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Biological Delignification and Enzymatic Saccharification of Diverse Lignocellulosic Biomass Substrates by the White-Rot Fungus *Trametes hirsuta* PSRF-29

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Abstract
India produces 998 million tonnes of ligno-cellulosic biomass annually, and its improper disposal, particularly through open-field burning, poses considerable environmental challenges. Hence, sustainable biological approaches for valorisation of these agro-residues are of utmost importance. The present study investigated the biodegradation and enzymatic saccharification of diverse lignocellulosic biomass substrates such as paddy straw, leaf litters, corn stover and sugarcane bagasse, using white-rot fungus *Trametes hirsuta* PSRF-29 (MTCC 13584). Following 14-days of fungal treatment, the isolate exhibited substantial lignocellulolytic activity as a result of the production of diverse array of extracellular enzymes. The saccharification of biomass was caused by a number of enzymes, including laccase, mannanase, cellulase, pectinase, and xylanase. The enzyme activity was 0.23, 0.38, 2.89, 5.08, and 13.15 IU/ml respectively. Correspondingly, the reducing sugar content exhibited a 39% increase, accompanied by 57.57% lignin, 47.22 % cellulose and 26.08% hemicellulose degradation, along with a 33% reduction in total phenolic content. These coordinated biochemical changes collectively confirm the efficient degradation of lignocellulosic biomass. Overall, the study highlights the strong biodegradation potential of *T. hirsuta* PSRF-29, demonstrating its applicability in sustainable biomass management. The efficient enzymatic conversion of lignocellulosic residues into simpler compounds underscores its potential for the development of value-added bioproducts, while simultaneously mitigating environmental pollution associated with biomass burning.

Keywords: Lignocellulosic biomass – Saccharification- *Trametes hirsuta* - Value added products - White rot fungi

1. Introduction

Lignocellulosic biomass (LCB) that is primarily composed of cellulose, hemicellulose, and lignin as structural components (Carroll and Somerville 2009). Lignocellulosic biomass is mainly composed of cellulose (30 to 50% of dry matter weight), hemicellulose (20 to 40% of dry matter weight), and lignin (15 to 25% of dry matter weight), with trace amounts of structural proteins, lipids, and ash (Hans et al. 2022). The main contributors of lignin-cellulosic rich biomass are crop residues remained after harvesting, grasses, and trees. It is the most plentiful biomass on Earth, producing roughly 181.5 billion tonnes annually worldwide (Paul and Dutta 2018). Currently, 7 billion tonnes of these come from farming, grass, or forest land and are used as fodder or for material and energetic purposes. Moreover, Dahmen et al. (2019) report that 7 billion tonnes of lignocellulosic biomass residues can be generated as agricultural residues, of which less than 25% are used significantly. Annually, approximately 96.54 million metric tonnes of agro-wastes are expected to be generated in Malaysia alone. It is expected that 991 million dry tonnes of lignocellulosic biomass will be produced annually in the United States of America by 2030 (Langholtz et al. 2020). Massive lignocellulosic biomass production has also been observed in the European Union, where 1 billion tonnes of lignocellulosic biomass is expected to be produced annually by 2030 (Ahorsu et al. 2018). The projected annual biomass production in India from agriculture, forest cover, and agroindustry is 998 MT/year (Raman et al. 2021). . Among Indian states, the maximum biomass is produced in Punjab, Uttar Pradesh, Maharashtra, and Madhya Pradesh. Lignocellulosic biomass (LCB), when indiscriminately burned, contributes significantly to environmental pollution through the release of particulate matter, greenhouse gases, and other toxic emissions. Therefore, the development of sustainable and eco-friendly strategies for its management has become an urgent necessity. Biological degradation of LCB using microorganisms such as fungi and bacteria offers a promising alternative, as these organisms possess specialized enzymatic systems capable of breaking down complex lignocellulosic polymers into simpler, value-added products. Among them, white-rot fungi are particularly efficient due to their ability to produce a wide range of ligninolytic and hydrolytic enzymes. Keeping this perspective in view, the present study was designed to investigate the potential of an isolated culture of white-rot fungi for the effective biodegradation of lignocellulosic biomass, aiming to provide an

environmentally benign solution for LCB management while contributing to waste valorization.

2. Materials & methods

Collection of wild samples

Wild samples of mushroom were collected from diverse region of Chandigarh and lower Shivalik hills of Himachal Pradesh (Figure 1). The fruiting bodies were wrapped in wax paper and transported to the laboratory for further macroscopic and microscopic examination. The important taxonomic characteristics of the fruiting bodies were noted at the collection site. The samples were labelled with information such as the collection number, names, procurement location, and collection date.

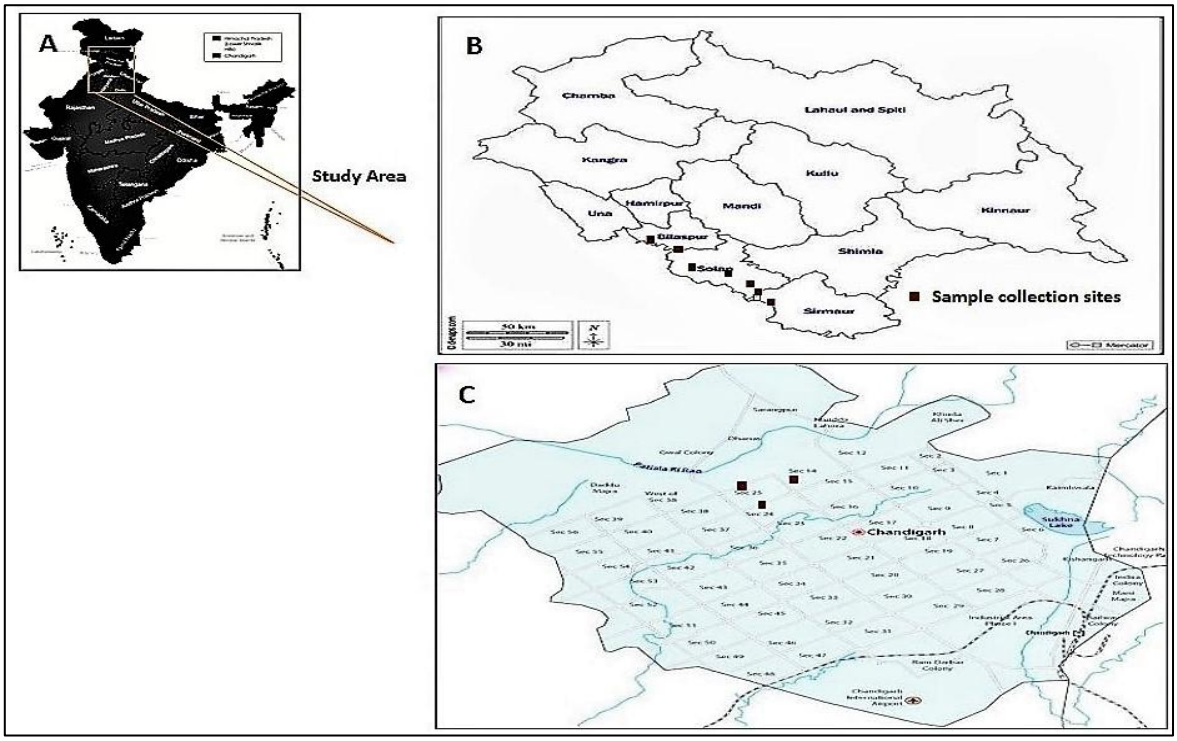


Figure 1. Study area map for mushroom sample collection A) India (Country) B Sample collecting site in Himachal Pradesh C) Sample sites in Chandigarh, India.

Isolation of pure culture of White rot fungi

All collected samples were isolated on Potato Dextrose Agar (PDA) plates using the tissue culture method (Stamets and Chilton 1983). Pure cultures of fungal species were preserved on PDA slants and stored at 4°C for further use.

Primary screening of culture for enzymes

Enzyme's presence was evaluated by zone of clearance around the colonies on staining with 0.2% Congo red dye and then destaining by 1N NaCl. The primary screening for the production of ligninolytic enzymes like laccase, lignin peroxidase was done by adding 0.5% guaiacol, 0.01% azure B dye respectively in PDA (Bains et al. 2003). The enzyme production was evaluated by zone of clearance. For cellulolytic enzymes, like endo-β-glucanase, exo-β-glucanase, FPase and β-glucosidase 0.1% of CMC, 0.1% Avicel,0.05% Whatmann filter paper and 0.1% salicin were added in PDA respectively and incubated at 28 ˚C for 7 days (Wood and Bhat 1988) .The zone of clearance around fungal colonies was shown the enzyme potential. For penctinolytic enzymes, 0.5% pectin was added to PDA plate. The presence of the enzymes was evaluated by zone of clearance around the colonies on staining with potassium iodide (KI) solution. For laccase guaiacol was added to PDA plate and halo zones were seen after 3 or 4 days cultivation.

Secondary screening of cultures for lignocellulolytic enzymes

5g of wheat bran with 60 % of moisture content was used as solid substrate for quantitative measurement of enzyme activity of isolated cultures of white rot fungi subsequently inoculated with mycelial discs of Trametes hirsuta having size of 10 mm, and then kept for incubation at 28 ºC for 14 days. After incubation, fungal colonised biomass was ground and mixed with buffers for enzyme extraction (Kachlishvili et al. 2012).

Enzyme extraction

The fungal enzymes were extracted by macerating the mycelial biomass from fully colonized lignocellulosic substrates after the designated incubation period. Extraction was carried out using buffers of varying pH, including citrate buffer (pH 4.8), sodium acetate buffer (pH 5.0), phosphate buffer (pH 6.0), and distilled water (pH 7.0).

For each extraction, 5 g of fermented biomass was mixed with 30 ml of the respective buffer and agitated at 120 rpm for 10 minutes. The mixture was then centrifuged at 6000 rpm at 4±1°C for 10 minutes, and the resulting supernatant was collected as the crude enzyme extract (Ghose 1987). The enzyme activity was subsequently determined through specific enzyme assays.

Enzyme assays

Laccase

Laccase (EC 1.10.3.2) activity was determined spectrophotometrically by measuring the oxidation of the substrate guaiacol in 10 minutes at 420 nm, as per Karp et al. (2012).The spectrophotometric blank contained 0.9 ml of sodium acetate buffer (pH 4.0) and guaiacol (10 mM) 0.1 ml. The reaction mixture contained 0.88 ml sodium acetate buffer pH 4 .0, guaiacol 0.1 ml and enzyme 0.02 ml. The above mixture was incubated at 32 ^{O}C in the dark for 10 minutes. After incubation the absorbance of each mixture was noted .The enzyme activity was calculated as:

Enzyme activity = OD×DF× Volume of reaction / Extinction coff. × Incubation time× volume of enzyme

One unit of enzyme activity was calculated as the amount of enzyme required for oxidizing 1 µmol of guaiacol per minute.

Assay for Xylanase

The xylanase assay was done by following the method devised by Bailey et al. (1992) which was based on measuring the reducing sugar liberated from xylan by 3, 5-dinitrosalicylic acid (DNSA). Crude enzyme extract was taken as xylanase source and assayed in 2.0 ml of reaction mixture containing 1.80 ml of 0.5% beech wood which was prepared in 0.05 M Na-citrate buffer (pH 5.0) and 200 µl of enzyme source and was incubated at 50 °C for 5 minutes. The reaction was terminated by the addition of 3.0 ml of DNSA and the contents was boiled for 15 minutes. After cooling, absorbance was measured at 540 nm. The amount of xylose liberated was quantified using xylose (0.50mg/ml) as standard. One International unit of xylanase activity (IU) was defined as the amount of enzyme which liberated 1 µmol of xylose per minute under the standard assay conditions.

Assay for Mannanase

Mannanase activity was determined based on the method of Xu et al. (2002) using a substrate solution of 0.5% guar gum (Sigma) in 0.1 M citrate buffer (pH 5.6). The reaction mixture was made up of 0.3 ml of appropriately mixed enzyme filtrate and 2.7 ml of the substrate solutions and incubated for 30 min in a water bath at 50 °C. The standard was made of 0.5mg/ml mannose. The reducing sugar released was measured as mannose equivalents by the method of Miller (1959). Absorbance of the reaction mixture was measured at 540 nm using a spectrophotometer.

Assay for cellulase enzyme (Cello bio hydrolases, Endoglucanases, β-glucosidases)

For endo-β-glucanase (CMCase), the reaction mixture contained 1ml of 1 % CMC, 250 µl of crude enzyme with addition of 250 µl of 0.05 mM citrate buffer and incubated at 50 °C for 30 minutes. The method of DNSA was used to measure the change of sugar (Ghose. 1987).For exo-β-glucanase, the reaction mixture contained 1ml of 1 % Avicel, 250 µl of crude enzyme with addition of 250 µl of 0.05 mM citrate buffer and incubated at 50^{0}C for 30 minutes. The method of DNSA was used to measure the change of sugar. Optical density was measured at 575 nm against a blank containing all the reagents minus the crude enzyme. For β-glucosidase, 1 % salicin was used as substrate in reaction mixture and rest of the procedure was same as followed for other cellulase enzymes. DNSA method was used for the measurement of enzyme activity. One enzyme unit was the amount of enzyme necessary to produce 1 µmol reducing sugar as glucose equivalent per minute under standard assay condition (Miller 1959).

Molecular identification of RDS-23

18 sRNA sequencing of RDS-23 was done to identify it up to species level and culture was deposited at Biokart India pvt. Ltd. After identification, the culture was deposited to Microbial Type Culture Collection and Gene Bank (MTCC) and assigned number MTCC 13584. The organism was maintained on potato dextrose agar (PDA) medium plates at 4 °C and sub-cultured at regular intervals of 4 week.

Assessment of LCB degradation by selected isolate under different culture conditions

RDS-23, identified as Trametes hirsuta PSRF29, was selected for further lignocellulosic biomass degradation. The degradative capability was assessed by determining the enzyme activities of crude enzyme extracts under varying physicochemical conditions, following the assay protocols described in the previous section. Spawns (inoculum) of *T. hirsuta* were prepared, and 4% (w/w) inoculum was introduced into the optimized biomass formulation, followed by incubation at 28 °C for 14 days under controlled conditions. Post-incubation, enzyme activity was analyzed across different parameters, including inoculum size (2–6%), pH range (3–8), temperature (24–40 °C), carbon source concentration (1–3%), and nitrogen source concentration (1–3%). Subsequently, the extent of lignocellulosic degradation was evaluated by quantifying the released sugars and determining changes in lignocellulosic components**.**

Spawns (Inoculum)
Pure culture of Trametes hirsuta was not used as such in the form of mycelium discs, firstly it was converted into inoculum where spawns of Trametes hirsuta were prepared. Sorghum grains were used for the production of spawns. 25 grams of sorghum grains were taken in 250 ml of conical flask with addition of water in 2:1 ratio or moisture content between was between 50 to 60%. After that, 0.5% of $CaCO_3$ and 0.5% of $CaSO_4$ were added in the grain followed by sterilization at 121°C for 15 minutes for 15 psi. After that, each flasks were inoculated with two discs of pure culture of selected isolates and subsequently incubated for 7 days at 28 °C. White growth were seen all over surface of grain and led to formation of spawn (Fig 5). Veena and Pandey (2012) had produced spawn of *Trametes versicolor* using sorghum grain.

Other parametrs evaluating LCB degradation by Trametes hirsuta
Lignin estimation
One gram of air-dried extractive-free, ground sample was weighed out accurately in a weighing bottle into a 50 mL beaker. Ten millilitres of 72% (v/v) sulphuric acid was carefully added with a pipette and with a tiny glass rod, the mixture was whisked. Subsequently the solution was left with irregular stirring at room temperature for 2 hours. The mixture was next transferred to 500 mL conical flask, and dilute with water until the final volume was 300 mL and covered with reversed smaller conical flask. The entire solution was heated on the hotplate for an hour. This process was continued for an extra 2 hours. A crucible (fine or medium porosity) was oven dried for an hour at 105ºC, then allowed to cool in a desiccator and weighed accurately. The insoluble lignin was recovered when the heating was completed, by filtration through the crucible. The lignin was washed with 250 mL of hot distilled water. The crucible containing the lignin was dried at 105ºC for a day, cooled in desiccator and weighed. The lignin content was reported as percentage by weighed of the dry sample (Carrier et al. 2011).
The percent yield of lignin from different extraction techniques was ascertained gravimetrically.
Lignin percentage = A (100)/W
A = Weight of lignin (in grams), W = Oven dry weight of test specimen (in grams)

Cellulose /Holocellulose/Hemicellulose estimation
One gramme of LCB powder was transferred to a test tube along with three millilitres of acetic/nitric reagent, and the mixture was vortexed. The test tube incubated for 30 minutes in a water bath set at 100 degrees Celsius. Centrifugation was used to separate the solid particles for 15 minutes at 10,000 revolutions per minute. After discarding the supernatant, distilled water was used to wash the residue. The residue was then mixed with 10 mL of 67% sulphuric acid and left to stand for one hour. A 100 ml sample was prepared by diluting 1 mL of the solution. After combining 1 millilitre of the diluted solution with 10 millilitres of anthrone reagent, the sample was heated to a boil for ten minutes. At 630 nm, absorbance was measured following cooling. Using a calibration graph with standard cellulose, the quantification of cellulose estimation was established (Shet et al. 2018). A dried sample of holocellulose (2 g) was taken in a 250 ml flask. Then 10 ml of 17.5% NaOH was added to the flask and a lid put on it. The flask was kept in a water bath at 20°C and the mixture was stirred. After 5 min, another 5 ml of the same 17.5% sodium hydroxide was added to it. The process was repeated and continued for 15 min. After that it was left for 30 min and 33 ml of water was added and kept at 25°C. The solid residue containing cellulose was filtered through a muslin cloth. It was washed again in 100 ml of 8.3% NaOH solution. The cellulose was subjected to acid treatment in 15 ml of 10% acetic acid. Thereafter washed it in distilled water and dried the residue containing hemicellulose till it attained the constant weight (TAPPI 2002).
Holocellulose = Cellulose+ Hemicellulose

The Folin–Ciocalteu method was used to determine the extract's total phenolic content (Kaur et al. 2002). In summary, 2 mL of 20% (w/v) sodium carbonate was added after 200 µl of crude extract (1 mg/mL) was fully combined with 0.5 mL of Folin-Ciocalteu reagent for three minutes. After a further 60 minutes of standing in the dark, the mixture's absorbance at 650 nm was determined. Using the calibration curve, the total phenolic content was determined. The results were expressed as milligrams of gallic acid equivalent per gramme of dry weight.

Carbohydrate/reducing sugar estimation
Carbohydrate content profile of the completely colonized lignocellulosic biomass at different interval of time was determined as per Dubois et al. (1956).Reducing sugar was estimated by DNSA method (Nwogwugwu et al. 2016)

Analytical characterisation of degraded LCB
Certain techniques such as FTIR, SEM and TGA were conducted for the evaluation of decomposed Lignocellulosic biomass. These analysis were performed at the Sophisticated Analytical Instrumentation Facility (SAIF), Panjab University, Chandigarh, to evaluate the structural alterations in lignocellulosic biomass following fungal treatment.

3. Results and Discussion
Collection of mushroom samples (white rot fungi)
Mushroom wild samples were collected across different habitat of Shivalik region of India and transported to a lab for processing. Sixteen samples were collected and tentative identification of genera was done on the basis of their morphology (Table 1).

Table 1: White rot fungi obtained in the multiple biodiverse areas of Chandigarh and the Shivalik region.

Isolates	Genera(Tentative identification) (Macroscopic examination)	Location
RDS1	*Schyzophyllum* sp.	Solan, Himachal Pradesh
RDS2	*Ganoderma* sp	Solan , Himachal Pradesh
RDS3	*Ganoderma* sp	Shimla, Himachal Pradesh
RDS4	*Ganoderma* sp	Arki, Himachal Pradesh
RDS5	*Pleurotus* sp.	Rose Garden, Sector 16,Chandigarh
RDS6	*Ganoderma* sp	Solan, Himachal Pradesh
RDS7	*Ganoderma* sp.	Sector 25, Chandigarh, Panjab University
RDS8	*Ganoderma* sp	Bilaspur, Himachal Pradesh
RDS9	*Trametes* sp	Solan, Himachal Pradesh
RDS10	*Ganoderma* sp	Chandi, Solan, Himachal Pradesh
RDS11	*Trametes* sp.	Arki, Solan, Himachal Pradesh
RDS12	*Trametes* sp	Solan, Himachal Pradesh
RDS13	*Ganoderma* sp	Kufri, Shimla ,Himachal, Pradesh
RDS14	*Trametes* sp	Sector 14,Chandigarh
RDS15	*Trametes* sp	Shimla, Himachal Pradesh
RDS 23	*Trametes* sp.	Sukhna wildlife sanctuary, Chandigarh,

Isolation of pure culture

Out of total 16 wild samples of white rot fungi, eight (8) pure culture were retained and preserved as slants for longer use at 4°C.These isolated pure culture were taken further for primary and secondary screening. Pure cultures of white rot fungi were shown below (Figure 2).

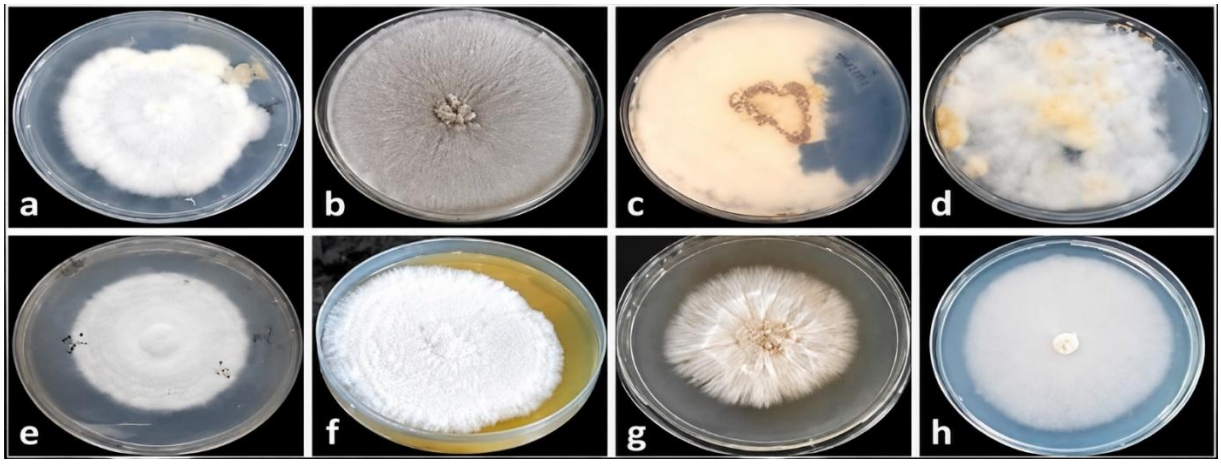


Figure 2. Pure cultures of eight different successfully isolated white rot fungi on Potato Dextrose Agar (PDA) plate. (a): RDS1: *Schyzophyllum* sp. (b): RDS 3: *Ganoderma* sp. (c): RDS4: *Trametes* sp.(d): RDS5: *Pleurotus* sp. (e): RDS 6: *Ganoderma* sp. (f): RDS9: *Trametes* sp. (g): RDS10: *Ganoderma* sp. (h): RDS 23: *Trametes* sp.

Screening of isolates for their potential to produce enzymes

Both qualitative and quantitative screening were done for isolates to screen out best isolate for lignocellulolytic enzyme production. Qualitative screening was done on the basis of halo zones around culture, further quantitative examination was done under solid state fermentation using wheat bran. On the basis qualitative and quantitative study, enzyme profile was designed for each and every isolate (Table 2).

Table 2: Screening of isolated strains for their efficacy to produce enzymes

Isolates	**Qualitative Screening for Enzymes**				
	Laccase	**Cellulase**	**Xylanase**	**Mannanase**	**Pectinase**
RDS1	√	√	×	×	×
RDS3	√	√	×	×	x
RDS-4	√	√	×	x	×
RDS-5	√	√	×	×	×
RDS-6	√	×	×	×	×
RDS-9	√	×	×	×	×
RDS-10	×	√	×	×	×
RDS -23	√	√	√	√	√
Isolates	**Quantitative Screening for Enzymes**				
	Laccase	**Cellulase**	**Xylanase**	**Mannanase**	**Pectinase**
RDS-1	0.05 ± 0.02	0.2 ± 0.08	nil	0.02±0.04	nil
RDS-3	0.20 ± 0.08	0.1 ± 0.06	nil	nil	nil
RDS-4	0.28 ± 0.11	0.13 ± 0.08	nil	nil	nil
RDS-5	0.21 ± 0.12	0.18 ± 0.04	nil	nil	nil
RDS-6	0.08 ± 0.03	nil	nil	nil	nil
RDS-9	0.03 ± 0.01	nil	nil	nil	nil
RDS-10	nil	0.4 ± 0.11	nil	nil	nil
RDS -23	0.45 ± 0.16	0.11 ± 0.08	2.3 ± 0.21	0.13 ± 0.08	2.8 ± 0.56

Values are mean ± SD of three observations.

$p < 0.05$

Enzyme activities were assayed on 14th day of incubation

As presented in Table 2, isolate RDS-23 exhibited the highest enzyme activities for laccase, cellulase, xylanase, mannanase, and pectinase among the tested isolates. Owing to its superior lignocellulolytic enzyme profile, RDS-23 was selected for subsequent studies on lignocellulosic biomass degradation.

Macroscopic and microscopic examination of RDS- 23

Macroscopic examination was conducted for the wild sample of RDS-23 collected from site and certain observation were made on the basis external appearance such as shape and size of basidiocarp, flesh appearance, stem ,pore surface etc while microscopic analysis was also done by studying mycelium and spore structure (Table 3). After examination, it was tentatively identified as a member of genus *Trametes*.

Table 3: Macroscopic and microscopic examination of Trametes RDS- 23

Characteristics	**Observation**
1. Macroscopic examination	
Basidiocarp	Saprobic on the deadwood of hardwoods (very rarely reported on conifer wood); annual; causing a white rot; growing in clusters on logs and stumps;3–18 cm across; 2.5–9 cm deep; 1.5–5 cm thick; semi-circular or kidney-shaped; convex to plano convex; dry; vaguely or distinctly zoned with to mentose and bald zones; whitish to grayish or whitish
Pore Surface	White to pale brownish; usually featuring slot-like pores

Stem	Absent
Flesh	White in color
2. Micrscopic examination	
Hyphae	Aseptate and coenocytic
Spore	Oval and cylindrical shaped

Selection and Identification of potential consortium organisms up to species level

Out of 8 strains, strain RDS-23, was chosen for further study because of its potential to degrade lignocellulosic biomass. The strains that were chosen on their ability to produce enzymes. The selected strain RDS-3 was identified as *Trametes hirusta* based on macroscopic, microscopic and by 18s rRNA sequencing (ITS sequencing). Culture was sent to Biokart India Pvt. Ltd., Bangalore for species identification.

Molecular Identification

The organism was identified as *Trametes hirsuta* PSRF-29 by molecular studies using 18S rRNA sequencing of the chosen test fungus RDS- 23. Based on the significantly conserved nucleotide sequence of the 18S ribosomal RNA gene, the species was identified. Using a fungal genomic DNA isolation kit (RKT 13; Biokart Pvt. Ltd. Bangalore, India), the DNA of *Trametes* RDS-23 had been extracted in preparation for sequencing. Using primer pairs, the 18s rRNA gene sequence was amplified by PCR. The following parameters were followed when conducting the PCR reaction: one cycle of heat treatment at 94 °C for three minutes; thirty cycles of denaturation at 94 °C for one minute; annealing at 50 °C for one minute; two cycles of initial extension at 72 °C and a final extension cycle at 72 °C for seven minutes. For amplification, 184 ng of extracted DNA and 10 pM of each primer are used. Two microliters of DNA in each of the 184 ng forward and reverse primers, 2.5 mM dNTPs, 1 µl Taq DNA Polymerase Enzyme (3 IU/ml), and 10µl 10X Taq DNA polymerase assay buffer were all included in the 100 µl PCR reaction mixture. Using agarose gel electrophoresis (0.8%), the PCR product was examined, and Biokart Pvt. Ltd., India provided forward and reverse primer sequencing using an ABI 3130 Genetic Analyzer. Using PCR, the 654 bp sized 18S rRNA of RDS-23 was successfully amplified (Figure 3). After obtaining the sequence, it was uploaded to GenBank for homology searching using BLAST (http://ncbi.nim.nih.gov) against the fungal DNA database.

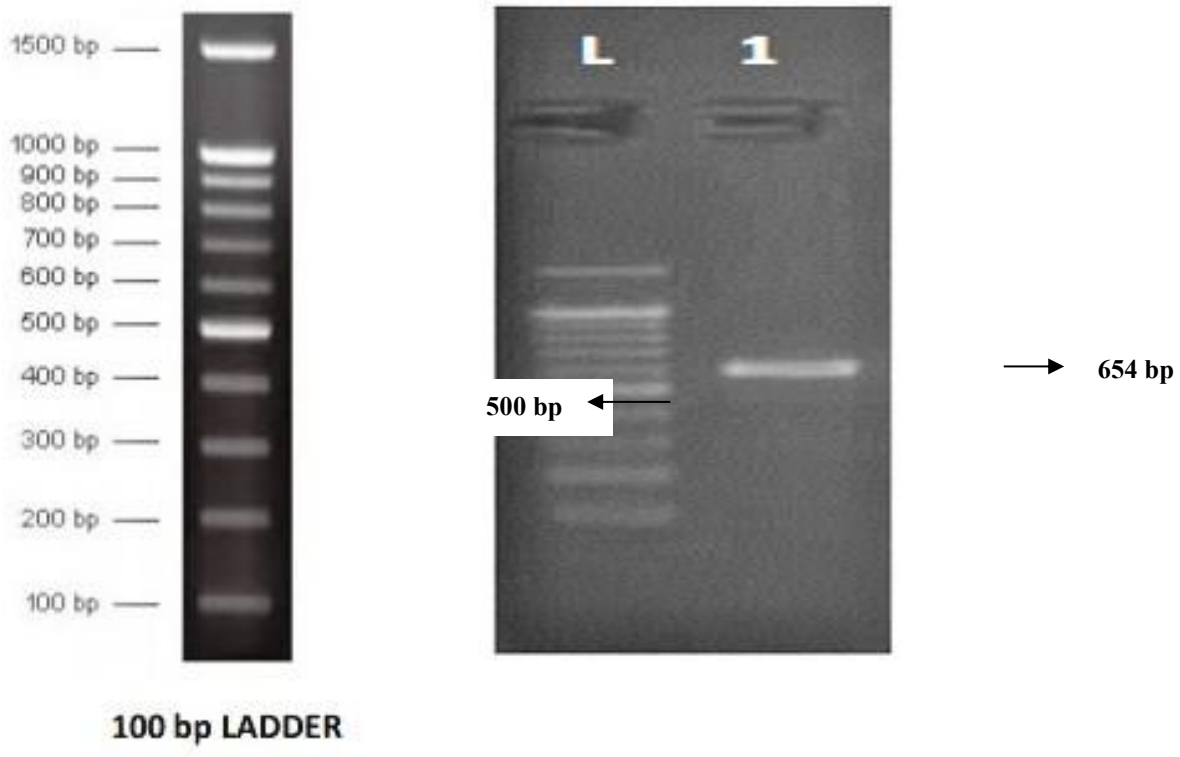


Figure 3. PCR amplification of ITS region from the test fungal sample RDS-23. The size of PCR amplified product is ~ 654bp.

According to the BLAST, this sequence is 99% similar to *Trametes hirsuta*, which is the closest match found in the result (Table 4). Sequences with over 99.84 percent identity shared were thought to belong to the same species.

The results of the BLASTn search for the chosen isolate agreed with the original identification. By using MEGA-11 (Molecular Evolutionary Genetics Analysis) software and the neighbour joining method to align homologous nucleotide sequences among these fungal species, a phylogenetic tree measuring 100 bp, 200 bp, 500 bp, 700 bp, and 650 bp was produced . This phylogenetic analysis revealed *Trametes hirsuta* to be the identity of isolate RDS-23(Figure 4).

Aligned sequence data of *Trametes hirsuta*.

AGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTTTTGA
AATGGGTTGTTGCTGGCCTTCCGAGGCATGTGCACGCCCTTGCTCATCCACTCTACACCTGTGCACT
TACTGTAGGTTGGCGTGGGTTTCTAGCCTCCGGGCTGGGAGCATTCTGCCGGCCTATGTACACTAC
AAACTCTAAAGTATCAGAATGTAAACGCGTCTAACGCATCTTAATACAACTTTCAGCAACGGATCT
CTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
TGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGT
GTCATGAAATTCTCAACCCATAAGTCCTTGTGATCTATGGGCTTGGATTTGGAGGCTTGCTGGCCCT
AGCGGTCGGCTCCTCTTGAATGCATTAGCTTGATTCCGTGCGGATCGGCTCTCAGTGTGATAATTGT
CTACGCTGTGACCGTGAAGCGTTTTGGCAAGCTTCTAACCGTCCATTAGGACAATCTTTCAACATC
TGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAG

Table 4: Closest species along with their GenBank accession number and % identity from BLASTn search

BLAST DATA:

Sl. No.	Organism Name	Accession No.	% Match
1	Trametes hirsuta strain PSRF29 small subunit ribosomal RNA gene	OP508351.1	99.84%
2	Trametes sp. strain RH8 small subunit ribosomal RNA gene	OP209778.1	99.84%
3	Trametes hirsuta isolate HH1 small subunit ribosomal RNA gene	MF377430.1	99.84%
4	Trametes hirsuta voucher M0137906 18S ribosomal RNA gene	KF573024.1	99.84%
5	Trametes sp. strain MA-X01 small subunit ribosomal RNA gene	MF687399.1	99.84%
6	Trametes hirsuta isolate HH2 small subunit ribosomal RNA gene	MF377429.1	99.84%
7	Trametes hirsuta strain SYBC-L8 18S ribosomal RNA gene	HQ891292.1	99.84%
8	Trametes hirsuta isolate 5154 18S ribosomal RNA gene	EF546240.1	99.84%
9	Trametes hirsuta strain BRPCT17 small subunit ribosomal RNA gene	MT658058.1	99.84%
10	Trametes hirsuta genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA	LC317799.1	99.84%

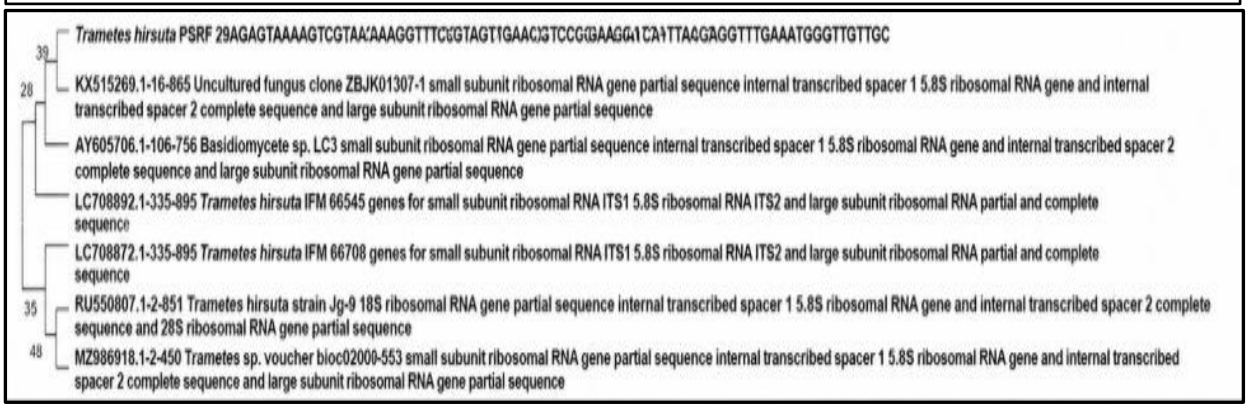


Figure 4. Phylogenetic tree produced using MEGA-7 (Molecular Evolutionary Genetics Analysis) software by the neighbour joining method

Assessment of LCB degradation by selected isolate under different culture conditions

First of all lignocellulosic biomass such as leaf litters, paddy straw, corn cob, corn stalk, sugarcane bagassae and rice husk were collected and carried to the lab. Further, these biomass were treated with 4% of sodium hypochlorite solution in water tub for 2 hours in order to get rid of unwanted microorganisms, then dried under sunlight and again washed with water to remove hypochlorite solution and dried. Dried biomass was chopped into smaller pieces, supplemented with basal salts such as 0.5 % glucose, 1 % yeast extract, 0.02% K_2HPO_4, 0.005% (w/v) $MgSO_4.7H_2O$. Medium was autoclaved at 121°C for 15 minutes at 15 psi. After medium cooled down to room temperature, it was inoculated with 4 % (w/w) spawns of T. hirsuta and incubated at 28 °C for 14 days (optimized conditions).

Inoculum Preparation of *Trametes hirsuta*: (spawn production)
The Spawn of *Trametes hirsuta* PSRF-29 was prepared and used as inoculum for the degradation of lignocellulosic biomass (Figure 5)



Figure 5. Spawns of Trametes hirsuta a) wild sample of *Trametes hirsuta*. b) pure culture of Trametes hirsuta. c) Spawn formation after 7 days of incubation at 28 ˚C.

Lignocellulosic biomass formulation
Trametes hirsuta was used for the degradation of lignocellulosic biomass and an optimized lignocellulosic biomass formulation was used for the growth of the *Trametes hirsuta* .LCB biomass formulation was given below (Table 5).

Table 5: Composition of various lignocellulosic biomass used in formulation

LCB component	Quantity (g, w/w) /100g
Leaf litters	45
Paddy straw	25
Sugarcane bagassae	10
Corn cob	5
Corn stalk	10
Rice husk	5

Sterilized LCBs were inoculated with 4 % of spawns (Inoculum) of *Trametes hirsuta* and kept for incubation up to 14 days. After 14 days, the enzyme activity, reducing sugar, carbohydrate concentration were measured to evaluate the degradation potential of *Trametes hirsuta*.

Lignocellulolytic enzyme production from Trametes hirsuta
Degradation potential of Trametes hirsuta was evaluated by assessing various lignocellulolytic enzyme production in LCB formulation. In formulation, 45 g of leaf litters, 25 g paddy straw, 10 g sugarcane bagassae, 5 g corn cob, 10 g corn stalk, 5g rice husk were taken as shown in the table 5, then chopped and maintained 60-70% of moisture content, subsequently autoclaved at 121ºC for 15 minutes at 15 psi. Decomposition potential of Trametes hirusta was evaluated after 14 days (optimized) of incubation as T. hirsuta had colonised the surface of LCB. Enzyme production was recorded at various parameters such as inoculum size, pH, Temperature, carbon and nitrogen described etc.

Effect of Inoculum size
Enzyme activity was recorded at various inoculum size ranges from 2 to 6 % (w/w), at 28 ˚C and pH 5. After 14 days of incubation, maximum lignocellulolytic enzyme activity was recorded at 5 % and least activity was recorded at 8 % of inoculum size (Table 6). Maximum enzyme activity for laccase, CMCase, exo-b-glucanase, β-glucosidase, mannanase, pectinase, xylanase was 0.14, 0.21, 0.14, 0.17, 0.37, 1.8 IU/ml respectively.

Table 6: Enzyme activity of Trametes hirsuta in LCB at different inoculum size

Inoculum Size (%)	Enzyme Activity (IU/ml)						
	Laccase	CMCase	Exo-β-glucanase	β-glucosidase	Mann-anase	Xylan-ase	Pectin-ase
2	0.03±0.08	0.03±0.02	0.10±0.04	0.08±0.012	0.20±0.08	2.16±0.12	0.44±0.11
3	0.035±0.01	0.09±0.03	0.12±0.01	0.12±0.032	0.23±0.05	3.83±0.22	0.56±0.15

4	0.10±0.07	0.15±0.01	0.11±0.03	0.15±0.01	0.28±0.12	4.06±0.16	1.11±0.31
5	0.14±0.04	0.21±0.04	0.14±0.05	0.17±0.09	0.37±0.10	8.02±0.26	1.80±0.26
6	0.12±0.03	0.06±0.08	0.09±0.05	0.16±0.06	0.34±0.04	7.02±0.34	1.24±0.24
7	0.035±0.01	0.03±0.02	0.06±0.01	0.13±0.06	0.28±0.05	6.97±0.42	1.07±0.08
8	0.032±0.03	0.02±0.04	0.04±0.03	0.21±0.08	0.26±0.32	4.98±0.51	0.98±0.12

Values are mean ± SD of three observations.
$p < 0.05$
Enzyme activities were assayed on 14th day of incubation at 28°C

Effect of temperature

Enzyme activity was recorded at different temperature ranges from 24 to 40 °C, after 14 days of incubation, maximum activity was recorded at 28°C and least activity was recorded at 40 °C (Table 7). Maximum enzyme activity for laccase, CMCase, Exo-β-glucanase, β-glucosidase, mannanase, pectinase, and xylanase was 0.10±0.05, 0.43±0.03, 0.14±0.08, 0.18± 0.04, 0.48 ± 0.09, 2.1 ± 0.08, 8.93 ± 0.98 IU/ml respectively. Chenthamarakshan et al. (2017) reported the maximum laccase production at 28°C by Marasmiellus palmivorus. Teshome and Melaku (2017) also reported 28°C as the best suited temperature for laccase production by *Pleurotus* species. Dinis et al. (2009) reported maximum cellulase activity (0.27 IU/ml & 0.13 IU/ml) at this temperature.

Table 7: Enzyme activity of Trametes hirsuta in LCB at different temperature ranges

Temperature (°C)	Enzyme Activity (IU/ml)						
	Laccase	**CMCase**	**Exo-β-glucanase**	**β-glucosidase**	**Mannanase**	**Xylanase**	**Pectinase**
24	0.027± 0.02	0.26± 0.12	0.12± 1.2	0.16± 0.03	0.36±0.11	7.42±0.13	1.2± 0.12
28	0.10± 0.05	0.43± 0.03	0.14± 0.08	0.18± 0.04	0.48±0.09	8.93±0.18	2.1± 0.08
32	0.06± 0.03	0.38± 0.04	0.11± 0.06	0.17± 0.03	0.42±0.08	8.45±0.21	1.8± 0.06
36	0.08± 0.01	0.38± 0.16	0.10± 0.01	0.13± 0.06	0.39±0.05	8.28±0.19	0.98±0.05
40	0.02± 0.03	0.20± 0.11	0.09± 0.03	0.10± 0.01	0.22±0.06	7.53±0.31	0.65±0.03

Values are mean ± SD of three observations.
$p < 0.05$
Enzyme activities were assayed on 14th day of incubation having Inoculum size 5%

Effect of pH

Enzyme activity was recorded at different pH ranges from 3 to 8. After 14 days of incubation, maximum activity was recorded at pH 6 and least activity was recorded at pH 8 (Table 8). Maximum enzyme activity for laccase, CMCase, Exo-β-glucanase, β-glucosidase, mannanase, pectinase, and xylanase was 0.16, 0.33, 0.12, 0.18, 0.48, 5.92, 2.24 IU/ml respectively.

Table 8: Enzyme activity of Trametes hirsuta in LCB at different temperature ranges

pH	Enzyme Activity (IU/ml)						
	Laccase	**CMCase**	**Exo-β-glucanase**	**β-glucosidase**	**Mannanase**	**Xylanase**	**Pectinase**
3	0.06 ± 0.02	0.11± 0.01	0.06± 0.01	0.08± 0.01	0.24± 0.02	2.77± 0.11	0.81 ± 0.01
4	0.08 ± 0.04	0.12± 0.03	0.09± 0.02	0.12± 0.03	0.33± 0.16	3.05± 0.13	0.92 ± 0.08
5	0.12 ± 0.012	0.29± 0.06	0.11± 0.03	0.13± 0.05	0.38± 0.02	4.8 ± 0.21	2.06 ± 0.14
6	0.16 ± 0.031	0.33± 0.01	0.12± 0.03	0.18± 0.03	0.48± 0.13	5.92± 0.31	2.24 ± 0.16
7	0.10 ± 0.050	0.30± 0.02	0.09± 0.02	0.12± 0.06	0.42± 0.16	5.33± 0.18	2.06 ± 0.12

8	0.06 ± 0.07	0.29± 0.06	0.05± 0.06	0.17± 0.04	0.41± 0.09	5.38± 0.12	2.02 ± 0.14

Values are mean ± SD of three observations.
$p < 0.05$
Enzyme activities were assayed on 14th day of incubation at 28 ºC with inoculum size 5% (w/w)

Effect of carbon source
Enzyme activity was recorded at various carbon sources such as cmc, glucose, glycerol, xylose, arabinose, sucrose, fructose, mannitol, maltose, and galactose. Maximum activity was recorded at 1.5 % of glucose. Laccase, CMCase, exo-β-glucanase, β-glucosidase, mannanase, pectinase, xylanase had 0.18 ± 0.06, 1.36 ± 0.21, 0.42 ± 0.12, 0.21 ± 0.12, 0.28 ± 0.21, 3.08 ± 0.12, 8.39 ± 0.24, IU/ml respectively.

Effect of nitrogen source
Enzyme activity was recorded at various nitrogen sources such as peptone, malt extract, yeast extract, beef extract, casein, potassium nitrite, ammonium sulphate, ammonium chloride, sodium nitrite. Maximum enzyme activity was recorded at 1.0 % of yeast extract. Laccase, CMCase, Exo-β-glucanase, β-glucosidase, mannanase, pectinase, xylanase had 0.23 ± 0.05, 1.7± 0.21, 0.78 ± 0.13, 0.41 ± 0.13, 0.38 ± 0.08, 5.08 ± 0.02, 13.15 ± 0.51 IU/ml respectively.

Other parameters indicating degradation of LCB

Lignin / Cellulose / Hemicellulose content
Lignin content was estimated by Klason process. In *T. hirsuta* treated biomass (test), the yield of lignin was 0.14g/g of biomass while in untreated biomass (control) it was 0.33g/g, corresponding to a 57.57 % reduction in lignin content. Cellulose content was estimated by spectrophotometric method. The overall yield in untreated biomass was 0.36 g/g or 36 % while yield was 0.19 g/g or 19 % in fungal treated biomass. Decrease in cellulose content was 47.22 %. Hemi -cellulose content was 0.23 g/g or 23 % while yield was 0.17 g/g or 17 % in fungal treated biomass. Decrease in hemi-cellulose content was 26.08 % (Figure 6).

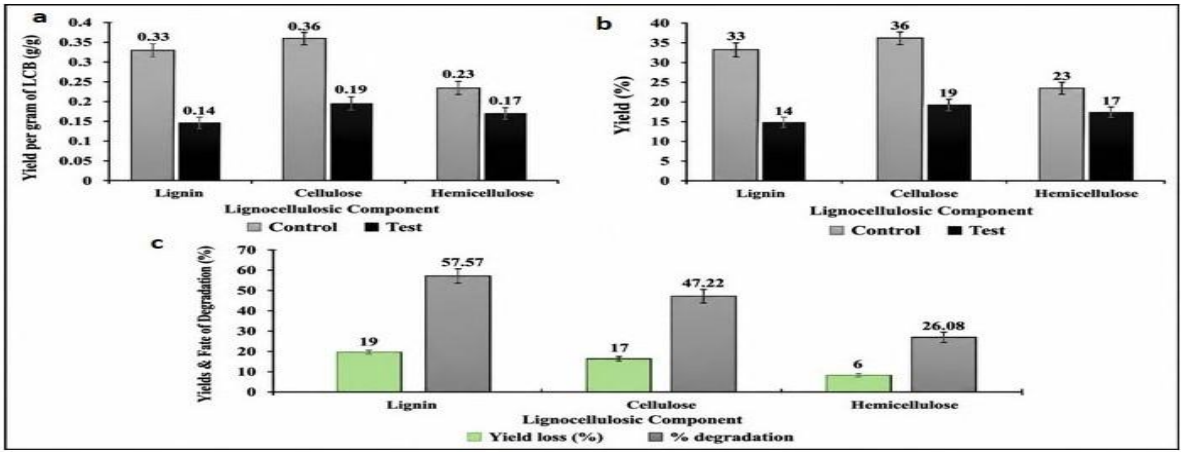


Figure 6. Yield and degradation ratio in untreated LCB (Control) and fungal treated LCB (Test) a) yield in gram per gram of untreated and fungal treated LCB, (b) percentage yield of lignin, cellulose and hemicellulose in untreated and treated LCB, and (c) percentage degradation of lignin, cellulose and hemicellulose of untreated and fungal treated lignocellulosic biomass.

Total phenolic content
Total phenol content of untreated lignocellulosic biomass or treated lignocellulosic biomass with Trametes hirsuta was determined. In untreated LCB the total phenolic content was 35.77 ± 4.11 mg/g while in treated LCB it decreased to 23.91±3.26 mg/g of sample. Therefore percentage decrease in phenol content was 33.16% indicating LCB decomposition.

Total carbohydrate/ reducing sugar
Carbohydrate concentration was estimated by Dubois (1983) method and carbohydrate concentration was reduced from 14.8 mg/20ml (Control, untreated biomass) to 7.4 mg/20ml (Fungal treated biomass).This decrease in carbohydrate concentration indicted breakdown of complex sugar into simpler one and hence proved decomposition of LCB. Total reducing sugar was found increased from 0.87 mg/20ml (Control, untreated biomass) to 1.43 mg/20ml (Fungal treated biomass). 39 % increase in reducing sugar was recorded. This increase in reducing concentration indicted breakdown of complex sugar into simpler one and hence proved decomposition of LCB. Taniguchi et al. (2010) has reported 33 % of sugar yield using Pleurotus as decomposer.

Analytical characterisation indicating LCB degradation

TGA analysis

LCB has lignin, cellulose, hemicellulose which make it a fire retardant biomass but after Trametes hirsuta treatment and decomposition led to disintegration of polymer which resulted into less fire resistant property as shown below in the figure. Approximately 75 % of weight loss was recorded in the control up to 600 ºC of temperature while 70% of weight loss was reported fungal treated lignocellulosic biomass up to same temperature, indicated polymer degradation and hence LCB decomposition (Figure 7).

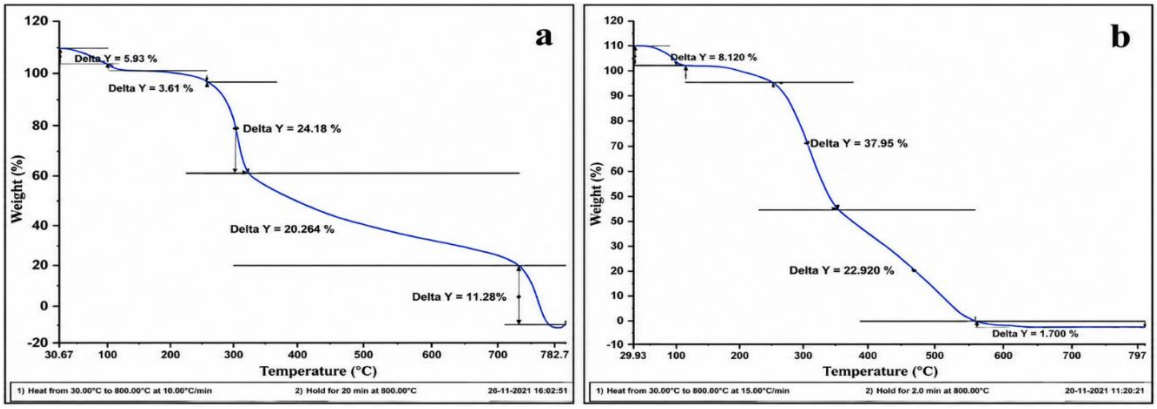


Figure 7. TGA images (a) Control (Untreated lignocellulosic biomass), and (b) Fungal treated LCB

FTIR (Fourier-transform infrared spectroscopy)

FTIR study has evaluated decomposition of lignocellulosic biomass by studying change in moiety of functional group in functional group and fingerpoint region. FTIR was done after 14 days of fungal treated with lignocellulosic biomass. Peak wavelength increased from 1629 to 1633 cm-^{1}, indicating-C=C-stretching and N-H-bending. The peak wavelength increased from 3331 to 3345 cm-^{1} indicated O-H-stretching, 2925 to 2921 cm-^{1} indicating stretching in N-H and O-H bond and did cause lot of disturbances in this region and subsequently biomass degradation. Following that, in finger print region peak decreased from 1041 to 1037 cm-^{1} indicating S=O, C-N,C-O stretching (Figure 8).

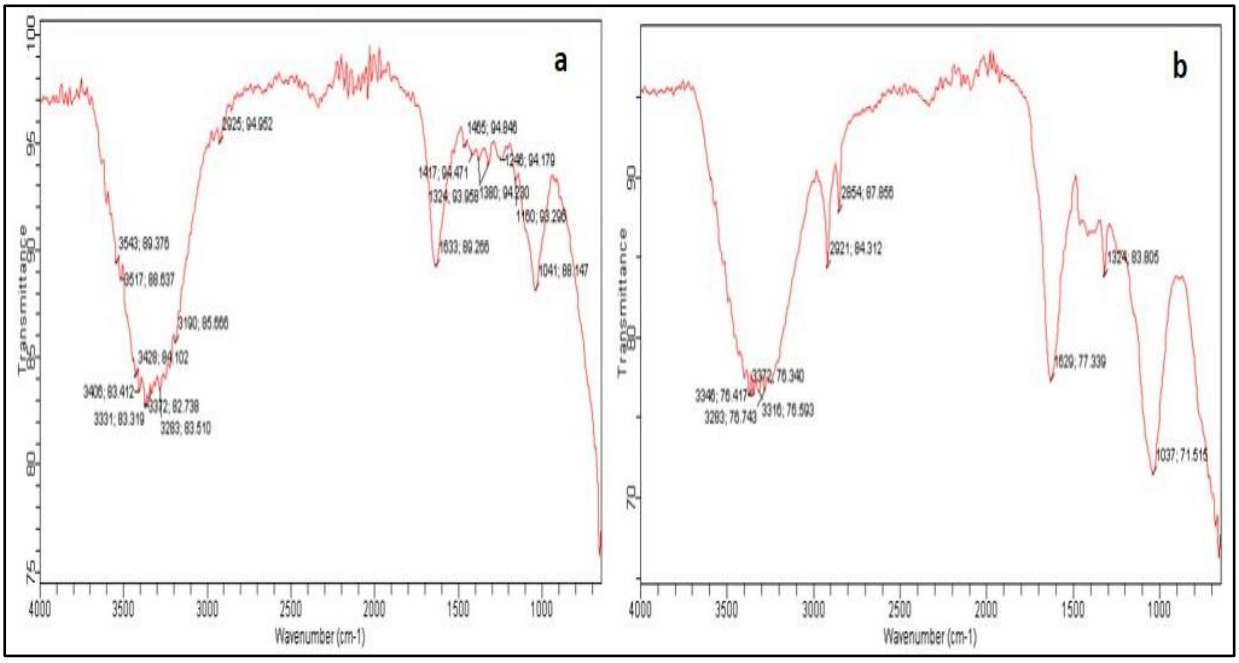


Figure 8 – FT-IR images (a) undecomposed LCB, and (b) decomposed LCB after treatment with Trametes hirsuta

SEM (Scanning electron microscopy)

SEM was employed to assess the degradation potential of white-rot fungi by comparing untreated (control) and treated biomass samples at a 14-day incubation interval. The untreated biomass exhibited an intact and well-organized surface morphology with no visible signs of structural disruption. In contrast, the fungal-treated samples displayed pronounced morphological changes, including surface erosion, fibrillation, pore formation, and disruption of the lignocellulosic matrix. These substantial ultra-structural modifications indicate an efficient

depolymerization and decomposition of the biomass, confirming the strong lignocellulolytic activity of the white-rot fungus (Figure 9)

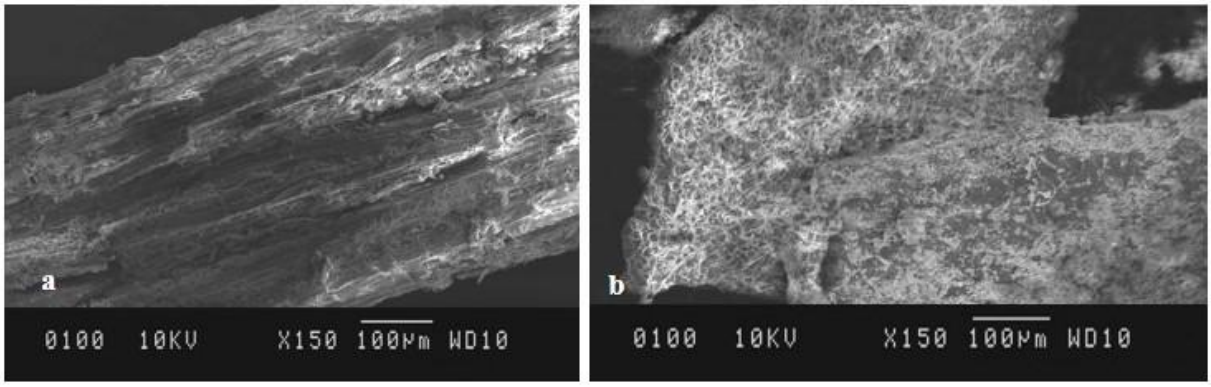


Figure 9 – SEM micrographs at an at an at an accelerating voltage of 10 kV with 100x magnification a) Control, untreated lignocellulosic biomass, b) degradation of biomass after 14 days of treatment with Trametes hirsuta.

4. Discussion

In the present investigation, isolate RDS-23, identified as *Trametes hirsuta* PSRF-29, demonstrated strong lignocellulolytic potential, evidenced by its ability to produce a complete suite of lignocellulose-degrading enzymes. The simultaneous production of laccase, CMCase, exo-β-glucanase, mannanase, pectinase, and xylanase suggests that this isolate possesses an intrinsic capability to degrade complex lignocellulosic biomass independently, without reliance on microbial consortia. This characteristic is particularly advantageous for streamlined and scalable bioconversion processes. Unlike earlier studies that primarily focused on single substrates or limited combinations of two biomasses, the present study employed a composite formulation of four to five lignocellulosic substrates, including leaf litter, paddy straw, corn stover, and sugarcane bagasse. This approach better simulates natural heterogeneous biomass conditions and provides a more comprehensive assessment of the degradation efficiency of T. hirsuta. The fungus exhibited notable enzymatic activities on the 14^{th} day of incubation, with laccase (0.23 ± 0.05 IU/ml), CMCase (1.7 ± 0.21 IU/ml), exo-β-glucanase (0.78 ± 0.13 IU/ml), mannanase (0.41 ± 0.13 IU/ml), pectinase (0.38 ± 0.08 IU/ml), and xylanase (5.08 ± 0.02 IU/ml), along with a peak xylanase activity of 13.15 ± 0.51 IU/ml under optimized conditions. These findings indicate a coordinated enzymatic system enabling efficient depolymerization of cellulose, hemicellulose, and lignin fractions**.** When compared with previous reports, the enzymatic performance of *T. hirsuta* in this study appears competitive or superior in several aspects. Datsomor et al. (2022) reported cellulase activity of 0.9 IU/ml using Pleurotus ostreatus, whereas higher CMCase activity was observed in the present study. Similarly, Tirado-González et al. (2016) and Dange et al. (2018) reported xylanase (3.3 IU/ml) and pectinase (1.2 IU/ml) activities, respectively, which are comparatively lower than or within the range of values obtained here. Elisashvili et al. (2008) reported xylanase activity of approximately 13 IU/ml in wheat straw, which aligns closely with the peak values observed in this study. In contrast, Pinto et al. (2022) documented comparatively lower enzyme activities using Trametes versicolor and Ganoderma resinaceum, further highlighting the efficiency of the present isolate**.** de Oliveira Junior et al. (2022) and Shankar et al. (2022) reported that a 5% spawn level is optimal for lignocellulolytic enzyme production. In contrast, Manavalan et al. (2012) observed that a higher spawn concentration of 10% (w/w) was more effective for lignocellulosic biomass degradation. In the present study, however, a 5% spawn resulted in substantial biomass degradation, indicating that lower inoculum size can be sufficient to achieve efficient lignocellulolytic activity under the given experimental conditions. Substantial compositional changes in lignocellulosic biomass were also observed, with reduction of 58% in lignin, 47.22% in cellulose, and 26.08% in hemicellulose content. These results confirm the effective breakdown of structural polymers, particularly lignin, which is typically the most recalcitrant component. The observed degradation patterns are consistent with the findings of Sun et al. (2011), who reported approximately 41% biomass weight loss using *T. hirsuta*. Additionally, a 33.16% reduction in total phenolic content was recorded, indicating significant breakdown of lignin-derived phenolic compounds. This reduction is markedly higher than the 10.80% reported by Su et al. (2018) and is comparable to the 33% decline reported by Alzagameem et al. (2018). TGA analysis shown 75 % of weight loss at 600 ^{o}C temperature wherever Jones et al. (2018) had reported maximum 70 % of weight loss in fungal treated LCB up to 600 ^{o}C of temperature indicating higher degradation potential of *Trametes hirsuta* PSRF 29. Furthermore, scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR) analyses provided structural and chemical evidence of biomass degradation. Earlier, Awoyale et al. (2021) had reported similar of functional group stretching. Onoji et al. (2016) and Ma et al. (2015) had reported similar peak intensity pattern in chemical treated LCB, hence validating LCB decomposition. SEM images revealed disruption and erosion of the lignocellulosic matrix, while FTIR spectra indicated modifications in functional groups associated with lignin, cellulose, and hemicellulose. These findings support previous research by Shirkavand et al. (2017), who documented comparable structural changes after Trametes-mediated

degradation**.** Collectively, the results demonstrate that *Trametes hirsuta* PSRF29 is a highly efficient lignocellulose-degrading organism capable of acting on complex, mixed biomass substrates within a relatively short incubation period. Its dual ability to effectively colonize and degrade diverse lignocellulosic materials underscores its potential for sustainable biomass management. This strain could be further exploited for the development of value-added products such as mycobricks, mycoboards, mycofoam, and biofertilizers, thereby contributing to circular bioeconomy initiatives and environmentally sustainable waste valorization strategies**.**

5. Conclusions

The present study establishes that the isolate RDS-23, identified as *Trametes hirsuta* PSRF 29, is a highly efficient lignocellulose-degrading fungus with significant biotechnological potential. The organism demonstrated the ability to produce a comprehensive spectrum of lignocellulolytic enzymes, enabling the effective and independent degradation of complex lignocellulosic biomass without the need for microbial consortia. The use of a multi-substrate system comprising diverse agricultural residues provided a realistic representation of natural biomass conditions and highlighted the adaptability and robustness of the fungal strain. The substantial reduction in lignin, cellulose, and hemicellulose content, along with decreased phenolic compounds, confirms the strong degradative capability of *T. hirsuta*. Enzymatic activity levels observed in this study were comparable to or exceeded those reported in previous studies, indicating the superior efficiency of the isolate. Furthermore, structural and chemical analyses through SEM and FTIR validated the breakdown of lignocellulosic architecture. Overall, the findings underscore the potential of *Trametes hirsuta* as a promising candidate for sustainable biomass valorization. Its application could be extended to the production of eco-friendly, value-added materials such as mycocomposite, as well as in waste management and bioconversion processes. This study contributes to advancing circular bioeconomy approaches by providing an efficient biological alternative for lignocellulosic biomass recycling.

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Data Availability

All of the data that support the findings of this study are available in the main text

Declarations

Conflict of interest The authors declare that no conflict of interests exist in relation to the published work

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