Innovative production of fungal pulp from *Trametes versicolor* and its application in a fungal paper box containing clove oil

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Abstract The aims of this study were to develop fungal pulp from *Trametes versicolor* (white-rot fungi) and apply it with clove oil in a paper box to inhibit mold growth on the surface of peanuts. Broken rice media with different sugar solutions (2–10% w w⁻¹) were prepared and then inoculated with *T. versicolor* mycelium at amounts from 0.5 to 1.5% w w⁻¹. Fungal pulp and commercial paper (50 g) at different ratios (100:0, 70:30, 50:50, 30:70 and 0:100) were mixed and prepared before being placed into a stainless box (5 cm long by 5 cm wide). For the antimicrobial activity against *Aspergillus flavus* on peanuts, a paper box was incorporated with 2.5, 5 and 7.5% w w⁻¹ of clove oil, eugenol, caryophyllene, and a combination of eugenol and caryophyllene at ratios of 7:1, 4:4, and 1:7. Results indicated that the highest fungal pulp biomass of *T. versicolor* in broken rice media was found when using 6% sugar with 1% mycelium inoculums. Fungal pulp and commercial paper at the ratio of 70:30 produced the highest value of hardness. The paper box containing clove oil at 7.5% w w⁻¹ inhibited *A. flavus* on peanuts for at least 28 days while the control had mold growth within 3 days. Combining eugenol and caryophyllene, the main components of clove oil, at the ratio of 7:1 (7.5% w w⁻¹) in the paper box should be a key factor to inhibit *A. flavus* during storage.

Keywords Fungal pulp · *Trametes versicolor* · Clove oil · *A. flavus* · Peanut · Eugenol

Introduction

Mold contamination has raised public concern about microbiological safety (Prakash et al. 2015). *Aspergillus flavus* was reported to be harmful to human health and has been found on the surface of peanuts (Shakerardekani and Karim 2013). It produces mycotoxins that can be very unsafe (Reddy et al. 2011). This fungus was also found to cause damage to other grains such as brown rice (Songsamoe et al. 2016). To solve this problem, the natural preservative clove oil has been selected for this study. The ability of clove oil against microorganisms has been reported earlier (Matan et al. 2006). Unfortunately, when essential oil is added on the surface of products to achieve effective antifungal activity, this could cause inappropriate flavors and odors. Part of this research was done to eliminate the effect on flavor. So, clove oil was incorporated in the packaging.

The global demand for paper production used in food packaging is high. In 2020, world production of paper and paperboard is expected to reach 490 million tons (Bajpai 2013). Environmental problems from the pulp and paper industry, such as toxic compounds and solid waste entering the marine environment, are of concern and focus in various parts of the world (Jawjit et al. 2006; Lindholm-Lehto et al. 2015). Wood is the main raw material needed to make a paper box. However, paper box processing consumes a lot of energy. For example, trees must be cut down, transported to the paper factory, made into wood chips and be processed (Chen et al. 2012). In addition, chemicals such as chlorine, sodium hydroxide, and sulfur are needed for wood bleaching (Nakamata and Ohi 2003; Saski et al. 1997). In this research, *Trametes versicolor* was selected to develop the fungal pulp. For the role it normally plays in the decomposition of all wood polymers (such as lignin and

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extractives) by releasing various enzymes, *T. versicolor* can be found in either living or dead wood (Jönsson et al. 1998). Moreover, *T. versicolor* can also produce exopolysaccharides in many culture media. These are used in medicinal or cosmetic applications (Tavares et al. 2005). In this research, fungal pulp from *T. versicolor* is developed by using broken jasmine rice media, low-cost substrate. This substrate is one of the most widely available polysaccharide materials in the world that can be used to create fungal pulp. Fungal pulp from *T. versicolor* is safe for humans. Therefore, the objective of this study was to develop fungal pulp from the agricultural substrate and to use the fungal pulp to develop a paper box containing clove oil against *A. flavus* on peanuts.

Materials and methods

Trametes versicolor

A white-rot decay fungus (*T. versicolor* WU 0704) was identified from bamboo. The code refers to the strains held in the culture collection of the Wood Science and Engineering Research Unit of Walailak University in the Nakhon Si Thammarat province of Thailand. Fungi were obtained from mycelia grown on a malt extract agar (MEA; Merck Ltd, Thailand) medium at 30 °C for 7 days.

Clove oil, eugenol and caryophyllene

Food-grade clove oil containing 72% eugenol, 10% caryophyllene derived by steam distillation was provided by the Thai China Flavors and Fragrances Industry Co., Ltd. Pure substances of eugenol and caryophyllene were purchased from Sigma-Aldrich.

Effect of sugar solution and amount of inoculums on fungal pulp

A statistical 3²-factorial design was used for the design of the experiment (Table 1). Two variables, X₁ (2, 6 and 10% sugar solution) and X₂ (0.5, 1.0, and 1.5% inoculum) were taken into consideration to yield 9 different experiments. Rice media was prepared by using the same method in previous section. The model generated as a function of these variables on the predicted responses of the fungal pulp yield is a second-order polynomial and is represented as follows;

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

where Y is the prediction of dry weight (g l⁻¹), β_0 – β_2 are the regression coefficients, and X₁, X₂ are the factors.

Table 1 Factor levels for a 3² factorial design and dry weight of fungal pulp

TRT	Sugar (%)	Inoculum (%)	Dry weight (g l ⁻¹)
1	2	0.5	0.9 ± 0.1
2	2	1.0	2.5 ± 0.2
3	2	1.5	1.3 ± 0.1
4	6	0.5	4.2 ± 0.1
5	6	1.0	4.5 ± 0.1
6	6	1.5	2.4 ± 0.2
7	10	0.5	1.1 ± 0.1
8	10	1.0	3.6 ± 0.2
9	10	1.5	3.3 ± 0.2

The optimal process parameters for fungal pulp production were estimated by Statistica software (StatSoft, USA). The coefficients in the second-order polynomial (Eq. 1) were calculated by using the analysis of variance (ANOVA) method. Each experiment was replicated 3× and the reported results indicate the average values of the replicated experiments.

Development of using a paper box fungal pulp

The fungal pulp was prepared from a 6% sugar solution with 1.0% inoculum. A paper box (5 cm wide × 5 cm long) was prepared from 50 g of fungal pulp and commercial paper (Commercial Cone Cup, Thailand) at ratios of 100:0, 70:30, 50:50, 30:70 and 0:100 by using a stainless box. All materials were sterilized at 121 °C for 15 min by an autoclave (Hirayama, Japan) and were mixed together. Then the paper box was dried in a tray at 60 °C for 12 h. After preparation, the box was conditioned in an environmental chamber (Binder, Germany) for 48 h at 23 °C with 50% relative humidity (TAPPI Committee 1996).

After conditioning, the weight of the paper box was recorded by using digital balance weight ±0.01 (Sartorius, Germany). The average thickness was determined with a hand-held vernier caliper (Mitutoyo, Japan) with an accuracy of 0.01 mm. Six random locations on the paper were chosen to calculate the thickness. A color measurement was performed on the paper by using a tristimulus color analyzer (ColourFlex colorimeter, Hunter Associates Laboratory Inc., USA). A contact angle goniometer (Kyowa Interface Science Co. Ltd, Japan) was used to measure the spreading of distilled water on the plate surface of the paper box. Drops of distilled water were made by using a micro-syringe. Then, during the 5 s after the water was dispensed, five images were captured for every second to analyze the contact angle. Each sample was

subjected to 8 measurements for each of the 4 angles: vertical left, vertical right, horizontal left and horizontal right. Finally, the images of the distilled water drops were analyzed with computer contact angle software (FAMAS, Japan). The hardness of the paper was measured with a texture analyser (Lloyd Materials Testing, Leicester, UK). A stainless knife blade with a flat 3 mm end was used to measure the hardness of specimens at a 90° cross section to the surface with an 80% cutting depth. To cut the treated specimens, the controlled speed was at 60 mm min⁻¹. Each measurement was done 8×. The maximum force was measured in Newtons (N) to report the hardness and Nexygen[®] software (Lloyd Instruments, UK) was used to do this.

Fourier transform infrared spectroscopy (TIR) was used to investigate changes in the chemical bond of the paper and was performed by using a Spectrum One FT-IR spectrometer (PerkinElmer, USA). A horizontal attenuated total reflectance accessory (HATR) was mounted into the sample compartment. The internal reflection crystal, which was made of zinc selenide, had a 45° angle of incidence. Spectra were acquired at a resolution of 4 cm⁻¹ and the measurements ranged from 600 to 4000 cm⁻¹ (mid-IR region) at room temperature. Automatic signals were collected in 16 scans at a resolution of 4 cm⁻¹ and were normalized against a background spectrum recorded from clean, empty cells at 25 °C. Analysis of the spectral data was carried out using the Spectrum One software.

Morphology on some selected specimens was examined with a scanning electron microscope (SEM, JEOL JSM-5800 LV, Japan). After critical point drying with CO₂, the specimens were mounted on stubs and coated with gold to be ready for the SEM analysis.

Preparation of antifungal paper box

A paper box (5 cm long × 5 cm) was prepared from a 50 g combination of fungal pulp and commercial paper at a ratio of 70:30. After mixing, the pulp was sterilized at 121 °C for 15 min before it was separated into 3 groups. For the first group, each combination was mixed with clove oil (Thai China Flavors and Fragrances Ltd, Thailand), eugenol, and caryophyllene (Merck Ltd, Germany) at 2.5, 5.0 and 7.5% w w⁻¹. For the second group, each combination was dipped into 2.5, 5.0 and 7.5% w w⁻¹ with eugenol and caryophyllene at ratios of 7:1, 4:4 and 1:7. For the last group, there was no adding of clove oil or main components (control). After mixing, the solution was poured onto stainless plates (5 cm wide × 5 cm long × 5 cm height, 1 mm thickness) and rolling machining was used to roll the specimen in the form of box. Then, the pulp was dried at 60 °C for 12 h.

Inhibition of *A. flavus* on peanuts by a fungal paper box containing clove oil or main components

Aqueous spore suspensions of *A. flavus* WU0501 obtained from 7 days old culture plates were incubated at 25 °C. Codes refer to the strains held in the culture collection of the Wood Science and Engineering Research Unit of Walailak University. Spores were harvested by flooding plates with 10 ml of sterile distilled water containing 0.01% (v v⁻¹) Tween 80. The spore concentration was determined with the aid of a haemocytometer and adjusted to 10⁶ spores ml⁻¹ with sterile distilled water.

Peanuts in shell (15% moisture) were purchased from the Nakhon Si Thammarat province in Thailand. After the shells were removed, 5 kg of peanuts (~wide 1.08 ± 0.07 cm × 3.61 ± 0.18 cm × 0.98 ± 0.03 cm, volume 3.08 ± 0.12 cm³) were then vacuum-packed and stored at 4 °C before using. Three replication were done for each analysis. They were dipped in 70% ethanol for 2 min, rinsed with tap water and air-dried. Each peanut was inoculated with 5 ml of spore suspension containing 10⁶ spores ml⁻¹ of *A. flavus* and held at room temperature (28 °C) for 8 h.

Then, the inoculated peanuts (25 g; ~15 peanuts) were packed into a paper box (5 cm long × 5 cm wide × 5 cm height) incorporated with clove oil or main components. Then, the paper lid (5 cm long × 5 cm wide × 0.5 cm height) was placed on the top of the box. After packing, the box with the peanuts was kept at 25 °C for 28 days. The number of colonies from the peanuts were assessed both before and after storage using MEA. In order to count *A. flavus* for storage times of 3, 7, 14, 21, and 28 days, the peanuts (25 g) were placed into sterile stomacher bags with 225 ml of buffered peptone water (BPW; Nissui Pharmaceutical CO., Ltd, Tokyo, Japan). Then, each count of the *A. flavus* was done by using MEA to count the colonies. The percent inhibition of mold growth was next computed by the following Eq. 2:

$$\text{Inhibition (\%)} = \frac{N_c - N_s}{N_c} \times 100 \quad (2)$$

where N_c is the colony count number (log₁₀ cfu ml⁻¹) in the control and N_s is the colony count number (log₁₀ cfu ml⁻¹) in the sample. Three replications were performed for each treatment.

Statistical analysis

All results are expressed as the mean ± standard deviation (n = 3). The data were statistically treated by one-way ANOVA and Duncan's post hoc test, with *p* < 0.05 being considered to be statistically significant. Statistical analysis was performed with Statistica software (StatSoft, USA).

Results and discussion

Optimization of fungal mycelium in rice media

The mycelium of *T. versicolor* seemed to be related to two main factors: sugar and the amount of inoculum. At medium levels, sugar (6%) and inoculum (1.0%) had a significant ($p < 0.05$) effect on the dry weight of fungal mycelium at $4.5 \pm 0.1 \text{ g l}^{-1}$ (Table 1). Looking at the results from this level indicates that sugar content was related to the mycelium production due to the high dry weight of fungal pulp. However, sugar at a higher level (10%) did not produce higher fungal pulp. When sugar (sucrose) was used in submerges, intracellular invertase enzymes produced by *T. versicolor* hydrolyzed sucrose to glucose and fructose. Then, the glucose and fructose were used as a carbon source for biomass production (Tišma et al. 2010). Zhou et al. (2007) suggested that after 2–3 days incubation, the fungi formed pellets and entered a slow growth period. In this step, glucose was consumed and stimulated ligninolytic enzymes, but after incubation for 5–6 days, glucose was not used to stimulate the enzymes and biomass growth. Therefore, optimized glucose from the first to fourth days might be the key booth factor to increase mycelium. In addition, from this study it was found that a medium amount of inoculum (1%) was also related to sugar concentration (6%). This relationship should be related to the enzyme production from fungi in the optimized glucose level for cell growth. Their interaction and effects in the 3^2 factorial designs are shown in Table 2. The response surface and contour plot are shown along with the effect of sugar concentration and amount of inoculum in Fig. 1. Here the maximum value of the dry weight of fungal pulp was 4.5 g l^{-1} at 6% sugar and 1% inoculum. This model (Eq. 3) contains both linear and quadratic effects. Estimated regression was estimated using un-coded units:

$$Y = -6.6 + 6.0X_1 + 4.4X_2 - 1.6X_1^2 + 0.5X_1X_2 - 1.3X_2^2 \quad (3)$$

Table 2 The analysis of variance (ANOVA) for response quadratic model from experimental designs

	Degree of freedom	Prob. > F
X_1	1	0.03*
X_2	1	0.00*
$X_1 \times X_1$	1	0.00*
$X_1 \times X_2$	1	0.04*
$X_2 \times X_2$	1	0.00*

R^2 0.990, R^2_{adj} 0.986

* $p < 0.05$

Fig. 1 Response surface (a), contour plot (b) for the effect of sugar solution and inoculum on biomass of fungal pulp

Quality of the paper box

Results showing the quality of the paper box made from various ratios of fungal pulp and commercial paper are presented in Table 3. Paper boxes made from 100% fungal pulp were the highest in dry weight, thickness, and lightness (L^*) while those cups made from 100% commercial paper were the lowest in dry weight, thickness, and lightness (L^*). Fungal pulp growth in rice media (non-pigmented) contained a good lightness and had a white color. Therefore, pigments in media are regarded as important for developing the color of fungal pulp. This is very helpful when selecting a target paper color when using fungal pulp in the early stage of paper making. In the pulping industry, the benefits of using white fungal pulp include the avoidance of using bleaching chemicals such as chlorine dioxide

Table 3 Quality of paper cone cup

Fungal pulp: paper	100:0	70:30	50:50	30:70	0:100
Weight (g)	6.67 ± 0.08 ^a	4.24 ± 0.04 ^b	3.23 ± 0.03 ^c	2.21 ± 0.04 ^d	1.53 ± 0.06 ^e
Thickness (mm)	0.28 ± 0.01 ^a	0.22 ± 0.01 ^b	0.19 ± 0.01 ^c	0.12 ± 0.01 ^d	0.11 ± 0.01 ^e
<i>Color</i>					
L*	94.11 ± 0.08 ^a	89.70 ± 0.05 ^b	89.36 ± 0.15 ^b	81.18 ± 0.29 ^c	70.49 ± 0.89 ^d
a*	0.65 ± 0.02 ^b	0.37 ± 0.02 ^c	−0.14 ± 0.04 ^c	1.10 ± 0.06 ^a	0.28 ± 0.04 ^d
b*	−1.07 ± 0.08 ^c	8.21 ± 0.13 ^c	3.43 ± 0.15 ^d	14.35 ± 0.59 ^b	17.55 ± 0.45 ^a
Contact angle (°)	0 ± 0 ^{cd}	74 ± 2 ^{oc}	90 ± 1 ^{ob}	92 ± 1 ^{oab}	108 ± 1 ^{oa}
Hardness (N)	13.1 ± 1.7 ^c	16.4 ± 1.1 ^a	15.4 ± 0.8 ^b	8.3 ± 0.9 ^d	5.9 ± 0.3 ^e

^{a–e} Different superscripts are significantly different ($p < 0.05$)

(Nakamata and Ohi 2003) or organic matter containing halogen and sulfur (Saski et al. 1997). This decreases environmental hazards (Singh et al. 2010). In addition, white fungal pulp could be produced from low cost broken rice substrates and harvested within a short time (~6 days). In sum, fungal pulping has diverse ecological and economic efficacies in the future.

In addition, the combination of fungal pulp and commercial paper at the 70:30 ratio had a significant ($p < 0.05$) effect on the hardness (~16 N). SEM images of a paper box at this ratio and a paper box made from commercial paper are shown in Fig. 2a–c. While the network structure of the paper box at the ratio of 100:0 (fungal pulp: commercial paper, Fig. 2a) had higher surface uniformity than those at the ratios of 70:30 (Fig. 2b) and 0:100 (Fig. 2c), complete wetting (the contact angle was 0°) was also found. When compared, it seemed that the 0:100 box had a rougher surface and a greater number of open pores than the 70:30 box. This result might suggest that hydrogen or ionic bonds between fungal pulp and commercial paper can induce a strong interfacial interaction.

From the FT-IR results, it appears that paper boxes at the fungal pulp: commercial paper ratios of 100:0 and 0:100 have prominent peaks in the region between 710 and 964 cm^{-1} . Bands at 814 and 870 cm^{-1} are represented by glucomannan in hemicellulose (Bjarnestad and Dahlman 2002). The band at 896 cm^{-1} is due to a polysaccharide β -glycosidic linkage (Mansfield et al. 1997), and the band at 964 cm^{-1} is from xylan in cellulose. When the fungal pulp: commercial paper ratio is 70:30, change in the chemical structure was noticed to be at 1150–1300 cm^{-1} due to C–O–C. The high peak of 896 cm^{-1} is due to C–H (Alemdar and Sain 2008) and glycoside linkages. In addition, the band at 3246 cm^{-1} represents O–H stretching (Mukherjee et al. 2014) and was also observed at 100:0 but a little peak could be found at 70:30. Therefore, the wettability measurements demonstrated that at 70:30, there was increase in the contact angles of the specimens from 0 (100% fungal pulp) to $74^\circ \pm 2^\circ$. Overall results suggested that the

combination of fungal pulp and paper at 70:30 may be of good value during the paper process.

Effectiveness of the paper box containing clove oil or its main components

The inhibition of *A. flavus* on peanuts packed inside a paper box containing clove oil and its main components eugenol and caryophyllene at various ratios is shown in Fig. 3a–c. The antifungal indices of clove oil (7.5%) in the paper box at and both of its main components (eugenol:caryophyllene, 7:1) at 7.5 and 2.5% of eugenol alone were 100% against *A. flavus* on peanuts. Results clearly showed that by decreasing the ratio of eugenol in the eugenol:caryophyllene combination to 4:4 and 1:7 could not inhibit the growth of *A. flavus* compared to that of the highest ratio of eugenol (7:1). With a higher clove oil concentration (7.5%), the growth of *A. flavus* was clearly eradicated. Moreover, the combination of main components at 7:1 (7.5%) showed about the same strong inhibition as using clove oil alone at 7.5%. In addition, peanuts packed inside the paper box with 7.5% clove oil or a 7.5% combination (at 7:1) could completely stop the spore germination of *A. flavus* for at least 28 days. On the other hand, the control box (without essential oil) could not protect mold growth due to the high *A. flavus* colony count of $\sim 4\log_{10}$ cfu g^{-1} which was found within 3 days and increased to $\sim 6\log_{10}$ cfu g^{-1} within 10 days of storage at 25 °C. Results also confirmed that using 2.5% or 7.5% of only caryophyllene could not effect mold growth.

Clove oil was reported to consist of many components such as eugenol, eugenyl acetate, and caryophyllene (Matan et al. 2006; Hemalatha et al. 2015). However, the major component of clove oil in this study was eugenol (72%) with only a small amount of caryophyllene (10%) added. Therefore, the combined influence of the two main components on mold was done for finding the best combination of inhibiting mold growth. Although, eugenol alone was reported to be capable of inhibiting the growth of

Fig. 2 SEM image of paper box by fungal pulp: commercial paper at **a** 100:0, **b** 70:30, **c** 0:100

molds such as *A. niger*, *Penicillium* sp., *Rhizopus* sp. (Matan et al. 2014), and *A. parasiticus* (Pillai and Ramaswamy 2011), evidence from this work clearly indicated that the synergic reaction between some components of clove oil (eugenol:caryophyllene) took place and, at the

Fig. 3 Effect of paper box containing clove oil (CO), eugenol (EU), caryophyllene (CP), and combination of eugenol and caryophyllene (EU:CP using ratio 7:1, 4:4, 1:7) at concentration of 2.5% (**a**), 5% (**b**), 7.5% (**c**) on a spore germination of *A. flavus* in peanut

ratio of 7:1, affected the growth of *A. flavus*. On the other hand, caryophyllene itself was also known to be used for the purpose of antimicrobial activity (Yang et al. 2000). But findings from this research suggest that caryophyllene alone at 7.5% in a paper box could not inhibit *A. flavus* on peanuts.

To sum up, a paper box made from fungal pulp could adsorb and release clove oil or its main components during storage at 25 °C for 28 days, resulting in complete

inhibition of *A. flavus* on peanuts. Compared to other research, clove oil alone at 100 $\mu\text{l ml}^{-1}$ and eugenol alone at 80–90 $\mu\text{l ml}^{-1}$ could also inhibit the growth of *A. niger*, *Penicillium* sp., and *Rhizopus* sp. on malt extract agar (Matan et al. 2014). Nevertheless, the amount of clove oil and eugenol put in a paper box was reduced to 7.5% w w⁻¹ and 2.5% respectively. Therefore, a paper box made from fungal pulp shows the possibility of adsorbing and releasing essential oil during storage.

Conclusion

Rice media with 6% sugar and the amount of *T. versicolor* at 1% was suitable for the production of fungal pulp (4.5 g l⁻¹). Furthermore, after producing a paper box from fungal pulp and commercial paper at a ratio of 70:30 ($p < 0.05$), higher quality paper properties of hardness were found. The paper box containing clove oil at 7.5% showed good results for inhibiting the growth of *A. flavus* on peanuts. Also, the combined effect between eugenol and caryophyllene at a ratio of 7:1 showed the same strong inhibition as using clove oil alone at 7.5%. It was found that by using a paper box incorporated with clove oil at 7.5%, the percentage of mold inhibition was 100% after at least 28 days of storage at 25 °C.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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