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Solid-state fermentation of spent mushroom substrate through a synergistic fungal consortium for enzymatic cocktail production

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ABSTRACT

This study aimed to develop a cost-effective and efficient enzymatic cocktail from spent mushroom substrate (SMS) while serving as a model to understand fungal-fermentative communities and characterize microbial diversity stability in SMS valorization. Co-cultivation of *Aspergillus fumigatus* IMCC2006 and *Trichoderma asperellum* IMCC2012 was optimized for cellulase production under varying moisture, pH, and fermentation time. Optimal enzyme yields were achieved at 60 % moisture, pH 5, and 96 h of fermentation, producing maximum CMCase (11.5 U g^{-1}) and FPase (4.35 U g^{-1}) activities, while the highest xylanase activity (13.8 U g^{-1}) occurred at 70 % moisture. Scanning electron microscopy and FTIR confirmed the consortium's cellulolytic capability in SMS degradation. Microbial community analysis revealed *Proteobacteria* and *Firmicutes* dominance at the end of fermentation, supporting system stability. The findings provided a plausible solution for valorization of agricultural residues for enhanced enzymatic production from the synergistic consortium, to catalyze biorefinery applications.

1. Introduction

Spent mushroom substrate (SMS) is a post-harvest byproduct waste from edible mushroom cultivation that consists of sawdust, wheat straw and even crop stalks. The increasing number of these wastes is steadily generated all year around, and has not yet been fully utilized. The mushroom industry has faced inefficient practice of SMS disposal, which is vastly underutilized (Khalil et al., 2024; Paula et al., 2017). Given that improper treatments taken and the issue of expensive commercial cellulase, this hardly difficult-to-degrade lignocellulosic waste could be transformed into fermentable sugars to catalyze biorefinery applications. Bioconversion of these nutrient-rich residues via fermentation into numerous new valuable chemicals could contribute to the resource efficiency, while closing the waste loop. Previously, many trials of SMS valorization have been focused on soil amendment but fewer studies were reported on the extended use of SMS as a substrate for enzymatic cocktail production. Undergoing partial decomposition by extracellular oxidative enzymes during the growth of edible mushroom has made SMS a good candidate to be used as a fermentable source without any washing steps and nutrients supplied.

Indeed, SMS could be considered an abundant and cheap lignocellulosic fibers source that is rich in cellulose, hemicellulose and lignin with up to 75 % carbohydrates, which offers a good prospect for enzyme recovery (Jordan et al., 2008). However, this cellulose serves as the main hindrance in the comprehensive SMS reutilization and requires complete enzymatic hydrolysis to obtain the desirable product, glucose. Despite its high abundance, the commercial production cost of cellulolytic enzymes becomes a major setback to flourish the biorefinery industries. In recent years, there has been a dramatic interest in using this feasible raw feedstock as a fermentable substrate with several pre-treatment choices for optimum enzymes to catalyze biorefinery applications. Of these approaches, biological pre-treatment is regarded as a

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promising technique applied to overcome problems associated with the SMS disposal through fungal solid-state fermentation (SSF) without causing serious environmental consequences. Through the SSF process, the SMS is cultivated under optimized parameters by cellulose-degrading fungi in the absence of free-flowing liquid as it offers a comparable quality with a low process energy requirement economically (Selvakumar et al., 1998). Therefore, any effort to accelerate the conversion rate of these lignocellulosic fibers is become prime significance towards the green solution of under-utilized SMS valorization, supporting the transition of circular bioeconomy.

In view of the cellulosic component of SMS, fungal cultivation has shown to be a better degrader of plant cell mass constituents rather than bacteria (Yoon et al., 2014). This is due to large quantities of enzymatic secretion that work in harmony with their hyphal penetration capability for complete hydrolysis. At present, the most potent fungal strain for cellulase production, namely as *Aspergilus* sp. and *Trichoderma* sp. are always referred for complete cellulose hydrolysis, but *Trichoderma reesei* is usually known to be deficient in β-glucosidase. Taking an example, a higher cellulase activity was recorded when *A. niger* was cultivated on wheat bran with 10.81 Ug^{-1} (Kumar et al., 2011). Similarly, SSF using oil palm leaves as a substrate involving *Trichoderma asperellum* UC1 and *Rhizopus oryzae* UC2 has been employed to co-produce cellulase and xylanase with enzyme efficiency effect on dough rising (Ezeilo et al., 2021). In this respect, cultivation by a fungal consortium consisting of *Aspergilus fumigatus* and *Trichoderma asperellum* was developed on SMS valorization to complement the deficiency of particular cellulase components if sole cultivation took place. Besides, applying SSF as an alternative production route for ligninolytic enzymes is a closer resemblance to the natural habitat of filamentous fungi and the titre of produced enzymes is superior than the titre produced from the conventional submerged fermentation.

There are few documented applications of fungal consortia for the production of cellulase through SMS fermentation. Thus, the utilization of steam-sterilized SMS by indigenous fungal co-culture via SSF offers further opportunities for reducing enzyme production costs and facilitating the demand gap driven by commercial biofuel production. Incubation time, temperature and moisture content are crucial parameters that need to be taken into consideration in SSF that will influence the efficiency of ligninolytic enzyme production. In this context, this work aims to test our hypothesis that the enhancement in cellulase production is correlated with the synergistic interaction between fungal co-cultivation and the SMS substrate used. Specifically, this study was designed to (1) evaluate cellulase and xylanase production by the co-culture of *Aspergillus fumigatus* IMCC2006 and *Trichoderma asperellum* IMCC2012 under optimized SSF conditions, (2) characterize the surface micrstructural and functional group modifications of SMS residues following fungal fermentation using SEM and FTIR analyses and subsequently, (3) assess the bacterial community diversity in fermented SMS via 16S rRNA amplicon sequencing to identify dominant phyla involved in post-fermentation processes.

2. Materials and methods

2.1. Materials

Spent mushroom substrate (SMS) was supplied by C&C Mushroom Cultivation Farm Sdn. Bhd. (Johor, Malaysia), which was the byproduct of *Pleurotus ostreatus* cultivation. It contained 30.35 % organic carbon, 0.89 % total nitrogen, 0.28 % total phosphorus with an organic matter of 52.32 %. The SMS was subjected to a sterilization process by autoclaving at 121 °C for 20 min to eliminate any possible pathogens. Then, the material was dried at 55 °C until constant weight and milled to an average size of 60–80 mesh. The processed material was kept in a dryer before subsequent experiments.

Chemicals including 3,5-dinitrosalicyclic acid (DNS), carboxymethyl cellulose (CMC), sodium hydroxide, sodium sulphite, potassium sodium tartarate, and xylan, were all of analytical grade and purchased from Sigma-Aldrich (USA) and Medchem Express (USA).

2.2. Isolation and inocula preparation of cellulase-producing fungal strains

Two fungal isolates were used, originating from a compost sample, which were identified and molecularly characterized (IMCC2006 and IMCC2012) in this work. Fungi were isolated using the serial dilution method. Appropriate dilutions were spread in triplicate on potato dextrose agar (PDA) plates and incubated at 30 °C for 5 days. The fungal isolates were selected based on distinct cellulase production features (cellulose hydrolysis zone) on CMC agar medium which had the following composition (gL^{-1}): $FeSO_4.H_2O$ 0.2, KCI 0.2, $MgSO_4.7H_2O$ 1.0, K_2HPO_4 4.0, yeast extract 1.0, peptone 5.0, CMC 10.0 and agar 15.0. Briefly, each spotted isolate on CMC agar medium was flooded with Congo red reagent, and allowed to stand for 30 min before washing with 1 M NaCI to remove excess dye. The isolates were incubated on PDA slants before being kept at 4 °C.

Fungal strains were extracted using PrimeWay Genomic DNA Extraction Kit (Apical Scientific, Malaysia) in accordance with the manufacturer's protocol. Next, the internal transcribed spacer (ITS) region from each isolated strain was amplified by PCR using universal primers ITS1, 5′- TCCGTAGGTGAACCTGCGG – 3′ and ITS4, 5′ – TCCTCCGCTTATTGATATGC – 3′. Amplication was performed in 25 μL reaction mixture using a TC-1000G PCR thermal cycler (DLAB, China). A PCR purification kit (NucleoSpin gDNA Clean-Up, Macherey-Nagel, France) was used to purify the PCR product, which was subjected to DNA sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Reference sequences retrieved from the GenBank database following BLAST searches were aligned with ITS gene sequence. Phylogenetic tree was constructed by using Neigbour-Joining methods in MEGA7.0 with the Maximum Composite Likelihood parameter model via bootstrap analysis of 1000 replicates. Their nucleotide sequences have been deposited at the National Center for Biotechnology Information (NCBI) database as *A. fumigatus* IMCC2006 and *T. asperellum* IMCC2012 (GenBank accession PP478203 and PP267700).

2.3. SSF and enzyme production

Matured spore suspensions from both *A. niger* IMCC2006 and *T. asperellum* IMCC2012 were prepared separately in 0.1 % Tween 80 solution at a concentration of 1.0×10^7 spores g^{-1} after 7-days cultivation in PDA. The diluted fungal spores were aseptically used as inocula for the SSF experiment. The assay was prepared by wetting 5 g of sterilized SMS with an appropriate volume of modified Mandel medium comprising of $(NH_4)_2SO_4$ 10.0 gL^{-1}, KH_2PO_4 4 gL^{-1}, $MgSO_4.7H_2O$ 0.5 gL^{-1}, $CaCI_2.2H_2O$ 0.5 gL^{-1} to reach relative 70 % moisture level. The pH of the prepared medium was 4.8. SSF was conducted in a 250 mL Erlenmeyer flask covered with cotton plugs at 30 °C for 4–6 days in duplication.

Enzyme production efficiency was optimized after SSF. Flasks were inoculated with mixed spore suspension and incubated for different moisture, time and pH levels. Medium without spore suspension was used as a negative control. For incubation time optimization, the substrate was inoculated with mixed spore suspension having 10^7 spores incubated at different times of 96 h, 120 h and 144 h. For screening of optimum moisture content, SMS was moistened with 3 different volumes of Mandel media (7 mL, 10 mL and 12 mL) to achieve a moisture content of 60 %, 70 % and 80 % (*w/v*), respectively. Fermentation medium was subjected to varying pH levels (pH 5.0, 6.0, 9.0) with mixed spore suspension at 30 °C for 5 days. During optimization, enzyme extraction was analyzed for cellulase and xylanase activities.

2.4. Enzyme extraction

At the end of fermentation, 50 mL of cold buffer solution (50 mM acetate buffer pH 5.0) in a 1:2 ratio (w/v) was added to the samples and stirred at 200 rpm for 1 h at 25 °C for enzyme extraction. After centrifugation (8000 rpm at 4 °C for 10 min), the resulting supernatant was obtained. Supernatant, in the form of crude enzyme extract was assayed for cellulase and xylanase activities. The crude enzyme was stored at −20 °C prior to enzyme assays.

In this study, endoglucanase (CMCase) and exoglucanase (FPase) represented cellulase activity of the crude enzymes, in units per gram U g^{-1} of SMS substrate with glucose used as a standard. Carboxymethyl cellulose (CMC, 1 % *w/v*) was used as the substrate to determine endoglucanase or CMCase activity using the procedure of the International Union of Pure and Applied Chemistry (IUPAC). CMCase activity was assayed by incubating 0.5 mL CMC in 0.05 M sodium acetate buffer (pH 5.0) with 0.5 mL of crude enzyme for 30 min at 50 °C. Liberated reducing sugars were measured using DNS method by 10 min boiling the mixtures with 1 mL 3,5-dinitrosalicyclic acid (DNS) and 2 drops of 0.1 M sodium hydroxide. Exoglucanase or FPase activity was assayed by incubating 0.5 mL of crude enzyme in 0.5 mL of 0.05 M sodium acetate buffer (pH 5.0) containing Whatman Filter Paper strip (1 × 6 cm dimension, approximately 50 mg) for 60 min (Ghose, 1987). Liberated reducing sugars were quantified by absorbance at 540 nm after DNS method. For xylanase activity, the assay was performed using 1 % xylan solution (*w/v*) in 0.05 M acetate buffer pH 5.0, as a substrate (Ezeilo et al., 2019). This assay defined that one unit of xylanase activity corresponded to the amount of enzyme liberating 1 μmol of xylose per min at 50 °C. Measurement of all colorimetric readings was taken at an absorbance of 540 nm (CMCase, FPase and xylanase) using a UV-VIS spectrophotometer (Cary 60, Agilent, USA). One unit (U g^{-1}) of enzyme activity was expressed as the concentration of enzyme required to liberate 1 μmol of product (glucose corresponding to CMCase and FPase, xylose equivalents for xylanase) in 1 min at 50 °C, under the assay conditions (Miller, 1959). All experiments were performed in duplicate. The data shown in the bar figures corresponded to mean values with error bars.

2.5. Morphological characterization

The physical changes of fermented SMS and uninoculated SMS were observed using scanning electron microscopy (SEM). Then, the samples were mounted on an aluminium stub with double-sided carbon tape and sputter-coated with gold under argon at 50 Å thickness (Q150RS, Quorum Technologies Ltd., United Kingdom). Following coating, images of the substrate surface before and after SSF were visualized at magnification powers of 500 to 2000× with acceleration voltage 5 kV using a field emission scanning electron microscope (SU8020, Hitachi, Japan).

2.6. FTIR analysis

Fourier Transform-Infrared (FTIR) spectra analysis was conducted on a Thermo Scientific (Nicolet iS5, USA) spectrometer ranging from 400 to 4000 cm^{-1}, to identify the possible functional group presence in each respective sample.

2.7. High-throughput sequencing

To determine the bacterial diversity, samples from uninoculated and fermented SMS were collected. Total DNA from 0.5 g of each sample was extracted using the following method of soil DNA Isolation Kit (Omega, Norcross, USA). DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) and DNA integrity was detected using 1 % agarose gel electrophoresis. PCR amplification and high-throughput sequencing were undertaken on 16S rRNA V3-V4 region using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′- ACTCCTACGGGAGGCAGCA-3′). PCR reactions were performed in triplicate at a final volume of 20 μL containing 4 μL of 5 x FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The PCR reaction procedure was performed as follows: 95 °C for 3 min, 30 cycles, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, 72 °C for 10 min. The amplification product was detected using 2 % agarose gel electrophoresis. After measuring the concentration of the purified product, the equimolar number was mixed. The high-throughput sequencing of 16S rRNA was performed by the Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocol of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Subsequently, operational taxonomy units (OTUs) were clustered at a 97 % similarity cut-off using USEARCH (version 7.1). Chimeric sequences were removed. OTUs were classified with RDP classifier (http://rdp.cme.msu.edu/) against the SILVA 16S rRNA database using a confidence threshold of 70 %. The functional prediction of metabolic pathway was obtained by comparing the OTU table with KEGG database using PICRUSt.

2.8. Statistical analysis

The results were initially collated with Excel 2010 (Microsoft, USA) before analysing using Statistical Packages for Social Sciences (SPSS, USA). One-way of variance (ANOVA) and Duncan's multiple comparisons were performed. Means comparisons between treatments were performed at a significance level of $p < 0.05$. The OTU dataset was used to calculate Chao 1 and Shannon-Simpson's diversity indices. All reported results are calculated in the average measurement of repeated experimental runs and presented as means ± standard deviation.

3. Results and discussion

3.1. Morphological and molecular identification

Generally, the co-cultivation of identified filamentous fungi for efficient valorization of lignocellulosic wastes has been considered widely, taking advantage of their possible synergistic interaction to yield the hydrolyzed products to be used in the sugar-based biorefineries. Employing more than one fungi as a consortium into SSF could boost and compensate for genomic variations since fungi were co-existed synergistically in nature settings (Salomão et al., 2019). Historically, co-cultivation of fungal isolates has always been mentioned in cellulose biodegradation, which involved synergistic interplay of enzymatic conversion of cellulose into glucose. Besides, white-rot fungi *Aspergillus* and *Trichoderma* species were commonly used due to wide selection of substrates and high cellulase activity. In this study, the cellulase-producing ability of natural isolates was examined by selective agar medium plates, in which CMC is one of the supplemental nutrients. Two fungal strains named as IMCC2006 and IMCC2012 that produced distinct hydrolysis rings on the screening agar plate were selected for further strain identification. After 7 days of incubation on PDA, IMCC2006 produced fluffy creamy aerial mycelia with blackish-grey conidial areas. Whereas, IMCC2012 had a white aerial hyphae texture with no pigmentation on the reverse side of colonies. Dark green spores appeared in the center of the plate after 7 days of incubation. These morphological characteristics were consistent and complementary with the other reported studies (Wu et al., 2017; Mandal et al., 2021). The appearance of isolates after purification on PDA solid media suggested that the most likely case was that, IMCC2006 and IMCC2012 belonged to a member of sporogenous fungi (Fig. 1B & C). Similar results were also obtained from studies documented by Akpomie et al. (2021), reported the occurrence of *Aspergillus fumigatus* and *Trichoderma asperellum* in their roles of producing lignocellulolytic enzymes.

Molecular identification was further conducted by sequencing ITS1/4 regions. The result of the PCR amplification of the ITS is shown in

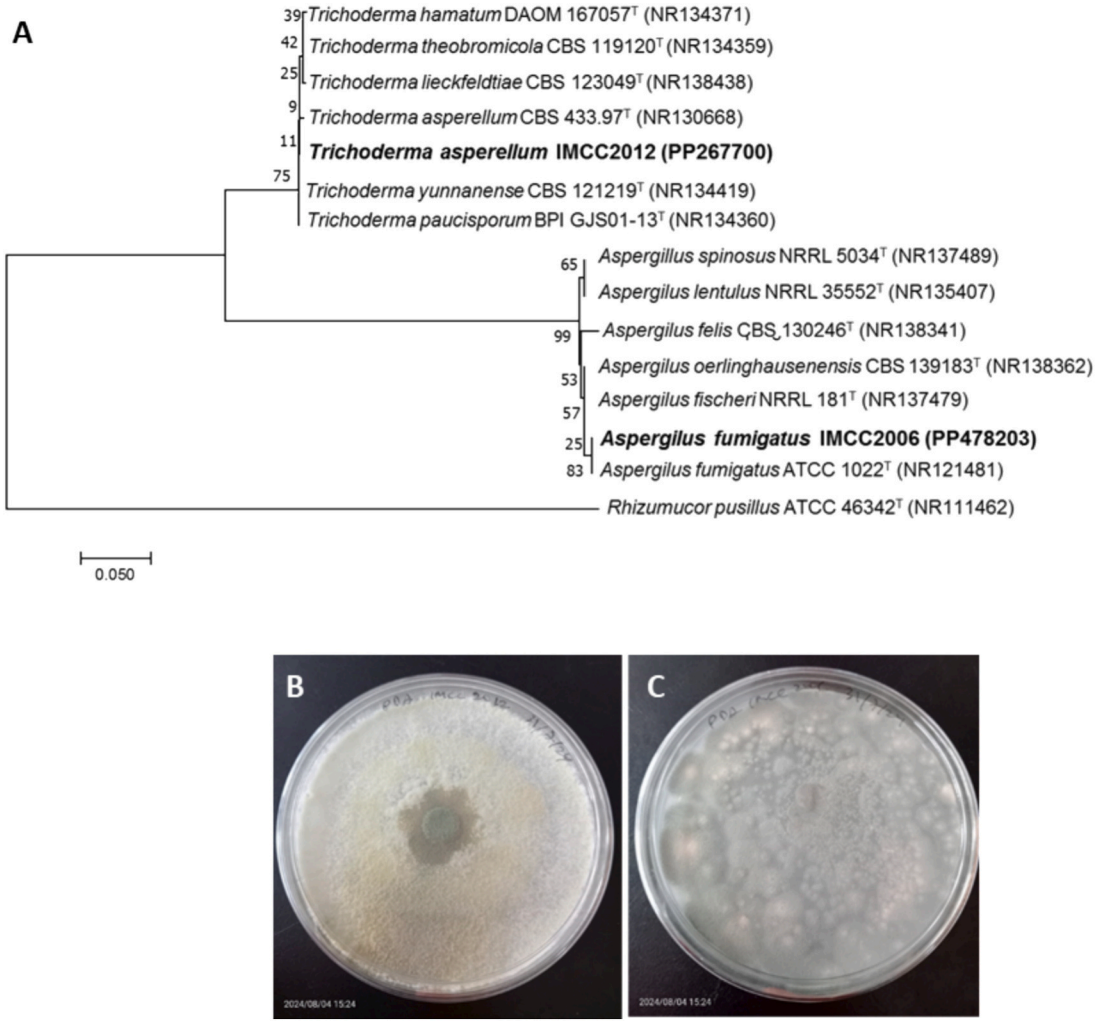

Fig. 1. Phylogenetic and morphological analysis of IMCC2006 and IMCC2012. (A) A phylogenetic tree based on ITS gene sequences was created using neighbor-joining method and showing the relationships among IMCC1007 and the members of the genus *Aspergillus* and *Trichoderma*. Bar 0.05 substitution per nucleotide position. *Rhizomucor pusillus* was used as the outgroup. The analysis was generated using maximum likelihood with 1000 bootstrap replications. (B & C) Upside fungal strains morphological image on PDA after 7 days.

Fig. 1A, with product sizes of 605 and 608 bp, respectively. The BLAST analysis of these fungal strains revealed 98–99 % identical similarity with ITS sequences of each type fungal strain-related species. IMCC2006 had a close affinity towards *A. fumigatus* ATCC 1022^T and IMCC2012 shared the highest homology with model strain *T. asperellum* CBS 433.97^T, respectively, with *Rhizomucor pusillus* ATCC 46342^T used as an outgroup. The ITS gene sequence of these fungal strains was deposited in the GenBank database with accession numbers PP478203 and PP267700, respectively. According to preliminary morphological observation and molecular characteristics of those fungal isolates, IMCC2006 and IMCC2012 were identified as *A. fumigatus* and *T. asperellum* strains. Crucially, lignocellulosic substrate with fungal cultivation is expected to be limitless *on-site* hydrolysed with one fermentation step of lower energy input operation. From the economic standpoint, yield of enzymatic recovery produced and readily available fermentable sugars from hydrolysis are reported to be higher than the chemically catalysed route. Although strain modifications and other approaches seem to offer an alternative way to improve enzymatic cocktail production, the most promising techniques yet convenient was the co-cultivation of two or more fungi, which facilitated the hydrolysis process. Hence, these isolates, exhibiting the cellulase activity were selected for fungal SSF using SMS as a substrate to produce a pool of lignocellulolytic enzymatic cocktail in-house at a low cost. Among the identified isolates, these cellulose-producing isolates served as a fungal-fermentative consortium model, bearing profound importance in the utilization of cellulose resources. Indigenous fungal isolates are presumed to be effective producers of ligninolytic enzymes, as compost is a suitable substratum in their natural ecological setting.

3.2. Enzymatic production

SMS served as an excellent fermentable source without any additional supplementation or prior treatment for the production of ligninolytic enzymes. In addition, the porous surface of SMS played a role in sustaining the release of inhibitory factors during the fermentation process, making them a viable option for being a fermentable substrate (Li et al., 2020). Simultaneous production of cellulase and xylanase became a key option for total hydrolysis of lignocellulosic biomass material. On the other hand, identifying highly efficient enzyme production with SSF, could reduce the overall cost challenges associated with the entire chain of biorefinery processess. Based on the SSF experiment, fungal cultivation gave the highest CMCase, FPase and xylanase activities corresponding to 12.06, 4.35 and 13.88 U g^{-1}. The effects of incubation time (96 h, 120 h and 144 h), pH (5.0, 6.0 and 9.0) and moisture (60 %, 70 % and 80 % moisture) on the CMCase, FPase and xylanase activities from *A. fumigatus* IMCC2006 and *T. asperellum*

IMCC2012 were assayed using SMS substrate (Fig. 2). Both cellulase and xylanase activities appeared to be highly imperative to co-produce synergistically for total hydrolysis of lignocellulose biomass. One-factor-at-a-time (OFAT) strategy used in the present study could be considered as a critical optimization process to maximize the yield of specific enzyme before proceeding into large scale production. Earlier researchers optimized the enzymatic cocktail production through independent variables with effect on fermentation conditions. Of these strategy, Response Surface Methodology (RSM) became effective to complement OFAT findings, which could purposely measure more than one response simultaneously and predict the variable interactions for improved result (Bhaturiwala et al., 2022).

Optimum pH for CMCase was 5.0, with the highest 11.57 ± 0.02 U g^{-1}, whereas 6.0 was the optimum pH for FPase activity (2.21 ± 0.01 U g^{-1}). A similar result was reported by Boondaeng et al. (2024), showing the optimal pH (5.0–7.0) for cellulase production through SSF process by *Aspergillus* sp. IN5. Therefore, an initial pH 5.0 is the preferable pH for accelerating the optimal cellulase activity as recommended by Yoon et al. (2014). The optimal SSF condition with 96 h incubation time produced the highest CMCase production (12.01 ± 0.02 U g^{-1}). In contrast, a time of 120 h appears to be optimal for FPase (Fig. 2B) and xylanase (Fig. 2C) activities. Whereas, the conditions of pH 6.0, 120 h incubation time and 70 % moisture were best at producing FPase. Additionally, the optimal pH for CMCase activity was 5.0 while the pH range between 4 and 6 did not significantly affect FPase activity, and pH 6.0 was found to be the best for xylanase activity (Fig. 2). The trends of CMCase and FPase indicate that enzyme activities reached the maximum of 11.55 ± 0.02 U g^{-1} and 4.35 ± 0.01 U g^{-1} respectively on day 5 and dropped dramatically afterward. This decline is probably due to irreversible inactivation or denaturation of enzymes as described by Hu et al. (2018). Even, any changes of pH fluctuation beyond alkaline state might affect the activation of enzyme stability. From here, the CMCase activity remained stable at pH and incubation time ranges of 5.0–6.0 and 96–120 h, respectively. Whereas the FPase activity maintained stable at pH 6.0 and 120 h incubation time. In a previous report of cellulase production through SSF of SMS from *Auricularia polytricha*, the maximum enzyme activity after 7–8 days was recorded at only 3.51 U g^{-1} at pH 6.0 by *Trichoderma reesei* ATCC56765, in which was 70.89 % lower than the present study (He et al., 2021). Previous reports from Narra et al. (2012) mentioned that the optimal pH for fungal enzymatic cellulase production was 5.0, which is in line with current findings. The optimal activity and stability at acidic pH assist fungal cellulase to become more attractive over bacterial one in food manufacturing applications (Silva et al., 2015). Ma et al. (2022) obtained cellulase activity with 18.82 U g^{-1} by using *Aspergilus niger* on pre-treated SMS with pH 5.0 and temperature of 50 °C. It was noticeable that chemical-free pre-treatment upon SMS sample did contribute to better assimilation of fungal mycelium, resulting in better production of cellulase activity. In the study by Arbaain et al. (2019), a cellulase activity produced by *Schizophyllum commune* on chemical-free oil palm fruit bunch substrate had reached a pattern of maximum level at optimal parameters.

It is worth mentioning that fungal-fermentative consortium has a greater capability in producing lignocellulolytic activities when compared to the monoculture mode. This might explain the synergistic interaction between strains as co-cultivation promotes faster colonization and superior substrate degradation. In the present study, the conjuction of cellulase and xylanase accelerate more hydrolytic activities over the available substrate. Metreveli et al. (2017) have reported the synergistic association between *Irpex lacteus* and *Pycnoporous coccineus*, which led to higher activities of cellulase and xylanase. Regarding moisture level, the highest level was recorded in FPase (3.83 ± 0.01 U g^{-1}) and xylanase (13.88 ± 0.03 U g^{-1}) activities at 70 % moisture, as shown in Fig. 2B and Fig. 2C. Whereas, a 60 % moisture level seemed to be optimal for CMCase activity. Notably, levels of xylanase produced by *Trichoderma* isolates cultivated on SMS from *Agaricus bisporus* cultivation were much higher than levels of xylanase secreted by isolates here (Grujić et al., 2015). The result is concurrently in harmony with Cheirsilp et al. (2023), mentioning that higher xylanase activity than cellulase activity might be due to substrate complexity and availability. It should be noted that xylanase has to be secreted first to degrade the

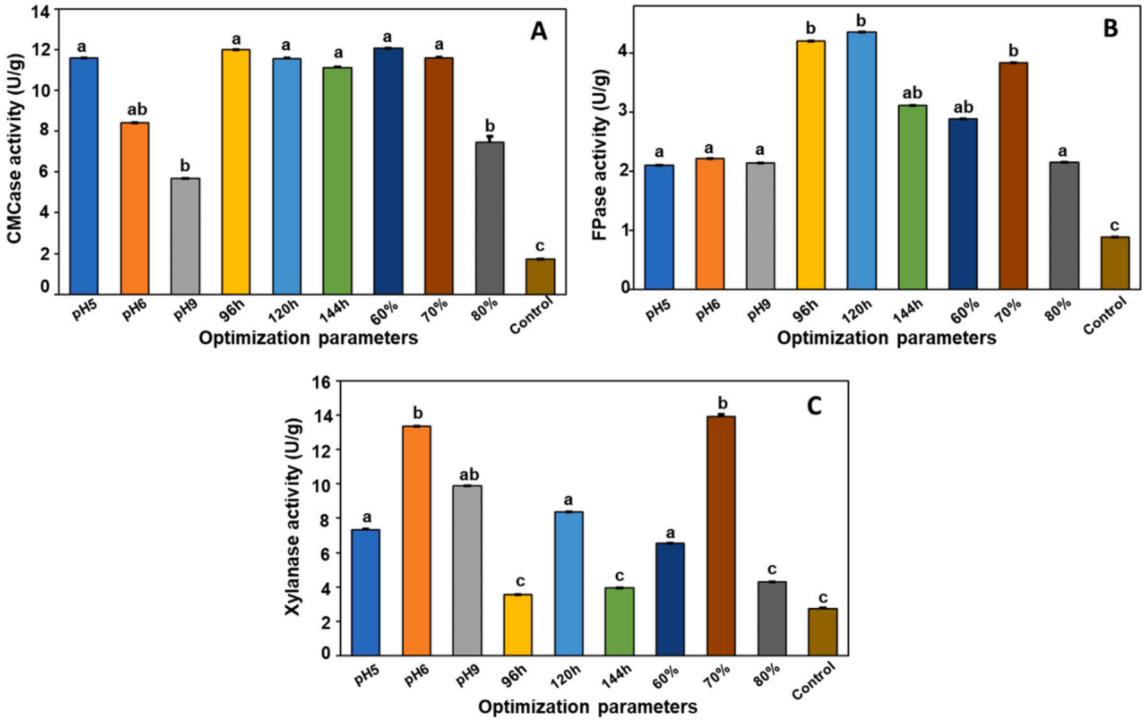

Fig. 2. CMCase (A), FPase (B) and xylanase (C) activities (U g^{-1} substrate) of fungal consortium (*Aspergillus fumigatus* IMCC2006 and *Trichoderma asperellum* IMCC2012) during SSF process of SMS at different pH, incubation time and moisture content. Vertical bars represent the mean of each treatment ($n = 2$) and the error bars indicate standard deviation. Some of the error bars are hidden by the vertical bars. Different letters in each bars indicate significantly different treatments.

outer layer before cellulase secretion takes place for inner layer degradation. In the other fungal SSF study of using pea pods waste to produce cellulase, the best CMCase cocktail secretion (24.7 IU mL^{-1}) was recorded at moisture content of 51 % along with temperature of 30 °C in 7 days incubation after co-cultivation with *Rhizopus* and *Cladosporium*. Additionally, Tripathi et al. (2024) agreed with concern of optimized moisture content amount in the fermentation, which has a role in maximizing enzyme production. Although SSF was operated without flowing water operation, a least minimal moisture has to be present to promote microbial growth. Meanwhile, SSF parameter with a long incubation time up to 144 h, alkaline condition and above 80 % moisture dampened the enzyme production, rendering them as an unsuitable condition for achieving higher yield of exoglucanase. This resulted in a decline in enzymatic production likely due to the presence of inhibiting factors. Knowing the concentration limit of inhibition sugar product, glucose, is beneficial to avoid any competitive inhibitor for enzymes. Earlier reports have addressed this point that cellulase activity could be recovered with the addition of *Lactobacillus* bacteria in the fermentation process, in which feedback inhibition was eliminated (Ma et al., 2022). Interestingly, the enzymatic cocktail production in this study showed higher quantities in CMCase, FPase and xylanase respectively as compared to *Trichoderma asperellum* RCK2011 cultivated in wheat bran under SSF (Raghuwanshi et al., 2014). Based on the findings from previous reports, parameter optimization processes including pH, moisture, carbon and nitrogen sources and even incubation time were proven to be essential for enzyme production.

To further explore the effectiveness of employing fungal cultivation for large-scale of enzymatic cocktails, Pan et al. (2025) proposed solid-state tray fermentation process of *Chaetomium globosum* DX-THS3 on raw rice straw with optimized parameters. Compared to unoptimized parameters, their findings particularly on CMCase activity and total secreted protein revealed a remarkable 63 % and 48 % increase, respectively. Although that study indicated the feasibility of employing optimized parameters for scalability in large-scale enzyme production, such factors of inadequate homogeneity issues and intense heat generation that led to enzyme denaturation required an attention. Cerda et al. (2017) reported that higher temperatures in 50 L bioreactor were achieved, leading to 50 % drop of cellulase production at coffee husk fermentation. Moreover, Ortiz et al. (2015) also observed an increase in temperature during pilot trials with its consequent drop in enzymatic productivity. Also, the absence of suitable bioreactors alongside current labor-intensive practices hindered large-scale industrial processes. Grajales et al. (2025) cultivated *Myceliophthora thermophila* I-1D3b in mechanical intensified rotary drum bioreactor using 40 % substrate loading, obtaining a reduction (23.57 U mL^{-1}) in enzymatic activity result. In light of these results, the consistency of the small-scale SSF process for scalability means has to consider the inherent variability of substrates used and the prior optimized parameters in maximizing the sustainable enzymatic yield recovery.

3.3. Compositional and structural modification analysis during fungal SSF

3.3.1. Scanning electron microscope of SMS substrate

Structural and morphological changes occurring in the substrate during SSF were monitored by using scanning electron microscopy (SEM) with uninoculated SMS was used as a comparison. Disrupted and porous microstructure of fermented SMS after fungal cultivation were visualized, indicating hydrolysis of lignocellulosic material. The surface of fermented SMS seems to be more corrugated and became evidently sparse, generated by enzymatic degradation (Fig. 3D-E). Such microscopic changes have been generally considered as a result from the significant decrease of cellulose content as concurrently in harmony reported by Xu et al. (2017). Moreover, bacterial attachment onto fungal hyphae was found to assist bacteria in getting the nutrient accessibility in return (Fig. 3E). It has been proven that the positive effect of fungal cultivation provides cooperative actions to achieve depolymerisation and keeps microbial stability. In this sense, it would be proper to mention that inter-kingdom interaction has existed and more commonly occurred in the SSF (Peters et al., 2010; Zhou et al., 2023). On the contrary, uninoculated SMS has a rigid structure with a smooth surface without cracks (Fig. 3A-C). Hansen et al. (2011) also demonstrated the utilization of enzymatic hydrolysis up to 144 h which leads to further change in lignified vascular tissue. Our SEM visualization also has a good correlation with Yoon et al. (2013) findings on sugarcane bagasse, indicating removal of lignin content and reduction in cellulose crystallinity during SSF treatment. Of note, a recent study of enzymatic saccharification with the addition of yeast inoculation by Mori et al.

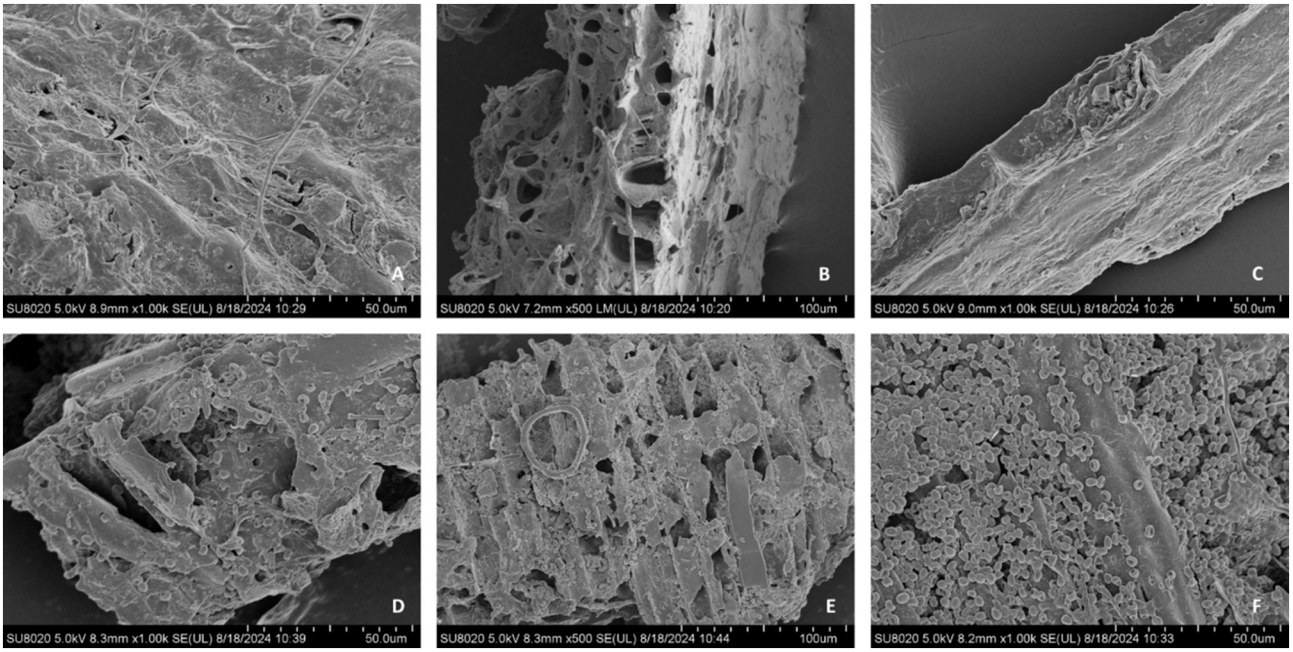

Fig. 3. Microscopic analysis of the surface of uninoculated SMS (A-C) and fermented SMS (D-E). Scale bar: 50–100 μm.

(2023) conducted on SMS substrate has resulted in a heavily decayed wood structure. From these findings, it was observed that fungal fermentation caused a collapsed cell wall layer morphologies. It has been reported that a similar observation was found by Xue et al. (2022) that the distortion structure changes in the fermented insoluble fiber of orange peel as a result of enzyme secretion by *Trichoderma reesei* and *Aspergillus niger*. Our observations reconfirmed the localization of 'lignin droplets' in the fermented substrate as none of these spherical structures were present in the untreated SMS. This suggested that fungal fermentation dramatically opened up the structure of the cell wall matrix, thus improving the accessibility of cellulose saccharification. Moreover, autoclaving the SMS substrate by pressurized steaming process not only improved the fermentation by structural modification, but also led to higher fungal cellulase activity and metabolism, in line with our findings (Donohoe et al., 2008). Even, Zhang et al. (2012) also found that a steam-pressurized approach in SSF of rice straw improved delignification potential. In particular, the SMS porosity structure could be improved with the reduction of cellulose crystallinity if steam explosion combined with fungal fermentation were adopted. Collectively, the SEM observation further suggested that fungal cultivation might help in increasing the specific surface area of fermented SMS structure during SSF.

3.3.2. FTIR analysis

FTIR analysis was performed to investigate the chemical structure of fermented SMS after SSF in comparison with untreated one. From Fig. 4, it was obvious that the spectra were similar for both samples. This indicates that the chemical composition and structure did not change very much after undergoing SSF. On the other hand, the broad peak at wavenumber 3327 cm^{-1} (peak I) in both samples could be associated with the hydroxyl (-OH) and CH groups stretching, indicating the presence of lignocellulosic structure in nature (Wang et al., 2024). Conversely, the spectra intensity at 1637 cm^{-1} (peak III) was more intense after SSF, which represents the stretching behavior in C═C groups in lignin. Such alterations indicated substantial lignin degradation. In the same trend, the peak spectra at 1243 cm^{-1} (peak I*V*) and 1031 cm^{-1} (peak V) associated with C—N and C—O groups stretching vibrations in cellulose and hemicellulose were relatively higher after being subjected to the SSF process. These peaks represented cellulose retention. In contrast, Nakason et al. (2018) mentioned that the cellulose crystallinity of cassava bagasse was decreased during hydrothermal treatment, which corresponded to the binding disruption in cellulose fraction. Another report demonstrated by Bhatia and Johri (2016) also explained the cellulase with fungus *Mucor indicus* MTCC 4349 could result in cellulose-related peak absorption changes of fermented pineapple peel as characterized by FTIR spectroscopy. In this regard, this peak was observed with reduced intensity, indicating the removal of amorphous cellulose. Apart from that, hemicellulose was characterized by the band at 1732 cm^{-1} (peak II) stretching at C═O unconjugated carbonyl groups with slightly reduced intensity spectra detected in fermented SMS after SSF. High cellulose content in SMS may collectively be responsible to produce sustainable enzymes in the SSF, as shown in the present result. Generally, the FTIR results were well correlated with the morphological structure changes result and assisted in comprehending the evident changes of functional group intensities.

3.4. Microbial community structure diversity in the fermentation substrate

A total of 27 phyla, 59 classes, 106 orders, 133 families, 165 genera, and 344 OTUs with more than 97 % sequence similarity were obtained from 2 samples (Control: uninoculated SMS and Consortium: fermented SMS with fungal-fermentative cultivation). A total of 322,663 bacterial sequences and alpha diversity indices, including Shannon-Wiener index, Simpson index and Chao 1 index were estimated in the both fermentation samples under two conditions (Table 1). Fungal cultivation led to significantly higher bacterial species and richness as compared to the

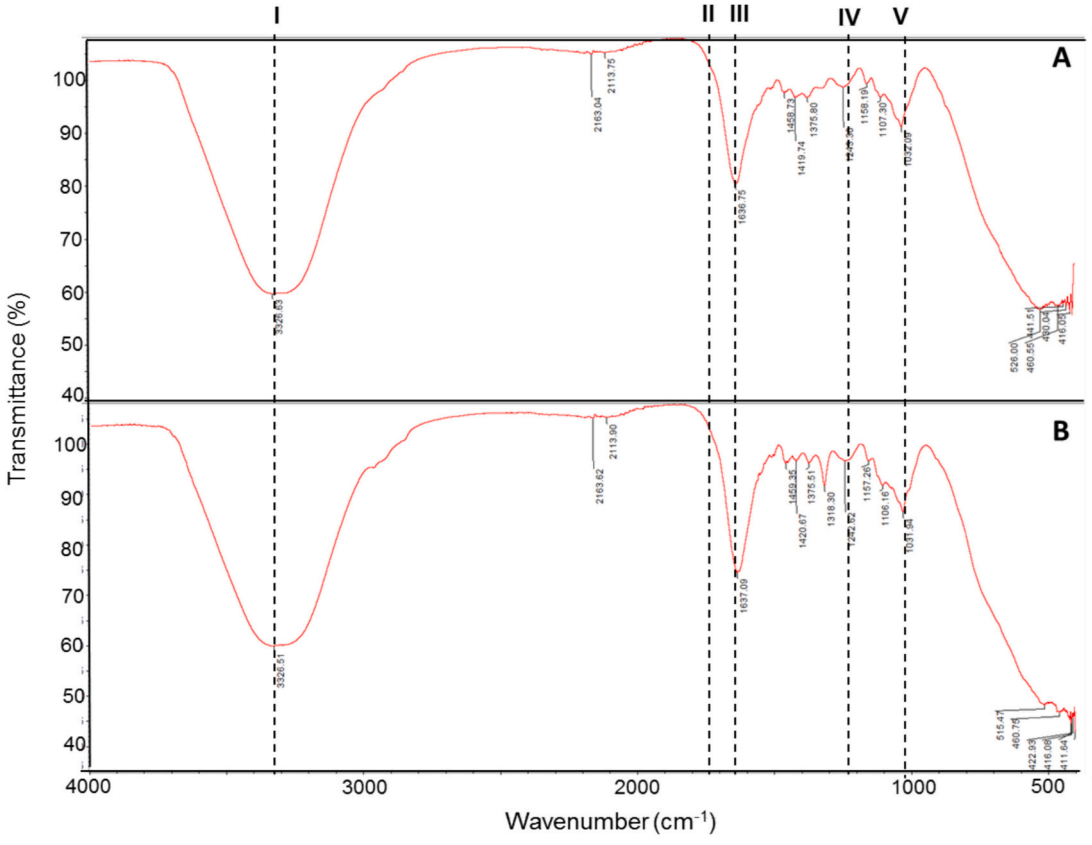

Fig. 4. FTIR spectra of untreated (A) and fermented SMS (B) before and after SSF.

Table 1
Summary of high-throughput sequencing data and diversity indices.

Sample name	No. of sequences	Observed species	Chao 1	Shannon	Simpson
Control	178,907	21	21.1 ± 0.42[a]	1.63 ± 0.04[a]	0.74 ± 0.15[a]
Consortium	143,756	337	337.1 ± 0.24[b]	3.66 ± 0.07[b]	0.93 ± 0.29[a]

Data are means of triplicate (n = 2) ± standard deviations. Different letters shown after values with the same column indicate significant differences ($p < 0.05$).

uninoculated control sample. Whereas the Shannon-Wiener index was significantly impacted by the fungal cultivation subjected to fermented SMS. However, no significant difference was recorded in bacterial diversity index in term of Simpson index, suggesting the dominant phyla were primarily expanding as the fermentation progress. These results demonstrated that the SSF process upon SMS, following fungal cultivation changes dramatically the diversity of the bacterial community in the samples. Gradually, the growth of non-dominant bacterial phyla was inhibited, leading to a decreased abundance diversity in the fermented SMS substrate. With higher bacterial species related to cellulose degradation, it was expected that SMS could be broken effectively and theoretically, a higher of fermentable sugars (glucose and xylose) produced as a result of *in situ* hydrolysis.

The proportion of shared and unique OTUs across samples was illustrated in Fig. 5. A shared of 14 bacterial OTUs was identified among these two samples. SMS with the fungal consortium and the uninoculated sample had 323 and 7 number of unique OTUs, respectively, indicating that the bacterial community was significantly increased by inoculation of *T. asperellum* IMCC2012 and *A. fumigatus* IMCC2006. Bacterial communities in fermented SMS after fungal cultivation were dominated by phyla abundance of *Firmicutes* (67.92 %), *Proteobacteria* (19.07 %), *Cyanobacteria* (3.52 %) and *Acidobacteriota* (3.11 %) (Fig. 6A). The relative abundance of *Firmicutes* was obviously reduced by 32.07 % while other phyla showed a sharp increase, after SSF for 144 h, revealing the microbial network formed resulting from inter-kingdom interactions. It has been reported that a similar observation found by Bianco et al. (2024) that the positive effect of specialized inoculum cultivation provided cooperative actions to achieve depolymerisation of raw brewers' spent grain and kept microbial stability. This suggested that the inter-kingdom interaction has existed and commonly occurred in the SSF processes, thus stabilizing the fermentation. Our observation possibly portrayed their co-existence condition by the fungal-fermentative consortium effectively broke down macromolecules into smaller beneficial biochemicals that could be readily utilized by native resident bacteria. In return, bacterial attachment onto fungal hyphae was observed as in Fig. 3E assisting bacteria in getting nutrient accessibility. The observation that *Firmicutes* dominated the entire SSF process was in accordance with Sun et al. (2021) report, which explains the presence of this phylum in degrading indigestible lignocellulose. These two phyla were similarly found in other rice-flavor Baijiu fermentation as the main dominant bacterial populations, but different in the abundance proportions (Zhou et al., 2023). The dominance of lignocellulose-degrading bacteria belonging to *Firmicutes* and *Proteobacteria* has been related to their higher tolerance to fluctuating environmental conditions. These resilient phyla mainly *Proteobacteria* convert water-soluble sugar to monosaccharides, which could imperatively enhance the whole cellulosic degradation process. Although *Firmicutes* showed a decreased abundance which was consistent with the observation of Gavande et al. (2021), this phylum remarkably contributed to hemicellulose decomposition with some of them were considered heat-labile microbes. This result was truly depicted the prevalent distribution of functionally phyla having the lignocellulolytic enzymes for efficient saccharification of plant-based biomass. Significantly, a relative abundance of phylum *Actinobacteria* has been associated with the degradation of lignocellulosic materials since they played a role to catalyze the hydrolysis of glycosidic bonds through glycosidase secretion (Meng et al., 2018). At the family level, the nine most abundant in SSF samples on SMS substrate were *Bacillaceae*, *Paenibacillaceae*, *Mitochondria*, *Aneurinibacillaceae*, *Brevibacillaceae*, *Enterobacteriaceae*, *Ktedonobacteraceae*, *Sporolactobacillaceae* and *Pedosphaeraceae* (Fig. 6B). By the end of fermentation, the bacterial community structures converged, with *Paenibacillaceae* increased sharply by 99.30 % and *Bacillaceae* decreased by 63.98 %, indicating that SSF process quickly altered the whole microbial community structure. It should be noted that their abundance exhibited a potential correlation with enzymatic production following the fungal SSF process. As fermentation progressed, the genera of *Paenibacillus* and *Bacillus* belonging to *Proteobacteria* phylum became dominant with relative abundance of 26.09 % and 34.01 %, respectively. The genus *Bacillus* was ubiquity in lignocellulosic degradation while *Paenibacillus* with the potential for cellulose secretion, which is of great interest in the current study. The increased abundance of genus *Brevibacillus* was helpful in the conversion of glucose into acid, explaining the acidic pH obtained. Here, a noticeable presence of the *Bacillus* genera, although SMS substrate was steam-sterilized. This findings however corroborated by the earlier reports, claiming that a number of spore-forming bacteria particularly from *Bacillus* genera could survive even the post-autoclaving process (Sahlström et al., 2008). In general, the evolutions of bacterial community structure at the family level after using specific fungal inoculum were consistent with those at the phylum level, ensuring cellulase and xylanase production. As shown in Fig. 7A, the genes of starch and sucrose metabolism, pyruvate metabolism, glyoxylate and dicarboxylate metabolism and fructose and mannose metabolism in the fermented SMS were abundant. Their relative abundances account for 37 %, 90 %, 80 % and 78 % of total carbohydrate metabolism, respectively. This indicated that the carbohydrates were preferentially produced during microbial metabolism as they played a significant role in the lignocellulosic degradation. The genes for amino acid metabolism from both samples were primarily glycine, serine and threonine metabolism and phenylalanine, tyrosine and tryptophan biosynthesis with relative abundances of 42–75 % and 31–49 %, respectively (Fig. 7B). As a result, the higher amino acid metabolism in

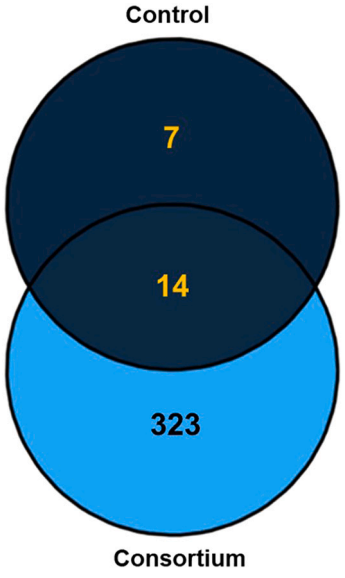

Fig. 5. Venn diagram for shared and unique 16S rRNA OTU between the different samples.

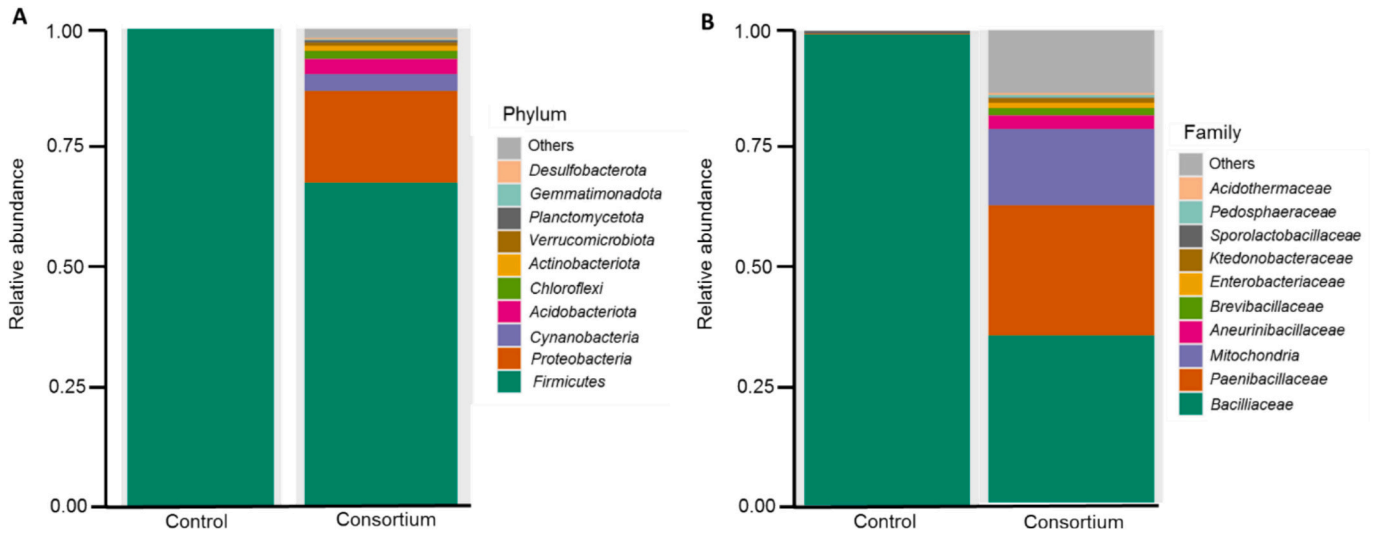

Fig. 6. Relative abundance (%) of dominant bacteria taxa. Dominant bacterial phyla (A) and family (B) in both samples, control (uninoculated) and consortium (fungal cultivation) refer to SMS subjected to solid state fermentation (SSF), respectively.

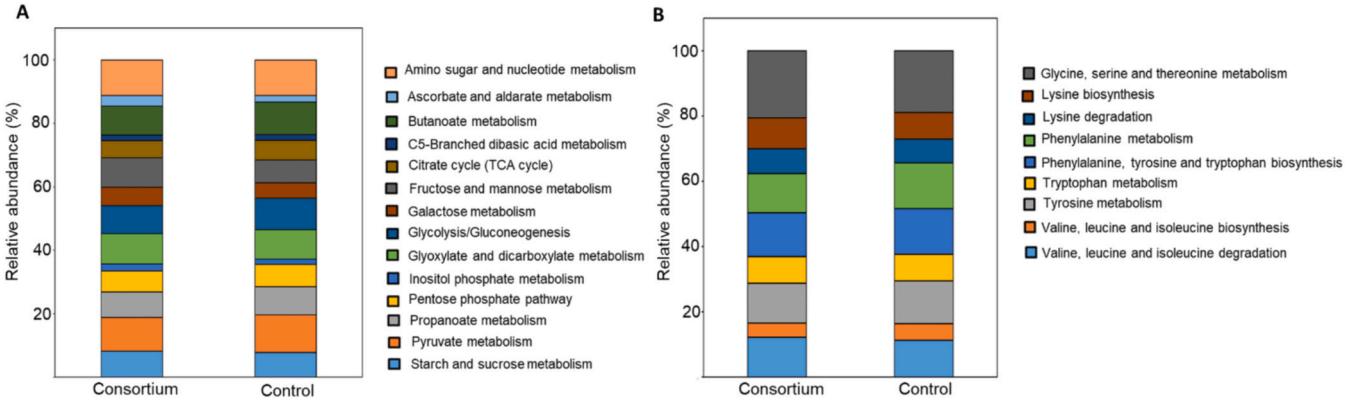

Fig. 7. Bacterial metabolism prediction by PICRUSt from both samples; control (uninoculated SMS) and consortium (fermented SMS by fungal cultivation). Carbohydrate metabolism (A) and amino acid metabolism (B).

SSF substrate was of great help in promoting fungal growth and cellulase stabilization.

As SSF technology continued to advance, its applications were considered essential and integrated with circular economic principles. These resource-efficient methods were designed to transform agricultural waste into greener industrial practices including food processing, pharmaceuticals and biofuel. Notable case studies and applications of SSF in various sectors illustrated how this technology is widely adopted to valorize waste residues into valuable biochemical products (Dessie et al., 2022; Sosa-Martínez et al., 2023). By leveraging the robust enzyme-producing capabilities of the fungal-fermentative consortium in conjunction with SMS as a substrate, future cost production processes could be optimized as there was no need to add commercial enzymes for hydrolysis. Currently, this study only analyzed the effect of several single variables on the enzyme activities out of the SSF process utilizing fungal co-cultivation, which makes this study have certain limitations. Future research should focus on the addition of lactic acid bacteria strains that could potentially co-regulate any feedback inhibition to maximize the enzyme capability, which is crucial for its scale-up application. Given the importance, further economic feasibility studies through process simulation are essential for technical readiness application. Besides, although this work has provided a piece of information about how fungal fermentation affected the bacterial communities, comparison on the microbial taxonomic succession and ecological network interactions in the time-lapse experiment across the samples should also be considered in the future. On the basis of above results, it can be concluded that this research provides a theoretical support for producing a high yield of sustainable enzymatic recovery with low operational cost.

4. Conclusion

The results highlighted that the co-cultivation of promising fungal-fermentative consortium consisting of *Aspergilus fumigatus* IMCC2006 and *Trichoderma asperellum* IMCC2012 under optimized SSF conditions effectively valorized spent mushroom waste (SMS) as a low-cost feedstock for enzymatic production. The maximum CMCase activity (12.06 U g^{-1}) was obtained using 5 g of substrate after 4 days of incubation at 30 °C and 60 % moisture content, with an optimal pH of 5.0. After 120 h, the highest FPase activity (4.35 U g^{-1}) was recorded at 30 °C, which was lower than the corresponding CMCase activity at the same incubation period. In addition, xylanase and FPase activities reached 13.89 U g^{-1} and 3.83 U g^{-1}, respectively, at 70 % moisture content. Structural and compositional analyses revealed substrate modifications, while bacterial community profilling indicated the dominance of *Firmicutes* and *Proteobacteria*, suggesting cooperative roles in the depolymerization of indigestible materials with cellulose degradation potential. The analysis of cellulase and xylanase activities, substrate decomposition and

bacterial community structure supported a robust fungal-fermentative strategy, offering an effective solution for SMS valorization and paving the way for potential biorefinery applications.

CRediT authorship contribution statement

Abd Rahman Jabir Mohd Din: Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tuan Liang Chua:** Software, Investigation, Formal analysis, Data curation. **Zaheda Mohamad Azam:** Resources, Investigation, Data curation. **Nor Zalina Othman:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Hesham Ali El-Enshasy:** Supervision, Resources, Methodology, Funding acquisition. **Jean Wan Hong Yong:** Writing – review & editing, Resources, Funding acquisition.

Ethical approval

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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