Review

A Review on Microbial Alkaline Protease: An Essential Tool for Various Industrial Approaches

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Abstract

Proteolytic enzymes are present in all living organisms and help in cell growth and differentiation. Proteases are the hydrolytic enzymes that act as biocatalysts for the cleavage of proteins into smaller peptides and amino acids. Microorganisms have turned out to be a competent and inexpensive source of alkaline protease enzymes that can produce a continuous and consistent supply of desired product. Alkaline proteases have extensive application in various industrial sectors especially in detergent and leather industries. However, their application in food has not been much exploited. This review summarizes all the reports of applications of alkaline protease in different sectors with a main view on food applications. The effect of various physiochemical parameters on alkaline protease is discussed. Different sources of isolation and optimum pH and temperature of alkaline protease producing bacterial and fungal species are also reported.

Keywords: proteolytic enzymes, alkaline protease, enzymes in food, isolation and characterization

Introduction

Enzymes are biocatalysts essential for life that catalyze almost every biological process.[1] Since ancient times, enzymes have been used in the manufacturing of different food products, including beer, wine, vinegar, cheese, and sourdough, and in the production of commodities like leather, linen and indigo.[2] Biocatalysis has evolved as a necessary tool in industrial production of active pharmaceuticals, agrochemical and pharmaceutical intermediates, bulk chemicals, and food ingredients.[3] Although enzymes were initially not used in pure form, fermentation processes producing pure enzymes from a specific strain were eventually developed at large scale.[2] Microorganisms have made an essential contribution in industrial biology.[4] Microbial enzymes play an important role as metabolic catalysts and hence are used in various industrial applications. Currently, most enzymes used in industrial processes are hydrolytic and are used for the degradation of various natural substances.[2]

Protease remains the dominant type of enzyme because of its extensive use in dairy and detergent industries. Proteases are very important enzymes, accounting for more than 60% of total global enzyme sales.[5] These enzymes can be broadly divided into two major groups: endopeptidases, which cleave internal peptide bonds, and exopeptidases, which cleave C- or N- terminal peptide bonds (*Fig. 1*). Applications of alkaline protease are shown in *Fig. 2*. Protease enzyme catalyzes the hydrolytic reaction, which brings about the breakdown of protein molecules to amino acids and peptides.[6] Microbes serve as a better source of proteases than plants and animals because they can be cultured in large amounts in a short duration, are relatively inexpensive, and can produce a continuous supply of the desired product.

Alkaline proteases are proteases that are active from neutral to alkaline pH range. These can be isolated from various microorganisms, as shown in *Tables 1–2*.[7–37] Alkaline proteases are widely used in detergents, food, and various other applications. Considerable literature is available on alkaline protease production, purification, and characterization. With continuous increases in demand for alkaline proteases, there is a need to explore information on methods to increase commercial production from newly isolated strains. The present study attempts to review various methods of alkaline protease production. The present review also discusses the effect of various optimization conditions on stability and activity of alkaline protease, with special emphasis on applications in the food and other industries.

Production of Alkaline Protease

Alkaline protease can be obtained from various sources, like fungi, bacteria, and insects. Alkaline protease is produced by most alkalophilic microorganisms, however, and interest is mainly focused on those that yield high amounts of enzyme. The feasibility of protease enzyme in different industrial applications relies on various factors. Media formulation and strain affect alkaline protease production. Alkaline protease can be produced by different fermentation techniques, including solid state fermentation and submerged state fermentation. To obtain commercially viable enzyme production, fermentation media has to be properly optimized. Different factors like pH, nitrogen source, carbon source, metal ions, temperature and inhibitors affect the production of enzymes. In the case of industrial production of proteases, technical media that contains increased concentrations (100–150 g dry weight/L) of proteins, complex carbohydrates and other components of media are used.[38]

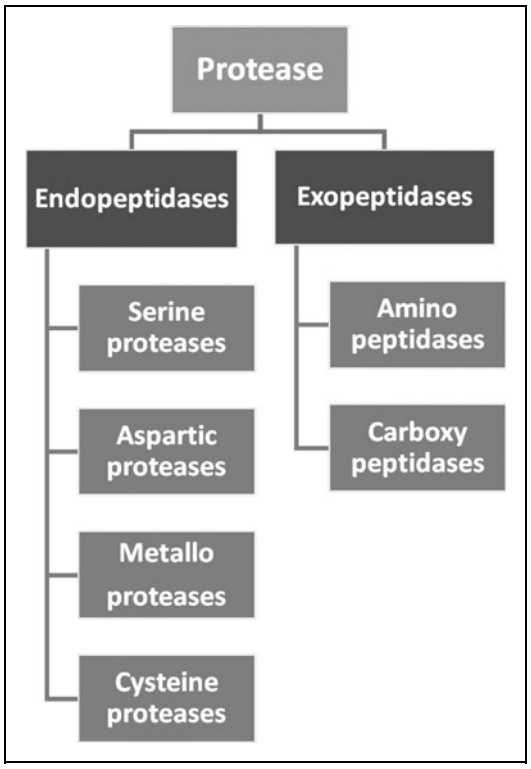

Fig. 1. Classification of protease enzyme.

Effect of Production Parameters on Activity and Stability of Alkaline Protease

EFFECT OF pH

Alkaliphilic microorganisms are strongly dependent upon extracellular pH for their cell growth and production of enzymes. These microorganisms require optimum pH of around 10 for their growth. The cytoplasmic pH of these microorganisms can either be estimated from optimum pH of intercellular enzymes or by measuring the distribution of inside and outside weak bases that are not transported by the cells. Generally, for alkaline protease production, optimum pH ranges from 9–11.[38] This includes different alkaline protease-producing bacterial and fungal species like *Bacillus firmus* 7728 (pH 9), *Bacillus sp.* JB-99 (pH 11), *Bacillus sp.*SSR1 (pH 10), *Aspergillus clavatus* (pH 9.5), and *Penicillium* sp. (pH 9). However, there are some exceptions, like *Bacillus pumilus* MK 6-5, which has an optimum pH of 11.5. The activity of the crude and purified protease can be determined by incubating it in buffer solutions of different pH.[39] The activity and stability of protease at distinct pH values varies from one organism to another. The molecular basis of pH affecting bacterial metabolism in culture broth is obscure. Because the pH value of the medium affects the proton motive force in chemiosmosis, under the optimum pH range it is possible to achieve high metabolic efficiency. Hence, pH is a very critical factor that should be optimized.[40] The optimum pH requirements of different microorganisms are shown in *Tables 1–2*.[7–37]

EFFECT OF TEMPERATURE

Temperature is another critical parameter that has to be controlled for maximum cell growth and protease enzyme production. Alkaline protease exhibits optimum activity at temperatures ranging from 50–70°C. This includes different bacterial and fungal species like *Micrococcus sp.* (50°C), *Bacillus clausii* I 52 (60°C), *Pseudomonas aeruginosa* MN1 (60°C), *Aspegillus oryzae* CH93 (50°C), and *Actinomycete* MA1-1 (50°C). However, there are many microorganisms whose optimum temperature does not fall in the given range, including *Aspergillus niger* (30°C), *Aspergillus nidulans* HA-10 (35°C), and *Bacillus amovivorus* (37°C). The optimum temperature requirements of different bacterial and fungal species are represented in *Table 1*[5–25] and *Table 2*,[26–37] respectively. To determine protease activity and stability at changing temperatures, crude enzyme is incubated at different temperature ranges. There is a relationship between enzyme synthesis and energy metabolism of bacilli that is governed by the uptake of oxygen and temperature.[41] *Bacillus* and *Streptomyces sp.* produce alkaline protease, which is quite stable at elevated temperatures. The addition of calcium ion further enhances the thermal stability of the enzyme.[38]

EFFECT OF PROTEASE INHIBITORS

Protease inhibitors are the molecules that antagonize the action of protease enzyme. These inhibitors are generally classified by the class of protease they inhibit. Types of protease inhibitors include PMSF (phenyl methyl sulfonyl fluoride), EDTA (ethylene diamine tetra acetic acid), β-mercatoethanol, DFP (Diisopropylflurophosphate), and dithreothreol inhibitor (DTT). Most alkaline proteases are completely inhibited by PMSF and DFP, which inhibit the activity of serine protease, indicating that serine protease is produced by the microorganism. The PMSF inhibitor sulfonates the serine residue present on the active site of protease enzyme, thus inhibiting its activity.[38] The protease inhibited by PMSF is produced from different species of fungi and bacteria, including *Bacillus subtilis* PE 11, *Aspergillus clavatus* ES1, *Myceliopthora species*, and *Bacillus megaterium*. The alkaline protease produced by these microorganisms is maximally inhibited by PMSF at 5 mM concentration, indicating that they are serine proteases. The concentration of protease inhibitor required for the maximum inhibition of protease activity varies from one organism to another. The alkaline protease enzyme that is dependent upon metal ions is inhibited by metal-chelating agent EDTA. This includes *Pseudomonas aeruginosa* MN1 and *Micrococcus species*. These are maximally inhibited by EDTA at a concentration of 5 mM, indicating they are metalloproteases. The effect of different concentrations of protease inhibitors on relative enzyme activity is presented in *Table 3*.[8,10,11,13–16,27,34–37,42,43] The effect of thiol inhibitors on alkaline protease produced by *Bacillus sp.* is minimal, although they show significant effect in the case of alkaline protease produced by *Streptomyces* species. The inhibition studies demonstrated the active site of the protease enzyme, cofactor requirement by protease, and the nature of protease enzyme.[38]

EFFECT OF METAL IONS

There are a number of metal ions that influence protease activity. For exhibiting maximal activity, alkaline protease requires

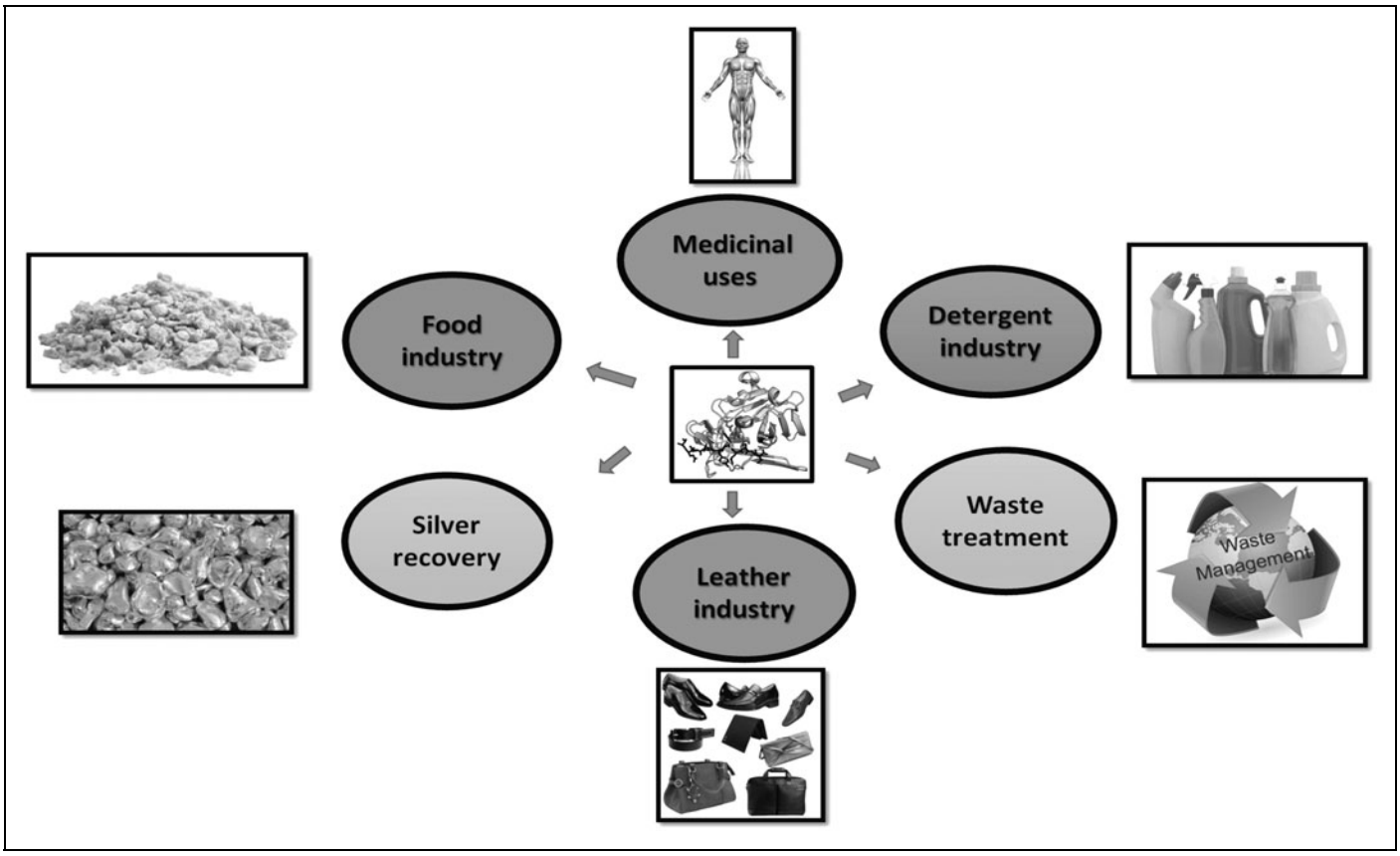

Fig. 2. Applications of alkaline protease enzyme.

different divalent ions like Ca^{2+}, Mn^{2+}, and Mg^{2+} or combinations of these divalent ions. These cations play an important role to maintain active confirmation of enzyme at very high temperatures. They also protect the enzyme from thermal denaturation.[38] Metal ions, like Mn^{2+}, Mg^{2+}, Ca^{2+} and Co^{2+}, at a concentration of 10mM, enhanced the relative activity of protease enzyme produced from *Bacillus sp*. JB 99 by 65, 29, 24, and 2%, respectively.[39] In the case of *Aspergillus clavatus* ES1, the increase in relative enzyme activity by addition of Ca^{2+} and Mg^{2+} ions was found to be 124 and 108%, respectively, at 5 Mm concentration.[44] In *Bacillus subtilis* PE-11, the protease activity was stimulated by Ca^{2+}, Mn^{2+}, and Mg^{2+}, at 5mM concentration, by 135%, 108%, and 116%, respectively. These metal ions also increased the thermal stability of alkaline protease enzyme. Other metal ions like Cu^{2+}, Zn^{2+}, Hg^{2+}, Al^{3+}, Co^{2+}, Cd^{2+} and Na^{2+} did not show any appreciable effect on the relative activity of alkaline protease.[45] However, metal ions can also inhibit protease enzyme activity. There was a decrease in protease activity due to Ba^{2+}, Cu^{2+}, and Zn^{2+} ions by 23%, 38%, and 84% at 5mM concentration, respectively.[45] In *Beauveria sp.,* the protease enzyme activity was inhibited by Cd^{2+}, Hg^{2+} and Mn^{2+} ions.[46]

Applications of Alkaline Protease

Microbial alkaline protease has numerous applications in different industrial sectors like leather, detergent, food, clinical, etc. The various applications of protease enzyme are presented in *Fig. 2*. Many industries have launched different products based on alkaline protease enzymes. In the food industry, alkaline protease plays an important role in the formation of value-added products. Alkaline protease also plays a significant role in waste management. The applications of alkaline protease enzyme are described below.

DETERGENTS

Enzymes are of great interest to the detergent industry because of their ability to remove a large variety of stains. The alkaliphilic enzyme has major applications in the detergent industry. The detergent industry uses several hydrolytic enzymes that work in the alkaline pH range. Apart from being used in laundry detergents, alkaline protease enzyme is also used in household dish washing detergents and also in the formulation of institutional and industrial cleaning detergents.[47] Enzymes can be used as a detergent additive if they possess an alkaline pH range and compatibility with the detergents.

Isoelectric point, or PI value, is one of the most critical parameters for selecting detergent proteases. Detergent proteases show the best performance when the pH value of the detergent solution is same as the PI value of the enzyme. An alkaline protease was isolated from *Bacillus pumilus* MP 27. The enzyme was stable under a broad range of pH and temperature. It

Table 1. Optimum pH and Temperature Required for the Production of Alkaline Protease Isolated from Different Bacterial Species

ORGANISM	SOURCE OF ISOLATION	OPTIMUM PH	OPTIMUM TEMPERATURE	REFERENCE
Bacillus firmus 7728	Soil samples taken from leather factories	9	40°C	7
Bacillus licheniformis YP1A	Crude oil contaminated soil	9.5	60°C	8
Bacillus megaterium	Fermented broth of Thai fish sauce	10	50°C	9
Bacillus clausii I-52	Heavily polluted tidal mud flat	~11	60°C	10
Micrococcus sp.	Marine sediment samples	10	50°C	11
Bacillus sp. SSR1	Soil	10	-	12
Bacillus pumilus MK6-5.	Soda soil sample	11.5	55–60°C	13
Bacillus horikoshii	Body fluid of the polychaeta, *Periserrula leucophryna*	9	Around 45°C	14
Bacillus sp. B001	Compost	10	60°C	15
Pseudomonas aeruginosa MN1	Tannery waste water	8	60°C	16
Serratia liquefaciens	Raw milk	8	-	17
Bacillus sp	Soil	9	40°C	18
Bacillus sp	Different wastes	8	50°C	19
Virgibacillus pantothenticus (MTCC 6729)	Chicken meat samples	10	50°C	20
Stenotrophomonas maltophilia strain SK	Slaughter house soil samples	9	40°C	21
Bacillus amovivorus	Degraded pulse	8.5	37°C	22
Elizabethkingia meningoseptica KB042	Decomposing feather samples	10	60°C	23
Halobacterium sp.	Soil	10	37°C	24
Bacillus cereus RS3	Desert soil	9	45°C	25

retained 70% of its activity at pH 11 and 50% of its activity at pH 9. It was highly compatible with commercial detergents. It showed 80% compatibility with Triton X 100 and 100% compatibility with Tide brand detergent. Washing performance showed good destaining at 50°C and 4°C. Hence, this alkaline protease can be used as an additive in commercial detergent, as it is also effective in removing stains in cold water washes.[48] Alkaline serine protease was isolated from *Bacillus licheniformis* NH1. The protease was active from pH 7–12 with an optimum pH of 10–11. The enzyme retained 90% of its initial activity after incubation with 7 mg/mL of Axion, Dixan, and new Dex for 60 min at 40°C.[49] Commercial detergents like Surf, Henko, Ariel, Mr. White, and Rin had maximum protease activity at 50°C. They showed poor protease activity at 40°C and very low activity at 60°C. The *Bacillus cereus* protease showed better stability with commercial detergents at alkaline pH range. The protease enzyme present in commercial detergent showed poor activity at 40°C, but *Bacillus cereus* protease has high activity at both 40°C and 50°C. Thus, *Bacillus cereus* protease is superior as compared to the protease enzyme used in laundry detergents.[50]

In recent years, protease has emerged from being a simple additive to a key ingredient in detergent formulations. Several commercially available enzymes are unstable in the presence of bleaching and oxidizing agents. This limitation can be overcome by using DNA recombinant technology to produce genetically engineered enzymes. An alkaline protease isolated from *Bacillus clausii* I 52 showed an optimum temperature of 60°C and optimum pH of 11. The protease was incubated with surfactants and oxidizing agents for 72 h at room temperature. The protease was stable towards nonionic surfactants like Triton-X-100 and Tween-2 (1% concentration). The enzyme retained 73% of its activity when treated with 5% SDS for 72 h. The enzyme also showed enhanced activity when treated with 2.5% sodium borate and 5% hydrogen peroxide. These properties of protease enzymes show that they are a suitable additive in detergent

Table 2. Optimum pH and Temperature Required for the Production of Alkaline Protease Isolated from Different Fungal Species

ORGANISM	SOURCE	OPTIMUM PH	OPTIMUM TEMPERATURE	REFERENCE
Streptomyces sp. CN902	Soil	9	45°C	26
Myceliopthora sp.	-	9	40–45°C	27
Penicillium sp	Soil	9	45°C	28
Streptomyces fungicidicus MML1614	Marine samples	9	40°C	29
A. oryzae CH93	-	8	50°C	30
Aspergillus niger	Soil	8	30°C	31
Aspergillus clavatus	-	9.5	40°C	32
Actinomycete MA1-1	Marine sediment	9	50°C	32
Aspergillus parasiticus	-	8	40°C	33
Aspergillus terreus gr.	Potato grown soil fields	11	50°C	34
Aspergillus nidulans HA-10	Poultry farm soil	8	35°C	35
Pleurotus citrinopileatus	-	10	50°C	36

industry.[10] For many years, protease enzyme has been used as a detergent additive. The main drawback to their use is the need to to obtain microbe-free enzyme, which requires cost-intensive filtration methods. However, in case of fungi, the mycelia can be easily removed by filtration techniques.[51] Caution must be taken in handling concentrated detergent enzymes, however, due to the potential for respiratory sensitization via inhalation. This is an industrial hygiene issue associated with handling high concentrations of enzymes under conditions when aerosols may be formed. This worker safety issue can be controlled by minimizing exposure with appropriate engineering controls such as ventilation, process controls, and product form to minimize aerosol formation, and personal protection equipment as appropriate.

LEATHER INDUSTRY

Alkaline protease enzyme has extensive applications in the leather industry. Different steps, like soaking, dehairing, bating, and tanning, are involved in leather processing. Traditionally, leather processing involved the use of chemicals such as sodium sulfide and lime. These chemicals are hazardous and expensive. The use of these chemicals is not considered eco-friendly due to the problems encountered with effluent disposal. An alternative is the use of enzymes, which have led to improved leather quality and decreased pollution. Previously, the varieties of enzymes used in leather industry were diverse, but they proved unsuccessful. Protease enzymes from certain bacterial species, however, have proved to be efficient in hair removal.[52] Proteases play an important role in the removal of non-fibrillar proteins like albumins and globulins. They also cause selective hydrolysis of non-collagenous proteins. Traditional methods of dehairing involved the use of lime and sulfide, which led to pollution hazards. Hence attempts were made to minimize the use of sulfite and develop eco-friendly methods. An alkaline protease enzyme from *Thermoactinomyces sp.* RM4 had an optimum pH and temperature of 10 and 80°C respectively. A fresh goat hide (5×5 cm) washed repeatedly with water was transferred into a petri dish containing enzyme preparation (buffer +5 U/mL enzyme) and incubated for 24 h. The enzyme was highly effective, as there was removal of hide hairs with mere washing of the hide after enzyme treatment.[52] Microbial alkaline protease hastens the dehairing process because hair roots swell up in alkaline conditions and the subsequent attack by a protease enzyme on hair follicle protein permits easy removal of hair.

Dehairing is followed by bating and then tanning. An alkaline protease enzyme was isolated from *Bacillus cereus* MCM B-326. This enzyme was used in the dehairing of buffalo hides. The protease enzyme was precipitated with ammonium sulphate. This ammonium sulphate precipitated enzyme was stable at ambient temperature (25–35°C). The enzyme showed visible dehairing activity after 12 h of incubation and complete dehairing of buffalo hide after 21 h. When the pelt was examined under the microscope, it showed empty hair follicles, concluding that there was complete removal of hair.

Bating aims to remove unwanted inter-fibrillary proteins,[53] and results in soft, silky, and stretchier leather.[54] Alkaline proteases isolated from selective microbial sources have proved to cause effective bating. The main aim of the bating process is to cause internal separation of collagens by degrading keratin protein, thus exposing maximum reactive surface for tanning agents to act upon.

Table 3. Effect of Protease Inhibitors on Relative Activity of Alkaline Protease Enzyme

ORGANISM	ORGANISM TYPE	PROTEASE INHIBITOR	% RELATIVE ACTIVITY (MAXIMALLY INHIBITED)	PROTEASE TYPE	REFERENCE
Pseudomonas aeruginosa MN1	Bacteria	EDTA (5mM)	0	Metalloprotease	16
Bacillus sp. B001	Bacteria	PMSF (15mM)	11	Serine protease	15
Bacillus megaterium	Bacteria	DFP (1mM)	0	Serine protease	9
Bacillus horikoshii	Bacteria	PMSF (1mM)	21.7	Serine protease	14
Bacillus pumilus	Bacteria	PMSF (1 mmol l^{-1})	1	Serine protease	13
Aspergillus terreus gr.	Fungi	PMSF (5mM)	16.3	Serine protease	34
Aspergillus parasiticus	Fungi	PMSF (5Mm)	2.9	Serine protease	33
Myceliopthora sp.	Fungi	PMSF (5mM)	0	Serine protease	27
Beauveria bassiana	Fungi	PMSF (10mM)	5.7	Serine protease	41
Aspergillus nidulans HA-10	Fungi	PMSF (1mM)	0	Serine protease	35
Pleurotus citrinopileatus	Fungi	PMSF (1mM)	14	Serine protease	36
B. clausii I-52	Bacteria	PMSF (1 mmol l^{-1})	2.4	Serine protease	10
Micrococcus sp.	Bacteria	EDTA (1 mmol l^{-1})	3.35	Possessed the characteristic of metalloprotease	11
Aspergillus niger	Fungi	EDTA (5mM)	11	-	42

SILVER RECOVERY

Silver is a crucial industrial metal and has applications in jewelry, photographic films, electronic items, silverwares, and X–ray films. During photography, silver is not demolished and thus can be recovered and reused. X–ray or photographic films consist of 1.5–2% (w/w) of silver in its gelatin layers. Alkaline protease plays a significant role in the bioprocessing of used X–ray films for recovering silver. The enzyme removes the gelatin layer embedded with silver in X–ray films. The conventional method of recovering silver involved burning X–ray films, which led to environmental pollution. Though the enzymatic process is slow, it is cost effective and pollution-free. About 18–20% of the world's silver requirement is fully filled by photographic waste recycling. Silver was recovered from X-ray films using an alkaline protease produced from a fungal strain *Aspergillus versicolor* PF/F/107. It was observed that the gelatin layer completely decomposed in 20 min at 50°C and pH 9.0, and 0.135 g of silver was retrieved with 0.335% yield.[55] Alkaline protease from *Conidobolus coronatus* removed the gelatin layer within 6 min at 40°C and 9.0 pH.[46]

WASTE TREATMENT

Fibrous proteins like horns, feather, hair, and nails are plentiful as waste in nature. Alkaline protease enzyme from microorganisms can be used in the conversion of waste to useful biomass. Proteinaceous wastes are solubilized by protease, which reduces biological oxygen demand (BOD) of aquatic systems. Alkaline protease has an action potential to degrade waste from food-processing industries and household activities, thereby playing a significant role in waste management. Alkaline protease enzyme from *Serratia sp.* HPC 1383 was able to degrade chicken feathers. The observations suggested that the large number of chicken feathers produced by the poultry industry can be converted into highly digestible animal feed.[56] The feathers, which were soaked in an inoculum of *Streptomyces spp. and B. licheniformis,* degrade more quickly compared to feathers that were not pre-soaked.[57]

FOOD INDUSTRY

Alkaline proteases have been used in the preparation of protein hydrolysate, which has high nutritional value. There are various kinds of protein hydrolysates, which can be prepared from food proteins, as described in *Fig. 3*. The protein hydrolysate plays an important role in blood pressure regulation and is used in infant food formulations, specific therapeutic dietary products, and the fortification of fruit juices and soft drinks.[58] The application of alkaline protease in the preparation of protein hydrolysate is described below.

Production and debittering of protein hydrolysate. Proteolytic enzymes are used in the modification and treatment of proteins. This results in improved solubility, heat stability, and resistance

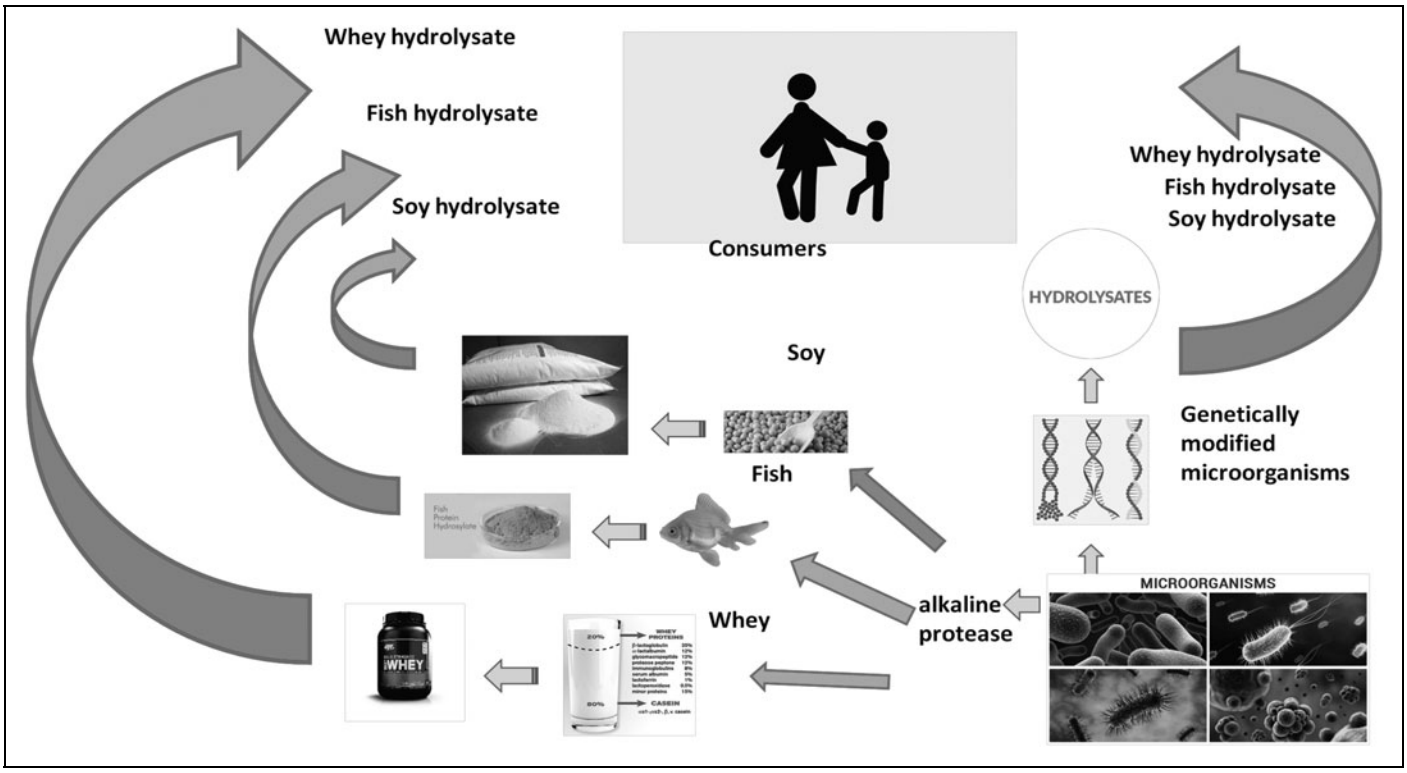

Fig. 3. Application of alkaline protease enzyme in food industry.

to precipitation in acidic environments. Protein hydrolysates are also used as an emulsifying agent in various applications like salad dressings, spreads, ice cream, coffee whitener, sausages, and luncheon meat products. Alkaline protease enzyme is used in the formation of protein hydrolysates. Hydrolysates are of high nutritional value and have various applications, such as a constituent in infant formula, clinical nutrition supplements, flavoring agents, and in dietic and health products. Various food proteins when subject to enzymatic treatment result in a bitter taste due to the formation of low molecular weight peptides, which are mainly composed of hydrophobic amino acids. Thus, this bitterness, due to the formation of bitter peptides, is the most serious hurdle to the practical use of food protein hydrolysate. The magnitude of bitterness depends upon the number of hydrophobic amino acids present in the particular hydrolysate. Debittering of hydrolysate comprises selective separation by various methods like isoelectric precipitation, extraction with alcohol, treatment with activated carbon, hydrophobic interaction chromatography, masking of bitter taste, and chromatography on silica gel. However, biobased methods include bitter peptide hydrolysis with enzymes like alkaline/neutral protease, amino peptidase, and carboxypeptidase, use of *Lactobacillus* as a debittering starter adjunct and condensation reactions of bitter peptides using protease. For the production of protein hydrolysate with less intensity of bitterness, a combination of endoprotease in primary hydrolysis and aminopeptidase enzyme in the secondary hydrolysis is used.

Fish hydrolysate. Every year, about 100 billion tons of fish are harvested, although from this only 29.5% is converted into fishmeal.[59] The seafood industry discards large amounts of byproducts rich in protein content without any recovery efforts. Due to a dramatic rise in world population and the threat of overfishing, there is the need to preserve sea resources. Enzyme technology helps recover and modify fish protein. This fish protein can then be used in different industrial products or food ingredients.[60] Enzymatic fish protein hydrolysis has been used as an alternative method for transforming underutilized fish biomass into consumable protein products.[61] Proteolytic enzymes form one of a major group of commercial enzymes used in various industrial processes.[62] Fish protein hydrolysates (FPH) are either produced by an autolytic process using endogenous enzymes or by a controlled and accelerated process utilizing exogenous enzymes.[63] FPH has shown their potential applications in the industrial and pharmaceutical sectors.[64] Many researchers have found that alcalase (an alkaline enzyme) produced by *Bacillus licheniformis* and papain (plant-based) are the most efficient enzymes for formation of fish protein hydrolysate.[65] Ovissipour et al. carried out chemical (at pH 3.3 and 12 and temperature 70°C and 85°C) and biochemical (neutrase, alcalase, protamax, trypsin, and flavourzyme) hydrolysis of a fish (*Persian sturgeon*).[66] The fish hydrolyzed by alcalase has the highest percentage of degree of hydrolysis (DH%). Alcalase-hydrolyzed fish showed high protein content as well as increased protein recovery (83.64%).[66] Fish hydrolysate prepared with the help of microbial enzyme has a greater degree of hydrolysis

compared to fish hydrolysate prepared with the help of animal enzymes. Chalamaiah et al. isolated protein hydrolysate from *Cirrhinus mrigala* (mergia) fish egg.[67] *Cirrhinus mrigala* is a fresh water herbivorous fish with the ability to produce 1,000,000 to 2,000,000 eggs/kg of body weight. Because there is limited consumption of these eggs by humans, they can be utilized in value-added products with high nutritional value and greater functional properties like protein hydrolysate. The hydrolysate was prepared by using commercial papain (latex of *C. papaya*) and alcalase *(B. licheniformis*). DH% for papain and alcalase was 17.1% and 62% after 90 min digestion at 60–65 and 50–55°C, respectively. The protein content of isolates prepared from papain was less than 70%, whereas hydrolysates prepared from alcalase were 85%.[67]

Whey hydrolysate. Proteins are crucial constituents of the human diet, as they are the chief source of essential amino acids. Milk proteins possess high nutritional value. The two primary proteins found in milk are whey and casein proteins. Among total milk proteins, caseins comprise approximately 80% and whey proteins approximately 20%. Whey proteins are referred as those milk proteins that remain soluble after rennet casein[68] or acid[69] precipitation. The former source of whey protein is referred to as acid whey while the latter is called rennet or sweet whey.[70] Much attention is centered on whey proteins because they have higher biological value compared to most other proteins. Whey proteins have a large amount of sulfur-containing amino acids that help in antioxidant functions. These proteins are soluble across a wide pH range and are globular.[71] The major proteins present in the whey are α-lactalbumin and β-lactoglobulin, which constitute about 70–80% of whey protein.[69,72] The remaining constituents of whey proteins are bovine serum albumin (BSA), protease peptones, and immunoglobulins.[68,70,72] Proteins can be modified by enzymatic, physical or chemical treatment, changing structure and confirmation and, consequently, functional and physiochemical properties. During protein hydrolysis, there is cleavage of the peptide bonds, and proteins are broken down to free amino acids and peptides of various sizes. The degradation can be carried out by alkalis, enzymes, or acids. Alkaline and acid hydrolysis results in the formation of product with reduced nutritional qualities; moreover, they are difficult processes to control. Chemical hydrolysis leads to the formation of toxic substances such as lysino–alanine. Enzymatic hydrolysis under mild temperatures (40–60°C) and pH (6–8) leads to the formation of bioactive nutritional components. Infants who are intolerant to protein of cow's milk can be given hydrolyzed whey protein-based infant formulas. Guadix et al. produced whey protein hydrolysate with reduced allergenicity in a stable membrane reactor.[73] Enzymatic hydrolysis of whey protein was achieved at pH 8.5 and temperature of 50°C using bacterial protease enzyme (protex from *Bacillus licheniformis*). About 80% of substrate conversion was achieved using this method. Reduction in antigenic whey proteins was also observed to be about 99.97%. These observations suggest that it can be incorporated into infant formulas as a good nitrogen source.[73]

Soy hydrolysate. Soybean is a very good source of plant protein. Soy protein can be hydrolyzed by two methods: enzymatic hydrolysis and acid hydrolysis. Presently, manufacturers prefer enzymatic hydrolysis over acid hydrolysis as it is considered a milder and safer method. Enzymatic hydrolysis improves nutritional value and can either increase or decrease functional properties. Soy protein hydrolysate can be used as protein substitutes, flavor enhancers, functional food ingredients, and clinical products. The chief problem with proteolytic hydrolysis of soy protein is that it produces a bitter taste. However, this bitter taste can be reduced by controlling the degree of hydrolysis, adding different components (such as adenosine monophosphate) to mask the effect of bitter taste, and exo-peptidases treatment to the hydrolysates. Agrawal et al. produced alkaline protease from *Penicillium sp.* by solid state fermentation and explained its suitability in soy protein hydrolysis.[28] Different parameters affecting the production of alkaline protease were optimized (pH 9.0 and temperature 45°C). There was a marginal increase in protease activity on pre-incubation with Ca^{2+}, Mg^{2+} and Mn^{2+}. However, there was a drastic decrease in enzyme activity on pre-incubation with Fe^{3+}, Hg^{2+} and Cu^{2+}. *Penicillium sp*. grown on wheat bran supplemented with soy protein indicated the possibility of developing good quality soy hydrolysate.[28]

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