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STUDIES ON PRODUCTION, CHARACTERIZATION AND APPLICATIONS OF MICROBIAL ALKALINE PROTEASES

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ABSTRACT

With the modern world focusing on eco-friendly products and product output, more and more chemical processes are being replaced by enzymatic methods. Alkaline proteases are one of the most important group of microbial enzymes that find varied uses in various industrial sectors such as leather, detergents, textile, food and feed etc. Industrially important alkaline proteases from bacterial sources have been studied extensively, of which *Bacillus* sp. is most reported. Most of the alkaline proteases that play a role in industries are thermostable as their optimal activity lies between 50^0C to 70^0C. The recently used statistical methods have given way to a more rapid optimization process for alkaline protease production. Other than traditional industrial uses, alkaline proteases have promising application in feather degradation and feather meal production for animal feed. This review highlights the alkaline proteases production, optimization of process parameters, characterization as well as their different applicability.

KEYWORDS: Alkaline proteases, *Bacillus*, Detergent, Keratin, Thermostable

INTRODUCTION

Proteases are enzymes occurring everywhere in nature be it inside or on the surface of living organisms such as plants, animals and microbes. These enzymes carry out proteolysis i.e. break down proteins by hydrolysis of the peptide bond that exists between two amino acids of a polypeptide chain. The proteases available today in the market are derived from microbial sources. This is due to their high productivity, limited cultivation space requirement, easy genetic manipulation, broad biochemical diversity and desirable characteristics that make them suitable for biotechnological applications [1]. The world enzyme market is currently at $5.1 billion and is expected to rise by 6.3% annually by 2013. Since proteases share a major part of the global enzyme market, an upward trend in their demand is

expected. Proteases can be classified according to their active pH range into neutral, acidic and alkaline proteases. Alkaline proteases (EC.3.4.21–24, 99) are those enzymes that are active at alkaline pH with optimum pH in between 9 to 11. Alkaline proteases are most widely used in industries. The largest application of proteases is in detergent industry where removal of proteinaceous substances occurs at alkaline pH. Another important application of alkaline proteases is in the leather industry for dehairing of hides and skins. Commercially successful uses of alkaline proteases are also mentioned in photographic, textile, chemical and food industry. They can be classified on the basis of chemical nature of the active site into: serine protease and metalloprotease.

Serine proteases are characterized by a serine residue forming a catalytic triad with aspartic acid and histidine in the active site. They are inactivated by organic phosphate esters. Organic phosphate esters acylate the active serine residue thereby rendering the enzyme inactive. They are strongly inhibited by phenylmethylsulfonyl fluoride (PMSF) and diodopropyl fluorophosphate (DFP) and chymostatin. Alkaline proteases from different microbial sources mainly belong to this group. Subtilisins are a major group of alkaline serine proteases mostly active at pH 10 that initiate the nucleophilic attack on the peptide (amide) bond through a serine residue at the active site. Subtilisin Carlsberg and subtilisin novo (sequence homology to subtilisin BPN') are two important types of alkaline proteases obtained from *Bacillus* sp. A subtilisin-like protease BPP-A produced by *Bacillus pumilus* MS-1 can be utilized as an industrial enzyme for the production of zein hydrolysates [2]. Serine alkaline proteases from halophilic sources *Natrialba magadii*, haloalkaliphilic bacterium sp. AH-6 and *Halobacterium* sp. have also been reported [3-5].

Metalloproteases are usually characterized by a catalytic divalent metal ion such as zinc ion in the active site. The ion coordinates to the protein via three ligands (histidine, glutamate, aspartate, lysine or arginine) and a labile water molecule. They are inhibited by ethylenediaminetetraacetic acid (EDTA). The neutral metalloproteases show specificity for hydrophobic amino acids, while the alkaline metalloproteases possess a very broad specificity [1]. A metalloprotease secreted by *Pseudomonas aeruginosa* MTCC 7926 isolated from solvent-contaminated habitat is suggested to be useful for dehairing of animal skin, anti-staphylococcal activity and processing of X-ray film [6]. Another metalloprotease from *Streptomyces olivochromogenes* was reported to be useful in organic solvent-based enzymatic synthesis and detergent formulation [7]. Alkaline protease purified from entomopathogenic bacterium *Photorhabdus* sp. strain EK1 (PhPrtPI) has been classified as metalloprotease and it is Ca^{2+} dependent. The broad substrate specificity of the enzyme towards different biologically important proteins and peptides suggests the possibility of PhPrtPI being involved in degradation of insect tissues for providing nutrients to the associated nematode [8]. A metallotype protease secreted by *Salinivibrio* sp. strain AF-2004 exhibiting broad pH ranges (5.0–10.0), moderate thermoactivity and halotolerance is suggested for its high commercial value, it being a thermophilic and halophilic alkaline protease [9].

SOURCES OF ALKALINE PROTEASES

Alkaline proteases are obtained from various microbial sources such as bacteria, fungi and certain yeasts. Of all the microbial sources, bacterial proteases are of particular interest due to their various applications in industries such as detergent, textile, leather, food and feed industry. A major source of bacterial alkaline proteases is *Bacillus* species, which has been studied extensively. Some fungal species are also known to produce alkaline proteases of industrial use, of which *Aspergillus* species has been extensively studied. A very few studies exist on yeast species. Alkaline proteases from *Aureobasidium pullulans*, *Yarrowia lipolytica*, *Issatchenkia orientalis* and

Cryptococcus aureus with optimum pH of 9-10 and optimum temp of 45-50^{o} C have been reported for their excellent bioactive peptide production properties [10]. Recently, alkaline proteases from mushrooms have also been studied and purified [11,12]. A list of various microorganisms producing alkaline proteases is shown in Table 1. Some halophilic sources have also been screened for the secretion of alkaline proteases (Table 2).

Halophilic enzymes find rapidly increasing use in biotechnological applications owing to their halo-tolerance, thermostability for long incubation periods and capability to retain activity in presence of high levels of organic solvents [13,14]. Extracellular alkaline proteases from halophilic bacteria with high pH and thermostability, organic solvent stability and compatibility with detergents have been reported [15]. Capiralla *et al*. [16]have reported alkaline protease from *Halobacterium halobium* S9 with potential application in debittering of protein hydrolysates.

PRODUCTION OF ALKALINE PROTEASES

For industrial use, only large scale production of alkaline proteases can suffice the requirement. Industrial production of alkaline proteases can be carried out by solid-state and submerged fermentations. Media composition [17], particularly carbon and nitrogen source [18] and process parameters such as temperature, pH, agitation speed [19], greatly influence the enzyme production. Each micro-organism producing alkaline protease requires a different medium and process conditions. The effect of carbon and nitrogen sources on the enzyme production has been studied extensively. Akhavan Sepahy and Jabalameli [20] studied the effect of various culture conditions on the production of an extracellular protease by *Bacillus* sp. and reported that sucrose and corn steep liquor are the best substrate for enzyme production. Some alkaline proteases also require metal ions in the form of salts in the production media. It has been shown that $FeSO_4.7H_2O$ and $MgSO_4.7H_2O$ enhanced the protease production by *Bacillus subtilis* RSKK96 [21]. Since cost of fermentation media is considerable, production of alkaline proteases have also been carried out using different agro industrial wastes (green gram husk, chick pea, wheat bran, rice husk, lentil husk, cotton stalk, crushed maize, millet cereal), tannery wastes, shrimp wastes, date wastes etc. [21-27]. Most of the microorganisms produce alkaline proteases at pH 8-9 and temperature of 32-45^{0}C. Table 3 presents different physiochemical parameters for maximum production of alkaline proteases. Researchers are in a continuous process of optimizing production to achieve maximum yield and economical use of available resources. Traditionally, scientists have adopted “one variable at a time” strategy where each variable is optimized independently [28,29]. This is very time consuming, expensive and does not reflect true optimum when a large number of variables are involved because of interference by interaction between them. Recently, a number of statistical methods (Table 4) have been developed such as Taguchi methodology, Plackett–Burman design and response surface methodology (RSM) for optimization to achieve rapid and better understanding of interaction between various variables using a minimum number of experiments [30].

CHARACHTERIZATION OF ALKALINE PROTEASES

Alkaline proteases from different sources have been characterized by various workers in order to use them for specific purposes. For example alkaline proteases with broad pH range activity, high thermostability and bleach stable find application in detergent and leather industry. The alkaline protease isolated from *Pseudomonas aeruginosa* is active at a broad pH range of 6–11 and a temperature range of 25–65^{0}C. The studies showed that the purified enzyme retains its activity in surfactants and bleaching agents. These properties indicate its possible use in the detergent industry [6]. The optimum pH for caseinolysis by

most of the alkaline proteases lies in the range 8.0-11.0 at a temperature range of 37-60^{0}C. Most of the bacterial alkaline proteases studied so far have been reported with optimum temperatures in the range of 50-70^{0}C in buffers of pH 9-10, whereas fungal proteases show a pH optimum of 9-10 with optimum temperature in the range of 37-50^{0}C. Alkaline proteases from bacterial sources exhibit higher thermostability than those obtained from fungal sources. Some alkaline proteases show exceptionally high thermostability with no fall in activity at 60-70^{0}C upto 3 hours [31-33].

It has been also observed that alkaline proteases also require a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations for maximum activity. Supposedly, cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures. Alkaline proteases are completely inhibited by phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphates (DFP) and ethylenediaminetetraacetic acid (EDTA). Sulfonation of the essential serine residue in the active site by PMSF results in the complete loss of activity (Kumar and Takagi 1999). DFP inhibits the serine alkaline proteases by reacting with the serine residue in the active site followed by formation of a covalent adduct. EDTA inhibits metallotype alkaline proteases by chelating the divalent metal ion in its active site.

The molecular mass reported for most of the alkaline proteases lie in the range 30-45 KDa. Bacterial alkaline proteases with molecular weight below or above this range have been rarely reported. Extracellular alkaline proteases with very low molecular mass of 23 kDa and 15 kDa have been isolated from *Streptomyces aurantiogriseus* EGS-5 and *Bacillus subtilis* PE-11 respectively [34,32]. Alkaline proteases with very high molecular masses of 51 and 66 kDa have also been reported [35,25]. The effect of various parameters on the enzyme characterization along with their molecular weight is summarized in Table 5.

IMMOBILIZATION OF ALKALINE PROTEASES

Despite of various advantages, the use of enzymes in industrial applications has been limited due to their high cost of production, instability and expensive recovery which restrict the repeated use of the enzyme. This led to technological developments in the field of immobilized enzymes/whole cells systems which offer the possibility of a wider and more economical use of enzymes. Several attempts have been made to increase stability to temperature, pH, and organic solvents; recovery and reuse of enzymes by using various techniques of enzyme immobilization. Whole cell immobilization technique has been employed for higher productivity of alkaline proteases by protecting the cells from shear forces, and advantage of reusability and easy recovery of products [36]. Cell free immobilization techniques have also been widely used for the production of proteases. The alkaline proteases are immobilized on solid support matrix by adsorption, covalent binding, ionic binding, cross linking and entrapment. A few examples of the immobilization of whole cells as well as cell free supernatants using various matrices are listed in Table 6.

MOLECULAR CHARACTERIZATION OF ALKALINE PROTEASES

Attempts have been made to deduce the nucleotide and amino acid sequences of alkaline proteases from various sources. These sequences not only help in deriving the primary structure, but also in identifying various functional regions of the proteases. Studies of DNA and protein sequence homology are important for a variety of purposes and have therefore become routine in computational molecular biology. They serve as a prelude to phylogenetic analysis of proteins and assist in predicting the secondary structure of DNA and proteins, and elucidating the structure-function relationship of proteases [1].

Alkaline protease from marine bacterium strain YS-80-122 with 463 amino acid residues and ANGTSSAFTQ as the N-terminal amino acid

sequence has been reported. Sequence alignment revealed that it displayed the highest percentage identity (91%) to an alkaline metalloprotease from psychrophilic *Pseudomonas* sp., with identity to serralysin-type metalloproteases of other strains, *Pseudomonas fluorescens* Pf-5 and *Dickeya zeae* Ech1591 at 66% and 53% respectively. These results indicate that the alkaline protease belongs to the serralysin-type metalloproteases [37]. Another alkaline protease BPP-A from *Bacillus pumilus* strain MS-1consisting of 275 amino acids (mature protein), with a very high homology with that of subtilisin from *Bacillus pumilus* strain TYO-67 and strain UN-31-C-42 (99% amino acid identity) has been studied. Sequence of mature BPP-A contained a conserved essential catalytic triad made up of Asp140, His175 and Ser329 as well as three serine protease (subtilase) family signatures. High sequence homology and conserved sequences of the catalytic triad indicate that BPP-A is a typical subtilisin, which belong to the serine protease (subtilase) family [2].

Similarly, amino acid sequence of halotolerant alkaline proteases from *Bacillus subtilis* FP-133 was >98% identical to that of alkaline serine proteases identified as firbinolytic enzymes. The mature protein consisted of 275 amino acid residues with a catalytic triad centre containing Asp, His and Ser residues, as identified by comparing the sequence with the homologous serine alkaline protease subtilisins of *Bacillus* and *Brevibacillus* sp.. Three dimensional modeling suggested that the acidic and polar amino acid residues located on the surface stabilize protein structure in the presence of relatively high NaCl concentrations [38].

Multiple sequence alignment of alkaline protease protein sequence of different *Aspergillus* species revealed a stretch of conserved region for amino acid residues from 69 to 110 and 130–204. A motif with a signature amino acid sequence of 50 amino acids was uniformly observed in proteases protein sequences indicating its involvement with the structure and enzymatic function. A phylogenetic tree was constructed which indicated that several *Aspergillus* species-specific clusters for alkaline proteases exist from *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus clavatus* [39]. A similar result from sequence homology of alkaline protease from *Aspergillus clavatus* with alkaline serine proteases from *Aspergillus fumigatus* and *Verticillium chlamydosporium* revealed 90% and 100% amino acid identity respectively. It also showed high identity with the subtilisin-like protease of *Ophiostomaulmi* and *Metarhizium anisopliae* (88% and 81% identity respectively), indicating that this enzyme belongs to a serine proteases group and it is a subtilisin-like proteases. These findings were supported by observations made by specific inhibitors and substrates specificity of the enzyme [40].

USES OF ALKALINE PROTEASES

Alkaline proteases are one of the most important classes of proteases from an industrial point of view, occupying a major share of the total enzyme market. Use of alkaline proteases as active ingredients in detergents is the largest application of this enzyme. They are also widely used in leather industry, medical diagnostics, recovery of silver from X-ray films, silk degumming, food and feed industry etc. Due to their vast applications in the industrial processes, many companies started manufacturing them at commercial level. The table 7 gives the commercial manufacturers of alkaline proteases with their product trade name and different applications.

Detergent industry

Alkaline proteases have contributed greatly to the development and improvement of modern household and industrial detergents. They are effective at the moderate temperature and pH values that characterize modern laundering conditions in industrial & institutional cleaning. Various enzymes used in laundry industry are proteases, lipase, cellulases, amaylses etc. [41]. Of these, alkaline protease find a major application as

detergent additives because of their ability to hydrolyze and remove proteinacious stains like blood, egg, gravy, milk etc in high pH conditions [42]. Proteases and other enzymes used in detergent formulations should have high activity and stability over a broad range of pH and temperature. The enzymes used should be effective at low levels (0.4–0.8%). One of the most difficult design challenges that biotechnologists face is that the protease should be compatible with various commercially available detergents and its function is not hampered in the vicinity of typical detergent ingredients, such as surfactants, builders, bleaching agents, bleach activators, fillers, fabric softeners and various other formulation aids. Recently, alkaline proteases from *Bacillus cereus*, *Bacillus pumilus* strain CBS, *Streptomyces* sp. strain AB1, *Bacillus licheniformis*, *Aspergillus flavus*, *Aspergillus niger*, *Bacillus brevis*, *Bacillus subtilis* AG-1 have exhibited excellent deteregent compatibily in the presence of certain stabilizers such $CaCl_2$ and glycine [43-49]. To survive the extremes of high alkalinity and chelator concentration in detergents, subtilisins have been improved with respect to their thermostability, resistance to chelators [50]. To prevent the loss of activity, several oxidatively stable serine proteases (OSPs) suitable for use in detergents have been isolated from alkaliphilic *Bacillus* strains [51].

Leather industry

Soaking, dehairing of hides and skins and bating have traditionally being carried out by using different chemicals which poses a high tannery waste pollution threat. Hence, proteases with a pH optimum around 9–10 are widely used in soaking to facilitate the water uptake of the hide or skin. Alkaline proteases with elastolytic and keratinolytic activity are used for dehairing and bating process to obtain a desired grain, softness and tightness of leather in a short time. Alkaline proteases with keratinolytic activity have been reported for remarkable dehairing properties [52-24]. A novel protease showing keratinolytic activity from *B. subtilis* has been studied as a potential for replacing sodium sulfide in the dehairing process of leather industry [55]. Verma *et al.* [31] showed the use of protease from *Thermoactinomyces* sp. RM4 for dehairing goat hides.

Chemical Industry

A high stability in the presence of organic solvents is a feature which is highly desired in applications involving biocatalysis in non-aqueous medium for peptide synthesis. Alkaline proteases from *Aspergillus flavus*, *Bacillus pseudofirmus* SVB1, *Pseudomonas aeruginosa* PseA have shown promising results for potential of peptide synthesis due to their organic solvent stability [46,56,57]. In addition to demonstrating high organic tolerance, alkaline proteases from *B. pumilus* strain CBS *and Streptomyces* sp. strain AB1 are potential strong candidates for use in peptide synthesis in low water systems [44]. Apart from peptide synthesis, they have also been reported for organic synthesis. Alcalase, an industrial alkaline protease has been used for the synthesis of Bz-Arg-Gly-$NH_{(2)}$ (N-benzoylargininylglycinamide), precursor dipeptide of RGDS (Arg-Gly-Asp-Ser)] catalysed in water/organic co-solvent systems [58]. Synthesis of 2H-1-benzopyran-2-one derivatives using alkaline protease from *Bacillus licheniformis* has been reported by Wang *et al.* [59]. Regioselective synthesizes of polymerizable vinyl guaifenesin ester has been studied by an alkaline protease from *Bacillus subtilis* [60].

Medical Uses

The use of immobilized alkaline protease from *Bacillus subtilis* possessing therapeutic properties has been studied for development of soft gel-based medicinal formulas, ointment compositions, gauze, non-woven tissues and new bandage materials [61]. Oral administration of proteases from *Aspergillus oryzae* has been used as a diagnostic aid to correct certain lytic enzyme deficiency syndromes [1]. Alkaline-fibrinolytic protease have been reported to preferentially

degrade fibrin suggesting its future application in thrombolytic therapy and anticancer drugs [62,35]

Waste management

Wastes from poultry processing industry and leather industry are recaliterant to commonly known proteolytic enzymes due to presence of keratin-rich wastes whose polypeptide is densely packed and strongly stabilized by several hydrogen bonds and hydrophobic interactions in addition to several disulfide bonds [63]. Chemical and mechanical hydrolysis of keratin wastes is successful but they have several disadvantages of being energy intensive, polluting and leading to loss of essential amino acids. Hence, enzymatic degradation using alkaline proteases with keratinolytic activity (keratinases) is an attractive method [64]. *Bacillus* species is the most widely reported bacterial source of keratinases for feather degradation [65-67]. Other reported bacterial sources of keratinases are *Pseudomonas* sp. MS21, *Microbacterium* sp., *Chryseobacterium* sp. and *Streptomyces* sp. [68-71]. Fungal keratinases from *Aspergillus Oryzae*, *Chrysosporium indicum*, *Trichophyton mentagrophytes*, *Microsporum* sp., *Trichophyton* sp., *Aspergillus terreus*, *Scopulariopsis* sp., *Fusarium oxysporum* have also been studied towards the degradation of keratin [72,73]. Feather hydrolysates obtained after feather degradation can be used as additives for feedstuffs, fertilizers, glues and films or used for the production of the rare amino acids -serine, cysteine, and proline [74].

Feed and Food industry

Alkaline proteases are widely used for production of protein hydrolysates for more than 40 years. Hydrolysates can be used as additives to food and mixed feed to improve their nutritional value. In medicine, they are administered to patients with digestive disorders and food allergies [75]. Protein hydrosylates can be obtained from a variety of substrates such as whey, meat, soy and casein. It is well known that meat hydrolysates usually taste bitter when the degree of hydrolysis (DH) is above the 10% required for satisfactory solubilization. With Novozymes's Flavourzyme®, it is possible to degrade the bitter peptide groups and obtain a degree of hydrolysis of 20% without bitterness. Alkaline proteases also find their use in meat processing. SEB Tender 70, commercially available proteases is extensively used in meat tenderization to break down collagens in meat to make it more palatable for consumption.

Silver Recovery

Silver is one of the precious and noble metals used in large quantities for many purposes, particularly in the photographic industry. The waste X-ray/photographic films containing black metallic silver spread in gelatin are very good source for silver recovery compared to other types of film. The amount of silver in the X-ray film varies between 1.5 and 2.0% (w/w). Various traditional methods for silver recovery are burning the films directly, oxidation of the metallic silver following electrolysis, stripping the gelatin-silver layer using different chemical solutions. But this method poses serious environmental considerations. For this reason, enzymatic hydrolysis of gelatin is an alternative option to minimize the impact on environment [127]. Successful recovery of silver from X-ray films have been reported by alkaline proteases derived from *Bacillus subtilis*, *Conidiobolus coronatus*, *Streptomyces avermectinus* [76-78]. Recovery of silver and polyethylene terephthalate from used lith film for printing using the thermostabilized mutant enzyme of the alkaline protease from alkaliphilic *Bacillus* sp. B21-2 has also been reported [79].

Silk Degumming

Threads of raw silk must be degummed to remove sericin, a proteinaceous substance that covers the silk fiber. Traditionally, degumming is performed in an alkaline solution containing soap. This is a harsh treatment because the fiber itself is attacked. It also have other disadvantages of high energy consumption, time consumption and also a loss in luster of silk due to the amounts of water used in

this process [80]. However, the use of selected alkaline proteolytic enzymes is a better method because they remove the sericin without attacking the fibre. Tests with high concentrations of enzymes show that there is no fiber damage and the silk threads are stronger than with traditional treatment. In a recent study by Romsomsa *et al.* [81], optimization of silk degumming protease production from *Bacillus subtilis* C4 has been carried out using Plackett-Burman design and response surface methodology.

CONCLUSION

Keeping in view the industrial importance of alkaline proteases, researchers are more and more focusing on discovering alkaline proteases with novel properties to meet the industrial requirements as well as increasing demand of global enzyme market. Other than traditional uses in detergent industry for removing protein stains, leather industry for dehairing, textile industry for silk degumming, photographic industry for silver recovery, food and feed industry, chemical industry for peptide synthesis, they are increasingly finding potential future applications in pharmaceutical industry. Recently, alkaline proteases with keratinolytic activity are finding an interesting application in the feather degradation for reducing poultry waste and feather meal production for fertilizers, glues, biodegradable films, and foils. Through this review, an attempt has been made to highlight the recent advances in the field of alkaline proteases and their applicability.

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Tables:

Table 1. Alkaline proteases from various sources.

Microorganisms	Applications	References
Pseudomonas aeruginosa PD 100	Collagen replacement therapy, waste treatment, removal of blood stains and dehairing	Najafi *et al.* [82]
Thermoactinomyces sp. RM 4	Leather industry for dehairing process	Verma *et al.* [31]
Bacillus sp. Tk1 and Tk2	Detergent industries, food industries and pharmaceutical industries	Kuberan *et al.* [83]
Bacillus pumilus Cbs	Detergents, dehairing	Jaouadi *et al.* [44]
Streptomyces sp. Ab1	Dehairing, feather hydrosylation	Jaouadi *et al.* [44]
Bacillus subtilis PE-11	Detergents	Adinarayana *et al.* [32]
Bacillus sp. SSR1	Detergent industry	Singh *et al.* [84]
Bacillus licheniformis RP1	Chitin extraction, chicken feather degradation and dehairing	Haddar *et al.* [26]
Bacillus coagulans and *lichenformis*	Industrial sector	Asokan and Jayanthi [85]
Bacillus clausii	Industrial sector	Vadlamani *et al.* [86]
Bacillus cereus	Detergents and removal of blood stains	Abou-Elela *et al.* [43]
Bacillus sp. K-30	Deproteinisation of rice bran and detergents	Naidu *et al.* [87]
Bacillus cereus 1173900	Dehairing	Ravindran *et al.* [25]
Bacillus circulans	-	Prakasham *et al.* [23]
Bacillus subtilis	Depilating agent in leather processing	Mukhtar and Haq [88]
Pseudomonas fluorescens	Detergent and textile industry	Kalaiarasi and Sunitha [89]
Microbacterium strain AR-68	Detergent and leather industry	Gessesse and Gashe [90]
Streptomyces pulvereceus	-	Jayasree *et al.* [29]
Stenotrophomonas maltophilia (MTCC 7528)	Detergent additive for cold washing and environmental bioremediation in cold regions	Kuddus and Ramteke [91]
Shewanella oneidensis MR-1	Industrial sector	Anbu *et al.* [92]
Streptomyces aurantiogriseus EGS-5	High temperature industrial process	Ahmad [34]
Aspergillus niger	Detergents	Devi *et al.* [47]

Genetically engineered *Aspergillus oryzae* U1521	Animal food and feed processing	Samarntarn *et al.*[93]
Aspergillus flavus and *Aspergillus terreus*	Leather and food industry	Chellapandi [94]
Aspergillus nidulans HA-10	Food processing, pharmaceutical and leather industries	Charles *et al.* [95]
Engyodontium album BTMFS10	Detergents and silver recovery	Jasmin *et al.* [96]
Aspergillus flavus	Detergent industry, peptide synthesis	Yadav *et al.* [46]

Table 2. Halophilic sources of alkaline proteases.

Microorganisms	Optimum pH	Optimum Temperature (^{0}C)	References
Chromohalobacter TVSP101	8	75	Vidyasagar *et al.* [7]
Virgibacillus marismortui NB2-1	10	50	Chamroensaksri *et al.* [98]
Natronococcus occultus	7-9	60	Studdert *et al.* [99]
Natrialba magadii	8-10	60	Giménez *et al.* [3]
Bacillus sp. HS-4	8	37	Sehar and Hameed [100]
Halobacterium sp Js1	7.5-8	40	Vijayanand *et al.* [101]
Haloalkaliphilic Bacteria	9	50	Dodia *et al.* [102]
Bacillus halodurans SK5	10	70	Ibrahim *et al.* [103]
Halobacterium mediterranei	8-8.5	55	Stepanov *et al.* [104]
Streptomyces clavuligerus strain Mit-1	9	-	Thumar and Singh [105]
Bacillus lichenformis	10	60	Öztürk *et al.* [106]

Table 3. Physiochemical parameters for alkaline proteases production.

Microorganisms	Carbon source	Nitrogen source	pH	Temperature (^{0}C)	Agitation speed (rpm)	References
Bacillus licheniformis N-2	Glucose	Defatted Soyabean meal	10	37	140	Nadeem *et al.* [22]
Bacillus sp. strain CR-179	Starch, Maltose	Corn steep liquor	8	45	150	Akhavan Sepahy and Jabalameli [20]
Aspergillus niger	Glucose	Ammonium sulphate	8.5	45	150	Devi *et al.*[47]
Halobacterium sp JS1	Rice bran	Defatted Soyabean meal	7	40	200	Vijayanand *et al.* [101]
Bacillus subtilis SVR-07	Glucose	Soyabean meal	9	55	150	Reddy *et al.* [107]
Bacillus brevis	Lactose	Soyabean meal	10.5	37	200	Banerjee *et al.* [108]
Bacillus licheniformis NCIM-2042	Starch	Soyabean meal	7	37	180	Bhunia *et al.* [28]
Bacillus subtilis RSKK96	Arabinose	Beef extract	9	37	150	Akcan and Uyar [21]
Pseudomonas fluorescens	Wheat bran	Peptone	9	37	--	Kalaiarasi and Sunitha [89]
Microbacterium strain AR-68	Glucose, Sucrose	Peptone	10.3	32	--	Gessesse and Gashe [90]
Streptomyces pulvereceus	Starch	Casein	9	33	--	Jayasree *et al.* [29]

Table 4. Statistical methods for optimizing production of alkaline proteases.

Optimization methods	Micro organism	Parameters Optimized	Yield improvement	References
Plackett–Burman design and RSM	*Bacillus licheniformis* NCIM 2042	Inoculum size, temperature, pH and agitation	1.71 fold	Bhunia and Dey [109]
Plackett–Burman design	*Bacillus subtilis* DKMNR	Carbon and nitrogen source	4.4 fold	Kezia *et al.* [110]
Plackett–Burman design and RSM	*Bacillus subtilis* C4	Medium components and agitation	2.2 fold	Romsomsa *et al.* [81]
Response surface methodology	*Bacillus subtilis* HB04	Medium components, temperature and agitation	8 fold	Venil and Lakshmanaperumalsamy [111]
Plackett–Burman design and RSM	*Aspergillus clavatus* ES1	Media constituents, pH and temperature	14 fold	Hajji *et al.* [30]
Plackett–Burman and RSM	*Bacillus* sp. RKY3	Inoculum size and medium components	2 fold	Reddy *et al.* [112]
Plackett–Burman method	*Halobacterium* sp. SP1	Medium components	3.9 fold	Akolkar *et al.* [113]
Taguchi design of experiments	*Bacillus clausii*	Medium components and agitation	4 fold	Oskouie *et al.* [114]
Box-Behnken design	*Aspergillus oryzae*	Carbon and nitrogen source, temperature, pH	3 fold	Babu *et al.* [115]

Table 5. Characterization of Alkaline proteases.

Sources	Mol. Wt. (kDa)	Optimum pH	Optimum Temp. (^{0}C)	Effect of metal ions		Inhibitors	References
				Stimulatory	Inhibitory		
Bacillus subtilis PE-11	11	10	60	Ca^{2+}, Mg^{2+}, Mn^{2+}	-	PMSF, DFP	Adinarayana *et al.* [32]
Aspergillus flavus	--	9	37	Fe^{3+}	Hg^{2+}, Cu^{2+}	PMSF, EDTA	Yadav *et al.* [46]
Aspergillus niger	38	10	50	Ca^{2+}	Zn^{2+}, Co^{2+}	EDTA	Devi *et al.* [47]
Bacillus cereus	31	10	50	Cu^{2+}	Ca^{2+}, Mg^{2+}, and Mn^{2+}	EDTA	Abou-Elela *et al.* [43]
Streptomyces aurantiogriseus EGS-5	23	10-11	40	-	-	DFP	Ahmad [34]
Aspergillus nidulans HA-10	42	8	35	-	-	mercaptoethanol, PMSF, EDTA	Charles *et al.* [95]
Bacillus cereus	66	8	40	Ca^{2+}, Mg^{2+}, Mn^{2+}	Zn^{2+}, Cu^{2+} and Fe^{2+}	EDTA and EGTA	Ravindran *et al.* [25]
Natrialba magadii	45	8	45	-	-	DFP, PMSF, chymostatin	Giménez *et al.* [3]
Streptomyces olivochromogen	--	7.5	50	Ca^{2+}	Co^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}	EDTA	Simkhada *et al.* [7]

es					and Fe^{2+}		
Bacillus sp.	--	9.5	60	Ca^{2+} and M^{2+}	Ag^{+} and Co^{2+}	DFP, PMSF, Iodoacetic acid	Saurabh *et al.* [116]

Table 6. Immobilization of alkaline proteases in different matrices.

Microorganisms	Matrix used	Stability and activity of enzymes	References
Bacillus subtilis#	Agar, gelatin, sodium alginate and polyacrylamide	Increased activity	Kumar and Vats [117]
Bacillus subtilis PE-11#	Calcium alginate, k-Carrageenan, polyacrylamide, agar-agar, and gelatin	Higher volumetric activities, long life-term stability, reusablility	Adinarayana *et al.* [36]
Bacillus circulans#	agar-agar and calcium alginate	Increased activity	Kocher and Mishra [118]
Bacillus subtilis-K 30#	calcium alginate, polyacrylamide, agar-agar and gelatin	Higher volumetric activities , long life-term stability, reusability	Naidu *et al.* [119]
Bacillus licheniformis#	Calcium alginate, k-Carrageenan, agar-agar	Increased activity	Ahmed and Abdel-Fattah [120]
Bacillus pumilis#	calcium alginate, polyacrylamide and agar-agar	Increased activity	Kumari *et al*. [121]
Bacillus sphaericus^	Sawdust, perlit and silica	Increased stability	Surendran *et al.* [122]
Myceliophthora sp^	Calcium alginate beads	Increase in optimum temperature , thermal stability and stability for up to 7 reuses	Zanphorlin *et al.*[123]
Streptomyces avermectinus^	Sponge	Increased thermostabilty, half life, activation energy , reuse after 5 cycles	Ahmed *et al.* [78]
Aspergillus oryzae^	Calcium alginate beads	Increase in Km value, optimum pH, enhanced stability in acidic as well as alkaline environments	Sharma *et al*. [124]
Bacillus licheniformis^	Loofa	Increased activity, half life, activation energy, stability in EDTA	Ahmed *et al*. [125]
Bacillus polymyxa^	Perlite	Increased stability	Emtiazi *et al.*[126]

whole cell immobilization, ^ cell free immobilization

Table 7. Commercial manufacturers, trade name and applications of alkaline proteases.

Manufacturers	Trade name of product	Industrial Uses	Applications
Novozymes, Denmark	RONOZYME ProAct	Feed industry	Maximize protein utilization and improving nutritional value of animal feed
	Novozym 37020	Meat and feed industry	Enhance protein quality
	Savinase, Everlase	Detergent industry	Remove protein based stains
	Ovozyme		Remove egg soils
	Polarzyme		Cold water hand wash
	Alcalase	Chemical industry	Resolution of N-protected amino acids , peptide synthesis
		Textile	Silk degumming

		Detergent	Remove protein based stains
	Novobate 1547	Leather industry	Bating
	NovoLime		liming
	NovoCor SG		Soaking
Genecor, USA	Purafast	Detergent industry	cold wash conditions
	Purafect Prime L		High alkaline performance at lower dose rate
	Purafect OX L		High alkaline protease with superior storage stability
	PrimaTan	Leather industry	Bating
	Purafect 4000L		Soaking
	Protex 6L	Food industry	Hydrolysis of milk fractions, baking, protein processing, pet food production
		Photographic industry	Recovery of silver from films
Advanced Enzymes, India	SEBsoak	Leather industry	Soaking
	SEBlime		Dehairing,liming
	SEBate Alakli		Bating
	Protosol	Detergent industry	Cleaning and washing
Amno Enzyme, Japan	Peptidase R, ProteAX	Feed industry	Bitterless protein hydrosylate
	THERMOASE PC10F PROTIN SD-AY10	Leather industry	Bating and dehairing
		Photographic industry	Recovery of waste silver in the film industry
Maps Enzyme Limited, India	Palkobate	Leather industry	Bating
	Palkosoak		Soaking
	Palkodehair		Dehairing
	Palkogent	Detergent industry	Removal of protein stains
AB Enzymes, Germany	COROLASE H-PH	Food and Feed industry	Protein hydrolysis
	COROLASE LAP		Bitterless Protein hydrolysis of animal or vegetable raw materials, e.g. whey, casein, soy
Speciality enzymes and biotechnologies, USA	SEBalase BP	Detergent industry	Removal of protein stains
	SEB Tender 70	Meat industry	Tenderization of meat
	SEBDigest	Food and Feed industry	Protein hydrolysis
	SEBpro A		develop savory flavors and debitterment of protein hydrolysates
Rossari Biotech, India	Lyserine ESD Powder	Silk industry	Degumming of silk
Kao Corp., Tokyo, Japan	Proteinase K-16	Detergent industry	Protein removal
Bachauna Technology, India	Verma Batzyme	Leather industry	Bating
Sunson, China	*PRAL800*	Leather, silk and food industry	Leather depilation, silk degumming and protein hydrolysis