Lyophyllum decastes, a new mushroom species for India and its extracellular enzymes

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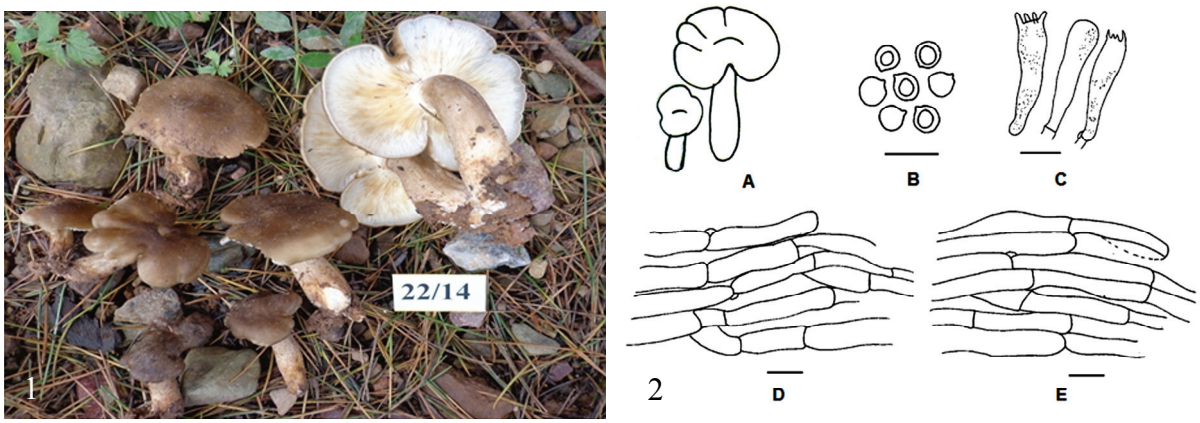
Abstract: *Lyophyllum decastes* of the genus *Lyophyllum* section *Difformia* was collected from North West Himalayas and is described and illustrated morphologically and evaluated using analysis of sequence data from the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. This is the first report of this taxon from North West Himalayas. Qualitative tests for various enzymes by spot test gave high laccase, peroxidase and phosphatase activity. Laccase and peroxidase were estimated on three culture media viz. Czapek Dox medium, modified Melin Norkran's medium and wheat straw medium. Maximum laccase activity (9.52 U/µg protein) was observed in modified Melin Norkran's medium and minimum in wheat straw medium (0.1 U/µg protein) on 20th day. Peroxidase activity was maximum in Czapek Dox medium (0.59 U/µg protein) on 5th day and minimum in wheat straw medium at 20th day (0.006 U/µg protein), whereas the total protein content was maximum in wheat straw medium (140.29 µg/ml) on 20th day and minimum in modified Melin Norkran's medium (8.84 µg/ml) on 5th day, while the maximum mycelial biomass was in Czapek Dox medium (3.65 mg/ml) on 20th day and least in wheat straw medium (1.14 mg/ml) on 10th day. The pH became more acidic (4.4) in Melin Norkran's medium, while in Czapek Dox and wheat straw medium it increased to 8.3 and 8.4 respectively on 20th day.

Zusammenfassung: *Lyophyllum decastes* der Gattung *Lyophyllum* Sektion *Difformia* wurde im Nordwest-Himalaya gesammelt, wird detailliert morphologisch beschrieben und unter Verwendung von Sequenzdaten der ITS1-Barcoding-Region der nukleären ribosomalen DNS eingeordnet. Dies ist der erste Nachweis dieses Taxons aus dem Nordwest-Himalaya. Qualitative Tests für verschiedene Enzyme durch Spot-Tests ergaben hohe Laccase-, Peroxidase- und Phosphataseaktivität. Laccase und Peroxidase wurden auf drei Kulturmedien, nämlich Czapek Dox-Medium, modifiziertem Melin Norkrans-Medium und Weizenstroh-Medium, untersucht. Maximale Laccase-Aktivität (9.52 U/µg Protein) wurde in modifiziertem Melin Norkrans-Medium und minimale in Weizenstroh-Medium (0.1 U/µg Protein) am 20. Tag beobachtet. Die Peroxidaseaktivität war am fünften Tag maximal im Czapek Dox-Medium (0,59 U/µg Protein) und minimal im Weizenstroh-Medium am 20. Tag (0,006 U/µg Protein), während der Gesamtproteingehalt im Weizenstroh-Medium am 20. Tag maximal war (140,29 µg/ml) und minimal in modifiziertem Melin Norkrans-Medium (8,84 µg/ml) am fünften Tag. Die Myzel-Biomasse war am 20. Tag in Czapek Dox-Medium (3,65 mg/ml) maximal und minimal im Weizenstroh-Medium (1,14 mg/ml) am 10. Tag. Der pH-Wert wurde im Melin Norkrans-Medium



Figs. 1, 2. *Lyophyllum decastes.* – Fig. 1. Basidiomata of in natural habitat. – Fig. 2. *A* Basidiomata, *B* basidiospores, *C* basidia, *D* pileipellis, *E* stipe cuticle. Bars: B–E = 10 µm.

saurer (4.4), während er sich im Czapek Dox- und Weizenstroh-Medium am 20. Tag auf 8.3 bzw. 8.4 erhöhte.

Lyophyllum decastes (FR.) SINGER is an edible mushroom well known in American and European countries. *L. decastes* s. l.: The original description by FRIES (1818) reads: "In ericetosis ad radices *Quercuum*". Interpreted as: "In *Erica*-heathland next to the roots of oaks". It is reported to occur in deciduous and coniferous forests but also found on the ground in parks, lawns, avenues etc. in August to October (KALAMEES 2003). There are about 40 species of *Lyophyllum* reported from the world (KIRK *et al*. 2008) and from India only 4 species have been reported namely *L. ambustum* as *Collybia* from Calcutta (BOSE 1919, BANERJEE 1947), *L. leucocelphalum* from Orissa as *Tricholoma* (Sinha & Padhi 1978), *L. subnigricans* from Tamil Nadu (MANJULA 1980) and *L. ulmarium* from Uttar Pradesh (SINGH & MEHROTRA 1974).

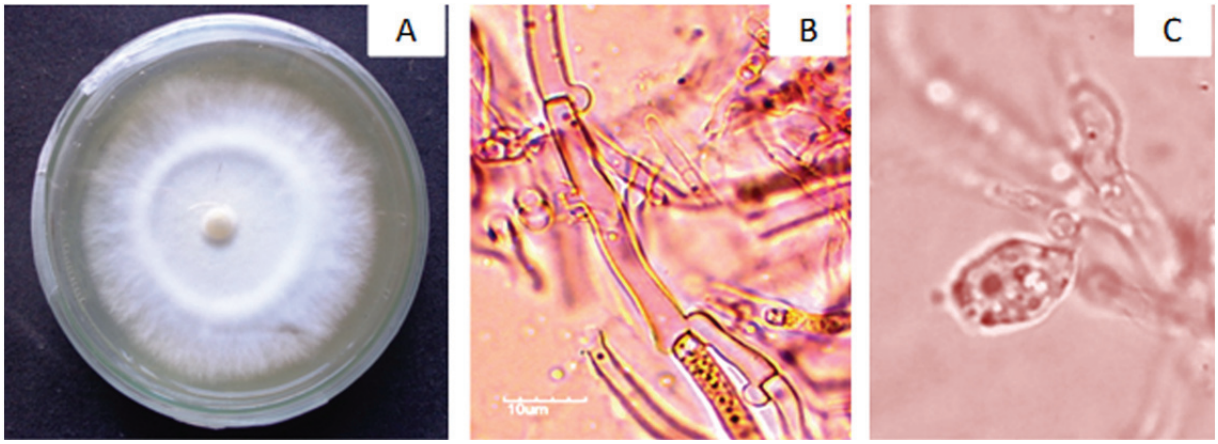


Fig. 3. A Pure culture of *Lyophyllum decastes,* B mycelium with clamp connection, C mycelium with chlamydospore. Bar: B–C = 10 µm.

Materials and methods

Morphological identification and pure culture isolation: The macroscopic details such as colour, shape and size of basidiocarp and change in colour of fruit body were studied from fresh specimens before preservation. The microscopic details and camera lucida drawings were made from material revived in 3 % aq. KOH, stained using 2 % congo red, 1 % cotton blue and MELZER'S reagent and examined under oil immersion (Motic BA 310). The spores were studied from the spore deposits. All colour citations are from KORNERUP & WANSCHER (1978).

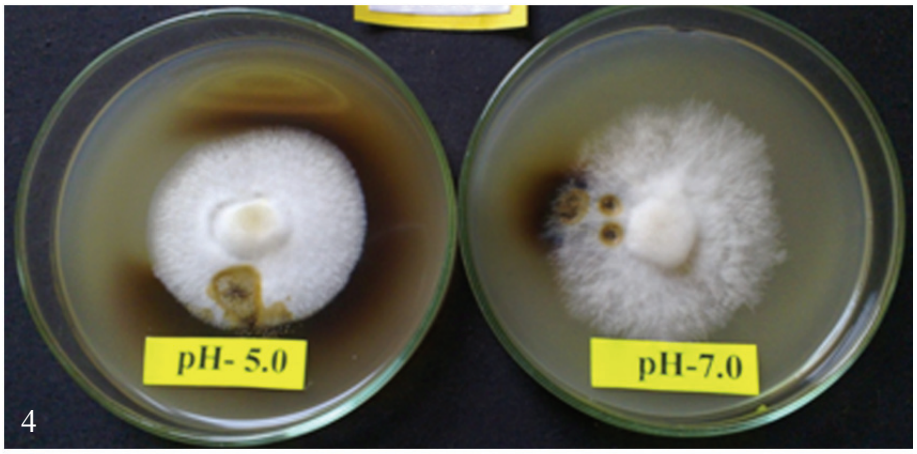


Fig. 4. Peroxidase spot test activity at different pH.

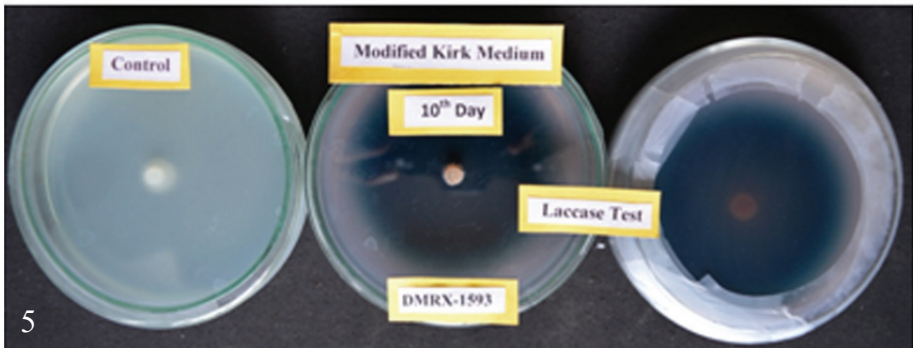


Fig. 5. Laccase spot test using ABTS as substrate.

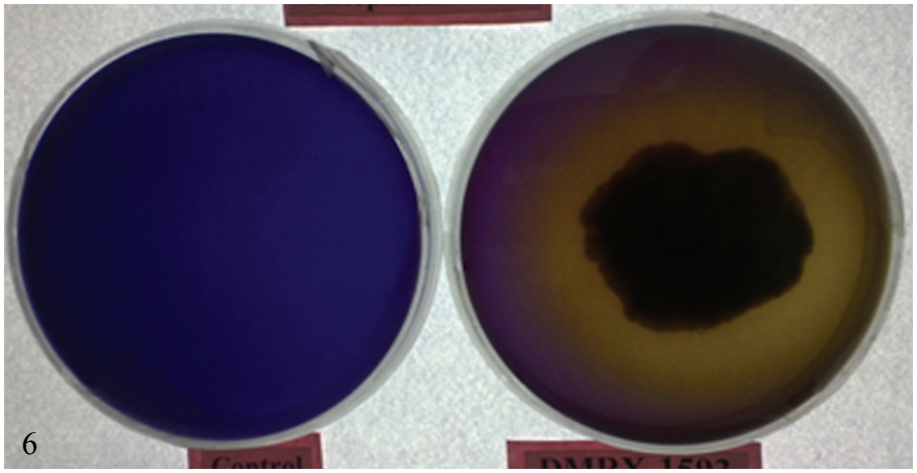


Fig. 6. Phosphatase activity in Pikovskaya agar containing bromocresol blue (clear zone).

Malt Extract (ME) agar (2 % w/v) medium supplemented with streptomycin (50 µg/ml) was used to isolate pure cultures. Surface of a fresh basidioma was sterilized with mercuric chloride and inner pileal context was used for tissue culturing and incubated at 25 °C for one month. The pure culture isolated for *L. decastes* has been designated as "Isolate DMRJU2" and deposited in the Gene Bank of Directorate of Mushroom Research Gene Bank, Chambaghat, Solan, India under the accession no. DMRX-1593.

Enzymatic studies: Three liquid culture media were used viz. Czapek Dox medium (Modified) ($NaNO_3$-3.0, K_2HPO_4-1.0, $MgSO_4.7H_2O$-0.5, KCl-0.5, $FeSO_4.7H_2O$-0.01, glucose-10.0 [g/l]), modified Melin Norkran's medium (Malt extract-3.0, D-Glucose-10.0, $(NH_4)_2HPO_4$-0.25, KH_2PO_4-0.5, $MgSO_4.7H_2O$-0.15, $CaCl_2$-0.05, $FeCl_3$-0.012, NaCl-0.02 [g/l]) and wheat straw medium (wheat straw-50 g/l). Each medium has 20 flasks and 5 flask of control for each medium. Samples were drawn on 5^{th}, 10^{th}, 15^{th} and 20^{th} days from the flasks for estimation of change in pH, proteins, laccase and peroxidase. The mycelial mat was used to quantify mycelium dry weight on pre weighed Whatman filter paper (No.1). A control having uninoculated medium was also run along with the study. All the enzymes and total protein were estimated using Perkin Elmer (Lambda-12) UV-VIS Spectrophotometer.

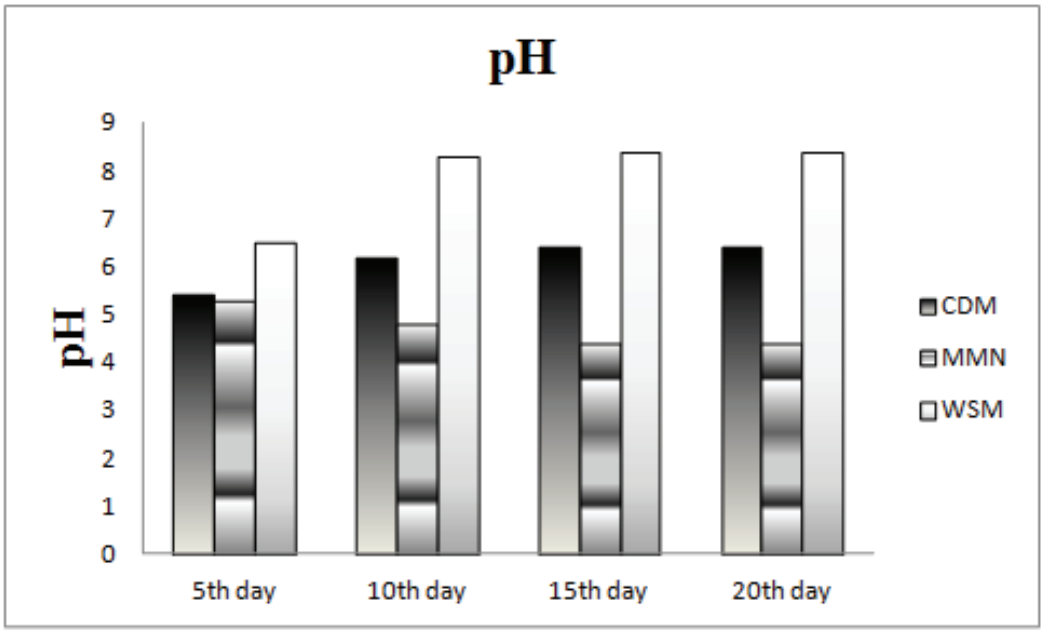


Fig. 7. Changes in pH (initial pH-5.5) in different culture media: Czapek dox medium (CDM), modified Melin Norkran's medium (MMN) and wheat straw medium (WSM) at different time intervals.

Qualitative screening of enzyme:

P e r o x i d a s e : Production was determined by the procedure of BERGMEYER & BERNDT (1962). Fungus was grown on 2 % malt extract medium at pH 5.0 and 7.0 for 5-10 days. One drop of freshly prepared 1 % w/v aqueous solution of pyrogallol and 0.4 % hydrogen peroxide was added to on actively growing mycelium; presence of a yellowish brown colour indicates peroxidase activity.

L a c c a s e : Laccase activity of the fungal strains to secrete extracellular laccase was visualized according to the method of NIKU-PAAVOLA & al. (1990). The assay plate contained 15 ml of modified KIRK agar medium amended with of 0.2g/l of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at pH 5.5. Observations were recorded upto 20th days. The medium becomes blue coloured in the presence of laccase.

P h o s p h a t a s e : The Phosphate solubilisation EMF was tested by inoculating the test fungus on the PIKOVSKAYA agar medium (PIKOVSKAYA 1948) containing tricalcium phosphate with a pinch of bromophenol blue. Plates were incubated at 25 °C, appearance of clear zone around the colony showed the presence of phosphatase.

Enzyme assay:

P e r o x i d a s e : Peroxidase activity was measured at 420 nm by using the method of BERGMEYER & BERNDT (1962). Reaction mixture contained 100 mM of phosphate buffer (pH-6.0), 0.5 % pyrogallol and H_2O_2 (0.49 M) and enzyme source. The blank contained all the assay constituents except the active enzyme. One unit of enzyme activity was equal to change of 1µmol of pyrogallol to purpurogallin.

L a c c a s e (EC.1.10.3.2) : Laccase activity was determined using ABTS as the substrate according to the method of (NIKU-PAAVOLA *et al*. 1990). The assay mixture contained sodium malonate buffer pH 5.0 (0.1 M), ABTS (5 mM). Enzyme activity (U) was defined as the amount of enzyme oxidized 1µmol ABTS per minute. The kinetic reaction was spectrophotometrically recorded at 420 nm for 10 min. The blank contained all the assay constituents except the enzyme sample. The enzyme activity was expressed as U/µg protein.

Total protein estimation: Total protein was estimated by using BRADFORD method (BRADFORD 1976) with a UV-VIS spectrophotometer at 595 nm. A control with distilled water was also run simultaneously. A graph was plotted by using ovalbumin as standard. The standard curve was used to estimate the amount of total protein µg/ml.

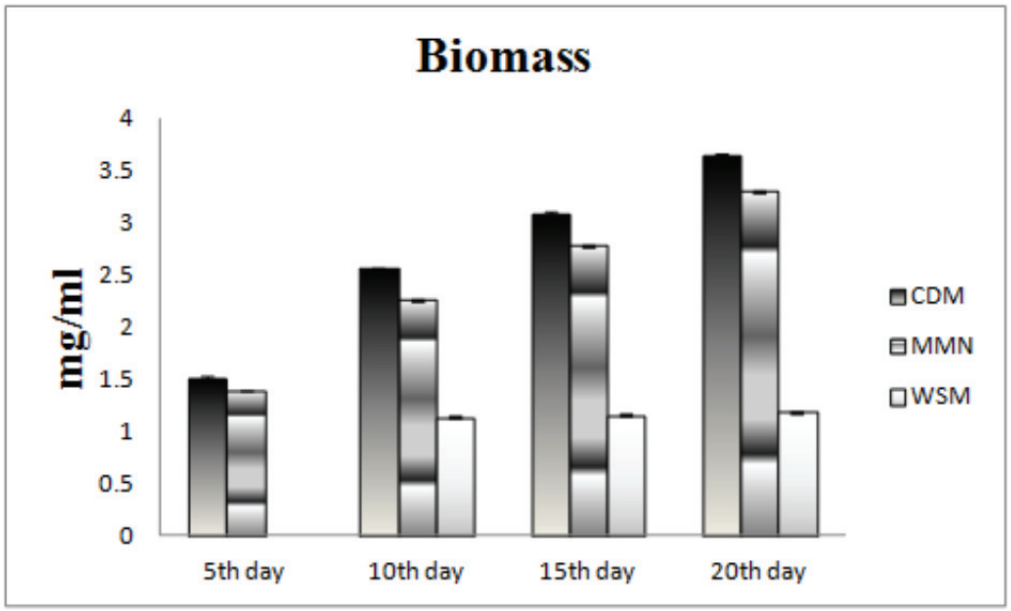


Fig. 8. Mycelial biomass of *L. decastes* in liquid media Czapek Dox medium (CDM), modified Melin Norkran's medium (MMN) and wheat straw medium (WSM).

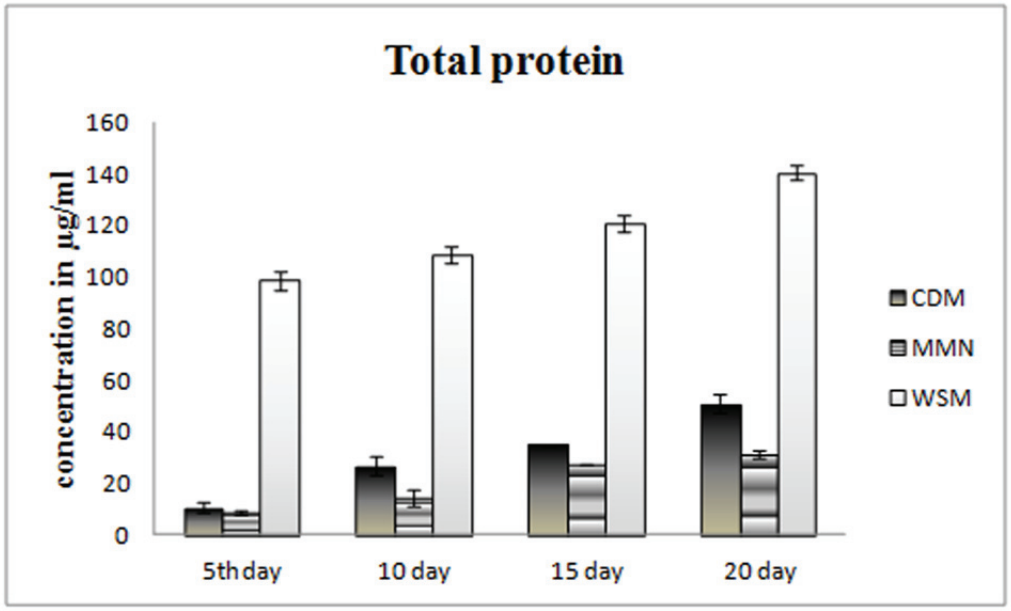


Fig. 9. Total extracellular protein of *L. decastes* in Czapek Dox medium (CDM), modified Melin Norkran's medium (MMN) and wheat straw medium (WSM).

Molecular characterization: Genomic DNA was extracted from the mycelium grown in (2 % malt) liquid broth medium following the standard cetyl trimethyl ammonium bromide (CTAB) isolation protocol slightly modified for higher fungi (Xu & al. 1994). Lyophilized mycelium was grinded with mortar and pestle to powder. Lysis was done using preheated CTAB buffer (2 % CTAB, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, and 0.2 % mercaptoethanol) 5ml per gram tissue followed by chloroform/iso-amyl alcohol (24:1) extraction twice. The supernatant was taken after centrifugation and DNA was precipitated with equal volume of pre-chilled isopropanol. DNA was washed and air dried briefly and 250–500 µl of TE was added and left overnight, treated with 2 µl RNase (10 ng/ml) and incubated for 45 min at 37 °C. DNA was spooled out, air dried and re-suspended in 0.5 to 1 ml TE and stored at –20 °C. DNA extracted was electrophoresed in a 0.8 % agarose gel using 1X TAE and visualised in gel documentation system using ethidium bromide. From the total genomic DNA, a DNA segment, 5.8S rDNA, was amplified using primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′)

and ITS 4 (5´ TCCTCCGCTTATTGATATGC 3´) (WHITE et al. 1990). PCR amplification was performed in a total volume of 50 µl. Amplification reactions: 25 µl master mix (EmeraldAmp® GT PCR Master Mix by Takara) and l µl each of both forward and reverse ITS (10 pmol/µl) were added with 1 µl of genomic DNA. Total volume of the mixture was made up to 50 µl by adding 22 µl of nuclease free water. The mixture was mixed gently. PCR was performed in a thermocycler (Eppendorf). Initial denaturation of 95 °C for 10 min was followed by 35 cycles of 95 °C for 1 min (denaturation), 54 °C for 1 min 20 sec. (annealing), 72 °C for 1 min (extension). A final extension at 72 °C for 10 min was incorporated, followed by cooling to 4 °C until recovery of the samples. Amplified PCR product was electrophoresed in a 1.2 % agarose gel using 1X TAE buffer. Sequencing of the PCR products was carried out by Chromous Biotech Pvt. Ltd. Bangalore. The ITS sequences acquired in this study were compared with those available in the National Center for Biotechnology Information (NCBI) GenBank database by using the BLASTn search algorithm (https://blast.ncbi.nlm.nih.gov/BLAST.cgi?PAGE_TYPE=BlastSearch). The ITS sequences obtained from isolate DMRJU2 of *L. decastes* have been deposited in the NCBI GenBank under the accession number KT804926.

Phylogenetic analysis: To find out the possible similarity with the sequenced taxon, a preliminary phylogenetic analysis was performed using MrBayes v.3.2.2 (http://mrbayes.sourceforge.net/; RONQUIST & al. 2012) by considering the ITS sequences representing *Lyophyllum* species. On the basis of preliminary analysis, a group comprising 20 *Lyophyllum* species including our taxon and outgroup were selected for phylogenetic analysis. ITS sequences for other 19 species were retrieved from GenBank. *Calocybe indica* (JN874408) was taken as an outgroup taxon. Alignment of the sequences was constructed using MAFFT ver. 7.0 (http://mafft.cbrc.jp/alignment/server/; KATOH & STANDLEY 2013) and edited with BioEdit 5.0.6 (http://www.mbio.ncsu.edu/bioedit/; HALL 1999). Phylogenetic analysis on the resulting alignment was performed using Bayesian Inference (BI). A Bayesian analysis was implemented in MrBayes v.3.2.2 over 300000 generations and the convergence of Bayesian analysis was observed by examination of the standard deviation of split frequencies <0.01. Trees were sampled every 100th generations resulting in total of 4,357 trees. The aligned data set has been deposited in TreeBASE (submission ID-18389).

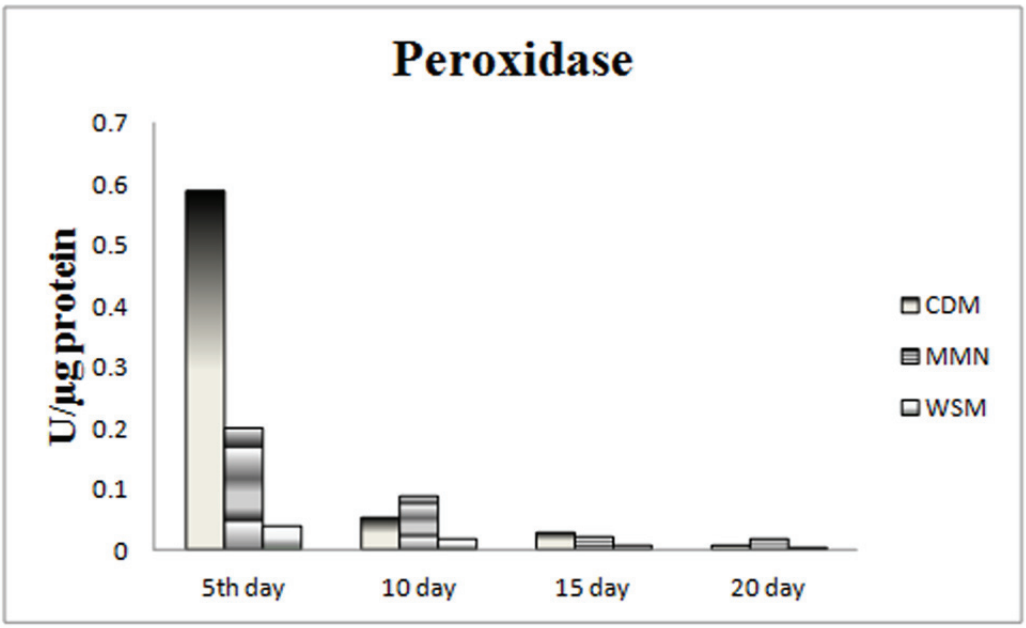


Fig. 10. Peroxidase activity of *L. decastes* on liquid media Czapek Dox medium (CDM), modified Melin Norkran's medium (MMN) and wheat straw medium (WSM).

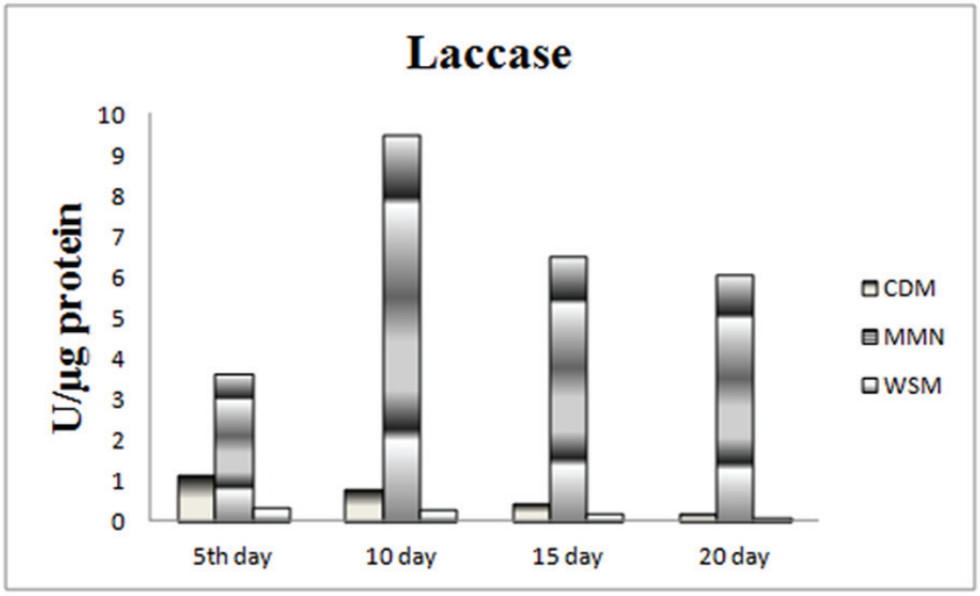


Fig. 11. Laccase activity of *L. decastes* in liquid media Czapek Dox medium (CDM), modified Melin Norkran's medium (MMN) and wheat straw medium (WSM).

Results and discussion

Description of the Indian collection:

P i l e u s : up to 7.0 cm in diam., umbonate, greyish brown, surface non hygrophanous, lobed to irregular, non-striate, moist, cuticle half peeling, pileus consistency fleshy, context white, up to 0.4 cm thick, no colour change on touch or handling, confluent.

L a m e l l a e : adnate, densely crowded, white to creamish, fleshy, breadth 0.1 cm, unequal, present in 4 sets of lamellulae.

S t i p e : central, equal to obclavate, 4.0–4.5 × 1.0–1.5 cm, stipe surface cream to light greyish, terate in cross section, blunt at the base, smooth, context stuffed, fleshy, stipe trama white, ring, veil and volva absent. No colour change in pileus, stipe or gills.

B a s i d i o s p o r e s : (5.4–)5.8(–6.2) × (4.3–)4.7(–5.1) µm, Q = 1.2, hyaline, smooth, globose, thin walled, apiculate, germ pore absent, oil globule present, cyanophilic and inamyloid, spore deposit white.

B a s i d i a : (21.7–)28.6(–30.8) × (6.3–)7.3(–8.7) µm Q = 3.9, clavate, 4-spored, sterigmata long up to (1.8–)2.8(–4.6) × (1.0–)1.3(–1.6) µm, thin walled, oil globule absent, siderophilous granules present, basal septa with clamp connections.

C y s t i d i a : absent.

P i l e i p e l l i s : (4.4–)6.4(–7.6) µm thick, hyphae parallel arranged, thin walled, septate, branched, septa with clamp connections and blunt ends.

H y m e n o p h o r a l t r a m a : (3.4–)5.9(–7.7) µm thick, regular. Subhymenium 5–10 µm. Hymenium 25–30 µm.

S t i p e c u t i c l e : made up of longitudinally arranged cylindrical cells, (6.4–)9.2(–16.3) µm wide, septate, branched, septa with clamp connections.

Growth manner and habitat: gregarious to caespitose, growing on grass on roadside under the canopy of *Cedrus deodara*.

Collection examined: India, Himachal Pradesh, Dharamshala, McLeod Ganj, Dal Lake: 1775 m a.s.l.; GPS 32°14'45"N, 76°18'49'E, 13. September 2014, Herbarium Acc. No.22/14.

Culture characteristics:

M y c e l i u m : off white to creamish strandy, creeping to slightly fluffy.

H y p h a e : (3.5–)4.7(–6.3) µm septate, with clamp connections, branched.

C h l a m y d o s p o r e s : (5.6–)8.5(–12.2) × (4.2–)6.2(–8.8) µm, broadly subglobose to globose, present singly.

Notes:

The present specimen belongs to *Tricholomataceae* due to lamellate basidiomata, white spore deposit, basidiospores without germpore, monomitic hyphae and regular lamellae trama. It was placed in *Lyophyllae* due to presence of siderophilous granules and clamp connections in the basidia and in mycelial culture. It is mostly found from non termitophilous habitat. It is placed in section *Differmia* (FR.) KUHNER of *Lyophyllum* due to basidiomata in large clumps, caespitose to connate, non discolouring flesh and adnate gills. The mycelial produces clamydospores in culture media characteristic of *Lyophyllum*. The morphology and anatomy of our specimen matches well with *L. decastes*.

Enzymatic study

Spot test for enzyme:

The petri plates inoculated with test fungus on modified Kirk medium incubated at 25 °C showed presence of deep blue colour around the mycelium indicating laccase secretion (Fig. 5). Peroxidase activity was shown by the presence of brown colour near the actively growing mycelium (Fig. 4). Phosphatase activity was shown by the appearance of a clear zone around the actively growing mycelium (Fig. 6).

pH and biomass:

The initial pH of all the media was adjusted to 5.5. On the 5th day pH changed to 6.5 and there was an abrupt change in pH in WSM on 5th and 20th day to 8.4 and the pH became more acidic in case of MMN on 5th day (5.3), 15th and 20th day (4.4) (Fig. 7). Maximum mycelial biomass was obtained in CDM on 20th day (3.65 mg/ml) and least biomass was observed in WSM on 10th day (1.14 mg/ml) (Fig. 8).

Total protein, peroxidase and laccase:

Maximum protein was recorded in WSM followed by CDM and MMN. All the media showed an increase in protein from 5th day to 20th day. Maximum protein production was recorded on WSM (140.29 µg/ml) on 20th day while minimum in MMN (8.84 µg/ml) on 5th day (Fig. 9). Peroxidase activity was observed maximum on 5th day in CDM (0.59 U/µg protein) followed by MMN (0.2 U/µg protein) and WSM (0.04 U/µg) (Fig. 10). The maximum laccase activity was reported in MMN and least in WSM on 20th day (0.1 U/µg). It was 9.52 U/µg protein in MMN on 10th day, followed

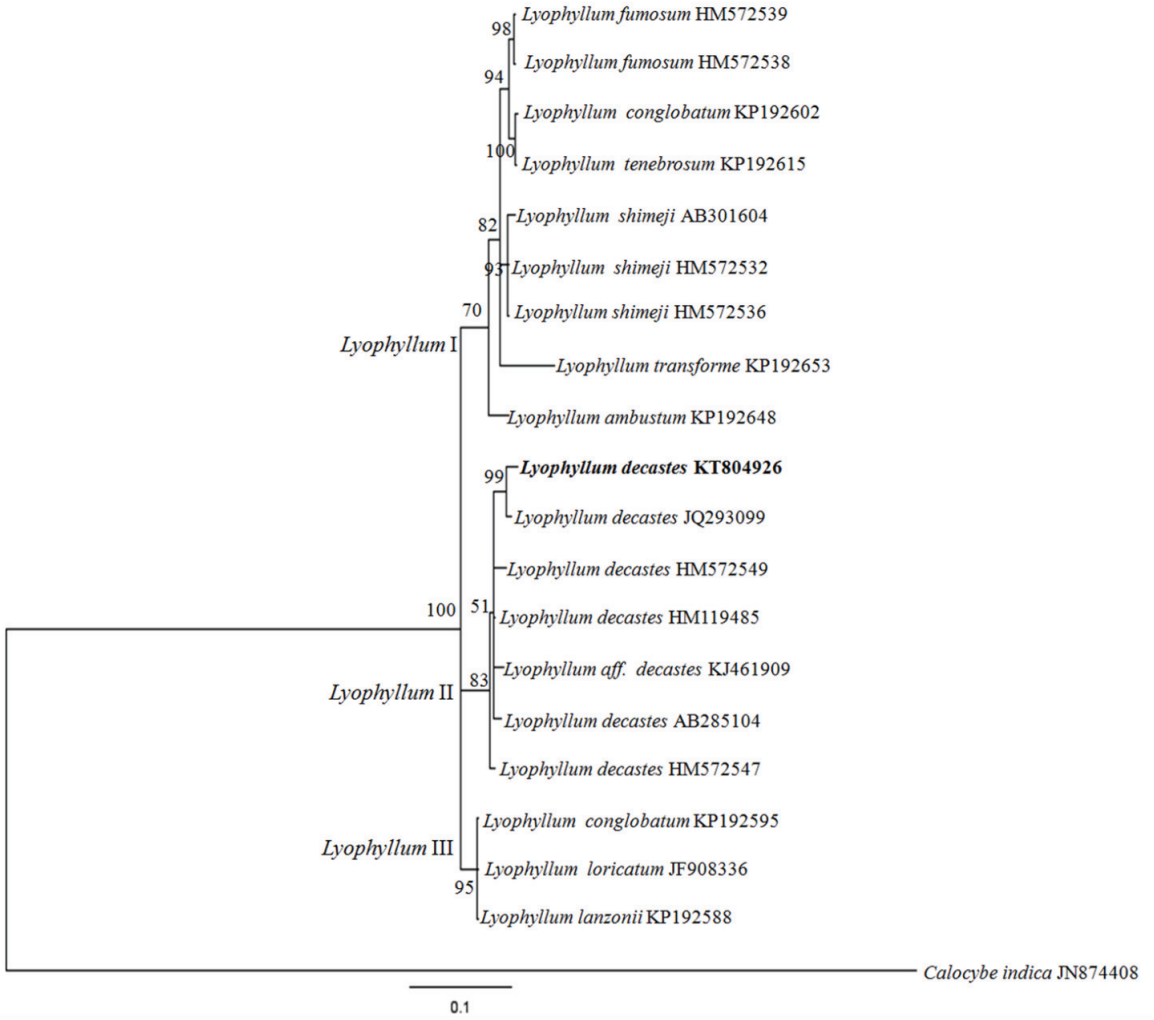


Fig. 12. Bayesian tree showing the relationships between the internal transcribed spacer (ITS) sequence of *L. decastes* isolate DMRJU2 and those of related species retrieved from GenBank. Numbers at nodes stand for the posterior probability percentages (>50%) of the Bayesian analysis (outgroup: *Calocybe indica*).

on 15th and 20th day by 6.5 and 6.07 U/µg protein, respectively, and least on 5th day (3.62 U/µg protein) while in the other two media it showed decreasing order (Fig. 11).

PALAEZ & al. (1995) screened the lignin degradation enzyme (AAO, laccase and MnP) for different groups of basidiomycetes, and observed laccase activity in *L. decastes* but no AAO and MnP activity. There was no laccase activity on 21st day. However in our studies we could see laccase activity up to 20th day. CASIERI & al. (2010) conducted a spot test for laccase, peroxidase and tyrosinase activity with *L. fumosum*, *L. semitale* and three strains of *L. decastes* and observed that *L. fumosum* showed the presence of tyrosinase but not laccase while all other showed the laccase activity and one of the *L. decastes* strain showed peroxidase activity. CLARA SALAHEDDIN & al. (2010) characterized the pyranose oxidase from *L. shimeji* (hon-shimeji). Two proteolytic enzymes protease I and II were purified from fruit body of *L. aggregatum* by NODA *et al*. (1999) and aminopepetidase was purified from extract of *L. cinerascens* by ABDUS SATTAR & al. (1989). In our studies also we found laccase production in MMN medium and peroxidases in CDM. Culture medium plays an important role in extra cellular enzyme production. Optimum pH for laccase production is at 5.5 and acidic pH favours more laccase. It was interesting to note that peroxidase

activity was maximum on 5th day and it goes on decreasing from 10th, 15th and 20th day in all the three culture media. It could be due to change in pH from acidic to alkaline in WSM and CDM and more acidic in MMN medium (4.4). On 15th and 20th day the optimum pH for peroxidase secretion could be between 5 and 6. The enzyme spot test for peroxidase also indicates strong reaction at pH 5 and less at pH 7.0 (Fig. 4).

Various methods have been successfully evaluated to test the presence of extracellular enzymes like cellulase, laccase, peroxidase. These tests are very simple and give indication of presence or absence of an enzyme. *L. decastes* showed most intense reactions and within 2 days showed deep blue colour. It showed that ectomycorrhizal fungi like *L. decastes* can secrete extracellular enzymes and can be capable of oxidizing a large number of anaerobic compounds present in the soil.

Molecular genetic study

The alignment of ITS sequences of selected *Lyophyllum* species resulted in a data matrix comprising 20 taxa and 644 characters including gaps. Bayesian analysis of ITS region for the selected *Lyophyllum* group yielded a consensus tree (Fig. 12) and divided the species broadly into three major clades (*Lyophyllum* I, *Lyophyllum* II, *Lyophyllum* III). The clade *Lyophyllum* II consisted of ITS sequences of *Lyophyllum decastes* including the present *L. decastes* (KT804926) and forms an independent subclade with *L. decastes* strain JZB2115005 (JQ293099) from China, with 99 % similarity and 96 % of query coverage, which is well supported by the Bayesian posterior probability percentage (99 %). It is closely related to an other Chinese *L. decastes* strain Ld418 (HM119485) with 98 % similarity, which is paraphyletic to *L.* aff. *decastes* (KJ461909) from New Zealand, *L. decastes* (AB285104) from Japan, and *L. decastes* (HM572549) from Sweden. *L. shimeji* and *L. fumosum* form a different subclade in clade *Lyphyllum* I. Hence our specimen matches well with *L. decastes* on the basis of morphology, anatomy and molecular studies.

Worldwide 15 species of *Lyophyllum* section *Difformia* have been reported, namely *L. brunneum* DÄHNCKE, CONTU & VIZZNI (Canary Islands), *L. calabrum* LAVORATO & CONTU (Italy), *L. cristophilum* VILA & LLIMORA (Spain and Italy), *L. conglobatum* (VITT.) BON (Europe), *L. decastes* (FR.) SING. (Europe), *L. fumosum* (PERS.) P. D. ORTON (Europe), *L. lanzonii* CANDUSSO (Italy), *L. multiforme* (PECK) BIGELOW (North Amarica and Turkey), *L. pergamenum* (COOKE) HORICEK (Europe), *L. pseudoloricatum* DÄHNCKE, CONTU & VIZZINI (Canary Islands), *L. shimeji* (KAWAMURA) HONGO (North Europe and East Asia), *L. subglobisporum* (Italy), *L. soniai* PICILLO & CONTU (Italy), *L. tucumanense* SING. (South America), and *L. turcium* SEsli, VIZZINI & CONTU (Turkey) (SESLI & al. 2015).

To the best of our knowledge this is the first report of *Lyophyllum decastes* from India and the molecular and anatomical structures and extracellular enzymes are in accordance with the diverse literature on this species.

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