Production of alkaline protease from halotolerant bacillus sp. through submerged fermentation-A review.

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Microbial proteases

Bacillus genus is a group of aerobic microorganisms that form endospores and produce hydrolytic enzymes like protease, mannanase, and glucanase [1]. These enzymes are crucial for the global enzyme market, accounting for nearly 60%. Alkaline proteases are particularly interesting from a biotechnological perspective, with applications in the food industry, pharmaceutical and medical diagnostics, detergent industry, and textile industry [2]. They are used in meat tenderization, peptide synthesis, infant formula preparations, baking, brewing, pharmaceutical and medical diagnoses, detergent industry additives, and dehairing and leather processing processes [3].

Classification of Microbial Proteases

The proteases from different microbial sources differ widely not only in functions but also in properties, based on which they can be classified into different groups. Depending upon the optimum pH for activity they can be classified into acid, neutral and alkaline types. Microbial proteases can be classified into four groups, based on the essential catalyt-c residue at their active site. They are the serine, thiol, acid (carboxyl) and metallo proteases.

Serine Proteases

Serine proteases are characterized by a catalytically active serine residue in the active centre. They are inactivated by organic phosphate esters which acylate the active serine residue. Alkaline proteases from different microbial sources mainly belong to this group.

Thiol Proteases

Enzymes in this group have cysteine at their active site. In general, they are activated by reducing compounds and inhibited by oxidising agents. They are susceptible to sulfhydryl (SH) reagents such as 4-hydroxy mercury benzoic acid (p-CMB) and are activated by reducing agents such as hydrogen cyanide. The best known microbial thiol proteases are clostripain obtained from Closbdium histolyticum and the streptococcal protease.

Acid (Carboxyl) Proteases

Many of them contain aspartate residues at their active site. They are insensitive to sulfhydryl agents and chelating agents. They include microbial rennets and microbial acid proteases from moulds and yeasts.

Metallo Proteases

They have metal ion involved in the catalytic mechanism and are consequently sensitive to chelating agents such as EDTA. They are insensitive to sulfhydryl agents and organic phosphate esters. Neutral proteases from many microbial sources belong to this group. Commercial Applications Microbial proteases constitute a complex range of enzymes with wide spectrum of properties that make them suitable for different commercial applications.

Proteases are essential components in various industries, including food, detergent leather, and leather. They improve the functional and taste properties of proteins, such as soya sauce, meat extract powder, and hydrolysates of soya protein, gelatine, casein, and whey proteins. In the meat industry, they are used alongside papain for meat tenderisation and conditioning. The tenderising actions of plant and microbial proteases vary, with plant protease acting on collagen and elastin, while bacterial and bacterial preparations have only slight action on collagen fibers but considerable effect on muscle fibres.

Microbial proteases are useful in baking processes, improving handling properties and increasing loaf volume, resulting in soft loaves with good crumb and crust structure. This is due to gluten hydrolysis occurring in doughs and allowing bakers to adapt flours of different qualities to a standard process. The protease commonly used in bread production is usually obtained from *Aspergillus oryzae*.

The dairy industry is the single major application of proteases, particularly in cheese making due to the scarcity of calf rennet, an enzyme used for coagulating milk during cheese making. Microbial rennets are the most important sources of microbial rennets, and their role in cheese ripening is also discussed. In brewing, microbial proteases can be used during cereal mashing to increase the yield of extract and level of amino nitrogen of the wort produced, and in the finishing stages of beer production to remove chill haze.

In the detergent industry, alkaline proteases are used during various stages such as soaking, dehairing, bating, and waste processing. Alkaline proteases reduce the requirement of lime by 50%, reduce the dehairing process time, lower waste

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***Received:** 02-Aug-2024, Manuscript No. AAMCR-24-144369; **Editor assigned:** 05-Aug-2024, PreQC No. AAMCR-24-144369 (PQ); **Reviewed:** 19-Aug-2024, QC No. AAMCR-24-144369; **Revised:** 23-Aug-2024, Manuscript No. AAMCR-24-144369 (R); **Published:** 28-Aug-2024, DOI:10.35841/aamcr-8.4.216*

***Citation:** Ray L. Production of alkaline protease from halotolerant bacillus sp. through submerged fermentation-A review. J Micro Curr Res. 2024; 8(4):216*

treatment costs, increase skin area, and facilitate dyeing. For the treatment of fine wooled skins, neutral bacterial protease formulations are used [4].

For the processing of wastes from the leather industry, alkaline proteases are useful, with a technique developed by Dalev and Simeonova for the total utilization of wastes from the leather industry. In addition to these applications, microbial proteases are used to a lesser extent for other purposes [5].

Production and immobilization of alkaline protease

Microorganisms, particularly bacteria of the *Bacillus* genus, are excellent sources for producing alkaline protease due to their broad biochemical diversity, simplicity in genetic manipulation, and feasibility in mass culture. *Bacillus bacterium* are active producers of extracellular alkaline proteases, making them commercially useful due to their high pH and thermal stability [6]. To reduce production costs and increase industrial utility, various measures have been taken, such as immobilization of enzymes and whole cells. This method offers numerous advantages over free enzymes, such as increased production rate, operational stability, reuse of enzymes, and recovery of final products free of enzyme contamination [7]. Whole cells can show modifications in mechanical, chemical, thermal resistance, and stability in changes in pH. Different *Bacillus* species, such as alkalophylic and thermophylic *Bacillus*, have been immobilized in various substances like calcium alginate, polyacrylamide gel, gelatin, chitosan, corn cab, and κcarrageenan. Calcium alginate has been reported as a better matrix for immobilizing *Bacillus* subtilis, while corn cab is suitable for *B. licheniformis*.

Immobilized *Bacillus* strains for effective production of protease

Microorganisms, particularly bacteria of the *Bacillus* genus, are ideal for producing alkaline protease due to their broad biochemical diversity, ease of genetic manipulation, and mass culture feasibility. *Bacillus bacterium* are active producers of extracellular alkaline proteases, making them highly useful in the industrial sector due to their high pH and thermal stability [8]. To reduce production costs and increase its industrial utility, various measures have been taken, including immobilization of enzymes and whole cells. This method offers numerous advantages over free enzymes, such as increased production rate, operational stability, reuse of enzymes, and recovery of final products free of enzyme contamination. Whole cells can show modifications in mechanical, chemical, thermal resistance, and stability in changes in pH. Different *Bacillus* species, such as alkalophylic and thermophylic *Bacillus*, have been immobilized in various substances, such as calcium alginate, polyacrylamide gel, gelatin, chitosan, corn cab, and κ-carrageenan. Calcium alginate has been reported as a better matrix for immobilizing *Bacillus* subtilis and corn cab for *B. licheniformis*.

Application of microbial protease

Microbial proteases are becoming increasingly important in biotechnological processes due to their lower production costs and use of renewable resources. Bacteria, including *Bacillus* sp., are active producers of extra cellular alkaline proteases, which are highly stable at high alkaline ranges and temperatures. These serine proteases have industrial importance and are particularly useful in the detergent industry, where laundry detergents typically have pHs between 9-12 [9].

Several studies have investigated the production, optimization, and partial purification of protease from *Bacillus* subtilis. One study identified a bacterial strain JRK-3 as a potential producer, with its activity comparable to available literature [10]. Another study found that GS-P4 isolate produced the highest protease activity, indicating its industrial potential.

Another study focused on the production of thermostable protease from *Bacillus* subtilis, a thermotolerant bacterium widely used for isolating protease enzyme. The presence of galactose and peptone in the medium enhanced enzyme production by 0.5% compared to other carbon and nitrogen sources.

A third study studied the protease-producing halotolerant bacterium from saltern pond sediment (Tuticorin) and its optimization of culture conditions, nutritional factors, and physical parameters. Results showed that xylose and urea enhanced enzyme production.

In recent years, there has been a potential increase in using alkaline protease as industrial catalysts, especially in food and textile industries and medical diagnoses. Cell immobilization techniques have been used for alkaline protease production using different carriers, such as chitosan, corn cob, and corn tassel. Corn cob, with 65% immobilization capacity and the highest enzyme activity, was selected as the best carrier. After immobilization, the enzyme activity was obtained at 119.67 U/ml.

Conclusion

The production of alkaline protease from halotolerant *Bacillus* species through submerged fermentation has significant potential for industrial applications. These proteases are highly adaptable and have high thermal and pH stability, making them valuable in industries like food, detergent, leather, and textiles. Submerged fermentation, combined with immobilization techniques, enhances the production yield and operational stability of these enzymes, facilitating enzyme reuse and product recovery, reducing production costs. Different carriers, such as calcium alginate and corn cob, are effective in immobilizing *Bacillus* species, increasing enzyme activity and stability. The wide range of applications for alkaline proteases, including meat tenderization, dairy processing, detergent formulation, and leather treatment, underscores their commercial significance.

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Citation: *Ray L. Production of alkaline protease from halotolerant bacillus sp. through submerged fermentation-A review. J Micro Curr Res. 2024; 8(4):216*