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Efficiency of *Bacillus Licheniformis* to Reduce Aflatoxin B1 Produced by *Aspergillus Flavus*

Mohsen H. Risan[*1] and Athraa H. Muhsin[**]
[*] College of Science, University of Al-Nahrain, Baghdad-Iraq.
[**] College of Science, University of Kufa, Najaf-Iraq.
[1]E-mail: Mohsen2000res@gmail.com.

Abstract

This study aimed to evaluate efficiency of the bioproduct of the bacterium *B. licheniformis* to degrade aflatoxin B1 produced by *A. flavus*. Results showed that 11 isolates belonging to the genus *Aspergillus* spp. Five isolates diagnosed as *A. flavus* and 5 isolates as *A. niger*. Colonies were identified by morphological characteristics, colony characterized of *A. flavus* were Yellow - Green color on PDA medium. The bacterium *B. licheniformis* is highly effective in inhibiting the fungus *A. flavus* p3 in the culture medium. Percentage of inhibition zone reached 85% caused by the fungus *A. flavus* compared with the control. The medium *Aspergillus flavus* parasiticus agar (AFPA) was used to test the ability of *A. flavus* for aflatoxin production. *A. flavus* varied in producing aflatoxin. Results revealed that the three isolates P2, P3 and P7 are aflatoxin producers, but P6 and P11 can't produce aflatoxin. On the other hand, results showed variation in *A. flavus* isolates in producing the aflatoxin. The isolate (P3) was more produce able of aflatoxin. Moreover, Results showed aflatoxin B1 after the treatment with the fungus *A. flavus*, recorded 32µg/kg, compared to treatment with *B. licheniformis* + *A. flavus* and the control treatment.

Keywords: *Bacillus licheniformis, Aspergillus flavus*, aflatoxin B_1.

Introduction

The mycotoxins produced by microorganisms are the most dangerous toxins, which are secondary metabolites produced by some species of fungi [1]. Aflatoxins cause significant economic losses as well as a series of risks to humans and animals[2]. Aflatoxins are carcinogenic to the liver and mutagenic [3]. Aflatoxin is the greatest contaminants of foodstuffs, that produced mainly by some fungi of the genus *Aspergillus*, especially *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. [1], [2], [4], [5]. Aflatoxins with low molecular weight are dissolved in some organic solvents such as methanol and chloroform, and the possibility of solubility in water [6]. Aflatoxin contains several important types of B_1, B_2, G_1 and G_2 [1], [4], [7]. Aflatoxin B1 (AFB1) is the most potent hepatocarcinogen in human and is classified by the International Agency of Research on Cancer (IARC) as a Group 1 carcinogen [8].

Turner[1]; Elshafie[4] refered that the toxin aflatoxin B1, B2, G1 and G2 diagnosed the aflatoxin depending on Ultra Violet (UV) and color (Blue or Green). They classified aflatoxin group into B and G depending on the brilliance under UV and the wavelength 365 nm. The numbers 1 and 2 that are written beside the letters refer to the sites of the separation of these compounds on the plates separation by special technology of Thin layer chromatography, there are (18) of aflatoxin, types B_1, B_2, G_1 and G_2 of the most important as well as M_1 and M_2 [9]. *A. flavus* risks, studied in several countries on cereal crops and food products. In Iran 51 samples of corn were mostly contain aflatoxin AFB1, AFB2, AFG1, and (AFG2) [7]. Studied [10], [11], ability the aflatoxin of food contaminating including corn, wheat, rice, lentils, sunflower and many of the crops, which may cause of illness or death of an animal or human, especially (B1, B2, G1 and G2). Aflatoxins are causing many diseases, particularly as a result of continuous exposure and their accumulation in human body and may lead to occurrence of cancers and damage to DNA [12]. Used several methods to reduce the aflatoxin. Microorganisms used in inhibition aflatoxin, *Bacillus* spp. Several isolates of *B. subtilis* were effectiveness in inhibition for fungi, *A. flavus*, *A. niger*, *Fusarium oxysporum*, *Rhizopus stolonifer*, [13]. *B. licheniformis*

isolated from soil samples and led to degradation of Zearalenone (ZEN) and reduce its concentrate and improve the digestion of nutrients in animal feed. [14]. Proved *B. licheniformis* (My75 strain) ability to inhibit *A. niger* and suppress of fungus sporulation, because its production to enzyme chitinase [15]. Therefore, this study aimed to use bioproduct of the bacterium *B. licheniformis* to reduce aflatoxin B1 produced by *A. flavus*.

Materials and Methods

Samples of barley grain were brought from local markets of Baghdad city, placed in sterile Polyethylene bags and kept at a temperature of 4 ºC until use.

Preparation of bioproduct

The use of the product which is a component from *B. licheniformis*, was obtained from the research fungi laboratory in the department of biology, College of Science, University of Kufa.

Isolation and identification of the fungus *A. flavus*

Samples of sunflower were purchased from local market, sterilized by sodium hypochlorite (2% for 2 minutes), then washed with distilled water and dried, seeds were cultured in petri dishes containing Potato Dextrose Agar (PDA) with 250 mg of the antibiotic Chloramphenicol per 1000 ml. The inoculated plates were incubated at 25 ºC for 5 days. The fungal isolates were purified and diagnosed by taxonomic features mentioned in [16].

Efficiency of bioproduct of the bacterium *B. licheniformis* in growth inhibition of *A. flavus* in culture medium

The bioproduct was mixed with PDA medium by dissolving 39g of the powder in 1000 ml of distilled water sterilized by autoclaving at 15 psi / inch^2 at 121 ºC for 15 min. After cooling, Chloramphenicol concentration at 250 mg/1000 ml was added, then dispense in 4 flasks each containing rate of 250 ml per flask, sterilized for 20 minutes and after cooling the bioproduct of the bacterium *B. licheniformis* 1 g/1000 ml was added, flasks were mixed well for the purpose of blending the product with the medium, then poured into 5 petri dishes and incubated 27 ºC for 24 hrs. The dishes were inoculated by disc diameter 0.5 cm of *A. flavus* at a rate of one disc in the center of each dish and concentration. Flask four was treated without bioproduct. Poured contents of PDA per flask in five sterile Petri dishes. Plates were incubated at 25 ºC for 24 hrs., inoculated by disc diameter 0.5 cm of *A. flavus* at a rate of one disc in the center of each plate. All plates incubated at 25 ºC for a week. Perpendicular colony diameters were measured and the percentage inhibition was calculated according to the formula.

$$\text{Inhibition } \% = R1 - R2 / R1 \times 100$$

Where R1: maximum radial growth of the pathogenic fungus colony of control treatment.

R2: maximum radial growth of the pathogenic fungus colony in dishes containing inoculate bacterial.

Determining fungi producing aflatoxin

The medium *Aspergillus flavus* parasiticus agar (AFPA) was used to differential isolates producing aflatoxin from *A. flavus* [16].

Cultured isolates on AFPA to test their ability to aflatoxin production and characterized isolates for aflatoxin producing ability to produce Aspergillic acid, which reacts with ferric ammonium citrate and gives bright orange-yellow colour in the background medium of developing colony within 48 hrs, and at 28 ºC as a characterized sign, while isolates non-producing to aflatoxin do not have the ability to produce Aspergillic acid.

The detection capability of *A. flavus* producing aflatoxin B1 by using Thin layer chromatography

Extraction of aflatoxin B1

Followed the method to [17], a quantity of 50g of barley seeds was taken and put in a blender with the amount 50 ml of chloroform then mixed for three minutes and filtrated by filter paper and the filtrate was concentrated in an oven at 60 ºC until drying.

The diagnoses aflatoxin B_1

To detect the aflatoxin, the layer chromatography was used Type (Glass sheets silica gel). According to the method used by [18], platelets activated for one hour before the

use with a separation system and component of chloroform and methanol (97:3) (attended the standard toxin B_1 dissolved 1 mg in 1 ml of chloroform. The standard aflatoxin B_1 was placed on for spots on a plate of silica gel with a distance of 1.5 cm from the edge by lattice tube at a rate of (10) microliters. Spots of fungus sample were placed beside standard aflatoxin at a distance 1.5cm between spots, then placed in a tank, developed plates were left to dry and checked under UV 360 nm to observe the starred with compared spots starred resulting from fungus extract and color of standard toxin. Aflatoxin concentration in micrograms per kg, was calculated using the equation described by [19]

Results and Discussion

Isolation and identification of *A. flavus*

Eleven isolates belonging to the genus *Aspergillus* spp, Table (1). (5) isolates diagnosed as A. *flavus*. Diagnosed isolates according to the qualities adopted in [16]. Colonies were identified by morphological characteristics, colony characterized of *A. flavus* were Yellow - Green Color on PDA. [2] They characterized by the microscopic and morphological features as *A. flavus* with addition of Lactophenol cotton blue. Presence of septate hyphae, colorless conidiophores, and conidiophores ends vesicle appeared in spherical shape (globose) with a series of one or two of sterigmata and conidia measured (3-6) µm and walls were rough [20]. It was isolated and others were undiagnosed. An isolate of the fungus *A. flavus* P3 was chosen be used in subsequent experiments.

Table (1)
Isolated species of the fungus Aspergillus spp. which isolated from sunflower seeds.

Isolate	*Fungal species*
P1	*A. niger*
P2	*A. flavus*
P3	*A. flavus*
P4	*A. niger*
P5	*A. niger*
P6	*A. flavus*
P7	*A. flavus*
P8	*A. niger*
P9	*Aspergillus* spp.
P10	*A. niger*
P11	*A. flavus*

Efficiency of the bacterium *B. licheniformis* bioproduct in growth inhibition of *A. flavus*

Table (2) shows that *B. licheniformis* is highly effective in inhibiting the fungus *A. flavus* P3 in the culture medium. Percentage of inhibition zone reached 85% caused by the fungus *A. flavus* compared with the control. These results are in agreement with Nabti, *et al*. [21] who isolated several isolates belonging to *B. licheniformis* from rhizosphere of the potato plant, and studied the antifungal activity of these isolates against several fungal species, including *A. flavus* and *A. niger*, compared with other species which belong to the genus *Bacillus*. Two isolates gave good efficacy in inhibiting more than 60%. This may due to the production of antibiotics or building of analyst enzymes or competition on nutrients or secretion of the chitinase enzyme which degrade fungi cell wall or production of siderophores that attract iron element. [22] Proved the effectiveness of B. *licheniformis* (MS3, MS1) in inhibiting the mycelium on PDA for many fungi including, *Rhizoctonia solani*, *Macrophomina phasiolina*, *F. culmorum*, *Pythium* sp, *Alternaria alternate*, *Sclerotium rolfsii*, [15].

They found that *B. licheniformis* strain MY75 has the ability to inhibit the growth of the fungus *Gibberella saubinetii*, *A. niger*, and suppression the germination of spores completely.

The results are simillar to those who studied [14] the ability of *B. licheniformis* to

deteriorate by zearalenone toxin (ZEN) produced by *F. graminearum, F.equiseti, F. crookwellense, F. semitectum, F. culmorum, F. cerealis*. On the other hand, [23] found that *B. licheniformis* is effective in suppressing the growth of *A. flavus* removing mycotoxins produced from *Aspergillus* sp with a reduction in AFB1 up to 74% and ochratoxin (OTA) up to 92%.

Table (2)
Efficiency of the bioproduct from B. licheniformis in the inhibition of the radial growth of A. flavus P3 in culture medium.

Treatments	*% Inhibition*
B. licheniformis A. flavus +	85
Control (*A. flavus*)	0.0

Determination of fungi producing Aflatoxin

AFPA was used to test the ability of *A. flavus* for aflatoxin production, Table (3). *A. flavus* varied in producing aflatoxin. Results revealed that the three isolates P2, P3 and P7 are aflatoxin producers, but (P6, P11), can't produce aflatoxin. This agrees with [30], who found that (AFPA) medium differentia between isolates producing aflatoxin, [24] showed the of ability isolated *A. flavus* in producing the aflatoxin (55%). Out of 43 isolates, 9 (20.93%) produced Aflatoxins (AFs) including (AFB1, AFB2, AFG1, AFG2). [25] found that differences between isolates in aflatoxin production, may due to the genes conferred by *A. flavus* responsible for the production of aflatoxin.

Table (3)
A. flavus isolates producing aflatoxin.

Isolates	*Producing of aflatoxin from A. flavus*
P2	+
P3	++++
P6	-
P7	++
P11	-

(+): Light brilliance (+ + +): High brilliance.
(++): Medium brilliance (-): No brilliance.

Detecting *A. flavus* P3 to produce aflatoxin B1

Results showed aflatoxin B1 after the treatment with the fungus *A. flavus*, reaching 32µg/kg, compared to treatment with *B. licheniformis* + *A. flavus* and the control treatment Table (3), Fig. (1).

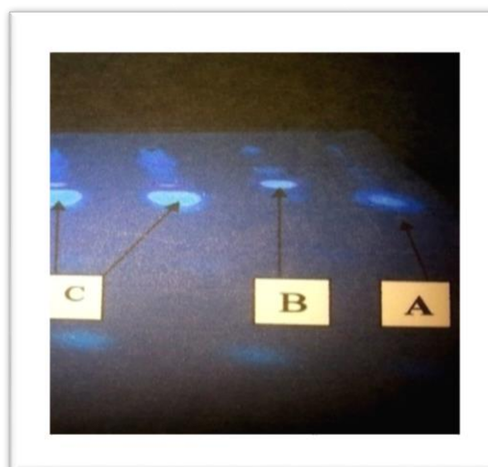

Fig. (1) Production of aflatoxin B_1 as detected by Thin layer chromatography.
A- Standard aflatoxin B_1.
B- Standard aflatoxin B_2 .
C- isolates produce aflatoxin B_1.

[26] Explained that aflatoxin in humans and animals food have certain limits, 20 µg/kg in human food, milk 0.5 µg/kg, beef cattle 300 µg/ kg, mature poultry 100 µg/kg, peanuts contamination by 33.3% in with aflatoxins B1 reaches up to (7-116) µg/kg. [27] referred that food products used by human, should not exceed 15 Nanograms of aflatoxin per kilogram of body weight. On the other hand, *B. licheniformis* has a high ability to secrete the chitinase enzyme, which is effective in inhibiting mycelium growth of *A. flavus* and *B. licheniform*. They remove all mycotoxins produced by *Aspergillus* sp. [21]. Toxicity removal may be due to transformation of AFB1 to metabolic products less toxic as (AFD1) [28], or mutation AFB1 to B2a as less toxic material and the motivate in aflatoxin deterioration [29].

Table (4)
Detecting A. flavus P3 as aflatoxin B1 producer.

Treatment	*aflatoxin concentration of toxins μg/kg*
A. flavus	32
A. flavus + B. licheniformis	0.0
B. licheniformis	0.0
Control	0.0

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الخلاصة

هدفت الدراسة الحالية إلى تقييم كفاءة المستحضر الحيوي المنتج من بكتريا *B. licheniformis* في تثبيط الافلاتوكسين B1 المنتج من قبل الفطر *A. flavus*. أشارت النتائج الحصول على ١١ عزلة تنتمي الى الجنس *Aspergillus* spp.، شخصت خمسة عزلات تعود للنوع *A. flavus* واخرى خمساً للنوع *A. niger*، تميزت مستعمرات الفطر *A. flavus* بلونها الاصفر– المخضر على الوسط الزرعي PDA. اثبت المستحضر الحيوي المنتج من البكتريا *B. licheniformis* كفاءة في تثبيط النمو الشعاعي للفطر *A. flavus*، اذ بلغت ٨٥% مقارنة مع معاملة السيطرة. استعمل الوسط التميزي للانواع الفارزة للافلاتوكسين (AFPA) ، اظهرت عزلات الفطر *A.flavus* تباين في انتاجها لسم الافلاتوكسين وكانت العزلة (P3) الاكثر انتاجاً له ، من جانب اخر أظهر المستحضر الحيوي المنتج من بكتريا *B. licheniformis* فعالية في تحطيم تركيز الافلاتوكسين اذا بلغت بلغت ٣٢ مايكروغرام/ كغم في معاملة الفطر *A. flavus* مقارنة مع معاملة الفطر *B. licheniformis + A. flavus*.