Isolation of Bacillus sp. for its Protease Activity and Detergent Compatibility

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Abstract: ***Alkaline and heat stable proteolytic enzymes were introduced into detergent products in the 1960's to improve the performance of the detergent via their ability to hydrolyse proteins imbedded in fabrics. A protease producing bacillus bacterium isolated from garden soil and bacillus licheniformis 8600 NCIM are used to investigate the protease activity and detergent compatibility with commercially available detergents. Four different detergents were used to test the activity. The maximum amount of enzyme production was observed around 48h of incubation at room temperature around 34-370c, when the bacteria were grown in kcl rich agar medium and nutrient agar media respectively. The protease activity was observed in 1% casein media. The protease enzyme was partially purified by filtration, centrifugation and ammonium sulphate precipitation. Partially purified enzyme was obtained. Test was conducted with protease obtained by centrifugation method. In compatibility studies protease showed considerable stability in the presence of detergents and retained more than 80% of activity.***

Keywords: proteases, bacillus, detergent, stain, removal.

1. Introduction

Protease is an enzyme that conducts proteolysis, which begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. It occurs naturally in all organisms. Proteolytic enzyme, also called proteinase, any of a group of enzymes that break the long chainlike molecules of proteins into shorter fragments (peptides) and eventually into their components, amino acids. Enzymes are proteins that are found in every living organism: man, animals, plants and microorganisms. Nature — including human digestive systems — relies on enzymes to break down proteins, starches and fats. The same types of enzymes can be used in detergents to break down the stains that bind to fabrics. High throughput screening, based on predictive micro screen assays, is used to identify the best enzyme candidates. For instance, Novozymes has developed a technology platform that allows it to scan the capabilities of more than a million enzymes each week. The most promising candidates are then subjected to more complex testing and additional screening to assure a strong correlation between specific micro screen assays and the real-life application. After a specific enzyme has been identified and the relevant genes isolated, an efficient expression system for the enzyme must be developed. ***Bacillus licheniformis:***

Bacillus licheniformis is a Gram-positive, motile, spore-forming, facultatively anaerobic rod belonging to the *B. subtilis* group of Bacilli. It is an apathogenic soil organism that is mainly associated with plant and plant materials in nature but can be isolated from nearly everywhere due to its highly resistant endospores that are disseminated with the dust.

Bacillus licheniformis is used by industry to produce proteases and amylases. Proteases are needed in huge amounts for example as additions to washing agents. An enzyme was first used to improve the effectiveness of a laundry detergent in 1913 by a German named Otto Rohm, the founder of the giant chemical company Rohm and Hass. The proteolytic enzyme he used derived from milled animal pancreases, was quite crude and contained many impurities which, in turn, sometimes stained the very textile it was supposed to clean. Neither was the process of enzyme extraction economical enough to include it routinely in household detergents. Commercially feasible quantities are now produced through fermentation of *Bacillus subtilis* or *Bacillus licheniformis.* This was made possible in the last two decades by the rapid advances in enzymology and fermentation.

2. Materials and Methods

2.1 Isolation and screening of bacteria producing proteases

Collection of Samples

The rhizosphere soil samples were collected from the garden area of ASTRA campus. The serially diluted 0.1ml of the appropriate dilutions was inoculated on casein agar plates and the plates were incubated at 370 C for 24-48 hrs. The isolated cultures were preserved in casein agar slant tubes for further study. The protease producing cultures were identified as per the standard procedure (Buchanan and Gibbons, 1974).

Bacillus licheniformis 8600 pure culture is obtained from MTCC, pune. This was in its lyophilized form to compare the protease activity.

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2.2 Composition of the media used for isolation media(selective/lit,wt/vol)

2.3 Isolation The bacterial cultures was streaked onto Nutrient agar plates and incubated at 37ºC for 24h. In order to obtain single colonies the mechanism of streaking employed was quadrant and simple streaking.

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2.4 Production of Protease Enzyme

Bacterial cultures were serially diluted and were plated on skimmed milk agar plates to get colonies. Plates were incubated at 37°C for 72h to visualize the zone of clearance. This is formed due to the degradation of casein by proteases that are produced by *Bacillus* and AB-1 strains.

2.5 Purification

Purification of the protein is done in three stages namely:
Ammonium sulphate precipitation
Filtration
centrifugation

2.6.1 Ammonium sulphate precipitation

A. Ammonium Sulphate Precipitation is a simple and effective means of fractionating proteins. It is based on the fact that at high salt concentrations the natural tendency of proteins not to aggregate is overcome, since the surface charges are neutralized. Charge neutralization means that proteins will tend to bind together, form large complexes and hence are easy to precipitate out by mild centrifugation. Since each protein will start to aggregate at a characteristic salt concentration, this approach provides a simple way of enriching for particular proteins in a mixture. Ammonium sulphate precipitation is the most commonly used salt precipitate because it is highly soluble in water, it stabilizes most proteins in solution, and helps reduce the lipid content of the sample.)50gms of ammonium sulphate is dissolved in distilled water which will get saturated. Nutrient broth of about 10ml was added in this saturated solution which results in precipitation of crude form of enzyme.

2.6.2 filtration. Filtration is one such method to partially purify the enzyme. Typical filtration was carried out by watt man's filter paper in which nutrient broth containing desired bacillus was filtered through filter paper. Two different bacteria i.e. *bacillus licheniformes* and AB-1 bacteria was separately filtered to purify enzyme.

2.6.3 Centrifugation Centrifugation is one such method were maximum protease purification was observed. Basic steps involved are:

Transfer 5ml nutrient broth containing *bacillus licheniformes* in four centrifuge tube and AB-1 bacteria in another four tubes. These tubes were placed in centrifuge by adjusting 1000 rpm for 10minn.Supernatent was collected and used for further steps for detergent activity.

Maximum protease was obtained through centrifugation method

3. Washing Test with Protease Preparation

Application of protease enzyme as a detergent additive was studied on white cotton cloth pieces stained with human blood, chocolate, coffee and turmeric. Four different commercially available detergents i.e. Ariel, surf excel (Hindustan lever), tide and Xtra where procure from the market and the compatibility test was carried out.

Maximum growth was observed at pH 7.4 and the highest protease activity was recorded at pH 7.4. 37° C was the optimal temperature or the bacterial growth and the maximum enzyme production was recorded at 37° C.

Bacillus usually produces extra cellular protease during late exponential phase (Ward, 1985). Maximum production of protease with 48 to 72 hours of incubation by bacteria.

4. Results and Discussion

Proteases are enzymes which catalyze the hydrolysis of peptide bond in proteins or peptides. They are present in bacteria, plant and animal tissues. The microorganisms are capable of producing these enzymes intracellular and extracellularly. The isolation of proteases especially the extracellular proteases of microbial origin is easy and economical. The diversity of proteases available from microorganisms is great. They belong to acid, neutral and alkaline types which possess wide spectrum of characteristics that make them utilizable for different commercial applications.

A total of 3 morphologically distinct colonies were isolated by using kcl,nutrient, agar medium and selective medium from the collected soil sample. The potentiality of the isolates was checked by measuring the clear zone formed around the colony. The proteolytic activity was assayed using casein agar and expressed as diameter of clear zone.

4.1. Clone using Zone Clearance Assay:

Bacterial cultures were serially diluted and were plated on casein agar plates to get colonies. Plates were incubated at 37°C for 72h to visualize the zone of clearance. This is formed due to the degradation of casein by proteasesA` that are produced by *Bacillus* and AB-1 strains.

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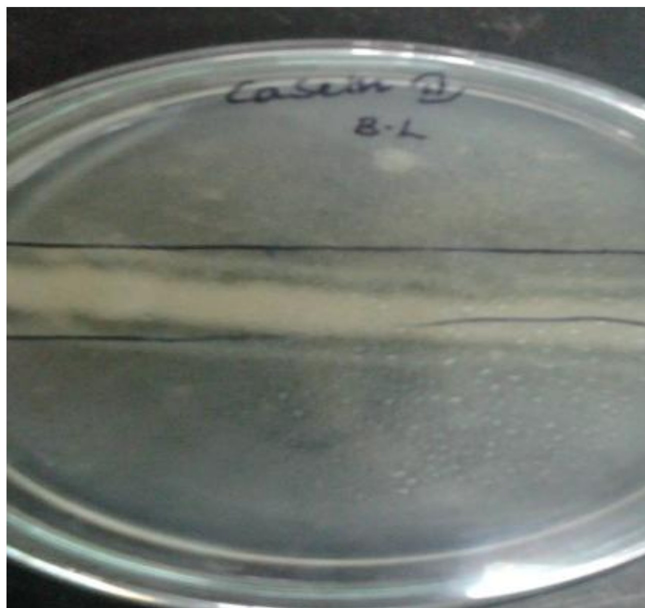

Figure 1: Zone of clearance

Table 1: Morphological tests for identification of *Bacillus*

Test	AB-1	NCIM-8600
Shape	Bacilli	Bacilli
Spore formation	+ +	+ +
Motility	+ +	+ +
Colony formation	++	++
Turbid layer formation	++	++

Washing performance of protease from *Bacillus licheniformis* and AB-1

5. Conclusions

A study was carried out with the objective of isolation, purification and compatibility detergent activity of alkaline protease from *Bacillus licheniformis and AB-1 expressed* alkaline protease was found to be most active at pH 8 and 40°C

The protease enzyme produced in the present study, removed the blood, chocolate, turmeric and coffee stains within 60 minutes. Thus the protease from *Bacillus licheniformis 8600 and AB-1* strain was compatible with different commercially available detergents and even retained the activity in the presence of detergents. We can conclude that it has potential to use in detergent industry and can be useful in detergent formulation

6. Future Scope

Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. The worldwide requirement for enzymes for individual applications varies considerably. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations; whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used.

Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure-function relationship, in the synthesis of peptides, and in the sequencing of proteins.

In essence, the wide specificity of the hydrolytic action of proteases finds an extensive application in the food, detergent, leather, and pharmaceutical industries, as well as in the structural elucidation of proteins, whereas their synthetic capacities are used for the synthesis of proteins.

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Author profile

G. Harathi received my B.Tech and M.Tech degrees in biotechnology from JNTU aurora scientific technological research academy (astra) and Osmania University in the year 2008-2012, 2012-2015 respectively. Now at present got eligible in PhD entrance test, awaiting for the rest of the process to persue my PhD.

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