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SHORT COMMUNICATION

Direct Use of Spent Mushroom Substrate from *Pleurotus pulmonarius* as a Readily Delignified Feedstock for Cellulase Production

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Abstract The feasibility of spent mushroom substrate (SMS) as an alternative fermentation feedstock for cellulase production has been demonstrated in this work. Utilization of SMS as a substrate has been attempted widely due to its high cellulose content and readily available in smaller particle size. On top of that, the availability of delignified SMS by the action of *Pleurotus pulmonarius* during mushroom cultivation offers another benefit to its use whereby no chemical pretreatment would be required prior to fermentation. The recovery of crude laccase and manganese peroxidase from delignified SMS were found to be 3 and 1.4 U/g, respectively. Further to this, the cellulase production from SMS by *Trichoderma asperellum* UPM 1 under solid state fermentation was optimized by applying central composite design, resulted in increment of 1.4-fold in CMCase (171.21 U/g) and 1.5-fold in β-glucosidase (6.83 U/g), with the optimum temperature of 27.5 °C, initial moisture content 81% and initial pH of fermentation 4.5. Therefore, this study showed that the direct utilization of SMS is feasible for promising cellulase production by *T. asperellum* UPM 1.

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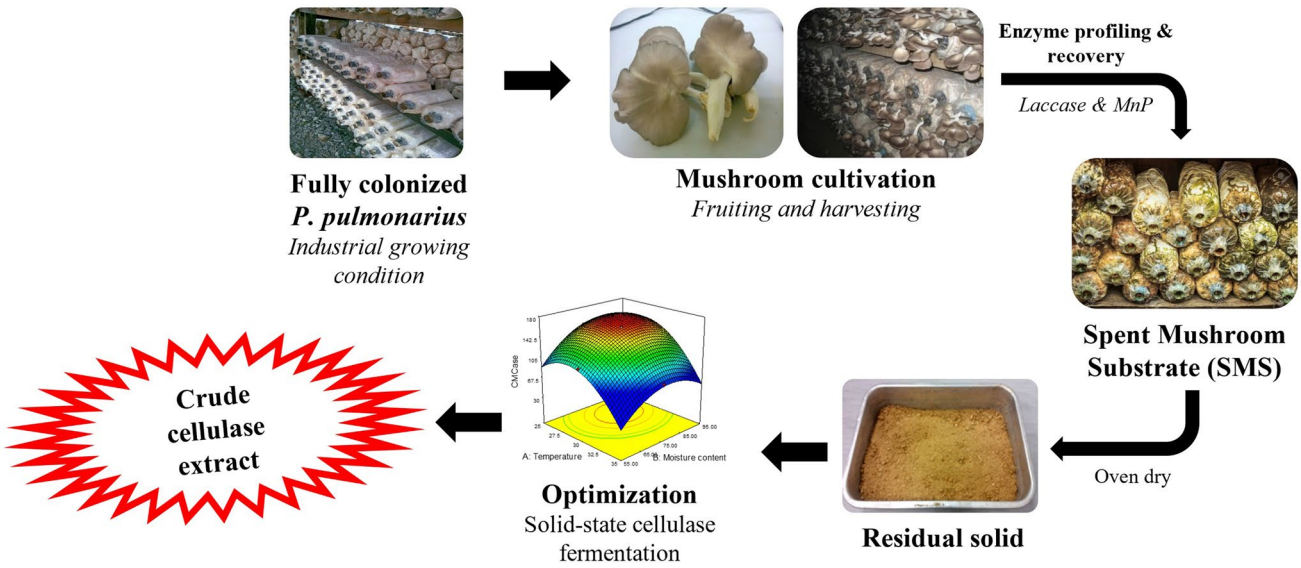
Graphical Abstract



Keywords Cellulase · Bioconversion · Lignocellulolytic enzyme · Filamentaous fungi · Spent mushroom substrate · Solid state fermentation

Introduction

Recently, mushroom demand in Malaysia as well as in the global market has escalated, leading to the increasing growth of the mushroom industry. The mushroom demand is expected to increase about 15% per year, with around 72,000 tonnes in 2020 [1]. The Malaysian government has declared mushroom farming as one of the eleven business opportunities under the National Key Economic Area (NKEA) [2]. Following this, the growing area for mushroom farming is estimated to reach 340 ha in 2020 compared to 78 ha in 2010 [1]. The nutritional values and medicinal properties of the mushroom which include anti-tumor, anti-diabetic and anti-oxidant [3] have contributed in the increasing demand for mushroom.

Along with the increased production of mushroom, spent mushroom substrate (SMS) generated has been increased over time. According to Phan and Sabaratnam [4], the annual average of mushroom production in Malaysia is approximately 100 tonnes of mushroom along with 438 tonnes of SMS. Majority of SMS were disposed in landfills and reused as agricultural fertilizers which cause leaching to the local water stream resulting in eutrophication [5]. Generally, SMS consists of fungal mycelia, extracellular enzymes and residual lignocellulosic biomass [6]. SMS of *Pleurotus* (oyster mushroom) is composed of single lignocellulosic biomass varying from wheat straw, cotton seed hull [7], sawdust, rice bran, beet pulp [6], corncob [8], and a combination of these lignocellulosic biomass [9]. The secretion of hydrolytic and oxidative enzymes during *Pleurotus* cultivation are responsible for the degradation of organic materials into soluble low molecular weight compounds which can be assimilated for fungal growth. Hence, these components collectively make SMS attractive and exhibit a great potential to be reused for various applications.

Many potential applications of SMS have been explored in the agricultural and industrial sectors including as a novel biosorbent [10], ruminant feed [11] and agricultural fertilizer [5]. The presence of crude enzymes especially laccase and peroxidases recovered from the SMS has resulted in the development of an economical approach for bioremediation of oil-contaminated industrial soils [12], phenols and polycyclic aromatic hydrocarbons (PAHs) [13]. Meanwhile, the rich population of heterotrophic fungi and bacteria in SMS have the ability to break down the xenobiotic compounds present in soil and water [14]. Despite its vast applications, very limited studies have been performed concerning the potential use of SMS as a feedstock for cellulolytic enzyme production.

Cellulase enzymes have gained consistent attention due to their extensive applications in the detergent and textile industry [15], pulp and paper industry [16], supplementation of animal feed [17], wine and brewing industry [18] as well as in the starch processing industry [19]. The recovery of lignocellulolytic enzymes from SMS and exploration of this inexhaustible supply for further commercial applications present very interesting research opportunities and continues to raise interest. In addition, application of SMS is the key to fully utilize the large amount of this waste in order to avoid

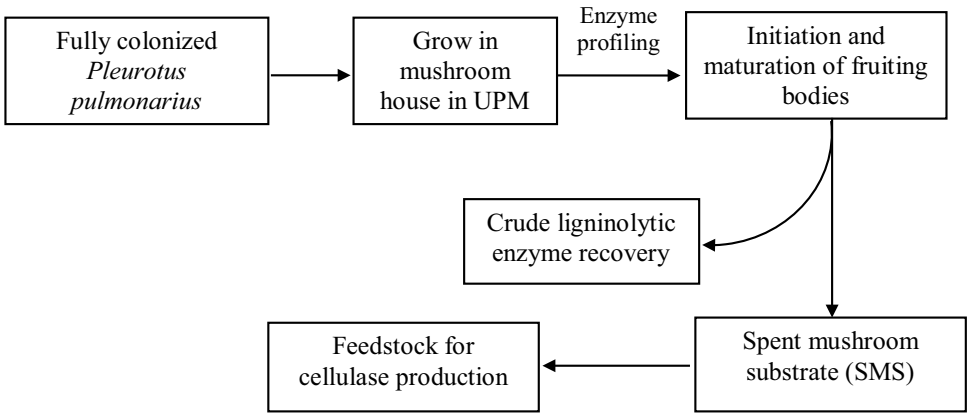


Fig. 1 A flowchart of spent mushroom substrate (SMS) from *Pleurotus pulmonarius* to be used as feedstock for cellulase production

disposal and environmental issues. Therefore, this study was carried out by adopting SMS as a potential feedstock for cellulolytic enzyme production. After the ligninolytic enzyme recovery, an experimental design using central composite design (CCD) was applied to determine the optimal condition for cellulase production and interaction among the process parameters.

Materials and Methods

Ligninolytic Enzyme Profile of *Pleurotus pulmonarius*

A fully colonized substrate of *P. pulmonarius* was obtained from the Vita Agrotech Mushroom Farm, Tanjung Sepat, Banting, Selangor, Malaysia. To initiate the fruiting stage, the mushroom bags were opened and grown in a mushroom house in Universiti Putra Malaysia (UPM). The conditions of the mushroom cultivation were as follow: temperature in the range of 20–25 °C; light intensity ranged from 800 to 1500 L 80 to 85% humidity; two times per day of watering. Pinheads appeared 4 days after opening of the mushroom caps, and the mushroom substrates were collected in the morning 10 am at every 2 days interval after the pinheads appeared. Sampling was prolonged during the resting period (when the cap of the mushroom bag was closed) until the start of the next cycle, whereby the samples were subjected to ligninolytic enzyme extraction. Experiments were carried out in triplicates and the results were presented as mean values.

Ligninolytic Enzymes Recovery

The SMS cultivated with *P. pulmonarius* was collected after 5 times harvesting, taken out from the polypropylene bag and mixed well in a tray. Then, SMS was mixed with 100 mL of 50 mM sodium citrate buffer (pH 4.8) in a ratio of 1:5 (w/v) and agitated for 30 min in an orbitary shaker. The crude enzyme extract was recovered by filtration through muslin cloth and centrifuged at 10,000 rpm for 15 min and subjected to ligninolytic enzyme assay [20], meanwhile the residual solid was dried in an oven at 85 °C overnight. The dried SMS (0.4–1.0 mm) was kept in a sealed plastic bag at room temperature prior to use as a substrate for cellulase production. A flowchart of the utilization of SMS from *P. pulmonarius* as a feedstock for cellulase production is represented in Fig. 1.

Cellulolytic Enzymes Production

Inoculum Preparation

Trichoderma asperellum UPM 1 was grown on potato dextrose agar (PDA) plate and incubated at 30 °C for 7 days. The spores were harvested using 5 mL of sterile 1% (v/v) Tween 80 and spore concentration was determined using a haemocytometer under light microscope. Then, the collected spores were stored at 4 °C prior to use.

Table 1 Process variables and assigned levels for central composite design for cellulase production from spent mushroom substrate

Process variables	Symbol	Coded and actual value				
		$-\alpha$	−1	0	+1	$+\alpha$
Temperature (°C)	X_1	25	27.5	30	32.5	35
Initial moisture content (%)	X_2	55	65	75	85	95
Initial pH of the medium	X_3	3	4	5	6	7

Solid State Fermentation

Cellulase production was carried out under solid state fermentation (SSF) in 100 mL Erlenmeyer flask using 4 g dried SMS (0.4–1.0 mm). The substrate was moistened with Mandel solution [21] containing (g/L); $(NH_4)SO_2$, 1.4; KH_2PO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.3; $CaCl_2$, 0.3; peptone, 0.75; Tween 80, 1% (v/v), 2 mL; urea (47%), 0.63 mL; and trace element, 1 mL (0.005 g $FeSO_4{\cdot}7H_2O$, 0.0016 g $MnSO_4{\cdot}7H_2O$, 0.0014 g, $ZnSO_4{\cdot}7H_2O$ and 0.002 g $COCl_2$). The initial moisture content was adjusted to 80% by adding 12 mL of Mandel solution. Substrates and Mandel solution were sterilized separately prior to fermentation at 121 °C for 15 min. Spore suspension of 1×10^6 spores/mL was introduced into the fermentation flask. The fermentation was carried out in a static incubator for 10 days at 30 °C and samplings were done for every 24 h interval.

Optimization of Cellulase Production Using Central Composite Design

The central composite design (CCD) was used in this study to develop a mathematical model from the independent variables on the production of CMCase, FPase and β-glucosidase. Three independent variables: temperature (X_1), initial moisture content (X_2) and initial pH (X_3) were chosen in this experiment at low, middle and high concentration levels, designated as − 1, 0, and + 1, respectively. Activities of CMCase, FPase and β-glucosidase were then selected as responses and coded as Y_1, Y_2 and Y_3, respectively. The design was represented by a second order polynomial regression model according to the following equation (Eq. 1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y is the measured response (CMCase, FPase and β-glucosidase, U/g), β_1, β_2, and β_3 are linear coefficients, β_{11}, β_{22}, and β_{33} are squared coefficients, and β_{12}, β_{13}, and β_{23} are interaction coefficients. The coded and actual values of the variables are given in Table 1. The design matrix consists of 20 experimental runs, including 6 center point runs. Statistical analysis of the data was performed using the Design Expert Software (version 7.0 Stat-Ease, Inc.).

Analytical Procedures

Determination of Lignocellulosic Content

Cellulose, hemicellulose and lignin content of the SMS were determined by the standard method of AOAC [22].

Scanning Electron Microscopy of Mushroom Substrate

Scanning electron microscopy (SEM) analysis was employed to examine the morphological changes of the mushroom substrate. Samples (raw and spent mushroom substrate) were oven dried at 90 °C for 24 h. The fibrous samples were placed onto carbon tape. The samples were sputter coated with platinum prior to morphological observations. SEM images were recorded at ×1000 magnification using an acceleration voltage of 15 kV.

Determination of Lignocellulolytic Enzyme Activities

Lignocellulolytic enzyme activities were determined using UV–Vis spectrophotometer (Shimadzu, Japan). All enzyme activities were expressed as U/g of cultivation substrate, whereby one unit activity was defined as the amount of enzyme oxidizing 1 µmole of substrate per minute. The sampling was done in triplicates and three replicates per enzyme sample were carried out at the same conditions.

Ligninolytic Enzymes Assay

Manganese peroxidase (MnP) activity was measured as the oxidation of Mn^{2+} to Mn^{3+} by following formation of Mn^{3+} tartrate complex at 465 nm ($\varepsilon_{465\ nm} = 12.1$ mM/cm). The reaction mixtures consisted of 100 mM DL-lactate

Table 2 Lignocellulosic composition of raw and pretreated mushroom substrate in comparison with other biomass samples

Materials	Lignin (%)	Cellulose (%)	Hemicellulose (%)	References
Rubber	28.0	39.0	29.0	[54]
Pine	28.4	58.2	NA	[55]
Spruce	28.1	57.6	NA	
Birch	20.2	51.7	NA	
Sawdust	28.3	39–44	25–34	[56]
SMS	25.0	38.0	19.0	[27]
SMS from *P. ostreatus*	11.0	48.7	22.1	[26]
SMS from *P. pulmonarius*	21.3	45.5	13.1	This study

NA not available

buffer (pH 4.5), 1 mM $MnSO_4$, 2 mM H_2O_2 and 500 µL samples [23]. Laccase activity was determined using ABTS [2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] as substrate at 420 nm ($\varepsilon_{420\ nm}$ = 36 mM/cm). The reaction mixture contained 150 mM sodium acetate buffer (pH 4.5), 0.03% (w/v) ABTS and 350 µL samples [24]. LiP activity was measured using veratryl alcohol as substrate at 310 nm ($\varepsilon_{310\ nm}$ = 9.3 mM/cm). The reaction mixtures consisted of 100 mM citrate phosphate buffer (pH 3.0), 20 mM veratryl alcohol, 20 mM H_2O_2 and 500 µL samples [25].

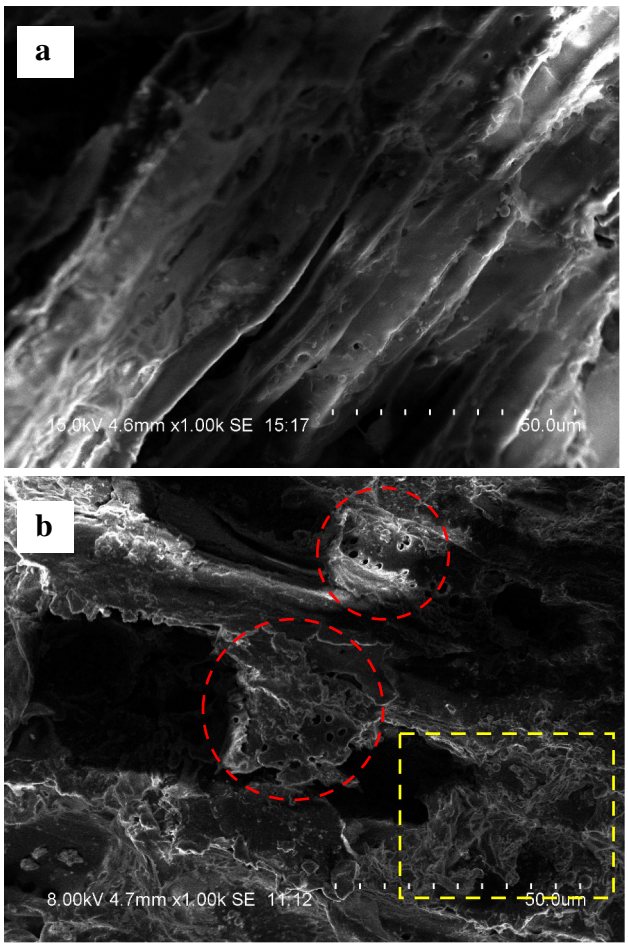


Fig. 2 SEM images of **a** raw mushroom substrate, **b** spent mushroom substrate by the action of *P. pulmonarius*

Cellulolytic Enzyme Assay

The activities of carboxymethylcellulase (CMCase) (EC 3.2.1.4), filter paper (FPase) (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) were assayed according to the methodology described earlier by Wood and Bhat [26]. The concentrations of reducing sugars liberated during reactions on FPase and CMCase were determined by the 3,5-dinitrosalicylic acid method at 575 nm wavelength. *p*-nitrophenyl-β-D-glucopyranoside (pNPG) was used as a substrate for determination of β-glucosidase activity. The *p*-nitrophenol released on pNPG was quantified using *p*-nitrophenol as a standard and absorbance was read at 400 nm wavelength. One unit of enzyme activity (U) was defined as the amount of enzyme required to generate 1 µmol of the product from their corresponding substrate per minute.

Results and Discussion

Chemical Composition of Spent Mushroom Substrate

The total cellulosic content in lignocellulosic biomass is one of the important criteria that need to be considered in selecting suitable substrate for cellulase production. Table 2 shows a significant content of holocellulose (hemicellulose and cellulose) and lignin in SMS supplemented with sawdust, as well as for other lignocellulosic biomass. As regards to the fibre constituents, SMS used in this study is presented to have high cellulose content (45.5%), in agreement with the studies

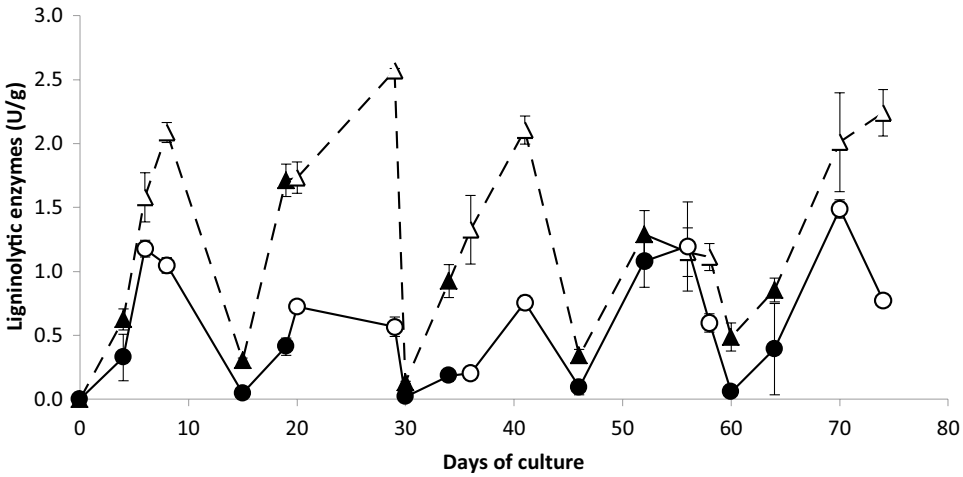


Fig. 3 Ligninolytic enzymes (laccase and MnP activities) of *Pleurotus pulmonarius* during fruiting and harvesting stages. Filled triangle: laccase, fruiting; open triangle: laccase, post-harvest; filled circle: MnP, fruiting; open circle: MnP, post-harvest

conducted by Koutratsios et al. [27] and Jordan et al. [28] which reported 48.7 and 38% of cellulose content in SMS, respectively. The high content of cellulose in SMS gives a profound effect particularly for cellulase induction [29].

One of the major constraints in utilizing any raw substrate for cellulase production is the presence of lignin which acts as a barrier and restricts the direct contact between enzyme and substrate materials [30]. In consequence, the raw substrate used for cellulase production has to undergo a pretreatment process in order to remove or modify the lignin structure. In the case of SMS, a significant amount of lignin removal had been achieved [31] during the period of mushroom cultivation [32], hence require no addition of chemical and less energy consumption for the pretreatment process. Lignin and hemicellulose showed significant loss of 6.7 and 25.9%, respectively during mushroom cultivation. Comparable results in lignin reduction were also presented by Koutrotsios et al. [27] in the cultivation of *P. ostreatus* in SMS of beech sawdust with 4.7% reduction from initial value of 15.7%. Selective lignin and hemicellulose degradations by *Pleurotus* spp. offer remarkable benefits in utilizing SMS as a substrate by exposing the cellulose component for further application [33]. In addition, the small particle size of SMS in the range of 0.4–1.0 mm is very suitable to be employed as a fermentation feedstock for saccharification as compared to the woody material [31].

The structural changes of raw mushroom substrate (sawdust) during mushroom cultivation of *P. pulmonarius* were then observed using scanning electron microscopy (SEM). The SEM image of raw mushroom substrate (Fig. 2a) shows rigid, highly fibrillar and ordered structure of fibre, as supported by Zhu et al. [34]. Upon the biological pretreatment during mushroom cultivation period, the microscopic image showed the modification of the SMS fibrillar to less ordered structures (Fig. 2b) with the detachment of the fibres, eroded, cracked, loose and collapsed cell walls (in rectangular) [35, 36]. More irregular pores (in circle) were also found on the SMS strand's surface which resulted from the breakdown of the lignocellulose structures due to fungal attack [37]. The presence of these pores would increase the substrate porosity and surface area of accessible cellulose for microbial enzyme attack [36, 37]. From the SEM image, it showed that biological pretreatment had occurred during the cultivation period and had met the purpose of pretreatment in removing lignin and increase porosity of SMS. Therefore, a direct route for cellulase production from SMS biologically pretreated with *P. pulmonarius* would be achieved without chemical pretreatment prior to fermentation.

Table 3 Crude enzyme extract from spent mushroom substrate

Mushroom strain	Crude enzyme extract (U/g)			References
	Laccase	MnP	LiP	
P. pulmonarius	3.00	1.40	ND	This study
P. floridanus	2.62	2.10	ND	This study
P. ostreatus	2.97	NA	NA	[6]
P. eryngii	8.01	NA	NA	
P. cornucopiae	3.77	NA	NA	
P. sajor-caju	1.62	NA	154.30	[20]

NA not available, *ND* not detected

Ligninolytic Enzyme Profile of *P. pulmonarius*

Figure 3 shows periodical bell shaped patterns of laccase and MnP activities produced by *P. pulmonarius*. Higher extracellular laccase activity up to twofolds (2.57 U/g) was recorded after the second harvest as compared to MnP activity. Laccase is the most common ligninolytic enzyme found in SMS, with varied amounts depending on the mushroom strains. Meanwhile, higher MnP activities were recorded after first and final harvests with value of 1.18 U/g and 1.20 U/g, respectively. From Table 3, lignin peroxidase (LiP) was not detected from the crude extract and from all mushroom strains except from *P. sajor-caju*. Hence, it is suggested that the type of enzymes produced during mushroom cultivation is mainly affected by the species of the mushroom and ingredients of the growth substrates [38].

All enzymatic activities increased as the *P. pulmonarius* reached its maturity and were maintained at high levels even after the harvesting period (a stage when the mycelia start to regenerate). The higher production of laccase and MnP activities during this stage suggested that ligninolytic enzymes were secreted as secondary metabolites [39]. The fruiting process caused depletion of the intramycelial substrate to a level which prevented the next fruiting body formation. Thus, accumulation of the mycelia is necessary for the initiation of fruiting body formation [40]. Therefore, based on this study, the high enzyme activities during mycelia accumulation are possibly associated with the secretion of extracellular enzymes for the uptake of the necessary carbon and nutrient sources. The bell shaped pattern of enzymes also proved that secretion of extracellular enzymes is in two different stages of mushroom production; vegetative and fruiting. Similar profiles have also been demonstrated by other white-rot Basidiomycetes such as *P. ostreatus* [41, 42] *Grifola frondosa* [43] and *P. pulmonarius* [41].

The crude laccase and MnP detected from the sawdust-based SMS after the final harvest of *P. pulmonarius* suggested that ligninolytic enzymes are the main components that responsible for the degradation of the lignin in SMS, hence could be a good potential feedstock for cellulase production. Furthermore, cellulose production would be achieved without chemical pretreatment prior to fermentation. Drying of SMS,

Table 4 Full factorial of central composite design and experimental results of cellulase production by *Trichoderma asperellum* UPM 1 from spent mushroom substrate

Run	Temperature (X_1)	Initial moisture content (X_2)	Initial pH (X_3)	Cellulase activities (U/g)		
				CMCase	FPase	β-glucosidase
1	−1	+1	+1	166.02	8.08	5.80
2	0	0	+α	125.73	7.03	2.89
3	−1	−1	−1	135.73	8.59	6.52
4	+1	−1	−1	101.84	7.20	1.44
5	−α	0	0	147.04	8.5	9.13
6	0	+α	0	142.4	7.9	4.05
7	0	0	−α	120.26	7.46	2.91
8	+1	−1	+1	103.70	7.72	1.18
9	−1	−1	+1	137.00	7.59	5.97
10	−1	+1	−1	158.00	9.27	6.41
11	+α	0	0	83.00	7.00	0.93
12	0	−α	0	109.66	7.13	3.07
13	+1	+1	−1	114.53	7.35	1.72
14	+1	+1	+1	128.28	7.86	1.87
15	0	0	0	159.33	8.43	4.09
16	0	0	0	161.57	8.59	3.95
17	0	0	0	158.03	8.38	4.17
18	0	0	0	159.7	8.4	3.88
19	0	0	0	163.01	8.48	4.18
20	0	0	0	162.02	8.65	3.85

Table 5 Analysis of variance (ANOVA) for response surface quadratic model obtained for CMCase, FPase and β–glucosidase production by *Trichoderma asperellum* UPM 1 in solid state fermentation

Source	p value Prob > F		
	CMCase	FPase	β-glucosidase
Model	< 0.0001	< 0.0001	< 0.0001
A-Temperature	< 0.0001	< 0.0001	< 0.0001
B-Initial moisture content	< 0.0001	0.0010	0.0224
C-Initial pH	0.0151	0.0116	0.2120
AB	0.1362	0.0866	0.1021
AC	0.4819	< 0.0001	0.1616
BC	0.0567	0.6751	0.6253
A2	< 0.0001	0.0005	0.0004
B2	< 0.0001	< 0.0001	0.0391
C2	< 0.0001	< 0.0001	0.0002
Lack of fit	0.0688	0.0973	0.0580

however is necessary prior to fermentation and it is costly and energy intensive treatment. Thus, appropriate drying method suitable for industrial scale should be developed to reduce the cost. Parameters that can influence dryer's energy use also should be considered with the aim to reduce the consumption of heat and electricity.

Optimization of Cellulase Production from SMS

Statistical optimization by CCD was adopted to investigate the optimal levels of the significant variables for cellulase production from SMS. The relationship between the variables and three corresponding responses; CMCase, FPase and β-glucosidase with mean values were analysed and shown in Table 4. The maximum CMCase activity was measured in run 1 (166.02 U/g), while the activities of FPase and β-glucosidase peaked in run 10 (9.27 U/g) and 5 (9.13 U/g), respectively. In general, middle levels of variables gave higher values for CMCase and FPase activities compared to other combinations of parameters except β-glucosidase. The lowest cellulase activities; CMCase (83 U/g), FPase (7 U/g) and β-glucosidase (0.93 U/g) were obtained under SSF conditions of 35 °C, initial moisture content of 75% and initial pH of 5 (Run 11). The results were then subjected to analysis of variance (ANOVA).

As shown in Table 5, significant *p* values and insignificant lack of fit for all three models suggested that the models were acceptable for predicting CMCase, FPase and β-glucosidase activities. Moreover, the adequacy of the models was determined based on the high R^2 values (0.97–0.99) with only 0.8–3.3% of the total variation not being explained by the regression model. The *p* values in the ANOVA table indicate the significance of each coefficient (Table 5). Variables with *p* values less than 0.05 specify that the model terms are significant and the smaller the *p* value, the higher the significance of

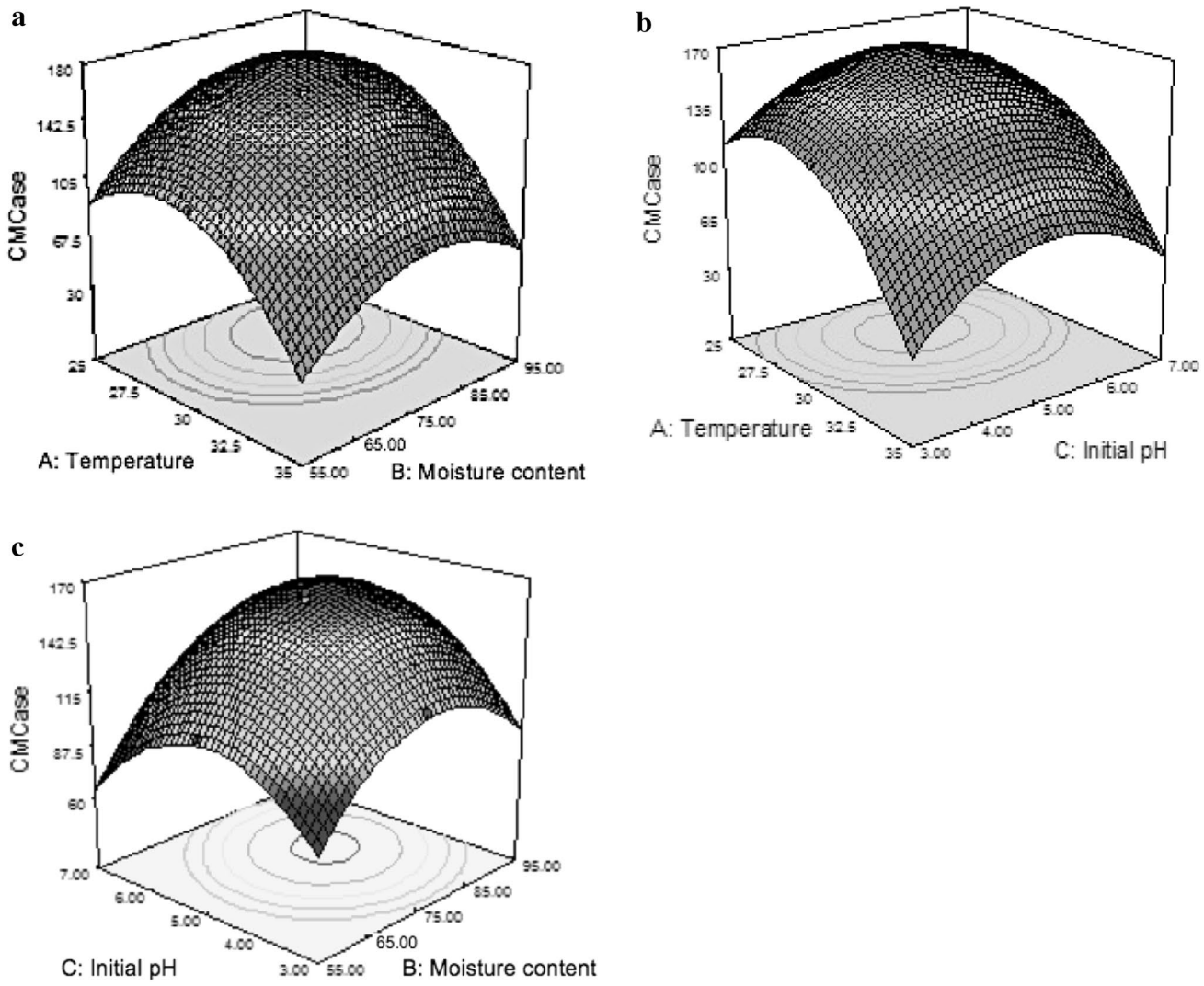


Fig. 4 D response surface plots showing the interactions of **a** temperature and initial moisture content, **b** temperature and initial pH and **c** initial moisture content and initial pH on CMCase production by *T. asperellum* UPM 1 in a solid state fermentation

the particular variable. Initial moisture content, initial pH and incubation temperature are identified as important parameters which affect cellulase production in the SSF system. Application of CCD in optimization of fermentation conditions for cellulase production by locally isolated *T. asperellum* UPM 1 allows rapid identification of the important parameters. In this study, temperature was found to be the most critical parameter for all cellulase enzymes, whereby the small changes of temperature could influence cellulase production. The final regression equations in term of coded factors for cellulase produced from SMS are as given below:

$$Y_1 = 160.51 - 17.28X_1 + 9.63X_2 + 2.24X_3 - 11.45X_1^2 - 8.69X_2^2 - 9.45X_3^2 - 1.75X_1X_2 + 0.79X_1X_3 + 2.33X_2X_3$$

$$Y_2 = 8.53 - 0.40X_1 + 0.19X_2 - 0.13X_3 - 0.17X_1^2 - 0.22X_2^2 - 0.29X_3^2 - 0.11X_1X_2 + 0.40X_1X_3 - 0.025X_2X_3$$

$$Y_3 = 4.02 - 2.18X_1 + 0.17X_2 - 0.082X_3 - 0.25X_1^2 - 0.12X_2^2 - 0.28X_3^2 + 0.16X_1X_2 + 0.13X_1X_3 + 0.044X_2X_3$$

where Y_1, Y_2 and Y_3 were predicted CMCase, FPase and β-glucosidase activities, respectively.

Three dimensional response surface curves were plotted from the experimental results of SSF to deduce the optimal levels of each parameter. The shapes of the contour plots stipulate the nature and extent of the interactions [44]. The understanding on the interaction between parameters is essential for attaining maximal cellulase activities. Figure 4a

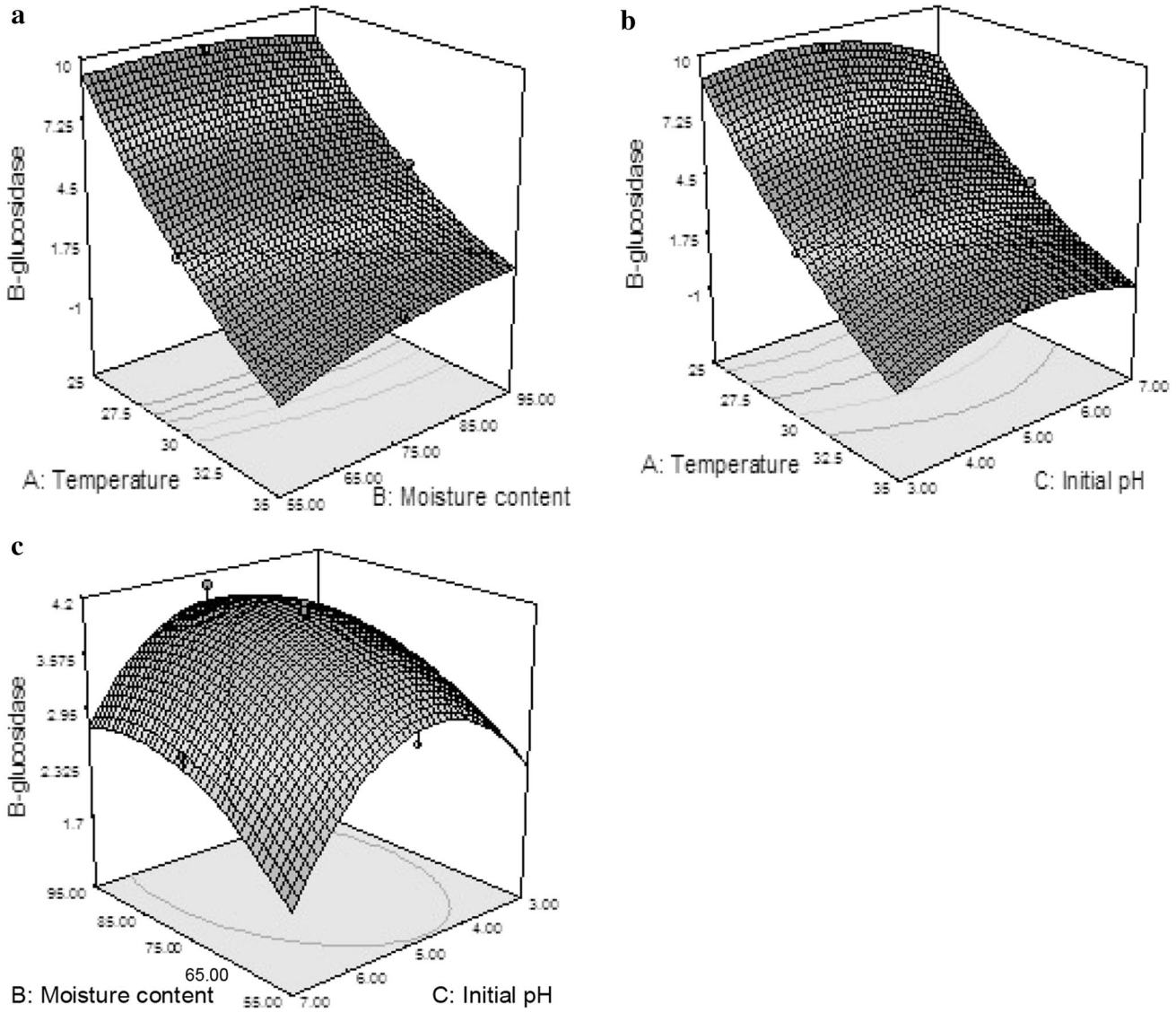


Fig. 5 D response surface plots showing the interactions of **a** temperature and initial moisture content, **b** temperature and initial pH and **c** initial moisture content and initial pH on β-glucosidase production by *T. asperellum* UPM 1 in a solid state fermentation

shows the maximal CMCase activity was obtained between 75 and 85% of initial moisture content regardless of incubation temperature. The response surface in Fig. 4b shows a minor shift towards lower incubation temperature with the highest CMCase activity being in the pH range of 4.0–4.5 and incubation temperature of 27.5 °C. Increase of incubation temperature to more than 30 °C was strongly affected the CMCase activity. Meanwhile, an increase in moisture content (optimum at 75–80%) led to an increase in CMCase activity till optimal values were reached (Fig. 4c).

Suitable range of moisture content which did not exceed the maximum binding capacity of the water and solid substrate is important for fungal growth and metabolism [45]. In this study, clumping of substrate was observed at higher moisture content of more than 85%, which suggested it was due to the thick layer of water which surrounded the substrate particles [46]. This would lead to substrate porosity reduction and limited oxygen transfer between particles and surroundings [47], thus, resulting in the decrease of fungal growth which in turn lowered the enzyme production. On the other hand, lower moisture content (Run 12) decreased the solubility of nutrients, thus hindering efficient nutrient uptake by the fungi [48]. As exemplified by Yoon et al. [46], moisture content between 60 and 80% are favorable for both microbial growth and cellulase production in SSF. However, it is necessary to take into account the nature of

Table 6 Comparison of cellulase production form various substrate and fungi in solid state fermentation

Substrate	Fungi	CMCase (U/g)	FPase (U/g)	β-glucosidase (U/g)	References
Oil palm trunk	*Aspergillus fumigatus* SK1	54.27	3.36	4.54	[57]
Rice straw	*Trichoderma*	23.01	NA	NA	[58]
Oak sawdust and corn bran	Grifola frondosa	12.3	16.2	2.3	[42]
Wheat bran	*Fomitopsis* sp. RCK2010	71.53	3.27	50.70	[59]
SMS from *P. pulmonarius*	*Trichoderma asperellum* UPM 1	171.21	9.85	6.83	This study

NA not available

the substrates applied in fermentation when determining the optimal moisture content [49]. Essentially, this differs due to different water holding capacity of each substrate which has significant effect on water activity [50].

Surface area of substrate is dependent on the particle size of the particular substrate with small particle size providing larger surface area. The availability of optimal surface area is important for nutrient absorption, fungal support and growth, gas exchange and enzyme production [51, 52]. The experimental results obtained in this study is in accordance with other studies which showed particle size < 1.0 mm providing the highest cellulase activity [51, 53]. Additionally, direct use of SMS without the milling and grinding steps prior to fermentation could be advantageous as a feedstock for cellulase production.

Figure 5a–c show the response surface and contour plots for β-glucosidase. Temperature was observed to act as a critical parameter in β-glucosidase production. It was noted that in this study, β-glucosidase production was critically affected by temperature. The incubation temperature at 25 °C improved the amount of β-glucosidase production by up to 2.28-fold (9.13 U/g), meanwhile low temperature (< 27.5 °C) was observed to strongly influence the production of β-glucosidase (Table 4). As reported by Singhania et al. [15], temperature is one of the important parameters that affecting cellulase yield and known to be a microorganism-dependent parameter. This study is in agreement with Sohail et al. [54] whereby the highest activity of β-glucosidase from corncob, grass and bagasse as substrates was recorded at 25 °C and showed a drastic effect at 40 °C. Hence, the variations of the temperature strongly affected the cellulase production.

The model predicted a maximum CMCase activity of 166.02 U/g, FPase activity of 9.06 U/g and β-glucosidase of 6.44 U/g following 7 days of cultivation and incubation at 27.5 °C with initial pH of medium at 4.5 and 81% initial moisture content. Validation runs with three repetitions were performed using the predicted optimal conditions. CMCase (171.21 U/g), FPase (9.85 U/g) and β-glucosidase (6.83 U/g) obtained in the validation runs, were in closed agreement with the predicted values. The accuracy and applicability of CCD to optimize the culture medium for cellulase production were proven with the high degree of similarity between the predicted and the experimental values with less than 20% error. The optimized conditions resulted in increment of 1.4-fold in CMCase and 1.5-fold in β-glucosidase. Moreover, the CMCase activity produced by *T. asperellum* from SMS is significantly higher compared to other fungi as shown in Table 6.

Conclusions

Over decade, one of the crucial issue faced by mushroom growers is waste management of SMS. Current disposal practice of SMS which is being disposed in landfill require high cost and has become burden to mushroom grower and municipality. Often think disposal only include cost at landfill, transportation cost is the actual component contributing to high disposal cost. Total of disposal cost will increase depending to the distance of disposal facility from mushroom farm. High moisture content of SMS (60–75%) also contributed to increase of transportation cost. Thus, in order to reduce the cost, the cost of disposal should be kept as low as possible. Extracting enzyme from SMS and producing beneficial enzyme using SMS will minimize the logistic problem of SMS. The direct utilization of spent mushroom substrate (SMS) without prior pretreatment which consists of small particle size of 0.4–1.0 mm and high cellulose content (45.5%) are the major advantages of SMS as a potential feedstock for cellulase production. In this study, Central composite design (CCD) was applied for the optimization of CMCase, FPase and β-glucosidase production from SMS by *T. asperellum* UPM 1, which showed the existence of correlations between the independent parameters and the response. Higher cellulase activities were achieved with the optimized fermentation condition which showed increment of 1.4-fold in CMCase (171.21 U/g) and 1.5-fold in β-glucosidase (6.83 U/g).

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