Ozone-Induced Kinetic Deactivation of *Aspergillus flavus* in Nutmeg Seed (*Myristica fragrans* Houtt)

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ABSTRACT

Aspergillus flavus is a fungal species frequently contaminating nutmeg seeds. Therefore, this study aimed to prevent sporulation by introducing gaseous ozone treatment. In the process, spore suspensions (4.8×10^7 spore/mL) were exposed to ozone (0-11 ppm) for 90 min. Approximately 0.5 mL of each suspension was plated on CDA and incubated at 28 °C (7 days; 24 hours for the remaining samples) alongside the remaining 4.5 mL for 24-60 hours. The results showed that the treatment has significantly delayed the spore germination up to 60 hours. A positive correlation exists between the increasing gas concentration and the lowering of germination. Furthermore, the reduction of *A. flavus* load after being treated was from 0.24-1.2 log spores/mL. The efficacy of this treatment is directly proportional to the concentration of ozone. The three proposed models, including the linear log regression, the Geeraerd shoulder, and the Weibull models, were fully suitable for describing spore inactivation kinetics, emphasizing the potential of ozone as an effective antifungal treatment for microbial control.

Keywords: Concentration; exposure; germination; ozone; reduction; spore

INTRODUCTION

Low quality grade, caused by fungal infection, is frequently reported in the international trade of nutmeg seed. In the worst case, this is strongly related to mycotoxin contamination fatality, impacting border rejections (Dharmaputra et al., 2022; Kabak & Dubson, 2016; Prasetia et al., 2024). It has led to approximately 25% of economic loss during the recent decade (Kabak & Dubson, 2016).

Aspergillus flavus has been reported to be a mold species that grows massively in humid and warm environmental conditions. Several previous studies showed that *A. flavus* has optimally grown between 28 and 35 °C. The fungal species was observed to enrich its hosts in the range of water activity (a_w) = 0.95–0.99 (Fleurat-Lessard, 2017; Mannaa & Kim, 2017). Therefore, a non-thermal approach to mitigate the microbiological threat should be introduced gradually to minimize the fungal growth.

Ozone treatment, a non-thermal method, is environmentally friendly and has less impact on human health. The substance is generally considered safe and has been approved by the FDA (Mastanjevic et al., 2017). Ozone is commonly used as a potential food preservation agent, serving as a microbiological barrier (Jin et al., 2021). It has been successfully reported to lower the sporulation of some fungi, such as *Aspergillus* spp.; *Fusarium* spp.; and *Penicillium* spp. The frequently attacked spice commodities include caraway, licorice, peppermint, chamomile, and lemongrass (Ouf & Ali, 2021).

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The predictions of microbial behavior alterations due to environmental changes are frequently determined through some mathematical models, as previously reported by Cattani et al. (2016) and Gare et al. (2017). The implementation of these models, including the effect of non-thermal treatment for microbial inactivation used in food preservation, has been explored and previously reported (Aspridou & Koutsoumanis, 2021). Several mathematical models have been proposed to describe the behavior of the inactivation. The logarithmic linear model has shown excellent suitability in describing the survival population after the treatment (Cattani et al., 2016). Meanwhile, the effects of several factors, such as variations expressed in terms of concentration, dosage, and time trying, are intimately related to the smooth and saturating transitions that occur in the initial and final phases of the microbial deactivation, as shown by the Geeraerd model (Maresca & Ferrari, 2017). In comparison, the Weibull distribution model has been used to analyze the occurrence of transformation among the survivors. Therefore, determining the size of microbial reduction and the susceptibility of survivors is essential (Buzrul, 2022).

Based on the knowledge obtained, the implementation of ozone exposure is essential, specifically to provide a new solution to the fungal contamination frequently reported in nutmeg. Therefore, this study focuses on isolating *A. flavus* from the nutmeg seed, followed by gaseous ozone exposure in varying concentrations within a finite period. The process continues by observing the suppression of spore germination and evaluating the kinetics-related microbial inactivation models.

METHODS

Materials

Nutmeg seed was purchased from the Beringharjo traditional market in Yogyakarta, Indonesia. The pure culture of *A. flavus* regularly enriched at the Biotechnology Laboratory, Department of Food and Agricultural Product Technology, Universitas Gadjah Mada, was used as the positive control for observing the colony culture during this study. Dichloran Rose Bengal Chlorotetracycline (DRBC) media and Potato Dextrose Agar (PDA) were purchased from HiMedia Laboratories LLC (PA, USA). Finally, Malt Extract Agar (MEA) and Czapex Dox Agar (CDA) were obtained from HiMedia Laboratories Pvt., Limited (Mumbai, India) and Sigma-Aldrich Inc. (Missouri, USA), respectively.

Isolation of *A. flavus* from Nutmeg Seed

Surface disinfection was conducted for two minutes with a chlorine solution. The sample was placed in a new 0.4% chlorine solution (prepared by dissolving 1:10, commercially available chlorine solution) and shaken rapidly for two minutes (Norlia et al., 2019). It was then rinsed four times with sterile distilled water and dried under aseptic conditions.

Five nutmeg seeds were chopped and planted in each petri dish containing DRBC media, a selective medium for Aspergillus species (Pitt & Hocking, 2022). Incubation was conducted for seven days at 28 °C, where checks were performed daily. Following this process, any fungal infections are recorded and determined. The colonies were then cultured again on PDA and MEA media containing 100 mg/L streptomycin as an antibacterial agent. When the mold's reproductive structure has been differentiated between strains, the species was identified based on morphological characteristics (Pitt & Hocking, 2022). Furthermore, the identification was equipped with the pure isolate of *A. flavus*. The fungus was used as the positive control to differentiate the colonies' color between *A. flavus* and *A. parasiticus*.

Ozone Exposure to Spore Suspension

The equipment used consists of: a.) ozone generator Aura PX-902 (Ozofresh, Northants, UK), b.) 0-1.5 L/min gas flowmeter for medical ozone generator flow setting (Generic Manufacturer, China), c.) Tupperware 2.2 L (Sistema Plastics Limited, Auckland, NZ), d.) ozone detector BOSEAN Trioxygen Tester Monitor Gauge Meter (Henan Bosean Electronic CO Ltd., China), e.) mini-fan, as well as f.) Hoses and collection tubes. First, a suspension of *A. flavus* spore was prepared as reported by Das et al. (2015), until the concentration 4.8×10^7 spores/mL was achieved. The ozone exposure treatment instruments were also prepared under aseptic conditions in a laminar airflow 1384G320OP1122 (Thermo Fischer Scientific, UK).

First, the ozone generator was turned on. Then, the gas flowmeter was gradually adjusted to a range of 0.5 to 1.2 L/min to match the produced ozone gas concentration, which varied from 0 (control) to 2, 3.5, 5, 6.5, 8, 9.5, and 11 ppm, and was maintained for 90 minutes. When ozone gas was introduced to the fungal spore suspension, bubbles formed, which were visible on the surface of the suspension. After the entire treatment series was completed, 500 µL of each of the treated samples was planted in a petri dish containing CDA until three replicates were obtained, as reported by Ali and Abdallah (2024), before incubation at 28 °C for seven days. The collecting spore was carried on by adding 5 mL of sterile water to each petri dish and rubbing the surface with a sterile diffuser. However, the remaining samples containing 4500 µL of ozone-

treated suspensions were further incubated at 28 °C for 20, 36, and 60 hours. The effect of exposure on suppressing spore germination was examined under a light microscope (OPTIKA Binocular digital microscope B-190TBPL, PHYWE, Göttingen, Germany). The germinating spore suppression was counted based on a formula (Equation 1) developed by Ouf & Ali (2021).

$$\% \text{ germinating spore suppression} = \frac{(gc-gt)}{gc} \times 100 \quad (1)$$

Where gc and gt are the means of the germinating spore in the control and the treated samples, respectively.

Data Analysis of Sporulating Inhibition Kinetics

The kinetics of fungal desporulation affected by various ozone concentrations, particularly to observe the decline in survival of previously inoculated spore in an agar medium, were approximated by the log-linear model. This was followed by the shoulder and tail of the Geeraerd model (Maresca & Ferrari, 2017). The Equation 2 describes the log-linear model.

$$\log\left(\frac{N}{N_0}\right) = -k_{max}C \quad (2)$$

N and N_0 are fungal spore concentrations at definite and initial times (spore/mL), k_{max} is the first-order inactivation rate (ppm^{-1}), and C is the ozone concentration (ppm). The shoulder length of the Geeraerd model represents the lag phase, where a particular transition occurred from the stable fungal population to the starting point of the initial deactivating process (Garre et al., 2017). The equation for representing this phase is expressed as follows (Equation 3)

$$N = N_0 e^{(-k_{max}C)}\left[\frac{e^{(k_{max}S_l)}}{1+(e^{(k_{max}S_l)}-1)e^{(-k_{max}C)}}\right] \quad (3)$$

Where S_l is the shoulder length (ppm). The tail of this model represented the saturation point which describes the residue of the sub-population (Shelake et al., 2022). The equation has been formulated as follows (Equation 4).

$$N = (N_0 - N_{res})\, e^{k_{max}C} + N_{res} \quad (4)$$

k_{max} is the particular activating rate (ppm^{-1}), and N_{res} is a part of the residual population (spore/mL). The tail portion carries on due to the sub-population N_{res}, in a particular case observed during the experiment (Shelake et al., 2022). Meanwhile, the distribution function known as Weibull was used to analyze the survival data of microbial species (Buzrul, 2022). This mode showed the non-linear part of the semi-logarithmic survival curve. Therefore, the first-order approach was a fascinating exploration of the Weibull model (Equation 5)

$$\log\left(\frac{N}{N_0}\right) = -\left(\frac{C}{\delta}\right)^p \quad (5)$$

Where δ is the concentration characteristics causing the reduction of 1 log microbial population (s), and *p* is the shape characteristics (no dimension). When *p* > 1, the curve tends to be convex, signifying that the survival population was less susceptible to the applied treatment. At *p* = 1, the curve tended to be linearly flattened, which implied equal resistance of the entire microbial population. In the case where *p < 1*, the curve tended to be concave, signifying the susceptibility rate inclined alongside time (Kocer & Keklik, 2021).

Statistical Analysis

The experimental approach used in this study is a completely randomized design with a dependent factor, namely ozone gas concentration of 0 (control), 2, 3.5, 5, 6.5, 8, 9.5, and 11 ppm, subsequently operated for 90 minutes. The obtained data were statistically analyzed using SPSS 27 (IBM Corporation, US), and the results are presented as means ± variance. Furthermore, the fungal deactivating model evaluations were performed by using the statistical parameters *viz.*, Root Mean Squared Errors (E_{RMS}), adjusted R^2 (R^2 adj). The determining coefficient (R^2) was the main criterion, while the suitability parameters were evaluated based on chi-square (χ^2), Mauchly's Test of Sphericity (MTS), and Bayesian Information Criteria (BIC).

RESULTS AND DISCUSSION

Identification of *Aspergillus* Species Isolated from Nutmeg Seed

Figure 1 shows the isolated molds from nutmeg seeds in MEA media. At least three *Aspergillus* species were identified as *A. flavus*, *A. parasiticus*, and *A. niger*. In line with a previous study reported by Nikolic et al. (2018), the green pigmentation of mycelia observed in the colony of *A. parasiticus* appeared to be darker compared to *A. flavus* (Figure 1. a-c). In contrast, the pigmentation of *A. niger*'s colony was distinctly observed to be dark-brown (Figure 1.d). Several *Aspergillus* species in the postharvest and storage periods produced mycotoxins, which were formed as secondary metabolite products. *Aspergillus* producing mycotoxins were reported in many legumes (Ali & Abdallah, 2022), cereals (Akinola et al., 2021), and spice products (Ouf & Ali, 2021). The growth and development of *A. flavus* and *A. parasiticus* and their relationship with the formation

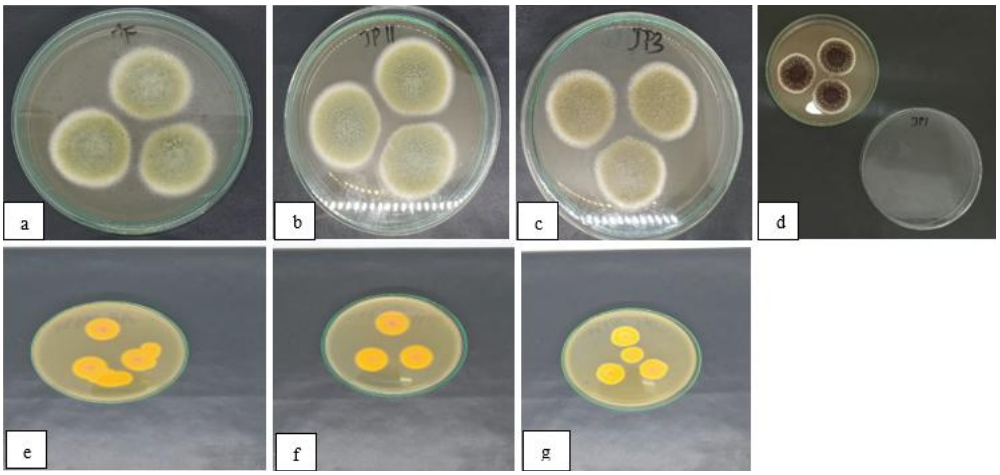

Figure 1. Mycotoxin-producing molds in MEA: pure *A. flavus* culture isolates as a positive control (a) and the extracted molds from nutmeg seeds, including *A. flavus* (b), *A. parasiticus* (c), and *A. niger* (d). Aflatoxin-producing mold colonies in AFPA: pure *A. flavus* culture as a positive control (e) and the extracted molds from nutmeg seeds: *A. flavus* (f) and *A. parasiticus* (g)

of aflatoxin were reported to be closely related to two environmental factors,mnamely temperature and water activity (aw) (Shi et al., 2023).

The presence of aflatoxigenic mold groups was further identified using *A. flavus* and parasiticus agar (AFPA), a selective medium. Figure 1 presents colonies with orange colouration on the back side, implying a powerful sign clusters, which have potentially formed aflatoxin (Barakat & Swaileh, 2022; Nikolic et al., 2018). Therefore, *A. flavus* and *A. parasiticus* were categorized as aflatoxigenic. The gradation of orange pigment in the *A. flavus* colony appeared to be more intense on the underside than that observed in *A. parasiticus* (Figure 1. e-g). This result strongly correlated with a previous study by Prasetia et al. (2024), where the reverse colony, specifically characterized by the predominant reddish-orange pigment, tends to be more aflatoxigenic. Additionally, the evidence deduced signifies that *A. flavus* was the focus of the present study.

A similar investigation reported that more than 55% of the molds had contaminated red chilies, cardamom, ginger, and a mixture of spices with aflatoxin levels ranging from 1.7 - 13.5 µg/kg. Furthermore, low levels of aflatoxin contamination remained a serious threat, specifically when the sanitary conditions of the storage environment are poor and inadequate. In this condition, the opportunity for growth and development of this group of molds becomes greater (Frisvad &

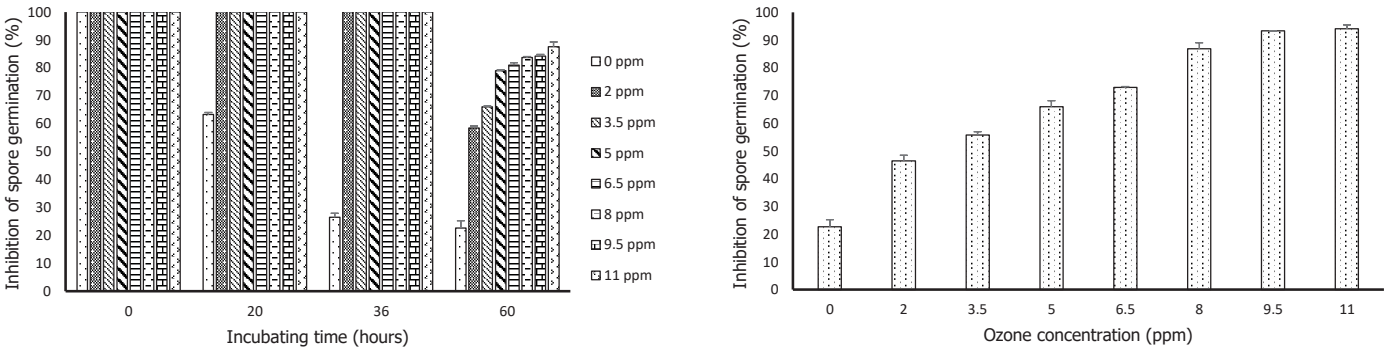

Figure 2. Effect of exposure to various concentrations of ozone (0, 2, 3.5, 5, 6.5, 8, 9.5, and 11 ppm) for 90 minutes to suppress the germination of *A. flavus* spore dissolved in suspension (A) and inoculum planted on CDA (B), followed by incubation for 7 days. The results are expressed as mean ± variance.

Larsen, 2015). *A. niger* is also known as a mold that produces ochratoxin A in many spice commodities, including nutmeg (Kabak & Dobson, 2017). It has been reported to attack onions (Khalifa et al., 2016), pomegranates (Jahani et al., 2020), and cumin and licorice (Ouf & Ali, 2021). The spore of *A. flavus*, which was isolated from nutmeg, was used to evaluate the efficacy of the ozone treatment.

Effect of Various Ozone Concentrations on Suppressing Spore Germination

Figure 2 shows the effect of various concentrations of ozone gas on the suppression of germination in spore suspensions and inoculum propagated in CDA media. The treatment had a significant effect ($p < 0.05$) on inhibiting the germination of *A. flavus* spore that dissolved in suspension, as detailed in Figure 2. A Finally, germination of ozonized *A. flavus* spore was delayed up to 60 hours post-incubation.

Increasing the ozone gas concentration to 8 ppm significantly affected ($p < 0.05$) the reduction of spore germination. The suppression rate was raised to 87.6% for spore exposed to treatment at 11 ppm for 90 minutes. The trend of inhibiting spore germination was similar to that reported by Baazeem et al. (2022), where there is a delay in germination of *A. flavus* spore for up to 48 hours after exposure to ozone at 200 ppm for 30 min. Bhavitha et al. (2024) stated that exposure to 43 ppm for 30 min had a significant effect on reducing the rate of biomass production. In this case, spore and

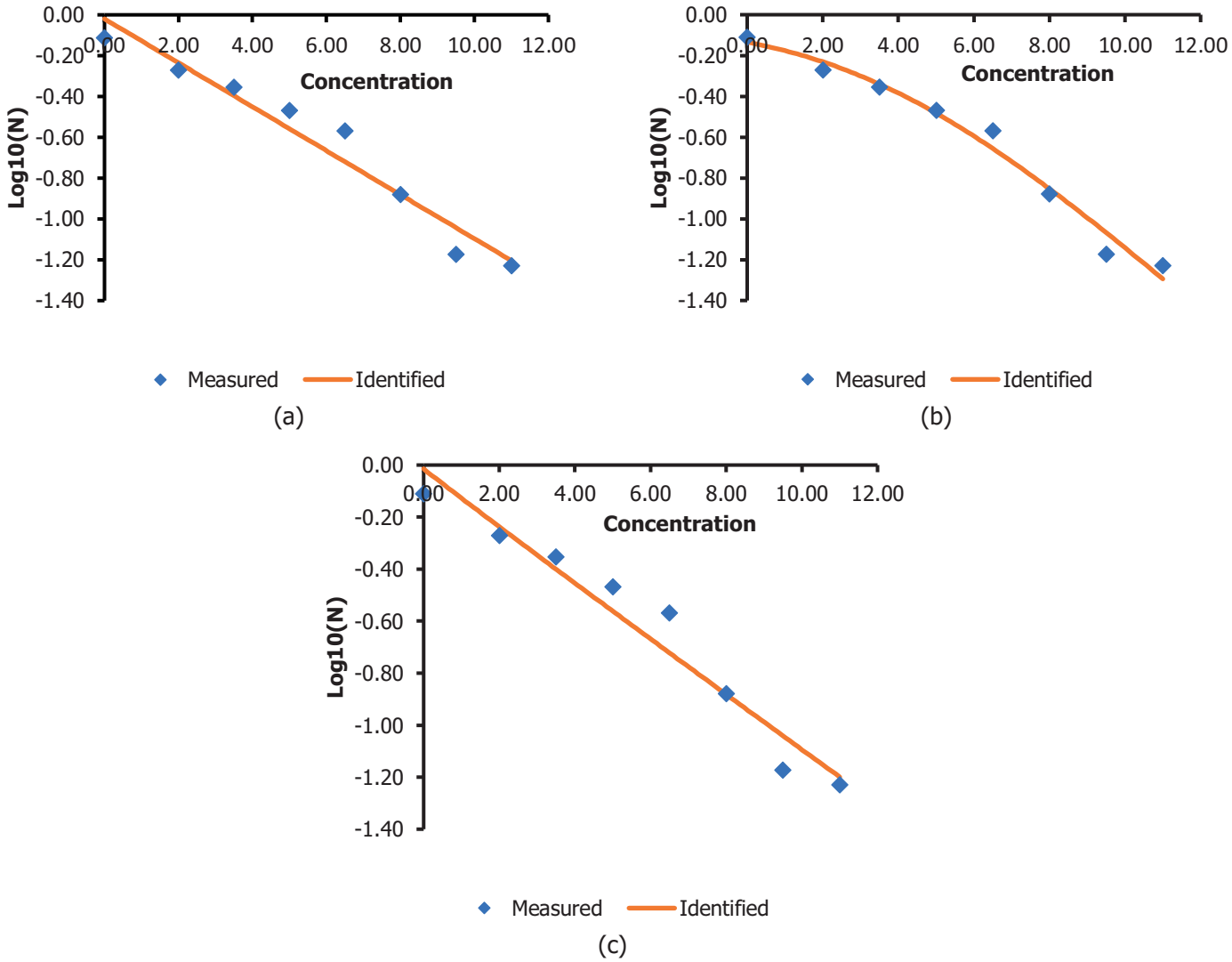

Figure 3. Kinetics deactivation of *A. flavus* due to various exposures to ozone concentrations (2 – 11 ppm) for 90 min in terms of log-linear sporulating reduction (A), followed by shoulder + tail (NA) Geeraerd models (B), and Weibull one (C).

aflatoxin production were suppressed and delayed for at least 72 hours after incubation.

A similar effect was observed in reducing the percentage of germination of *A. flavus* spore inoculum grown on CDA media, as detailed in Figure 2. B, after incubation for up to seven days. The reduction in germination was visible ($p < 0.05$) up to 89.3% at a concentration of 11 ppm. The results of the present study are similar to the reports of Choudhary et al. (2020), who stated that there is a decrease in the number of *A. flavus* spore conidia. This war is characterized by a reduction in biomass growth in the solid phase to 14% in the lag phase.

Ali and Abdallah (2022) reported a reduction in the quantity of *A. flavus* spore and aflatoxin production by 95% and 85% after exposure to ozone gas at four ppm for 180 min to suppress the viability of *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. In spice commodities, including cumin, coriander, licorice, and peppermint, the treatment was effective in inhibiting spore growth and reducing mycotoxin contamination by 90% and 80% at concentration of 3 ppm for 210 minutes (Ouf & Ali, 2021).

Effect of Various Ozone Concentrations on The Deactivation Rate

A wide range of concentrations for sporulating inhibition was determined by observing the survival of spore after gaseous ozone exposure. The log reduction of *A. flavus,* accompanied by shoulder + tail Geeraerd models and the Weibull trend, is shown in Figure 3.

The log reduction of sporulation was observed at 0.24 – 1.2 log spore mL^{-1}. This was a distinct trend that described the significant decrease in sporulating rate ($p < 0.05$) with the increase of ozone concentration (Figure 3A). The result was distinctly supported by the fact that rapid inclination occurred, particularly after gaseous exposure at four ppm, summarized as shoulder length. The final phase of this desporulation was not adequately accounted for since the residual sub-population (N_{res}) was less than the minimal measured value (Figure 3B). Meanwhile, the last third curve was slightly concave rather than linear, signifying much more lethality of spore when exposed to higher dose levels of ozone (Figure 3C).

The inclining of the de-sporulation rate (Figure 3. A-C), was strongly related to rising germination inhibition as presented in Figure 2. A-B. Further observation has proved that exposure to higher ozone concentrations at certain levels significantly impacts the levelling-up sensitivity of spore, leading to a much faster deactivation rate (Shelake et al., 2022). Additionally, penetrating gaseous ozone molecules into microbial cells has caused instability of the cell's structure, gradually triggering the leakage of several main components (protein, fats, etc). This minimizes spore germination and increases the cell's death (Gibson et al., 2019; Liu et al., 2022).

Based on the summary of work-performance parameters shown in Table 1, the Geeraerd shoulder model was the best-fit for the *A. flavus* survival. The model had the lowest value of ERMS compared to the three others, although MTS values were similar, except for the Geeraerd tail model. It has not been accounted

Table 1. Suitability of parameters of the *A. flavus* spore inactivating rate

Model	R^2_{adj}	$E_{RMS}\ 10^{-1}$	x^2	MTS	BIC
Log-linear regression	0.942	1.002	0	1	0.147
Geeraerd shoulder model	0.970	0.725	0	1	0.148
Geeraerd tail model	NA	NA	NA	NA	NA
Weibull	0.923	1.158	0	1	0.148

NA: not accounted

Table 2. Essential values of model parameters in the inactivation rate of *A. flavus* spore

Model	Critical values of main parameters	R^2
Log-linear regression	1.) k_{max} = 0.25 ppm^{-1}; 2.) log (N_0) = 0.09	0.951
Geeraerd shoulder model	1.) S_l = 4 ppm; 2.) k_{max} = 0.37 ppm^{-1}; 3.) log (N_0) = -0.02	0.979
Geeraerd tail model	NA	NA
Weibull	1.) δ = 9.25 ppm; 2.) p = 0.98	0.945

NA: not accounted

for, since the number of spore-resistant organism observed after the treatment was not fully determined, implying that a further extended trial of the experiment needs to be conducted. Except for the Geeraerd tail one, BIC values were relatively insignificant ($p > 0.05$) among the three remaining models. The following Table was used to determine the crucial parameters calculated for each of the four proposed kinetics models.

As summarized in Table 2, the Geeraerd shoulder model was the best fit, supported by the highest R^2 value. By excluding the Geeraerd tail, the Log-linear regression and the Weibull models were subsequently in the second and the third positions, as listed in Table 1. When deeply observed, the δ-value gained from the Weibull showed that at least 9.25 ppm of ozone concentration was required to reduce 90 % of the survival spore, which is equivalent to a 1-log spore/mL. Based on observations , a *p*-value equal to 0.98 impresses the concave curve. This implied that the sub-resistant population would gradually decrease alongside the rise of ozone concentration applied.

The result has been positively related to some previous studies (Hua et al., 2018; Liu et al., 2022; Shelake et al., 2022), mentioning the accumulated rise of ozone concentration. This drastically reduces microbial survival through oxidative stress triggered by the release of several reactive oxygen species, significantly impacting the distraction of the spore. However, the result was still lower than a standard determined by the Food and Drug Administration (FDA), suggesting that additional treatment can be categorized as an antimicrobial agent when the reduction of the microbial population achieves at least 2 log (Akata et al., 2015).

CONCLUSION

In conclusion, at least three species of Aspergillus producing mycotoxins were identified from nutmeg seed, namely *A. flavus*, *A. parasiticus*, and *A. niger*. Through a series of experimental trials, exposure to ozone gas significantly delayed the dissolution of the spore of *A. flavus* in the suspension. This phenomenon was primarily related to the suppression of spore germination, subsequently monitored on the suspension and the inoculum grown on agar, implying the elimination of the fungal population ranging from 30.8% to 92.4 %. Furthermore, linear log regression, the Geeraerd shoulder model, and the Weibull model can be suitable for predicting the survival of *A. flavus* spore, primarily after being treated by gaseous ozone exposure. In the future, various time exposures shall occur in further experimental trials alongside a range of selected ozone concentrations to obtain improved models, including the Geeraerd tail model.

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CONFLICT OF INTEREST

The authors declare that they have no competing financial interests or personal relationships that could be perceived as influencing the work reported in this paper

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