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A REVIEW ON INDUSTRIAL APPLICATIONS OF MICROBIAL PROTEASE

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ABSTRACT

Proteases catalyze hydrolysis of peptide bonds in proteins and are one of the most widely used industrial enzymes. Though they are ubiquitously found in a wide diversity of sources such as plants, animals, and microorganisms but microbial sources are preferred for the production of proteases due to technical and economic advantages. Microbial proteases have potential for application in different industries including detergent, leather, silver recovery, dairy, baking, beverages and pharmaceutical industries. These hydrolytic enzymes are efficiently involved in food industry for enhancing nutritional value, digestibility, palatability, flavor and reducing allergenic compounds as well as in management of domestic and industrial wastes. Furthermore, they are also involved in synthesis and structural elucidation and characterization of proteins. The present communication is an overview of the proteases produced from bacterial and fungal sources and their role in various industrial applications.

KEYWORDS: Protease; Microbial; Alkaline; Hydrolytic; Palatability; Characterization.

INTRODUCTION

Proteases are known as an important class of enzyme which have a key role in biological fields' i.e. physiological as well as commercial fields (Rao et al. 1998). Later on, protein hydrolyzing enzymes catagorized into different classes on the basis of their origin and site of action for enzymatic reactions (Sawantam and Nagendran 2014). The enzyme classification on basis of mode of action is shown (Fig.1) (Rao et al. 1998; Ramesh et al. 1994)). Proteolytic enzymes i.e. proteases are also referred as peptidases or proteinases (Motyan et al. 2013). Economically, primary storage complex i.e. Protein are the components of large number of important crops, fruits and vegetables. Moreover, this macromolecule plays many critical roles in the structure, function, and regulation of the body's tissues and organs.

Protease	Mode of action[a]	EC no.
Exopeptidases		
Aminopeptidases	●↓○-○-○-○---	3.4.11
Dipeptidyl peptidase	●-●↓○-○-○---	3.4.14
Tripeptidyl peptidase	●-●-●↓○-○---	3.4.14
Carboxypeptidase	---○-○-○-○-○↓●	3.4.16–3.4.18
Serine type protease		3.4.16
Metalloprotease		3.4.17
Cysteine type protease		3.4.18
Peptidyl dipeptidase	---○-○-○-○↓●-●	3.4.15
Dipeptidases	●↓●	3.4.13
Omega peptidases	*-●↓○-○---	3.4.19
	---○-○-○↓●-*	3.4.19
Endopeptidases	----○-○-○↓○-○-○---	3.4.21–3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24
Endopeptidases of unknown catalytic mechanism		3.4.99

Figure 1: Classification of proteases.

Amino acids are abundantly formed by Proteins. Proteins mainly consist of linear chain of amino acid residues i.e. polypeptide linked by peptide bond. Protease is an highest acceptable useful enzyme due to its action specifity and it is used rapidly in different application of biotechnology (Rani et al. 2012). Therefore; trends of protease processing industry are increasing from recent century. In enzyme market, 60% industrial enzyme used is 'Proteases' (Sawantam and Nagendran 2014). Protease (hydrolases, having EC 3.4) is an enzyme that cause hydrolysis or proteolysis of peptide bond between amino acids in the polypeptide chain that forms protein (Gupta and Khare 2007; Devi et al. 2008). This process of enzyme also hydrolyzes (breaks down) the other associated products (in an endo manner) and further synthesizes free amino acids. Proteases fall into four main mechanistic classes: serine, cysteine, aspartyl and metalloproteases as given in Figure 2 (Erez et al. 2009). Depending on enzyme source, the action, properties and hydrolysis products of enzymes can be somewhat different.

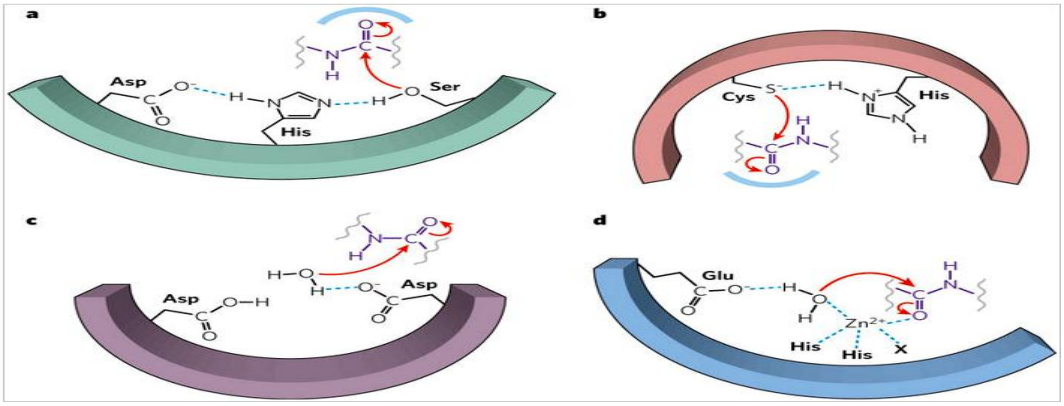

Figure 2A, 2B, 2C and 2D: Serine proteases; cysteine proteases; aspartyl proteases and metalloproteases.

Reactions Catalyzed By Proteases

According to Janos and Ferenc (Sawantam and Nagendran 2014), the reactions catalyzed by protease are grouped into several types on the basis of their classifications. Suitable substrates proteases are involve in enzyme catalyzing reactions. The reaction catalyzed by aminopeptidases, carboxypeptidases and endopeptidases are shown in Figure 3 (Rao et al. 1998).

I) Aminopeptidases

Aminopeptidases form single amino acid residue, a dipeptide, or a tripeptide depending on the polypeptide chain by attacking on N terminus end. In heterologously expressed proteins, methyl group on N terminus end is present but in mature protein this group is absent. Aminopeptides can also remove the methyl group on N terminus end. Many species of bacteria and fungi form this class of enzyme. In addition to it, aminopeptide has an extra attribute of becoming intracellular as well as extracellular enzyme but its function as an extracellular enzyme is rare. Its production is recently reported in *Aspergillus oryzae* (Rani et al. 2012).

ii) Carboxypeptidases

Carboxypeptidase form single amino acid residue or a dipeptide depending on the polypeptide chain by attacking on C terminus end. Carboxypeptidases are further subgroup into different carboxypeptidases on the basis of amino acids nature (Rani et al. 2012).

iii) Endopeptidases

Endopeptidases form amino acid residues depending on the polypeptide chain by attacking on inner regions of polypeptide chain i.e. away from N and C terminal end. If free carboxyl or amino group is present at the respective ends then they can influence a negative effect of endopeptidases activity (Rao et al. 1998).

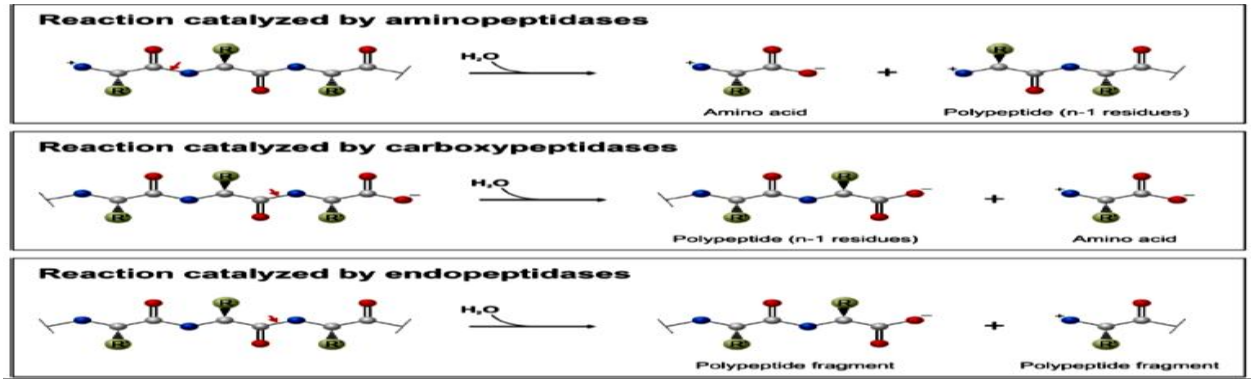

Figure 3: Reactions catalyzed by Protease.

Industrial Uses of Protease

Protease is known as fastest growing enzyme due to its importance. In industrial production, microbial Protease considered among one of the best enzyme. The use of different techniques involving enzyme production from various sources of microorganisms is intensively competitive for industrial use, because it is connected with health, welfare, and prosperity of mankind. Many commercially important enzymes i.e. Protease and their derivatives are produced from different bacterial species. To use this enzyme, many industrial processes are important. These industrial processes involve industrial, environmental processes and food biotechnology manufacturing. The biochemical parameters determine the application of protease in industry apart from other factors, which include the cost of production and development, markets and the economy of application (Mahajan and Badgujar 2010). Summarizations of major applications of protease are presented in Figure 4 (Kumar et al. 2008). Application of protease is extend to various fields of routine life. Some of the commercial protease available is given in Table 1.

Food industry

From the distant past, proteases are constitutively used in the food industry i.e. dairy industry, baking industry, preparation of soy sauce, and meat tenderization.

Dairy industry

Protease is mainly used in the dairy industry for production of cheese. Due to cheese production, protease is also known as milk-coagulating enzymes. On this basis, it is divided into four types. These are: animal rennets, microbial milk coagulants, vegetable rennet and genetically engineered chymosin. The class of animal and microbial milk coagulating proteases is actually acid aspartate proteases class. Due to it, microbial enzymes comprise of drawbacks i.e cheese bitterness and a low yield. The nonspecific and heat-stable proteases when cross a certain limit after storage they result a change in cheese flavour. To avoid this, pasteurization is required to inactivate non specific protease. The mechanism of action of protease in cheese production is to form para – casein and macropeptides by breakdown i.e. hydrolyze the Phe105-Met106 peptide bond. In all these proteases, Chymosin gives good result in cheese production because of its action specifity for casein. The process of cheese making from milk is given in Figure 5.

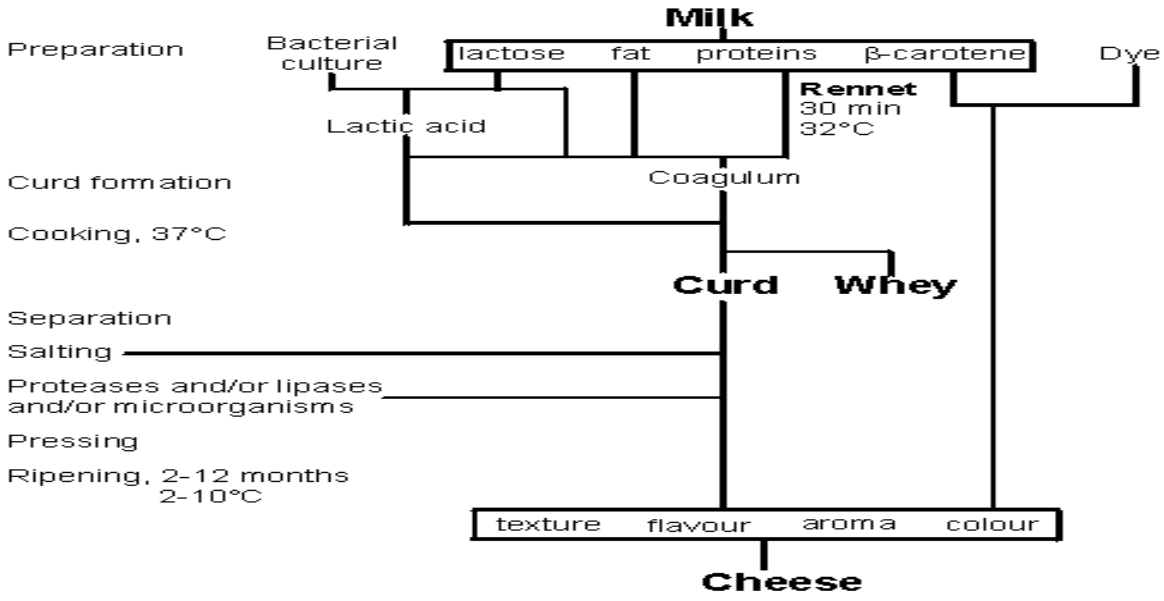

Figure 4: Outline method for the preparation of cheese.

Baking industry

The major part of baking is comprised on Flour (Wheat). Bakery dough properties can be determined by gluten which includes insoluble protein. Limited proteolysis leads to modification in wheat gluten by endoproteinases and exoproteinases that are produced from *Aspergillus oryzae*. Handling of the can be dough facilitated by treatment with enzymes and it results in various products. Moreover, reduction in mixing time and enhancement in loaf volumes can be done by protease. In addition to it, protease also results in strength and elasticity of dough. Furthermore, the use of microbial proteases during bread-making processes has also emerged to reduce immunogenic gluten, especially in baked goods. The use of microbial protease in bread baking process is given in Figure 6 (Heredia et al. 2016). Actually in modified baked food by microbial protease, there is no immunogenic recognition for pathogenesis of celiac disease. Therefore, this disease is not developed. Moreover, this modification also increases quality and quantity of bread (Liang et al. 2009).

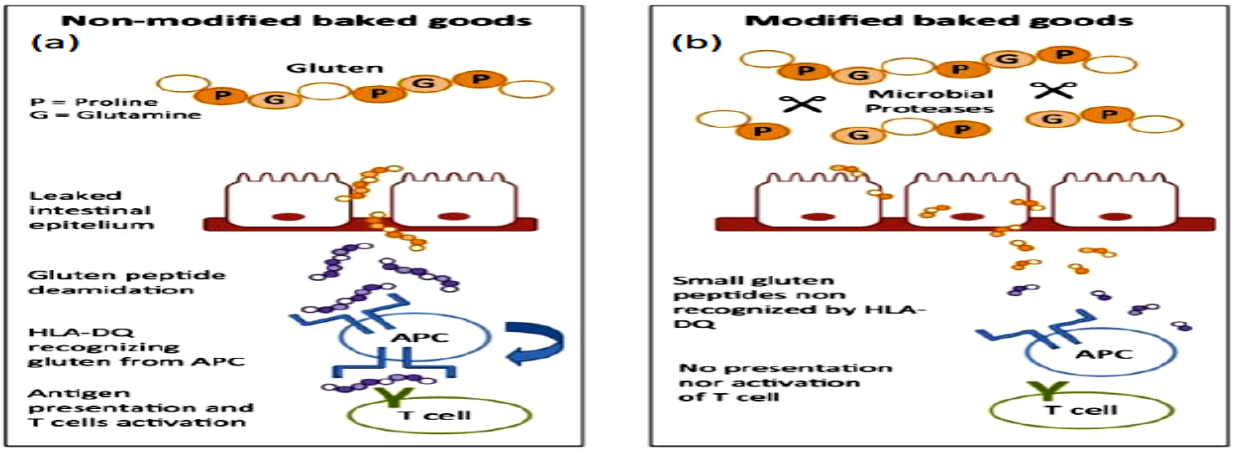

Figure 5A and 5B - Adaptive immune response to non-modified baked foods and modified baked foods i.e. protease results in non-activation of T cells to modified baked goods. (Abbreviation: APC: antigen-presenting cell).

Soy sauce production

Soybeans are a source of premium quality protein in high amount. Proteases have been extensively used for production of soy sauce and other soy related products from decades. Specifically, proteases of fungal source having alkaline and neutral nature play an important role in the processing of soy sauce. Proteases treatment modifies soy proteins result in improved functional properties. Soy proteins are treated with alcalase at pH 8 results in high yield and low bitterness soluble with high solubility. Uses of hydrolysate are included in protein-fortified soft drinks and in the formulation of dietetic feeds. Moreover, microbial protease can use different sources of substrate to form soy sauce products. The flow sheet describing soy products production is shown in Figure 7 (Liang et al. 2009).

Figure 6: Common soy sauce production.

Meat tenderization

Among cattles cows and buffaloes are major source of meat. Animals are slaughtered for meat source but other by products from these animals has also extensive applications. Buffalo tripe has high nutritional value but its processing at commercial scale is very limited due to excessive collagen which makes it inherently tough. Meat should be first tenderize before further processing and for this purpose various methods can be used among which chemical and enzymatic treatment by using proteolases is most common (Fig.8) (Ashie et al. 2002). On commercial level tenderization of meat cuts is done by using papain which is very active hydrolysing fibrous protein present in connective tissues. But enzymatic tenderizations also make this process difficult.

Figure 7: Reaction in meat tenderization.

Brewing industry

The brewing industry has vast applications of proteases. Brewing wort is produced by using proteases which increases solubility of protein present in barley adjuncts resulting in release of small peptides and protein monomers that can be used as nitrogen supplements. The second major application of proteolytic enzymes is in chill proofing that resists precipitate during cold storage.

Hazes formation in beer can be inhibited by using proteolysis. The mechanism involve in hazes inhibition involves hydrolysis of proteinaceous components which precipitate polyphenols and oligosaccharides and causes hazes formation. There is schematic diagram showing various steps in beer formation by using proteases as key enzyme (Fig.9) (Pokharkar 2002).

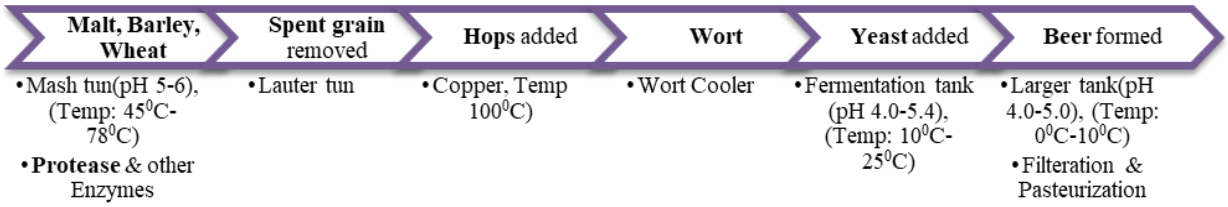

Figure 8: Simplified flow chart on beer production.

Synthesis of aspartame

Aspartame is a commonly used non calorific artificial sweetener and approved by Food and Drug Administration (FDA). Structurally, it is a dipeptide composed of L-aspartic acid and the methyl ester of L-phenylalanine. The sweet taste of aspartame is due to its L Amino Acids configuration. Stereospecificity is maintained by chemical methods in its synthesis that increases its cost of production. Therefore enzymatic synthesis is preffered over chemical method. The major enzymes used in this process are proteolyses due to its hydrolytic reaction. The catalysis takes place in specified and controlled condition. The synthesis of aspartame by proteolytic reaction is shown in Figure 10 (Yang 1998). An immobilized preparation of thermolysin from *Bacillus thermoprotyolyticusis* used for the enzymatic synthesis of aspartame. The major industrial producers of aspartame are Toya Soda (Japan) and DSM (The Netherlands).

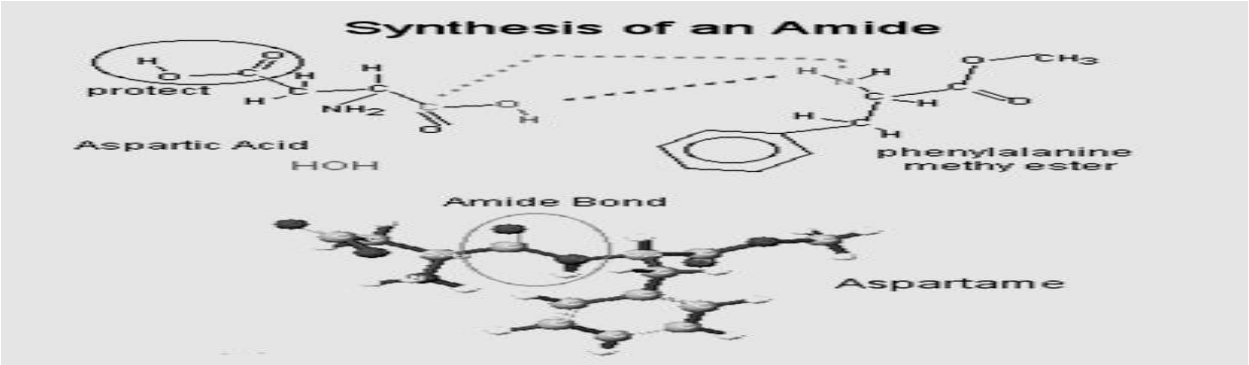

Figure 9: Reaction mechanism of aspartate synthesis.

Detergent industry

Proteases are one of the standard ingredients of detergents due to its broad substrate specificity activity and stability at high pH and temperature, and compatibility with other chelating and oxidizing agents to facilitate the cleaning of variety of stains. The ionic strength or pI is a key parameter for the best performance of a protease in a detergent. On commercial level serine proteases by Bacillus strains are most commonly used in detergents. Alkaline proteases isolated from fungal are beneficial due to the simple downstream processing to prepare a microbe-free enzyme. Mixture of lipase, amylase, and cellulose is used to improve the performance of protease in washing detergents.

Table 1: Sources & Industrial applications of Protease available commercially.

Types	Sources	Applications as	Companies Marketing	References
Fungal Protease	*Aspergillus sp.*	Protease P	Amano Pharmaceutical Ltd Japan	(Ellaiah et al. 2002)
Bacterial Protease	*Bascillus licheniformis*	Alcalase	Novo Nordisk, Denmark	(Ellaiah et al. 2002)
	Protein engineered variant of *Savinase*	Durazyme	Novo Nordisk, Denmark	
	Protein engineered variant of Alkhalophilic *Bascillus sp.*	Maxapem	Solvay Enzymes GmbH, German	
	Alkhalophilic *Bascillus sp.*	Savinase, esperase	Novo Nordisk, Denmark	
	Alkhalophilic *Bascillus sp.*	Maxacal, maxatase	Gist-brocades, The Netherlands	

	Alkhalophilic *Bascillus sp.*	Opticlean, optimase	Solvay Enzymes GmbH, German	
	Alkhalophilic *Bascillus sp.*	Proleather	Amano Pharmaceutical Ltd Japan	

Leather industry

Leather processing involves the uses of proteases in various steps incluuding soaking, dehairing, bating and tanning. Chemical have side-effect on leather and in increasing environmental pollution so the best alternative is the use of enzymes (Gupta et al. 2002]. As skin and hair have high amount of non-collageneous constituents and non-fibrillar proteins like albumins and globulins which are hydrolysed by proteases in pre-tanning operations. Traditionally soaking and dehairing were performed with alkali but recently these steps are performed by alkaline proteases along with hydrated lime and sodium chloride result in less amount of wastewater produced (Miller et al. 1981; Drivdahl et al, 1977). Enzymes for leather industry are selected on the basis of its specificity for matrix proteins and their amount depends on the type of leather (soft or hard) to be produced. For bating bacterial and fungal proteases along with trypsin are used for saving energy and reducing pollution. Aquaderm, NUE, and Pyrase are three different types of proteases produced by Novo Nordisk forsoaking, dehairing, and bating respectively. The Leather processing by using microbial protease is given in Figure 11 (Ruttloff et al. 2013).

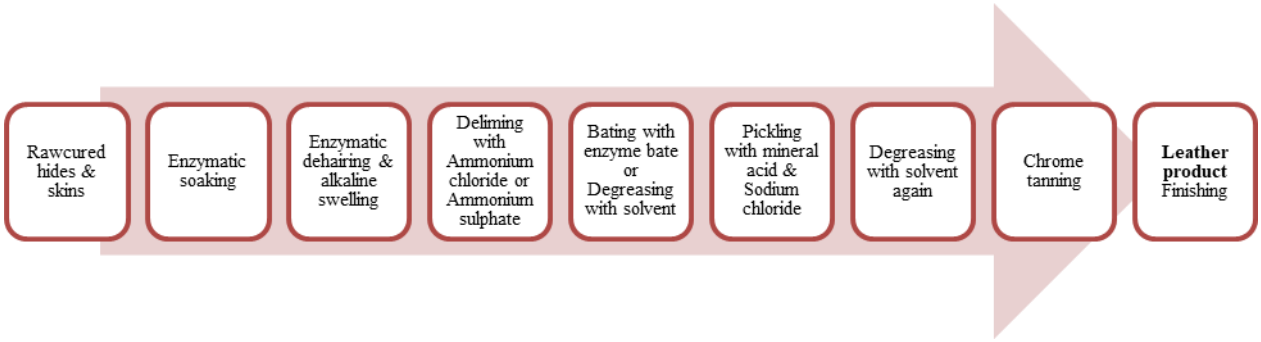

Figure 10: Leather processing by Microbial protease.

Therapeutics

Proteolytic enzymes are potent digestive agent so used to assist digestion process. In human, tissue inflammation and pain can be reduced by injecting some distant proteases. In lactating women proteolytic enzymes are used to lower the distress of breast engorgemen. After surgery or any injury these enzymes have been reported to be used effectively for pain killing, swelling and inflammation. Furthermore, proteases have been also used various Proteomic applications where aim is identification, characterization and quantification of the required samples specifically by using mass spectrometric (MS) analysis. MS an important element in medicinal field can be used to study various properties of proteins like its composition, chemical properties (Steen and Mann 2004; Nieri et al. 1998). Mass-spectrometry is used to analyse proteins of interest after its separation and in-gel digestion. Various steps of this analysis are shown in Figure 12 (Granvogl et al. 2007; Raghunath T. et al. 2010).

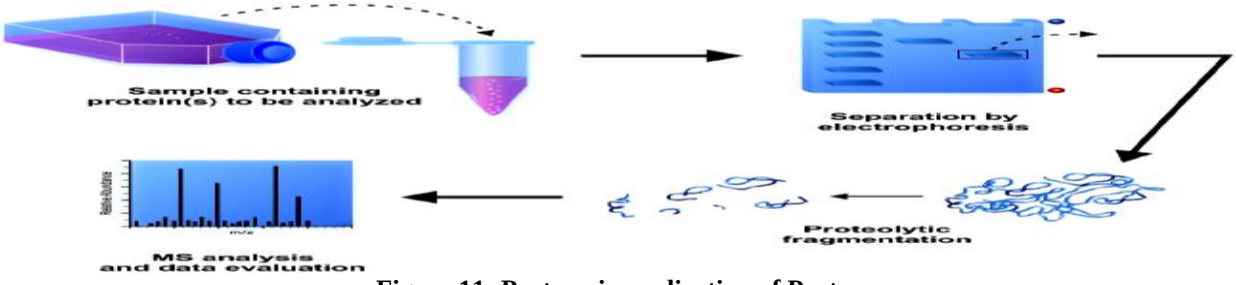

Figure 11: Proteomic application of Protease.

Photography industry

In photography industry silver is used in large quantity to produce light sensitive emulsion. After processing of this film, this silver must be recovered for reusing by separation of silver particles containing gelatin from film base. The silver particle along with gelatin comes into aqueous solution but due to presence of protein silver cannot be separated from this mixture. Therefore, use of proteolytic enzymes at very high temperature (500°C) and at alkaline pH (8.0) quickly degrades the gelatin and the silver particles are separated out. The use of proteases in gelatine disappearances shown in Figure 13 (Masui et al. 1999). *B.subtilis 18'* and *B. coagulans PB-77* yield alkaline proteases which are effectively use in

decomposition of the gelatinous coating to recover silver on used X-ray films.

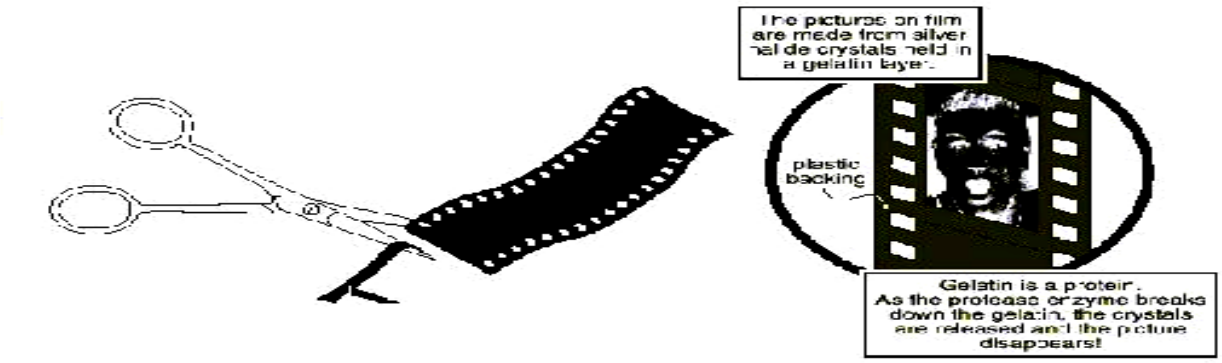

Figure 12: Treatment of photography film by microbial protease.

Management of industrial waste

Proteases have enormous applications in waste degradation. Alkaline proteases are recently found useful in waste management from various food processing industries. The mechanism behind waste management of proteases is its solubilization of proteinaceous waste residues result in less BOD (biological oxygen demand) of aquatic systems. In poultry about 5% body weight is occupied by feathers which have high proteionacious contents and have high amount of rigid keratin. Therefore, keratinolytic proteases are used to degrade keratin and protein in waste feathers, as well as to remove hair from the drains as a depilatory agent (Takami et al. 1992; Rawlings et al. 1993). Commercially proteolytic enzymes from *B. subtilis*, *B.amyloliquefaciens* and *Streptomyces* spare used in mixture along with thioglycolate a disulfide reducing agent result in more efficient hair degradation and remove clog's of hair deposits result in clearing pipes. Another study was performed for waste degradation efficiency test of proteases produced from 6 strains In this study it was concluded that among six strains of proteases BM1 has the highest productivity of proteases so it have maximum degradation potential followed by BM3 as shown in Figure 14 (Zaved et al. 2008).

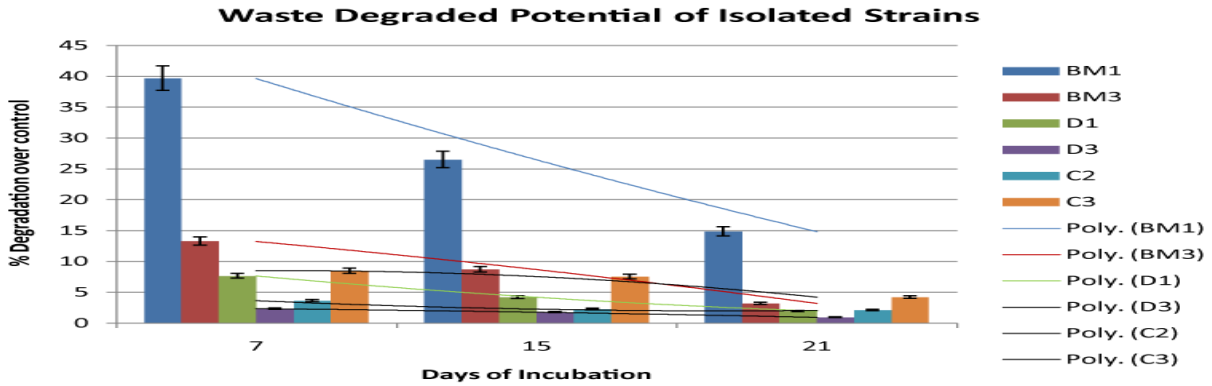

Figure 13: Waste degradation potential of the isolated strains.

Chemical industry

Enzymes has vast applications in chemical industry especificcaly in organic chemistry and synthetic chemistry due to their biocatalysis. But this approach also have a major However, a major disadvantage that enzyme's activity is reduced under anhydrous conditions. Thus, it is important to discover ways to keep enzymeactivated in organic solvents. Use of alkaline protease has been reported to catalyze synthesis of peptide bond in organic solvents. In some other studies immobilized proteases have been used to synthesize peptides. Proleather,a commercial alkaline protease preparation from Bascillus sp was used in synthesis of sucrose polyester in anhydrous pyridine. Moreover, Racemic mixtures of DL-Phenyalanine and DL-Phehylglycinecan be resolved by using proteolytic enymes (Cabrera and Barca 2010).

Pharmaceutical industry

Proteases have wide diversity and specificity so they have numerous useful applications in pharmaceutical and theraputical industry for production of medicinal components (Beg et al. 2001). Proteases from *Aspergillus oryzae* (Luizym and Nortase) have been used in digestive disorders and to cure lytic enzyme deficiency syndromes by oral administration (Pakpahan et al. 2009). Mixture of Clostridial collagenase or subtilisin with broad-spectrum antibiotics in burns and

wounds treatment. Lymphocytic leukemia has been treated by asparginase that is isolated from E. coli to eliminate aspargine from the bloodstream (Saiga et al. 1993; Rivero et al. 1991). Trypsin has been replaced in animal culture by an alkaline protease isolated from Conidiobolus. Curcain a plant protease has found to be work as wound healing agent. It was purified from the latex of Jatropha curcusso named as curcain (Benson 2001).

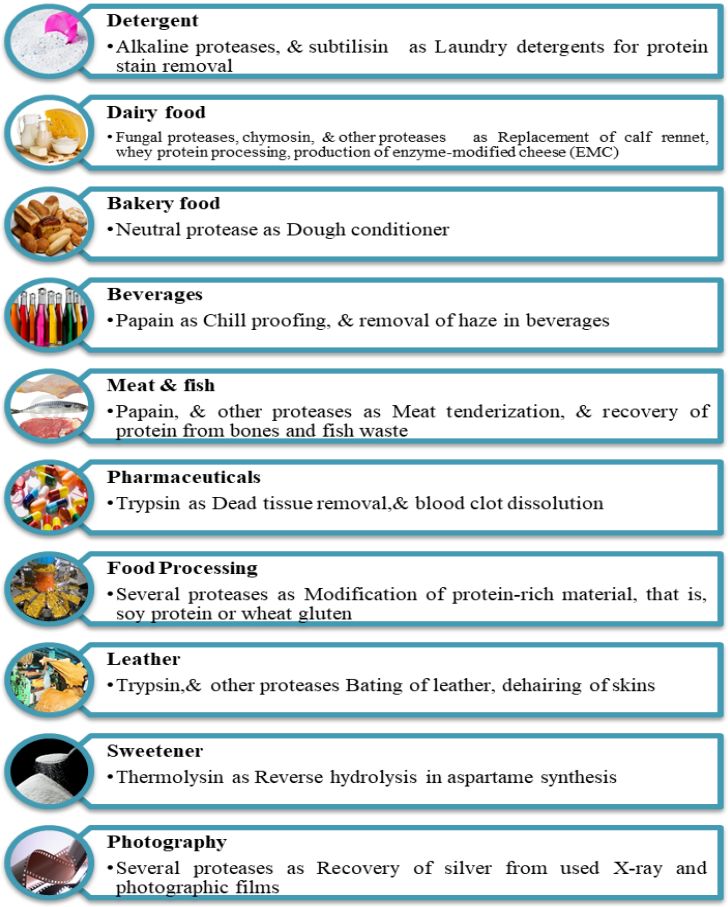

Figure 14: Microbial protease- Industrial applications.

Paper industry

In paper industry enzymes are used for conservation of art works on paper along with other conservators. Hydrolyases enzymes are most commonly used in conservation treatment of art work on paper by degradation of adhesive residues, from primary repairs and thus they help in removal of facilitate the removal of linings or mounts which are secondary supports (Sumardi et al. 2018). Enzymes have advantages over other paper conservators as of their specific nature and high catalytic activity in hydrolytic cleavage of polymers like proteins, polysaccharides, and lipids. They are also economically feasible and work in optimal conditions. The paper conservation treatment by protease is shown in Figure 15 (Van 2005).

Figure 15: Before treatment(with Protease), upper left quadrant & After treatment(with Protease), upper left quadrant.

Degumming of silk

The use of proteases is the silk industry is one of the least explored areas for. The traditional degumming of silk is an expensive process therefore an alternative enzymatic method is used on commercial level. Enzyme preparations of proteases are used for degumming of the silk prior to dyeing (Mala et al. 1998). Raw silk has rough texture due to presence of Sericin which is about 25% of the total weight of raw silk. Conventionaly, this sericin can be removed by conducting shrink-proofing and twist-setting for the silk yarns, using starch which removed all sericin residues from the inner core of fibroin (Kanehisa et al. 2000; Sanna et al. 2001). Enzymatic removal of sericin by the degumming process yields heavy and shiny texture of silk. Volume of raw silk fibres is also increased by enzymatic treatment. Alkaline protease from Bacillus sp. RGR-14 has been studied for its silk-degumming efficiency. The comparison of enzymatic treated and untreated silk fibre is shown in following Figure 16 (Freddi et al. 2003).

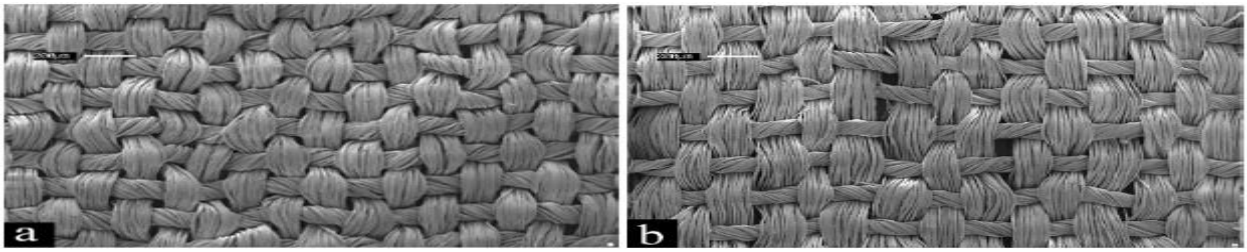

Figure 16A and 16B: Enzymatic treated raw silk fabric and untreated raw silk fabric.

Other applications

In addition to their industrial and pharmaceutical applications, proteases enzymes are also used in basic research. In research they are used to study structure function relationship of proteins by selective peptide bond cleavage and synthesis and in the proteins sequencing. Due to hydrolytic action of proteases they have wide application in the food, detergent, leather, and pharmaceutical industries.

CONCLUSION

The main theme of this review concentrates around the applications of microbial proteases enzymes of bacterial and fungal origin and among number of microbial sources genus *Bascillus* is of most importance due to ease of cultivation, faster generation time and its genetic manipulation for enzyme overproduction. The role of proteases has been extensively studied in different industries related to food processing, beverage production, leather, textiles, detergents, etc. Their role with the advent of biotechnology is also expanding into many new fields such as clinical, pharmaceutical analytical and synthetic chemistry.

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