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Enhanced Mushroom Cultivation Using Oil Palm Empty Fruit Bunch Waste: Insights from Transcriptomic and Lignocellulose Degradation Studies

(Peningkatan Hasil Pengkulturan Cendawan Menggunakan Tandan Kosong Kelapa Sawit: Pemerhatian daripada Kajian Transkriptomik dan Penguraian Lignoselulosa)

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ABSTRACT

Volvariella volvacea is a nutritional and pharmacologically valuable tropical mushroom. Yet, its potential as a sustainable food source and bioremediation agents remains underutilised. This study evaluated the efficiency of oil palm empty fruit bunches (OPEFB) as a lignocellulosic substrate to enhance the growth and biological efficiency (BE) of *V. volvacea*. Among the tested formulations, F3 (OPEFB supplemented with black soil) produced the highest yield (11 kg) and a BE of 17.75%. F3 also showed a high cellulose degradation rate (34.14%) and reduced lignin content (5.55%). Transcriptomic profiling showed that key lignocellulolytic genes were strongly expressed during the pinhead (Stage 1) developmental stage, correlating positively with the improved substrate conversion observed in F3. Overall, the findings highlight the synergistic interaction between fungal and soil-associated microbial communities in promoting enzymatic degradation and advancing sustainable utilisation of OPEFB for mushroom cultivation.

Keywords: Agricultural waste valorization; bioeconomy; empty fruit bunch fibre; *Volvariella volvacea*

ABSTRAK

Volvariella volvacea ialah cendawan tropika yang mempunyai nilai nutrisi dan farmakologi yang tinggi. Namun, potensinya sebagai sumber makanan lestari dan agen bioremediasi masih belum dimanfaatkan sepenuhnya. Kajian ini menilai keberkesanan tandan kosong kelapa sawit (OPEFB) sebagai substrat lignoselulosa untuk meningkatkan pertumbuhan dan kecekapan biologi (BE) *V. volvacea*. Daripada semua formulasi yang diuji, F3 (OPEFB yang ditambah tanah hitam) menghasilkan hasil tertinggi (11 kg) dan BE sebanyak 17.75%. F3 juga menunjukkan kadar degradasi selulosa yang tinggi (34.14%) dan kandungan lignin yang rendah (5.55%). Profil transkriptomik menunjukkan bahawa gen lignoselulolitik utama diekspreskan dengan ketara pada peringkat pinhead (Peringkat 1) dan pengekspresan ini berkait rapat dengan peningkatan kecekapan penukaran substrat yang diperhatikan dalam F3. Secara keseluruhannya, keputusan ini menonjolkan interaksi sinergistik antara kulat dan komuniti mikrob tanah dalam merangsang degradasi enzim dan memajukan penggunaan OPEFB secara lestari untuk penanaman cendawan.

Kata kunci: Bioekonomi; peningkatan nilai sisa pertanian; tandan buah kelapa sawit kosong (OPEFB); *Volvariella volvacea*

INTRODUCTION

Volvariella volvacea, commonly known as the rice straw mushroom, is valued for its nutritional and pharmacological properties. Yet, its commercial production remains limited due to low yield and biological efficiency. As a result, optimisation of substrate composition is essential to

enhance its cultivation potential. Malaysia, being one of the world's largest producers of oil palm, generates substantial quantities of OPEFB. OPEFB is a lignocellulosic by-product rich in cellulose and hemicellulose. These characteristics make OPEFB a promising substrate for mushroom cultivation and an attractive candidate for agricultural waste valorisation.

Current interest in OPEFB highlights studies related to substrate amendments, such as soil, organic matter, and nutrient-based supplements. Such amendments can promote microbial synergy and improve lignocellulose degradation, thereby supporting fungal growth. Recent transcriptomic advances also provide insights into how fungi regulate lignocellulolytic enzyme expression across developmental stages. In this study, the focus aimed to integrate the cultivation performance along with the transcriptomic profiling. Research objectives involved the following: (i) to evaluate the effectiveness of OPEFB-based substrate formulations; (ii) to determine lignocellulose degradation patterns and identify key genes and pathways associated with substrate bioconversion. The findings contribute to improved cultivation strategies for *V. volvacea* and further support sustainable utilisation of palm oil agricultural residues.

MATERIALS AND METHODS

The experimental workflow is outlined in Figure 1. The first phase involved the optimisation of formulation media for growth, whereas the second phase involved analysing lignocellulolytic degradation and the lignocellulolytic pathway using transcriptome data.

PREPARATIONS OF SPAWN AND OPEFB PELLETS SUBSTRATE

The OPEFB pellets were purchased from a local supplier as raw material (Ecobed Sdn. Bhd., Puchong, Malaysia). The spawn was purchased from local farmers in Kampung Musa Pedu, Kuala Nerang, District Kedah State, Malaysia. Preparation of spawn and OPEFB pellet substrate followed the methods described in Amir et al. (2025). To prepare the substrate, water was mixed with the OPEFB pellets at a ratio of 1:1.5. The mixture was left to stand for about 24 h to allow the pellets to soften. The cultivation of *V. volvacea* on the OPEFB pellet substrate was carried out the following day at a ratio of 250 g of spawn per 1 kg of substrate.

SUBSTRATE COMPOSITIONS FOR THE CULTIVATION OF *Volvariella volvacea*

Four substrate formulations were used for the study, which were prepared and standardised for the cultivation process (Table 1). Cultivation was carried out in plastic dishes using 2 kg of OPEFB pellets for each formulation used and for each replicate. All experiments were carried out in duplicate to check the reproducibility of the results (Ahlawat & Tewari 2007). The reason for choosing these formulations was to evaluate the differences in nutrient availability, microbial cooperation, and bioconversion efficiency.

CULTIVATION AND MEASUREMENT PRODUCTIVITY OF *Volvariella volvacea*

During spawn-running, temperature was maintained at 28-35 ºC, whereas during fruiting and monitoring phases,

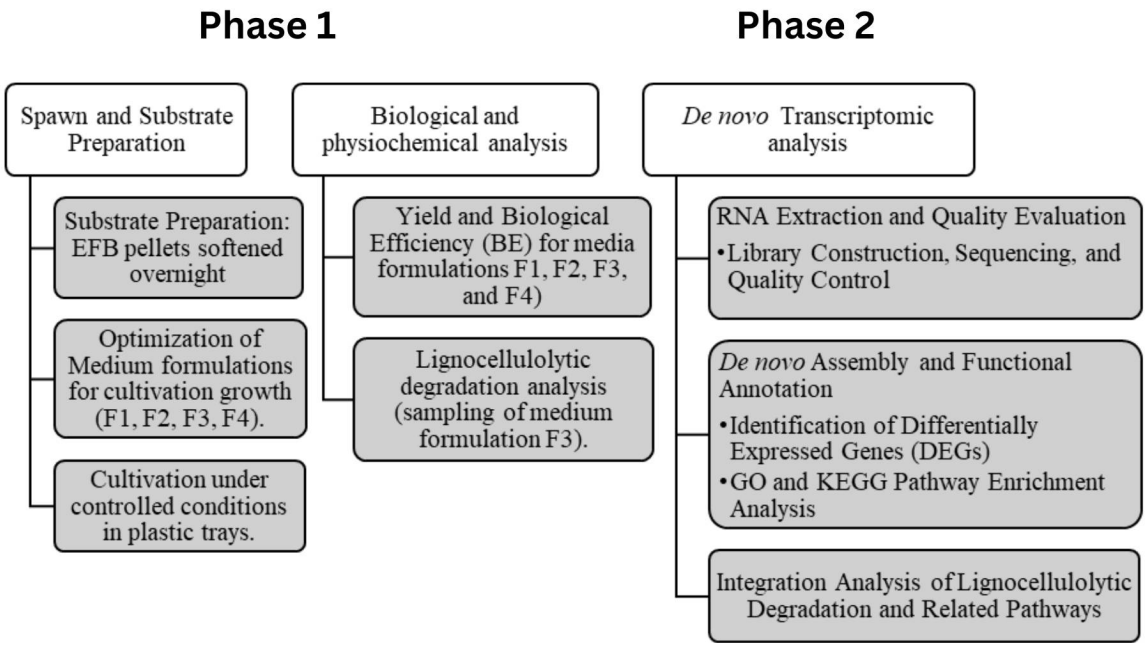

FIGURE 1. Schematic diagram of the experimental workflow for this study

TABLE 1. Substrate formulations for the cultivation of *Volvariella volvacea*

Sample	Substrate formulation
F1	2 kg OPEFB pellet (control)
F2	2 kg OPEFB pellet + 8% (w/w) rice bran + 7% (w/w) organic fertilizer + 5% (w/w) calcium carbonate (Triyono et al. 2019)
F3	2 kg OPEFB pellet + 0.5 kg black soil (proposed by this study)
F4	2 kg OPEFB pellet + oligochitosan (proposed by this study)

temperature was controlled at 23-27 ºC and relative humidity at 85-90%. The ambient relative humidity was measured using a digital hygrometer. The method for cultivation (Amir et al. 2025) and the biological efficiency (BE) of each sample were calculated using the formula from Alam and Singha (2020), as described previously.

$$\text{Biological efficiency (BE) (\%)} = \frac{\text{Total weight of fresh } \textit{V. volvacea} \text{ (kg)}}{\text{Dry weight of OPEFB substrate (kg)}} \times 100$$

The total yield of the species *V. volvacea* was measured daily during the harvest phase. The mean yield quantified the average total number of fruiting bodies harvested per OPEFB substrate growth block during the 28-day cultivation period (Ahlawat & Tewari 2007). The mean yield for each OPEFB substrate block was calculated using the following formula:

$$\text{Mean yield} = \frac{\text{Total yield of mushroom of each substrate block}}{\text{Total number of days of cultivation}}$$

LIGNOCELLULOLYTIC DEGRADABILITY ANALYSIS

The results of lignocellulolytic degradation from the optimised medium formulation with *V. volvacea* were tested using the methods for neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) as explained by Goering and Van Soest (1970). These analyses are used to estimate the amount of cellulose, hemicellulose, and lignin degraded in the OPEFB substrates. Substrate samples of 1.0 g (dry weight) were harvested weekly. Cellulose was determined from the difference between the ADF and ADL values, hemicellulose from the difference between NDF and ADF values, and lignin from the residual ADL value. These methods are widely recognised for their accuracy in assessing lignocellulosic bioconversion.

TRANSCRIPTOMIC PROFILING ANALYSIS

Transcriptomic analysis was performed using several recent approaches that have provided a detailed understanding of the specific genes involved in lignocellulose degradation and the enzymes that catalyse the process (Gruninger et al. 2023; Zhang, Liu & Zhao 2023). RNA was extracted from *V. volvacea* at two developmental stages: pinhead (stage 1) and button (stage 2) from fungi grown in the optimised media formulation. Total RNA was extracted from the collected samples while maintaining RNA quality using RNAlater (Thermo Fisher Scientific, USA). RNA isolation was carried out using TRIzol reagent, and quality and quantity were assessed using the NanoPhotometer spectrophotometer and Agilent 2100 Bioanalyzer.

Clean reads were obtained through standard QC processes, including adapter trimming, removal of low-quality reads (Q<30), and filtering of ambiguous bases. Genes related to lignocellulolytic enzymes were analysed using the following criteria: log 2-fold > 1 and padj < 0.05. Their biological functions and regulatory mechanisms were investigated based on the metabolic pathways associated with these enzymes.

STATISTICAL ANALYSIS

All statistics were performed using one-way ANOVA, and Tukey's test was used in cases where the analysis showed significant differences between means (IBM SPSS Statistics, version 22). The significance level of $p < 0.05$ was used as the threshold for statistical significance. Sequencing data from two different libraries were submitted to the NCBI database (http://www.ncbi.nlm.nih.gov/sra). These data are publicly available in the NCBI SRA database under accession numbers SRR31527688 and SRR31527687.

RESULTS AND DISCUSSION

Volvariella volvacea FRUITING BODIES FORMATION

Figure 2 shows the development of *V. volvacea* fruiting bodies starting from pinhead, button, and egg. Fruiting bodies are formed when mycelium transitions to pinheads (also known as primordia), as seen in the arrow in Figure 2(a). This is the first visible sign of fruiting bodies, starting with the pinning stage where the hyphae are clumped into hyphal knots and develop pinhead structures. The pinhead increases in size, becoming either a small round or egg-shaped button structure (Figure 2(b)). At this point, stalk and cap structures are not externally visible but are covered

completely with a wrap of a layer of tissue known as the universal veil (or volva). Then, the button continues to develop an egg (Figure 2(c)). At the egg stage, the pileus or cap starts to protrude through the veil and leaves behind an open cup-like structure (the volva) at the bottom of the garment (cross section of *V. volvacea* in Figure 2(d)). The mushroom proceeds to enlarge and stretch out, later breaking the veil entirely to reveal the fully grown cap and gills to release spores (Figure 2(e)). A closer inspection using a microscope showed the basidiospores on the inner folds of *V. volvacea* volva, as depicted in Figure 2(f).

Using different formulations, the productivity of *V. volvacea* was determined. It was found that the total mass of fruiting bodies of *V. volvacea* yielded the highest in formulation F3, followed by F4, F2, and finally F1 (Table 2). Based on the statistical analysis, the results depicted a different level of significance against all tested formulations for yield and total mass production. These results were also confirmed by the highest BE percentage of F3 (17.75%), followed by F4 (12.75%), F2 (10.9%) and the lowest BE percentage for F1 (7.45%) (Table 2). Statistically, there are also significant differences between F3 and the gross weight of F1 and F2. However, no significant differences between F1, F2, and F4. This result confirms that the formulation of culture media with F3 components increases fungal yields.

This study suggests that supplementation increases the amount of fruiting body yield, BE, and total mass of *V. volvacea*, similar as shown in Nannapaneni and Subbiah (2016). Nevertheless, rice bran nutrients, fertiliser and soil (F2), and oligochitosan (F4) could have an influence on the growth of mycelia per se. Another assumption could be attributed to the presence of the microbiome in the substrate (as the substrate used in the experiment was not sterilised prior to cultivation). This is indicated by the fact that substrate F3 contains black soil and other bacterial taxa that may play an important role in fungal synthesis. This is consistent with the statement by Noble, Dobrovin-Pennington and Hobbs (2009) that the soil bacteria, *Pseudomonas putida*, may be involved in the fructification of fungi. Liu et al. (2017) also pointed out that some microbial groups such as protobacteria, chloroflexi, bacteria, and various other soil microbes were also important for the induction of fungal primordia in the cultivation of fungi.

LIGNOCELLULOLYTIC DEGRADATION CONTENT

The degradation of cellulose, hemicellulose, and lignin varied among substrate formulations and seemed to be related to differences in mushroom growth and total production of biomass (Table 3). Formulation F3 demonstrated greater cellulose (34.14%) and lignin

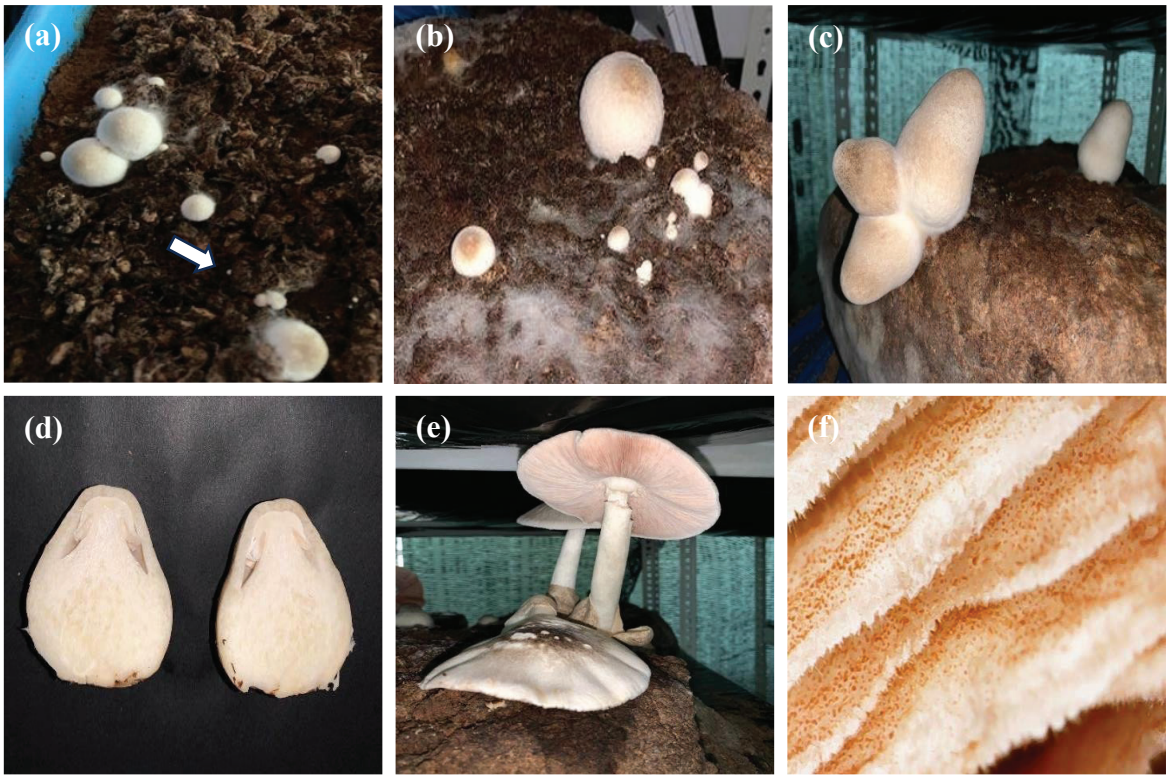

FIGURE 2. The primary developmental stages in the life cycle of *V. volvacea* from its vegetative state to a visible pinhead, button, egg, and harvestable fruiting body

TABLE 2. Yield of fruiting bodies, total mass (kg) and biological efficiency (%) of *V. volvacea* after growth in different substrate formulations

Formulation	Mean yield (Fruit Body)	Mean of mushroom mass (kg)	Biological efficiency (%)
F1	4	0.14	7.45
F2	6	0.17	10.9
F3	11	0.32*	17.75*
F4	8	0.22	12.75

*The mean difference is significant at <0.05 level

TABLE 3. Degradation of the cellulose, hemicellulose, and lignin content as a function of the different substrate formulations

Substrate formulation	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)	Yield (kg)	Total mass (kg)
F1	24.32	65.5	1.89	5	0.149
F2	19.27	81.5	4.38	8	0.218
F3	34.14	60	5.55	11	0.355
F4	22.75	46	4.91	9	0.255

degradation (5.55%). This improvement is accompanied by increased yield and total mass, indicating that a better degradation of these structural components might result in better nutrient accessibility to the mushroom. On the other hand, F2 had an increased inclination towards the degradation of hemicellulose, which was linked to moderate yield performance. Formulations F1 and F4 showed relatively reduced or more discerning degradation of substrate components, and this effect was manifested in the reduced biomass accumulation.

All these observations suggest that substrate composition is a key factor affecting the degradation behaviour of lignocellulosic components as well as the ultimate productivity of *V. volvacea*. These results imply that the degradation effect of the substrate components such as cellulose, hemicellulose, and lignin is highly dependent on the type of formulation that is being used. This variation suggests that F3, which was a mixture of OPEFB and soil, is most efficient in the degradation of cellulose and lignin. F2, which comprises OPEFB mixed with organic materials, is most effective in the degradation of hemicellulose.

In formulation F1, only OPEFB pellet was added, which serves as control to the amount of lignocellulosic content degradation that can be degraded by *V. volvacea* independently. This shows that $CaCO_3$, rice bran and organic fertiliser added in the OPEFB might help in the bioconversion of the lignocellulosic materials. The fact that the $CaCO_3$ provides the best living condition for mycelial growth, the source of nitrogen from rice bran and nutrients from organic fertiliser could have contributed to *V. volvacea* in degrading the lignocellulosic content. Formulation F3 which consisted of OPEFB pellet mixed with black soil degraded the highest percentage of cellulose and lignin compared to the other formulations. This implies that the bioconversion of the lignocellulosic material in OPEFB by *V. volvacea* is highest in F3.

Since black soil may harbour soil microbes that could help in fungal fruiting, they could be involved in the secretion of extracellular enzymes that promote the degradation of the lignocellulosic materials in OPEFB. Payapanon et al. (2011) conducted a study and found that bacterial isolates of *Paenibacillus* and *Bacillus* improved compost and fungal yield. Formulation F4 includes OPEFB pellets with regular incorporation of oligochitosan. While oligochitosan can prevent diseases that could harm *V. volvacea*, it does not provide a food source for the substrate. This may explain why the percentage of lignocellulose degradation is probably higher in F1 and F2 compared to F4. This finding agrees with the results of Choudhary et al. (2009), where hemicellulose was reduced more than cellulose content by the action of xylanase.

It is known that OPEFB contains a reasonable amount of hemicellulose, which is a heterogeneous polymer composed of several different sugar monomers. The *V. volvacea* secretes cellulase, hemicellulase, xylanase, and mannans, all of which contribute to the depolymerisation of hemicellulose to simpler sugars. The evidence of hemicellulose degradation observed in this experiment therefore suggests that hemicellulose is an essential nutrient for the growth and development of *V. volvacea*. As shown in Table 3, the hemicellulose degradation is highest in formulation F2, while the yield and mass of *V. volvacea* are among the lowest in formulation F2. However, hemicellulose degradation in the F3 formulation was 21.5% lower than in the F2 formulation, although it had the highest yield and biomass of *V. volvacea*. Considering that

the OPEFB normally has large quantities of hemicellulose (14-37%) (Mahardika et al. 2024; Mondylaksita et al. 2020) the findings suggest that the presence of hemicellulose and its association with other structural elements, instead of the degradation level of hemicellulose determine overall mushroom performance.

TRANSCRIPTOME PROFILING AND QUALITY CONTROL

Transcriptomic analysis was chosen to be conducted on pinhead and button stages of development in *V. volvacea* to reflect two distinct physiological stages of development. Thus, comparison of these stages allows determining the stage-specific patterns in gene expression that control the conversion of the substrate to the product and affect the cultivation efficiency. High-throughput sequencing showed a total of 48,547 transcripts and 17,762 unigenes, whereas the transcripts longer than 2 kbp contain 14,459 different transcripts and 2,550 unigenes. This indicates the power of the assembly, as long transcripts represent complete genes and multiple complex splice isoforms. In addition, the quality of the data collected was very high, as shown by the Q30 scores, which are over 94% for both samples, and an error rate of 0.02% (Table 4). The average values of GC content were almost the same at both stages; the average at Stage 1 (pinhead stage) was 50.56% and at Stage 2 (button stage) was 50.22%, indicating that GC content does not change with developmental stages. The reliability of these data is supported by Kukurba and Montgomery (2015), who highlighted that high Q30 scores and consistent GC content attributed to effective sequencing and accurate downstream analyses.

FUNCTIONAL ANNOTATION AND GENE ENRICHMENT

Functional annotations in seven databases showed that 70.2% of the unigenes were assigned to at least one database, with most of them being annotated in the NR database (60.78%), as summarised in Table 5.

Based on the Gene Ontology (GO), the unigenes were divided into three categories: biological processes, molecular functions, and cellular components (Figure 3). The biological processes category accounted for the largest proportion; the genes are mainly related to carbohydrate metabolism and lignin degradation. These processes are important for the degradation of lignocellulosic materials, which is a unique feature of the *V. volvacea* niche. The corresponding bar chart shows the degree of success with which the unigenes were assigned to the overlapping databases. The results are shown for the minimum and maximum only for the following databases: non-redundant protein database (NR), eukaryotic orthologous groups (KOG), gene ontology (GO), nucleotide sequence database (NT), and protein family (PFAM).

The classification of molecular functions showed that there are many more hydrolases, oxidoreductases, and transferases, all of which are necessary for the enzymatic hydrolysis of lignocellulosic biomass. A similar observation was made by Xie, Li and Zhang (2022), who found that these enzymes play a central role in the degradation of lignocellulose in fungi. The category of cellular components showed that most of the enzymatic activities occurred in the extracellular space and cytoplasm, which is consistent with previous observations that lignocellulolytic processes occur in fungi.

DIFFERENTIALLY EXPRESSED GENES AND LIGNOCELLULOLYTIC ACTIVITIES

Comparison of gene expression at both stages showed that 2,723 genes were upregulated, and 2,661 genes were downregulated. This volcano plot focuses on the statistically significant DEGs, of which the upregulated genes are shown in red and the downregulated ones in green (Figure 4). This figure also shows the *p*-values (the statistically significant differences indicated by red dots), and the sum of the numbers in each circle is the number of genes expressed within a specific group, and the overlapping area represents the number of genes expressed in more than one group.

Some of these genes are involved in the production of lignocellulolytic enzymes; for example, genes from cluster-275.0 (glucose-1-phosphate adenylyltransferase) and cluster-1759.0 (β-glucosidase L) showed strong upregulation at Stage 1 (Supplementary file-data not shown). This suggests that the pinhead stage has an increased metabolic readiness for the onset of lignocellulose degradation, which may be required for rapid growth and substrate acquisition. On the other hand, genes related to β-glucosidase E (cluster-6223.0) and pyranose dehydrogenase (cluster-13922.1) were downregulated at the second stage, indicating a metabolic shift. Such patterns of developmental stage specificity are consistent with data from Yan et al. (2018), where similar shifts in fungi in response to substrate type were observed.

TABLE 4. Output in sequencing data of the transcriptomic analysis

Sample	Library	Raw_ Reads	Raw_ Bases	Clean_ Reads	Clean bases	Error Rate (%)	Q20	Q30	GC (%)
Volva_P	DRRA 230012812-1a	11,590,990	3.5	11,365,982	3.4	0.02	98.23	94.93	50.56
Volva_E	DRRA 230012811-1a	9,108,525	2.7	8,957,606	2.7	0.02	98.23	95.00	50.22

TABLE 5. Percentage of genes successfully annotated in each functional database searched

Annotation	Number of unigenes	Percentage (%)
Annotated in NR	10796	60.78
Annotated in NT	1715	9.65
Annotated in KO	4375	24.63
Annotated in SwissProt	6551	36.88
Annotated in PFAM	8033	45.22
Annotated in GO	7950	44.75
Annotated in KOG	3370	18.97
Annotated in all databases	829	4.66
Annotated in at least one database	12469	70.2
Total unigenes	17762	100

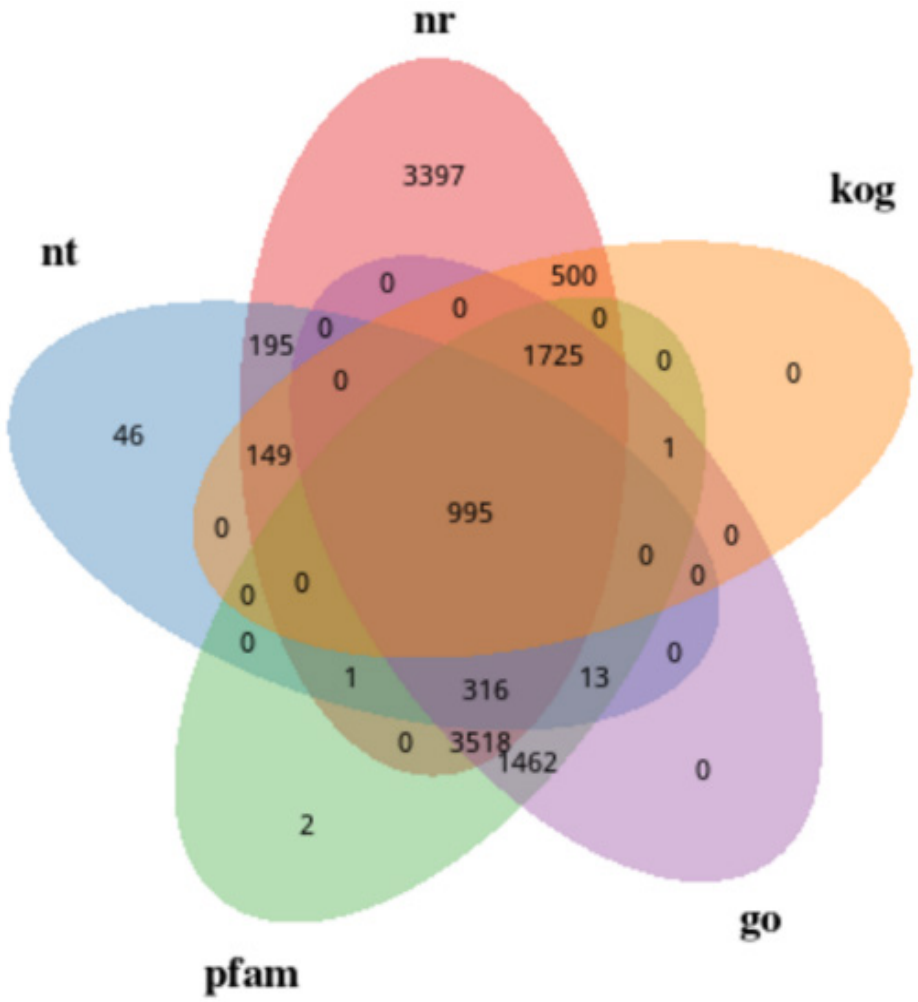

FIGURE 3. Mapping of unigenes using a Venn diagram

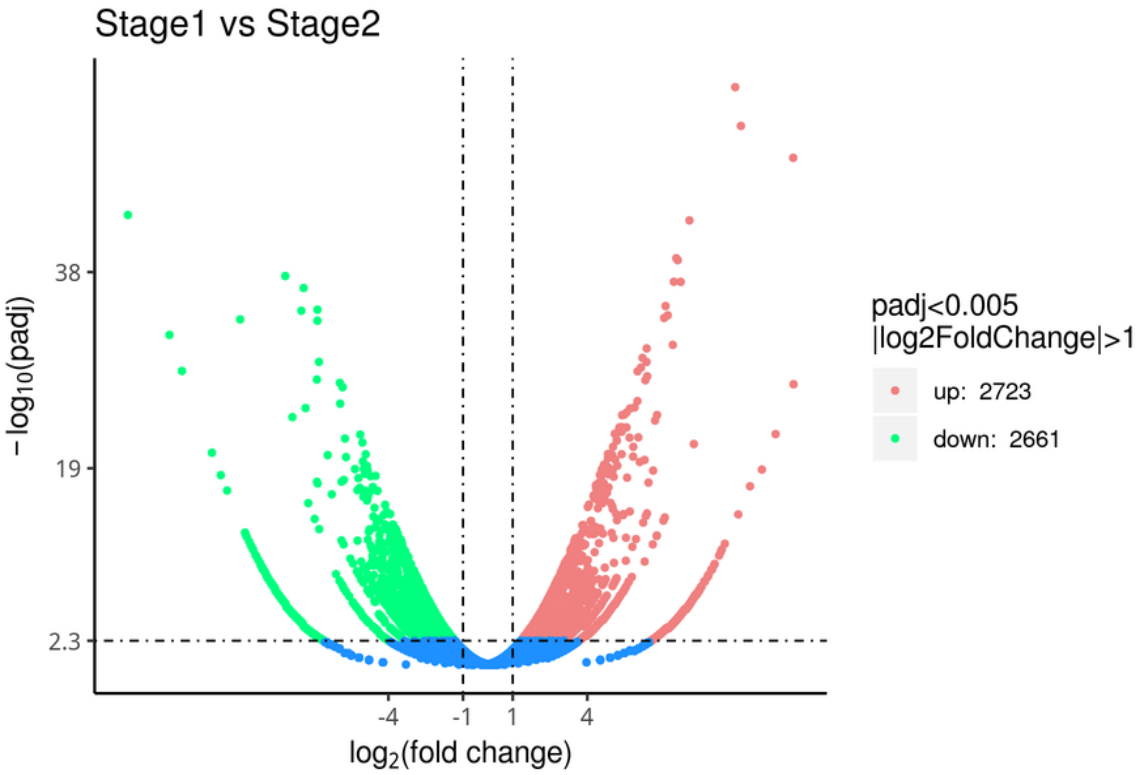

FIGURE 4. Volcano plot shows the fold change in gene expression between different samples (x-axis), and statistical significance of the differences (y-axis). In the volcano plot, red dots indicate significantly up-regulated genes, green dots indicate significantly down-regulated genes, and grey dots represent non-significant expression changes

Surprisingly, glucose-6-phosphate-1-dehydrogenase and β-glucosidase are the genes associated with lignocellulose degradation among the major upregulated genes (Table 6). These three genes were important for sugar metabolism, indicating the importance of enzymatic activities in substrate conversion.

In Figure 5, the integration of lignocellulose by-products into the pentose phosphate and glycolytic metabolic pathways is evident from the enrichment analysis. Overall, the transcriptomic analysis showed that significantly higher transcriptional activity was observed in the pinhead stage (Stage 1) than in the button stage (Stage 2). This is shown by the increase in genes encoding synthetases such as cluster-275.0 (glucose-1-phosphate adenylyltransferase) and cluster-1759.0 (β-glucosidase L), which are involved in the synthesis of lignocellulolytic enzymes (Table 6). These enzymes play an important role in cellulose and lignin degradation, which are inevitable steps in the bioconversion of plant biomass into fermentable sugars.

This stage-specific upregulation is consistent with the results of lignocellulose degradation (Table 3). Cellulose degradation was most decreased in F3, which consists of OPEFB pellets and black soil. Higher enzymatic activity in F3 might be attributed to the microbial communities in the soil, which may mimic *V. volvacea* by releasing extracellular enzymes as demonstrated in Payapanon et al. (2011). The high lignin degradation in F3 of 5.55% also supports this synergy, as lignin degradation is normally difficult and requires the synchronised action of laccases and peroxidases. In another study, supplementing the oil palm frond (OPF) fermentation process with P 2000, Ca 2000, and Mn 150 ppm led to the highest ligninase enzyme activity and reduced lignin content (Pazla et al. 2020).

Of all formulations, the highest hemicellulose degradation (81.5%) was observed in F2, indicating the presence of hemicellulolytic enzymes, xylanases, and mannans (Table 3). This result is in good agreement with the study by Choudhary et al. (2009). However, at the button stage (Stage 2), the specific hemicellulolytic genes were downregulated, including cluster-6223.0 (β-glucosidase E) and cluster-13346.1 (pyranose dehydrogenase). This could be the reason why the yield and mass of *V. volvacea* in F2 were relatively low, as degradation of hemicellulose alone did not increase biomass productivity. In this formulation, the degradation rates of cellulose, hemicellulose, and lignin were evenly distributed as in F3. This resulted in the highest fungal mass growth and yield of 0.355 kg and 11 kg, respectively.

The high metabolic activity at this stage confirms the hypothesis that the pinhead stage is very important for the degradation of lignocellulose. The upregulated enzymes

TABLE 6. List of upregulated key genes in lignocellulose degradation

Gene ID	Enzyme	Function
Cluster-344.0	Glucose-6-phosphate 1-dehydrogenase	Sugar metabolism
Cluster-1759.0	β-glucosidase	Cellulose hydrolysis
Cluster-765.0	Xylanase	Hemicellulose degradation

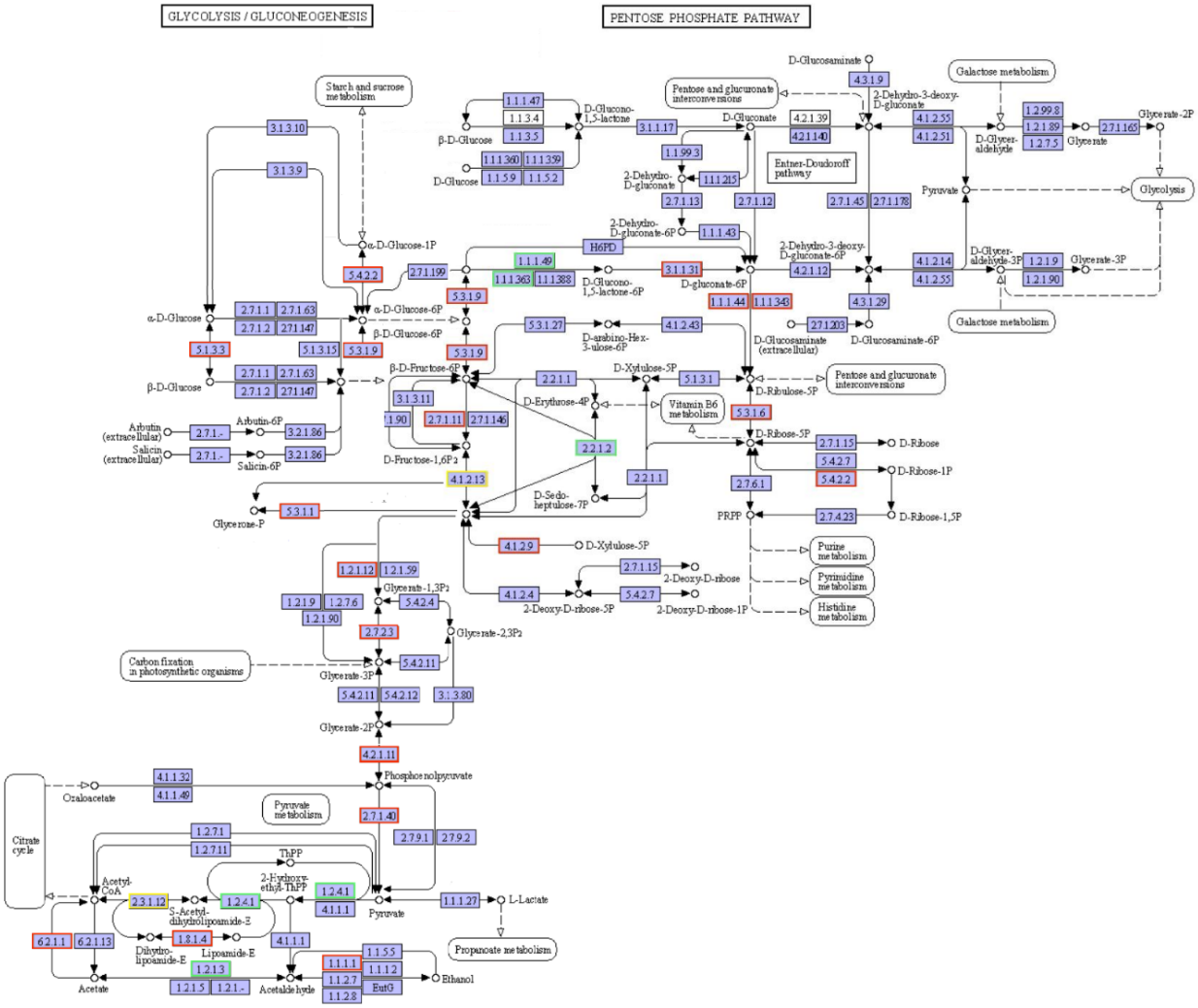

FIGURE 5. Proposed integration pathway of metabolic integration of lignocellulosic by-products into the pentose phosphate and glycolytic pathways. Box with yellow outline: Up-regulated and down-regulated genes were detected in specified KEGG orthology (KO) number. Box with red outline: Up-regulated genes were detected in specified KEGG orthology (KO) number. Box with green outline: Down-regulated genes were detected in specified KEGG orthology (KO) number

were cluster-3698.0 glucose phosphate isomerase and cluster-10621.0 β-glucosidase M (Supplementary file-data not shown). This was consistent with the increased degradation of cellulose and lignin observed in F3 (Table 3). Consequently, the present study elucidates the metabolic profile of *V. volvacea* to improve the conversion efficiency at different stages.

Significantly, F2 contains tremendous potential for the degradation of hemicellulose, but its contribution to yield is not measurable as in the case of F1. However, the hemicellulolytic enzymes in F2 play the key role in the conversion of complex hemicelluloses into simpler sugars for primary fungal growth. This is consistent with previous studies that hemicellulose can be broken into

monosaccharides such as xylose and mannose, which are important substrates for fungal metabolism (Dolah et al. 2021). Our findings also agree with Zhang, Liu and Zhao (2023), which demonstrated that the degradation of cellulose, hemicellulose, and lignin should occur in an even ratio to ensure high substrate-fungal efficiency. The transcriptomic data also supports this idea, but with an even stronger focus on genes associated with cellulose and lignin degradation in Stage 1 (pinhead stage).

Thus, our findings significantly remark on the importance of substrate formulation and growth stage in maximizing the lignocellulolytic efficiency of *V. volvacea*. The integration of black soil in F3 not only enhanced lignocellulose degradation but also potentially fostered microbial interactions that amplified enzymatic activities that produced the highest yield and mass. Besides, it was also found that hemicellulose degradation alone may not be sufficient to sustain optimal fungal growth.

INTEGRATION ANALYSIS OF THE TRANSCRIPTOMIC AND LIGNOCELLULOLYTIC RESULTS

Integration of the transcriptomic profiles with lignocellulolytic degradation patterns provides a clearer understanding of how *V. volvacea* optimises substrate utilisation. The strong up-regulation of lignocellulose-degrading genes during Stage 1 (pinhead stage) indicates an early metabolic commitment toward rapid substrate conversion. This aligns with the higher degradation of cellulose and lignin observed in formulation F3, suggesting that early enzymatic activation is critical for productive fruiting.

Formulation F3, which incorporated black soil, consistently demonstrated enhanced bioconversion efficiency. Soil-associated microbial communities, such as *Paenibacillus* and *Bacillus* spp., were likely involved in the process. Their presence may have supported extracellular enzyme release, thereby complementing fungal lignocellulolytic activity. These synergistic interactions may explain the superior yield and BE observed in F3. Transcriptomic data also showed developmental stage-specific regulations. Stage 1 showed strong expression of β-glucosidase, xylanase, and glucose-6-phosphate dehydrogenase, supporting a metabolic shift favouring carbohydrate mobilisation. In contrast, down-regulated genes in Stage 2 (button stage) reflect a transition from active substrate breakdown to morphological development.

Figure 5 visualizes the integration of these metabolic pathways. The upregulated and downregulated pathways highlight key enzymatic nodes responsible for converting lignocellulosic by-products into central carbon metabolites such as glucose-6-phosphate and pentose phosphate intermediates. Overall, the integrated analysis suggests that microbial synergy and early enzymatic activation enhanced cellulose and lignin degradation. Therefore, it underpins the superior performance of OPEFB + black soil (F3). These findings further validate the importance of substrate formulation and developmental timing in designing improved cultivation strategies for *V. volvacea*.

Our findings suggest that the promising results of cellulose and lignin degradation, but not hemicellulose, are more directly related to the positive performance of *V. volvacea*. The highest cellulose and lignin degradation gave the highest yield and BE in F3 (Table 3). Conversely, F2 showed the greatest hemicellulose breakdown but with a relatively lower yield and BE. These correlations are further supported by the transcriptomic findings described earlier. Taken together, these findings indicate that the early-stage activation of enzymes and microbial-fungal interaction is a preeminent factor of cultivation efficiency due to its role in the balanced lignocellulose degradation.

CONCLUSIONS

This study demonstrates that OPEFB supplemented with black soil (F3) significantly enhanced the yield, BE, and lignocellulose degradation capacity of *V. volvacea*. The integration of productivity assessments with transcriptomic analysis provides a mechanistic explanation for this improvement, showing early activation of key lignocellulolytic enzymes. Particularly, β-glucosidase, xylanase, and glucose-6-phosphate dehydrogenase were associated with rapid substrate conversion during the pinhead developmental stage. The synergistic contribution of soil-associated microbial communities likely strengthened enzymatic breakdown processes, further improving substrate utilisation efficiency. These interactions point out the potential role of microbial–fungal partnerships in advancing sustainable mushroom cultivation systems. Findings from this research offer valuable insights into optimising substrate formulations for *V. volvacea* cultivation and demonstrate the relevance of transcriptomics in guiding substrate enhancement strategies. Furthermore, findings from this study contribute to sustainable agricultural waste management by promoting the valorisation of OPEFB. By manipulating the information, it can be applied to support both circular These findings may support circular bioeconomy initiatives and contribute to environmental impact reduction. It is recommended for future studies that may investigate the specific microbial taxa involved in supporting lignocellulose degradation and validate their functional contributions across different cultivation environments.

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