ISOLATION AND IDENTIFICATION OF PROTEASE-PRODUCING *BACILLUS* STRAIN FROM COLD CLIMATE SOIL AND OPTIMIZATION OF ITS PRODUCTION BY APPLYING DIFFERENT FERMENTATION CONDITIONS

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Abstract. Proteases have received a lot of attention in recent years thanks to their use in a variety of industries, including the food, feed, leather, textile, and pharmaceutical sectors. The soil sample from a cold climate was used in the current experimental study of protease production. In order to identify the protease-producing bacterial species, morphological, physiological, and biochemical analyses were carried out, and the isolated bacterial strain's ability to produce proteases on skim milk agar was assessed. As a result of casein hydrolysis, a zone formed surrounding the bacterial colony suggested a protease-positive strain. To increase the production of protease by isolated *Bacillus* species, various sources of nitrogen and carbon were examined. Yeast extract (309.32 IU/ml) was the nitrogen source that supported growth the best among the other sources studied, and fructose (269.4 IU/ml) was the carbon source that produced the most protease activity. The fermentation time of 72 h (393.7033 IU/ml) was anticipated to be the best setting for protease synthesis. When 2% (w/v) soybean meal was employed, protease activity was measured at 494.92 IU/ml. The influence of pH was investigated, and it was discovered that 7.5 was the best and optimal pH for *Bacillus* species to produce 511 IU/ml of protease. When the temperature range was discovered to be 40°C, the highest protease activity of 542.93 IU/ml was recorded.
Keywords: *protease, Bacillus species, yeast extract, physiological, biochemical analysis, casein hydrolysis*

Introduction

Enzymes are proteins and are used in a variety of industrial processes as biological catalysts (Asha and Palaniswamy, 2018). Proteases, the largest class of enzymes that function as proteinases, peptidases, and amidases, are important industrial biocatalysts with a variety of industrial uses (Naveed et al., 2021; Ullah et al., 2022). This enzyme has the ability to hydrolyze peptide bonds (ROOC-NH-R) and peptide groups. Proteases

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are classified by the Enzyme Commission as being in hydro-lase group 3 and subgroup 4 (peptide bond hydrolysis) (EC) (Contesini et al., 2018). Specific amino acid chains in the form of protein fragments or peptides make up bioactive peptides that regulate biological functions but become inactive when incorporated into the chain of a protein (Kaur et al., 2020). For commercialization purposes, in vivo and in vitro studies are widely used to assess the degree of protein breakdown during separation, which is the primary determinant of the bioavailability of isolated peptides. Protein fragments or peptides made from certain amino acid chains are used to regulate the body's natural processes. Bioactive peptides can alter how the body responds to life-threatening diseases like cancer and strengthen the immune system (Quintal-Bojórquez and Segura-Campos, 2021). The sales of industrial enzymes in several industrial market sectors, including detergent, food, pharmaceuticals, leather, diagnostics, waste management, and silver recovery, represent the largest product segment in the worldwide industrial enzymes market, which is growing annually (Gimba et al., 2021). Due to their employment in numerous industries, proteases have gained a lot of notoriety recently, including the food, leather, pharmaceutical, and other industries (Tsado et al., 2022). One of the enzymes with several industrial uses is protease (Balachandran et al., 2021). The commercial sector makes extensive use of proteases that can remain active in settings of high temperature, acidity, and salt concentration. The bulk of proteases become ineffective in severe industrial environments. Therefore, it is necessary to look for novel proteases that can endure and work in challenging environments, increasing their commercial value (Hashmi et al., 2022). Proteases can be used in many different industries, including Production of deter-gents, food, silk, leather, dairy products, beverages, brewing, textiles, bioremediation, and medicines, be-cause of their high activity and stability over a broad range of temperature and pH (Hakim et al., 2018). Due to their rapid production rates and cheap cultivation needs, microorganisms are now the primary source of the majority of proteases available on the market. The employment of proteases in the wastewater treatment, food, deter-gent, leather, pharmaceutical, cosmetic, silk degumming, and pharmaceutical industries is also briefly mentioned. The methods for enhancing the protease's catalytic abilities through protein engineering and immobilization are then carefully thought out. To meet their ever-increasing demands for industrial exploitation, the search for new sources of protease enzyme has been emphasized (Naveed et al., 2021; Ullah et al., 2022). It is well known that 40–60% of all enzyme sales worldwide are made up of proteases derived from microorganisms (Kumar, 2020). Over the past ten years, it has become clear that using cold adapted bacteria in food industry operations rather than organisms and their enzymes that demand higher temperatures offers numerous economic and ecological benefits (Far et al., 2020). These environments foster the growth of bacteria that serve as sources for a number of enzymes, including lipases and proteases, which are used in the food and pharmaceutical industries, among other sectors (Elyasi et al., 2020). Microorganisms cannot develop in icy environments due to a number of factors, including chemical reactions, freezing related damage, and membrane stiffness. These unfavorable conditions have led to the evolution of adaptive adaptations in cold-modified organisms, such as the development of cold active enzymes and the production of proteins that regulate the formation of ice crystals (Bar Dolev et al., 2020). As a result, researchers have started to refine and use enzymes in a variety of businesses. These enzymes have garnered interest because of their distinctive benefits, which include the capacity to accelerate chemical reactions at low temperatures in order

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to minimize microbial load, low cost, high catalytic efficiency, lastly, the ability to catalyze chemical reactions at low temperatures, making it simple to inactivate using heat rather than chemical inactivators in order to protect nutrients that are sensitive to heat. Due to these characteristics, enzymes are one of the substances that are widely utilized in the medicine and food industries (Bruno et al., 2019). Numerous fascinating extracellular enzymes produced by the Bacillus species genera have been used commercially in the detergent, textile, food, feed, beverage, and detergent industries. Recently, the development of strains and production procedures has been affected by or made possible by the application of molecular biology techniques. Bacillus species are excellent industrial organisms because of their quick development, capacity to secrete proteins, and safety for humans. Enzymes that are used in a wide range of industrial processes can be released by numerous Bacillus species (Ortiz and Sansinenea, 2022). The primary genus that generates neutral proteases is Bacillus because they function and remain stable at neutral or slightly acidic/alkaline pH levels, neutral protease family enzymes include metalloproteases, several serine proteases as well as cysteine proteases. The active site and subsequent enzymatic catalysis process are related to the neutral proteases' zinc ion. Bacterial proteases are helpful in the food industry because they can hydrolyze proteins. Neutral protease has long been employed in bakeries (Razzaq et al., 2019).

Materials and methods

Collection of soil sample and strain isolation

To find the bacteria that produce proteases, soil samples were taken in winter season from the upper most 0–5 cm layer consisted by compost comprising dead animal's leftovers from the various places in Parachinar, Khyber Pakhtunkhwa Pakistan. After being collected, the samples were put in a clean zipper bag. 200 µL of the diluted solution was applied to nutrient agar plates after dilution of the samples up to a 10-10 concentration. Bacteria were isolated using a repeated subculturing technique after the plates had been incubated at 37°C for 48 h (Bishop et al., 2018).

Screening protease production

The bacteria isolates were initially tested in sterile conditions for their capacity to create proteases on skim milk agar medium plates. After the purified isolates were streaked on skim milk agar plates and incubated at 37°C for 24-48 h, zones of hydrolysis were then identified. A quantitative assay was then performed on the isolates that produced bigger zones (Hashmi et al., 2022).

Screening of proteolytic strain

Selected protease-producing bacterial isolates' overnight cultures were transferred to a 500 mL nutrient broth flask, augmented with skim milk (10 g), $MgSO_4$, sodium chloride (0.5 g), glucose (5 g), and potassium phosphate (10 g/l), and cultured at 37°C for 48 h, while being shaken at 150 rpm. By centrifuging the culture containing media at 8000 rpm for 15 min, the crude enzymes were extracted. The crude proteinase extract from the cell-free supernatant was obtained and used to test for proteinase activity (Ahmed et al., 2016).

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Protease specific activity assay

To 10 mL of Luria-Bertani (LB) broth media, a 1% inoculum of bacteria was introduced and cultivated for 24 h at 37°C while being shaken. Centrifuge the pallet at 6000 rpm after removing it. Check the supernatant's optical density (OD) at 660 nm by Spectrophotometer SP-300 Optima-Japan for better bacterial growth. Using a micropipette, add 0.5 ml of casein solution to 0.5 ml of supernatant. After adding the TCA solution, centrifuge it at 6000 rpm for 10 min at 55°C. Remove the pallet and add 1 ml folin solution, 5 ml of carbonate solution, and 5 ml of used supernatant. After 30 min, read the absorbance at 660 nm using a spectrophotometer. Different temperatures, carbon sources, nitrogen sources and incubation times were used to test the characteristics of the proteases (Hashmi et al., 2022).

Fermentation medium

The composition of fermentation medium was MgSO4 0.5, NaCl 0.5, glucose 5, KH2PO4 10 g/l and soya bean meal 10 g/l as a substrate in an Erlenmeyer flask with a capacity of 250 ml and 100 ml of the production medium. The medium was autoclaved at 121℃ for 15 min; inoculated a 48-h incubation of a 24-h fresh culture at the completion of fermentation period the culture medium the enzyme was extracted by centrifugation at 9000 rpm for 10 min. the pellets was discarded and the supernatant was a used as crude enzyme to estimate the proteolytic activity.

Screening of nitrogen and carbon sources

The production of proteases was studied using a variety of organic and inorganic nitrogen sources, such as peptone, yeast extract, urea, and $NaNO_3$, as well as carbon sources like glucose, fructose, starch, and sucrose. Under submerged fermentation, bacteria used soybean meal as a substrate to create protease (Gul et al., 2015).

Optimization of process parameters

The parameters were optimized using a one factor at a time approach while using various variables. For the synthesis of protease, the effects of pH 7, 7.5, 8, 8.5, and 9 as well as incubation period and temperatures of 25, 30, 35, 40, and 45°C were investigated. Various concentrations of soya bean meal (1.0, 2.0, 3.0, 4.0, and 5.0%) were optimized as a substrate (Homaei and Izadpanah Qeshmi, 2022).

Results

Isolation and screening of proteolytic strain

Bacterial strains were isolated by pour plate method on the medium of skim milk agar. After 24 h incubation the colonies having clear zone on the utilizing the skim milk agar media were subjected for secondary screening. The 16 colonies exhibited zones of clearance on the medium of skim milk agar and were transferred to the screening medium (*Table 1*). Out of these, 1 bacterial isolate of serial no 9 exhibit maximum protease activity (163.68 ± 11.78 IU/ml/min) and was selected for further study.

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Table 1. *Protease activity of different bacterial isolates*

S. No	Enzyme activity IU/ml ± SD	Clear zone mm	S. No	Enzyme activity IU/ml ± SD	Clear zone mm
1	83.02 ± 3.83^{bc}	10	9	163.68 ± 11.78^{g}	33
2	76.06 ± 7.93^{b}	08	10	118.95 ± 2.78^{de}	21
3	59.33 ± 2.84^{a}	ND	11	87.77 ± 5.18^{bc}	18
4	127.92 ± 7.26^{ef}	17	12	138.87 ± 1.99^{f}	24
5	140.87 ± 2.23^{f}	30	13	121.80 ± 1.96^{de}	19
6	95.94 ± 5.87^{c}	13	14	117.85 ± 1.37^{de}	16
7	120.37 ± 4.65^{de}	19	15	84.34 ± 5.51^{bc}	ND
8	74.75 ± 6.88^{b}	07	16	110.75 ± 1.35^{d}	15

Each value is the mean of three replicates and the ± specifies the standard deviation (SD) between the replicates. Values in each column that are followed by different letters vary significantly from each other

Identification of proteolytic strain

The selected strain was grown on nutrient agar medium to study its morphological and biochemical characterization. Gram staining results indicated that the strain is gram positive rod as shown in *Figure 1,* and the various biochemical results are indicated in *Table 2.*

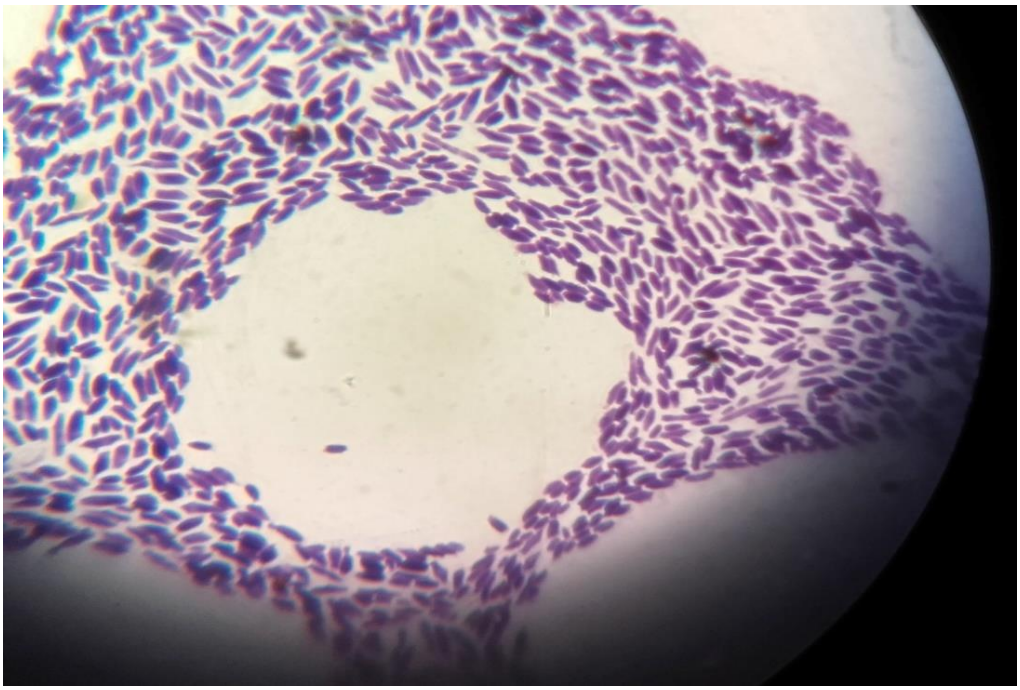

Figure 1. *Showing the microscopic image of identified Bacillus species*

Screening of nitrogen and carbon source

The organic and inorganic sources of nitrogen were screened for the maximum production of proteases. Maximum proteolytic activity 309.32 IU/ml was achieved by using the yeast extract an organic nitrogen source in the production medium as denoted in *Figure 2.* Among various carbon sources the fructose was found to be a best source of carbon for the production of bacterial proteases exhibited 269.4 IU/ml represented in *Figure 3.*

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Table 2. *Morphological and biochemical characterization*

Morphological features	
Color	White
Shape	Rods
Gram staining	Positive
Endospore staining	Positive
Biochemical features	
Urease	Positive
Catalase	Positive
Oxidase	Positive
Indole test	Negative
Casein hydrolysis test	Positive
Methyl red test	Positive

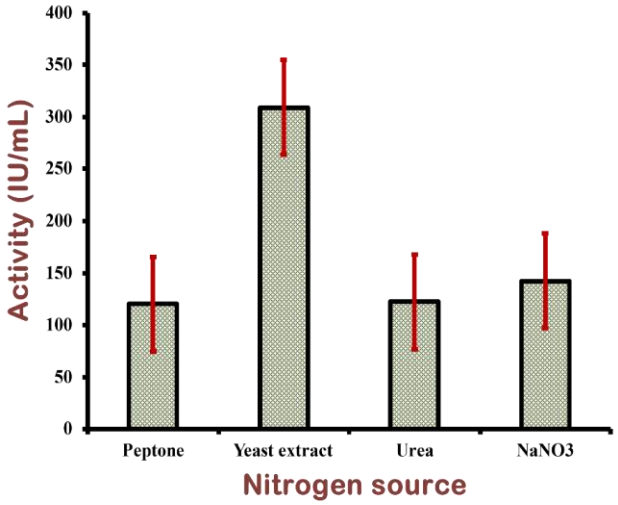

Figure 2. *Screening of nitrogen sources for protease activity. The error bars indicate standard deviation ± between the replicates*

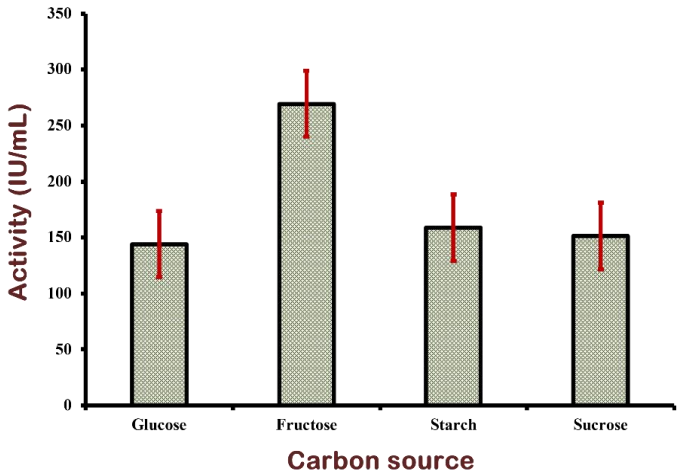

Figure 3. *Screening of carbon sources for protease activity. The error bars indicate standard deviation ± between the replicates*

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Optimization of parameters

Bacillus protease production and the impact of incubation time were investigated, and the optimum cultivation time was found 72 h. The maximal protease activity 393.7033 IU/ml was achieved by 72 h of fermentation time as expressed in *Table 3*. The optimum level of soybean meal was found 2% (w/v) in the basal medium having 494.92 IU/ml of activity as represented in *Table 4*, the impact of pH was studied and 7.5 was found to a best and optimal condition for protease yield by *Bacillus sp.* The maximum protease activity 511 IU/ml was obtained shown in *Table 5* the optimum temperature range was found 40℃ given in *Table 6*.

Discussion

Microorganisms found in their natural habitats must be isolated and put to the test because they can contain enzymes with unusual properties. A variety of microorganisms, such as bacteria, yeasts, and fungus, produce the protease enzymes, are one of the types of enzymes that are essential to industry on a commercial level. In this study, soil samples from arctic regions were collected. Microorganisms that make proteases were found using a specific culture medium that contained skim milk. For bacteria to grow, they must be able to manufacture the protease enzymes. 16 colonies that produce a clean zone on skim milk were chosen for this experiment and placed through a further screening. Additionally, in earlier investigations, protease-producing bacteria were screened using skim milk (Omidinia, 2012; Ali et al., 2016; Homaei and Izadpanah Qeshmi, 2022).

Table 3. *Effect of incubation period on protease production*

S.No	Incubation period	Enzyme activity ± SD
1	24	210.1067 ± 17.32^{a}
2	48	265.6733 ± 9.37^{b}
3	72	393.7033 ± 11.68^{d}
4	96	348.2167 ± 28.78^{c}

Each value is the mean of three replicates and the ± specifies the standard deviation (SD) between the replicates. Values in each column that are followed by different letters vary significantly from each other

Table 4. *Effect of substrate concentrations on protease production*

S.No	Substrate Conc. %age	Enzyme activity ± SD
1	1	398.8767 ± 9.00^{a}
2	2	494.9200 ± 26.63^{a}
3	3	402.2500 ± 58.01^{a}
4	4	448.3633 ± 16.02^{a}
5	5	424.1067 ± 91.13^{a}

Each value is the mean of three replicates and the ± specifies the standard deviation (SD) between the replicates. Values in each column that are followed by different letters vary significantly from each other

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Table 5. *Effect of pH on protease production*

S.No	pH	Enzyme activity ± SD
1	7	415.33 ± 34.78^{b}
2	7.5	511.00 ± 9.53^{d}
3	8	452.66 ± 37.60^{cd}
4	8.9	340.82 ± 20.50^{a}
5	9	362.42 ± 37.85^{ab}

Each value is the mean of three replicates and the ± specifies the standard deviation (SD) between the replicates. Values in each column that are followed by different letters vary significantly from each other

Table 6. *Effect of Temperature on protease production*

S.No	Temperature	Enzyme activity ± SD
1	25	247.61 ± 46.23^{a}
2	30	323.02 ± 19.07^{b}
3	35	474.31 ± 32.80^{c}
4	40	542.93 ± 21.28^{d}
5	45	423.75 ± 7.37^{c}

Each value is the mean of three replicates and the ± specifies the standard deviation (SD) between the replicates. Values in each column that are followed by different letters vary significantly from each other

One isolate was chosen for further study out of the 16 that were tested in the subsequent stage because it had the highest level of protease activity. These strains were known as *Bacillus* species due to their physical and biochemical characteristics. They were gram-positive, motile rods. The similar result was also obtained by another investigation (Ullah et al., 2021). The composition of the media has a considerable impact on how enzymes are synthesized by microorganisms. For protease to grow and be produced in large volumes that are also commercially viable, it is essential to optimize the fermentation conditions. No standardized medium has been developed for the best production of alkaline proteases from varied microbial sources. To maximize the synthesis of enzymes, each organism or strain has certain needs (Sharma et al., 2017).

When testing various nitrogen sources for the generation of proteases, all of the sources permitted a significant amount of protease production, although they were still less than yeast extract. The use of yeast extract as an organic nitrogen source in the production medium resulted in the maximum proteolytic activity of 309.32 IU/ml. Another study came to the same conclusion (Hammami et al., 2018). Among the several carbon sources, glucose was found to be the best one for the development of bacterial proteases. When bacteria were exposed to fructose, 269.4 IU/ml IU/ml of protease were generated. Similar results obtained from a different study (Gul et al., 2015). In addition to these, environmental parameters such as temperature, pH, and incubation period have a significant impact on microbial metabolism. To encourage, stimulate, improve, and maximize the synthesis of proteases, these elements are crucial (Abusham et al., 2009; Abidi et al., 2018).

Among different incubation times, 72 h of fermentation resulted in the highest amount of protease activity (393.7033 IU/ml). Another study came to the same conclusion (Srividya, 2011). The rate of protease production was shown to be two times

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higher in the optimized medium as compared to the unoptimized reference medium when soyabean was used as the basal media, a finding supported by a previous investigation (Shabbiri et al., 2012).

These enzymes demonstrate a broad range of pH activity and catalytic activity at both high and low pH values, making them appealing for both fundamental sciences and industrial applications. Additionally, the effect of pH was investigated, and 7.5 was discovered to be the greatest and most ideal setting for *Bacillus* sp. to produce protease. A maximum of 477.03 IU/ml of protease activity was attained (Homaei and Izadpanah Qeshmi, 2022). When multiple temperatures were examined, 40°C produced protease enzyme at the best rate. Identical conclusion reached by earlier research (Lingouangou et al., 2022).

Conclusions

The present study was conducted on the isolation and identification of protease producing *Bacillus* strain from cold climate soil and optimization of its production by applying different fermentation conditions. Soil is the most common habitat for all types of microorganisms that produces various types of secondary metabolites. In this research work protease was produced and various physiochemical parameters were optimized to enhance the yield of enzymes. Soya bean meal an agro industrial waste and a protein source was utilized as a substrate. Yeast extract and fructose were used as nitrogen and carbon source. One factor at a time technique was used for optimization. Optimization of parameters increases the production of protease to 3.5 folds as compared to un optimized condition.

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Conflict of interests. The authors declare no conflict of interests.

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