# Analysis of Phenolic Compounds in Empty Fruit Bunches in Oyster Mushroom Cultivation and in Vermicomposting

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Analyses of total phenolic compounds were carried out for oil palm empty fruit bunches (EFBs) vermicomposting in oyster mushrooms cultivation. The oyster mushrooms (*Pleurotus sajor-caju*) were cultivated according to the large-scale vermicomposting trial (LSVT) methods. Both oyster mushrooms cultivation and vermicomposting of EFB with earthworms enhanced the lignin degradation of EFB. Analysis of total phenolic compounds EFB vermicomposting treated with earthworms showed a decrease in total phenolics concentration from 31.1 GAE/100g extract (raw EFB) to 5.66 g GAE/100g extract (after oyster mushroom cultivation) and to less than 1.5 g GAE/100g extract at the end of vermicomposting. Gas chromatography–mass spectrometry (GC-MS) analysis of the mushroom fruiting body, spent mushrooms, and vermicompost showed no trace of phenolphenol, pyrocatechol, 4-hydrobenzoic acid, or antioxidant and flavonoid phenolics, *e.g.*, phenol, 3,4-dimethoxy-, vanillic acid, and cinnamic acid. This indicates that the mushroom fruiting body is fit for human consumption and the final vermicompost is a useful agricultural product without the detrimental effects of spreading phenolics-loaded EFBs on the land.

*Keywords: Phenolic compounds; Empty fruit bunch; Vermicomposting; Oyster Mushroom*

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## INTRODUCTION

Malaysia is one of the largest palm oil producers in the world, accounting for approximately 37% of the total world palm oil production in 2011 (Sharma *et al.* 2012). Although the production of palm oil by Malaysian palm oil industries has boosted the national economy, it also generates a vast amount of oil palm biomass, including oil palm empty fruit bunches (EFBs), during processing of fresh fruit bunches for palm oil production (Tye *et al*. 2016). Oil palm EFBs are produced in large quantities in localized areas. The traditional practice has been to use it as fuel for small-scale generation of steam at the palm mills. However, this method has been prohibited since Malaysia strengthened air quality regulations, so a new method of disposal for EFBs is needed. Thus, there is an opportunity to develop a low-cost method to produce vermicompost using unsterile/ untreated EFB.

Several phenolic acids can be found in EFBs, such as *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, ferulic acid, and vanillin (Mohamad Ibrahim *et al*. 2008; Multari *et al.* 2016). Phenolic compounds in other lignocellulosic materials, like wheat and

flax straws, include ferulic acid, coumaric acid, vanillin, and vanillin acid (Buranov and Mazza 2008; Oliva-Taravilla *et al.* 2016).

The edible mushroom *Pleurotus sajor-caju* has a known capability to degrade lignin compounds in lignocellulosic waste (Corrêa *et al.* 2016). Laccase, phenolic oxidase, and manganese oxidase are enzymes released by *P. sajor-caju* to oxidize phenolic compounds to free radicals (Jeon *et al.* 2012). *Pleurotus sajor-caju* has been used in solid-state fermentation of pasteurized EFBs to produce a feed supplement for ruminants such as cows and goats (Awang *et al.* 1993; Jeon *et al.* 2012). However, no studies have been undertaken to determine the concentration of phenolic compounds in the vermicomposted EFBs.

Sabrina *et al.* (2012) studied the effect of direct composting of EFBs and palm fronds by earthworms in an oil palm plantation and found that *Pontoscolex corethrurus* and *Amynthas rodoricensis* died immediately when added to EFBs. It is believed that the death of both species was due to phenolic compounds, as phenols are the most toxic chemicals to earthworms (Bi *et al.* 2016). Phenolic compounds in EFBs are expected to be tannin, chlorogenic and benzoic acid derivatives, and procyanidin oligomers, which are all phenolic compounds found in lignocellulosic residues (Sabrina *et al.* 2012; Arshadi *et al.* 2016). These authors also suggested that intensive insecticide spraying in oil palm fields might leave EFBs with chlorinated phenols as well as phenolic compounds from the lignin. The degradation products of lignin may leach from the litter into the ecosystem and have a high potential for environmental pollution (Wang *et al.* 2002).

The aim of the present work was to determine phenolic compounds in EFB vermicomposting cultivated with oyster mushroom using the Folin-Ciocalteu method, and characterize phenolics in EFBs by GC-MS analysis before and after *P. sajor-caju* cultivation and vermicomposting by *Eisenia fetida* and *Dendrobaena veneta.*

## EXPERIMENTAL

### Sample Collection

All EFBs were originally collected in the form of short and long fibers from Sabutek Sdn. Bhd (Kuala Lumpur, Malaysia). Plant health movement documents and plant passports (phytosanitary certificates) were produced by the Malaysian government before shipment to the UK, as required by EU Legislation and the Food and Agriculture Organization (FAO) International Plant Protection Conventions. The EFBs were ground to sizes of 5 to 10 mm using a Glen Creston Beater mill (Retsch GmbH, Germany) soaked for 2 weeks, and drained well before use.

### Cultivation of Oyster Mushroom

*Pleurotus sajor-caju* mycelium was cultivated according to the large scale trial methods (LST) on potato dextrose agar in Petri dishes for stock, and wheat grain was used to prepare the spawn of the fungus. Ten percent fresh spawn was added to 5 kg of EFBs in breathable polyethylene bags and placed in a temperature-controlled propagator in darkness at 25 °C for three weeks. When fungal pinheads started to develop, the bags were placed in a propagator in a light box. After 12 h, the propagator was ventilated, and the first flush of fruiting bodies (mushrooms) were harvested after 2 to 3 days. The interval between flushes was then 5 to 7 days, and the harvest lasted for 25 weeks. Four trials were

conducted for large scale vermicomposting trials (LSVT), such as LSVT1, LSVT2, LSVT3 and LSVT4.

## Vermicomposting by *Eisenia fetida* and *Dendrobaena veneta*

One kilogram of *Eisenia fetida* and *Dendrobaena veneta* was added to 5 kg of decomposed EFBs after the fungal cultivation, and the mixture was placed in a four-level vermicompost reactor. Vermicomposting was monitored for 30 weeks. Moisture content and temperature were maintained at 80% and 19 to 24 °C, respectively.

## Total Phenolic Compound Analysis

Determination of total phenolic compounds and their characterization were carried out separately following the method reported by Waterhouse (2002). This procedure was modified to minimize the amount of sample needed and waste produced in determining total phenolic compound concentrations. For a high recovery of phenolic compounds in the extract, high-purity methanol (≥ 99.9%) was used as the extraction solvent (Waterman and Mole 1994). Air-dried samples (5 ± 0.01 g) were placed in cellulose extraction thimbles in the extraction chamber of a Buchi-B811 LSV automated extractor (Flawil, Switzerland). Sample flasks were filled with 100 mL of 99% AR-grade methanol ($CH_3OH$) from Fisher (Fisher Scientific, Loughborough, UK). The extraction mode was set for a combination of Soxhlet and hot extraction for 2 h with unlimited cycles. Nitrogen gas was supplied at 0.35 $L \cdot min^{-1}$ during the extraction. The system purified extracts and reduced the sample to 2 mL using preprogrammed rinsing and drying by the automated extractor. Extracts were filtered through 0.45-µm fiberglass Whatman filter paper. Samples were stored at 4 °C for further analysis.

A stock solution of 5000 $mg \cdot L^{-1}$ gallic acid was prepared by dissolving 5 ± 0.01 g of anhydrous gallic acid in 100 mL of ethanol and then adding 750 mL of distilled water. The dissolved solution was transferred to a 1-L volumetric flask, and distilled water was added until the total volume reached 1 L. From the stock solution, different standard solutions were prepared, from 50 to 3000 $mg \cdot L^{-1}$.

A stock solution of 200 $g \cdot L^{-1}$ $Na_2SO_3$ was prepared by dissolving 200 ± 0.1 g $Na_2SO_3$ in 800 mL of distilled water and boiling at 60 °C. After all the $Na_2SO_3$ was dissolved, a few crystals of $Na_2SO_3$ were added to the solution and left at room temperature for 24 h. Whatman no. 1 filter paper was used to filter the solution, and distilled water was added to make up 1 L. Then, 20-µL standard solutions were pipetted into 2-mL plastic cuvettes together with 1.58 mL of distilled water. After this, 100 µL of Folin-Ciocalteu reagent (Fisher Scientific, Loughborough, UK) was added to each cuvette. Within 8 min, 300 µL of sodium carbonate (200 g/1000 mL) was added and the cuvettes were incubated in the dark at 40 °C for 30 min. Absorbance was measured at 765 nm against a blank using a DR 2800 HACH Dr. Lange spectrophotometer (Thermofisher; Loughborough, UK). Absorbance was plotted against a standard solution concentration. Extracts were analyzed by replacing the standard solution with extracts. The concentration of total phenolic gallic acid equivalent (GAE) of the extracts was calculated from the graph using the linear regression equation.

## Characterization of Phenolics by GC-MS Analysis

Isolation and recovery of phenolics were carried out following the method developed by Sabrina *et al.* (2012), with slight modifications, as shown in Table 1, where total phenolics was extracted using dichloromethane (DCM, 99%). For the procedure, 2

mL of concentrated extract was cleaned by passage through a ready-made Florisil/$Na_2SO_4$ syringe tube. Hexane was used to wash and activate the column, and acetone was used to collect the eluate. The eluate was concentrated to dryness in a rotary evaporator with a nitrogen stream. Then, 2 mL of ethanol was added and the eluate was filtered with a fiberglass Whatman 0.22-µm syringe filter and stored in vials for gas chromatography mass spectrometry (GC-MS) (Agilent Technologies 789OA GC System MS 5975C VL MSG) (California, USA) analysis.

For samples with no derivative, an aliquot of 0.5 µL was injected using the splitless mode for 30 s into a Thermo Finnigan GC-MS. For processing data, Thermo Xcalibur-Qual Browser software was used. Phenolic compounds were identified by comparison with the MS in the National Institute of Standard and Technology data libraries (NIST 2.0). Helium gas was used as carrier gas with 99.99% purity. All the analyses were carried out in triplicate, and the data reported are the mean values from the triplicate experimental run.

**Table 1.** GC-MS Experimental Conditions

| Gas Chromatography | Mass Spectrometry |
|---|---|
| Column (30 m X 0.32 mm, 0.25 µm film thickness) | Transfer line temperature 300 °C |
| Head column pressure 10 kPa (5 min), 70 kPa (25.47 min) + 1.0 kPa·min$^{-1}$ | Electron impact ionization 70 eV<br>Scan mode (*m/z*) 50 – 500, rate 1.5 scans/s |
| Injector temperature 300 °C | |
| Injected volume 1 µL splitless mode 30 s | |
| Oven program 80 °C to 250 °C at 10 °C/min, remained for 2 min<br>Injector temperature 260 °C<br>Carrier gas Helium (99.999% purity) | |

A blank extract was produced (without EFBs), and a standard solution of phenol (purity ≥ 98%) was used to develop the calibration curve to quantify the phenol concentration in EFBs. Standard solutions with 0.1 µg·L$^{-1}$, 0.05 µg·L$^{-1}$, 0.033 µg·L$^{-1}$, and 0.00067 µg·L$^{-1}$ were added to blank extracts in different vials. All samples were analyzed using the same conditions as for non-derivatized samples. Phenol peak areas (MA) were plotted against phenol concentration. The concentration of phenol in EFB extracts was read from the linear regression line on the graph. Gas chromatography mass spectrometry (GC-MS) (Agilent Technologies 789OA GC System MS 5975C VL MSG) with a triple axis detector was used to analyze derivatized samples.

## RESULTS AND DISCUSSION

The total phenolic compounds in raw EFBs, analyzed using the Folin-Ciocalteu method, were found to be 31.10 g GAE/100 g extract, as presented in Table 2. The total phenolic compounds in EFBs in this work were higher than those reported by Anyasi *et al.*

(2015) using the Folin-Denis method (total phenolic compounds concentration of 10 g GAE/100 g extract). These differences in total phenolic compounds might be due to application of the different methods. It can be presumed that the total phenolic compounds found in this work were more accurate, as Folin and Ciocalteu improved upon the Folin-Denis reagent by making it more sensitive to reduction by phenolics, less prone to precipitation, and giving 30% more color for spectrophotometric absorption (Waterman and Mole 1994) .

**Table 2.** Determination of Phenol and Total Phenolic Compounds in EFBs

| Parameter | Unit | Mean | SE |
|---|---|---|---|
| Total Phenolic Compounds | g GAE/100 g extract | 31.10 | 0.131 |
| Phenol | mg/kg | 1.35 | 0.036 |

Silane, trimethylphenoxy-, or phenol TMS was found in the GC-MS analysis at a retention time (RT) of 3.98 min using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as a derivative added to the EFB extract (Table 2). Analysis of non-derivative EFB extracts also found phenol at a RT of 9.68 min and other phenolic acids (*e.g.*, pyro-catechol, 4-hydrobenzoic acid, phenol 3,4-dimethoxy, and cinnamic acid), as shown in Fig. 1 and Table 3. These findings are consistent with studies reported by Ibrahim *et al.* (2008) and Sabrina *et al.* (2012), which found these phenolic acids in EFBs. Phenol TMS (derivatized sample) had a lower retention time (appearing at a RT of 3.984 min) compared with a non-derivatized sample (RT of 9.68 min, Fig. 1).

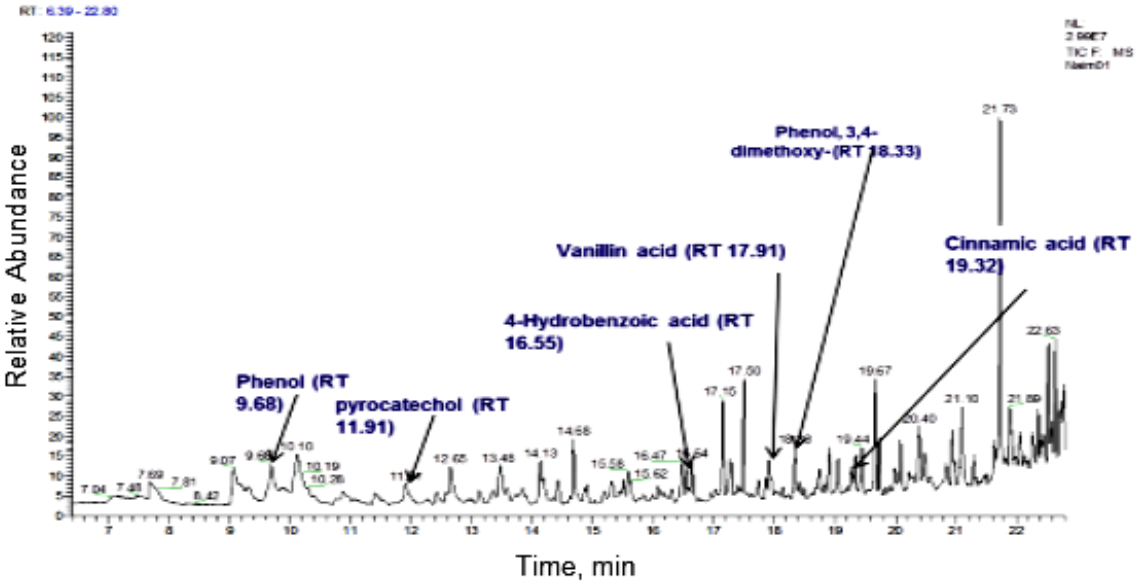


**Fig. 1.** GC-MS analyses for the determination of the phenolic acids in EFBs using iso-octane as the background solvent for the development of the calibration curve

This shows that BSTFA modifies phenol to TMS-ether compounds, which are volatile derivatives (Waterman and Mole 1994). The presence of phenols in both derivatized and non-derivatizated samples confirms that phenols are present in EFBs, which indicates that raw EFBs may not be a good feedstock for vermicomposting because phenols are toxic to earthworms (Neuhauser *et al.* 1986). The retention time for phenol in

the sample extracts (non-derivatized) was shortened from 9.68 to 5.57 min when the background solution was changed from iso-octane to ethanol, as presented in Fig. 2. Ethanol therefore may be regarded as the better background solution for GC-MS analysis (Table 3). The total concentration of phenol in EFBs in this work was 1.35 mg·kg$^{-1}$ (Table 2).

**Table 3.** Determination of the Background Solution for GC-MS Analysis

| Compound | Background Solution | Retention Time (min) |
|---|---|---|
| Phenol | Iso-octane | 9.68 |
| Pyro-catechol | Iso-octane | 11.91 |
| 4-Hydrobenzoic acid | Iso-octane | 16.55 |
| Vanillin acid | Iso-octane | 17.91 |
| Phenol,3,4-dimethoxy | Iso-octane | 18.33 |
| Cinnamic acid | Iso-octane | 19.32 |
| Phenol TMS/Silane, trimethylphenoxy- | Iso-octane | 3.984 |
| Phenol | Ethanol | 5.57 |
| Phenol (standard, 0.1 μg·L$^{-1}$) | Ethanol | 5.52 |
| Phenol (standard, 0.05 μg·L$^{-1}$) | Ethanol | 5.42 |
| Phenol (standard, 0.033 μg·L$^{-1}$) | Ethanol | 5.44 |
| Phenol (standard, 0.033 μg·L$^{-1}$) | Ethanol | 5.42 |
| Phenol (standard, 0.00067 μg·L$^{-1}$) | Ethanol | 5.43 |

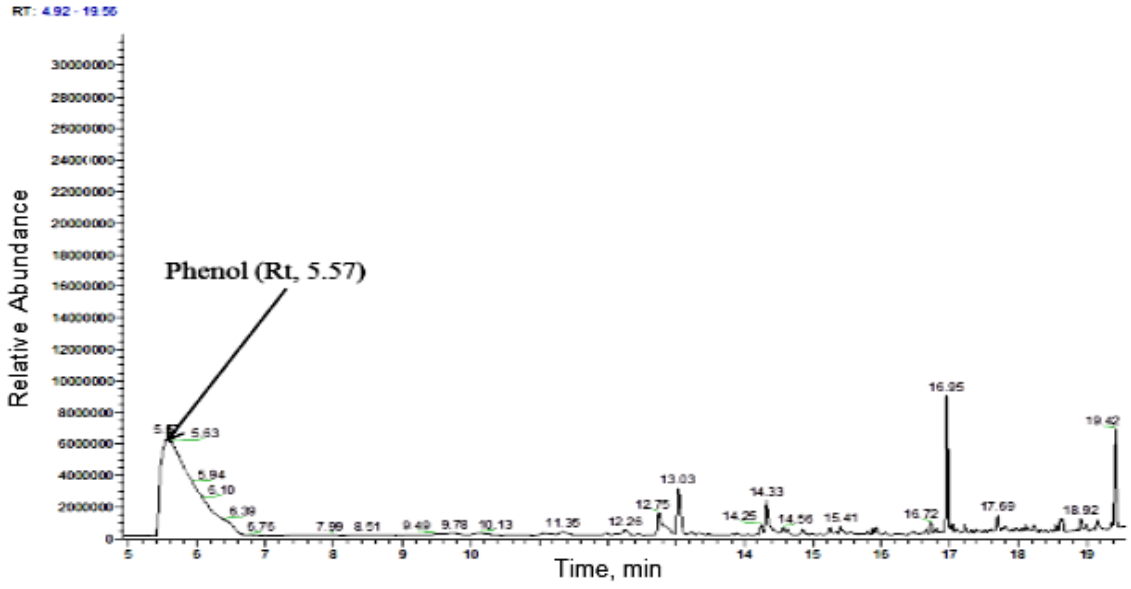


**Fig. 2.** GC-MS analyses for the determination of the phenolic acids in EFB using ethanol as the background solvent for the development of the calibration curve

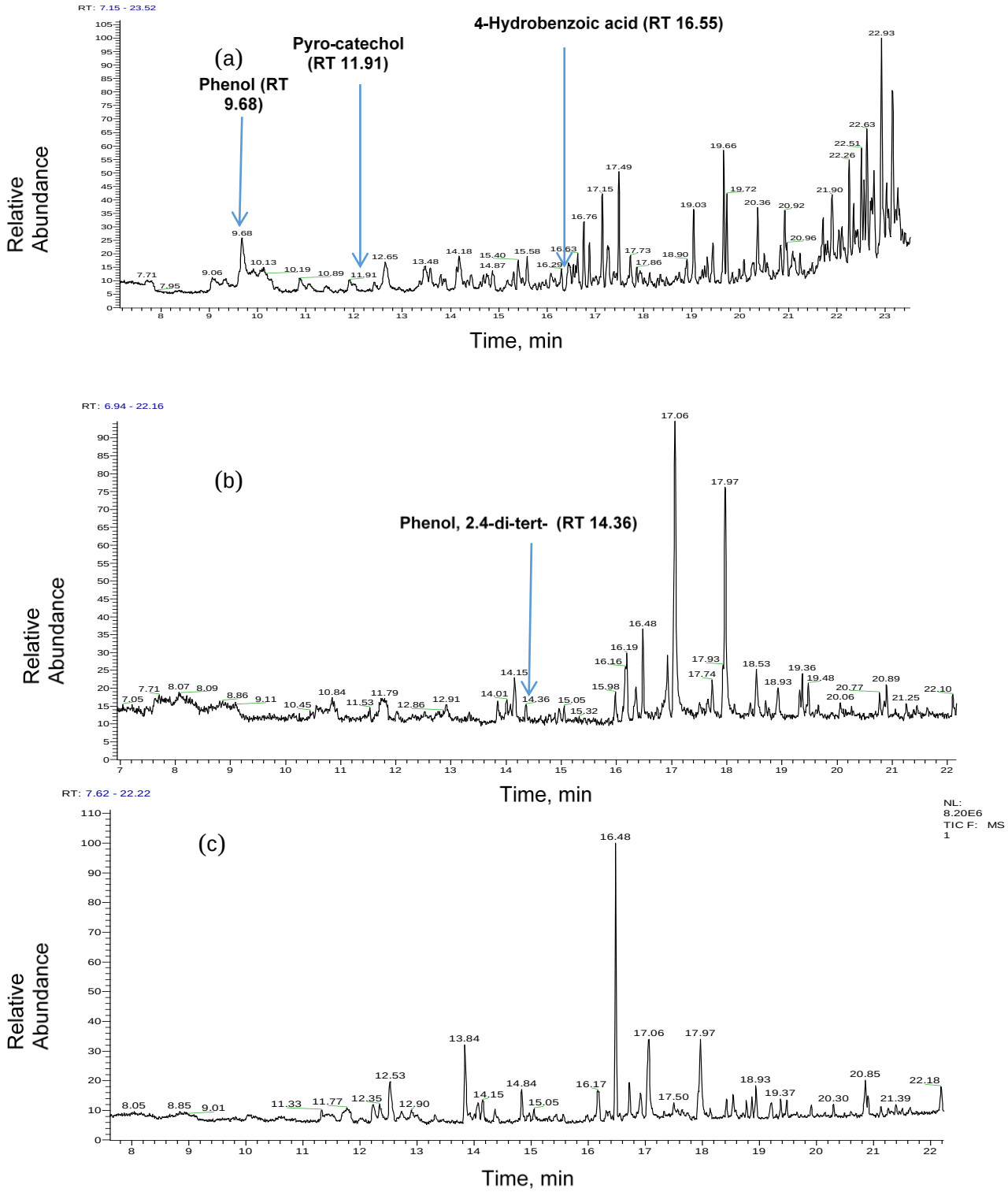


**Fig. 3.** Chromatographs of the phenolic acids in (a) after middle of cultivation, (b) at the end of cultivation, and (c) showing no trace of phenolic acids in the vermicompost

Figure 3 shows chromatographs of the phenolic acids in raw unsterile EFBs, cultivated EFBs, and in the vermicompost. However, the absence of phenolic acids in

vermicompost was observed. Changes in total phenolic compound (TP) concentration were monitored over 25 weeks of cultivation of *P. sajor-caju.* The total reduction in TP was between 85% and 92%, as shown in Table 4 and Fig. 4. Total phenolics sharply decreased at the end of primordial initiation, after five weeks of cultivation (Fig. 4). There was a correlation between spawn running and primordial initiation during weeks 3 to 4. Rani *et al.* (2008) showed high cellulase, laccase, and polyphenol oxidase activity during this period and attributed it to the need for carbohydrates by fungal mycelia for sporophore and fruiting body formation. The TP bounce back (but not by more than 25 g GAE/100 g) may have been caused by active mycelial growth, which increases carbon dioxide in the substrate, suppressing other organisms involved in the degradation of phenolic derivatives. This might cause temporary accumulation of degraded total soluble phenol in the EFBs (Rani *et al.* 2008). Reduction of TP and the absence of phenol in samples at the end of cultivation, after 25 weeks (end of Stage IV in Fig. 4), suggest that there is a strong lignin degradation by *P. sajor caju*.

GC-MS analysis was carried out at three points in the cultivation: before cultivation (raw EFBs or control) (Table 5), in the middle of cultivation (after five harvests and 10 weeks of cultivation), and at the end of cultivation (after another 15 weeks, at week 25). GC-MS analysis of the mushrooms was carried out on samples from every harvest. Traces of phenol (RT of 9.68 min), pyro-catechol (RT of 11.91 min), and 4-hydrobenzoic acid (RT of 16.55 min) were found in the middle stage (Table 5). Vanillin acid was not found in these samples, but it was in the control (Table 4). Figures 3a to 3c highlight compounds found in GC-MS chromatograph plots from the large simple trials (LSTs) after the first 10 weeks (end of stage 2 in Fig. 4) out of a total of 25 weeks cultivation.

There was no trace of phenol, pyro-catechol, or 4-hydrobenzoic acid at the end of cultivation (Table 5). However, Fig. 3b does show traces of phenol, 2,4-di-tert (RT of 14.36 min) in these samples. This is similar to results reported by Anyasi *et al.* (2015), who still found phenol, 2,4-di-tert after four weeks of vermicomposting following one month of pre-composting EFBs with cow manure. According to Baldrian *et al.* (2005), *Pleurotus* spp. have two extracellular enzymatic systems (hydrolytic and oxidative lignolytic systems), which degrade hemicellulose and cellulose, and lignin, respectively. These enzymes may be responsible for the degradation of lignin and subsequent reduction in phenolic compounds in the EFBs (Table 18). No phenol was present in any of the mushroom fruiting body throughout the cultivation (Table 5 and Fig. 3c). This is an indication that oyster mushrooms produced on EFB substrates are safe for human consumption.

**Table 4.** Chemical Composition of Treated EFBs after 15 Weeks

| Trial | Lignin (%) | Reduction of Lignin (%) | C:N ratio | Reduction of C:N Ratio (%) | TP (g GAE/100g Extract) | Reduction of TP (%) | Phenol (mg/kg) |
|---|---|---|---|---|---|---|---|
| LST1 | 8.64 | 30.20 | 30.82:1 | 51.52 | 9.83 | 68.40 | NT |
| LST2 | 9.23 | 25.42 | 31.78:1 | 49.74 | 5.90 | 84.64 | NT |
| LST3 | 9.79 | 20.91 | 32.53:1 | 48.35 | 1.66 | 95.66 | NT |
| LST4 | 8.91 | 28.03 | 31.40:1 | 50.44 | 5.24 | 86.36 | NT |
| *NT = No trace in GC-MS analysis | | | | | | | |

**Table 5.** GC-MS Analysis of Phenolic Acids in Control at the Middle and End of Cultivation

| Compound | Retention Time (min) | Raw EFBs (Control) | Middle Stage of Cultivation [a] | | | | End of Cultivation [b] | | | | *P.sajor-caju* Fruiting Body |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | LST1 | LST2 | LST3 | LST4 | LST1 | LST2 | LST3 | LST4 | |
| Phenol | 9.68 | √ | √ | √ | √ | √ | - | - | - | - | - |
| Pyro-catechol | 11.91 | √ | √ | √ | √ | √ | - | - | - | - | - |
| 4-Hydrobenzoic acid | 16.55 | √ | √ | √ | √ | √ | - | - | - | - | - |
| Vanillin acid | 17.91 | √ | - | - | - | - | - | - | - | - | - |
| Phenol, 3,4-dimethoxy | 18.33 | √ | √ | √ | √ | √ | - | - | - | - | - |
| Cinnamic acid | 19.32 | √ | √ | √ | √ | √ | - | - | - | - | - |
| Phenol, 2,4-di-tert | 14.36 | - | - | - | - | - | √ | √ | √ | √ | - |

a: Harvest within 5 to 10 weeks of cultivation; b: Harvested within 15 to 25 weeks of cultivation period; √: Present; -: absent

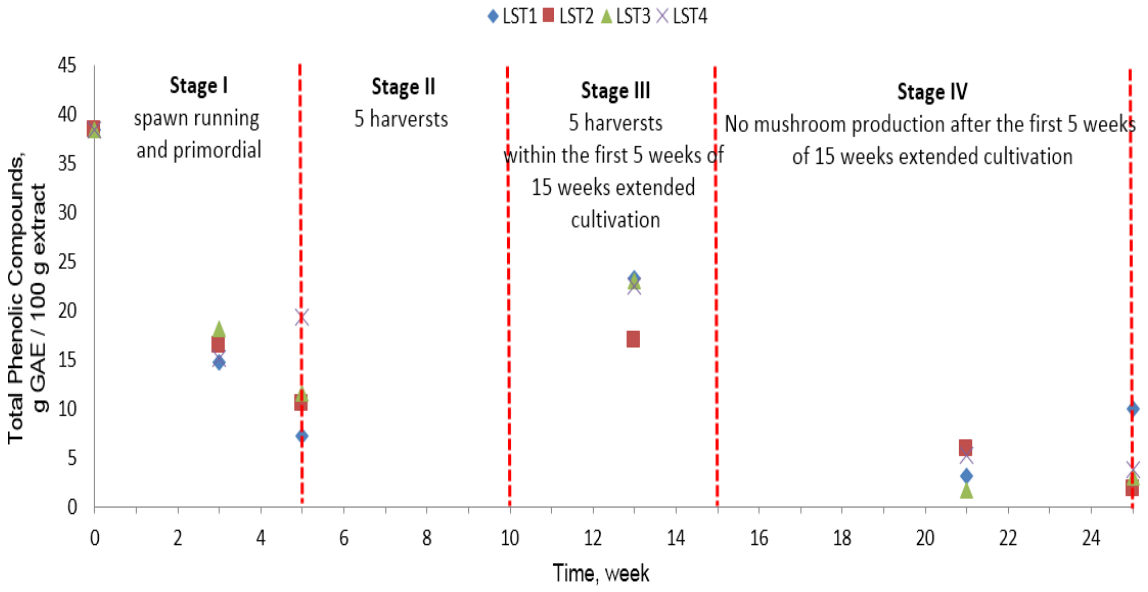


**Fig. 4.** Total phenolic compound concentrations throughout LST trials

Figure 5 shows total phenolic compound concentrations during vermicomposting. The concentration of total phenolic compounds reached a maximum value of 4.5 g GAE/100 g extract for LSTV1 and LSTV3 at week 10. For LSTV2 and LSTV4, there was no increase in total phenolic compounds during vermicomposting. Total phenolics in all trials was less than 1.5 g GAE/100 g extract after 30 weeks of vermicomposting. It is

presumed that there was some enzymatic activity left by *P. sajor-caju* in the vermicomposted EFBs.

GC-MS analysis of vermicomposted EFBs shows no trace of phenol, and the low levels of TP indicates that the lignin content of vermicomposted EFBs was very low (Fig. 5). This is consistent with results reported by Lazcano *et al.* (2008) and Anyasi *et al.* (2015), who found no phenol by GC-MS analysis and 15 g GAE/100 g of phenolic compounds in vermicomposted EFBs pre-composted with cow manure (12 weeks vermicomposting with *E. fetida*). However, Hayawin *et al.* (2011) still found 15% lignin and 24% hemicellulose-cellulose in vermicomposted EFBs (pre-composting with POME and cow manure).

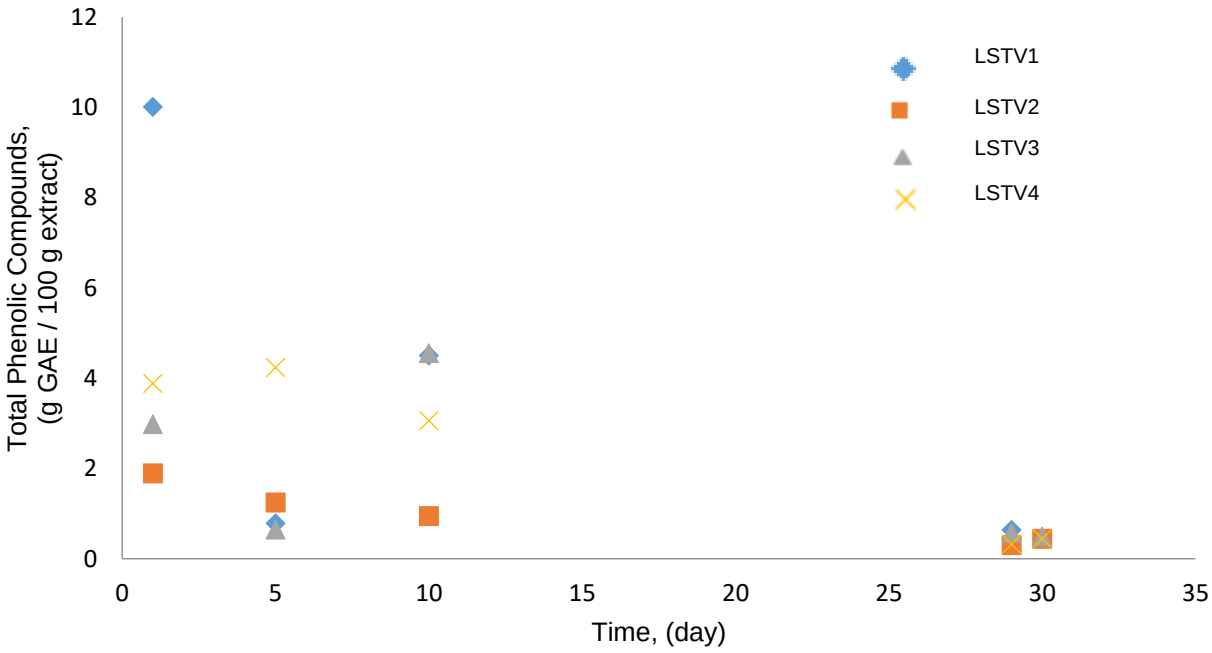


**Fig. 5.** Total phenolic compound concentration distributions throughout 30 weeks of vermicomposting

## CONCLUSIONS

1. The presence of phenol in EFBs confirmed that raw EFBs may not be a good feedstock for vermicomposting because phenol is toxic to earthworms.
2. Traces of phenol (*retention time* 9.68 min), pyro-catechol (*retention time* 11.91 min), and 4-hydrobenzoic acid (*retention time* 16.55 min) were found in the middle stage of mushroom cultivation. However, there was no trace of phenol, pyro-catechol, or 4-hydrobenzoic acid at the end of cultivation.
3. The absence of phenol in the fruiting body of the mushrooms throughout the cultivation indicates that oyster mushrooms produced on EFB substrates are safe for consumption.
4. Vermicomposted EFBs showed no trace of phenol, and the low levels of total phenolic compounds indicates that the lignin content of vermicomposted EFBs is very low.
5. This study shows that fungi can be used to degrade the phenolic compounds in EFBs.

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