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Aflatoxin B1 elimination in low-grade maize by co-influence of heat and chemical treatment

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Abstract

This research investigated the optimal conditions of heat and chemical treatment for elimination of aflatoxin. NaOH showed the highest inhibition percentage (75.44%) of *Aspergillus flavus* growth that was isolated from low-grade maize. Low-grade maize contaminated with *A. flavus* was treated with NaOH by varying three factors in three levels: NaOH concentration (0, 2.5 and 5% w/v), temperature (25, 50 and 75°C) and time (24, 48 and 72 h). Aflatoxin was removed from low-grade maize after sprinkling with 5% w/v NaOH at 50°C for 24 h that reduced aflatoxin B1 content to 4.25 μg/kg with 60.35% reduction from initial value. The use of NaOH solution with simple heat influenced the reduction in fungal contamination and elimination of aflatoxin B1.

Keywords: aflatoxin; aflatoxigenic fungi; chemical concentration; fungal inhibition; maize; temperature

Introduction

Aflatoxins are the main group of mycotoxins commonly produced by *Aspergillus* species such as *Aspergillus flavus*, *A. nomius* and *A. parasiticus*. Agricultural commodities, food and feed that enrich in carbohydrates such as maize, rice, wheat and barley are susceptible to aflatoxin contamination due to carbohydrates acting as carbon source for fungal growth. The entry of fungus in crops causes contamination and leads to the production of aflatoxin. These toxic compounds are produced under suitable humidity and temperature conditions. The main types of aflatoxins are AFB1, AFB2, AFG1 and AFG2, which are found in common contaminations. AFB1 is the most toxic and is classified under primary group of carcinogen compounds as the first group, with liver being the primary target organ, and it affects humans and animals and also lead to economic losses. AFB1 is a stable compound that cannot be destroyed during most of the food processes (Eslami *et al*., 2015; Heshmati *et al*., 2021; Kumar *et al*., 2021; Nejad *et al*., 2014a).

Aflatoxins are rarely eliminated in food-chain process due to their heat-stable property; they are highly resistant to heat treatments. Therefore, simple drying process cannot significantly reduce their contamination rates in stored grains. However, heat treatment for a prolonged time seems to have a beneficial effect on the decontamination (Pankaj *et al*., 2018; Peng *et al*., 2018; Sipos *et al*., 2021). Several studies have widely used chemical and physical treatments to minimize the risk of aflatoxin contamination in feeds and foods, which usually focussed on the inhibition of mycelia spore development and the consequent elimination of aflatoxins (Tian and Chun, 2017).

The effects of heating methods on aflatoxin detoxification in many crops depend on temperature and time such as in steam under pressure and dry roasting (Jalili, 2016; Peng *et al*., 2018). Kumar *et al*. (2021) reported that AFB1 contents in maize were reduced by 80% when the seeds were exposed to sunlight for 10–12 h. The usage of chemical additives on contaminated foods has also become a popular choice (Rushing and Selim, 2019). Several chemicals, acids, alkalis and oxidizing agents have proved to mitigate the aflatoxigenic fungal growth and aflatoxin production when used in appropriate quantity (Kumar *et al*., 2021). Some food preservatives such as propionic, benzoic and boric acids, and sodium acetate also inhibit the growth of *A. flavus* thereby eliminating aflatoxin. The chemical treatment converts AFB1 into degradation products and thereby reduces the toxicity of AFB1 molecules (Aiko and Mehta, 2015). Recent researches have found that physical and chemical methods can be applied safely with high efficiency for the elimination of aflatoxin, which makes feeds or foods much more acceptable to consumers (Sipos *et al*., 2021).

This study aimed at the elimination of aflatoxin B1 that contaminated low-grade maize using both physical and chemical methods. We investigated the actions of seven kinds of chemicals, namely sodium hydroxide (NaOH), ammonium carbonate [$(NH_4)_2CO_3$], calcium hydroxide [$Ca(OH)_2$], sodium sulfite (Na_2SO_3), potassium hydroxide (KOH), sodium chloride (NaCl) and hydrogen peroxide (H_2O_2) on aflatoxigenic fungal growth. The elimination of aflatoxin B1 in low-grade maize was carried by optimising chemical concentration (0, 2.5, and 5% w/v), temperature (25, 50, 75°C) and time (24, 48, 72 h) through sprinkling method.

Materials and Methods

Raw material preparation

Low-grade maize (*Zea mays* L.) grains with high moisture content, discolouration and kernel damage (kernels were broken and incomplete) were obtained from Phatananikom Kaset Ltd. Company, Phrae, Thailand. Maize grains were ground and sieved to around to 2 mm particle size. The ground maize was kept in double polypropylene plastic bags and stored at room temperature (25–30°C) until use (Boonma *et al*., 2018).

Process of chemicals selection for chemical treatment

Isolation of fungi from low-grade maize

The aflatoxigenic fungi were isolated from low-grade maize sample by using pour plating method. One gram low-grade maize was added to 9 mL sterile peptone (1% w/v) solution and performed serial dilution up to 10^{-6}. One mL of each serial dilution solutions was placed on to three sterile petri dishes (replicas), and molten potato dextrose agar (PDA) was poured over the inoculum. All plates were rotated manually and incubated at 30°C for 7 days. The differential mycelial fungi that could be observed by naked eyes were isolated by transferring on to fresh PDA plates under sterile conditions and incubated at 30°C for 7 days (Sanders, 2012). The morphological features of each isolated fungus were observed under the microscope 40X (Olympus, Japan). The identification of different forms of fungi were confirmed by comparing with the published data or descriptive key (Barnett and Hunter, 1987). Moreover, one of the fungal strains that was expected to produce aflatoxin was selected to confirm the strains using nucleotide sequence analysis. Molecular identification was carried out by DNA barcoding using the ITS region sequencing and compared to those in the database using NCBI-BLAST.

Selection of chemicals to inhibit the mycelial fungal growth

The growth of aflatoxigenic fungi isolated from low-grade maize through the above procedure was inhibited by seven kinds of chemical reagents, namely sodium hydroxide (NaOH), ammonium carbonate [$(NH_4)_2CO_3$], calcium hydroxide [$Ca(OH)_2$], sodium sulfite (Na_2SO_3), potassium hydroxide (KOH), sodium chloride (NaCl) and hydrogen peroxide (H_2O_2). The fungal growth inhibitions were determined using three agar plating methods.

Fungal hyphal extension method

The antifungal activity of chemical solutions was determined by examining the fungal hyphal extension using the method by Ali *et al*. (2020). The 7-days old, 6 mm plug of old aflatoxigenic fungi was placed on the center of the PDA medium supplemented with 10% w/v of the seven chemical solutions. The PDA plate without chemical solution was used as control. The experiments replicate in the three individual experiments and all test plates were incubated at 30°C. Fungal growth or hyphal extension was performed by measuring the diameter of fungal mycelial every day for 7 days. The inhibition percentages of each chemical were calculated from the fungal growth diameter on the chemical-supplemented plates and that of the control plate (PDA) using following equation:

$$\% \text{ Inhibition of fungal mycelial growth} = (C-T)/C \times 100$$

Where, C is the average of the three replicates of hyphal diameter (mm) of control, and T is the average of three replicates of hyphal diameter (mm) of chemical-treated plate.

Hole-plate diffusion method

The hole-plate diffusion on PDA growth medium followed the method by Nejad *et al*. (2014) with some modification. 100 μL of fungal suspension (10^6 spore/mL in 0.85% sterile saline solution) was spread on the PDA plates. After resting for 10 min, 6 mm diameter holes of uniform distance were punched on to the plates, and 20 μL each of seven sterile chemical solutions (10% w/v) and water (as control sample) were filled in each hole. The antifungal capacity was conducted after 7 days at 30°C by observing the clear zone around the holes.

Disc diffusion method

The disc diffusion method with some modifications was followed by Gaziano *et al*. (2018). 100 μL of fungal suspension (10^6 spores/mL in 0.85% sterile saline solution) was spread on the PDA plates. After resting them for 10 min, sterile Whatman No.1 filter papers of 6 mm diameter, soaked in 10% w/v of each of the seven chemical solutions, were placed on the fungal-spread PDA at uniform distance. The antifungal capacity was determined after 7 days at 30°C by observing the clear zone around the filter paper discs.

Aflatoxin B1 elimination by co-influence of heat and chemical

The chemical reagent that mostly inhibited the growth of aflatoxigenic fungi from the chemical selection methods discussed above was used for the elimination of aflatoxin B1 in low-grade maize, along with heat and chemical. Low-grade maize (15 g) and fungal spores (10^6 spores/mL) were added to a polypropylene plastic bag (15 × 22 cm) and pluged with cotton wool. The procedure followed the method described by Thanaboripat *et al*. (1997) with some modifications. Each experiment sample was treated with selected chemical through sprinkling method based on a three-level, three-variable of 10 mL chemical concentration (0, 2.5 and 5% w/v), temperature (25, 50 and 75°C) and time (24, 48 and 72 h). The experimental design was generated with 3^3 full-factorial design with the experimental data, which were analysed from the means of triplicate determinations.

The optimum operational conditions were determined based on the moisture content and number of contaminated fungi. The optimal data was then determined using statistical analysis. Selected samples from the experiments were analysed for determining the optimal conditions that are able to reduce the aflatoxin B1 content.

Moisture content

Treated low-grade maize samples (3–5 g) were weighed to four decimal places with a moisture can of known weight. The sample in the moisture can was heated in a hot air oven at 105°C for at least 3–6 h and left at room temperature in a desiccator until a constant weight was attained (AOAC, 2000).

Number of fungi

The number of contaminated fungi in the low-grade maize sample was determined using pour plating technique, same as that of the method used in the isolation of fungi. The plates were incubated at 30°C for 24–48 h in duplication in an inverted position. The number of fungi was counted and was recorded in the range of 30–300 cells in the spores-forming plate.

Determination of aflatoxins

Aflatoxin B1 was analysed using a high-performance liquid chromatography-fluorescence detector (HPLC-FD), a modification of the method reported by AOAC 991.31 (2002). 25 g of ground maize samples, 5 g NaCl and 125 mL methanol and water (7:3, v/v) were added to blender and homogenised at high speed for 2 min. The mixture was filtered through pre-folded paper. 15 mL of the filtrate was then added into 125 mL glass-stopper Erlenmeyer flask and mixed with 30 mL bromine stopper in water. The solution was filtered through glass microfibre paper ≤ 30 min before affinity column chromatography; 10 mL of second filtrate (equivalent to 1 g test portion) was passed through IAC Aflatest™ at a flow rate of 1.0 mL/min. The AFs were subsequently eluted from the column with 2.0 mL of methanol and collected in a glass vial, and 1.5 mL of pure water was then added to the vial. 50 μL of this test eluted solution was injected to the HPLC-FLD (Alilent Technologies, USA, series 1100). The mobile phase used during HPLC analysis was methanol, acetonitrile and water, and the flow rate was 1 mL/min. The quantity of aflatoxin in each eluate injected was determined from standard curves.

Statistic analysis

The statistical analysis of data and mathematical calculations of models generated were performed by employing the Design Expert software version 7.0.0 (Stat-Ease). The optimal data was analysed by means of Response Surface Methodology (RSM), using analysis of variance and significant F-values indicated a confidence level of 95%. A probability test of $P < 0.05$ was used to estimate the statistical significance of variation in the observed responses using analysis of variance (ANOVA).

Results and Discussions

Process of chemicals selection for chemical treatment

Isolation of fungi from low-grade maize

Low-grade maize isolated fungi appeared in four different morphologies and colours of fungal mycelium determined by visual observation (agar plate and microscope, 40X) on 7 days of incubation as shown in Figure 1. Different morphologies dispersed from filamentous cluster (Figures 1a and 1c) and spherical pellets (Figures 1b and 1d), which consisted of aggregated hyphal structures. Different colours were observed as follows: black–green, black, green and white mycelium (Figures 1a, 1b, 1c and 1d, respectively), and fungal hyphae were presented in a light-colour that turned dark with age. In Figure 1a, the morphology of black–green mycelium revealed dark green and black powdery mycelium on agar plate, and the microscopic photograph showed the arrangement of various round conidia. In Figure 1b, black fungus dispersed and grew as fluffy mycelium and showed single round conidia. In Figure 1c, green-coloured mycelium colony developed, which formed a prominent growth ring with powdery appearance, and the colour changed from yellow to olive green with white border encircling covered the sporulating mycelia as presented on the agar plate. The colonies showed round conidia from brush arrangement of phialospores-like globose vesicles under the microscope. Morphology of the green fungus on agar plate was similar to the reproductive spore of *A. flavus* as described by Afzal *et al.* (2013), who reported that the morphological identification of *A. flavus* from the soil of Larkana district (Sidh, Pakistan) showed yellow to green or dark green colony. The microscopic features of green fungus were same as the morphology of *A. flavus* from groundnut kernels according to the study by Okayo *et al.* (2020). The round conidia from brush arrangement of phialospores were biseriate with radial phialides. In Figure 1d, white mycelial fungus showed deep cottony texture and became more full cotton with age. The colony of white mycelia showed sickle-shaped macroconidia under the microscope.

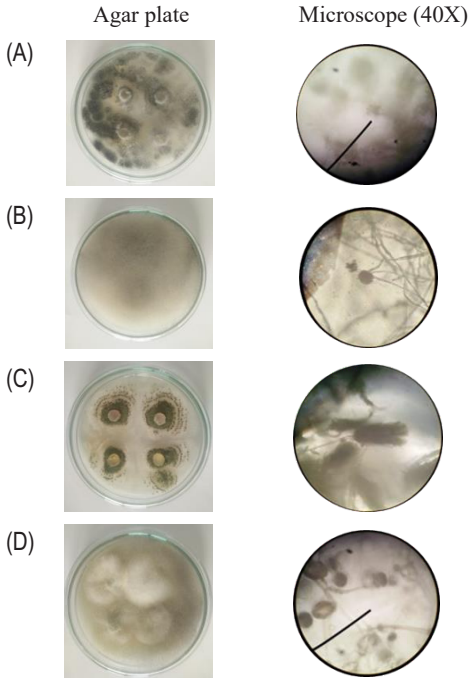

Figure 1. Morphological and characteristic features of mycelia of fungi isolated from low-grade maize. (A) Black-green; (B) Black; (C) Green; (D) White.

The morphology of green fungus was most likely colony and microscopic feature of *A. flavus* was aflatoxin-forming fungi according to the morphological identification, which was similar to that of previous study findings. The typical morphology of *A. flavus* are dark yellow conidia which then transforms to a dominant olive-green and surrounds with white circle. Their borders of colonies are generally plain and flat, which are raised from the centre. Under the microscope, the conidiophores of *A. flavus* are rough and bearing vesicles, the vesicle appears as globose to sub-globose with numerous uniseriate and biseriate columnar *Aspergillus* heads (Khan *et al.*, 2020; Makhlouf *et al.*, 2017; Thathana *et al.*, 2017). Therefore, the isolated green fungus was selected to confirm the classification into species level by ITS region of nucleotide sequence analysis. The nuclear-encoded rDNA of isolated green fungi investigated significant alignments of 97–99% with *Aspergillus* sp. as in the species of *A. flavus*. According to the morphology and nucleotide sequence analyses, green fungus was identified as *A. flavus*, which normally contaminate agricultural products during storage and produce aflatoxin B1 as reported by Jaibangyang *et al.* (2021). Thus, the isolated green fungus from low-grade maize was used in the further experiments of chemicals selection for aflatoxin B1 elimination by heat and chemical treatments.

Selection of chemicals to inhibit the mycelial fungal growth

Seven chemicals, NaOH, $(NH_4)_2CO_3$, $Ca(OH)_2$, Na_2SO_3, KOH, NaCl and H_2O_2, were investigated and one among them was selected as the chemical for heat and chemical treatments. The selection of chemical was performed through three agar plate methods.

Fungal hyphal extension method

The growths of green fungi on seven chemical-supplemented agar plates through fungal hyphal extension method were different, which resulted in different

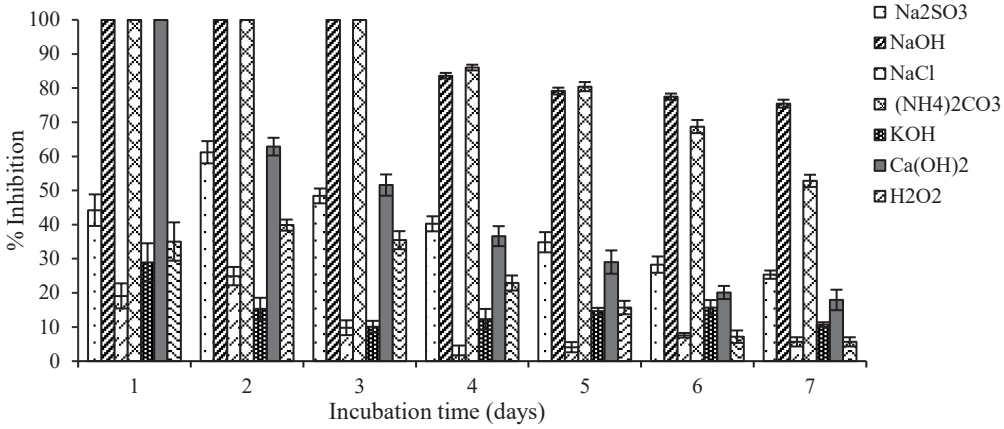

Figure 2. Fungal growth inhibition percentages of each agar plate supplemented with chemical.

fungal growth inhibition percentages during the incubation as shown in Figure 2. The percentage of inhibitions were less than 50% on the supplemented plates of Na_2SO_3, NaCl, KOH, $Ca(OH)_2$ and H_2O_2 since 4 days of incubation. At Day 7 of incubation, the lowest inhibited percentages were observed with NaCl- and H_2O_2-supplemented plates. While the inhibition percentage was from Days 1–3, NaOH and $(NH_3)_2CO_3$ plates were completely inhibited, which indicated that green fungus did not generate spores for 3 days after inoculation. However, after 3 days, the inhibition percentages of NaOH and $(NH_3)_2CO_3$ plates gradually decreased to 75.44 and 52.83%, respectively at Day 7. Ammonium carbonate was found to be effective in reducing the radial growth and completely inhibited the colony growth of *A. flavus*. As demonstrated by Shekhar *et al.* (2009), *A. flavus* that contaminated post-harvest maize is highly sensitive to $(NH_3)_2CO_3$, which reduced its radial growth at a concentration of 20 mM. Samapundo *et al.* (2007) found that 1% of $(NH_3)_2CO_3$ completely inhibited the growth of Aspergillus isolates, which resulted in a large reduction of aflatoxin B1-production in corn. From these results, agar plate supplemented with NaOH presented the highest fungal growth inhibition as confirmed by the fungal hyphal growth at Day 7 in Figure 3c. The diameter of fungal mycelium on NaOH plate was shorter than that of the other chemical plates. The control plate (without chemical in Figure 3a) and the plates supplemented with Na_2SO_3, NaCl, $(NH_4)_2CO_3$, KOH, $Ca(OH)_2$ and H_2O_2 plates, as shown in Figures 3b, 3d and 3e–h, respectively, found that fungi grew and hyphae expanded from the original plug which then distributed over the agar plates.

Hole-plate diffusion method

In case of hole-plate diffusion for seven chemicals selection, the clear zones appeared around test holes of NaOH and H_2O_2 addition as shown in Figure 4. The appearance

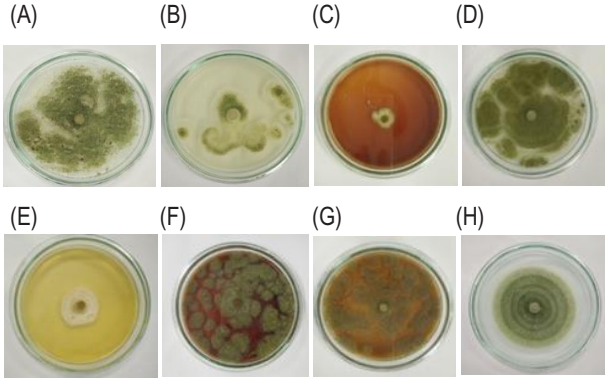

Figure 3. Fungal growth on agar plate supplemented with 10%w/v chemical (A) control (without chemical) (B) Na_2SO_3 (C) NaOH (D) NaCl (E) $(NH_4)_2CO_3$ (F) KOH (G) $Ca(OH)_2$ and (H) H_2O_2.

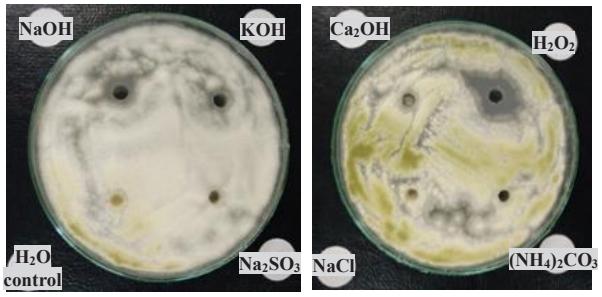

Figure 4. Fungal growth inhibition by hole-plate method.

of clear zone around the test hole indicated that the fungi could not produce the reproductive spore and could not consequently grow in these areas with presence of chemical solutions. Thus, the aflatoxigenic fungal spore formations were inhibited by addition of NaOH and H_2O_2 on hole-plate diffusion. *A. flavus* growth is inhibited by H_2O_2 as per the study by Kure *et al.* (2020), and hydrogen peroxide is effective in reducing the spores of *A. flavus* isolated from food waste.

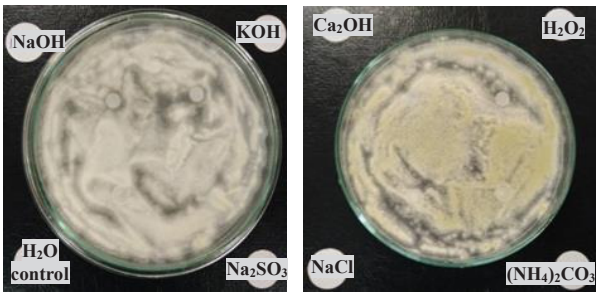

Figure 5. Fungal growth inhibition by disc diffusion method.

Disc diffusion method

The results for seven chemicals selection through disc diffusion are shown in Figure 5; fungi could produce mycelium with fully bloom on sterilized water disc (control disc), and no clear zones were observed around the discs of $(NH_4)_2CO_3$, $Ca(OH)_2$, Na_2SO_3 and NaCl. But the discs of NaOH, KOH and H_2O_2 revealed the clear zone. Fountain *et al.* (2015) reported that the growths of *A. flavus* and *A. parasiticus* were inhibited at a high H_2O_2 concentration. Therefore, in case of disc diffusion method, it was found that NaOH, KOH and H_2O_2 inhibited the aflatoxigenic fungal growth.

From the seven chemicals selected through three methods of agar plating as shown above, NaOH was the best chemical for aflatoxigenic fungal growth inhibition. The direct exposure of pathogenic fungi to NaOH could destruct the internal structures of fungi or even loss their normal shape after block the metabolic functions of fungal cells. Moreover, *A. flavus* could not exhibit the conidia production in the strong alkalinity inform of hydroxyl ion such as in NaOH and KOH media (Al-Janabi, 2011; Monzur *et al.*, 2016). Therefore, NaOH was selected for aflatoxin B1 elimination in low-grade maize by heat and chemical treatments.

Aflatoxin B1 elimination by co-influence of heat and chemical

In this experiment, aflatoxigenic fungi and aflatoxin B1 in low-grade maize were eliminated by treatment with heat and selected chemical (NaOH) from the results of *A. flavus* growth inhibition in agar plates. The treatment samples were added with NaOH solution at difference concentrations 0, 2.5 and 5%(w/v) and kept at three differential temperatures 25, 50 and 75°C for 24, 48 and 72 h. The suitable conditions for aflatoxigenic fungi and aflatoxin B1 elimination in low-grade maize were evaluated through moisture content, number of aflatoxigenic fungi and aflatoxin B1 content.

Effects of temperature and chemical concentration on moisture content

The initial moisture content of low-grade maize was 5.31 ± 0.25%, which was increased to 18.09 ± 1.58% after inoculation with 10^6 spores/mL fungal spores and 25.28 ± 1.15% after addition of NaOH solution.

The effect of temperature on moisture and changes in moisture content during heat and chemical treatments are shown in Figure 6. The moisture contents were significantly different, which were clearly divided into three levels when treated with differential temperatures of 25, 50 and 75°C. The highest moisture content level was observed in the range of 25.57 to 27.81% when treated at 25°C, which was the minimum temperature provided. In low-grade maize samples that were heated at 50°C, the moisture content decreased from 11.34–20.00% and 1.20–8.15% at 75°C. The lowest moisture content level was observed at 75°C, and moreover the moisture content at 75°C decreased faster than that at 50°C. Under conditions of 0% w/v NaOH for 24 h, the moisture contents at 25, 50 and 75°C were observed as 25.57, 22.00 and 8.15%, respectively. Moreover, moisture contents decreased at high temperature (50 and 75°C), under conditions of 0% w/v NaOH; their levels when kept for 24,

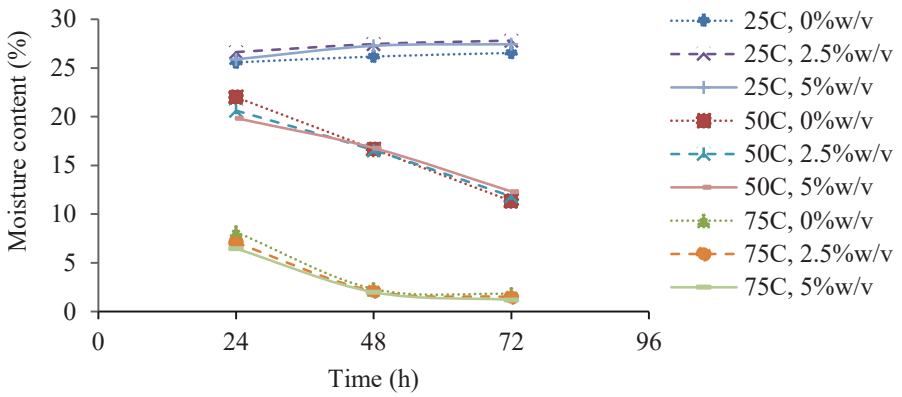

Figure 6. Moisture content of heat- and NaOH-treated low-grade maize.

48 and 72 h were 22.00, 16.66 and 11.34%, respectively. Therefore, it was observed that high temperature significantly decreased the moisture content levels, which tended to decrease even more when the time of heat was extended. The heat provided to low-grade maize caused the moisture content to decrease rapidly from the initial value to the lowest level. According to the report of Srikiatden and Robert (2007), when the grain receives heat energy during the drying process, the water in grains continuously evaporates by moving to the surface and into the atmosphere. The surface of grains become too dry, and the moisture content gradually decreases to the lowest level at zero drying rate.

Analysis of variance (ANOVA) and response surface of individual effect and two-factor interactions of temperature, NaOH concentration and time on moisture content is shown in Table 1 and Figure 7. Temperature (A) and time (C) had individual effects on moisture content in low-grade maize at $P < 0.05$. The two-factor interaction of temperature × time (AC) also had a significant influence on the moisture content at $P < 0.05$. The increase in temperature and time resulted in the decrease of moisture content in the response surface (Figure 7b). Schultz (2016) reported that fungal growth and reproductive fungal spores could be prevented by keeping the moisture content lower than 14% (w/w) or by lowering the water activity. As shown in Figure 7b, a moisture content level lower than 14 % (w/w) was maintained by high levels of temperature (A) and time (C), which were maintained at higher than 60°C and 30 h.

The effect of NaOH concentration on moisture content is presented in Figure 6. NaOH solution with differential concentration 0, 2.5 and 5% w/v had no change on the moisture content. The moisture content levels of low-grade maize samples that were treated at 25°C for 24 h with NaOH concentrations of 0, 2.5 and 5% w/v

Table 1. ANOVA of individual effect and two-factor interactions on moisture content.

	P; Prob > F					
Model	**A**	**B**	**C**	**AB**	**AC**	**BC**
< 0.0001*	< 0.0001*	0.8123	< 0.0001*	0.4983	0.0038*	0.4605

A = temperature B = NaOH concentration C = time

AB = temperature × concentration AC = temperature × time BC = concentration × time

*Significant level, $P < 0.05$ $R^2 = 0.9741$ adjust $R^2 = 0.9663$

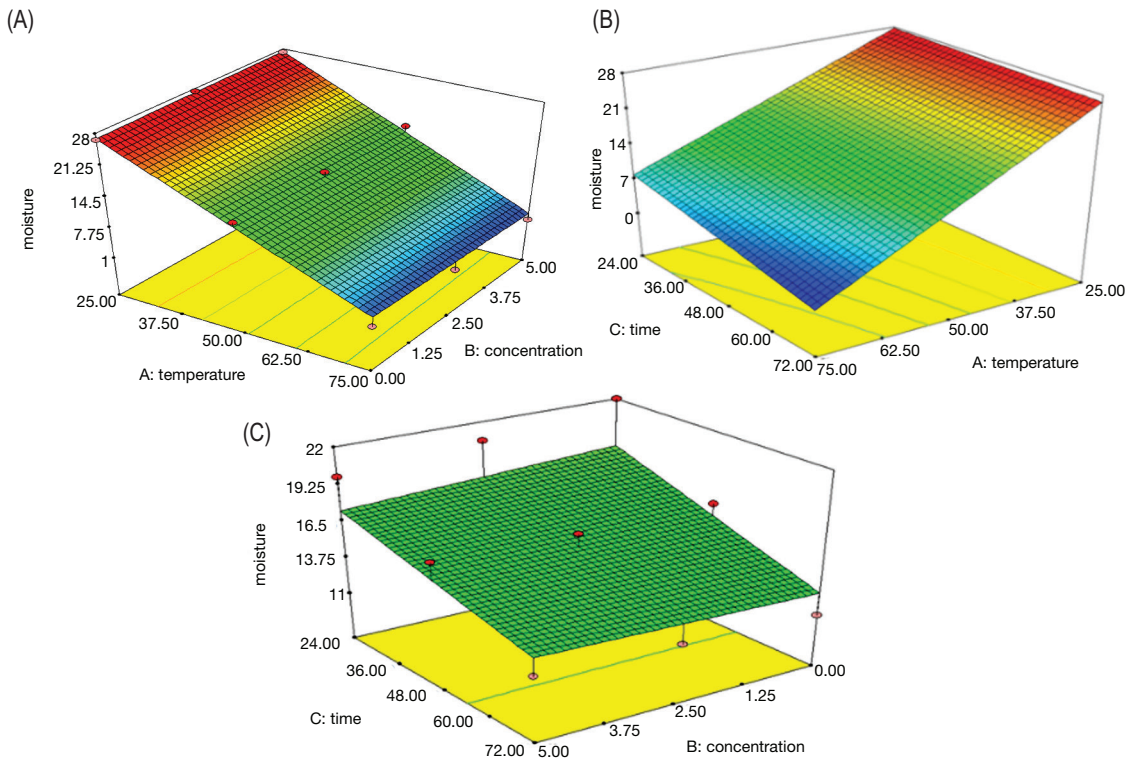

Figure 7. Response surfaces two factors interaction model of moisture content. (A) Factors interaction of temperature × concentration (AB); (B) temperature × time (AC); (C) concentration × time (BC).

were 25.57, 26.63 and 25.89%, respectively; and at 50°C for 24 h with NaOH concentrations of 0, 2.5 and 5% w/v were 22.00, 20.00 and 19.84%, respectively. Thus, NaOH concentrations without temperature, had no significant effect on the moisture content ($P < 0.05$) as shown in ANOVA (Table 1) and response surface (Figure 7.). The interactions between temperature and NaOH concentration (AB) and NaOH concentration and time (BC) did not have any affect on the moisture content ($P < 0.05$) as shown in Figures 7a and 7c, respectively. Our results found that NaOH concentration did not have any profound effects on the moisture content of low-grade maize. However, the study by Shi *et al.* (2008) reported that NaOH pretreatment at high temperature resulted in slight diffusion of the moisture; the moisture diffusion coefficient of the surface showed a trend to increase when NaOH treatment was used.

Effects of temperature and chemical concentration on fungal number

The initial fungal number of aflatoxigenic fungi contaminated in low-grade maize was 2×10^4 cfu/g spores, which changed upon heat and chemical treatments as presented in Figure 8. Aflatoxigenic fungi were able to grow and produce more mycelial spores during treatment at 25°C, and the highest fungal number (8.2×10^4 cfu/g) was observed at 25°C with 0% w/v NaOH for 72 h. The moisture contents at 25°C were in the range of 25.89–27.44%. The moisture content of low-grade maize was higher than 14% which resulted in fungal contamination and production of mycotoxins. It was reported that fungal growth and mycotoxin production in grains could be prevented by maintaining the maximum moisture content level below 14% (Vallverdú *et al.*, 2022). At various temperatures, when low-grade maize samples were treated with 0% w/v of NaOH for 24 h at 25, 50 and 75°C, the fungal numbers were 4.7×10^3, 2.2×10^3 and 0 cfu/g, respectively. The fungal number tended to decrease when the temperature was increased, which confirmed the effect of temperature (A) on fungal number ($P < 0.05$), as presented in Table 2. Fungi in low-grade maize samples treated at high temperatures (50 and 75°C) found to produce less number of spores. These results were consistent with the findings of Rajarajan *et al.* (2021), who reported that fungi favoured to grow in temperature ranging from 12–48°C. *A. flavus* can grow at 37°C and produce the highest levels of mycotoxins at 28°C. Furthermore, heat treatment at 50°C with 0% w/v NaOH for 24, 48 and 72 h found that the number of fungi increased to 2.2×10^3, 3.9×10^3 and 7.3×10^3 cfu/g, respectively. At 50°C without NaOH, fungal spores were observed in low-grade maize because low heat treatment could not destroy the fungal spores. However, fungal spore formation was not observed on addition of NaOH at temperatures 50 and 75°C. Most fungi have an optimal growth rate within a comfortable temperature, and when the temperature reaches a certain point, the growth stops and cell components begin to damage as a result of the heat (Kamil *et al.*, 2011). The interaction term of temperature × time (AC) also had a significant influence on fungal contamination in low-grade maize at $P < 0.05$, as presented in Table 2. The fungal number tended to decrease when the

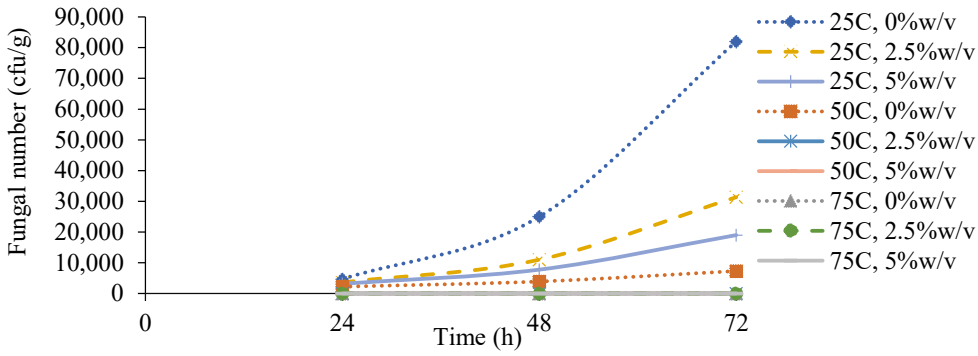

Figure 8. Fungal number of heat- and NaOH-treated low-grade maize.

Table 2. ANOVA of individual effect and two-factor interactions on fungal number.

P; Prob > F						
Model	**A**	**B**	**C**	**AB**	**AC**	**BC**
< 0.0001*	0.0003*	0.0369*	0.0077*	0.0290*	0.0024*	0.0698

A = temperature B = NaOH concentration C = time

AB = temperature × concentration AC = temperature × time BC = concentration × time

*Significant level, $P < 0.05$ $R^2 = 0.7315$ adjust $R^2 = 0.6509$

factors of temperature and time were increased as presented in the response surface (Figure 9b). Through the heat–chemical treatment process, the fungal numbers might increase or decrease based on the conditions of each factor, which affected the induction or inhibition of fungal sporulation.

Fungal numbers from low-grade maize after treatment with various NaOH concentrations are shown in Figure 8. At 25°C for 24 h with 0, 2.5 and 5% w/v NaOH, fungal numbers were 4.7×10^3, 3.6×10^3 and 3.2×10^3 cfu/g, respectively. The number of fungi after treatment with 2.5 and 5% w/v of NaOH decreased from 0% w/v NaOH. Due to this, NaOH and other alkali are proved to mitigate the aflatoxigenic fungal growth and aflatoxin production (Kumar *et al*., 2021). According to the finding of Lefyedi and Taylor (2006), the reduction in microbial counts of the green malts is directly related to the NaOH concentration up to 0.2%, because most fungi do not survive at higher pH values. Furthermore, at higher temperature (50°C), the fungal numbers significantly decreased when NaOH concentration was increased, as observed in the treatment of 0, 2.5 and 5% w/v NaOH for 72 h that resulted in 2.2×10^3, 0 and 0 cfu/g, respectively. From Table 2, it can be observed that NaOH concentration was the individual effect on fungal number that led to a significance in the fungal formation ($P < 0.05$).

In addition, the two-factor interactions were evaluated by ANOVA to verify their significances on fungal number as presented in Table 2 and respond surface of interaction effect presented in Figure 9 (a–c). The interaction effect of NaOH concentration × time (BC) did not significantly affect the fungal numbers, but the interactions of temperature × NaOH concentration (AB) and temperature × time (AC) significantly influenced the fungal numbers and contamination in low-grade maize at $P < 0.05$. The interaction between two factors was observed by comparing the two edges of the surface that was not parallel. In case of interaction between temperature (A) and NaOH concentration (B), the high temperature (A) influenced the decrease of fungal number in a more pronounced way when NaOH concentration (B) was increased (Figure 9b). Thus, the co-effects of high temperature and NaOH concentration were effective to reduce the contamination of fungi by destruction of fungal spores, and fungi did not multiply after this treatment. Thermal and alkaline treatments can increase the efficacy of reduction in mycotoxin contamination by mitigating the aflatoxigenic fungal growth (Karlovsky *et al*., 2016; Kumar *et al*., 2021). The shape of the response surface suggested that a displacement towards higher concentrations of NaOH and temperature when treatment time was extended led to less number of fungal spore contamination. Within this experimental domain, the lowest predicted number

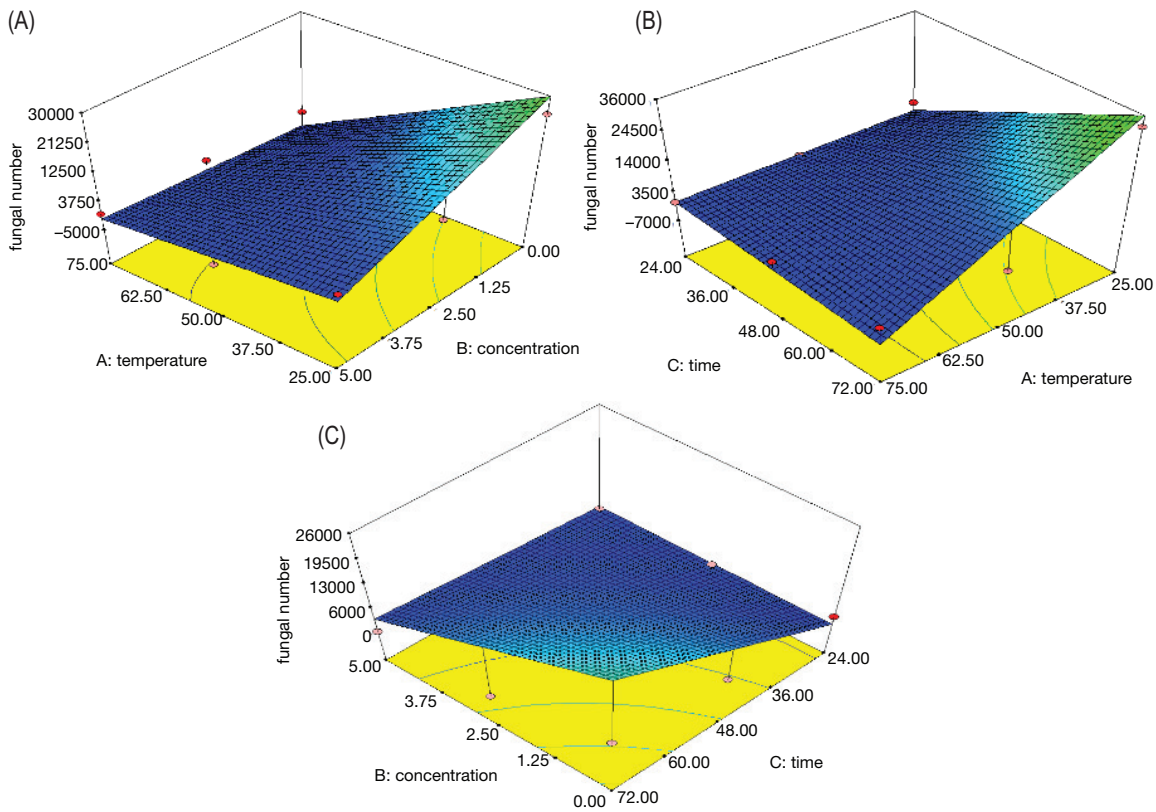

Figure 9. Response surfaces two-factor interaction model of fungal number. (A) Factors interaction of temperature × concentration (AB); (B) temperature × time (AC); (C) concentration × time (BC).

of fungal spore contaminate was 0 cfu/g or fungal spore contamination not found, which was achieved using the following conditions: NaOH concentrations of 2.5 and 5% w/v at 50 and 75°C, with a treatment time of more than 24 h, which corresponded to the conditions in Figure 8. Figure 9 shows that fungal number was not found in low-grade maize in the conditions of tempeature higher than 60°C and heating time higher than 30 h with addition of at least of 2.5% w/v of NaOH.

Relation of moisture content and fungal number

The number of living fungal spores correlated with the moisture content or water activity in low-grade maize; the decrease in moisture content levels resulted in the reduction of fungal spore number as presented in Figure 10. Low-grade maize containing a moisture content level above 11% was contaminated by fungal spores. Thus, moisture is one of the factors that promote fungal growth. These results were similar to the findings of Genkawa *et al.* (2008), who reported that fungi were observed in rice with more than 14.4% moisture content at 25°C, however no forms of fungi were observed on rice with less than 12.8% moisture content. Furthermore, the lowest limit for growth of *A. flavus* happened at moisture ranging from 18 to 25% (Hassane *et al.*, 2017).

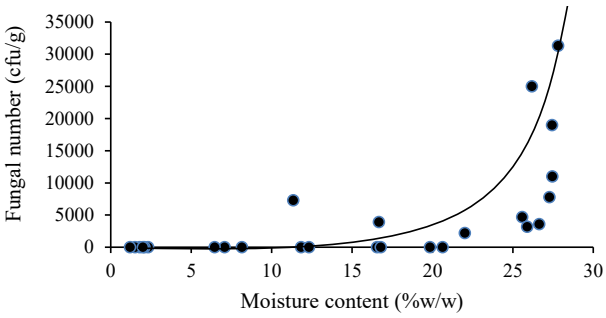

Figure 10. Moisture content and fungal number.

Effect of temperature and chemical concentration on aflatoxin B1

Some conditions of heat and NaOH treatment were selected to analyse aflatoxin B1 levels (Table 3). The initial aflatoxin B1 content in low-grade maize before treatment with NaOH was 10.72 µg/kg. After treatment with 2.5 and 5% w/v NaOH at 50 and 75°C, AFB1 decreased. The lowest AFB1 level was found to be 4.25 µg/kg with 60.35% reduction after treatment with 5% w/v NaOH at 50°C for 24 h. Low-grade maize treated with 2.5% w/v NaOH at 75°C for 24 h also showed reduction in aflatoxin B1 content (55.69%).

Increasing temperatures (25, 50 and 75°C) with 0% w/v NaOH, moisture contents and numbers of fungi in low-grade maize decreased, and AFB1 was also decreased from 10.72 µg/kg to lower values. The moisture content in low-grade maize was reduced by about 20% from the initial value to 1.80–8.15% w/w at 75°C for 24–72 h. These moisture content levels were not suitable for fungal growth and therefore no contamination with aflatoxigenic fungi was observed. Our findings were in agreement with that of Hassane *et al.* (2017) who noticed a correlative effect of moisture on fungal growth and aflatoxins production in wheat flour. The growth of *A. flavus* happen at moisture contents ranging from 18 to 25% and temperature range of 7.5–40°C with optimal growth at around 25°C. These conditions, *A. flavus* favours to germination and thereby production of aflatoxin. In a study by Lahouar *et al.* (2016) *A. flavus* that produced aflatoxin B1 isolated from sorghum seeds, most favoured growth temperature was 25–37°C and water activity 0.91 a_w.

Table 3. Moisture content, fungal number and AFB1 content in low-grade maize.

NaOH (% w/v)	Temperature (°C)	Time (h)	Moisture (% db)	Fungal spores (cfu/g)	AFB1 (µg/kg)	% reduction of AFB1
1. Low-grade maize without fungi addition			2.68	-	7.52	-
2. Low-grade maize with fungi addition			25.62	20,000	10.72	-
3. Fungi treatment low-grade maize						
0	25	48	26.17	25,000	11.98	-
5	25	48	27.27	7,767	8.84	17.54
0	50	24	22.00	2,200	9.82	8.40
0	50	48	16.66	3,900	9.75	9.05
2.5	50	24	20.62	0	6.72	37.31
2.5	50	48	16.56	0	6.37	40.58
5	50	24	19.85	0	4.25	60.35
0	75	24	8.15	0	8.21	23.41
0	75	72	1.80	0	8.15	23.97
2.5	75	24	7.07	0	4.75	55.69

Interestingly, after treatment with NaOH at conditions 2.5 and 5% w/v, fungal spores and aflatoxin B1 were absent, and aflatoxin B1 also decreased to a lower than initial value in low-grade maize (7.52 μg/kg). An extension of time on NaOH-treated low-grade maize resulted in decreased amount of aflatoxin B1. Because the molecule of aflatoxin B1 was breaken by NaOH during treatment. This result correlated with the previous research findings that alkaline treatment leads to the opening of the lactone ring of aflatoxin and its transformation to another form called the beta-keto acid compound, a less toxic aflatoxin D1 (Jalili, 2016). Therefore, aflatoxin contamination in low-grade maize was eliminated by heat and NaOH treatment. Heat helps decrease the moisture content, which results in fungus being unable to produce spores and therefore aflatoxin B1 is not produced. NaOH could inhibit the reproductive spores and destruct mycelial spores of aflatoxigenic fungi thereby decreasing AFB1 (Sheikh and Awad, 2022). Furthermore, the study by Memdes *et al*. (2013) found that thermal alkaline treatment reduced 98% of aflatoxin contamination during roasting process at 250°C for 15 min by adding 20–30 g/kg NaOH in cocoa liquor products. Most aflatoxins are chemically and thermally stable with temperatures up to 100°C, and therefore these factors have little effect on mycotoxin contamination reduction. However, the thermal and chemical treatments increase the efficacy of mitigation of aflatoxin contamination (Karlovsky *et al*., 2016).

Conclusions

In this study, the aflatoxigenic fungi isolated from low-grade maize with high frequency of occurrence was identified as *A. flavus*. The growth of *A. flavus* was used for selection of the chemical, which could inhibit its spore formation; the seven chemicals used were NaOH, $(NH_4)_2CO_3$, $Ca(OH)_2$, Na_2SO_3, KOH, NaCl and H_2O_2. Sodium hydroxide had the potential to inhibit the growth of *A. flavus* through three agar plate methods. Thus, NaOH was applied as the antifungal growth and aflatoxin B1 detoxifying agent in low-grade maize by heat–chemical treatment. The contaminated aflatoxigenic fungal low-grade maize was sprinkled with NaOH solution. The treatments were done by varying three factors in three levels of NaOH concentration (0, 2.5 and 5% w/v), temperature (25, 50 and 75°C) and time of exposure (24, 48 and 72 h). Aflatoxin B1 levels were decreased to 17.54% on treatment with 5% w/v NaOH at 25°C (at room temperature) for 48 h, while the treatment using heat at 75°C for 72 h without NaOH addition resulted in the reduction of aflatoxin B1 to 23.97%. However, the highest reduction of aflatoxin B1 (60.35%) was presented in the treatment condition of 5% w/v NaOH at 50°C for 24 h, in which the aflatoxin B1 content decreased to 4.25 μg/kg. Thus, it was observed that treatment using both heat and chemical was better. Heat treatment along with NaOH addition provided more efficiency and significantly reduced the level of fungal contamination, and consequently eliminated the aflatoxin B1 in low-grade maize.

Acknowledgements

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