Folia Microbiologica
https://doi.org/10.1007/s12223-022-00991-4

ORIGINAL ARTICLE

# Isolation and characterization of thermostable and alkali-tolerant cellulase from litter endophytic fungus *Bartalinia pondoensis*

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Received: 19 April 2022 / Accepted: 5 July 2022
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**Abstract**
Endophytic fungi in plant tissues produce a wide range of secondary metabolites and enzymes, which exhibit a variety of biological activities. In the present study, litter endophytic fungi were isolated from a fire-prone forest and screened for thermostable cellulases. Among nine endophytic fungi tested, two isolates, *Bartalinia pondoensis* and *Phoma* sp., showed the maximum cellulase activity. *Bartalinia pondoensis* was further selected for its cellulase production and characterization. Among the carbon and nitrogen sources tested, maximum cellulase production was observed with maltose and yeast extract, and the eucalyptus leaves and rice bran served as the best natural substrates. The cellulase activity increased with increasing temperature, with maximum activity recorded at 100 °C. The maximum CMCase activity was observed between pH 6.0 and 7.0 and retained 80% of its activity in the pH range of 8–10. Partially purified cellulase of *B. pondoensis* retained 50% of its activity after 2 h of incubation at 60 °C, 80 °C and 100 °C. These results suggest that litter endophytic fungus *B. pondoensis* is a potential source for the production of thermostable and alkali-tolerant cellulase.

**Keywords** Litter endophytic fungi · Thermal stability · Agni fungi · Cellulase · *Bartalinia pondoensis*

## Introduction

Enzymes are biocatalysts and play a vital role in various biological processes such as signal transduction, macromolecules degradation and cell regulation. Enzymes play a crucial role in developing enormous biotechnology products, such as food, cleaning supplies, clothing, paper products, fuels, pharmaceuticals and monitoring devices (Singh et al. 2016). Many microbial sources have been explored to produce enzymes like yeast, bacteria and fungi (Silva 2017). Filamentous fungi were essential in producing industrial enzymes and were considered the preferred source among other organisms (Yang et al. 2017). Some of the commercial enzymes produced by filamentous fungi are cellulase, lipase, laccase, catalase, glucoamylase, glucose oxidase, pectinase, phytase and proteases. Though the significant heterogeneity of filamentous fungi exists in nature, very few species such as *Trichoderma reesei*, *Aspergillus niger* and *A. oryzae* have been explored as enzyme producers (Cherry and Fidantsef 2003; Corrêa et al. 2014). The quest for new microorganisms to produce industrially essential enzymes is increasing worldwide.

Fungi are ubiquitous in plants and are categorized as epiphytes and endophytes. The epiphytes live on the plant surfaces, and endophytes inhabit the healthy tissues. Most endophytes live inside the plant tissues without causing any sign of infection during all or part of the plant life cycle (Schulz and Boyle 2005). Endophytic fungi have been explored for various bioactive compounds for therapeutic purposes. Endophytic fungal research mainly focuses on producing secondary metabolites with applications in the environment, agriculture, medicine and the food industry (Manganyi and Ateba 2020). Though endophytic fungi are explored to produce bioactive metabolites and novel drugs, little attention has been paid to industrial enzymes. Endophytic fungi have been recognized as enzyme producers

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Published online: 29 July 2022

for their natural needs, specifically for penetrating and colonizing their plant hosts (Suryanarayanan et al. 2012). Endophytic fungi produce different enzymes such as amylases, lipases and proteases to overcome the host's defences against invasion and obtain nutrients for their development (Mishra et al. 2019).

The enzymes required for industrial processes should be robust and capable of resisting a wide range of conditions such as extreme pH, temperature, osmolarity and pressure. Endophytic fungi isolated from harsh environments may produce such enzymes for industrial processes (Bhadra et al. 2022; Mishra et al. 2019). To produce biofuel from biomass, cellulases are required, which convert cellulosic polymers of the lignocellulosic biomass (LCB) to monomers. Although many fungi, including basidiomycetes, produce cellulases, the ascomycete fungus *T. reesei* is considered the primary source for cellulase production. However, the cellulase of *T. reesei* may not be universally efficient for converting LCB to monosaccharides. This underscores the need to explore the highly species-rich fungal kingdom members for novel LCB degrading enzymes (Singh et al. 2021). In this context, those foliar endophytic fungi living asymptomatically inside intact leaves and switching to a litter degrading lifestyle when the leaf is shed appear to hold promise (Reddy et al. 2016). We coin the term 'litter endophytes' to denote such fungal species. Generally, a leaf harbours 15–20 species of endophytic fungi; while some of these decline after the leaf falls, a few survive as pioneer litter degraders (Kumaresan and Suryanarayanan 2002). Earlier, we isolated some of the litter endophytes whose spores can tolerate up to 110 °C for 2 h (Suryanarayanan et al. 2011). These fungi could utilize the toxic furaldehydes produced during biomass pretreatment, making them nontoxic in lignocellulosic liquors (Govinda Rajulu et al. 2014; Prakash et al. 2015). Enzymes produced by these fungi might tolerate extreme environmental conditions and serve as a potential source for their production.

Cellulase has massive industrial requirements and is produced by many industries worldwide through solid-state fermentation and submerged fermentation technologies. Using lignocellulosic biomass for enzyme production resolves waste management and environmental degradation problems due to dumping this waste. Furthermore, research is required to improve cellulase production, yield, cost reduction and utilization of various types of lignocellulosic biomass. Though much work has been done on other fungi for thermostable and pH tolerant cellulases, endophytic fungi have not been explored with respect to cellulases. Hence, the litter endophytic fungi capable of surviving at high temperatures might serve as a potential source for the production of thermostable and pH tolerant cellulase.

## Materials and methods

### Isolation and thermotolerance

Litter endophytic fungi were isolated from the leaf litter of various hosts from private land in the semi-arid zone of the Nilgiris, Western Ghats. Twenty-five different endophytic fungal morphotypes were isolated, mostly in their anamorphic states and belonging to Ascomycota. The fungi were grown on potato dextrose agar (PDA) and incubated under a 9-h light:15-h dark cycle at 26 °C for 7 days to induce sporulation. The spores were tested for their ability to survive at 100 °C by incubating them in preheated hot air oven (Suryanarayanan et al. 2011). Among these, the spores of nine fungal isolates could survive at 100 °C for a minimum of 2 h.

### Identification of litter endophytic fungi

The nine fungal isolates were identified to genus and, where possible, to species by microscopic examination. The identity was confirmed by comparing the internal transcribed spacer (ITS) sequence analysis. Genomic DNA was extracted from the nine fungal isolates according to the method described in Van Kan et al. (1991). The ITS region was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCC GCTTATTGATATGC-3') (White et al. 1990) as described in Suryanarayanan et al. (2011). The PCR products were purified and sequenced using ITS1 and ITS4 primers. The obtained sequence was searched for homologous sequences from the NCBI database by performing BLASTN analysis. The homologous sequences were aligned using multiple sequence alignment, and the phylogenetic tree was reconstructed by MEGA 11 software (Tamura et al. 2021). The neighbour-joining (NJ) method was used to construct the phylogenetic tree. The bootstrap of 1000 replications was used to analyze the definitive level of the tree's nodes. The cultures were deposited in the National Fungal Culture Collection of India (NFCCI), a national facility at Agharkar Research Institute, Pune, India. The ITS sequences obtained in this study were submitted to the NCBI database and obtained the accession numbers. The culture and sequence accession numbers are presented in Table 1.

### Screening for cellulase

The nine fungal isolates were tested for their ability to produce thermostable cellulases. Cellulase activity was performed by growing the fungi on GYP agar plates containing 0.5% Na-carboxy methylcellulose (CMC) (yeast

**Table 1** Different litter endophytic fungi, their hosts, GenBank accession numbers, culture accession numbers and production of cellulase activity by plate assay

| Fungal isolates | Host | GenBank accession no | Culture deposition accession no | Cellulase activity |
|---|---|---|---|---|
| *Bartalinia pondoensis* | *Bridelia retusa* | HQ909075 | NFCCI2307 | +++ |
| *Chaetomella raphigera* | *Pterocarpus marsupium* | HQ909076 | NFCCI2308 | + |
| *Curvularia sp.* | *Holoptelia integrifolia* | HQ909079 | NFCCI 2311 | - |
| *Exserohilum rostratum* | *Bridelia retusa* | HQ909080 | NFCCI2312 | + |
| *Leptosphaerulina sp.* | *Holoptelea integrifolia* | HQ909074 | NFCCI4854 | + |
| *Montagnulaceae (Pithomyces)* sp. | *Sapindus emarginatus* | HQ909081 | NFCCI4855 | ++ |
| *Pestalotiopsis sp.* | *Maytenus emarginatus* | HQ909077 | NFCCI 2309 | ++ |
| *P. microspora* | *Syzygium cumini* | HQ909082 | NFCCI 2313 | ++ |
| *Phoma sp.* | *Butea monosperma* | HQ909078 | NFCCI 2310 | +++ |

+++: maximum activity; ++: moderate activity; +: low activity; -: no activity

extract, 0.1 g/L; peptone, 0.5 g/L; Agar 1.5%) (Rohrmann and Molitoris 2002). After 7 days of incubation at 25 °C, the plates were flooded with 0.2% aqueous congo red solution and de-stained with 1.0 M NaCl for 15 min. The appearance of a yellow zone around the fungal colony in an otherwise red medium indicated cellulase activity. One of the isolates, *Bartalinia pondoensis* with maximum cellulase activity, was selected for further studies.

## Characterization of cellulase

### Crude enzyme preparation and cellulase assay

The fungus was grown in a Czapek Dox medium containing carboxy methyl cellulose (CMC) as a carbon source and incubated at 25 ± 1 °C under shaking conditions (120 rpm). After incubation, the cultures were centrifuged, and supernatants were used as a source of crude enzyme. The crude enzyme solution was utilized for the determination of enzyme activity. The cellulase activity was measured by the DNS (3,5-dinitrosalicylic acid) method (Ghose 1987). The enzyme activity was determined by measuring the amount of reducing sugars liberated from CMC solubilized in 0.1 M citrate buffer at pH 5.0. Samples without CMC served as a control. The reaction was initiated by incubating the mixture at 50 °C for 60 min, then adding 3 mL of 1% DNS reagent. The contents were incubated in a water bath at 95–100 °C for 15 min, and subsequently, 1 mL of 20% (w/v) potassium tartrate was added. The absorbance was recorded at 540 nm, and a standard graph was prepared with known quantities of glucose. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose from the appropriate substrates per minute under the assay conditions.

### Effect of time

The fungus was grown in a Czapek Dox medium containing CMC as a source of carbon and incubated at 25 ± 1 °C for 15 days under shaking conditions (120 rpm). The culture filtrate was extracted at three days intervals for up to 15 days and assayed for CMCase activity. Since a 9-day-old culture exhibited the highest cellulase activity, the effect of temperature, pH, carbon and nitrogen sources on the enzyme activity was studied using the culture supernatant of 9-day-old cultures.

### Effect of carbon and nitrogen sources

The fungus was grown in Czapek Dox medium replacing glucose with different carbons sources such as fructose, maltose, lactose, malt extract or Na-CMC at a concentration of 1.0%. Similarly, the fungus was grown in various nitrogen sources such as peptone, yeast extract, beef extract, casein, sodium nitrate or ammonium chloride at a concentration of 0.3%. After 10 days of incubation at 30 °C, the culture filtrate was used to assess the cellulase activity.

### Solid-state fermentation

*Bartalinia pondoensis* was grown on agricultural residues such as wheat straw, rice straw, sugarcane bagasse, rice bran, eucalyptus leaves and bamboo leaves. These substrates were washed with water to remove the residual sugar, dried, chopped, milled to 40 mesh powder and treated with 2% NaOH as alkaline pretreatment. Then, the substrates were washed thoroughly with tap water followed by distilled water till pH became neutral. The treated substrates were dried in an oven at 70 °C. Fermentation was performed in 250-mL Erlenmeyer flasks containing 5.0 g of each pretreated substrate moistened with 15 mL Mandel and Waber medium

(Mandels and Weber 1969). The flasks were sterilized at 121 °C, cooled and inoculated with 5-mL mycelial suspension of *B. pondoensis*. The flasks were incubated at 28 °C for 15 days. Fivefold autoclaved distilled water was added to each flask after cultivation, and then, flasks were placed on a rotator shaker for 1 h at 300 rpm. The enzyme extracts from different substrates were obtained by filtering the mixtures through nylon cloth followed by centrifugation for 15 min at 10,000 × *g* to remove fungal and substrate residues. The obtained clarified extracts were tested for cellulase activity.

### Partial purification of cellulase

About 20 mL of the crude enzyme prepared was brought to 80% saturation with solid ammonium sulphate. The precipitate obtained after saturation point was dialyzed in a cellulose membrane bag against 20 mM sodium citrate buffer (pH 4.8) for 24 h at 4 °C (Haung and Monk 2004). After dialysis, the precipitate was collected by centrifugation (9000 × *g* for 10 min at 4 °C), and the 40–60% fraction, which showed maximum cellulase activity was collected and used for enzyme activity and protein content. The protein content was estimated by Lowry method (Lowry et al. 1951).

### Effect of pH

The partially purified enzyme was tested at various pHs ranging from 3.0 to 10.0 using different buffers. The buffers used were sodium acetate buffer (pH 3.0 or 4.0), citrate buffer (pH 5.0), potassium phosphate buffer (pH 6.0 or 7.0), Tris–HCL (pH 8.0) and glycine–NaOH (pH 9.0 or 10.0). The contents were incubated for 60 min at 50 °C and assessed for cellulase activity.

### Effect of temperature

A temperature gradient was maintained to determine the cellulolytic activity of the extract. The reaction mixture consists of the partially purified enzyme, substrate and MES buffer (pH 5.0). The contents were incubated for 60 min at 30 °C to 100 °C with an interval of 10 °C (Ariffin et al. 2006). After 60 min, the samples were assessed the cellulase activity.

### Thermostability

A partially purified enzyme was tested for its thermal stability by incubating the enzyme at 60 °C, 80 °C and 100 °C for 1, 2, 3 and 4 h. The samples were assessed for cellulase activity at the end of each incubation period.

### Zymographic analysis

The proteins present in the culture filtrate were precipitated by mixing with cold acetone (1:4 v/v) and kept for 1 h at − 20 °C. The proteins were precipitated by centrifugation, and the pellet was air-dried and dissolved in a minimum volume of distilled water. The CMCase zymogram was prepared using SDS-10% polyacrylamide gels containing 0.2% CMC (Laemmli 1970). The CMC was incorporated into the separating gel before the addition of ammonium persulphate to avoid polymerization. After electrophoresis, the gel was washed with solution A (sodium phosphate buffer, pH 7.2 containing isopropanol 40%) for 1 h, followed by solution B (sodium phosphate buffer, pH 7.2) for 1 h to remove SDS. Staining was done with Coomassie Blue R dye in methanol-acetic acid–water solution (4:1:5, v/v) for 1 h and de-stained in the same solution without dye. Renaturation of the enzyme proteins was carried out by leaving the gel in solution C (sodium phosphate buffer, pH 7.2, containing 5.0 mM β-mercaptoethanol and 1.0 mM EDTA) at 4 °C overnight. The gel was then transferred onto a glass plate, sealed in a film and incubated at 37 °C for 4–5 h. The gel was then stained in a 1% Congo red solution for 30 min and de-stained in 1 M NaCl for 15 min. A clear zone around the bands indicated CMCase activity (Ratanakhanokchai et al. 1999).

## Statistical analysis

The data were analyzed by analysis of variance, and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using GraphPad Prism software v5.1 (GraphPad Software Inc., La Jolla, CA).

# Results

## Isolation, identification and thermotolerance

Twenty-five different morphotypes of litter endophytic fungi were isolated. Most of these fungi belong to Ascomycota. When the spores of these fungi were tested for their ability to survive at 100 °C, only the spores of nine fungal isolates survived at 100 °C for a minimum of 2 h. These fungi were further identified based on ITS sequence analysis. BLASTN sequence analysis revealed high-sequence similarity of these isolates with *B. pondoensis*, *Chaetomella raphigera*, *Curvularia* sp., *Exserohilum rostratum*, *Leptosphaerulina* sp., *Pithomyces* sp., *Pestalotiopsis microspora* and *Phoma* sp., respectively. Phylogenetic analysis also clustered these isolates with their respective groups of fungi (Fig. 1). The culture accession

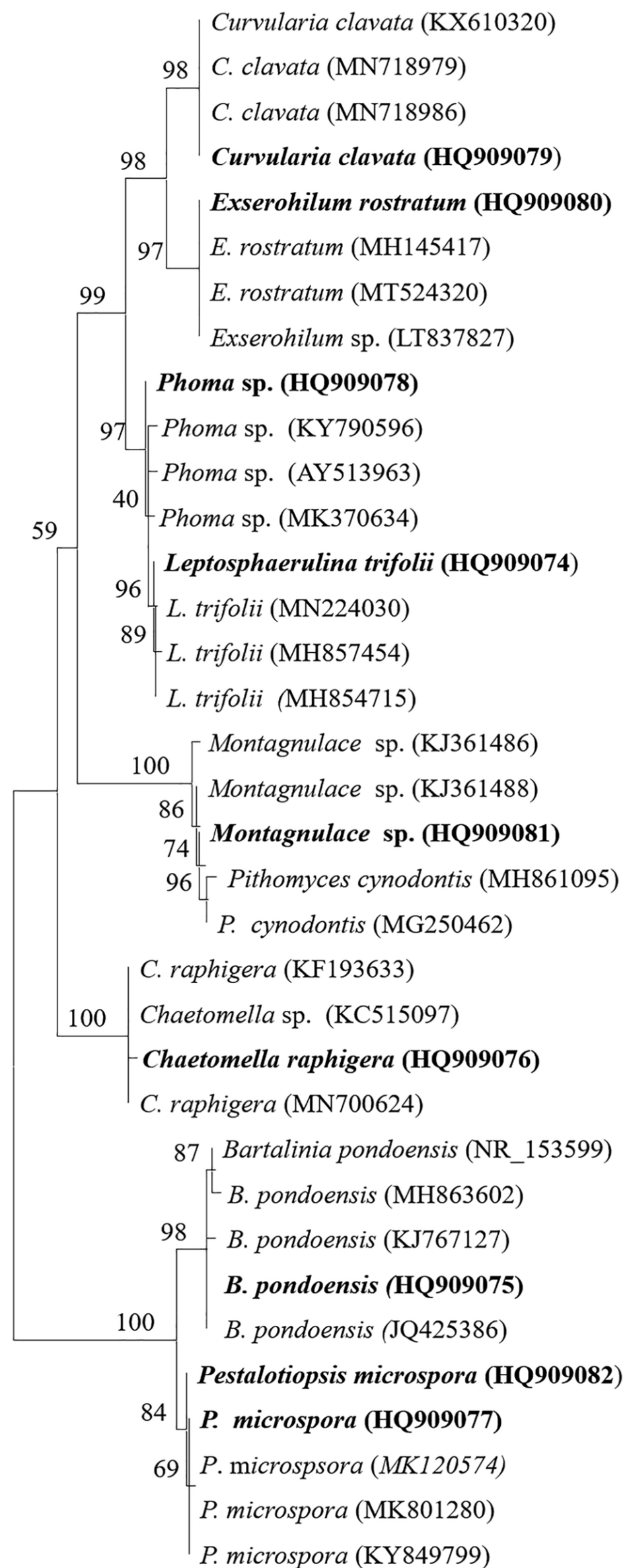


**Fig. 1** Neighbour-Joining phylogenetic tree showing the relationship between internal transcribed spacer sequences of litter endophytic fungi of the present study and those of related sequences obtained from Genbank of NCBI. Accession numbers are mentioned in parenthesis. Numbers at the nodes are bootstrap values

numbers from National Fungal Culture Collection of India (NFCCI) and ITS sequence accession numbers of GenBank along with their host plants are presented in Table 1.

## Screening for cellulase

All the litter endophytic fungi except the *Curvularia* sp. showed CMCase activity as determined through plate assay. *Bartalinia pondoensis* and *Phoma* sp. showed maximum cellulase activity, while *Montagnulaceae* sp. and isolates of *Pestalotiopsis microspora* showed moderate CMCase activity. *Chaetomella raphigera*, *Exserohilum rostratum* and *Leptosphaerulina* sp. showed lower CMCase activity (Table 1). One of the isolates, *B. pondoensis* was selected for further studies based on its faster growth and high cellulase activity than *Phoma* sp.

## Effect of time

To test the optimum time for maximum cellulase activity, *B. pondoensis* culture was grown for different time intervals in the presence of CMC. The CMCase activity in the culture filtrate of *B. pondoensis* increased up to 9 days and decreased after 12 and 15 days. The maximum cellulase activity was observed at nine days (Fig. 2).

## Effect of carbon and nitrogen sources

Among the various carbon sources tested, maximum CMCase activity was observed in presence of maltose, followed by lactose and CMC. Though the enzyme activity was significantly higher in presence of maltose than in lactose and CMC. However, no significant difference between lactose and CMC was observed. The minimum CMCase activity was recorded when the fungus was grown in presence of fructose (Fig. 3a). When different nitrogen sources were tested, yeast extract showed significantly higher CMCase activity, followed by $NaNO_3$ (Fig. 3b). Casein and beef extract also induced enzyme production, while peptone showed lower CMCase activity (Fig. 3b).

## Solid-state fermentation

Various agricultural residues were tested for the production of cellulase by inoculating *B. pondoensis* in the pretreated substrates. The CMCase activity differed significantly among the different residues. The fungus grown in eucalyptus leaves

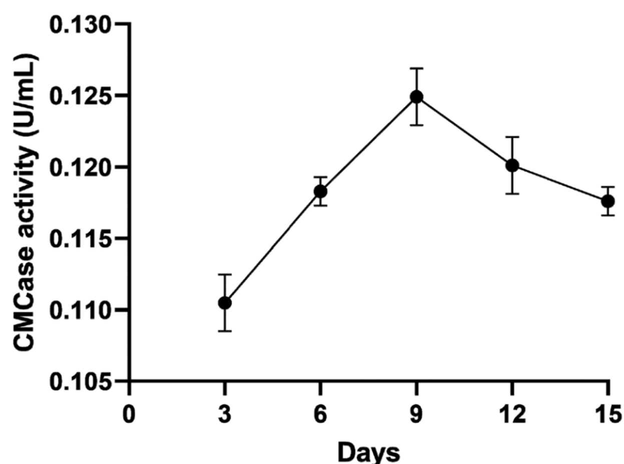


**Fig. 2** Effect of different incubation periods on CMCase activity of *Bartalinia pondoensis* grown in the presence of CMC

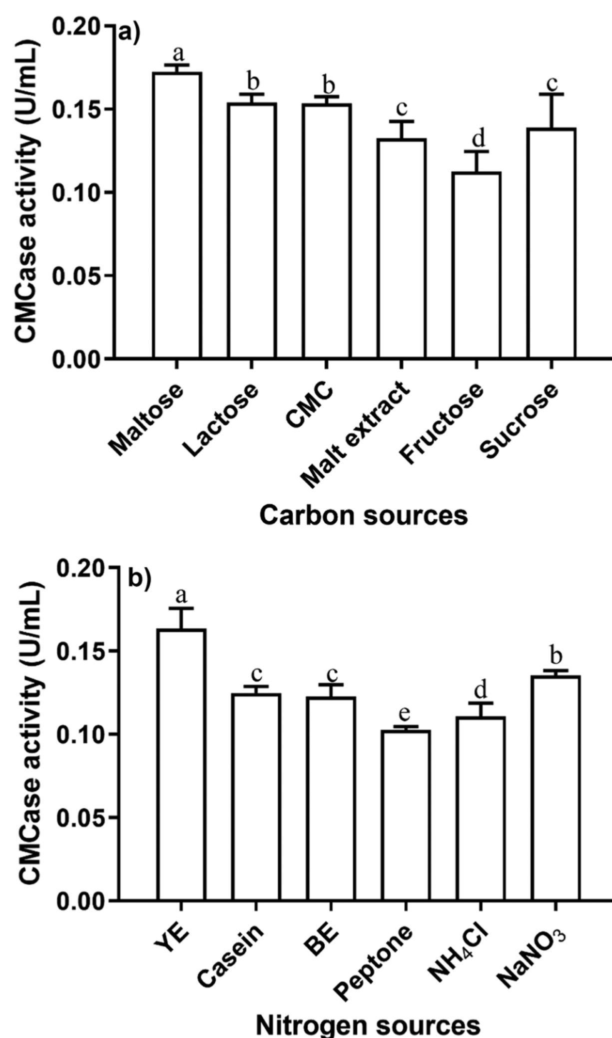


**Fig. 3** Effect of different **a)** carbon and **b)** nitrogen sources on CMCase activity of *Bartalinia pondoensis* (YE, yeast extract; BE, beef extract). Bars sharing a common letter among the treatments are not significant at $p<0.05$ ($n=3$)

showed significantly higher CMCase activity, followed by rice brawn. Wheat straw and rice straw also induced CMCase activity, and the least enzyme activity was observed in banana leaves and sugarcane bagasse (Fig. 4).

## Effect of pH

The partially purified enzyme was tested at different pH (pH 3–10) levels to test the CMCase activity. The maximum CMCase activity was observed at pH 6.0, followed by 7.0. However, more than 80% of the CMCase activity was retained in the pH range of 8–10. The enzyme activities were lower in the acidic pH range than in alkaline pH (Fig. 5a).

## Effect of temperature

The CMCase activity was tested at different temperatures from 30 to 100 °C with an interval of 10 °C. The CMCase activity increased significantly with increasing temperature, and maximum activity was observed at 100 °C, followed by 90 °C. The enzyme activity was twofold higher at 100 °C than at 30 °C (Fig. 5b).

## Thermostability

A partially purified enzyme was tested for its stability by incubating the enzyme at 60 °C, 80 °C and 100 °C for different time periods. The enzyme lost 50% of its activity after 2 h of incubation, while it retained 30% of its activity even at 4 h of incubation. The maximum activity of the enzyme was retained at 100 °C compared to other temperatures in all time periods (Fig. 6).

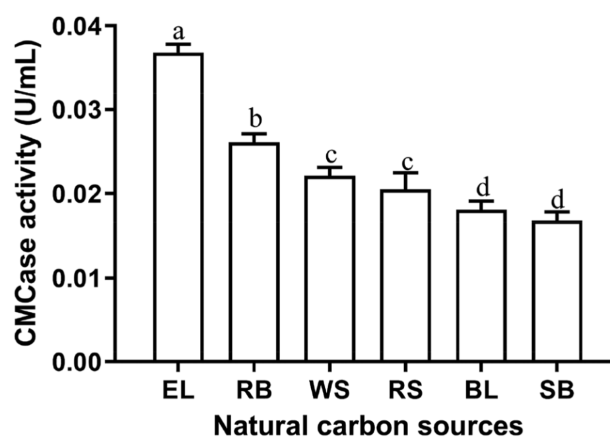


**Fig. 4** Cellulase activity of *Bartalinia pondoensis* using different agro-residues (EL, *Eucalyptus* leaves; RB, rice brawn; WS, wheat straw; RS, rice straw; BL, banana leaves and SB, sugarcane bagasse). Bars sharing a common letter among the treatments are not significant at $p<0.05$ ($n=3$)

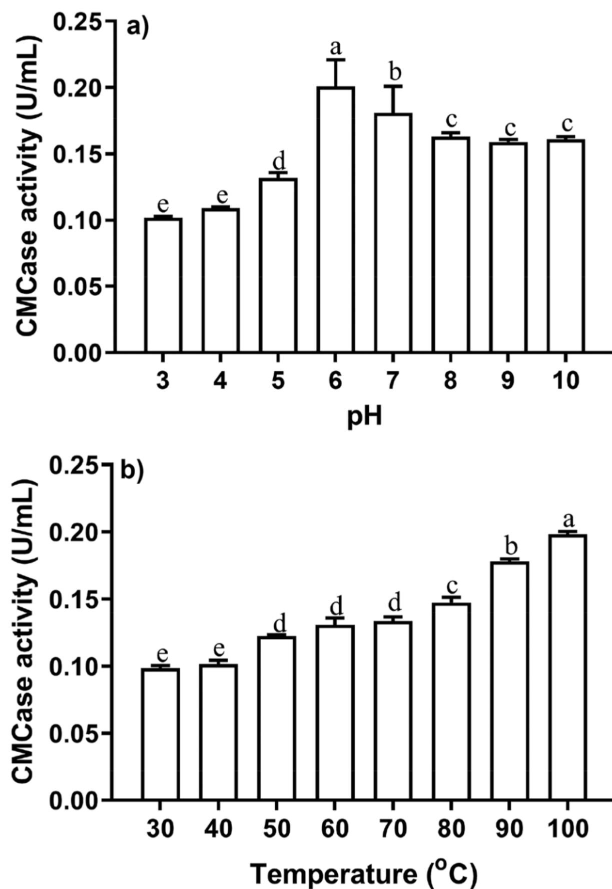


**Fig. 5** Effect of different **a)** pH and **b)** temperature on CMCase activity of *Bartalinia pondoensis*. Bars sharing a common letter among the treatments are not significant at $p < 0.05$ ($n = 3$)

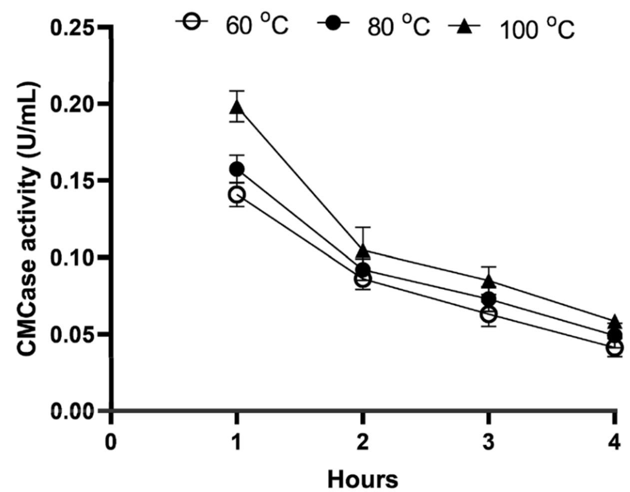


**Fig. 6** Thermal stability of cellulase produced by *Bartalinia pondoensis* incubated at 60 °C, 80 °C and 100 °C for different time periods

## Zymographic analysis

The qualitative analysis of the enzyme activity was done using zymography. The presence of cellulase on the gel was confirmed by performing zymography using CMC as a substrate. When the SDS-PAGE gel was stained with Congo red solution, the hydrolysis zone was observed, showing the cellulase enzyme activity in the crude protein extract (Fig. 7).

## Discussion

The conversion of LCB to biofuels using microbes is a sustainable alternative to fossil fuels (Chen et al. 2015). However, biofuel production is fraught with difficulties, the major one being the recalcitrance of LCB to degrade. Hence, LCB is treated with alkali or acidic pretreatment to shear the lignin cover and expose cellulose and hemicellulose degradation (Chen and Liu 2015; Kim et al. 2016). Saccharification involves the enzymatic conversion of celluloses and hemicelluloses to monomeric sugars which are mainly dependent on mutants or engineered *T. reesei* strains (Bischof et al. 2016). However, the cellulases of such *T. reesei* strains may not equally be efficient for saccharification of biomass of different plant residues due to variations in their cell wall chemistry (Shrestha et al. 2011). Furthermore, some of the saccharification enzymes of *T. reesei* show low activity under industrial conditions (Druzhinina and Kubicek 2017). Hence, exploring fungi from specific ecological niches for novel cellulases would be worthwhile.

Although not of higher plants, a few studies on endophytic fungi show that they could be a good source of novel biomass-degrading enzymes (Mishra et al. 2019). The endophytic fungi seamlessly enter a saprotrophic lifestyle mode as litter degraders in senesced plant parts endorse their ability to produce several LCB degrading enzymes (Prakash et al. 2015). Indeed, endophytic fungi secrete several enzymes, including amylase, cellulase, laccase, lipase, pectinase, pectin lyase, protease, L-asparaginase and tyrosinase (Mishra et al. 2019). In the present study, the litter endophytic fungus *B. pondoensis* produced cellulase, which is thermotolerant and thermostable. The cellulases produced by some thermophilic fungi such as *Acremonium thermophilum*, *Chaetomium thermophilum*, *Humicola grisea*, *Melanocarpus albomyces* and *Talaromyces emersonii* are active at pH range of 4.0–7.0 and exhibited maximum activity at 50–80 °C (Patel et al. 2019). Kumar et al. (2018) reported that the cellulase of leaf litter fungus *Schizophyllum commune* NAIMCC-F-03379 is stable over a wide range of temperatures (25–55 °C) and pH (3–10), endorsing its potential for application in LCB hydrolysis. Temperature, pH, carbon and nitrogen sources affected cellulase production or cellulase activity. pH was the critical factor affecting cellulase production and activity (Shafique and

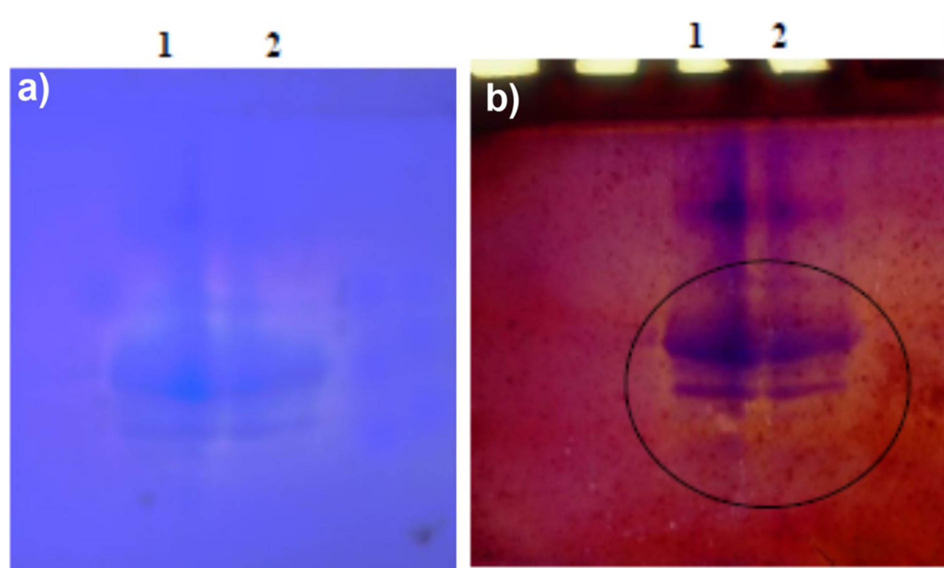


**Fig. 7** SDS-PAGE analysis and zymography. **a)** Cellulase protein bands in lanes 1 and 2 were observed with Coomassie brilliant blue staining. **b)** Activity staining with 1% Congo red was done after removing SDS and renaturation of the enzyme. The activity of the CMCase enzyme was observed around the protein bands as a yellow hydrolysis zone

Bajwa 2009). In the present study, CMCase activity produced by *B. pondoensis* showed a wide pH range (pH 3–10) and thermostability (30–100 °C). CMCase activity from *B. pondoensis* was more stable to pH and temperature variations when compared to those reported by other researchers (Song and Wei 2010; de Almeida et al. 2011; Marques et al. 2018). The optimum pH and temperature of the crude extract obtained from *Trichoderma* sp. IS-05 were 3.0 and 60 °C, respectively (Andrade et al. 2011). Two *Penicillium* species showed optimum temperature, and pH of CMCase activity at 65 °C and pH 4.5, respectively, and activity remained stable after incubation at 60 °C and pH 4.5 for three hours (Picart et al. 2007; Rosa et al. 2009) reported that *Bjerkandera adusta* and *Pycnoporus sanguineus* showed maximum CMCase activity at pH 5.0 and 60 °C but remained active even at alkaline pH and temperature of 70 °C. These results indicate that the cellulase produced by *B. pondoensis* can tolerate high temperatures and a wide pH range.

Carbon and nitrogen are essential sources for the growth and production of enzymes. Some carbon sources such as maltose, lactose, CMC and nitrogen sources like yeast extract, $NaNO_3$ induced cellulase production compared to other carbon and nitrogen sources. Previous reports also indicated that carbon sources such as sophorose, lactose, CMC and sucrose induced cellulase production (Dashtban et al. 2011; Narasimha et al. 2006). Submerged and solid-state fermentation is used for the production of cellulase enzymes. Agro-industrial residues are generally considered the best substrates for the solid-state fermentation processes. Cellulosic substrates like wheat bran, wheat straw, rice straw, corn cob, cotton flower shell, groundnut shell, water hyacinth blend, sawdust and many other substrates were used by researchers for cellulase production (Bhavna and Jayant 2010; Ali and Saad 2008; Acharya et al. 2008; Kang et al. 2004; Yang et al. 2004). In the present study, among the different substrates tested, eucalyptus leaves and rice brawn substrates produced more CMCase production than other substrates. This variation may be attributed to the substrate's chemical nature and nutrient availability. Also, pretreatment methods significantly impact the weakening of cellulosic structure and lignin removal. Abostate et al. reported that among 16 isolated strains of *Aspergillus* species, maximum CMCase activity was reported on rice straw followed by wheat straw (Abo-state et al. 2010).

Thermostable cellulases perform hydrolysis at elevated temperatures, which eventually increases cellulose hydrolysis efficiency. Though many developments have been made in cellulases research, including thermostable cellulases (Bok et al. 1998), not many thermostable cellulases are available in the market. The present study on process development involves optimizing different fermentation conditions (physical and nutritional) toward enhancing cellulolytic enzyme production. The optimum temperature and pH and cellulase enzyme production from *B. pondoensis* were 100 °C and 5–6, respectively. Among the various carbon sources, maltose, lactose and nitrogen sources yeast extract showed higher CMCase activity. *Bartalinia pondoensis* grown in eucalyptus leaves as substrate showed higher cellulase production under solid-state fermentation. The cellulase of *B. pondoensis* was thermostable, and alkali tolerant, maintaining almost 50% of the initial activity when kept for 2 h at 100 °C. Furthermore, investigations are required to use this organism's full potential for cellulase production by employing genetic, biochemical and microbial engineering techniques.

In conclusion, the litter endophytic fungus *B. pondoensis* isolated from the fire-prone forest area is capable of producing cellulase with thermostability and pH tolerance. The

cellulase produced by this fungus maintained 50% of its initial activity for two h at 100 °C. These results suggest that litter endophytic fungi isolated from fire-prone forests have the potential to produce industrially important thermostable and alkali-tolerant cellulases.

**Acknowledgements** The authors are thankful to Thapar Institute of Engineering & Technology, Patiala and Swami Shukadevananda, Secretary, Ramakrishna Mission Vidyapith, Chennai, for the facilities provided.

**Author contribution** T. S. S. and M. S. R. conceived the study and designed experiments; R. Y., M. V. R. and T. R. performed experiments; M. V. R. and T. R. analyzed data, prepared figures and wrote the draft. T. S. S. and M. S. R. edited the manuscript, interpreted the results and edited the final version of the manuscript. All authors reviewed the manuscript.

**Data availability** The authors declare that all data supporting the findings of this study are available within this article and the ITS sequence information at the NCBI database.

## Declarations

**Conflict of interest** The authors declare no competing interests.

## References

Abo-state MAM, Hammad AI, Swelim M, Gannam RB (2010) Enhanced production of cellulase(s) by *Aspergillus spp.* isolated from agriculture wastes by solid state fermentation. American-Eurasian J Agr Environ Sci 8:402–410

Acharya PB, Acharya DK, Modi HA (2008) Optimization for cellulase production by using sawdust as substrate. Afr J Biotechnol 7:4147–4152

Ali UF, Saad H (2008) Production and partial purification of cellulase complex by *Aspergillus niger* and *A. nidulans* grown on water Hyacinth blend. J Appl Sci Res 47:875–891

Andrade JP, Bispo AS, Marbach PA, do Nascimento RP (2011) Production and partial characterization of cellulases from *Trichoderma* sp. IS-05 isolated from sandy coastal plains of Northeast Brazil. Enzyme Res 167248. https://doi.org/10.4061/2011/167248

Ariffin H, Abdullah N, Umi Kalsom MS, Shirai Y, Hassan MA (2006) Production and characterization of cellulase by *Bacillus pumilus* EB3. Int J Engineer Technol 3:47–53

Bhadra F, Gupta A, Vasundhara M, Reddy MS (2022) Endophytic fungi: a potential source of industrial enzyme producers. 3Biotech 12:86. https://doi.org/10.1007/s13205-022-03145-y

Bhavna M, Jayant M (2010) Use of agricultural waste of cellulose production by *Aspergillus niger* with submerged and solid state fermentation. Bionano Frontier 2:189–192. https://doi.org/10.1590/S1517-83822011000300033

Bischof RH, Ramoni J, Seiboth B (2016) Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei*. Microb Cell Fact 15:106. https://doi.org/10.1186/s12934-016-0507-6

Bok JD, Yernool DA, Eveleigh DE (1998) Purification, characterization and molecular analysis of thermostable cellulases CelA and CelB from *Thermologa neapolitana*. Appl Environ Microbiol 64:4774–4781. https://doi.org/10.1128/AEM.64.12.4774-4781.1998

Chen H, Liu J, Chang X, Chen D, Xue Y, Liu P, Lin H, Han S (2015) A review on the pretreatment of lignocellulose for high-value chemicals. Fuel Process Technol 160:196–206. https://doi.org/10.1016/j.fuproc.2016.12.007

Chen HZ, Liu ZH (2015) Steam explosion and its combinatorial pretreatment refining technology of plant biomass to bio-based products. Biotechnol J 10:866–885. https://doi.org/10.1002/biot.201400705

Cherry JR, Fidantsef AL (2003) Directed evolution of industrial enzymes: an update. Curr Opinion Biotechnol 4:438–443. https://doi.org/10.1016/s0958-1669(03)00099-5

Corrêa RCG, Rhoden SA, Mota TR, Azevedo JL, Pamphile JA, de Souza CGM, Polizeli MLTM, Bracht A, Peralta RM (2014) Endophytic fungi: expanding the arsenal of industrial enzyme producers. J Ind Microbiol Biotechnol 41:1467–1478. https://doi.org/10.1007/s10295-014-1496-2

Dashtban M, Buchkowaski R, Qin W (2011) Effect of different carbon sources on cellulase production by *Hypocrea jecorina (Trichoderma reesei*) strains. Int J Biochem Mol Biol 2:274–286

de Almeida MN, Guimarães VM, Bischoff KM, Falkoski DL, Pereira OL, Gonçalves DS, de Rezende ST (2011) Cellulases and hemicellulases from endophytic *Acremonium* species and its application on sugarcane bagasse hydrolysis. Appl Biochem Biotechnol 165:594–610. https://doi.org/10.1007/s12010-011-9278-z

Druzhinina IS, Kubicek CP (2017) Genetic engineering of *Trichoderma reesei* cellulases and their production. Microb Biotechnol 10:1485–1499. https://doi.org/10.1111/1751-7915.12726

Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268. https://doi.org/10.1351/pac198759020257

Govinda Rajulu MB, Lai LB, Murali TS, Gopalan V, Suryanarayanan TS (2014) Several fungi from fire-prone forests of southern India can utilize furaldehydes. Mycol Progress 13:1049–1056. https://doi.org/10.1007/s11557-014-0992-0

Haung XP, Monk C (2004) Purification and characterization of a cellulase from a newly isolated thermophilic aerobic bacterium *Caldibacillus cellulovorans* gen. nov.sp. World J Microbiol Biotechnol 20:85–92

Kang SW, Park YS, Lee JS, Hong SI, Kim SW (2004) Production of cellulases and hemicellulases by *Aspergillus niger KK2* from lignocellulosic biomass. Bioresource Technol 91:153–156. https://doi.org/10.1016/s0960-8524(03)00172-x

Kim JS, Lee YY, Kim TH (2016) A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. Bioresour Technol 199:42–48. https://doi.org/10.1016/j.biortech.2015.08.085

Kumar B, Bhardwaj N, Alam A, Agrawal K, Prasad H, Verma P (2018) Production, purification and characterization of an acid/alkali and thermotolerant cellulase from *Schizophyllum commune* NAIMCC-F-03379 and its application in hydrolysis of lignocellulosic wastes. AMB Exp 8:173. https://doi.org/10.1186/s13568-018-0696-y

Kumaresan V, Suryanarayanan TS (2002) Endophyte assemblages in young mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. Fungal Divers 9:81–91

Laemmli UK (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227:680–685. https://doi.org/10.1038/227680a0

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Mandels M, Weber J (1969) Production of Cellulases Adv Chem Ser 95:391–414

Manganyi MC, Ateba CN (2020) Untapped potentials of endophytic fungi: a review of novel bioactive compounds with biological applications. Microorganisms 8:1934. https://doi.org/10.3390/microorganisms8121934

Marques NP, Pereira JS, Gomes E, Silva R, Araújo AR, Ferreira H, Rodrigues A, Dussán KJ, Bocchini DA (2018) Cellulases and xylanases production by endophytic fungi by solid state

fermentation using lignocellulosic substrates and enzymatic saccharification of pretreated sugarcane bagasse. Ind Crop Product 122:66–75. https://doi.org/10.1016/j.indcrop.2018.05.022

Mishra R, Kushveer JS, Revanthbabu P, Sarma VV (2019) Endophytic fungi and their enzymatic potential. In: Singh BP (ed) Advances in endophytic fungal research, fungal biology. https://doi.org/10.1007/978-3-030-03589-1_14

Narasimha G, Sridevi A, Viswanath B, Subhosh M, Reddy R (2006) Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger*. Afr J Biotechnol 5:472–476

Patel AK, Singhania RR, Sim SJ, Pandey A (2019) Thermostable cellulases: current status and perspectives. Bioresource Technol 279:385–392. https://doi.org/10.1016/j.biortech.2019.01.049

Picart P, Diaz P, Pastor FI (2007) Cellulases from two penicillium sp. strains isolated from subtropical forest soil: production and characterization. Lett Appl Microbiol 45:108–113. https://doi.org/10.1111/j.1472-765X.2007.02148.x

Prakash CP, Thirumalai E, Govinda Rajulu MB, Thirunavukkarasu N, Suryanarayanan TS (2015) Ecology and diversity of leaf litter fungi during early-stage decomposition in a seasonally dry tropical forest. Fungal Ecol 17:103–113. https://doi.org/10.1016/j.funeco.2015.05.004

Ratanakhanokchai K, Kyu KL, Tanticharoen M (1999) Purification and properties of a xylan-binding endoxylanase from alkaliphilic *Bacillus* sp strain K-1. Appl Environ Microbiol 65:694–697. https://doi.org/10.1128/aem.65.2.694-697.1999

Reddy MS, Murali TS, Suryanarayanan TS, Govinda Rajulu MB, Thirunavukkarasu N (2016) *Pestalotiopsis* species occur as generalist endophytes in trees of Western Ghats forests of southern India. Fungal Ecol 24:70–75. https://doi.org/10.1016/j.funeco.2016.09.002

Rohrmann S, Molitoris HP (2002) Screening for wood-degrading enzymes in marine fungi. Can J Bot 70:2116–2123. https://doi.org/10.1139/b92-263

Rosa EQC, Edgar BL, Edgar DG, Alfredo M, Jorge FM, Claudia MA (2009) Characterization of cellulolytic activities of *Bjerkandera adusta* and *Pycnoporus sanguineus* on solid wheat straw medium. Electronic J Biotechnol 12. https://doi.org/10.2225/vol12-issue4-fulltext-3

Schulz B, Boyle C (2005) The endophytic continuum. Mycol Res 109:661–686. https://doi.org/10.1017/S095375620500273X

Shafique S, Bajwa R (2009) Cellulase biosynthesis by selected *Trichoderma* species. Pak J Bothechn 41:907–916

Shrestha P, Szaro TM, Bruns TD, Taylor JW (2011) Systematic search for cultivatable fungi that best deconstruct cell walls of *Miscanthus* and sugarcane in the field. Appl Environ Microbiol 77:5490–5504. https://doi.org/10.1128/AEM.02996-10

Silva RR (2017) Bacterial and fungal proteolytic enzymes: production, catalysis and potential applications. Appl Biochem Biotechnol 183:1–19. https://doi.org/10.1007/s12010-017-2427-2

Singh A, Bajar S, Devi A, Pant D (2021) An overview on the recent developments in fungal cellulase production and their industrial applications. Bioresource Technol Rep 14:100652. https://doi.org/10.1016/j.biteb.2021.100652

Singh R, Kumar M, Mittal A, Mehta PK (2016) Microbial enzymes: industrial progress in 21st century. 3Biotech 6:174. https://doi.org/10.1007/s13205-016-0485-8

Song JM, Wei DZ (2010) Production and characterization of cellulases and xylanases of *Cellulosimicrobium cellulans* grown in pretreated and extracted bagasse and minimal nutrient medium M9. Biomass Bioenergy 34:1930–1934. https://doi.org/10.1016/j.biombioe.2010.08.010

Suryanarayanan TS, Govinda Rajulu MB, Thirumalai E, Reddy MS, Money NP (2011) Agni's fungi: heat–resistant spores from the Western Ghats, southern India. Fungal Biol 115:833–838. https://doi.org/10.1016/j.funbio.2011.06.011

Suryanarayanan TS, Thirunavukkarasu N, Govinda Rajulu MB, Gopalan V (2012) Fungal endophytes: an untapped source of biocatalysts. Fungal Divers 54:19–30. https://doi.org/10.1007/s13225-012-0168-7

Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol 38:3022–3027. https://doi.org/10.1093/molbev/msab120

Van Kan JAL, van den Ackerveken GFJM, de Wit PJGM (1991) Cloning and characterization of the cDNA of avirulence avr9 of the fungal pathogen Cladosporium fulvum, the casual agent of tomato leaf mold. Mol Plante Microbe Interact 4:52–59

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCRProtocols: a guide to methods and applications. Academic Press, New York, pp 315–322

Yang H, Li J, Du G, Liu L (2017) Microbial production and molecular engineering of industrial enzymes: challenges and strategies. In: Brahmachari G (ed) Biotechnology of microbial enzymes, production, biocatalysis and industrial applications. Academic Press, pp 151–165

Yang YH, Wang BC, Wang QH, Xiang LJ, Duan CR (2004) Research on solid-state fermentation on rice chaff with a microbial consortium. Colloids Surf B Biointerfaces 34:1–6. https://doi.org/10.1016/j.colsurfb.2003.10.009

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