are found ubiquitously in microbes, animals and plants. However, microbes are goldmine of proteases because of their fast growth, limited area needed for cultivation and their accessibility to the genetic manipulation. Microbial proteases have been greatly used in leather, food, dairy and detergent industries from ancient times.[66]

CONCLUSION: Proteases assume a part of driving catalysts having an incredible business esteem these are utilized broadly in industry and furthermore has a considerable measure of remedial applications. Proteases from fungi are not limited for applications in industry, their therapeutic potentials have been classified in last few years it constitutes one of the biggest groups of commercially important enzymes and modern techniques are needed to find these amazing and useful molecules for humans. Thermophilic fungi are the vital components of the micro-flora that are developed in stacked masses of materials including, plants, heaps of items that are forestry or rural, and other gathered natural matter in which the warm, muggy, aerobic condition gives the essential conditions to their advancement.

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