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Biodegradation of Aflatoxins by Bacteria

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AFLATOXINS (AFs) have great side effects on human, animals and crops. It causes severe illness to both human and animals and play a major role in economic loss. Biological decontamination seems to be attractive. The present investigation aimed to found new and safe microorganisms able to degrade AFs and inhibit its producing fungi. Forty three bacterial isolates were isolated from different cultivated soils and animal feces. All isolates were screened for their ability to degrade AFs using thin layer chromatography (TLC). Nine bacterial isolates were able to degrade AFs using TLC and HPLC. HPLC results showed that AFs degradation ratio higher than 90% occurred by 3 bacterial cultures. The identification of the highly degrading isolate CaG6 (with 98.1% degradation ratio) was established by using 16S rRNA gene sequencing as *Bacillus cereus*. Then sequence was acquiesced to GenBank in accession number MG 751322 with 99% similarity to *Bacillus cereus*. Bacterial isolates CaG7 and IsW1 (with 94.6, 90.7% degradation ratio, respectively) has been identified by MALDI-TOF MS using the VITEK MS system (bioMérieux) as *Brevibacillus* sp1 and *Brevibacillus* sp2. Results showed that cell cultures (viable cells) were more effective in the degradation of AFs than cell free supernatant (CFS), while ppt exhibited no degradation. The three bacteria not only reduced AFs but it could also inhibit *Aspergillus flavus* NRRL 3145 with inhibition zone ≥20mm after incubation for 96h at 28°C. The AFs degradation by *B. cereus*, *Brevibacillus* sp1 and *Brevibacillus* sp2 enhanced by addition Mn^{+2} ions to the liquid media.

Keywords: Aflatoxins (AFs), *Bacillus cereus*, *Aspergillus flavus*, TLC and HPLC.

Introduction

Outside of 400 kinds of mycotoxins have been recognized, only about 10–15 have a public health interest (Turner et al., 2015). Aflatoxins (AFs) are toxic secondary metabolites of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Zhu et al., 2016). From 18 different types of aflatoxins, as B_1, B_2, G_1, G_2, P, Q, M_1, M_2, B_2a, etc., were recognized, the most distributing ones in fungal cultures are aflatoxins B_1, B_2, G_1 and G_2, then aflatoxins M_1 and M_2 in milk (Wu et al., 2009). B- and G-type AFs has been classified by The International Agency for Research on Cancer (IARC) as Group 1 mutagens, while AF-M_1 has been classified in Group 2B (IARC, 2015).

Corn, peanuts and cotton seed are the most affected crops in addition to rice, soybean and pistachio that can be extremely contaminated (Oplatowska-Stachowiak et al., 2016). It is expected to cause more than 28% of the total global cases of hepatocellular carcinoma (HCC) (Wu, 2014). AFs affect a wide diversity of animals comprising poultry, turkey, fish, rodents, swine, cattle and humans (Afsah-Hejri et al., 2013). Strong exposure to AFs causes growth delay in young children (Lombard, 2014). Recent studies carried out on the mice showed that the early exposure to the AFB_1 in particular at the embryonic period is amutagen (Chawanthayatham et al., 2015).

Harmful effects caused by this dangerous toxin have directed researchers towards finding new strategies for prevention and detoxification in order to preserve the safety of products intended for human consumption (Ben Salah-Abbes et al.,

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2015). The efficiency of aflatoxin detoxification relys on different factors, including food conditions (food constituents, moisture content and pH conditions), detoxification technologies and conditions (Pankaja et al., 2018).

Therefore, several approaches have been applied to detoxify AFs in crops and during postharvest (Spadaro & Garibaldi, 2017), as physical, chemical and biological methods (Siciliano et al., 2016). The chief problems with chemical and physical methods are their restricted range of effect on different mycotoxins and some chemicals may lead to drop in animal health. Moreover, there is a common drawback of physical and chemical treatments, since they may also cause a significant decline in the quality of food products (Prettl et al., 2017).

Over the past decades, The use of selected microorganism to control mycotoxins toxification and postharvest disease has greatly increased, providing an attractive alternative tool for removing toxins and safeguarding the value of food and feed (Wambacq et al., 2016). Some strains of lactic acid bacteria, *Aspergillus parasiticus*, *Trichoderma viride*, *Mucor ambiguus* and other fungi were able to degrade AFB_1 with different ranges (Cao et al., 2011). Terrestrial bacteria are a group of antagonistic microorganisms able to inhibit toxigenic fungus growth and AFs production (Siahmoshteh et al., 2016).

The aim of this study was to achieve biological degradation of aflatoxins by bacteria and fungi using the following approach: 1- Isolation of bacterial isolates from cultivated soils and animal feces, 2- Purification of bacterial isolates and Screening the ability of its fractions (cell free supernatant, cell precipitate (ppt) and viable cells) on the degradation of aflatoxins, 3- Identification of the most potent bacterial isolates in degradation of aflatoxins by TLC and HPLC and 4- Examine the factors affecting degradation of aflatoxins by the best fraction.

Materials and Methods

Sample collection

Bacterial species were isolated from soils and animal feces from different governorates in Egypt including Sharkia, Dakahlia, Cairo, Ismalia and Tanta. These soils were cultivated with wheat, trifolium, cabbage, garlic, onion and potato, respectively. Also samples from different animal feces including feces of horse, pigeon, sheep and buffalo were collected. Fourty-three bacterial isolates were screened for their ability to degrade aflatoxins.

Culture media

Coumarin nutrient agar medium (CMNAM) consisted of 5.0g peptone, 2.0g yeast extract, 1g lab-lemco powder, 5.0g NaCl, 15.0g agar and 2.5g coumarin per liter (pH=7.0). Nutrient agar (NA) and nutrient broth (NB) were prepared according to Monica (1985) for bacterial colonies preservation. Czapek – Dox's agar medium (CD) which prepared according to Oxoid (1982) for preservation of AFs producing fungi.

Isolation and purification of bacterial isolates

Bacterial colonies isolated and counted using standard dilution plate procedure (El-Shirbiny, 1990). About 25g of each soil sample and animal feces were placed in 500ml Erlenmeyer flask. Sterile water was added to the soil and feces until the total volume of 250ml reached 10^{-1} soil dilution. This soil and feces dilution were stirred by stirrer until suspension obtained. The suspension was left to stand for sedimentation of soil and feces matter. The supernatant was serially diluted till reached 10^{-7}. One ml aliquot of each dilution was transferred aseptically to petri dishes containing 15ml of CMNAM. Colonies that were able to grow on the medium were selected, purified and preserved as pure isolates on nutrient agar and tested for aflatoxins degradation.

Screening the ability of bacterial isolates on the degradation of aflatoxins (B_1, B_2, G_1, G_2) by TLC analysis

The standard mixture of four types of aflatoxins (AFs) were kindly provided by Dr. Khaled El-Meligy from Regional Centre for Food and Feed, Ministry of Agriculture, Giza, Egypt. Bacterial isolates were cultured NB, (pH 7.0) at 37°C in shaker incubator for 48h, then 200µl of each bacterial culture was transferred to 5ml NB medium. The medium inoculated with 100µl of AFs methanol stock solution from standard AFs (500, 150, 500 and 150 ppb of AFB_1, AFB_2, AFG_1 and AFG_2, respectively) (Shehata, 2002).

The detoxification tests were conducted in the dark at 37°C on a rotor shaker for 24h. The AFs was extracted from the cultures by

adding 5ml chloroform, mixed well for 5min, transport to separatory funnel, left until stand, the bottom layer was drained in clean flask and the chloroform extracts were evaporated to dryness then AFs detected by TLC.

Screening the ability of bacterial cultures on the degradation of aflatoxins by HPLC

The residues of dried AFs extract were dissolved in 0.5ml of water:methanol:acetonitril (54:29:17, v/v/v) and analysis. The total AFs content was determined according to AOAC (2006) method using monoclonal antibody columns for total AFs (VICAM Science Technology, Watertown, MA, USA). Aflatoxin identification was performed by a modification of the HPLC-Afla test procedure Agillent 1200 Series USA. HPLC equipment with two pumps, column C18, Lichrospher 100 RP-18, (5µm * 25cm) was used. The mobile phase consisted of water:methanol:acetonitrile (54:29:17, v/v/v) at flow rate of 1ml/min. The excitation and emission wavelengths for all AFs were 362 and 460nm (Flourcenses detector), respectively.

Degradation of AFs by bacterial cell free supernatant (CFS) and cell extract using TLC and HPLC analysis

Fresh NB was inoculated with 12h pre-cultured bacterial isolates (200µl). Inoculated cultures were incubated at 37°C with agitation at 200rpm for 24h in a shaker incubator. The preparation of supernatant was carried out following the method of Teniola et al. (2005) with slight modifications. After incubation, bacterial isolates were centrifuged with 4000rpm for 20min, The precipitate (ppt) was harvested and the resulting culture supernatant was filtered aseptically using sterile cellulose pyrogen free disposable filters of 0.2µm pore size. AFs methanol stock solution (100µl) was added to 2ml culture supernatant and ppt then the reaction mixtures were for 24h in the dark with agitation at 200rpm in a shaker incubator. The AFs degradation tests were performed as described before.

Effect of the most potent bacterial isolates on antifungal activity by disc diffusion assays

Aspergillus flavus NRRL 3145 was kindly provided by Dr. Khaled ElMeligy, Regional Centre for Food and Feed, Ministry of Agriculture, Giza, Egypt. Sterilized discs of filter paper (6mm diameter) were soaked in 1ml of each isolated bacterial suspension, for 2min; they were then placed onto agar plates that were previously seeded by cell suspension of the indicator fungi used (*A. flavus*). After incubation for 4 days, diameter of inhibition zones (mm) were measured after subtracting diameter of paper disc **(**Saeed & Tariq, 2007).

Characterization of B. cereus MG751322

Determination of 16S rRNA gene sequence

DNA extraction was done by using (QIAamp DNA Mini Kit Catalogue no.51304) according to the manufacturer's instructions. PCR– mediated amplification of the 16S rDNA, purification and sequencing of the PCR products were done by QIAquick Gel Extraction Kit Protocol: Using QIA quick gel extraction kit. (Qiagen Inc. Valencia CA). The primers used for amplifying and sequencing were: 27f (5'AGAGTTTGATCMTGGCTCAG - 3') and 1492r (5'TACGGYTACCTTGTTACGACTT -3') (Source: Metabion, Germany).

Phylogenetic analyses

A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, which was designed by Thompson et al. (1994) and Phylogenetic analyses were done using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

Effects of incubation period, temperature, pH, and metal ions on AFs degradation by B. cereus MG751322

Bacterial isolates cultivated in nutrient broth (NB) for 24h, after incubation, viable cells were tested for AFs degradation. AFs methanol stock solution (100µl) was added to (200µl) pre-culture bacterial isolates in a 5ml NB. The reaction mixtures were incubated in the dark at 37°C without shaking for 1, 12, 24, 48, 72 and 90h, respectively. To determine the effect of temperature, the mixtures were incubated at 20, 30 and 37°C, respectively for 24h. Controls were set at this temperatures by using NB medium (according to Guan et al., 2008 with modification).

In the pH tests, initial pH value was obtained by adjusting pH to 4.0, 5.0 and 6.0 with 1.0N HCl buffer, and to 7.0, 8.0 and 9.0 by 1.0N NaOH buffer. Controls were set by adjusting NB medium to different pH values.

The effects of different metal ions on degradation were determined by adding Mg^{2+}, Zn^{2+}, Cu^{2+}, $Mn^{2+}$and Co^{2+} in the form of $MgSo_4$, $ZnSO_4$, $CuSO_4$, $MnSo_4$ and $Co(No_3)_2$, respectively, to the reaction mixtures, respectively resulting in a final ion concentration of 10mM. NB was used to substitute culture supernatant in the control. The AFs degradation tests were performed as described above previously.

Results

Isolation and purification of bacterial isolates

In the present study, a medium containing coumarin (CM) was used for selection of aflatoxins (AFs) degrading microbes. The microorganisms grew and very few colonies appeared on the medium. Pure isolates only were subcultured on slants of NA. From 43 culture isolates about 9 bacterial cultures were able to degrade aflatoxins as shown in Table 1.

Screening the ability of bacterial cultures on the degradation of aflatoxins by HPLC

The isolates of bacterial cultures able to degrade AFs on TLC were taken for further analysis on HPLC. Nine bacterial isolates were capable of decreasing AFs concentrations in the liquid medium with different values. Three isolates reduced AFs in the medium by ratio up to 90% (Table 2). HPLC analysis showed the absence of peaks in the samples treated with isolated cultures corresponding to standard AFs sample (Fig. 1).

Screening of the ability of CFS and cell extract (ppt) of selected bacterial isolates on AFs degradation using TLC analysis

The CFS of bacterial isolates which showed high degradation ratio (code's CaG6, CaG7 and IsW1) were screened for their ability to degrade AFs using TLC. As observed from TLC analysis, Fig. 2 and 3, respectively, showed no or few degradation of AFs by bacterial CFS.

Degradation of aflatoxins by bacterial CFS using HPLC analysis

Bacterial CFS were subsequently investigated for their ability to degrade AFs using HPLC analysis. Different levels of AFs were observed. Results from Table 3 showed a percent of about (65, 76.3, 81.1%) of AFs degradation as compared to standard sample. As shown in Table 4 CFS of bacterial isolates exhibited less degradation activity compared to degradation by cell culture. The highest degradation rate was observed by bacterial isolate (CaG6) CFS with degradation rate 65% compared to 86% by bacterial cell culture while degradation rate by CFS of bacterial isolate (IsW1) was 76.3% compared to 85.5% by cell culture, and bacterial isolate (IsW1) showed degradation activity reached 81.1% by CFS compared to 85.7% by cell culture.

Inhibition of Aspergillus flavus NRRL 3145 (aflatoxin producing strain) by selected bacterial isolates

The three bacterial isolates CaG6, CaG7 and IsW1 inhibited the aflatoxin producing fungi to varying extents (Fig. 4). Highest inhibition (30mm) was observed with CaG6**.**

Molecular characterization of the most potent aflatoxins degrading bacterial isolate (code CaG6)

The identification of the highly AFs degrading bacterial isolate CaG6 was confirmed by using 16S rRNA gene sequencing as *Bacillus cereus*. Then sequence was submitted to GeneBank in accession number MG751322 with 99% similarity to *Bacillus cereus* category (Fig. 5).

Screening the ability of CaG6 on under optimum conditions of temperature, pH and metal ions for degradation of AFs by Bacillus cereus using HPLC analysis

From TLC results the optimum conditions for AFs degradation was observed during incubation at pH 8.0 for 48h at 37°C with addition of Mn^{2+} ions. These results were taken for further analysis on HPLC. *Bacillus cereus* showed more degradation ability at optimum conditions compared to isolates without optimization (Table 5).

Discussion

Aflatoxins (AFs) are furano-coumarin compounds and considered the most studied mycotoxin group, comprising aflatoxin B_1 (AFB_1) (Williams et al., 2004). Foods contaminated with AFB_1 have a high carcinogenic potency for humans. It is therefore important to find a microbe or an enzyme possessing wide exclusion activity against most of mycotoxins.

TABLE 1. Screening of aflatoxins biodegradation by different bacterial isolates using TLC method.

Isolate No.	Isolate code	Cultivated plant	Region	Morphology of isolates	TLC results
1	Shw1	wheat	Sharkia	Creamy colony	-
2	ShW2	wheat	Sharkia	Creamy colony	-
3	ShW3	wheat	Sharkia	Creamy colony	-
4	ShT1	Trifolium	Sharkia	Creamy colony	-
5	ShT2	Trifolium	Sharkia	Creamy colony	-
6	ShT3	Trifolium	Sharkia	Creamy colony	-
7	ShT4	Trifolium	Sharkia	Creamy colony	
8	ShC1	Cabbage	Sharkia	Creamy colony	-
9	ShC2	Cabbage	Sharkia	Creamy colony	-
10	ShC3	Cabbage	Sharkia	Creamy colony	-
11	IsW1	Wheat	Ismalia	Creamy colony	+
12	IsW2	Wheat	Ismalia	Creamy colony	-
13	IsW3	Wheat	Ismalia	Creamy colony	-
14	IsW4	Wheat	Ismalia	Creamy colony	-
15	IsW5	Wheat	Ismalia	Creamy colony	+
16	IsW6	Wheat	Ismalia	Creamy colony	-
17	IsW7	Wheat	Ismalia	Creamy colony	-
18	CaG1	Garlic	Cairo	Creamy colony	+
19	CaG2	Garlic	Cairo	Creamy colony	+
20	CaG3	Garlic	Cairo	Creamy colony	-
21	CaG4	Garlic	Cairo	Creamy colony	-
22	CaG5	Garlic	Cairo	Creamy colony	-
23	CaG6	Garlic	Cairo	Creamy colony	+
24	CaG7	Garlic	Cairo	yellow colony	+
25	DaO1	Onion	Dakahlia	Creamy colony	-
26	DaO2	Onion	Dakahlia	Creamy colony	-
27	DaO3	Onion	Dakahlia	Creamy colony	-
28	TaPo1	Potato	Tanta	Creamy colony	-
29	TaPo2	Potato	Tanta	Creamy colony	-
30	TaPo3	Potato	Tanta	Creamy colony	-
31	H1	Horse feces	Sharkia	Creamy colony	-
32	H2	Horse feces	Sharkia	Creamy colony	+
33	H3	Horse feces	Sharkia	Creamy colony	-
34	H4	Horse feces	Sharkia	Yellowcolony	-
35	H5	Horse feces	Sharkia	Yellowcolony	+
36	P1	Pigeon feces	Sharkia	Creamy colony	-
37	P2	Pigeon feces	Sharkia	Creamy colony	-
38	P3	Pigeon feces	Sharkia	Creamy colony	-
39	C1	Cheap feces	Sharkia	Creamy colony	-
40	C2	Cheap feces	Sharkia	Creamy colony	-
41	B1	Buffalo feces	Sharkia	White colony	-
42	B2	Buffalo feces	Sharkia	Creamy colony	+
43	B3	Buffalo feces	Sharkia	Creamy colony	-

Sh= Sharkia Governorate, Is= Ismalia Governorate, Ca= Cairo Governorate, Da= Dakahlia Governorate, Ta= Tanta Governorat, W= Farm soil cultivated withwheat, T= Farm soil cultivated with trifolium, G= Farm soil cultivated with garlic, O= Farm soil cultivated with onion, P= Farm soil cultivated with potato, H= Horse feces, P= Pigeon feces, C= Sheep feces, B= Buffalo feces. 1,2,3,….= No. of bacterial isolates.

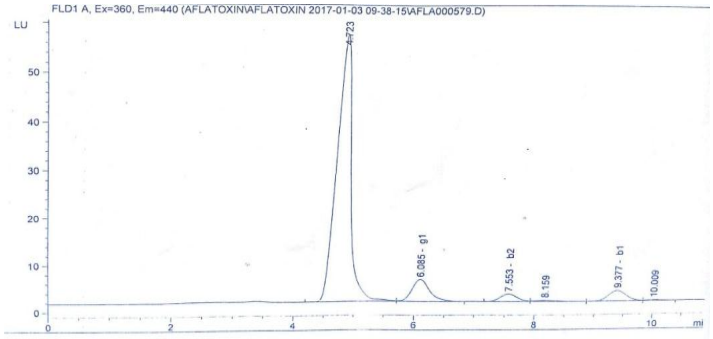
-= No Afs degradation += There is degradation

TABLE 2. Bacterial isolates reduced AFs concentration in liquid medium by HPLC.

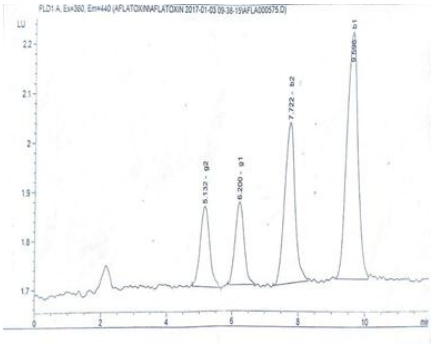
Isolate no.	Active isolate	Total concentration Of AFs + isolate culture	Ratio of total degradation %
1	B2	243.81	81.2
2	CaG1	344.88	73.4
3	H5	160.9	87.6
4	H2	385.77	70.3
5	IsW5	245.63	81.1
6	IsW1	120.08	90.7
7	CaG6	69.18	98.1
8	CaG7	24.62	94.6
9	CaG2	205.21	84.2
Standard aflatoxin (P.P.b)		1300	

Is= Ismalia Governorate, Ca= Cairo Governorate, W= Farm soil cultivated with wheat, G= Farm soil cultivated with garlic, H= Horse feces, B= Buffalo feces, H= Horse feces, F=Fungi.
1, 2, 3,…. =No. of isolates.

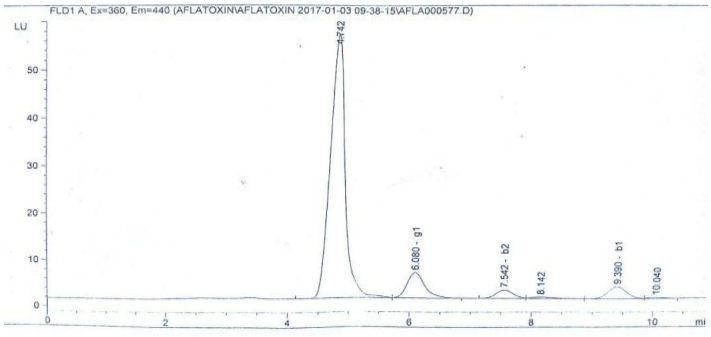
Data of HPLC of aflatoxins + different bacterial isolates

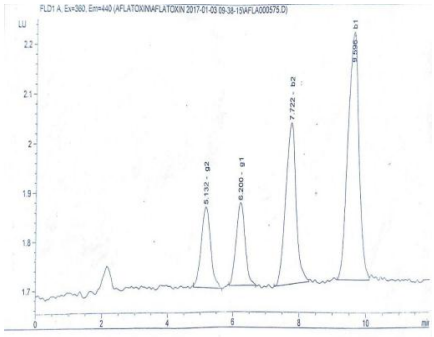


Data of HPLC of standard aflatoxins.



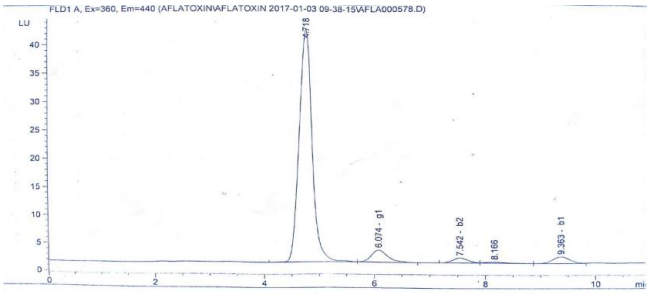
Biodegradation ofaflatoxins (AFs) by isolate 1 (code B2).

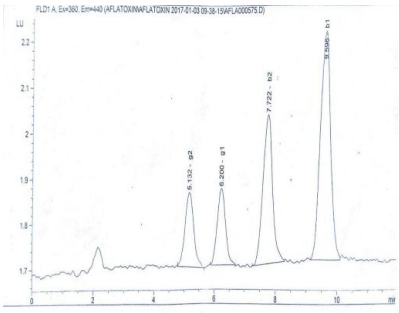




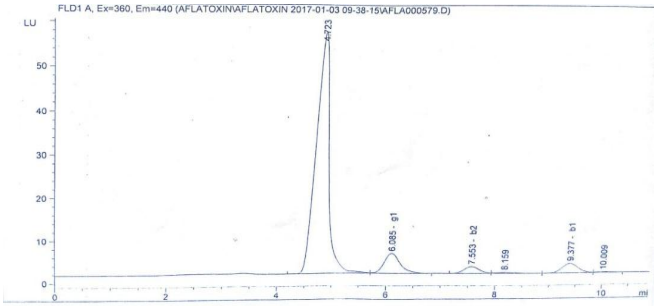
Biodegradation of aflatoxins (AFs) by isolate 2 (code H2).

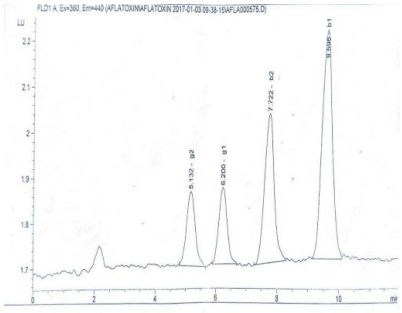
Biodegradation of aflatoxins (AFs) by isolate 2 (code H2).



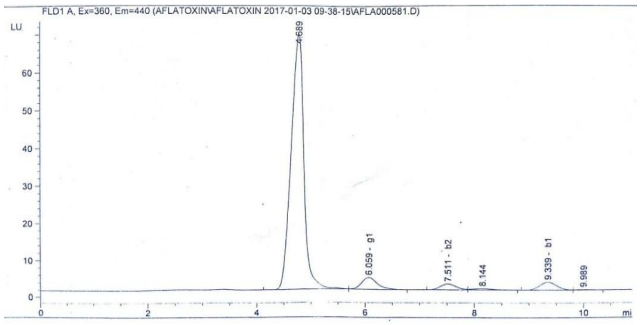


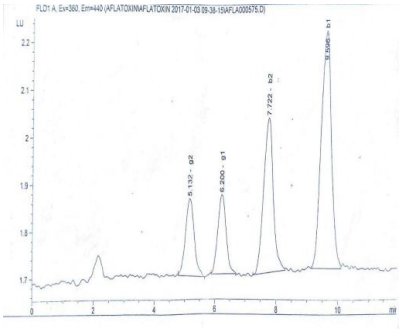
Biodegradation of aflatoxins (AFs) by isolate 3 (code H5).



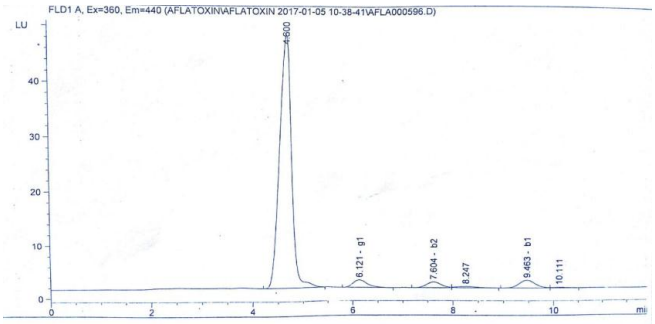


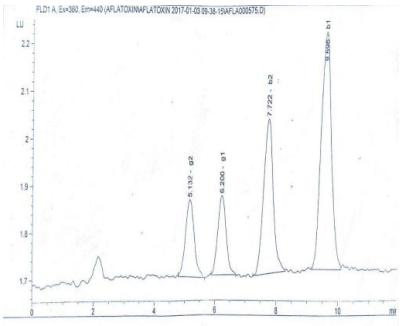
Biodegradation of aflatoxins (AFs) by isolate 4 (code CaG1).



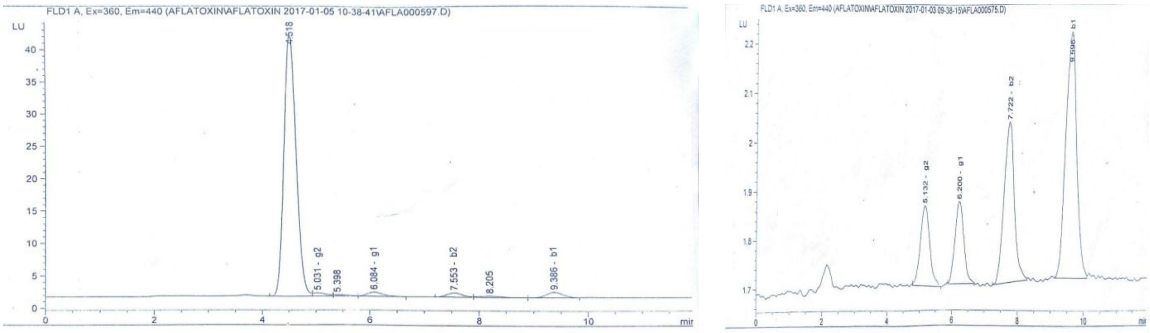


Biodegradation of aflatoxins (AFs) by isolate 5 (code IsW5).

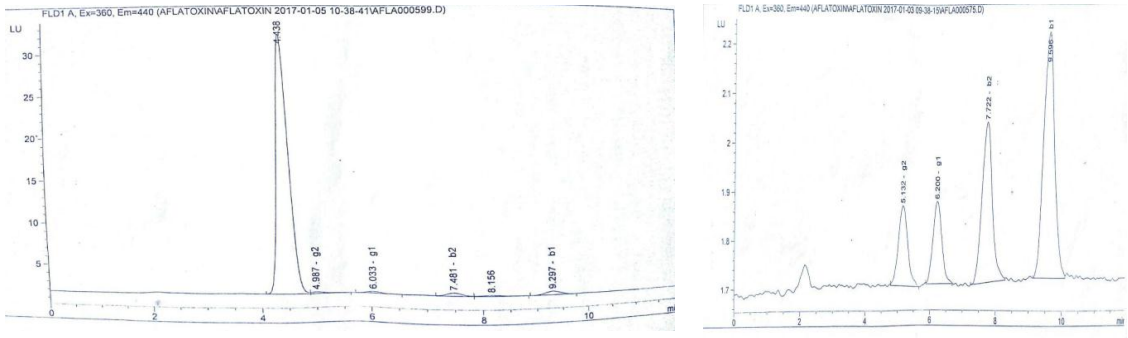




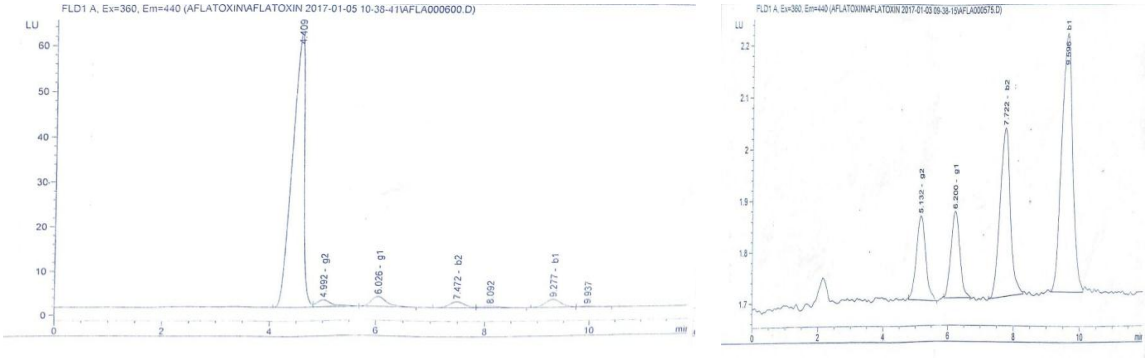
Biodegradation of aflatoxins (AFs) by isolate 6 (code IsW1).



Biodegradation of aflatoxins (AFs) by isolate 7 (code CaG7).



Biodegradation of aflatoxins (AFs) by isolate 8 (codeCaG6).



Biodegradation of aflatoxins (AFs) by isolate 9 (code CaG2).

Fig. 1. HPLC chromatogram of AFs degradation by bacterial isolates.

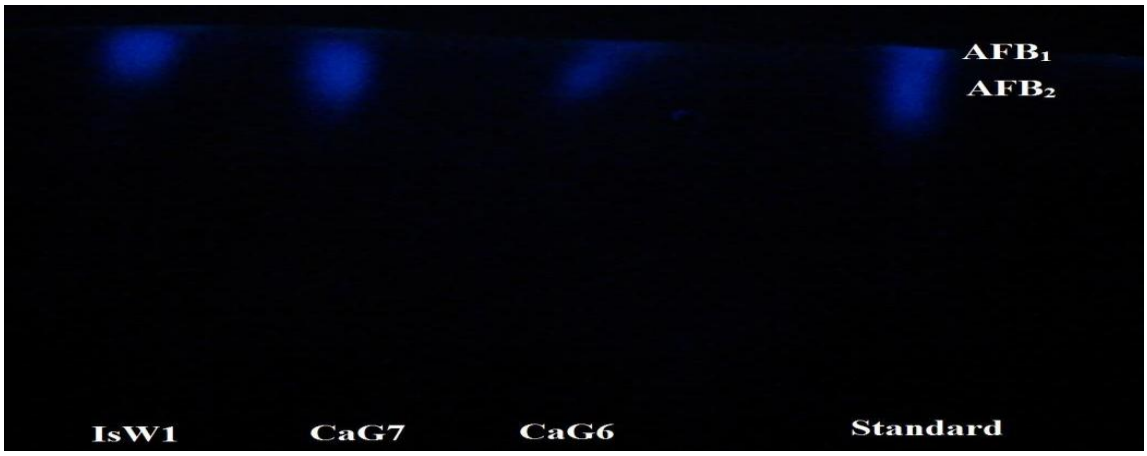


Fig. 2. TLC analysis indicate the activity of aflatoxin-degradation by bacterial CFS.

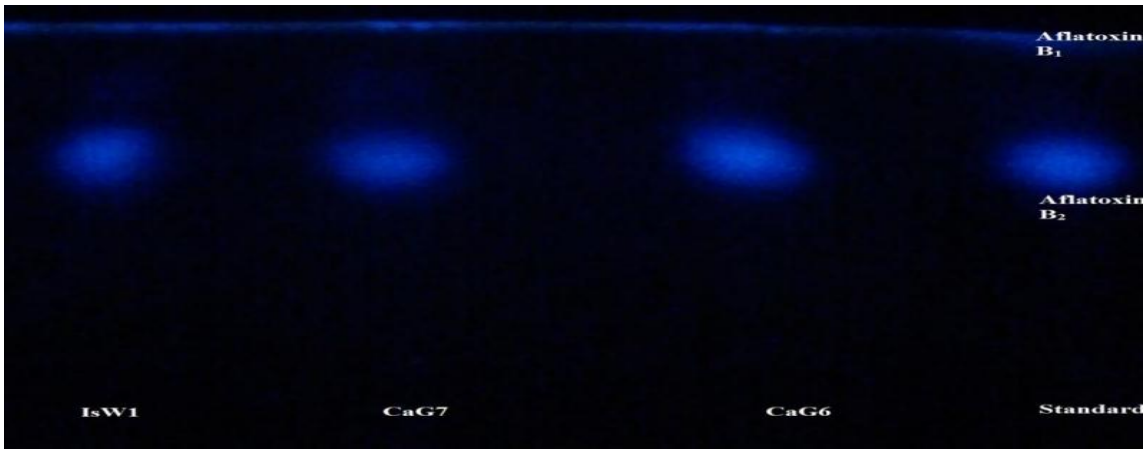


Fig. 3. TLC analysis indicate the activity of aflatoxin-degradation by bacterial ppt.

TABLE 3. Biodegradation of aflatoxins (AFs) by CFS of bacterial isolates.

Type of aflatoxins	Concentration of AFs of control (ppb)	Concentration of AFs (ppb) of control+isolate1 (CaG6)	% of bio-degradation of AFs	Concentration of AFs (ppb) of control +isolate2 (CaG7)	% of bio-degradation of AFs	Concentration of AFs (ppb) of control +isolate3 (IsW1)	% of bio-degradation of AFs
1- AFB_1	22.8	3.4	85	5.5	75.8	3.2	85.9
2-AFB_2	7.6	1.4	81.5	0.5	93.4	1.3	82.8
3-AFG_1	206.6	34.8	83.1	45.6	77.9	30.1	85.4
4-AFG_2	72.9	68.8	5.6	21.6	70.3	21.6	70.3
5-Total AFs	310.1	108.4	65	73.2	76.3	56.2	81.1

TABLE 4. Biodegradation of aflatoxins (AFs) by CFS of bacterial isolates (with degradation activity over than 90%) compared to degradation by culture of isolates using HPLC.

Isolate No.	Isolate code	Total conc. of AFs+CFS of isolates	% of degradation by CFS	Total conc. Of AFs+cell culture of isolates	% of degradation by cell culture
1	CaG6	108.4	65	43.3	86
2	CaG7	73.2	76.3	44.7	85.5
3	IsW1	56.2	81.1	44.3	85.7
Standard AFs for bacteria (P. P. b.)		310.1		310.1	

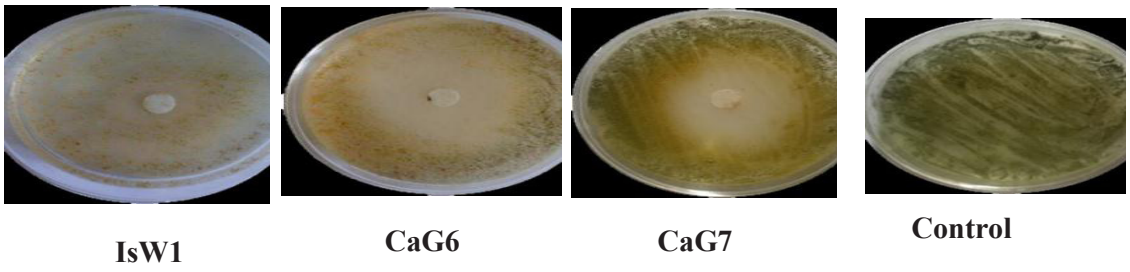


Fig. 4. The inhibition effect of bacterial isolates and fungal isolate on *A.flavus* NRRL 3145.

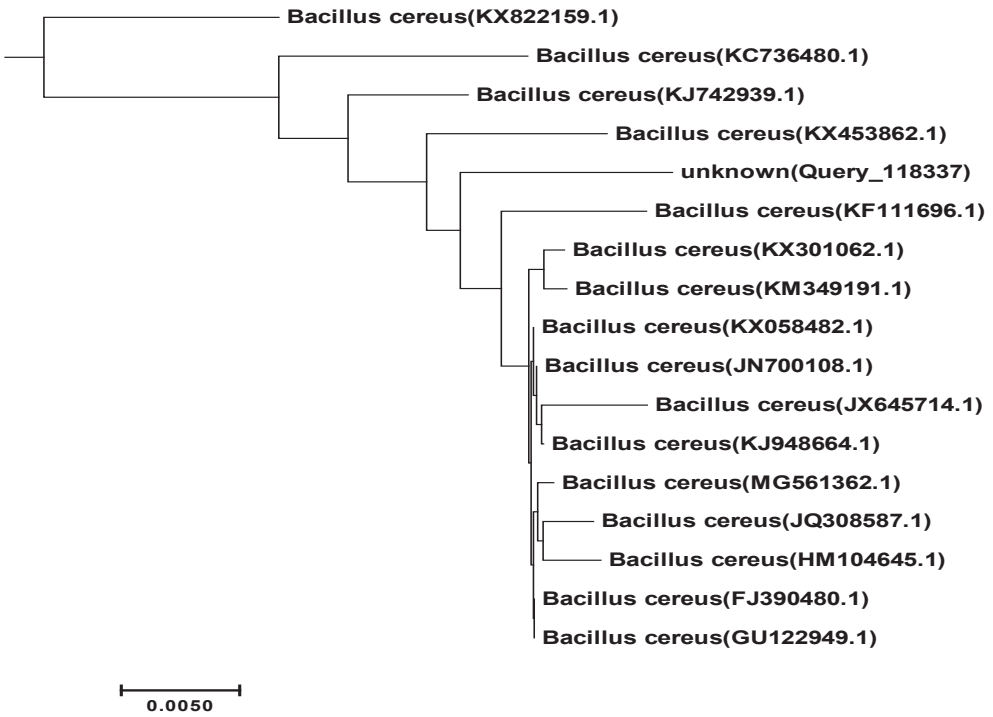


Fig. 5. Phylogenetic tree of *Bacillus cereus* CaG6 strain.

TABLE 5. Biodegradation of aflatoxins (AFs) by *Bacillus cereus* CaG6 under optimum conditions.

Type of aflatoxins	Concentration of AFs of control (ppb)	Concentration of AFs (ppb) of control + *B. cereus* CaG6 (without optimization)	% of biodegradation of AFs (without optimization)	Concentration of AFs (ppb) of control + *B. cereus* CaG6 (at optimum conditions)	% of biodegradation of AFs(at optimum conditions)
1-AFB_1	22.8	2.6	88.5	2.4	89.4
2-AFB_2	7.6	1.03	86.4	0.9	88.1
3-AFG_1	206.6	15.4	92.5	6.2	96.9
4-AFG_2	72.9	24.3	66.6	2.6	96.4
5-Total AFs	310.1	43.3	86	12.03	96.1

In this study, fourty three bacterial isolates isolates could be isolated from various sources including cultivated soils and animal feces from different governorates in Egypt. The isolation was occurred on media contain coumarin as a part of its composition. Hence, microorganisms that could consume coumarin and use it as their carbon source might be able to use AFs (Guan et al., 2008).

This process called cometabolism which is the process through which a contaminant is accidentally decomposed by an enzyme or cofactor secreated during microbial metabolism of another complex. Typically, the microorganism involved has no benefit. Only indigenous microbes that is abile to degrade the contaminant and cosubstrate is stimulated by this strategies. This greatly targeted stimulation assures that only those microbes that is capable of degrading the contaminant are targeted, so reducing amendment costs, well and formation plugging, etc. (Hazen, 2010). So, using coumarin provided a cheap, viable, and effective tool for selecting aflatoxin B_1 degrading microorganisms (Zhang et al., 2014).

In this study, the fourty three different isolates were screened for their ability to degrade AFs using thin layer chromatography analysis (TLC). Out of 43 isolates, Nine isolates showed reduction activity of AFs by different ratios. The degradation by different isolates was taken for further analysis using high performance liquid chromatography (HPLC). The analysis indicated that the different isolates reduced AFs by high ratio that reached 98.1%.

In the present study, cell culture, cell free supernatant (CFS) and cell ppt of isolates were tested for their degradation ability. In contrast to results obtained by Guan et al. (2008) who found that AFB_1 degradation by Culture supernatant of *S. maltophilia* 35-3 was greater than degradation by viable cells and cell extracts, cell culture showed strong AFs degradaing activity compared to CFS while cell extract couldn't exhibit any degrading effect.

Further more, the most potent bacterial and fungal isolates which show high degradation activity were selected. Bacterial isolates with the highest degradation identified as *Bacillus cereus* MG751322 by investigation of 16S rRNA gene sequences. *Bacillus cereus* is an significant cause of food borne disease and food poisoning. However, *B. cereus* has been used as a probiotic in human medication and livestock production, with low standards of safety assessment (Zhu et al., 2016). *B.cereus* MG751322 had the strongest ability to detoxify AFs, where the degradation percentages of AFs was 98% after 24h of incubation.

The results from TLC showed that the optimum conditions for AFs degradation by *B. cereus* MG751322 was at pH 8.0. The

degradation was not observed at pH 4.0 and 5.0 and increase gradually till reached maximum degradation rate at pH 8.0. This result agreed with AFB_1 degradation by of *S. maltophilia* 35-3 CFS in which the maximum degradation (84.8%) was observed at pH 8.0 (Guan et al., 2008) and degradation of AFB_1 by cell extracts of *F. aurantiacum* under different pH values showed a similar result (Smiley & Draughon, 2000), while the highest degradation rate ofAFB_1 (91.24%) by *B. licheniformis* CFR1was obtained at pH of 7 (Rao et al., 2017).

The optimum temperature for AFs degradation by *B. cereus* MG751322 was observed at 37°C. Also low degradation obtained at 20°C. The same results obtained by Guan et al. (2008) who stated that the AFB_1 degradation by culture supernatant of *S. maltophilia* 35-3 was higher at 37°C (78.7%) ($P<0.05$) and also Rao et al. (2017) reported that a maximum degradation by *B. licheniformis* was at 37°C. By increasing temperature the degradation rate increase, till reach 37°C. AFB_1 degradation by *R. erythropolis* and *M. fluoranthenivorans* cell extracts were around the same in between 10-40°C (>90%) (Teniola et al., 2005).

The maximum degradation rate of AFs by *B. cereus* MG751322 was obtained after incubation for 24h, and by addition of Mn^{2+} ions, Also combined microbes as *Lactobacillus casei*, *Bacillus subtilis* and *Pichia anomala* showed maximum degradation of AFB_1 was between 24 and 48h (Zuo et al., 2012) while 78.7% of AFB_1 degraded by the culture supernatant of S. maltophilia 35-3 after 72h (Guan et al., 2008) and 93.57% of AFB_1 degraded by *B. licheniformis* after 72h (Rao et al., 2017).

AFs degradation by the cell culture of *B. cereus* MG751322 could be stimulated by Mn^{2+} ions, also Mg^{2+} showed high reduction effect on AFB_1 degradation and on the four types of AFs generally, and addition of Zn^{2+} showed no effect on degradation rate while, Cu^{2+} ions reduced AFs obviously. The same results obtained by D'Souza and Brackett about the effects of Mg^{2+} on AFB_1 degradation by *F. aurantiacum.* Also, AFB_1 degradation ability by *B. licheniformis* stimulated by addition of Cu^{2+} and Mg^{2+} (Rao et al., 2017).

Conclusion

This study showed more than 90% degradation of AFs by *Bacillus cereus*. Thus, *Bacillus cereus* might be an excellent candidate for bioremediation and detoxification of AFs from both field and food matrices. The results reported in this study could contribute towards the development of food and feeds additives for the detoxification of AFs to improve, safeguard and ensure the quality of foods and feeds.

References

Afsah-Hejri, L., Jinap, S., Hajeb, P., Radu, S. and Shakibazadeh, S. (2013) A review on mycotoxins in food and feed: Malaysia case study. *Compr. Rev. Food Sci. Food Saf,* **12**(6), 629-651.

Association of official Analytical Chemists (AOAC) (2006) "*Official Methods of Analysis*", 18th ed., Association of Official Analytical Chemists, Washington, D.C.

Ben Salah-Abbes, J., Abbes, S., Jebali, R., Haous, Z. and Oueslati, R. (2015) Potential preventive role of lactic acid bacteria against aflatoxin M1 immunotoxicity and genotoxicity in mice. *J. Immunotoxicol.* **12**(2), 107-14.

Cao, H., Liu, D.L., Mo, X.M., Xie, C.F. and Yao, D.S. (2011) A fungal enzyme with the ability of aflatoxin B1 conversion: Purification and ESIMS/MS identification. *Microbiol. Res.* **166**, 475-483.

Chawanthayatham, S., Thiantanawat, A., Egner, P.A., Groopman, J.D., Wogan, G.N., Croy, R.G. and Essigmann, J.M. (2015) Prenatal exposure of mice to the human liver carcinogen aflatoxin B1 reveals a critical window of susceptibility to genetic change. *Int. J. Cancer,* **136**(6), 1254-62.

El-Shirbiny, E.A.A. (1990) Studies on the antimicrobial activities of some bacteria belonging to Actinomycetales. *Ph.D. Thesis.* Bot. Depat. Zagazig Univ., Egypt.

Guan, S., Ji, C., Zhou, T., Li, J., Ma, Q. and Niu, T. (2008) Aflatoxin B1 degradation by *Stenotrophomonas maltophilia* and other microbes selected using coumarin medium. *Int. J. Mol. Sci.* **9**, 1489-1503.

Hazen, T.C. (2010) Cometabolic Bioremediation. In: "*Handbook of Hydrocarbon and Lipid Microbiology*", Timmis, K.N. (Ed.). Springer, Berlin, Heidelberg.

IARC, (2015) Mycotoxin control in low - and middle income countries. Lyon, France: International Agency for Research on Cancer (WHO). *Report No. 9*. pp. 31-42.

Lombard, M.J. (2014) Mycotoxin exposure and infant and young child growth in Africa: What do we know? *Ann. Nutr. Metabol.* **64**, 42-52.

Monica, C. (1985) Medical laboratory manual for tropical countries. English language book society editions. *Tropical Health Technology,* **42**, 250-290.

Oplatowska-Stachowiak, M., Sajic, N., Xu, Y., Haughey, S.A., Mooney, M.H., Gong, Y.Y., Verheijen, R. and Elliott, C.T. (2016) Fast and sensitive aflatoxin B1 and total aflatoxins ELISAs for analysis of peanuts, maize and feed ingredients. *Food Control,* **63**, 239-245.

Oxoid, L. (1982) "*The Oxoid Manual of Culture Media Ingredients and Other Laboratories Services*", 5th ed. Turnergrabhic, Ltd. England.

Pankaja, S.K., Shib, Hu. and Kevin, M. K. (2018) A review of novel physical and chemical decontamination technologies for aflatoxin in food. *Trends Food Sci. Technol.* **71**, 73-83.

Prettl, Z., Dési, E., Lepossaa, A., Kriszt, B., Kukolya, J. and Nagya, E. (2017) Biological degradation of aflatoxin B1 by a *Rhodococcus pyridinivorans* strain in by-product of bioethanol. *Anim. Feed Sci. Technol.* **224**, 104-114.

Rao, K.R., Vipin, A.V., Hariprasad, P., Anu Appaiah, K.A. and Venkateswaran. G. (2017) Biological detoxification of Aflatoxin B1 by *Bacillus licheniformis* CFR1. *Food Control,* **71**, 234-241.

Saeed, S. and Tariq, P. (2007) Antimicrobial activities of *Emblica officinalis* and *Coriandrum sativum* against Gram-positive bacteria and *Candida albicans. Pak. J. Bot.* **39**(3), 913-917.

Shehata, S.A. (2002) Detoxification of mycotoxin contaminated animal feedstuffs. *Ph.D Thesis*, Zagazig Univ., Fac. Agric., Egypt.

Siahmoshteh, F., Hamidi-Esfahani, Z. and Razzaghi-Abyaneh, M. (2016) Antifungal activity, biodegradation and production inhibition of aflatoxins B1 and G1 by a soil isolate of *Bacillus subtilis* against *Aspergillus parasiticus* NRRL 2999. *J. Pure Appl. Microbiol.* 10(4): 2541-2549.

Siciliano, I., Spadaro, D., Prelle, A., Vallauri, D., Cavallero, M., Garibaldi, A. and Gullino, M. (2016) Use of cold atmospheric plasma to detoxify hazelnuts from aflatoxins. *Toxins (Basel),* **8**, 125-204.

Smiley, R.D. and Draughon, F.A. (2000) Preliminary evidence that degradation of aflatoxin B1 by *Flavobacterium aurantiacum* is enzymatic. *J. Food Prot.* **63**, 415-418.

Spadaro, D. and Garibaldi, A. (2017) Containment of mycotoxins in the food chain by using decontamination and detoxification techniques. In: "*Practical Tools for Plant and Food Biosecurity*", Gullino, M.L., Stack, J., Fletcher, J. and Mumford, J. (Ed.), p. 163-177. Springer, Dordrecht, The Netherlands.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725-2729.

Teniola, O.D., Addo, P.A., Brost, I.M., Färber, P., Jany, K.D., Alberts, J.F., van Zyi, W.H., Steyn, P.S. and Holzapfel, W.H. (2005) Degradation of aflatoxin B1 by cell-free extracts of *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* sp. nov. DSM44556T. *Int. J. Food Microbiol.* **105**, 111-117.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**(22), 4673-4680.

Turner, N.W., Bramhmbhatt, H., Szabo-Vezse, M., Poma, A., Coker, R., and Piletsky, S.A. (2015) Analytical methods for determination of mycotoxins. *Anal. Chim. Acta*, **901**, 12-33.

Wambacq, E., Vanhoutte, I., Audenaert, K., De Gelder, L. and Haesaert, G.(2016) Occurence, preventive and remediation of toxigent fungi and mycotoxins in silage. *J. Sci. Food Agric.* **96**, 2284-2302.

Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M. and Aggarwal, D. (2004) Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. *Am. J. Clin. Nutr.* **80**, 1106-1122.

Wu, Q., Jezkova, A., Yuan, Z., Pavlikova, L., Dohnal, V. and Kuca, K. (2009) Biological degradation of aflatoxins. *Drug Metab. Rev.* **41**(1), 1-7.

Wu, F. (2014) Perspective: Time to face the fungal threat. *Nature*, **4**, 516(7529):S7.

Zhang, W., Xue, B., Li, M., Mu, Y., Chen, Z., Li, J. and Shan, A. (2014) Screening a strain of *Aspergillus niger* and optimization of fermentation conditions for degradation of aflatoxin B1. *Toxins,* **6**(11), 3157-3172.

Zhu, K., Hölzel, C.S., Cui, Y., Mayer, R., Wang, Y., Dietrich, R., Didier, A., Bassitta, R., Märtlbauer, E. and Ding, S. (2016) Probiotic *Bacillus cereus* strains, a potential risk for public health in China. *Front. Microbiol.* **7**, 718.

Zuo, R., Chang, J., Yin, Q., Wang, P., Cheng, W., Wang, X., Liu, J. and Zheng, Q. (2012) Inhibiting *Aspergillus flavus* growth and degrading aflatoxin B1 by combined beneficial microbes. *Afr. J. Biotechnol.* **11**(65), 12903-12909.

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االتكسير الحيوي للأفلاتوكسينات بواسطة البكتريا

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إن الأفلاتوكسينات لها اثار جانبية كبيرة علي الإنسان والماشية والمحاصيل. فهي تسبب أمراض خطيرة للإنسان والحيوانات علي حد سواء، وتلعب دورا رئيسيا في الخسارة الأقتصادية. لذا جهود هائلة أصبحت موجهة نحو منع وإزالة هذه السموم. هذا البحث يهدف إلى العثور على كائن دقيق جديد وأمن قادر علي تكسير الأفلاتوكسينات وتثبيط الفطر المنتج لها. تم عزل 43 عزلة بكتيرية من أراضي زراعية مختلفة ومن روث الماشية. وتم اختبار قدرة جميع العزلات على تكسير الأفلاتوكسينات باستخدام (TLC), ووجد ان تسع عزلات بكتيرية قامت بتكسير الأفلاتوكسينات باستخدام TLC. تم أخذ هذه العزلات لمزيد من التحليل باستخدام (HPLC). وأظهرت النتائج أن 3 عزلات بكتيرية استطاعت تكسير الأفلاتوكسينات بنسبة تكسير وصلت إلى أكثر من 90%. تم تعريف أكثر البكتريا قدرة على تكسير الأفلاتوكسينات CaG6 (بنسبة تكسير 98.1%) بواسطة 16Sr RNA جين ثم تم وضع التتابع الجيني لها على الجين بانك على موقع NCIB تحت رقم MG 751322ويصل إلى حد من التشابه لبكتيريا باسيلس سيريس*Bacillus cereus* بدرجة 99%. كما تم تعريف العزلتين CaG7, IsW1 (لهما نسبة تكسير 90.7، 94.6%, على التوالي) باستخدام نظام VITEK MSعلى أنهما من رتبة بريفيباسيلس *Brevibacillus* sp.

تم اختبار قدرة السائل الرائق (CFS)ومستخلص الخلايا (ppt) (للعزلات القادرة على التكسير بنسبة أعلى من 90%) على تكسير الأفلاتوكسينات باستخدام TLC و HPLC ، وأظهرت النتائج أن الخلايا الحية للعزلات كانت أكثر فعالية في التكسير من السائل الرائق, في حين أن مستخلص الخلايا لا يظهر أي تكسير. وقد تم اختبار قدرة العزلات البكتيرية على تثبيط الفطر المنتج للأفلاتوكسين. وكانت البكتريا ليست قادرة فقط على تكسير الأفلاتوكسين ولكن على تثبيط الفطر المنتج أيضا بعد تحضين لمدة 96 ساعة عند درجة حرارة 28°م ووصلت مساحة التثبيط إلى أكبر من 20 مم. كانت الظروف المثلي للنمو البكتيري لبكتريا *Brevibacillus* , *B. cereus* sp1 عند الرقم الهيدروجيني 8 (pH 8.0) ولبكتريا *Brevibacillus* sp2 عند الرقم الهيدروجيني 7 (pH 7.0) بعد تحضين لمدة 24 ساعة عند درجة حرارة 37°م. أوضحت النتائج أن التكسير بواسطة الخلايا البكتيرية يتحسن بعد إضافة أيونات المنجنيز Mn^{2+}.