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Molecular Characterization and Screening of Protease Production from indigenous thermophilic fungi *Malbranchea cinnamomea* isolated from dairy farm compost soil

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ABSTRACT

Compost environments represent a rich reservoir of microbial diversity, including thermophilic fungi with the potential to produce proteases. The dynamic and nutrient-rich nature of compost provides a conducive habitat for the growth and metabolism of diverse fungal species, including Malbranchea cinnamomea. In this study, Malbranchea cinnamomea was isolated from dairy farm compost soil and characterized based on its morphological characters and subsequently confirmed molecularly using internal transcribed spacer (ITS) sequencing. The Phylogenetic tree was inferred using Neighbour- joining method. The evolutionary distances were computed using the maximum composite likelihood method. The identified fungi was primarily screened for protease and further used for enzyme production in submerged fermentation for 4-6 days at 50^{O}C. Culture filtrate obtained were used for the assessment of enzymatic activity. This study revealed thermophilic fungus Malbranchea cinnamomea holds significant potential for protease production. The enzyme activity was estimated at 50 °C, pH 7, six days incubation time and using 1.0 ml inoculum. Under submerged substrate cultivation, the best substrate lodge for maximal fungal biomass production was YPSs broth. Hence this isolate could be further exploited for numerous industrial applications that require thermophilic enzymes.

Keywords: Thermophilic fungi, protease, enzyme, compost, molecular characterization

INTRODUCTION

Microbes perform the aerobic and biochemical process that leads to hydrolysis of the organic fraction to stable humus (Wei *et al.,* 2017). Organic matter breaks down to release macro and micronutrients, improve organic carbon and enhance the soil's beneficial microbial load. In addition to promoting the synthesis of humified compounds and the release of nutrients during the decomposition of plant and animal residues. Soil organic matter also activates fauna and microorganisms in the soil**.** Organic matter boosts soil microbial activity and soil enzyme activity, which are indicators of soil fertility and microbial activity (Elayaraja *et al.,* 2019). The presence of high organic matter and elevated moisture levels provide a suitable substrate for the growth of thermophilic fungi (Jaitly and Rai, 1982). Research has shown that many thermophilic microorganisms were primarily isolated from composts: prevalence of these microbes in compost sites is due to their high temperatures, aerobic and humidity conditions present in the compost. Additionally, compost also serves as a source of nutrients for the development of microorganism (Lee *et al.,* 2014)

Thermophilic fungi isolated and identified from various composting materials, including agricultural waste, animal manure, and municipal solid waste. Thermophilic fungi are an excellent source of new, thermostable enzymes essential to industrial and biotechnological uses. (Moretti *et al*, 2012). These fungi can thrive in extremely harsh temperatures ranges (20–50°C). The ability of thermophilic fungi to endure in extremely hot environments, such as the deep sea, hydrothermal vents, hot springs, and volcanic environments, increases the possibility that they will produce novel and bioactive secondary metabolites. The ability of fungi to grow on inexpensive substrates and secrete large amounts of the enzyme into the culture medium has drawn the interest of environmental biotechnology specialists in fungus protease. (Abdalla *et al*.,2018) The filamentous fungus can grow on a wide range of substrates as nutrients and have ability to grow in a variety of environmental conditions including temperature, pH, and time course (Haq *et al*.,2006) .These fungi can produce unique metabolites or enzymes that exhibit thermostability between 40 - 70 degrees Celsius (Maheshwari *et al.,* 2000). Because of their proficiency in producing notable metabolites and thermostable enzymes such as

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xylanases, proteases, phytases, and cellulases that are relevant to industry, as well as their ease of use in anaerobic fermentative processes and their adaptability to high temperatures, they are chosen for their enormous biotechnological potential. (Van den Brink, 2011). Enzymes produced by these thermophiles are thermostable, extreme pH tolerant and also possess high activity at other extreme environmental or industrial conditions (Ahirwar *et al.,* 2017; Gulmus and Gormez, 2020). Based on their pH range, the proteases can be categorized into three primary groups: neutral, acidic, and alkaline proteases. The ideal pH range for acidic protease is 2.0 to 5.0. While alkaline protease prefers a pH of greater than 7.0, neutral protease has optimum pH of 7.0. Because of their wide range of uses in the food and detergent industries, alkaline proteases are extremely significant (Naeem *et al*.,2022) Proteases are used in brewing, meat tenderization, and milk coagulation because they are known to catalyze the hydrolysis of peptide bonds in proteins. The proteases have additionally been utilized to support the emulsification and coagulation processes, enhance food flavor, nutritional value and improve digestion (Aruna *et al.,* 2014). In the present study, thermophilic fungi *Malbranchea cinnamomea* was isolated from dairy compost which was further molecularly identified and screened for proteases production, for future biotechnological applications.

MATERIALS AND METHODS

This study comprised collection of compost soil samples from dairy farms in Gokul Nagar, Bhilai, Chhatisgarh, for the isolation of thermophilic fungi, as well as morphological and molecular characterization and screening of the fungi for extracellular protease production.

Collection of Samples

Samples of dairy farm compost soil were collected randomly from four locations in Gokul Nagar, Bhilai, region of Chhattisgarh. A soil sample was taken in sterile polythene bags from 5 to 10 cm deep layer of soil and it was then brought into the laboratory to isolate the fungi.

Isolation of fungi

The collected soil samples underwent a pretreatment at 45^0 C to promote the growth of thermophilic fungi and inhibit the growth of mesophilic ones. The serial dilution method was used to inoculate soil samples. The petri plates were incubated for 4 to 6 days at 50^0C. Fungal growth was regularly observed during the incubation period. Morphological characteristics of an isolated fungal culture were examined using lactophenol cotton blue stain.

Morphological characterization

The isolates were observed for macroscopic as well as microscopic characters like colony colour, reverse coloration, hyphal structure, septation, shape and spore bearing structure, pigmentation were observed. (Gilman 1957, Ainsworth 1965)

Molecular Identification

Genomic DNA was isolated from the sample. The ITS1 –5.8S—ITS2 rDNA was amplified using primers ITS4 (forward primer) and ITS5 (reverse primer) (White *et al*. 1990). Amplification was achieved in a vial containing 10x buffer, MgCl2 15 mM, dNTP 0.2 mmol, Forward primer and Reverse primer 10 picomolar, Taq Polymerase 2µl, DNA sample 50-100 ng/µl and Milli Q water. The PCR reaction was carried out using a Thermal Cycler with conditions as follows: Initiation for 10 minutes at 94^0C, denaturation for one minute at 94^0C, annealing for 30 seconds at 55^0C, extension for 1 minute at 72^0C. The Thermal Cycler was run for 35 cycles and then a final extension cycle was run at 72^0C for 10 minutes. The PCR products were analyzed by electrophoresis on 1.2% agarose. The fungal strain was identified according to a molecular biological protocol by DNA amplification and sequencing of the internal transcribed spacer (ITS) region. After alignment into GenBank database, the similarities of the target sequences were analyzed to identify the fungi and its closest neighbors.

Growth optimization of fungal isolate

After incubating the fungal isolate in broth containing the 50ml of yeast extract starch without agar in 100 ml flask for different incubation periods, under the suitable condition at respective temperature, pH, and by using different synthetic media included Yeast extract starch (YPSs), Waksman (glucose, casein, K2HPO4, MgSO4,7H2O) and M5 (sucrose, NaNO3, KCl, FeSO4, K2HPO4).The optimum growth time required for fungus was examined at

various incubation periods (2nd day,4th day,6th day) by measuring the dry weight of biomass at the end of each time interval (Ajmera *et al*,2019).

Screening for protease production

Protease production by fungal isolates was detected by using casein agar media by plate assay method (Suryawanshi and Pandya 2017).The fungal isolate were spot inoculated on the medium and incubated at 50^0C. Following incubation, presence of a clear zone around the colony was an indication of a positive reaction for extracellular protease activity of the fungal strain. Further cell free culture filterate was prepared for enzyme activity assessment.

Cultivation and preparation of cell free culture filterate (CFCF)

YPSs broth was prepared and sterilized in 250 ml Erlenmeyer conical flasks. Each flask was filled with 100ml broth medium. After sterilization, flasks were inoculated with 1 ml spore suspension of the selected fungi, which contained approximately 10^6 spores/ml. The flasks were then incubated at a temperature of 50^0C for a duration of 6-7 days. Subsequently, the culture broth was centrifuged at 8000 rpm for 10 min., supernatant was collected and pelleted cells were discarded (Sethi and Gupta, 2015). The cell free supernatants collected were further used for evaluation of protease enzyme activity. All microbial cultivation was performed in triplicate for each selected isolate.

Enzyme Assay

Protease activity was assayed by the method of Keay et al. (1970) with minor modifications. One milliliter of enzyme was mixed thoroughly with 1 mL 2 % of casein solution. The mixture was incubated at 50°C for 30 min. The reaction was terminated by the addition of 2 mL 0.4M Trichloroacetic acid, incubated for 20 min at 50°C and filtered. Then 1 mL from the filtrate with 5 mL 0.4 M Na_2CO_3 and 1 mL 2 M Folin phenol reagent were added and mixed thoroughly. The mixture was incubated at 37°C for 20 min and the final solution was measured at 660 nm. Tyrosine was used as standard. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol tyrosine per minute per gram substrate under experimental conditions. Protein content of the extract was estimated by the method described by Lowry *et al.,* (1951)

Morphological characterization

RESULTS AND DISCUSSIONS

Four different sites: siteA, siteB, siteC, siteD were selected in Gokul nagar Bhilai for isolation of *Malbranchea cinnamomea*. Maximum no. of colonies were observed in site C followed by siteA, siteD and siteB respectively. (Table: 1) Fungal Colony was whitish yellow in colour, hyphae was septate and colourless and conidia were smooth walled and curved shape. (Fig:1, Fig:2)

Table: 1 Occurance of *Malbranchea cinnamomea* in Gokul nagar ,Bhilai site

S.No.	Name of Site	No.of Colonies observed
1.	Site A	04
2.	Site B	02
3.	Site C	07
4.	Site D	03

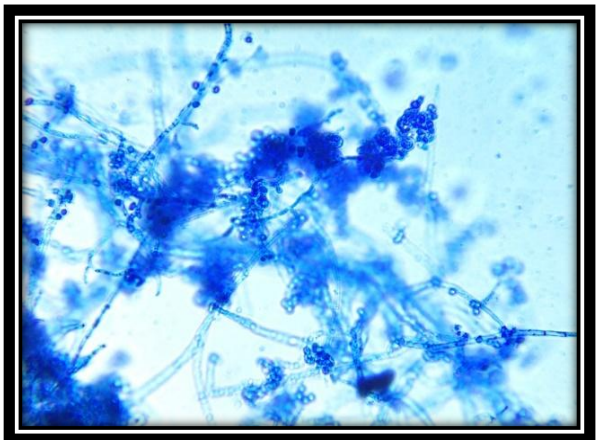

Fig1: Culture plate of *Malbranchea cinnamomea* colony Fig2: Microscopic view of *Malbranchea cinnamomea*

Molecular identification

Aligned Sequence of fungal isolate *Malbranchea cinnamomea* (590 bp)

ATTTTTATTTAGGCTTTCGCGAACTGATCCGAGGTCATTACCGTGCCAACGGGGACGCGACACACGGGGTGTCGCGGC
CCCTCCCGGCCAGCGCCGTGTCCTACCGAACCATCGTTGCCTCGACGGAGCCCCATGCACTCGGGCGATCGAGGATG
ACCGCGACGCCGCCACTGCCTTTCGGGCCCGTCCCGCCCAGTTCGGGGGGACGCGCGCCCATCACACAAGCCGCGC
TTGAGGGTTGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGAGGGCGCCATGTGCGTTCAAAGATTCGAT
GATTCACGGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGGGAACCAAGAGATCCGTT
GTTGAAAGTTTTGACGATTTTCCAGTCGACTCAGACTCGCTCACGGATCGACAGAGGTTCGGTCGAACTCCTCGGGCG
GGCGCGGGCCCGGGGCCGTCGGCGCAGCAGGGCGCCGGCGAGGCCCCCGGCGGTCCCCCGGGGCGATCCCGGG
GGACGGGCCCGCCGAGGCAACGTGGTTCGGTAGACACGGGTGGCCGGGAGG

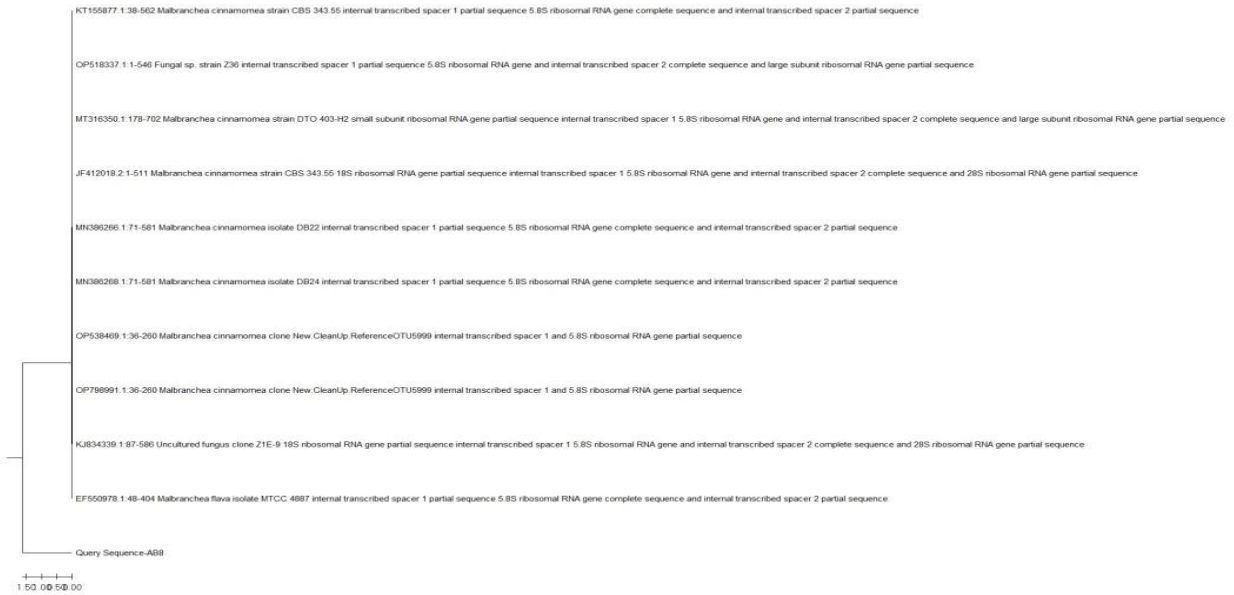

Fig 3: BLAST analysis showing phylogenetic tree of *Malbranchea cinnamomea along with closest relatives*

The Microbe was identified as *Malbranchea cinnamomea* as it showed highest similarity of 95.25% with *Malbranchea cinnamomea* culture CBS:343.55 strain CBS 343.55 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 with accession no. MH857506.1

Fig. 4: Plate showing screening of *Malbranchea cinnamomea* for Protease production on casein agar media

Table 2: Showing zone of clearance diameter (mm) of *Malbranchea cinnamomea* at different incubation time

Sample site	Fungal Isolate	Zone of clearance diameter(mm)		
		2nd Day	4th Day	6th Day
Gokul Nagar,Bhilai	*Malbranchea cinnamomea*	24	43	43

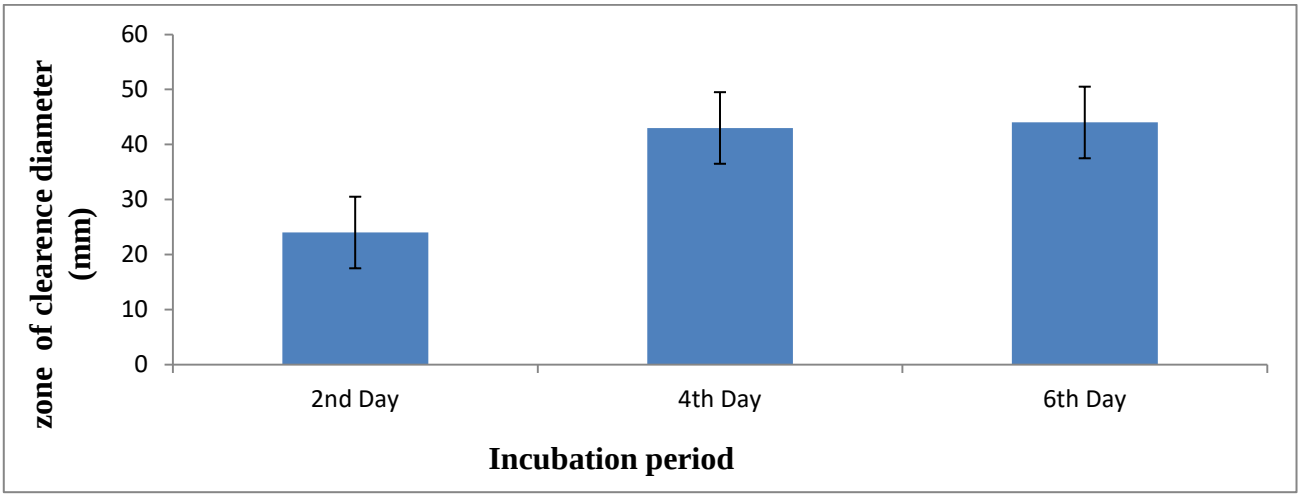

Fig. 5: Zone of clearance of *Malbranchea cinnamomea*

Fungal isolate exhibited maximum zone of clearance (43mm) at 4th day of incubation. At 2nd day isolate zone was 24 mm and increased to 43 mm at 4th day. After that it did not show any significant change in protease production. (Table: 2, Fig: 5) Similar results were reported by Abdalla *et al.,* 2018 in his study on extracellular protease from fungal isolates of soil.

Table 3: Showing effect of incubation period on growth of fungal isolate

Sample site	Fungal Isolate	Incubation period	Biomass (Dry wt)(g/ml)		
			YPSs broth	Waksman broth	M5 broth
Gokul Nagar,Bhilai	*Malbranchea cinnamomea*	2nd Day	2.46	2.48	2.44
		4th Day	3.2	2.50	2.44
		6th Day	2.9	2.40	2.40

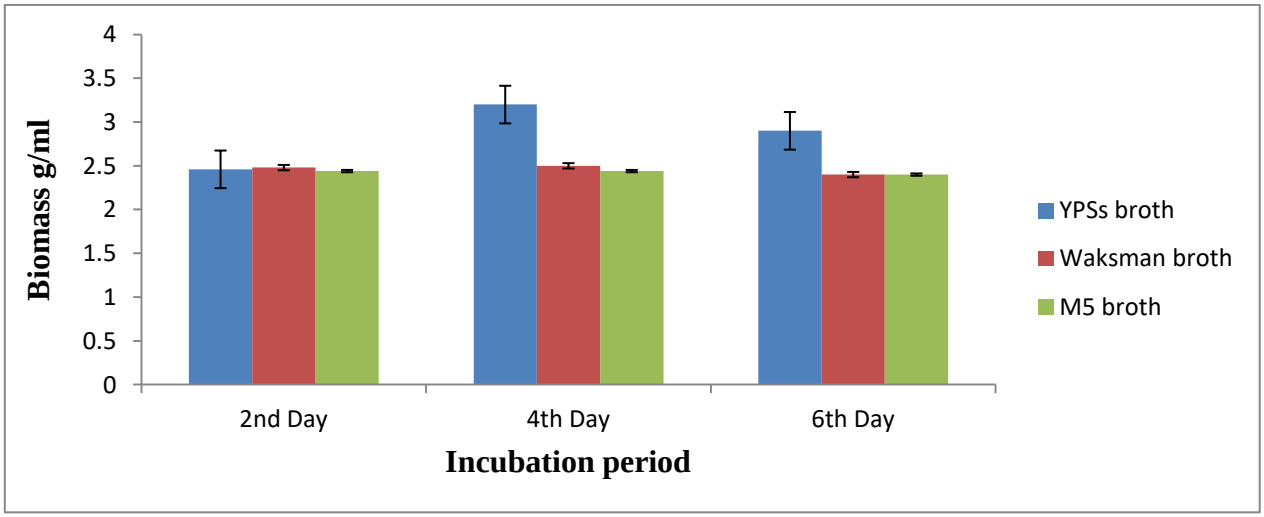

Fig 6: change in biomass of *Malbranchea cinnamomea* at different incubation period

Fungal growth period was also investigated at different intervals ranging from 2 to 6 days in three different media composition represented in Fig 6, Table:3. Maximum growth was observed in YPSs broth at 4th day of incubation. Ajmera *et al*.2019 also reported optimum growth of thermophilic fungal isolate *Malbranchea cinnamomea* in YES media at 10th day of incucbation period.

Quantitative enzyme activity

The enzymatic analysis was carried on supernatant after growing isolated fungi in Erlenmeyer flask incubated at 50^{0}C. The culture filtrate were used for quantitative estimation of crude protease enzyme activity: results are depicted in Table :4 All the experiments were done in triplicates and values are expressed as mean ± SD (n=3).

Table 4: Protein concentration and enzyme activity of protease in culture filtrate

Fungal isolate	Protein concenteration (mg/ml)	Crude enzyme activity (U/ml)	Incubation temperature	Optimum pH
Malbranchea cinnamomea	0.365±0.015	2.156±0.018	50ºC	7.0

In present study extracellular protease activity of fungal isolate was recorded 2.156±0.018 U/ml at 50^{0}C whereas protein concentration was found 0.365±0.015mg/ml. (Table:4) Ibraheem *et.al*, 2021 also investigated screening study for protease production from thermophilic fungi and reported highest protease activity 3.003±0.009 U/ml and protein concentration 0.369±0.008mg/ml at 50^{0}C . Such studies provide insights into the unique enzymatic capabilities of thermophiles and their potential advantages over mesophiles in various applications.

CONCLUSION

From the present study it can be concluded that compost soil harbors a diverse group of fungi which can be used as a source of industrially important protease enzyme. *Malbranchea cinnamomea* showed positive result for protease production. The crude protease enzyme activity evaluated at optimum temperature and pH. It can be concluded that *Malbranchea cinnamomea* represents a promising candidate for industrial protease production. Further research is needed to fully exploit their capabilities and address challenges related to enzyme production, purification, and commercialization.

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