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An Overview of Microbial Proteases for Industrial Applications.

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ABSTRACT

Microorganisms are attractive sources of proteases as they can be artificially cultured in large quantities in a relatively short time by established fermentation methods. Microbial alkaline proteases dominates the world enzyme market, accounting for a nearly two-thirds share of the detergent industry. Screening and characterization of these proteases from different sources serves many advantages from both environmental and industrial points of view. Most of the microbial proteases of significant application in detergent industry are bacterial alkaline proteases from genus Bacillus. The major bottleneck has been the screening of wild-type biocatalyst that would be detergent compatible with increase efficiency than what is seen today. Given their role in commercial industries; it is deemed imperative to gather the disperse literature on the current state of the art describing the sources, classification, application and biosynthetic regulation of bacterial proteases. Special emphasis has been given to bacterial alkaline proteases that are detergent compatible.

Keywords: Bacillus spp, Biocatalyst, Detergent industry & Microbial alkaline proteases.

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INTRODUCTION

Microorganisms regarded as an important source of proteases because they can be obtained in large quantities using cultural techniques within a shortest possible time by established fermentation methods, and they produce a regular and abundant supply of the desired product. Furthermore, microbial proteins have a longer shelf life and can be stored under less than ideal conditions for weeks without significant loss of activity [1]. Microbial proteases generally have been pointed as to be extracellular in nature and directly express in the fermentation medium. This help in simplicity of downstream processing of the enzymes relative to their plants and animal counterparts. The appropriate producers of these enzymes for commercial exploitation are non-toxic and non pathogenic that are designated as safe [1]. Bacteria are known to produce alkaline proteases with genus *Bacillus* as the prominent source. Different exotic environment has been the sources of different *Bacillus* species with alkaline protease production abilities. A large number of microbes belonging to bacteria, fungi, yeast and actinomycetes are known to produce alkaline proteases of the serine type [2].

Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most prominent source. A myriad of Bacillus species from many different exotic environments have been explored and exploited for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B. licheniformis, B. subtilis, B amyloliquifaciens*, and *B. mojavensis* [1-3]. Another bacterial source known as a potential producer is *Pseudomonas* sp as reported by [4], are considered to be another source of alkaline protease.

Alkaline proteases produced are of special interest as they could be used in manufacture of detergents, food, pharmaceuticals and leather [5,6]. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, 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 [3].

In recent years a number of studies have been conducted to characterize alkaline protease from different microorganisms. However, many of these proteases applied to industrial purposes faces some limitations such as low stability towards high temperature, high NaCl concentration, and commercial detergent stability, alkaline pH stability and production cost of the enzymes with regard to various carbon and nitrogen sources from growth medium.

Genus *Bacillus* for Industrial Applications

Bacillus species have been major workhorse industrial microorganisms with roles in applied microbiology, which date back more than a thousand years, since the production of natto by solid-state fermentation of soybeans using *Bacillus subtilis* (natto) was first practiced in Japan [7]. *Bacillus* species are of particular interest when it comes to industrial applications because of they have peculiar features, owing from their high growth rates leading to short fermentation cycle times, their ability to secrete proteins into the

extracellular medium, and the GRAS (generally considered as safe) status with the Food and Drug Administration for species, such as *B. subtilis* and *Bacillus licheniformis* [8].

In the same vein, greater advances has been documented about the biochemistry, physiology, and genetics of *B. subtilis* and other species, which facilitates further development and greater exploitation of these organisms in industrial processes. We recently reported Bacillus cereus BM1 protease with potential industrial applications [9]. Advances seen today in the knowledge and interpretation of *Bacillus* genomics will open doors to the further development of products or processes as the genomic information is interpreted and exploited. Recent applications of phylogenetic analysis, DNA–DNA hybridization, and other molecular techniques to bacterial classification are leading to the reclassification of a range of *Bacillus* species and to the creation of new genera [8]. For example, the former *Bacillus stearothermophilus* and *Bacillus brevis* have been reclassified as *Geobacillus stearothermophilus* and *Brevibacillus brevis*, respectively [8].

Industrial Enzymes from Genus Bacillus

Bacillus spp. produces the most detergent proteases currently used in the market, example is serine proteases [3]. It is estimated that *Bacillus* spp. enzymes make up about 50% of the total enzyme market. There are three dominant enzyme suppliers, Novo Nordisk, Genencor International, and DSN N.V., having reported market shares of 41%–44%, 21%, and 8%, respectively, with smaller producers in North America, Europe, Japan, and China making up the remaining 27%–30% [8].

The alkaline serine proteases (subtilisins) are produced by various *Bacillus* species having its fundamental application in household detergents. *Bacillus licheniformis*, *Bacillus pumilus*, and *B. subtilis* produce the subtilisin Carlsberg-type enzyme [8]. The enzyme production annually amounts to about 500 metric tonnes of pure enzyme protein [3]. Alkalophilic species produce enzymes with greater alkaline tolerance, used in heavy-duty detergent formulations (e.g., esperase from Novo Nordisk). Neutral *Bacillus* spp. proteases are zinc metalloproteinases, with pH optima around 7, which are used in milk protein modification, nitrogen control, mash extraction and chill-haze removal in brewing, soy modification for use as flavours, and in animal feeds [8].

Proteases from Alkalophilic Bacillus sp

Most alkaliphile bacilli produce various alkaline proteases. Numerous species of *Bacillus* have been explored for alkaline protease production, but most potential alkaline protease producers are strains of *B. amyloliquefaciens*, *B. licheniformis*, *B. mojavensis*, and *B. subtilis* [3]. The proteases from these strains, having pH optima in the range 9–12 and good thermostability, have found commercial applications in detergents, in abating and (or) dehairing of leather [1]..

Classification of Microbial Proteases

As reported by Pushpam [10], proteases are classified into six types based on the functional groups in their active sites. They are aspartic, cysteine, glutamic, metallo, serine,

and threonine proteases. They are also classified as exo-peptidases and endo-peptidases, based on the position of the peptide bond cleavage. Proteases are also classified as acidic, neutral or alkaline proteases based on their pH optima. The largest share of the enzyme market is occupied by detergent proteases, which are mostly alkaline serine protease and active at neutral to alkaline pH range. Alkaline serine proteases have Aspartate (D) and Histidine (H) residues along with Serine (S) in their active site forming a catalytic triad [11]. Serine proteases contribute to one third of the share in the enzyme market and are readily inactivated by Phenyl Methane Sulfonyl Fluoride (PMSF) [11]. Based on the sequence and structural similarities, all the known proteases are classified into clans and families and are available in the MEROPS database [12]. Broad classifications of proteases are of endo or exo enzymes on the basis of their protein substrate site of action [3]. Depending on their catalytic mechanism, they are further classified as serine proteases, aspartic protease, cysteine proteases or metalloproteases. The classification can be viewed from the perspectives of their amino acid sequence and evolutionary relationship.. Neutrality, alkalinity and acidity are used as another form of classification based on pH requirements and pH optimum.

Exopeptidases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as aminopeptidases- and carboxypeptidases, respectively [3]. The former act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide while the later act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide [3].

Endopeptidases

The peculiar characteristics of endopeptidases are by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. Endopeptidases are categorized into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases, and (iv) metalloproteases [3].

Serine Proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. Based on their structural similarities, serine proteases have been grouped into 20 families, which have been further subdivided into about six clans with common ancestors [13]. The primary structures of the members of four clans, chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC), and *Escherichia* D-Ala–D-Ala peptidase A (SE) are totally unrelated, suggesting that there are at least four separate evolutionary origins for serine proteases [3] . Clans SA, SB, and SC have a common reaction mechanism consisting of a common catalytic triad of the three amino acids, serine (nucleophile), aspartate (electrophile), and histidine (base) [3].

The peculiar characteristics of serine protease is its ability to be irreversibly inhibited by Phenylmethylsulfonyl (PMSF), di-isopropylflourophosphate (DFP) and tosyl-L-lysine chloromethyl ketone (TLCK). Generally, the activity of serine proteases is higher at neutral and alkaline pH with best optimum at pH 7 and 11 [3].

Serine Alkaline Proteases

Microbial alkaline serine protease emanates from molds, yeast, bacteria and fungi.Serine alkaline proteases are produced by several bacteria, molds, yeasts, and fungi. They are deactivated by DFP or a potato protease inhibitor. They hydrolyze a peptide bond which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. The optimal pH of most alkaline proteases is around pH 10, and their isoelectric point is around pH 9 [3]. The range of 15 to 30kDa has been found to be molecular masses of alkaline serine proteases. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacter*, *Streptomyces*, and *Flavobacterium* spp. [14]. *Bacillus* spp. is the best known producers of subtilisin [3].

Subtilisins

BPN9 is designated as subtilisin Novo and it is being produced by *Bacillus amyloliquefaciens*. The widely used Subtilisins in detergent is called Carlsberg, their properties such as optimal temperature such as 60oC and pH optima of 10 are similar [3]. Both enzymes exhibits characteristics of an active site triad made up of similar properties such as an optimal temperature of 60°C and an optimal pH of 10. Both enzymes exhibit characteristics of an active-site made up of Ser221, His64 and Asp32 with with broad substrate specificity [3]. The enzyme carlsberg posses unique property of not depending on Ca^{2+} for its stability and hence has broad substrate specificity. In addition the conformation in the active site of subtilisins is similar to that trypsin and chymotrypsin, but dissimilarity exists in their molecular arrangement as a whole [3].

Aspartic Proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. They are not most often found in bacteria; instead they occur mostly in viruses. Because of their acidic nature, they show maximal activity at low pH (3-4) and have isoelectric points in the range of pH 3 to 4.5 [3].

Cysteine/Thiol Proteases

Proteases of cysteine type occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. The order of Cys and His (Cys-His or His-Cys) residues differ among the families [13]. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin-with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv)

others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents such as EDTA [3].

Metalloproteases

The diversity in catalytic type of protease is seen in metalloproteases. The divalent metal ions are their characteristics requirement for activity [15]. Enzymes from different origins are included in this group which entails higher organisms.

Applications of Proteases

Generally microbial proteases have a large variety of applications, in various industries. These include food industries, detergent, pharmaceutical industries. The application of these enzymes varies considerably.

Applications of Proteases in Detergents

Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. The preparation of the first enzymatic detergent, "Burnus," dates back to 1913; it consisted of sodium carbonate and a crude pancreatic extract [3]. The first detergent containing the bacterial enzyme was introduced in 1956 under the trade name BIO-40. In 1960, Novo Industry A/S introduced alcalase, produced by *Bacillus licheniformis*; its commercial name was BIOTEX. This was followed by Maxatase, a detergent made by Gist-Brocades [3]. A *Bacillus cereus* BM1 protease reported recently Mienda [9] has been found to be detergent compatible. The enzyme retained its activity in a solution containing up to 10% w/v commercial detergent (Fabs Perfect). This suggest that it can find application in commercial industry [9].

Proteases key performances in detergent are its PI. The PI of the detergent for best performing proteases should coincide; this supports the enzymes application in detergent. Proteases from alkalophilic *Bacillus* sp with very high isoelectric points (PI) and hence hence they can withstand higher pH ranges. Lipases, amylase and cellulase when combined together is expected to increase the performing efficiency of laundry detergent proteases [3].

All detergent proteases that are presently used in market are serine proteases produced by Bacillus strain. The advantage of fungal alkaline proteases over its bacterial counterpart is the ease of downstream processing to prepare a microbe-free enzyme. *Conidiobolus coronatus* produces alkaline protease that is compatible with commercial detergents used in India [16] and retained 43% of its activity at 50°C for 50 min in the presence of Ca^{2+} (25 mM) and glycine (1M) [17].

Regulation of Protease Biosynthesis

Production of protease is a general property of almost all living organisms and these enzymes are generally considered as constitutive; sometimes they are partially inducible [1]. Stationary phase are generally considered as protease production phase and these are acconted by are by carbon and nitrogen stress. A number of methods in submerged fermentations have been applied to regulate protease synthesis with approaches combining fed-batch, continuous, and chemostat cultures [1, 18]. Those approaches or ways can achieve greater yield of alkaline protease in the fermentation medium over a longer period of incubation during prolonged stationary state [1] . Protease production is often related to the sporulation stage in many bacilli, such as *B. subtilis* [19], and *B. licheniformis* [20].

In addition, Gupta [1], reported that production of extracellular protease is related to manifestation of nutrient deficiency at the beginning of stationary phase. Manipulation of the growth medium is needed to improve growth and subsequently protease yields. Though a huge number of factors interfere with protease production, complexity of interaction exists among these parameters that can be studied by following response surface methods [11].

Properties of Alkaline Proteases

Microbial alkaline proteases have been studied and explored and, based on their unique properties; they become useful in various industries. Information regarding their individual properties has been briefly highlighted below:

pH and Temperature kinetics

The thermostable nature of alkaline proteases made them detergent-compatible proteases with a high pH optimum. The laundry detergents pH is generally in the range of 8 to 12 and has uniqueness in their thermostabilities at laundry temperatures (50–70ºC). Commercially available subtilisin-type proteases are mostly known to be active in the pH and temperature ranges 8–12 and 50–70ºC, respectively [1].

Effect of Stabilizers/Additives and Metal Ions

Some of the major commercial uses of alkaline proteases necessitate high temperatures, thus improving the thermal stability of the enzyme is distinctly advantageous. Improvement in thermo stability can be achieved either by adding certain stabilizers (PEG, polyhydric alcohols, starch) to the reaction mixture or by interfering with the tertiary structure of enzyme by protein engineering [1]. The ion Ca^{2+} is also known to play a major role in enzyme stabilization by increasing the activity and thermal stability of alkaline protease at higher temperatures [21, 22]. Stability of proteases can also be achieved by the use of metal ions such as Ba^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{3+} and Zn^{2+} [1]. These metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at higher temperatures [1].

Substrate Specificity

Because of their broad substrate specificity, allkaline proteases have been found to be active against a number of natural proteins and synthetic substrates. Furthermore, the literature conclusively suggests that they are more active against casein than against azocasein, hemoglobin or BSA [1]. In addition, different types of alkaline proteases exist, i.e. collagenase, elastase, keratinase and insect cuticle-degrading protease, which are active against specific protein substrates (such as collagen, elastin, keratin, and cuticle) [1].

Kinetic Parameters

Enzyme-based development process requires prior knowledge about kinetic parameters of the enzyme in question. This fundamental knowledge is extremely important with respect to efficient and sustainable process development. To be precise, kinetic properties like *Vmax, Km, Kcat*, and *Ea* are important, being not only enzyme-specific, but also substrate- and environment specific, and knowledge of these is essential for designing enzyme reactors or quantity of the enzyme under different conditions for its applications. Interms of popularity, the synthetic substrates are more than the enzyme substrate for defining *Km* and *Vmax* as regard to convenience [21, 23]. For an alkaline protease from *B. mojavensis*, the Km for casein decreased with corresponding increase in *Vmax*, as the reaction temperature was raised from 45 to 60ºC [24]. In comparison, the Km and Vmax for *Rhizopus oryzae* alkaline protease increased with an increase in temperature from 37ºC to 70ºC [25].

CONCLUSIONS AND FUTURE PERSPECTIVES

Microbial proteases are becoming as ubiquitous as bacteria and fungi [26]. The industrially important producers of bacterial proteases belong to the genus *Bacillus*. The sources, classification, properties, application and biosynthetic regulation have been briefly highlighted. Various types of microbial proteases were characterized by different researchers and their potential industrial applications have been explored. Although microbial proteases already play a significant role in industry, still researchers are looking for more efficient newer wild type biocatalyst that will be compatible with process parameters and industrial applications. Given the commercial success of these enzymes, their potential is much greater and their application in future process is likely to increase.

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