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# APPLICATION OF *Bacillus safensis* LAU 13 METABOLITE FOR THE CONTROL OF GROWTH AND AFLATOXIN PRODUCTION BY *Aspergillus flavus* ON STORED MAIZE GRAINS

**[1] Salau N. O., [2] Adelere I. A., [3] Bello, U. M., [2] Abdulameen S. A., [2] Adabara N. U., Ilyasu, U. S.**

1 Africa Centre of Excellence for Mycotoxin and Food Safety, Federal University of Technology, Minna, Nigeria
2 Department of Microbiology, Federal University of Technology, Minna, Nigeria
3 Department of Biochemistry, Federal University of Technology, Minna, Nigeria
*Corresponding author: isiaka.ade@futminna.edu.ng
+2348062187217

## ABSTRACT

*The uncontrolled rise in the use of synthetic chemicals for post-harvest preservation of farm produce has resulted in ecological deterioration and contamination of crop products with harmful chemical residues. Thus, the use of bioactive materials as a substitute for common synthetic chemicals in controlling post-harvest microbial contamination of crops has gained tremendous attention due to their eco-friendliness, cost-effectiveness, and sustainability. This study aims to evaluate the antifungal effect of Bacillus safensis LAU13 metabolites (METOX) for the control of Aspergillus species growth and aflatoxin production on stored maize grains. The METOX was produced by Bacillus safensis LAU 13 during a three-day submerged fermentation cultivation. Characterisation of METOX by gas chromatography-mass spectrometry (GC-MS) revealed the presence of bioactive compounds, predominantly aldehyde derivatives, 4-Dodecen-1-al (13.5%) and Octanal,3,7-dimethyl (13%). The METOX exhibited a potent inhibitory effect against the aflatoxin-producing strains of A. flavus and A. niger isolated from stored maize grains, as it induced 62% and 16% fungal growth inhibition against the isolated strains of A. flavus*

*and A. niger, respectively. Hence, the results obtained herein suggest that the METOX produced by B. safensis LAU 13 has promising applications as a bioactive material for the sustainable control of aflatoxin-producing fungal species in stored grains.*

**Keywords**: *Bacillus safensis*, *Aspergillus species*, aflatoxin, METOX, eco-friendly

## INTRODUCTION

Aflatoxins are one of the types of mycotoxins produced primarily by Aspergillus species (Peles et al., 2019). They are highly toxic, carcinogenic food poison that mainly causes pathological dysfunction of the liver, kidneys, gastrointestinal tract, immune and reproductive systems in both humans and livestock (Peles et al., 2019). Contaminations by aflatoxins have been reported in various food and feed products, including groundnuts, millet, sesame seeds, maize, wheat, rice, spices, and cocoa, due to fungal infections during pre- and post-harvest conditions (Mahato et al., 2019). Aflatoxin contamination of food crops poses a serious health threat to humans and livestock. Additionally, it incurs a significant global economic burden, as it is responsible for the annual destruction of a substantial quantity of the world's food crops. Aflatoxins significantly limit the development of international trade due to strict regulations in high-value markets. Several cases of liver cancer incidences in Sub-Saharan Africa have been attributed to dietary aflatoxin exposure. Conventionally, physical and chemical methods are used to control post-harvest aflatoxin contamination; however, limitations such as the presence of residues of synthetic fungicides in food and the environment, the emergence of resistant strains, and the development of secondary pests necessitate the development of better and sustainable alternative approaches. In recent times, biocontrol of mycotoxins and phytopathogens using microbial metabolites as well as the use of biogenic nanoparticles are receiving tremendous research interest and have been recommended as a suitable alternative to the traditional physical and chemical approaches owing to their biocompatibility, cost-effectiveness and eco-friendliness (Lagogianni and Tsitsigiannis, 2019; Lateef et al., 2024).

Maize is a staple food for more than 1.2 billion people in sub-Saharan Africa (SSA) and Latin America, and it is the most important cereal crop in SSA (IITA, 2020). It is a versatile crop that is not only consumed domestically but also used industrially by confectionery and animal feed producers, flour mills, breweries, and bakeries (Sadiq et al., 2020). The global production of maize averages 785 million tons; the United States of America is the largest producer, accounting for 42%. Africa produces 6.5% of its goods and imports 28% from countries outside the continent. Additionally, Africa accounts for 30% of global maize consumption. Africa as a whole use 95% of its maize, compared to other world regions, which typically use most of their maize as animal feed (IITA, 2020). In Nigeria, maize accounts for between 60% and 65% of poultry feed constituents, and approximately 6.5% is used by brewing companies.

In comparison, 13% is used for the manufacturing of industrial flours, cornflakes, and other confectionery products (IITA, 2020). The quality of maize products depends on agronomic practices and climatic conditions (Sadiq et al., 2020), and unfortunately, it is one of the most susceptible crops to aflatoxin contamination, thereby causing significant food safety issues and hindering exportation (Foley, 2019). In fact, due to high aflatoxin content, maize and other related food items were denied from developing countries like Nigeria (Sadiq et al., 2020).

Bacillus safensis is a Gram-positive, mesophilic, spore-forming, aerobic and chemo-heterotrophic bacterium (Lateef et al., 2015). It is a rod-shaped, motile bacterium with high tolerance to salt, heavy metals, and ultraviolet and gamma radiation (Satomi et al., 2006). B. safensis LAU 13 is a non-pathogenic bacterium whose mutant and wild strains' enzymes and hydrolysates have demonstrated potent multifunctional activities, ranging from keratin waste management, destaining of blood-stained fabric, dehairing of animal hide, biofertilizer and very remarkable application in nanobiotechnology (Lateef et al., 2015; Adelere and Lateef, 2016; Adelere and Lateef, 2023a, b). Investigation into its potential for preventing and controlling mycotoxins in food grains is likely to open a new vista of research in the area of food safety. Therefore, this study investigates the antifungal effect of its metabolites (METOX) for the control of growth and aflatoxin production by Aspergillus flavus and Aspergillus niger on stored maize grains.

## MATERIALS AND METHODS

### Collection of Maize Grains

The maize grain sample was purchased from Kure Ultramodern Market, Minna, in a clean plastic container and kept under ambient conditions in the Microbiology Laboratory of Federal University of Technology, Minna, until further use.

### Source of Bacterial Isolate

*Bacillus safensis* LAU 13 was obtained from the culture collection of the Department of Microbiology, Federal University of Technology, Minna. It was subcultured on a fresh nutrient agar plate and stored on an agar slant for further use.

### Production of *B. safensis* LAU 13 METOX

The production of METOX was carried out by inoculating 1 mL of a 24 h-old *B. safensis* LAU 13 inoculum into 19 mL of nutrient broth in 100 mL flasks and incubating at 37 °C and 100 rpm for 3 days. After the incubation period, the flask contents were centrifuged in a refrigerated

centrifuge (10,000 rpm, 15 min, 4 °C) to obtain supernatant (Lateef *et al*., 2015). The supernatant was collected, freeze-dried, and stored at 4 °C until required for use. This served as METOX.

**Isolation of *Aspergillus species* from Stored Maize Grains**

Stored maize grains were ground into powder form using an electric blender, and 1 g of the powder was suspended in 9 ml of sterile distilled water and serially diluted. An Aliquot of 0.5 ml of 10-4 dilution was plated out on Saboraud Dextrose Agar (SDA) using the pour plate technique. The plate was incubated at ambient conditions (27 ± 2°C) for 48 h. Thereafter, distinct colonies were sub-cultured on fresh SDA plates to obtain pure cultures, which were stored on agar slants for further use (Abd El-Aziz *et al.,* 2021).

**Identification of Fungal Isolates Obtained from Stored Maize Grains**

Macroscopic features of the isolates, including colony growth, colour, texture, and conidia, will be observed after 3 days of incubation. The slide culture was prepared for microscopic examination. The 18 × 18 mm cover slip was placed gently at a 45° angle on the inoculated SDA. Upon fungus sporulation, the cover slip was gently removed and placed on the microscope slide. A drop of lacto-fuchsin was added, and a small coverslip was placed on top. Another drop of lactofuchsin was placed on top of the small cover slip before completing the assembly with a 22 × 22 mm cover slip. The microscopic features, such as conidiophores, vesicles, metulae, philiades, conidium shape, and texture, were observed under a basic biological light microscope (BA 210, 100x objective) using immersion oil (Okayo *et al.,* 2020).

**Quantification of Aflatoxins on Stored Maize Grains**

Aflatoxins were quantified using the AOAC method (AOAC 999.07, 20th edition, 2016), where a 50g powder form of the maize sample was dispersed into a 1-litre capacity solvent-resistant blender jar. 200 ml of 60% acetonitrile was added, and the mixture was mixed at high speed for 2 minutes. This was centrifuged at 4000 rpm for 10 minutes. The filtrate obtained was diluted with 22 ml of phosphate-buffered saline (PBS). The diluted filtrate was passed through the column at a flow rate of 2 ml. The column was washed with 20 mL of PBS, and air was blown through it to remove residual liquid. The toxin was eluted at a flow rate of 1 drop per second using acidified methanol (acetic acid: methanol (2:98 v/v)). Then, 100 microlitres of this was injected into the HPLC system.

**Characterisation of METOX**

This was achieved using Gas Chromatography-Mass Spectrometry (GC-MS) analysis to identify the biomolecules that comprise the product. The METOX was prepared by extracting its compounds using methanol, followed by filtration to remove any particulate matter, before being injected into the GC-MS instrument. Peaks were matched to compounds based on their molecular weights and retention times.

**Antifungal Activity of METOX Against the Aflatoxin-Producing *A. flavus* and *A. niger***

This was carried out using the cup disc technique, where 1 mL of METOX was introduced into SDA media before solidification. A 6mm hole was punched into the agar with a sterile cork borer, as explained by Dasari *et al.* (2014), and a mycelial mass of 6mm was placed into the hole. The percentage of fungal growth inhibition was calculated relative to the control.

$$\text{Percentage (\%) growth inhibition} = \frac{\text{Diameter of control} - \text{Diameter of test}}{\text{Diameter of control}} \times 100$$

**Data Analysis**

The data obtained in this study were analyzed using SPSS (version 25). Results were expressed as mean inhibition (%) ± standard deviation, and differences among treatment means were evaluated using one-way ANOVA at a 5% significance level ($p < 0.05$), followed by Duncan's multiple range test.

**RESULTS AND DISCUSSION**

**METOX Production**

The cultivation of *B. safensis* LAU 13 in nutrient broth resulted in very profuse growth after three days of incubation (Fig. 1). The freeze-dried supernatant, regarded as METOX, is a yellow solid pellet (Fig. 2). *Bacillus* spp. have remarkable biosynthetic potential. Some of their metabolites have demonstrated tremendous applications as therapeutic agents, food preservatives, and plant-pathogen control agents (Iqbal *et al*., 2023). Moreover, several strains of *B. safensis* have been found to produce metabolites that can be harnessed industrially for diverse biotechnological applications (Adelere and Lateef, 2016).

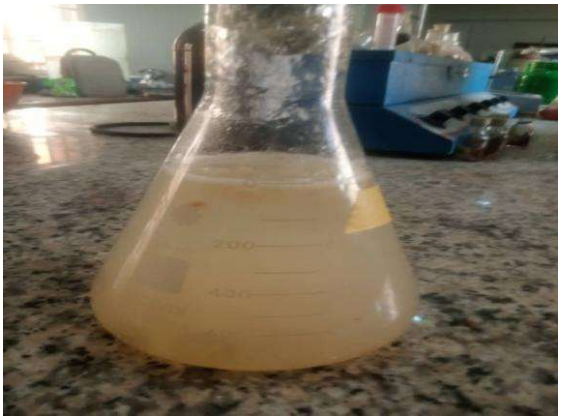

Fig. 1: Cell Suspension of *B. safensis* after cultivation for three days in nutrient broth

Fig. 2: Freeze-dried cell-free extract of the *B. safensis*

**GC-MS Characterization of METOX**

The result of analysis of METOX is shown in GC-MS chromatogram in Fig. 3. The analysis revealed the presence several bioactive compounds in the METOX. Aldehydes, including 4-Dodecen-1-al (13.5%) and Octanal (13%), were the most abundant, while the likes of monoterpenes, phenols, and fatty acids were detected in low quantity (Table 1). The result obtained herein correlate with the study of Koilybayeva *et al*. (2023) that reported the production of 69 volatile organic compounds by five *Bacillus* species. The authors affirmed that all the five species were found to share different chemical classes of volatile organic components, which have a variety of pharmacological applications. The remarkable metabolic capacity and adaptive biochemistry of *Bacillus* species might be responsible for the production these diverse volatile

organic compounds making them a promising organisms for commercial, pharmaceutical and biotechnological applications.

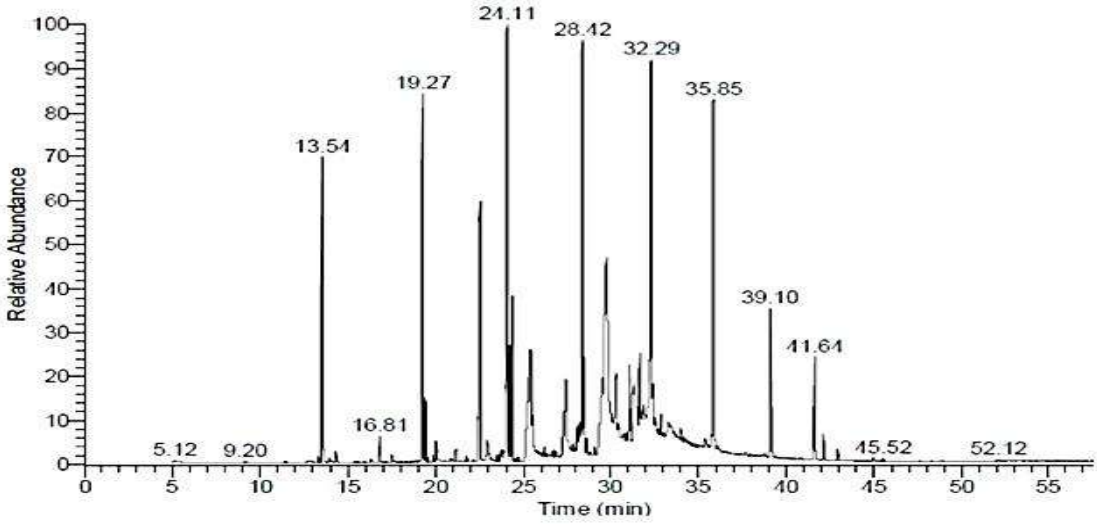


Fig. 3: GC-MS Chromatogram of *Bacillus safensis* LAU 13 metabolite (METOX)

**Fungal Identification**

Aflatoxin-producing strains of A. flavus and A. niger were isolated from stored maize grains. The A. flavus appeared greenish and velvety with a powdery surface on the SDA plate. The colonies are dense with a granular appearance and slightly raised. At the same time, the A. niger strain has a woolly texture, black in appearance and are more granular (Fig. 4). Microscopically, the Aspergillus flavus strain shows rough, radiating conidia attached to vesicles with Aspergillus niger exhibiting large, smooth conidial heads with dense arrangement of conidia (Fig. 5). Contamination of maize grains by aflatoxin-producing fungal species have been reported by authors. For instance, Dadzie et al. (2019) isolated aflatoxin-producing strains of A. flavus, A. niger, Rhizopus sp., Penicillium sp., and Fusarium sp. as the major fungal contaminants on stored maize grains. The authors affirmed that Aspergillus flavus was the most predominant contaminant among the isolated fungal species.

**Table 1: Chemical Composition of *Bacillus safensis* LAU 13 Metabolites (METOX)**

| Compound Groups | Identified Compounds | Molecular Weight | Molecular Formula | Retention Time | Peak Area % |
|---|---|---|---|---|---|
| Monoterpene Derivatives | L-(−)-Menthol | 156 | $C_{10}H_{20}O$ | 13.29 | 1 |
| | Decitol,1,2:4,5:9,10-trianhydro | 178 | $C_{10}H_{10}O_3$ | 13.53 | 5.84 |
| Cyclic Ketone Derivatives | Tert-Butyl-p-benzoquinone | 164 | $C_{10}H_{12}O_2$ | 14.3 | 0.5 |
| | 2,5-Cyclohexadiene-1,4-dione | 220 | $C_{14}H_{20}O_2$ | 21.17 | 0.8 |
| | 7-Ethyl-4,6-heptadecandione | 296 | $C_{19}H_{36}O_2$ | 30.42 | 1 |
| | Cyclopentadecanone, 2-methyl | 238 | $C_{16}H_{30}O$ | 30.76 | 1.2 |
| Alkaloid Derivatives | 1-Amino-7-methylpyrrolo[1,2a]pyrazine | 147 | $C_8H_9N_3$ | 17.47 | 0.5 |
| | Glycozolicine [5-methoxy-3-methylcarbazole] | 211 | $C_{14}H_{13}NO$ | 24.35 | 2.87 |
| Alkane Derivatives | Pentanenitrile, 4-methyl | 97 | $C_6H_{11}N$ | 19.26 | 6.79 |
| | Tetradecane | 198 | $C_{14}H_{30}$ | 19.42 | 0.8 |
| | Octadecane | 254 | $C_{18}H_{38}$ | 24.19 | 1.22 |
| | Tridecane, 3-methylene | 196 | $C_{14}H_{28}$ | 28.21 | 0.7 |
| | (cis)-2-nonadecene | 266 | $C_{19}H_{38}$ | 32.29 | 10.52 |
| | 10-Heneicosene (c,t) | 294 | $C_{21}H_{42}$ | 32.53 | 1.5 |
| | Cyclopentane, 1,2-dibutyl | 182 | $C_{13}H_{26}$ | 35.84 | 7 |
| | Cyclotetracosane | 336 | $C_{24}H_{48}$ | 42.12 | 0.5 |
| Phenol Derivatives | Phenol,2,4-bis(1,1-dimethylethyl) | 206 | $C_{14}H_{22}O$ | 22.49 | 7.5 |
| | 1,4-Benzenediol,2-(1,1-dimethylethyl) | 166 | $C_{10}H_{14}O_2$ | 23.74 | 0.6 |
| Fatty Acids | Dodecanoic acid | 200 | $C_{12}H_{24}O_2$ | 22.96 | 0.55 |
| | Tridecanoic acid | 214 | $C_{13}H_{26}O_2$ | 25.44 | 2.7 |
| | Tetradecanoic acid | 228 | $C_{14}H_{28}O_2$ | 27.45 | 2.52 |
| | 9-Octadecenoic acid (Z) | 282 | $C_{18}H_{34}O_2$ | 28.11 | 0.8 |
| | Hexadecanoic acid | 256 | $C_{16}H_{32}O_2$ | 25.54 | 0.7 |
| | 9,12-Octadecadienoic acid (Z,Z) | 280 | $C_{18}H_{32}O_2$ | 35.35 | 0.6 |
| | 9-Hexadecenoic acid | 254 | $C_{16}H_{30}O_2$ | 31.87 | 0.4 |
| Aromatic Derivatives | 1-methoxymethyl-4-methylnaphthalene | 186 | $C_{13}H_{14}O$ | 19.84 | 0.4 |
| Aldehyde Derivatives | 4-Dodecen-1-al | 182 | $C_{12}H_{22}O$ | 24.09 | 13.5 |
| | Octanal, 3,7-dimethyl | 156 | $C_{10}H_{20}O$ | 28.38 | 13 |
| Alcohols | 1-Tetradecanol | 214 | $C_{14}H_{30}O$ | 26.76 | 0.6 |

| | | | | | |
|---|---|---|---|---|---|
| | n-Tetracosanol-1 | 354 | $C_{24}H_{50}O$ | 39.1 | 2.5 |
| Carboxylic Acids Ester Derivatives | Sebacic acid, 2,6-dimethoxyphenyltridecyl ester | 520 | $C_{31}H_{52}O_6$ | 31.68 | 1.5 |
| | Glutaric acid, di-(−)-menthyl ester | 408 | $C_{25}H_{44}O_4$ | 42.93 | 0.9 |
| Alkene Derivatives | 1-Octadecene | 252 | $C_{18}H_{36}$ | 28.64 | 0.5 |
| | 10-Methyl-E−11-tridecen-1-ol-propionate | 268 | $C_{17}H_{32}O_2$ | 29.06 | 0.4 |

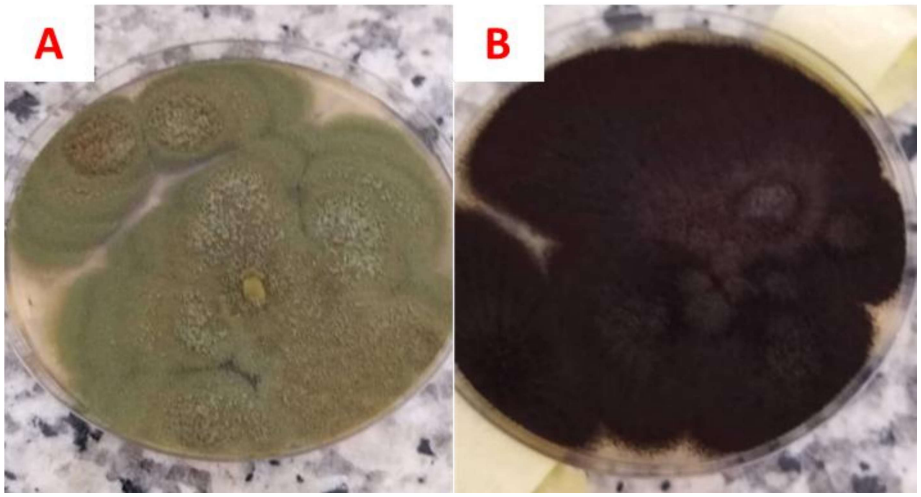


Fig. 4: Colonial morphologies of aflatoxin-producing strains of *Aspergillus* isolated from stored maize grains: *Aspergillus flavus* (A) and *Aspergillus niger* (B)

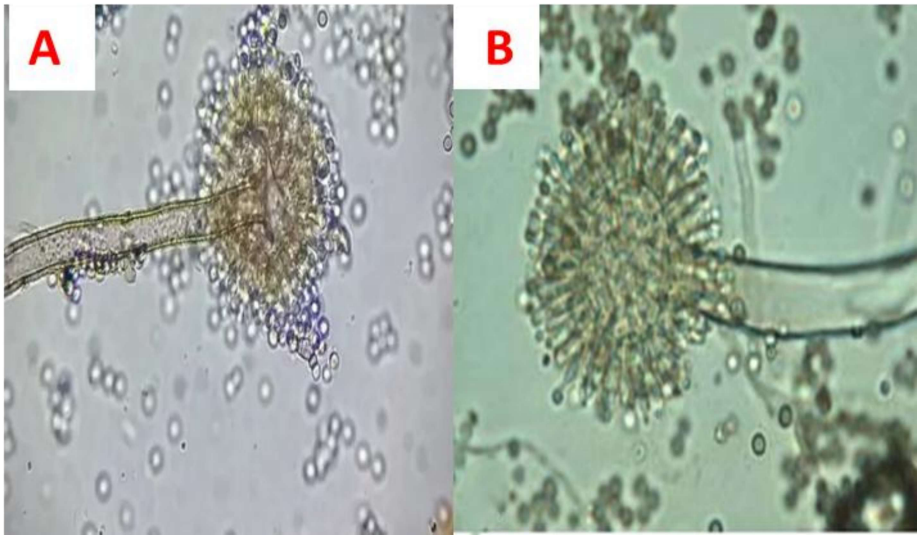


Fig. 5: Microscopic morphologies of aflatoxin-producing strains of *Aspergillus* isolated from stored maize grains: *Aspergillus flavus* (A) and *Aspergillus niger* (B)

**Aflatoxin Quantification**

Fig. 6 is the chromatogram of varieties of aflatoxins detected in the stored maize grains. Aflatoxin B1 was found to be most predominant with a concentration of 204.39 ng/mL, exceeding permissible limits for human consumption. The amount of aflatoxin obtained in this study falls within the range of values of aflatoxin (23-945 ng/g) reported in stored maize grains by Dadzie et al. (2019). Moreover, Perrone et al. (2014) reported high levels of aflatoxins in maize grains collected from open markets in Ghana and Nigeria. Due to the toxic and health-threatening nature of aflatoxin, it is therefore very pertinent to devise means of attenuating the activity of aflatoxin-producing organisms in our stored food items.

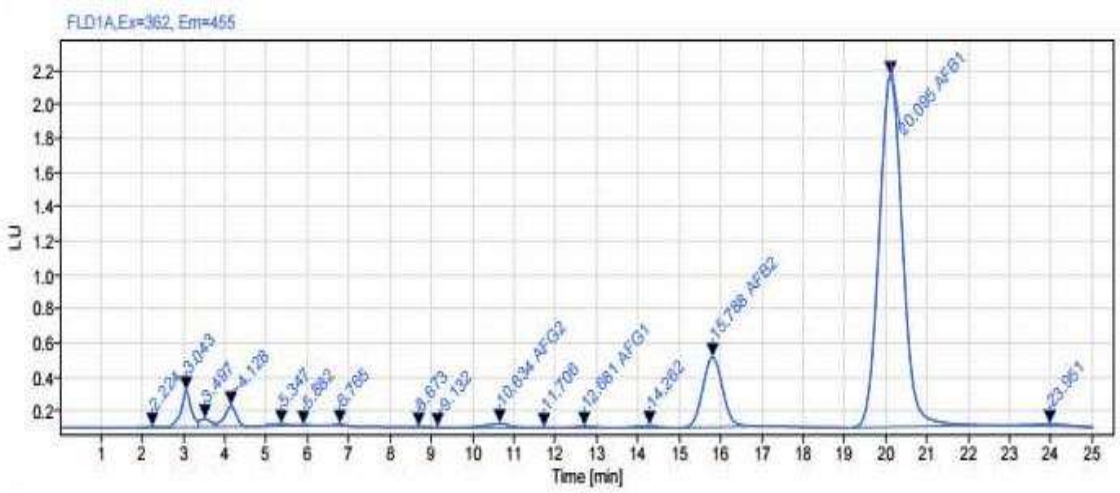


Fig. 6: HPLC Chromatogram of Aflatoxins in Stored Maize Grain

**Antifungal Activity**

METOX demonstrated significant antifungal activity against A. flavus (61.50% inhibition) and A. niger (16 % inhibition) (Fig. 7). Statistical analysis confirmed a significant difference ($p < 0.05$) in growth inhibition between the two fungi (Table 2). Many bacteria exhibit antifungal activity, producing substances or using other mechanisms to inhibit fungal growth. These mechanisms include the production of antifungal compounds, the activity of lytic enzymes, and competitive growth for nutrients. Bacteria, such as Bacillus species, are known for their antifungal properties, producing substances like lipopeptides and other compounds that effectively inhibit fungal

growth. Bharose and Gajera (2018) reported that a metabolite produced by Bacillus species exhibited desirable antifungal activity against an aflatoxin-producing strain of Aspergillus flavus. Similarly, Siahmoshteh et al. (2018) reported potent antifungal activities demonstrated by particular species of Bacillus against Aspergillus parasiticus. The Bacillus species were able to suppress A. parasiticus growth (up to 92%) and aflatoxin production (up to 100%). Most recently, Abdel-Nasser et al. (2024) reported the production of bioactive metabolites obtained from Bacillus cereus DSM 31T, which exhibited strong antifungal capabilities against an aflatoxin-producing strain of A. Flavus. The authors affirmed that the bioactive metabolites displayed antifungal efficiency against A. flavus growth and caused morphological alterations in fungal conidiophores and conidiospores. Also, data obtained indicated that the metabolites reduced aflatoxin B1 production by 99.98%. The results report herein indicate that Bacillus species metabolites could be harnessed as biological control agents to control aflatoxin contamination in crops and agricultural commodities.

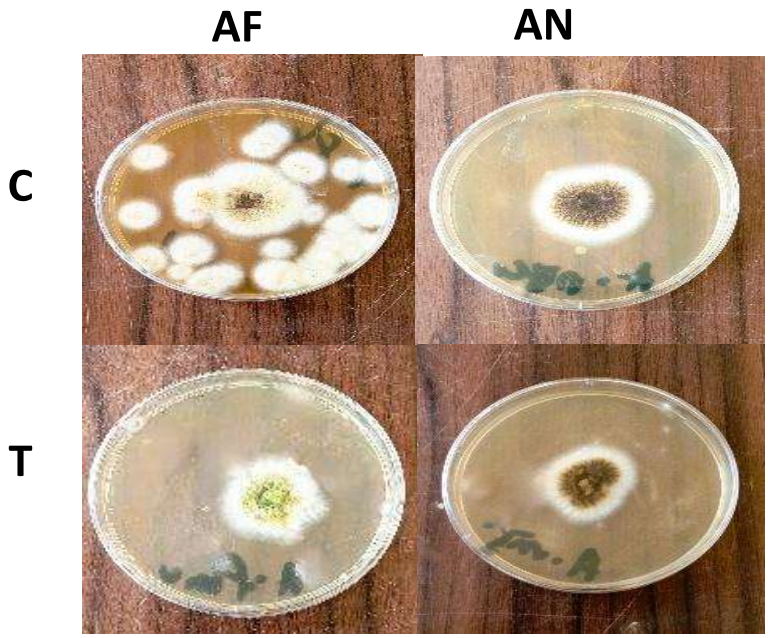


Fig. 7: Inhibitory effect of METOX on mycelial growth of *A. flavus* (AF) *and A. niger* (AN). The control is C (treatment without METOX) and T represents test (treatment with METOX

**Table 2: Antifungal Activity of METOX Against A. flavus and A. niger**

| Fungal Species | Mean Inhibition (%) ± SD | Degree of freedom (between, within) | F-value | p-value | Significance |
|---|---|---|---|---|---|
| *Aspergillus flavus* | 61.50 ± 5.20 | (1,4) | 122.36 | 0.0005 | Yes ($p < 0.05$) |
| *Aspergillus niger* | 16.00 ± 3.50 | | | | |

Values are expressed as mean inhibition (%) ± standard deviation of three replicates

## CONCLUSION

This study established the production of antifungal metabolite (METOX) by *B. safensis* LAU 13 during 3 days of cultivation through submerged fermentation. The metabolite was rich in bioactive compounds predominantly aldehyde derivatives, 4-Dodecen-1-al (13.5%) and Octanal,3,7-dimethyl (13%). The METOX exhibited potent inhibitory effect against the aflatoxin-producing strains of *A. flavus and A. niger* isolated from stored maize grains as it induced 62% and 16% fungal growth inhibition against the isolated strains of *A. flavus and A. niger,* respectively. Hence, the result obtained herein suggests that the METOX produced by *B. safensis* LAU 13 has promising application as a bioactive agent for sustainable control of the growth of aflatoxin-producing fungal species in stored grains.


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