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Bacterial Protease Enzyme: Safe and Good Alternative for Industrial and Commercial Use

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Abstract

Proteases are present in all forms of life, they are produced by microorganisms, various plants and animals. Among them bacterial proteases secure most important place due to their enormous industrial applications. They represent one of the largest groups of industrial enzymes and find application in detergents, leather, food, pharmaceutical and textile industries as well as silver recovery and bioremediation processes. The largest application of protease is in laundry detergents and leather industry, where they remove protein based stains from clothes and dehairing purpose, respectively. As the modern world focuses on ecofriendly products and product output, subsequently more chemical processes are being replaced by enzymatic methods. Today there is a need for new, improved and/or more versatile enzymes in order to develop more novel, sustainable and economically competitive production processes. In this review, some protease producing bacteria and protease classification as well as the techniques to enhance productivity of protease are discussed. The production of bacterial proteases using cheap and readily available substrates is delineated. The use of bacterial proteases in diverse industrial applications is also discussed. Finally, future prospects are proposed.

Keywords

Bacteria, Protease, *Bacillus,* Enzyme, Industry

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1. Introduction

Proteases are essential constituents of all form of life on earth including prokaryotes, fungi, plants and animals. However, proteolytic enzymes from microbial sources are preferred over the enzymes derived from plant and animals since they possess almost all characteristics desired for their biotechnological applications [1]. Hence microbial proteases represent one of the largest groups of industrial enzymes and account for approximately 60% of the total industrial enzyme sale in the world [2, 3].

Microorganisms elaborate a large array of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products. At the same time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes [4].

The global market for industrial enzymes is increase from year to year, it constitute the largest product segment in the global industrial enzymes sales in various industrial market sectors such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery. Considering this market demand, there is a need to effort to solve this problem by investigating new microorganisms because they are the major sources of all commercially

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important alkaline proteases, which have unlimited industrial applications.

Bacterial proteases are one of the most important groups of enzymes, used in various industrial processes as food, silvery recovery, pharmaceutical and detergent industries, as well as in the preparation of leather, textile and wool, among others [5, 6]. Another area of promising application in medical usage and management of industrial and household waste [7, 8].

Microbial proteases have been reviewed several times; with emphasis on different aspects of proteases. Excellent review were published in terms of molecular biology, biochemical and genetic aspects of microbial, animal and plant proteases. In this review, some aspects of bacterial proteases are discussed with reference to their industrial applications and an emphasis on the detergent, silver recovery, pharmaceutical, food, waste management, textile and leather industry along with the upcoming approaches for feasible production and developing novel proteases using new technologies.

2. Source of Proteases

Proteases are physiologically necessary for almost all living organisms such as bacteria, fungi and viruses, because of that they are ubiquitous found in a wide diversity of sources such as plants, animals and microorganisms. The use of plants as a source of proteases is governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process. Papain, bromelain, keratinase, and ficin represent proteases of plant origin. The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennin. These are prepared in pure form in bulk quantities. However, their production depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies.

The incapability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Due to these proteases from microbial sources are preferred over the enzymes derived from plant and animals. In addition, they possess almost all characteristics desired for their biotechnological applications. Among microorganisms, Bacteria and fungi are the most prominent one. However, compared to the bacterial protease, fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity, for instance *Aspergillus oryzae* produces the three proteases; acid, neutral and alkaline. However, they have a lower reaction rate and not as good as heat tolerance than do the bacterial enzymes. The advantages of fungal proteases are conveniently produced in a solid-state fermentation process [4]. Among bacteria, *Bacillus spp* is attractive industrial tools for a source of proteases (See Table 1).

Table 1. Some of industrially important protease producing bacteria and corresponding applicable area.

Bacteria Genus	Protease producing bacterial species and strains	Industries	References
Bacillus Spp	Bacillus cereus strain AT	Leather	[9]
	Bacillus megaterium RR2	Detergent	[10]
	Bacillus cereus FJ10	Detergent	[11]
	Bacillus licheniformis N-2	Detergent	[12]
	Bacillus amyloliquefaciens	Food and feed	[13]
	Bacillus isolate C45	Leather	[14]
	Bacillus licheniformis KBDL	Silvery recovery	[15]
	Bacillus subtilis	Medical usage	[16]
	Bacillus cereus	Detergent	[17]
	Bacillus subtilis	Detergent	[18]
Pseudomonas Sp.	Pseudomonas auroginosa PD100	Detergent	[19]

3. Classification of Proteases

Proteases are classified based on chemical nature of the active site, the reaction they catalyse, and their structure and composition. They are mainly classified into endoproteases and exoproteases based on the catalytic site on the substrate. Exoproteases act at the end of the polypeptide chain, while endoproteases preferably act at the inner region of the polypeptide chain. Exoproteases are further classified in to amino peptidases and carboxypeptidases proteases which act at the free N-terminus of the polypeptide substrate and free C-terminal of the polypeptide chain respectively. The whole classification system of proteases are summarized in figure 1.

In the same way, endoproteases are also classified based on their side chain specificity and functional group present in characteristic active site. They may also be classified on the basis of their active site into the following:

(i) Serine proteases: Serine proteases are proteases having a serine group (-OH) in their active site.

(ii) Cysteine proteases: The activity of these depends on the

presence of an intact-SH group in their active site.

(iii) Metalloproteases: These depend on the presence of more of less tightly bound divalent cations for their activity.

(iv) Aspartic proteases: Acid proteases contain one or more side chain carboxyl groups in their active site. Commonly known as acid protease [20, 21].

Further endoproteases proteases are classified according to their maximal activity in a particular pH range as:

1) Acidic proteases. Acid proteases are proteases which are active in the pH ranges of 2-6

2) Neutral proteases. Neutral proteases are proteases which are active at neutral, weakly alkaline or weakly acidic pH.

3) Alkaline proteases. Alkaline protease can be defined as the protease those are active in alkaline range of pH (8-12) [22].

Alkaline proteases are a physiologically and commercially important group of enzymes used primarily as detergent additives. They play a specific catalytic role in the hydrolysis of proteins [23]. Several alkaline proteases from different sources are currently in the market, but almost all are products of microbial origin. Alkaline proteases are produced by both neutralophilic and alkaliphilic microorganisms. Alkalophilic microorganisms are widely distributed in nature and can be found in almost all environments without restriction of alkalinity [15].

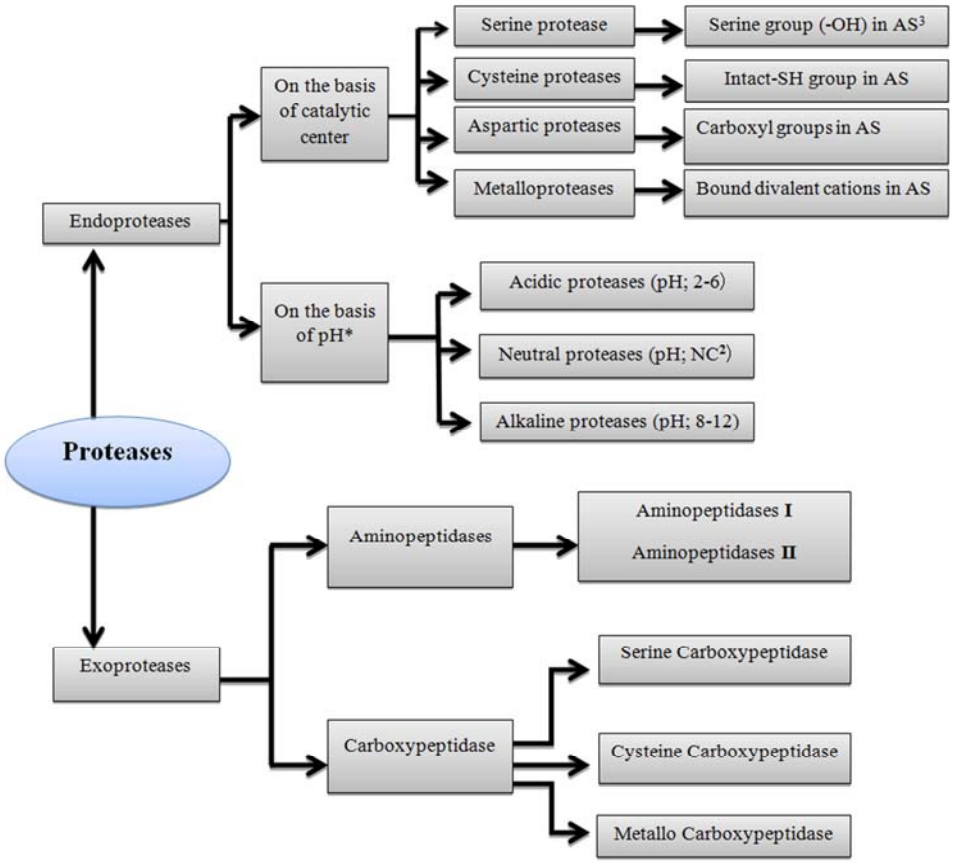

Figure 1. Diagram summarizing classification of proteases.

Key: pH[1]- Power of hydrogen; NC[2]-Neutral condition and AS[3]- Active site.

4. Industrial Applications of Bacterial Protease

Proteases are one of the most important group of enzymes used in various industries such as detergent, silver recovery, food, pharmaceutical, leather and textile industries. Accordingly proteases account for about 60% of the total industrial enzyme sale in the world.

4.1. Proteases Application in Protein Hydrolysis

Protease use in food industries for protein modification and to improve palatability and storage stability of the available protein sources. Alkaline proteases have been used in the preparation of protein hydrolysates of high nutritional value with well-defined peptide profile. It also plays a great role in meat tenderization, especially of beef [2].

4.2. Application of Proteases in Leather Industry

Proteases are useful in dehairing for the purpose of leather manufacture. Since, the beginning of human civilization the conventional method of dehairing involves the use of lime and sodium sulphide as the lack of technology. But currently it is possible to replace chemical dehairing with enzyme based dehairing processes using proteases. This avoiding the use of lime and sulphide are being developed because of their environmental benefits. Thus, enzyme based dehairing processes using proteases help to reduce or even avoid those chemical and offer enormous environmental benefits [24]. In addition, to improve the quality of leather produced [3].

The only alternative to manage and control the pollution caused by leather processing is substitution of the used chemicals with enzymes especially proteases. Degradation of unwanted protein by simple eco-friendly and inexpensive method is one of the central requirements in several industries especially leather industries [24]. Proteases active in the pH range of 8-12 and stable at alkaline pH are known as potential candidates for dehairing of hides [10, 14, 25].

4.3. Application of Proteases in Waste Management

The scarcity of natural resources and the accumulation of pollution caused by human activity have required the development of production technology that is less harmful to the environment. One well-established application of modern biotechnology is the use of bacterial protease for treatment of waste or the bioremediation of hydrocarbons. In addition to that these enzymes also offer advantages over the use of conventional chemical catalysts for numerous reasons. Bacterial proteases have interesting potential applications in the management of wastes from households and processing industries, also cleaning of hair clogged pipe lines containing hairs. It also used for degrading waste keratinous material in household refuse and to remove bad odors in houses and in public places. These proteases can solubilize proteins in wastes especially waste feather from poultry slaughterhouses through a multistep process to recover liquid concentrates or dry solids of nutritional value for fish or livestock [26].

Many industrial and household processes cause adverse changes in the immediate environmental change and therefore being challenged by society. Cleaner production should be used in many industries and household in order to mitigate their impact and reduce the loss of chemicals, water and raw materials and thus minimize the risks of our environments (See Figure 2). Solid waste from the tannery process, large quantities of sludge and effluent from treatment plants is a major source of environmental pollution. Nowadays, bacterial proteases used for commercially in bioremediation process as well as probiotic agent in aquaculture by incorporating them into fish/shrimp diet [27].

4.4. Pharmaceutical Application of Proteases

Proteases of the subtilisin group are used for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses to speed up healing process by producing anti-inflammatory response in patients have been reported. It is possible to use protease as a therapeutic agent for the treatment of pulmonary emboli and degradation of elastin, collagen [7]. The purified protease from bacteria could be used for various purposes like antibacterial activity against clinical pathogens as well as it degrade slime and bio films to limit gram negative bacteria [27]. In addition it digest debris in blood like bacterial and viral proteins and as medicine in the field of oncology [16].

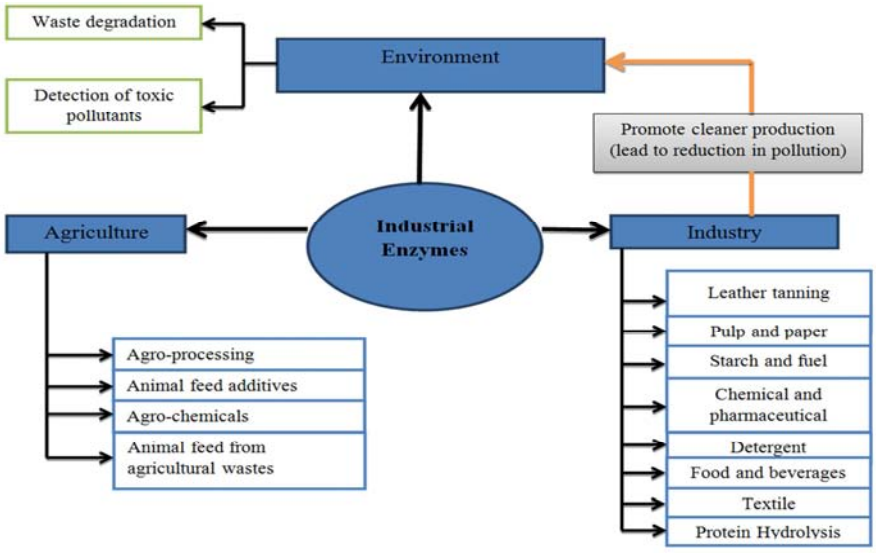

Figure 2. Flow chart summarizing application of enzymes and clean production strategy.

4.5. Application of Proteases as Detergent Additive

The idea of using detergent enzymes dates back to 1914 when two German scientists, Rohm and Haas, used pancreatic proteases and sodium carbonate in washing detergents. However, it was only in 1963 alkaline protease was effectively incorporated in detergent powder. Due to this its economic importance became well known [28]. However, currently proteases constitute the largest product segment in the global industrial enzymes market. The detergent enzyme market has grown nearly 10-fold during the past 20 years [10].

Removal of proteinaceous stains such as blood, milk, egg, grass and chocolate is very difficult using conventional detergent method. However, removal of such stain is achieved by using bacterial proteases [19, 29, 30]. In addition the use of protease supplementation to detergent formulation significantly improves the cleansing of proteinaceous stain and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies [16, 31].

The use of enzymes in detergent formulations is now common in developed countries, with over half of all detergents are contain enzymes. Most of these enzymes are produced from different bacterial species. In this regard, *Bacillus sp.* have a very useful and profound application in textile and detergent industries [32]. See Table 1 and 2. Currently, detergent protease account for more than 30% of the world enzyme market. Over the past 30 years, the proteases in detergents have changed from being minor additives to being the key ingredients. As result, protease is one of the standard ingredients of all kinds of detergents ranging from household laundering to reagents used for cleaning contact lenses or dentures [10].

The major prerequisites for the application of protease as detergent additive are its activity and stability at alkaline pH, temperature, compatibility with other chelating and oxidizing agents. Good detergent proteases exhibit: i) optimum activity at alkaline pH; ii) effectiveness at low wash temperatures of 20-40ºC; iii) stability at up to 60ºC wash temperatures; iv) stability in the presence of other detergent ingredients, such as surfactants, builders and activated bleach, both during storage and use; and v) broad specificity, enough to enable the degradation of a large variety of stain due to blood, food and other body secretion. From the different classes of known proteases, alkaline proteases are suitable for industrial applications based on their properties such as high stability and activity under harsh conditions [17, 33].

Table 2. Some commercial bacterial alkaline proteases, sources, applications and their industrial suppliers, adopted from [2, 28].

Supplier	Bacterial source	Application
Novo Nordisk, Denmark	*Bacillus sp.*	Detergent
Genencor International, USA	*Bacillus lentus*	Detergent
	Bacillus alcalophilus	Detergent
Gist-Brocades, The Netherlands	*Bacillus sp.*	Detergent
	Bacillus sp.	Detergent
	Bacillus alcalophilus	Detergent
Solvay Enzymes, Germany	*Bacillus licheniformis*	Detergent
	Bacillus sp.	Detergent
Wuxi Synder Bioproducts, China	*Bacillus sp.*	Detergent
Advance Biochemical, India	*Bacillus sp.*	Detergent

4.6. Application of Proteases in Silver Recover from Waste X-ray Films

It is known that silver is one of the precious and noble metals used in large quantities for many purposes, particularly in the photographic industry. The waste X-ray films (1.5-2.0% silver by weight) containing black metallic silver spread in gelatin are very good source for silver recovery compared to other types of film. With an increasing demand for silver in the world, recent attention is focused on used X-ray or photographic films as one of the sources [34].

Various conventional methods are carried out to recover the silver from X-ray film wastes. This are burning the films directly and stripping the gelatin-silver layer using different chemical solutions. Stripping the gelatin-silver layer by chemical methods using ammonium thiosulphate, sodium thiosulphate, nitric acid or reagents such as sodium cyanide, NaOH, organic compounds cause environmental hazards (generation of foul smell), time consuming and very expensive. In addition polyester film on which emulsion of silver and gelatin is coated cannot be recovered. These all poses a serious industrial safety problem.

Proteases play a crucial role in the bioprocessing of used X-ray films for silver recovery. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled [34, 35]. Use of proteases that degrade the gelatin in few minutes for silver recovery has been reported [36]. Using such enzyme treatment, about 99% pure silver recovery has been achieved. This contributes zero/minimum to environmental pollution. So that bacterial proteases which is stable in the environment of silver recovery has application in silver recover industry (See Table 3).

Table 3. Application of microbial protease for silver recovery process.

S.no	Microbial	Optimum pH and Temperature	Application	References
1	*Bacillus licheniformis* KBDL	n.s*	Silver recovery	[15]
2	*Aspergillus Versicolor* PF/F/107	50°C (pH 9)	Silver recovery	[30]
3	*Bacillus sp. B21-2*	50°C (pH 9)	Silver recovery	[35]
4	*Bacillus subtillis* ATCC6633	50°C (pH 8)	Silver recovery	[36]
5	*Bacillus subtilis*	40°C (pH 10)	Silver recovery	[37]

Key: n.s* not specified.

4.7. Miscellaneous Applications of Bacterial Protease

Silk degumming

The conventional method to remove sericin from silk is generally expensive and therefore an alternative method suggested is the use of proteases for degumming the silk prior to dyeing. Protease based silk degumming have higher efficiency, expressed over removal of sericin and energy save [38].

Scale removal from wool fiber

Wool fiber exhibit felty and shrinking feature because of the presence of scales that are found toward fiber tips. Conventionally, chlorine-Hercosett is applied to remove the scales by oxidation, or coating of fiber in polymers used. Wool treatment with hair and keratin degrading alkaline protease provide an option in the removal of scale tips from wool fibre [20].

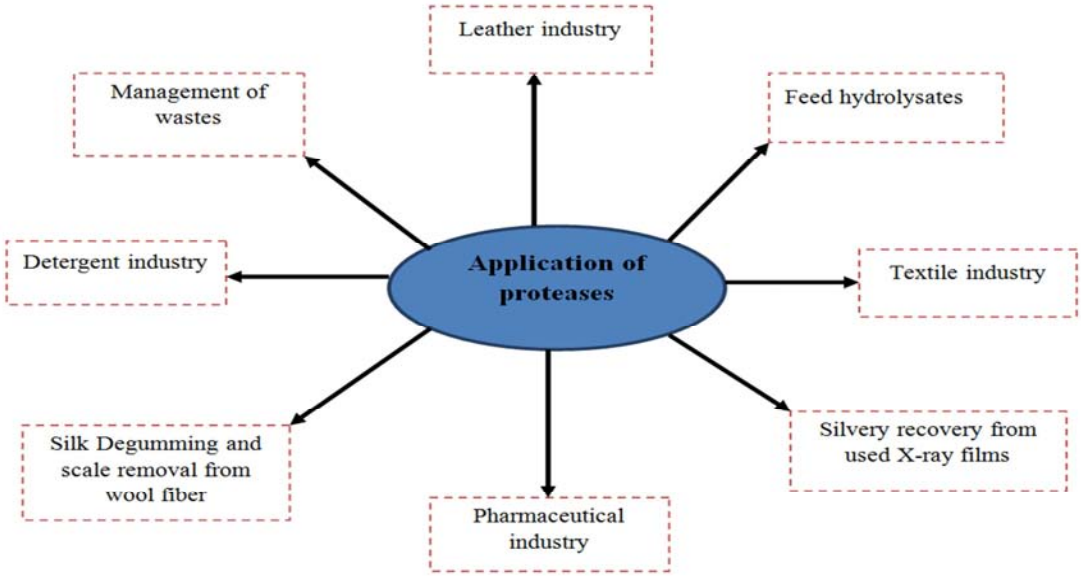

Figure 3. Summary on application of bacterial proteases in different sectors.

5. Production of Protease Using Cheap Substrates

The use of microorganisms to produce enzymes has a number of technical and economic advantages and in recent years it has become the predominant mode of enzyme production [39]. The exploration of protease producing bacteria is still underway to achieve high quality industrial grade enzyme to produce goods [40]. However, the cost of producing this enzyme is the main problem. Almost all proteases are derived from expensive growth substrate. Upto 40% of the total production cost of enzymes is due this growth substrate [2]. But currently, significant interest to produce enzymes using cheap and readily available substrate. This is achieved by using both solid-state and submerged fermentation techniques. Production of protease from bacteria using cheap and readily available substrate like wheat bran was found to be promising industrial application and environmental restoration such as treatment of chromium contaminated proteinaceous saline wastes [41].

Substrates from agro-industrial wastes can be used for low-cost production of bacterial proteases [42]. Bacterial protease remarkable produced by using cow dung, groundnut shell and wheat bran as substrate [9, 43, 44]. In addition production of value-added products like enzymes using wastes released from processing industries like dairy sludge and molasses is possible [33]. Interestingly, use of agro-industrial waste and reuse of industry sludge and effluents as substrate for enzyme production could result in reduce production cost primarily and at the same time help to mitigate environmental pollution. Commercial production of economically valuable proteases by utilizing agro-industrial

wastes expected in near future.

6. Protease Yield Improvement Techniques

Advance in genetic manipulation of microorganisms opens new possibilities for the introduction of predesigned changes resulting in the production of tailor-made proteases with novel and to meet the requirements of the multitude of protease applications with desirable properties. Microbial diversity represents an invaluable resource for biotechnological innovations and plays an important role in the search for improved strain of microorganisms used in the industry [20].

Researchers and process engineers have been used several methods to increase the yield of proteases with respect to their industrial requirements. Approaches for increasing protease yield include screening for hyper-producing strains, cloning and over-expression, fed-batch, chemostat fermentations, and optimization of the fermentation medium through a statistical approach, such as response surface methodology. Bacterial strain improvement by either conventional mutagenesis (UV or chemical exposure) or recombinant DNA technology (rDNA) to selectively generate mutants exhibiting higher protease production is also used for improving protease production in microorganisms. A number of desired protease-encoding bacterial genes have been cloned and expressed in new hosts [45]. Some of the industrially important enzymes are now produced from genetically engineered microorganisms with the aim of (i) enzyme overproduction by gene dosage effect and (ii) protein engineering to locate the active site residues and/or to alter the enzyme properties to suit its commercial applications [46].

The latest trend in enzyme-based detergents is the use of recombinant DNA technology to produce bioengineered enzymes with better stability. Bleach and oxidation stability has been introduced through protein engineering by the replacement of certain amino acid residues. Further, it is recommended to improve the production and its stability under extreme conditions by expressing the gene coding for protease in different vector systems [46, 47].

7. Crude Protease Preparation and Its Purification Techniques

Enzyme purification is a complex process and a number of methods are usually applied in sequence for the purification of enzyme. To get the pure enzyme after the production of protease, several purification techniques can be carried out. A wide range of techniques is available for the recovery of the product from the fermented substrate and the choice depends on the source of enzyme, *i.e.* intracellular or extracellular, scale of operation and enzyme stability [48]. When isolating enzymes on industrial scale for commercial purposes, the prime consideration has been the cost of production in relation to the value of the end product. Therefore, the methods for enzyme extraction and purification is should be economical feasible. The methods of protease production and purification are summarized in the following in Figure 4: in addition this figure show clean industrial production processes in order to mitigate the risk of our environment.

Concentration

The primary objective in enzyme downstream processing is the removal of water because the amount of enzyme present in the cell-free filtrate is usually low so as to the concentration of enzyme become high. Recently, membrane separation processes have been widely used for downstream processing and ultrafiltration is one such membrane process that has been largely used for the recovery of enzymes and formed a preferred alternative to evaporation. This pressure-driven separation process is inexpensive, results in little loss of enzyme activity, and offers purification and concentration as well as diafiltration for salt removal or for changing the salt composition [49].

Precipitation

Precipitation is the most commonly used method for the isolation and recovery of proteins from crude biological mixtures. It also performs both purification and concentration steps. It is generally affected by the addition of reagents such as salt or an organic solvent, which lowers the solubility of the desired proteins in an aqueous solution. Although precipitation by ammonium sulphate has been used for many years, it is not the precipitating agent of choice for detergent enzymes. Ammonium sulphate found wide utility only in acidic and neutral pH values and developed ammonia under alkaline conditions. Hence, the use of sodium sulphate or an organic solvent formed the preferred choice. Despite better precipitating qualities of sodium sulphate over ammonium sulphate, the poor solubility of the salt at low temperatures restricted its use for this purpose [16, 18]. Protease from *Bacillus licheniformis* ATCC21415 and *Pseudomonas aeruginosa* were fold purification obtains for ammonium sulphate [19].

Gel filtration chromatography is convenient technique for protease purification from many bacterial strains like *Bacillus cereus* AT and *Bacillus circulans* [9]. Such purification procedures are also efficient ways to deduce the molecular mass of the enzyme protein using a mixture of proteins of known molecular mass as a reference standard.

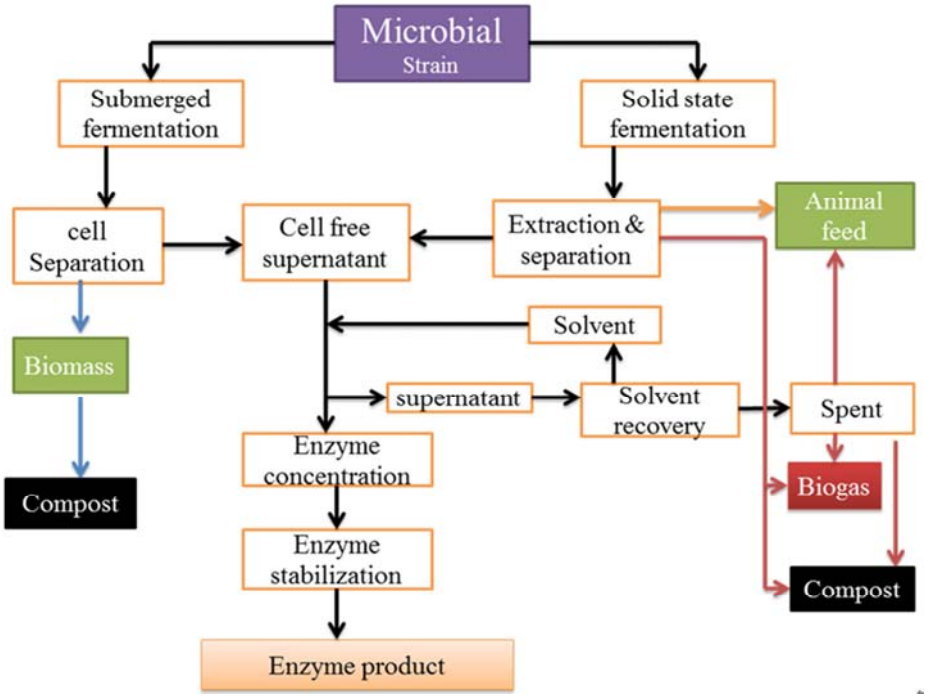

Figure 4. Process flow chart for clean enzyme production and purification.

8. Future Prospects

Several aspects of proteases have stimulated research on the study of biochemical, regulatory and molecular aspects of proteolytic enzyme systems. Regarding the commercial realization of this enzyme class, researchers and process engineers have now started aiming at the discovery and engineering of novel enzymes that are more robust with respect to their pH and temperature kinetics, using genetic and protein engineering techniques.

In the future, protein engineering will offer possibilities of generating proteases with entirely new functions. Hence, although microbial alkaline proteases already play an important role in several industries, their potential is much greater and their applications in future processes are likely to increase in the near future. The pursuit of other, newer approaches, such as novel discovery, metagenomics strategies targeting new dimensions of molecular diversity (ecology), protein/genetic engineering, computational biology and technologies to improve performance of proteases by *in vitro* evolutionary changes of protein primary structures will certainly be the major field of development in next few years. As result ideal proteases will be produced with desired application for specific industry in the near future.

9. Conclusions

Bacterial proteases are among the important hydrolytic enzyme and used extensively since the advent of enzymology. It has great importance due to its wide spectrum applications in detergent industries, bioremediation, food industries, and leather processing and have been widely commercialised by various companies throughout the world. The production of these enzymes has been improved significantly by the utilisation of cheaply available agro-industrial wastes, hyper-producing strains and genetically modified bacteria.

New technology implementation is necessary to promote cleaner production in every industry especially leather and treatment industries by replacing most traditional chemical methods with inexpensive and eco-friendly alternative especially proteases. The higher official and any responsible body give more attention and enforce the investor for cleaner production in order to mitigate the risk of our environments.

References

[1] Balakrishnan, P., Thamaraichelvan, R., Rajendran, N. and Flanet, R. (2012). Production and purification of alkaline serine protease from marine Bacillus species and its application in detergent industry. *European Journal of Applied Sciences,* 4:21-26.

[2] Gupta, R., Beg, Q. K. and Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology,* 59:15-32.

[3] Madhavi, J., Srilakshmi, J., Raghavendr, V. M. and Rao, K. R. S. S. (2011). Efficient leather dehairing by bacterial thermostable protease. *International Journal of Bio-Science and Bio-Technology,* 3:11-26.

[4] Kumar, D. and Takagi, H. (1999). Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnology Advances,* 17:561-594.

[5] Saha, M. L., Begum, K. J. M. H., Khan, M. R. and Gomes, D. J. (2011). Bacteria associated with the tannery effluent and their alkaline protease activities. *Plant Tissue Culture Biotechnology*, 21:53-61.

[6] Younes, G., Sara, R., Alireza, E., Aboozar, K., Maryam, S. and Najme, T. (2011). Screening and isolation of extracellular protease producing bacteria from the Maharloo salt Lake. *Iranian Journal of Pharmaceutical Sciences,* 7:175-180.

[7] Vadlamani, S. and Parcha, S. R. (2011). Studies on industrially important alkaline protease production from locally isolated superior microbial strain from soil microorganisms. *International Journal of Biotechnological Applications*, 3:102-105.

[8] Josephine, F. S., Ramya, V. S, Devi, N., Suresh, B. G., Siddalingeshwara, K. G., Venugopal, N. and Vishwanatha, T. (2012). Isolation, production and characterization of protease from *Bacillus Sp*. isolated from soil sample. *Journal of Microbiology and Biotechnology Research,* 2:163-168.

[9] Vijayaraghavan, P., Lazarus, S. and Gnana, P. V. S. (2013). De-hairing protease production by an isolate *Bacillus cereus* strain AT under solid-state fermentation using cow dung: biosynthesis and properties. *Saudi Journal of Biological Sciences*, 1:1-8.

[10] Rajkumar, R., Ranishree, K. J. and Rengasamy, R. (2011). Purification and characterization of a protease produced by *Bacillus megaterium* RRM2: application in detergent and dehairing industries. *Journal of Basic Microbiology*, 51:614-624.

[11] Jabeen, F. and Qazi, J. I. (2011). Production and optimization of detergent compatible thermostable alkaline protease from *Bacillus cereus* FJ10. *Journal of Scientific and Industrial Research*, 70:1042-1048.

[12] Nadeem, M., Qazi, J. I., Baig, S. and Syed. (2007). Studies on commercially important alkaline protease from *Bacillus licheniformis* N-2 isolated from decaying organic soil. *Turkish Journal of Biochemistry*, 32:171–177.

[13] Aguilar, C. N., Gutierrez, G., Lilia, P. A., Rado, R. H. R., Martínez, J. L. and Contreras, J. C. (2008). Perspectives of solid state fermentation for production of food enzymes. *American Journal of Biochemistry and Biotechnology*, 4:354-366.

[14] Haile, G. and Gessesse, A. (2012). Properties of alkaline protease C45 produced by alkaliphilic *Bacillus Sp*. isolated from Chitu, Ethiopian Soda Lake. *Journal of Biotechnology and Biomater*, 2:1-4.

[15] Pathak, A. P., and Deshmukh, K. B. (2012). Alkaline protease production, extraction and characterization from alkaliphilic *Bacillus licheniformis* KBDL4: A Lonar Soda Lake isolate. *Indian Journal of Experimental Biology,* 50:569-576.

[16] Prabhavathy, G., Rajasekara, M., Pandian and Senthilkumar, B. (2013). Identification of industrially important alkaline protease producing *Bacillus subtilis* by 16s rRNA sequence analysis and its applications. *International Journal of Research in Pharmaceutical and Biomedical Sciences,* 4:332-338.

[17] Kanmani, R., Dhivya, S., Jayalakshmi, S. and Vijayabaskar, P. (2011). Studies on detergent additives of protease enzyme from an estuarine bacterium *Bacillus cereus*. *International Research Journal of Biotechnology*, 2:157-163.

[18] Kumar, D. J. M., Rajan, R., Lawrence, L., Priyadarshini, S. and Kalaichelvan, P. T. (2012). Destaning and dehairing capability of partially purified *Bacillus subtilis* protease from optimized fermentation medium. *Asian Journal of Experimental and Biological Science,* 3:613-620.

[19] Najafi, M. F. (2005). Potential application of protease isolated from *Pseudomonas aeruginosa* PD100. *Electronic Journal of Biotechnology*, 8:197-203.

[20] Rao, M. B., Tanksale, M. S., Ghatge and Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Research*, 62:597-635.

[21] Nduka, O. (2007). Modern industrial microbiology and biotechnology: biocatalysts: immobilized enzymes and immobilized cells. *Published by Science Publishers*, South Carolina, USA, 398-416.

[22] Rao, k., and Narasu, L. M. (2007). Alkaline protease from *Bacillus firmus* 7728. *African Journal of Biotechnology,* 6:2493-2496.

[23] Kumari, D., Sharma, N., Pandove, G., Achal, G. (2009). Alkaline protease production by immobilized cells of *Bacillus pumilis* MTCC 2296 in various matrices. *Life Science Journal*, 6:8-10.

[24] Khan, F. (2013). New microbial proteases in leather and detergent industries. *Innovation Research of Chemistry,* 1:1-6.

[25] Sundararajan, S., Kannan, C. N. and Chittibabu, S. (2010). Alkaline protease from *Bacillus cereus* VITSN04: Potential application as a dehairing agent. *Journal of Bioscience and Bioengineering,* 3:11-22.

[26] Pandian, S., Sundaram, J. and Panchatcharam, P. (2012). Isolation, identification and characterization of feather degrading bacteria. *European Journal of Experimental Biology,* 2:274-282.

[27] Harish R. and Chauhan, B. J. (2017). Isolation, characterization of protease producing microbes from soil of agriculture land and purification of protease. *International Journal of Pharma Research and Health Sciences*, 5 (1):1581-1585.

[28] Kumar, D., Savitri, Thakur, Verma, R. and Bhalla, T. C. (2008). Microbial proteases and application as laundry detergent additive. *Research Journal of Microbiology,* 3:661-672.

[29] Raj, A., Khess, N., Pujari, N., Bhattacharya, S., Das, A. and Rajan, S. S. (2012). Enhancement of protease production by *Pseudomonas aeruginosa* isolated from dairy effluent sludge and determination of its fibrinolytic potential. *Asian Pacific Journal of Tropical Biomedicine,* 3:1845-1851.

[30] Chaudhari, S. G., Chaudhari, S. D., Khobragade, R. M. and Deshmukh, A. M. (2013). Studies on production of alkaline proteases from *Bacillus species* and its application in detergent industry. *DAV International Journal of Science,* 2:44-48.

[31] Bayoumi, R. A., Atta, M. H., Swelam, S. M. and El-Hemiany, A. (2010). Microbial production of thermo alkaliphilic enzymes for application in biodetergent technology. *European Journal of Applied Sciences,* 2:85-93.

[32] Rupali, D. (2015). Screening and isolation of protease producing bacteria from soil collected from different areas of Burhanpur Region (MP) India. *International Journal of Current Microbiology and Applied Sciences*, 4(8):597-606.

[33] Kumar, D. J. M., Premavathi, V., Enkatachalam, N., Govindarajan, M. D., Balakumaran and Kalaichelvan, P. T. (2012). Production and purification of alkaline protease from *Bacillus sp*. MPTK 712 isolated from dairy sludge. *Global Veterinaria,* 8:433-439.

[34] Shankar, S., More, S. V. and Seeta, L. R. (2010). Recovery of silver from waste x-ray film by alkaline protease from *Conidiobolus coronatus*. *Kathmandu University Journal of Science, Engineering and Technology*, 6:60-69.

[35] Masui, A., Fujiwara, N., Takagi, M. and Imanaka, T. (1999). Feasibility study for decomposition of gelatin layers on X-ray films by thermostable alkaline protease from alkaliphilic *Bacillus sp*. *Biotechnological Techniques,* 13:813-815.

[36] Nakiboglu, N., Toscali, D. and Yasa, I. (2001). Silver recovery from waste photographic films by an enzymatic method. *Turkish Journal of Chemistry*, 25:349-353.

[37] Annapurna, S. A., Singh A., Garg, S., Kumar, A. and Kumar, H. (2012). Screening, isolation and characterisation of protease producing moderately halophilic microorganisms. *Asian Journal of Microbiology, Biotechnology and Environmental Science,* 14:603-612.

[38] Rajasekhar, A., Ravi, V., Reddy, M. N. and Rao, K. R. S. (2011). Thermostable bacterial protease - anew way for quality silk production. *International Journal of Bio-Science and Bio-Technology,* 3:122-131.

[39] Singh, P., Rani, A. and Chaudhary, N. (2015). Isolation and characterization of protease producing Bacillus sp from soil. *International Journal of Pharma Sciences and Research*, 6(4):633-639.

[40] Priya, V. V. G., Preethi, S., Karthikeyan, S. and Babu, R. N. G. (2016). Isolation and identification of protease producing bacteria from soil. *International Journal of Research in Engineering and Technology,* 3(8):1362-1365.

[41] Verma, T. and Agarwa, S. (2016). Isolation and screening of haloalkaline protease producing bacteria from tannery solid waste. *International Journal of Research in Engineering and Technology,* 5(1):237-244.

[42] Krishnaveni1, D. J. K., Kumar, M., Balakumaran M. D., Ramesh, S. and Kalaichelvan, P. T. (2012). Production and optimization of extracellular alkaline protease from *Bacillus subtilis* isolated from dairy effluent. *Pharmacia Letter*, 4:98-109.

[43] Prabhavathy, G., Rajasekhar, M., Pandian and Senthilkumar, B. (2012). Optimization and production of extracellular alkaline protease by solid state fermentation using *Bacillus subtilis*. *Journal of Academic Indian Research,* 1:428-430.

[44] Rathakrishnan, P. and Nagarajan, P. (2012). Optimizing factors affecting protease production by a *Bacillus cereus* using groundnut shell under solid substrate fermentation. *International Journal of Science Inventions Todays*, 1:114-129.

[45] Rani, M. R., Kumari, B. L., Sri, M. H. and Prasad, D. S. (2012). Isolation and screening of alkaline protease producing bacteria and induction of overproducing *Bacillus licheniformis* mutants through UV irradiation. *Journal of Pharmacy*, 1:1-14.

[46] Mienda, B. S. and Yahya, A. (2011). Engineering of microbial proteases: Improving stability and catalytic performances. *IIOAB Journal,* 2:10-15.

[47] Singha, P. Nigam, V. K. and Vidyarthi A. S. (2012). Studies on production, characterization and applications of microbial alkaline proteases. *International Journal of Advanced Biotechnology and Research*, 3:653-669.

[48] Sumantha, A., Larroche, C., and Pandey, A. (2006). Microbiology and industrial biotechnology of food-grade proteases: a perspective. *Food Technology and Biotechnology*, 44:211-220.

[49] Naidu, K. S. (2011). Characterization and purification of protease enzyme. *Journal of Applied Pharmaceutical Science,* 1:107-112.