# PRODUCTION AND CHARACTERIZATION OF PROTEASE ENZYME FOR BLOOD STAINS REMOVAL IN SURGICAL WITH GROUNDNUT OIL CAKE AS A SUBSTRATE

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## ABSTRACT

Protease is a vital extracellular enzyme produced by bacterial and fungal species to utilize the protein from its substrate. Groundnut oil cake is a rich and cheap source of protein, which makes it as an excellent substrate for protease production. During surgery maintaining sterile condition in surgical instrument and clothes is a vital to avoid infection. Detergents in combination of alkaline protease can be used to remove the blood stains completely as blood is pretentious. Production of peptone as a substrate makes it costlier restricting its commercial application in large scale such as detergents. Stains isolated from the dairy effluents was identified to be bacillus cereus by 16rRNA partial sequencing and further confirmed by biochemical tests. Comparative study of production media with peptone and groundnut oilcake as substrate showed maximum protease production in 36hr and 48hr respectively. Groundnut oil cake as substrate showed maximum protease production than production media with peptone as substrate. Optimization of partially purified enzyme showed maximum activity at PH of 9.0$\pm$0.2 and temperature of 60°C$\pm$0.2. Physical observation of washed clothe stained with the blood stained showing maximum washing performance with detergent and enzyme combination treated for 30 min

**KEYWORDS**: groundnut oil cake, 16s rRNA partial sequencing, *Bacillus cereus* and detergents

## INTRODUCTION

Bulk production is a vital process in any industrial processes, to make the production cost effective, raw material required for the production should be cheap, readily available and high efficiency in the production. Protease is a vital enzyme and has many commercial applications like leather tanning, food industry, detergents etc. These applications require bulk production in

cheaper means. It can be done by the supply of cheap substrate, better strain of protease producer and optimized condition for the production [1]. The main aim of the article is the production of protease with cost effective groundnut oil cake as a substrate by the organism isolated from dairy effluent and its application in removal of blood stains from the surgical cloths. The objective is the isolation of different bacterial colonies from soil effluents and screening of colonies for protease activity using skim milk casein agar .Characterization of the isolated strain using 16s rRNA sequencing and biochemical tests .Optimization of the purified enzyme with PH, temperature, fermentation , time as variable.

Protease is an enzyme that conducts proteolysis, i.e. protein catabolism by hydrolysis of the peptide bond that link amino acids together in the polypeptide chain forming the protein. This enzymes are also known as peptidase or proteinase. Protease, belong to the class of enzyme known as hydrolases, which catalyze the reaction of hydrolysis of various bonds with the participation of water molecules. These enzymes are involved in multitude physiological reactions from simple digestion of proteins to highly regulated cascades .Bacteria also secrete protease to hydrolyze the peptide bonds in proteins to their constituent monomers. Bacterial proteases are of high importance to the global carbon and nitrogen cycle in the re-cycling of proteins [2].

Protease action in removing stains includes stains caused by blood, food which is made of protein will bind to the wardrobe fibers strongly reducing the action of non-enzymatic detergents which further leads to permanent stains due to oxidation and denaturation action of bleaching agents, and for example bloods stains will leave rust colored. Protease helps in cleaning surgical instruments. Surgery is a complex process which is performed by several surgical instruments which is costly. During surgical process blood from the patients will come in contact with instruments and clothes invariably. Such instruments has to be washed thoroughly to avoid potential danger . Non enzymatic detergents cannot remove stains from the corner of instruments and in surgical clothes. Moreover surgical chemicals are costly, an alternative usage of enzymatic detergents i.e. protease enzyme + detergents.

## METHODOLOGY

**Isolation of bacterial colonies from soil sample:** Serial dilution was carried out to enumerate bacterial population from the soil sample. It will reduce the organism count in logarithmic pattern for each dilution carried out bringing the bacterial population within the countable range

**Screening for colonies on nutrient agar:** After incubation, colonies were counted in each plate, and plates which were within the countable range(30-300colonies) are considered (cappuccino and Sherman,2004) .From the selected plates different types of colonies were isolated based on its morphological character like size and shape of colony. The isolated colonies were grown in fresh nutrient agar plates.

**Screening for protease producing bacteria by skim milk casein agar:** Protease producers when grown in skim milk casein agar media will release the protease enzyme to utilize the protein in media thereby forming clear zone surrounding the colony. This confirms the protease activity of the organisms.

**Groundnut oil cake agar media:** Groundnut oil cake was considered in the place of casein and skim milk to check the protease activity in oil cake

**Gram's staining:** Smear was made from the isolated strain in clean, grease free glass slide. Smear was then stained with few drops of crystal violet for 2min and excess stain was washed with distilled water, followed by gram's iodide for 1min.70%ethanol was used as a decolorizing agent .Finally, the smear was treated with safranin for 10seconds.After air drying the smear ;It was observed under the microscope.

**Biochemical Tests:** The isolated strain was subjected to various biochemical tests such as indole, Methyl red, Voges-proskaeur, citrate, catalyze, nitrate , haemolysis and gelatinase tests for the identification of strain species by the principle of biochemical reaction

**Production media for protease production:** Production media was prepared for the production of protease enzyme with peptone as substrate and other nutrients and salts in the following composition. Media is was prepared in 250ml conical flasks and sterilized this media is considered as media 1. Production media was prepared for the produced of protease enzyme with crude groundnut oil cake as substrate in the place of peptone. Other nutrients and salts were used in the following composition . It was prepared in 250 ml conical flasks and sterilized . This media was referred as media 2. Protease enzyme produced in the production media, was

analyzed for its protein concentration and enzyme activity at various time interval of fermentation. Protease is the extracellular enzyme hence the supernatant was collected from the production media sample.10ml of culture was collected in centrifugal tube in sterile condition Collected sample was centrifuged using REMI R-24 centrifuge 10000rpm for 20 min. The supernatant was collected in a sterile vial. Protein content of the collected supernatant (enzyme) was estimated by lowry's method for samples collected at different time intervals. Activity of enzyme was quantified by the modified Anson method .Enzyme present in the sample will react with casein to release tyrosine. One unit of protease activity was defined as the amount of enzyme which releases1mueM of tyrosine per min under assay condition with tyrosine as standard.

**Downstream Processing:** Enzyme has to be processed to obtain the purified enzyme, and its done by: Ammonium Sulphate Precipitate and Dialysis

## RESULTS AND DISCUSSION

**Isolation of Bacterial Colonies from Soil Sample:** After serial dilution , 24 different types of bacterial colonies were isolated from the nutrient agar plates of various dilutions. These colonies were screened for protease activity using skim milk casein agar.

**Screening for protease producing bacteria by skim milk casein agar:** The isolated colonies were screened for protease activity on skim milk casein agar media. After 24 hours incubation, only two colonies were found to produce maximum protease activity i.e this two colonies (referred as B8 and B9) produced protease enzyme to digest the protein available in the media ,thereby forming a clear zone which indicates the presence of protease producing organism.

**Screening for protease using different media:** The two colonies (B8 and B9) isolated from skim milk casein agar media were screened for maximum protease activity using different media namely skim milk agar media and groundnut oil cake agar media. After 24 hour incubation, B8 strain produced maximum zone in both the media when compared withB9 strain. Hence, B8 strain is selected for further work.

**Identification of isolated strain:** Gram staining was performed for the B8 strain and it was found to be gram positive rod shaped bacillus strain. The isolate was further subjected to various biochemical test for identifying its species and genera.

**Biochemical Tests:** Biochemical test were carried out to narrow down the genus name. Result showed positive for the Catalase, Nitrate and Gelatinase test . According to Cappucino and Sherman, 2004"Manual of Microbiology" the genus may be bacillus, it is further confirmed by 16s rRNA sequencing.

**Estimation of protein and total carbohydrate in groundnut oil cake:** Protein concentration and carbohydrate (Phenol Sulphuric Method) was estimated .Similar results showed maximum protein concentration of 49.5%.In our sample protein content was high when compared to his study [3]

**Optimization of Fermented Product:** Protease activity was estimated by Anson method at various time intervals was plotted in graph and maximum activity was observed at 48hrs for peptone as substrate and at 60hrs for groundnut oil cake as substrate. Since Groundnut oilcake is a complex substrate, it takes more time to be broken down into simple molecules and so the time taken for maximum production rate increases when compared to peptone. Maximum activity was observed at 48hrs for peptone as substrate. Maximum enzyme activity was observed at 60 hrs for groundnut oil cake as substrate.

Protein concentration of crude enzyme collected at various time intervals was estimated and tabulated .Maximum concentration was observed at 48hrs for peptone as substrate and 60 hours for Groundnut oil cake as substrate. The production rate of groundnut oil cake is high in samples collected every 12hrs when compared with peptone, this is due to high protein content of groundnut oil cake which provides high nutrition to the organism, thereby the utilization is more and invariably increases the production rate.

The partially purified protein sample is electrophorized in SDS PAGE along with protein marker to obtain the molecular weight of unknown protein sample .After electrophoresis, the unknown protein sample band formed is compared with that of protein marker and the molecular weight was found to be 20kDa.In the work done by Nilegaonkar,2006 the molecular weight was 45kDa and 24kDa.Certain alkaline protease was 15-20 kDa ( 2).

**Application in removal of blood stains in surgical clothes:** Protease enzyme cleaves the blood stains and detergents as a surfactant removes the stain completed from cloth .Washing performance of blood stained cloth was maximum with the detergent and enzyme combination

incubated at room temperature and 60℃, for 30 min. Blood stains was partially removed in the detergents treated cloth. In work carried by the washing performance was maximum at 25 min with the enzyme and detergents combination. And stains are not removed in the detergent treated cloth.

## SUMMARY AND CONCLUSION

The strain isolated from the dairy effluents was found to be bacillus cereus and was confirmed by Gene Sequencing and Various biochemical tests. Bacillus cereus is a known producer of protease enzyme. The isolate is grown in two production media, groundnut oil cake and basal media. Groundnut oil cake was found to be a better substrate for protease production on comparison with the commercial peptone based on the following results: Maximum protein production rate and enzyme activity for Groundnut oil cake was at 60 hours and for basal it as at 48hrs respectively. Thus Groundnut oil cake can be a viable and cheap substrate for protease production in large scale. Protease produced by bacillus cereus was optimized to find the stability of the enzyme . The enzyme was found stable over a broad range of PH (8 to10).Maximum activity was determined at PH 9$\pm$0.2. Enzyme was stable at temperature of 40℃ to 60℃. Maximum stability was detected at 60℃, and residual activity sharply reduced at 80℃. This increase in compatibility of he enzyme with the detergents which is alkaline in nature and also stable at room temperature. Washing performance of detergents in removal of blood stains was enhanced with the action of enzymes. On immobilizing the enzyme in micro carriers, it may increase the shell life of enzyme for commercial usage.

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