

Role of Proteases in Bioremediation of Temple Protein-Containing Waste with Special Reference to Mangalnath, Ujjain (M.P.) – India

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ABSTRACT

The aim of present study is to isolate and screening of protease producing fungi from the proteinaceous waste of Mangalnath Temple, Ujjain. Fungi were isolated from the soil samples and screened by growing on Czapek Dox Agar medium supplemented with pasteurized skimmed milk and zone of protein degradation was noted. A total 23 soil fungi were isolated, out of those 8 proteases producing fungi belonging to 4 different genera were screened out. Soil protein was extracted from the soil sample and protein quantification was done by Bicinchoninic Acid (BCA) Protein Assay. *Aspergillus* showed the highest protein degradation and after *Penicillium*. Followed by *Cladosporium* and *Trichoderma* showed protein degradation nearly at a low level.

Keywords: Soil Fungi, Proteinaceous Waste, Proteases, Bioremediation.

INTRODUCTION

Waste can be defined as- waste is everything that no longer has a use or purpose and requirements to be disposed of people generate a lot of wastes by their daily activities and these wastes adversely affect the human and environmental health (Yadav et al., 2015; Rao et al., 2010). Along with different types of wastes, proteinaceous wastes are key pollutants of the environment. Proteins are one of the most important and essential component for the sustainability of living thing. But when end of these proteins as a waste, it cause environmental problems. Protease enzymes are participated in the primary hydrolysis of

protein of wastes in to simple amino acids. Proteinaceous waste are produced from different sources, including temple waste, plant by-products, dairy waste, as well as blood, hides, skin, and visceral proteins after removal from animals (Adhikari et al., 2018). Places of historical, religious and touristic significance around the world are often prone to a great amount of waste leftovers by the visitors. This is responsible for numerous environmental concerns but also serves as a source of inoculums for several unwanted diseases or various other health problems (Singh et al., 2013).

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In India, worshiping is the path of living and people offer several offerings to the deities, which generally consist of coconuts, fruits, flowers, leaves, clothes etc. out of which floral and coconut offerings are found in huge amount. People also offer some special types of offerings to some kind of deities (Aruna et al., 2016). There are many temples where people do worship with only oil, milk, curd, rice, etc. The famous Mangalnath Temple is one of the well-known temples of India, which is situated on the bank of Kshipra River in holy city Ujjain, Madhya Pradesh. It is believed that this temple is situated on the center point of the earth and the famous karka line (Tropic of Cancer) also passes away from here. Here the pilgrims do worship of deity Mangalnath with rice and curd. Thousands of pilgrims come here every day and do worship. The number of pilgrims is very high on Tuesday and special (worshiping) days than on other days. After the worship the mixture of rice and curd is washed out which mixes directly in the soil which causes soil pollution.

Unconventional technologies like microbial waste treatment have become progressively attractive in the light of their greater relative economical (Sharma, 2010; Gareth et al., 2003). Using the conventional methods for pollutants degradation is generally expensive and cannot adopt for the long term. Bioremediation is a less energy-consuming and cost-effective alternative to the traditional methods (Seth et al., 2016). Inappropriate disposal or handling of waste results in unsanitary conditions which further leads to pollution in the environment. Although, the management of wastes is considered as essential part of better living habits (Singh et al., 2017). The present study will reveal the importance of fungal proteases in bioremediation of proteinaceous waste originated from temple and which fungi can do it more efficiently.

MATERIALS AND METHODS

Soil Sample Collection

For this study ancient Mangalnath temple, Ujjain (M.P.) (23°13'18"N 75°47'7"E) was

selected. Wastes of this temple contain proteinaceous liquid waste along with solid waste which is generally generated by worship (Abhishek, Pooja etc.) in the temple. Five soil samples were collected in clean and sterile zipper polythene bags from the polluted soil near the temple (Gaddeyya et al., 2012). Before collecting the soil sample top soil layer was removed. The collected soil samples were brought to the microbiology laboratory for the further study.

Screening of Proteolytic Fungi

For the screening and isolation of proteolytic fungi from the soil sample, Czapek Dox Agar media was used. The soil dilution plate method was performed to isolate fungi from the soil samples. From the collected soil samples 10g soil was diluted in 90ml of sterile distilled water and dilution prepared up to 10^{-6} . 1ml of suspension was taken from each dilution of 10^{-4} , 10^{-5} , and 10^{-6} and added to sterile petri plates (duplicate of each dilution) (Waksman, 1922). Just before the pouring 5ml pasteurized skimmed milk (as a protein source) and Streptomycin antibiotic was added to the medium and mixed it well. After solidification plates were incubated at $28^{\circ}\text{C} \pm 1$ for 7 days. After incubation zone of proteolysis surrounding the colony was observed on medium. Production of proteases was exhibited as clear zone due to hydrolysis of protein around the fungal growth. Such type of fungi were screened and transferred on fresh Czapek Dox Agar medium to obtain pure culture. Pure culture was maintained and stored at low temperature (Chandrasekaran et al., 2015; Seeley & VanDemark, 1981).

Characterization of Isolates

Isolates were identified on the basis of cultural and morphological characters with the help of manuals and literatures (Waksman, 1922; Rapper & Thom, 1949; Gilman, 2001; Alexopoulos, 2018).

Protein Extraction and Quantification

For the quantification of protein, Bicinchoninic Acid (BCA) protein assay was performed with BCA protein assay kit (23227) of Thermo Fisher Scientific. Soil protein was extracted for the purification and

quantification. 3gram of air-dried, grinded, sieved, and well-mixed soil was taken into the pressure and heat-stable glass screw-top tube. Thereafter 24ml of Sodium Citrate Buffer (Himedia- R014) was mixed with the soil sample and the mixture was shaken to dissolve all aggregates (Tunsisa et al., 2018). Subsequently, the tube was autoclaved at 121⁰C and 15 psi for 30 minutes and then cooled. 2ml of the mixture was taken and centrifuged it on 10,000xg for 3 minutes to remove soil particles (Wright & Upadhyaya, 1996). Extract was collected in a separate tube.

BCA working reagent was prepared in Falcon tube by mixing of reagent A (a clear reagent mixture) and reagent B (a blue-green copper sulfate solution) provided by Thermo Fisher Scientific with BCA protein assay kit. These two reagents were taken in the ratio of 50:1. For the preparation of working BCA reagent, 0.5ml (500 microliter) reagent B and 25 ml reagent A were mixed gently (Walker, 1996). Thereafter 0.01ml (10 microliter) of pre-prepared Bovine Serum Albumin (BSA) standards (0, 0.25, 0.5, 1.0, 1.25, 1.5, 1.75, and 2.0) was pipette out into the first column of the reaction plate (microtiter plate). Same amount of standards were also dispensed into second column as a duplicate. Then 0.01 ml sample was added into the third column of the microtiter plate. Subsequently 0.2 ml working BCA reagent was added to the all well and swirled gently for proper mixing. Afterward plate was sealed and kept in incubator on 37⁰C for 30 minutes. Thereupon, the plate was cooled by removing it from the incubator and it was placed in the plate reader and absorbance (ABS) was measured at 562nm.

In next step, 5ml protein extract was taken into 4 different test tubes and isolated

fungi were inoculated respectively and incubated them at 28⁰C in shaking incubator. After 48 hours tubes were removed from the incubator and mixture was centrifuged at 10,000xg for 3 minutes to remove fungal mycelium and extract was collected in separate tubes. Subsequently, the absorbance of the extract was measured the same as described earlier.

BSA standard curve was prepared by using BSA standards. The absorbance of BSA standards was measured at 562nm. The average absorbance was calculated by addition of ABS1 with ABS2 (duplicate) and dividing by 2 (ABS1+ABS2/2). Net absorbance was calculated by subtraction of average absorbance value of blank from all average absorbance value of BSA standards (Average absorbance of BSA standards - Average absorbance of blank).

The net absorbance of extract was calculated by subtracting the blank value of standards from the initial absorbance value of sample. The protein concentration was calculated by the formula (Y= mX+C), where Y= absorbance of unknown sample, X= unknown protein concentration.

RESULTS AND DISCUSSION

From the collected 5 soil samples 23 fungi were isolated. Among those only 8 fungi were found proteases producing. These protease producing fungi were belongs to 4 different genera. On the basis of microscopic observation, colony characteristics and literature review, 4 common type fungi were identified, which are *Penicillium*, *Trichoderma*, *Cladosporium* and *Aspergillus*. Out of each common genus, only one fungus was selected for study.

Table 1: Occurrence Percentage of Proteolytic Fungi

S.no.	Isolated Proteolytic Fungi	No. of fungi	% of Isolates
1	<i>Penicillium</i>	02	8.69
2	<i>Trichoderma</i>	02	8.69
3	<i>Cladosporium</i>	01	4.34
4	<i>Aspergillus</i>	03	13.04

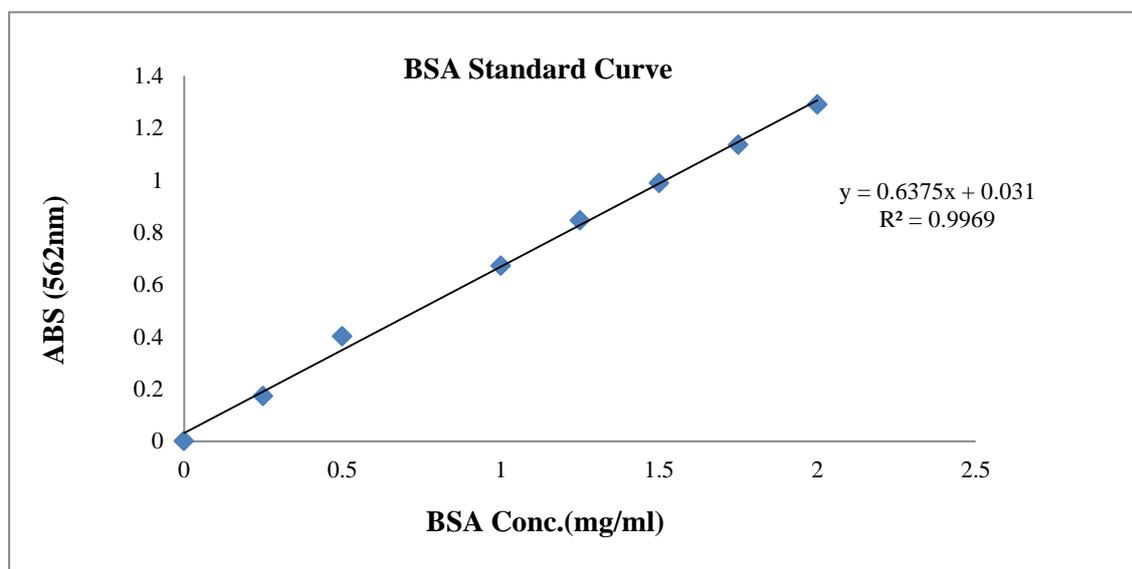
BSA Standard Curve

BSA standard curve was prepared by using the absorbance value of BSA standards (Table 2).

Table 2: Absorbance of BSA Standards at 562nm

BSA Standards (mg/ml)	ABS1 (562nm)	ABS2 (562nm) (Duplicate)	Average ABS	Net ABS (562nm)
0 (Blank)	0.370	0.375	0.372	0 (Blank)
0.25	0.546	0.544	0.545	0.173
0.5	0.776	0.771	0.773	0.401
1.0	1.046	1.042	1.044	0.672
1.25	1.235	1.201	1.218	0.846
1.5	1.368	1.355	1.361	0.989
1.75	1.516	1.501	1.508	1.136
2.0	1.669	1.655	1.662	1.290

Protein Concentration in Extract before Fungal Inoculation



Protein extract showed higher absorbance at 562nm. Higher Absorbance is directly proportional to protein content. The absorption and total protein content of the extract before

fungal inoculation is shown in Table 3. The protein concentration calculated using the formula ($y = 0.6375x + 0.031$) shown on the scatter graph.

Table 3: Absorbance and Protein Concentration in Extract before Fungal Inoculation.

Protein Extract ABS (562nm)	Net ABS (562nm)	Protein Conc. (mg/ml)
0.800	0.428	0.622745

Protein Concentration in Extract after Fungal Inoculation

The fungus produced proteases and degraded available protein content in the extract. The

absorbance and protein content in extract of each test tube progressively reduced (Table 4).

Table 4: Absorbance and Protein Concentration in Extract after Fungal Inoculation

Test Tube Number	Fungus Inoculated	Protein Extract ABS (562nm)	Net ABS (562nm)	Protein Conc. (mg/ml)
1	<i>Aspergillus</i>	0.404	0.032	0.001569
2	<i>Cladosporium</i>	0.423	0.051	0.031373
3	<i>Penicillium</i>	0.410	0.038	0.010980
4	<i>Trichoderma</i>	0.427	0.055	0.037647

Table 5: Net Protein Degradation by Fungal Isolates

Proteolytic Fungi	Protein Conc. Before Fungal Inoculation(mg/ml)	Protein Conc. After Fungal Inoculation(mg/ml)	Net Protein Degradation(mg/ml)
<i>Aspergillus</i>	0.622745	0.001569	0.621176
<i>Cladosporium</i>	0.622745	0.031373	0.591372
<i>Penicillium</i>	0.622745	0.010980	0.611765
<i>Trichoderma</i>	0.622745	0.037647	0.585098

CONCLUSION

The present study showed that proteases play a best role in the degradation of proteinaceous waste originated from the temple. In this study, a total of 4 protein degrading fungal isolates: *Aspergillus*, *Penicillium*, *Cladosporium* and *Trichoderma* were tested for the production of proteases and their ability to biodegrade protein content present in temple waste, out of which 2 fungal strains demonstrated higher proteases production and perfect biodegradation ability: *Aspergillus* and *Penicillium* as shown in Table 4. *Cladosporium* and *Trichoderma* showed protein degradation comparatively at a low level. Among these four strains, *Aspergillus* showed higher degradation ability.

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