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# Microbial Proteases and Their Applications

Stephen Ameho

(*Department of Biotechnology, Lovely Professional University, Phagwara*)

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## Abstract:

Proteases are ubiquitous and their exploitation began several centuries ago. They play vital roles in our daily lives depending on the type and sources involved. Proteases form the largest group of enzymes and can be classified based on their line of mechanism, the types of reactions they catalyse as well as their active sites. Even though some proteases (especially, viral protease) are hazardous to health, research has shown that the merits of proteases outweigh its demerits. Proteases have the ability to catalyse several reactions with great efficiency and specificity which makes them potentially essential for biochemical research. The applications of proteases were limited due to their complex structure as well as functional diversity. Nowadays, proteases are vastly used in the food industry, detergent production, leather industry, and also in pharmaceutical approaches to managing health conditions such as coronary thrombosis, cancers, hyperacidity, bloating, flatulence, stomach cramps, and indigestion. This study is focused on a brief outline of the microbial sources of proteases, their general, physiological, and pharmaceutical applications.

*Keywords* **—Protease, thermophiles, hyperthermophiles, acidophiles, alkaliphiles, barophiles, prodrugs, hydoxylates, chelating agents, plasminogen, plasmin, fibronectin, protein turnover, and kallikreins.**

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## I. INTRODUCTION

Proteases (also termed peptidases, Proteolytic enzymes, and proteinases) can be defined as groups of enzymes that play catalytic roles in the hydrolysis of peptide bonds within protein molecules. They can be found in diverse groups of organisms ranging from bacteria, archaea, viruses, fungi, and eukaryotes. According to Mala and his associates (1998), "proteases represent about 60% of the total enzymes sold across the world, representing one of the largest groups of industrial enzymes" [52]. In addition to analytical and medicinal enzymes, trypsin, rennin, alkaline proteases, other proteases, lipases, and other carbohydrases, amylases showed sales in the global market around 3 percent, 10 percent, 25 percent, 21

  

percent, 3 percent, 10 percent, 18 percent, and 10 percent respectively [52].

Godfrey and West (1996), projected that 75% of the industrial enzymes are hydrolytic and contribute to approximately $1 billion of the total value of global enzyme sales [34]. Proteolytic enzymes have attracted worldwide attention due to their diversity in industrial and physiological applications [33, 64]. The role of proteolytic enzymes in the life cycle of pathogenic microorganisms has triggered an interest in the development of therapeutic agents that will combat these microbial diseases such as typhoid fever and HIV AIDS. Proteolytic enzymes perform several functions which encompass functions from the cellular level, through the tissue and organ level to the system level to enhance systemic mechanisms which including the homeostatic and the inflammatory system mechanisms.

## II. SOURCES OF MICROBIAL PROTEASES

There are several sources from which proteolytic enzymes can be isolated. These sources include animal sources, plant sources, and microbial sources. Even though proteases can be extracted from all the above-mentioned sources, microbial sources have the greatest prospect because they are ubiquitous, hence, this review will focus on microbial sources of proteases. Figure 1 shows a summary of the sources of microbial proteases.

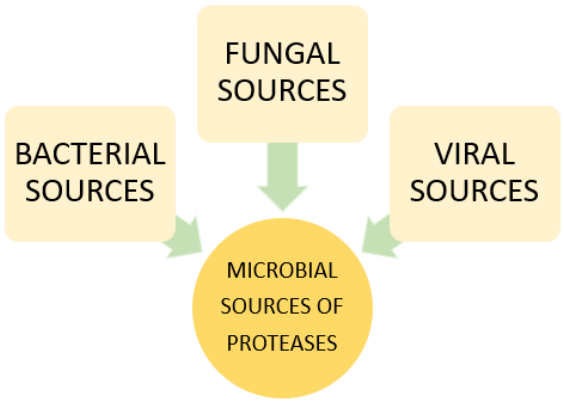


Fig.1 Microbial Sources of Proteases

### A. *Bacterial Source of Proteases*

Bacterial proteases are the most exploited proteases across the world due to their scope of diversity compared to animal and plant proteases. As a result, the interest in microbial proteases have been in high demand for research because, bacterial proteases are easy to manipulate both genetically and biochemically [52]. Also, bacterial proteases possess properties such as limited space for growth and rapid growth rate which are desirable industrial properties [65]. The Industrial activities take place at extreme conditions at which plant and animal proteases cannot be able to withstand. This intrigues researchers to probe into the potentials of microbial enzymes such as those produced by thermophiles, hyperthermophiles, acidophiles, alkaliphiles, and barophiles, as enzymes produced by these microorganisms are stable at unfavourable conditions. Mostly, alkaline and neutral proteases

  

are used on a commercial basis and are usually found in the genus of bacillus as a result of their remarkable characteristics [19]. According to Mala and his colleagues (1998), neutral proteases are very useful in the food industry since they do not produce any awful taste such as bitterness or sourness when they are been used to hydrolyze dietary proteins due to their intermediary rate of reaction (pH 5 to pH 8) [52]. Some neutral proteases such as neutrase can be used in the brewery industries since they are not sensitive proteolytic enzymes produced by plants. Microbial neutral proteases are usually preferred to other sources of proteases because their reactions can be easily manipulated due to their activity at low temperature, hence, hydrolysates can be produced by these proteases at a low rate of breakdown [35, 71]. Since bacterial alkaline proteases are stable at high temperatures up to 60°C and pH of about 10, it makes these enzymes appropriate for use in the detergent industry [65].

B. ***Fungal Source of Proteases***

Different fungal species, most of which belong to the genera *penicillium, ThermoascusRhizopus*, *Aspergillus*, *Humicola*, *Muc or*, and *Thermomyces* have been found to produces proteases [25]. Fungi forms one of the largest groups of microorganisms that produce proteases and can be easily produced in a solid-state fermentation process for use in food industries etc. [85]. Literature has shown that, fungal species such as *Aspergillus oryzae*has the capacity to produce alkaline, acid, and neutral proteases [60, 81, 82]. Fungal proteases have a wide range of pH (usually pH 4 ≥ pH 11) as well as substrate specificity [25, 52]. Fungal acid proteases have pH ranges of 4 ≥ 4.5 but can be stable at pH ranges of 2.5 ≥ 6.0, hence, they can be conveniently used in the cheese industry; fungal neutral proteases have an optimal pH of 7.0 of which their activity can be inhibited by chelating agents, and fungal alkaline proteases are stable at pH range of 9.5 ≥ 10.0 [52]. Despite these characteristics, fungal proteases are unable to withstand extreme temperature condition, hence, they have a low rate of reaction [25, 52]. This makes thermophilic bacterial proteases more applicable in some industrial processes compared to fungal proteases. The advantage of neutral and alkaline fungal proteases over bacterial, animal, and plant proteases is their capacity to reduce bitterness in food protein hydroxylates during the hydrolysis of hydrophobic bond in proteins.

C. ***Viral Source of proteases***

Viral proteases are pathologically involved in the mechanization of viral proteins in order to cause deadly diseases such as cancer and HIV AIDS. According to Rawlings and Barrett (1993), viruses encode aspartic, serine, and cysteine peptidases and endopeptidases [68]. Homodimers such as retroviral aspartyl proteases help in the assembly

  

and replication of the viral parts and subsequently help in the expression of its proteins, acting as a polyprotein precursor which is released via autolysis [48]. Extensive investigation into viral protein structures and their modes of interaction can be an avenue to the discovery of an effective cure for the HIV/AIDS virus. The three-dimensional structure of viral proteases, their mechanism of infection, and how they interact with other synthetic inhibitors have become the focal point of research with the view of developing potent inhibitors to curtail the menace of viral diseases such as chickenpox, AIDS, measles, and smallpox [52].

## III. PHYSIOLOGICAL ROLES OF PROTEASES

Proteolytic enzymes have demonstrated varieties of physiological functions of which some are very complex because some of the mechanisms involved in these functions are not yet fully understood. On the basis of physiological functions, proteases can be divided into two categories namely intracellular proteases and extracellular proteases which play major roles in controlling metabolic pathways as well as catalysing the breakdown of large molecules proteins into simple absorbable forms respectively. Proteases play important roles in nutrition, protein turnover, enzyme modification, breaking of dormancy, transmembrane movement of secretory proteins such as hormones, etc. "They are also actively involved in some pathophysiological processes such as inflammation and tumor growth" [4, 65]. Some physiological roles of proteases are summarized in figure 2.

### A. *Breaking of Dormancy in Seeds*

According to Chahtane and his associates (2018), seeds are exceptional structures that preserve the embryos of plants in a state of desiccation and resistance as well as enhancing the growth of multiple plants [12]. These seeds are usually in a state of dormancy and lack the amino acid that will enhance the germination process. Proteases such as serine endoproteinases have been found to breakdown dormant seeds and subsequently synthesize the required amino acids as well as nitrogen required for germination without having negative impacts on the seeds [52]. Leighton and Stock (1970) have also reported that, specific alkaline serine proteases are involved in hyphal fusion and the germination of microconidal [49]. It is believed that Extracellular acid proteases catalyse the hydrolysis of the peptide bond within the cell walls on seeds during the germination of *Polysphondylium pallidum* microcysts and *Dictyosteliumdiscoideum*spores [41, 58].

### *B. Enhancement of Digestion*

After the consumption of complex protein food products, the proteins need to be hydrolysed into simple absorbable forms (amino acids) in order that, the villi can absorb and assimilate the nutrients in

  

complex proteins. This requires the action of proteases. According to literature, minor proteolysis begins in the stomach by the action of proteases known as renin (in infants) and pepsin (in adults) whereas the major protein breakdown catalysed by trypsin and chymotrypsin (produced by the pancreas) takes place in the small intestine [42, 84]. The depolymerizing activity of the proteolytic enzymes produced by the pancreas is also primarily involved in the nourishment of cell by providing them with the required amino acids in order to keep them alive [52].

***C. Promotion of Growth and Development***

Protein turnover enhances growth and development, hence occurs in all living cells. The amino acid molecules formed as a result of the hydrolysis of the peptide bonds within the protein molecules are used as precursors for the synthesis of other proteins. This balance plays a key role in maintaining homeostasis within cells [6]. Van der Hoorn (2008) also projected that proteases are also essential components of the body's regulatory system as they promote growth and development, significant in programmed cell death, boost the immune system, and photosynthesis in plants [79]. It has also been shown that metalloproteases take part in cell division (meiosis) and meristem growth regulation [10, 79]. According to Mala and his colleagues (1998), appropriate protein turnover for cellular activity is enhanced by intracellular proteases [52]. Pathways including ATP-dependent proteases affect the turnover of intracellular proteases, for example, "in E. coli, ATP-dependent protease La (lon gene product) catalyses the breakdown of abnormal proteins" [17, 40].

***D. Formation of reproductive structures in microorganisms***

Sporulation, conidial discharge, fruiting bodies, and ascospores in bacteria, fungi, slime mold, and yeast respectively are involved in protein turnover [30, 45, 57, 62]. Dancer and Mandelstam (1975) have shown the prerequisite for the sporulation of protease inhibitors [22]. An increase in protease A activity has also been linked to ascospore formation in diploid yeasts while the formation and differentiation of fruiting bodies and stalks was respectively attributed to extensive proteolysis in slime mold [30]. Alkaline serine protease isolated from *Conidioboluscoronatus*has revealed the discharge of conidia without getting to the stage of maturity through the isolation of mutant with fewer conidia formation, hence autoproteolysis of these mutants can be a novel source of physiological regulation of protease activity in *Conidioboluscoronatus*[62, 63].

## IV. GENERAL APPLICATIONS OF PROTEASES

Proteases as one of the largest groups of exploited enzymes have a wide range of applications in the production of detergents which are used for

  

decomposing proteins such as bloodstains in fabrics; leather industry for the softening of leather and removal of hair from animal hides; baking industry for improving the workability through the modification of dough rheology and handling characteristics; manufacture of soy products; de-bittering of protein hydrolysates;synthesis of aspartame; and many other applications. Some general applications of proteases are summarized in figure 2.

A. ***Production of detergent***

"Proteases are one of the major enzymes used in the detergent industry, accounting for about 25% of the total worldwide sales of enzymes used for household laundry and other reagents" [65]. Alcalase produced by *Bacillus licheniformis* was used in 1960 by BIOTEX to produce detergent after which Maxatase was used. The major requirement to enhance protease activity in detergent is the isoelectric points (pI), that is the pI of the protease, and the pH of the detergent must be compatible [52]. Alkalophilic *Bacillus spp.* have been used by the Novo Industry to produce proteases such as Savinase T and Esperase for commercial production of detergents because they have high isoelectric points of about pI 11 and also high pH ranges [52].

High-alkaline proteases that are produced in alkalophilic Bacillus species, including *Bacillus clausii* and *Bacillus halodurans* have shown stability at high temperatures of up to 60 °C in the absence of calcium as well as high pH ranges of about 11–12 pH, making them suitable for detergent processing. Several alkaline detergent proteases have also shown high activity at low temperatures, between the ranges of 10–20 °C and an example of such protease is Kannase (Novozymes' detergent alkaline protease). Proteases that require low temperatures for activity are appropriate for energy conservation amidst the energy crisis [52, 83].

B. ***Production of flavour and rise of baking dough***

Gluten is a major component of wheat, found in bread baking flour which contains complex proteins with characteristics of both elasticity and viscosity (viscoelastic). Since the major component of baking dough is protein, it requires a proteolytic enzyme for its hydrolysis, for instance, neutral proteolytic enzyme obtained from bacteria such as *Bacillus subtilis* has the capacity to be used in the production of cookies, biscuits, etc. [29]. Protease-catalysed degradation of gluten flour weakens the flour and this is a requirement for biscuit production. When gluten is broken down, it causes the bread flour to rise during baking, for example, *Aspergillus oryzae* has demonstrated the ability to hydrolyse gluten in wheat [11]. The advantages of using microbial proteases for proteolytic degradative activities of gluten include the production of flavour loaves; increase in nutritional

  

values of bread; increase in the volume of loaves; and reduction in mixing time of flour. It must be noted that, proteolytic enzymes used for these purposes should be liable to high temperature, that is, denaturation of the protease used during baking [52, 83].

C. ***Production of Soy and its derivatives***

Soy products such as tofu and soymilk have been used as meat alternatives due to their rich content of proteins of high quality. In the olden days, proteolytic enzymes have been used to cook soy products such as soy sauce. Proteases have been proven useful in the food industry for having a positive impact on the foaming and emulsifying characteristics of proteins as bioactive peptides become active and made available from complex proteins as a result of proteolysis [1, 44]. Fungal proteases such as neutral and alkaline proteases are used in the processing of soy sauce and this helps in enhancing the functional characteristics of soy protein products.

Soy protein hydroxylates with enhanced properties of antioxidants can be produced on large scales using proteolytic enzymes such alcalase, validase, and neutral protease which can be obtained from *Bacillus licheniformis*, *Aspergillus oryzae*, and *Bacillus subtilis* respectively [80, 87]. Novel proteolytic enzymes such as keratinolytic bacterium *Chryseobacterium sp.* kr6 has been found to produces extracellular proteases, using feathers as a substrate in submerged culture and this could be used to produce hydroxylates and peptides [24]. Hence, the ability of proteases to catalyse the hydrolysis of casein, albumin, keratin among other proteins is evident of their capacity to produce hydroxylates [9, 75]. In context, hydrolysates are exploited in preparing dietetic feeds and protein-fortified drinks when soy proteins are treated with alcalase (pH 8) resulting in the solubility, reduced bitterness, and increased yield [52].

D. ***Leather and Fabric Processing Industry***

Soaking, dehairing, bating, and tanning are the four major steps in the processing of leather. Alkali solutions are added during the soaking stage which causes the animal hide to swell [52]. When the hide is treated with hydrogen sulfide combined with high concentrations of alkali, the root hairs become soluble, leading to the removal of hair/ wool from hides. These chemicals pose threats to the environment and its habitats by increasing the costs of effluent treatment and waste disposal as well as pollutions and spills [52]. As a result, proteases present an environmentally friendly, cost-efficient approach to handling and leather products as well as means of disposing of chemical contaminants. Again, the proteolysis of non-collagenous protein constituents increases the quality of leather produced as compared to the chemical method. Proteases also play prominent roles in the

  

manufacture of silk where they are used to degrade silk protein sericin which gives a rough texture to the silk in its raw state instead of using a more expensive process involving starch application in shrink proofing and twist setting [52].

## V. PHARMACEUTICAL APPLICATIONS

Proteases have been found to have a wide range of applications in the pharmaceutical industry. Proteases used in the pharmaceutical industry are usually produced in small quantities, since medical products require much carefulness and purification steps compared to proteases used in other industrial activities such as leather production, detergent production, etc. "Proteases are used in the pharmaceutical industry for preparation of medicines such as antibiotics, ointments for debridement of wounds, treatment of infectious diseases and cancers, etc" [65]. Some pharmaceutical applications are summarized in figure 2.

### A. *Treatment of infectious diseases*

The wide range of protease activities, their specificity, and their role in the life cycle of pathogens have made them very useful in developing therapeutic agents against lethal diseases such as cancer, AIDS, bacterial infections, and other viral infections. Administration of Nortase and Luizym orally (a protease obtained from *Aspergillus oryzae*) can help in the treatment of hyperacidity, bloating, flatulence, stomach cramps, indigestion, as well as other problems associated with digestion. In context, proteases can be used as alternatives for deficiencies in digestive lytic enzymes. "For example, alkaline protease from *Conidioboluscoronatus*has been efficiently used as a substitute for trypsin in animal cell cultures" [14]. According to Mala and his colleagues (1998), several antibiotics have been used together with subtilisin or Clostridial collagenase to heal injuries such as wounds and burns while aspargine in the bloodstream in forms like lymphocytic leukemia has been treated with asparginase isolated from *E. coli* [52].

### B. *Therapeutical applications as anticoagulation agents*

Microbial proteolytic enzymes such as xiaflex, lysostaphin, serrapeptase, streptokinase-streptodornase, L-asparaginase, streptokinase, L-glutaminase, and serrazime have demonstrated the capacity of treating respiratory tract disorders, and cardiovascular disease [13]. According to Rijken and Lijnen (2009), proteases such as t-PA have the potential for treating thromboembolic disorders [70]. t-PA has five domains namely a growth factor domain, a catalytic serine protease domain, a fibronectin finger domain, and two kringle domains which perform different roles in converting activated plasminogen into plasmin and subsequently breaking down the fibrin mesh of embolus [21]. Plasminogen activation is achieved

  

through the binding of t-PA to fibrin which is mediated by EGF2 and fibronectin finger domains [40]. Craik and his colleagues (2011) projected that Alteplase marketed as Activase® (Genentech) was the first fibrinolytic drug used for the treatment of acute myocardial infarction (AMI) in 1987 under the approval of the FDA as research has shown that Recombinant t-PA provides a basis for therapeutic intervention in cases of fibrinolysis and clot dissolution, adding that reteplase and tenecteplase are the first generation products of alteplase [21]. Bode et al 1996 also added that the potency of reteplase has been shown in several therapeutic interventions.

Streptokinase (protease activator produced by β-haemolytic streptococci) can be used indirectly as a protease cofactor to stimulate and activate endogenous protease plasminogen into plasmin (active protease [21, 47]. The line of action of streptokinase is by the formation of a dual complex with plasminogen, causing a change in the structure of the protease's active site and circulates antibodies to proteins from previous streptococcal infections [21]. The initial dose of streptokinase should be high and subsequently administered multiple times in order to overcome the antibodies with a half-life of about an hour and a few minutes after eliminating the antibodies and deplete circulating plasminogen [21, 78]

C. ***Activation of the immune system***

The activation of the immune system in response to inflammation follows a complex pathway which involves mediators such as coagulation and complement proteolytic systems, NF-κB-transcribed cytokine induction, acute phase proteins, matrix metalloproteinases (MMPs), Toll-like receptors, etc. [36, 43, 68]. The lytic action of proteases controls the complement system as a result of interaction between each other [5, 7]. For example, proteases control (activate or inactivate) pathways accountable for inflammatory cell response, resulting from chemokines, cytokines as well as their binding proteins and receptors [20, 37, 55]. Enough diagnostic tests and drugs have not been developed due to the lack of in-depth knowledge in the crosstalk and pathways involved during infection [53]. Posttranslational modifications (PTMs) and the lytic activity of proteases account for a change in bioactivity while neutrophil elastase andinflammatory cell matrix metalloproteinases (MMPs) are responsible for the activation and inactivation of chemokines [8, 26, 51,54]. According to Fortelny and his associates (2014), the proteases activate alpha-1 antitrypsin which is achieved by MMP8 mediated elastase activation of chemokines in vivo [32].

D. ***Treatment of prostate cancer***

Serine proteases have been found to have great potential for the treatment of various cancers.

  

Example of serine proteases that are conventionally associated with poor clinical prognosis of human carcinoma is kallikreins (KLKs) [16]. Human kallikrein 3 (hK3) which is recognized as prostate-specific antigen (PSA) is the most common diagnostic tool used for determining the biological state of prostate cancer [16]. According to Cleutjens and his associates (1996), despite the fact that there are post-translational activities that regulate the expression of KLK genes, the expression of these genes are also controlled by sex-steroid hormones in instances such as KLK2 and KLK3 being regulated by androgen. Kallikreins can be associated with the secretions from their sources, for example, kallikreins are found in seminal plasma, cerebrospinal fluid, sweat, saliva, and milk in humans are secreted by testes, brain, epithelial cells of the skin, salivary glands, and breast respectively [16, 38].

Research has shown that, the determination of substrates for "Human tissue kallikreins" (HKs) has been a challenge because HKs are capable of regulating (inhibit or enhance) the development of tumor with respect to the type of tissue involved and hormone balances [86]. An investigation conducted on HKs, including HK (3, 8, 9, 10, 13, and 14) has shown that, Overexpressed HKs in different cancers can be used as workable targets for drug delivery [16, 50]. Different types of HKs can interact particularly with serine proteases (including uPAR and uPA) during cancer progression such as angiogenesis, invasion and metastasis despite more attention is directed towards PSA (hK3) and hK2 due to their role in prostate cancer [16]. The uPA-uPAR pathway is a prospective anti-cancer therapy, because, it gives details of the mechanisms involved in cancer progression as well as offering chances for target proteases such as MMPs, hK2, PSA, plasmin, and uPA in the therapy [3, 31, 65]. It has also been reported that, levels of serine proteases and their associated uPA have been found to increase in various cancers such as prostate, cervical cancer, gastric, and colorectal [28, 23, 56, 76].

**Table I: Protease-activatable pharmaceutical prodrugs (as adopted from Choi et al., 2012) [16]**

| Protease | Trade Name | Indication | References |
|---|---|---|---|
| Cathepsin B | Brentuximab vedtin (SGN-35) | Hodgkin lymphoma | *[2, 72]* |
| Cathepsin B | OPAXIO™ (CT-2103) | NSCLC (Non-Small-Cell Lung Cancer) | *[15, 27, 59, 61]* |
| Cathepsin B | PK1 (FCE 28068) | Cancer | *[74]* |
| Cathepsin B | PK2 (FCE 28069) | Liver cancer | *[73]* |
| Prostate-specific antigen (PSA) | L-377,202 (C1853) | Prostate cancer | *[27]* |

  

## VI. OTHER APPLICATIONS OF PROTEASES

Proteases have also been found to be applicable in the purification of both animal and plant extracts such as mucopolysaccharides and carbohydrate gums. Bromelain, papain (plant proteases), and neutral protease isolated from *Bacillus subtilis* are very useful in the meat industry as tenderization agents. Proteases can also be used in breaking down complex bonds in both animal and plant by-products which are used for animal feed in order to increase the palatability and the degradability of the feed. Apart from the pharmaceutical and industrial applications of proteases, they have shown relevance in the field of research, as they can be used to explain the principles underlying protein bond formation, peptide bond cleavage, and proteinsequencing. In view of the diversity and specificity of proteases, they can be extensively applied in the pharmaceutical industry for mass production of antibiotics as they have proven potent in other industries. Proteases can also be used to design protease-activatable prodrugs which are basically chemically-caged derivatives of medicines or drugs that can be converted by chemical agents, enzymes, or physiological stimuli to in order to release the active elements of the drug in vivo [67]. Table 2 outlines some prodrugs, enzymes used in their designing processes as well as their target diseases.

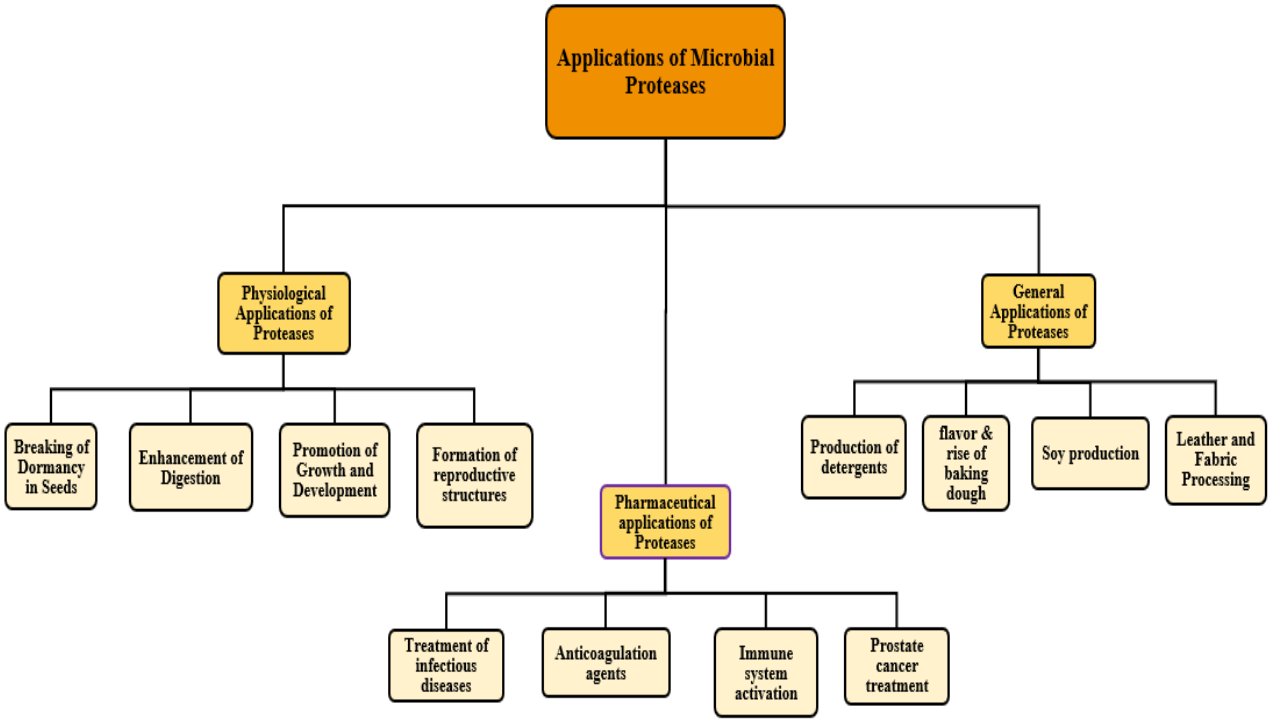


Fig.2 Summary of some Applications of Microbial Proteases

  

## VII. CONCLUSION

The expansion of expertise to improve the applications of proteolytic enzymes with the aid of protein engineering techniques and chemical enzyme modification techniques can contribute to the discovery and expansion of other potential applications of these enzymes. Apart from the above-stated applications, proteolytic enzymes can be extensively applied in fields such as microbiology, molecular biology, and recombinant DNA technology for cell isolation and culturing as well as dissociation of tissues. The applications in this review intends to broaden the scope of understanding the roles of known proteolyticenzymes in industrial operations (especially pharmaceutical industry) to develop novel proteases with altered properties for therapeutic, industrial, or research fields.

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