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Characterization and application of bioactive compounds in oil palm mesocarp fiber superheated steam condensate as an antifungal agent†

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Lignocellulosic degradation products from superheated steam (SHS) pretreatment of oil palm mesocarp fiber (OPMF) at 190 °C to 240 °C for 1 hour were recovered as a condensate. Compositional analysis of the condensate was carried out and subsequently its potential application as an antifungal agent was investigated. GCMS analysis revealed a total of 62 compounds in the condensate sample from SHS pretreatment at 240 °C, which were identified and classified into ten different groups of aromatic phenolics, furans, pyrans, dioxoles; and open chains ketones, esters, alcohols, aldehydes, alkenes and alkanes. The presence of carboxylic acids was identified by HPLC and the condensate contained acetic, formic, levulinic and succinic acids at concentrations of 1671 mg L^{-1}, 12 320 mg L^{-1}, 831 mg L^{-1} and 435 mg L^{-1}, respectively. Complete suppression of *Ganoderma boninense* UPM13 mycelial growth was observed in the agar dilution test, while spore germination of *Aspergillus fumigatus* UPM2 and *Trichoderma asperellum* UPM1 was completely inhibited in the spore germination test. The growth suppression of *G. boninense*, a fungal causing basal stem rot (BSR) disease in oil palm plantation shows the condensate potential benefit to combat the disease.

Introduction

The concept of 'Waste to Wealth' focuses on utilization of unused resources to produce useful and high-end products. It has gained a lot of interest especially involving lignocellulosic biomass as the most abundant non-food biomass.[1] In Malaysia, palm oil industries produce about 80 million dry tonnes of lignocellulosic biomass annually.[2] Six types of biomass from oil palm industries are oil pam frond (OPF), oil palm trunk (OPT), oil palm empty fruit bunch (OPEFB), palm kernel shell (PKS), palm oil mill effluent (POME) and oil palm mesocarp fiber (OPMF).

To begin with, the utilization of these vast amounts of biomass as alternative sources to produce various chemicals[1,3] and purposive products such as fermentable sugars, biofuel and biocomposite filler in various applications requires pretreatment in order to modify and alter its complex lignocellulosic materials structure. This could be achieved by biological, chemical and physical pretreatments. For instance, it has been reported that acid and alkaline pretreatment of OPEFB managed to improve bioconversion of OPEFB as substrate for cellulase production due to alteration of lignin by the pretreatment.[4] Hydrothermal pretreatment through hot water extraction has been reported to reduce hemicellulose and lignin content which in turn caused structural changes on the cellulose–hemicellulose–lignin matrix, resulting in the opening and expansion of specific surface area and pore volume and hence, provided better condition for hydrolysis of sugars.[5]

Recently, a physical pretreatment using superheated steam (SHS) has been reported as one of the pretreatment methods for oil palm biomass meant for sugar[6,7] and biocomposite[8,9] production. SHS can be defined as a type of dry, unsaturated steam with temperature higher than its boiling point at given pressure.[8,9] The advantages of using SHS for pretreatment of lignocellulose biomass are due to its effectiveness in altering the component in lignocellulose, non-chemical pretreatment method which is more eco-friendly compared to chemical pretreatment, safe to be used and energy saving as it does not involve the use of high pressure and easy to scale-up as it is widely used in food industries for drying. It was reported that SHS pretreatment caused hemicellulose removal from OPMF, which eventually provided better surface for polymer–fiber interaction in biocomposite making.[9] In other reports, it was

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exhibited that the application of SHS for pretreatment had partially degraded the lignocellulosic structure of the biomass into smaller compounds.[6–9] This indicates the possibility of having lignocellulosic components degradation products as by-products during SHS pretreatment.

It is postulated that low molecular mass compounds of lignocellulosic degradation products can be easily volatile and eventually being released out to the environment along with the exhaust steam. Nevertheless, the degradation mechanism of lignocellulosic by SHS is unclear and thus, the degradation products are also unknown. As lignocellulosic materials contained carbohydrate and lignin, it is expected that major degradation products from the material would be acids, furans and phenolics. The release of these products to the environment along with the exhaust steam will not be a favorable option to the environment and hence recovery of these products is crucial. Lignocellulose degradation products are generally known to have antimicrobial property which can be used for controlling microbial infection in the environment. In this paper, SHS degradation products of oil palm mesocarp fiber (OPMF) are characterized and identified. The potential of the degradation products as an antifungal agent is also discussed.

Results and discussion

Characterization of OPMF condensate

OPMF condensate obtained was characterized for its color appearance, pH and chemical composition. A light yellowish-brown color condensate was obtained from steam condensation (OS sample) during SHS treatment of OPMF at 240 °C temperature (ESI Fig. S1(a)†). The resulted color of the condensate is in agreement with published report of wood vinegar samples from several studies.[10–12] It was reported that carboxylic acid and phenolic compounds in the samples contributed to its color appearance.[12] Upon concentration of OS sample, a dark brown liquid (RF fraction) was obtained as shown in ESI (Fig. S1(b)†). Color intensity of OPMF condensate was increased with the increase in SHS treatment temperature (ESI Fig. S2†), along with the increment in absorbance (Abs) value.

This observation could be contributed by increased concentration of degradation products of lignocellulosic component in OPMF at higher temperature. It was previously reported that an increased in SHS treatment temperature resulted in more degradation of hemicellulose in OPMF. The composition of hemicellulose reduced to 26% from 33% when treated with SHS at temperature 190 °C for an hour and further reduced to 9% after treated at 240 °C.[9] Accordingly, the percentage of OPMF weight reduction at different temperature was found to be increased with increased in SHS temperature (shown in ESI Fig. S3†). The percentage of OPMF weight reduction after treatment at 190 °C to 240 °C were 13.6 ± 2.8%, 17.1 ± 2.4%, 23.0 ± 4.8%, 28.7 ± 5.4%, 31.6 ± 6.1% and 39.5 ± 6.8%, in order.

Both OS sample and RF fraction of OPMF condensate from 240 °C had acidic pH value at 3.03 ± 0.02 and 2.87 ± 0.02, respectively. In consideration whereby SHS pretreatment degrades lignocellulosic materials specifically cellulose and hemicellulose into compounds such as acetic acid, formic acid, levulinic acid and succinic acid, identification of those acids concentration within OPMF condensate samples was analyzed by HPLC (Fig. 1). The peak of each acid was identified by reference of its retention time to standard compound.

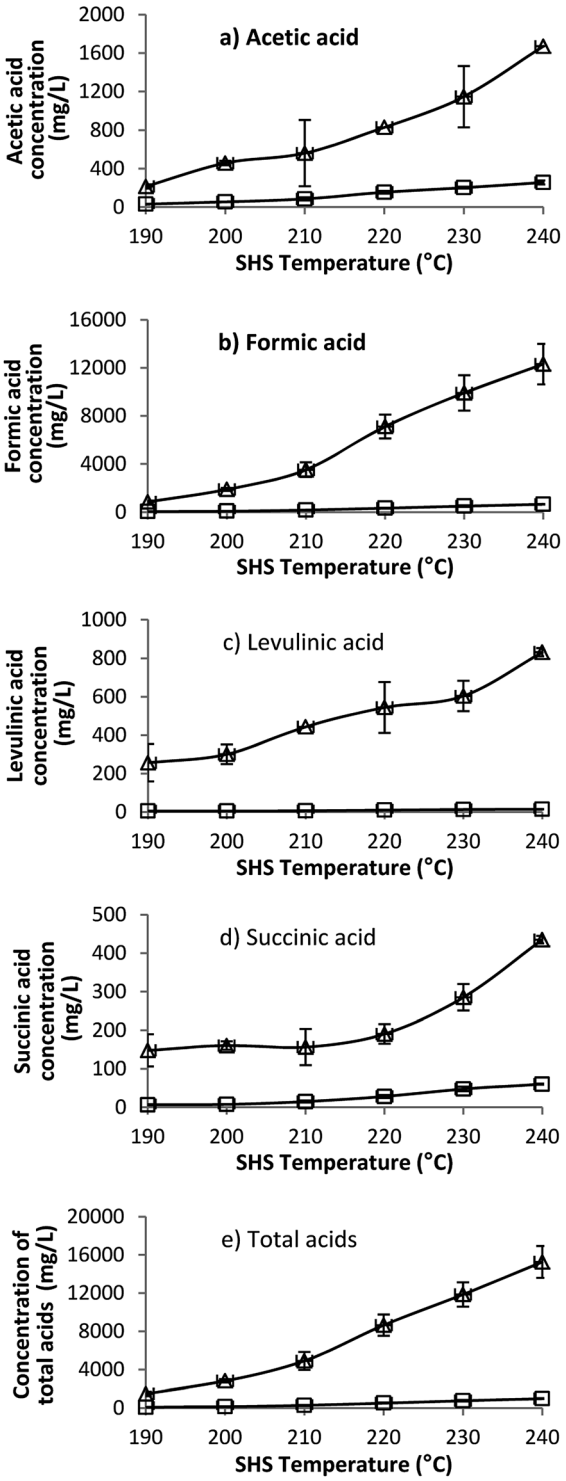


Fig. 1 The HPLC analysis of organic acids concentration of OPMF condensates from different superheated steam temperature [(a) formic acid, (b) levulinic acid, (c) acetic acid, (d) succinic acid, (e) total acids; OS sample (□), RF fraction (△)].

Formic acid concentration was found to be the highest among all four acids in both OS sample and RF fraction, at 657 mg L^{-1} and 12 320 mg L^{-1}, correspondingly. Remarkable presence of acetic acid was also found with concentration of 257 mg L^{-1} and 1671 mg L^{-1} in OS sample and RF fraction, respectively. Meanwhile, little amount of levulinic and succinic acids were found in both samples. Total carboxylic acids concentration was 988 and 15 255 mg L^{-1}, respectively for OS sample and RF fraction. Originated from polysaccharides degradation, acetic acid was from cleavage acetal linkage of hemicellulose, formic was from decomposition of both hexose and pentose sugar, while levulinic and succinic acids were from hexose sugar degradation.[13,14] It was exhibited that the concentration of these acids were gradually increased with the increase in SHS temperature (Fig. 1). These findings were further supported by the reduced pH value of each OS sample and RF fraction from different SHS temperature as shown in Table 1.

Gradual increment of acetic, formic, levulinic and succinic acids concentration explains that more lignocellulose was degraded as higher treatment temperature was used. This finding could be explained by the fact that an increase in reaction temperature could enhance thermal hydrolysis reaction of lignocellulose components during the treatment.[15] Higher energy apparently meant higher supply of energy; the chemicals molecules get more excited and move faster hence inducing the reaction to undergo hydrolysis and degradation. The chemical compositional analysis by GCMS revealed that a total of 62 peaks of compound were found from RF condensate (240 °C) (ESI Fig. S4†). These compounds were classified into: (i) aromatic compounds of furans, phenolics, pyrans and dioxoles; and (ii) open chain compounds of ketones, esters, alcohols, aldehydes, alkenes and alkanes. The number of identified compounds was highest in RF fractions obtained from 240 °C SHS temperature. It was decreased by decreasing of SHS temperature, of which 61, 58, 53, 15 and 9 compounds were found in RF condensate from 230 °C, 220 °C, 200 °C, 210 °C, and 190 °C, respectively. They were listed as in Table 2.

The presence of lignocellulose degradation products in all RF fractions indicated that partial degradation of OPMF occurred even at SHS treatment of 190 °C. The increasing profile of compounds relative area (%) as shown in Fig. 2 supports our earlier discussion on the relationship between color intensity and concentration of degradation products. It is seen that phenolic was the most prominent type of compounds among the ten types of compounds classified. Additionally, the relative area percentage of phenolic compounds was increased with the increases in temperature. This observation is expected to be contributed by degradation of lignin component in OPMF. As mentioned before, lignin has a broad range of thermal degradation temperature which is from 160 °C to 900 °C.[9] At elevated SHS temperature, this degradation continuously occurred and thus explains the obtained results of increasing phenolic degradation products in the condensate.

From the chemical profile obtained, aside from phenolics, other compounds identified were expected to be contributed by degradation of carbohydrates in OPMF (cellulose and hemicellulose), except for some ketones, alkenes and alkanes which were contributed by degradation of fatty acids and/or waxes.[16] Fatty acids were found due to residual palm oil within the OPMF,[17] while waxes were from the external membrane of palm fruit (exocarp).[18,19] To the extent of our knowledge, details on lignocellulose degradation mechanism by SHS treatment is not widely reported. It is expected that hydrothermal degradation initiated by auto-ionization of water molecules producing hydronium ion had catalyzed the degradation process, similarly to steam explosion or liquid hot water pretreatments.[20,21] Nevertheless, the characteristic of SHS which is known as dry steam and the presence of upper and lower heaters in SHS oven (Fig. 5) which are meant for controlling the temperature inside the oven has provided a condition similar to an oven whereby it would dry out any moisture within the OPMF along with removal of waxes or extractives on the surface of the fiber. The heat energy exerted on the OPMF would degrade the lignocellulose structure and hence, degradation occurring in SHS oven is also expected to follow thermal degradation pathway.

It has been reported earlier that thermal degradation of cellulose is initiated by 1,4-glycosidic linkage cleavage due to acetal reaction between C1–C6 of the cellulose unit.[22] The disrupted glycosidic bond on C4 position will react with free –OH radical to form anhydrous sugar. Intramolecular rearrangement and ring opening of the monomer will take place followed by possible reactions such as dehydration, hydration, fission, decarbonylation and decarboxylation, forming various degradation products such carboxylic acids, alcohols, aldehydes, ketones, alkenes, furans, pyrans and esters[16] which were also found in this study (Table 2). Research have shown that these degradation products are potent antimicrobial agents, hence the RF samples were tested for antifungal activity as being discussed in the next section.

Table 1 The pH value of OS sample and RF fraction of OPMF condensate

SHS temperature (°C)	OS sample	RF fraction
190	3.60 ± 0.03	3.37 ± 0.10
200	3.35 ± 0.02	3.23 ± 0.06
210	3.28 ± 0.01	3.12 ± 0.11
220	3.12 ± 0.01	2.92 ± 0.01
230	3.03 ± 0.04	2.88 ± 0.06
240	3.03 ± 0.02	2.88 ± 0.03

OPMF condensate as an antifungal agent

A marked inhibition reaction of OPMF condensate RF fraction (240 °C) on spore germination of *T. asperellum* UPM1 and *A. fumigatus* UPM2 was observed. There was no germination occurred even after 72 hours of prolonged incubation (Table 3). Generally, fungal growth starts with spore germination, whereby the spore will rapidly germinate in the presence of nutrients and water, as well as absence of biocides, specifically fungicides. Water uptake by the spores will lead to spore 'swelling' in which would increase its size due to water uptake.

Table 2 Presence of compounds from lignocellulosic degradation in RF fraction of OPMF condensates from different superheated steam temperature by GCMS analysis[a]

		SHS temperature (°C)						
No.	Name of compounds	190	200	210	220	230	240	Source
Alcohols								
6.	3-Butenol	—	—	✓	✓	✓	✓	C, HC
19.	Isopropyl alcohol	—	—	✓	✓	✓	✓	C, HC
Aldehydes								
8.	Acetaldehyde	—	—	✓	✓	✓	✓	C, HC
20.	3-Hydroxybutanal	—	—	✓	✓	✓	✓	C, HC
Ketones								
7.	4,4-Dimethyl-2-pentanone	—	—	—	✓	✓	✓	FAs/Ws
9.	Methoxyacetone	—	—	✓	✓	✓	✓	C, HC
10.	Hydroxyacetone	—	—	✓	✓	✓	✓	C, HC
13.	2-Butanone	—	—	✓	✓	✓	✓	C, HC
15.	2,3-Pentanedione	—	—	✓	✓	✓	✓	C, HC
22.	3-Methylcyclopentanone	—	—	✓	✓	✓	✓	C, HC
26.	1-Oxiranyl ethanone	—	—	✓	✓	✓	✓	C, HC
31.	Cyclotene	✓	—	✓	✓	✓	✓	C, HC
35.	3-Methyl-1,2-cyclopentanedione	✓	✓	✓	✓	✓	✓	C, HC
44.	2-Methyl-3-butanone	—	—	✓	✓	✓	✓	C, HC
47.	3-Octanone	—	✓	✓	✓	✓	✓	FAs/Ws
50.	2,4-Pentanedione	—	—	✓	✓	✓	✓	C, HC
51.	2,5-Hexanedione	—	—	—	✓	✓	✓	C, HC
54.	2-Pentanone	✓	✓	✓	✓	✓	✓	C, HC
Ester								
2.	Ethenyl acetate	—	—	✓	✓	✓	✓	C, HC
3.	Oxiran-2-yl-methyl 2-methylprop-2-enoate	—	—	✓	✓	✓	✓	FAs/Ws
4.	Methyl prop-2-enoate	—	—	✓	✓	✓	✓	C, HC
5.	Methyl acetate	—	✓	✓	✓	✓	✓	C, HC
14.	2-Propenyl acetate	—	—	✓	✓	✓	✓	C, HC
23.	2-Oxopropyl acetate	—	—	—	—	✓	✓	C, HC
27.	*n*-Hexyl acrylate	—	—	✓	✓	✓	✓	FAs/Ws
32.	3-Methyl-2-butenyl acetate	—	—	✓	✓	✓	✓	FAs/Ws
33.	Propyl formate	—	—	✓	✓	✓	✓	C, HC
39.	Octyl acetate	—	—	—	✓	✓	✓	FAs/Ws
40.	2-Propenyl butanoate	—	—	✓	✓	✓	✓	FAs/Ws
41.	Ethyl propenoate	—	—	✓	✓	✓	✓	C, HC
Alkenes								
17.	4,4-Diethoxy-2-methyl-1-butene	—	—	—	—	—	✓	FAs/Ws
37.	Ethenoxyethene	—	—	✓	✓	✓	✓	C, HC
46.	1-Octene, 3,7-dimethyl-	—	—	✓	✓	✓	✓	FAs/Ws
Alkanes								
12.	2,4-Dimethylpentane	—	—	✓	✓	✓	✓	Ws
38.	3,3-Dimethyloctane	—	—	✓	✓	✓	✓	Ws
60.	Heptadecane	—	—	✓	✓	✓	✓	Ws
Furans								
11.	Furfural	—	✓	✓	✓	✓	✓	HC
16.	5-Ethyltetrahydro-2-furanone	—	—	✓	✓	✓	✓	C, HC
18.	Butyrolactone	—	✓	✓	✓	✓	✓	C, HC
21.	3-Methylfuran-2,5-dione	—	—	✓	✓	✓	✓	C, HC
24.	Tetrahydro-2-furanmethanol	—	—	✓	✓	✓	✓	C, HC
28.	Tetrahydro-3-furanol	—	—	✓	✓	✓	✓	C, HC
36.	2-Methyltetrahydro-3-furanone	—	—	✓	✓	✓	✓	C, HC
42.	2-Furancarboxylic acid	—	✓	✓	✓	✓	✓	C, HC
43.	Furan	—	—	✓	✓	✓	✓	C, HC
52.	5-Hydroxymethylfurfural	✓	✓	✓	✓	✓	✓	C, HC

Table 2 (*Contd.*)

No.	Name of compounds	SHS temperature (°C) 190	200	210	220	230	240	Source
Phenolics								
30.	Phenol	—	✓	✓	✓	✓	✓	Lignin
34.	1,2-Dichlorobenzene	—	—	✓	✓	✓	✓	Lignin
48.	2,5-Dihydroxybenzaldehyde	—	—	✓	✓	✓	✓	Lignin
49.	Benzoic acid	—	✓	✓	✓	✓	✓	Lignin
53.	4-Methylbenzaldehyde	—	—	✓	✓	✓	✓	Lignin
55.	2-Methoxyhydroquinone	—	—	✓	✓	✓	✓	Lignin
56.	1,3-Di-*tert*-butylbenzene	✓	—	✓	✓	✓	✓	Lignin
57.	Pyrocatechuic acid	✓	✓	✓	✓	✓	✓	Lignin
58.	Vanillin	✓	✓	✓	✓	✓	✓	Lignin
59.	Dinoseb acetate		—	—	—	✓	✓	Lignin
61.	Dihydroeugenol		—	—	—	✓	✓	Lignin
62.	Butylated hydroxytoluene	✓	✓	✓	✓	✓	✓	Lignin
Pyrans								
29.	2-Methoxy-3,4-dihydro-2*H*-pyran	—	—	—	✓	✓	✓	C, HC
45.	Maltol	✓	✓	✓	✓	✓	✓	C, HC
Dioxoles								
1.	1,3-Dioxol-2-one	—	—	✓	✓	✓	✓	C, HC
25.	2-Methyl-1,3-dioxolane	—	—	—	✓	✓	✓	C, HC
Total		**9**	**15**	**53**	**58**	**61**	**62**	

[a] '✓': indicates presence of compound; '—': indicates absence of compound; HC: cellulose and hemicellulose; Fas/Ws: fatty acids or waxes; Ws: Waxes; HC: hemicellulose.

Following this stage is the development of germination tube; emerged and elongated from the spore. This structure would further developed into hyphae and mycelia. The development of spores according to these stages was observed in the experiment.[23,24]

While there were no spore structure changes observed in media containing condensates, development such as spore swelling and elongation of germination tube in control media can be seen as early as 12th hour of incubation. The appearance of mesh network of hyphae with no trace of non-germinated spore was observed in control media (Table 4). As spore of fungi will not be able to germinate in an unsuitable environment, these findings prove that incorporation of RF fraction 240 °C from OPMF condensate into PDA as media for growth created toxic environment and eventually halted any spore germination and fungal growth process.

Subsequently, the inhibitory effect of the condensate was tested on the mycelia growth of the fungi. Fig. 3 shows mycelia growth profile of *T. asperellum* UPM1 and *A. fumigatus* UPM2 on PDA plate containing RF fraction 240 °C and control plate (PDA without RF sample). Both *T. asperellum* UPM1 and *A. fumigatus* UPM2 showed slower mycelia growth in RF plate compared to control plate. *T. asperellum* UPM1 in RF plate showed full plate growth on the 5th day, two days later compared to the control plate. On the 3rd day of incubation, the mycelia growth diameter was only 45% of the control plate (Table 5). Similar observation was found for *A. fumigatus* UPM2; whereby the mycelia reached its maximum diameter only on the 10th day for RF plate compared to control plate which took only 6 days. The mycelia growth in RF plate was nearly two times slower (52% of control plate on 6th day of incubation).

Fungi mycelia is a meshwork of hyphae extended after its development from germination tube of spore.[24] As seen in Fig. 3, initiation of mycelia growth for *T. asperellum* UPM1 and *A. fumigatus* UPM2 was also halted and delayed in RF fraction media compared to control media. This could be related to the inhibition in spore germination as shown in Table 4, which was later affected the growth of the hyphae and mycelia of both fungi. A fungistatic effect was initially seen whereby no mycelia growth was observed after a day and two days of incubation for *T. asperellum* UPM1 and *A. fumigatus* UPM2 on RF plate (Fig. 3). Based on the observation, it can be concluded that incorporation of RF fraction of OPMF condensate (240 °C) in the PDA suppressed the hyphae extension and spore germination at the beginning of growth stage, and later managed to slowly overcome the inhibitory effect, as seen in Fig. 3. This is an interesting finding as this may suggest that the fungi developed an acquired resistance towards inhibitory compounds in the OPMF condensate.

Acquired resistance is an occurrence whereby a particular microorganism obtains the ability to resist the activity of a particular antimicrobial agent to which it was previously susceptible such as mutation, conjugation, transformation, transduction and transposition.[25] The fungi could acquire resistance by modification of antifungal target sites or produce antifungal compounds degrading enzyme such as acetyltransferases,

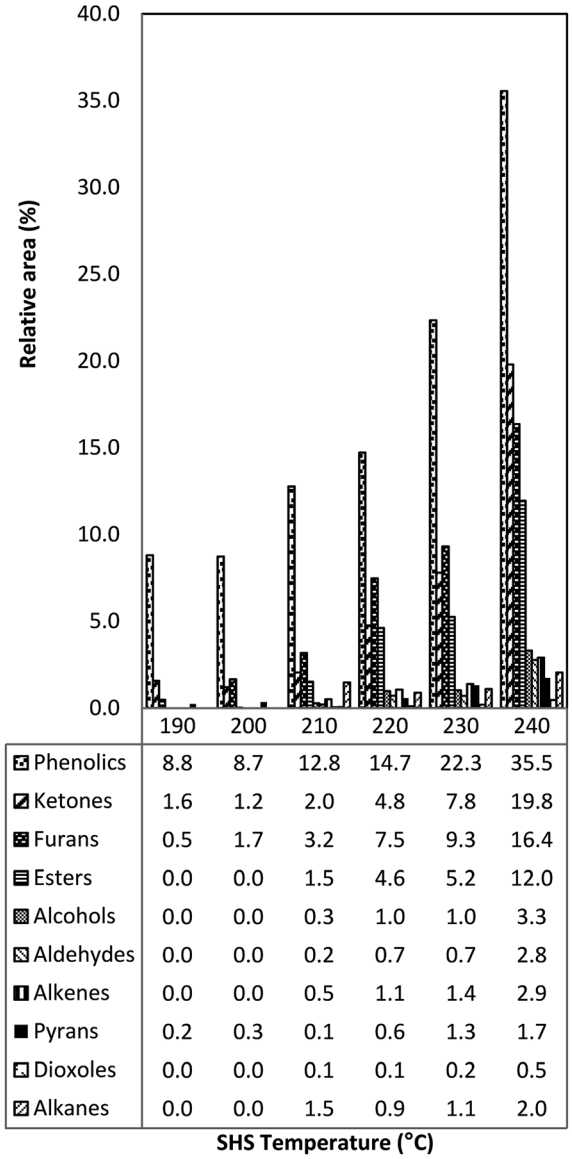


Fig. 2 GCMS relative area percentage of different type of compounds in RF condensate from 190 °C to 240 °C superheated steam temperature.

hydrolases, hydratases, demethylases and formate oxidase.[24] Studies have shown that *Trichoderma* sp. exhibited resistance towards chemical fungicides and xenobiotic compounds of which are mainly phenolic compounds.[26–28] However, little is known about the resistance mechanism, particularly at molecular level. Detailed study is needed in order to clarify this.

Another test was conducted to determine inhibitory effect of the OPMF condensate RF fraction (240 °C) on *Ganoderma boninense* UPM13. *G. boninense* is a white rot fungus which is the causal agent of basal stem rot (BSR), the most destructive disease of oil palm plantations.[29] Interestingly unlike partial inhibition against *T. asperellum* UPM1 and *A. fumigatus* UPM2, 10% v/v RF fraction was able to completely suppress the mycelia growth of *G. boninense* UPM13 (Table 5). The control plate (without RF fraction) on the other hand showed full plate growth on the 8th day. There was no mycelia growth observed even after 14 days of incubation (Fig. 4(a)). The observation shows inability of *Ganoderma* sp. as basidiomycota fungus (of which its asexual reproduction is absent[30]), to withstand the toxic environment caused by incorporation of RF fraction in the media, nor exhibiting any defense mechanism against it.

In the same figure (Fig. 4(b)), the effect of RF fractions (different SHS temperature) on mycelia growth of *G. boninense* UPM13 is shown. It was found that RF fractions of SHS pretreatment at 220 °C to 240 °C completely suppressed the growth of the fungus, while RF fractions of SHS treatment at 190 °C to 210 °C did not suppress the *G. boninense* UPM13 growth at all. This is an interesting observation as it indicates that concentration and types of OPMF degradation products in the OPMF condensate contributed significantly on the inhibitory effect of the condensate. As seen in both Fig. 1(e) and Table 2, the concentration of total carboxylic acids and number of degradation products were markedly increased, respectively, with the increment in temperature. These could be the factors affecting the inhibition of mycelia growth of *G. boninense* UPM13 in RF fraction 220 °C to 240 °C.

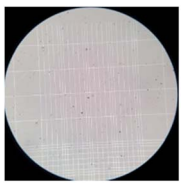
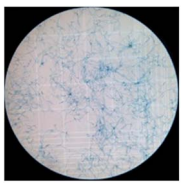
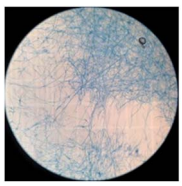
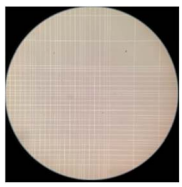
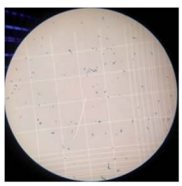
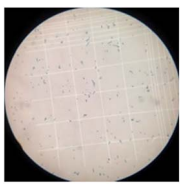
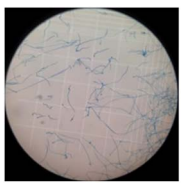
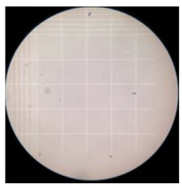
Antimicrobial activity depends on the type of antimicrobial compounds and its concentration.[25] The presence of various compounds with increased concentration (relative area percentage) along with increased acids concentration found in RF fraction from condensates from 190 °C to 240 °C could

Table 3 Spore germination percentage of *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 in PDA with and without RF fraction[a]

	Spore germination percentage, %			
	Trichoderma asperellum UPM1		*Aspergillus fumigatus* UPM2	
Incubation time (hour)	PDA*	PDA with RF**	PDA*	PDA with RF**
0	NG	NG	NG	NG
12	95.7 ± 1.2	NG	18.0 ± 0.6	NG
24	100.0 ± 0.0	NG	97.2 ± 0.6	NG
36	100.0 ± 0.0	NG	100.0 ± 0.0	NG
48	100.0 ± 0.0	NG	100.0 ± 0.0	NG
60	100.0 ± 0.0	NG	100.0 ± 0.0	NG
72	100.0 ± 0.0	NG	100.0 ± 0.0	NG

[a] *Media without RF fraction of OPMF condensate (control); **media containing RF fraction of OPMF condensate; NG: no germination.

Table 4 Spore germination development of *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 in PDA with and without RF fraction[a]

	Incubation time (hour)		
Germination media	0	12	24
***Trichoderma asperellum* UPM1**			
PDA*			
PDA with RF**	No spore development	No spore development	
***Aspergillus fumigatus* UPM2**			
PDA*			
PDA with RF**	No spore development	No spore development	

[a] *Media without RF fraction of OPMF condensate (control), **media containing RF fraction of OPMF condensate.

contribute to its efficiency as antifungal agent. As stated earlier, the number of compounds found in the condensates from 190 °C to 240 °C SHS temperature was 9, 15, 53, 58, 61 and 62, in increasing order (Table 2). For instance, taking phenolic group as example, condensate from 240 °C contained 12 types of phenolic compounds while condensates from 190 °C had only 4 compounds. The eight new compounds formed and found in condensates from 240 °C SHS temperature could contributes to its stronger antifungal activity. As such, the new compounds include aldehydic aromatic compounds such as 4-methylbenzaldehyde and 2,5-dihydroxybenzaldehyde which had been reported as one of ten compounds (from 70 phenolic compounds) showing most active inhibition against four pathogens; *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica*.[31] Besides, 2-methoxyhydroquinone found in condensate from 240 °C which was also absence in condensate from 190 °C had also been reported as one of compounds found in fragrance with antimicrobial activity.[32]

Moreover, in term of concentration, the relative area percentage of compounds in condensates was increasing with the increased of SHS temperature (Fig. 3). All other compounds in condensates from higher SHS temperature were found to have higher relative area percentage. Additionally, a distinct increment of relative area percentage for furan compounds was found in condensates from 220 °C which was 7.5% as compared to 210 °C which was only 3.2% could be the factors affecting the inhibition of mycelia growth of *G. boninense* UPM13 in RF fraction 220 °C to 240 °C. The results obtained shows that condensates from higher temperature (220 °C to 240 °C) shows higher inhibition on fungal growth as compared to condensates from lower temperature (190 °C to 210 °C). Likewise, the total of four organic acids analyzed (formic, acetic, levulinic and succinic) was proportionally increasing with increased of SHS temperature (Fig. 2). Presence of higher concentration of these inhibitory compounds including furans and acids within RF fraction of OPMF condensates increase the antifungal activity of the condensates. Higher concentration of inhibitory compounds (antimicrobial agents) cause higher inhibition on microbial growth including fungi.[25]

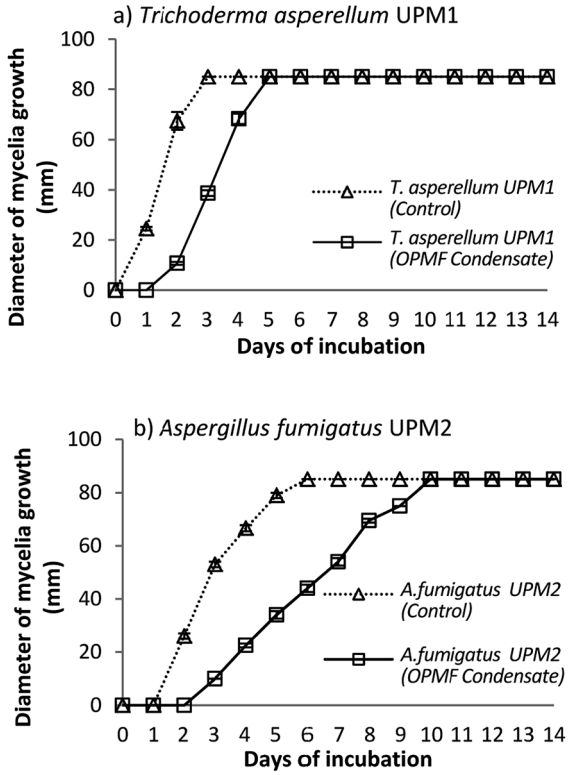
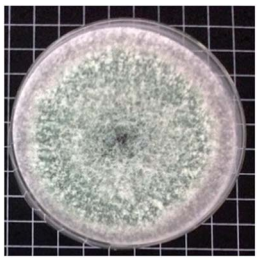
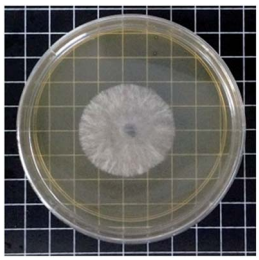
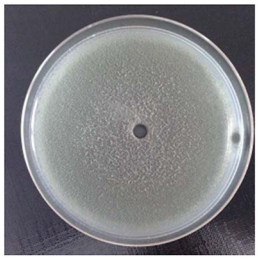
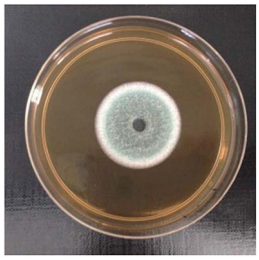
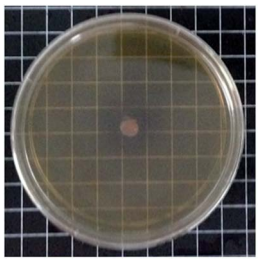


Fig. 3 Mycelia growth of *Trichoderma asperellum* UPM1 (a) and *Aspergillus fumigatus* UPM2 (b) on PDA with and without (control) RF fraction of OPMF condensate.

Table 5 Mycelial growth of fungi on control and antifungal plate[a]

Control plate*	Antifungal RF plate**
***Trichoderma asperellum* UPM1 (on 3rd day of incubation)**	
	
***Aspergillus fumigatus* UPM2 (on 6th day of incubation)**	
	
***Ganoderma boninense* UPM13 (on 8th day of incubation)**	
	

[a] *Media without RF fraction of OPMF condensate (control), **media containing RF fraction of OPMF condensate.

As OPMF is a plant-origin material, the composition of OPMF condensate is very much similar to plant essential oil (bio-oil). According to McDonnell (2007),[25] most essential oils act as fungistat and halted progression of fungal growth even at relatively low concentration.

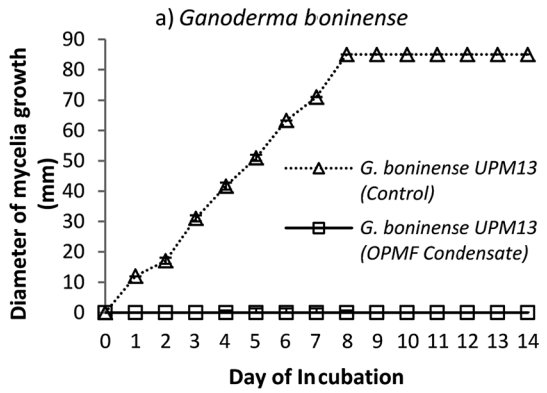
It has been shown that various chemicals toxic to microbial growth were presence in the OPMF condensate RF fraction. Carboxylic acids (acetic, formic, levulinic and succinic acids) found in the condensate could cause interference of cellular proton motive force, and eventually disturb microbial cell wall and membrane essential function.[33,34] In addition, acids will cause pH imbalance and accumulation of toxic anions within the bacterial cytosol.[33,35]

Alcohols such as 3-butenol and isopropyl alcohol, through its reactive hydroxyl group may cause precipitation and denaturation of protein and other macromolecules of microbial cell by formation of hydrogen bond. For aldehyde compounds such as acetaldehyde and 3-hydroxybutanal, their –CHO functional group would cross-linked with free exposed amino group of proteins such as lysine and arginine, eventually lead to inhibition of cellular essential function.[36]

Meanwhile, various furan compounds in RF fraction may cause interference in various enzymatic functions of cell and consequently, cell death. This is due to the presence of its aldehyde moiety such as in furfural. As for phenolics, they promote loss of integrity in biological membranes, and thus affect their ability as selective barriers, as enzyme matrices, decrease cell growth and further sugar assimilation.[37–39] For instance, a development of biomaterials consisting natural phenols was proven to demonstrate antimicrobial activity against Gram-positive and Gram-negative bacterial strains, such as *B. subtilis* NCTC 3610, *S. aureus* NCTC 6571, *E. coli* NTCT 10418 and *P. aeruginosa* NCTC 10662.[40]

The presence of numerous furanic and phenolic aldehyde compounds within RF sample enhanced inhibitory activity of the sample against fungal growth. Moreover, the existence of furan and phenolic as aldehyde such as furfural and hydroxy benzaldehyde isomers was reported to be more toxic to microbial growth compared to its presence as acids such as furan carboxylic acid and benzoic acid.[37,39,41] This is due to the reaction of their aldehyde (CHO) group which was more active than the carboxyl (COOH) group whether or not OH groups were present.[31]

Since RF sample of OPMF condensate has the above mentioned potent antimicrobial compounds, it is suggested



(b)

	SHS Temperature (°C)					
	190	200	210	220	230	240
	Diameter of Mycelia Growth (mm)*					
Ganoderma boninense UPM13	85.0[a]	85.0[a]	85.0[a]	NG**	NG**	NG**

*Diameter on the day when the growth of fungal mycelia on control plate reach maximum (85.0 mm); **NG: No growth;
[a]Fungi reached maximum growth on the 8th day of incubation

Fig. 4 (a) Mycelia growth of *Ganoderma boninense* UPM13 in RF fraction (240 °C) and (b) the effect of RF fractions from various SHS treatment temperature on the mycelia growth of *Ganoderma boninense* UPM13.

that these compounds acted synergistically and contributed to the suppression of the fungal growth, particularly *G. boninense* UPM13. Results obtained in this study provide promising solution to the issue as it is shown that the OPMF condensate is potentially applicable to combat BSR disease in oil palm plantations. As being mentioned earlier, the OPMF condensate RF fraction (220 °C to 240 °C) completely suppressed *G. boninense* UPM13 growth and this shows great potential of this condensate to be an option for treatment of BSR disease in oil palm plantation caused by the fungi. According to a published report, chemical treatment on plant infected by BSR was inadequate. The uses of substances such as fungicides and hexaconazole only extended the life span of affected plant but unable to cure the disease.[42] Therefore, application of RF fraction of OPMF SHS condensate containing lignocellulose degradation products should be an advantageous option as it is not only inhibiting the growth of the fungus effectively, but it also helps in solving biomass waste issue at the oil palm plantation as the biomass can be used in the production of antifungal agent.

Experimental

Materials

The OPMF used in the experiments was supplied by Seri Ulu Langat Palm Oil Mill Sdn Bhd in Selangor, Malaysia. It was washed by using water to remove crushed kernel, shell and debris before been sun dried and stored at room temperature (±24 °C) before use.

Recovery of OPMF lignocellulosic degradation product

Degraded lignocellulosic components of OPMF from SHS pretreatment were recovered as condensate. The process comprised of two steps: (i) SHS treatment of OPMF; and (ii) steam collection and condensation. The whole process is depicted in Fig. 5.

(i) SHS treatment of OPMF. SHS treatment of OPMF was done in accordance with Nordin *et al.* (2013).[9] A batch type SHS oven (QF-5299C, Naomoto Corporation, Osaka Japan), with heating space (treatment chamber) dimension of 300 mm × 265 mm × 100 mm was used in the experiment. According to manufacturer's manual, the boiler with electric capacity of 3.30 kW produces about 4.95 kg h^{-1} steam when the steam supply is controlled at 100% (ESI – Fig. S5†). The saturated steam produced was further heated by a super-heater (1.02 kW) to produce SHS.

The temperature of SHS in treatment chamber was maintained by the presence of upper and lower heater installed inside the oven. Approximately 35 g of OPMF was placed inside the heating chamber for the treatment. The SHS treatment was conducted at temperature range between 190 °C to 240 °C for 60 minutes.[9] The weight of OPMF treated was measured for weight reduction analysis.

(ii) Collection and condensation of steam from SHS oven. Exhaust steam generated during SHS treatment of OPMF was collected and condensed as depicted in Fig. 5. A rubber tube connecting SHS oven's chimney and stainless steel product collector was used to trap exhaust steam during the SHS treatment process. High temperature of exhaust steam was cooled down and condensed into condensate inside the stainless steel product collector surrounded with ice. The accumulated condensate was collected at the end of the treatment and stored at 4 °C until further use.

Concentrating OPMF condensate

A 500 mL OPMF condensate obtained was concentrated to 99% water removal by using rotary evaporator (Rotavapor® R-215 Buchii, Switzerland), resulting in 5 mL concentrated fraction. The process was conditioned to 55 °C temperature at 50 mbar pressure. The non-concentrated original OPMF condensate and its concentrated fraction were regarded as OS sample and RF fraction, respectively.

Analytical methods

pH. Acidity level of condensate sample was determined through pH detection by using a pH meter (Thermo Scientific, USA). Ultraviolet-Visible (UV-Vis) spectroscopy analysis of each condensate sample was carried out by using a UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan) at 275 nm.

High performace liquid chromatography (HPLC). The presence of organic acids were identified according to the analytical methods NREL/TP-510-42623.[43] A 10 μL sample of condensate was pre-filtered using nylon membrane 0.2 μm and injected into the high-performance liquid chromatography (HPLC) system equipped with an ultraviolet (UV) detector (Shimadzu, Kyoto,

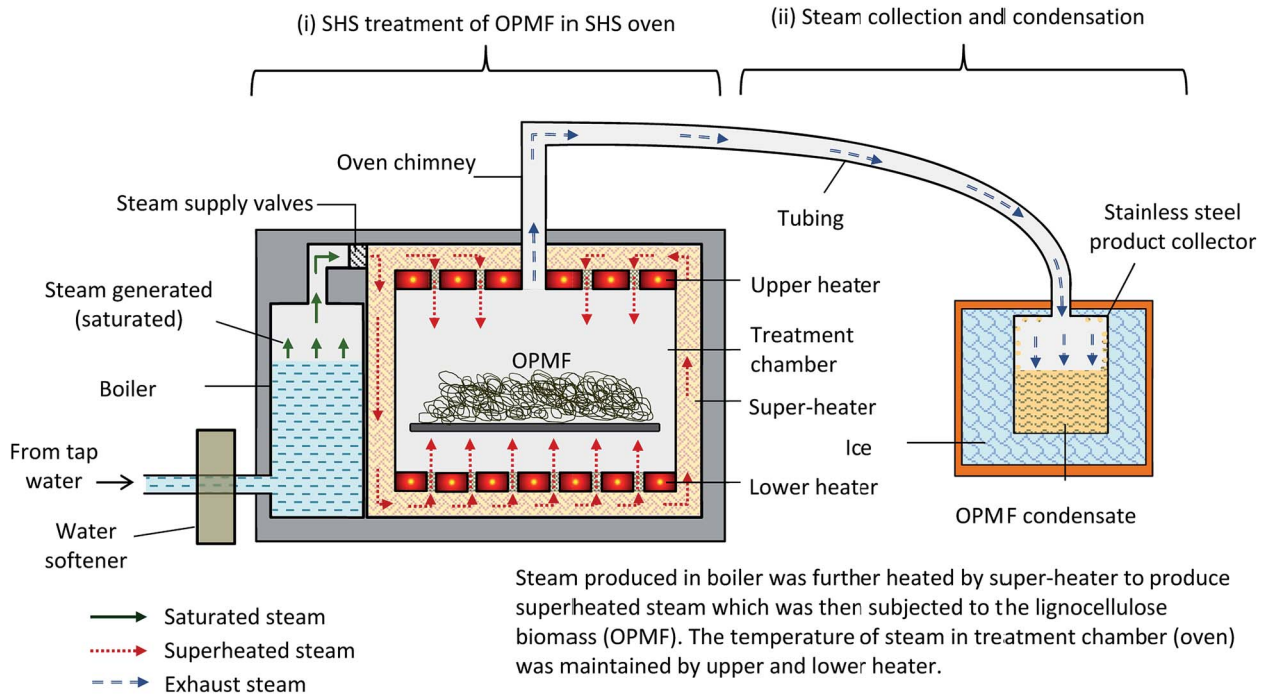


Fig. 5 The overall process of OPMF SHS condensate production.

Japan). Compounds were separated on an HPX-87H column, 300 mm × 7.8 mm (Bio-Rad, California, USA) using 0.08 M sulphuric acid as mobile phase at a flow rate of 0.6 mL min^{-1}. Detection was carried out using UV at 210 nm, while peak of each acid was identified by referring the retention time obtained to that of standard compounds, using a standard curve. Standard chemicals used were acetic acid and formic acid of HPLC grade purchased from Sigma-Aldrich (Missouri, USA), while levulinic acid and succinic acid were from Wako Pure Chemical Industries Ltd. (Tokyo, Japan).

Gas Chromatography-Mass Spectrometry (GCMS). Detailed compositional analysis of other lignocellulosic degradation products from SHS was conducted using a Gas Chromatography-Mass Spectrometry (GCMS). Liquid–liquid extraction was carried out prior to GCMS analysis.[44] Five mL condensate was extracted with diethyl ether at ratio of 1 : 2 for five times. The extract-contained ether layer was concentrated back to its original volume; 5 mL afterwards before been analyzed. Compounds identification was done by using Shimadzu GC-MS Parvum 2, Zebron ZB-1 capillary column of 30 m length, 0.25 mm internal diameter and 0.25 μm thickness. Sample injected at 250 °C, at split ratio of 20 : 1 was carried by helium gas with 0.88 mL min^{-1} constant flow. The column temperature was maintained at 50 °C for 3 minutes and programmed to increase to 100 °C, held at 100 °C for 3 minute and increased up to 200 °C and held for 10 minutes. The heating rate used was 20 °C min^{-1} 70 eV ionizing energy was supplied at 300 °C with mass range of 35–200 amu s^{-1}. The composition of condensate was qualitatively identified through comparison of sample's mass spectrum with library and literature. Quantitative identification of the compounds was estimated from the relative area concentration's obtained (ESI – eqn (S1)†).

Antifungal test

OPMF condensate RF sample was preceded to antifungal tests againts *Trichoderma asperellum* UPM1, *Aspergillus fumigatus* UPM2 and *Ganoderma boninense* UPM13. Two methods were used: (i) spore germination method (only for *Trichoderma* sp. and *Aspergillus* sp.), and (ii) antifungal agar dilution.

(i) Spore germination method. The spore germination test was adopted from Yulia (2005).[45] An agar–spore film was prepared by mixing 10^6 spore suspensions with PDA solution at a ratio of 1 : 1. Mixed agar–spore solution was poured onto 150 mm diameter Petri plate at a fixed volume (≈35.4 mL) to achieve a uniform ±2 mm agar–spore film thickness. Ten millimeter diameter size of cork borer was used to make an agar–spore film of respective diameter size. The film was then placed on a glass slide inside a container and 25 μL condensate samples were dropped onto each agar–spore film. The same amount of distilled water was dropped on a set of agar–spore as negative control. Finally, the container was incubated at 28 ± 2 °C for 72 hours. Germination process was observed evey six hours by dropping two drops of lacto phenol-cotton blue dye onto of the agar–spore film.

Preparation of lacto phenol-cotton blue. The lacto phenol-cotton blue dye was prepared over two days.[46] Cotton blue (aniline blue) of weight 0.05 g was dissolved in 20 mL deionized water and stirred overnight. Phenol–glycerol–lactic acid solution was prepared the next day, in which 20 g phenol crystals was added to 20 mL 100% lactic acid. The solution was stirrer until the phenol was dissolved, before 40 mL of 100% glycerol was added. The cotton blue solution was later filtered into the phenol–glycerol-acid solution, mixed well and stored at room temperature until used.

Spore was deemed to have germinated when the length of its germ tube exceeded one and half of the diameter measurement of the largest spore.[47] The germinated spore was counted under light microscope at 40× magnifications from a total of 500 to 600 spores observed. Illustration of the method is shown in Fig. S6 in ESI.† Germination percentage was counted as follows:

$$\text{Germination percentage} = \frac{\text{germinated spore}}{\text{total spore counted}} \times 100$$

(ii) Agar dilution method. The agar dilution method was adopted from Alizadeh *et al.* (2013)[48] with some modifications. Antifungal plate was prepared by preparing mixture of filter-sterilized RF sample with autoclaved potato dextrose agar (PDA) solution at ratio of 1 : 9, in which a 100 mL PDA–RF mixture solution contained 10 mL RF sample and 90 mL PDA solution. pH of the mixture was 5.05 ± 0.02. Approximately 25 mL PDA-condensate solution was poured onto 85 mm diameter Petri dish plate to get uniform thickness of agar. A hole of size 6 mm was made in the middle of the agar.

Spore suspension prepared from seven-day old fungi cultured on potato dextrose agar (PDA) plate was used as inoculum for *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2, while mycelia plug from seven-day old fully grown culture was used as inoculum for *Ganoderma boninense*, UPM13. For *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2, 20 μL of spore suspension with concentration 1×10^5 spore count was inoculated into the prepared hole. As for *Ganoderma boninense* UPM13, mycelia plug of same size with the hole diameter was used as inoculum, and place on top of the agar.

The inoculated agar plate was incubated for two weeks at 30 ± 2 °C. Growth diameter was measured daily. Determination of inhibitory potential was identified by comparing the mycelia growth of fungi on antifungal plate with control plate (PDA with no OPMF condensate). Illustration of the method is shown in Fig. S7 in ESI.† The percentage of growth in respect to growth in control plate was calculated as follows:

$$\text{Growth percentage} = \frac{\text{diameter of mycelia grown on antifungal plate}}{\text{diameter of mycelia grown on control plate}} \times 100$$

The entire antimicrobial experiments were carried out aseptically.

Conclusions

Superheated steam treatment of lignocellulosic biomass aimed for altering the composition of the biomass prior to biocomposite and renewable sugars production generating exhaust steam which can be condensed and collected as condensate. The condensate contains degradation products of lignocellulosic biomass consists of bioactive compounds beneficial as antifungal agent. Oil palm mesocarp fiber SHS condensate tested herewith showed a superior inhibitory effect on the growth of *G. boninense*, a white rot fungus causing BSR disease in oil palm plantations. It is important to highlight that SHS treatment temperature played a role in determining the concentration and types of bioactive compounds in the condensate, which eventually affecting the condensate effectiveness as an antifungal agent, particularly towards *G. boninense*. The finding from this study exhibits a good example on the efficient utilization of biomass generated at the oil palm plantation, for the benefit of the plantation itself. The collection of the SHS exhaust steam as condensate will not only avoiding the occurrence of environmental pollution, but it also contributes to the generation of bioactive compounds with antifungal property.

Acknowledgements

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