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RESEARCH ARTICLE

A review on microbial proteases

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Abstract

The focus of the present review is to provide an updated overview on the major sources and important applications of the microbial Proteases produced by a wide range of microorganisms that could resist extreme environments. In recent years there has been a phenomenal increase in the use of microbial proteases as industrial catalysts. These enzymes offer advantages over the use of conventional chemical catalysts for exhibiting high catalytic activity , high degree of substrate specificity, economically viable and can be produced in huge amounts etc. The selection of this topic is mainly based on the different characteristics of proteases to address wide application in various industrial sectors. Protease enzymes constitute one of the most important groups of industrial enzymes being extensively used in the food, pharmaceutical, protein hydrolysis, detergent, cheese-making, brewing, photographic, baking, meat, leather industries , inclusions in animal and human food as digestive aids etc. It can be obtained commercially from plants, animals and microbial sources.

Introduction:-

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms and are essential for cell growth and differentiation. Extracellular proteases are of commercial value and find multiple applications in various industrial sectors. Protease refers to a group of enzymes whose catalytic function is to hydrolyze proteins. They are also called proteolytic enzymes or proteinases. Proteases are classified according to their structure or properties of the active site. Accordingly there are several kinds of proteases such as serine, metallo, carboxyl, acidic, neutral and alkaline proteases. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers (Gupta, *et al*., 2002b). Of these, strains of *Bacillus sp*. dominate the industrial sector (Gupta *et al*., 2002a).

Protease enzyme accounts for about 70% of total enzyme sales market (Rao *et.al*, 1998). A protease (also called peptidase or proteinase) is any enzyme that perform proteolysis, that is it begins protein catabolism by hydrolysis of peptide bonds that links amino acids together in a polypeptide chain. Protease represents the largest category and constitutes a very large complex group of enzymes with extensive variation in physicochemical and catalytic properties (Purva *et.al*, 1998b). These enzymes have attracted considerable attention during the last decade. About 500 tonnes of these enzymes are produced per annum. The world market for industrial enzymes is estimated to be 1.6 billion US$, split between food enzymes (29%), feed enzymes (15%) and general technical enzymes 56%.

Most detergent proteases currently used in the market are serine proteases (Rao *et.al*, 1998). It is estimated that *Bacillus* sp enzymes make up about 70% of total enzyme market (Schallmey *et.al*, 2004). Among the proteases available, alkaline proteases are the most significant segment representing 25% of the total enzyme sales and are equivalent to 89% of the total microbial protease used as detergent additives (Purva *et.al*, 1998a).

Research on proteolytic enzymes has been carried out worldwide since last century. Earlier works around 1960s were mostly the isolation and characterization of protease enzymes. Microbial proteases are preferred to plant and animal sources due to various advantages (Ward, 1985). The ability of different species to ferment in the acidic, neutral and alkaline pH ranges, combined with the presence of thermophiles in the genus, has led to the development of a new variety of commercial enzyme products with the desired temperature, pH activity and stability properties to address a variety of applications (Schallmey *et.al*, 2004).

Numerous moulds especially those belonging to genera *Aspergillus, Penicillium, Rhizopus* and *Mucor* are known to produce a variety of proteases (Mulimani *et.al*, 2002). However these fungal proteases have lower reaction rate and low heat tolerance than bacterial proteases. Microbial proteases, which are of interest for application in the food industry, are of endopeptidase type and are all extracellular enzymes.

The most common class of bacterial proteases is serine proteases, which shares a common catalytic mechanism, similar structural features involving the catalytic triad and inhibition by phospho and sulphonyl fluoride. *Bacillus* sp continue to be the dominant bacterial work horses in microbial fermentations. The capacity of selected *Bacillus* strains to produce and secrete large quantities (20-25 mg/L) of extra cellular enzymes has placed them among the most important industrial enzyme producers.

Many of the organisms produce more than one kind of protease. Under a wide range of growth conditions *Bacillus* sp produce extra cellular proteases during the post exponential growth phase (Schaeffer, 1969). Other *Bacilli* synthesize proteases during the exponential growth phase (Chaloupka and Kreckova, 1966). Proteases from bacteria of the genus *Bacillus* are so far the most important group of enzymes produced commercially with major application in industries. Alkaline and acid proteases are more widely applicable than neutral proteases.

Serine alkaline proteases are used because of their superior economic importance. Certain strains of *Bacillus*, showing alkalophilic properties, synthesize serine alkaline proteases that are most active at pH 11-12 (Aunstrup *et.al* 1972). Neutral proteases reduce dental plaques. Alkaline proteases are used to decompose the gelatinous coating of X-ray films, from which silver is recovered (Ishikawa *et.al*, 1993).

Proteases are also an useful and important component in biopharmaceutical products such as contact-lens, enzyme cleaners and enzymatic debriders (Anwar and Saleemuddin, 2000).
The history of detergent enzymes dates back to 1914, when two German scientists, Rohm and Haas used pancreatic proteases and sodium carbonate in washing detergents. The product was named Burnus. The first detergent containing the bacterial enzyme was introduced into the market in 1956 under the trade name Bio-40. An alkaline protease, alcalase, was effectively incorporated in detergent powder and was marketed by Novo Industry, Denmark under the trade name Biotex in 1963. Today, detergent enzymes account for 89% of the total protease sales in the world and a significant share of the market is captured by *subtilisins* and alkaline proteases from many *Bacillus* sp.

Proteases have been used in the hide-dehairing process, where dehairing is carried out at pH values between 8 and 10 (Najafi *et.al*, 2005). Proteases find applications at various steps of leather processing, neutral protease in soaking (Deshpande *et.al*, 2004), alkaline protease in dehairing (Dayanandan *et.al*, 2003) and acid protease in bating (Padmavati *et.al*, 1995).

Proteases from bacteria of the genus *Bacillus* are so far the most important group of industrial enzymes produced commercially with major application in detergent industry (Purva, 1998b). Largest application of protease is in laundry detergents, where they help removing protein based stains from clothing (Banerjee *et.al*, 1999). Commercial protease variants of the *Bacillus clausii* enzyme have been developed through protein engineering for the detergent market in response to the need for enzymes with improved performance, for example in low temperature washes, in soft water and in bleach-containing detergents etc (Schallmey *et.al*, 2004).
Next to amylases currently the second most important industrial enzymes are the proteases. There are different types of proteases produced by an extraordinarily large number of microorganisms. Microorganisms secrete a wide variety of proteolytic enzymes belonging to four mechanistic types or classes, which are also found in mammalian systems. They are Serine proteases, Thiol proteases, Metallo proteases and Acid proteases.
Proteases are currently classified in to six broad groups.

1. *Serine Proteases* – they are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the enzyme active site. They are found ubiquitously in both eukaryotes and prokaryotes. (serine endopeptidases) [serine is an amino acid with the formula $HO2CCH(NH2)CH2OH$]
2. *Threonine Proteases* –These are a family of proteolytic enzymes harbouring a theronine residue within the active site.(Threonine proteases use the secondary alcohol of their N-terminal threonine as a nucleophile to perform catalysis)
3. *Cysteine Proteases* – Also known as thiol proteases, are enzymes that degrade proteins. These proteases share a common catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic triad or dyad. Cysteine proteases are commonly encountered in fruits including papaya, pine apple, fig and kiwifruit.
4. *Aspartase Proteases* – These are catalystic type of protease enzymes that use an activated water molecule bound to one or more aspartate residues for catalysis of their peptide substrates. In general, they have two highly conserved aspartates in the active site and are optimally active at acidic pH. Nearly all known aspartyl proteases are inhibited by pepstatin.
5. *Glutamic acid proteases* – uses a glutamate carboxylic acid for its activity.
6. *Metalloproteases* – It is a type of protease enzyme whose catalytic mechanism involves a metal. Most metalloproteases require zinc, but some use cobalt.

Major sources of Proteases:-

Plant, animal, microbial, fungal and bacterial sources are employed in protease production.

1. **Plant Proteases:–** Papain, Bromelin, keratinases and ficin are some of the well-known proteases of plant origin. But plant proteases production is a time consuming process. The latex of *Carica papaya* fruits provides papin while bromelain is obtained from the stem and juice of pineapple. But these are very time consuming and tedious processes.

2. **Animal Sources:-** Pancreatic trypsin, chymotrypsin, pepsin and rennin are the most animal originated proteases. One of the main intestinal digestive enzyme trypain is responsible for the hydrolysis of food proteins. Chymotrypsin an expensive enzyme prepared from the pancreatic extract of animals is only used for diagnosis and analytical applications. Pepsin an acidic protease mainly found in the stomach of all vertebrates had been used in laundry detergents. Rennin is another major protease that is mainly found in stomach of animals as an inactive precursor called rennet which is later converted to active rennin by the action of pepsin.

3. **Microbial Proteases:–** Microbial community is mostly preferred over the other for large scale production of proteases because of their fast growth and they can be easily manipulated for the production of new recombinant enzymes with desired properties. Microbial proteases provide a major share for the worlds commercial protease needs.

4. **Fungal Proteases:–** Many of the Aspergillus species like A. *candidus* ,A. *flavus,* A. *fumigatus* etc. and species like *cephalosporium* , *chrysosporium* etc. are the major sources of fungal proteases. It can be also used in for modifying food proteins.

5. **Bacterial Proteases:–** Bacterial proteases have more commercial importance in laundry, food, leather and silk industries because of their high production capacity and catalytic activity. *Alteromonas* sp, *Brevibacterium linens, Lactobacterium helveticus etc*. are the main sources of bacterial proteases.

Applications of Proteases:-

Proteases are used in industry, medicine and also as a biological research tool. The microbial and fungal proteases have a lot of industrial applications. Proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes.

In essence, the wide specificity of the hydrolytic action of proteases finds an extensive application in pharmaceutical industries as well as in the structural elucidation of proteins, whereas their synthetic capacities are used for the synthesis of proteins.

Medicines and Pharmaceuticals:-

A variety of proteases are used medicinally either for their native function (e.g. for controlling blood clotting) or for completely artificial functions (e.g. for the targeted degradation of pathogenic proteins). Highly specific proteases such as TEV protease and thrombin are commonly used to cleave fusion proteins and affinity tags in a controlled fashion. Proteases are used as immune–stimulatory agents.

Proteases are used extensively in pharmaceutical industry for the preparation of medicines such as ointments for debridement of wounds. It is also used in denture cleaners and as contact-lens enzyme cleaners. Proteases are widely used in diagnosis and treatment of intra vascular clots in myocardial infraction, stroke, deep vein thrombosis etc. by haemolytic action of serine proteases.

Proteolytic enzymes are very important in digestion as they breakdown the peptide bonds in protein foods to liberate the amino acid needed by the body. Additionally, proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is notable based on several clinical studies indicating their benefits in oncology, inflammatory conditions, blood rheology control and immune regulation. Proteases find extensive applications in the formulation and production of pharmaceutical products, health products and peptide synthesis.

Food industries:-

Proteases are also used in a wide range of food and food processing applications. It also have applications like fish and sea food processing such as fish meals, enhanced oil recovery and aqua culture. It is also used in animal protein processing for improved digestibility, allergenicity, solubility, improved flavor and meat tenderization. Proteases are a powerful tool for modifying the properties of food proteins. They modify the functional properties such as emulsification, fat-binding, water-binding, foaming properties, gel strength, whipping properties *etc.*

Digestive proteases are used extensively in bread industry in improving the texture of bread . Major application of proteases in dairy industry is in the manufacture of cheese. Acid proteases have application in beer chill proofing, extraction of fruit juice , fruit pulp, clarification and cheese production. Microbial rennet is substituted with lipase to avoid rancidity of cheese. These protease enzymes are used for clotting of milk in cheese manufacture, production of fermented foods by moulds from soya beans, rice and other cereals. These are used in baking industries for the modification of wheat protein in bread dough. Neutral proteases have application in bakeries. The use of proteases in the food industry dates back to antiquity.

Leather industries:-

Leather processing involves several steps such as soaking, de hairing, bating and tanning. Conventional methods of leather processing involve hazardous chemicals such as sodium sulfide, which create problems of pollution and effluent disposal. The use of enzymes as alternatives to chemicals has proved successful in improving leather quality and in reducing environmental pollution. Proteases are used for selective hydrolysis of non-collagenous constituents of the skin and for removal of non-fibrillar proteins such as albumins and globulins; at present, alkaline proteases with hydrated lime and sodium chloride are used for dehairing, which resulted in a significant reduction in the amount of waste water generated. In addition, studies carried out by different workers have demonstrated the successful use of alkaline proteases in leather tanning from *Aspergillus flavus, Streptomyces* sp, *B. amyloliquefaciens and B. subtilis.*

Alkaline proteases are used for dehairing, dehiding, softening of the skin, testing the elasticity and texture of skins in leather industry.

Detergent industries:-

Digestive proteases are part of many laundry detergents. Alkaline proteases are used to remove stains and other dirts by breaking down the macromolecules. Washing powders containing enzymes are used in dry cleaning industries as stain and spot removers.

Textile industries:-

Pulping and thinning of yarns, improving the texture of yarns and cotton fibres *etc.* are done with the help of protease enzymes. In textile industry, proteases may also be used to remove the stiff and dull gum layer of sericine from the raw silk fibre to achieve, improved luster and softness.

Proteases are used in wool finishing. It is aimed at increasing comfort (reduce prickle, greater softness) as well as improved surface appearance and pilling performance. Other application is degumming of silk, to produce sand-washed effect on silk garments. Treatment of silk-cellulosic blends is claimed to produce some unique effects. Proteases are also used to wash down printing screens after use in order to remove the proteinaceous gums which are used for thickening of printing pastes.

Research:-

Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure function relationship, in the synthesis of peptides and in the sequencing of proteins. The field of protease research is enormous. Proteases have applications in molecular biology and genetic engineering. For studying sub cellular organelles likes nucleus, golgi bodies, mitochondria, lysosomes, endoplasmic reticulum *etc.* dissolution of cell wall membranes using proteases is essential.

How protease deficiency can affect our health???

- Acidity is created through the digestion of protein. Therefore a protease deficiency results in an alkaline excess in the blood. This alkaline environment can cause anxiety and insomnia.
- In addition, since protein is required to carry protein- bound calcium in the blood, a protease deficiency lays the foundation of arthritis, osteoporosis and other calcium- deficient diseases.
- Because protein is converted to glucose upon demand, inadequate protein digestion leads to hypoglycemia, resulting in moodiness, mood swings and irritability.

Protease also has an ability to digest unwanted debris in the blood including certain bacteria and viruses. Therefore, protease deficient people are immune compromised, making them susceptible to bacterial, viral and yeast infections and a general decrease in immunity. The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents.

Silver recovery:- Alkaline proteases are used in silver recovery from used X-ray films. Used X-ray film contains approximately 1.5% to 2.0% (by weight) silver in its gelatin layers. Silver recovery by burning film causes a major environmental pollution problem; hence enzymatic hydrolysis of gelatin layers on the X-ray film enables the recycling of both silver and poly-ester film base.

The activity of proteases is inhibited by protease inhibitors. One example of protease inhibitors is the *serpin* super family which include *alpha 1- antitrypsin, c1 – inhibitor, antithrombin, alpha 1- antichymotropsin, plasminogen activator inhibitor-1 and, neuroserpin.* Natural protease inhibitors include the family lipocalin (the lipocalin family of proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids, and lipids) proteins, which play a role in cell regulation and differentiation. Other natural protease inhibitors are commonly used in defense mechanisms. Eg. trypsin inhibitors found in the seed of some plants..

Conclusion:-

Proteases are a unique class of enzymes, since they are of immense physiological as well as commercial importance. They possess both degradative and synthetic properties. Since proteases are physiologically necessary, they occur ubiquitously in animals, plants, and microbes. However, microbes are a goldmine of proteases and represent the preferred source of enzymes in view of their rapid growth, limited space required for cultivation, and ready accessibility to genetic manipulation. Microbial proteases have been extensively used in the food, dairy and detergent industries since ancient times. There is a renewed interest in proteases as targets for developing therapeutic agents against relentlessly spreading fatal diseases such as cancer, malaria, and AIDS. The development of recombinant rennin and its commercialization by Pfizer and Genencor is an excellent example of the successful application of modern biology to biotechnology. Analysis of sequences for acidic, alkaline and neutral proteases has provided new insights into the evolutionary relationships of proteases. Despite the systematic application of recombinant technology and protein engineering to alter the properties of proteases, it has not been possible to obtain microbial proteases that are ideal for their biotechnological applications. Industrial applications of proteases have posed several problems and challenges for their further improvements. Biodiversity represents an invaluable resource for biotechnological innovations and plays an important role in the search for improved strains of microorganisms used in the industry. A recent trend has involved conducting industrial reactions with enzymes reaped from exotic microorganisms that inhabit hot waters, freezing Arctic waters, saline waters or extremely acidic

or alkaline habitats. Proteases isolated from extremophilic organisms are likely to mimic some of the unnatural properties of the enzymes that are desirable for their commercial applications. The existing knowledge about the structure-function relationship of proteases, coupled with gene-shuffling techniques, promises a fair chance of success, in the near future, in evolving proteases that were never made in nature and that would meet the requirements of the multitude of protease applications.

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