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Inhibitory impact of vapor-phase ethanol on conidia germination and mycelial growth of *Aspergillus fumigatus* on bread

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ABSTRACT

Mold contaminated on bread sold in local market was isolated and identified as *Aspergillus fumigatus*. Then, the bio-control of ethanol on *A. fumigatus* for significantly reducing consumer health risk was investigated. Susceptibility of the mold to evaporated vapor-phase ethanol (EVE) was examined *in vitro*. Complete elimination of viable spore and inhibition growth spread on Potato Dextrose Agar at 30 ± 2 °C of were found with exposure for 15 min to EVE (10.80 ± 0.3 mmol/L). The effect of EVE on the susceptibility of artificial inoculated *A. fumigatus* on bread were further examined. While exposure to EVE affected the survival conidia, the degree of reduction depended on the inoculation level. Complete reduction was achieved for the low inoculation level (2.08 ± 0.04 log conidia/g) by EVE (60 min exposure, 19.27 ± 0.3 mmol/L) but susceptibility was reduced with the high inoculation level (5.4 ± 0.06 log conidia/g). Simultaneously, total aflatoxin production decreased, as EVE exposure increased. Scanning electron microscope images of inoculated bread, confirm the effects of EVE in reducing levels of viable conidia. Hence, EVE is shown to be an effective bio-fumigant for *A. fumigatus* mycelial growth and aflatoxin formation on bread, so effectively reducing the potential for consumer health risks due to this widespread fungus.

1. Introduction

Vapor-phase ethanol has been proven as an alternatively effective bio-fumigant. Normally, ethanol is a familiar natural substance and widely recognized by consumers as safe. Numerous investigations have reported the beneficial application of ethanol dips or vapor-phase treatments for various kinds of horticultural produces. There are many of different ways in which ethanol can be used as an ethanol-vapor (EV) enriched atmosphere (Corcuff, Arul, Hamza, Castaigne, & Makhlouf, 1996), EV from alcohol powder (Suzuki, Uji, & Terai, 2004), EV with hot air (Wang et al., 2011), hot water and ethanol treatments (Wang, Nie, & Cantwell, 2014), and evaporated EV vapor-phase ethanol (EVE) treatments (Krusong, Teerarak, & Laosinwattana, 2015). Some investigation reports that heat and ethanol combinations have marked the synergistic effect in toxicity on some fungi (Dao, Bensoussan, Gervais, & Dantigny, 2008).

Bakery products as a rich source of nutrients are normally subjected to the mold spoilage problem due to their low water activity. Normally, filamentous mold involved in spoilage of bread consists of *Mucor* spp., *Rhizopus* spp., *Penicillium* spp., *Monilia sitophila* and *Aspergillus* spp. Sources of mold spoilage on bread usually identify as post-baking process contamination, slicing machine, bread cooler, conveyor belts and air surroundings (Saranraj & Geetha, 2012). Mold spoilage of bread usually happens when it is stored under high relative humidity and temperature. However, to reduce this spoilage, the fumigation with EV is recommended during cooling of bread which is proved effective but should be optimized (Dao & Dantigny, 2011).

Mold, *Aspergillus fumigatus*, is a common found in the environment, grown rapidly on building materials such as wood, chipboard, mineral wool and gypsum board (Nieminen et al., 2002). It remains airborne for very long periods due to the hydrophobicity, small size (2–3 μm) of conidia and the resistance to UV radiation and temperature (O'Gorman, 2011). Among the human pathogenic mold species, *A. fumigatus* is a crucial causative agent of human infections (Morgan et al., 2005). Besides the infections, we could find preliminary evidence of work on aflatoxin produced by this mold. Therefore, the aims of the present work were to investigate the susceptibility of *A. fumigatus* to evaporated vapor-phase ethanol (EVE) *in vitro*, and to evaluate the efficacy of EVE

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as a bio-control agent to inhibit the growth and aflatoxin production of the artificially inoculated *A. fumigatus* on fresh bread without evaporated water vapor. However, in this present work we use the EVE without heat treatment for controlling spore germination and growth of mold isolated on bread.

2. Materials and methods

2.1. Materials

Bread without added preservatives was purchased from a bakery in the local market in the Ladkrabang area of Bangkok, Thailand. The samples were brought to laboratory within 2 h and, then, transferred into sterile polythene bags before using in the study.…."

The ethanol (95% aqueous) was purchased from the Excise Division, Thailand.

2.2. Microorganism

Isolation and identification of contaminated mold on bread were conducted. Firstly, the contaminated bread was obtained after keeping the purchased bread at room temperature (32 ± 2 °C) for 1 week. Then, the mold was isolated by culturing on the surface of sterile Potato Dextrose Agar (PDA; Merck KGaA, Darmstadt, Germany) in a petri dish before purifying by re-culturing on the PDA using the point inoculation technique. The mycelia at the edge of the colony was cut by cork borer with 0.5 cm in diameter, then, put at the center of the plate. After repeating for three times, the isolated and purified mold culture was kept in the PDA slant. Identification was based on macro and micro morphological characteristics to species level together with taxonomic keys according to Pitt and Hocking (1985) and Raper and Fennell (1965). The isolated namely *A. fumigatus* exhibited a surface growth showing various shades of green, most commonly a blue-green to a grey-green with a narrow white border. Then, the pure culture was maintained on PDA slants at 4 ± 1 °C and later formally confirmed to be *A. fumigatus* by the Thailand Bioresource Research Center, National Center for Genetic Engineering and Biotechnology, Thailand, by using nucleotide sequence.

For preparation of *A. fumigatus* inoculum, a pure culture of *A. fumigatus* was spread on a PDA plate and incubated at 37 °C for 3 d. Next, several conidia were flooded the surface of the plate with sterile 0.1% Peptone water (PW) containing 0.1% (v/v) Tween 80 (Merck KGaA, Darmstadt, Germany) to obtain conidia suspension. The amount of conidia was determined by a haemacytometer and this was used as inoculum.

2.3. Susceptibility of Aspegillus fumigatus to evaporated vapor-phase ethanol in vitro

An *in vitro* investigation of the susceptibility of *A. fumigatus* on sterile PDA to direct contact with evaporated vapor-phase ethanol (EVE) was carried out in a vapor exposure box (Fig. 1; modified method of Krusong, Pornpukdeewatana, & Teerarak, 2016). This consisted of a 37.5 L plastic box (0.25 × 0.30 × 0.25 m) with a slide cover to prevent pressure build up during vapor treatment. The filter-sterilised ambient air was pumped to 500 mL of 95% v/v liquid ethanol in a 1000 mL closed bottle at 0.5 L/min and, then, the delivery of EVE from the headspace of the bottle was pump at the same rate (0.5 L/min) before spreading in the box via a spreader manifold. The actual EVE content was calculated based on weight loss of liquid ethanol used during treatment and expressed as mmol/L (Krusong et al., 2016).

The investigation of susceptibility of *A. fumigatus* conidia with EVE, the volumes of 1 mL of inoculum were spread on plates of 10 mL PDA agar and allowed to solidify for 20 min at room temperature. The inoculated plates were uncovered and placed aseptically on a sterile perforated stainless steel rack in the exposure box. Five durations of

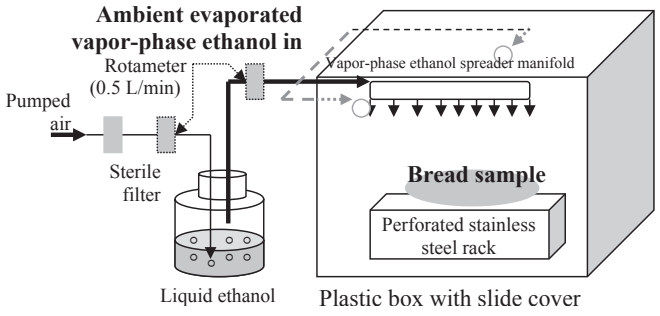

Fig. 1. Schematic representation of vapor exposure box (0.25 × 0.30 × 0.25 m) for exposing bread to evaporated vapor-phase ethanol (modified method of Krusong et al., 2016).

EVE exposure were examined: 0, 5, 10, 15, and 20 min. After vapor treatment, the plate samples were incubated at 37 °C for 3 d. The survival of *A. fumigatus* conidia was recorded and susceptibility to EVE was expressed as the proportion (%) of reduction.

The effect of EVE on mycelial growth *A. fumigatus* was also determined. The mycelial growth on a PDA plate was cut by cork borer with 0.5 cm in diameter was placed on the centered of PDA plate. Five durations of EVE exposure were also examined as mentioned above. The diameter of mycelial production of *A. fumigatus* was measured by vernier caliper and recorded while susceptibility to EVE was expressed as the proportion (%) of reduction.

2.4. Evaporated vapor-phase ethanol effects on Aspegillus fumigatus on fresh bread

Two levels of *A. fumigatus* inoculum were examined: 2 log conidia/mL (low) and 6 log conidia/mL (high). Volumes of 0.2 mL of a conidia suspension of each level of inoculum were single dropped on fresh bread in five positions (totally 1 mL suspension for each piece of bread). Bread was aseptically air-dried in a laminar flow for 5 min before exposing to EVE for five durations: 0, 20, 40, 60 and 90 min in the vapor exposure box (Fig. 1). The contents of ethanol in the box were determined during fumigation period used. The EVE treatment without the water evaporation were operated. The treatments were conducted at room temperature (32 ± 2 °C). After EVE treatment, the bread samples were examined for the evidence of survival of *A. fumigatus*. The treated samples were placed in a sterile bag, added with 100 mL of sterile 0.1% PW and, then, gently mixed and mashed for 15 min for recovering all viable conidia. The suspension of survived *A. fumigatus* was enumerated by spreading on PDA and incubated at 37 °C for 3 d. Results were expressed as the survival of *A. fumigatus* and the proportion of reduction.

Aflatoxin estimation following each treatment was carried out at the same time. As recommended with many studies, the immunoenzymatic method (ELIZA) can be successfully used for the detection of pollutant contaminations such as aflatoxin in food, especially at a very low level (Leszczyńska, Maslowska, Owczarek, & Kucharska, 2001; Lupo, Roebuck, Dutcher, Kennedy, & Abouzied, 2010). The ELIZA is one of the rapid, easy-to-use, and more affordable methods for determining aflatoxins, and can be measured a large number of samples at once. Commercial 96-well assays and strip-tests are available. In addition, Veratox aflatoxin test kit, which was developed by Neogen and has been cited in several peer-reviewed publications (Abbas et al., 2002; Bruns & Abbas, 2006; Harper, Zhao, Meldrum, & Estienne, 2006). Bread samples were dried at 60 °C for 3 h, then ground after keeping cool in the desiccator and put in a clean bottle. The ground sample was vigorously shaken and mixed well for 3 min (Lupo et al., 2010) before taking for aflatoxin extraction. Extraction of aflatoxin from bread employed a modification of the method of Sidhu, Chandra, and Behl (2009). A sample (5 g) of ground bread powder was extracted for 3 min