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INVESTIGATE THE OPTIMAL PRODUCTION CONDITIONS OF FUMONISIN B1 FROM LOCAL ISOLATION OF *FUSARIUM VERTICILLIOIDES*

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ABSTRACT
Fumonisin B1 (FB1) is a mycotoxin produced mainly by the fungus *Fusarium verticillioides* in food and feed and it has been related with high rates of human esophageal cancer and with increased incidences of neural tube defects in infants of mothers consuming maize-based products contaminated with this toxin. Therefore the study aimed to investigate the optimal condition of FB1 production by *F. verticillioides.* Thirteen local isolates of *F. verticillioides* isolated from maize screened for their ability to produce FB1 using ELISA and TLC techniques. Among these isolates, FV1was the most efficient one in production of FB1 (175.39 ppb) on patty maize media, while the concentration of FB1 for the other isolates were ranged between (21.31-170.51ppb). The optimum production conditions of FB1 using solid state fermentation included: patty maize medium with 30% moisturizing ratio at 20ºC incubation temperature for twenty one days.

KEYWORDS: *Fusarium*, FB1, maize, optimal condition.

INTRODUCTION

Several *Fusarium* species are capable of producing fumonisins, the most important species are *F. verticillioides* (formerly: *moniliforme*) and *F. proliferatum*, both included in the *Gibberella fujikuroi* species complex. Also, fumonisins can be produced by *F. oxysporum*, *F. beomiforme*, *F. napiforme*, *F. dlamini*, *F. globosum*, *F. nygamai*, *F. anthophilum*, *F. polyphialidicum*, *F. subglutinans* and *F. thapsinum*, and *Alternaria alternata* (WHO, 2000; Kumar *et al.,* 2008; Yazar & Omurtag, 2008). As well as, *Aspergillus niger* has been found to produce fumonisins such as FB2, FB4 and new series FB6 have been identified from this fungus (Huffman *et al.,* 2010). Fumonisins are polyketide mycotoxins found mainly in corn crop, and in other grains such as: rice, wheat and oat (Mallmann *et al.,* 2001; Park *et al.,* 2005). Fumonisins consumption has been related with esophageal cancer in humans and some other tumors in animals (Creppy, 2002; Zain, 2011). FB1 produced by *Fusarium* species have been first isolated from maize by Bezuidenhout *et al.* in 1988, and then maize based products (Sydenham *et al.,* 1991), such as tortillas (Stack, 1998), and beer (Scott & Lawrence, 1995), as well as other commodities like rice (Park *et al.,* 2005), black tea leaves (Martins *et al.,* 2001), asparagus (Logrieco *et al.,* 1998), and pine nuts (Marin *et al.,* 2007). Many factors affect the production of FB1 by *Fusarium spp.* have been well studied including; solid substrates and liquid substrates (Vismer *et al.,* 2004), temperature (Marin *et al.,* 1999; Dilkin *et al.,* 2002), water activity (a_w) (Marin *et al.,* 1999; Samapundo *et al.,* 2005), pH (Keller *et al.,* 1997), addition of nitrogen repressor (Shim & Woloshuk, 1999), aeration of the substrate (Keller *et al.,* 1997), and addition of FB1 precursors (Branham and Plattner, 1993). The study aimed to investigate optimal condition of FB1 production by *F. verticillioides* using ELISA and TLC technique.

MATERIALS & METHODS

Isolation of *F. verticillioides*

Thirteen local isolates of *F. verticillioides* isolated form maize samples that collected from local markets and silos in Baghdad. Identification of these local isolates based on colony morphology and microscopic appearance on potato dextrose agar (PDA), Spezieller Nährstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA) according to the fungal keys of Booth (1977) and Leslie & summerell (2006) and the diagnosis confirmed by species specific PCR according to Mule *et al.,* (2004) primers.

Spore Suspension

Spore suspension for *F. verticillioides* prepared according to the method described by Acharlyakul (2000).

Screening of *F. verticilioides* isolates for FB1 production

FB1 production on patty maize medium was achieved according to Vismer *et al.* (2004). Whole grains were ground into a fine powder by coffee grinder. Thirty gram of each grains was taken in Pyrex Petri dishes (15cm diameter) and thirty ml of distilled water was added. The preparation was autoclaved at 121ºC for 30min. and allowed to stand overnight. The sterilization was repeated next day for 30min. Each of the thirteen isolates was cultured on patty maize medium in duplicate for each isolate; media were inoculated

with 1ml of spore suspension of each tested isolates. Inoculated Patties were incubated in dark at 25ºC for four weeks. After which they were dried in a hot air oven at 50ºC for overnight. Harvested dry patties were grinded by using a coffee grinder to a fine powder stored in deep freezer (-20°C) and used for FB1 analysis.

DETERMINATION CULTURE CONDITIONS FOR FB1 PRODUCTION

1. Effect of different substrates on FB1 production

Selected isolate of *F. verticilliodes* was grown in each of three different media (maize, rice and wheat patties). Duplicate cultures of each media were inoculated with one ml of spore suspension and then incubated at 25°C for 28 days. At the end of incubation period, they were dried in a hot air oven at 50ºC for overnight. Harvested dry patties were grinded by using a coffee grinder to a fine powder stored in deep freezer (-20 °C) and used for FB1 analysis.

2. Effect of Different Temperature on FB1 production

Three different temperatures (20, 25 and 30) ºC were used to determine the optimum temperature for FB1 production on patty maize. Each plate was inoculated with one ml of spore suspension. All plates were incubated for 28 day, at the end of incubation period; they were dried in a hot air oven at 50ºC for overnight. Harvested dry patties were grinded by using a coffee grinder to a fine powder stored in deep freezer (-20 °C) and used for FB1 analysis.

3.Effect of Different Incubation Period on FB1 Production

Four different time period (7, 14, 21 and 28) days were used to determine the optimum incubation period for FB1 production on patty maize. Each plate was inoculated with one ml of spore suspension and incubated at 20ºC for specific day. At the end of incubation period; they were dried in a hot air oven at 50ºC for overnight. Harvested dry patties were grinded by using a coffee grinder to a fine powder stored in deep freezer (-20°C) and used for FB1 analysis.

4.Effect of Different Moisturizing ratio on FB1 production

Various amount of sterile water (30, 40 and 50%) were used to determine the optimum moisturizing ratio for FB1 production on patty maize. Each plate was inoculated with one ml of spore suspension. All plates were incubated for 21 day at 20ºC, after the end of incubation period; they were dried in a hot air oven at 50ºC for overnight. Harvested dry patties were grinded by using a coffee grinder to a fine powder stored in deep freezer (-20 °C) and used for FB1 analysis.

DETECTION & QUANTIFICATION OF THE FB1

1. Thin Layer Chromatography analysis

Ten grams of grinded patty maize culture sample was weighed and transferred into a 250ml beaker and mixed with 50ml of acetonitrile: water (50:50, v/v). The beaker was covered with aluminum foil and shaken for 30min. The mixture was filtered through Whatman No. 4 filter paper. The filtered solution was evaporated to dryness at 50°C. Dried extracts stored at 4°C until TLC analysis (Sreenivasa *et al.*, 2012). TLC plate was activated at 110˚C for 1hr., 2cm from the bottom and 2cm from the other sides of the plate was left. Each of crude extract and FB1 standard (5mg) were dissolved in acetonitrile: water (50:50, v/v) separately. Ten µl of each standard and crude extract were applied separately on the same plate. Two replicates were done and allowed the spots to dry.

The plate was placed in a developing tank which developed with a solvent system of acetonitrile: water (85:15 v/v) as described by (Desjardins *et al.*, 1994). When the solvent system reached to 2cm from the bottom of the plate, the plate was removed from the tank and allowed to dry. The plate was sprayed with; 0.5% P-anisaldehyde in (methanol: sulfuric acid: acetic acid, 90:5:1, v/v/v) and heated at 100ºC for 5min (Bailly *et al.*, 2005).

2. ELISA analysis

Sample extraction and measure the concentration of FB1 according to manufacture instruction of Biooscientific/ USA.

Preparation of standard FB1

Standard FB1 (Enzolife Science/ USA) was prepared by dissolving 5 mg of FB1 in 5 ml of acetonitrile: water (50:50, v/v) and then kept at -20°C.

Purification of FB1

all extracts were combined, evaporated and dissolved in 4ml of ACN: H_2O (50:50, v/v) and filtered through a millipore filters 0.45 µm and then two ml of filtered extract was added to 6ml of 1% KCl and loaded into a C18 clean-up column, which preconditioned with 2ml ACN followed by 1% KCl. The column was rinsed with 2ml of 1% KCl followed by 2ml of ACN/ H2O (85+15, v/v). The rinses were discarded, and air was forced through the column to expel all the rinse solution. FB1was eluted from the column with 2ml of ACN: H2O (70+30, v/v), and then the eluted was transferred to small glass vial and evaporated to dryness, then freeze-stored until analysis (Sreenivasa *et al.*, 2012).

Statistical analysis

All analysis was performed using the statistical package (SPSS) version thirteen; the data were expressed as mean, standard deviation SD, percentage. ANOVA was used to analyze repeated measurement. Results were determined as very high significant at ($P \leq 0.001$), high significant ($P < 0.01$) and significant at ($P < 0.05$) and non significant at ($P > 0.05$).

RESULTS

The ability of thirteen isolates of *F. verticillioides* for FB1 production was determined using patty maize medium as a solid state fermentation as shown in Figure (1), the production of FB1 in thirteen isolates was detected by ELISA. Results in Table (1) showed that all *F. verticillioides* isolates were FB1 producer. The high level of FB1 production was 175.39 ppb for isolate FV1 on patty maize media, while the other isolates were ranged between (21.31-170.51ppb). According to these results, isolate FV1 was selected to study optimal production condition of FB1, since it gave the highest productivity.

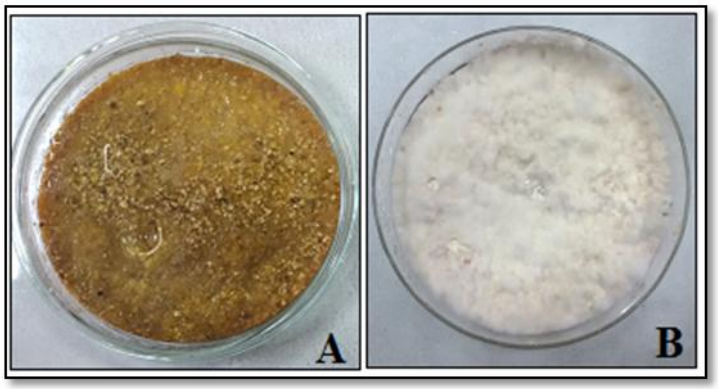

FIGURE 1: Patty maize culture; A- before inoculation, B -after inoculation

TABLE 1: FB1 production by of *F. verticillioides* isolates on patty maize media after 28 days, growth at 25ºC.

Isolates	Source*	FB1 ppb (Mean+SD)**
FV1	M	175.39+3.55[a]
FV2	S	170.51+4.27[b]
FV3	M	168.30+5.68[b]
FV4	S	167.16+5.09[b]
FV5	M	167.19+2.84[b]
FV6	M	165.49+3.88[c]
FV7	S	124.27+3.04[d]
FV8	S	124.29+3.66[d]
FV9	M	121.54+2.27[d]
FV10	S	86.19+2.91[d]
FV11	S	62.46+2.26[e]
FV12	S	34.31+2.58[f]
FV13	S	21.31+2.18[g]

*M= local markets, S= Silo

** Different letter within the same column are significantly different (P≤0.05)

Optimal Condition for FB1 Production

1. Effect of the Substrate

Three substrates were used: rice, wheat and maize, which have been used as carbon source able to support the growth and stimulate the production of FB1 by *F. verticillioides* strain FV1. The results in Table (2) revealed that the substrates varied in their ability to induce FB1 production. In this study high production of FB1 occurs in maize (169.48 ppb) followed by wheat (155.55 ppb) and then rice (148.06).

TABLE 2: FB1 production on different substrate media after 28 days, growth at 25ºC

Substrate	FB1 ppb (Mean+SD)**
Maize	169.48+4.74[a]
Wheat	155.55+5.43[b]
Rice	148.06+3.55[c]

**Different letter within the same column are significantly different (P≤0.05).

2. Effect of Temperature

Temperature is one of important factors that influence on FB1 production, therefore it needs to be optimized, and FB1 production was achieved at various temperatures (20, 25 and 30 º C). The optimum temperature for FB1 production by FV1 isolate was found to be 20°C (157.02 ppb). However, the increase in the incubation temperature lead to decrease toxin production as it was illustrated in Table (3).

Table (3): FB1 Production at different temperature after 28 days, on patty maize media

Temperature (ºC)	FB1 ppb (Mean+SD)**
20	157.02+7.59[a]
25	154.46+4.94[ab]
30	147.29+3.74[b]

**Different letter within the same column are significantly different (P≤0.05)

3. Effect of Incubation Period

FB1 production by FV1 isolate was observed during; 7, 14, 21, 28 days. The results revealed that maximum FB1 production (154.16 ppb) was achieved after 21 days; however it decreased down to (149.29 ppb) when prolong incubation to 28 days as shown in Table (4).

TABLE 4: FB1 production for different incubation period at 20°C on patty maize media

Incubation period (day)	FB1 ppb (Mean+SD)
7	138.21+2.61[a]
14	145.40+3.54[b]
21	154.16+4.26[c]
28	149.29+3.06[b]

**Different letter within the same column are significantly different (P≤0.05)

4. Effect of Moisturizing Ratio

Table (5) showed the effect of different moisturizing ratio on FB1 production by the isolate FV1. A moisturizing ratio of 30% allowed higher levels of toxin accumulation in patty maize cultures than 40% (123.18ppb) and 50% (115.15ppb).

TABLE 5: FB1 production in different water content on patty maize media at 20°C for 21 Days

Water content (%)	FB1 ppb (Mean+SD)**
30	154.13+4.96[a]
40	123.18+2.83[b]
50	115.15+3.21[c]

**Different letter within the same column are significantly different (P≤0.05)

Production and Purification of FB1

FB1 have been produced under optimal condition, using patty maize culture with moisturizing percentage of 30%, and incubated at 20ºC for twenty one days. The extracts of inoculated patty maize substrate with FV1 were analyzed for FB1 content using both TLC and ELISA kit techniques. Figure (4-11) showed TLC plate revealed the FB1 spot after spraying with a mixture of 0.5% p-anisaldehyde, having R_f values of 0.46 appeared as brown spots under visible light.

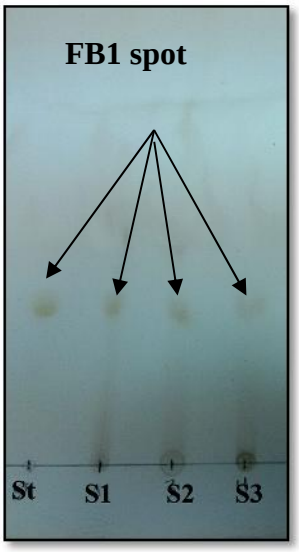

FIGURE 2: Detection of crude and standard FB1 by TLC under visible light, **St:** standard FB1, **S1, S2** and **S3:** samples of purified FB1

DISCUSSION

The ability of thirteen *F. verticillioides* isolates for FB1 production was determined using patty maize medium as a solid state fermentation at 25°C after 28 days of incubation. ELISA technique was used to measure the concentration of FB1. A number of studies found that ELISA is suitable for the rapid detection of FB1 in food and feed samples, because it is sensitive, quick and accurate (Barna-Vetro *et al.*, 2000; Wang *et al.*, 2011). The study observed that all thirteen isolates of *F. verticillioides* had the ability to produce FB1 in different concentration, because these isolates had *fum1* gene (PKA gene), which required for FB1 biosynthesis (Proctor *et al.*,1999). The high level of FB1 production by *F. verticillioides* FV1 isolate was used to study the factors that affect the production of FB1.

In this study the first factor studied was the effect of different substrates (maize, rice and wheat) were used as cultures media to support the growth and stimulate the production of FB1 by *F. verticillioides* FV1 isolate. According to ELISA analysis found that patty maize medium gave good production of FB1 as compared with rice and wheat patties media. The highest production of FB1 by *F. verticillioides* was obtained from patty maize medium, (Vismer *et al.,* 2004; Bailly *et al.* 2005), therefore, patty maize medium is the best medium for screening large numbers of *F. verticillioides* isolates which produce FB1, because it is simple, inexpensive and available. The concentration of FB1 depends on the origin of isolates including: a host plant and a geographic region. Under laboratory conditions; isolates of *F. verticillioides* which obtained from maize synthesis more FB1on maize culture than these which obtained from wheat or barley (Visconti & Doko, 1994). The research study by Nelson *et al.* (1991) also showed that strains of *F. verticillioides* isolated from various substrates and geographic areas, for FB1 production, found that the most strains from maize-based feed (16/20) were high producers, while among the strains isolated from millet and sorghum grain, were low producers (4/15). Temperature is one of important factors that affect FB1 production, so it need to be optimized; FB1 production was achieved at various temperature (20, 25and 30 ºC), the optimum temperature for FB1 production by FV1 isolate was found to be 20 ºC, as noticed in Table (3), the same result obtained by Hinojo *et al.* (2006). Several studies found the optimum temperature for FB1 production range between 20-25ºC (Marin *et al.,* 2004; Bailly *et al.,* 2005; Mogensen *et al.,* 2009; Medina *et al.,* 2013). The increase in the incubation temperature lead to decrease FB1 production because temperature can effects all vital events in the cell directly through influence in the genetic material, enzymes and lipids in the cell membrane and lead to influence in; fungal growth, germination, metabolites formation and sporulation, also the activity of fungi declined exponentially when the temperature for growth, reached more than optimum temperature (Raghvarao *et al.,* 2003). The study observed that incubation period, also effect on FB1 production. The optimum FB1 production was 21 days at 20 ºC and then decrease when prolonged the incubation period to 28 days. Similar with Alberts *et al.*(1990) that found the higher FB1 production at 21days.. Other studies reported that the incubation period for FB1 production by *F. verticillioides* on corn cultures was range between fourteen days to five weeks (Vismer *et al.,* 2004; Bailly *et al.,* 2005).The decrease in FB1 contents after several weeks of culture may be due to both; the decrease of metabolic precursors and enzymatic cleavage of the toxins within the culture medium (Le Bars *et al.,* 1994). Another important factor that effect on the FB1 production observed in this study was moisturizing ratio. The optimum moisture content obtained at 30%. Solid substrates used in solid state fermentation are insoluble in water therefore; water will have to be absorbed on to the substrate particles, which could be used by the fungi for growth and metabolic activity. Therefore the degree of hydration of the substrate plays an important role on the growth fungi and then the toxin production (Pandey, 1992). The importance of water for solid state fermentation is attributed to the fact that the majority of microbial cells require about 70-80% moisture content for new cell biosynthesis. Furthermore, moisture level is very important factors affecting stability, biosynthesis and secretion of fungal metabolites (Pandey *et al*., 1999).

The optimum moisture is depended on some other parameters such as nature of substrate, microorganism and studied metabolite. Low moisture may reduce the solubility and swelling capacity of substrate causing high-water tension, decreasing growth and metabolite production. A reduction in metabolite biosynthesis at higher moisture than the optimum is due to hindrance of microorganisms growth through reduction in inter particle space, decreased porosity, gummy texture, alteration in particles of substrate structure and impaired oxygen transfer (Rathakrishnan and Nagarajan, 2011).

Marin, *et al.* (2004) found that temperature and moisturizing ratio of substrate are important factors for growth of mycotoxigenic *Fusarium* spp., that are mostly mesophilic and hygrophilic fungal strains and acting as parasites on living plants.Infection of maize with *Fusarium* spp. and contamination by different mycotoxins are generally affected by many factors including environmental conditions (temperature, humidity) and pre-and postharvest handling. These factors do not influence infection independently but most often there are complex interactions. The higher concentration of nutrients and the loss of consistency due to the temperature treatment may enable the moulds to colonize the corn easily also, the moisture conditions during the growing season as well as during storage are often pointed out to affect maize infection by *Fusarium* spp. and mycotoxins synthesis (Fodor *et al.,* 2006). Acetonitrile and methanol are the best extraction solvents for the fumonisins, because FBs are very polar, therefore the solubility of FBs are consistent in methanol, acetonitrile and their aqueous forms (WHO, 2000). In this work, the extraction of FB1 was performed with acetonitrile-water (50:50). The major important of using ACN:H_2O for extraction is due to lower toxicity of this solvent compared with other solvent, therefore could be used as oral administration for experimental animals without further purification of FB1 (Bailly *et al.,* 2005).TLC considered an economical analytical method that was used for the detection of FB1 production by *Fusarium* species. It is an important tool in countries that often produce and export agricultural commodities, because do not have expensive equipment at their disposal, it is a relatively simple and useful technique (Maheshwar and Janardhana, 2010).

CONCLUSION

Our findings demonstrate that the optimal production conditions for FB1 by using patty maize culture with 30% moisturizing ratio at 20ºC incubation temperature for twenty one days.

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