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Introduction

Soil is the superficial material of earth's surface which supplies nutrition and mechanical strength to plants. Microbiologically, soil is one of the most effective sites of biological reactions in the nature and a universe of microorganisms where most of the physical, biological and biochemical reactions related to decomposition occur. These micro-flora include bacteria, fungi, actinomycetes, algae and viruses (Abiola and Oyetayo, 2016).

Of all the divergent soil micro-flora, bacteria occupy 80% of the total microbial population, followed by actinomycetes (13%), fungi (3%) and by algae and viruses (0.2 - 0.8%). However, occupying less than 1% of the total soil content, each microorganism is complicated. It is responsible for bringing about a specific change or modification in the soil, consequently, giving a typical nature to the soil. Soil microorganisms and enzymes are crucial in soil functioning and also play a vital role in breaking down organic matter, which converts complex natural nutrients into simpler inorganic forms. Generally, these enzymes include hydrolases and oxidases, which decompose substrates and also release plant nutrients in soil which help in good growth of plants. Thereby resulting in increased fertility (Sekaran *et al.*, 2018; George *et al.*, 2019).

Several microorganisms produce powerful biocatalysts as enzymes that differ in their microbial sources, chemical properties and mechanisms. Microorganisms are predominant sources of enzymes because in a short period of time they can be grown in huge amounts. It is possible to make genetic modifications on microorganisms to increase the enzyme production. In addition, attributable to their dynamic and firm nature, microbial enzymes have been given more recognition than animal and plant enzymes. Nearly all the microorganisms, in harsh habitats are inadequate to grow and produce enzymes that cause harm to organisms. However, some microorganisms undergo multiple modifications that enable them to grow under severe circumstances and generate enzymes. Recently, several lines of research have been launched to isolate new bacterial strains from

hostile settings such as extreme temperature, pH, salinity, organic solvent and heavy metal to produce different enzymes with greater yielding characteristics (Anbu *et al.*, 2017).

At present the worldwide sale of enzymes in industries is estimated to be 4.20 billion dollars (Suberu *et al.*, 2019; Singh *et al.*, 2016a). Of this, proteases constitute the biggest class of commercial enzymes anticipated to achieve a worldwide demand of about 2.21 billion dollars by 2021 at a 6 percent Compound Annual Growth Rate (CAGR) for five years between 2016 and 2021. Among all the proteases produced, those secreted by microorganisms have the highest demand, followed by proteases of animal and plant sources (Proteases Market by Source., 2016).

Enzymes are significant for the existence of life itself. They were used for thousands and thousands of years without knowing their mechanism of action. Science, however, has unlocked the mystery of enzymes and their applications in different areas over the previous several years. Microbial enzymes generally promote hydrolysis, oxidation, or reduction responses. They have various active site motifs that target a variety of substrates. Even if they belong to the same class, distinct responses can be catalyzed. Microbial enzymes are generated primarily through the fermentation method. The variety of these microbial enzymes makes them an exciting group of products for application in many fields such as agricultural, chemical, food processing, textile, pharmaceutical, timber processing, cosmetics sectors and also for analytical applications and control of environmental pollution such as bioremediation and biodegradation (Liu and Kokare, 2017; Marathe *et al.*, 2018).

Microorganisms are an important source of proteases, a precious source of manufacturing as well. Eventhough proteases can be obtained from animals and plants, microbes are the major producers due to their diverse biochemical nature and possibility of genetic manipulation owing to their financial and technical benefits. These microbial enzymes have many physiological, biochemical and regulatory functions (Aguilar and Sato, 2018).

Current approaches in biotechnology, especially for the manufacturing of hydrolyzed protein products, have assumed a significant improvement in the field.

Enzymatic breakdown of molecules makes use of various sources of food protein that can become a source of bioactive peptides after hydrolysis. In the context of low manufacturing cost, outstanding stability and specificity, microbial origin proteases are chosen. It represents a powerful tool that can be explored industrially for the manufacture of new protein hydrolysates. Researchers look proteases from microorganisms because they are tolerant to extreme conditions, ways to prevent auto-proteolytic activity, stability at optimal pH and specificity of substrates (Kaur *et al.,* 2019).

In various industries, the use of chemicals has increased dramatically thereby causing health hazards. In order to improve life on the planet, the current scenario calls for replacing these damaging chemicals with eco-friendly products. Scientists primary goal was to replace enzymatic processes with chemical processes. Different enzymes, particularly microbial proteases, are the most commonly used in commercial sectors to meet cost-effective manufacturing (Smith, 2019).

Proteases related to physiological and commercial roles are crucial. Proteases are discovered everywhere as they conduct synthetic functions, such as in crops, livestock and microbes. *Bacillus* sp. are the most commercially exploited microbes for the protease production. Proteases are regarded effectively as an alternative to chemicals and are not environmentally harmful (Razzaq *et al.,* 2019).

The most vital steps in the industrial production of enzymes are the identification and selection of the best microbe that produces potent enzymes (eg. proteases). Industries require enzymes that can perform over a broad range of conditions and henceforth the selection of appropriate enzymes with best performance under suitable conditions is of main importance (Moradian *et al.,* 2009). The need for protein-degrading enzymes with increased stability and activity at extreme pH and temperature ranges was the prime driving force in the quest for new enzyme sources (Ariyaei *et al.,* 2019).

Because of their enormous commercial and physiological significance, proteases are a distinct class of enzymes. These hydrolytic enzymes can cause proteins to degrade into small peptides and amino acids. They also have anabolic as well as catabolic characteristics. They play a crucial role in physiological mechanisms such as blood

coagulation, zymogen activation through proteolysis, secretory protein membranal transport, tumor and cell growth, inflammation, tissue arrangement, developmental morphogenesis and breakdown of protein molecules. Microbial proteases are the most abundant group of all industrial enzymes, accounting to almost 60 percent worldwide sale of industrial enzymes (Souza *et al.,* 2015).

Based on their protein sequences, the genetic relationship between the various proteases (acidic, neutral and alkaline) has been evaluated, but there is still a lack of information that regulates the diversity in their specificity. The hydrolysis of proteins by alkaline proteases show peak activity in a range of 7.0-11.0 neutral to alkaline pH. Presently, there is a growing interest in proteases with new characteristics and a steady urge to optimize their manufacturing based on their prospective use (Sharma *et al.,* 2017).

The production of enzymes with high activity can be accomplished by optimizing the medium conditions such as pH, temperature, carbon and nitrogen sources or by offering extra treatments such as exposure to a magnetic field of the medium (Sumardi *et al.,* 2018).

Enzyme purification is more essential for a better understanding of the functioning of the enzyme. The different enzyme purification methods taken are similar to those of proteins. Inspite of the different sources, all enzymes are purified in a similar manner involving initial protein recovery, their concentration and ultimately the chromatographic purification of high-resolution. The purified enzyme is also needed for property studies and understanding of the structure and functional relationship. Traditionally, protein purification methods were employed to isolate and purify specific proteins in order to facilitate studies of their physical, chemical, enzymatic and structural properties. These kinds of studies are necessary to elucidate the biological function of individual proteins present in the cell and to comprehend the mechanism by which the specific enzyme activity is controlled. A number of alkaline proteases from different sources have been purified and characterized. In the purification and characterization of enzymes, factors influencing the circumstances of culture, productivity and characteristics of protease are regarded important (Lakshmi *et al.,* 2018).

The purified enzymes are subjected to a battery of characterization studies which include functional characteristics, evidence of purity, structural studies and the like purified protein can be further characterized by Matrix Assisted Laser Desorption Ionization/Mass Spectroscopy (MALDI/MS) analysis. The combination of electrophoretic separation and mass spectrometric analysis is considered to be a very powerful tool for protein analysis (Ozacar *et al.*, 2018).

The properties of the enzyme identified during these studies help in determining the areas of their possible potential application. As the recovery costs of enzymes are nearly 70 percent of the total manufacturing costs, it is necessary to identify the characteristics of an enzyme to determine whether it has the ability for being adopted as a commercial enzyme or not (Robinson, 2015 and Li *et al.,* 2017).

Utilization spectrum of proteases is limited despite their wide application potential due to lack of industrially desirable characteristics among the available proteases. Proteases intended for an industrial application must have stability towards surfactants, solvents, oxidants and stability at high temperatures and pH, considering that most of the industrial processes are accomplished under hostile conditions including extremes of temperatures and pH, presence of inhibitors etc. (Rao *et al.*, 2009a). Proteases that are thermostable, pH-stable and organic solvent resistant may find novel applications in industries. Hence attention is focused currently on finding new protease-producing microorganisms to meet the industrial demands (Singh *et al.*, 2014).

Protein engineering was used to regulate and manipulate genes to develop proteases which show unique specificity and improved stability. It also helps to understand the enzyme's structure and functional relationships. But research into enormous microbial diversity to target novel protease manufacturers with industrially desirable features is still an important research / green area (Singh and Bajaj, 2015). Microbial proteases have been isolated and characterized from several bacterial and fungal species. Enzymes from these *Bacillus* species are known to be capable of functioning in adverse ecological conditions (Choudhary, 2013).

Bacillus sp. such as *B. subtilis, B. amyloliquefaciens, B. licheniformis* and *B. cereus* has been reported to produce proteases and are becoming the most popular in biotechnological processes for protease production due to their excellent fermentation qualities, high product yields and lack of toxic by-products (Joshi and Satyanarayana, 2013). Furthermore, the wide diversity of proteases in contrast to the specificity of their action has attracted worldwide attention in an attempt to exploit their physiological and biotechnological applications in various industries (Sanatan *et al.,* 2013). Due to the growing trend towards the development of environmentally friendly techniques, out of all proteases, alkaline proteases have an excellent scope for implementation in the detergent and leather industries. The goal of genetic engineering is to produce large quantities of specific proteins or to visualize the image of the gene of interest by homology modeling. Gene expression is a method that helps scientists to understand the function of proteins. For example, *Escherichia coli* is widely used successfully as a mesophilic host to produce recombinant proteins because of its well-known genetics, cultivation simplicity, high transformation efficiency and rapidity (Drejer *et al.,* 2018).

Over time, researchers have succeeded in finding wide use of proteolytic enzymes in the pharmaceutical field. Various formulas in medicine, such as gauze, non-woven tissues and ointment compositions containing *Bacillus subtilis* produced alkaline proteases which demonstrate promising therapeutic properties (Awad *et al.,* 2013). Some syndromes of lytic enzyme deficiency are found to be helped by oral alkaline protease administration. It has been revealed that alkaline fibrinolytic proteases have attained degradation of fibrin. The use of this fibrinolytic enzyme indicates its future use in thrombolytic therapy and as an anticancer medication (Jaouadi *et al.,* 2012). In therapeutic applications, slow-release dosage form preparations containing alkaline proteases with collagenases are widely used (Suwannaphan *et al.,* 2017).

Silver is a significant precious metal, due to its unique thermal conductivity, optical reflectivity and its photosensitivity. Its innumerable medical and industrial applications have made studies in silver an attractive area of research related to both

extraction and recovery from various wastes. The waste X-ray films contain 1.5 - 2 percent (w/w) black metallic silver which is retrieved and reused. Around 18 - 20 percent of the world's silver needs are supplied by recycling photographic waste. Since silver is associated with gelatin in the emulsion layer, it is possible to break the same and release the silver using proteolytic enzymes (Canda *et al.,* 2018).

One of the top customers of enzymes is the detergent sector. Proteases are used in different industries and constitute a significant ingredient in the laundry detergent. Stains made of proteins, involving milk, egg white, dirt from human bodies and others are easily removed from fabrics, after they are digested by the enzymes. In the liquid laundry detergent, the undesirable self-digestion of proteases occurs as they are included in the active form as opposed to the natural proteases being synthesized in the pre-mature form. Thus, incorporation of enzymes in liquid detergent raises a severe problem with regard to their stability during the storage period (Kumari *et al.,* 2019 and Osamura *et al.,* 2019).

The leather industry is one among the most important industrial sectors for economic development, contributing to high earnings. However, tanning of hides and skins is constrained by primitive technology and the use of hazardous chemicals that contribute to environmental pollution. Microbial processing is a good alternative to simplify leather processing by reducing processing steps into a few significant ones and making the products more safe, attractive and durable. Alkaline proteases have excellent potential for implementation in leather industry and there is a growing trend to develop environment-friendly techniques (Chander and Puir, 2019; Zekeya *et al.,* 2019).

A proteinaceous material, ‘sericin or silk gum’, must be separated by the process of degumming from raw silk in an alkaline solution of soap conventionally. Protease is the right choice to remove sericin while not attacking the fibre. It has been proven that fibre break is not amenable and silk threads have been discovered to be much stronger than prior traditional treatments (Yadav *et al.,* 2011, Silva *et al.,* 2017 and Radha *et al.,* 2017).

In addition to the essential industrial application of proteases, they are also used to cleavage a peptide bond to elucidate the association between structure and functions of

peptides and proteins. Alkaline proteases isolated from *Vibrio metschnikovii* RH530 can be used as an alternative to proteinase K in DNA isolation (Narasimhan *et al.*, 2015 and Vijayaraghavan & Vincent, 2015). Proteases can therefore be seen as an alternative to many chemicals involved in different procedures of biochemistry and physiology.

Researchers are looking for novel and robust enzymes from microorganisms, because of the realization of the market value of these enzymes. Keeping this in view, the present study focuses on the current problems faced during production and application at the industrial level. Deciphering these issues would enable us to promote microbial proteases economically and commercially around the world.

The objectives of the present work were:

- Isolation and screening of protease-producing bacteria from different environmental soil samples
- Optimization of media components for the production of extracellular protease
- Purification and characterization of the isolated protease
- Immobilization studies of purified protease
- Application studies of protease

An assemblage of the background information available in the literature relevant to the present study is reviewed in the next chapter.