Int.J.Curr.Microbiol.App.Sci (2017) *6*(5): 249-255

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 5 (2017) pp. 249-255**
Journal homepage: http://www.ijcmas.com

EXCELLENT PUBLISHERS

Original Research Article

https://doi.org/10.20546/ijcmas.2017.605.030

Screening and Isolation of Protease Producing Bacteria from Rhizospheric Soil of Apple Orchards from Shimla District (HP), India

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Keywords

Protease activity, Rhizosphere soil, Apple orchard, *Pseudomonas* sp., Skim milk.

Article Info

Accepted:
04 April 2017
Available Online:
10 May 2017

A B S T R A C T

The objective of present study was to screen and isolate protease producing bacteria from soil samples collected from rhizospheric soil of apple orchard from Kotkhai of Shimla District. Collected soil samples were serially diluted and 0.1ml of sample was spread on skim milk agar plates at 37^0C for 48 hrs. Out of fifteen, six bacterial colonies from rhizospheric soil samples showed clear zone around the colony indicating protease activity. Among these, two isolates *i.e.* KK_3 and KK_4 produced highest protease activity and was identified as *Pseudomonas Sp*. by physiological, morphological and biochemical test, the isolated protease producing bacteria also having antifungal activity against different plant pathogenic fungi. The above results indicate that these bacterial isolates can be used as a biocontrol agent against different phytopathogens.

Introduction

To produce environmental eco-friendly products and product out puts chemical process are being replaced by enzymes like proteases (Abebe *et al.,* 2014). The production of enzymes is central to the modern biotechnology of industrial and agricultural fields. The technology for producing and using commercially important enzyme products combines the discipline of microbiology, genetics, biochemistry and engineering. Enzymes are biocatalysts produced by living cells to bring about specific biochemical reaction generally forming parts of the metabolic processes of cells (Mohammad *et al.,* 2013). Proteases which include proteinases, peptidases or proteolytic enzymes break peptide bonds between amino acids of proteins. They use a molecule of water for this and are thus classified as hydrolases. Proteases are of two types exopeptidases and endo-peptidases (Grewal *et al.,* 2010).

Proteases play a crucial role in numerous pathological processes. Microbial proteases have been proposed as virulence factors in a variety of diseases caused by microorganisms. The virulence of *Pseudomonas aeruginosa* is

multifactorial, but it is partly determined by exoproducts such as alkaline protease and elastase, which are responsible for the damage of tissues by degrading elastin, collagen and proteoglycans. These enzymes have been also shown to degrade proteins that function in host defense *in vivo* (Sakata *et al.,* 1993).

In bacteria, serine and metallo-proteases are the principal classes of proteases found in several species such as *Bacillus subtilis, B. amyloliquefaciens, Pseudomonas* sp., *Lysobacter enzymogenes* and *Escherichia coli* (Fujishige *et al.,* 1992).

Identification and characterization of microbial proteases are prerequisites for understanding their role in the pathogenesis of infectious diseases as well as to improve their application in biotechnology (Lantz and Ciborowski, 1994). Bacterial Proteases are preferred as they grow rapidly, needless space, can be easily maintained and are accessible for genetic manipulations (Odu *et al.,* 2012).

In the present study, soil samples were collected from different area of Khotkhai for screening of Protease producing bacteria and study of their morphological and physiological characters.

Materials and Methods

Source of sample collection

Soil samples were collected from rhizosphere of apple orchard at Rauni (Kotkhai) of Shimla district. Soil samples were taken from five different plant rhizosphere and mixed to make composite sample. Each soil sample was collected around 10 cm apart and from a depth of 1½ - 2 feet. Rhizosphere soil samples along with root pieces were collected and stored in plastic bags at 4^0C temperature until further processing.

Isolation of proteolytic bacteria

The techniques used for isolation of bacteria were serial dilution and spread plate method. Ten gram of rhizoplane soil sample was shaken vigorously in 90 ml of sterile water blank in 150 ml flask as a stock for further dilutions. Serial dilution (10^{-1} to 10^{-6}) of each soil sample was carried out 0.1 ml of each aliquot was spread on skim milk agar (1%) plate at temperature 28ºC for 48 hour. 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 plate repeatedly and preserved on nutrient agar slant at 4^oC. Based on the morphological and biochemical tests the bacterial isolate was identified (Sneath *et al.,* 1986).

Identification of bacteria

The identification of bacteria was carried out by morphological and physiological studies *i.e.* staining including Gram staining. Cultural characterization on agar plates like colony morphology that is shape, size, margin, elevation, opacity, texture and pigmentation and also growth in different temperatures that is 4ºC, 25ºC, 37ºC and 41ºC and biochemical test includes catalase test, oxidase test, carbohydrate fermentation test, Starch hydrolysis test, Gelatin liquification, denitrification test, Tween 80 hydrolysis and Lecithinase test (Aneja).

Quantitative assay of protein

The total protein content of the samples were determined by Lowry's method (Lowry and Bessey, 1946). The protein standard used was Bovine Serum Albumin (BSA) (.1mg/ml).

Preparation of casein solution

Casein was used as substrate. It was prepared from alkali soluble casein which was

dissolved in 10 ml distilled water. The insoluble portion was dissolved by addition of the alkali. The pH was adjusted to 8.0 with 0.1 M sodium hydroxide.

Crude enzyme preparation

The protease producing bacterial colony was inoculated in Nutrient broth medium. It was incubated at 28°C for 72 hours. The culture broth was subjected to centrifugation at 12,000 rpm for 20 minutes to remove unwanted particles. The supernatant was used as crude enzyme preparation for further studies.

Qualitative assay (Proteolytic activity)

All bacterial species were screened out for proteolytic activity by well plate assay method on skim milk agar plates. 100 µl of 72 h old cell free culture supernatant of each bacterial species was added to each well already cut on skim milk agar plate in which 1% of separately autoclaved skim milk is added to nutrient agar medium. Plates were incubated at 28$\pm$2°C for 24-48 h. Proteolytic activity was expressed in terms of mm diameter of clear zones produced around the well (Kaur *et al.,* 1989).

Quantitative method (Protease enzyme assay)

To study proteoloytic activity, supernatant was used as enzyme source. 1%casein in 0.1 M phosphate buffer and pH 7.0) was used as substrate. 1ml enzyme and substrate was incubated at 50°C for 60 min. To stop the reaction 3ml trichloroacetic acid was used. One unit of protease activity was defined as the increase of 0.1 unit optical density at 1 hr incubation period. Then it was centrifuged at 5000 rpm for 15 min. From this 0.5ml of supernatant was taken, to this 2.5ml of 0.5 M sodium carbonate was added, mixed well and incubated 20 min. Then it was added with 0.5ml of folin phenol reagent and the absorbance was read at 660 nm using Spectrophotometer (Bharat *et al.,* 2014). The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions. Based on the tyrosine released the protease activity.

Antifungal activity

Antifungal activity of each test isolate of *Pseudomonas* sp. was checked by standard well/bit plate assay method (Vincent, 1947). Fresh culture bits (10 mm dia) of 5 days old indicator fungi were cut with the help of sterile well borer and placed on the one side of prepoured malt extract agar (MEA) plates with the help of sterile inoculating needle. On the other side of plates, 10 mm well was cut with the help of sterile cork borer. 100 µl of 72 h old cell free culture supernatant of each test bacterial isolates was added to each well (10 mm). For control culture bit of indicator fungi kept in the centre of MEA plate and incubated at 28±2°C for 4 days. Antifungal activity expressed in terms of mm diameter of mycelial growth and that in turn expressed as per cent inhibition of fungal mycelia growth as calculating from equation:

$$\text{Percent inhibition (\%I)} = \frac{C-T}{C} \times 100$$

C : growth of mycelium in control
T : growth of mycelium in treatment

Results and Discussion

In the present study various isolates were screened for protease activity on the casein agar plates in terms of mm diameter of zone of hydrolysis. Six bacterial isolates showed proteolytic activity. Protease activity was

observed in the range of 11 to 23 mm from the zone of hydrolysis observed on agar surface mentioned in table 1. Among these six isolates, maximum protease activity was shown by KK_3 *i.e.* 23 mm. The protease producer was identified as genus *Pseudomonas* sp. a gram negative bacteria. The identification was done on the basis of their morphological, physiological and biochemical characterization. The isolate KK_3 which shown maximum protease production also having antifungal activity in terms of mm diameter *i.e.* 40.0 and percent inhibition in terms of % I *i.e.* 42.85 % against fungal pathogen *Pythium ultimum*. For further study the strain showing largest zone of hydrolysis was considered and designated as KK_3 were preserved and maintained at 4^0C by repeated sub culturing.

Table.1 Zone of inhibition (mm)

Sr.No	Isolates	Zone of Inhibition(mm)
1.	**Kk_1**	16
2.	**Kk_2**	15
3.	**Kk_3**	23
4.	**Kk_4**	19
5.	**Kkr_1**	11
6.	**Kkr_2**	13

Table.2 Colony morphology on agar plates

S.No.	Isolates	Sites	Shape	Elevation	Edge	Opacity	Pigment
1	**Kk_1**	Kotkhai	Circular	Flat	Entire	Transparent	Yellowish
2	**Kk_2**	Kotkhai	Circular	Flat	Entire	Transparent	Yellowish
3	**Kk_3**	Kotkhai	Circular	Raised	Entire	Transparent	Yellowish
4	**Kk_4**	Kotkhai	Iregular	Raised	Entire	Transparent	Yellowish
5	**Kkr_1**	Kotkhai	Circular	Raised	Entire	Transparent	Yellowish
6	**Kkr_2**	Kotkhai	Circular	Raised	Entire	Transparent	Yellowish

Table.3 Physiological and biochemical characteristics

Sr. No.	Isolates	Gelatin Liquification	Denitrification	Catalase	Tween 80 hydrolysis	Gram staining	Oxidase test	4^0C	25^0C	37^0C	42^0C	Starch hydrolysis	Lecitinase
1	**Kk_1**	+	+	+	–	–	+	+	+	+	–	–	+
2	**Kk_2**	+	+	+	–	–	+	+	+	+	–	+	+
3	**Kk_3**	–	+	+	–	–	+	+	+	+	–	–	–
4	**Kk_4**	+	+	+	–	–	+	+	+	+	–	–	–
5	**Kkr_1**	–	–	+	–	–	+	+	+	+	–	–	–
6	**Kkr_2**	+	+	+	–	–	+	+	+	+	–	–	–

(+) sign indicates positive test
(-) sign indicates negative test

Table.4 Antifungal activity by Protease producing *Pseudomonas* isolate KK_3 against *Pythium ultimum*

Sr. No.	Isolate	Percent inhibition of fungal pathogen *Pythium ultimum* (Control=70mm)	
		mm diameter	% Inhibition
1.	KK_3	40	42.85

Figure.1 Isolated colony of *Pseudomonas* isolate KK_3 on nutrient agar plate

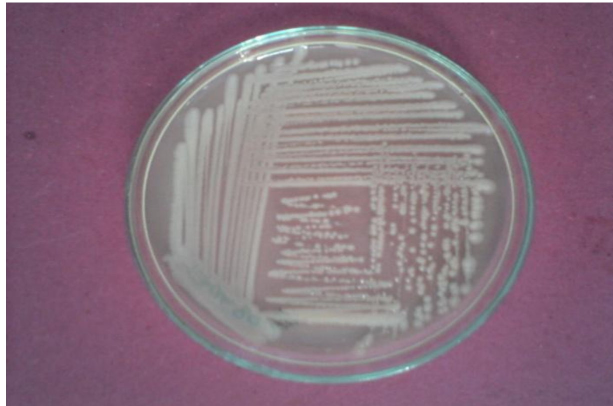

Figure.2 Zone of inhibition on skim milk agar plate by isolate KK_3

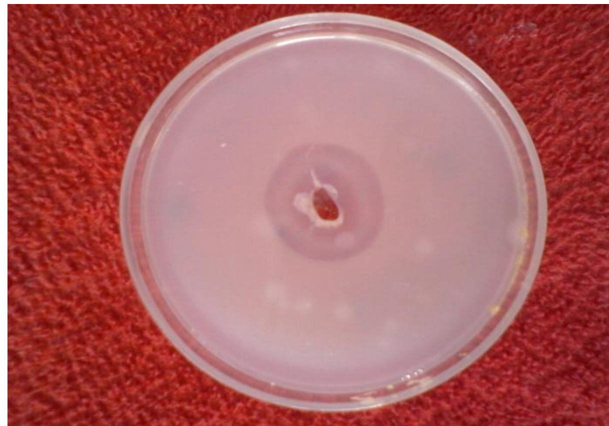

Figure.3 Antifungal activity showed by *Pseudomonas* isolate KK_3 against *Pythium ultimum*

It was found that the rhizospheric soil of apple orchard at Kotkhai of Shimla District shows the presence of protease producers. The bacteria were screened and identified on the basis of their morphological, physiological and biochemical characteristics as *Pseudomonas* sp. Qualitative and qualitative estimation were also done. Antifungal activity of *Pseudomonas* isolates against pathogenic fungi was also done. These bacteria can be very useful in agricultural fields due to their antagonistic properties against phytopathogens and can be used as a biocontrol agent. Further experiments were carried out to enhance enzyme production and their purification for further studies.

Acknowledgement

The authors are highly thankful to the department of Basic Science (Microbiology) and department of Plant Pathology of Dr Y S Parmar University of Horticulture and Forestry, Nauni-Solan (HP)-173230, India to carry out the present work.

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How to cite this article:

Kumari Manorma, Shweta Sharma, Himani Singla, Kirti Kaundal and Mohinder Kaur. 2017. Screening and Isolation of Protease Producing Bacteria from Rhizospheric Soil of Apple Orchards from Shimla District (HP). *Int.J.Curr.Microbiol.App.Sci.* 6(5): 249-255.
doi: http://dx.doi.org/10.20546/ijcmas.2017.605.030