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Endophytes Producing Proteases from Custard Apple (*Annona squamosa* L.) Leaves

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Abstract **Original Research Article**

The cost of the proteolytic enzymes and their inability to remove "fine" hairs from the animal hide has been the main obstacles to the commercialization of an enzymatic dehairing procedure. Endophytic microbes that grow inside various plant tissues without causing tissue damage in the host plant are the important sources of bioactive compounds. They are rich in secondary metabolites and known to produce wide range of enzymes. Several endophytes showing different properties have been reported from Annona leaves and hence this study was performed to isolate endophytes having proteolytic properties. Endophytes from custard apple leaves were isolated and screened for proteolytic activity on casein agar plates. The protease enzyme activity of the promising isolates was determined using casein substrate and tyrosine release assay. Present study conducted on the bacterial endophytes from the Custard apple leaves showed that the plant-associated bacterial endophytes are a good reservoir of proteases. Two promising isolates were obtained on the casein agar plates. These two isolates C and D possessed 113U/ml and 102 U/ml enzyme activity respectively. The isolate C showed more dehairing capacity than isolate D. Purified forms of these enzymes can prove to be potential candidates in biotechnological applications.

Keywords: Endophytes, proteolytic, Annona leaves, dehairing.

INTRODUCTION

Endophytes are fungi, bacteria, or yeast symbionts that live in the intercellular spaces or vascular tissues of host plants. They colonize the inside tissue of the plant and demonstrate no outer sign of disease or negative impact on their host (Schulz and Boyle, 2006). The endophytic microorganisms in plants function in growth promotion and disease control. They help plant directly by producing phytohormones or by delivering the chemical. Endophytes and plants have mutualism, endophytes supporting the host plant and in return getting food and protection from them. Every single vascular plant has endophytic life forms in all parts like leaves, stem, and bark, organic product and roots (Zhang *et al.,* 2006). It has been reported that many secondary metabolites produced by plants are enhanced by endophytic microorganisms present inside them (Abdelkader *et al.,* 2022).

Protease enzymes are occurring in every living cell. Proteases break peptide bonds between amino acid of proteins. Among the different types of proteases, alkaline proteases have wide applications in different industries such as detergent, leather, pharmaceutical, protein processing, foods, diagnostic reagents, soy processing, peptide synthesis industries, extraction of silver from used X-ray film and wastes treatment (Asha and Palaniswamy, 2018). The protease is a one of the three largest groups of industrial enzyme, account for about 60% of the worldwide sale of enzymes (Ningthoujam *et al.,* 2009). Proteolytic enzyme has great value in the pharmaceutical industry. Hence it is customary to select strains for the commercial large scale production of desired enzymes. Only the keratinases (EC 3.4.21) family of proteases has a broad temperature and pH range that permits complete breakdown of complex and resistant proteins (Gupta and Ramnani, 2006). The distinctive capacity of keratinases to bind to the complex and insoluble substrates like hair, feather, wool etc., sets them apart from other proteases.

Feather waste is a byproduct of the poultry industry and the slaughter house. This waste is an environmental pollutant which is not easily degraded by common proteolytic enzymes. A feather / skin hair is mainly composed of 90% keratin protein which is

highly resistant to proteases due to its molecular conformation (Mazotto *et al.*, 2011). Keratinolytic microbes are known to degraded them to smaller peptides and amino acids, which can be further used in various applications like dietary supplements in animal feed. A vast variety of bacteria, actinomycetes and fungi are known to be keratin degraders (Wang *et al.*, 2003). To produce environmental eco- friendly products outputs, chemicals processes are being replaced by enzymes like proteases (Ma *et al.*, 2016).

The current study aimed to screen organisms possessing keratinolytic activity for biodegradation of skin hair waste, thereby help in finding a solution to a global concern issue of environmental pollution.

MATERIAL AND METHODS

Collection of Leaves

The leaves were collected from fresh and healthy plant of Custard apple and transported to Microbiology Research laboratory, Dr. D. Y. Patil Arts, Commerce & Science College, Pimpri, Pune, in plastic bags. All leaves were washed under running tap water to remove soil and dust and finally washed with sterile distilled water (Fig. 1 and 2).

Figure 1: Collected Custard Apple Leaves

Figure 2: Washed leaves

Isolation of Microbial Endophytes

The isolation of endophyte microbes from of Custard apple leaves was been carried out as described by Hallmann *et al.*, 2006. Briefly, surface sterilization was performed by immersing the leaves in 70% ethanol for 3 minutes separately. Further, the leaves were treated with 1% of sodium hypochlorite solution for 1minutes and finally treated with 70% ethanol. Thereafter, each leaf was separately washed thrice in sterile distilled water.

These leaves were cut with sterile blade and then with the help of a sterile forcep, these pieces were placed on NA plate. All the NA plates were incubated at 37^0 C for 24 hrs. After incubation, the colonies grown on nutrient agar were considered as endophytic bacteria.

All the isolated endophytic bacteria were screened for protease production.

Screening for Protease Producing Endophytes

Isolated colonies were spot inoculated on casein agar plate and plates were incubated at 37^0 C for

24 hours. Zone of casein hydrolysis by the protease enzyme activity was measured (Sekar *et al.*, 2014).

Quantitative Estimation of the Protease Enzyme Produced by the Endophytes

The bacterial isolates were grown in casein broth for 24 hrs at 37^0 C. Supernatant containing protease was then collected by centrifugation. Protease activity in the supernatant was determined by Sigma's Non-specific Protease Activity Assay as described by Carrie Cupp-Enyard, 2008. Briefly, 5mls of 0.65% casein solution (0.65% weight/volume casein solution in the 50 mM potassium phosphate buffer) was added to three tubes and equilibrated in a water bath at 37°C for about 5 minutes. 1ml of different enzymes to be tested was added to the respective tubes and one kept as blank (no enzyme). These tubes were mixed by swirling and then incubated for 37°C for exactly ten minutes. After incubation, 5 ml of the 110 mM TCA was added to each tube to stop the reaction. Then, 1ml of enzyme solution was added to each tube, even to the blank. This was done to account for the absorbance value of the enzyme itself and to ensure that the final volume in each tube is equal. The tubes were then incubated at 37°C for 30 minutes. Then the solution in each tube was filtered using a 0.45 um syringe filter. Filtration was done to remove any insolubles from the samples. To the 2ml filtrate, 5ml of 500 mM sodium carbonate was added and 1 ml of 0.5 mM Folin's reagent was added immediately after that. The tubes were mixed by swirling and then incubated at 37°C for 30 minutes. 2ml of these solutions were filtered using a 0.45 um syringe filter. The absorbance of the samples was measured at 660nm. For the tyrosine standard graph, 1.1 mM tyrosine standard stock solutions with the following volumes in ml: 0.05, 0.10, 0.20, 0.40, and 0.50 was added to five tubes and one kept empty as blank. Volume of each tube was then made up to 2ml with sterile distill water. To this 2ml standard solution, 5ml of 500 mM sodium carbonate was added and 1 ml of 0.5 mM Folin's reagent was added. Similar protocol thereafter was followed for the standard tubes also.

The change in absorbance in the test samples was determined by calculating the difference between the test sample absorbance and the absorbance of the test blank. Then by inserting the absorbance value for the test samples into the slope equation of the standard graph, the micromoles of tyrosine liberated during this particular proteolytic reaction was obtained. To get the activity of enzyme in units per/ml, following formula used:

$$\text{Units/ml Enzyme} = \frac{\text{(umole tyrosine equivalents released) x (a)}}{\text{(b) x (c) x (d)}}$$

a= Total volume (in milliliters) of assay
b= Time of assay (in minutes) as per the Unit definition
c= Volume of Enzyme (in milliliters) of enzyme used
d= Volume (in milliliters) used in Colorimetric Determination

$$\text{Units/ml} = \frac{\text{µmole of Tyr x reaction volume}}{\text{sample vol x reaction time x vol assayed}}$$

Keratin Degrading Efficiency of the Isolates

In order to check the keratin degrading efficiency of the isolates, the isolates were grown in nutrient broth for 48hrs at 37^0C, cell free extract was obtained by centrifugation at 6000 rpm for 15min, and supernatant thus obtained was treated as crude enzyme. Goat skin was used to check the dehairing property of the crude enzyme. Briefly, the goat skin was cut in to small pieces and these pieces were added to 5ml supernatant. The tubes were incubated at room temperature. Sterile distilled water used as negative control. Loosening of the hair was visually monitored for 2hrs.

RESULTS AND DISCUSSION

Isolation of Microbial Endophytes

The endophytic bacteria residing in the custard apple leaves were allowed to grow on the nutrient agar plate. The leaves placed on these plates were thoroughly sterilized before placing them on the NA plates thus ensuring the growth of endophytes. The Figure 2 shows the growth of these bacteria surrounding the leaves.

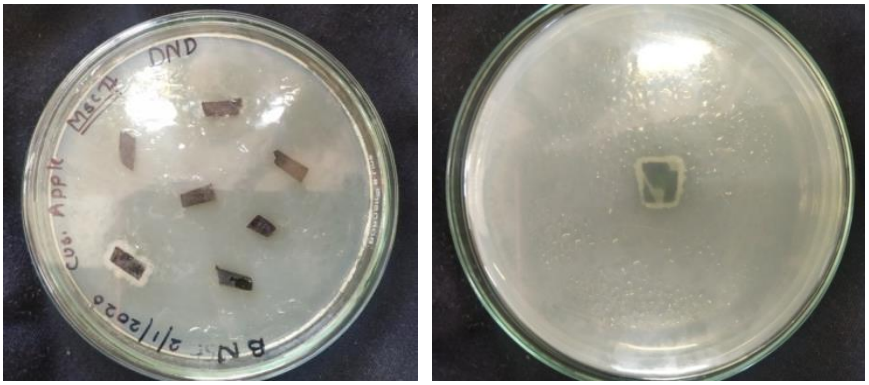


Figure 2: Endophytic bacterial growth on NA Plate after 24 hour

Screening for Protease Producing Endophytes

The endophytic bacteria isolated from the custard apple leaves were screened for protease activity by spotting them on the casein agar plates. The zone of hydrolysis indicated the protease production by the isolate. Isolate C and D showed maximum zone of hydrolysis and is shown in the Figure 3.

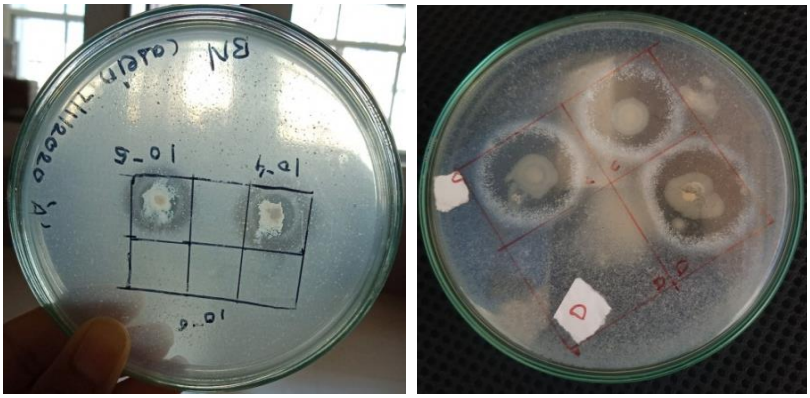


Figure 3: Zone of hydrolysis on casein agar plate

Quantitative Estimation of the Protease Enzyme Produced by the Endophytes

Absorbance values for the standards were obtained by subtracting the blank from them (Table 1) and then these were used to plot a standard graph (Fig. 4). Similarly the blank was also subtracted from the absorbance of the test sample and plotted on the standard curve in order to obtain the µM of tyrosine generated in each test sample. The more the protease activity, higher was the µM of tyrosine generated.

Table 1: Absorbance of the standard (tyrosine)

Volume of Tyrosine Standard	uMoles Tyrosine	A_{660}
0.05	0.055	0.056
0.1	0.111	0.1
0.2	0.221	0.22
0.4	0.442	0.39
0.5	0.553	0.49

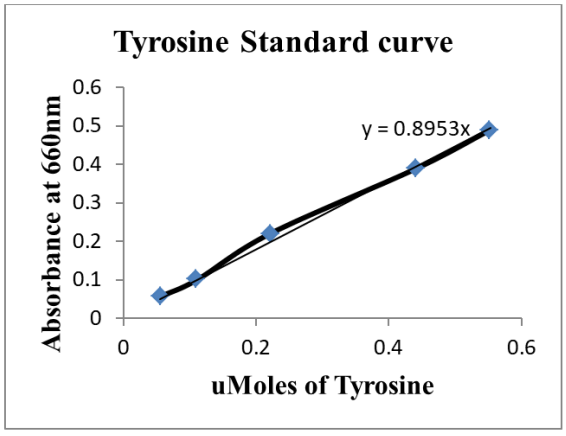


Figure 4: Standard graph for tyrosine

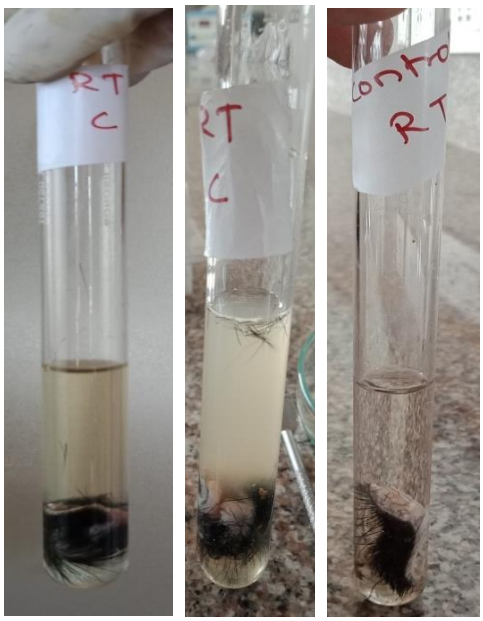
After plotting the test absorbance in the above standard graph for tyrosine, 113U/ml and 102 U/ml enzyme activity was calculated for C and D respectively.

Keratin Degrading Efficiency of the Isolates

The goat skin when was incubated with the crude enzyme from isolate C and D, the hair loosening began after one hour incubation and few hair actually got detached from the skin which were observed at the top of the suspension as seen in fig 5 and 6. As a negative control, the crude enzyme was replaced by water and showed no loosening or detachment of hair.

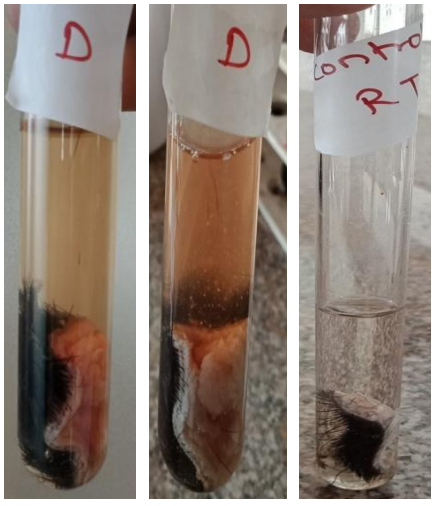
Table 2: Dehairing activities of protease produce by isolate C and D

Isolate	Loosening of hair
C	++
D	+



A B C

Figure 5: Dehairing efficiency of isolate C, A) after 1hr incubation B) after 2 hrs of incubation C) control without enzyme



A B C

Figure 6: De-hairing efficiency of isolate D, A) after 1hr incubation B) after 2 hrs of incubation C) control without enzyme

The present study, 2 endophytes possessing proteolytic activity were obtained from leaves of custard apple. The caseinolytic activity of trypsin/bacterial protease was determined using casein agar plates. A protease activity (U/mL) for both isolates was determined using a standard curve of trypsin activity assay. Isolate C possessed more activity than isolate D.

Traditional ways for feather disposal includes incineration or chemical treatment (Papadopoulos *et al.*, 1986; Cai *et al.*, 2008). Economical and eco-friendly technique for the conversion of feather waste is use of microorganisms. Microbes use enzymes to degrade keratin and produce peptides / amino acids that can be further used in various ways (Gupta and Ramnani, 2006). Isolation of keratinolytic bacteria from various sources have been reported earlier (Sekar *et al.*, 2014; Sharma *et al.*, 2019).

Thus, these endophytes isolate C and D can be further considered for different and inexpensive

methods, such as commercial waste management, enzyme production, agricultural purpose, etc.

CONCLUSION

Two proteolytic endophytic bacterial strains C and D were isolated from the custard apple. Proteases produced from the two bacterial isolates showed promising enzyme activity. Isolate C showed more dehairing capacity that isolate D. Purified forms of these enzymes can prove to be potential candidates in biotechnological applications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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