"An Insight on Fungal Alkaline Proteases: An Ecofriendly Approach for Industrial Application"

Adarsh Kachhi[1] and Roshni Choubey[2]

[1]M. Sc IV Sem Microbiology, Dept. of Microbiology, St. Aloysius College (Auto) Jabalpur

[2]Asst. Prof. Dept. of Microbiology, St. Aloysius College (Auto) Jabalpur

DOI: https://doi.org/10.51584/IJRIAS.2025.100700009

Received: 01 July 2025; Accepted: 03 July 2025; Published: 27 July 2025

ABSTRACT

Enzymes are high molecular weight proteins which are well known to increase the rate of a reaction. These enzymes are the real key for the success of an industry. Proteases are one such well known industrially useful biocatalyst. They held the largest share among all industrial enzymes. Alkaline proteases find their applications in wide industries including detergent, leather, textile, pharmacy, food, beverages, agriculture, paper and pulp and many more. They are produced by organisms including plants, animals, and microbes. Microbial proteases especially fungal proteases are advantageous over others due to simple requirements including solid state and submerged fermentation and easy and cheap down streaming processes.

The current study deals with the study of best known fungi for producing alkaline proteases, their isolation, optimization, qualitative and quantitative estimation, purification, immobilization methods and applications of fungal proteases in various fields for human welfare. Our study supports the fact that with further advancement in the technology, these fungal isolates could be proved as the economic factory for enzyme production and also, they can replace certain chemicals and hence offers a green approach.

Keywords: Enzymes, Alkaline Proteases, Fermentation, Industrial application, Human welfare, Green approach.

INTRODUCTION

Industrial enzymes are necessary for the proper operation of bioprocesses. The spectrum of their production methods has been substantially enlarged by the emerging enzyme applications. (Chander, Mukesh, 2019). Three application segments comprise the industrial enzyme market: food enzymes, animal feed enzymes, and technical enzymes. Arora and Changer (2014). Enzymes are used in a wide range of sectors worldwide, and their utilization has grown significantly. Proteases, in particular, are widely utilized in a variety of industries, including textile, leather, feed, waste, dairy, and detergent. Proteases are present in all living things, including bacteria, plants, and mammals (Sharma, N., 2019). Nearly all biological processes are catalyzed by enzymes, which are biocatalysts (figure 1 BocSciences) that are necessary for life (Hinnemann, B., & Nørskov, J. K., 2006). Enzymes have been utilized in the creation of goods like leather, linen, and indigo as well as a variety of food products like beer, wine, vinegar, cheese, and sourdough (Kirk, O., Borchert, T. V., & Fuglsang, C. C. 2002). The industrial manufacture of bulk chemicals, food additives, agrochemical and pharmaceutical intermediates, and active pharmaceuticals has made bio-catalysis an indispensable technique (Schoemaker, H. E., Mink, D., & Wubbolts, M. G., 2003). Enzymes have been identified as valuable catalysts for a variety of organic transformations and the manufacturing of fine chemicals and pharmaceuticals. They are a major resource used by the food, chemical, and related sectors to produce a wide range of biotechnology products (Gupta, R., Beg, Q., & Lorenz, P. 2002).

Protease

Proteases play a critical role in a wide range of application fields, including home and leather processing, nutraceutical applications, healthcare product development, diagnostic kit development, and value-added

product manufacturing. (Subba Rao, C., Sathish, T., Pendyala, B., Kumar, T. P., & Prakasham, R. S. (2009). Protease is an enzyme produced by a broad range of bacterial species. It has been carefully applied and utilized in a number of biotechnological contexts, including the environmental and industrial domains. (Masi, C., Gemechu, G., & Tafesse, M. 2021). All living things include proteolytic enzymes, which aid in the development and differentiation of cells. The hydrolytic enzymes known as proteases function as biocatalysts when proteins are broken down into smaller peptides and amino acids. (Sharma, M., Gat, Y., Arya, S., Kumar, V., Panghal, A., & Kumar, A. 2019). Figure

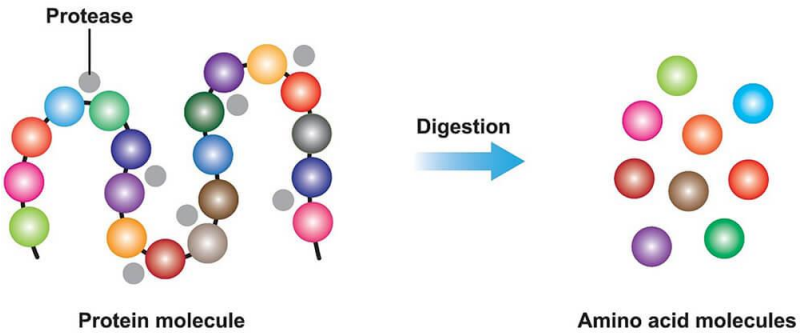

Figure 1 showing mechanism of protease enzyme (BOCSCIENCES, 2025)

The endopeptidases, which cleave internal peptide bonds, and the exopeptidases, which cleave C- or N-terminal peptide bonds, are the two main families into which these enzymes can be roughly classified. The hydrolytic reaction that breaks down protein molecules into amino acids and peptides is catalyzed by the protease enzyme. (Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. 1998). Because of its wide application in the dairy and detergent industries, protease continues to be the most common type of enzyme. More than 60% of all enzyme sales worldwide are made up of proteases, which are extremely significant enzymes. (Ningthoujam, D. S., Kshetri, P., Sanasam, S., & Nimaichand, S. 2009).

Alkaline protease

Alkaline proteases have many applications in detergent and leather industries and there is an increasing need to develop environment friendly technologies (Oberoi, R., Beg, Q. K., Puri, S., Saxena, R. K., & Gupta, R. 2001). Plants, animals and microbes are the main sources for protease production. The most preferred one being microbes are the best source as they grow rapidly and the ease with which they can be genetically modified to generate new enzymes with altered properties and are currently being utilized by the detergent industry e.g. Serine proteases produced by *Bacillus strains* (Harwood, C. R., & Cranenburgh, R. 2008).

Alkaline proteases are most commercially important. 60–65% of the world market for industrial enzymes is made up of proteases, of which 25% is made up of alkaline protease (Bhosale, S. H., Rao, M. B., Deshpande, V. V., & Srinivasan, M. C. 1995, Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. 1998). Proteases are frequently sourced from a variety of species, including microbes, plants, and animals. Because of their quick development, low cost, and potential for genetic modification, microbial proteases are among the most significant hydrolytic enzymes that have been thoroughly investigated (Tremacoldi, C. R., & Carmona, E. C. 2005). At least 25% of all enzymes sold worldwide are alkaline proteases, with microbiological sources accounting for two thirds of the commercially manufactured proteases.

Classification of proteases

Proteases belong to sub-group 4 (hydrolyzing peptide bonds) and group 3 (hydrolases) of the Enzyme Commission's (EC) categorization. Based on their capacity to hydrolyze internal peptide bonds (endopeptidases) or N- or C-terminal peptide bonds (exopeptidases), proteases have been classified into two major categories. While endopeptidases are more significant in the industrial sector than exopeptidases, the former are nevertheless employed in some commercial applications. According to Chander M. and Puri P. 2019), exopeptidases are separated into aminopeptidases, which break the N-terminal peptide linkage, and carboxypeptidases, which cleave the C-terminal peptide bond.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

Morihara in the year 1999 has classified serine proteases as trypsin-like proteinases, alkaline proteinases, Myxobacter lytic proteinases and Staphylococcal proteinases. Hartley (2000) on the basis of active site and sensitivity to various inhibitors classified endo-proteases into four groups.

Serine Proteases

Serine proteases have a serine group at their active site. Serine proteases are generally active at neutral and alkaline pH between 7 to 11.

Cysteine/Thiol Proteases

They have cysteine at their active site and have pH optima in the range of 6 to 8. They are activated by reducing agents such as hydrogen cyanide and inhibited by oxidizing agents**.**

Aspartic Proteases

They are commonly known as acidic proteases having aspartic acid residues at their active sites. They show specificity towards aromatic or bulky amino acids residues on both sides of the peptide bond and have pH optima 5 between 3-4. Microbial aspartic proteases are further divided into two groups, produced by molds and yeasts.

1.Pepsin like proteases and

2.Rennin-like protease

Metalloproteases

Metalloprotease are characterized by the requirement of divalent ion for their activity they are insensitive to sulfhydryl agents and phosphate ester as they are involved in catalytic mechanism but these metalloproteases are sensitive to chelating agent.

Proteases Producing organism

Proteases are commonly obtained from diversified sources such as plants, animals, and microorganisms (Tremacoldi, C. R., & Carmona, E. C. 2005).

Plant proteases

Plant require protease throughout their lifespan so plants are the important source of protease. Plant protease are present in almost all part of the plants and thus can be extracted (by aq. maceration) easily from any plant part and also can be prepared using in vitro techniques. Pure enzymes can be obtained from the crude extract, this protease have the ability to coagulate milk proteins so these enzymes are used in cheese making from decades to make different types of cheese in West Africa, Mediterranean, Southern European countries These proteases are used as crude or in purified form; they are a substitute to the calf rennet. (Shah, M. A., & Mir, S. A. 2019).

Aspartic, cysteine, and serine proteases have been successfully produced using in vivo methodology from a broad variety of tissues, such as seed (*Arabidopsis thaliana*, rice, and barley), leaves (tomato and potato), and flowers (cardoon). Cardosin A (*Cynara cardunculus*) is an aspartic protease whose accumulation in pistils tissue has been increased through in vivo production. In this way, the proteases show higher milk-clotting activity and their specificity could be also enhanced to the substrate target (k-casein) (Castanheira, P., Samyn, B., Sergeant, K., Clemente, J. C., Dunn, B. M., Pires, E., ... & Faro, C. 2005).

Animals proteases

An organism uses proteases for a variety of metabolic functions. We can digest protein in food thanks to serine proteases found in the duodenum and acid proteases produced into the stomach (such pepsin and chymotrypsin). Proteases found in blood serum, such as plasmin, thrombin, and Hageman factor, are crucial for blood clotting,

clot lysis, and proper immune system function. Leukocytes also include other proteases, such as cathepsin G and elastase, which have various functions in the regulation of metabolism. Certain snake venoms, like pit viper hemotoxin, are also proteases and prevent the victim's blood coagulation cascade from working properly.

Other proteins with significant physiological functions, such as hormones, antibodies, or other enzymes, have their lifespan regulated by proteases. The physiology of an organism has one of the fastest "switching on" and "switching off" regulation systems. Proteases can catalyze cascade reactions through a complicated cooperative action that leads to a quick and effective amplification of an organism's response to a physiological stimulus. (Rawlings, N. D., & Barrett, A. J. 1993).

Microbial proteases

Alkaline protease is derived from a variety of organisms, including bacteria, fungus. The majority of alkalophilic microbes do, however, produce alkaline protease; among them, those with large enzyme yields are of particular interest. (Ellaiah, P., Srinivasulu, B., & Adinarayana, K. 2002).

Microbial proteases are among the most significant hydrolytic enzymes that have been thoroughly investigated because of their quick development, low cost, and capacity for genetic modification. (Tremacoldi, C. R., & Carmona, E. C. 2005).

Bacterial

The most active species that produces extracellular proteases is *Bacillus* sp., that have found extensive use in the food processing, leather, washing, and pharmaceutical industries. (Bhunia, B., Basak, B., Bhattacharya, P., & Dey, A. 2012).

Fungal

Fungi are found to be the most potent group which is widely used for the protease production commercially, it has variety of application and its downstream processing is also simple. Fungi producing protease can be found natural as well as in artificial environment (Souza, P. M. D., Bittencourt, M. L. D. A., Caprara, C. C., Freitas, M. D., Almeida, R. P. C. D., Silveira, D., & Magalhães, P. O. (2015). Tremacoldi, C. R., & Carmona, E. C. 2005). It can be found in waste water, garden soil, agriculture soil, decomposing wheat straw, cheese, meat and can be isolated using casein as the protein substrate on Reese agar media. (Choudhary, V., & Jain, P. C. 2012). Ex. *Aspergillus flavus (*Malathi, S., & Chakraborty, R. (1991).

Actinomycetes

Actinomycetes are also known to have protolytic activity as they produce rich amount of secondary metabolites with diverse biological activity, having high GC content, gram-positive bacteria with fungal morphology. (Dilip, C. V., Mulaje, S. S., & Mohalkar, R. Y. 2013).

METHODS OF ISOLATION AND IDENTIFICATION

Methods of isolation

Bacterial isolation

Proteolytic bacteria can be isolated from the tannery soil taken from the CLRI tannery. Using the serial dilution procedure, the salt-tolerant proteolytic bacteria were separated from the samples. Using the conventional pour plate procedure, the samples were serially diluted with sterile distilled water to isolate the bacteria on saline skim milk agar plates. (APHA 1992).

Fungal isolation

Different types of fungi can be isolated from the decaying and rotting food. Injera which is a fermented food

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

was further kept at room temperature for 5 days to get the fungal growth over it. Moldy sample was collected from the injera surface and was grown on Potato dextrose agar media by dilution plate method. It was then incubated at 30°C for 5 days until mycelial growth was observed. The grown fungi were identified using microscopic identification method by staining with lactophenol cotton blue, it was identified using available reports and reference microscopic slides (Chauhan, N. M., Gutama, A. D., & Aysa, A. 2019). The isolated fungi were then examined from proteolytic activities. Protease-producing fungi were identified by the presence of a clear zone on the skimmed milk agar around the colonies. (Omrane Benmrad, M., Mechri, S., Zaraî Jaouadi, N., Ben Elhoul, M., Rekik, H., Sayadi, S., ... & Jaouadi, B. (2019).

A. Protease producing organism

The *Penaeus monodon* (Tiger prawn) is the original home of *P. aeruginosa*, dissected the guts of *Penaeus monodon* in an aseptic manner to isolate and characterize protease-producing bacteria. Colonies with varying morphologies were chosen and purified on nutrient agar plates using the dilutions. The isolated *P. aeruginosa* was found to produce protease on skim milk agar which forms a zone of clearance of 19mm (Kumar, R. S., Pmbhu, D., Shankar, T., Sankaralingam, S., & Anandapandian, K. T. K. 2011).

B. Effect of incubation period on enzyme production

The effect of incubation period was studied by Kumar et al., 2002, for neutral and alkaline protease production, *Pseudomonas sp.* was found to produce alkaline protease. The growth was determined by UV- VIS Spectrometer at 600 nm and enzyme activity was estimated for every 6 hrs. interval until the decline phase. (Kumar, S., Dang, T. D., Arnold, F. E., Bhattacharyya, A. R., Min, B. G., Zhang, X., ... & Willis, P. A. 2002).

Molecular Identification of Fungi

Pure fungus isolate that had a high ability to hydrolyze casein in the skimmed milk agar (SMA) plate was identified via 18S rRNA gene sequencing (Hinrikson, H. P., Hurst, S. F., De Aguirre, L., & Morrison, C. J. 2005).

Methods of qualitative assessment

Identification of fungi*:*

Characterization method employed for the fungal isolates were made by both the inspection of colonial features, cellular characteristics at X100 and X40 microscopic magnification. Identification was done by employing the method of Barnet and Hunter, 1972 and conventional techniques of isolating individual microorganisms and allowing them to grow and produce colonies (Barnet HL, Hunter BB. 1972).

Screening for proteolytic activity

Proteolytic activity was detected by casein hydrolysis on agar plates containing yeast nitrogen base, medium supplemented with 0.5% casein, 0.5% glucose and 2% agar (w/v), pH 7.0. The plates were incubated at 28°C for 7-8 days. Activity was observed by forming clear zone around colonies after precipitation with 1 $molL^{-1}$ HCL solution (Larsen, M. D., Kristiansen, K. R., & Hansen, T. K. 1998).

Screening for proteolytic activities done for the isolated colonies, clear zone was formed around the protease producing fungi on skimmed milk agar. (Omrane Benmrad, M., Mechri, S., Zaraî Jaouadi, N., Ben Elhoul, M., Rekik, H., Sayadi, S., ... & Jaouadi, B. (2019).

Proteolytic fungi were recovered by (Choudhary, V., & Jain, P. C. 2012). from soil using a Reese agar plate containing casein as a protein substrate. After staining, the fungi were inspected under a microscope, samples were filtered, and scraping was seeded on Potato Dextrose Agar (PDA) and allowed to grow for seven days at room temperature. After the fungus was incubated and intense mycelial development with abundant brown and black spores was noticed, it was chosen and purified before continuing to grow in order to obtain axenic cultures (Bhalla et al., 1999).

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

On skimmed milk agar plates, the synthesis of proteases by T. reesei, both wild type and mutant, was examined. Using the caseinolytic test technique, protease activity was ascertained. For thirty minutes, the mixture of 1 ml of the supernatant and 4 ml of casein (0.625% w/v) was kept at 40°C. The addition of 5 ml of 5% trichloro acetic acid halted the reaction. Using casein treated with an inactive enzyme as a blank, the amount of casein hydrolyzed enzymatically was determined using a modified Folin Ciocalteu method (Kanekar, P. P., Nilegaonkar, S. S., Sarnaik, S. S., & Kelkar, A. S. 2002).

Plate Assay by Agar Well Diffusion Method

The proteolytic activity was measured by the agar well diffusion method using casein, gelatine, and skimmed milk agar plates. On casein, gelatine, and skimmed milk agar plates, a precise volume of 0.5µl of crude enzyme was added to each well in that sequence. Plates were incubated at room temperature for 48 hours. There was evidence of a proteolysis zone around the agar well, as well as clear zones of hydrolysis. Casein agar plates were flooded with 5% Bromocresol green while gelatin agar plates were flooded with acidic HgCl2 to achieve more zone removal. A zone measuring scale was used to measure the zones (Vijayaraghavan, P., & Vincent, S. G. P. 2013).

The enzymes were tested using a slightly modified Yang and Haung approach (Yang, S.S.; Huang, C.I. 1994). One unit of enzyme activity is equal to one micro mole of amino acid generated by the per ml/per minute of enzyme source.
1 mL of crude enzyme was mixed with 2 mL of 1% casein solution in a glycine-NaOH buffer (pH 10) and incubated for 30 minutes at 40 °C. 3 ml of a 10% tri-chloroacetic acid (TCA) solution is added to stop the reaction. Subsequently, the mixture was centrifuged at 9000 rpm for 10 minutes. The absorbance was measured at 280 nm in comparison to the blank. Tyrosine standard curves were utilized at different concentrations to calculate the standard factor. As per the recommendation of Lowery et al (Lowry, O. H., Rose Brough, N. J., Farr, A. L., & Randall, R. J. 1951) the total estimation of proteins was evaluated by employing bovine serum albumin, or "BSA" as a standard protein.

METHOD OF QUANTITATIVE ESTIMATION

The culture supernatant (250 mL) was incubated for 20 minutes at 40 ◦C with 500 mL of 1% (w/v-1) casein sodium salt (Sigma) in 50 mmol L-1 buffer (pH 7). This allowed for the determination of enzyme activity. 1 ml of 20% (w/v-1) trichloroacetic acid was added, and the reaction was allowed to sit at room temperature for fifteen minutes. The non-reacted casein was then separated from the reaction mixture by centrifuging it for five minutes at 10,000 rpm. 2.5 mL of 0.4 molL-1 Na2CO3 and 1 mL of 3-fold diluted Folin-Ciocalteu phenol reagent were combined with the supernatant.

After 30 minutes of dark incubation at room temperature, the resultant solution's absorbance of the blue color is measured at 660 nm using a tyrosine standard in comparison to a reagent blank (Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. 1951).The amount of enzyme that, under the assay conditions stated, results in an increase of 0.1 units of absorbance in 1 hour at 30 ◦C is defined as one unit (U) of enzyme activity (Tremacoldi, C. R., & Carmona, E. C. 2005).

Methods of Optimization

Physical parameters-

Physical parameters like pH and temperature of the fermentation medium play a vital role in the growth and metabolic production of fungal population.

Optimum pH

Reports state that the production of proteases is significantly influenced by the pH of the fermentation medium. By affecting the surfaces of the cells, it can directly or indirectly affect the proliferation of fungal growth (Yadav et al., 2011, Sindhu et al., 2009, Hajji et al., 2008, Hajji et al., 2007).

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

By pre-incubating the enzyme solution without substrate at different pH values between 6 and 12, the stability of pH was ascertained. Different buffer solutions were made, including sodium phosphate (pH 6, 7), tris-HCl (pH 8, 9), and glycine NaOH (pH 10–12). The enzyme solution was individually combined in the aforementioned buffers at a ratio of 1:1, and it was then incubated for 12 hours at 40°C in a water bath. Then, the enzymes' activity was examined in accordance with the standard test protocol (Ullah, N. et al., 2022).

1% casein solution (w/v) produced in various buffers (0.05 M), pH 6–7 phosphate, pH 8–9 tris–HCl, and pH 10–12 glycine–NaOH were employed independently to carry out the protease activities at different pH values (6–12). The mixture's reactions were incubated at 40 °C for 30 minutes. After that, the activities were calculated using the enzymes assay methodology (Ullah, N., Rehman, M. U., Sarwar, A., Nadeem, M., Nelofer, R., Shakir, H. A., ... & Alqahtani, F. 2022).

Temperature

The ideal temperature range for alkaline protease activity is between 50 and 70°C. The metabolic processes of bacteria that produce proteolytic enzymes may be negatively impacted by higher temperatures (Tunga, R. B. (1995). Over 24 hours at 28°C and 1 hour at 40°C, the fungal alkaline protease maintained about 60% activity; at 50°C, it preserved about 50% activity, but at this temperature, a total loss was observed (Nirmal, N. P., & Laxman, R. S. (2014).

Temperature and oxygen uptake control the link between energy consumption and enzyme synthesis (Ellaiah, P., Srinivasulu, B., & Adinarayana, K. 2002). Alkaline protease is produced by *Bacillus* and *Streptomyces* species and is fairly stable at high temperatures. The enzyme's heat stability is further improved by the addition of calcium ions (Frankena, J., Koningstein, G. M., van Verseveld, H. W., & Stouthamer, A. H. 1986).

Chemical parameters

Effect of carbon sources on Protease enzyme production:

Effects of various carbon compounds namely fructose, glucose, sucrose, lactose and maltose were used for studying. The broth was distributed into different flasks and 1.0% of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 5 days at 28°C (Sethi, S., & Gupta, S. 2015).

Effect of nitrogen sources on Protease enzyme production:

The fermentation medium was supplemented with organic and inorganic compounds (ammonium sulphate, urea, yeast extract and peptone) replacing the prescribed nitrogen source of the fermentation medium (Sethi, S., & Gupta, S. (2015).

Effect of metal ions on protease activity-

A number of peptidases contain or require metal atoms, without which they are inactive. For example, carboxypeptidase specifically requires cobalt for its activity and leucine aminopeptidase requires manganese or magnesium.

Alkaline proteases isolated from different fungal sources behaved differently in the presence of metal ions. At a concentration of 1mM Co^{+2}, Fe^{+2} increased the activity of alkaline protease from *T. Koningii*, whereas Na^{+}, Ag^{+}, K^{+} and Pb^{+2} inhibited its activity (Ashour, S. A., El-Shora, H. M., Metwally, M., & Habib, S. A. 1996).

Salts of heavy metals such as silver, copper, mercury and lead inactivate most enzymes in high concentration. Tsuchiya *et al*., (1987) reported protease isolated from *Cephalosporium sp.* KM 338 inhibited by Hg^{+2} Mn^{+2}, Cu^{+2} and Ca^{+2}. (Nehra, K. S., Singh, A., Sharma, J., Kumar, R., & Dhillon, S. (2004) reported that Mg^{+2} were found to be an activator of the alkaline protease enzyme produced by *Aspergillus sp.*

Fungal alkaline proteases are completely inhibited by phenylmethylsulphonyl fluoride (PMSF). Protease

activity completely lost in presence of PMSF because it sulfonates the essential serine residue in the active site (Gold, A. M., & Fahrney, D. 1964).

Methods Of Purification

Purification of protease enzyme

An extracellular alkaline protease is partially purified from the culture filtrate of *S. griseorubens* E44G, using the ammonium sulphate precipitation method. Maximum precipitation of protease content was reached at saturation of 70% of ammonium sulphate, giving the highest proteolytic and specific activities compared with the crude protease and other concentrations. Therefore, precipitation of protease with this percentage of saturation was adopted as standard precipitation. By using the ammonium sulphate method for protease precipitation, proteases from *S. albidoflavus* are precipitated by 45% saturation and proteases from *S. alboniger*, by 40% saturation (Kang, S. G., Kim, I. S., Rho, Y. T., & Lee, K. J. 1995).

After ammonium sulphate precipitation, the pellet is dialyzed and loaded onto a Sephadex G-100 column. The fractions were tested for protease activity and the active fractions were pooled, the elution profile of the enzyme solution is shown in reports on the purification of proteases by different affinity chromatographic methods show that Sephadex is commonly the method of choice for separation of proteases (Rifaat, H. M., Hassanein, S. M., El-Said, O. H., Saleh, S. A., & Selim, M. S. 2005) Hatanaka et al. reported that Sephadex G-75 was used for the purification of proteases from *S. albidoflavus*.

Sds-Page

Purified sample of protease from FPLC is examined on 12% of SDS-PAGE by running under the denaturing condition. Purified proteases exhibit single band, the crude and precipitates showed numerous bands after with commissive blue R-250 staining we understand that the crude and pellets have numerous bands on SDS-PAGE represents multi proteins in the mixtures and the fraction filtrate have a single band of 35 k Da (Ullah, N., Rehman, M. U., Sarwar, A., Nadeem, M., Nelofer, R., Shakir, H. A., ... & Alqahtani, F. 2022).

Applications of Alkaline Protease

There are several uses for microbial alkaline protease in the food, pharmaceutical, leather, and detergent industries, among other industries. Alkaline protease enzymes have been the basis for several product launches across numerous sectors. Alkaline protease is crucial for the creation of value-added goods in the food sector. In addition, alkaline protease is important for waste management (Sharma, M., Gat, Y., Arya, S., Kumar, V., Panghal, A., & Kumar, A. 2019).

Detergents Industry-

- *Conidiobolus coronatus's* fungal alkaline protease retained 43% of its activity in the presence of calcium (25 mM) and glycine (M), while remaining consistent with commercial detergents used in India. These findings suggest that *C. coronatus* protease may find application in laundry detergents (Lourdes,M., Polizeli, TM,Rai, M., 2014).

- Modern industrial and domestic detergents have considerably benefited from the invention and enhancement of alkaline proteases. They work well in the current industrial and institutional cleaning environments of moderate temperature and pH levels. The laundry business uses a variety of enzymes, including lipases, amylases, cellulases, and proteases (Ito, S., Kobayashi, T., Ara, K., Ozaki, K., Kawai, S., & Hatada, Y. 1998).

- Because these alkaline proteases can hydrolyze and remove proteinaceous stains such as blood, eggs, gravy, milk, etc. under high pH environments, they are widely used as detergent additives (Saeki, K., Ozaki, K., Kobayashi, T., & Ito, S. 2007).

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

Leather Industry-

- In the processing of leather, alkaline proteases possessing elastolytic and keratinolytic activity have been used, particularly in the dehairing and debating of skins and hides. The enzymatic method is eco-friendly since it is simple to regulate, takes less time, and aids in waste management.

- Furthermore, the enzymatic process eliminates unwanted colors and expands the skin's surface area, resulting in a clean hide (Arora, D. K. 2003).

- Alkaline proteases accelerate the dehairing process by allowing the hair roots to enlarge. This is made possible by the protease's subsequent attack on the hair follicle protein, which makes hair removal simple (Varela, H., Daniel Ferrari, M., Belobrajdic, L., Vázquez, A., & Lyliam Loperena, M. 1997). It has been investigated if a new protease from B. subtilis exhibiting keratinolytic activity may take the place of sodium sulphide in the leather industry's dehairing procedure. (Arunachalam et al., 2009).

- According to Laxman et al., a protease derived from *C. coronatus* has the ability to be used for soaking, dehairing, and bating animal skin or hide. (US Patent No. 6, 777, 219, 2007)

Photographic Industry

- Silver has been effectively recovered from X-ray films using alkaline proteases generated by *B. subtilis, Streptomyces avermectnus*, and *Conidiobolus coronatus*. This makes the procedure more environmentally friendly than using chemicals. (Godfrey and West, 1996b; Wolff et al., 1996; Yang Y. et al., 2000).

- The possibility for silver recovery through the effective use of Bacillus sp. B21-2's thermally stable mutant alkaline protease has also been documented. (Bettiol and Showell, 2002; Dhawan and Kaur, 2007; Araujo et al., 2008).

- A good source of silver recovery is used X-ray photographic film trash, which contains 1.5%–2% black metallic silver dissolved in gelatin. Given that silver is bonded to gelatin, it is feasible to extract the silver by using fungal alkaline protease to hydrolyze the gelatin. (Shankar *et al*., 2010, Choudhary, 2013).

Medical Field

- Scientists have successfully discovered a wide range of applications for proteases in the medical profession over time. Various formulations, including gauze, non-woven tissues, and ointment compositions containing *B. subtilis* produced alkaline proteases, exhibit promise therapeutic characteristics in medicine. (Sen et al., 2011; Anbu, 2013; Awad et al., 2013).

- Research has been done on the application of immobilized alkaline protease from *Bacillus subtilis* with therapeutic qualities for the creation of novel bandage materials, non-woven tissues, soft gel-based medication formulations, and ointment compositions. (Davidenko, T. I. 1999).

- Proteases from *Aspergillus oryzae* have been given orally to treat certain lytic enzyme deficiency diseases as a diagnostic tool. According to reports, alkaline fibrinolytic protease preferentially breaks down fibrin, which may lead to its use in thrombolytic treatment and anticancer medications in the future. (Mukherjee, A. K., & Rai, S. K. 2011).

- Fungal protease's enormous variety and selectivity are fantastic tools for creating therapeutic medicines that work. Protease from *Aspergillus oryzae* has been used orally to treat certain lytic enzyme deficiency diseases as a digestive aid (Rani, K., Rana, R., & Datt, S. 2012).

Soy Product

- Since ancient times, soy sauce and other soy products have been made using proteases.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

- The breakdown of soy protein is significantly aided by the fungal-derived alkaline protease. (Agrawal, D., Patidar, P., Banerjee, T., & Patil, S. 2005).

Waste Management

- The surroundings and the environment suffer when chemicals are used in industry. An eco-friendly substitute for this risky chemical use is desperately needed for trash management.
- Poultry's 5% body weight is made up of feathers with a very stiff keratin structure, which is a rich source of proteins for food and feed.
- The keratinolytic process may break down poultry waste into feed and food (Neklyudov et al., 2000; Lasekan et al., 2013).
- The keratin-rich waste from the leather and poultry industries is made up of polypeptides that are tightly packed and stabilised by a number of weak contacts in addition to disulfide bonds.
- *Aspergillus oryzae*, *Chrysoporium inducum*, Trichophyton sp., *A. terreus*, *Microsporum gypseum*, and *Fusarium oxysporum* fungal keratinases have also been investigated for their ability to degrade keratin. (Ali *et al*., 2011, Sharma *et al*., 2011, Kim, 2003).

Silk Degumming

- A proteinaceous material known as "sericin or silk gum" needs to be eliminated by the traditional method of degumming raw silk in an alkaline soap solution. The greatest option for removing sericin without damaging the fiber is alkaline protease.
- Silk threads are discovered to be far stronger than when earlier standard treatments were applied, and it has been demonstrated that fiber break is not susceptible. (Yadav et al., 2011; da Silva et al., 2017; Radha et al., 2017).
- The silk's shine, color, and texture all enhance when the gum is removed. The literature reports on the use of fungal alkaline protease for degumming silk. (Gulrajani *et. al*, 2000, More *et al*, 2013).

Feed And Food Industry

- In order to speed up dough preparation, a heat-labile fungal protease partially hydrolyzes the gluten in dough before it inactivates early in subsequent baking. Protein hydrolysate preparation with outstanding nutritional value has been accomplished by the addition of microbial alkaline proteases.
- The bioactive peptides are useful molecules in harsh environments and are essential to the synthesis of many pharmaceuticals.
- Making hydrolysate is crucial for strengthening juices and soft drinks and creating infant meals. (Ray, A. 2012; Singhal et al., 2012; Mótyán et al., 2013; Singh et al., 2016).
- Alkaline proteases have been widely used to create protein hydrolysates for over 40 years. Hydrolysates can be added to meals and mixed feed to raise their nutritional value.
- Individuals with food allergies and digestive problems are given them in medication (Neklyudov, A. D., Ivankin, A. N., & Berdutina, A. V. 2000).

Table 1 showing role of few important fungi in industries (Rao et al.)

Name of organism	Application	Source
Conidiobolus brefeldianus	Dehairing of skins/hides	Khandelwal et al. (2015)
Conidiobolus coronatus ATCC PTA–4132	Silver recovery from photographic film	Shankar et al. (2010)
Penicillium sp.	Soy protein hydrolysis	Agrawal et al. (2004)
Aspergillus niger LCF 9	Collagenolytic activity	Barthomeuf et al. (1992)
Fusarium sp. BLB	Fibrinolytic activity	Ueda et al. (2007)
Aspergillus niger DEF 1	Fibrinolytic activity	Lanka et al. (2017)
Aspergillus strain KH 17	Fibrinolytic activity	Palanivel et al. (2013)
Scopulariopsis spp.	Detergent formulation	Niyonzima and More (2014)
Penicillium godlewskii SBSS 25	Detergent formulation	Sindhu et al. (2009)
Trametes cingulata CTM10101	Detergent formulation	Omrane Benmrad et al. (2016)
Graphium putredinis, *Trichoderma harzianum*	Detergent formulation	Savitha et al. (2011)
Aspergillus sp. DHE7	Detergent formulation	Suresh and Dass

Dairy Industry

- Bovine rennet is still the most frequently used enzyme in the dairy industry to make cheese. Proteases produced by fungi such *Rhizomucor miechie, R. pusillus, and A. oryzae* are widely employed as milk coagulants (Neelakantan, S., Mohanty, A. K., & Kaushik, J. K. (1999).

- Neves Souza and Silva (2005) report that a further extract generated from the fugus *A. niger var. awamori* contains powdered coagulating milk, which has already been made industrially.

Brewing Industry

- Proteases are used primarily in two applications in brewing. To boost the production of extract, they can be added during the mashing of cereals.

- Microbial proteases have also been shown to be beneficial, despite the fact that papain, bromelain, and papsin are the proteases that are often employed in the cold proofing process.

- It has been observed that *Mucor pusillus* produces rennet that is useful for clarifying beer (Nelson, JH and Witt, PR 1973).

Other Perspectives of Proteases

- Proteases have important industrial uses, but they are also employed to break down peptide bonds and clarify the relationship between the structure and function of proteins and peptides. Proteinase K can be substituted by alkaline proteases obtained from *Vibrio metschnikovii* RH530 for DNA isolation (Mukherjee and Rai, 2011; Narasimhan et al., 2015; Vijayaraghavan and Vincent, 2015).

- In animal cell cultures, it was discovered that trypsin could be substituted by the alkaline protease from *C. coronatus* (Chiplonkar, J. M., Gangodkar, S. V., Wagh, U. V., Ghadge, G. D., Rele, M. V., & Srinivasan, M. C. 1985).

CONCLUSION

This paper gives us deep insights about the significance of alkaline protease enzymes. Being proteolytic in nature, these enzymes are of utmost importance in food, medical, dairy, pharmaceutical, detergent, leather, and other industries. Proteases play a critical role in a wide range of application fields, including home and leather

processing, nutraceutical applications, healthcare product development, diagnostic kit development, and value-added product manufacturing ETC.

Soil being a natural habitat of a diverse group of organisms proves to be a good source of fungi. With the advanced research and technology, the fungal isolates could be used as a promising producer strain in several industries requiring alkaline protease enzymes.

The secret to the success of bioprocesses is industrial enzymes. These enzymes' novel and developing uses have greatly expanded the field of their manufacturing techniques. With about half of the market, the industrial technical enzymes used in the production of detergent, pulp, and paper make up the largest category. Alkaline proteases are the most valuable commercial enzyme since they have the biggest share among these enzymes. Alkaline proteases are used in the leather and detergent industries, and the development of environmentally friendly technology is becoming more and more popular. Since microorganisms grow quickly and can be easily genetically modified to produce new enzymes with desired altered features in accordance with the needs of the relevant industry, they are the greatest source for producing proteases.

Alkaline proteolytic enzymes are the main product of the gram-negative bacteria Pseudomonas. The benefit of fungal alkaline proteases is their simplicity of downstream processing, which makes it possible to affordably create an enzyme free of microbes. Future research can focus on improving the technology for producing proteases from a lab setting to a large-scale procedure that involves treating protein fibers like silk, wool, and hair with enzymes. New formulas for cleaning wool carpets in homes and businesses, washing clothes, and dyeing protein fibers can be developed by pre-treating them with proteases. The present review elaborates the microbial protease production technologies and also studies their applications in various industries. This overview explains the technologies used to produce microbial proteases and examines how they are used in different sectors.

REFERENCES

1. Adesh Kumar, A. K., Archana Sachdev, A. S., Balasubramanyam, S. D., Saxena, A. K., & Lata, L. (2002). Optimization of conditions for production of neutral and alkaline protease from species of Bacillus and Pseudomonas.
2. Agrawal, D., Patidar, P., Banerjee, T., & Patil, S. (2005). Alkaline protease production by a soil isolate of Beauveria felina under SSF condition: parameter optimization and application to soy protein hydrolysis. *Process Biochemistry*, *40*(3-4), 1131-1136.
3. Ali, T. H., Ali, N. H., & Mohamed, L. A. (2011). Production, Purification And Some Properties Of Extracellular Keratinase From Feathers-Degradation By Aspergillus Oryzae NRRL-447. *Journal of Applied Sciences in Environmental Sanitation*, *6*(2).
4. Anbu, P. (2013). Characterization of solvent stable extracellular protease from Bacillus koreensis (BK-P21A). *International Journal of Biological Macromolecules*, *56*, 162-168.
5. Araujo, R., Casal, M., & Cavaco-Paulo, A. (2008). Application of enzymes for textile fibres processing. *Biocatalysis and Biotransformation*, *26*(5), 332-349.
6. Arora, D. K. (2003). *Handbook of fungal biotechnology*. CRC press.
7. Arunachalam, C., & Saritha, K. (2009). Protease enzyme: an eco-friendly alternative for leather industry. *Indian Journal of Science and Technology*, 29-32.
8. Ashour, S. A., El-Shora, H. M., Metwally, M., & Habib, S. A. (1996). Fungal fermentation of whey incorporated with certain supplements for the production of proteases. *Microbios*, *86*(346), 59-69.
9. Awad, H. M., Mostafa, E. S. E., Saad, M. M., Selim, M. H., & Hassan, H. M. (2013). Partial purification and characterization of extracellular protease from a halophilic and thermotolerant strain Streptomyces pseudogrisiolus NRC-15.
10. Barnet HL, Hunter BB. 1972. Illustrated Genera of Imperfect Fungi. 3th Edition. Burgess Publishing Comp, Minnesota
11. Bettiol, J. L. P., & Showell, M. S. (2002). *U.S. Patent No. 6,376,445*. Washington, DC: U.S. Patent and Trademark Office.
12. Bettiol, J.-L. P., And Showell, M. S. (2002). *Detergent Compositions Comprising A Mannanase And A Protease*. Google Patents.

13. Bhattacharya, S., & Das, A. (2022). Extremophilic Fungal Proteases: Screening, Purification, Assay, and Applications. In *Extremophilic Fungi: Ecology, Physiology and Applications* (pp. 439-464). Singapore: Springer Nature Singapore.
14. Bhosale, S. H., Rao, M. B., Deshpande, V. V., & Srinivasan, M. C. (1995). Thermostability of high-activity alkaline protease from Conidiobolus coronatus (NCL 86.8. 20). *Enzyme and Microbial Technology*, *17*(2), 136-139.
15. Bhunia, B., Basak, B., Bhattacharya, P., & Dey, A. (2012). Kinetic studies of alkaline protease from Bacillus licheniformis NCIM-2042.
16. Boc Sciences, Best of Chemicals (2025), https://www.bocsci.com/resources/proteases-classifications-applications-and-inhibitors.html?srsltid=AfmBOooDWazI7KJ6fN1B-FGjNViZULu-CvcWD-PfISuoztRjJx1a7pQF.
17. Castanheira, P., Samyn, B., Sergeant, K., Clemente, J. C., Dunn, B. M., Pires, E., ... & Faro, C. (2005). Activation, proteolytic processing, and peptide specificity of recombinant cardosin A. *Journal of Biological Chemistry*, *280*(13), 13047-13054.
18. Chander, M., & Arora, D. S. (2014). Biodegradation of a Dye by Different White–rot Fungi on a Novel Agro Residue Based Medium. *Lignocellulose*, *3*(1), 37-50.
19. Chander, Mukesh, 2019/03/15,65Z, Recent Advances in Microbial Production of Proteases,6, International Journal of Environmental Analytical Chemistry
20. Chauhan, N. M., Gutama, A. D., & Aysa, A. (2019). Endophytic fungal diversity isolated from different agro-ecosystem of Enset (Ensete ventericosum) in Gedeo zone, SNNPRS, Ethiopia. *BMC microbiology*, *19*, 1-10.
21. Chiplonkar, J. M., Gangodkar, S. V., Wagh, U. V., Ghadge, G. D., Rele, M. V., & Srinivasan, M. C. (1985). Applications of alkaline protease from Conidiobolus in animal cell culture. *Biotechnology letters*, *7*, 665-668.
22. Choudhary, V. (2013). Recovery of silver from used X-ray films by Aspergillus versicolor protease. *J Acad Ind Res*, *2*(1), 39-41.
23. Choudhary, V., & Jain, P. C. (2012). Screening of alkaline protease production by fungal isolates from different habitats of Sagar and Jabalpur district (MP). *Journal of Academia and Industrial Research*, *1*(4), 215-220.
24. Da Silva, O. S., Gomes, M. H. G., de Oliveira, R. L., Porto, A. L. F., Converti, A., & Porto, T. S. (2017). Partitioning and extraction protease from Aspergillus tamarii URM4634 using PEG-citrate aqueous two-phase systems. *Biocatalysis and Agricultural Biotechnology*, *9*, 168-173.
25. Davidenko, T. I. (1999). Immobilization of alkaline protease on polysaccharides of microbial origin. *Pharmaceutical Chemistry Journal*, *33*(9), 487-489.
26. Devi, M. K., Banu, A. R., Gnanaprabha, G. R., Pradeep, B. V., & Palaniswamy, M. (2008). Purification, characterization of alkaline protease enzyme from native isolate Aspergillus niger and its compatibility with commercial detergents. *Indian journal of science and technology*, 1-6.
27. Dhawan, S., & Kaur, J. (2007). Microbial mannanases: an overview of production and applications. *Critical reviews in biotechnology*, *27*(4), 197-216.
28. Dhawan, S., & Kaur, J. (2007). Microbial mannanases: an overview of production and applications. *Critical reviews in biotechnology*, *27*(4), 197-216.
29. Dilip, C. V., Mulaje, S. S., & Mohalkar, R. Y. (2013). A review on actinomycetes and their biotechnological application. *International Journal of pharmaceutical sciences and research*, *4*(5), 1730.
30. Ellaiah, P., Srinivasulu, B., & Adinarayana, K. (2002). A review on microbial alkaline proteases.
31. Frankena, J., Koningstein, G. M., van Verseveld, H. W., & Stouthamer, A. H. (1986). Effect of different limitations in chemostat cultures on growth and production of exocellular protease by Bacillus licheniformis. *Applied microbiology and biotechnology*, *24*, 106-112.
32. Free A. I. (2014). Optimization of alkaline protease production by Streptomyces ambofaciens in free and immobilized form. *American Journal of Biochemistry and Biotechnology*, *10*(1), 1-13.
33. Godfrey, T., and West, S. (1996b). "Introduction to industrial enzymology," in *Industrial Enzymology*, eds T. Godfrey and S. West (London: Mac. Millan Press).
34. Gold, A. M., & Fahrney, D. (1964). Sulfonyl fluorides as inhibitors of esterases. II. Formation and reactions of phenylmethanesulfonyl α-chymotrypsin. *Biochemistry*, *3*(6), 783-791.
35. Gulrajani, M. L., Agarwal, R., & Chand, S. (2000). Degumming of silk with a fungal protease.

36. Gupta, R., Beg, Q., & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied microbiology and biotechnology*, *59*, 15-32.
37. Hajji, M., Kanoun, S., Nasri, M., & Gharsallah, N. (2007). Purification and characterization of an alkaline serine-protease produced by a new isolated Aspergillus clavatus ES1. *Process Biochemistry*, *42*(5), 791-797.
38. Harwood, C. R., & Cranenburgh, R. (2008). Bacillus protein secretion: an unfolding story. *Trends in microbiology*, *16*(2), 73-79.
39. Hatanaka, T., Uesugi, J. A. Y., & Iwabuchi, M. (2005). Purification, characterization cloning, and sequencing of metalloendopeptidase from Streptomyces septatus TH-2. *Archives of biochemistry and biophysics*, *434*(2), 289-298.
40. Hinnemann, B., & Nørskov, J. K. (2006). Catalysis by enzymes: the biological ammonia synthesis. *Topics in Catalysis*, *37*, 55-70.
41. Hinrikson, H. P., Hurst, S. F., De Aguirre, L., & Morrison, C. J. (2005). Molecular methods for the identification of Aspergillus species. *Medical mycology*, *43*(Supplement_1), S129-S137.
42. Hou, R. Z., Yang, Y., Li, G., Huang, Y. B., Wang, H., Liu, Y. J., ... & Zhang, X. Z. (2006). Synthesis of a precursor dipeptide of RGDS (Arg-Gly-Asp-Ser) catalysed by the industrial protease alcalase. *Biotechnology and applied biochemistry*, *44*(2), 73-80.
43. Ikram-Ul-Haq, H. M., & Umber, H. (2006). Production of protease by Penicillium chrysogenum through optimization of environmental conditions. *J. Agric. Soc. Sci*, *2*(1), 23-25.
44. Ito, S., Kobayashi, T., Ara, K., Ozaki, K., Kawai, S., & Hatada, Y. (1998). Alkaline detergent enzymes from alkaliphiles: enzymatic properties, genetics, and structures. *Extremophiles*, *2*(3), 185-190.
45. Kanekar, P. P., Nilegaonkar, S. S., Sarnaik, S. S., & Kelkar, A. S. (2002). Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. *Bioresource technology*, *85*(1), 87-93.
46. Kang, S. G., Kim, I. S., Rho, Y. T., & Lee, K. J. (1995). Production dynamics of extracellular proteases accompanying morphological differentiation of Streptomyces albidoflavus SMF301. *Microbiology*, *141*(12), 3095-3103.
47. Kim, J. D. (2003). Keratinolytic activity of five Aspergillus species isolated from poultry farming soil in Korea. *Mycobiology*, *31*(3), 157-161.
48. Kirk, O., Borchert, T. V., & Fuglsang, C. C. (2002). Industrial enzyme applications. *Current opinion in biotechnology*, *13*(4), 345-351.
49. Kumar, R. S., Pmbhu, D., Shankar, T., Sankaralingam, S., & Anandapandian, K. T. K. (2011). Optimization of alkalophilic protease production by Pseudomonas aeruginosa. *World*, *3*(5), 371-375.
50. Kumar, S., Dang, T. D., Arnold, F. E., Bhattacharyya, A. R., Min, B. G., Zhang, X., ... & Willis, P. A. (2002). Synthesis, structure, and properties of PBO/SWNT Composites&. *Macromolecules*, *35*(24), 9039-9043.
51. Larsen, M. D., Kristiansen, K. R., & Hansen, T. K. (1998). Characterization of the proteolytic activity of starter cultures of Penicillium roqueforti for production of blue veined cheeses. *International Journal of Food Microbiology*, *43*(3), 215-221.
52. Lasekan, A., Bakar, F. A., & Hashim, D. (2013). Potential of chicken by-products as sources of useful biological resources. *Waste management*, *33*(3), 552-565.Shankar, S., More, S. V., & Laxman, R. S. (2010). Recovery of silver from waste X-ray film by alkaline protease from Conidiobolus coronatus. *Kathmandu university journal of science, engineering and technology*, *6*(1), 60-69.
53. Lopes, A., Coelho, R. R., Meirelles, M. N. L., Branquinha, M. H., & Vermelho, A. B. (1999). Extracellular serine-proteinases isolated from Streptomyces alboniger: partial characterization and effect of aprotinin on cellular structure. *Memórias do Instituto Oswaldo Cruz*, *94*, 763-770.
54. Lourdes, M., Polizeli, TM, Rai, M. (2014), Fungal Enzymes, Taylor & Francis Group, LLC, pg.106.
55. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J biol Chem*, *193*(1), 265-275.
56. Malathi, S., & Chakraborty, R. (1991). Production of alkaline protease by a new Aspergillus flavus isolate under solid-substrate fermentation conditions for use as a depilation agent. *Applied and Environmental Microbiology*, *57*(3), 712-716.
57. Masi, C., Gemechu, G., & Tafesse, M. (2021). Isolation, screening, characterization, and identification of alkaline protease-producing bacteria from leather industry effluent. *Annals of Microbiology*, *71*, 1-11.

58. More, S. V., Khandelwal, H. B., Joseph, M. A., & Laxman, R. S. (2013). Enzymatic degumming of silk with microbial proteases. *Journal of natural fibers*, *10*(2), 98-111.
59. Morihara, K. (1974). Comparative specificity of microbial proteinases. *Advances in enzymology and related areas of molecular biology*, *41*, 179-243.
60. Mótyán, J. A., Tóth, F., & Tőzsér, J. (2013). Research applications of proteolytic enzymes in molecular biology. *Biomolecules*, *3*(4), 923-942.
61. Mukherjee, A. K., & Rai, S. K. (2011). A statistical approach for the enhanced production of alkaline protease showing fibrinolytic activity from a newly isolated Gram-negative Bacillus sp. strain AS-S20-I. *New Biotechnology*, *28*(2), 182-189.
62. Narasimhan, M. K., Chandrasekaran, M., & Rajesh, M. (2015). Fibrinolytic enzyme production by newly isolated Bacillus cereus SRM-001 with enhanced in-vitro blood clot lysis potential. *The Journal of General and Applied Microbiology*, *61*(5), 157-164.
63. Neelakantan, S., Mohanty, A. K., & Kaushik, J. K. (1999). Production and use of microbial enzymes for dairy processing. *Current Science*, 143-148.
64. Nehra, K. S., Singh, A., Sharma, J., Kumar, R., & Dhillon, S. (2004). Production and characterization of alkaline protease from Aspergillus sp. and its compatibility with commercial detergents. *Asian Journal of Microbiology Biotechnology and Environmental Sciences*, *6*, 67-72.
65. Neklyudov, A. D., Ivankin, A. N., & Berdutina, A. V. (2000). Properties and uses of protein hydrolysates. *Applied Biochemistry and Microbiology*, *36*, 452-459.
66. Nelson, JH and Witt, PR (1973), Mucorpusillus Lind tenzyme for chillproofing Beer,USPatent,3740233
67. Neves-Souza, R. D., & Silva, R. S. S. F. (2005). Study of cost and yield of minas like fresh cheese produced with added fat freee soybean hydro-soluble extract powder with curd formed by different coagulants agents. *Food Science and Technology*, *25*, 170-174.
68. Ningthoujam, D. S., Kshetri, P., Sanasam, S., & Nimaichand, S. (2009). Screening, identification of best producers and optimization of extracellular proteases from moderately halophilic alkalithermotolerant indigenous actinomycetes. *World Appl Sci J*, *7*(7), 907-916.
69. Nirmal, N. P., & Laxman, R. S. (2014). Enhanced thermostability of a fungal alkaline protease by different additives. *Enzyme research*, *2014*, 109303.
70. Nout, M. J. R., & Rombouts, F. M. (1990). A review: Recent developments in tempeh research. *Journal of Applied Bacteriology*, *69*, 609-633.
71. Oberoi, R., Beg, Q. K., Puri, S., Saxena, R. K., & Gupta, R. (2001). Characterization and wash performance analysis of an SDS-stable alkaline protease from a Bacillus sp.
72. Omrane Benmrad, M., Mechri, S., Zaraî Jaouadi, N., Ben Elhoul, M., Rekik, H., Sayadi, S., ... & Jaouadi, B. (2019). Purification and biochemical characterization of a novel thermostable protease from the oyster mushroom Pleurotus sajor-caju strain CTM10057 with industrial interest. *BMC biotechnology*, *19*, 1-18.
73. Radha, S., Sridevi, A., HimakiranBabu, R., Nithya, V. J., Prasad, N., & Narasimha, G. (2012). Medium optimization for acid protease production from Aspergillus sp. under solid state fermentation and mathematical modelling of protease activity. *Journal of Microbiology and Biotechnology Research*, *2*(1), 6-16.
74. Rani, K., Rana, R., & Datt, S. (2012). Review on latest overview of proteases. *Int J Curr Life Sci*, *2*(1), 12-18.
75. Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and molecular biology reviews*, *62*(3), 597-635.
76. Rawlings, N. D., & Barrett, A. J. (1993). Evolutionary families of peptidases. *Biochemical Journal*, *290*(1), 205-218.
77. Ray, A. (2012). Protease enzyme-potential industrial scope. *International Journal of Technology*, 1-5.
78. Rifaat, H. M., Hassanein, S. M., El-Said, O. H., Saleh, S. A., & Selim, M. S. (2005). Purification and characterisation of extracellular neutral protease from Streptomyces microflavus. *Arab J Biotechnol*, *9*, 51-60575.
79. Saeki, K., Ozaki, K., Kobayashi, T., & Ito, S. (2007). Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. *Journal of bioscience and bioengineering*, *103*(6), 501-508.
80. Schoemaker, H. E., Mink, D., & Wubbolts, M. G. (2003). Dispelling the myths--biocatalysis in industrial synthesis. *Science*, *299*(5613), 1694-1697.
81. Sen, S., Dasu, V. V., Dutta, K., & Mandal, B. (2011). Characterization of a novel surfactant and organic

solvent stable high-alkaline protease from new Bacillus pseudofirmus SVB1. *Research Journal of Microbiology*, *6*(11), 769.

82. Sethi, S., & Gupta, S. (2015). Optimization of Protease Production from Fungi Isolated from Soil.
83. Shah, M. A., & Mir, S. A. (2019). Plant proteases in food processing. *Bioactive molecules in food*, 443-464.
84. Shankar, S., More, S. V., & Laxman, R. S. (2010). Recovery of silver from waste X-ray film by alkaline protease from Conidiobolus coronatus. *Kathmandu university journal of science, engineering and technology*, *6*(1), 60-69.
85. Sharma, M., Gat, Y., Arya, S., Kumar, V., Panghal, A., & Kumar, A. (2019). A review on microbial alkaline protease: an essential tool for various industrial approaches. *Industrial Biotechnology*, *15*(2), 69-78.
86. Sharma, N., & De, K. (2011). Production, purification and crystallization of an alkaline protease from Aspergillus tamarii [EF661565. 1]. *Agriculture and Biology Journal of North America*, *2*(7), 1135-1142.
87. Sindhu, R., Suprabha, G. N., & Shashidhar, S. (2009). Optimization of process parameters for the production of alkaline protease from Penicillium godlewskii SBSS 25 and its application in detergent industry. *Afr J Microbiol Res*, *3*(9), 515-522.
88. Singh, J., Batra, N., & Sobti, R. C. (2001). Serine alkaline protease from a newly isolated Bacillus sp. SSR1. *Process Biochemistry*, *36*(8-9), 781-785.
89. Singh, R., Mittal, A., Kumar, M., & Mehta, P. K. (2016). Microbial proteases in commercial applications. *J Pharm Chem Biol Sci*, *4*(3), 365-374.
90. Singhal, P., Nigam, V. K., & Vidyarthi, A. S. (2012). Studies on production, characterization and applications of microbial alkaline proteases. *International Journal of Advanced Biotechnology and Research*, *3*(3), 653-669.
91. Souza, P. M. D., Bittencourt, M. L. D. A., Caprara, C. C., Freitas, M. D., Almeida, R. P. C. D., Silveira, D., ... & Magalhães, P. O. (2015). A biotechnology perspective of fungal proteases. *Brazilian Journal of Microbiology*, *46*(2), 337-346.
92. Souza, P. M. D., Bittencourt, M. L. D. A., Caprara, C. C., Freitas, M. D., Almeida, R. P. C. D., Silveira, D., ... & Magalhães, P. O. (2015). A biotechnology perspective of fungal proteases. *Brazilian Journal of Microbiology*, *46*(2), 337-346.
93. Subba Rao, C., Sathish, T., Pendyala, B., Kumar, T. P., & Prakasham, R. S. (2009). Development of a mathematical model for Bacillus circulans growth and alkaline protease production kinetics. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, *84*(2), 302-307.
94. Tremacoldi, C. R., & Carmona, E. C. (2005). Production of extracellular alkaline proteases by Aspergillus clavatus. *World Journal of Microbiology and Biotechnology*, *21*, 169-172.
95. Tsuchida, O., Yamagata, Y., Ishizuka, T., Arai, T., Yamada, J. I., Takeuchi, M., & Ichishima, E. (1986). An alkaline proteinase of an alkalophilic Bacillus sp. *Current Microbiology*, *14*, 7-12.
96. Tunga, R. B. (1995). Influence of temperature on enzyme production. *Tech M Thesis II T. Indian Institute of Technology Kharagpur, India*.
97. Ullah, N., Rehman, M. U., Sarwar, A., Nadeem, M., Nelofer, R., Shakir, H. A., ... & Alqahtani, F. (2022). Purification, characterization, and application of alkaline protease enzyme from a locally isolated Bacillus cereus strain. *Fermentation*, *8*(11), 628.
98. Varela, H., Daniel Ferrari, M., Belobrajdic, L., Vázquez, A., & Lyliam Loperena, M. (1997). Skin unhairing proteases of Bacillus subtilis: production andpartial characterization. *Biotechnology letters*, *19*, 755-758.
99. Vijayaraghavan, P., & Vincent, S. G. P. (2013). A simple method for the detection of protease activity on agar plates using bromocresolgreen dye. *Journal of Biochemical Technology*, *4*(3), 628-630.
100. Vijayaraghavan, P., & Vincent, S. G. P. (2013). A simple method for the detection of protease activity on agar plates using bromocresolgreen dye. *Journal of Biochemical Technology*, *4*(3), 628-630.
101. Vijayaraghavan, P., & Vincent, S. P. (2015). A low cost fermentation medium for potential fibrinolytic enzyme production by a newly isolated marine bacterium, Shewanella sp. IND20. *Biotechnology Reports*, *7*, 135-142.
102. Wolff, A. M., Showell, M. S., Venegas, M. G., Barnett, B. L., & Wertz, W. C. (1996). Laundry performance of subtilisin proteases. *Subtilisin enzymes: Practical protein engineering*, 113-120.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

103. Yadav, S. K., Bisht, D., Shikha, S., & Darmwal, N. S. (2011). Oxidant and solvent stable alkaline protease from Aspergillus flavus and its characterization. *African Journal of Biotechnology*, *10*(43), 8630-8640.
104. Yang, Y., Jiang, L., Zhu, L., Wu, Y., & Yang, S. (2000). Thermal stable and oxidation-resistant variant of subtilisin E. *Journal of biotechnology*, *81*(2-3), 113-118.