Alkaline Protease Producing Bacteria: Classification and Characterization of Protease

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Abstract

A wide variety of bacterial species produces protease enzyme, and the application of the same enzyme has been manipulated precisely and used in various biotechnological areas including industrial and environmental sectors. The main aim of this research study was to isolate, screen, and identify alkaline protease-producing bacteria that were sampled from leather industry effluent present in the outer skirts of Addis Ababa, Ethiopia. Cells of every living organism consist of a chemical substance that possesses the ability to catalyse or speed up a biochemical reaction and acts as biocatalysts, which are known as enzymes. Enzymes have better catalytic efficiency, adjustable activity, and high specificity when compared to catalysts of chemical or synthetic origin.

Introduction

Biotechnology uses living cells from plants, animals and microorganisms such as yeasts, moulds and bacteria as well as enzymes from living cells to produce value-added products and services. The living cells are considered as "cell factories" to produce enzymes, antibiotics, vitamins, vaccines and proteins for different applications. Successful application of biotechnology involves an integrated approach encompassing different disciplines such as microbiology, biochemistry, genetics, molecular biology, chemistry and chemical engineering. Biotechnology can reduce pollution and wastes, decrease the use of fossil fuels, raw materials, lead to better quality food products, create new bio-based materials and provide environmentally friendly alternatives to certain chemical and physical processes. Efficient uses of natural biological resources as a strategy for sustainable industrial growth are of paramount significance in achieving economic, social and environmentally sustainable human development. In living cells, the metabolic reactions are mediated by enzymes which catalyze the reactions with greater specificity and rate enhancement. Such catalytic properties together with environmental compatibility and easy disposal of wastes provide tremendous opportunities for industrial applications. Enzymes are increasingly used in industries employing harsher processes which employ thermal, chemical and mechanical technologies to convert raw materials into products. Enzymes are also used to catalyse many industrial processes as diverse as textiles, paper manufacture, food processing, diagnostic kits, washing liquids and medicine. Industrial enzymes can be obtained from various sources, which include plants, animals and microorganisms. Nowadays many enzymes are being produced in large-scale from microorganisms, because of myriad number of

advantages (Many microorganisms and their enzymes have been discovered by means of extensive screening and are now commonly used in food and other industrial applications. The discovery of new microbial enzymes through persistent and extensive screening has proved to be antidote to many of the industrial and environmental problems.

Protease

Proteases are also known as peptidyl-peptide hydrolases, they are industrially useful enzymes which catalyse the hydrolysis of peptide bond from protein molecule. Proteases constitute 50 – 65% of the global industrial enzyme market, most of which are alkaline protease Given the wide application of this enzyme, it is reported that the global proteolytic enzyme demand will increase dramatically to 5.0 – 5.2 billion dollars in years to come .Proteases being ubiquitous are found in plants, animals, and microorganisms. Despite the large number of protease producing microorganisms, only a few are considered as appropriate producers for commercial exploitation being "generally regarded as safe" Currently, a large proportion of the commercially available proteases are derived from Bacillus. Though many different exotic environments have been explored and exploited for different protease-producing microorganisms, the marine ecosystem is one among the least studied.

Classification of Protease

Proteases are classified based on chemical nature of the active site, the reaction they catalyze, and their structure and composition .The major classes are again classified into sub classes based on pH, catalytic site on polypeptide, occurrence, and so on. Based on the catalytic site on the substrate, proteases are mainly classified in to end proteases and exoproteases. End proteases preferably act at the inner region of the polypeptide chain. By contrast, exoproteases preferentially act at the end of the polypeptide chain. Exoproteases are further classified into amino peptidases, those proteases which act at the free N-terminus of the polypeptide substrate and carboxypeptidases, those proteases which act at the free C-terminal of the polypeptide chain Similarly, end proteases are also classified based on the functional group present in active site and pH optimum. The different classes of proteases based on their catalytic active site include:

Serine proteases	Cysteine proteases	Metalloproteases	Aspartic protease	Other rare proteases
• Serine proteases are proteases having a serine group (-OH) in their active site.	• Cysteine proteases are proteases having a thiol (-SH) group in their active site.	• Metalloproteases are proteases requiring divalent metal ion for their catalytic activity.	• Aspartic proteases are proteases with aspartic residue at their catalytic active site.	• They also contain amino acid residues at their active site, such as threonine and glutamic acid.

Based on their optimal pH, proteases are also classified as:

a) Acid Proteases

Acid proteases are proteases which are active in the pH ranges of 2-6 (Rao et al. 1998) and are mainly of fungal in origin (Aguilar et al., 2008). Common examples in this subclass include aspartic proteases of the pepsin family. Some of the metalloprotease and cystein proteases are also categorized in as acidic proteases

b) Neutral Proteases

Neutral proteases are proteases which are active at neutral, weakly alkaline or weakly acidic pH. Majority of the cystein proteases, metalloproteases, and some of the serine proteases are classified under neutral proteases. They are mainly of plant in origin, except few fungal and bacterial neutral proteases (Aguilar et al., 2008).

c) Alkaline Proteases

Alkaline proteases are optimally active in the alkaline range (pH 8-13), though they maintain some activity in the neutral pH range as well (Horikoshi, 1999). They are obtained mainly from neutralophilic and alkaliphilic microorganisms such as Bacillus and Streptomyces species. In most cases, the active site consists of a serine residue, though some alkaline proteases may have other amino acid residue in their active site.

Proteases can be classified according to 3 major criteria as given below:

i) The reaction catalysed

ii) The chemical nature of the catalytic site

iii) The evolutionary relationship, as revealed by the structure.

They are currently classified into six broad groups: Serine proteases, threonine proteases, cysteine proteases, aspartate proteases, metalloproteases and glutamic acid proteases depending on their catalytic mechanism. They are also classified into different families and class depending on their amino acid sequences and evolutionary relationships. Based on the pH of their optimal activity, they are referred to as acidic, neutral, or alkaline proteases.

Applications of Halophile Organic Solvent Tolerant Proteases

Alkaline protease are robust enzymes with considerable industrial potential in detergents, leather processing, silver recovery, medical purposes, food processing, feeds and chemical industries, as well as waste treatment. The different applications of alkaline proteases currently employing are:

a) Detergent Additives Microbial alkaline proteases dominate on commercial applications with significant share of the market captured by subtilizes and or alkaline proteases from Bacillus sp for laundry detergent applications (Ward, 1985). Alkaline proteases added to laundry detergents enable the release of pertinacious material from stains (Masse and Van Tilburg, 1983). In addition to

improve washing efficiency, the use of enzymes allows lower wash temperatures

b) Tannery Industry

Alkaline proteases possessing elastolytic and keratinolytic activity offer an effective biotreatment of leather, especially dehairing and bating of skins and hides (Taylor et al., 1987). The alkaline conditions enable the swelling of hair roots and subsequent attack of proteases on the hair follicle protein allowed for easy removal of the hair. The strong alkaline conditions is pleasant and safer than traditional methods using sodium sulfide treatment, which contributes to 100% of sulfide and over 80% of the suspended solids in tannery effluents .

c) Medical Uses

Collagenases with alkaline protease activity are increasingly used for therapeutic applications in the preparation of slow release dosage forms. A new semi alkaline protease with high collagenolytic activity was produced from Aspergillus niger LCF9. The enzyme hydrolyzed various collagen types without amino acid release and liberated low molecular weight peptides of potential therapeutic use.

d) Waste Treatment

Alkaline proteases provide potential application for the management of wastes from various food processing industries and house hold activities. These proteases can solubilize proteins in wastes through a multi-step process to recover liquid concentrates or dry solids of nutritional value for fish or livestock (Shoemaker, 1986). De Castro et al. (2006) reported an enzymatic process using a B. subtilis alkaline protease in the processing of waste feathers from poultry slaughter houses.

e) Photographic Industries

Proteases find potential application in the bioprocessing of used X-ray films for silver recovery. Used X-ray film contains approximately 1.5-2.0 % (by weight) silver in its gelatin layers. The enzymatic hydrolysis of the gelatin layers on the X-ray film allows the silver as well as the polyester film base, to be recycled. Alkaline proteases can also be used for silver recovery (Kumar and Takagi, 1999).

Objective of Study

1. Identification of protease producing bacteria from soil
2. Isolation and screening of protease producing bacteria from soil .
3. Quantitative estimation of alkaline protease produced by bacteria.
4. Optimization of the most potential strain for various physicochemical parameters.

Review of Literature

Razak et al., (1997) isolated thermophilic Bacillus sp. in Sampah-sarap island of Malaysia and observed the physico chemical parameters affecting the protease production at pH-8, 50oC and 150

rpm. Mohamed et al., 1998 isolated alkaline protease in the submerged culture of Bacillus mycoides. The optimal conditions for enzyme production were pH 9.0, 3% (w/v) dextrin as carbon source, (NH4) So4 (0.1%, w/v) and peptone (0-1%, w/v) as nitrogen sources and KH2SO4 at 0.1% g/L. The enzyme was immobilized on various carriers by different methods of immobilization. The optimal reaction temperature of the immobilized enzymes was shifted from 50 to 55°C.

Kumar (1999) reviewed on the proteases that can resist extreme alkaline environments produced by a wide range of alkalophilic microorganisms. They reviewed on different isolation methods, strain improvement, optimisation aspects, purification and practical application of alkaline proteases.

Yanga et al., 2000 isolated the protease-producing microorganism in northern Taiwan and identified a strain of Bacillus subtilis. B. subtilis Y-108 was used for deproteinization of crustacean wastes in the preparation of chitin. Liquid phase fermentation of untreated shrimp shell, crab shell, and lobster shell wastes were done with this microbe which showed protein removal of 88, 67, and 83%, respectively. In contrast, the protein removal of the acid treated wastes was 76, 62, 56%, respectively.

Gupta et al., (2002) reviewed on protease enzyme which included the types and sources of proteases, protease yield-improvement methods, the use of new methods for developing novel proteases and applications of alkaline proteases in industrial sectors, with an overview on the use of alkaline proteases in the detergent industry.

Park et al., (2003) isolated four proteolytic bacteria from a rotating biological contactor based on Bacillus. The four isolates, Ni 26, 36, 39 and 49 were identified as B. vallismortis, B. subtilis, Aeromonas hydrophila and B. amyloliquefaciens, respectively based on their biochemical properties and 16S rDNA sequence analyses. The optimal proteolytic activity was observed in the temperature and pH ranges of 40-70ºC and 8.0-8.5, respectively. Zymographic analyses of the culture supernatants revealed the presence of at least two proteases in all isolates and inhibitory studies revealed that it was a metalloproteases.

Kanlayakrit and Bovornreungroj (2005) studied the fermented fish samples from fish sauce factories in the eastern part of Thailand which were used as sources for the isolation of salt-loving protease-producing bacteria. Two hundred and eighty five strains of halophilic bacteria were isolated and a total of ten strains were of extremely halophilic bacteria exhibited the highest salt-loving proteases producing ability. Maximal growth and salt-loving proteases of these strain occurred in modified M73 (mM73) agar medium at 4M (24 %, w/v) NaCl. It was concluded that the extremely halophilic bacterial strain PB407 belonged to the genus Halobacterium.

Kanmani et al., (2011) produced the protease enzyme from the bacterial strain Bacillus cereus. The strain was isolated from the estuarine sediment sample on casein agar medium. Based on the results obtained from optimization, the mass scale culture was made with pH 9.0; 40oC temperature; 35ppt of salinity; 3% of the casein as the suitable substrate. Ammonium sulphate saturation was made and the enzyme was partially purified and lyophilized. 80% saturation has given the maximum saturation of 0.9g. SDS-PAGE resulted in the separation 55 KDa and 20 KDa, both representing the enzyme

protease. Application study was carried out with the enzyme recovered from Bacillus cereus. The purified enzyme was included in to the stain removal procedure. There was the better stain removing observation at the time of using the protease enzyme.

Ghasemia et al., (2011) screened and identified moderately halophilic protease producing bacteria from different regions of Maharloo, a hypersaline lake in the southern area of Iran, which were able to grow comfortably in the media containing 7-15% of salt. Protease activity was assessed through in vitro colorimetric assays for general proteases and the strains were identified by 16S rDNA molecular marker. Among the strains tested, Bacillus sp. BCCS 041 was found as the highest protease producing strain with 0.43 U/ml of supernatant activity.

Material and Methodology and Characterization

Determination of molecular weight of the enzyme by SDS-PAGE- (Laemmli, 1970)

Introduction

Sodium dodecyl polyacrrylamide gel electrophoresis (SDA-PAGE) is probably the most widely used technique for analysing mixtures of proteins with a high resolving power. With SDS, an ionic detergent, proteins loose their individual charges and a net negative charge is formed due to complexing of SDS with protein. The update of detergent is the same per unit mass (1.4g SDS per gm of protein) for all proteins and hence the mobility on electrophoresis is proportional to the molecular mass. Depending upon the size of the proteins to be separated, the concentration of acrylamide is selected to optimize the sieving effect. In addition, a stacking gel of low acrylamide concentration allows rapid movement of sample to the top of separating gel in the form of narrow zone of proteins. In this method the proteins are separated by SDS-PAGE electrophoresis and size of polypeptide chains of given protein is determined by comparing its electrophoresis mobility in SDS-PAGE gel with standard marker proteins of known molecular weight.

Gel Casting

Chemistry Involved in Gelling

Polyacrylamide gel results from the polymerization of acrylamide monomers into long chains and cross linkages are brought by N-N-methylene bisacrylamide. Polymerization of acrylamide is initiated by the addition of either ammonium per sulphate or riboflavin. Tetra methylene diamine (TEMED N-N-N-N) acts as an accelerator of polymerization. Effective pore size of polyacrylamide gel is greatly influenced by the total acrylamide concentration in the polymerization mixture. Buffer system in PAGE is designed in such a way that the protein is separated into individual polypeptide.

Sample Preparation and Electrophoresis

The samples were mixed with equal amount of sample buffer and were boiled in a boiling water bath for 5 min. After that, the samples were loaded into the wells and allowed for electrophoresis at 50V

initially. After the dye front had reached the end of the stacking gel, the voltage was increased to 100V and proteins were allowed to migrate through resolving gel.

Procedure

The casting apparatus was assembled and the gel volume was determined using distilled water. Then the solution for the resolving gel of 15% concentration was prepared. The solution was poured into the gel plate and overlaid it with a layer of butanol: water (1:1) to prevent exposure of the gel to air, allowed to solidify and then the solution for the stacking gel was prepared. The butanol layer was removed and washed twice or thrice to remove traces of butanol. The components of the stacking gel (5%) were added; the gel solution was poured over the separating gel and allowed to solidify after placing the comb.

Sample loading

Protein marker and samples were loaded into the wells of the stacking gel. The electrophoresis buffer was added in to the top and bottom reservoir. The samples were electrophoresed at 100V until the tracking reached 0.5cm from the bottom of the plate. Power supply was turned off and the gel was kept in the staining solution.

Statistical Analysis

Statistical analysis was run using Statgraphics Centurion XVI version 16.1.18 software (Statgraphics Centurion for Windows, Statpoint Technology, Inc., USA). Analysis of variance (ANOVA) of each variable of soil characterization was performed by a completely randomized block design, where each block was the soil sample. Block means were compared using the least significance difference (LSD) multiple range test, calculated at 0.05 probability level ($P < 0.05$). Principal component analysis (PCA), a conventional multivariable technique, was performed for soil physicochemical variables to identify groups of variables contributing most to microbial diversity between study areas. PCA is based on the correlation (covariance) matrix, which measures the interrelationships among multiple variable.

Conclusion

Thus in the present study a soil originated B. cereus strain was characterized and it was found to be an ideal producer of serine alkaline protease. This study also revealed the potential for the industrial scale protease production using this strain. The abundance of protease producers in dairy plant soil sample also indicated it as a new source for the search of industrially important serine alkaline proteases. Enzymes are the best catalysts known for their high efficiency in biosystems and in many of the emerging industrial processes. Lipase is one such enzyme having the great potential for the conversion of lipid molecules in and out of the biological systems and thus help the environment to sustain the life from the time immemorial. This vital property of lipase has been recognized and been explored for many of the industrial processes of dairy products, detergents, textiles, leather and so on

to serve the mankind. In this endeavor these lipases were extracted and produced from various biological sources and one of such sources being the bacteria has made the research to be focused in search of novel bacterial strains.

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References:

1. Abdulwahed et al., S.P., J.D. Kim, S. Machida and K. Hayashi, 2019. Overexpression and protein folding of a chimeric ß-glucosidase constructed from Agrobacterium tumefaciens and Cellvibrio gilvus. Indian J. Biochem. Biophy.
2. Abou-Elela et al., G., M. R. Pandian and B. Senthilkumar, 2011. Identification of industrially important alkaline protease producing Bacillus subtilis by 16s rRNA sequence analysis and its applications. Inter. J. Res. Pharmaceu. Biomed. Sci., 4 (1).
3. Bajaj, Jamwal and R. Seeta Laxman, 2014. Purification and characterization of an alkaline protease by a new strain of Beauveria sp. Process. Biochem., 46: 579–585.
4. Banerjee et al., H. S., 1999. Isolation and screening of extracellular proteases produced by new isolated Bacillus sp. J. Appl. Pharmaceut. Sci., 2 (9) : 71-74.
5. Banerjee et al., M., and E.C. Webb, 1999. Enzymes, 3rd edn. Longmans, Green & Co., London, and Academic Press, New York.
6. Boominadhan et al., M.F., D. Deobagkar and D. Deobagkar, 2009. Potential application of protease isolated from Pseudomonas aeruginosa PD100, Electronic J. Biotechnol., 8:
7. De Coninck et al., C. G. and H. Takagi, 2000. Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnol. Adv., 17: