2

Review of Literature

Soil is a mixture of organic matter, minerals, gases, liquids and microorganisms. Microorganisms in particular play an important role in the decomposition of organic matter. Different types of microbes are specialized to different types of organic matter. Soil microbes play a crucial role in returning nutrients to their mineral forms (https://sesl.com.au/blog/the-role-of-soil-microorganisms/). Soil enzymes are central to the ecosystem processes because they catalyze innumerable reactions in soils that have biogeochemical significance. Catalytic soil enzymes can exist internally on surface membranes of viable cells, be excreted into soil solution or be complexed in the soil matrix or microbial debris. Extracellular enzymes may play an ecological role for some microbial community by hydrolyzing substrates that are too large or insoluble for direct absorption by microbial cells. More than 100 enzymes have been characterized in soils. Many studies have addressed soil enzyme activity with the goal of understanding soil nutrient cycling and decomposition biochemistry (Dotaniya *et al.*, 2019; Dick and Kandeler, 2005).

There is a growing need to develop a cost-effective, environment-friendly method for synthesizing such enzymes, to replace traditional carbon and nitrogen sources in various media. Use of agro-industrial residues as carbon and nitrogen sources has become popular in the synthesis of eco-enzymes (Rathod and Pathak, 2014). A number of sources are available to produce the enzymes including microorganisms, plants and animals. However, more efforts are directed towards extremophiles and symbiotic microorganisms.

In recent years, a number of studies have been conducted to characterize protease from different microorganisms. Thus, although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications.

The review of literature pertaining to the present study **‘Characterization, Immobilization and Applications of Extracellular Protease from *Bacillus* sp. ASASBT isolated from Termite Soil’** is discussed under the following headings.

2.1 Importance of soil
2.2 Microorganisms and microbial sources
  2.2.1 Microorganisms
    2.2.1.1 *Bacillus* sp.
  2.2.2 Microbial sources
    2.2.2.1 Termite Mound Soil
    2.2.2.2 Organic Waste degraded Soil
    2.2.2.3 Textile Effluent degraded Soil
    2.2.2.4 Marine Soil
2.3 Enzymes
  2.3.1 Classification of enzymes
    2.3.1.1 EC1: Oxidoreductases
    2.3.1.2 EC2: Transferases
    2.3.1.3 EC3: Hydrolases
    2.3.1.4 EC4: Lyases
    2.3.1.5 EC5: Isomerases
    2.3.1.6 EC6: Ligases
  2.3.2 Proteases
  2.3.3 Sources of proteases
    2.3.3.1 Plant proteases
    2.3.3.2 Animal proteases
    2.3.3.3 Microbial proteases
  2.3.4 Classification of proteases
    2.3.4.1 Serine proteases (EC: 3.4.21)
    2.3.4.2 Cysteine proteases (EC: 3.4.22)
    2.3.4.3 Aspartic proteases (EC: 3.4.23)
    2.3.4.4 Metallo proteases (EC: 3.4.24)

2.1 Importance of soil

The organic matter present in soil are plant, animal and microbial residues in various states of decomposition, which forms as a critical ingredient. The percentage of soil organic matter indicates the better quality of the agricultural soil. There are a host of small, medium and large organisms that live in soils, including mammals, birds, insects and protozoa. But the greatest biodiversity lies in the soil microbes - the bacteria, fungi, and archaea. A teaspoon of rich soil can contain one billion bacteria. Soil microbiologists are applying advanced molecular techniques to understand the diversity and function of soil microbes. It is an exciting field of exploration with new biological taxa frequently being discovered (Hoorman, 2016 and Jacoby *et al.,* 2017).

2.2 Microorganisms and microbial sources

2.2.1 Microorganisms

Microorganisms is a broad term used to encompass bacteria, yeast, fungi and in some definition viruses. The classification is broad and includes both microorganisms that are capable of replication outside of any host and those that require a host to survive (Gupta *et al.*, 2002). Microorganisms comprise about half of the biomass on our planet and play a key role in the biogeochemical cycling of elements like carbon, nitrogen and sulfur. Furthermore, due to their small size and short generation time, microorganisms provide ideal model systems for the study of many universal ecological processes. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Screening of microorganisms from unexplored natural and manmade environments will significantly facilitate the search for these enzymes. Exploring these habitats will provide access to novel bacteria and their robust enzymes that can act under multiple extreme conditions (Raval *et al.,* 2013, Jadhav *et al.,* 2014; Almas *et al.,* 2009).

2.2.1.1 *Bacillus* sp.

Bacteria belonging to *Bacillus* sp. are by far the most important source of several commercial microbial enzymes. They can be cultivated under extreme temperature and pH conditions to give rise to products that are in turn stable in a wide range of harsh environments. *Bacillus* is a rod shaped, gram positive, spore forming, aerobic, usually catalase positive, chemoorganotropic bacterium. Alkaliphilic *Bacillus* can be found mostly in alkaline environments such as soda soils, soda lakes, neutral environments and deep-sea sediments. Animal manure, man-made alkaline environments such as effluents from food, textile, tannery, potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry are also good sources. Many species of *Bacillus* can produce copious amounts of enzymes which are used in various industries, such as the production of protease subtilisin used in detergents, alpha-amylase used in starch hydrolysis, natural antibiotic protein barnase (a ribonuclease) and BamH1 restriction enzyme used in DNA research. *B.subtilis* is a valuable model for bacterial research. Some *Bacillus* species can synthesize and secrete lipopeptides, in particular surfactants and mycosubtilins

(Alcaraz *et al.*, 2010). They can occur in extreme environments such as high pH (*B. alcalophilus*), high temperature (*B. thermophilus*) and high salt concentrations (*B. halodurans*). *B. thuringiensis* produces a toxin that can kill insects and thus has been used as insecticide. *B. siamensis* has antimicrobial compounds that inhibit plant pathogens, such as the fungi *Rhizoctonia solani* and *Botrytis cinerea* and they promote plant growth by volatile emissions. Some species of *Bacillus* are naturally competent for DNA uptake by transformation (Keen *et al.*, 2017).

2.2.2 Microbial sources

Microorganisms are found everywhere - air, soil, water, farms, animals, feces, processing plants, retail stores, restaurants, homes and people. Indeed, people are one of the main sources responsible for transmission and control of microbial growth and contamination. Most microorganisms cause spoilage, illness, disease and even death, while some microorganisms are harmless, beneficial and are actually used in the production of various industrial products (Lonergan, 2019).

Most soil microorganisms work in the “recycler” role. These are the decomposers that take dead plant and animal matter and break it down. The microbes that work in the recycling role, use the organic carbon in the organic matter as an energy source. The minerals and microbes in soil are responsible for filtering, buffering, degrading, immobilizing and detoxifying organic and inorganic materials, including industrial and municipal waste by-products and atmospheric deposits.

2.2.2.1 Termite Mound Soil

Termites have both beneficial and harmful effects on the soils. The beneficial effects are addition of plant nutrients (organic matter) from the subsoil which is used for mound construction, the soil becomes enriched with subsequent erosion and leaching, those nutrients are distributed to the surrounding soils. The harmful effects include - removal of organic matter present in the soil and nutrients held up in the termite gut (Jouquet *et al.*, 2015). Termites go through a sequence of actions, from fetching, carrying, to cementing mineral particles into mounds by using their salivary secretion, which in turn the microbial density in mound soil will be increased (Fall *et al.*, 2004). There are high proportion of phosphorus, calcium, potassium, magnesium and sodium in termite mound soil. Termite mounds are common features of agricultural landscape of the tropics and relatively not much attention has been given to their potential influence on agricultural production (Semhi *et al*., 2008).

2.2.2.2 Organic Waste degraded Soil

A variety of organic wastes generated through different household, agricultural and other activities in our day to day life including vegetable residues in the form of biomass, animal wastes and by products like dung, urine, bones, fish processing wastes and human habitation wastes like garbage, sewage and sludge etc. A considerable part of which remains unutilized and are either burnt or dumped at nearby sites that create pollution, pathogen for diseases and causes severe problem of disposal. These accumulated wastes left on the field side causes major unpleasant odours and furthermore, they are often been the source of contamination of ground water. However, most of these potentially nutritious wastes are recyclable. The positive impact of organic waste application on the improvement of physical properties of soil and the microorganisms present in those degraded soil can be used for various industrial needs thereby reducing the pollution problem (Chatterjee *et al.*, 2017).

2.2.2.3 Textile Effluent degraded Soil

In textile industries, during dyeing process approximately 50% of the applied dyes are discharged as waste effluent to the environment without proper disposal of toxic matters (Asad *et al.*, 2007). Because of its resistance to degradation by light, chemicals and microbes, textile industry wastewater treatment is very challenging (Shah *et al.*, 2013, Marchis *et al.*, 2011). The use of microbial techniques to deal with pollution or bioremediation is a key research area in the environmental sciences (Kumar *et al.*, 2011, Najirad *et al.*, 2012). In such approach microbes involves themselves to the toxic wastes and promoting the capacity of degrading different toxic chemicals. Several reports demonstrate the degradation of complex organic substances which can be brought about by enzymatic mechanisms. A number of biotechnological approaches have attracted interest with regard to tackling textile industrial dye pollution in an eco-efficient manner, mainly with the utilization of bacteria for wide industrial applications (Karthik *et al.,* 2014).

2.2.2.4 Marine Soil

The world's ocean's coastline is of 312,000 km and it has been used since long period of time for variety of purposes. Marine microbes are found to a potential source for commercially available enzymes. The oceans cover many ranges of extreme environmental factors. Viable microorganisms have been recovered from environments with temperatures ranging from as low as -32°C (Breezee *et al.,* 2004; Cassell and Mekalanos, 2001; Price, 2000; Price and Sowers, 2004) to the surroundings of deep-sea hydrothermal vents where temperatures approach 400°C (Eecke *et al.,* 2012; Gerday *et al.,* 2007). The specialization of the inhabitants has also yielded biosynthetic pathways that have just begun to be analyzed. The sampling of bacterial communities of extreme niches in marine environments carries great potential to be a major approach for unearthing new natural products (Maldonado *et al.,* 2005; Thornburg *et al.,* 2010).

2.3 Enzymes

Enzymes are specialized proteins or macromolecular biological catalysts produced in an organism which is capable of catalyzing specific chemical reactions. The molecules upon which enzymes may act are called substrates and the enzymes convert the substrates into different molecules known as products. Almost all metabolic processes in the cell need enzyme catalysis in order to occur at rates fast enough to sustain life (Stryer *et al.,* 2002). They are known to catalyze more than 5,000 biochemical reaction types. A few enzymes are catalytic RNA molecules called ribozymes. Specificity of enzymes comes from their unique three-dimensional structures. Like all catalysts, enzymes increase reaction rates by lowering their activation energy. Some enzymes can make their conversion of substrate to product occur many millions of times faster (Schomburg *et al.,* 2013).

2.3.1 Classification of enzymes

The International Union of Biochemistry and Molecular Biology have developed a nomenclature for enzymes the EC numbers. Each enzyme is described by a sequence of four numbers preceded by "EC", which stands for "Enzyme Commission". The first number broadly classifies the enzymes based on mechanism (Nomenclature Committee, 2015). Enzymes are classified into six major groups. Each of the groups has many subgroups (Veerakumari, 2005).

2.3.1.1 EC1: Oxidoreductases

These catalyze oxidation and reduction reactions between two substances. They are concerned with reactions of biological oxidation and involve transfer of hydrogen atoms or electrons from one substrate to another. Example: pyruvate dehydrogenase which catalyzes the oxidation of pyruvate to acetyl coenzyme A.

2.3.1.2 EC2: Transferases

These catalyze the transfer of a chemical group from one compound to another. An example is a glutamate oxaloacetate transaminase which transfers an amine group from one molecule to another, transacylases which transfer acyl and acetyl groups; transpeptidases-which transfer amino acids or peptides from one peptide to another.

2.3.1.3 EC3: Hydrolases

These enzymes catalyze the hydrolysis of a bond. For example, the enzyme pepsin (protease) hydrolyzes peptide bonds in proteins.

2.3.1.4 EC4: Lyases

These enzymes catalyze breakage of bonds without catalysis, e.g. aldolase (an enzyme in glycolysis) catalyzes the splitting of fructose-1, 6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Depending on the nature of the chemical groups added or removed they are further classified as dehydratases catalyzing reversible removal and addition of water molecule, desulphydratases - removal of hydrogen sulphide and decarboxylase-removal of carbondioxide.

2.3.1.5 EC5: Isomerases

These are the enzymes, which catalyze the formation of an isomer of a compound. Eg. phosphoglucomutase catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate (transfer of a phosphate group from one position to another in the same compound) in glycogenolysis (conversion of glycogen to glucose for quick release of energy).

2.3.1.6 EC6: Ligases

Ligases catalyze the joining of two molecules. For example, DNA ligase catalyzes the joining of two fragments of DNA by forming a phosphodiester bond.

2.3.2 Proteases

Proteases is an important group of enzymes which conducts proteolysis by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. It comes under the class hydrolases.

Protease plays an essential role in the growth and survival of all living organisms. Protease production is an inherent capacity of all microorganisms. Bacteria are the most predominant group of alkaline protease producers. The genus *Bacillus* being the most common source (Singh *et al.*, 2015). The history of proteolytic enzymes or peptidases can be traced back at least to the late eighteenth century. However, in recent times the research work in this area has accelerated greatly, fueled by numerous practical applications in biotechnology and the realization of the fact that they are among major therapeutic targets (Homaei *et al.*, 2016).

2.3.3 Sources of proteases

Proteases of commercial importance are produced from plant, animal and microbial sources. They constitute a very large and complex group of enzymes with different properties of substrate specificity, active site and catalytic mechanisms, pH and temperature activity and stability profiles. The preferred sources of proteases are microbes because of their rapid growth and the ease with which they can be genetically manipulated to generate new enzymes with altered properties. The most common and widely used bacteria for industrial proteases belong to the genus *Bacillus* (Maurer, 2004; Saeki *et al.*, 2007; Krishna *et al.*, 2011). Industrial proteases have applications in a range of process as taking advantage of the unique physical and catalytic properties of individual proteolytic enzyme types. This vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Naik *et al.*, 2013). Some intracellular enzymes are used commercially without isolation and purification but the majority of commercial enzymes are either produced extracellularly by microbes or plants or are released from the cells into solution and further processed.

2.3.3.1 Plant proteases

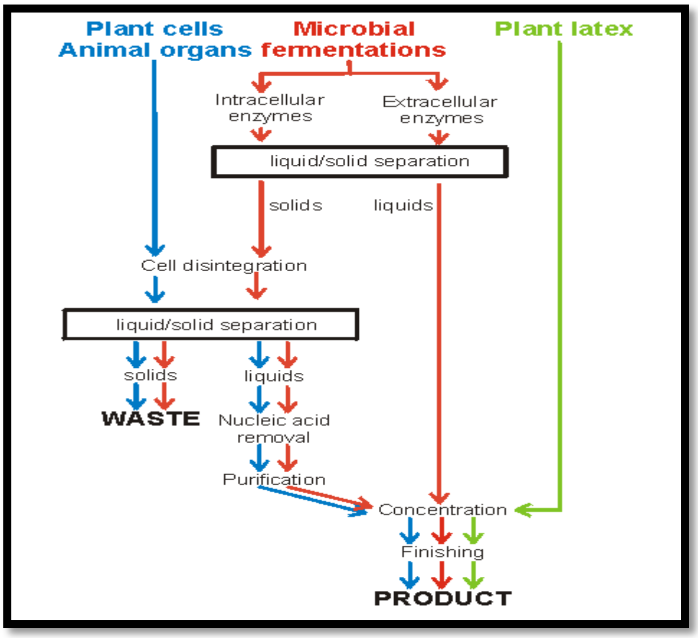
The use of plants as a source of proteases is governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process. Papain, bromelain, keratinases and ficin are some of the well-known proteases of plant origin.

Papain is a traditional plant protease with a long history of use especially in tonics, which is active between pH 5.0 and 9.0 (Schechler and Berger, 1967). It is extracted from the latex of *Carica papaya* fruits. It is extensively used in industry for the preparation of highly soluble and flavoured protein hydrolysates.

Bromelain is prepared from the stem and juice of pineapples. The major supplier of the enzyme is Great Food Biochem., Bangkok, Thailand. The enzyme is characterized as a cysteine protease and is active from pH 5.0 to 9.0. Its activation temperature is 70°C, which is lower than that of papain.

Keratinases are produced by some plants which degrade hair. Digestion of hair and wool is important for the production of essential amino acids such as lysine and for the prevention of clogging of wastewater systems (Secor *et al*., 2005).

Figure 1
Flow diagram for the preparation of enzymes
(http://www1.lsbu.ac.uk/water/enztech/preparation.html**)**



2.3.3.2 Animal proteases

The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and renin.

Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. It is a serine protease and hydrolyzes peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues. It has limited applications in the food industry, since the protein hydrolysates generated by its action have a highly bitter taste and also used in the preparation of bacterial media and in some specialized medical applications.

Chymotrypsin is found in pancreatic extracts of animals. Pure chymotrypsin is an expensive enzyme, which is used only for diagnostic and analytical applications. It is specific for the hydrolysis of peptide bonds in which the carboxyl groups are provided by one of the three aromatic amino acids, i.e., phenylalanine, tyrosine, or tryptophan. It is used extensively in the deallergenizing of milk protein hydrolysates. It is stored in the pancreas in the form of a precursor, chymotrypsinogen and is activated by trypsin in a multistep process

Pepsin is an acidic protease that is found in the stomach of all vertebrates. The active enzyme is released from its zymogen, i.e., pepsinogen, by autocatalysis in the presence of hydrochloric acid. Pepsin is an aspartyl protease and resembles human immunodeficiency virus type 1 (HIV-1) protease, responsible for the maturation of HIV-1. The enzyme catalyzes the hydrolysis of peptide bonds between two hydrophobic amino acids. Pepsin has been used in laundry detergents as early as 1913, which is now being replaced by a mixture of serine and metal microbial proteases, which appears to be less degradable by detergents, alkaline conditions and high temperatures.

Rennet is a pepsin-like protease that is produced as an inactive precursor, prorennin in the stomach of all nursing mammals. It is converted to active rennin by the action of pepsin or by its autocatalysis. It is being used extensively in the dairy industry to produce a stable curd with good flavour (Adinarayana and Ellaiah, 2002). The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in k-casein to generate insoluble para-k-casein and C-terminal glycopeptide.

2.3.3.3 Microbial proteases

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications and for the large-scale production of proteases due to their fast growth and simplicity of life for the generation of new recombinant enzymes with desired properties. It accounts for approximately 40% of the total worldwide enzyme sales. Proteases play a decisive role in detergent, pharmaceutical, leather, food and agricultural industries. Currently, the estimated value of the global sales of industrial enzymes is over 3 billion USD (Rao *et al.,* 1998; Kumar and Takagi, 1999).

Neutral and alkaline proteases hold great potential for application in the detergent and leather tannery industries due to the increasing trend in developing environment-friendly technologies. Alkaline proteases have numerous applications in the food industries, silver recovery from X-ray films and several bioremediation processes. There are two types of secreted proteases-intracellular and extracellular. Intracellular proteases are vital to sustain various cellular and metabolic processes such as sporulation and cell differentiation, protein turn over, enzyme maturation and hormones. Extracellular proteases carry out protein hydrolysis in fermented media and enable the cell to absorb and utilize hydrolytic products (Jisha *et al.*, 2013).

Bacteria - Most commercial proteases, mainly neutral and alkaline are produced by organisms belonging to the genus *Bacillus.* Bacterial neutral proteases are active in a narrow pH range (pH 5.0 to 8.0) and have relatively low thermotolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do animal proteinases and hence are valuable for use in the food industry. Neutrase, a neutral protease, is insensitive to natural plant proteinase inhibitors and is therefore useful in the brewing industry. The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity, while others are serine proteinases which are not affected by chelating agents. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10.0 and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao *et al.*, 1998).

Fungi - Fungi elaborate a wider variety of enzymes than do bacteria. For example, *Aspergillus oryzae* produces acid, neutral and alkaline proteases. The fungal proteases are active over a wide pH range (pH 4.0 to 11.0) and exhibit broad substrate specificity. Fungal enzymes can be conveniently produced in a solid-state fermentation process. Fungal acid proteases have an optimal pH between 4.0 and 4.5 and are stable between pH 2.5 and 6.0. They are particularly useful in the cheesemaking industry due to their narrow pH and temperature specificities. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating agents. Fungal alkaline proteases are used in food protein modification (Rao *et al.*, 1998).

Viruses - Viral proteases have gained importance due to their functional involvement in the processing of proteins of viruses that cause certain fatal diseases such as AIDS and cancer. Serine, aspartic and cysteine peptidases are found in various viruses (Rawlings and Barrett, 1993). All of the virus-encoded peptidases are endopeptidases. There are no metallopeptidases. Retroviral aspartyl proteases that are required for viral assembly and replication are homodimers and are expressed as a part of the polyprotein precursor (Kuo and Shafer, 1994). Extensive research has focused on the three-dimensional structure of viral proteases and their interaction with synthetic inhibitors with a view to designing potent inhibitors that can combat the relentlessly spreading and devastating epidemic of AIDS.

2.3.4 Classification of proteases

Proteases are classified based on the chemical nature of the active site, the reaction they catalyse and their structure and composition. They are mainly classified into endopeptidases and exopeptidases based on the catalytic site on the substrate. In EC system for enzyme nomenclature, all proteases belong to subclass 3.4, which is further divided into 3.4.11-19, the exopeptidases and 3.4.21-24, the endopeptidases. Exopeptidases act at the end of the polypeptide chain, while endopeptidases preferably act at the inner region of the polypeptide chain. Exopeptidases are further classified into amino peptidases and carboxypeptidases proteases which act at the free N-terminus of the polypeptide substrate and free C-terminal of the polypeptide chain respectively (Hamza, 2017). The overall classification system of proteases is summarized in Figure 2.

In the same way, endopeptidases are also classified based on their side chain specificity and functional group present in the characteristic active site. They may also be classified on the basis of their active site. Serine proteases (EC.3.4.21), Cysteine proteases (EC.3.4.22), Aspartic proteases (EC.3.4.23), Metalloproteases (EC.3.4.24) (Bizuye, *et al.*, 2014).

2.3.4.1 Serine proteases (EC: 3.4.21)

Serine proteases are most widely distributed among microorganisms and eukaryotes and are characterized by the presence of a reactive serine (-OH) residue in the active site. These proteases are optimally active over a wide pH range 7.0 – 11.0 and have broad substrate specificities including amidase and esterolytic activity. Serine alkaline proteases have the largest commercial application owing to their high activity and stability in extreme reaction conditions. This group of enzymes are generally inhibited by di-isopropyl fluorophosphates, 3,4-dichloroisocoumarin (3,4-DCI), 1-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), tosyl-1-lysine chloromethyl ketone (TLCK) and phenylmethylsulfonyl fluoride (PMSF) (Nigam, 2013).

2.3.4.2 Cysteine proteases (EC 3.4.22)

Cysteine proteases are proteins that constitute a catalytic dyad consisting of cysteine and histidine for their activity. Though reducing agents such as HCN or cysteine, DTT, EDTA are required for stimulation of the catalytic activity of cysteine proteases but inhibited by sulfhydryl (SH) reagents such as 4-hydroxy mercuri benzoic acid (p-CMB), iodoacetic acid, iodoacetamide, etc. They have high potential in food and pharmaceutical applications due to their activity over a wide range of temperature and pH. They are involved in metabolic degradation of proteins and peptides such as scrapie protein degradation dendritic and neuronal cells. Papain is one of the best-known microbial cysteine proteases which is widely used in food industry (Souza *et al.*, 2015; Singh *et al.*, 2016b).

2.3.4.3 Aspartic proteases (EC 3.4.23)

Aspartic proteases contain one or more side chain carboxyl groups in their active site and are commonly known as acid proteases. It has been obtained from a variety of organisms including plants and animals. These enzymes have two aspartic acid residues at their active sites which can optimally be activated at acidic pH values (pH 3.0 to 4.0) and they are specifically inhibited by pepstatin and in the presence of copper ions by diazoketone compounds. Amino acid sequences of aspartic proteases show high level of homology. These proteases preferentially cleave peptide bonds between non-polar amino acid residues and the three-dimensional structure of these enzymes have been preserved (Domingos *et al.,* 1992; Salehi *et al.,* 2017).

2.3.4.4 Metallo proteases (EC 3.4.24)

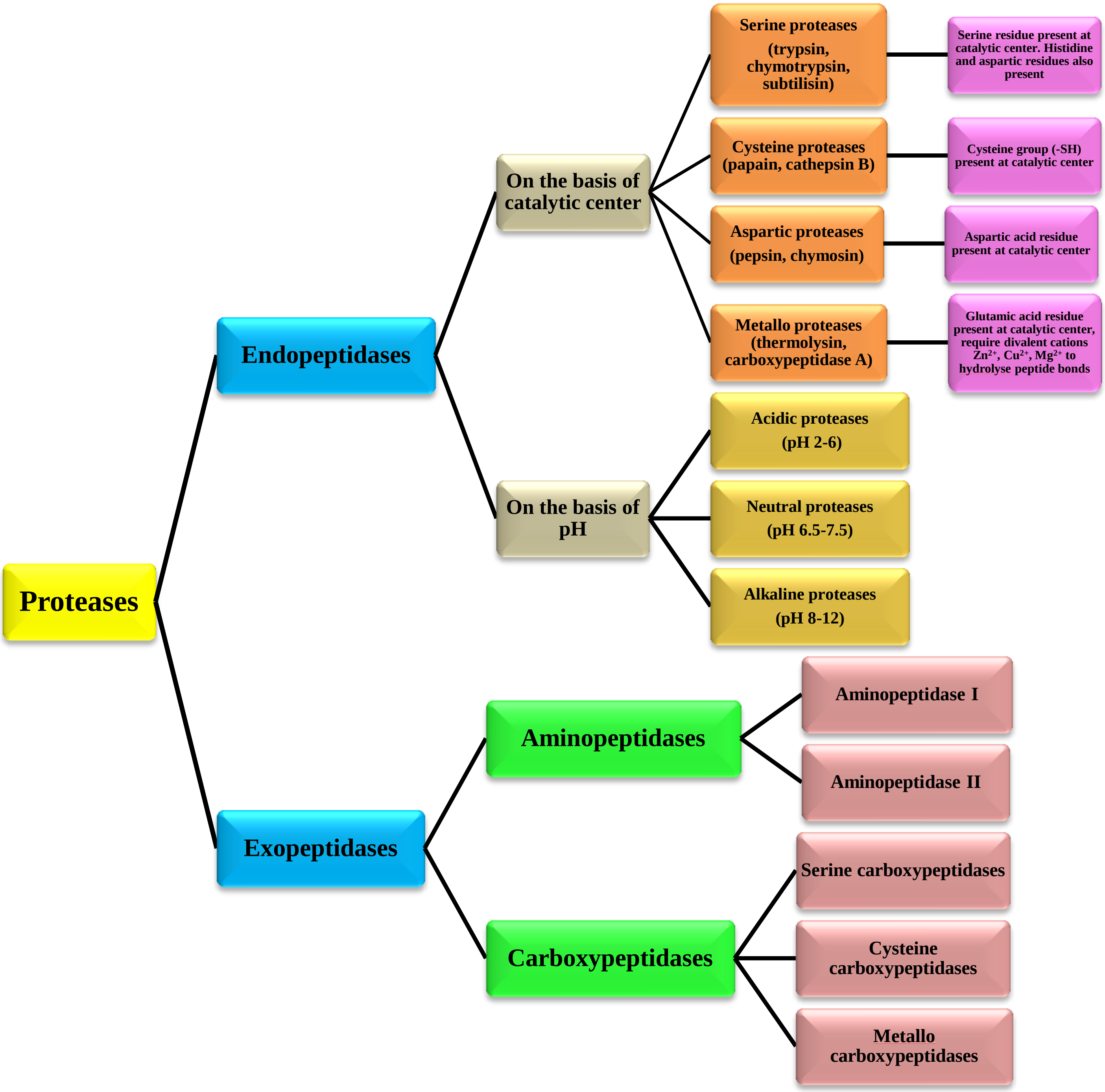
This group of enzymes require divalent metal ions, such as zinc, cobalt, manganese or nickel for their catalytic activity. These proteases are sensitive to chelating agents, such as EDTA, due to sequestering effect of chelating agents on the metal ions involved in the catalytic mechanism. They have a wide range of substrate specificity. The water molecule serves as a nucleophile in catalysis and also coordinates with the metal ion as a fourth ligand. The catalytic metal ion is usually coordinated by three conserved amino acid side chain ligands that can be His, Asp, Glu, or Lys amino acid and at least one other residue, which may play an electrophilic role. Metalloproteases exhibit deviant physiological and biochemical properties that account for their wide range of applications in different industries including therapeutical, pathophysiological and drug development (Vranova *et al.,* 2013).

In addition, proteases are also classified into three other important groups, namely threonine proteases, glutamic acid proteases and asparagine proteases. Threonine proteases (EC 3.4.25) are represented by the presence of a threonine residue at the catalytic sites. The classic examples of this group are acyltransferases and proteasome. Glutamic acid (EC 3.4.19) and asparagine proteases are characterized by the presence of glutamic acid and asparagine residue at their active site, respectively. Glutamic acid proteases have potential applications in food industry and therapeutic management (Srilakshmi *et al.,* 2014; Singh *et al.,* 2016b).

Figure 2

Classification of proteases

(Hamza, 2017)



Further, endopeptidase proteases are classified according to their maximal activity in a particular pH range as - Acid proteases which are active in the pH ranges of 2.0 - 6.0. Neutral proteases are proteases which are active at neutral, weakly alkaline or weakly acidic pH. Alkaline proteases can be defined as the protease those are active in alkaline range of pH (8.0 - 12.0) (Rao and Narasu, 2007).

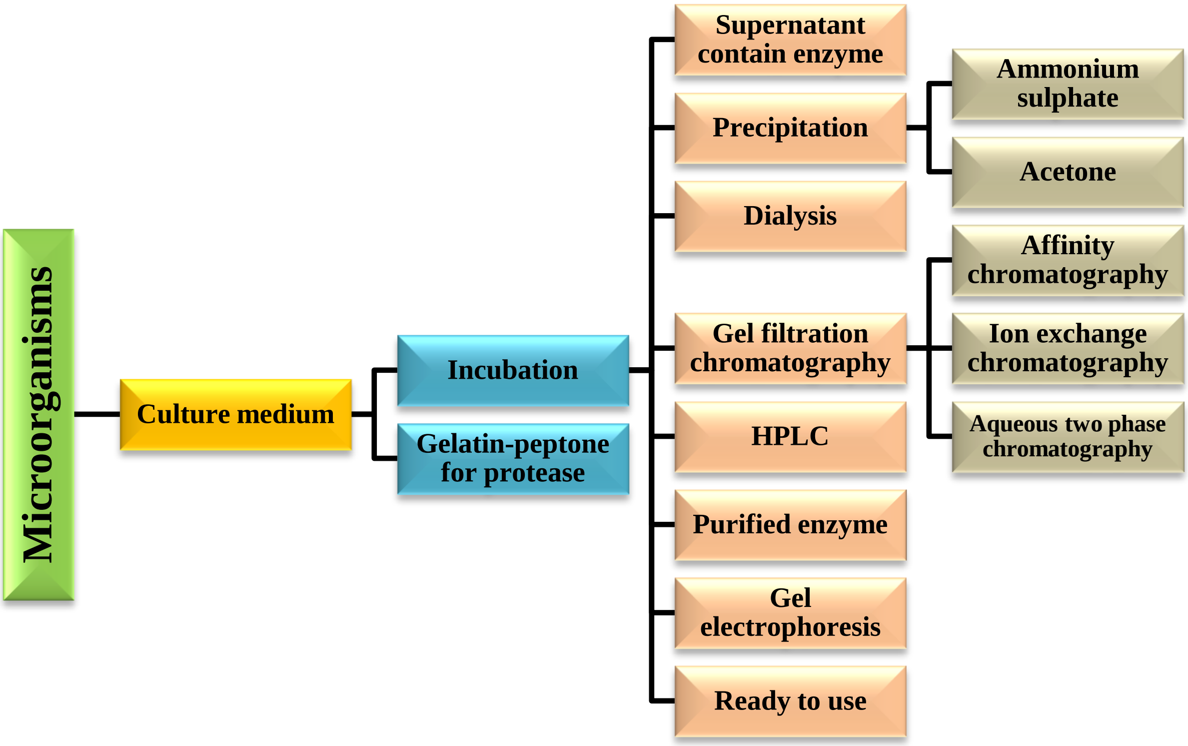
2.4 Purification of protease

Biotechnological industries produce different types of microbial enzymes in bulk amount and do not require high purity. However, enzymes used in pharmaceutical, medical and research purpose require high purity. Several types of classical methods have been employed for enzyme purification from microbial sources.

Figure 3

Microbial protease purification

(Patel *et al.*, 2017)



Enzyme purification is not a single step procedure. It requires combination of several technologies and methods. Since the first alkaline protease Carlsberg from *Bacillus licheniformis* was commercialized as an additive in detergents in the 1960s (Saeki *et al.*, 2007), a number of *Bacillus*-derived alkaline proteases have been purified and characterized and significant proteolytic activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification, and low cost have been demonstrated (Haddar *et al.*, 2010). Purification details of proteases from several microbial sources are given in Table 1. Few important techniques that are generally used for microbial enzyme purification are described below.

Table 1

Purification details of proteases from several microbial sources (Banerjee and Ray, 2017)

Microbial sources	Method of purification	Purification fold	Recovery (%)
Lactobacillus plantarum A6	50-70% $(NH_4)_2SO_4$, DEAE-Cellulose	19.5	35.6
Penicillium expansum	Acetone, Sephadex G-100, DEAE-Sephadex A-50	96.23	48
Bacillus subtilis	80% $(NH_4)_2SO_4$, DEAE-Sepharose, Sephacryl S-200	37	18
Chaetomium thermophilum	90% $(NH_4)_2SO_4$, DEAE-Sepharose, Phenyl-Sepharose (PRO33)	19.3	4
Aspergillus niger	74% $(NH_4)_2SO_4$, DEAE-Cellulose	34.42	32
Chromohalobacter sp.	Ethanol, Phenylsepharose 6B column, Gel permeation G-100	180	22
Aspergillus flavus	70% $(NH_4)_2SO_4$, DEAE-Cellulose	5.8	3.2
Bacillus cereus	60% $(NH_4)_2SO_4$, Sephadex G-200, SephadexG-100	-	-
Bacillus megaterium	60% $(NH_4)_2SO_4$, DEAE-Cellulose, Sephadex G-200	7.72	11
Bacillus licheniformis B18	70% $(NH_4)_2SO_4$, DEAE-Cellulose, Sephadex G-100	26.33	10.6
Corynebacterium alkanolyticum	60% $(NH_4)_2SO_4$, Sephadex G-50, DEAE Cellulose	26.03	15.21

2.4.1 Ion exchange chromatography

In ion exchange chromatography, separation or purification of enzymes depends on ionic nature (positive or negative). Generally, two types of beads are used, DEAE-sephadex / cellulose (anion) and CM-sephadex/cellulose (cation). Mostly microbial enzymes are positively charged and require anion exchanger. Elution of bounded molecules is done by increasing salt concentration (usually NaCl and $CaCl_2$) or pH gradient.

2.4.2 Gel filtration chromatography

It is a very common method of enzyme purification. Several types of gels or beads have been used for column packing such as Sephadex (G-25, G-50, G-75, G-100, G-200), Sepharose, Sephacryl, Superdex and so on. The selection of packing material depends on the molecular weight of the enzymes. Low molecular weight enzymes (10 - 80 kDa) require Sephadex G-25 to G-75. Whereas, enzymes having higher mass require G-100 to G-300. Packing of column is very important for proper purification. Bubble in the column hampers the flow rate and thus before packing, gel should be properly de-gassed.

2.4.3 Fast Protein Liquid Chromatography (FPLC)

It is one of the most popular chromatography instruments, usually used to purify the proteins and enzymes. Like liquid chromatography, it also contains a liquid mobile phase or buffer (phosphate buffer and citrate buffer) and a stationary phase which contains resin. The flow rate of buffer is controlled by a positive-displacement pump. Selection of column is important in Fast protein liquid chromatography and may be varied (size exclusion, hydrophobicity, ion exchange, reverse phase and the like according to the nature of separating molecules.

2.5 Characterization of protease

The characterization of protease is an important step that leads to the development of novel tools for therapeutic and biotechnological applications. The effects of varying pH, temperature, substrate concentration, metal ions and inhibitors on the activity of the enzymes are needed to find out the optimum conditions (Amaral *et al.,* 2006). There is a particular range of pH over which the activity of an enzyme is generally maximum. Excessive acidity or alkalinity causes the destruction of enzymes. The rate of enzyme action increases with increase in temperature, where the enzyme action is maximum is called optimum temperature, beyond which the enzyme being protein in nature, gets denatured and as a result, the rate of reaction decreases constantly (Veerakumari, 2005). The speed of any reaction being catalyzed by a particular enzyme can reach a certain maximum value based on the substrate concentration. Proteases can be classified based on their sensitivity to various inhibitors. Metal ions, organic solvents and oxidizing agents also play an important role in the applications of enzymes (Oliveira *et al.*, 2010)

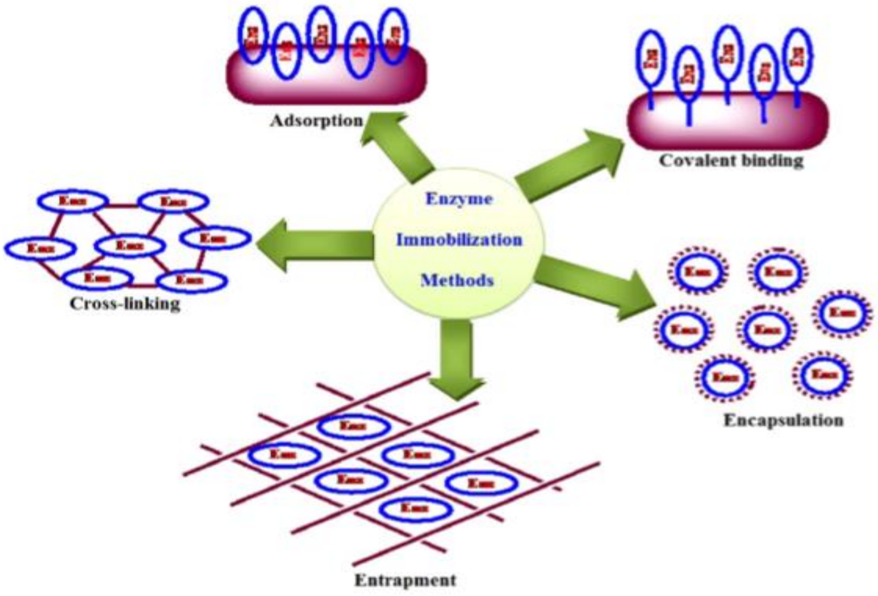
2.6 Immobilization of protease

The use of free enzymes has been limited, because they easily denature and have a short lifetime and are unstable. The immobilization of enzymes can increase their stability and protect their chemical and biological functions from degradation (Chen *et al.*, 2013). Enzyme immobilization is a technique specifically designed to restrict the freedom of movement of an enzyme. Immobilization of enzymes is a common practice, mainly in order to minimize enzyme costs on the process economics by making it possible to reuse the enzyme many times and also minimize the operation cost as the immobilization technique may be modify the enzyme behavior, thus reducing the enzyme and product costs significantly (Mohamed *et al.*, 2016).

Figure 4

Basic enzyme immobilization methods

(Asgher *et al.*, 2014)



For industrial applications, the immobilization of protease on a solid support can offer several advantages, including repeated usage of enzyme, ease of product separation, improvement of enzyme stability and continuous operation in packed-bed reactors (Abdel-Naby *et al.,* 1998).

The general methods employed for immobilization are entrapment, microencapsulation, copolymerization, cross linking, physical adsorption, chemical attachment and covalent binding (Hasirci *et al.,* 2006). Figure 4 represents the different methods of immobilization. Among different immobilization techniques, entrapment in calcium-alginate, agar-agar and alginate-chitosan methods offer many advantages due to its simplicity and non-toxic character (Gangadharan *et al.,* 2009).

2.7 Applications of enzymes

2.7.1 General applications of enzymes

Currently, most enzymes used in industrial processes are hydrolytic and are used for the degradation of various natural substances. Over the past few years, the use of enzymes in industrial processes have been significantly increased. Due to the looming climate catastrophe, the search for green industrial processes is more urgent and popular and finally the search for robust enzymes is a key feature of this urgent effort (Raval *et al.,* 2014). Enzymes are fundamental to life and are used in many biotechnological processes (Kumar and Barth, 2010). Currently, enzymes are used in four distinct fields- industrial catalysts, therapeutic agents, analytic reagents, manipulative tools. The global enzyme market is currently dominated by the hydrolases, especially the proteases, together with amylases, cellulases and lipases supplied either as liquid concentrates or as powders or granules that release the soluble enzyme on dissolution (Adrio and Demain, 2014).

2.7.2 Applications of protease

About 75% of industrial enzymes are hydrolytic enzymes, proteases representing one of the three largest groups of industrial enzymes and it allocated about 60% of the total worldwide sale of enzymes. Proteases are one of the most important group of enzymes used in various industries such as detergent, pharmaceutical, textile, leather, photographic and food industries.

Figure 5

Application of bacterial proteases in different sectors

(Tavano *et al.*, 2018)

2.7.2.1 Detergent industry

The thermostability and activity at high pH are the characteristics that have made proteolytic enzymes an ideal candidate for laundry applications (Gupta *et al.*, 2002).

Detergents are very important in fabric cleaning process because they disperse well in water and do not damage the fabric or our body on exposure and it cleanse different types of stains. It can clean stains of protein, chemical, fat, carbohydrate or any other origin. A laundry detergent formulation contains a multitude of components and comes in solid powder form to liquid formulations (Divya and Tyagi, 2007). Enzymes constitute an important part of detergent formulations. Enzymes are highly beneficial because they reduce activation energy of a reaction thereby making a reaction process more efficient with reduced energy consumption. By optimizing the parameters we can ensure that enzymes function most efficiently and deliver the desired (Parameswaran *et al.*, 2013).

2.7.2.2 Leather industry

Proteases are useful in dehairing for the purpose of leather manufacture. Since, the beginning of human civilization, the conventional method of dehairing involved the use of lime and sodium sulphide as the lack of technology. Most of the tannery industries used chemicals for dehairing that led to great environmental and health problem. The tannery pollutants cause heavy damage to water resources, agriculture, fisheries and finally to avoid the deleterious effects of chemical agents in tannery industries, eco-friendly process are being takes place. But currently it is possible to replace chemical dehairing with enzyme dehairing using proteases. In the back drop of this scenario enzymes, started replacing poisonous chemicals from tannery industries (Khan, 2013).

With the advent of enzymes, leather processing in various countries has become environment friendly and in addition to improve the quality of leather produced. Enzymatic dehairing is suggested as an environment friendly alternative to the conventional chemical process. Proteases play an important part in biotechnological applications in the leather industry. Since protease are active in the pH range of 8.0 - 12.0 and stable at alkaline pH, they are potential candidates for dehairing hides (Madhavi *et al.,* 2011, Uddin *et al.,* 2015).

2.7.2.3 Textile industry

The use of enzymes in the textile industry is an example of white / industrial biotechnology, which allows the development of environmentally friendly technologies in fibre processing and strategies to improve the final product quality (Araujo *et al.,* 2008). Textile processing has been benefited greatly in both environmental impact and product quality through the use of enzymes. From the 7000 enzymes known, only about 75 are commonly used in textile industry processes (Quandt and Kuhl, 2001).

The principal enzymes applied in textile industry are hydrolases and oxidoreductases. The group of hydrolases includes amylases, proteases, cellulases, pectinases and lipases/esterases. The potential of proteolytic enzymes was assessed for the removal of wool fibre scales, resulting in improved anti-felting behaviour and degumming of silk. Raw silk must be degummed to remove sericin, a proteinaceous substance that covers the fibre. Degumming is typically performed in an alkaline solution containing soap, a harsh treatment that also attacks fibrin structure. Several alkaline, acidic and neutral proteases have been studied as degumming agents since they can dissolve sericin, but are unable to affect silk fibre protein. Alkaline proteases seem to be the best for removing sericin and improving silk surface properties like handle, shine and smoothness (Arami *et al.*, 2007)

2.7.2.4 Pharmaceutical industry

Proteases of the subtilisin group are used for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses to speed up healing process by producing anti-inflammatory response in patients have been reported (Vadlamani and Parcha, 2011). It is possible to use protease as a therapeutic agent for the treatment of pulmonary emboli and degradation of elastin, collagen. The purified protease from bacteria could be used for various purposes like antibacterial activity against clinical pathogens as well as it degrades slime and bio films to limit gram negative bacteria and also it digests debris in blood like bacterial and viral proteins and act as medicine in the field of oncology (Prabhavathy *et al.*, 2013; Harish and Chauhan, 2017).

Blood clotting in vascular pipeline block blood supply and it is a major problem that causes cerebral and cardiac attack. Researchers have reported that fibrinolytic activity exhibited by several bacterial and fungal species are important in cardiovascular treatment. The molecules which dissolve these clots are some kind of proteases called thrombolytic agents. In the last few years, several thrombolytic agents from microbial sources like streptokinase and staphylokinase were developed to overcome this critical situation (Mane and Tale, 2015). Protease also plays a critical role as an anti-inflammatory agent. The bacterium *Serratia* sp. produced a special protease called serratiopepetidase, which is able to reduce inflammation rapidly.

2.7.2.5 Photographic industry

Silver is one of the precious and noble metals used in large quantities. The waste X-ray film content approximately 1.5 to 2.0 % silver in its gelatin layer. Recovery of silver from used X-ray film is quite difficult and laborious. There are several chemical methods for silver recovery such as burning of film and oxidation of metallic silver, but these are not environment friendly. Alkaline proteases are used for this particular purpose, which actively degrade the gelatin layer of the film and release the bounded silver (Nakiboglu *et al.,* 2001; Kumaran *et al.,* 2013).

Proteases play a crucial role in the bioprocessing of used X-ray films for silver recovery. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled. Various conventional methods are carried out to recover the silver from X-ray film wastes. The films are being burning directly and stripped off the gelatin-silver layer using different chemical solutions, which cause environmental hazards is time consuming and very expensive. In addition, the polyester film on which emulsion of silver and gelatin is coated cannot be recovered. All these pose a serious industrial safety problem. Therefore, bacterial proteases which are stable in the environment of silver recovery has applications in the silver recover industry (Shankar *et al.,* 2010).

2.7.2.6 Food industry

In recent years, microbial proteases have been randomly used in food industries like meat tenderization and cheese preparation (Adrio and Demain, 2014). Milk protein coagulation is the first step in cheese preparation, which is accomplished with the help of protease enzyme. Cheese making industries use microbial source of protease. However, it has two drawbacks - low yield and bitterness upon long storage. Among several classes of proteases, aspartic proteases are considered to be best for cheese industries (Martin and Hernàndez, 2007; Siota *et al.,* 2014).

Microbes produce several types of proteases which are non-specific and thus cause bitterness in storage food. Nissen (1986) has reported that Alcalase (an alkaline protease) removes bitterness from food stuff and make it sweeter. Protease produced by *Bacillus amyloliquefaciens* is useful for making methionine-rich protein hydrolysate from chickpea protein (Wang *et al.,* 2016). Proteases obtained from microbial sources are used for the fortification of fruit juices or soft drinks and in preparation of protein rich therapeutic diets also (Amore and Faraco, 2015). Wheat flour is the major component of baking process which contains an insoluble protein, gluten that determines the bakery dough’s properties. Protease produced by *Aspergillus oryzae* hydrolyzes the gluten and increases the product quality in terms of colour, softness, and texture (Sawant and Nagendran, 2014; Souza *et al.*, 2015).

2.7.2.7 Waste management

The scarcity of natural resources and the accumulation of pollution caused by human activity have required the development of production technology that is less harmful to the environment. One well-established application of modern biotechnology is the use of bacterial protease for treatment of waste or the bioremediation of hydrocarbons (Pandian *et al.*, 2012).

In addition to that, bacterial proteases have interesting potential applications in the management of wastes from households and processing industries and also cleaning of hair clogged pipe lines containing hairs. Microbial sources of keratinases are very useful in waste management due to their broad substrate specificity. In general, keratinases convert the feather or other waste materials (such as scale, hair, wool, etc.) into karatin, which has high demand in poultry and aquaculture industries as a feed ingredient (Motyan *et al.*, 2013). Due to special characteristics, keratinases have been widely used as nematicidal agents, biofuel production and fertilizer production from poultry waste (Verma *et al.*, 2017). Solid waste from the tannery process, large quantities of sludge and effluent from treatment plants is a major source of environmental pollution. Nowadays these bacterial proteases are being used commercially in bioremediation processes as well as probiotic agents in aquaculture by incorporating them into fish or shrimp diet (Harish and Chauhan, 2017).

2.8 Microbial protease production

In recent years a number of studies have been conducted to characterize protease from different microorganisms. The production of proteases by microorganisms is greatly influenced by media components, especially carbon, nitrogen sources and physical factors such as temperature, pH, incubation time, agitation speed and inoculum concentration. Much research must be done to establish the proper medium in a fermentation process (Zheng *et al.*, 2001; Sharma *et al.*, 2017). Aeration is used in the submerged fermentation to provide sufficient oxygen to meet the metabolic needs of the growing microorganism. Stirring is necessary to ensure the homogeneity of the cells in suspension and the nutrients of the medium (Ellaiah *et al.,* 2002; Aguilar and Sato, 2018). Although protease production is an inherent property of all organisms. Only those microbes that produce a substantial amount of extracellular protease have been exploited commercially (Balachandran *et al.,* 2012).

With this background information, the experimental design for the study was formulated as given in the following chapter.