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Review paper

Alkaline Proteases: A Review on Production Optimization Parameters and Their Physicochemical Properties

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Abstract

Proteolytic enzymes are available in the entire livelihood and facilitate in growth of cells along with cell differentiation. Proteases are the hydrolytic enzymes that act as biocatalysts for the degradation of proteins into smaller peptides and amino acids. Microorganisms are capable and provide economical source of alkaline protease enzymes that are able to generate a constant and reliable supply of desired products. Alkaline proteases comprise the major group of enzymes from bio-industrial point of view with large number of applications. They play a significant position in diverse biotechnological industries, viz. in leather, detergent, food & feed and pharmaceutical areas. This review draws attention on different sources of proteases with particular consideration on bacterial alkaline proteases. A variety of nutritional as well as environmental parameters influencing the production of alkaline proteases with a foremost view from *Bacillus* species in submerged fermentation conditions are discussed. The biochemical characterization such as purification aspects along with the physicochemical properties of alkaline proteases from several *Bacillus* species are also addressed in brief which could facilitate to recognize enzymes with higher stability and activity above extreme temperature and pH, in order that they can be exploited for industrial applications.

Key Words: Proteolytic enzymes, Alkaline Proteases, optimization and characterization, Industrial uses.

Introduction

Proteins the indispensible part of all living forms has been mastered by a molecule of its own kind enzyme "**Protease**" (catalytic protein scissor). The world market for enzymes is expected to record Compound Annual Growth Rate (CAGR) of approximately 7.8% during the forecast period of 2015–2020 and reach USD 6.30 Billion in terms of value (1). Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total global enzyme market (2). The most basic classification for proteases is: Neutral, Acidic and Basic (Alkaline protease). According to the Enzyme Commission (EC) classification, proteases belong to third class (hydrolases), and sub-group four (which hydrolyse peptide bonds) (3). Thus, Microbial proteases (EC 3.4.21-24 and 99, peptidyl-peptide hydrolases) are

among the most important hydrolytic enzymes that hydrolyse proteins via the addition of water across peptide bonds (4). Four mechanistic classes are documented by the EC and in these classes; six families of proteases are recognized till date: serine proteases (EC 3.4.21), serine carboxy proteases (EC 3.4.16), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23), metallo peoteases I (EC 3.4.24) and metallo carboxy proteases (EC 3.4.17) (5). In contrast to plants and animals, microbes represent as a better source of proteases because they can be cultured in large volume in a short duration, are comparatively inexpensive, and can produce a continuous supply of metabolites. Being extracellular in nature and are directly secreted by the producer organism into the fermentation medium, simplified the downstream processing of the enzyme (6). The kind & volume of contribution made by researchers towards all the three type of proteases, it's difficult to summarize all in one article. Hence, the present study attempts to review alkaline proteases from microbes in general and *Bacillus* species in particular.

Among microorganisms, the genus "*Bacillus*" is an important source of industrial alkaline proteases and is probably the only genus being commercialized for alkaline protease production (7-8). The reason for this is their wide temperature, pH tolerance and thermal stability (9). The first alkaline protease Carlsberg (BIOTEX) from *B. licheniformis* was commercialized as an additive in detergents in the 1960s (10). *Bacillus* derived alkaline proteases are of immense utility in different sectors. The potent avenues that established with use of alkaline proteases are detergent, leather, pharmaceuticals, food, textile, silk, bakery, soy processing, meat tendering, brewery, protein processing, peptide synthesis, ultra filtration membrane cleaning and recovery of silver from photographic films (11-13). Owing to their immense demand in industries, researchers are persistently investigating diverse aspects of proteases (14).

Laboratories engaged in alkaline protease production studies are making efforts to improve upon existing potential alkaline protease producing bacteria, particularly belonging to *Bacillus* sp. In this regard, different biotechnological approaches like immobilized cells, gene amplification etc. have been employed (8, 15-16). Therefore, keeping in view the importance of alkaline proteases in different sectors, the review will focus on potential emerging strains for alkaline protease along with production, purification and properties of alkaline proteases.

Potential Emerging Strains for Alkaline Protease

Emerging strains are need of the hour for the fulfillment of the high demand of alkaline protease in present scenario. Potential microbes has been screened, studied and established for extracellular alkaline protease production. Amongst microbes bacteria has been appraised for its potential to produce extracellular protease production. More than 50 species of *Bacillus* has been established for alkaline protease production. The percentage distribution of various microbes for alkaline protease production (**Fig 1**) are as follows bacteria, fungus and actinomycetes are 81, 11 and 8%, respectively.

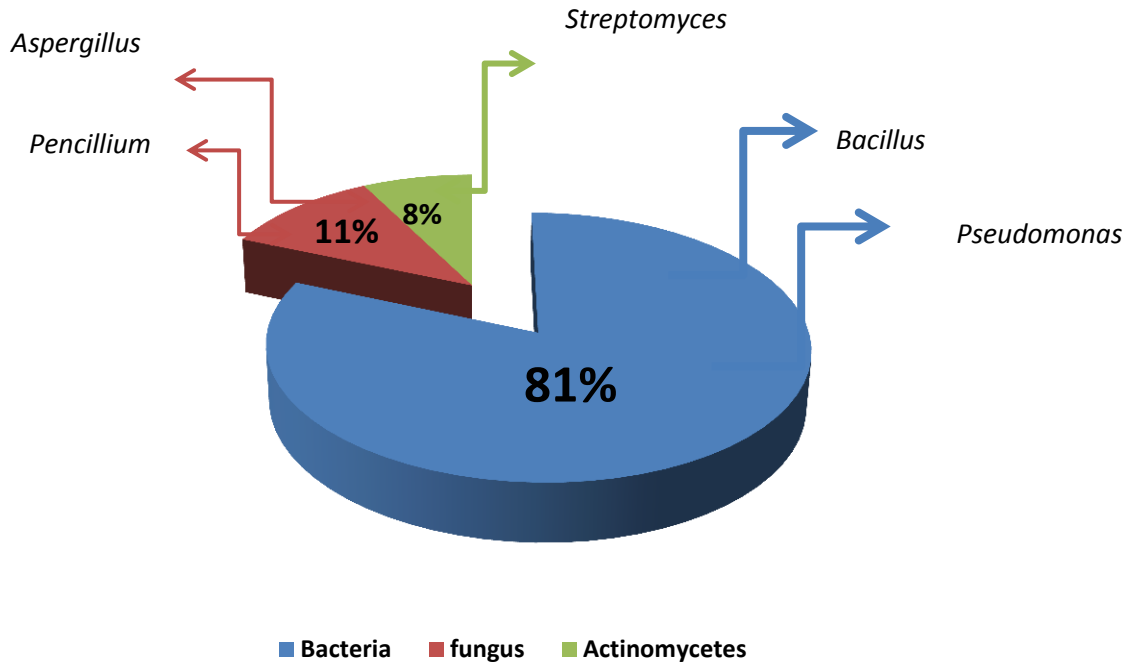

Fig: 1 Percentage distribution of alkaline protease producing microorganisms

Bacteria

Bacterial strains have been the major source of alkaline proteases with the genus *Bacillus* being the most predominant source (**Table 1**). The most potential alkaline proteases producing bacilli are the strains of *B. licheniformis*, *B. subtilis*, *B. amyloliquifaciens*, *B. mojavensis* and *B. circulans* (17- 20). Microbial proteases, especially from *Bacillus* sp. have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergent formulations (21).

The first report about commercial use of an alkaline protease enzyme was that of an extracellular alkaline serine protease from alkalophilic *Bacillus* strain 221 during 1971. Bacterial alkaline proteases are in general characterized by their high activity at alkaline pH, for instance, 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.

Table 1. Different alkaline protease producing bacterial species

***Bacillus* sp.**	**Source (Reference)**
Oceanobacillus iheyensis O.M.A18	(22)
B. alcalophilus ATCC 21522	(23)
B. amyloliquefaciens	(19)
Bacillus sp.	(24)

Bacillus sp. SSR1	(25)
Bacillus sp. MIG	(26)
Bacillus sp. L21	(27)
Bacillus sp. GPA4	(28)
B. subtilis	(29-30)
B. subtilis MTCC N0-10110	(31)
B. subtilis SVR-07	(32)
Bacillus subtilis RSKK96	(33)
B. firmus	(34)
B. firmus Tap5	(35)
B. proteolyticus	(36)
B. thuringiensis	(37)
B. licheniformis	(38)
B. licheniformis NCIM-2042	(39)
B. pseudofirmus AL-89	(40)
B. circulans	(20)
B. cereus	(41)
B. cereus VITSN04	(42)
B. amovivorus	(43)
B. proteolyticus-CFR3001	(11)
B. aquamoris	(44)
Bacillus clausii	(45)
Bacillus sphaericus	(46)
Thermophilic strains	**Source**
Bacillus sp. JB-99	(47)
Bacillus sp. Strain SMIA 2	(48)
Bacillus RV.B2.90	(49)
B. licheniformis RP1	(50)
B. thermoruber BT2T	(51)
B. stearothermophilus TLS33	(52)
B. pumilus CBS	(53)
Thermomonospora fusca	(54)
Thermoactinomycetes sp.	(55)
A. stearothermophilus	(56)
Geobacillus	(57)

Pseudomonas aeruginosa	(58)
*Vibrio fluvialis*strain VM10	(59)
Gamma-Proteobacterium	(60)
Bacillus sp. Ve1	(24)
Bacterium O.M.E12	(22)

Fungi and Actinomycetes

Fungi present a wide variety of proteases than do the bacteria (Table 2). They are active over a wide range of pH eg., *Aspergillus oryzae* produces acidic, neutral and alkaline proteases which have broad substrate specificity but they have low reaction rate and lesser heat tolerance than bacterial proteases. Fungal alkaline proteases are used in food protein modification. Among fungi, *Aspergillus* sp. (61), *Conidiobolus* sp. and *Rhizopus* sp. (62) also produce alkaline proteases. Among yeast, *Candida* sp. has been studied in detail as a potent alkaline protease producer (63).

Among actinomycetes (Table 3), strains of *Streptomyces* are preferred protease sources (64). Partial purification and characterization of a noval alkaline protease produced by *Nocardiopsis* sp. was reported by Moreira *et al* (65). The serine and metalloproteases produced by *Streptomyces* sp. 594 in submerged (SF) and solid-state fermentation (SSF), using feather meal, an industrial poultry residue (keratinous waste) and corn steep liquor (corn processing by-product), were found to be active over a wide range of pH (5.0-10.0) and high temperatures (55-90°C) (66).

Table 2 Alkaline protease producing fungal species

Fungi	Source Reference
Penicillium charlesii	(67)
P. lilacinus	(68)
P. griseofulvim	(69)
Fusarium graminearum	(70)
Chrysoporium keratinophilum	(71)
Scedosporium apiosermum	(72)
A. melleus	(73)
A. niger	(74)
A. fumigatus	(75)
A. flavus	(76)
A. terreus	(61)
A. clavatus	(77)
A. nidulans HA-10	(78)
A. oryzae	(79)
A. awamori	(80)
Conidiobolus coronatus	(81)
Botrytis cinerea	(82)

Table 3 Alkaline protease producing Actinomycetes

Actinomycetes	Source reference
Streptomyces sp. CN902	(83)
Streptomyces sp. DP2	(64)
Streptomyces NRRLB-8165	(84)
Streptomyces fradial	(85)
Streptomyces penutius	(86)
Streptomyces sp. 594	(66)
Streptomyces pseudogrisiolus NCR-15	(87)
Nocardiopsis Alkalophila sp Nov.	(88)
Nocardiopsis TOA-1	(89)
Nocardiopsis sp. *NCIM* 5124	(90)
Saccharomonospora virdis SJ-21	(91)

Microbial Production of Alkaline Protease

Protease production is an inherent property of all organisms and these are generally constitutive however at times they are partially inducible (92). The proteases are largely produced during stationary phase and thus are generally regulated by carbon and nitrogen stress. Different methods in submerged fermentations have been used to regulate the protease synthesis by combinations of either of the strategies, such as fed-batch, continuous, and chemostat cultures (93). These strategies have resulted in high yields of alkaline protease in the fermentation medium (94).

Protease being associated with the onset of stationary phase, their production is often related to the sporulation stage in many bacilli, such as *B. subtilis* and *B. licheniformis* (95). On the contrary, few reports also suggest that sporulation and protease production though may co-occur, but are not related as the spore-deficient strains of *B. licheniformis* (96) and *B. stearothermophilus* (97) were not protease-deficient. A similar observation has also been reported in *B. licheniformis* by analysis of nucleotide pools (GTP and ATP) in the cells (98). These results strongly suggested that the protease production is under stringent response to amino acid deficiency and is related to the Gppp ratio in the cell. The transitions between different growth phases or different nutritional limitations can be discerned by the alterations in the nucleotide pool as a marked decrease in the GTP content of the cells (after addition of mycophenolic acid in the exponential phase) was found to increase the protease production during the stationary phase (95).

Hence, it conclusively suggests that extracellular protease production is a manifestation of nutritional limitation, at the onset of stationary phase. However, final protease yield during this phase is also determined by the biomass produced during exponential phase. Therefore, media manipulations are needed to maximize growth and hence protease yields.

Effect of carbon sources on alkaline protease production

Carbon sources used in the culture media vary greatly in type and concentration for different types of bacteria and other microorganisms. Among various types of

carbon sources, carbohydrates are used predominantly. Majority of the microorganisms utilize glucose and starch as the preferred source of carbon, though other types of carbohydrate and non-carbohydrate sources have been reported to be utilized by protease producing microorganisms (51, 70). In different studies, glucose, glycerol, starch, sucrose, fructose, maltose, galactose and lactose have been reported as optimum carbon sources for alkaline protease production (92, 99). Mukherjee *et al* (100) used glucose, fructose, galactose, maltose, sucrose and lactose (10% w/w) for alkaline protease production by thermophilic *B. subtilis* with maltose supporting maximum alkaline protease production under solid state fermentation as co-carbon source. Lactose was reported as the best carbon source for protease production by *B. halodurans* with a specific activity of 135.5 U/mg protein, which indicated that, this bacterium was able to produce β-galactosidase for consuming lactose (101). Among other carbon sources, *B. alcalophilus* TCCC11004 exhibited highest productivity of alkaline protease in medium containing maltodextrin (102), *B. cereus* MTCC 6840 utilized fructose as preferred carbon source (157) while whey and sucrose were used preferably by *Serratia marcescens* ATCC 25419 (104).

Glucose, the most commonly used carbon source has been shown to be inhibitory for alkaline protease production in some bacteria such as *Geobacillus caldoproteolyticus* strain SF03 (105), *B. horikoshii* (106) and *B. nesternkonia* sp. AL-20 (40). This negative effect of glucose on protease production is attributed to catabolite repression as it has been established that the catabolite control protein (Ccp A) is responsible for the regulatory mechanism of glucose catabolism, and acts as a signal for the repression in protease synthesis (107). On the other hand, in some *Bacillus* strains such as *Bacillus* sp. AR 009 (108), *B. licheniformis* ATCC 21415 (109), *B. thuringiensis* cc7 (110), *B. licheniformis* N-2 (111), *Bacillus* sp. EL31410 (112) and *B. cereus* strain 146 (113), enhanced protease yields have been reported upon supplementation of glucose in the production medium.

Besides glucose, starch is another widely used carbon source and has been shown to induce protease synthesis in alkalophilic *B. pumilus*, *B. cereus* MCM B-326 and *Bacillus* sp. 2-5 (114-116), but was found to be dependent on starch type. However, supplementation of potato starch, corn starch, and pearl millet flour exerted an inhibitory effect on alkaline protease production in *Bacillus* sp. Y (117). This negative influence on enzyme synthesis was probably due to the presence of protease inhibitors in these carbon sources (118).

Natural agricultural materials such as Rice bran, soybean, wheat flour, wheat bran, corn bran, corn starch, orange peels have been reported to produce alkaline proteases (119-120). Ahmed *et al* (121) also observed the effect of different carbon substrates i.e. sunflower meal, soybean meal, cotton seed meal, rice husk, rice polish, rice bran and wheat bran on alkaline protease production where by maximum activity was found on rice husk (110.42 U/ml). Furthermore, Kumar *et al* (122) observed wheat bran as the best carbon source with the maximum protease activity of 1160 U/ml. Molasses as a supplement was also found to be effective for protease production in *Bacillus* species (123, 124). In another report, wheat bran, rice bran and Cotton Deoiled Meal (CDM) was used in the production medium (125). The use of CDM revealed a maximum alkaline protease production (589.20 U/ml) with glucose as carbon source.

Non-carbohydrate sources such as organic acids as sole carbon sources have also been found to increase the yield of alkaline protease by *Bacillus* sp. B1 (126). Researchers studied the effect of citrate, fumarate, succinate, glucose and fructose on protease production by *B. alcalophilus* and found that citrate, fumarate and succinate can be effectively utilized for protease production with citrate at 1.0% level being the most potent carbon source (127). Calik *et al* (128) used citric acid as a sole carbon source for alkaline protease production by *B. licheniformis*. On the other hand, the presence of citric acid and trisodium citrate in the medium repressed the growth and yield of alkaline protease by *B. licheniformis* N-2 (111). The citric acid inhibition is attributed to chelation of divalent ions from the medium, resulting in ion depletion in the growth medium (129).

Effect of nitrogen sources on alkaline protease production

Synthesis of the alkaline protease by microorganisms is strongly stimulated by the presence of the nitrogen sources (proteins and peptides) in the culture medium, which have regulatory effects on enzyme synthesis (24). Different bacteria have diverse preferences for either organic or inorganic nitrogen for growth and protease production, although complex nitrogen sources are usually employed for alkaline protease production (100, 130).

Mukherjee *et al* (100) has shown a preference for organic nitrogen sources (beef extract followed by yeast extract) compared to inorganic nitrogen for protease production by *B. subtilis* DM-04. The inducing effect of nitrogen sources (peptone, soybean meal and beaf extract) on bacterial alkaline protease production has also been reported by (131-132). A mixture of nitrogen sources i.e. yeast extract in addition to casamino acid, peptone or L-glutamate were used and achieved highest protease activity was observed with *Bacillus* sp. 2-5 (116). In addition to this, a combination of yeast extract and cotton seed meal induced maximum alkaline protease production by *B. alcalophilus* TCCCC11004 (102). In a sharp contrast to these observations, organic nitrogen sources like peptone and yeast extract were found to suppress the protease production by an alkalophilic strain of *Arthrobacter ramosus* MCM B351 (133). However, Patel *et al* (24) observed higher protease production with organic nitrogen sources. Similarly, free amino acids and inorganic nitrogen sources, such as NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$ have been reported to repress enzyme synthesis in *Bacillus* sp. (134, 116, 136). The repression of growth and protease biosynthesis is thought to be due to the fast release of ammonia from these inorganic nitrogen sources (111). Inorganic nitrogen sources such as ammonium nitrate (48), $(NH_4)_2SO_4$ (110) have been successfully used as optimal nitrogen sources.

Skim milk exhibited a prominent effect on protease production by *B. subtilis* (121). Furthermore, *B. mojavensis* produced maximum alkaline protease when casein and casamino acids are used as nitrogen sources (94). The maximum protease production was exhibited with gelatin concentration at 1% (w/v) for *B. anthracis* S-44 and *Bacillus sp*. K 30 (137), 0.5 % (w/v) for *B. firmus* 7728 (138). Saurabh *et al* (139) observed maximum alkaline protease production in the presence of casein from *Bacillus* sp. and *B. pseudofirmus* SVB1, respectively. While, Kanekar *et al* (140) used soya-cake as the exclusive carbon and nitrogen source for protease production. Groundnut cake as the main nitrogen source gave maximum protease activity of 940

U/ml by *Bacillus* sp. (122). Kanchana and Padmavathy (141) observed Bengal gram powder as the best substrate for highest enzyme activity.

Among the natural complex substances employed as nitrogen sources, Corn Steep Liquor (CSL) was found to be a cheap and suitable source of nitrogen by some workers (142). Apart from serving as a nitrogen source, CSL also provides several micronutrients, vitamins, and growth-promoting factors. However, its use is limited by its seasonal and interbatch variability. While, Moreira *et al* (65) found that *B. pumilus* UN-31-C-42 showed maximum protease production on 2% bean powder preferably, when supplemented to the medium, *Bacillus* sp. JB-99 used KNO_3 as the preferred nitrogen source (47).

Now days, there has been increasing trend to utilize raw/unprocessed carbon and nitrogen sources so as to cut down the cost of production. Hence, there are many reports where agricultural or industrial by/waste products are being used for cultivation of microorganisms. Soybean contains 40% of protein, 17% carbohydrate, 18% oil, traces of metals, moderate amount of vitamins and amino acids, thus supplying almost all the nutrients required for the growth of bacilli. Besides that, soybean also contains small amount of enzymes such as protease, urease and lipoxidase (139). Soybean meal has been used as inducer for protease production from *Conidiobolus coronatus* (143), *B. cereus* MCM B-326 (115), *B. licheniformis* N-2 (111), recombinant *B. subtilis* (144), *B. subtilis* BS1 (145) and *Bacillus* sp. I-312 (146). The protease production was considerably enhanced (4771 U/ml) when *B. licheniformis* NH1 was grown in medium containing hulled grain of wheat as nitrogen source (147). Chu (148) used a combination of wheat flour and soybean meal for optimized alkaline protease production (2560 Uml^{-1}) by *Bacillus* sp., rice bran @ 1 per cent (120), wheat bran and lentil husk (149), wheat bran @ 2.5 per cent (150) and combination of glucose and soybean meal (125) were also found suitable for production of alkaline protease in different studies.

A comprehensive account of cultural conditions for maximum alkaline protease production from various *Bacillus* sp. has been listed in **Table 4**.

Table 4: Fermentation conditions for production of alkaline proteases from bacteria

Microorganism	pH	Temperature (°C)	Agitation (rpm)	Incubation period (h)	Nitrogen sources	Carbon sources	Reference
Bacillusmycoides	7	30	180	48	$(NH_4)_2SO_4$, peptone	Glycerol	(151)
Bacillus polymyxa	9	50	n.s.	72	Casein	Glucose	(132)
Bacillus licheniformis ATCC 21415	7	30	250-400	48	Soybean, $(NH_4)_2PO_3$	Lactose, Glucose	(109)
Bacillus sp.	8	37	120	18	Peptone, yeast extract	Glucose	(152)
B. horikoshii	9	34	250	16-18	Soybean, casein	*	(106)
Bacillus pumilus MK6-5	9.6	35	250	60	Corn-steep liquor	Glucose	(153)
Bacillus subtilis PE-11	9	37	140	48	Peptone	Glucose	(154)
B. mojavensis	10.5	50	250	10-12	Casamino acid	Glucose	(155)
Bacillus sp. RGR-14	7	37	250	96	Soybean meal, casamino acid	Starch	(156)
Bacillus sp. MIG	7.5	30	120	48	Yeast extract	Wheat bran	(26)
B. cereus MTCC 6840	9	25	150	24	Yeast extract + Peptone	Fructose	(157)
Bacillus sp.	9	50	n.s.	48	Soybean meal	Wheat flour	(148)
B. cereus MCM B-326	9	30	100	36	Soybean meal	Starch	(115)
B. circulans	10.5	25-30	200	96	Soybean meal	Glucose	(125)
B. licheniformis RPk	9	37	200	48	Yeast extract	Chicken fearhers	(136)
B. alcalophilus TCCC11004	9	34	180	50	Yeast extract, cotton seed meal	Glucose, maltodextrin	(102)
B. thuringiensis cc7	8.5	29	150	48	Casein, urea,	Glucose	(110)
B. licheniformis NCIM-2042	7.1	37	180	86	$(NH_4)_2SO_4$	Starch	(39)
Streptomyces sp. DP2	5	50-100	250	72-96	Soybean meal	fructose	(158)
Bacillus licheniformis ATCC 12759.	7	37	-	-	Mustard cake	Wheat flour	(159)
Bacillus licheniformis P003	9.0	25	150	72	Urea, Sodium nitrate Beef extract	Rice flour Glucose	(160)
Bacillus circulans MTCC 7906	9.5	28	200	96	Potato peel	glucose	(161)

Biochemistry of Alkaline Protease

Preparation of extracellular alkaline protease by enzyme concentration and purification

Purification of microbial proteases has received a great attention. A crude protein extract derived from some types of physical or chemical manipulation of the source will typically contain several types of contaminating molecules such as carbohydrates, lipids, nucleic acids, proteins, salts and other cellular debris. Separation of the protein fraction of the crude protein extract containing these contaminants is usually referred to as the capture step. One of the more popular methods of protein capture is the bulk precipitation from the crude extract (162). Precipitation also performs both purification and concentration steps. It is generally affected by the addition of reagents such as salt or an organic solvent that lower the solubility of the desired proteins in an aqueous solution.

Generally, purification procedures include 3-4 main steps e.g. Ammonium sulfate fractionation followed by dialysis (to remove excess salts and impurities) and then applying different sephadex chromatographic columns, using ion-exchange or adsorption chromatography and many cases performing the so called polyacrylamide gel electrophoresis technique (163). This protocol has been successfully employed in *Bacillus* sp. and *Pseudomonas* sp. (95). Joshi (35) used phenyl sepharose instead of DEAE-sephacryl where by the protease was completely retained by the matrix and all other impurities went into washing. The resultant bound protease was further eluted with 50% ethylene glycol.

Infact, Chromatography has been protease specific as Durham *et al* (164) purified proteases (AS and HS) from an alkalophilic *Bacillus* sp. strain GX6638 (ATCC 53278) by ion exchange chromatography that were distinguishable by their isoelectric point, molecular weight and electrophoretic mobility. A thermostable alkaline protease was purified from an alkaliphilic thermophile *Bacillus* sp. B18 by using DEAE and CM-Toyopearl 650M column chromatographies (165). Mane and Bapat (166) isolated an alkaline protease from culture filtrate of *B. subtilis* NCIM 2713 by ammonium sulphate precipitation and was purified by gel filteration. Two novel extracellular serine proteases was reported in *B. sphaericus* (25). The crude enzyme was purified to homogeneity from the cell free culture filtrate by a combination of ultrafiltration, ammonium sulphate precipitation and chromatographic methods. Partial purification of protease enzyme which was performed using chilled acetone for enzyme precipitation (47) is one of the different methods that were applied for the protease purification.

Different workers have reported variable yield of purified (by enzyme precipitation followed by chromatographic separation) proteases. Kumar (153) purified an alkaline protease from *B. pumilis* MK6-5 by using ammonium sulphate precipitation, ion exchange and gel-filtration chromatographies, where in a 26.2% recovery of enzyme with 36.6 fold purification was recorded. Tang *et al* (167) treated crude enzyme of engineered strain of *Bacillus* sp. BA071 with ammonium sulphate fractionation and further purified it with CM-Sephadex-C-50 and Sephadex-G-75. The purity of enzyme was increased by 76.2 times. Thermostable serine alkaline protease from a newly isolated *B. subtilis* PE-11 was purified in a two step procedure involving ammonium sulphate precipitation (between 50%-70%) and sephadex G-200 gel permeation chromatography. The enzyme was purified 21-fold with a yield of 7.5% (154). The enzyme from *B. pseudofirmus* AL-89 grown on chicken feather was purified to electrophoretic homogeneity following ammonium sulphate precipitation (60% w/v), ion exchange, hydrophobic interaction and gel filtration chromatography. The yield and fold enzyme

purification was 44.1% and 43.7 times, respectively (40).

An alkaline protease producing haloalkaliphilic bacteria was isolated from west coast of India by Gupta et al (95). The protease secreated by this bacterium was purified 10 fold with 82% yield by a single step method on phenyl sepharose 6 fast flow column. The alkaline protease from *B. cereus* was purified to homogeneity by ammonium sulphate precipitation, concentrated by ultrafiltration followed by its anion exchange chromatography (168). Joo and Chang (119) carried out simple purification of an oxidant and SDS-stable alkaline protease produced by *B. clausii* I-52. The enzyme was purified to homogeneity with overall recovery of 79% and 10-fold purification from culture supernatant using Diaion HPA75, phenyl-Sepharose and DEAE-Sepharose column chromatographies. Kazan *et al* (169) observed 16-fold purification from culture filtrate of *B. clausii* GMBAE 42 by DEAE-cellulose chromatography, with a yield of 58%. In another report, a thermophilic neutral protease was purified and characterized from a thermophilic *Bacillus* strain HS08. The purification steps included ammonium sulphate precipitation (80% saturation), with columns of DEAE-Sepharose anion exchange chromatography and sephacryl S-100HR on AKTA purifier 100 protein liquid chromatography. This method gave a 4.25 fold increase of the specific activity and had a yield of 5.1% (170). Banik and Prakash (171) carried out ammonium sulphate fractionation at 80% saturation, concentration by ultrafiltration, anion exchange chromatography and gel filtration to have a 16-fold purification of alkaline protease. In another report, two alkaline proteases produced by marine *Bacillus* sp. MIG were observed. Two proteases were purified to homogeneity using acetone precipitation (two volumes of cold acetone), cation exchange chromatography on CM-Sepharose CL-6B, followed by gel filtration on Sephadex G-75 superfine. These steps were very effective and combined to give overall purification of 19.3 and 16.1 fold for the protease 1 (Pro 1) and protease 2 (Pro 2), respectively (26). Darani *et al* (116) reported a new strain of *Bacillus* sp. from alkaline soil, whose purification was conducted by fractionation (55%), concentration and certain exchange chromatography. The yield and fold of enzyme purification was 24% and 50 times respectively. Alkaline protease enzyme from *B. pumilis* CBS was purified by using salt precipitation (between 40% and 60%) and gel filtration HPLC. The yield and fold enzyme purification were 12% and 38 times, (53) respectively.

Protease from *B. subtilis* KO strain (172) was precipitated by using ammonium sulphate fractionation (20%) and further purified by column chromatography (Sephadex G-200, mesh 200 μ) techniques. Fakhfakh *et al* (136) purified protease produced by *B. licheniformis* RPk to homogeneity from the culture supernatant by a 3-step procedure. The keratinolytic protease was precipitated by $(NH_4)_2SO_4$ between 40 and 60% (32,142 U/mg of protein) which was then subjected to gel filtration on a Sephadex G-100 column and DEAE-cellulose. The elution profiles of the protease and proteins from Sephadex G-100 yielded a single peak of protease activity with an increase of 4.4 fold in specific activity and 52.8% recovery. A 6.8 fold purification of TC4 protease from *B. alcalophilus* TCCC11004, 5.34 fold alkaline protease purification of *B. firmus* Tap5 and 13 fold purification of a thermophilic alkaline protease from *Bacillus* sp. were reported by Cheng *et al* (102), Joshi (35), (141), respectively by employing gel filtration, ammonium sulphate or acetone precipitation protocols.

The molecular weights of alkaline proteases generally range from 15 to 35 kDa (132, 154, 173) with few reports of higher molecular weights of 36.0 kDa (164), 42 kDa from *Bacillus* sp. PS719 (174) and a very high (90 kDa) from *B. subtilis* (175). Recent reports on different isolated species for alkaline

proteases production and its purification strategies along with results of respective studies are summarized in table 5.

Table 5 Recent purification strategies adopted for alkaline proteases from different microorganisms

Microorganism	Isolation /Procurement	Purification Technique	Fold purific-taion	Molecular weight	Reference
B. licheniformis MP1	shrimp waste	Ultrafilteration + sephadex G-100 gel filteration + Mono Q-sepharose ion exchenge chromatography	3.9	30	(176)
B. subtilis	Enzyme Biotechnology laboratory, University of Agriculture Faisalabad, Pakistan	Ammonium sulphate precipitation (40-70% saturation) + dialysis + sephadex G-100 column chromatography	1.9	27	(177)
B. cereus 1173900	Waste water from tanning industry	Ammonium sulphate precipitatipn (70%) + dialysis + sephadex G-100	53.64	66	(178)
Serratia marcescens S3-R1	Korean ginseng rhizosphere.	Ammonium sulfate precipitation + DEAE-Sepharose anion-exchange chromatograph + Mono Q chromatography	-	50	(179)
Bacillus megaterium	soil	Ammonium sulfate precipitation + DEAE-cellulose ion-exchange chromatography + gel filteration sephadex G-200	P1=13.63 P2= 7.72	P1=28 P2=25	(180)
Saccharomycopsis fibuligera strain R64	Tape Indonesian fermented food	50% ammonium sulfate precipitation + dialysis + gel filteration G 100	20	97	(181)
Planomicrobium sp. L-2	digestive tract of *Octopus variabilis*	ammonium sulfate precipitation, dialysis and enrichment, DEAE-Sephadex A50 anion-exchange chromatography, and Sephadex G-100 gel chromatography	1.7	61.4	(182)
Geobacillus stearothermophilus B-1172	---	Ammonium sulfate precipitation + dialysis + ion exchange chromatography	13.7	39	(183)
Bacillus circulans MTCC 7906	Vegetable waste	Ammonium sulphate precipitation + dialysis + DEAE cellulose ion exchange chromatography + PEG concentrated	22	40	(20)

Characterization of purified alkaline protease for kinetic parameters

Effect of pH

Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly located on their surface. The charges on these groups vary according to their acid dissociation constants and pH of their environment. This affects the net charge on the enzymes and the distribution of charges on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighborhood of the active sites, which will overall affect the activity, structural stability and solubility of the enzyme (184). In general, all currently used detergent-compatible proteases are alkaline in nature with a high pH optimum, therefore they fit into the pH of laundry detergents, which is generally in the range of 8 to 12. Therefore, most of the commercially available subtilisin-type proteases are also active in the pH range of 8-12 (92). A good example for this is the well-known detergent enzymes, subtilisin Carlsberg and subtilsin Novo or BPN which show maximum activity at pH 10.5 (185).

Alkaline proteases of the genus *Bacillus* show an optimal activity and a good stability at high alkaline pH values (186). The optimum pH range of *Bacillus* alkaline proteases is generally between pH 9 and 11, with a few exceptions of higher pH optima of 11.5 (153), 11-12 (25, 107) and 12-13 (187).

Effect of temperature

The heat stability of enzymes is affected by at least two factors, either alone or in combination. The first one is the primary structure of the enzyme. A high content of hydrophobic amino acids in the enzyme molecule provides a compact structure, which is not denatured easily by a change in the external environment. In addition, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation. Secondly, specific components such as polysaccharides and divalent cations, if any, can stabilize the molecule (188).

Even though there is no firm evidence to suggest that thermostable enzymes are necessarily derived from thermophilic organisms, there is a greater chance of finding thermostable proteins from non-thermophilic bacteria (56). Therefore, a wide range of microbial proteases from thermophilic species has been extensively purified and characterized. These include *Thermus* sp., *Desulfurococcus* strain Tok12S1 and *Bacillus* sp. Among these, alkaline proteases derived from alkalophilic bacilli, are known to be active and stable in highly alkaline conditions. The earliest thermophilic and alkalophilic *Bacillus* sp. was *B. stearothermophilus* strain F1 isolated (189), which was stable at 60^{o}C (190). Further studies on microbial alkaline proteases have been done in view of their structural-function relationship and industrial applications, as they need stable biocatalysts capable of withstanding harsh conditions of operation (56).

Generally alkaline proteases produced from alkaliphilic *Bacillus* are known to be active over a wide range of temperature. The optimum temperatures of alkaline proteases range from 40 to 80^{o}C. In addition, the enzyme from an obligatory alkalophilic *Bacillus* P-2 showed an exceptionally high optimum temperature of 90^{o}C. The protease also had good thermostability being stable at 90^{o}C for more than 1 h and retained 95 and 37% of its activity at 99^{o}C (boiling) and 121^{o}C (autoclaving temperature), respectively. *Bacillus* P-2 was the only mesophile reported until 2001, which produced a proteolytic enzyme that was stable even at autoclaving (121^{o}C) and boiling temperatures (135).

Effect of enzyme and substrate concentration

Enzyme concentration is an important factor in determining the rate of reaction. The enzyme activity is directly proportional to

enzyme concentration at a particular substrate concentration. Hence, when all enzyme sites are saturated for the substrate, there is no further increase in enzyme activity even with increase in enzyme concentration.

A number of studies revealed that concentration of enzyme and substrate dependent on the type and speed of alkaline protease producing microorganisms. Researchers found 50% protease (of the reaction mixture) as the optimum for alkaline protease activity in the reaction mixture (2.0 ml) containing 0.05 M Tris-HCl buffer (pH 7.5), casein and enzyme (1.0%, 1.0 ml) in case of *B. polymyxa* B-17 (191). Huang *et al* (192) also revealed that 1.0 ml (50%) is optimum for protease activity at 2.0% casein in Bongsuan-NaoH buffer (pH 9.8) and temperature of 50°C in *B. pumilis*. Caseinolytic activity was estimated by using different enzyme concentrations (0.05-0.5 ml) in a reaction mixture of 3 ml, 0.1 ml of enzyme that constitutes 3.33% of reaction mixture produced maximum protease activity (173). Similarly, in another report, Ahmed *et al* (177) reported 33.3% protease as optimal for alkaline protease acitivity in *Bacillus* sp. GUS1 and *B. subtilis*, respectively.

Enzymes are natural catalysts that speed up the chemical reactions. However, the speed of any fastidious reaction being catalysed by a particular enzyme can only reach a certain maximum value. This rate is known as Vmax while, Kmcan define the concentration of substrate at which half of the maximal velocity (Vmax) is obtained. The relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate and is usually expressed as the Km (Michaelis constant) of the enzyme. An enzyme with low Km has a greater affinity for its substrate. An alkaline protease is highly substrate specific and exhibits maximum activity towards casein as substrate (193). Adinarayana *et al* (154) reported that proteases have a high level of hydrolytic activity for casein as substrate and poor to moderate hydrolysis of BSA and egg albumin, respectively.

Among the different studies on alkaline protease kinetics, Mane and Bapat (166) reported a Km value of 2.5 mg/ml for alkaline protease from *B. subtilis* NCIM 2713. Kumar (153) observed Km and Kcat values for alkaline protease of *B. pumilis* MK6-5 with synthetic substrates at 37°C and pH 8.0 as 1.1 mmol l^{-1} and 624 s^{-1} for Glu-Gly-Ala-Phe-pNA and 3.7 mmol l^{-1} and 826 s^{-1} for Glu-Ala-Ala-Ala-pNA, respectively. The kinetic data revealed that small aliphatic and aromatic residues were the preferred residues at the P1 position. Gupta *et al* (95) reported an alkaline protease producing haloalkaliphilic *Bacillus* sp. that had Km and Vmax of 2 mg/ml and 289.8 µg/min, respectively. Joo and Chang (119) determined the Km (83.9 micromol l^{-1}) and Kcat (238.6 s^{-1}) values for alkaline protease from a halo-tolerant *B. clausii* I-52 and Kazan *et al* (169) observed Km (0.655 µM) and Kcat (4.21×10^3 min^{-1}) values for *B. clausii* GMBAE 42. Patel *et al* (24) reported the Km and Vmax of an extracellular alkaline protease from a novel haloalkaliphilic *Bacillus* sp. (Ve1) as 0.153 g/100 ml and 454 U/ml, respectively. The kinetic parameters of serine alkaline protease from *B. pumilus* CBS (SAPB) was determined (53). The Km, Vmax and Kcat values of SAPB for casein (natural substrate) were 0.4 mM, 27,160 U/mg and 18,106 min^{-1}, respectively. Its deduced catalytic efficiency (kcat/Km) was found to be 45,265 min^{-1} mM^{-1}, which was 4.77, 2.73 and 2.11 times higher than those of SC, SB 309 and BPN', respectively. In addition, the Km and kcat values of SAPB for N-succinyl-L-Ala-Ala-Pro-Phe-p-nitroanilide (AAPF), as synthetic substrate, were 0.3 mM and 44,100 min^{-1}, respectively with a deduced Kcat/Km of 147,000 min^{-1} mM^{-1}, which was 2.21, 1.88 and 1.68 times higher than those of SB 309, SC and BPN', respectively. These results strongly suggest that SAPB is the most promising candidate

for cleansing power of laundry detergent and various other biotechnological applications.

Recently, Deng *et al* (18) observed the kinetic parameters of purified recombinant AprB towards different substrates. Alkaline protease enzyme of *Bacillus* sp. B001 indicated its maximum catalytic velocities (Vmax) towards casein (natural substrate), azocasein (modified substrate) and AAPF (synthesic peptide substrate) of 12.54, 102.54 and 242.09×10^3 U/mg respectively with a substrate preference of azocasin > casein > AAPF. The deduced Kcat/Km (catalytic efficiency) values of AprB for casein, azocasein and AAPF were found to be 57.42, 906.31 and 151.01×10^3 min^{-1} mM^{-1} respectively. These high catalytic efficiencies of AprB strongly indicate that it is a potential candidate for use as a laundry additive and other commercial applications (194).

The protease from *B. circulans* showed Km of 0.597 mg ml^{-1} and Vmax of 13825 µM min^{-1} towards casein as a substrate at 70°C (195). The Km, Kcat values of *B.licheniformis* MP1 purified alkaline protease were 0.53 mM and 12.7×10^3 min^{-1} respectively using casein as substrate (176). Using casein as substrate, the alkaline protease enzyme from *B. subtilis* showed maximum activity (Vmax) of 148 U/ml with its corresponding Km value of 58 µM (177). Furthermore, kinetic constants (Km and Vmax) for extracellular purified alkaline protease of *B. licheniformis* NCIM-2042 was determined (39) using Lineweaver-Burk plot as 0.01078 g/100 ml and 182.9 U, respectively.

Hence, there is a large variation in Km and Vmax values, revealed that optimum concentration of substrate is species specific.

Effect of metal ions and inhibitors on the alkaline protease activity

Various kind of metal ions, reagents and osmolytes have been reported to influence the enzyme activity of proteases, their pH and temperature stability. Alkaline proteases isolated from different fungal and bacterial sources behave differently in the presence of mono or divalent ions. Cu^{2+}, Co^{2+} have been found to enhance the enzymatic activity of alkaline protease isolated from *B. licheniformis* (196). It was found that Hg^{2+}, Zn^{2+} and Fe^{3+} ions inhibit the proteolytic activity of alkaline protease of *Bacillus* sp. JB99 (47) whereas Ca^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} and Mg^{2+} had stimulating effect on the enzyme activity. Ca^{2+} ion dependent activity is thought to be attributed to its involvement in stabilization of the protease molecular structure derived from *Bacillus* sp. (60, 174).

In case of alkaline protease of *Bacillus* sp. RGR 14, a two-fold enhancement in protease activity was observed in the presence of Mn^{2+} (1mM), however, the enzyme was strongly inhibited up to 90% in the presence of 10 mM Hg^{2+} and Cu^{2+} (173). Many alkaline proteases are reported to be inhibited by Hg^{2+} and Ag^{+} (26). Fe^{2+} was found to be the most effective stimulator while Cu^{2+} was least effective for the alkaline protease of *Bacillus* sp. (197). The activity of protease DHAP of *B. pumilus* was enhanced by Ca^{2+}, Mg^{2+} and Na^{2+} and inhibited by Cu^{2+} and Zn^{2+} (192). Ca^{2+}, Mg^{2+} and Mn^{2+} ions positively regulated the enzyme activity in *B. circulans* (195) and *B. alcalophilus* TCCC11004 (102). It is believed that these cations protect the enzyme against thermal denaturation and play a role in maintaining the active conformation of the enzyme at higher temperatures.

Most of the fungal and bacterial alkaline proteases have been found to be serine proteases as these are inhibited by PMSF and DFP, which sulfonated the essential serine residue in the active site and resulted in the complete loss of its activity (102,154,172). Some of these serine proteases have been found to be metal ions dependent as these are inhibited by certain metal chelating agents such as EDTA etc. (173,193). Contrary to these reports, a few alkaline proteases have not been found to be

inhibited by both or either PMSF or EDTA (25, 31,177).

Scale Up Studies of Alkaline Protease Production

In order to scale up protease production from microorganisms, biochemical and process engineers use several strategies to obtain high yields of proteases. Controlled batch and fed batch fermentation using simultaneous control of glucose, ammonium ion concentration, oxygen tension, pH and salt availability (93) and chemostat cultures have been successfully employed for improving protease production by a number of microorganisms.

Among the different case studies, *B. subtilis* from tannery waste was isolated (93), produced alkaline protease (pH 8.5) in a stirred tank fermenter at 37°C with the dissolved oxygen tension at 40% air saturation. Alkaline protease yield from *B. mojavensis* was improved upto 4-fold under semi batch and fed batch operations by separating biomass and protease production phases using intermittent de-repression and induction during the growth of organism (94). Moreover, an enhanced production of alkaline protease by an engineered strain BA071 in a 5L fermentor was reported. It was found that the maximum activity of alkaline protease reached 24,480 U/ml in 40 hours of fermentation by combination of enhancing aeration and regulating the agitation rate (167). Kumar *et al* (198) found a 5-fold increase in alkaline protease yields (31899 U/ml) at a lower production time of 45h, aeration of 1vvm and agitation of 400 rev/min at 37°C using arrow root starch casein medium (pH 10.2). Besides the improved yield, time of production was also reduced by 27 hours. Earlier, protease production in bioreactor at high agitation rate of 360-600 rpm have also been reported by Moon *et al* (86) in *B. firmus*. Alkaline protease production by a marine haloalkalophilic *B. clausii* was optimized in a bioreactor. The isolate produced maximum protease yields (15000 U/ml) under submerged fermentation conditions at 42°C for 40 hours with aeration of 1.5 vvm and agitation of 400 rev/min.

Similarly, Haq and Mukhtar (199) used a 7.5-L bioreactor for alkaline protease production (pH 9.0, 35°C, 175 rpm) by *B. subtilis* and Saurabh *et al* (139) employed a 10 L fermentor (Bio-flow IV, NBS, USA) for alkaline protease production (pH 7.0, 37°C, 350 rpm, 0.5 0.5 vvm) by *Bacillus* sp. and reported enzyme production of 3208 U/ml in 18 h. Thereafter, a decline in the protease production was observed in the bioreactor. Similar cessation in protease production has been reported (86, 92), once a maximum amount of the enzyme is produced during the run. Although, there are several theories such as autoproteolysis (200) and protease degradation by some proteases present on the cell surface on nitrogen starved cells (134, 155), the exact mechanism is yet not known. Laxman *et al* (81) reported the production of alkaline protease from *Condibolous coronatus* in 100 L fermenter with soyabean as optimum in concentrations of 2-3% as best inducer with diammonium hydrogen phosphate, casamino acids and Hi-media peptone gave activities comparable to yeast extract. The ammonium sulphate saturated enzyme was found to be stable upto two years. Researchers (139) revealed maximum of 3208 U/mL of protease from *Bacillus* was produced in 18 h in a 10L bioreactor. The enzyme has temperature and pH optima of 60°C and 9.5, respectively. However, the temperature stability range is from 20-90 °C and pH stability range is from 6.0–12.0. The protease was completely inhibited by PhenylMethylSulfonyl Fluoride (PMSF) and Diodopropyl FluoroPhosphate (DFP), with little increase (10-15%) in the production of upon addition of Ca^{2+} and Mg^{2+}. Irfan *et al* (201) showed that *Bacillus subtilis* M-21 was used for the production of alkaline protease in 2 L jar fermenter with working volume 1.5 L. Soybean meal was used as choice of substrate due to its high nitrogen contents. The effect of different process parameters

(pH, temperature, agitation and aeration) and their relationship with each other was studied during fermentation process. Highest protease production (896.5 ± 1.5 PU/ml) was obtained at optimum pH 10, temperature 37°C, agitation rate 300 rpm and at aeration 2 ml/min. The maximum protease activity was obtained from *P. aeruginosa* MCM B-327 with soybean meal 1%, tryptone 1%, initial medium pH 7, agitation rate 250 rpm, aeration rate 0.75 vvm and fermentation temperature 30 °C, under submerged fermentation conditions (SmF). The protease productivity at 10 and 120L fermenters was found to be 16,021 and 9,975 $UL^{-1}h^{-1}$ respectively (202).

Conclusion

In a moderately small time, recent biotechnological approaches have developed significantly from laboratory inquisitiveness towards commercialization. Recent trends in microbiological ideas and biotechnology have fashioned an encouraging niche for the advancement of proteases and would sustain their applications to give a sustainable environment for the improvement of mankind. Alkaline protease carries vast potential in diverse industries such as detergent, leather, food and pharmaceutical industry. In the present scenario, it's need of an hour to explore novel microorganisms for enzyme production which should have adaptable capacity to accomplish demand of industry. For industrial uses, enzyme production requires: isolation and characterization of novel microbes (strains) by utilizing cheaper carbon and nitrogen sources. Furthermore, the enzyme (alkaline protease) activity is influenced by various environmental factors, for instance pH, Temperature and ionic strength of the production medium. Besides, keeping in mind the stringent conditions of industrial processes, the genetic and protein engineering could participate an essential role for modifying the enzyme's properties in order to produce enzymes at large scale.

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Conflict of Interest

Authors declare no conflict of interest.

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