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Efficacy of Soil *Paraburkholderia fungorum* and *Bacillus subtilis* on the Inhibition of *Aspergillus niger* Growth and its Ochratoxins Production

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BIOLOGICAL control of toxigenic fungi and their mycotoxins by using antagonistic bacteria has currently gained attention as a non-toxic replacement for harmful chemical substances. The present work aimed to study the inhibitory effects of five strains of bacteria recovered from soil on the growth of twenty isolates of *Aspergillus niger* obtained from Arabian coffee by using a dual-culture method and cell-free bacterial supernatant. *Paraburkholderia fungorum* and *Bacillus subtilis* were the two active strains used against three *Aspergillus niger* isolates with the highest inhibition percentage (86.81% and 62.97%, respectively). Biochemical tests partially identified the effective bacteria, and complete identification was confirmed by using the 16S rRNA sequence. *P. fungorum* and *B. subtilis* completely biodegraded the ochratoxins produced by *A. niger*-22 and *A. niger*-24, respectively. Interestingly, commercial amylase can suppressed ochratoxins biosynthesis by *A. niger*-28 with percentage of 44.7% using high-performance liquid chromatography (HPLC). A large number of bio-active compounds was detected in the bacterial filtrate by gas chromatography–mass spectroscopy (GC-MS). *In vivo*, the selected bacteria significantly biodegraded ochratoxins in contaminated grains using fluorometric method, with 52.9% as the highest inhibition percentage in maize contaminated by *B. subtilis*. This is the first report of ochratoxins biodegradation by *P. fungorum* and amylase.

Keywords: Active components, Amylase, Antagonistic activity, Ochratoxins biodegradation, Soil bacteria.

Introduction

Mycotoxins are poisonous chemical substances excreted by field and storage fungi (Tola & Kebede, 2016). They pose a serious risk to human and animal health (Bryden, 2012). Now, hundreds of mycotoxins and other metabolites have been recorded, and scientists continue to focus on mycotoxins that were proven to be toxic (Miazzo et al., 2000; Oueslati et al., 2012). *Aspergillus ochraceus, A. carbonarius, A. niger* and *Penicillium verrucosum* are the main producers of ochratoxin A (OTA) (Bui-Klimke & Wu, 2016). Ochratoxin A is one of the most important mycotoxins due to its toxicity and wide distribution in food and feed products, such as coffee, cereals, date palm fruits, and spices (Hua et al., 2014; Sun et al., 2017; Abdallah et al., 2018; El-Dawy et al., 2019). OTA causes many critical diseases to human such as kidney diseases, deep-seated interstitial nephropathy, and Balkan endemic nephropathy (BEN) (Bui-Klimke & Wu, 2016).

Biological control of ochratoxins appears to be safer than chemical and physical methods (Shi et al., 2013). Biological degradation involves toxin binding by adsorptive materials and inhibition by microorganisms and enzymes, such as lactic acid bacteria (Fuchs et al., 2008), *Bacillus* sp., (Petchkongkaew et al., 2008), *Alternaria alternata* (Khalil & Yousef, 2020) and *Trichoderma* sp. (Elnahs et al., 2020)

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The genus *Burkholderia* was first defined by Yabuuchi et al. (1992) as Gram-negative, aerobic and non-sporulating β-proteobacteria. It commonly occurrs in soil (Dalmastri et al., 1999). *Burkholderia* species have been reported to produce a wide variety of antibiotics, toxins, lipolytic, proteolytic, and hemolytic enzymes; and siderophores which makes them highly useful in biochemical and pharmaceutical industrial processes. In addition, they secret large-scale of antifungal compounds in an antagonistic interaction with fungi (Vial et al., 2007). This genus serves as a biocontrol agent of phytopathogens, mycotoxigenic fungi, and OTA detoxification (Zeidan et al., 2019).

Bacillus species are aerobic or facultative anaerobic, Gram positive, rod shaped, spore-forming bacteria that is found in high concentrations in soil (Al-Janabi, 2006; Graumann, 2007), and are considered to be extremely useful microorganisms for producing antimicrobial agents (Amin et al., 2012). *B. subtilis* is considered to be an important biocontrol agent against several mycotoxigenic and pathogenic fungi (Thakaew & Niamsup, 2013; Shkula et al., 2018).

In this study, two selected isolates of *Paraburkholderia fungorum* and *Bacillus subtilis* were isolated from soil and used as antagonists to control the growth of mycotoxigenic *Aspergillus niger* isolated from Arabian coffee. Bacterial isolates were also examined on the biodegradation of *A. niger* total ochratoxins by HPLC. Active components in the bacterial supernatant were monitored by GC-MS. The antagonistic behavior and ochratoxins biodegradation exhibited by amylase were also evaluated. *In vivo*, biodegradation of ochratoxins in contaminated maize and lentil grains was estimated.

Materials and Methods

Isolation and identification of tested microorganisms

Isolation of bacteria

A total of fifty soil samples were collected from South Valley University campus at Qena Governorate, Egypt, under complete aseptic conditions (Abdulkadir & Waliyu, 2012). To determine the colony forming unit (CFU), one gram of each sample was added to tryptic soy broth (Oxoid®) and was incubated at 37°C for 24hrs. Then, serial dilutions up to 10^{10} were prepared. Samples were streaked on tryptic soy agar media (Oxoid®) using glass beads. All colonies that appeared on the plates were harvested and further subcultured to obtain pure colonies (Seeley & Van Demark, 1981). Isolates were identified by Gram staining, spore formation, and biochemical tests (Cruickshank et al., 1975; Sneath 1984; Koneman et al., 1992; Feng et al., 2015). Samples were preserved in glycerol 70% for further uses. Samples which were selected for biocontrol and ochratoxins biodegradation were then identified by 16sRNA gene sequencing, for which PCR products were purified using the QIAquick PCR Product extraction kit (Qiagen, Valencia). For the sequence reaction, the Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used and then purified by the Centrisep spin column. DNA sequences were achieved by an Applied Biosystems 3130 Genetic Analyzer (Hitachi, Japan) through analysis Basic Local Alignment Search Tool (BLAST®) (Altschul et al., 1990), which was initially performed to establish sequence identity to GenBank accessions. The MegAlign module of Lasergene DNAStar version 12.1 was applied to create the phylogenetic tree (Thompson et al., 1994). Phylogenetic analyses were performed using the maximum probability, neighbour retraction, and maximum parsimony in MEGA6 (Tamura et al., 2013).

Isolation of Aspergillus niger

Aspergillus niger was isolated from thirty Arabian coffee samples collected from different markets at Taif city, KSA by using the dilution plate method as mentioned by Kurtzman et al. (1971). Ten grams of coffee was suspended in 90 ml diluents of 0.1% peptone water and shaken for 5 min. Then, 1 ml aliquot was added to a sterile Petri dish, into which 20 ml of melted rose Bengal chloramphenicol agar (RBCA) medium was poured. The plates were incubated at 28 °C for 7 days. The obtained *Aspergillus niger* isolates were identified according to their macro-and microscopic characteristics (Raper & Fennell, 1965) and maintained on potato dextrose agar (PDA) slants.

Antagonistic behavior of selected bacteria against the growth of A. niger

Two methods were applied to study the antagonistic interactions between five isolates of amylase-producing bacteria and twenty isolates of *A. niger*. The anatagonstic efficacy of commercial amylase (Techno Pharmchem Bahadurgarh, Haryana, India) was also evaluated.

Dual-culture technique

The method described by Shi et al. (2013) was used to study the antagonistic behavior on PDA medium (Sisco Research Laboratories Pvt. Ltd, New Mumbai, India). First, 20 ml of melted PDA medium was poured into sterilized Petri dishes. One-third of the plate diameter was inoculated with a single colony of tested bacteria, and each strain of *A. niger* was inoculated at two-thirds of the diameter (Petchkongkaew et al., 2008). The plates were incubated for 7 days at 28°C. Triplicates for each treatment were prepared. Inhibition (%) was calculated according to the equation:

$$\text{Inhibition (\%)} = [(r-r')/r] \times 100$$

where r (mm) is the fungus growth from the colony midpoint to the edge of the Petri dish and r' (mm) is the fungus growth from the colony midpoint to the center of the selected bacteria.

Cell-free supernatant

Agar well assay was used to study the inhibitory effect of bacterial strains using cell-free supernatant. The tested bacterial strains were inoculated into potato dextrose broth (PDB) supplemented with starch, as the tested isolates were high amylase producers according to the starch hydrolysis ratio (SHR) test (Pranay et al., 2019). After inoculation, the strains were incubated at 37°C overnight. The bacterial growth was then centrifuged at 6,000 × g for 5min at 4°C. A plug of tested fungi was plated at the center of the PDA Petri dish and incubated for 2 days at 28°C. After this incubation period, three wells (8mm) diameter were prepared and supplemented with 100µL of the cell free bacterial supernatant. The plates were then incubated for further 5 days. Triplicates were prepared, and the mycelial inhibition was calculated as described above.

Detection of ochratoxin biosynthesis genes

Four published primers (*Ocra* and *Aopks*) were supplied from Biobasic Canada for detection ochratoxin genes in tested *A. niger* isolates. The sequences of the used primers were listed in (Table 1). To determine ochratoxin biosynthesis genes, 25µL was employed as a polymerase chain reaction (PCR) mixture comprising 12.5µL Emerald Amp Max PCR Master Mix (Takara, Japan), 1µL of each used primer of 20pmol concentration, 4.5µL water, and 6µL of the DNA template. The reaction was performed in Applied Biosystems 2720 Thermal Cycler. Next, 1.5% agarose gel electrophoresis was used to separate the PCR products (Applichem, Germany, GmbH) in 1× Tris-borate-EDTA (TBE) buffer at room temperature using gradients of 5V/cm. For gel analysis, 15µL of the products was laden in each gel slit. The fragment sizes were determined using GelPilot 100 bp DNA Ladder (Qiagen, Germany, GmbH). Photos of the gel were taken with a gel documentation system (Alpha Innotech, Biometra), and the data were analyzed with computer software (Automatic Image Capture, USA) (Patiño et al., 2005; Yassein et al., 2020).

Ochratoxins biodegradation by P. fungorum, B. subtilis, and amylase

In this experiment, the method described by Kocic´-Tanackov et al. (2012) was applied with little modification. Fifty ml of sterile yeast extract sucrose broth (YES) medium supplemented with starch (yeast extract, 20g; sucrose, 40g; starch, 10g; 1,000mL distilled water) was inoculated with two discs (8mm) of each isolate of tested *A. niger* and an inoculum of tested bacteria or 1 mg of commercial amylase. Medium without bacteria or amylase were used as control. Incubation was carried out at 28°C for 15 days to study the efficiency of the tested bacteria and amylase for ochratoxins biodegradation. Ochratoxin levels were determined by HPLC at Central Laboratories Network, National Research Centre, Cairo, Egypt. HPLC analysis was carried out using an Agilent 1260 series. A C18 column (4.6mm × 100mm i.d., 3.5µm) was applied during the separation phase. The mobile phase was composed of water : acetonitrile : acetic acid (43:55:3) at a run rate of 1mL/min with a ran time of 5 min. The fluorescence was detected at 330nm excitation and 450nm emission. The injection volume was 20µL for each of the sample solutions. The column temperature was maintained at 40°C.

Determination of the bioactive components of P. fungorum and B. subtilis by GC-MS

Sample derivatizations

The trimethylsilane (TMS) derivatization was based on the optimized protocol described by Villas-Bôas et al. (2006). The dried samples were resuspended in 20µL pyridine and 100µL N-methyl-N-(trimethylsily) trifluoroacetamide (MSTFA) and incubated in a dry block heater at 70°C for 60min.

TABLE 1. Primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Sequence	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			
				Secondary denaturation	Annealing	Extension	Final extension
OcrA genes	CTTCCTTAGGGGTGGCACAGC GTTGCTTTTCAGCGTCGGCC	400	94˚C 5min	94˚C 30sec	59˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.
Aopks gene	CAGACCATCGACACTGCATGC CTGGCGTTCCAGTACCATGAG	549	94˚C 5min	94˚C 30sec	46˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.

*: The specific sequences that were amplified for each of the used primers (Biobasic, Canada).

Gas chromatography–mass spectrometry (GC-MS) analysis

The GC-MS chromatography procedure was carried out at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC-MS system (Agilent Technologies) was equipped with a gas chromatograph (7890B), mass spectrometer detector (5977A) and HP-5MS column (30m × 0.25mm internal diameter and 0.25μm film thickness). Helium was used as the carrier gas at a run rate of 1.0mL/min fragmented at 50:1. The injection volume was 0.5µL bacteria with a temperature cycle of 50°C for 1min, increasing to 300°C (8°C/min) and held for 20min. The injector and indicator were detained at 250°C. The mass spectra conditions were as follows: Electron ionization (EI) at 70eV, using a spectral range of 30-700m/z and 8 min solvent delay. The mass temperature was 230°C and Quad 150°C. Different components were recognized by comparing the spectrum fragmentation pattern with those kept in the Wiley and NIST Mass Spectral Library databases.

Efficacy of P. fungorum and B. subtilis on ochratoxins biodegradation of contaminated grains

The modified method mentioned by Liang (2008) was used. *Aspergillus niger*-22 was chosen as the toxin-producing fungus. Spore suspension of *A. niger*-22 was obtained by cultivation the fungus on a PDA plate at 28°C for 7 days. Then, 10mL sterile water was added to the plate and gently joggled to dislodge spores into a 50mL Erlenmeyer flask with glass beads. The Erlenmeyer flask was shaken for 1hr., and the spore suspension was filtered using two layers of disinfected cheese cloth to eliminate the mycelial remains. The spore concentration was set to 10^7 spores/mL using a hemocytometer. Next, 30g of autoclaved maize and lentil purchased from a local supermarket at Qena Governorate was inoculated with one mL of the spore suspension (10^7 spores mL^{-1}). The final moisture content of the grains was adjusted to 180g/kg with sterile water. Triplicates for each treatment were prepared. After incubation in the dark at 28°C and 200rpm for 2 weeks, the grains were sterilized at 121°C for 20min. Then, the grains were mixed with 50mL of the overnight culture of *P. fungorum* and *B. subtilis*. The mixture was incubated at 30°C and 200rpm for 72hrs. OTs were extracted using the method described by El-Dawy et al. (2019). Thirty grams of grains was mixed with 3g NaCl and 100mL methanol:water (80:20, v/v) in a blender for 1min at high speed. Extracts were filtrated through Whatman filter paper (Whatman 2V; Whatman plc), 10mL aliquots was diluted with 60mL PBS buffer (at pH 7.4). Micro-fiber filter paper was used for filtration; 10 ml filtrate was passed through the OTs Test RWB SR Column (VICAM) and allowed to elute at 1–2 drops/s. The columns were washed two times with 20mL water, and the ochratoxins were extracted with HPLC-grade methanol (1mL). Next, 1.5mL of ochratoxin eluting agent was added and the Ochratoxins levels were measured with a recalibrated VICAM Series-4 fluorometer set at 360 nm excitations and 450nm emissions.

Statistical analysis

The degree of variability in the results was expressed as the means ± standard Deviation (Mean ± S.D) based on three independent determinations (n= 3). The data were statistically analyzed by one-way ANOVA analysis and compared using the least significant difference (LSD) test at 0.05 (*) levels, which was performed to compare the difference between control and the treatment groups.

Results

Microorganisms recovered in the present work

Thirty isolates of *A. niger* have been recovered from 30 Arabian coffee samples that were collected from different markets in Taif City, KSA. Twenty isolates were chosen randomly

for this study. One hundred colonies of bacteria were isolated from soil collected from South Valley University campus at Qena Governorate, Egypt. The results of the bacterial identification revealed that (47 isolates) *B. subtilis*, (22) *B. cereus*, (19) *P. fungorum*, (7) *B. thurnigiesis* and (5) *B. polymyxa*. The CFU of bacteria in fifty soil samples ranged from $215 \times 10^5 - 109 \times 10^8$CFU/mL (Table 2). The identification of the active bacterial isolates (*P. fungorum* and *B. subtilis*) was completely confirmed by the 16S rRNA gene sequences and deposited into the GenBank database under accession numbers MT903310 and MT898543 and showed 100% and 99.9% sequence similarity to *P. fungorum* CP010027and *B. subtilis* HQ718411, respectively (Fig. 1).

TABLE 2. Prevalence of bacterial species isolated from soil

Parameters / Isolates[a]		No. of isolates[b]	Percentage (%)	CFUmL^{-1}[d]	SHR[e]
B. subtilis		47	47	**215x10^5 – 109x10^8**	5.5
B. cereus		22	22		5
P. fungorum		19	19		6
B. thuringiesis		7	7		5.1
B. polymyxa		5	5		1.9
Total	5	100	100		

[a]: The isolated bacteria from soil, [b]: number of each isolated type from the total number of isolates, [c]: percentage of each isolate, [d]: Average of colony forming unit of bacteria per ml of 50 soil samples (highest value- lowest value), [e]: Starch hydrolysis rate (mm), the base of bacterial selection for ochratoxin biodegradation.

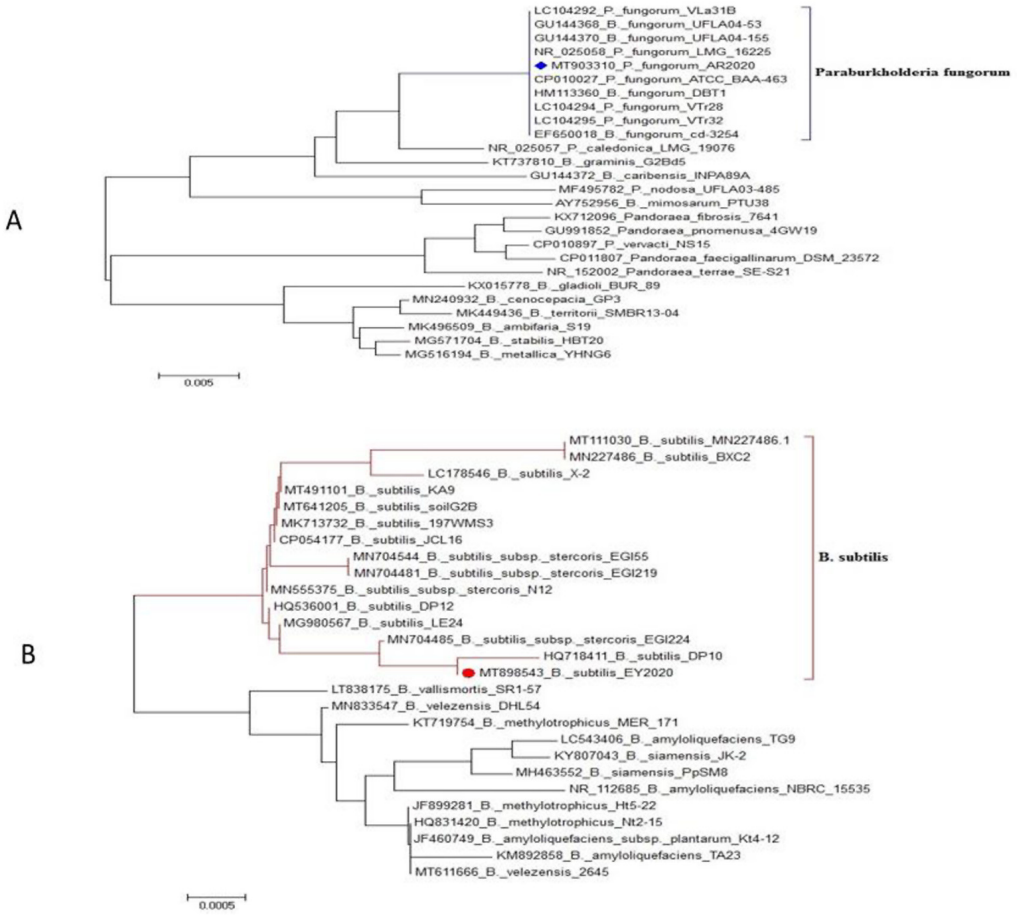

Fig. 1. Phylogenetic relationship between the bacterial strains A: *P. fungorum*, B: *B. subtilis* and other 16sRNA sequences of published strains belonging to *Parabulkholderia* and *Bacillus* sp.

Antagonistic behavior of the tested bacteria and commercial amylase against the growth of A. niger

Three isolates of *A. niger* were highly inhibited by the selected bacteria (Fig. 2). *P. fungorum* significantly inhibited the growth of *A. niger*-22 at a percentage of 86.81% and 53.69% when using dual-culture plate method and the cell-free supernatant method, respectively. Moreover, it exerted a significant impact on the inhibition of *A. niger*-24 at a percentage of 37.50% and 57.93% using the dual-culture plate method and the cell-free supernatant method, respectively. Finally, the significant inhibition percentages against *A. niger*-28 were 74.06% and 51.28% by using the dual-culture plate method and the cell-free supernatant method, respectively (Figs. 2, 3).

B. subtilis also showed a significant inhibition against all tested isolates of *A. niger* in both dual-culture plate method and cell-free supernatant method. The percentages of inhibition were as following: 56.15% and 53.02% for *A. niger*-22, 54.17% and 62.97% for *A. niger*-24 and 62.54% and 46.15% for *A. niger*-28 in dual-culture plate method and cell-free supernatant method, respectively. Interestingly, amylase did not exhibit antifungal activity against the *A. niger* isolates (Figs. 2, 3).

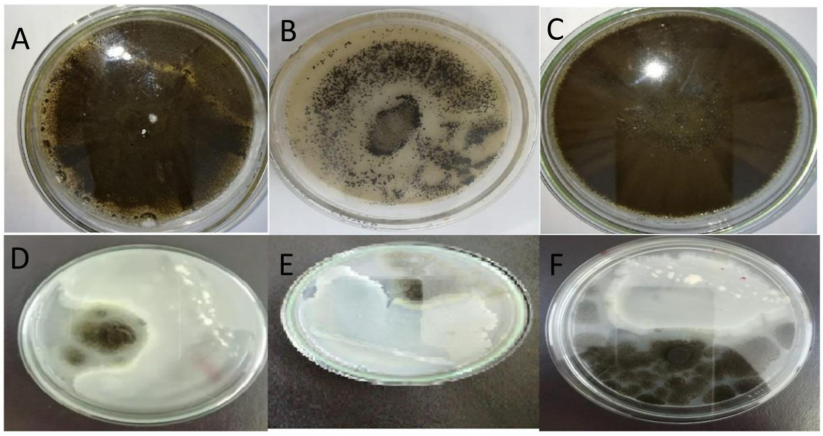

Fig. 2. Morphological characterization of *A. niger* isolates from Arabian coffee on malt extract agar medium (A, B, C), A: *A. niger*-22, B: *A. niger*-24, C: *A. niger*-28; Dual culture technique (D, E, F), D: *A. niger*-22, E: *A. niger*-28, F: *A. niger*-24 with *P. fungorum*.

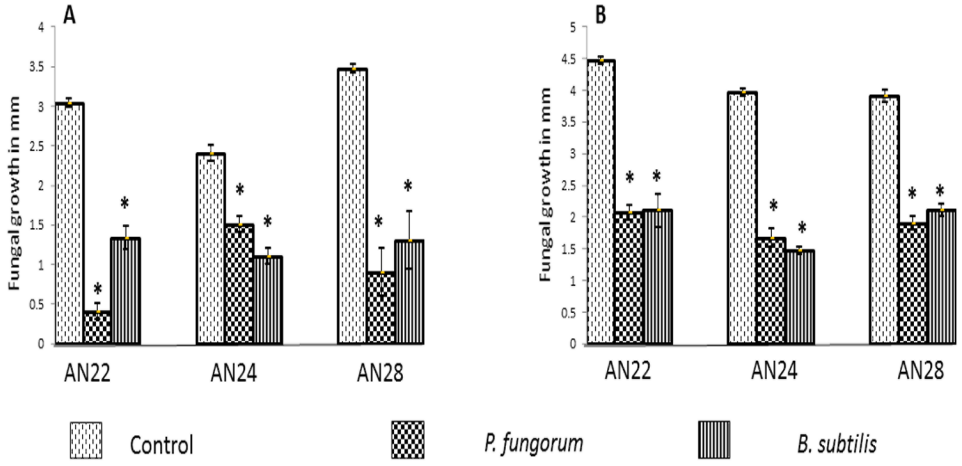

Fig. 3. Antagonistic efficacy of *P. fungorum* and *B. subtilis* isolated from soil against different isolated *A. niger* from Arabic coffee, (A): Dual culture method; (B): Cell free supernatant method [AN-22: *Aspergillus niger*-22; AN-24: *Aspergillus niger*-24; AN28: *Aspergillus niger*-28. The error bars indicate the standard deviations using the least significant difference (LSD). (A): Dual culture method, LSD at 0.05 of AN22 was 0.185 and 0.262 for *P. fungorum* and *B. subtilis*, respectively. AN24 was 0.227 for both *P. fungorum* and *B. subtilis.* AN28 was 0.489 and 0.585 for *P. fungorum* and *B. subtilis*, respectively. (B): Cell free supernatant method, LSD at 0.05 of AN22 was 0.207 and 0.434 for *P. fungorum* and *B. subtilis*, respectively. AN24 was 0.262 and 0.131 for *P. fungorum* and *B. subtilis,* respectively. AN28 was 0.227 for both *P. fungorum* and *B. subtilis*. *: means values are significant compared with control]

Ochratoxin biosynthesis genes

The *OcrA* gene was detected at 400 bp only in *A. niger*-22, whereas the *Aopks* gene was detected in all tested *A. niger* isolates at 549bp (Fig. 4).

Ochratoxins biodegradation by P. fungorum, B. subtilis, and commercial amylase

Total ochratoxins degradation by isolated *P. fungorum*, *B. subtilis* and commercial amylase were simultaneously confirmed by HPLC analysis. The results revealed complete degradation (100%) of ochratoxins production in *A.niger*-22 and *A.niger*-24 by *P. fungorum* and *B. subtilis*, respectively. In addition, *P. fungorum* exhibited high percentage of ochratoxins degradation produced by *A.niger*-24 with a percentage of 72.2%. *B. subtilis* also showed that 49.2% ochratoxins degradation was produced by *A.niger*-22. Also, ochratoxins produced by *A.niger*-28 was degraded by *P. fungorum* and *B. subtilis* by 41% and 40.4%, respectively. Interestingly, commercial amylase recorded great degradation efficacy against ochratoxins produced by *A.niger*-22, *A.niger*-24 and *A.niger*-28 with percentages of 38.4%, 33.3%, and 44.7%, respectively (Table 3, Fig. 5).

The active components of P. fungorum and B. subtilis by GC-MS

Detection of bioactive compounds of isolated bacteria plays an important role in its efficacy as an antifungal agent and its participation in ochratoxins biodegradation. Hence, one of the vital aims of the current study was to elucidate the bioactive compounds that are present in *P. fungorum* and *B. subtilis* by using gas chromatography-mass spectroscopy. The results revealed that the presence of twenty-six bioactive compounds in *P. fungorum.* The highest percentages content of the compounds were as follows: 9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester (20.45%); Palmitic Acid (20.29%); Oleic Acid, (Z)- (11.83%). GC-MS of *B. subtilis* also showed presence of 27 bioactive compounds as follows: Palmitic Acid (19.85); 9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester (18.96%); Oleic Acid, (Z)- (11.4%). Peak number, concentration (peak area%), and retention time (RT) of other effective compounds are presented in (Table 4, Fig. 6).

Ochractoxins biodegradation in contaminated grains by the selected bacterial isolates

Data in Table 5 showed that a significant effect of *P. fungorum* on ochratoxins biodegradation in contaminated lentil grains with an inhibition percentage of 41.2%. On the other hand, a slightly affect of *P. fungorum* on the ochratoxins level in contaminated maize grains, with 18.7% inhibition percentage. The ochratoxins level in contaminated maize grains was highly inhibited by *B. subtilis* and recorded as 52.9% inhibition percentage, whereas the effect on lentils was feeble with an inhibition percentage of 5.9% (Table 5).

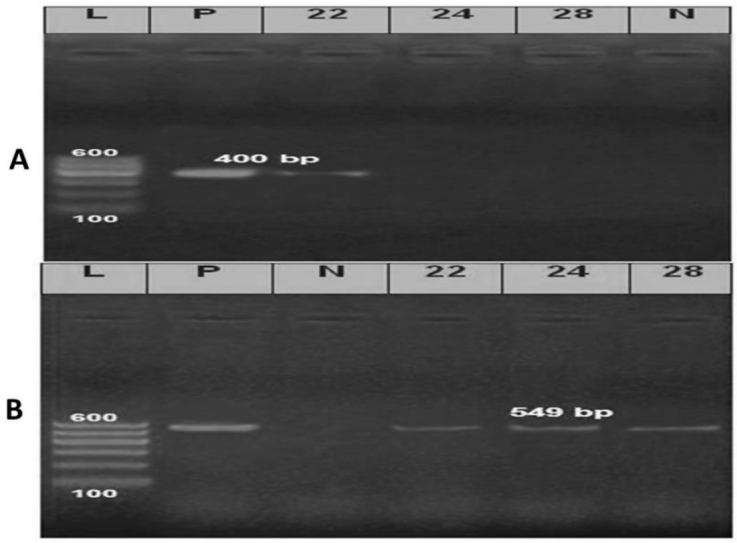

Fig. 4. 1.5% agarose gel electrophoresis of uniplex PCR of *OcrA* and *Aopks* genes of different isolated *Aspergillus niger* from Arabian coffee. A: *OcrA* (400bp); B: *Aopks* (549bp) [Lane L: A gelpilot 100bp DNA Ladder (Qiagen, Germany, GmbH) to determine the fragment sizes, Lane P: Positive control of DNA confirmed by reference laboratory for quality control of poultry production, Lane 22, 24 and 28: Selected *A. niger* isolates, Lane N: Negative control for detected genes]

TABLE 3. Biodegradation of *A. niger* total ochratoxins by *P. fungorum*, *B. subtilis*, and amylase using HPLC

Treatments		Conc.(ng/mL)	Area	Percentage of inhibition (%)
OTA standard		5	1.90862	--------
AN-22	Control	0.057	0.0219	--------
	P. fungorum	0.000	0.000	100
	B. subtilis	0.029	0.0112	49.2
	Amylase	0.035	0.0135	38.4
AN-24	Control	0.052	0.0200	--------
	P. fungorum	0.015	0.0056	72.2
	B. subtilis	0.000	0.000	100
	Amylase	0.035	0.0133	33.3
AN-28	Control	0.046	0.0177	--------
	P. fungorum	0.027	0.0104	41
	B. subtilis	0.028	0.0106	40.4
	Amylase	0.026	0.0098	44.7

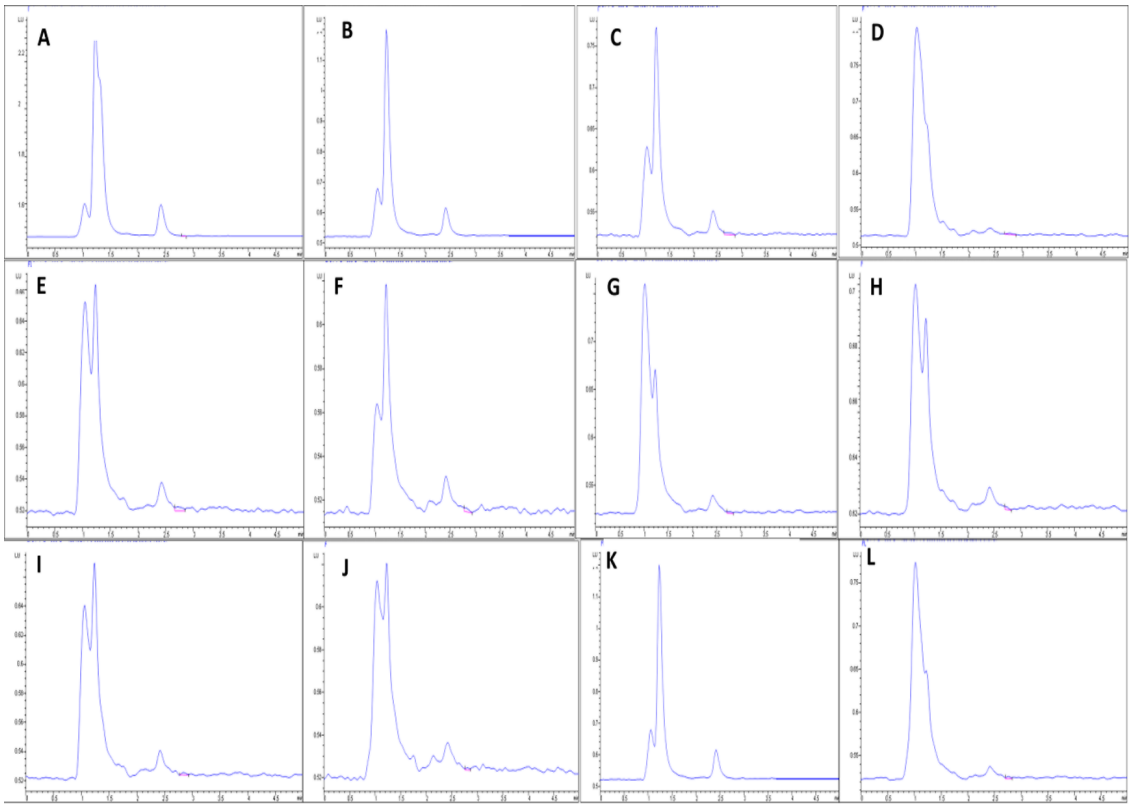

Fig. 5. HPLC analysis of ochratoxin biodegradation; A: *A. niger*-22 (control); B: *A. niger*-22 with *P. fungorum*; C: *A. niger*-22 with *B. subtilis*; D: *A. niger*-22 with amylase; E: *A. niger*-28 (control); F: *A. niger*-28 with *P. fungorum*; G: *A. niger*-28 with *B. subtilis*; H: *A. niger*-28 amylase; I: *A. niger*-24 (control); J: *A. niger*-24 with *P. fungorum*; K: *A. niger*-24 with *B. subtilis*; L: *A. niger*-24 with amylase.

TABLE 4. GC-mass spectra of *P. fungorum* and *B. subtilis* showing different active compounds

Peak	RT	Compound	Formula	Area	Area sum %
		P. fungorum			
1	12.066	3-Aminobutyric acid, 2TMS derivative	C10H25NO2Si2	3392857.8	2.87
2	13.196	Glycerol, 3TMS derivative	C12H32O3Si3	2715477.8	2.3
3	14.462	2-Hexenal	C6H10O	976559.06	0.83
4	14.718	2-Hexyn-1-ol	C6H10O	938132.41	0.79
5	15.931	1-Octyn-3-ol	C8H14O	1514585.8	1.28
6	16.12	2-Nonenal, (E)-	C9H16O	1626332.3	1.38
7	17.175	Oxetane, 2-methyl-4-propyl-	C7H14O	843658.24	0.71
8	17.348	3-Pentanol, 2,4-dimethyl-	C7H16O	1813839.2	1.53
9	19.556	Tetradecane	C14H30	5161571.5	4.37
10	20.106	1-Octanol, 2-butyl-	C12H26O	1775695.9	1.5
11	21.214	Methoxyacetic acid, 3-tridecyl ester	C16H32O3	738503.13	0.62
12	21.349	Myristic acid, TMS derivative	C17H36O2Si	7604244.7	6.43
13	21.598	5-Dimethyl(trimethylsilyl)silyloxytridecane	C18H42OSi2	1126376.2	0.95
14	22.269	Decane, 2,3,5,8-tetramethyl-	C14H30	7900845.6	6.69
15	22.344	1-Nonene, 4,6,8-trimethyl-	C12H24	1091354.2	0.92
16	22.751	1-Iodo-2-methylundecane	C12H25I	2052882.6	1.74
17	23.248	4-Hydroxy-4-methylhex-5-enoic acid, tert.-butyl ester	C11H20O3	1453662.3	1.23
18	23.746	Palmitic Acid, TMS derivative	C19H40O2Si	23981986	20.29
19	24.371	5-Tridecene, (Z)-	C13H26	800037.12	0.68
20	24.71	Nonadecane	C19H40	4694139.9	3.97
21	25.147	Hexadecane	C16H34	1029163.8	0.87
22	25.599	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	C21H40O2Si	24169155	20.45
23	25.637	Oleic Acid, (Z)-, TMS derivative	C21H42O2Si	13979115	11.83
24	25.84	Octadecanoic acid, trimethylsilyl ester	C21H44O2Si	4924923.6	4.17
25	28.983	Disulfide, di-tert-dodecyl	C24H50S2	667244.55	0.56
26	29.292	1-Monopalmitin, 2TMS derivative	C25H54O4Si2	1207590.9	1.02
		B. subtilis			
1	9.391	1,3-Dioxolane, 2-(1-methylpropyl)-	C7H14O2	408689.8	0.43
2	13.196	Glycerol, 3TMS derivative	C12H32O3Si3	2321412.2	2.44
3	14.47	2-Hexenal	C6H10O	435257.34	0.46
4	14.726	2-Hexyn-1-ol	C6H10O	666443.18	0.7
5	15.939	1-Octyn-3-ol	C8H14O	1962036.6	2.06
6	16.12	2-Nonenal, (E)-	C9H16O	2546222.7	2.67
7	17.182	Oxetane, 2-methyl-4-propyl-	C7H14O	624681.95	0.66
8	17.348	Benzoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, ethyl ester	C17H26O3	1010212	1.06
9	18.184	3-(Prop-2-enoyloxy)dodecane	C15H28O2	725596.56	0.76
10	18.524	Octadecane, 6-methyl-	C19H40	430205.08	0.45
11	19.548	Tetradecane	C14H30	4326579.2	4.54

TABLE 4. Cont.

Peak	RT	Compound	Formula	Area	Area sum %
12	20.106	1-Octanol, 2-butyl-	C12H26O	1556283.3	1.63
13	21.244	Methoxyacetic acid, 3-tridecyl ester	C16H32O3	471957.34	0.5
14	21.349	Myristic acid, TMS derivative	C17H36O2Si	6896086.7	7.24
15	22.261	Decane, 2,3,5,8-tetramethyl-	C14H30	6807550.3	7.15
16	22.344	1-Nonene, 4,6,8-trimethyl-	C12H24	784221.6	0.82
17	22.751	1-Iodo-2-methylnonane	C10H21I	1949268.9	2.05
18	23.241	4-Hydroxy-4-methylhex-5-enoic acid, tert.-butyl ester	C11H20O3	893665.36	0.94
19	23.731	Palmitic Acid, TMS derivative	C19H40O2Si	18911109	19.85
20	24.371	5-Tridecene, (Z)-	C13H26	862518.8	0.91
21	24.703	Nonadecane	C19H40	4381264.3	4.6
22	25.14	Hexadecane	C16H34	1274521.7	1.34
23	25.584	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	C21H40O2Si	18061652	18.96
24	25.622	Oleic Acid, (Z)-, TMS derivative	C21H42O2Si	10857080	11.4
25	25.84	Octadecanoic acid, trimethylsilyl ester	C21H44O2Si	3172947	3.33
26	28.983	Disulfide, di-tert-dodecyl	C24H50S2	1002455.2	1.05
27	29.292	1,3-Dioxolane, 4-[[(2-methoxy-4-octadecenyl) oxy]methyl]-2,2-dimethyl-	C25H48O4	1907309.3	2

RT: Retention time per minute; active compounds detected by GC mass; area (%): Percentage of compound; M. formula: Molecular formula; M. wt: Molecular weight of the compound.

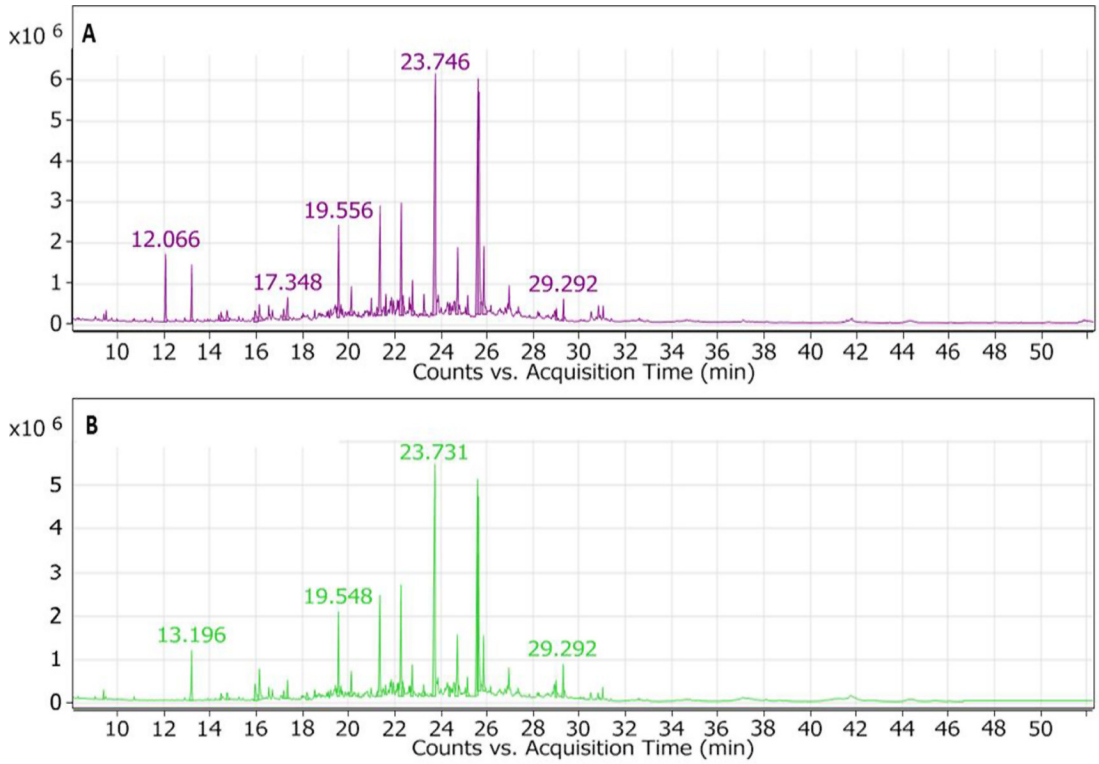

Fig. 6. Gas chromatography mass spectra (GC- MS) of biologically active compounds of *P. fungorum* (A) and *B. subtilis* (B)

TABLE 5. Ochratoxins biodegradation in contaminated maize and lentil grains by *P. fungorum* and *B. subtilis*

Contaminated grains	Control	*P. fungorum*	*B. subtilis*
Maize	10.56±2.60	8.7±0.87	4.9±0.75*
Lentils	5.1±0.66	3±0.46*	4.8±0.76

- LSD at 0.05 of ochratoxin biodegradation in contaminated maize was 4.39 and 4.34 by *P. fungorum* and *B. subtilis*, respectively.
- LSD at 0.05 for contaminated lentil was 1.28 and 1.60 by *P. fungorum* and *B. subtilis*, respectively.
- *: Means values are significant compared with control.

Discussion

Soil is an excellent culture media for the growth and development of various biocontrol microorganisms. *Bacillus* species are the predominant soil bacteria (Amin et al., 2015). Our results confirmed that *Bacillus* sp. were isolated with a percentage of 81% compared with 19% for *P. fungorum* (Table 2). The literature described the antagonistic behaviors of several microorganisms, which are often denoted as latent biocontrol agents against a variety of phytopathogenic fungi (Leifert et al., 1995; Podile & Prakash, 1996). Among the microorganisms producing metabolites with antifungal activity, bacteria of the genera *Bacillus* have been shown to be effective against wood blue-stain fungi (Silva & Morrell, 1998). The genus *Burkholderia* has been well defined phylogenetically, consisting of species that are functionally and abnormally diverse (Coenye & Vandamme, 2003). In fact, *Burkholderia* species have been obtained from many different environmental vocations, such as soil and water, and can form relationships with plants, animals, and humans. Several *Burkholderia* spp. are widespread in nature, and some of are useful (Compant, 2008). In the current study, the antagonistic efficacy of *P. fungorum* and *B. subtilis* isolated from soil against *A. niger* isolated from Arabian coffee were examined with the dual-culture technique and cell-free supernatant. The results revealed that the two selected bacterial isolates significantly reduced *A. niger* growth. Furthermore, the efficacy of dual-culture technique was better than that of the cell-free supernatant method for all *A. niger* isolates except for *A. niger*-24. The cell-free supernatant showed high inhibition percentages in comparison with the dual-culture technique (Fig. 3), which may be attributed to existence of a big number of bioactive compounds in bacteria that obtained by GC-MS analysis. This analysis confirmed the presence of high contents of palmitic acid; 9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester, and Oleic Acid, (Z)- (Table 4). These compounds were confirmed previously to be excellent antifungal agents (McGaw et al., 2002; Seidel & Taylor, 2004; Agoramoorthy et al., 2007; Jung et al., 2013; Elamary et al., 2020). Recently, it was verified that volatile organic compounds (VOCs), such as hydrocarbons, alcohols, ketones, aldehydes, ethers, esters, terpenes, terpene derivatives, and several heteroaromatic compounds that were formed by certain bacteria exhibit antifungal activity (Alstrom, 2001; Wheatley, 2002; Schalchli et al., 2011). Many of these compounds were obtained via a GC-MS analysis of the tested bacteria (Table 4, Fig. 6). In a study by Elshafie et al. (2012), *Burkholderia gladioli pv. agaricicola* were reported to have antagonistic activity against the growth of some phytopathogenic fungi. They also clarified that this antifungal activity could be reverted to the production ,of some extracellular hydrolytic enzymes, such as chitinase, protease, and glucanase. Hydrolytic enzymes cause degradation in fungal cell walls, which reflects their ability to inhibit the growth of phytopathogenic fungi (Cherif et al., 1992). Zeidan et al. (2019), who revealed that Qatari *Burkholderia cepacia* significantly inhibited the growth of twenty-one fungal species of mycotoxigenic and phytopathogenic fungi belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium*. *Aspergillus carbonarius* was the most sensitive species in both PDA and PDB media. Yara et al. (2006), discovered that the *Burkholderia cepacia* complex possessed potential mycophagous traits against *Pleurotus ostreatus* fungus. *Burkholderia gladioli pv. agaricicola* was demonstrated to cause rapid degradation of *Agaricus bitorqis* sporocarps (Chowdhury & Helnemann, 2006). Das et al. (2018), tested the antifungal activity of 6 endophytic bacteria isolated from leaf, stem, and root tissues of *Dryopteris uniformis* and found that *Burkholderia* sp. (UR 1-07) was the most effective isolate against *Candida albicans* (KACC 30062), with the highest inhibition zone diameter (47.67± 0.47mm). The antagonistic behavior of six *Bacillus* strains obtained from a

fig orchard soil sample against *Aspergillus niger* EGE-K-213 was confirmed by Öztopuz et al. (2018). Recently, the incidence of ochratoxins has been given more consideration (Trucksess & Diaz-Amigo, 2011). Thus, strategies to remove or disable ochratoxins in food and feed are needed. Numerous microorganisms are mentioned in the literature for their ability to detoxify, degrade, and adsorb OTAs (Abrunhosa et al., 2010). *B. subtilis* is one of most important microorganisms involved in ochratoxins biodegradation (Shi et al., 2013). Ochratoxin A produced by *Aspergillus carbonarius* was inhibited by Qatari *Burkholderia cepacia* at 100% of the bacterial supernatant (Zeidan et al., 2019). In our study commercial amylase in addition to *P. fungorum* isolated from soil were used for the first time in the biodegrdation of ochratoxins produced by *A. niger* isolated from Arabian coffee. *B. subtilis* isolated from soil were also used in biodegradation (Table 3, Fig. 5). Numerous previous studies have discoursed ochratoxins degradation using enzymes such as protease, lipase, carboxypeptidase, and a recombinant enzyme (Stander et al., 2000, 2001; Abrunhosa et al., 2006; Azam et al., 2019). *In vivo*, ochratoxins biodegradation in contaminated grains by bacteria was discussed in a few studies. Shi et al. (2013), found that *Bacillius subtilis* CW14 degraded 47.1% of OTAs in contaminated maize without any detectable degradation products. *Bacillus licheniformis* MZH-11 degraded 0.1, 0.5, and 5 µg/g of OTAs in corn flour after 3 days of incubation at 84.4%, 78.3%, and 73.5%, respectively (Guan et al., 2009). In corn-soybean feed contaminated by 0.02µg/g of OTA, *Lysobacter* sp CW239 degraded OTA at the percentage of 68.7% after 2 days of incubation (Jiang et al., 2016). Our data on ochratoxins biodegradation in contaminated maize and lentils exhibited 52.9% and 5.9% biodegradation by *B. subtilis* but the *P. fungorum* biodegradation percentages were 18.7% and 41.2%, respectively (Table 5).

Conclusion

The growth of *A. niger* isolates and their production of ochratoxins was significantly inhibited by *P. fungorum* and *B. subtilis*. Commercial amylase had no effect on *A. niger* growth but showed positive results against ochratoxins production. The selected bacteria significantly suppressed ochratoxins in contaminated grains. In future studies, we will isolate and purify the active components from the effective bacteria to determine which is the most effective compound.

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Authors contribution: Asmaa S. Yassein conceived, designed the manuscript, performed the practical work, and wrote some parts of the article. Rokaia B. Elamary shared in the manuscript design, performed practical work, wrote some parts of the article, analyzed the data. The authors revised the manuscript.

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فعالية بكتريا البارابروكلادوريا فنجورم والباسيلس ساتلس المعزولة من التربة على تثبيط نمو الاسبرجيللس نيجر وانتاجه من سموم الاوكرا

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المقاومة البيولوجية للفطريات السامة وانتاجها من السموم باستخدام البكتريا المضادة اكتسب الاهتمام حديثا كبديل غير سام للمواد الكيميائية الضارة. يهدف هذا العمل إلى دراسة التأثير المثبط لخمس انواع من البكتريا المعزولة من التربة لنمو 20 عزلة من الاسبرجيللس نيجر المعزولة من القهوة العربى باستخدام طريقة الزراعة المزدوجة ورشيح الخلية البكترية. بكتريا البارابروكلادوريا فنجورم والباسيلس ساتلس كانوا انشط عزلتين ضد 3 عزلات من الاسبرجيللس نيجر وكانت اعلى نسب تثبيط (86,81% و 62,97%, على التوالى). البكتريا الفعالة تم تعريفها جزئيا باستخدام الاختبارات البيوكيميائية وتم تأكيد التعريف باستخدام الحامض النووى الريبوسومى 61 اس ار ار ان ايه. البارابروكلادوريا فنجورم والباسيلس ساتلس حللت كليا الاوكراتوكسين المنتجة بواسطة اسبرجيللس نيجر-22 و اسبرجيللس نيجر-24, على التوالي. ومن المثير للاهتمام, مقدرة انزيم الاميليز على تثبيط انتاج الاوكراتوكسين بواسطة فطر اسبرجيللس نيجر-28 بنسبة 44,7% باستخدام الكروماتوجرافيا السائلة عالية الاداء (اتش بى ال سى). تم الكشف عن وجود عدد كبير من المركبات الفعالة في رشيح البكتريا المختبرة باستخدام الكروموتوجرافيا الغازية -مطيافية الكتلة (جى سى-ام سى). حيويا حللت البكتريا المختارة الاوكراوكسين الملوثة للحبوب باستخدام طريقة الفلورومتر وكانت اعلى نسبة تثبيط 52,9% للذرة الملوثة بالباسيلس ساتلس. هذا اول تقرير عن تحلل الاوكراتوكسين باستخدام البارابروكلادوريا فنجورم والاميليز.