Research Article

Isolation and Characterization of *Rhizopus* Producing Acid Proteases

Gururaj B. Tennalli*, Anil R. Shet, Veeranna S. Hombalimath, Laxmikant R. Patil
Department of Biotechnology, K.L.E. Technological University, Hubballi, Karnataka, India.
***Corresponding author's E-mail:** gururaj_tennalli@kletech.ac.in

Received: 12-06-2019; **Revised:** 23-07-2019; **Accepted:** 01-08-2019.

ABSTRACT

Proteases play an important role in the industrial enzyme market and have wide applications in many industries. In this study, two *Rhizopus* strains P-11 and P-15 were isolated from slaughter waste contaminated soil and were characterized for their proteolytic activity. Solid state fermentation was conducted and enzyme activity was calculated. The *Rhizopus* strain P-11 showed enzyme activity of 354 U/gdfs after 24hrs of incubation, while the strain P-15 and standard *Rhizopus oligosporous* strain showed an activity of 245U/gdfs and 235U/gdfs respectively. The P-11 strain was found to be the highest acid protease producer after 24hrs of incubation, when compared to P-15 strain and standard strain.

Keywords: *Rhizopus*, Acid protease, Proteolytic activity, Wheat bran, Solid state fermentation.

INTRODUCTION

Enzymes have extensive applications in a range of industrial processes. Proteases account for approximately 60% of all enzyme sales because of their applications in food, pharmaceutical and number of other industries.[1] Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms and essential for cell growth and differentiation. Extracellular proteases have high commercial value and multiple applications in various Indus tries such as detergent, food, pharmaceutical and leather.[2] Proteases are capable of cleaving proteins into peptides and amino acids; they are characterized by their optimal pH (acid, neutral and alkaline) and temperature. Also their ability to hydrolyze specific proteins as their homology is well characterized enzymes such as chymosine, chymotrypsin, pepsin and trypsin.[3]

Microorganisms represent an exceptional source of proteases owing to their extensive biochemical diversity and susceptibility to genetic manipula tion.[7] Acid proteases (EC 3.4.23) on the other hand, find applications in food processing. They are produced in much smaller quantities and are commonly obtained from animal or fungal sources. Fungal acid proteases (EC 3.4.23.6) have the potential to substitute the role of three important proteases involved in food processing, many fungi produce acid proteases. *Rhizopus oligosporous* which is highly proteolytic, has potential as an acid protease producer since it does not produce toxins, and produces a satisfactory calf rennet substitute at laboratory scale.[1] Also, the scarcity and high price of traditional calf rennet has promoted research towards the isolation of novel potent acid protease producer. Therefore the aim of this work was isolation and characterization of novel and potent acid protease producing fungal species, specifically the *Rhizopus* species isolated from different local soil samples and slaughter house waste contaminated soils.

MATERIALS AND METHODS

Materials

Media ingredients chemicals required for the isolation of fungal species were purchased from Himedia Lab (Mumbai) Ltd. The substrate used in this study, wheat bran, was obtained from the local market.

Isolation of microorganisms

Two different *Rhizopus* species were isolated from the local soil samples collected from slaughter waste contaminated soil. In laboratory, the fungal species were isolated by culturing on fungal specific sabouraud's agar and further maintained by weekly sub culturing.

Identification of fungi

Isolated fungal colonies were stained with Lactophenol Cotton Blue stain by Wet-Mount method and the mycelial and spores structure were studied microscopically.

Screening for proteolytic activity

Proteolytic activity for isolated organisms was done by Skim Milk agar plate test. In this test the different fungal species isolated were separately plated on Skim Milk agar plate and incubated at room temperature and after 24 Hrs the zone of clearance around the fungal colonies was recorded.

Quantification of Proteolytic activity

The quantification of proteolytic activity of two isolated *Rhizopus* species was performed by initiating a lab scale Solid state fermentation[11] in Erlenmeyer flask using wheat bran as sole carbon source. Later the acid protease produced by fungal was recovered by leaching and same

was used as crude enzyme source for calorimetric estimation of proteolytic activity of isolated fungi.

Inoculum preparation

The Inoculum was prepared by suspending the spores from a week-old fungal slant culture in 0.1 % Tween-80 solution with a sterile inoculation loop.[8, 12]

Solid-state fermentation

Five grams wheat bran was taken in a 250 ml Erlenmeyer flask, moistened with salt solution [composition (% w/v): ammonium nitrate 0.5, potassium dihydrogen orthophosphate 0.2, sodium chloride 0.1, and magnesium sulphate 0.1] to achieve the desired moisture content, sterilized at 121°C for 15 min, cooled, inoculated with 1 ml of fungal spore suspension (10^6 spores/ml) and incubated at 30°C for 72 hours. All experiments were carried out in two sets.

Extraction of crude enzyme

A solution of Tween-80 (0.1%) in distilled water was added to the fermentated substrate and the substrate was homogenized on a rotary shaker at 180 rpm for 1 hour. The solids were removed by centrifuging the homogenate at 8000x g at 4°C for 15 min and the resultant clear supernatant was used for analytical studies

Analytical methods

Assay for Acid protease

Acid protease activity was performed by method as described by Arima[7] with little modifications. To 2.5ml of 1% (w/v) casein in 0.02M Acetate buffer (pH 4.0) 0.5ml of crude enzyme extract was added. The reaction mixture was then incubated at 37°C in a water bath for 10 mins and the reaction was terminated by adding 2.5ml 0f 0.44M TriChloroacetic acid solution. The precipitate formed was removed by filtration through Whatman No 1 filter paper. 1 ml Folin Ciocalteau reagent and 2.5 ml 0f 0.55M Sodium carbonate solution was added to 1 ml of above clear filtrate. This further incubated for 20 mins at 37°C for color development. The optical density (OD) at 660nm expressed the activity of enzyme in terms of proteolytic units. One unit enzyme activity was defined as the amount of enzyme that liberated 1 µg of tyrosine from substrate (casein) per minute under assay conditions and reported in terms of protease activity per gram of dry fermentated substrate (gdfs).

RESULTS AND DISCUSSION

Isolation of Microorganisms

Isolation of acid protease producing microorganisms was aimed from fungal sources as fungal acid proteases (EC. 3. 4. 23. 6) have the potential to substitute three different proteases involved in food processing.[1] The slaughter house waste contaminated soil was used and fungal species were isolated by culturing on fungal specific Sabouraud's agar. Numerous fungal colonies appeared, the prominent ones were *Aspergillus, Fusarium, Penicillium* and *Yeasts* along with *Rhizopus* colonies. The acid protease activity was reported from various fungal species such as *Penicillium, Neurospora, Mucor and Aspergillus.*[3-6] The present study was focused on *Rhizopus* species, as the acid proteases produced from these are highly proteolytic, non toxic and can be a better calf rennet substitute.[1, 9, 10] The plate cultured with slaughter house waste contaminated soil resulted in profused *Rhizopus* colony development with white fungal mat with dark brown spores. This indicated that the slaughter house waste was a good source for *Rhizopus* isolation. Further, all fungal colonies which appeared on different plates were cultured on separate plates and processed for proteolytic activity.

Identification of fungi by staining

The *Rhizopus* colonies identified by their characteristic dark brown colour with white mycelial mat were further confirmed by Lactophenol-Cotton blue staining characterized by white to dark gray nonseptate mycelium with root like rhizoids; black columellate, sporangiophores, in clusters (Fig 1).

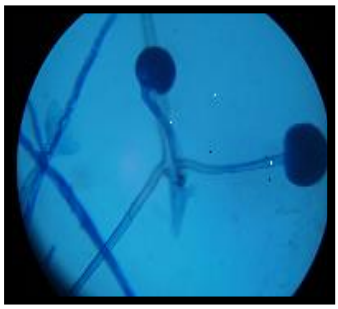

Figure 1: *Rhizopus* mycelium and sporangiophores

Screening for Proteolytic activity

The isolated *Rhizopus, Aspergillus and Penecillium* strains were further screened for their proteolytic activity. For this the spores of each fungal species were separately inoculated on Skim Milk Agar plate and incubated at 25-27°C for about 24 hrs. After incubation we found two *Rhizopus* strains P-11 and P-15 which effectively showed the proteolytic activity by distinctly forming the clear zone around their colonies (Fig2) in the skim milk agar plate. Later the *Rhizopus* strains were identified as proteolytic positive.

Figure 2: *Rhizopus* (P-11) showing proteolytic activity on Skim Milk Agar plate by forming clear zone around its colony

Quantification of proteolytic activity

The proteolytic activity which was observed for *Rhizopus* species P-11 and P-15 was further quantified by caseinolytic assay as described by Arima[7] with little modifications. For enzyme production, a lab scale solid state fermentation was set up using wheat bran as sole source of Carbon and after 72 Hrs of incubation the crude enzyme extract was prepared by leaching the fermented substrate with known volume of 0.1% Tween-80 solution. Later this crude enzyme solution was assayed for caseinolytic assay. Among P-11 and P-15, P-11 showed highest enzyme activity of 354U/gdfs leached at 24 hrs as compared to standard *Rhizopus oligosporous* (235U/gdfs) and P-15 (245U/gdfs) culture. Whereas, both P-15 and standard *Rhizopus oligosporous* strains produced almost equal enzyme activity at 72 hrs while, P-11 showed slightly lesser activity. (Table1, Fig.3). The *Rhizopus* strain P-11 with its highest acid protease activity at 24 Hrs would be of greater advantage in achieving high enzyme production within short period of time in large scale production.

Table 1: Comparison of protease activity between isolated and standard *Rhizopus* culture

Time in hrs	Enzyme Activity (U/gdfs)		
	R.oligosporous	**P-11**	**P-15**
20	154	172	154
24	235	354	245
48	125	240	222
72	263	206	269

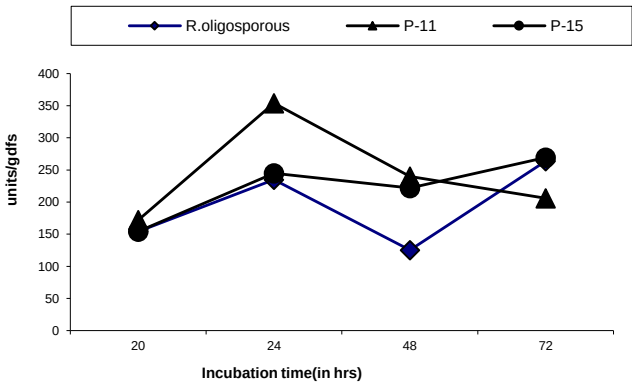

Figure 3: Change in Enzyme activity with Incubation time for isolated and standard *Rhizopus* culture.

CONCLUSION

The proteases are the most important industrial enzymes, accounting a major volume of the total worldwide enzyme sales. The fungal acid proteases have the potential to substitute the existing proteases of animal origin. The isolation and identification of suitable fungal source for acid protease production is an important area of research. In this study two *Rhizopus* strains P-11 and P-15 were isolated and characterized for their proteolytic efficiency. The strain P-11 showed highest acid protease production (354U/gdfs) at 24 hrs of incubation under solid state fermentation. Further this strain can be exploited for large scale acid protease production.

Acknowledgements: The authors thank Department of Biotechnology, K.L.E. Technological University, Hubballi for their support in completing this research work.

REFERENCES

1. Ikasari L and Mitchell D A, Leaching and characterization of *Rhizopus oligosporous* acid protease from solid-state fermentation, Enzyme Microb Tech, 19, 1996, 171-175.

2. Gupta R, Beg Q K, and Lovenz P, Bacterial alkaline proteases: molecular approaches and industrial applications, Applied Microbiology and Biotechnology, 59, 2002, 15-32.

3. Chekireb and Djamel, Acid protease production by isolated species of *Penecillium*, European journal of scientific research, 25(3), 2009, 469-477.

4. Lindberg R A, Rhodes W G, Eirich L D and Drucker H, Extracellular acid proteases from *Neurospora crassa,* Journal of Bacteriology, 150(3), 1982,1103-1108.

5. Wang H L, Release of proteinase from mycelium of *Mucor hiemalis,* Journal of Bacteriology, 93, 1967, 1794 -1799.

6. Tremacoldi C R, Watanabe N K and Carmona E C, Production of Extracellular acid proteases by *Aspergillus clavatus*, World Journal of Microbiology and Biotechnology, 20, 2004, 639 - 642.

7. Arima K, Yu J and Iwasaki S, Milk clotting enzyme from Mucor pusalis var Lindt In: Perlmann G, Lorald L editors. Methods in Enzymology, Vol 19, New York Academic Press, 1970, 446- 459.

8. Alagasamy, Sumantha, Paul Deepa, Chandra Sandhya, George Szakaes, Carlos, Ricardo Soccol and Ashok Pandey, Rice Bran as a substrate for proteolytic enzyme production, Brazilian Archives of Biology and Technology, 49(5), 2006, 843-851.

9. Gumbira Sa id E, Doelle H W, Greenfield P F and Mitchell D A, Protein enrichment of sago starch by solid state fermentation with *Rhizopus spp*, World J Microbiol Biotechnol,7, 1919, 419-427.

10. Thakur M S, Karanth N G and Nand K, Production of fungal rennet by *Mucor miehei* using solid state fermentation, Appl Microbiol Biotechnol,32, 1990, 409 -413.

11. Shet A R, Desai S V and Achappa S, Pectinolytic enzymes: classification, production, purification and applications, Res J Life Sci Bioinform Pharm Chem Sci, 4, 2018, 337-348.

12. Hombalimath V S, Udapudi B B, Patil L R, Shet A R and Tennalli G, Isolation and characterization of Lipolytic microorganisms from oil contaminated soil, International Journal of Advances in Engineering, Science and Technology, 2(3), 2012, 293-297.

Source of Support: Nil, **Conflict of Interest:** None.