ISOLATION AND IDENTIFICATION OF AFLATOXIN B1 PRODUCING FUNGI FROM STORED WHEAT IN SOME SILOS OF BAGHDAD

Mohamed A. M. **E. I. Al – Shamary**
Researcher **Prof.**
Dept. Food Sci, Coll. of Agric. Eng. Sci. University of Baghdad
mohamedayden142@gmail.com **elham.ismael@coagri.uobaghdad.edu.iq**

ABSTRACT

The aim of this study was isolation and identification of fungi producing aflatoxin B1 from wheat which was stored for four months in some silos in Baghdad , including Al Taji Silo, Khan Bani Saad Silo and Khan Dhari Silo. Those Silos were included in the agricultural plan for the marketing season 2020. Different types of fungi were obtained. They were initially identified according to cultural and morphological characteristics as *Alternaria spp*, *Aspergillus flavus* , *Aspergillus niger*. The ability of fungal isolates to produced aflatoxin was tested using ammonia vapor. All of the *Aspergillus flavus* isolates showed their ability to produce aflatoxin, unlike the other types which showed negative result. Aflatoxin produced by *Aspergillus flavus* was estimated using (HPLC) technique, and results showed that the highest concentration (978.5 µg / ml) was obtained from an isolate in Al-Taji Silo. This isolate was genetically identified by genetic analysis of 5.8S rRNA gene. Gene sequencing identity of local isolate was 99% in compassion to sequencing of *Aspergillus flavus* strain USMG09 recorded in the gene bank as ID: MK992254.1 .

Keywords:, *Aspergillus flavus* , HPLC, Al Taji , Khan Bani Saad , Khan Dhari
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عزل وتشخيص الفطريات المنتجة لسموم الافلاتوكسين B1 من الحنطة المخزنة في بعض سايلوات بغداد

محمد آيدن محمد الهام اسماعيل الشمري
باحث أستاذ
قسم علوم الأغذية، كلية علوم الهندسة الزراعية، جامعة بغداد

المستخلص

هدف البحث الى عزل وتشخيص الفطريات المنتجة لسموم الافلاتوكسين B1 من الحنطة المخزنة لمدة اربعة اشهر في بعض سايلوات بغداد والتي شملت سايلو التاجي وسايلو خان بني سعد وسايلو خان ضاري المشمولة بالخطة الزراعية لموسم التسويق 2020، تم الحصول على انواع مختلفة من الفطريات، شخصت بصورة اولية وأعتماداً على الصفات المزرعية والمورفولوجية على انها *Aspergillus flavus , Aspergillus niger, Alternaria spp*، تم الكشف عن قدرة العزلات الفطرية لأنتاج سموم الافلاتوكسين بأستعمال بخار ألامونيا، أظهرت جميع عزلات *A. flavus* قدرتها على الانتاج وبنسب متفاوتة، عكس الانواع الاخرى التي اعطت نتيجة سالبة للفحص. قدرت سموم الافلاتوكسين B1 المنتجة من عزلات *A.flavus* بأستعمال تقنية (HPLC) وأظهرت النتائج ان أعلى تركيز كان 978.5 مايكروغرام /مل للعزلة *A.flavus* المعزولة من سايلو التاجي، شُخصت هذه العزلة على المستوى الجزيئي للتأكد من هويتها بواسطة تتابعات الجين 5.8S rRNA وبنسبة تطابق 99% مع تتابعات السلالة *Aspergillus flavus* strain USMG09 والمسجلة في بنك الجينات العالمي تحت ID: MK992254.1 .

الكلمات المفتاحية: *Aspergillus flavus*، HPLC، التاجي، خان بني سعد، خان ضاري.
بحث مستل من رسالة ماجستير للباحث الاول

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INTRODACTION

Wheat (Triticum aestivum L) is the most important cereal crop, representing the staple food for 40% of the world's population(23). Wheat provides the human body with about 20% of the daily caloric requirement and 21% of the daily protein in the diet(29). The importance of wheat as a staple food because it contains 11.3% protein, 13.2% dietary fiber, 1.8% fat, 59.4% carbohydrates, minerals 1.7% and 12.5% moisture (17,18). Wheat is used in the manufacture of bread, flour, pasta, confectionery products, cosmetics, bio-fuel, and as animal feed (11). Storing wheat under good conditions and strategically to maintain its quality is necessary to ensure its continued supply throughout the year and to maintain stable food security (3,31). Where the properties of wheat are affected in terms of nutritional value, shape, color and moisture content during storage under bad conditions such as high temperatures, humidity and lack of ventilation, and thus it is vulnerable to attack by fungi because wheat is a good source of carbohydrates and protein is necessary for the growth of fungi, which negatively affects the quality of wheat and its lack of acceptance by Consumer, causing economic losses (6,22). In addition to the harm of human and animal health as a result of the ability of some fungi genetically produce mycotoxins and make them unacceptable as a food source(8,12) The most common contaminating grains fungi belong to the genera *Aspergillus, Alternaria, Penicillium, and Fusarium* (6,28), where these species invade grain in the field, after harvest, or during storage and remain alive for long periods (33). Aflatoxin is one of the main and important mycotoxins worldwide mycotoxin produced by *Aspergillus flavus* on wheat during storage (14,23). Aflatoxin B1 is the most dangerous type due to its health risks, which was ranked 1st by the International Agency for Research on Cancer (IARC) due to its carcinogenic effect (16). This mycotoxin is closely related to long-term exposure to hepatocellular carcinoma in humans, immunodeficiency and malnutrition (16,35). Despite the use of modern food safety applications, wheat contamination represents a serious challenge due to its infection with harmful fungi that produce mycotoxins, and methods of detection are still ongoing and widespread (28) . Accurate and sensitive detection of the types of fungi that produce mycotoxins is necessary because some fungi produce a different group of dangerous mycotoxins (30).The aim of the study is to conduct a survey of fungi contaminated wheat grains stored in Baghdad silos, which were covered by the agricultural plan for the marketing season 2020, to identify the most common types of fungi and to reveal the ability of fungi to produce Aflatoxin B1.

MATERIALS AND METHODS

Sources of isolates Thirty samples of local wheat were drawn randomly, weighing 1 kg, which belong to the 2020 marketing season and stored for a period of four months from the date of harvest in silos of Baghdad governorate, represented by Khan Dhari silo, Khan Bani Saad silo and al-Taji silo. the samples were placed in clean polyethylene bags with a special code for each model and were transferred to the laboratory for isolation.

Culture medium

Potato dextrose agar (PDA)

Medium was prepared as described (by Himedia limited laboratories) by adding 39 grams of the prepared medium to a liter of distilled water. Medium was autoclaved at 121° C and 15 pound/inch^2 for 15 min. After sterilization, medium was cooled to 45 ^{o} C process, antibiotic and chloramphenicol with (0.025%) was added to prevent bacterial growth. This medium was used for isolation and examining of fungi

Potato dextrose broth (PDB)

Medium was prepared according to(7). 250 gm. were boiled of well-washed and cut potatoes without removing their peel in a liter of distilled water for 45 minutes, then filtered with a dull cloth then 20 gm of dextrose were added. Volume was completed to 1 liter with distilled water and distributed in 250 ml beakers at 100 ml in each one. Medium was autoclaved at 121° C and 15 pound/inch^2 for 15 min. This medium was used for isolation and examining of fungi, more over Production of Aflatoxin

Coconut extract agar (CEA)

Medium was prepared according to(34).100gm coconut powder was mixed with 600 ml of biolled distilled water and filtered with a piece

of cloth. The semi clear extract was transferred to separated funnel. 1.5% agar was added to the lower layer (470ml). The mixture (media) was autoclaved at 121° C and 15. pound/inch^2 for 15 min.. This medium was used for Aflatoxins assay.

Methodology

Isolation and purification : Isolation process was conducted in two ways first is a pour plate method which was described by(37). After preparing the decimal dilutions for the samples one ml of each dilution was poured in a dish containing PDA media and incubated at 30^oC for 5 days . The purification process of isolates was carried out by sub culturing into PAD media and incubation at 30^oC for 5 days. The percentage of fungi frequency associated with the wheat samples was calculated according to Agarwal and Sinclair by the following equation:

%Fungi appearance = number of samples in which the fungus appeared / number of total samples x 100

Identification of fungi isolates

Cultural and morphological characteristics of Fungi isolates were carried out on PDA using lactophenol blue dye (according to the taxonomic keys) which were described, (10,13).

Detecting the ability of isolates to produce aflatoxin:

Detection of Aflatoxin Ammonia vapor method, according to (21), was used to examine the ability of fungal isolates to produce Aflatoxin, using coconut media (which was inoculated by stabbing method from young culture of 5 days). plates were incubated at 25^oC for 5 days, then filter paper was immersed in 25% ammonia solution and placed on the internal surface of the growth plate cover . The plates were incubated upside-down at 25^oC and the results were recorded after 24 hours.

Identification of aflatoxin:

spore suspension preparation Spore suspension of 16 producing Aflatoxin isolates was prepared as described by (36). The spores were counted according to (9), using hemocytometer and the amount of spores for all isolates was adjusted to 10^7 spore/ml.

Extraction of aflatoxin Extraction of toxin was carried out according to method of (22). which contents of the flasks were separately filtered using filter paper Whatman No.1 to get rid of fungal biomass, then 25 mL of filtrate was transferred to a 250 mL separation funnel, then 100 ml of chloroform was added, the mixture was shaked, expelling gases accumulated in separating funnel as needed, and leaving the separation funnel for 15 minutes. Then the lower layer was passed on a filter paper containing 10 g of anhydrous sodium sulfate (Na_2SO_4), then added 10 ml of Chloroform to upper layer in separation funnel. shake well with the expelling of the accumulated gases and leave funnel on the holder until two layers are separated where the lower layer was taken and passed over a filter paper containing anhydrous sodium sulfate obtained chloroform layer was collected and evaporated to dryness in rotary evaporator. Then it was re-dissolved using 5 mL of chloroform and kept in small tubes and wrapped in aluminum foil after being closed well to prevent exposure to light and kept at -18 ° C in the freezer.

Identification of aflatoxin using HPLC Detection process of aflatoxin was performed in Food Safety Laboratories - Department of Environment and Water - Ministry of Science and Technology, according to method mentioned by (24) using a high-performance liquid chromatography HPLC (SYKAMN) model of German origin, where the mobile phase was used. Acetonitrail: distilled water (70:30) and the stationary phase. used a C18 - ODS column with dimensions (25cm * 4.6 mm * 5um) to separate the phenols, and the mobile phase flow speed was: 0.7 ml / min. Florescence Detector used to detect mycotoxins according to wavelengths (Ex = 365nm, Em = 445nm). Diagnosis was made based on a match between retention time (RT) between extracted Aflatoxin and Aflatoxin standard. Concentration of Aflatoxin was calculated according to the following equation:

$$\text{Conc .= } (\mu g/ml) = \frac{\text{standard toxin concentration x Peak area of sample}}{\text{Peak area of standard toxin}} \times \text{Dilution factor}$$

Molecular identification of the fungal isolate *A. flavus* is the most aflatoxins production

DNA extraction Local isolate of *Aspergillus flavus* (AM-1) was cultured on PDB and incubated at 30°C for 5 days. 1 ml of the broth media culture was centrifuged at 14000 xg for 1 min. (the supernatant was discarded). 400 mg of precipitated cells was taken and smashed under liquid nitrogen using a mortar. smashed cells was transferred to a 1.5 ml Eppendorf tube. A Genomic DNA Mini Kit (from Geneaid Inc, Taiwan) was used to extract the DNA from fungal isolate according to kit extraction method (plant Gp-100 spin Column).DNA purification was estimated by Nano drop.

Polymerase chain reaction (PCR)

PCR was used to amplify Internal Transcribed Spacer (ITS) of 5.8S rRNA ribosomal gene in order to confirm the selected isolate type which includes two locus ITS1and ITS2 (using the primers in table (1) as described by (11). Concentration of the primer was 17 pecomole and numbers of nitrogen bases were 20 in forward primer and 22 in reverse one. amplification was done in 20 µl which then added to the master mix that was supplied by the Bioneer Company as shown in Table(2).

Table 1. The sequence of PCR primers

Primer	Sequence	GC %	Temp C°
Forward (5.8s)	TCCTCCGCTTATTGATATGC	45	55.7
Reverse (ITS1F)	GGAAGTAAAAGTCGTAACAAGG	40.9	60.7

Table 2. The compounds of the master mix

Compounds in the master mix	Vol.(µl)
10 P mole Forward primer	1
10 P mole Reverse primer	1
DNA extract	3
Deionized water	12
Total volume	20

Master mixture was mixed for few seconds using vortex. tube was placed in PCR thermo cycler. device was programmed according to table (3) and amplification was taken place to amplify extracted DNA. Then 5 µl of ampliocn was assayed.

Table 3. Conditions of the PCR master mixture

No.	Step	Temperature	Time	Cycles
1	Pre denaturation	95°C	3 min.	One cycle
2	Denaturation	95°C	20 sec	
3	Annealing	60°C	30 sec.	
4	Extension-1	72°C	40 sec.	
				35 cycle
5	Extension -2	72°C	5 min.	One cycle
6	Holding	4°C	∞	

Electrophoresis of DNA amplification products on agarosegel: PCR products were loaded on 1.5% agarose gel using a horizontal electrophoresis. 5 µl of PCR products and 2 µl of loading buffer for every each sample was add and mixed well. Samples were loaded into gel wells and subjected to electrophoresis assay for 1 hour, 5 volt \ cm at 70 milliamps in order to initiate the movement toward positive pole. The DNA bands were detected by UV light Tran's illuminator device.

Determination of nitrogen bases sequence Amplifying gene products have been sent to korean Macrogen Company in order to determine the nitrogen bases (DNA sequencing) for the pure isolate ITS zone. Sequence was compared with available information on NCBI website using BLAST Nucleotides software to identify the species of the chosen isolates.

RESULTS AND DISCUSSION

Thirty two fungal isolates were obtained from different sources have been grown on PDA medium (Table 4) . Sixteen isolates were primarily identified as *Aspergillus flavus*, Twelve isolates as *Aspergillus niger*, and four isolates as *Alternaria sp* (Table 5). according to following cultural and morphological characteristics (Table 6). *Aspergillus flavus* was characterized in the beginning by its white color, after 3 days it turned into a greenish-greenish color (Fig. 1a). Conidiophres approximately 1000 µm, of *Aspergillus niger*, It has a black growth on the top, with a sulfur-yellow color from bottom (Fig. 1 B) is divided

mesalem and its conidial heads are black, spherical and radial branching, with a spherical vesicle in shape and brown in color and diameter of conidia is 4.5 µm and the length of the conidia is 4 µm. *Alternaria ssp.* gray in color after 3 days of growth at a temperature of 25 ° C on PDA medium (Fig. 1 C) with possession of a multi-mycelium and its conidia-brown heads are oval vesicle is semi-spherical, while the diameter of conidia reached 4.42 µm and the length of the quinidia vesicle 10 µm.

Table 4. Fungal isolates and places of isolation

Isolation place	Symbol	No.
Khan Dari Silo	Ha(1,2,3,4)	4
	Hb(1,2,3)	3
Khan Bani	Ma(1,2,3,4,5)	5
Saad Silo	Mb(1,2,3)	3
	Mc(1,2)	2
Al-Taji Silo	Sa(1,2,3,4,5,6,7)	7
	Sb(1,2,3,4,5,6)	6
	Sc(1,2)	2

Table 5. Numbers of fungal isolates and their isolation place, and their type , Symbol

Isolation place	Isolated Type	Symbol	No.
Khan Dari Silo	*Aspergillus flavus*	Ha(1,2,3,4)	4
	Aspergillus niger	Hb(1,2,3)	3
khan Bani Saad Silo	*Aspergillus flavus*	Ma(1,2,3,4,5)	5
	Aspergillus niger	Mb(1,2,3)	3
	Alternaria sp	Mc(1,2)	2
Al-Taji Silo	*Aspergillus flavus*	Sa(1,2,3,4,5,6,7)	7
	Aspergillus niger	Sb(1,2,3,4,5,6)	6
	Alternaria sp	Sc(1,2)	2

Percentage of occurrence of fungal isolates obtained (Fig. 2), as it is noticed that highest incidence rate was for *A. flavus*, reaching 50%, followed by *A. niger*, with an

Percentage of occurrence of fungal isolates obtained (Fig.2).As it is noticed that highest incidence rate was for *A. flavus*, reaching 50%, followed by *A.niger*, with an emergence rate of 37.50%, and finally *Alternaria sp.* An appearance rate of 12.50%.

Table 6. The most important cultural and morphological characteristics of the local fungal isolates

Fungal isolation	Characteristic				
	Colonies	Mycelium	Conidial heads	Vesicle	Conidia
A. flavus	White is the beginning of growth and after 3 days becomes green olive	Septated	Dark green	Spherical or semi-spherical shape	The mature is large
A. niger	Black, heavy growth on PDA at 30° C after 5 days	Septated	Black	Spherical shape	The mature is large, immature is small
Alternaria sp.	Transparent at the beginning of growth and after 3 days it becomes dark gray	varied	Dark brown	Oval or semi-spherical shape	The mature is large

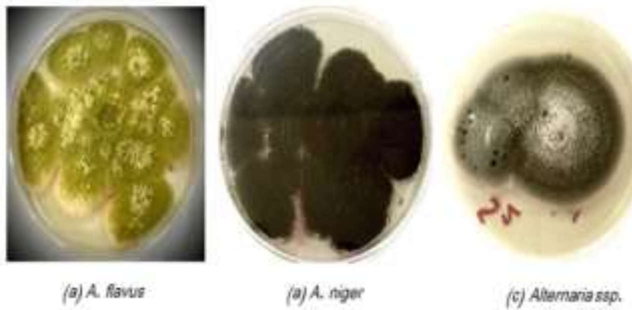

Fig 1.(a, b, c) Forms of fungal isolates obtained in PDA medium

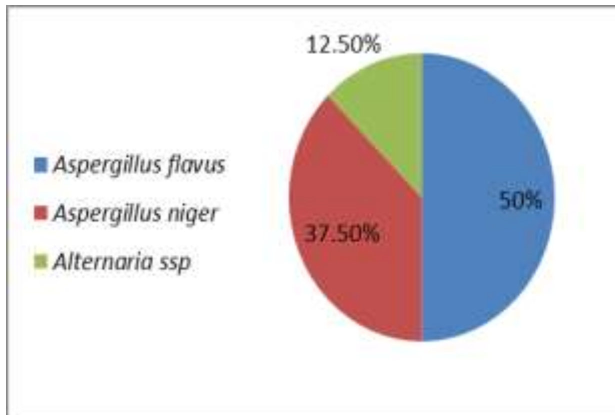

Fig. 2. Percentage of fungal isolates obtained from isolation of wheat samples drawn from silo in Baghdad

Assay of aflatoxin produced by fungal isolates.

Results show a positive response all isolates of *A .flavus* through color change of the base of the coconut extract from white to pink indicates its production of Aflatoxin at a temperature of 25 ° C for a period of 24 hours, while all isolates of *A. niger* and *Alternaria sp* showed their inability to produce Aflatoxins doesn't change in color from white to pink in the presence of ammonium hydroxide. , While noting a difference in the intensity of the pink color produced by different isolates (Table7) (Fig. 3). A dark pink color was obtained for *A. flavus* isolates isolated from wheat drawn from Al-Taji Silo, while the color formed as a result of ammonia testing was less dark with isolates obtained from wheat stored in Khan Dari Silo and Wheat Khan Bani Saad Silo, respectively. These results attributed to difference in the genetic ability of these isolates to produce toxins. The results were agree with (1,4) which indicate that aflatoxins production were negative for *A. niger*, *A. fumigates*, *Alternaria sp.*; *Fusarium sp.*; *Trichoderma sp.*; *Penicillium sp.*; but were positive for *Aspergillus flavus* and *Aspergillus terreus*

Table 7. Fungal isolates producing Aflatoxin using the ammonia hydroxide method

Isolation place	Isolated Type	The test result
Khan Dari Silo	***Aspergillus flavus***	+
	Aspergillus niger	-
Khan Bani Saad Silo	***Aspergillus flavus***	+
	Aspergillus niger	-
	Alternaria sp	-
Al-Taji Silo	***Aspergillus flavus***	+
	Aspergillus niger	-
	Alternaria sp	-

Fig. 3. Color change of coconut extract medium in ammonium hydroxide assay due to the ability of *A. flavus* isolates to produce aflatoxin

Identification of Aflatoxin Using HPLC

Fig. 4 show the identification of Aflatoxin produced from isolates of *A. flavus* by HPLC compared to the standard (Aflatoxin B1) are shown in (Fig, 5) depending on retention time for each of them. Retention time of all toxins produced from local isolates was identical with the Aflatoxin B1 standard there was a difference between the concentration of produced toxins, (Fig, 6) the concentration of Aflatoxin B1 (AFB1) produced by the fungal isolate *A. flavus* obtained from coronary silo samples were 978.5 µg / ml, while in Khan Bani Saad silo were 920.9 µg / ml. And 889.2 µg / mL for the Khan Dari silo, The highest concentration of Aflatoxin B1 toxins were obtained from *A. flavus* isolated from wheat grass samples in Al-Taji silo. it has been reported that *A. flavus* has high capability of producing Aflatoxin B1 at high conc. ranging from 18.6 -740 ppm (5).

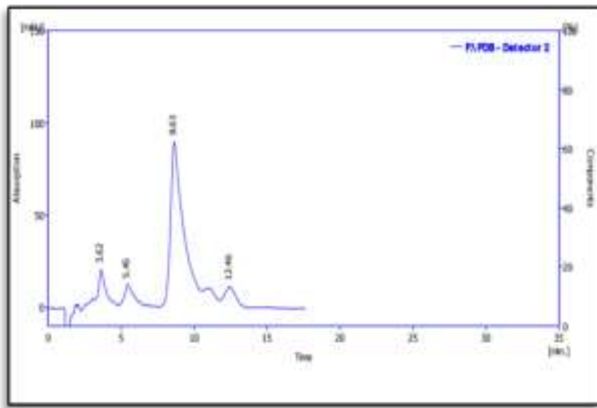

Fig. 4. HPLC diagram Aflatoxin B1 extracted from isolate *A.flavus*

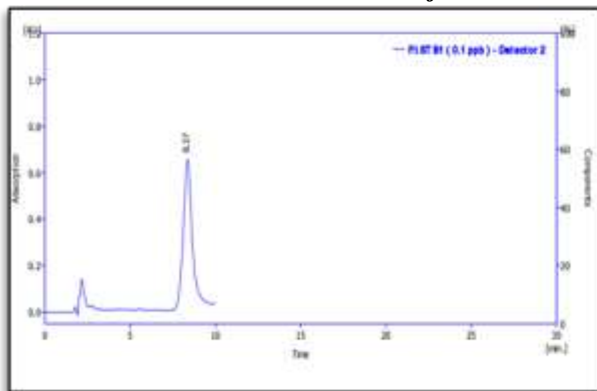

Fig. 5. HPLC diagram of Aflatoxin B1 standard

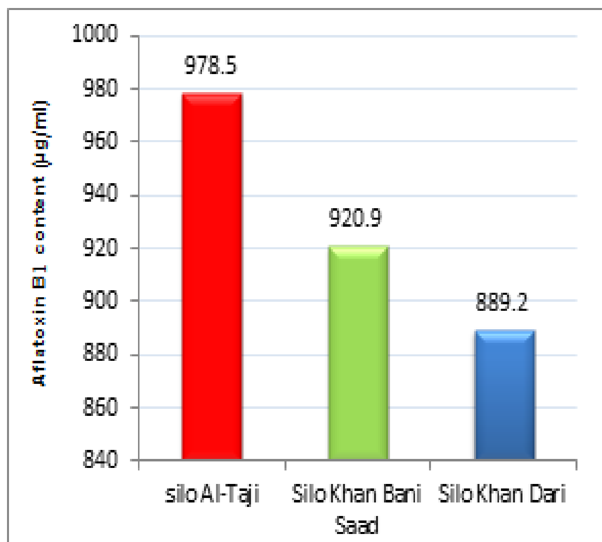

Fig. 6. Concentration of aflatoxin B1 produced from fungal isolation *A. flavus*

Molecular Identification

DNA extraction DNA was extracted from *the local fungal isolate A. flavus* (AM-1) initially examined as *A. flavus,* depending on cultural and morphological characteristics. purity of DNA was examined by Nano Drop and attained to purity of 1.9 which is adequate for Polymerase Chain Reaction (PCR) assay. (31) reported that the PCR did not need a large quantity of DNA which may instead produce unlimited amplifying products. On other hand, an adequate quantity of DNA may reduce the accuracy.

Polymerase Chain Reaction (PCR) Amplifi cation of ITSI &ITS4 in 5.8S rRNA gene for local isolate of *Aspergillus flavus* was carried out by PCR technique. The electrophoresis on 1.5% agaros show (by using U.V detector), that there was a clear band represents the genes amplifications (Fig, 7). The molecular size of gene amplification band was 530bp comparing with ladder size at the same conditions, which refers to the prime binding to the complete sequence in DNA pattern. The interference space, ITS1and ITS4 ribosomal unit 5.8s rRNA was used to distinguished the various species of fungi, with accuracy identification results.

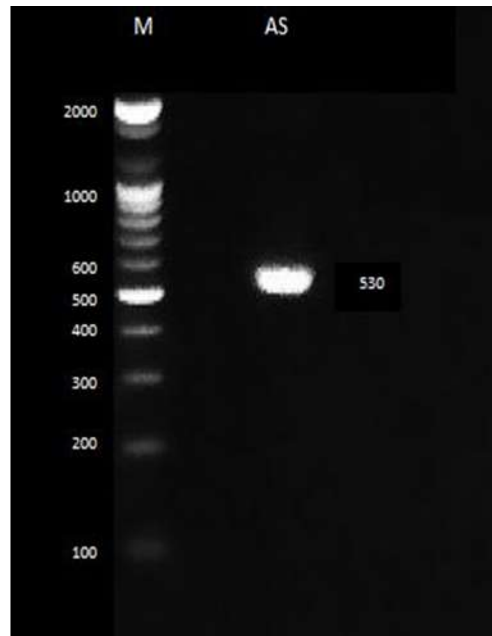

Fig. 7. Electrophoresis for the local *Aspergillus flavus* isolate (AM-1) for the internal transcribed spacer (ITS) on agaros gel (1.5%).

Sequence analysis of amplification products

The sequence of nitrogen bases, of the internal transcribed spacer ITS1, for the local fungi isolate *Aspergillus flavus* isolate (AM-1) was studied by sending the PCR amplicon to the Korean company Macrogen. The nitrogen bases sequence (608 base-pair) which was taken from the local isolate sequence (of the present study) is shown in (Fig, 8) . The BioEdit ver, 7.1 program has been used to find out the similarity of gene with the bank information (NCBI). The results showed that

Identity of 5.8S rRNA gene sequencing of local isolate gene sequence. was 99% with global isolate sequence ID: MK992254.1 Registered on the NCBI website and registered in the Malaysia Which belong to *Aspergillus flavus* strain USMG09 (Fig, 9)

FORWARD (5.8s):

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CCTCGGTAATGACTTCCGTAGGTGAACCTGCGGAAGGATCA
TTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCG
TGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATG
GCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAG
ACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTG
TATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCC
GGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAAT
TGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGC
CCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTG
CCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCG
GGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATC
CTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCG
GCGCTTGCCGAACGCAAATCAATCTTTTCCAGGTTGACCTCGGAT
CAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
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Fig. 8. The sequence of gene ITS from isolate *A. flavus*.

Aspergillus flavus isolate AM_1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK992254.1

GenBank FASTA

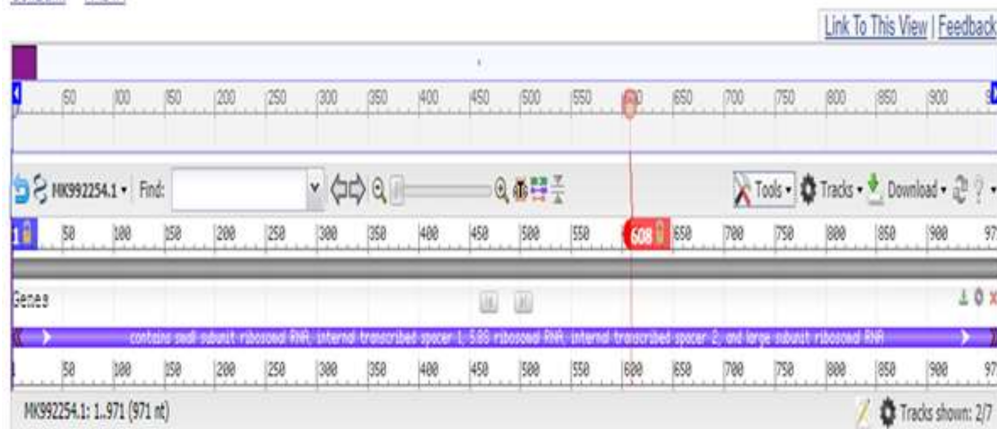

Fig. 9. The exact position of the retrieved 608 bp ampicons that covered a portion of ribosomal locus within the Aspergillus flavus genomic sequences (GenBank accession number MK992254.1).

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