# Understanding the Role of *aflS* Gene Expression in *Aspergillus flavus* and *Aspergillus oryzae* *Regarding* the Aflatoxin Production

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**Abstract:** The fungus *Aspergillus flavus* and *Aspergillus oryzae* are morphologically similar to each other and both belong to Aspergillus Section Flavi. Genetic study and genome sequencing of *A. flavus* and *A. oryzae* also revealed that both are closely related species**.** But, *A. flavus* is harmful for both human and animal due to its aflatoxins production, while *A. oryzae* is safe for both human and animal because it does not produce aflatoxins**.** Hence**,** *A. flavus* is known as the evil twin of *A. oryzae***.** Aflatoxins are carcinogenic secondary metabolites. They cause liver cancer and Aspergillosis in both human and animal. In this study, genomic DNA of *A. flavus* and *A. oryzae* were extracted. Conventional PCR was used to amplify the aflS gene and for more conformation, Real-Time PCR was also used to detect the expression of *aflS* gene in both mold. Expression of *aflS* gene was detected in the genome of *A.flavus*, but not detected in the genome of *A. oryzae*. The *aflS* gene is responsible for the formation of many compounds such as NOR, AVN, DMST and OMST, and these are the main precursor compound for the synthesis of aflatoxins. So, based on obtained result, it can be concluded that lack of *aflS* gene in *A. oryzae* might be responsible for its non-aflatoxigenic property, and production of aflatoxins from *A. flavus* might be blocked by inhibiting the expression of *aflS* gene.

**Keywords:** *Aspergillus flavus, Aspergillus oryzae,* Aflatoxins, *aflS* gene, NOR, AVN, DMST and OMST.

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**Introduction:**

The most serious problem of *Aspergillus flavus* infection is the production of aflatoxin. Aflatoxin was first recognised as carcinogenic, just after the outbreak of the "Turkey X" disease in England in 1960. Aflatoxin is also known as toxigenic, mutagenic, and teratogenic in nature (Kumar, Topno *et al*. 2018). It causes mutation (transversion) at 249th codon of P53 gene ((Kurtzman, Horn *et al*. 1987, Richard 2007). It was named "Class 1 Human Carcinogen" by the International Agency for Research on Cancer (IARC)

(Williams, Phillips *et al.* 2004). *A. flavus* produces two types of aflatoxins like aflatoxin B1 and B2 (Singh and Singh 2005). Aflatoxin causes aflatoxicosis and liver cancer in humans and animals (Payne and Widstrom 1992). It increases the risk of liver cancer 30 fold higher in the individuals, who was previously affected with Hepatitis (Henry, Bosch *et al*. 1999). In human, aflatoxin also causes chronic cavitary pulmonary aspergillosis, aspergilloma (clump of fungus in body cavity), allergic bronchopulmonary aspergillos, allergens, keratitis, endophthalmitis, cutaneous infection, wound infection, central nervous system (CNS) infection, rhinosinusitis, allergic fungal sinusitis, sinus aspergilloma, osteoarticular infection, and urinary tract infection (Hedayati, Pasqualotto *et al.* 2007). Aflatoxin contamination causes the death of affected animals or fast reduction in the body weight (Miller and Wilson 1994, Wogan 2000, Scheidegger and Payne 2003, Williams, Phillips *et al.* 2004) Aflatoxin affects crops globally, especially in tropical and sub tropical regions ((Park, Liang *et al. 1993)*. Approximately 25% of the world's food products are facing the problem of aflatoxin contamination (Moreno and Kang 1999). The presence of aflatoxin even in small amounts in food grains or foodstuff gives severe damage to human health (Chen, Brown *et al.* 1998). Therefore, in the United States, grains with aflatoxin levels more than 20 ng/g, have been banned by interstate commerce, and the grains with aflatoxin level more than 300 ng/g are not allowed to fed the Animals (USDA, 1994). Aflatoxin B1 inhibits seed germination of crop seeds such as wheat, corn, mustard, mung and gram (Klich 2007). Aflatoxin contamination is a major problem in the US (Payne and Widstrom 1992, Betrán, Isakeit *et al*. 2002). It limits the marketability of contaminated food grains and foodstuff because of harmful risk to human life (Betrán, Isakeit *et al*. 2002) The world losses hundreds of millions of dollars per year due to aflatoxins contamination (Moreno and Kang 1999). Aflatoxin contamination depends on biotic and abiotic stresses (Gorman, Kang *et al.* 1992, Payne and Widstrom 1992, Betrán, Isakeit *et al*. 2002). Its synthesis is accelerated by drought conditions and above normal temperatures (Widstrom, Snook *et al*. 1995). Many other factors such as soil fertility, weed related stresses and insect damage to developing ears of maize are responsible for aflatoxin synthesis (Windham, Williams *et al.* 1999, Munkvold 2003). Insect creates a favourable environment for the aflatoxin infection by dehydrating the kernels of Maize (Widstrom, McMillian *et al*. 1987). Environmental condition like drought is more effective than insect damage for the production of aflatoxin (Munkvold 2003). Researchers of more than 50 countries are doing research to get rid of the problem aflatoxin contamination in food grains and food stuff (Cleveland, Dowd *et al*. 2003). Only a limited amount of germplasms have been reported to resistance of aflatoxin contamination(Gorman, Kang *et al*. 1992, Campbell and White 1995). Genetic approaches may be the most effective and powerful techniques to control the *A. flavus* as well as aflatoxin related disease such as ear and kernel rot disease in Maize (Campbell and White 1995).

Genetic study and genome sequencing of *Aspergillus flavus* and *Aspergillus oryzae* revealed that both are closely related species (Machida, Yamada *et al.* 2008). *A. flavus* is known as the evil twin of *A. oryzae*. *A. oryzae* is named GRAS (Generally regarded as safe) by the FDA. *A. flavus* and *A. oryzae* have the same unique repeat telomeric sequences, TTAGGGTCAACA (Chang, Horn *et al*. 2005)). Both species have aflatoxin gene clusters but *A. oryzae* fails to produce aflatoxin because aflatoxin gene clusters are not

expressed in this mold due to various types of mutations such as deletions, rearrangements in clusters, and mutations in aflatoxin biosynthesis pathway genes (Lee, Tominaga *et al*. 2006)). Hence, many mycologist reported that *A. oryzae* is a natural variant of *A. flavus*, which got modified by itself into *A. oryzae* due to cultivation through thousands of years for fermenting of food.

Genetic approaches may be the most effective and powerful techniques to arrest the production of aflatoxin from the mold. So, present investigation was designed to find out the gene which is responsible for production of aflatoxin in *Aspergillus flavus.*

**Materials and Methods:**

**Preparation of pure cultures of *Aspergillus flavus* and *Aspergillus oryzae* :**

Strains of *Aspergillus flavus* and *Aspergillus oryzae* were purchased from Institute of Microbial Technology (IMTECH), Chandigarh, India, and their cultures were maintained on Potato Dextrose Agar (PDA) media at the Department of Botany and Biotechnology, College of Commerce Arts and Science, Patna, 800020, Bihar, India. Freshly prepared PDA medium was transferred into different petri plates (20 ml in each). Thereafter, the strains of *A. flavus* and *A. oryzae* were inoculated at the centre of different petri plates separately. All petri plates were incubated for 10 days at $28^0$C.

**Detection of Aflatoxins in *Aspergillus flavus* and *Aspergillus oryzae* :**

To evaluate the aflatoxigenic properties of *Aspergillus flavus* and *Aspergillus oryzae,* freshly prepared CDB (Czapek Dox Broth) medium was transferred into two conical flasks (25 ml in each flask), and pH of the medium was adjusted to 5.5 in each flask. The capacity of each conical flask was 100 ml. Thereafter, fungal discs (5mm diameter) of 10 days old culture of *A. flavus* and *A. oryzae* were cut with the help of sterilized cork borer. The fungal disc (5mm diameter) of *A. flavus* was inoculated into one flask, and the fungal disc (5mm diameter) of *A. oryzae* was inoculated into another flask. Both flasks were incubated for 10 days at 28°C. The fungal mycelium of each flask was filtered through Whatman no.1 filter paper, and filtered mycelium was autoclaved to kill the harmful spores of *A. flavus* and *A. oryzae*. Mycelium of both fungi were dried in hot air oven for 12 hours at $80^0$C, and weighed. The filtrates of the both molds were extracted with 20 ml of chloroform separately, and the chloroform extracts of the both molds were kept on water bath separately to evaporate till complete dryness. Residue of dried chloroform extracts of both molds were redissolved in 1ml of chloroform separately. Thereafter, 50µl chloroform extract of the *A. flavu*s was spotted on one thin layer chromatography (TLC) plate, and 50 µl chloroform extract of the *A. oryzae* was spotted on another TLC plate following Turner *et al.* methods (Turner, Subrahmanyam *et al.* 2009). Chloroform and acetone were taken, and mixed in the ratio of 9: 1 (v/v) to use as solvent system for both TLC plates. After running the samples, both TLC plates were air dried and then kept under UV transilluminator (360 nm) to detect the presence of aflatoxin. The standard of aflatoxin was purchased from Sigma Aldrich Company.

**Isolation of genomic DNA:**

The 50 mg fungal mycelium was taken from the petri plate with the help of spatula, and kept into a 2ml tube. The tube was containing ceramic pestle, 60- 80 mg sterile glass beads. Lysis buffer containing 100

mM Tris HCl (pH 8.0), 50mM EDTA & 3% SDS was added to the tube, and followed by homogenization with the help of homogenizer. Thereafter, the fungal mycelium was centrifuged at 13,000 rpm for 10 min, and then supernatant was transferred into a micro centrifuge tube. RNase A (10mg/ml) was added into the supernatant and followed by incubation at 37°C for 15 min. Equal volume of phenol: chloroform: isoamyl alcohol in the ratio of 25:24:1 were added and mixed well, and centrifuged at 13,000 rpm for 10 min. This step can be repeated many times to complete get rid of proteins and cell debris. Thereafter, the upper aqueous layer was transferred into a fresh micro centrifuge tube, and equal volume of ethanol was mixed. In next step, precipitation of sample was done at -20°C for 30 minutes and followed by centrifugation at 12,000 rpm for 10 minutes to pellet down the DNA contents. The DNA pellet was washed with the help of 70% ethanol and subsequently centrifuged at 12,000 rpm for 5 minutes, and then DNA pellet was air dried and dissolved in 1× TE buffer. The qualitative analysis of isolated DNAs was done on agarose gel (0.8%) electrophoresis. The extracted genomic DNA was quantified by measuring the absorbance at 260 nm with the help of Nano-drop Spectrophotometers (ND 1000, Nano Drop Technologies, Inc, Wilmington, DE, USA). Here, the calf thymus DNA was used as control.

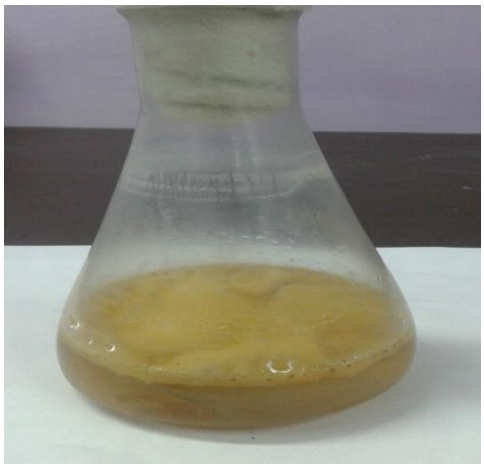
**PCR analysis:**

Thermal Cycler PCR was used to amplify the *aflS* gene of both *Aspergillus flavus* and *Aspergillus oryzae*. Gallo *et al*. (Gallo, SteA *et al.* 2012) proposed primer (F:5′-TGAATCCGTACCCTTTGAGG-3′ & R:5′-GGAATGGGATGGAGATGAGA-3′) of *aflS* gene was used in the reaction. The reaction was conducted using 25µl total reaction volume containing 1X Buffer, 0.2 µml$1^{-1}$ of each Primers, 1µl template DNA, 800 µmol $1^{-1}$ dNTPs, 1.25 U Taq DNA polymerase and 12 µl distilled H2O ( (RNase and Dnase free). Thermal cycler PCR was set up for initial denaturation of DNA at 95ºC for 5 minutes. Thereafter, 40 repeating cycles of denaturation, annealing and extension were set up at 94ºC for 30 second, 65ºC for 60 second and 72ºC for 90 second respectively, and finally the reaction was ended with 7 minutes extension at 72ºC. The qualitative analysis of *aflS* gene amplification was done on agarose (0.6%) gel electrophoresis.

**Real- time PCR analysis:**

Real-time PCR analysis of *aflS* gene for both *Aspergillus flavus* and *Aspergillus oryzae* was done simultaneously. The Real-time PCR was performed in 25µL reaction volume containing 12.5µl TaqMan Universal Master Mix (Applied Biosystems), 3 µl solution of primer and probe (0.2 nmol$1^{-1}$ probe and 0.5 nmol$1^{-1}$ primer), 2.5µl template DNA, 7µl Nuclease free water. Positive and Negative controls were also taken during the reaction. Amplifications were completed using the Bio Rad CFX96 platform (Bio Rad, Hercules, CA) with following condition: In first step temperature was 95ºC for 10 minutes followed by 45 repeating cycle of denaturation, annealing and extension with 95ºC for 15 second, 55ºC for 20 second and 72ºC for 30 second respectively. Positive result was monitored by sigmoid curves, and confirmed by the appearance of band of 684 bp on 2.0 % agarose gel.

## Result and Discussion:



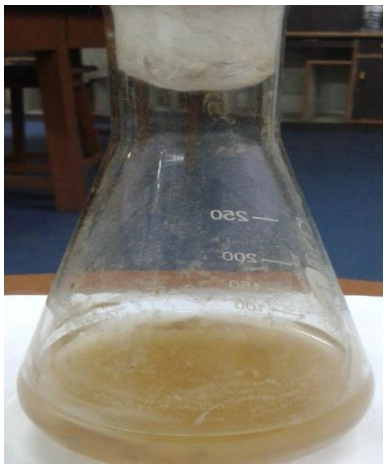


**Fig 1: Growth of *A. flavus in* CDB media** **Fig. 2: Growth of *A. oryzae* in CDB media**

Figure 1and 2 show the growth of *Aspergillus flavus* and *Aspergillus oryzae* on Czapek Dox Broth (CDB) media respectively. Both molds showed yellow colony color on CDB medium. The Presence of Nitrogen and carbon in CDB media play an important role in various metabolisms pathways of *A. flavus* and *A. oryzae*. Nitrogen also promotes synthesis of aflatoxin (Wang, Chen *et al. 2016)* Other ingredients of CDB media such as $KNO_3$ and $NaN0_3$ stimulate the growth of *A. flavus* and *A.* oryzae. The Presence of phosphorus in CDB media stimulates the production of averufin, which is an intermediate compound in the aflatoxin biosynthesis (Kachholz and Demain 1983). Potassium of the CDB media promotes the growth of fungal mycelium.

**Fig.3: TLC plate of *A. flavus* is showing the band of aflatoxin**

**Fig.4: TLC plate of *A. oryzae* is not showing the band of aflatoxin**

Figure 3 shows the image of TLC plate treated with the sample of *Aspergillus flavus.* This TLC plate is showing the bands (blue colour) of aflatoxin under the UV transilluminator (360 nm). But, the TLC plate treated with the sample of *Aspergillus oryzae* is not showing the bands of aflatoxin under the UV transilluminator (360 nm) in figure 4. It supports the hypothesis of kiyota *et al*. (Kiyota, Hamada *et al.* 2011) who has reported that aflatoxin is not found in *A.oryzae.* Saito and Tsuruta (1993) reported that the genes responsible for the synthesis of aflatoxin in *A. oryzae* were inactivated by mutation during the course

of evolution. This concept supports the hypothesis of other mycologists, Wicklow (Wicklow, Hor*n et al. 1984),* Samson and Pit (1985).

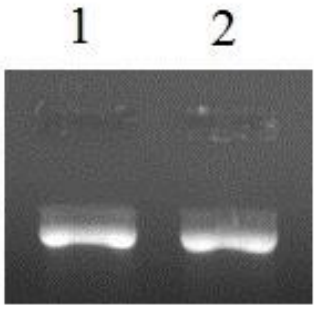



**Fig. 5: Isolation of DNA from both fungus at 10th day of mycelial growth: Lane1: *Aspergillus flavus*; Lane2: *Aspergillus oryzae***

Figure 5 shows the bands of DNA, isolated from the *Aspergillu flavus* and *Aspergillus oryzae*. The lane1 presents the band of *A. flavus*, and lane 2 presents the band of the *A. oryzae*. The result suggested that *A. flavus* and *A. oryzae* are approximately similar in genome size. Machida *et al.* ((Machida, Yamada *et al*. 2008)) reported that genome sequencing of *A. flavus* and *A. oryzae* suggests both are closely related species. The genome size of *A. oryzae* is 36.7 Mb with 12079 predicted numbers of genes and the gen*ome size of A. flavus is* 36.8 Mb with 12197 predicated numbers of genes (Payne, C Nierman *et al. 2006)*. Kusmo *et al.* (2003) observed that both *A .flavus* and *A. oryzae* have the same telomeric sequence, TTAGGGTCAACA.

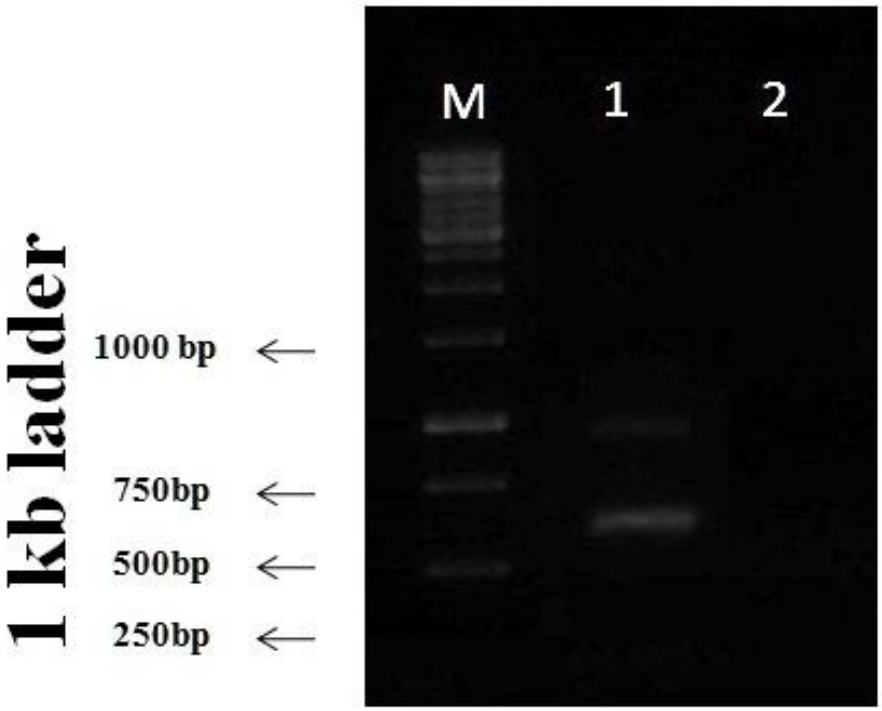



**Fig. 6: Gel electrophoresis of PCR product. Lane M: Molecular size marker (Invitrogen 1kb ladder); Lane1: *Aspergillus flavus*; Lane2: *Aspergillus oryzae***

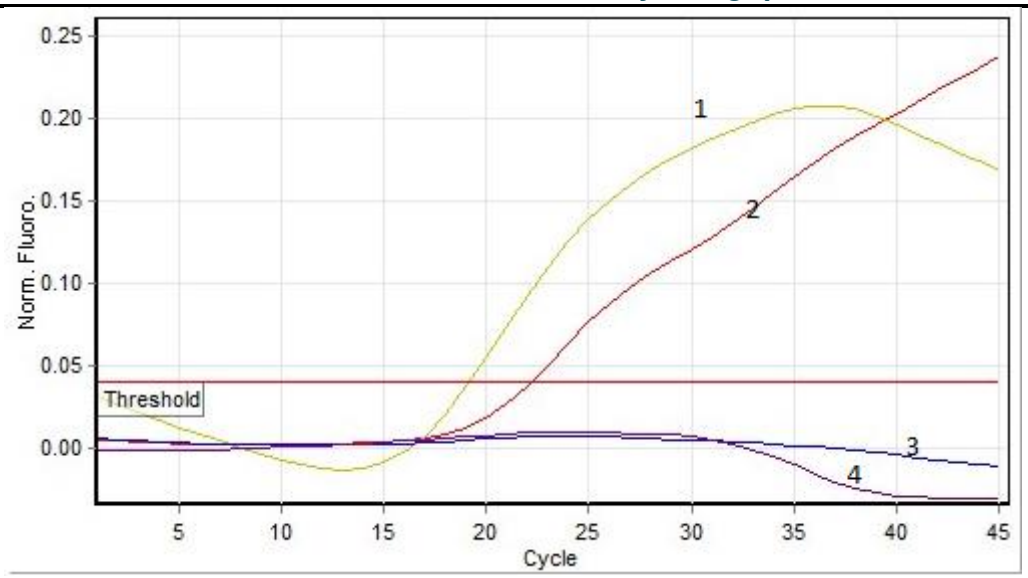



**Fig. 7: Result of Real- time PCR analysis is showing expression of aflS gene in *A. flavus* but not in *A. oryzae.* Amplification curve1: positive control; Amplification curve 2: *A. flavus* sample; Amplification curve 3: *A. oryzae* sample & Amplification curve 4: negative control.**

Bioinformatics analysis showed 99.5% gene homology between the *Aspergillus flavus and Aspergillus oryzae* pointing towards a large coherence in the secondary metabolite production (Rank, Klejnstrup *et al.* 2012). Both species produce kojic acid, ergosterol and other minor metabolites. It suggests high gene homology between the *A. flavus* and *A. oryzae* particularly for the secondary metabolite genes (Rank, Klejnstrup *et al.* 2012)). But, *A. flavus* produces aflatoxin and *A. oryzae* does not produce aflatoxin. It might be possible due to inactivation of gene, which is responsible for aflatoxin production in *A. oryzae* (SAITO and TSURUTA 1993).

Figure 6 displays the result of PCR (Conventional) analysis of *aflS* gene. The result revealed that *aflS* gene is found in the genome of *A. flavus*, but not found in the genome of *A. oryzae*. For more conformation, we have also done Real-time PCR for *aflS* gene of both *A. flavus* and *A. oryzae*. Here, we also found expression of *aflS* gene in *A. flavus* but not in *A. oryzae* (Fig. 7). The *aflS* gene is known as regulator gene of aflatoxin synthesis (Meyers, Obrian *et al.* 1998). It is found adjacent to the *aflR* gene (also known as regulator gene for aflatoxin synthesis) in the aflatoxin gene cluster. Meyers *et al.* ((Meyers, Obrian *et al. 1998)* reported that knock out of the *aflS* gene from the genome of *A. flavus* inhibits the conversion of three important intermediates norsolorinic acid, sterigmatocystin, and *O*-methylsterigmatocystin into aflatoxin. Yu *et al.* ((Yu, Chang *et al.* 2004) reported that *aflS* mutant *A. flavus* leads to 5- to 20-fold reduction in the expression of four aflatoxin pathway genes such as *aflC* (*pksA*), *aflD* (*nor-1*), *aflM* (*ver-1*), and *aflP* (*omtA*). The reductions of expressions of these genes lead to loss of the ability to synthesize aflatoxin by the mold. The *aflC* gene converts polyketide precursor into norsolorinic acid (NOR) (Brown, Adams *et al. 1996)*. The *aflD* gene is involved in the conversion of NOR into AVN (Averantin) (Skory, Chang *et al. 1993)*. The *aflM* gene converts versicolorin A (VERA) into D-emethylsterigmatocystin (DMST) (Liang, Wu *et al. 1997)*, and the aflP gene involves in conversion of sterigmatocystin (ST) into O-methylsterigmatocystin (OMST) (Yu *et al.*, 1993). The compounds such as NOR, AVN, DMST and OMST are known as main precursor

compounds for the aflatoxin biosynthesis. Based on the above data, we can hypothesize that lack of *aflS* gene in *A. oryzae* might be responsible for the non-aflatoxigenic property of this mold.

**Conclusion:**

Overall experiment revealed that *aflS* gene is found in *Aspergillus flavus* but not found in *Aspergillus oryzae*. This gene is very important for the production of aflatoxin because it regulates the expression of other aflatoxin pathway genes such as *aflC* (*pksA*), *aflD* (*nor-1*), *aflM* (*ver-1*), and *aflP* (*omtA*). Expression of these genes make aflatoxin precursor compounds like Norsolorinic acid, Averantin, D-emethylsterigmatocystin and O-methylsterigmatocystin, which are main compounds for the biosynthesis of aflatoxins. Hence, we may conclude that *A. oryzae* does not produce afltoxin due to absent of *aflS* gene, and aflatoxin production can be inhibited in *A. flavus* by silencing the expression of *aflS* gene.

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**References:**

*Betrán, F., et al. (2002). "Aflatoxin accumulation of white and yellow maize inbreds in diallel crosses." **42**(6): 1894-1901.*

*Brown, D., et al. (1996). "Aspergillus has distinct fatty acid synthases for primary and secondary metabolism." **93**(25): 14873-14877.*

*Campbell, K. and D. J. P. D. White (1995). "Evaluation of corn genotypes for resistance to Aspergillus ear rot, kernel infection, and aflatoxin production."*

*Chang, P.-K., et al. (2005). "Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic Aspergillus flavus isolates." **42**(11): 914-923.*

*Chen, Z.-Y., et al. (1998). "Resistance to Aspergillus flavus in corn kernels is associated with a 14-kDa protein." **88**(4): 276-281.*

*Cleveland, T. E., et al. (2003). "United States Department of Agriculture—Agricultural Research Service research on pre-harvest prevention of mycotoxins and mycotoxigenic fungi in US crops." **59**(6-7): 629-642.*

*Gallo, A., et al. (2012). "Molecular characterization of an Aspergillus flavus population isolated from maize during the first outbreak of aflatoxin contamination in Italy." 198-206.*

*Gorman, D., et al. (1992). "Combining ability for resistance to field aflatoxin accumulation in maize grain." **109**(4): 296-303.*

*Hedayati, M., et al. (2007). "Aspergillus flavus: human pathogen, allergen and mycotoxin producer." **153**(6): 1677-1692.*

*Henry, S. H., et al. (1999). "Reducing liver cancer--global control of aflatoxin." **286**(5449): 2453-2454.*

*Kachholz, T. and A. L. J. J. o. n. p. Demain (1983). "Nitrate repression of averufin and aflatoxin biosynthesis."* ***46****(4): 499-506.*

*Kiyota, T., et al. (2011). "Aflatoxin non-productivity of Aspergillus oryzae caused by loss of function in the aflJ gene product."* ***111****(5): 512-517.*

*Klich, M. A. J. M. p. p. (2007). "Aspergillus flavus: the major producer of aflatoxin."* ***8****(6): 713-722.*

*Kumar, M., et al. (2018). "Biological conversion of Carcinogenic Aflatoxin B1 to AFB1-8, 9-exo-epoxide and its potential prevention."*

*Kurtzman, C., et al. (1987). "Aspergillus nomius, a new aflatoxin-producing species related to Aspergillus flavus and Aspergillus tamarii."* ***53****(3): 147-158.*

*Lee, Y.-H., et al. (2006). "Aspergillus oryzae strains with a large deletion of the aflatoxin biosynthetic homologous gene cluster differentiated by chromosomal breakage."* ***72****(2): 339-345.*

*Liang, S.-H., et al. (1997). "Analysis of mechanisms regulating expression of the ver-1 gene, involved in aflatoxin biosynthesis."* ***63****(3): 1058-1065.*

*Machida, M., et al. (2008). "Genomics of Aspergillus oryzae: learning from the history of Koji mold and exploration of its future."* ***15****(4): 173-183.*

*Meyers, D. M., et al. (1998). "Characterization of aflJ, a gene required for conversion of pathway intermediates to aflatoxin."* ***64****(10): 3713-3717.*

*Miller, D. M. and D. M. Wilson (1994). Veterinary diseases related to aflatoxins. The toxicology of aflatoxins, Elsevier****:*** *347-364.*

*Moreno, O. and M. J. P. B. Kang (1999). "Aflatoxins in maize: the problem and genetic solutions."* ***118****(1): 1-16.*

*Munkvold, G. P. J. A. r. o. p. (2003). "Cultural and genetic approaches to managing mycotoxins in maize."* ***41****(1): 99-116.*

*Park, D. L., et al. (1993). "Perspectives on aflatoxin control for human food and animal feed."* ***4****(10): 334-342.*

*Payne, G., et al. (2006). Whole genome comparison of Aspergillus flavus and A. oryzae.*

*Payne, G. A. and N. W. J. C. R. i. P. S. Widstrom (1992). "Aflatoxin in maize."* ***10****(5): 423-440.*

*Rank, C., et al. (2012). "Comparative chemistry of Aspergillus oryzae (RIB40) and A. flavus (NRRL 3357)."* ***2****(1): 39-56.*

*Richard, J. L. J. I. j. o. f. m. (2007). "Some major mycotoxins and their mycotoxicoses—An overview."* ***119****(1-2): 3-10.*

*SAITO, M. and O. J. J. M. TSURUTA (1993). "A new variety of Aspergillus flavus from tropical soil in Thailand and its aflatoxin productivity."* ***1993****(37): 31-36.*

*Scheidegger, K. and G. J. J. o. T. T. R. Payne (2003). "Unlocking the secrets behind secondary metabolism: a review of Aspergillus flavus from pathogenicity to functional genomics."* ***22****(2-3): 423-459.*

*Singh, I. and V. P. J. I. J. o. M. Singh (2005). "Effect of plant extracts on mycelial growth and aflatoxin production by."* ***45****(2): 139-142.*

*Skory, C. D., et al. (1993). "Regulated expression of the nor-1 and ver-1 genes associated with aflatoxin biosynthesis."* ***59****(5): 1642-1646.*

*Turner, N. W., et al. (2009). "Analytical methods for determination of mycotoxins: a review."* ***632****(2): 168-180.*

*Wang, B., et al. (2016). "Aptamer induced assembly of fluorescent nitrogen-doped carbon dots on gold nanoparticles for sensitive detection of AFB1."* ***78****: 23-30.*

*Wicklow, D., et al. (1984). "Sclerotium dispersal of Aspergillus flavus and Eupenicillium ochrosalmoneum from maize during harvest."* ***83****(2): 299-303.*

*Widstrom, N., et al. (1987). "Segregation for Resistance to Aflatoxin Contamination among Seeds on an Ear of Hybrid Maize 1."* ***27****(5): 961-963.*

*Widstrom, N. W., et al. (1995). "Silk maysin content and resistance of commercial corn [maize] hybrids to kernel contamination by aflatoxin."* ***67****(3): 317-321.*

*Williams, J. H., et al. (2004). "Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions."* ***80****(5): 1106-1122.*

*Windham, G., et al. (1999). "Effects of the southwestern corn borer on Aspergillus flavus kernel infection and aflatoxin accumulation in maize hybrids."* ***83****(6): 535-540.*

*Wogan, G. N. (2000). Impacts of chemicals on liver cancer risk. Seminars in cancer biology, Elsevier.*

*Yu, J., et al. (2004). "Clustered pathway genes in aflatoxin biosynthesis."* ***70****(3): 1253-1262.*