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RESEARCH ARTICLE

Optimizing agro-waste substrates for enhanced enzymatic production in ethnomedicinal mushroom *Lentinus squarrosulus* Mont.

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Abstract

The ethnomedicinal subtropical mushroom *Lentinus squarrosulus* Mont. is a polypore with high wood-degrading potential. This study aimed to collect *L. squarrosulus* isolates, analyze lignocellulolytic enzymes - Laccase (Lac), Lignin peroxidase (LiP) and Manganese peroxidase (MnP) and evaluate substrates for mass production. Sporophores of *Lentinus sp.* (Isolate L1 to L5) were collected from different regions of Tamil Nadu and characterized morphologically and molecularly. Qualitative screening tests with guaiacol substrate demonstrated the production of laccase by all the isolates; however, isolate L3 showed maximum reddish-brown zonation. Additionally, isolate L3 produced the highest green colouration on the ABTS (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) substrate. This confirmed the oxidation of ABTS to ABTS-azine in the presence of laccase, outperforming the other *L. squarrosulus* isolates. All the isolates tested with Azure-B agar for LiP and MnP showed faint discolouration, suggesting lower secretion of peroxidase enzymes. Quantitative analysis using a lignolysis basal medium also confirmed that isolate L3 secreted Lac predominantly, followed by LiP and MnP enzymes extracellularly. All isolates showed peak Lac activity at pH 4.5, while LiP and MnP production were highest at pH 5.0. The optimal temperature for all lignolytic enzymes was 28±2°C. Maximum laccase secretion occurred between the 5th - 7th day after inoculation (DAI), with LiP and MnP peaking at 7 DAI. Nine agro-waste substrates were evaluated, with isolate L3 showing the highest biological efficiency on Paddy straw (99.62%), followed by Arecanut sheath (97.43%) and Groundnut shell (95.15%). This study enhances our understanding of *L. squarrosulus* enzymatic capabilities. It provides insights for mass production, which has strong potential for industrial bioremediation and waste management due to its high laccase production.

Keywords

agricultural waste; ethnomedicinal mushroom; laccase; mass production; peroxidase

Introduction

Edible mushrooms are prized worldwide for their distinct flavor, nutritional composition and therapeutic benefits (1). Despite not being a staple in most

diets, their consumption remains steady due to their significant nutritional advantages (2). Mushrooms are rich in proteins, vitamins B and D, minerals, carbohydrates and fibers (3). Numerous studies have demonstrated that edible mushrooms contain bioactive compounds with diverse health-promoting effects, including antiulcer, antioxidant, antiviral, anticancer, antibacterial, hepatoprotective, immunomodulatory antitumor, anticholesterol, anti-inflammatory and antihaemorrhagic activities (4). *L. squarrosulus* is one among the edible mushrooms considered for its ethnomedicinal values mainly used in Asia and Africa. It is traditionally used to treat anaemia, ulcer, fever, cough, infertility and fungal infections and it is also believed to reduce the risk of metabolic diseases (5-7).

L. squarrosulus, a member of the *Polyporaceae* family, thrives on the decaying trees in subtropical conditions (8). It shows a natural preference for degrading various lignocellulosic materials, particularly agricultural wastes. Due to its ability to degrade lignocellulosic materials, *L. squarrosulus* is a promising candidate for bioconversion processes in industries focused on biomass utilization for biofuel production or bioremediation. It produces diverse enzymes that facilitate the breakdown of complex lignocellulosic substrates. The primary enzymes involved are cellulases, hemicellulases including xylanases, which play a crucial role in the bioconversion of lignocellulosic materials by specifically targeting the hemicellulose component of biomass and lignin-modifying enzymes like laccases and peroxidases. Cellulases break down cellulose into simple sugars, while xylanases convert hemicellulose into sugars (9). Lignin polymers are covalently linked to the structural polysaccharides, making it difficult for carbohydrate-acting enzymes (CAZymes) to hydrolyze cellulose and hemicellulose (10). The production of three main lignocellulolytic enzymes -laccase, LiP and MnP-by a single organism suggests its potential to break down lignocellulose into simple sugars for other value-added products. Several wood-decaying fungi are known to have lignolytic solid machinery for lignin degradation by producing combinations of extracellular ligninolytic enzymes like Laccase (Lac), Lignin peroxidase (LiP), Manganese peroxidase (MnP), versatile peroxidase (VP), Aryl alcohol oxidases, Glycol oxidases (11).

Evaluating agricultural waste substrates for *L. squarrosulus* cultivation addresses economic and environmental concerns (12). Utilizing waste materials as growth substrates reduces cultivation costs and contributes to sustainable waste management practices (13). Assessing parameters such as mycelial growth rate, fruiting body initiation time and overall yield provides valuable information for scaling up production (14).

Hence, this addresses the lack of research on *L. squarrosulus* lignolytic secretion using quantitative and qualitative methodologies to measure Lac, LiP and MnP production at various pH levels, temperature and time intervals. This work establishes the foundation for the mass production of *L. squarrosulus* by selecting high-performing isolates.

Materials and Methods

Morphological and molecular characterization of *Lentinus* sp. isolates

Five isolates of ethnomedicinal mushroom *Lentinus* sp. were collected from distinct sites across Tamil Nadu, India and identified morphologically based on stipe colour, pileus character, gills attachment and presence of squamules (15).

The obtained *Lentinus sp.* isolates were cultured in potato-dextrose broth (PDB). The mycelial mat was then ground with lysis buffer for DNA extraction and the isolated DNA was subjected to PCR amplification as per the method (16). The size of amplified products was determined using a molecular weight marker on a 2% agarose gel followed by electrophoresis. The product was sent for sequencing to Biokart India Ltd., Bengaluru, India. The partial 18s rRNA sequence was compared with deposited sequences in the National Centre for Biotechnology Information (NCBI) database of the BLAST algorithm. The nucleotide sequences of *L. squarrosulus* were then submitted to GenBank to obtain accession numbers.

Qualitative screening tests for secretion of ligninolytic enzymes

Qualitative assays for the secretion of lignolytic enzymes such as Laccase (Lac), Lignin peroxidase (LiP), and Manganese peroxidase (MnP) of *L. squarrosulus* were tested as per (17). Laccase assay was performed on potato-dextrose agar (PDA) plates amended with 0.02% guaiacol, 0.1% ABTS (2,2'- azino-bis (3- ethylbenzthiazoline-6-sulfonic acid) and 0.1% syringaldazine. Each substrate was inoculated with the 9 mm mycelial disc of *L. squarrosulus* and incubated at 28±2°C for 7 days. Laccase activity was confirmed by the oxidative polymerization of guaiacol, forming reddish-brown zones in the medium. Similarly, in the ABTS medium, laccase production was indicated by the formation of a green colour, resulting from the oxidation of ABTS to ABTS-azine.

Peroxidase (LiP and MnP) assays were conducted using the AzureB agar clearance method on lignin-modifying enzyme basal medium supplemented with 0.01% w/v Azure B and 1.6 % w/v agar. Plates were inoculated with a 9 mm mycelial disc of *L. squarrosulus* and examined over 10 days. Peroxidase production (LiP and MnP) was recorded with the clearance of the blue-coloured medium, indicating enzyme activity.

Quantitative assessment of ligninolytic enzymes at different pH, temperature and time interval

Production of Lac, LiP and MnP by *L. squarrosulus* was evaluated under *in vitro* conditions at different day intervals, temperatures and pH. The prepared PDB served as a basal medium and a 9 mm mycelial disc of *L. squarrosulus* was inoculated. The laccase activity was assayed using 2,2'-azino-bis (3-ethylbenzthiazoline-6- sulfonic acid) (ABTS) as the substrate (18). The assay for the LiP activity was estimated using the method of (19) with pyrogallol as substrate. The MnP activity was assayed using the method of (20) with sodium malonate as a substrate. The secretion of lignolytic enzymes was assessed at different pH (3.5, 4.0,

4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) and at different temperatures (20, 25, 30, 35, 40, 45, 50 and 55°C) and different time interval (1 to 15 day).

Evaluating different agricultural waste substrates for cultivation of *L. squarrosulus*

This study employed the effectiveness of nine different agricultural waste substrates for mushroom cultivation. Paddy straw, Arecanut sheath, Lawn grass, Sawdust, Groundnut shell, Sugarcane bagasse, Corn cob, Garlic peel and Palm oil bunches were prepared by drying, cutting or shredding into small pieces, moistened to approximately 70% moisture content and sterilized at 121°C for 45 minutes. The substrates were placed in polypropylene bags (12x24 inch) in alternative layers of *L. squarrosulus* (isolate - L3) spawn at 10%. The bags were maintained at 28±2°C, 85±5% relative humidity and incubated for three weeks. The growth parameters viz., DFSR - days for spawn run, DFPI - days for pinhead initiation, AWOS - average weight of sporophore, BE -biological efficiency and total yield were determined (21).

Statistical Analysis

The experimental design, i.e. CRD and statistical analyses, adhered to Gomez and Gomez's recommendations (22). The statistical software used for the analysis of data is AGRES.

Results

Morphological and Molecular characterization of *Lentinus* sp. isolates

The study revealed distinct morphological characterization of five isolates of *L. squarrosulus* collected from different sites across Tamil Nadu, indicating potential genetic diversity within the species (Fig. 1). The observed variations suggest adaptability to diverse environmental conditions and ecological niches. Geographic location, substrate composition and climate may significantly influence the morphological traits of *L. squarrosulus*. Differences in pileus colour, stipe length, gill attachment, presence of squamules suggest that the species may exhibit significant genetic diversity. Molecular analysis showed amplification of genomic DNA at approximately 560bp for all isolates using the ITS1/ITS4 primer pair (Fig. 2). The sequences were deposited in the GenBank database and percent identity with the available sequences is shown in Table 1.

Qualitative screening of lignolytic enzymes secretion by *L. squarrosulus*

The solid-state screening for Lac, LiP and MnP secretion showed positive laccase activity in all substrates amended with guaiacol, ABTS and syringaldazine. All five isolates of *L. squarrosulus* exhibited laccase production in a guaiacol-amended medium, with isolate L3 showing the largest reddish-brown zone (Fig. 3).

In ABTS and syringaldazine amended medium, all isolates produced greenish-blue and light-yellow colourations, respectively, with isolate L3 showing the largest zones. Compared to syringaldazine, a more robust reaction for laccase was observed in the guaiacol and ABTS media. In the Azure B agar test, faint decolourization was observed in *L. squarrosulus,* indicating low LiP and MnP production levels.

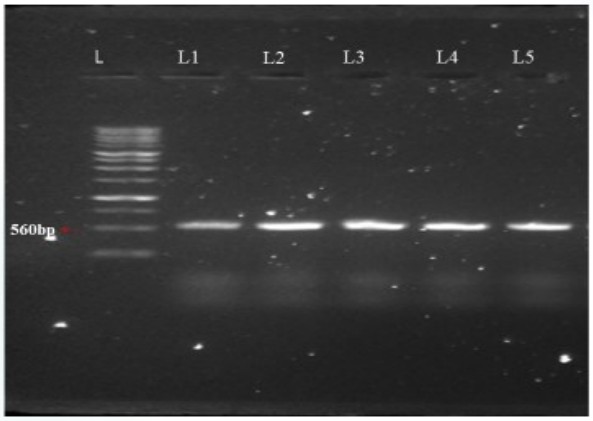


Fig. 2. Visualization of ITS region of *Lentinus sp. via* Gel Electrophoresis

PCR products amplified from the ITS Region in *L. squarrosulus* and the L-1kb ladder displayed substantial amplification in all samples (L1, L2, L3, L4 and L5), with bands of approximately 560bp in size.

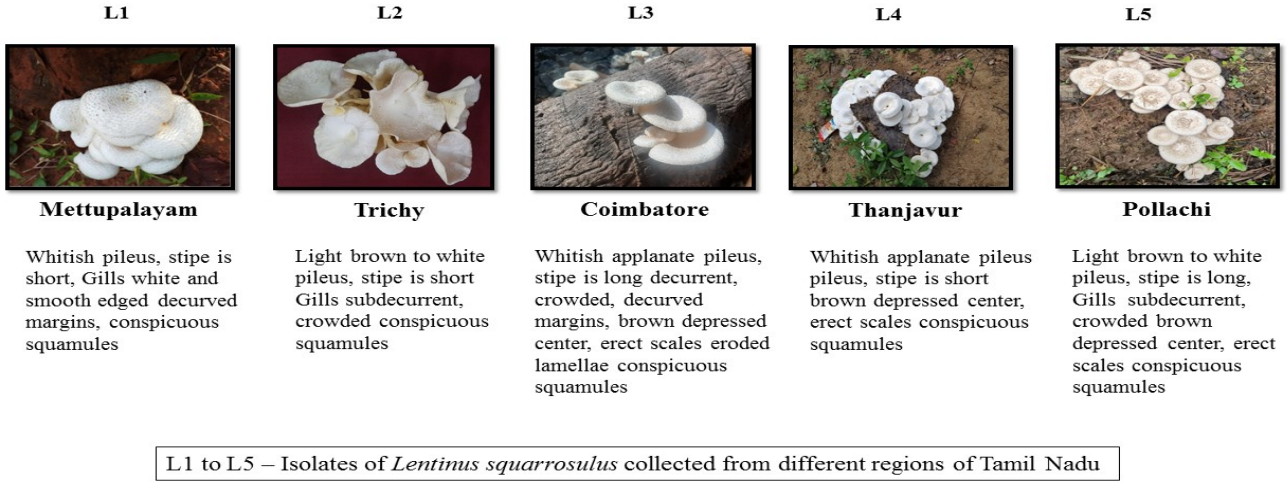


Fig. 1. Field Observations of *Lentinus sp.* Isolates

Table 1. Molecular confirmation of *Lentinus sp.* isolates

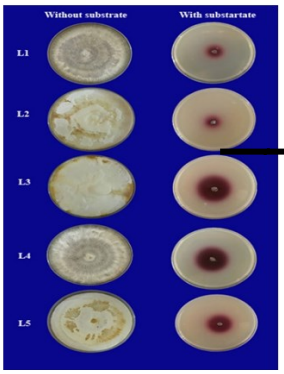
Isolates	Scientific name	Accession number	Query coverage	Percent identity
L1	*Lentinus squarrosulus*	PP479153	99	98.13
L2	*Lentinus squarrosulus*	OR177848	99	99.37
L3	*Lentinus squarrosulus*	OR077745	100	99.37
L4	*Lentinus squarrosulus*	OR574414	100	98.71
L5	*Lentinus squarrosulus*	PP479157	100	99.84

Query Coverage: This represents the percentage of the query sequence that aligns with the subject sequence in the NCBI database, indicating the extent of overlap between the two sequences. A higher query coverage suggests a more complete match.

Percent Identity: This indicates the percentage of identical nucleotides (or amino acids) between the query and matched sequences in the NCBI database. A higher percent identity reflects a closer genetic relationship, suggesting a more similar or identical sequence.

Quantitative analysis of ligninolytic enzyme

In this study, *L. squarrosulus* was cultured in a liquid medium to test for enzyme production under varying pH, temperature and time intervals to identify optimal conditions for lignolytic enzyme secretion. Laccase secretion was highest in isolate L3 (0.60 U/min/ml), followed by isolate L4 (0.59 U/min/ml) at a pH of 4.5. LiP and MnP activity was highest in isolate L3 at a pH of 5.0, with values of 0.069 U/min/ml and 0.197 U/min/ml, respectively (Table 2). Maximum Lac, LiP and MnP activity was recorded at 28±2°C (Fig. 4). Laccase secretion was noticed in isolate L3 peaked on the 5th (0.69 U/min/ml) and 7th (0.65 U/min/ml) DAI. LiP (0.059 U/min/ml) and MnP (0.199 U/min/ml) secretion peaked on the 7th DAI in isolate L3 (Fig. 5).



Reddish brown zonation, indicates the laccase enzyme production

- L1 to L5 - Isolates of *Lentinus squarrosulus*
- Without substrate – *L. squarrosulus* isolates axenic culture grown on PDA media as a control for the study
- With substrate - Reddish-Brown colour zone formation in PDA amended media with guaiacol indicates the presence of Laccase enzyme activity in *L. squarrosulus* isolates

Fig. 3. Secretion of laccase enzyme by *L. Squarrosulus* isolates in guaiacol-amended medium

Evaluating different agricultural waste substrates for mass production of *L. squarrosulus*

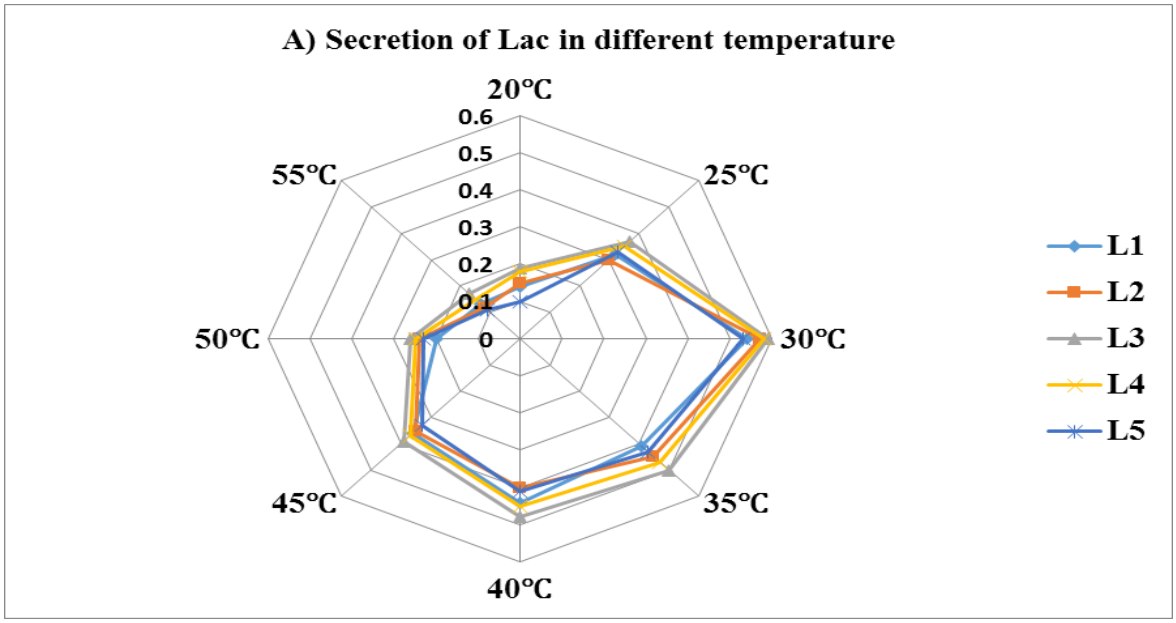
As shown in Table 3 and Fig. 6, paddy straw was the most effective substrate for mushroom cultivation, yielding 498.12 g/500g substrate with a biological efficiency (BE) of 99.62%. This was followed by an arecanut sheath, with a total yield (487.19 g/500g substrate) and BE of 97.43%. In contrast, garlic peel and palm oil bunches yielded 409.38 g/500g substrate (BE 81.87%) and 400.38 g/500g substrate (BE 80.07%), respectively, demonstrating lower yields and BE values. Overall, paddy straw was the most suitable substrate, while alternative substrates like arecanut sheath and groundnut shell were also influential.

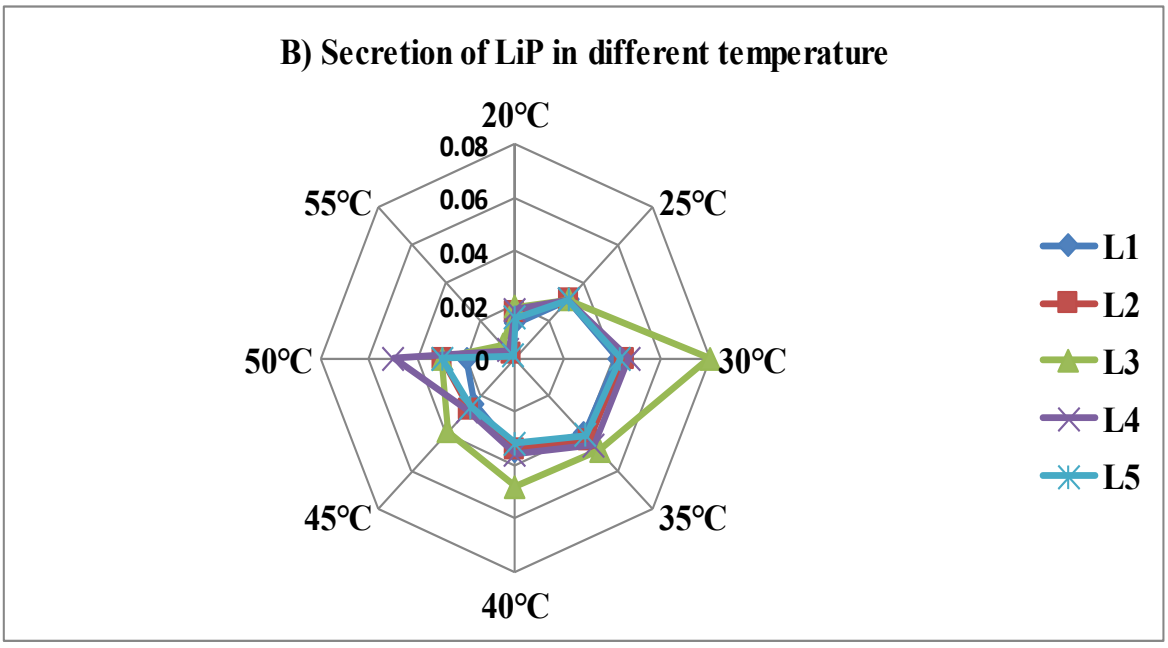
Table 2. Lignolytic enzyme production by *L. squarrosulus* isolates at different pH

pH	Lac (U/ml/min)					LiP (U/ml/min)					MnP (U/ml/min)				
	L 1	L2	L3	L4	L5	L 1	L2	L3	L4	L5	L 1	L2	L3	L4	L5
3.5	0.37^{e}	0.35^{f}	0.38^{f}	0.30^{g}	0.33^{e}	0.029^{d}	0.033^{f}	0.036^{g}	0.034^{e}	0.037^{e}	0.121^{e}	0.123^{g}	0.127^{e}	0.126^{g}	0.124^{g}
4.0	0.45^{b}	0.47^{c}	0.49^{cd}	0.46^{e}	0.48^{c}	0.045^{b}	0.043^{c}	0.049^{c}	0.046^{c}	0.042^{d}	0.177^{b}	0.174^{c}	0.178^{b}	0.176^{c}	0.175^{c}
4.5	0.55^{a}	0.58^{a}	0.60^{a}	0.59^{a}	0.58^{a}	0.068^{a}	0.062^{b}	0.060^{b}	0.065^{a}	0.063^{b}	0.188^{a}	0.183^{b}	0.189^{a}	0.186^{b}	0.184^{b}
5.0	0.53^{a}	0.56^{a}	0.59^{a}	0.54^{b}	0.57^{a}	0.065^{a}	0.064^{a}	0.069^{a}	0.061^{b}	0.067^{a}	0.193^{a}	0.192^{a}	0.197^{a}	0.195^{a}	0.190^{a}
5.5	0.54^{a}	0.53^{b}	0.55^{b}	0.52^{c}	0.51^{b}	0.046^{b}	0.044^{c}	0.047^{d}	0.048^{c}	0.045^{c}	0.161^{c}	0.164^{d}	0.169^{b}	0.167^{d}	0.166^{d}
6.0	0.44^{bc}	0.46^{cd}	0.50^{c}	0.49^{d}	0.47^{c}	0.044^{c}	0.040^{d}	0.041^{e}	0.042^{d}	0.044^{cd}	0.143^{d}	0.141^{e}	0.148^{c}	0.145^{e}	0.144^{e}
6.5	0.42^{cd}	0.44^{de}	0.48^{d}	0.45^{e}	0.43^{d}	0.033^{d}	0.035^{e}	0.038^{f}	0.036^{e}	0.031^{f}	0.140^{d}	0.146^{e}	0.149^{c}	0.147^{e}	0.144^{e}
7.0	0.40^{d}	0.43^{e}	0.46^{e}	0.41^{f}	0.42^{d}	0.021^{e}	0.023^{g}	0.029^{h}	0.025^{f}	0.026^{g}	0.134^{d}	0.130^{f}	0.138^{d}	0.135^{f}	0.132^{f}
SEd	**0.025**	**0.036**	**0.04**	**0.12**	**0.08**	**0.081**	**0.03**	**0.05**	**0.07**	**0.025**	**0.036**	**0.031**	**0.024**	**0.04**	**0.036**
CD (0.05%)	**0.021**	**0.024**	**0.013**	**0.019**	**0.016**	**0.005**	**0.002**	**0.002**	**0.002**	**0.003**	**0.009**	**0.007**	**0.009**	**0.006**	**0.006**

The values represent the means of three replications. Values with the same letter are not significantly different according to DMRT (P=0.05).

Lac - Laccase, LiP - Lignin peroxidase, MnP - Manganese peroxidase.





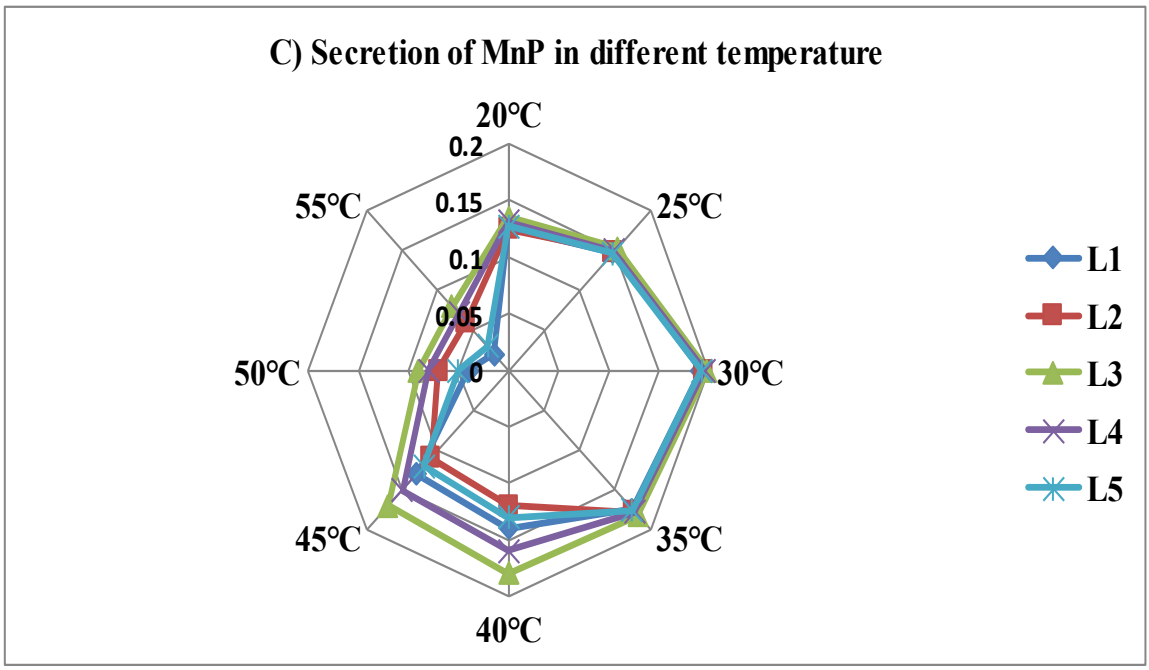


Fig. 4. Secretion of ligninolytic enzymes by *L. squarrosulus* isolates at different temperatures (A) Lac production; (B) LiP production; (C) MnP production

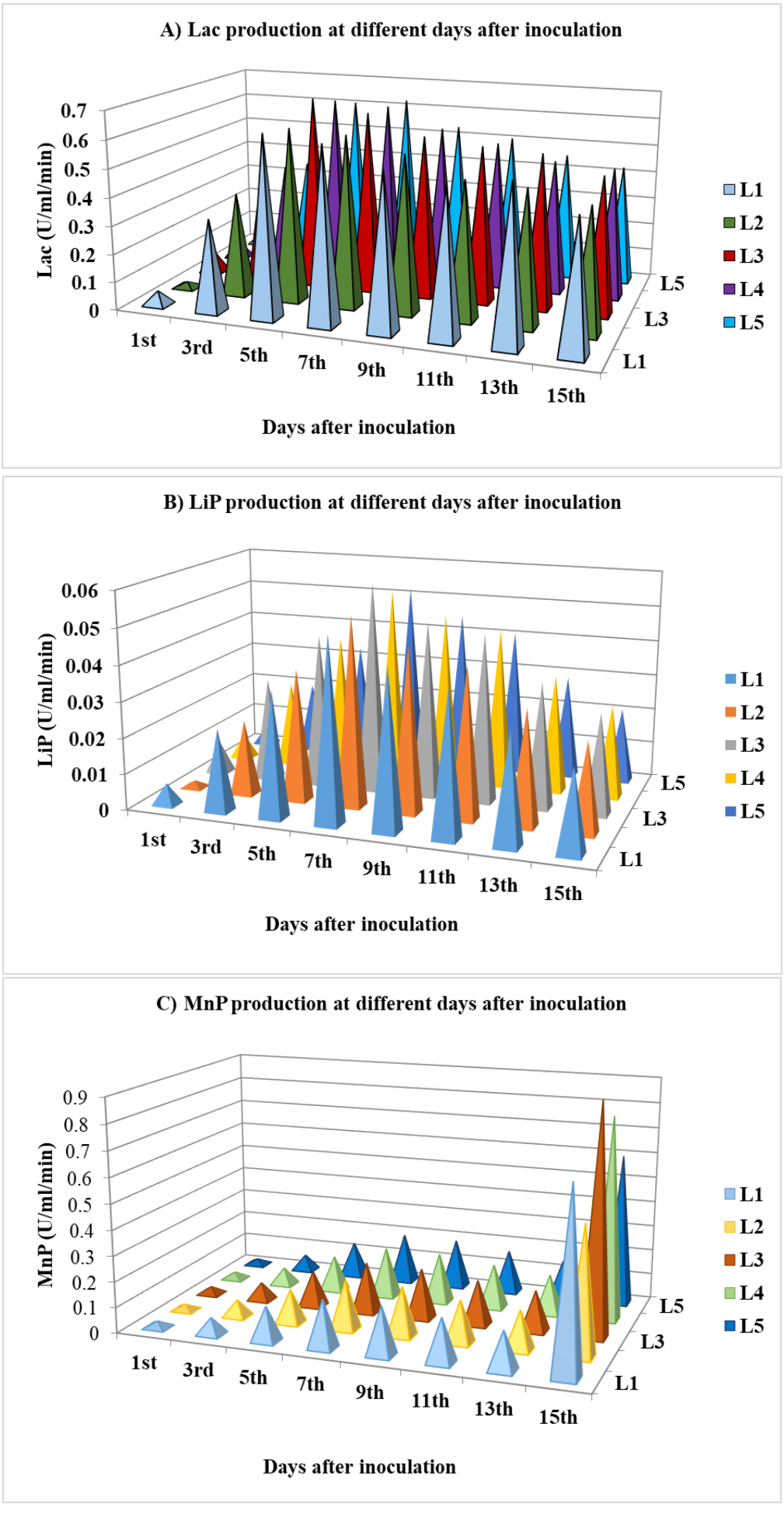


Fig. 5. Secretion of ligninolytic enzymes by *L. squarrosulus* isolates on different days after inoculation (A) Lac production; (B) LiP production; (C) MnP production

Table 3. Evaluating different agricultural waste substrates for mass production of *L. squarrosulus* (Isolate L3)

Treatments	DFSR	DFPI	AWOS	Total Yield (g) / 500g substrate	BE (%)
Paddy straw	10.08a	12.68a	19.89a	498.12a	99.62a
Arecanut sheath	11.24a	13.64a	18.21b	487.19ab	97.43ab
Groundnut shell	13.92bc	15.42b	17.89b	475.79abc	95.15abc
Lawn grass	13.36b	15.76b	17.64b	470.99abcd	94.19abcd
Corn cob	15.09c	17.19c	16.22c	459.23bcd	91.84bcd
Sugarcane bagasse	14.96c	18.06d	15.87c	441.12cde	88.22cde
Saw dust	16.73d	18.86d	13.59d	438.97de	87.79de
Garlic peel	18.81e	19.41e	12.02de	409.38ef	81.87ef
Palm oil bunches	18.49e	20.33e	12.63e	400.38f	80.07f
SEd	**0.18**	**0.66**	**0.62**	**17.51**	**3.50**
CD (0.05%)	**1.22**	**1.39**	**1.31**	**36.80**	**7.35**

The values represent the means of three replications. Values with the same letter are not significantly different according to DMRT (P=0.05).

DFSR - Days for spawn run, DFPI - Days for pinhead initiation, AWOS - Average weight of sporophore, BE - Biological efficiency.

Fig. 6. Selection of suitable substrates from agricultural waste for mass production of *L. squarrosulus* (isolate L3)

Discussion

Mushroom cultivation supports sustainable practices by utilizing agro-waste substrates, such as crop residues and sawdust, thus promoting eco-friendly farming and waste recycling (15). As a sub-tropical species, *L. squarrosulus* offers small-scale farmers and local communities a lucrative opportunity for additional income.

The morphological differences among the *L. squarrosulus* isolates suggest potential genetic diversity, likely influenced by environmental factors such as geographic location, substrate composition and climate. These factors affect fungal traits, including pileus colour, stipe length, gill attachment and squamules. These outcomes were similar to a study on *Lentinula edodes* (shiitake mushroom), which demonstrated that strains from different geographic regions exhibited significant variations in fruiting body morphology, which were linked to environmental conditions (16)

Molecular techniques, specifically the amplification of genomic DNA using the ITS1/ITS4 primer pair, provided valuable genetic insights into the *L. squarrosulus* isolates. The successful amplification of a 560bp fragment suggests that these primer pairs effectively targeted the internal transcribed spacer (ITS) region, a key marker in fungal taxonomy and phylogenetic studies. The sequences were compared to existing entries in the GenBank database to determine genetic identity and relationships with other *Lentinus* species. Similar studies on *Pleurotus* species have used primers for ITS sequences to identify the mushroom and to differentiate between closely related taxa and phylogenetic relationships (17).

Screening of lignolytic enzyme production in basidiomycetes, including *L. squarrosulus*, is essential for identifying suitable laccase-producing organisms for industrial applications. Effective screening strategies aim to find fungal strains capable of functioning under industrial conditions. Fungi are typically screened on solid media containing colorimetric indicators or liquid cultivations monitored for enzyme activity. Our study used synthetic phenolic reagents like guaiacol and syringaldazine as indicators, replacing traditional reagents such as gallic acid and tannic acid (11).

The assay results showed positive laccase production by *L. squarrosulus* f in all three substrates: guaiacol, ABTS and syringaldazine. The laccase assay with guaiacol showed the oxidative polymerization of guaiacol, forming a reddish-brown zone, indicating laccase production. In ABTS medium, an intense greenish-blue colour was observed in isolate L3, confirming laccase activity. Although laccase can oxidize various substrates, such as phenolic compounds and aromatic amines, indicators like guaiacol are commonly used for detecting laccase production (18). Inducers like ABTS also result in a green colour due to the oxidation of ABTS to ABTS-azine by laccase.

Among the three enzymes, laccase is the most abundantly produced by *L. squarrosulus,* while LiP and MnP are made at lower levels. Similar results were obtained with *Pleurotus pulmonarius*, *P.sajor-caju* and *Phanerochaete chrysosporium* where laccase was also the predominant enzyme (19). In addition, a high level of laccase enzyme production was observed in *L. squarrosulus,* and it also is noticed that minimum levels of LiP and MnP were produced. These enzymes are significant in quantities for the decolourization of azo and textile dyes and for lignin degradation (20, 23).

The production of Lac, LiP and MnP by *L. squarrosulus* was evaluated on different days after inoculation (DAI), as well as temperatures and pH under *in vitro* conditions. Enzyme expression varied depending on medium composition and culture conditions. Fermentation parameters such as cultivation time, culture type (stationary or submerged), concentrations of organic and inorganic compounds, inducer, aeration and protease degradation all significantly impact laccase production. White-rot fungi have varying physiological requirements and many studies have examined the effects of agitation, pH, temperature, carbon, nitrogen sources and microelements concentrations. In nature, the colonization of white rot fungi depends on substrate composition, the secretion of lignolytic enzymes, pH and the substrate's prevailing temperature. Studying the extracellular secretion of the major enzymes, viz., Lac, LiP and MnP under optimal pH and temperature conditions over time could enhance their production for industrial applications, such as biodegradation and bioremediation (6).

The results of different pH, temperature and DAI evaluated for lignolytic enzyme secretion by *L. squarrosulus* revealed that the L3 isolate recorded the highest Lac secretion at pH 4.5, temperature 28±2°C and 5th DAI. LiP and MnP activities peaked at pH 5.0, temperature 28±2°C and 7th DAI. Perusal of the literature also shows that the majority of white rot fungi grow optimally at acidic pH 4 - 4.5 (21). Temperature also plays a crucial role in the growth of mycelium and enzyme production, both of which influence the substrate decolourization rate by enzymes. Although enzyme secretion for dye decolourization may increase at higher temperatures, enzyme degradation must also be considered, as it varies among different fungal species. An optimal temperature range of 27-30°C is deemed suitable to enzyme production as observed by (19), who reported that *Pleurotus pulmonarius*, *P. sajor-caju and Schizophyllum commune* produced Lac, LiP and MnP at 30°C. In addition, *P. pulmonarius* and *P. sajor-caju* showed significant Lac activity on the 4th day after inoculation, while LiP and MnP peaked on the 7th and 8th days. For *S. commune*, Lac activity was highest on the 10th to 11th days, with LiP and MnP peaking on the 6th and 7th days.

Mushroom cultivation is an excellent approach to utilizing agricultural waste. For cost-effective cultivation, it is important to use locally, easily accessible and seasonally abundant agro-wastes. This study revealed that paddy straw was the most efficient in increasing the yield and sporophore formation of *L. squarrosulus* among various agro-wastes. This may be due to the substrate's higher availability of nitrogen, carbon and minerals. Similar results were also obtained by Mago et al. (24) found paddy straw to be the best substrate for the cultivation of *P. florida* and *P. sajor-caju*. Another study reported a spawn run period of 15 days and a biological efficiency of 156% for *H. ulmarius* grown on paddy straw (25). The lower performance of substrates such as garlic peel and palm oil bunches align with the findings that indicate substrate composition significantly impacts mushroom productivity. The structural composition of these substrates can also hinder enzymatic action; for example, overly complex lignocellulosic structures or high lignin content can impede degradation.

Moreover, these substrates might not retain moisture effectively, impacting the ideal growing conditions for the mushrooms (26). Thus, while paddy straw remains the optimal choice, other substrates may require additional processing or supplementation to enhance their efficacy. This study reinforces the importance of substrate selection in optimizing mushroom cultivation and suggests areas for further research to improve less effective substrates.

Overall, this research contributes to the current understanding of *L. squarrosulus* and provides a foundation for its industrial cultivation and application in sustainable waste management and biotechnological processes, such as bio-remediation and lignocellulose degradation.

Conclusion

This research provided critical insights into enzymatic and cultivation characteristics of the ethnomedicinal mushroom, *L. squarrosulus*, highlighting its considerable industrial potential. Morphological and molecular studies revealed genetic diversity among isolates, with isolate L3 showing significantly higher laccase production than the others. Quantitative assays confirmed that laccase was the primary enzyme secreted, with optimal activity at pH 4.5 and temperature 28±2°C. These conditions highlight its potential for application in bio-remediation and lignocellulose degradation. Paddy straw showed the highest biological efficiency of the nine agricultural waste substrates tested for mass production. Other substrates, such as arecanut sheath and groundnut shell, were also effective but less so than paddy straw. These results emphasize the importance of proper substrate selection and growth condition standardization in optimizing mushroom production with reducing costs. Overall, this research contributes to the current understanding of

L. squarrosulus and provides a foundation for industrial cultivation and application in sustainable waste management and biotechnological processes, such as bio-remediation and lignocellulose degradation.

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Authors' contributions

KGS - Methodology, Investigation and Writing original draft; GT - Conceptualization, Supervision, Writing - Review and Editing; KA, TP, NR and RA and MJ - Review and Editing

Compliance with ethical standards

Conflict of Interest: Nil.

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