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Proteolytic Activity of Microbial Isolates from Plant Rhizosphere

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A B S T R A C T

The soil quality is different as per location wise. The content comprises the broad factors which improved during the microbial consortia. Microbes having important role in soil nutrient content and solubilization of complex nutrient. The plant rhizosphere having variety of microorganisms play important role in soil root nutrient uptake and protection. Microbes and their enzymes solubilizing complex insoluble material into simple form, the rhizospheric soil isolates are studied for their plant root and microbial interaction. Soil health is more conserved for agriculture field for quality and quantity of the plant. Here proteolytic activity and their partial characterization is carried using rhizosphere isolates. Microbial isolates also reported for L-Aparginase and L-glutaminase activity from different plant rhizosphere their further investigation under progress. Here report of proteolytic activity of microbial isolate from *Trigonella foenum-graecum* root plant reported. The proteolytic activity may role as biocontrol agent to infection to the plant pathogen. Microbial enzymes and microbial transformation important role in microbes and plant interaction. This proteolytic activity is important role in nutrient cycling and plant growth promoting, as it helps release essential nutrients like nitrogen from dead organic matter. Also proteases produced by rhizosphere microorganisms can act as biocontrol agents

Introduction

Proteases are enzymes that break the proteins to smaller peptides or amino acids (Patil *et al.,* 2015). Protease is an important industrial enzyme, generally obtained from microbes, animal and plant sources (Harish and Chauhan, 2017). Proteases from microbial sources are preferred over the enzymes from plant or animal sources since they possess almost all the characteristics desired for their biotechnological applications. Microbial proteases represent a good source of enzymes due to a number of characteristics like their broad biochemical diversity, their rapid growth, the limited space required for cell cultivation and the ease with which the enzymes can be genetically manipulated to generate new enzymes for various applications. Production of proteases from microbial sources depends on several factors such as media composition, temperature, pH, incubation, moisture (Harish and Chauhan, 2017). Proteases are produced by a wide

range of microorganism including bacteria, moulds and yeasts, actinomycetes etc. In bacteria, this enzyme is produced mainly by a member of strains belonging to genus *Bacillus* especially, *B. licheniformis; B. horikoshii, B. sphaericus, B. furmis, B. alcalophilus, B. subtilis. Bacillus* species are the main producer of Extracellular proteases, and industrial sectors frequently use *B. subtilis* for production of various enzymes (Rahman *et al.,* 2018).

The significance of root proteases in N- mineralisation presents a knowledge gap. Additionally, microbes play a crucial role in the cycling of other essential elements like phosphorus, sulfur, and potassium, ensuring their availability for plant growth. The ecological significance of *Bacillus* spp. in soil ecosystems, for instance, cannot be overstated. Their ability to carry out multiple ecological functions, including nutrient cycling and stress tolerance, makes them essential in maintaining soil health and supporting plant growth (Hanan S. Alnahdi, 2012, Saxena *et al.,* 2020). The proteolytic fibrinolytic activity, purification and characterization from isolated thermophilic *Streptomyces sp* is reported (Chitte *et al* 2011).

Rhizosphere proteases play a crucial role in soil mineralization by facilitating the breakdown of proteinaceous organic matter, releasing nitrogen and other nutrients, which are then available for plant uptake nutrients

Materials and Methods

Sample Collection

Soil sample was collected from Rhizosphere Soil of methi. Rhizosphere soil samples along with root pieces was collected and stored in plastic bags at 4°C temperature until further processing.

Figure.1 Sample collected from rhizosphere sample of methi in agriculture plot

Screening of Protease Producing Isolates

The techniques used for isolation of bacteria were serial dilution and spread plate method. 1 gm of soil sample was weighed and serial dilution (10^{-1} to 10^{-6}) of soil sample was carried out. 0.1 ml of each aliquot was spread on skim milk agar. The plates were incubated at 37°C for 24-48 hr. The Zone of hydrolysis was noted for each sample. The colony showing highest zone of inhibition was selected for further study. The colony was grown on nutrient agar slant at 4°C.

Identification of Bacteria

For obtaining isolates, the swab Samples were cultured on St. Nutrient agar, St. Mac Conkey's agar, St. Mannitol salt agar plate and St. Superimposed Blood agar plates. The plates were incubated aerobically at 37ºC for 24 hours. Nutrient agar slants were incubated at 37°C for 18 – 24 hours before storage in the refrigerator at 4°C pending biochemical analysis.

Identification

Morphological Characterization of Isolates

Morphological examinations of the bacterial isolates were done on the basis of culture characteristics and colony morphology.

Microscopic Characterization of Isolates

Cellular shape, Gram reaction was determined by bright field microscopy.

Gram staining

In order to determine the Gram reaction of the organism, an even smear, (neither too heavy nor too light) of the test bacterium, was prepared on a clean glass slide, air dried and heat fixed. The smear was flooded with crystal violet reagent and allowed to stand for 1 minute followed by rinsing gently with water using a wash bottle. Gram's iodine was added and allowed to stand for 1 minute and then washed with water. The smear was then decolorized with 95 % alcohol drop by drop for 5-10 seconds until the excess stain washes off and then immediately rinsed with water. The smear was then counterstained with safranin for 30 seconds and then washed. The slides were observed under bright field microscope to determine the Gram reaction of the organism

Biochemical Characterization of Isolates

A loop-full of sample fro was streaked on to nutrient agar plate and incubated at 37 °C for 24 h. From the resulting agar culture, a loop-full of culture was again added to media containing different biochemical and incubated at appropriate temperature for 24 hours. Presence or absence of changes in the media was recorded as positive and negative, respectively

Carbohydrate fermentation test

The carbohydrate fermentation test checks for the ability of a particular organism to ferment a specific carbohydrate. The test also checks for the production of gas during fermentation. Microorganisms fermenting carbohydrate, produces acid or acid with gas, which is detected by the change in colour of the medium and gas formation can be demonstrated by the use of Durham's tube, which collects the gas. Utilization of different sugars was tested by inoculating the test organism in sterile broth containing a specific sugar (Glucose, Xylose, Mannitol, Lactose, Maltose and Sucrose) and inverted Durham's tube. Following incubation, the tubes were examined for colour change and bubble formation. 2.

Methyl Red (M-R) test

Sterile MR-VP broth tubes were prepared and inoculated with the selected isolates and incubated at a temperature of 37 0C for 24 - 48 hrs. Post-incubation, the tubes were added with 5 drops of methyl red and were observed for any colour change.

Voges-Proskauer (V-P) test

Glucose phosphate broth was autoclaved and cooled to room temperature. The selected isolates were inoculated using a sterile loop followed by incubation at 37 0C for 24-48 hrs. Post-incubation, 1 ml alpha-naphthol was added and shaken then followed by addition of 0.5 ml of 40 % KOH to the broth and shaken

Indole production test

The peptone broth medium was prepared and autoclaved. After cooling the medium, it was inoculated with the selected isolate and incubated at 37°C for 24-48 hrs. Few drops of Kovac's reagent were added step by step. The tube was shaken and allowed to stand for 10 min, to form a layer

Triple Sugar Iron (TSI) test

Gas production was detected using TSI agar slants which are prepared from a mixture of agar, a pH-sensitive dye (phenol red), 1% lactose, 1 % sucrose, 0.1 % glucose, sodium thiosulfate and ferrous sulphate. The bacterial isolate to be studied was inoculated both by streaking on slant and stabbing the butt. After inoculating, incubate TSI agar slant tubes at 37°C for 24-48 hours, presence of H_2S, colour change on the slant and in the butt were observed and Production of H_2S was indicated by the blackening of the TSI medium.

Citrate Utilization test

Preparation and sterilization of Simmons citrate agar tube agar tube are done and inoculation of selected isolates and incubated at 37°C for 24-48 hrs

Quantitative determination of protease activity

Crude enzyme preparation

The protease producing bacterial colony was inoculated in Nutrient broth medium. It was incubated at 37 0C for 24 hrs. The culture broth was subjected to centrifugation at 10,000 rpm for 20 minutes to remove unwanted particles. The supernatant was used as crude enzyme preparation for further studies (Manorma *et al.,* 2017).

Protease assay

Spectrophotometric analysis was carried out to determine protease activity, using casein as a substrate.

Two test tubes were taken and labeled as the test (T) and blank (B).1 ml of 1 % casein in 50 mM sodium phosphate buffer (pH 7) and 1 ml enzyme solution was added to the test sample (not to the blank) and incubated at 37 0C for 20 min. After 20 min of incubation at 37 °C, the reaction was terminated by adding 2 ml of 10 % Trichloroacetic acid (TCA) to both blank and test sample. 1 ml of enzyme was now added to blank such that the total volume in test and blank reaches 4 ml. Both the tubes again incubated at 37 °C for 20 min. Then centrifuged at 10,000 rpm for 15 min. 0.5 ml of clear supernatant was mixed with 2.5 ml of 0.5 M Na2CO3 and 0.5 ml of 1N Folin-Ciocalteau's phenol reagent. The solution was mixed properly by swirling and incubated at 37 0C for 20 min. The absorbance of the test and blank solutions were taken at 660 nm. Readings were calibrated against a standard curve of tyrosine.

One unit of protease activity was defined as the amount of enzyme that releases 1 µg amino acid equivalent to tyrosine per min under the standard assay conditions.

Units / ml = μ *mole of tyrosine X reaction volsample volume X reaction time X vol assay*

Determination of total protein content

The crude enzyme (supernatant) that collected is processed further to determine the total protein concentration present in the medium by Lowry's method (1951). Bovine serum albumin (BSA) was used as a standard for protein estimation

Specific activity

Specific activity is the activity of an enzyme per milligram of total protein. It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of total proteins.

The specific enzyme activity (U/mg) was calculated by the following formula specific enzyme activity=Enzyme Activity Total protein content

Results and Discussion

Screening of Protease Producing Isolates

The serial dilution procedure was used for the isolation of the pure bacterial colonies from the samples. The Skim milk agar media was used for the growth of the bacteria. More than 25 different bacterial colonies were isolated and studied during the experiment.

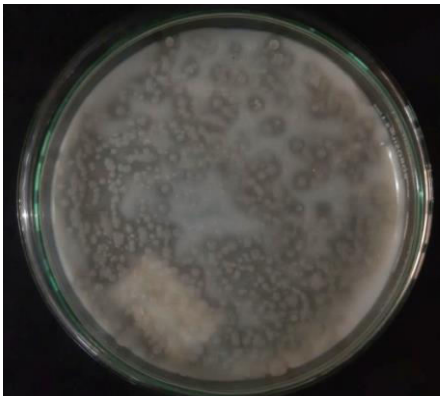


Figure.2 Isolated bacterial colonies

More than 30 different bacterial colonies were isolated (Figure # 2) and studied during the experiment. After observation total 6 colonies were selected and inoculated for the Zone of hydrolysis on the skim milk agar plate

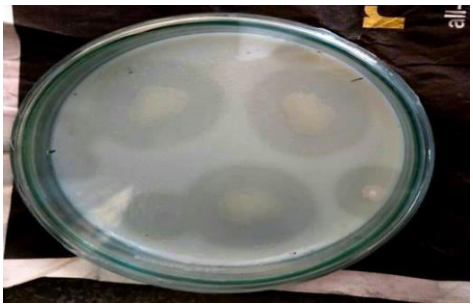


Figure.3 Zone of clearance on Skim milk agar plate

More than 30 different bacterial colonies were isolated and studied during the experiment. After observation total 6 colonies were selected and inoculated for the Zone

of hydrolysis on the skim milk agar plate (Figure # 3). The zone of diameter of colonies shown in the table.1.

Effect of the growth conditions for Production of Protease

Effect of Time on the Production of Protease Activity

The effect of Time period (1-9 Days) was tested for P4 and P6. It is observed that with the increase in time duration, the Protease activity (U/ml) also increases.

The maximum Protease Activity 0.29 (U/ml) for P4 was recorded at 7th day. The maximum Protease activity 0.28 (U/ml) for isolate P6 was recorded at 7th day (Table.2).

Effect of Temperature on the Production of Protease

The optimum temperature for P4 and P6 was found to be 37°C; Results showed that temperature between 37°C has been suitable for enzyme activity. As the maximum protease activity for isolate P4 was 0.21 (U/ml) and for isolate P6 was 0.22 (U/ml) at Temperature 37°C.

Table.1 Zone of diameter

S.No	Isolate	Zone of diameter (mm)
1	**P1**	20 mm
2	**P2**	20 mm
3	**P3**	17 mm
4	**P4**	25 mm
5	**P5**	23 mm
6	**P6**	25 mm

Table.2 Quantitative assay for protease enzyme production by the two selected

S. No.	Enzyme producers	Protease Activity(U/ml) (Crude Enzyme)	Specific Activity (U/mg) (Crude Enzyme)	Total Protein content (µg/ml) (Crude Enzyme)
1	**P4**	0.28	3.04	92.69
2	**P6**	0.32	3.20	109.80

Table.3 Incubation time with protease activity

Effect of Incubation time on protease activity		
Incubation period in days	**P4**	**P6**
1	0.17	0.20
2	0.18	0.21
3	0.21	0.23
4	0.22	0.24
5	0.25	0.26
6	0.27	0.28
7	0.29	0.28
8	0.26	0.27
9	0.22	0.24

Figure.4 Effect of Incubation Time on Protease Activity

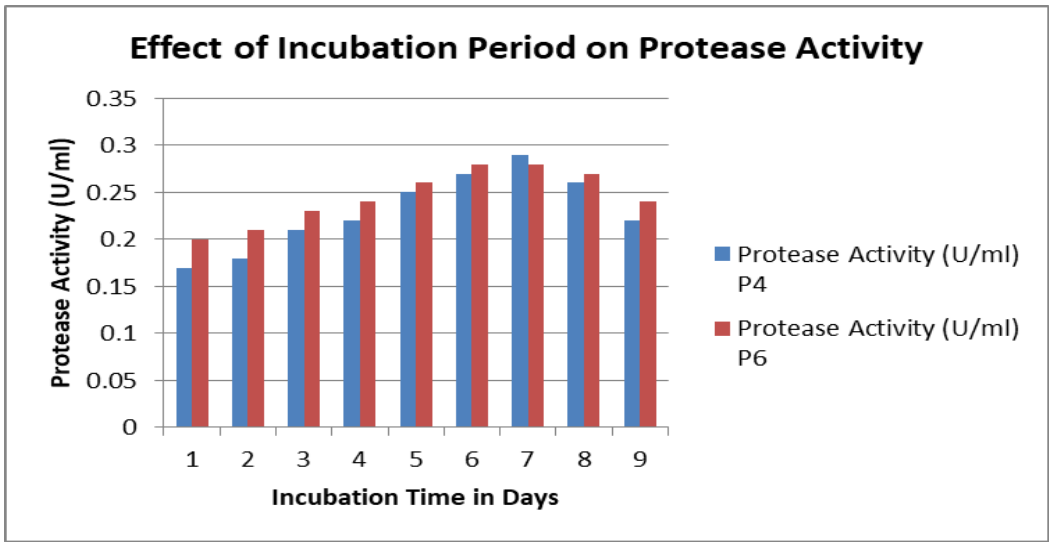


Table.4 Effect of Incubation Temperature on Protease Activity

Temperature (◦c)		Protease Activity (U/ml)
P4	**P6**	**P6**
30	0.18	0.20
37	0.21	0.22
40	0.13	0.14
45	0.12	0.13
50	0.04	0.09

Table.5 Effect of Incubation time on Protease Activity

Incubation Period (Days)	Protease Activity (U/ml)	
	P4	**P6**
1	0.17	0.20
2	0.18	0.21
3	0.21	0.23
4	0.22	0.24
5	0.25	0.26
6	0.27	0.28
7	0.29	0.28
8	0.26	0.27
9	0.22	0.24

Figure.5 Effect of Incubation Time on Protease Activity

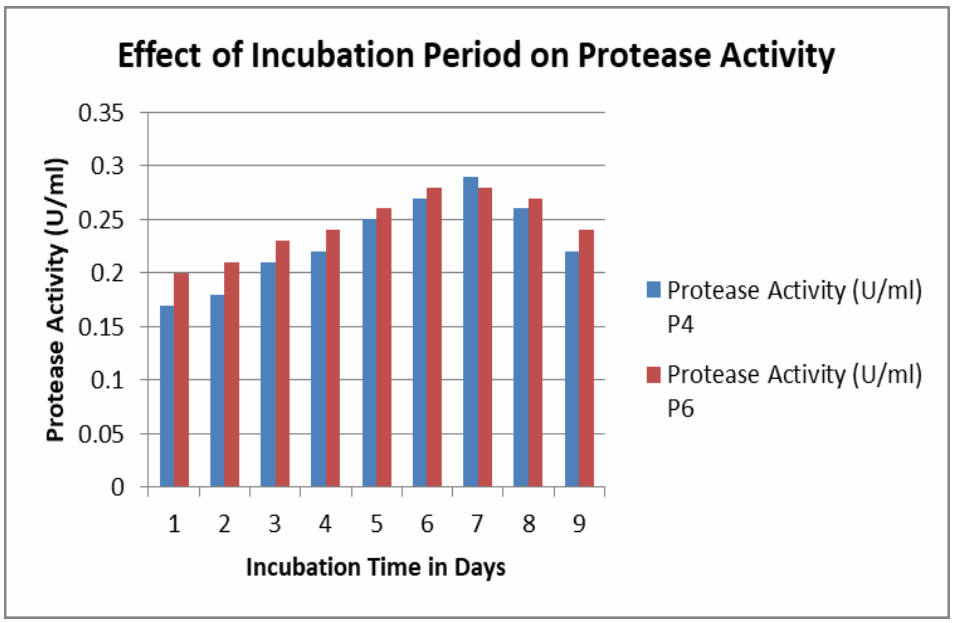
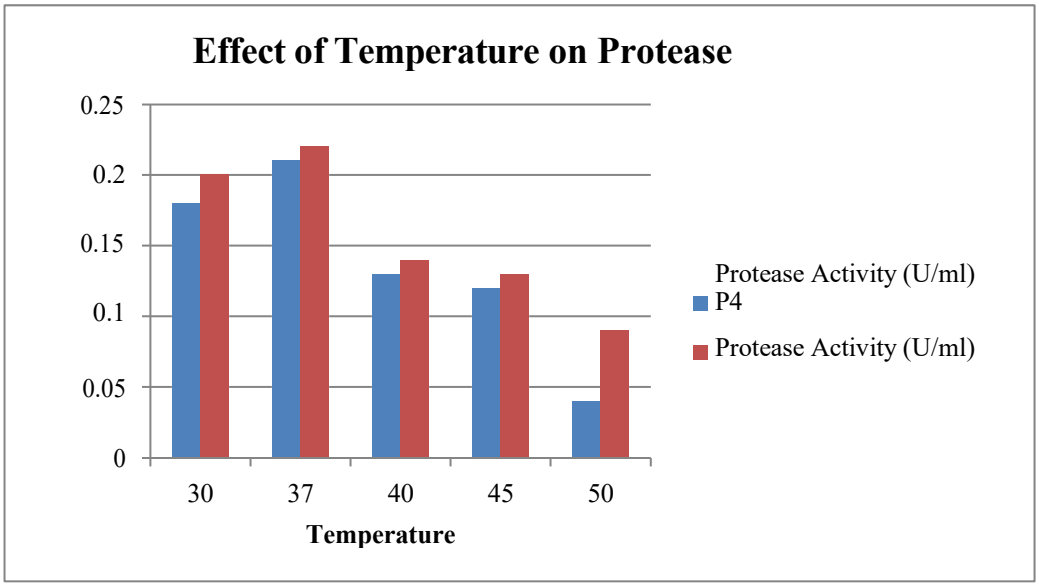


Table.6 Effect of Incubation Temperature on Protease Activity

Temperature (◦c)	Protease Activity (U/ml)	
30	0.18	0.20
37	0.21	0.22
40	0.13	0.14
45	0.12	0.13
50	0.04	0.09

Figure.6 Effect of Temperature on Protease Activity



Effect of Temperature on the Production of Protease

The optimum temperature for P4 and P6 was found to be 37 °C; Results showed that temperature between 37 OC has been suitable for enzyme activity. As the maximum protease activity for isolate P4 was 0.21 (U/ml) and for isolate P6 was 0.22 (U/ml) at Temperature 37 OC (Figure # 6)

Effect of pH on the Production of Protease

The optimum pH for protease production for the Isolates P4 and P6 was at pH-7 to 9, as the maximum protease activity for isolate P4 was 0.28 and for isolate P6 was 0.28 at pH-9. The pH 7 to 9 was found suitable for both bacterial isolates. (Table # 7 & Figure # 7).

Effect of Substrate Concentration on Protease Activity

Protease activity was measured at different substrate concentration level. Result showed that with the increase in the substrate concentration enzyme activity also increases (Table # 8 and Figure # 8).

Table.7 Effect of pH on the Protease Activity

pH	Protease Activity (U/ml)	
	P4	P6
5	0.20	0.21
6	0.20	0.22
7	0.23	0.25
8	0.25	0.27
9	0.28	0.28
10	0.19	0.19

Figure.7 Effect of pH on Protease Activity

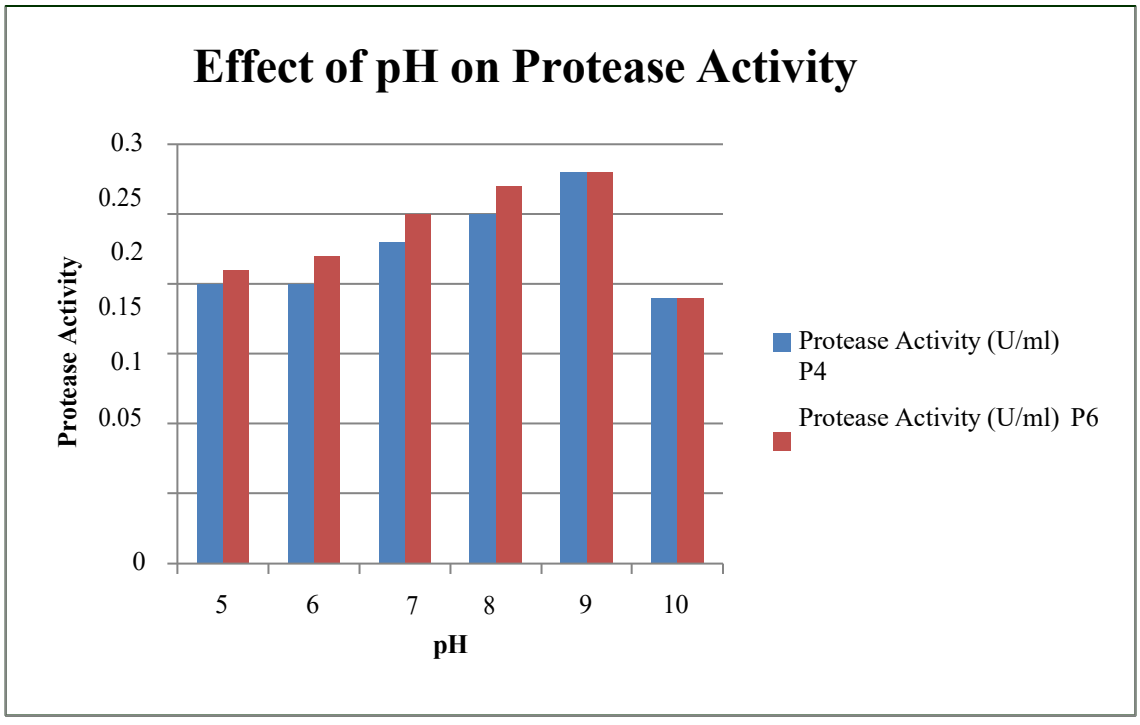
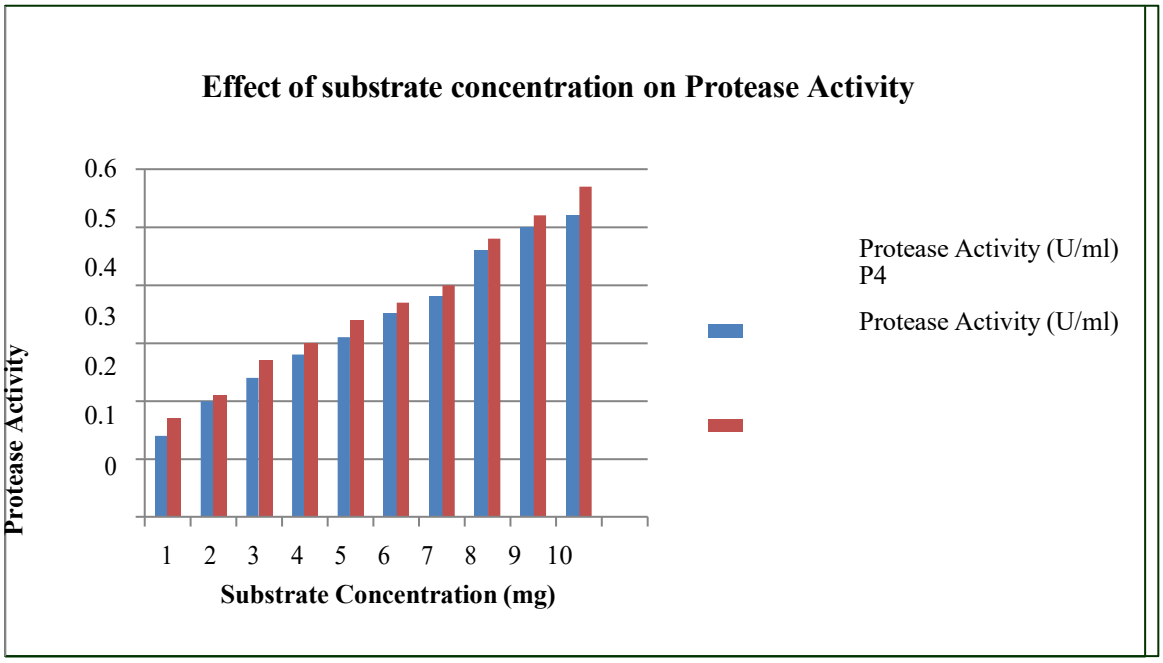


Table.8 Effect of Substrate Concentration on Protease Activity

Substrate concentration (mg)	Protease Activity (U/ml)	
	P4	P6
1	0.14	0.17
2	0.20	0.21
3	0.24	0.27
4	0.28	0.30
5	0.31	0.34
6	0.35	0.37
7	0.38	0.40
8	0.46	0.48
9	0.50	0.52
10	0.52	0.57

Figure.8 Effect of substrate concentration on Protease Activity



In conclusion, Proteases are one of the most important groups of industrial enzymes with considerable application in the animal feed processing, leather industry, medical activity, beverage industry and others sectors. In the Industrial sector, bacillus species are widely used to produce extracellular proteases, Bacillus subtilis is most frequently used species for this purpose, it is rod shaped bacterial species; it can survive in extreme environmental condition.

The first 6 bacterial isolates were identified from the Rhizosphere soil. Among the, two bacterial isolates showed potent Proteolytic protease activity on skim milk agar plate. Two bacterial isolates P4 and P6 are further characterized. On the basis of morphological and biochemical characteristics, these 2 isolates were identified to belong to genus Bacillus. These proteases is reported as biocontrol agent for pathogenic infection to the plant root. Further microbial intereaction to plant root need to investigate further

Author Contributions

Dharti Kurkutia: Investigation, writing—original draft. Jhanvi Chaudhari: Validation, methodology, writing-reviewing, Ratnakar Chitte: Formal analysis, writing-review and editing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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