Proteases:

Proteases are the enzymes which catalyse the hydrolysis of peptide bonds of the proteins. The amino acid composition of proteins is very diverse so the proteases responsible for their hydrolysis are also diverse. Microbes have both intracellular and extracellular proteases, the intracellular proteases are responsible for the maintenance of amino acid pool inside the cell by degrading the unwanted proteins and the extracellular proteases hydrolyse proteins outside the cells into peptides and amino acid required by the cells for their growth. Proteases are classified into two major groups: the exopeptidases (peptidases) and the endopeptidases (proteinases). The peptidases hydrolyse the protein from C- or N-terminus releasing single amino acid and the endopeptidases as the name suggests hydrolyses the peptide bond in the middle of the amino acid chain. Further the proteases are also classified into alkaline, acid and neutral proteases based on their pH optima of activity. On the basis of the functional group present at the catalytic site these proteases are classified as serine proteases, cysteine proteases, aspartic proteases, threonine proteases, glutamic acid proteases and metalloproteases. The classification of proteases has been summarized in Fig 4.

The proteases represent one of the three largest groups of industrial enzymes others being the amylases and lipases. The proteases find their application in detergents, leather, food, pharmaceutical industries and bioremediation processes. The major uses of microbial proteases have been listed in Table 4.

Both bacterial and fungal proteases are produced commercially and their production conditions are very different from each other. Some organisms are thermophilic especially grown to obtain thermostable proteases for use in detergents. In the proceeding sections alkaline, neutral and acid proteases have been described along with their major producers and important applications.

Alkaline proteases:

Strains of *Bacillus*, Steptomyces, *Aspergillus* are the major producers of alkaline proteases. Proteases from *Bacillus* (Bacillopeptidases) are mainly used in detergents. Subtilisin carlsberg (protease from *B. licheniformis*) and Subtilisin Novo (protease from *B. amyloliquefaciens*) are the best know proteases used in detergents. These proteases have serine at the active site, and are not inhibited by EDTA (ethylene diamine tetra acetic acid), but are inhibited by DFP (diisopropyl flurophosphate). These proteases are stable at high temperature, active in alkaline pH (9-11) and stable in presence of chelating and perborates, which is an important characteristic of these enzyme for use in detergents.

Screening of organism producing alkaline proteases is done using strongly basic media and the colonies are tested on protein agar plates (pH 10). The wild strains have been improved by altering the amino acid sequences of the proteases by genetic engineering tools, changing their substrate specificity, pH optimum, and stability to the bleaching agents. Extensive genetic manipulations have also been carried out to improve the yields of the proteases during the fermentation.

Proteases to be used as detergent additive should be stable and active in the presence of surfactants, bleaching agents, bleach activators, fillers, fabric softeners and various other formulation of a typical detergent. 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. Protease treatment also modifies the surface of wool and silk fibers to provide unique finishes. The alkaline proteases also have potential application in removal of gelatin from the used photographic films vis-à-vis recovery of silver from them.

Neutral protease:

Neutral proteases are obtained from plants e.g. papain (from *Carica papaya*), bromelain (from *Ananas comorus*) and ficin (from *Ficus* spp.), which are cysteine proteases. Neutral proteases are produced by bacteria (*Clostridium histolyticum*, *Streptococcus* spp., *Bacillus subtilis*, *B. cereus*, *B. megaterium*, *B. stearothermophilus*, *B. thuringiensis*, *B. pumilus*, *B. polymyxa*, *B. licheniformis*, *B. amyloliquefaciens*. *B. stearothermophilus*, *Pseudomonas aeruginosa*, *Streptomyces griseus*) and fungi (*Aspergillus oryzae*, *A. sojae*, *Penicillium* spp., *Pericularia oryzae*). These microbial neutral proteases are either cysteine or metalloproteases. Neutral metalloproteases have specificity towards peptide linkages that contain hydrophobic amino acids to the amino side. The neutral proteases are unstable and require calcium, sodium, and chloride ions for their stability. Not only the pH range for these proteases is small, but they also get inactivated at elevated temperatures.

Commercial fungal neutral proteases are used in baking, food processing, protein modification, and in leather, animal feeds and pharmaceutical industries.

Acid proteases*:*

Rennins from calf stomach, pepsin of humans are the well known examples of acid proteases catalysing hydrolysis of protein around pH 2-4. Some of the fungi also produce acid proteases which are rennin-like and used mainly in cheese production. Acid proteases are also used in the preparation of digestive syrup, soy protein digestion during sauce preparation, hydrolyzing the gluten from wheat dough used for preparing biscuits in bakery making them crispy. Silver from the film roll is recovered by digesting the gelatin by acid proteases. *Alcaligens*, *Bacillus*, *Corynebacterium*, *Lactobacillus*, *Pseudomonas*, *Serratia*, *Streptococcus*, and *Streptomyces* are the bacteria, and *Aspergillus*, *Candida*, *Coriolus*, *Endothia*, *Endomophthora*, *Irpex*, *Mucor*, *Penicillium*¸*Rhizopus*, *Sclerotium*, and *Torulopsis* are the some of the fungi producing rennin like proteases which find applications in cheese processing. There are three strains being used for acid protease production and divided into two groups on the basis of culture conditions cloned into *E. coli* for production and use of rennin enzyme for avoiding the problems encountered with microbial rennin.

Cellulases:

Cellulose is the most abundant organic macromolecule on earth and mainly constitutes the cell wall of plant cells. This macromolecule is a linear polymer of glucose residues linked by β-1,4 glycosidic linkage and utilized as carbon source by many microorganisms present in soil and guts of ruminants. These organisms produce cellulases, the enzymes that hydrolyze the β-1,4 glycosidic bond of cellulose. There are three different types of cellulases; endo-1,4-β-D-glucanase (EC-3.2.1.4), exo-1,4-β-glucanase (EC-3.2.1.91) and β-D-glucosidase (EC-3.2.1.21) having specific hydrolyzing properties (Table 5). The cellulase has three types of functionally different domains,

(a): catalytically active core.

(b): cellulose binding domain.

(c): a linker domain which is flexible connecting both the domains.

Table 5: Types of cellulases and their activities *(Source : Lynd et al. 2002)*

Type	Specific name	Mode of action
Endoglucanases (EG)	1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4)	Hydrolyses amorphous cellulose randomly at internal sites generating oligosaccharides of various length generating new ends
Exoglucanases or cellobiohydrolases (CBH)	1,4- β-D-glucan glucanohydrolases (cellodextrinases) (EC 3.2.1.74) and 1,4- β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91).	Hydrolyse cellulose polysaccharide in a sequential manner from reducing or nonreducing ends, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products
β–Glucosidases or cellobiase	β-Glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21)	β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose

Industrial production of cellulase started in early 1980s for treating animal feed along with other enzymes to degrade non-starch polysaccharide to improve nutritive values, later applied in food industry for juice extraction and vegetable processing. Further cellulases were used in the textile, laundry as well as in the pulp and paper industries. The use of cellulases, along with hemicellulases and pectinases has increased considerably sharing approximately 20% of the world enzyme market. The market of cellulase alone is about 190 million US $ in a year. Presently cellulase is being produced mainly by the mutant strains of *Trichoderma reesei*.

***Cellulase producing microorganisms*:**

Bacteria:

Aerobic thermophilic

Acidothermus cellulolyticus, Caldibacillus cellovorans, Cellulomonas flavigena, Rhodothermus marinus, Thermobifida fusca and *Thermomyces lanuginosus.*

Aerobic mesophilic:

Bacillus pumilis, Cellulomonas uda, C. flavigena, Cellvibrio fulvus, C. gilvus, Cytophaga hutchinsonii, Erwinia carotovora, Micromonospora chalcae, Pseudomonas fluorescens var. cellulosa, Sporocytophaga myxococcoides and *Streptomyces reticuli.*

Anaerobic thermophilic :

Anaerocellum thermophilum, Caldicellulosiruptor saccharolyticum, Clostridium thermocellum, C. cellulolyticum, Fervidobacterium islandicum, Spirochaeta thermophila and *Thermotoga neapolitana.*

Anaerobic mesophilic:

Acetivibrio cellulolyticus, Butyrivibrio fibrisolvens, Eubacterium cellulosolvens, Fibrobacter succinogenes, Halocella cellulolytica, Ruminococcus albus and *R. flavefaciens*

Fungi :

Acremonium cellulolyticus, Aspergillus acculeatus, A. fumigatus, A. niger, Humicola insolens, Fusarium solani, Irpex lacteus,

Penicillium funmiculosum, Phanerochaete chrysosporium, Schizophyllum commune, Sclerotium rolfsii, Sporotrichum cellulophilum, Talaromyces emersonii, Thielavia terrestris, Trichoderma koningii, T. reesei and *T. viride.*

Cellulase production:

Cellulose is the best substrate for the production of cellulase by various organisms (bacteria, actinomycetes and fungi). The purified cellulose is very expensive to be used for the cellulase production so different media based on cheaper cellulosic materials (sugarcane bagasse, corn cob and straw, cattle dung, municipal and agricultural waste, sawdust, etc.) are employed for cellulase production. These complex substrates require preliminary mechanical, chemical or biological treatment to expose the crystalline cellulose to be acted upon by the cellulase excreted by the microbe. Production conditions (temperature, pH, aeration, etc.) vary from organism to organism. The co-culture of two species (*Trichoderma reesei* and *Aspergillus wenti*, *T. reesei* and *A. phaenicis*, and *Chaetomium cellulolyticum* and *Sporotrichum pulveruleutum*) has better utilization of cellulosic biomass and cellulase production in respect of the pure cultures. In comparison to the submerged or solid state fermentation, combined (submerged and solid state) fermentation has higher production of cellulase and cell biomass. *Trichoderma reesei* strains used for the production of cellulase secrete up to 40 g/L of protein in the production media under optimized condition of which cellulases are the major component. The proportion of CBH I is about 60%, CBH II 25% and endoglucanases 15%.

In bacteria, cellulase is constitutively produced, whereas fungi produce cellulase only in the presence of cellulose as substrate. The induction in fungi is believed to be triggered by the soluble cellooligosacharides formed during cellulose hydrolysis by a basal level of constitutive cellulase. Cellobiose (β-1-4, linked disaccharide of glucose), sophorose (β-1-2, linked disaccharide of glucose) and gentibiose ((β-1-6, linked disaccharide of glucose) are some of the natural inducers for cellulase expression. These are easily metabolized, thus some stable analogues (thiocellobiose, thiogentobiose) have been successfully used for high titer of cellulase. The bottle neck in industrial use of these analogues is their complicated synthesis procedure.

***Application of cellulases*:**

Saccharification of cellulosic waste materials:

The cellulosic waste has complex structure formed by cellulose, hemicellulose, lignin, and ash. The saccharification of cellulosic waste requires pre-treatment with alkali to remove the lignin and other inhibitors, which increases the crystalline nature of cellulose thus enhancing its reactivity with the cellulase. Mutant strains of *Trichoderma reesei* QM-9414 producing cellulases are used for saccharification of cellulosic waste materials.

Large quantities of free enzyme is produced in plug flow reactor in comparison to batch, continuous stirred tank reactor (CSTR) and exceeds the saccharification rate by 80% at a high saccharification concentration. The cellulase is recovered and reused for

saccharification by centrifugation and ultrafiltration techniques. Reverse osmosis is employed to concentrate the sugars obtained by the saccharification process. This saccharified product is used for supplementing fermentation media for ethanol production.

Extraction of fruit/vegetable juices and olive oil:

Cellulases along with other macerating enzymes (pectinases and hemicellulases) are employed for extraction and clarification of fruit and vegetable juices. The use of enzymes increase juice yield without additional capital investment. The macerating enzymes are responsible for partial or complete liquefaction of fruit/vegetable pulp, which enhances the juice yield, reduces the processing time and to some extent cause the release of valuable fruit components in to the juices.

The macerating property of the cellulase, pectinases and hemicellulases are now being commercially utilized for the extraction of olive oil extraction. This has improved the yield of olive oil (1-2 kg per 10 kg olives), increased anti-oxidants and vitamin E content of oil, and also reduces oil content in the waste water.

Beer and wine industry:

During malting of barley, the seed germinates and produces α- and β-amylases, carboxypeptidases and β-glucanses which hydrolyse the seed reserve for further fermentation by yeast. Sometimes the malting is not proper due to poor quality of barley resulting in gel formation, poor filtration, and haze development in the final

product. Gel formation in wort is due to the presence of β-glucan. β-glucanases, endoglucanses, cellobiohydrolase II are used for digesting β-glucan and reducing the wort viscosity. The enzyme improves the quality of beer produced from poor quality of barley.

Commercial macerating enzymes (cellulases, pectinases and hemicellulases) are used for extraction of grape juice during wine making. The enzymatic treatment improves the filtration rate, wine stability, colour extraction, and reduces the must viscosity.

Feed industry:

The treatment of feed materials (grains, vegetable, forage) with cellulases and other hydrolytic enzymes is practiced to improve the nutritive values. Better digestibility, weight gain, and milk production have been observed in the animals fed on enzymatically treated feed. Attempts are also there to clone the cellulase and xylanase in animals for improving their digestion efficiency.

Paper Processing :

The cellulases along with hemicellulases find application in the pulping process of the woody raw material referred as biopulping, reduces the energy consumption and also improves in the paper strength. The cellulase treatment of pulp improves the betability and drainage property which increases the speed of paper mill. The cellulases are commercially used for deinking of xerographic and laser inks from paper which are otherwise difficult to remove by chemical methods. The de-inking of paper helps in recycling of paper without reducing the fibre strength. All these enzymatic

process have greatly reduced the energy consumption and chemical usage in paper manufacture.

Production of ethanol from cellulosic (Bio ethanol):

The conversion of cellulosic waste to fermentable sugars with the use of efficient cellulases has promising process technology fermentation processes for the production of ethanol. This ethanol is referred as bio ethanol and is a great achievement in the area of non-conventional sources of energy. The production process involves pretreatment of the cellulosic waste (agricultural and municipal wastes) by steam explosion process to expose the cellulose to be acted by the cellulases and other enzymes producing C6 and C5 sugars. Finally fermentative microorganisms convert both C6 and C5 sugars into ethanol. The use of the bio ethanol decreases the 90% CO_2 emmision in comparison to gasoline.