Isolation Of Protease Producing Bacteria

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***Abstract*:** One of the most significant industrial enzymes, proteases find extensive use in biotechnology, pharmacology, and therapeutic procedures, especially in thrombolytic treatments. The isolation, generation, purification, and characterisation of protease-producing bacteria from soil samples taken from heavy metal-contaminated industrial areas in Coimbatore, Tamil Nadu, India, are the main objectives of this work. For the initial screening of proteolytic activity, soil samples were serially diluted and plated on skim milk agar. Protease synthesis was demonstrated by sixteen bacterial isolates that showed distinct zones of casein hydrolysis. Isolates BP12, BP15, and BP16 were chosen for additional research because they showed noticeably greater protease activity.

The chosen isolates were cultivated in protease production media, and the effects of several environmental and nutritional factors, such as temperature, pH, incubation duration, and various sources of carbon and nitrogen, were assessed in order to maximize enzyme synthesis. After 72 hours of incubation, the highest level of protease synthesis was seen. The ideal pH was between neutral and slightly alkaline, and higher enzyme activity was encouraged by temperatures between 47 and 57 degrees Celsius. The most efficient suppliers of carbon and nitrogen among the nutrients examined were sucrose and ammonium nitrate, respectively.

Protease test and protein estimation were performed on the crude enzyme extracts, and then ammonium sulfate precipitation, dialysis, and gel filtration chromatography were used for partial purification. There were different levels of thrombolytic and proteolytic activity in the isolated enzyme fractions. The chosen isolates showed considerable clot lysis potential in in vitro thrombolytic experiments, with some fractions exhibiting activity above 90%, indicating the presence of strong fibrinolytic enzymes. Nevertheless, it was found that increased thrombolytic effectiveness was not always correlated with higher general protease activity, indicating the existence of functionally different enzyme components.

Subsequent analysis showed that the enzymes' molecular weight varied from 15 to 31 kDa, which is in line with previously documented bacterial fibrinolytic proteases. In the presence of EDTA, the action of metal inhibitors demonstrated a decrease in enzyme activity, suggesting that the enzymes were likely metalloproteases. An increase in the purification fold suggested better enzyme specificity and activity, even if the purification technique produced lower yields.

The study concludes by showing that contaminated soil bacteria are an important source of proteases with substantial industrial and medicinal potential. The chosen isolates' potent proteolytic and thrombolytic capabilities demonstrate their potential use in enzyme-based businesses and the creation of therapeutic medicines for the management of thrombotic diseases.

I. INTRODUCTION

The capacity of microorganisms, especially bacteria, to produce a wide variety of enzymes into their surroundings makes them notable. The ability of Bacillus species to produce a variety of extracellular enzymes, including proteases, makes them very valuable in industrial biotechnology (Mesbah, 2022). Proteases are specialized proteins that play crucial roles in many biological and industrial processes by catalyzing the breakdown of peptide bonds in other proteins. According to Sujitha and Shanthi (2023), these enzymes are essential for processes including cellular development, differentiation, and nutrition cycling. Proteases are categorized as acidic, neutral, or alkaline based on their ideal pH. Because they function best at higher pH

levels, alkaline proteases are especially crucial in sectors like waste management, food production, medicines, leather processing, and detergent formulation. Although both plants and animals may produce proteases, microbial sources are favored because of their quick growth, simplicity of genetic modification, and affordability for large-scale production (Gimenes et al., 2021).

Because of their excellent specificity, catalytic efficiency, and durability, enzyme-based therapies have drawn interest recently as prospective substitutes for traditional thrombolytic treatments (Hazare et al., 2024). Microbial-derived enzymes, particularly fibrinolytic enzymes, are among the many possible sources of thrombolytic medicines that have a lot of potential for treating arterial occlusions. These microbial enzymes have a number of benefits, such as a good cost-benefit ratio and large-scale production (Diwan et al., 2021). A major source of morbidity and mortality globally, thrombotic illnesses such deep vein thrombosis, myocardial infarction, stroke, and pulmonary embolism are brought on by obstructions in blood arteries (Lichota et al., 2020). The ability of microbial thrombolytic enzymes to break up blood clots makes them a useful substitute for traditional therapies, which are frequently expensive and have unfavorable side effects (Abdul Rahim and Rengaswamy, 2022).

One essential physiological mechanism that keeps thrombosis at bay is fibrinolysis, the breakdown of fibrin in blood clots. The capacity of microbial fibrinolytic enzymes, including plasminogen activators, to effectively hydrolyze thrombi with few side effects has drawn a lot of attention. In addition to being efficient in thrombolysis, these enzymes can be manufactured quickly and in huge quantities at a low cost. Beyond thrombolysis, they may also be used for antibacterial activity, blood pressure control, and cosmetic purposes such stain removal (Altaf et al., 2021). Finding new, reliable, and effective fibrinolytic medicines is still difficult, despite advancements in the use of microbial enzymes for thrombolytic therapy. These enzymes have a complicated purification procedure that calls for sophisticated methods to guarantee high activity and specificity. However, new developments in overexpression, genetic modification, and strain enhancement have increased the output of enzymes, increasing their therapeutic potential. It is becoming more widely acknowledged that microorganisms, especially Bacillus species, are dependable sources of fibrinolytic enzymes, providing prospective treatments for cardiovascular conditions like myocardial infarction and stroke (Leite et al., 2022).

Due to their high enzyme yield, stability, and wide substrate specificity, Bacillus strains are leading the market for microbial proteases, which are in great demand worldwide. Since these proteases are mostly secreted extracellularly, it is easier to extract and purify them from fermentation media. In order to improve both yield and activity, industrial production of these enzymes entails regulating a number of fermentation parameters, such as pH, temperature, nutrition sources, and incubation time (Danilova and Sharipova, 2020). Fibrinolytic enzymes, a noteworthy subclass of proteases with increasing medical importance, are attractive candidates for thrombolytic therapy because they can degrade fibrin, the primary component of blood clots.

The development of cardiovascular diseases including heart attacks and strokes is significantly influenced by thrombosis, which is caused by the abnormal accumulation of fibrin within blood vessels. Despite the widespread use of synthetic thrombolytic medications like urokinase and streptokinase, their high cost and

possible side effects, especially the danger of bleeding, have led to a search for better and more affordable therapy options (Alkarithi et al., 2021).

Fibrin clots can be broken down by microbial fibrinolytic enzymes, particularly those derived from Bacillus species. In addition to providing an affordable therapeutic alternative, these enzymes may lead to the development of oral thrombolytic medicines, which are less intrusive than conventional intravenous treatments (Cioni et al., 2022). In this regard, investigating bacteria from unusual and harsh settings, including metal-contaminated soils, offers a fascinating chance to find new protease-producing strains with improved stability and activity. With an emphasis on their potential for thrombolysis, this study attempts to separate and describe protease-producing bacteria from metal-polluted soils in Coimbatore, Tamil Nadu, India. This research aims to contribute to the development of environmentally acceptable, efficient, and economical substitutes for traditional thrombolytic medications by improving culture conditions and assessing these enzymes' capacity to dissolve clots.

II. PROTEASE PRODUCING BACTERIA

Numerous studies have been conducted on the diversity of protease-producing bacteria, with a focus on Bacillus species. These bacteria are extensively found in nature and are well-known for their capacity to generate strong, highly stable, and active enzymes in industrial settings.

1. BACILLUS SUBTILIS

One of the most researched and frequently used bacterial species for the manufacture of proteases is Bacillus subtilis. It is a rod-shaped, endospore-forming, Gram-positive bacteria that is frequently found in soil and other natural settings. The organism is a perfect option for industrial enzyme production because of its great adaptability and ability to thrive in a variety of environmental circumstances. The capacity of B. subtilis to release substantial amounts of extracellular proteases is one of its most important characteristics. These enzymes are very helpful in detergent compositions since they are mostly alkaline in nature and show strong activity in alkaline pH ranges. Enzyme recovery and purification are made easier by the extracellular secretion of proteases, which removes the need for intricate cell disruption procedures.

Numerous studies have shown that a variety of parameters, such as temperature, pH, incubation duration, and food availability, affect B. subtilis ability to produce proteases. Temperatures between 30°C and 37°C and slightly alkaline pH levels are typically associated with optimal enzyme synthesis. Enzyme yield and activity may be decreased if these ideal conditions are not met. Protease synthesis is also crucially regulated by nutritional variables. It has been demonstrated that carbon sources including glucose, sucrose, and galactose have a major impact on the synthesis of enzymes. Galactose is one of these that is frequently said to increase protease output. In a similar vein, compared to inorganic nitrogen sources, organic nitrogen sources like yeast extract and peptone are known to promote greater enzyme synthesis.

Another crucial component of protease synthesis is substrate selectivity. Proteolytic activity is assessed using various protein substrates, such as gelatin, casein, and skim milk. Larger zones of hydrolysis during screening tests show that gelatin is frequently linked to increased enzyme activity. This implies that the productivity and efficiency of enzymes can be directly impacted by the makeup of the substrate.

2. BACILLUS LICHENIFORMIS

Another well-known bacterial species that is widely employed in the manufacturing of industrial proteases is Bacillus licheniformis. It is well known that this organism produces thermostable alkaline proteases that continue to function in challenging environments, such as high temperatures and alkaline pH values. Because of these characteristics, the enzymes are especially well suited for use in the textile, leather, and detergent industries. In the presence of surfactants, oxidizing agents, and other substances frequently employed in industrial processes, the proteases generated by B. licheniformis show exceptional stability. Their commercial worth and applicability are increased as a result. These enzymes' capacity to work well in harsh environments lessens the need for stringent process control, which lowers operating expenses.

Similar to B. subtilis, a number of physicochemical and nutritional parameters affect B. licheniformis's ability to produce proteases. To maximize enzyme yield, fermentation variables such as temperature, pH, aeration, and agitation must be optimized. In order to lower production costs and encourage sustainable practices, studies have also looked into the use of alternative substrates, such as industrial and agricultural waste materials.

3. MARINE BACTERIA

Because marine bacteria can adapt to harsh environmental circumstances including high salinity, pressure, and temperature variations, they are a unique and prospective source of proteases. These microbes generate unique enzymes that are uncommon in terrestrial bacteria. Marine bacterial proteases are frequently salt-tolerant and active in a wide pH and temperature range. These qualities make them ideal for industrial procedures that need for the stability of enzymes under difficult circumstances. Salt-tolerant proteases, for example, are useful in bioremediation and food processing. Utilizing marine waste materials as substrates for microbial growth and enzyme synthesis, such as fish processing leftovers and cuttlefish by-products, has been the focus of recent study. This method not only improves enzyme yield but also tackles waste disposal-related environmental issues. In contemporary biotechnology, combining waste management with enzyme synthesis is a sustainable approach.

ISOLATION OF PROTEASE RPODUCING BACTERIA

An essential stage in the investigation of microbial enzyme synthesis is the isolation of bacteria that produce proteases. In order to obtain pure cultures of microorganisms with the appropriate enzymatic characteristics, environmental samples are collected and then microbiological procedures are applied. Protein-rich sources like soil, compost, and industrial waste are commonly used to gather environmental samples. Because there are protein substrates available, these settings are likely to have microbes that can produce proteases. The microbiological variety and quantity of soil samples in particular make them extensively utilized.

To lower microbial density and make it easier to isolate individual colonies, the serial dilution approach is frequently used. Using sterile diluents, the material is gradually diluted in this procedure, and aliquots of each dilution are plated on appropriate growth substrate. As a result, individual microbial colonies can be separated and subjected to additional analysis.

Samples are diluted, then plated on selective media or nutrient agar and incubated in a controlled environment. Based on their physical traits, distinct colonies are chosen and then further screened for protease activity. For protease-producing bacteria to be accurately identified and characterized, pure cultures must be isolated.

SCREENING OF RPOTEASE PRODUCING BACTERIA

Finding bacterial strains that can produce proteases requires screening. Enzyme activity is assessed using both qualitative and quantitative techniques, and strains that perform well are chosen for additional research. Agar plates with protein substrates like casein or skim milk are commonly used for primary screening. These substrates are hydrolyzed by bacteria that produce proteases, creating clean zones around the colonies. When choosing possible strains, the size of the clear zone serves as a preliminary indicator of the degree of proteolytic activity.

Enzyme activity is quantitatively analyzed utilizing biochemical tests in secondary screening. The release of amino acids or peptides as a result of protein hydrolysis is frequently measured using spectrophotometric techniques. These techniques yield precise and repeatable findings, making it possible to compare the enzyme activity of several strains. Proteases can also be further characterized using sophisticated methods including electrophoresis and fluorescence-based tests. These techniques make it easier to choose strains with desired characteristics by offering insights into the structure, molecular weight, and activity of enzymes.

FERMENTATION OF PROTEASE PRODUCTION

A crucial step in the large-scale synthesis of microbial proteases is fermentation. In order to enhance the yield of enzymes, microorganisms are cultivated under carefully monitored conditions. The most popular technique for producing proteases is submerged fermentation. By growing microorganisms in liquid media, this method enables effective mixing and control of environmental factors including pH, temperature, and aeration. Utilizing liquid media promotes microbial growth and makes it easier for nutrients to be distributed uniformly. In contrast, bacteria thrive on solid substrates with low moisture content during solid-state fermentation. Agricultural leftovers are used as substrates in this economical process. Because it lowers production costs and encourages sustainability, it is especially appropriate for areas with a lot of agro-industrial waste. Both fermentation methods have their advantages and limitations, and the choice of method depends on the specific requirements of the production process.

FACTORS AFFECTING PROTEASE PRODUCTION

Numerous factors, such as dietary availability and environmental circumstances, affect the generation of proteases. Optimizing enzyme output requires an understanding of these parameters. Both microbial growth and enzyme activity are significantly influenced by temperature. The majority of bacterial proteases are most active between 30°C and 50°C. Deviations from this range may result in denaturation or reduced enzyme production. Another important element that influences the stability and function of enzymes is pH. The pH range of 7 to 10 is usually where alkaline proteases exhibit their highest activity. High enzyme yields during fermentation depend on maintaining the ideal pH.

Protease production is also influenced by incubation time. The late exponential or early stationary phases of microbial development are often when maximum enzyme activity is seen. Due to the build-up of metabolic byproducts, prolonged incubation may cause enzyme degradation. Enzyme production is greatly impacted by nutritional factors, such as supplies of carbon and nitrogen. Organic nitrogen sources such as peptone and

yeast extract are generally more effective in promoting protease production. To maximize the fermentation process, the right nutrients must be chosen.

APPLICATION

Because they can effectively hydrolyze proteins, proteases are used in many different sectors. Proteases are employed in the detergent industry to eliminate protein-based stains from textiles. They are perfect for detergent compositions since they can operate in alkaline environments. Proteases are utilized in food industry activities such protein hydrolysis, meat tenderization, and cheese making. They improve food products' flavor, texture, and nutritional content. Proteases are employed in place of harsh chemical treatments in the leather industry to dehair and prepare animal skins. Proteases are employed in the pharmaceutical sector to create therapeutic medicines and to treat wounds. They are useful in many medicinal therapies because of their function in breaking down proteins.

III. METHOD AND METHODOLOGY

In this regard, investigating bacteria from unusual and harsh settings, including metal-contaminated soils, offers a fascinating chance to find new protease-producing strains with improved stability and activity. With an emphasis on their potential for thrombolysis, this study attempts to separate and describe protease-producing bacteria from metal-polluted soils in Coimbatore, Tamil Nadu, India. This research aims to contribute to the development of environmentally acceptable, efficient, and economical substitutes for traditional thrombolytic medications by improving culture conditions and assessing these enzymes' capacity to dissolve clots.

3.1 MATERIAL USED

In Vellakinar, Mettupalayam, Thudiyalur, P.N. Palayam, Karamadai, Idayarpalayam, Kanuvai, and G.N. Mills in Coimbatore, Tamil Nadu, India, soil samples were taken from locations close to metal industry. These locations were chosen because industrial areas frequently have a wide variety of microbial populations that can produce crucial industrial enzymes like proteases.

The serial dilution technique was used to isolate protease-producing bacteria from the collected soil samples. Nine milliliters of sterile distilled water were used to suspend about two grams of dirt. Aliquots were then transferred into new sterile water up to a dilution of 10^{-2}. After spreading roughly 100 µL of each dilution onto skim milk agar plates, the plates were incubated for 48 hours at 39 °C. Protease activity was suggested by the clear zones that surrounded the bacterial colonies, which showed that casein had been hydrolyzed. Colonies displaying these zones were determined to be protease-producing bacteria and were chosen for more investigation. For preservation, these isolates were subcultured on nutrient agar slants and kept at 4 °C.

A loopful of the master plate's bacterial culture was added to nutrient broth to create a pre-inoculum for the synthesis of enzymes. To guarantee proper growth, this culture was shaken and incubated at 39 °C for a whole day. After that, 20 mL of freshly made protease production media was added to 2 mL of the pre-inoculated culture, and the mixture was shaken for 48 hours at 39 °C. Following incubation, the supernatant was recovered by centrifuging the culture broth for five minutes at 10,000 rpm. For additional examination, this supernatant was utilized as the crude extracellular enzyme extract.

Fresh human blood samples were used to assess the crude extract's thrombolytic activity. To enable clot formation, about 6 mL of blood was put into sterile microcentrifuge tubes that had been previously weighed. The tubes were then incubated at 39 °C for 45 minutes. Following clotting, the tubes were weighed once more to ascertain the clot weight after the serum was carefully extracted without upsetting the clot. 100 µL of the bacterial crude extract was applied to each clot-containing tube, with distilled water serving as a negative control. For ninety minutes, the tubes were incubated at 39 °C. The tubes were weighed once more to determine the degree of clot lysis after the discharged fluid was removed during incubation. The difference in clot weight before and after treatment was used to calculate the percentage of clot lysis.

Gram staining and a number of biochemical tests were used to further describe the bacterial isolates. Motility testing, oxidase and catalase activity, indole generation, methyl red and Voges-Proskauer reactions, citrate utilization, urease activity, and tests for the fermentation of` carbohydrates utilizing glucose, lactose, and sucrose were among them. Important details about the isolates' physiological and metabolic traits were revealed by these testing.

Casein was used as a substrate in a Tris-HCl buffer solution to measure protease activity. Trichloroacetic acid was used to stop the reaction after the substrate and enzyme extract-containing reaction mixture was incubated at room temperature. Tyrosine was used as a standard in the Lowry method analysis of the filtrate after the mixture was filtered. To create a standard curve, different tyrosine concentrations were made. A spectrophotometer was used to detect the absorbance at 670 nm, and the amount of tyrosine released was used to compute the enzyme activity. The amount of enzyme needed to release one microgram of tyrosine per minute under the assay conditions was determined as one unit of enzyme activity.

Bovine serum albumin was used as the standard in the Lowry method to measure the protein content of the crude enzyme extract. The test sample was handled similarly, and various concentrations of the standard were created. By comparing the observed absorbance values with the standard curve, the protein content was determined.

To assess the variables influencing protease production, more research was done. In order to identify the ideal incubation period, the impact of incubation time was evaluated by monitoring enzyme activity at regular intervals over a number of days. By incubating the production medium at various temperatures and measuring the enzyme activity to determine the ideal temperature, the impact of temperature on enzyme production was investigated. Additionally, by adding alternative substrates to the production medium and assessing their effects on enzyme synthesis, the impact of diverse carbon and nitrogen sources on protease production was examined.

Ammonium sulfate precipitation, dialysis, and gel filtration chromatography were among the procedures used to purify the crude enzyme extract. A protein precipitate was created in the precipitation step by adding ammonium sulfate to reach a particular saturation level. The precipitate was then collected by centrifugation and dissolved in buffer. To get rid of extra salts and small molecules, the partially purified enzyme was dialyzed against phosphate buffer. Sephadex G-50 column chromatography was used for additional purification, separating the enzyme according to molecular size and gathering fractions for examination. To track the effectiveness of purification, protein concentration and enzyme activity were evaluated at each stage.

To ascertain the molecular properties of the purified fractions, electrophoretic methods were employed. Proteolytic activity was detected by fibrin zymography, and molecular weight was estimated using SDS-PAGE on a polyacrylamide gel. Fibrin-containing gels were made for zymography, and the gels were modified

to permit enzyme activity following electrophoresis. After staining, areas of protease activity were shown by clear zones.

The same method used for the crude extract was also used to assess the thrombolytic activity of the partially purified fractions. By comparing the weight before and after treatment, the degree of clot lysis was ascertained, giving information about the isolated enzyme's efficacy.

The impact of metal inhibitors was investigated in order to better understand the nature of the enzyme. Enzyme activity was assessed in the presence of various inhibitor doses. To find out if the enzyme needed metal ions to function, ethylenediaminetetraacetic acid was used as a chelating agent. The enzyme was identified as either a metalloprotease or a serine protease based on changes in activity when this inhibitor was present.

SPSS software was used to statistically assess all of the experimental outcomes. The mean values and standard deviation from several experimental replicates were used to express the data. Analysis of variance and suitable post hoc testing were used to establish statistical significance; a p-value of less than 0.05 was deemed significant.

RESULT

1 Screening of Protease Producing Bacteria

Nine soil samples polluted with metals were gathered in order to isolate bacterial colonies that produce proteases. For additional testing, a total of sixteen bacterial colonies that formed different zones on skim milk agar plates (Fig.1) were chosen and kept on nutrient agar slants (Table 1).

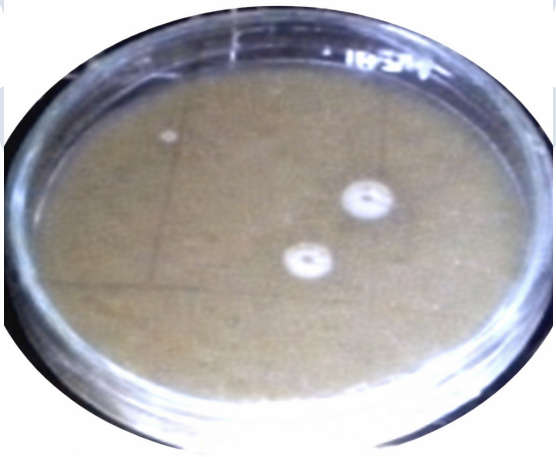

Fig. 1 Skim milk agar plate

Table 1. Isolation of protease producing bacterial colonies on skim milk agar.

S.No.	Place of Soil Sample Collection	Isolates	Colonies Obtained from Serial Dilution	Total no. of colonies isloated
1.	Kiran metal industry, Vellakin	11,14	10^{-2}	2
2.	Vinayaga metal industry, Mettupalayam	6	10^{-4}	1
3.	Amman metal industry, Thudiyalur	9,5	10^{-4}	3
4.	Deva mateal industry, P.N palayam	2,7	10^{-4}	2
5.	Dhana metal industry, Karamadai	3,15	10^{-4}	1
6.	Thambi metal transporting company, Idayarpalayam	5,10	10^{-4}	2
7.	Sivagami company, Kanuvai	1,6	10^{-3}	1
8.	Texmo comapny, G.N Mills	12	10^{-3}	1
9.	Ragu metal industry, Vellakinar	14,18	10^{-3}	2

2 Screening of Effective Protease Producer

Using protease-producing broth, the presumed protease-producing colonies from skim milk agar plates were exposed to increased protease production. Protease activity and protein quantification were carried out on the crude extract made from the inoculated broth. BP12 (8.23 ± 0.11 mg/mL), BP15 (7.28 ± 0.13 mg/mL), and BP16 (9.18 ± 0.15 mg/mL) showed considerably higher protease activity among the 16 isolates assessed for protease generation, with statistical significance at $P < 0.001$. (Table.2).

Table 2 In vitro clot lysis and specific activity of the crude bacterial extracts.

Isolates	Clot Lysis (%)	Protease (mg/mL)	Protein (µg/mL/min)	Specific Activity (µg/mg/min protein)
BP1	6.5 ± 0.1	6.0 ± 0.1	1.10 ± 0.03	2.75 ± 0.1
BP2	6.1 ± 0.1	3.32 ± 0.13	1.32 ± 0.02	2.50 ± 0.1
BP3	4.6 ± 0.1	3.64 ± 0.13	1.28 ± 0.04	2.86 ± 0.1
BP4	13.8 ± 0.2	3.95 ± 0.13	1.42 ± 0.05	2.88 ± 0.1
BP5	8.0 ± 0.1	4.99 ± 0.13	1.48 ± 0.06	3.10 ± 0.1
BP6	4.9 ± 0.1	3.01 ± 0.13	1.33 ± 0.03	2.30 ± 0.1
BP7	12.3 ± 0.1	3.64 ± 0.13	1.05 ± 0.01	3.60 ± 0.1
BP8	38.5 ± 0.3	4.16 ± 0.13	1.85 ± 0.03	2.35 ± 0.1
BP9	29.1 ± 0.2	6.18 ± 0.13	2.18 ± 0.13	2.85 ± 0.1
BP10	42.0 ± 0.3	3.33 ± 0.13	1.65 ± 0.13	2.10 ± 0.1
BP11	29.0 ± 0.2	6.80 ± 0.13	2.60 ± 0.1	2.70 ± 0.1
BP12	63.5 ± 0.3	8.23 ± 0.11	2.75 ± 0.07	3.10 ± 0.07
BP13	12.0 ± 0.1	3.64 ± 0.1	1.02 ± 0.01	3.60 ± 0.03
BP14	56.2 ± 0.2	3.9 ± 0.1	1.58 ± 0.04	2.40 ± 0.1
BP15	94.8 ± 0.1	7.26 ± 0.13	2.83 ± 0.08	2.60 ± 0.08
BP16	56.6 ± 0.1	9.15 ± 0.15	3.09 ± 0.1	2.97 ± 0.1

Control - 2.4 ± 0.05 Standard - 97.4 ± 0.1

3. In Vitro Thrombolytic Activity of Crude Extract

The inoculated broth was used to make the crude extract of each of the sixteen isolates, and its in vitro thrombolytic activity was assessed. Three of the sixteen bacterial crude extracts that were evaluated (BP12, BP15, and BP16) showed efficient clot lysis of 62.8 ± 0.3%, 94.5 ± 0.1%, and 58.6 ± 0.1%, respectively, with statistical significance at $P < 0.001$. (Fig.4. 2 and Table 4.2).

Fig 2 In vitro thrombolytic activity of bacterial crude extract.

IV. Characteristics of Protease Producer

Following morphological and biochemical evaluation of the three isolates with efficient protease production and clot lysis activity, the following findings were noted (Table 3). It was discovered that all three isolates were non-motile, Gram-positive spore-forming rods. According to Bergey's manual, BP12's extensive biochemical traits—such as catalase, oxidase, methyl red, Voges-Proskauer, urease activity, starch hydrolysis, and sugar fermentation—strongly suggest that the organism is Bacillus cereus. Its categorization within this species is further supported by the production of acid from glucose, lactose, and sucrose during the fermentation of carbohydrates. The isolate may be a member of a non-saccharolytic Bacillus species, likely Bacillus alcalophilus or a non-fermentative variation of Bacillus subtilis, based on the biochemical tests and non-fermentative carbohydrate profile of BP15 and BP16. It's interesting to note that these isolates tested positive for indole, which is unusual for Bacillus species and could indicate strain variations.

SR. NO.	Biochemical Tests	BP12	BP15	BP16
1	Oxidase test	+	+	-
2	Catalase test	+	+	+
3	Indole test	+	-	+
4	Methyl red(MR) test	+	+	-
5	VP test	-	+	+
6	Citrate utilization test	-	+	-
7	Urease test	+	+	-
8	Starch hydrolysis	+	-	+
9	Carbohydrate fermentation test	Acid	No Reaction	No Reaction
	Glucose:	Acid	Acid	No Reaction
	Lactose:	No Reaction	No Reaction	Acid
	Sucrose:	No Reaction	No Reaction	No Reaction

Table 3 Biochemical characterization of the Isolates.

V. OPTIMIZATION OF PRODUCTION MEDIUM

5.1 Effect of Incubation Days on Protease Production

For growth and protease synthesis, an incubation period of three days (72 hours) was shown to be optimal, with a significance of $p < 0.05$ (Fig. 5.5.1).

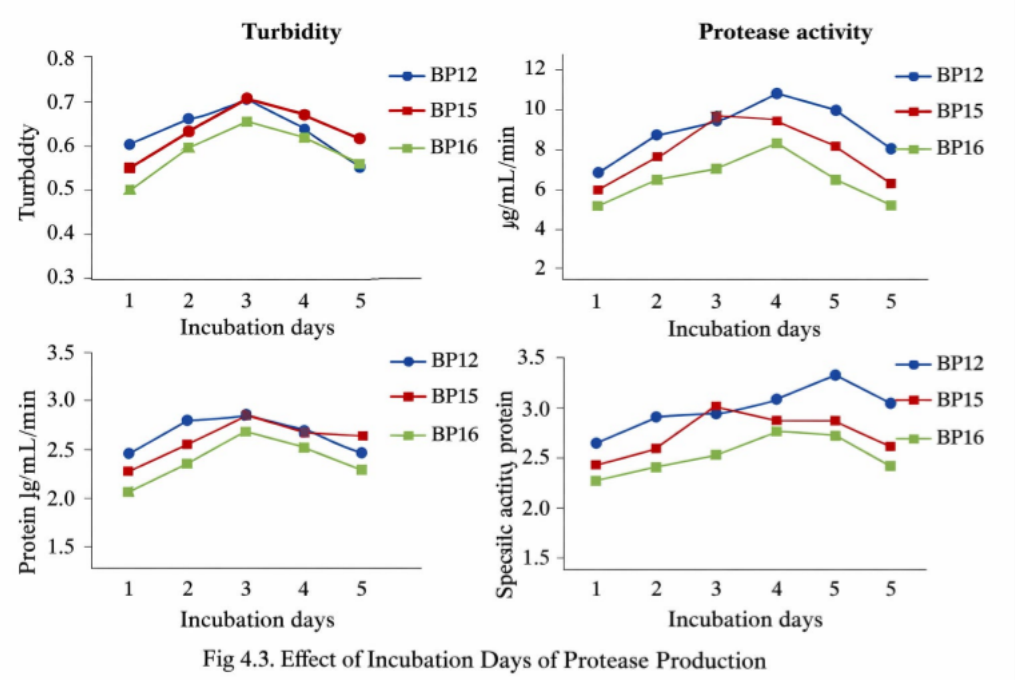

Fig 4.3. Effect of Incubation Days of Protease Production

Fig 5.5.1 Effect of Incubation Days of Protease Production.

5.2 Effect of pH on Protease Production

The production medium with a pH of 7 was determined to be the best for growth and protease production among the different pH values examined, with significance of $p < 0.05$ for BP15, BP16, and pH of 9 for BP12 (Fig.5.5.2).

Fig. 5.5.2 Effect of pH on Protease Production.

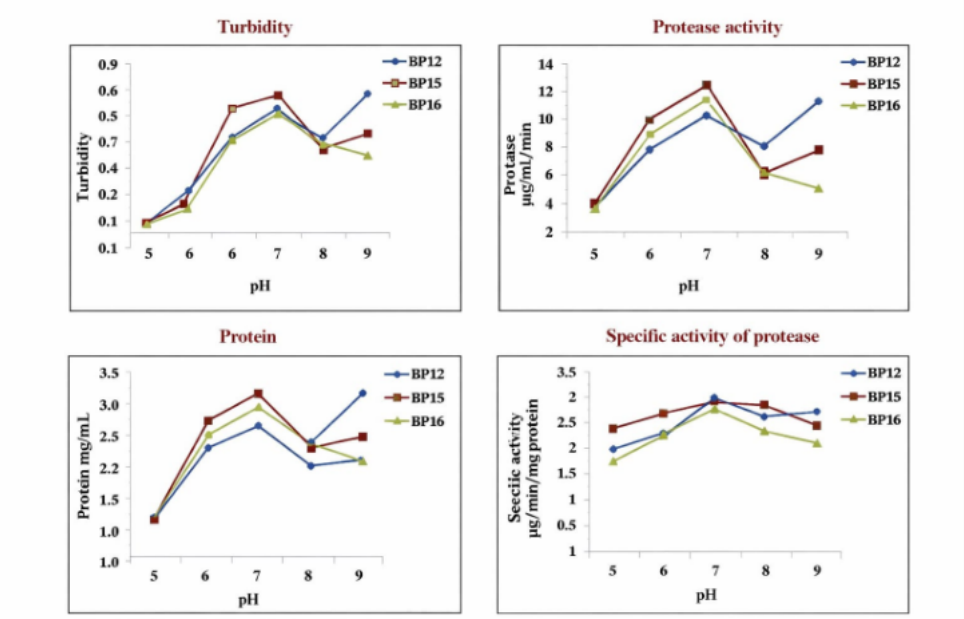

Fig. 4.4 Effect of pH on Protease Production

5.3 Effect of Temperature on Protease Production

The ideal temperature for growth and protease synthesis was determined to be between 47 °C and 57 °C (BP12, BP16) and 57 °C (BP15) out of all the temperatures studied (Fig. 5.5.3). They are hence thermostable. Growth and protease production do not significantly differ at the various temperatures that were examined.

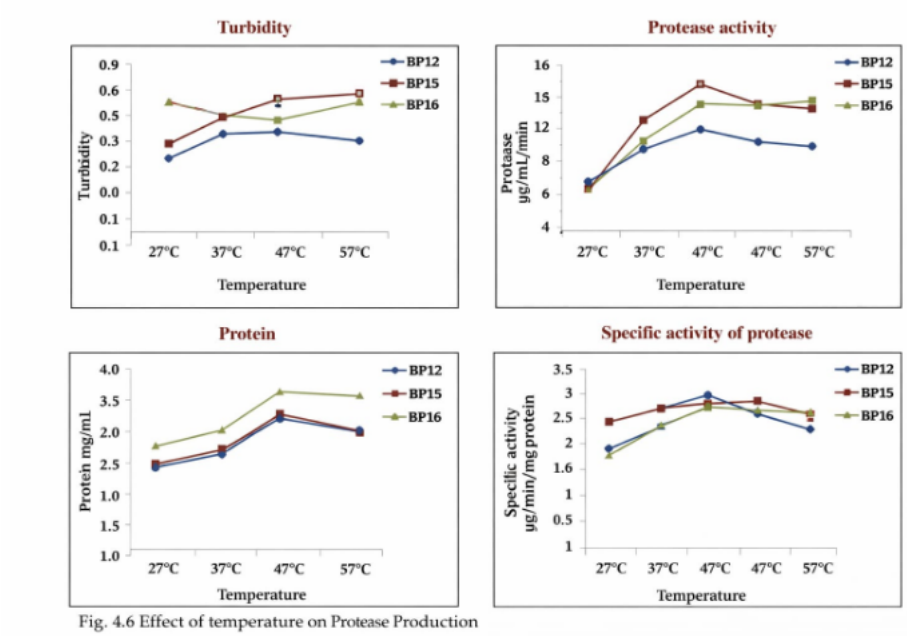

Fig. 4.6 Effect of temperature on Protease Production

Fig. 5.5.3 Effect of Temperature on Protease Production.

5.4 Effect of Carbon Source on Protease Production

It was discovered that sucrose (0.5%) produced significantly ($p < 0.001$) more growth and protease than the other carbon sources (Fig. 5.5.4).

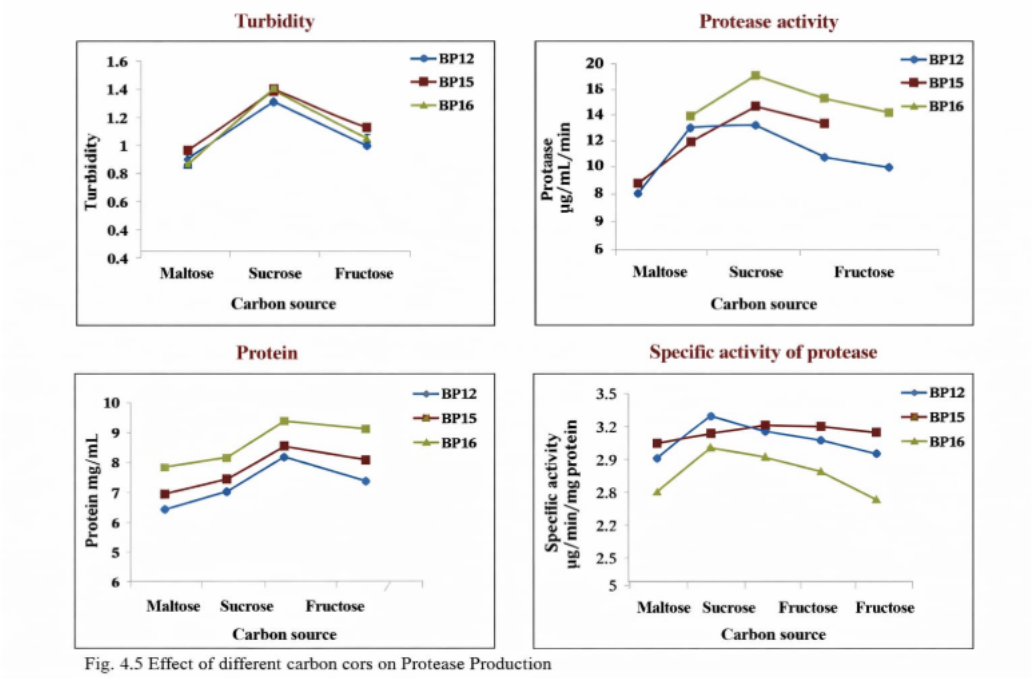

Fig. 4.5 Effect of different carbon cors on Protease Production

Fig. 5.5.4 Effect of Carbon Source on Proteae Production

5.5 Effect of Nitrogen Source on Protease Production

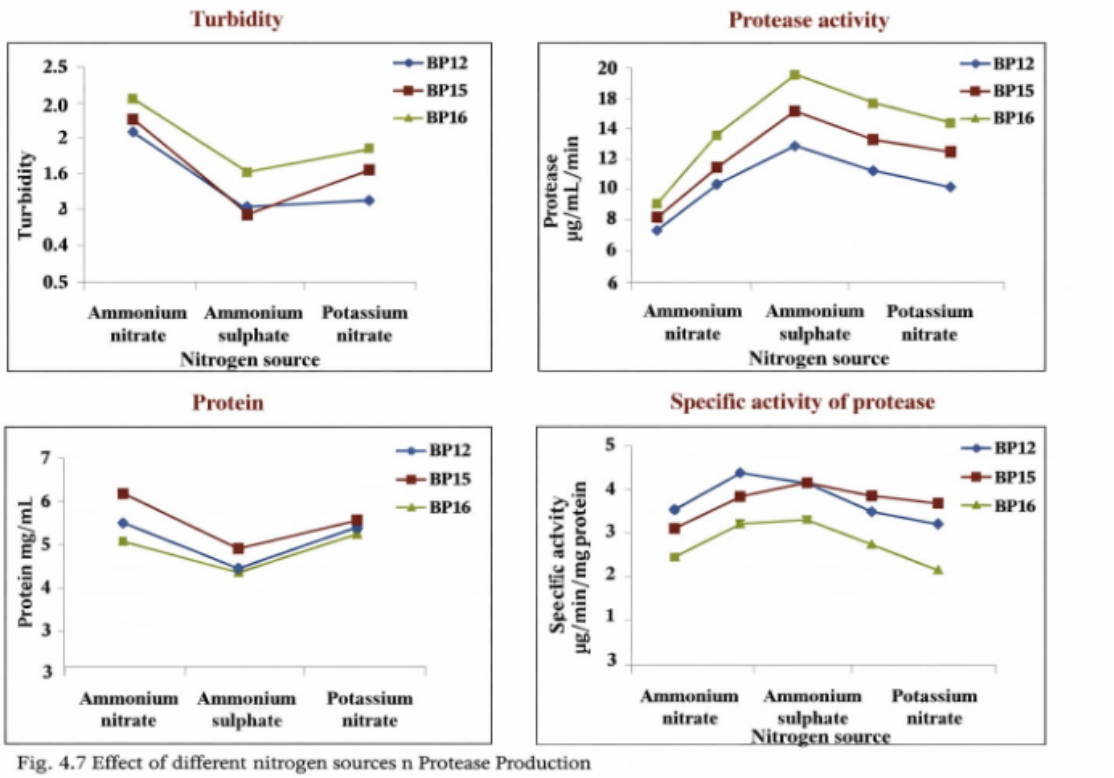

Fig. 4.7 Effect of different nitrogen sources n Protease Production

Fig. 5.5.5 Effect of Nitrogen Source on Protease Production

Ammonium nitrate (0.5%) was shown to provide significantly ($p<0.001$) greater growth and protease production than the other nitrogen sources studied (Fig. 5.5.5).

VI. Purification of Protease

Fractions 1 and 2 of the ten fractions extracted from the column for BP12 and BP15, respectively, demonstrated the highest levels of specific activity. The fractions 1, 3, and 4 of BP16 demonstrated the highest specific activity. The isolated fractions showed defined zones that corresponded to molecular weights of roughly 29 kDa (BP12-F2), 31 kDa (BP15-F1), 15 kDa (BP15-F9), and 25 kDa (BP16-F3) according to fibrin zymography (Fig. 6). One proteolytic band was seen in each fraction, indicating that the enzymes were comparatively uniform and pure.

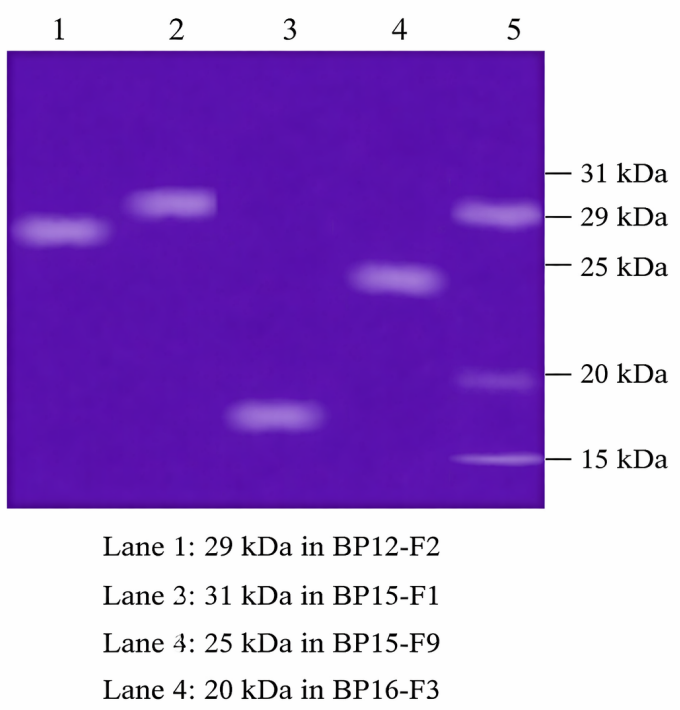

Fig. 6. Fibrin Zymogram of partially purified fractions of BP12, BP15 and BP16.

VII. In Vitro Thrombolytic Activity of the Partially Purified Fractions

In vitro clot lysis activity was tested for fractions F1, F2 of BP12, F1, F8, F9 of BP15, and F1, F3, F4 of BP16. Clot lysis was 37.9% in fraction 1 and 97.6% in fraction 2 of BP12. Clot lysis was 99.7% in fraction 1 and 85.7% in fraction 9 of BP15. In contrast, BP16's fractions 1 and 3 demonstrated clot lysis of 96% and 92.5%, respectively (Fig. 4.9). When compared to the crude extract, the partially purified fractions of BP12, BP15, and BP16 showed a much higher clot lysis percentage (Fig. 7). The rise in particular activity following partial purification would be the cause of this. The fractions F2 of BP12, fraction F1 of BP15, and fraction F1 of BP16 demonstrated the greatest clot lysis among the three fractions with elevated specific activity chosen for in vitro thrombolytic activity for BP12, BP15, and BP16. When compared to fraction F2, fraction F1 of BP12 did not exhibit efficient clot lysis, although having a higher specific activity. In a similar vein, BP15 fractions F1 and F9 exhibit efficient clot lysis in comparison to fraction F8. Fractions F1 and F3 of BP16 exhibit greater clot lysis activity than fraction F4. Because certain clot lysis proteases are present in the corresponding

fractions that exhibit effective clot lysis, the fractions exhibiting higher specific activity in each of the three examples do not exhibit effective clot lysis.

S – Standard, C – Control, 1-12F1, 2-12F2, 3-15F1, 4-15F8, 5-16F1, 6-16F1, 7-16F3, 8-16F4

Fig. 7 *In vitro* thrombolytic activity of partially purified fractions of BP12, BP15 and BP16.

VIII. Effect of Metal Inhibitor

The relative activity of the partially purified fractions treated with EDTA (0.1 mM, 1.0 mM, and 10 mM) was computed. Table 4 displayed the outcomes. Because of the chelating action of EDTA, the relative activity of proteases in the fractions exhibiting efficient clot lysis significantly decreased. This suggests that the metalloproteases found in the fractions F2 of BP12, F1, F9 of BP15, and F1 and F3 of BP16 would be in charge of the efficient clot lysis activity. In comparison to the other fractions, the fractions F1 of BP12 and F8 of BP15 had greater relative activity. Furthermore, there is no effective clot lysis in these fractions (F1 of BP12, F8 of BP15, and F4 of BP16).

Fractions	Control	10mM EDTA	1mM EDTA	0.1mM EDTA
BP12F1	100	56.45	75.30	87.45
BP12F2	100	29.39	55.89	64.77
BP15F1	100	26.95	69.27	84.70
BP15F8	100	38.5	77.7	81
BP15F9	100	14.32	42.90	77.78
BP16F1	100	25	61	100
BP16F3	100	13.0	28	62.5
BP16F4	100	55.65	76.83	85.7

Table 4 Relative Activity of Partially Purified Fraction.

IX. PURIFICATION FOLD

The yield percentage was found to decline with each purification step, yielding a total yield of 18.13%, 8.20%, and 17.31%, respectively. However, the protease's purity rises by 3.0931, 8.89, and 12.95 times, respectively (Table 5, Table 6, and Table 7).

Table 5 Purification fold for F1, F2 of BP12

Purification Step	Total Volumne (mL)	Total Amount of Protein (mg)	Total Protease Activity (µg)	Specific Activity (µg/mg)	Purifica-Tion Folds	Yield (%)
Crude extract	25	27.5	251.207	9.052	1	100
Dialysate	2.5	5.7361	155.9544	27.16	2.99	62.54
Sephadex G 50 F1 fraction	1	1.55	55.6968	35.73	3.5014	22.34
F2	1	1.615	45.2	30.03	3.0931	18.13

Table 6 Purification Fold from F1, F8, F9 & BP15

Purification Step	Total Volumne (mL)	Total Amount of Protein (mg)	Total Protease Activity (µg)	Specific Activity (µg/mg)	Purifica-Tion Folds	Yield (%)
Crude extract	25	118.65	601.25	5.067	1	100
Dialysate	2.5	5.9875	234.95	39.24	7.74	39.07
Sephadex G 50 fraction 13 F1	1	2.155	88.92	41.26	8.14	14.78
F8	1	1.235	54.43	44.07	8.697	9.05
F9	1	1.095	49.36	45.08	8.89	8.20

Table 7 Purification Fold for Fraction F1, F3, F4 & BP16

Purification Step	Total Volumne (mL)	Total Amount of Protein (mg)	Total Protease Activity (µg)	Specific Activity (µg/mg)	Purifica-Tion Folds	Yield (%)
Crude extract	25	86.25	336.25	3.89	1	100
Dialysate	2.5	4.8875	198.575	40.629	10.44	59.05

Sephadex G 50 fraction 14 F1	1	1.62	76.58	47.27	12.15	22.77
F3	1	1.29	62.65	48.38	12.43	18.63
F4	1	1.15	58.22	50.41	12.95	17.31

X. RESULT

Soil samples taken from habitats contaminated with heavy metals were effectively used to extract bacterial strains that produce proteases. To identify the ideal variables affecting enzyme production, such as pH, temperature, incubation period, and nutrient sources, these isolates were assessed under various environmental and nutritional circumstances. The enzymes were isolated and partially purified after protease activity was tracked during growth on production media. In vitro thrombolytic activity was used to evaluate their functional effectiveness and establish that they could break up fibrin clots.

BP12, BP15, and BP16 showed the highest protease activity out of the sixteen isolates that were collected. Significant thrombolytic capability was also shown by these isolates, suggesting prospective uses in biological domains. Enzyme production varied with ambient conditions, according to optimization studies, with maximal activity seen after 72 hours of incubation. Higher enzyme synthesis was favored by neutral to slightly alkaline pH and moderate to high temperatures; ammonium nitrate and sucrose were shown to be efficient suppliers of nitrogen and carbon, respectively. These results are in line with previous research that found comparable ideal conditions for protease activity.

The thrombolytic activity of the partially purified enzyme fractions varied, indicating the presence of certain fibrinolytic components in some fractions. The fact that some fractions had high protease activity did not necessarily translate into high clot lysis suggests that different types of enzymes have different functions. The enzymes' molecular weight varied from 15 to 31 kDa, which is consistent with fibrinolytic proteases from bacterial sources—specifically, Bacillus species—that have been previously reported.

The enzymes are probably metalloproteases, according to additional characterisation employing metal inhibitors, which showed a drop in enzyme activity in the presence of chelating chemicals. Even though the overall yield was relatively lower, purification experiments revealed higher enzyme purity. Overall, the findings show that bacterial isolates from soil are viable sources of proteases with potential use in industry and medicine. They may be used in the treatment of thrombotic diseases and other enzyme-based biotechnological processes due to their notable proteolytic and thrombolytic capabilities.

XI. CONCLUSION

Protease-producing bacterial strains were successfully isolated and identified in this study from soil samples polluted with heavy metals. Three isolates (BP12, BP15, and BP16) were discovered to have noticeably high protease activity after environmental and nutritional parameters like pH, temperature, and carbon and nitrogen sources were systematically optimized. These isolates' fibrinolytic qualities were further highlighted by their significant in vitro clot-dissolving potential. Strong thrombolytic activity was found in some fractions after enzyme purification and additional characterization; this was probably caused by the presence of metalloproteases, as shown by sensitivity to EDTA.

The results highlight the potential of these soil-derived bacteria to provide useful enzymes for industry and medicine, especially for thrombolytic applications. The use of these proteases in biopharmaceutical and therapeutic domains may be made possible by additional research on their molecular identification and processes.

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