RESEARCH ARTICLE

Isolation, Identification, and Screening of Alkaline Protease from Thermophilic Fungal Species of Raipur

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ABSTRACT- Proteases are protein degrading enzymes that catalyse the hydrolytic reaction in which protein molecules are degraded into peptides and amino acids. Thermostable alkaline proteases are of particularly great interest for industrial application because they are stable and active at a temperature above 60–70˚C. Thermophiles are found in a wide array of environment such as mushroom compost material, nest, hay, wood chips, grains, soil, manure, coal mines etc. Alkaline proteases are the most important industrial enzymes and they occupy about 60% of the total enzyme market. From the soil samples, eight different fungal species were isolated through soil dilution plate method. In the present study, two fungi *Aspergillus nidulans* and *A. glaucus* from mushroom compost and two fungi *A. terreus,* and *A. fumigates* from cow manure, showing alkaline protease activity, were isolated. The zones of clearance were observed in *A. nidulans, A. glaucus, A. terreus,* and, *A. fumigatus* species of fungi isolated from cow manure and mushroom compost. The best enzyme production was observed in *A. terreus* (1.005±0.057 IU/mg protein) obtained from cow manure and the minimum enzyme activity was observed with *A. glaucus* (0.278±0.026 IU/mg protein). However, more studies are required to assess the potential of *A. nidulans, A. glaucus, A. terreus,* and *A. fumigatus* species.

Key-words- Alkaline protease, Thermophiles, Trichloroacetic acid, Zone of clearance

INTRODUCTION

Enzymes are the biocatalyst that perform a multitude of chemical reactions and are commercially exploited in the different industries. Protease, pectinase, lipase, cellulase, renin and papain are some of the important enzymes that have been used commercially worldwide. More than 3000 enzymes are described to date which has been used in industries mainly from the mesophilic microorganism. The enzymes isolated from the mesophilic Microorganisms get denatured and lose their activities at the temperature over 50–60˚C. However, enzymes obtained from thermophilic Microorganism are dynamic at a high temperature, have unique characteristics of high reactivity and long term stability [1]. Thermophilic fungi were isolated from soil sample [2].

Thermophilic fungi are a small assemblage in group Mycota that grow at or above 45ºC Thermophilic fungi are mainly used for the production of such enzymes.

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The main drawback with the production of bacterial enzyme is the requirement of cost intensive procedures for separation of enzymes. Fungal origin of the enzyme offers an advantage of separation of mycelium by simple filtration [3]. Proteases are the enzyme that hydrolyzes the peptide linkage of proteins into simpler proteins, peptides and free amino acids. Unlike other enzymes, they are considered as mixture of enzymes [4] and include proteinases, peptidases and amidases, which hydrolyze intact proteins, peptides or peptones and amino acids respectively. Proteases are commonly classified according to their pH: Acid Proteases (pH 2.0–6.0), Neutral Proteases (pH 7.0 or around 7.0), and Alkaline proteases (pH 8–11). They are also classified on the basis of critical amino acid required for their catalytic functions (e.g. Serine proteases); the chemical nature of the catalytic site (e.g. Amino peptidases); or their requirement of a free thiol group (e.g. Thiol proteinases) [5]. Maximum activity of protease was observed at pH 8.5 and at 37˚C. Purified protease was active between pH 5.5-9.5. It was found to be stable upto 60˚C [6]. The adsorption and desorption pattern of alkaline protease was studied using different aliphatic and aromatic hydrophobic ligands. Overall, higher adsorption was obtained on ligands coupled to 6% cross-linked gel than the 4% gel [7,8]. Alkaline protease was totally inhibited by the phenyl methanesulphonyl fluoride, antipain,

chymostatin, and α-2-microglobulin [9]. *Penicillium fellutanum* was isolated from mangrove sediments and was studied for production of alkaline protease in submerged fermentation [10].

Thermostable proteases are advantageous because higher processing temperature can be employed, resulting in faster reaction rates, increase in the solubility of non gaseous reactant and products and reduced incidence of microbial contamination by mesophilic organisms. Thermophilic fungi *Mucor persillus* and *M. miechi* produce a protease enzyme having a high milk clotting activity .Alkaline protease is the main protease enzyme constituting about 60-65% of the global industrial enzyme market [5]. Alkaline proteases have the optimum pH 8-11 [11,12]. *Humicola lanuginosa* and *Malbranchea pulchella* were first identified to produce alkaline protease enzyme). Molds of the genera *Aspergillus, Penicillum, Rhizopus* are mostly used for the production of industrially important alkaline protease enzyme [3]. It has numerous applications in our daily life such as in food industries, bakery, waste water refinement, medicinal formulation, detergent formulation, alcohol production, beer production, leather industries, meat tenderization, dairy industry, silver recovery and oil manufacturing industries [13]. In the present study was performed to evaluate an identification of thermophilic fungi from mushroom compost and cow manure from Raipur city and Screening of the isolates for their alkaline protease activity followed by Quantitative assay of proteases from four isolates of fungi following Nakagawa method.

MATERIALS AND METHODS

Collection of Samples- Cow manure and mushroom compost sample were collected from Raipur city, for isolation of thermophilic fungi. The samples were mainly collected from the stable manure and mushroom compost material.

Isolation and Identification of Fungi- The samples were treated at 45˚C for 24 hrs to activate the thermophilic fungi present in the Cow manure and mushroom compost sample Dilution plate method [14] was used for isolation of fungi. Potato dextrose agar media was used for the isolation of fungi. After plating of the samples, the petri-dishes were kept at 45ºC in the incubator. The plates were examined daily, after 6th-day mycelial growth was observed over the medium in the plates. Fungi isolated from the sample identified by staining with lactophenol cotton blue stain. Stain and mold were mixed gently and coverslip was placed over the preparation. The slide was examined under microscope at low and high magnification. Identification of fungal species was made with the help of available literatures [15,16] and reference slides.

Screening of Fungi- The isolated fungi were screened for their proteolytic activity [17]. Czapek-Dox agar media containing gelatin was used for screening. The organism was inoculated on the plates and incubated at 45˚C for 48 h for the growth of organism. After the growth, the plate was flooded with mercuric chloride solution. Presence of a clear zone around the colony was an indication of a positive reaction.

Alkaline Protease Assay- The alkaline proteases activity was measured according to the method of Nakagawa [18]. An aliquot of 1 ml of hemoglobin solution in a test tube was equilibrated at 45ºC for 5 min. The assay was initiated by adding 0.5 ml of enzyme sample and mixture was incubated at the 45˚C for 10 min. The reaction was terminated by addition of 2 ml of 5% Trichloroacetic acid (TCA) at the end of the incubation period. In the control tube, 2 ml of TCA was added before the addition of enzyme sample. Test and Control tubes were allowed to stand for 5 min in ice-cold condition and centrifuged at 3000 rpm for 15 min. 1 ml supernatant was allowed to react with 5 ml alkaline solution for 10 min followed by addition of 0.5 ml Folin's Ciocaltaeu Reagent (FCR) and the color developed after 15 min was read against a blank at 750 nm. Enzyme activity was calculated by measuring the amount of tyrosine released and compared with that of the standard (standard curve was made by using tyrosine as a standard).

Protein Estimation- Protein concentration of the enzyme extract of the fungus was measured by the method of Lowry [19] using Bovine Serum Albumin (BSA) as the standard. 1ml of enzyme sample was allowed to react with 5 ml alkaline solution for 10 min, followed by the addition of 0.5 ml FCR. The color developed after 15 min was read against blank at 750 nm. The protein was estimated with reference to standard (BSA) and expressed as mg/ml.

RESULTS AND DISCUSSION

Isolation and identification of microorganisms from soil sample- From the soil samples, eight different fungal species were isolated through soil dilution plate method. Four isolates were recovered from the cow manure and four isolates were noticed from the mushroom compost. The isolated colonies are shown in Fig. 1 & Fig. 2. The colony morphology, texture of the isolated fungal species and details of spore structure were used to identify the species (Table 1 & Table 2).

Table 1: Fungal strains isolated from mushroom compost and Cow manure

S. No.	Samples	Isolates	Fungus identified
1.	Cow manure	CM1	*A. terreus*
2.		CM2	*A. fumigatus*
3.		CM3	*A. fischeri*
4.		CM4	*P. herquei*

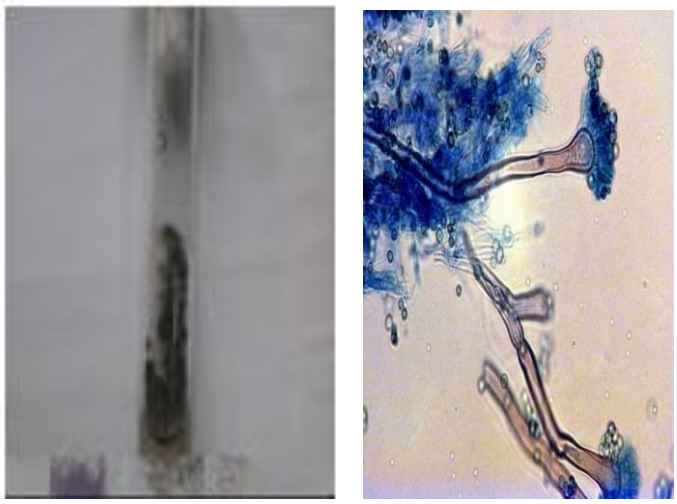
5.	Mushroom compost	MC1	*A. nidulans*
6.		MC2	*A. glaucus*
7.		MC3	*A. fumigatus*
8.		MC4	*A. flavus var columanris*

CM- Cow manure; MC- Mushroom compost

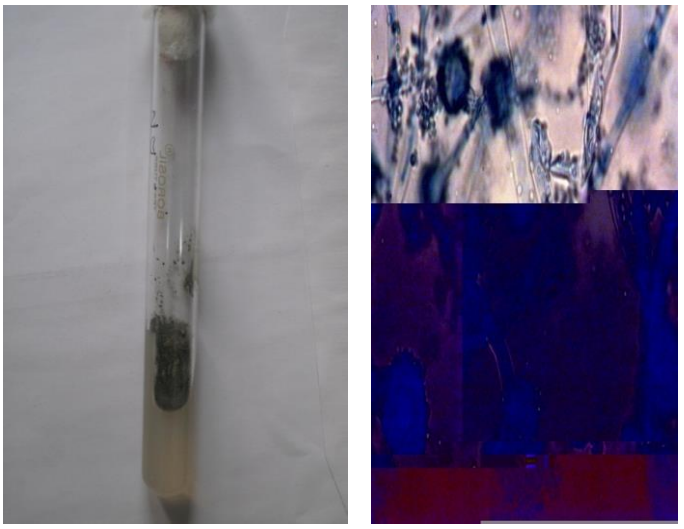
Table 2: Colony morphology of the isolated strains from Mushroom compost and Cow manure

S. No.	Isolates	Colony Morphology	Texture
1.	CM1	Green color colony	Rough
2.	CM2	Green color colonies	Granular
3.	CM3	Grayish color colonies	Cottony
4.	CM4	Yellowish green color colony	Smooth
5.	MC1	White color colonies turn to green color when mature	Granular
6.	MC2	Green color colonies, reverse yellow color	Powdery
7.	MC3	Green color colonies	Granular
8.	MC4	Green color colonies, reverse cream color	Granular

CM- Cow manure; MC- Mushroom compost



CM1



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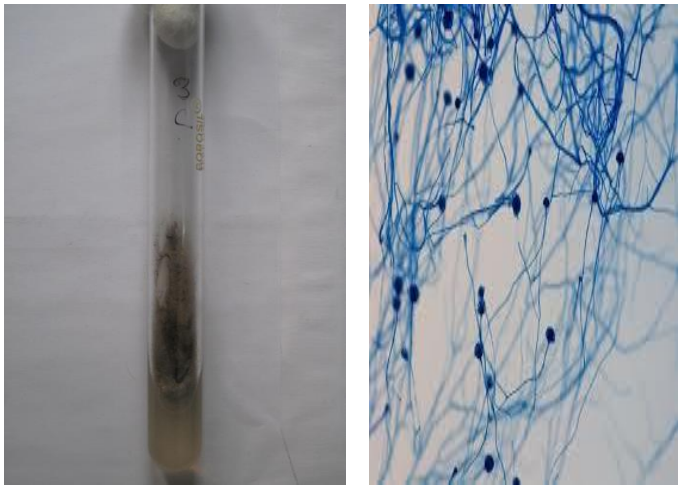

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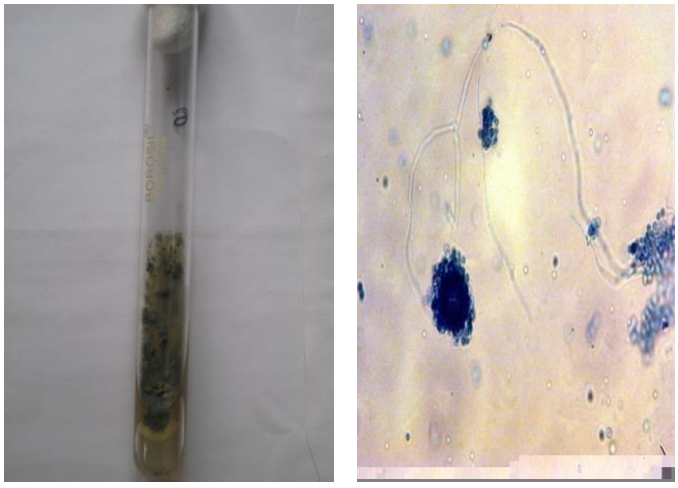
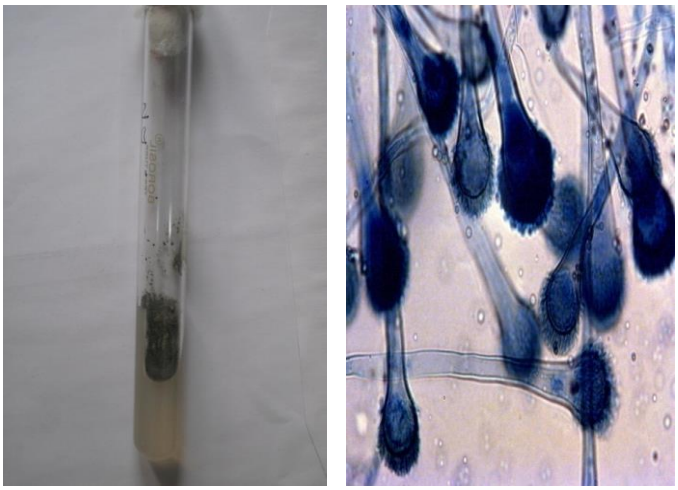
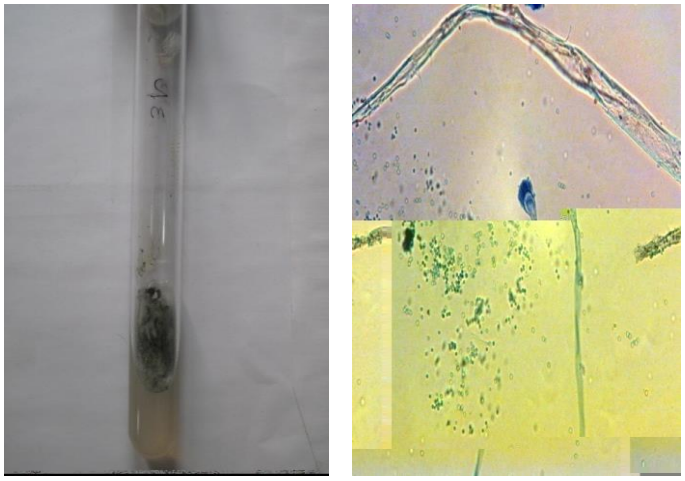


CM4

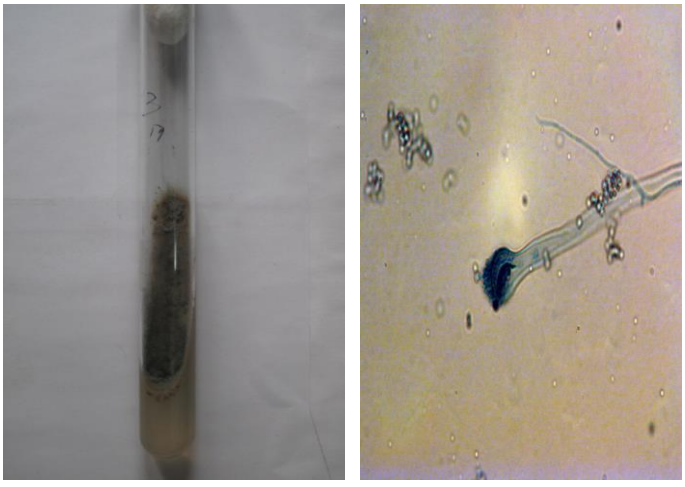
Fig. 1: Thermophilic fungi isolated from the cow manure the growth of colony in the slant and microscopic view of the fungus at x400 magnification are CM1, CM2, CM3 and CM4, respectively



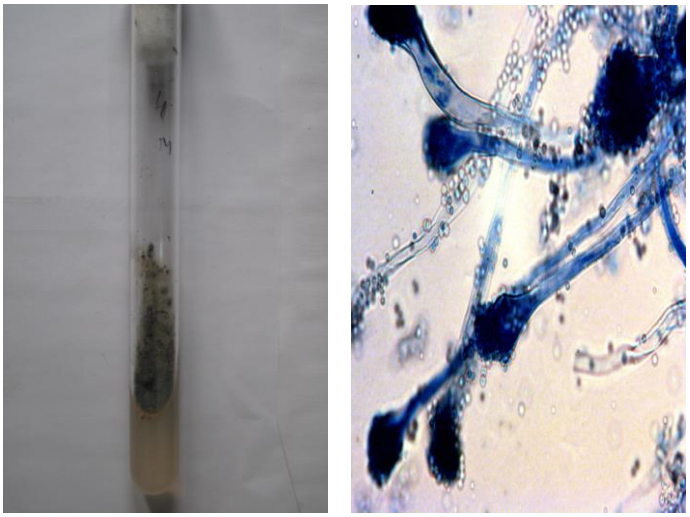
MC1



MC2



MC3



MC4

Fig. 2: Thermophilic fungi isolated from the mushroom compost the growth of colony in the slant and microscopic view of the fungus at x400 magnification are MC1, MC2, MC3 and MC4 respectively

Screening for protease producing microorganisms- Screening of protease producing fungi usually involves growth on the medium that contains protein as the selective substrate. In the present investigation, gelatin was used as selective substrate, in which the isolated fungal species were streaked. The fungal species, *A. terreus*, *A. fumigatus* isolated from cow manure and *A. nidulans*, *A. glaucus* isolated from mushroom compost produced the clear zone in the media and hence were confirmed to be protease producing species as depicted in Fig. 3. *A. fumigatus*, *A. flavus var collumanris* isolated from mushroom compost and *A. fischeri*, *Penicillium herquei* recovered from the cow manure did not produce the clear zone and hence were considered negative for protease enzyme.

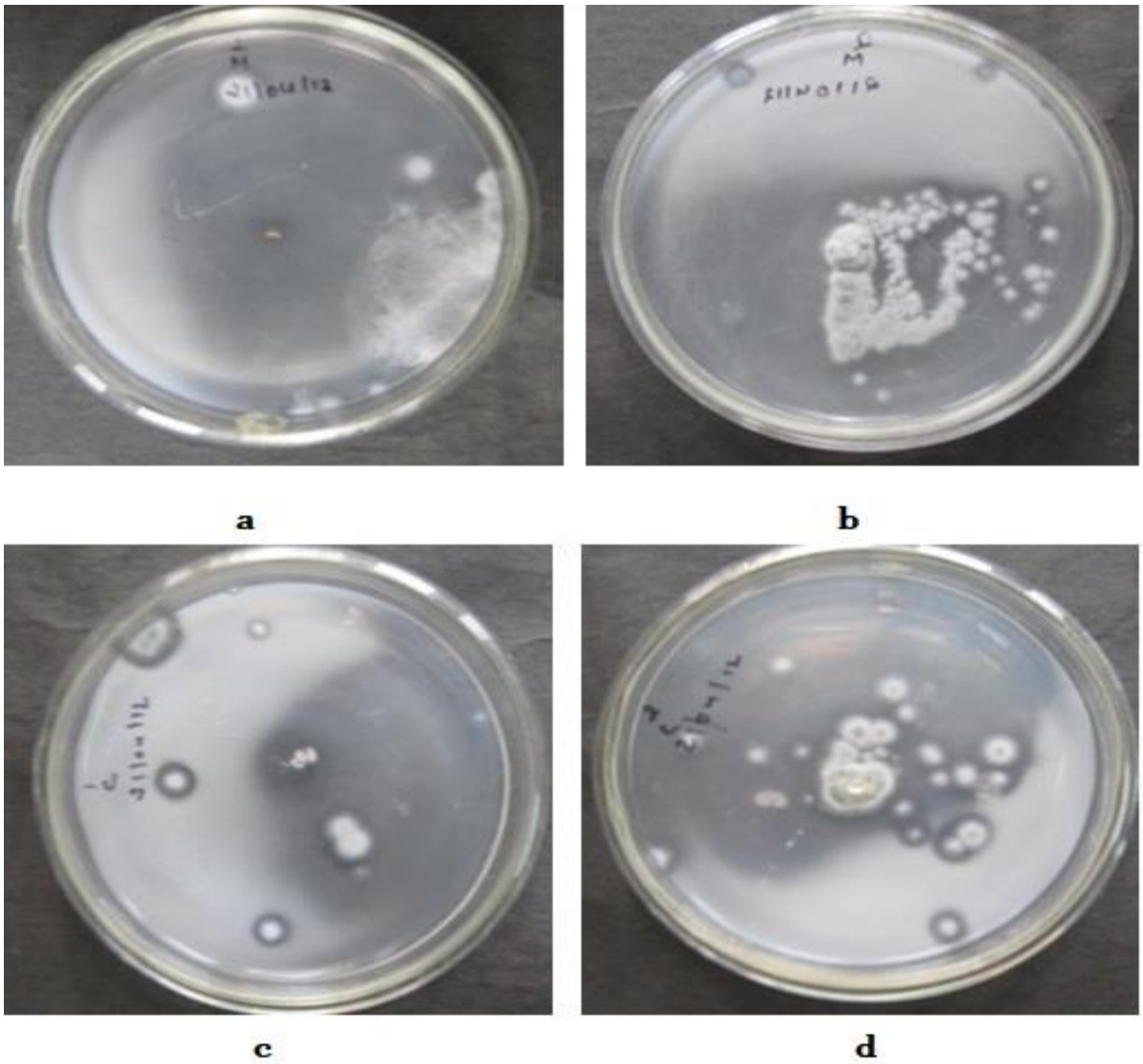


Fig. 3: Fungal strains showing positive result for protease production
(a) *Aspergillus nidulans* **(b)** *Aspergillus glaucus* isolated from mushroom compost, **(c)** *Aspergillus terreus* **(d)** *Aspergillus fumigatus* isolated from cow manure

Quantitative assay of alkaline protease enzyme producing microorganisms- Alkaline protease activity was measured following the method of Nakagawa [18]. The standard curve of tyrosine was constructed by plotting concentration on X-axis and optical density observed at 750 nm on Y-axis is presented in Fig. 4. The enzyme activity of all the fungal strains was carried out in five replicates and are tabulated in Table 3 and presented in Fig. 5 & Fig. 7. The maximum enzyme activity was observed in *A. terreus* (0.130±0.003 IU/ml and 1.005±0.057 IU/mg protein) isolated from cow manure and minimum enzyme activity was observed in *A. glaucus* (0.085±0.002 IU/ml and 0.278±0.026 IU/mg protein) isolated from mushroom compost.

Table 3: Quantitative assay of alkaline protease enzyme produced by the fungus isolated from the Mushroom compost and Cow manure

S. No.	Isolates	Enzyme Activity	
		IU/ml	IU/mg protein
1.	*A. terreus*	0.130±0.003	1.005±0.057
2.	*A. fumigatus*	0.096±0.001	0.291±0.026
3.	*A. nidulans*	0.111±0.009	0.455±0.039
4.	*A. glaucus*	0.085±0.002	0.278±0.006

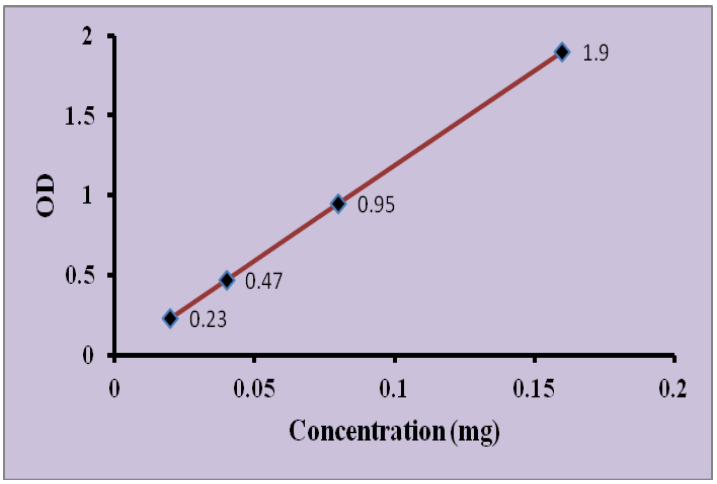


Fig. 4: Standard graph of tyrosine at 750 nm

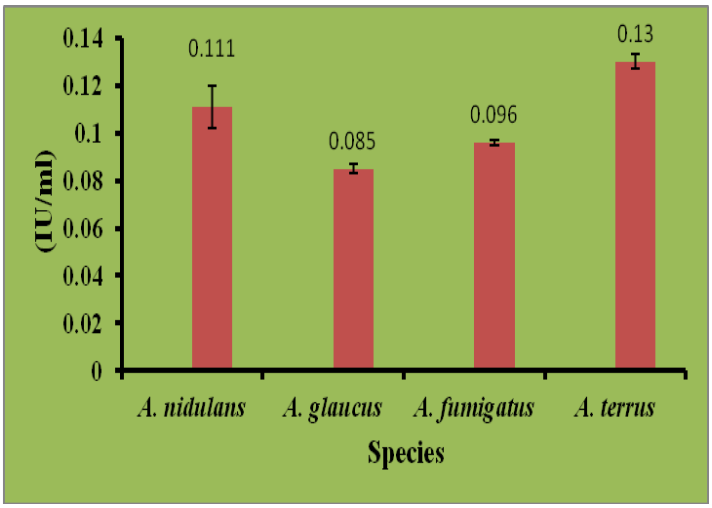


Fig. 5: Activity of Alkaline protease (IU/ml) by *A. nidulans*, *A. glaucus* isolated from mushroom compost, *A. fumigatus* and *A. terreus* isolated from the Cow manure

Protein Estimation- Protein was estimated following Lowry [19] method. The standard curve for protein was constructed by plotting concentration on X-axis and optical density at 750 nm on Y-axis and is presented in Fig. 6. The quantity of protein observed in enzyme extract was used to express the protease activity per mg protein and is shown in Table 3 & Fig. 7.

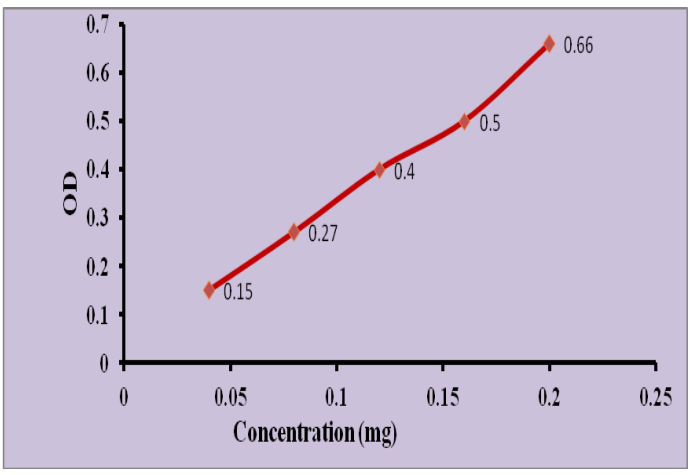


Fig. 6: Standard graph of BSA at 750 nm

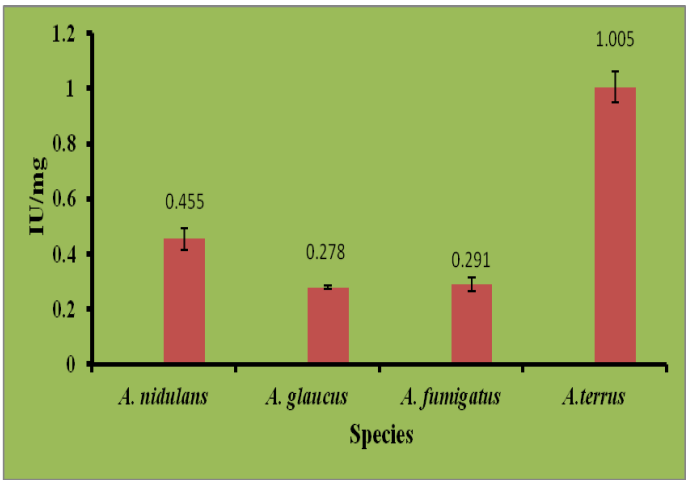


Fig. 7: Activity of Alkaline protease (IU/mg protein) by *A. nidulans*, *A. glaucus* isolated from mushroom compost, *A. fumigatus* and *A. terreus* isolated from the Cow manure

The quantity of protein observed in enzyme extract was used to express the protease activity per mg protein. The maximum enzyme activity was observed in *Aspergillus terreus* was 1.005 IU/mg protein isolated from cow manure and minimum enzyme activity was observed in *A. glaucus* is 0.278 IU/mg protein isolated from mushroom compost.

Preliminary attempt was made to isolate protease producing thermophilic fungi from the mushroom compost and cow manure from Raipur city. Four fungi were obtained from mushroom compost. When grown on the standard growth medium after protein hydrolysis by plate assay only two of them showed alkaline protease productions. However, in cow manure also four fungi were recovered of which only two were showing enzyme production.

The ability of protein hydrolysis by fungal strains was represented by the clear zone produced around colonies. This loss of opacity is result of a hydrolytic reaction, yielding soluble non-colloidal amino acids, representing the positive reaction. While non-producing colonies remained opaque containing, non-degraded gelatin (protein substrate) representing the negative reaction. The zone of clearance was observed in *A. nidulans*, *A. glaucus*, *A. terreus*, and *A. fumigatus*. The strains that did not show protease hydrolysis were found to be *A. fischeri* and *P. herquei* present in cow manure. *A. flavus var columnaris* and. *A fumigatus* present in mushroom compost. The enzyme activity shown by the fungal strain gave a quantitative comparison between different strains.

CONCLUSIONS

In the present study, the zone of clearance was observed in *A. nidulans*, *A. glaucus*, *A. terreus*, and *A. fumigatus*. In total, only four isolates from the respective sources are useful for further study. The maximum enzyme activity was observed in *A. terreus* (0.130±0.003 IU/ml and 1.005±0.057 IU/mg protein), followed by the *A. nidulans* (0.111 ±0.009 IU/ml and 0.455±0.039 IU/mg protein). The minimum enzyme activity was observed in *A. glaucus* (0.085±0.002 IU/ml and 0.278±0.026 IU/mg

protein). The best enzyme production was observed in *A. terreus* (1.005±0.057 IU/mg protein) that was obtained from cow manure. However, more studies are required to assess the potential of these species. Also, efforts to optimize the culture conditions would help in understanding the ability of these species. Thermostable alkaline proteases are of particularly great interest for industrial application because they are stable and active at a temperature above 60–70˚C.

REFERENCES

[1] Synowiecki J. Some applications of thermophiles and their enzymes for protein processing. African J. Biotechnol., 2010; 9: 7020-25.

[2] Ellis DH, Keane PJ. Thermophilic fungi isolated from some Australian soils. Australian J. Bot., 1981; 29: 689-704.

[3] Devi MK, Rasheedha Banu A, Gnanaprabhal GR, Pradeep BV, et al. Purification, characterization of alkaline protease from native isolates Aspergillus *niger* its compatibility with commercial detergents. Indian J. Sci. Technol., 2008; 1: 1-6.

[4] Lee H, Suh DB, Hwang JH, Suh HJ. Appl. Biochem. Biotechnol., 2002; 97: 123-33.

[5] Rao MB, Aparna M, Tanksale M, Ghatge, S, Deshpande VV. Molecular and Biotechnological aspects of microbial proteases. Microbiol. Molecular Biol. Rev., 1998; 62: 597-35.

[6] Chakrabarti SK, Matsumura N, Ranu RS. Purification and characterization of an extracellular alkaline serine protease from Aspergillus *terreus* (IJIRA 6.2). Curr. Microbiol. 2000; 40: 239-44.

[7] Adikne HV, Barhate RS, Thakar DM, Nene SN. Optimization of single-step purification of alkaline protease using different hydrophobic ligands. Bioseperation, 2001; 10: 133-37.

[8] Sharma N, Kantishree De. Production, purification and crystallization of an alkaline protease from *Aspergillus tamari* [EF661565.1]. Agriculture Biol. J. North America, 2011; 2: 1135-42.

[9] Monod M, Togni G, Rahalison L, Frenk E. Isolation and characterization of an extracellular alkaline protease of *Aspergillus fumigatus*. J. Medical Microbiol., 1991; 35: 23-28.

[10] Manivannan S and Kathiresan K: Alkaline protease production by Penicillium *fellutanum*, isolated from Mangroove sediments. Int. J. Biological Chem., 2007; 1: 98-103.

[11] Charles P, Devanathan V, Anbu P, Ponnuswamy MN, Kalaichevalan PT, et al. Purification, characterization and crystallization of an extracellular alkaline protease from *Aspergillus nidulans* HA-10. J. Basic Microbiol., 2008; 48: 347-52.

[12] Abbas CA, Groves S, Gander JE. Isolation, purification and properties of *Penicillium charlesii*. J. Bacteriol., 1989; 171:5630-5637.

[13] Anwar A, Saleemuddin M: Alkaline Proteases. A Review. Bioresource Technol., 1998; 64: 175-83.

[14] Wakesman SA, Fred EB. A tentative line of the plate method for determining the number of Microorganism in the soil. Soil Sci., 1922; 14: 27-28.

[15] Ellis MB. *Demitiaceous Hyphomycetes*. Common Wealth Mycological Institute, London, 1949.

[16] Barnett H. Illustrated genera of imperfect fungi. Burgess Publication Company Minneapolis 1969; Minnesota.

[17] Jain R, Agarwal SC, Jain PC. Proteoloytic actinomycetes from Indian habitats. J. Culture Collections, 2008-09; 6: 28-37.

[18] Nakagawa Y. Methods in enzymology proteolytic enzymes, 19. Academic Press, New York and London, 1970; 581-91.

[19] Lowry OH, Rosebrough NJ, Farr AC, Randall RJ. Protein measurement with Folin-phenol reagent. J. Biol. Chem., 1951; 193: 265-75.

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