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INTEGRATED STUDY ON THE PRODUCTION, CHARACTERIZATION AND INDUSTRIAL SIGNIFICANCE OF PROTEASE ENZYME

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ABSTRACT

The proteins represent one of the most commercially significant groups of enzymes because of their extensive usages in various industries. This combined research is devoted to the production, characterization, and industrial role of the protease enzymes with a focus on their functional adaptability and biochemical diversity. Microorganisms that produce proteases were culture is isolated and screened of various environmental sources then optimized conditions in order to increase enzyme yield. pH, temperature, substrate concentration, incubation time was put to systematic evaluation to optimize production efficiency. Protease obtained was purified by common biochemical methods and then determined by gram staining and then described in terms of its molecular weight, ideal activity parameters and stability characteristics. The enzyme exhibited active over a wide pH and temperature range, which makes the application in different possible industrial processes. Kinetic

analysis also indicated its catalytic activity and substrate specificity. The article places importance on the industrial application of proteases in the detergent industry among other sectors Such as leather processing, food and dairy production, pharmaceuticals and waste management. Their environmentally friendly and their capacity to substitute harsh chemical working methods highlight the fact that they are increasingly becoming significant in environmentally friendly industrial practices. Overall, this research leads to the knowledge on protease production and functionality, providing information on their improved use in industrial processes and biotechnology.

KEYWORDS: Protease enzymes, Microbial production, Enzyme characterization, Industrial applications, Biochemical properties.

INTRODUCTION

Proteases form some of the most commercially significant enzyme types, and since their application extends to a very large number of industrial applications, take up a substantial share of the global enzyme market.[1-3] The present investigation focuses on integrated research of the productions of proteases, their description, and industrial application. Proper environmental samples were collected and tested using casein hydrolysis tests with the loss of protease producing bacteria. The strong strain of the bacteria under ideal cultural conditions such as pH, temperature, incubation period, and nutritional composition was subject to submerged fermentation in the production of enzymes, followed by partial purification and characterization to determine the biochemical properties of the crude enzyme such as maximum pH, temperature stability, substrate specificity, and effects of metal ions and inhibitors on the enzyme activities. In addition, the protease exhibited extreme heat stability and optimum activity in alkaline conditions hence indicating its relevance in industrial practices.[4] Moreover, the industrial significance of the enzyme was also tested using it in the treatment of waste materials, in the hydrolysis of proteins, and in the making of detergents. The enzyme has been shown to have a good ability to remove the stain and also showed promise in the degradation of protein-based substrates and as such, emphasized its biologically friendly and affordable nature.[5,6] By highlighting the importance of protease enzymes in the fields of biotechnology and manufacturing industries, this systemic study offers informative understanding on the effectiveness of their manufacturing processes and functional properties as well as the real-world uses of the enzyme. In this combined paper, attention is given to the synthesis of protease enzymes, their characterization, and industrial

importance, with the emphasis made on their biochemical diversification, adaptability of functions, and increased importance in the context of sustainable industrial operations. The study is expected to help in the growth of effective, cost effective and environmentally friendly biotechnological applications by delving into microbial sources, optimization of production parameters, and enzyme characterization.

MATERIALS AND METHODS

Sample collection and isolation of Protease producing microorganisms

The sample was collected in the form of soil samples in the sterile polythene bags and delivered to the laboratory in a state of aseptic conditions. The refrigeration was done at low temperatures until the samples were processed. In case of microbial isolation, one gram of soil was poured into a test tube with 0.85 percent sodium chloride (NaCl) solution. Then, 9 mL of sterile distilled water were put in the tube to prepare a suspension. This mixture was homogenised well by the use of vortex mixer so that the microorganisms could be evenly distributed.

The preparation of skim milk agar was done by the following method; 2.5 g of skim milk powder was dissolved in 25 mL of distilled water and 0.375 g of agar was added. The medium was sterilized through autoclaving under the normal conditions and subsequently transferred to sterile Petri plates under aseptic condition. The volume of the prepared soil suspension, 10 uL, was uniformly smeared on the plates of the skim milk agar. The plates that were inoculated were incubated at 37degC after 24 hours.

After incubation, different colonies were counted and well isolated colonies were selected. The colonies were aseptically transferred and streaked in fresh skim milk agar plates of identical composition in order to get pure cultures. A Nutrients broth with composition of NaCl 0.1g Beef Extract 0.04g, Peptone 0.1g in 20ml distilled water prepared for further studies. The plates were incubated and then observed under the presence of clear zones around the colonies and hence casein hydrolysis. The size of the hydrolysis areas was compared and isolates with a clear zone prominent were termed as potent protease-producing isolates and were further selected to study.

Characterization of Effective Protease Producing Microorganisms

Strong protease producing isolates are defined and this is a mandatory step following the first screening and selection. The stage is pertinent in being aware of the biological and functional

properties of the microorganisms, which include, biological, physiological adaptability, biochemical behavior and enzyme production capacity, very crucial in the application of the microorganisms in industry.[7]

Identification and Characterization of Isolated microorganisms

Morphological analysis is selected during the preliminary step taking as a basis the isolates that show the most important areas of casein hydrolysis on skim milk agar plates. This involves examination of both size, shape, and microscopic examination of Gram reaction and cells character and structure. These features provide a major identification pattern of the isolates.[8] After that, the typical biochemical characterization is carried out with assistance of standard tests available, including Catalase, Urease, Citrate, Nitrate, MR, VP, Lipase, H2S, Indole, Lactose, Fructose, Maltose, Sucrose, Mannitol, Sorbitol, Dextrose usage patterns. The tests are employed to identify the metabolic profile and it was also employed to assist in taxonomic classification of the isolates. In the enzyme-specific analysis, there is usually the introduction of identifiable strains to the quantitative protease tests which typically use casein as a substrate. The most significant parameters (optimum pH, temperatures, incubation time, and substrate concentration) are analytically studied in the effort of finding the conditions under which the enzyme can be most active. Physiological research is also conducted to determine the effects of environmental factors such as pH variation, temperature, and salinity on the growth of microorganisms and generation of protease. Overall, such a broad characterization can be used to select highly effective protease-producing isolates with useful properties and implement them as promising agents in various industrial processes.

Optimization of Microbial Growth Conditions

Temperature, pH, and the various nutrient sources were tested under controlled laboratory conditions to select the best growth conditions for the selected microorganism.

Temperature Optimization

The culture was incubated at various temperatures, namely, 3°C, 27°C, 37°C, and 50°C, over a given period of 30 minutes to determine the optimum growth temperature for the microbe. Growth was checked after incubation to determine which temperature favored maximum proliferation.

pH Optimization

The effect of pH on microbial growth was examined by subculturing 1 ml of the microbial

culture into 5 ml of nutrient medium adjusted to varying pH levels (3, 5, 7, and 9). The cultures were allowed to grow at 37°C over a period of 30 minutes, and the growth was assessed to indicate the best pH environment.

Nitrogen Source Optimization

The model optimizes the food supply as a source of nitrogen to avoid overuse of fertilizer. Nutrient broth was prepared to study the influence of nitrogen sources. 1% ammonium ferrous sulfate, ammonium dihydrogen orthophosphate, ammonium sulfate, and ammonium acetate were used to supplement the nutrient broth. The media were incubated at 37°C for 48 hours. Microbial growth was studied after incubation to determine the most appropriate source of nitrogen.

Carbon Source Optimization

The microbial growth was assessed by the ability of different carbon sources to stimulate the growth of microbes in nutrient media by adding 1% dextrose, sucrose, lactose, fructose, and each individually into the nutrient media. The test organism was inoculated into each medium and allowed to incubate at 37°C for 48 hours. The growth after incubation was measured to establish the carbon source that is most favorable to microbial growth.

Production of protease enzyme

The protease enzymes are developed by growing the desired microbial isolates in controlled laboratory conditions. The first step in growing an actively growing culture involves the inoculation of the organism in nutrient broth and the incubation of the same over a period of approximately 18-24 hours.[9]

A prepared medium of production with the composition of yeast extract 0.25gm skim milk powder 0.25gm KH_2PO_4 0.1gm Na_2CO_3 0.5gm and maintain pH to 7-8 and sterilized. The ready inoculum is added to this shake flask medium in a minimal ratio. The culture is incubated at an ideal temperature (usually about 37degC) over 48-72hours under constant movement to aid in suitable growth and enzyme release.

When the microorganism is incubating, it releases protease into the surrounding medium. The culture is then centrifuged at 8000rpm for 5 minutes after the fermenting period in order to isolate the microbial cells. The resulting clear liquid (supernatant) is a crude enzyme extract, which can be used further to determine the enzyme activity, and other forms of analysis.

Protease enzyme assay

The simple and effective technique of identifying the microorganisms that produce protease enzymes is the skim milk agar assay.[1] It is founded on the disintegration of the casein which is the primary protein found in milk. Under this technique, the microorganisms are inoculated on agar plates with skim milk by making three holes using core borer and pour 50microlitre centrifuge protease enzyme in it. Upon incubation at appropriate temperature (usually 30-37degC), protease producing organisms release the enzyme into the medium around it. This enzyme decomposes casein and a clear and transparent zone is formed around the colony and the rest of the medium is opaque.

The emergence of a clear zone is an indication of positive activity of protease whereas the lack of a clear zone is an indication that the organism is not a protease producer. The assay is a common screening technique applied as a first line screening method to identify effective protease-producing strains to be studied further and used in industries. All in all, skim milk agar technique is fast, economical and dependable in identifying proteolytic activity in microorganisms.

Application of protease enzyme: Detergent industry

Proteins are also a major enzyme that is employed in the detergent formulation because of its capability to degrade protein-based stains. These enzymes help in breaking down the complex protein molecules to the simpler ones like peptides and amino acids, which are easy to get rid of in a washing procedure. In order to show the efficiency of any of the systems to be used in a detergent, the following experimental setup may be adopted:

Experimental Procedure

It takes four little pieces of clean white cotton cloth and artificially stained with various dyes such as malachite green, bromophenol blue, phenol red and crystal violet. The pieces of cloth stained are left to dry thoroughly in order to make the stains adhere well.

The four distinct bottles of culture are then made to have various treatment conditions:

- Bottle 1 (Control): Only 50 mL of distilled water is added to it.
- Bottle 2 (Detergent only): A mixture of 5 g of detergent and 50 mL of distilled water.
- Bottle 3 (Detergent + Enzyme): 50 mL of distilled water, 5 g of detergent and 500 uml of protease enzyme.
- Bottle 4 (Enzyme only): 50 ml of distilled water and 500 0.005 mL of protease enzyme.

Every piece of cloth covered with the stain is placed in one of the prepared solutions and incubated under the conditions (e.g., room temperature or slight heating) over a given period of time.

RESULTS AND DISCUSSION

Screening and isolation of protease enzyme producing microorganisms

Sterile soil samples were obtained in an agricultural field and subjected to isolation of microbes. The samples were diluted in series and placed on skim milk agar plates (figure 1a) which are a selective medium of protease activity. Following the incubation, the colonies surrounded by clear zones were observed, which means that the production of proteases led to the hydrolysis of casein.

Clear colonies with distinct colonies with clear zones were then identified and streaked again on new plates of skim milk agar (figure 1b) to verify their proteolyticity. The emergence of identical clear areas on re-streaking confirmed the existence of protease-producing microorganisms. These pure isolates were then inoculated on nutrient agar plates (figure 1c) to maintain these isolates, further characterize them and identify them. From Nutrients agar, inoculated colony transfer to the nutrient broth (figure 2) for further identification.

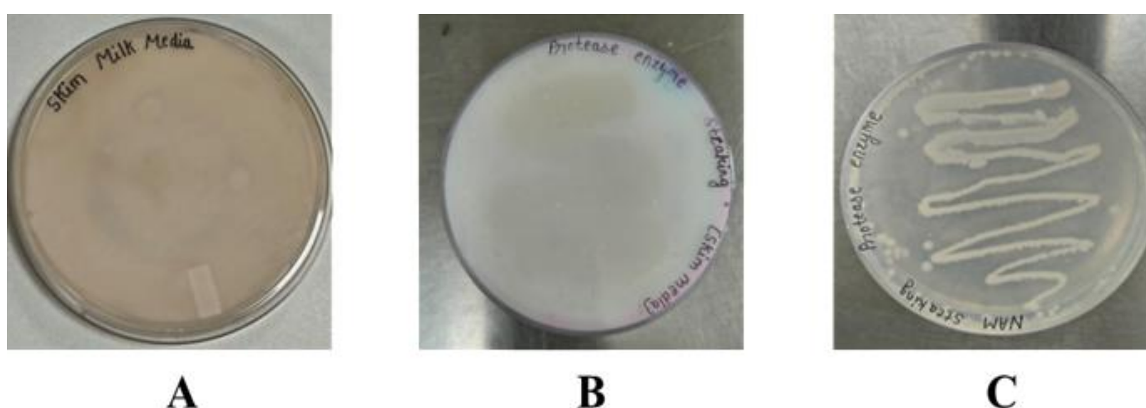


Figure 1: A Spreading on skim milk agar plate, B Streaking on skim milk agar, C Streaking on NAM plate.

Detection of Protease Producing Microorganism

After a preliminary screening on the skim milk agar, the strong protease producing isolate was identified by means of undergoing an elaborate morphological and biochemical characterization (table 1).

The chosen isolate had clear colonies of round, well-circumscribed colonies on nutrient agar of smooth margins and opaque, color. Gram staining followed by microscopic observation showed Gram-positive, rod-shaped cells which were singly arranged or in short chains (figure 3). These features are in line with the representatives of the genus Brevibacillus.

A set of standard biochemical tests (figure 4) were conducted to put in further confirmation on the identity of the isolate. The organism exhibited a positive reaction to catalase activity, which means that the organism is able to break hydrogen peroxide down into water and oxygen. It was also positive in proteolytic activity as it showed clear zones on skim milk agar which is a confirmation of casein hydrolysis.

Figure 2: Nutrient broth.

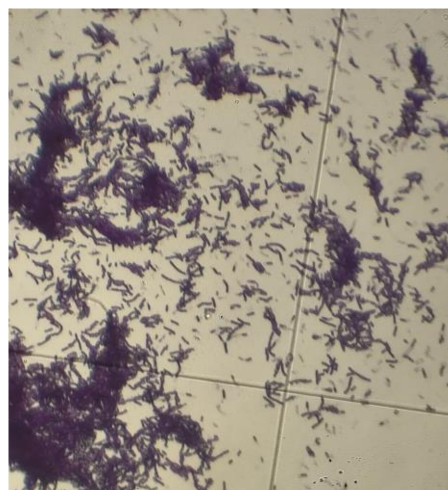


Figure 3: Gram staining.

It had mixed patterns of utilization in carbohydrate fermentation tests based on the source of carbon supplied. The organism was identified to be aerobic and this was able to grow under normal laboratory conditions. The negative outcomes of some of the tests like Voges proskauer and methyl red test were helpful in eliminating its classification. The biochemical profile was generally in accordance with the existing knowledge on *Brevibacillus laterosporus*.

Referring to the integrated morphological data (Gram-positive, rod-shaped,) and the outcomes of the biochemical tests (positive catalase, protease, and Nitrate), the isolate could be determined as Brevibacillus laterosporus. This organism has the characteristics of the extracellular production of enzymes and especially proteases, and it can be used in the future in the industry.

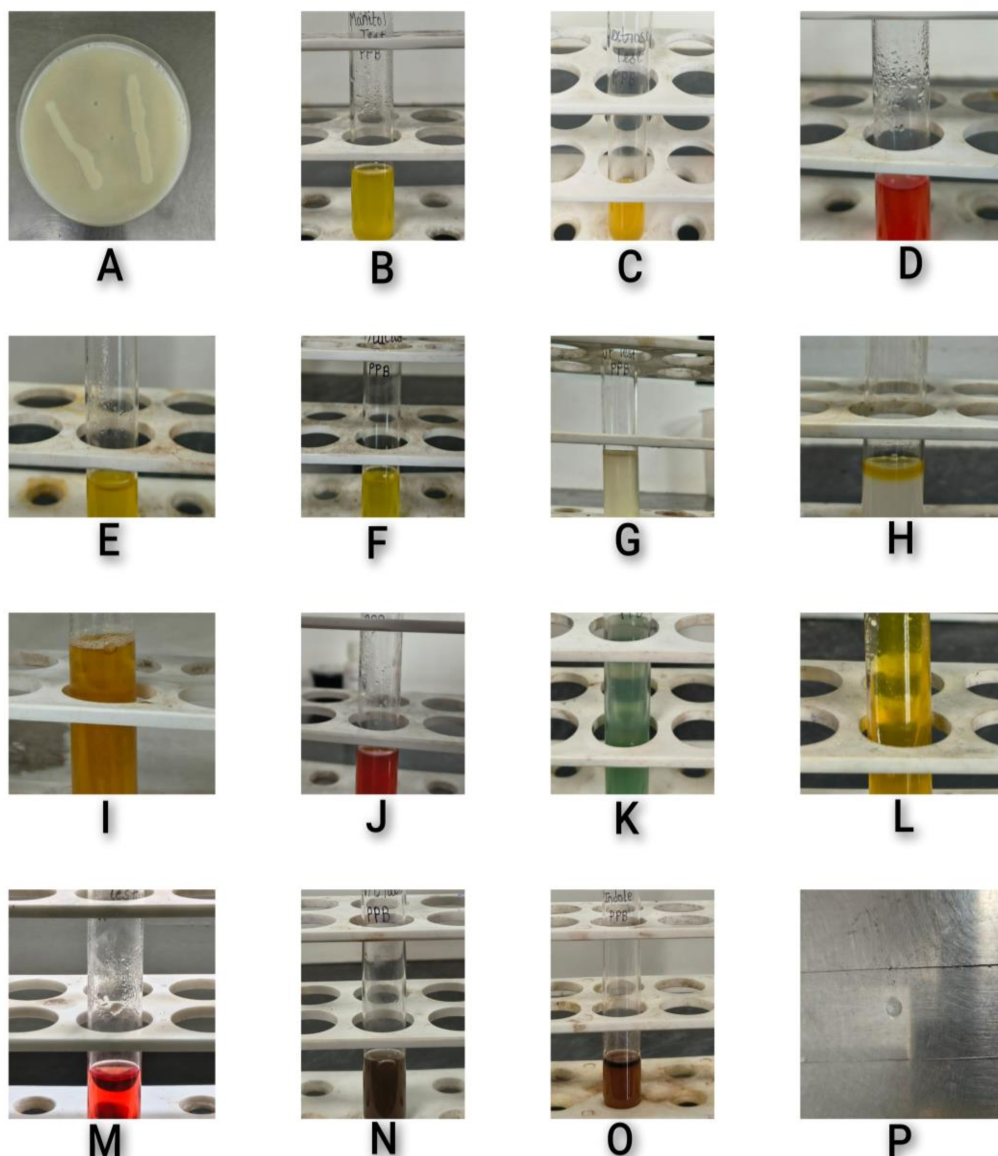


Figure 4: Biochemical tests A-Lipase, B-Mannitol, C-Dextrose, D-Sorbitol, E-Maltose, F-Fructose, G-VP, H-MR, I-H_2S, J-Lactose, K-Citrate, L-Urease, M-Sucrose, N-Nitrate, O-Indole and P-Catalase.

Table 1: Comparison of Morphological and Biochemical Characteristics of *Brevibacillus laterosporus*.

Test Category	Test/Parameter	Observed Result	Standard Characteristics	Inference
Morphological	Colony morphology	Irregular, rough colonies	Irregular, rough colonies	Matches
	Cell Shape	Rod shaped	Rod shaped	Matches
	Gram staining	Gram positive	Gram positive	Matches
Biochemical	Catalase	Positive	Positive	Matches
	Protease	Positive	Positive	Matches

	Methyl Red	Negative	Negative	Matches
	Voges proskauer	Negative	Variable	Matches
	Lipase	Positive	Positive	Matches
	Nitrate	Positive	Positive	Matches
	Indole	Positive	Variable	Matches
	Urease	Negative	Negative	Matches
	Citrate	Negative	Negative	Matches
	H2S	Negative	Negative	Matches
	Sucrose	Negative	Variable	Matches
	Dextrose	Positive	Positive	Matches
	Lactose	Negative	Negative	Matches
	Mannitol	Positive	Positive	Matches
	Maltose	Positive	Positive	Matches
	Sorbitol	Negative	Variable	Matches

Note: Observations are based on experimental results and compared with standard characteristics reported for *Brevibacillus laterosporus.*

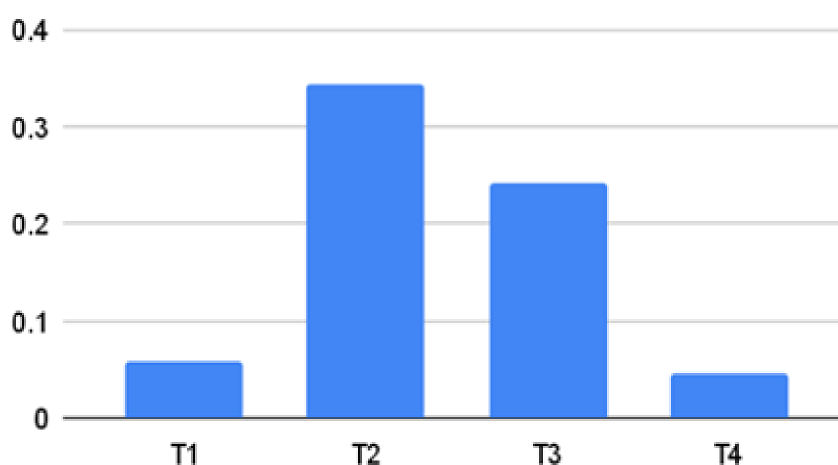
Effect of temperature on microbial activity

The temperature is also a vital factor that affects the development of microbes as well as their metabolism (Sharma et al., 2017). This experiment involved testing the growth of microorganisms under four temperature environments (T1, T2, T3, and T4). Optical density (OD) was used to test the growth of microorganisms. The following results were recorded regarding the observed OD values: T1 had OD of 0.059, T2 had the greatest values of 0.343, T3 had an intermediate growth with an OD of 0.243, and T4 had the least growth with an OD of 0.043 (graph 1). The results here show that the microbial activity was optimum at T2 that is room temperature. The fact that the OD value at this condition is significantly higher implies better cell growth and efficiency of the cell in terms of metabolism. Conversely, the OD values were lower at T1 and T4, indicating that moisture at either of the extremes (low or too low) or at the extremes high or too high) can suppress the growth of microbes. T3 exhibited moderate growth and this shows that it is quite permissible. The results reveal that room temperature is the most suitable environment to provide microbial proliferation of the chosen environments.

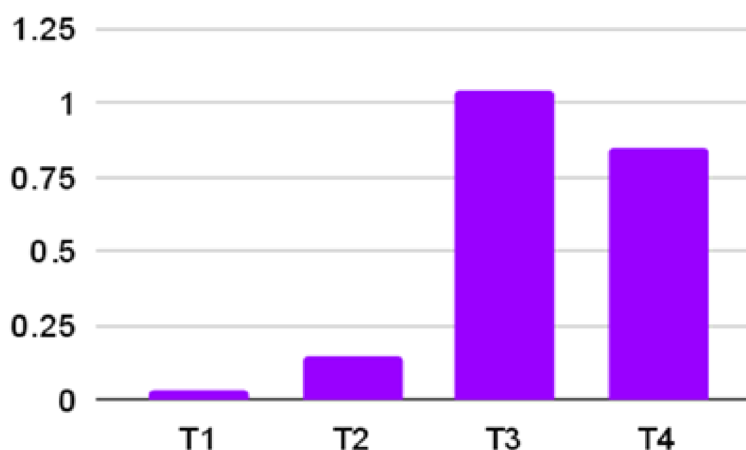
Effect of pH on microbial activity

The pH is also a vital factor that affects the development of microbes as well as their metabolism.[9] This experiment involved testing the growth of microorganisms under four pH environments (T1 pH 7, T2 pH 9, T3 pH 5, and T4 pH 3). Optical density (OD) was used to test the growth of microorganisms. The following results were recorded regarding the observed OD values: T1 had OD of 0.035, T2 had the OD values of 0.148, T3 had an highest

growth with an OD of 1.047, and T4 had an OD of 0.850(graph 2). The results here show that the microbial activity was optimum at T3 that is pH5. The fact that the OD value at this condition is significantly higher implies better cell growth and efficiency of the cell in terms of metabolism. Conversely, the OD values were lower at T1 and T2, indicating that pH at the extremes low can suppress the growth of microbes. T3 exhibited highest growth and this shows that it is quite permissible. The results reveal that pH5 is the most suitable environment to provide microbial proliferation of the chosen environments.



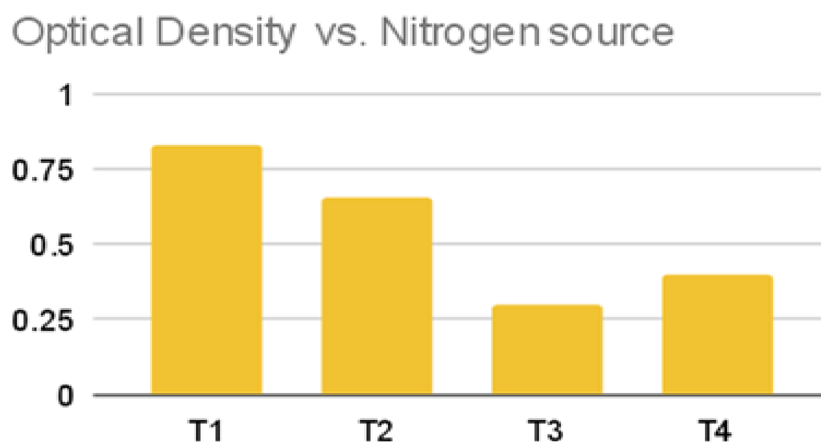
Graph 1: Bacterial growth at different temperature.



Graph 2: Bacterial growth at different pH.

Ffect of nitrogen source on microbial activity

The Nitrogen source is also a vital factor that affects the development of microbes as well as their metabolism. This experiment involved testing the growth of microorganisms under four different Nitrogen sources (Ammonium ferrous sulfate T1, Ammonium dihydrogen orthophosphate T2, Ammonium sulphate T3, and Ammonium acetate T4). Optical density (OD) was used to test the growth of microorganisms. The following results were recorded regarding the observed OD values: Ammonium ferrous sulfate had OD of 0.826, Ammonium dihydrogen orthophosphate had OD of 0.651, Ammonium sulphate had OD of 0.301, and Ammonium acetate had OD of 0.402 (graph 3). The results here show that the microbial activity was highest in Ammonium ferrous sulfate. The fact that the OD value at this condition is significantly higher implies better cell growth and efficiency of the cell in terms of metabolism. Conversely, the OD values were lower in Ammonium sulphate and Ammonium acetate, indicating lowest activity and microbial growth. Ammonium dihydrogen orthophosphate exhibited moderate growth and this shows that it is quite permissible. The results reveal that ammonium ferrous sulfate is the most suitable nitrogen source to provide microbial proliferation of the chosen environments.

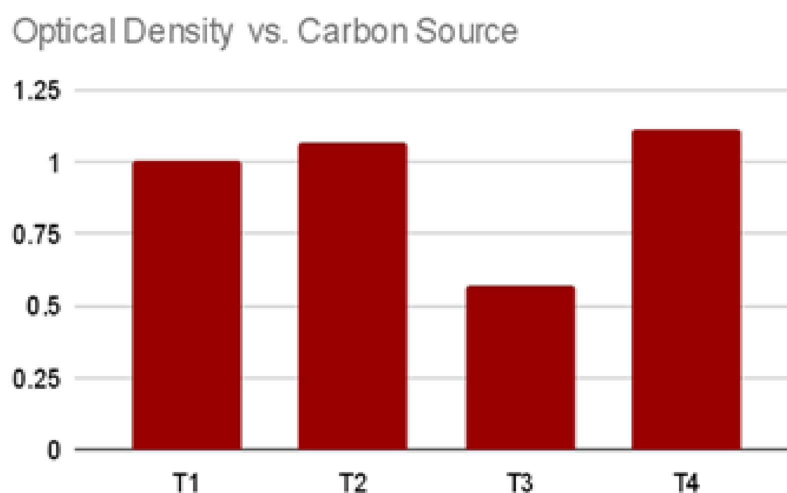


Graph 3: Bacterial growth at different Nitrogen sources.

Effect of carbon source on microbial activity

The Carbon source is also a vital factor that affects the development of microbes as well as their metabolism. This experiment involved testing the growth of microorganisms under four different Carbon sources (Dextrose T1, Sucrose T2, Lactose T3, Fructose T4). Optical density (OD) was used to test the growth of microorganisms. The following results were recorded regarding the observed OD values: Dextrose had OD of 1.001, Sucrose had OD of

1.065, Lactose had OD of 0.571, and Fructose had OD of 1.114 (graph 4). The results here show that the microbial activity was highest in Fructose. The fact that the OD value at this source is significantly higher implies better cell growth and efficiency of the cell in terms of metabolism. Conversely, the OD values were lower in Lactose indicating lowest activity and microbial growth. Dextrose and Sucrose exhibited moderate growth and this shows that it is quite permissible. The results reveal that fructose is the most suitable Carbon source to provide microbial proliferation of the chosen environment.



Graph 4: Bacterial growth at different carbon sources.

Protease enzyme assay on skim milk agar plate

The skim milk agar assay is a qualitative method acceptable in the determination of the activity of protease enzyme and it is quite effective in enzyme detection of protein hydrolysis. Proteins are made up of complex molecules, which are broken down into smaller molecules (peptides and amino acids) catalyzed by proteins known as proteases. The best medium to be used in this purpose is skim milk agar as it has casein, which is a milk protein and gives a medium an opaque, milky look.

In this test, the microorganism under test is inoculated on the skim milk agar plate and allowed to incubate, usually 24 hours with the appropriate conditions. In incubation, in case the organism releases outside cellular protease enzymes, the enzymes diffuse into the adjacent medium and disintegrate the casein available in the agar.

This enzymatic activity causes the opaque character of the medium within the area of microbial growth to be lost resulting in a clear area, also known as a zone of hydrolysis

(figure 5). The direct measure of proteolytic activity is this halo of clear colony. The size of the clear zone may be semi-quantitatively determined as an indicator of enzyme production, where bigger clear zones mean the presence of a higher activity of protease.

The skim milk agar assay is therefore a relatively easy, dependable and visual screening and identification technique of protease producing microorganisms, which is based on their capacity to fragment protein substrates.

Application of protease enzyme in stain removal

Protease enzymes have been known to play an important role in the elimination of protein-based stains and therefore, are also of great use in the cleaning industry and detergent industry. These enzymes facilitate the hydrolysis action of the peptide bonds of the proteins and cause their disruption into small, soluble water-soluble peptides and amino acids which are easily washed away. Proteinaceous stains like blood, sweat, milk, and food residues are especially difficult to remove with the traditional cleaning techniques, however, protease enzymes increase the effectiveness of their removal.[5]

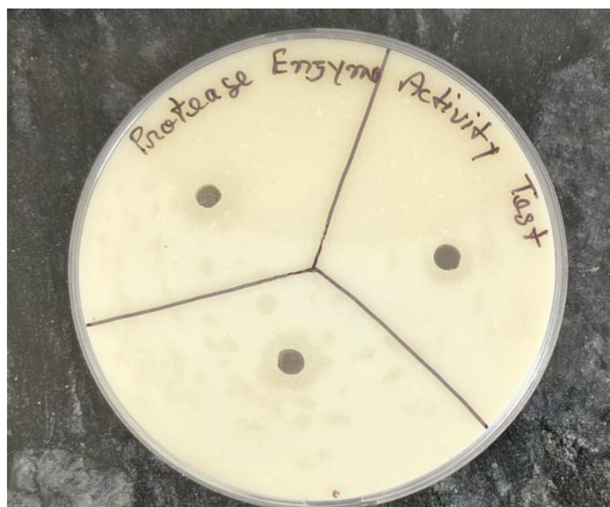


Figure 5: Protease enzyme activity.

The Protease was tested on stain removal by performing a controlled laboratory experiment. Four bottles with various treatment solutions were prepared and their cleaning performance was compared in this study. In the first bottle, there was distilled water only and it was used as the control. The second bottle was filled with distilled water and detergent which was the standard condition of cleaning. The third bottle contained distilled water, detergent, and protein enzyme, which was meant to test the effect of protein enzyme in the presence of

detergents. In the fourth bottle, there was distilled water together with the enzyme protease and this was done to determine the independent action of the enzyme.

Equal portions of the white cloth that had been stained with different substances were placed in each of the four solutions and left to be incubated at the same environmental conditions within an hour. The incubation period was followed by the removal of the cloth samples that were observed carefully in terms of the degree of removing the stains.

The experiment outcomes were clear to show that the bottle of third culture which had distilled water, detergent and protease enzyme had the highest stain removal ability (Figure 6). The cloth that was subjected to this solution registered a great deal of decrease in stain intensity when compared to other treatments. The second bottle which had detergent only recorded moderate cleaning ability, whereas the fourth bottle which contained protease enzyme only showed partial removal of the stain. The control system using distilled water only had little to no cleaning effect.

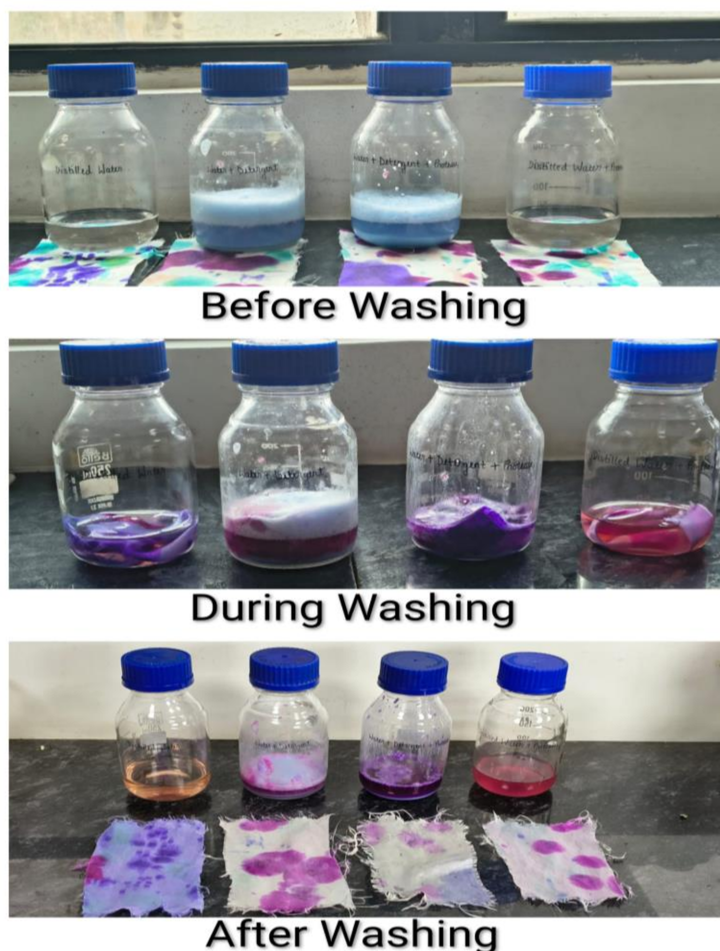


Figure 6: Application of protease enzyme in detergent industry.

These observations indicate that there is a synergetic interaction between protease enzyme and detergent. Although detergents aid in the solubilization and dispersal of particles of dirt, protease enzymes selectively target and degrade protein components of stains thus increasing cleaning performance at large. The fact that the enzyme can operate at mild conditions is another factor that has helped in making the enzyme to be industrially applicable.

To sum up. The paper has shown that addition of protease enzymes in detergent preparations results in a great increase in the ability to remove stains. This underscores the significance of the use of enzymatic additives in the contemporary cleaning products and justifies its extensive usage in the household and industrial laundry industries.[11]

CONCLUSION

This study successfully isolated and characterized a protease-producing microorganism identified as *Brevibacillus laterosporus* from agricultural soil. Optimization of growth conditions revealed that microbial activity was highest at room temperature, pH 5, with ammonium ferrous sulfate as the best nitrogen source and fructose as the most suitable carbon source. The organism showed strong proteolytic activity on skim milk agar, confirming efficient casein hydrolysis.

Application studies demonstrated that the protease significantly enhanced stain removal efficiency when combined with detergent, highlighting its industrial potential. Overall, *Brevibacillus laterosporus* shows promise for use in eco-friendly detergent formulations and other biotechnological applications, with further studies needed for large-scale production and characterization.

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