INHIBITION OF AFLATOXIN PRODUCTION BY *BACILLUS SUBTILIS*

SALEH,N.A.[1], M. S. ABDEL-AZIZ[1], NASHWA M. HELMY[2] AND IBTESAM A. ABDEL-GHANY[2]

1. *Biochemistry Department , Faculty of Science , Ain Shams University, Cairo*
2. *Biotechnology Department, Animal Health Research Institute, ARC, Ministry of Agriculture, Dokki, Giza*

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Abstract

Aflatoxins are toxic metabolites mostly produced by two fungal species *(Aspergillus flavus* and *Aspergillus parasiticus)* in cereal grains, groundnuts, corn and rice. In the present investigation the HPLC technique was used for estimation of aflatoxins produced by each *Aspergillus* species either without or with co-cultivation of *B. subtilis*. The antitoxin production activity of *Bacillus subtilis* was detected when being co-cultivated with *Aspergillus flavus* and *Aspergillus parasiticus* under different cultural conditions (*p*H, temperatures , incubation periods and two types of media). The thermal stability of the antitoxin production element by bacterium was post-heating to 80, 100 ºC and autoclaving at 121 ºC. Maximum antiaflatoxigenic effect of this bacterium was observed at 25 ºC and *p*H 10 after four days cultivation with *Aspergillus flavus*, while, the activity appeared at 25 ºC and *p*H 10 after ten days when cultivated with *Aspergillus parasiticus*. The antitoxin production metabolites are shown to be thermostable at 80, 100ºC and autoclaving at 121ºC for 30 minutes.

INTRODUCTION

Aflatoxins are toxic metabolites mostly produced by two fungal species as *Aspergillus flavus* and *Aspergillus parasiticus* in cereal grains, groundnuts, corn and rice. They include aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2. They are considered as potent carcinogens, teratogens, mutagens and immunosupressives. They also contribute in the development of many human diseases including hemorrhagic liver necrosis, hepatocellular carcinoma, lung and breast cancers, bile duct proliferation, Reyes syndrome and Kwashiorkor disease (*Dvorackova,1990 and Hendrickse, 1991*).

They also cause stunted growth in children who are exposed to aflatoxins at neonatal stages, due to their capacity to cross placental barrier causing genetic defects at fetal stages itself (Gong *et al.* 2002). It is reported as one of the major causes of liver cancer in Egypt (Hassanane, *et al.* 2009).

The aflatoxins were extensively found in many Egyptian grains and feed stuffs including wheat, corn, cereal grains, groundnuts and rice. Even they were discovered

in some processed meat, air dust and in the secreted breast milk of some Egyptian mothers *(Polychronaki et al., 2007* and Hassanane *et al.,* 2009).

Several preventive measures to minimize aflatoxins contamination in agricultural commodities have been attempted. These can be divided into three broad categories: as trials to develop plant strains resistant to fungal contamination, good system in harvesting, manipulation, storage of the grains, and finally trials to find some reagents to inhibit the fungal growth on plants and the detoxification of their toxins if the fungi are grown. These reagents are known as aflatoxin inhibitors (Hassanane *et al.,* 2009).

Some microorganisms were early reported to be capable of degrading AFB_1 such as as *Flavobacterium aurantiacum* by enzymatic way. Several *Bacillus* species produce various antibiotics, some of which have antifungal activity. These antifungal antibiotics play a major role in biological control of plant pathogens and post harvest spoilage fungi (Klich *et al.,* 1994 and Leifert *et al.,* 1995). Many of these antitoxinogen substances are cyclic such as Mycosubtilins produced by *Bacillus subtilis*, while, *Rhizocticins* are linear (Kluger *et al.,* 1990).

These antifungal compounds contain amino acid residues which are unique and not commonly found in proteins, and most of them are resistant to hydrolysis by proteases, peptidases and many other enzymes, for example, Fungicin M-4 is resistant to proteinase K, trypsin, carboxypeptidase A and lipase (Besson and Michel ,1990).

The present study aimed to clarify the optimal conditions in which *Bacillus subtilis* could be used as antitoxinogen against *Aspergillus flavus* and *Aspergillus parasiticus* . The HPLC technique was used to estimate the aflatoxins produced by each *Aspergillus* species when co-cultivated with *B. subtilis* under different cultivation conditions of *p*H, temperature, incubation period, two types of media and the thermal stability of its antifungal substances at 80, 100 ºC and autoclaving at 121 ºC.

MATERIALS AND METHODS

1-Organisms

I-Fungi :Two fungal strains: *Aspergillus flavus* and *Aspergillus parasiticus* were obtained from Department of Mycotoxins ,National Research Center (NRC, Dokki, Giza, Egypt).

II- *Bacillus subtilis* was obtained from Al- Azhar University, Faculty of Agriculture, Microbiology Department. The bacterial strain was maintained on Nutrient agar and stored at 5ºC.The inoculum of *Bacillus subtilis* was obtained by growing it in 5 ml of yeast extract (2%) (Mikrobiologie, MERCK), sucrose (20%) (YES broth, *p*H 6.5) at 30 ºC for 24 hours .The inocula of *Aspergillus flavus* and *Aspergillus parasiticus*

were obtained by growing them at 25 ºC on slants of potato dextrose agar (PDA) (Difco Laboratories) for 7 days until well sporulation occurred, YES broth was used as the growth medium for assessement of inhibition of aflatoxin production (*Munimbazi and Bullerman, 1998).*

Inhibition assay: The simultaneous antagonism assay was used, *Bacillus subtilis* was co-cultivated with *Aspergillus flavus* and *Aspergillus parasiticus* in 125 ml - Erlenmayer flasks containing 50 ml of sterilized YES broth , where the medium was inoculated with spores of each fungal strain and bacterial inoculum. Cultures were incubated at 28 ºC for 7 days in a static mode incubator, then analyzed for aflatoxins production. The cell –free supernatant fluids were prepared by centrifuging cultures at 5000 rpm for 15 minutes at 5 ºC in Beckman cooling centrifuge (Munimbazi and Bullerman, 1998).

Extraction of aflatoxins:. At the end of the incubation period, 25 ml of chloroform were added in separator funnel to each culture broth, then shaken on wrist –action shaker for 15 minutes , after phase separation the chloroform layer was removed and extraction repeated twice with an additional 25 ml of chloroform, then the combined extracts (lower layer) were passed through granular anhydrous sodium sulphate (Laboratory Chemicals Co) and evaporated under vacuum to dryness by rotary evaporator.

Determination of fungal growth (mycelia dry weight): The mycelia were collected from YES and washed twice with about 100 ml of distilled water, dried over night at 100ºC and then weighed, this step was repeated until getting constant weight.

Extraction of antifungal metabolites: The antifungal metabolites produced by *Bacillus subtilis* grown in 500 ml YES were precipitated by 40% ammonium sulphate (Laboratory Chemicals Co.) followed by centrifugation at 5000 rpm in Beckman cooling centrifuge for 15 minutes (*Munimbazi and Bullerman, 1998).*

Quantitative measurement of aflatoxins: Aflatoxins were determined using HPLC according to the procedure adopted by Rafaat (2008) . The mobile phase consisted of water: methanol: acetonitrile (70:17:17, v/v/v) at flow rate of 1 ml/min. The extraction and emission wave lengths for all aflatoxins were 362 and 360 nm, respectively. All chemicals used here were of HPLC grade (Aldrich Chem. Co.).

Many factors were affecting the production of antiaflatoxigenic substances from bacteria ,they include *p*H, incubation temperature, incubation period and composition of growth media (*Munimbazi and Bullerman, 1998).*

1- Initial *p*H of growth medium: Growth media (YES) of adjusted variable *p*Hs (4, 7, 9 and 10) were prepared, they were inoculated and incubated at 28 ºC for fixed

incubation period (7days), then aflatoxins content were extracted and determined using HPLC as a marker of antiaflatoxigenic activity of the bacterial secondary metabolites

2- . Incubation temperatures : Three incubation temperatures (25, 30 and 37 ºC) were tested for their effect on the production of antiaflatoxigenic substances from bacteria, YES broth was used as growth medium ,inoculated with spores of fungi and bacteria, then incubated for 7 days then, aflatoxins were extracted and quantified using HPLC.

3- Incubation periods (IPs): The production of antiaflatoxigenic substances from bacteria at three incubation periods (4, 7, and 10 days) was tested, YES broth was used as growth medium, inoculated and incubated at 28 ºC then, aflatoxins (B and G types) were extracted and HPLC-estimated as mentioned above.

4- Composition of growth media : two types of media yeast malt extract "YEM" broth and potato dextrose broth "PDB "were tested for their effect on the production of antiaflatoxigenic substances from bacteria , they were inoculated with spores of fungi and bacteria then incubated at 28 ºC for 7days, then aflatoxins were extracted to be HPLC determined.

5- Thermal stability: The thermal stability of antiaflatoxigenic substances of bacteria was tested by exposing the cell – free supernatant fluid of 72 h-old cultures of the bacteria to 80, 100 ºC for 30 minutes in a water bath and to121 ºC for 15 minutes in an autoclave and followed by rapid cooling with tap water , then inoculated with fungal spores and incubated at 28 ºC for 7 days, then aflatoxins extracted and tested for the antiaflatoxigenic activity of bacteria .

RESULTS AND DISCUSSION

The HPLC was the applied technique used for the estimation of aflatoxins produced by each *Aspergillus* species with or without the co-cultivation with *B. subtilis*. HPLC has been used in recent years because of its ease of operation and better quantification (Rafaat, 2008)

In the present study, four *p*H values were tested (4, 7, 9 and 10) for their effect on the amount of aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus,* when these fungi were co-cultivated with *Bacillus subtilis*. The amount of aflatoxins in control cultures of *A.flavus* and *A. parasiticus* (without bacteria) were higher at *p*H4 and *p*H7, but the amount of aflatoxins were reduced at strong alkaline *p*Hs (*p*H9 and *p*H10)

Concurrent cultivation of *Bacillus subtilis* with fungi led to relatively reduced production of these aflatoxins at *p*H 4, 7, 9 reaching minimum at *p*H10 (Table 1).

These results are in agreement with *Phae et al .,(1990),*who showed that antifungal metabolites produced by *Bacillus* species as *Bacillus subtillis* and *Bacillus pumilus* are very heat stable , active and resistant over a wide range of *p*H from 2 to 9.

Munimbazi and Bullerman *(1998)* reported that *Bacillus pumilus* inhibited aflatoxins production of *Aspergillus parasiticus* NRRL 2999, and the inhibitory activity was due to an extra cellular metabolites produced by bacterium in the growth medium. They also reported that the inhibitory effect was due to competition of nutrients and /or space between the bacterium and the mold or lowered *p*H, since the supernatant fluids and control of yeast extract sucrose (YES) had the same *p*H. Moreover, the inhibitory effect due to organic acids such as lactic acid which may be produced in the growth medium and cannot be excluded , these metabolites were heat stable and stable over a wide range of *p*H

Three incubation temperatures 25, 30 and 37ºC were tested for their effect on aflatoxins production. The antifungal metabolites of *Bacillus subtilis* showed higher antiaflatoxigenic activity at 25 ºC, when co-cultivated with *A. flavus*, and *A .parasiticus* (Table 2). In control cultures of *A .flavus*, and *A. parasiticus* (without bacteria), the amount of aflatoxins increased by increasing the temperatures ,also there were gradual increasing in mycelial growth,

These results agreed with Gibson and Gordon (1974), who reported that minimum temperature for growth of *Bacillus pumilus* was 25ºC. Moreover, Fiddaman and Rossall (1993) found that temperature had a considerable effect on production of antifungal volatiles by *Bacillus subtilis* against *Pythium ultimum* and *Rhizoctoni solani* . Munimbazi and Bullerman (1998) showed that *Bacillus pumilus* did not produce antifungal metabolites when grown at 4ºC, there is evident inhibitory activity observed in supernatant fluids of cultures grown at 25, 30 and 37ºC.

Three incubation periods 4,7 and 10 days were tested. In control cultures of *A flavus*, and *A .parasiticus* ,the amount of aflatoxins were reduced at incubation for 4 days, then increased at 7 days, by increasing incubation period the amount of aflatoxins were decreased, *Bacillus subtilis* showed higher antiaflatoxigenic activity at incubation for 4 and 10 days when co-cultivated with *A.flavus*, and *A.parasiticus* respectively (Table 3).

Doyle and Marth (1978) reported that total aflatoxins content after incubation for 6 days was maximum, but extended incubation cause decreasing in amount of aflatoxin G_1 , due to faster degradation than B_1.

Results obtained from Table 4 demonstrate the effect of two types of culture media (Potato dextrose broth and Yeast –malt extract broth) on production of

antifungal metabolites by *Bacillus subtilis* and aflatoxins production from *Aspergillus flavus* and *Aspergillus parasiticus*. Reduction of antiflatoxigenic activity was observed when *A. flavus* and *A. parasiticus* cultivated with *Bacillus subtilis* on PDB. On the other hand , when they were grown on yeast malt extract broth , there was high antifungal activity.

These results were in agreement with numerous findings of: Fiddaman and Rossall (1993), who found that PDB or potato dextrose agar(PDA) had a negative effect on inhibitory substances produced by *Bacillus* species.

Leifert *et al.,* (1995) tested the effect of various media including nutrient broth and PDB on the production of antifungal metabolites by *B. pumilus* and *B. subtillis.* They found very low antifungal activity when bacteria were cultured on nutrient broth, low or no antifungal activity was detected when potato dextrose broth (PDB) was used as growth medium, and the activity decreased rapidly when concentration of dextrose and potato extract were increased in the medium.

Munimbazi and Bullerman *(1998)* tested different media YES, nutrient broth+ yeast extract +dextrose (NYDB) , potato dextrose broth + yeast extract(PDBY) and Czapek-dox broth +yeast extract (CZKY), for detecting their effect on antiaflatoxigenic metabolites production by *Bacillus pumilus*. All media tested for production of antiaflatoxigenic metabolites supported the growth of *B. pumilus* and production of inhibitory metabolites. The Percentages of inhibition of aflatoxin production in supernatant fluids were 97.4 % for YES broth, 99.7 % for PDBY, 96.3 % for CZKY and 98.5% for NYDB , while the percentages of inhibition of mycelial growth were 30.2% for YES, 53.5 % for CZKY, 53.3% for PDBY and 32.9% for NYDB.

Table 5 showed the effect of thermal pre-treatments at 80, 100ºC and autoclaving at 121ºC on the thermal stability of antitoxinogen metabolites of *Bacillus subtilis* when co-cultivated with *A. flavus* and *A. parasiti*cus. The antifungal metabolites were highly thermostable and not affected by such thermal pre-treatment.

These results were in agreement with Munimbazi and Bullerman (1998) who mentioned that antifungal metabolites of *Bacillus pumilus* were thermostable at 80, 100ºC and autoclaving at 121ºC for 30 minutes.

CONCLUSION

Bacillus subtilis produced bioactive compound active against the plant pathogenic fungi, *A. flavus* and *A. parasiti*cus in simultaneous antagonism assays. The inhibitory activity was likely due to extracellular metabolites produced by the bacterium in the growth medium. The inhibitory metabolites were stable over a wide range of pH and were heat stable at 121 °C for 30 minutes. The potential use of the metabolites to control fungal should be investigated since many chemical fungicides are being removed from the commercial market. The chemical nature and biological characteristics of these antiaflatoxigenic metabolites are under investigation.

Table 1. Effect of different *p*H values on production of antifungal metabolites by *Bacillus subtilis* co-cultivated with *Aspergillus flavus* and *Aspergillus parasiticus* .

Cultivated organisms	*p*H 4			*p*H 7			*p*H 9			*p*H 10		
	Amount of aflatoxin		Mycelial dry weight g/100ml	Amount of aflatoxin		Mycelial dry weight g/100ml	Amount of aflatoxin		Mycelial Dry weight g/100ml	Amount of aflatoxin		Mycelial Dry weight g/100ml
	Toxin	µg/100ml		Toxin	µg/100ml		Toxin	µg/100ml		Toxin	µg/100ml	
Control *A. flavus*	B1	448.12	4.62	B1	570.65	5.82	B1	178.08	4.72	B1	97.14	4.42
	B2	10.11		B2	18.54		B2	7.17		B2	2.15	
	G1	18.26		G1	23.95		G1	16.51		G1	4.24	
	G2	6.23		G2	8.46		G2	3.82		G2	1.47	
A. flavus+ Bacillus subtilis	B1	97.12	3.92	B1	17.50	4.24	B1	0.05	2.04	B1	1.31	1.14
	B2	<LOD <LOD		B2	5.31		B2	3.16		B2	1.25	
	G1	<L OD		G1	<LOD		G1	0.42		G1	<LOD	
	G2			G2	0.72		G2	1.06		G2	0.375	
Control *A. parasiticus*	B1	427.12	4.02	B1	595.16	5.81	B1	314.09	3.61	B1	124.87	3.24
	B2	11.08		B2	13.21		B2	9.61		B2	23.75	
	G1	53.12		G1	76.21		G1	26.46		G1	7.23	
	G2	9.48		G2	11.48		G2	8.21		G2	11.25	
A. parasiticus + Bacillus subtilis	B1	128.09	4.02	B1	95.43	5.81	B1	185.21	3.61	B1	81.21	3.25
	B2	4.86		B2	5.3		B2	3.74		B2	1.32	
	G1	9.48		G1	40.21		G1	14.53		G1	4.27	
	G2	1.51		G2	0.62		G2	0.94		G2	0.31	

Organisms are cultivated under the same conditions of: temperature =28°C , cultivation medium: Yeast Extract Sucrose(YES) and incubation period =7 days

<LOD = under limit of detection of HPLC (1ng).

Table 2. Effect of different incubation temperatures on production of antifungal metabolites by *Bacillus subtilis* against *Aspergillus flavus* and *Aspergillus parasiticus* .

Cultivated organisms	Incubation temp.25°C			Incubation temp.30°C			Incubation temp.37°C		
	Amount of aflatoxin		Mycelial dry weight g/100ml	Amount of aflatoxin		Mycelial dry weight g/100ml	Amount of aflatoxin		Mycelial dry weight g/100ml
	Toxin	µg/100ml		Toxin	µg/100ml		Toxin	µg/100ml	
Control *A. flavus*	B1 B2 G1 G2	369.27 13.63 31.32 3.92	5.17	B1 B2 G1 G2	594.87 24.12 51.20 4.56	5.69	B1 B2 G1 G2	615.27 31.26 72.01 8.32	6.06
A. flavus+ Bacillus subtilis	B1 B2 G1 G2	<LOD <LOD 0.58 <L OD	4.10	B1 B2 G1 G2	<L OD 0.58 15.88 0.81	5.63	B1 B2 G1 G2	<LOD <LOD 3.16 0.20	6.21
Control *A. parasiticus*	B1 B2 G1 G2	249.24 15.61 19.22 3.84	4.28	B1 B2 G1 G2	487.25 17.62 25.23 6.42	5.24	B1 B2 G1 G2	530.18 26.81 48.63 9.87	5.87
A. parasiticus + Bacillus subtilis	B1 B2 G1 G2	18.10 9.02 7.43 0.92	4.28	B1 B2 G1 G2	116.61 <LOD 12.05 0.03	5.24	B1 B2 G1 G2	78.3 6.04 <LOD 0.86	5.87

Organisms are cultivated under the same conditions of incubation period =7 days , cultivation medium: Yeast Extract Sucrose(YES) and *p*H=7.
<LOD = under limit of detection of HPLC (1ng).

Table 3. Effect of different incubation periods on antifungal metabolites production by *Bacillus subtilis* against *Aspergillus flavus* and *Aspergillus parasiticus* .

Cultivated organisms	4 days			7 days			10 days		
	Amount of aflatoxin		Mycelial dry weight g/100ml	Amount of aflatoxin		Mycelial dry weight g/100ml	Amount of aflatoxin		Mycelial Dry weight g/100ml
	Toxin	µg/100 ml		Toxin	µg/100 ml		Toxin	µg/100ml	
Control *A. flavus*	B1	389.21	4.31	B1	540.25	5.82	B1	314.27	4.43
	B2	19.52		B2	28.41		B2	9.64	
	G1	35.21		G1	60.15		G1	49.26	
	G2	9.21		G2	20.52		G2	12.83	
A. flavus+ Bacillus subtilis	B1	12.51	3.32	B1	30.42	4.61	B1	43.62	4.06
	B2	8.09		B2	1.62		B2	0.24	
	G1	13.88		G1	7.36		G1	3.46	
	G2	1.44		G2	6.45		G2	<LOD	
Control *A. parasiticus*	B1	113.61	5.28	B1	337.24	5.62	B1	281.21	4.68
	B2	14.82		B2	24.05		B2	15.12	
	G1	29.27		G1	57.23		G1	38.62	
	G2	10.8		G2	18.83		G2	7.46	
A. parasiticus + Bacillus subtilis	B1	77.61	4.63	B1	76.42	5.02	B1	21.67	4.16
	B2	8.61		B2	0.21		B2	4.16	
	G1	15.24		G1	19.81		G1	12.13	
	G2	4.96		G2	8.62		G2	3.62	

Organisms are cultivated under the same conditions of: temperature=28°C , cultivation medium: Yeast Extract Sucrose(YES)

and *p*H=7. <LOD = under limit of detection of HPLC (1ng).

Table 4. Effect of two types of media on antifungal metabolites production by *Bacillus subtilis* against *Aspergillus flavus* and *Aspergillus parasiticus.*

Cultivated organisms	Potato dextrose broth (PDB)			Yeast malt extract broth		
	Amount of aflatoxin		Mycelial dry weight g/100ml	Amount of aflatoxin		Mycelial dry weight g/100ml
	Toxin	Mg/100ml		Toxin	µg/100ml	
Control *A. flavus*	B_1	350.22	2.52	B1	435.41	6.12
	B_2	20.14		B2	35.28	
	G1	63.63		G1	50.35	
	G2	13.88		G2	6.04	
A. flavus + Bacillus subtilis	B1	71.2	2.13	B1	26.81	6.02
	B2	8.61		B2	5.20	
	G1	31.2		G1	10.14	
	G2	1.24		G2	1.81	
Control *A.parasiticus*	B1	293.63	3.21	B1	240.65	4.72
	B2	17.52		B2	14.24	
	G1	43.62		G1	36.21	
	G2	13.21		G2	9.14	
A. parasiticus + Bacillus subtilis	B1	91.24	1.62	B1	71.12	4.26
	B2	6.02		B2	5.13	
	G1	14.21		G1	9.34	
	G2	3.65		G2	<LOD	

Organisms are cultivated under the same conditions of: temperature=28°C , incubation period =7 days and *p*H=7.

<LOD = under limit of detection of HPLC (1ng).

Table 5. Effect of thermal pre-treatment and autoclaving on the biostability of antifungal metabolites of *Bacillus subtilis* against *Aspergillus flavus* and *Aspergillus parasiticus.*

Cultivated organisms	80ºC			100ºC			121º C(Autoclaving)		
	Amount of aflatoxin		Mycelial dry weight g/100ml	Amount of aflatoxin		Mycelial dry weight g/100ml	Amount of aflatoxin		Mycelial dry weight g/100ml
	Toxin	µg/100ml		Toxin	µg/100ml		Toxin	µg/100ml	
Control *A.flavus*	B1 B2 G1 G2	360.15 32.41 60.64 20.01	4.81	B1 B2 G1 G2	360.15 32.41 60.64 20.01	4.81	B1 B2 G1 G2	360.15 32.41 60.64 20.01	4.81
A. flavus + Bacillus subtilis (cell free supernatant)	B1 B2 G1 G2	92.83 20.27 <LOD <LOD	4.32	B1 B2 G1 G2	<LOD 3.1 <LOD 16.1	3.51	B1 B2 G1 G2	61.67 17.13 18.02 12.07	4.13
Control *A. parasiticus*	B1 B2 G1 G2	295.24 37.08 65.67 16.27	5.22	B1 B2 G1 G2	295.24 37.08 65.67 16.27	5.22	B1 B2 G1 G2	295.24 37.08 65.67 16.27	5.22
A. parasiticus + Bacillus subtilis (cell free supernatant	B1 B2 G1 G2	0.28 30.1 1.58 5.65	3.84	B1 B2 G1 G2	5.76 2.05 0.94 1.69	5.01	B1 B2 G1 G2	74.26 2.47 9.81 5.61	2.12

Organisms are cultivated under the same conditions of: *p*H=7 , medium: Yeast Extract Sucrose(YES) , incubation period =7 days and temperature =28°C.

<LOD = under limit of detection of HPLC (1ng).

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تثبيط إنتاج الأفلاتوكسين بأحد الفطريات العضوية

نبيل عبد القادر صالح[١] ، محمد سمير عبد العزيز[١] ، نشوى محمد حلمى[٢]، إبتسام أحمد عبد الغنى[٢]

١ قسم الكيمياء الحيوية –كلية العلوم – جامعة عين شمس –القاهرة

[٢] *معهد بحوث صحة الحيوان– مركز البحوث الزراعية– وزارة الزراعة – الدقى – جيزة*

تعتبر الافلاتوكسينات من السموم الفطرية المنتجة بواسطة العديد من الفطريات مثل اسبيرجلس فلافس وباراسيتيكس حيث تنمو علي الحبوب مثل الفول السوداني ، الأرز والذرة مسببة العديد من الأمراض مثل سرطان الكبد والرئة وايضا العديد من الطفرات و تشوهات الأجنة .

تم فى هذه الدراسة استخدام جهاز الكروماتوجرافى السائل ذى الكفاءة العالية (HPLC) لقياس مستوى الأفلاتوكسين الناتج من فطريات الاسبيرجلس فلافس والباراسيتيكس و بعد زرعها مع إضافة بكتريا باسيلس سابتلس(*Bacillus subtilis*) *تحت معاملات معملية مختلفة* مثل قيمة الأس الهيدروجيني، فترة و درجة حرارة التحضين ،تأثير بيئات التنمية وايضا الثبات الحرارى عند درجات حرارة ١٠٠°م ،٨٠°م و المعاملة فى الأتوكلاف عند درجة حرارة١٢١°م.

وقد وجد أن لهذه البكتيريا نشاطاً مضاداً للأفلاتوكسين المنتج بواسطة اسبيرجلس فلافس عند الأس الهيدروجيني ١٠و تحضين لمدة ٤ أيام عند درجة حرارة ٢٥°م بينما ظهر هذا النشاط المضاد للأفلاتوكسين المنتج بواسطة اسبيرجلس باراسيتيكس عند الأس الهيدروجيني ١٠و تحضين لمدة ١٠ أيام عند درجة حرارة ٢٥°م و ايضا وقد وجد ان لهذه المادة ثبات حرارى عند درجات حرارة ١٠٠°م ،٨٠°م و المعاملة فى الأتوكلاف عند درجة حرارة ١٢١°م .