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RESEARCH ARTICLE

A study biological control of *Aspergillus flavus* using *Psudomonas fluorescens* and *Bacillus subtilis*

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ABSTRACT

Aspergillus flavus is a cosmopolitan fungus that uses to grow almost every type of environmental and nutritional conditions. It produces a group of toxins called aflatoxins. These aflatoxins are the primary cause of liver cancer and immunosuppressant in the peoples that consume the aflatoxins rich diet. Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. The majority of the cases are in south East Asia and Africa. Prospective epidemiological studies have shown a multiplicative interaction between the hepatic virus B (HBV) and aflatoxins in term of HCC risk. In the present study we are trying to isolate microbes from the soil that can potentially be used for the biocontrol of *Aspergillus flavus* toxigenic strain. A microbial library was formed and each microbe was tested under various varied nutritional changes. We have taken soil samples from the various soil locations including the outer skirts of Himalaya river Yamuna, some garden soil and soil from extreme environmental conditions. Using serial dilution and agar plating methodes we tried to isolate the microbial flora present in the soil and these flora were tested against the *A. flavus* if that isolated microbe have some potential to control the *A. flavus*. That microbe is identified using the 16srRNA gene sequence analysis..

Keywords: *A. flavus,* HBV, Aflatoxin, HCC, 16srRNA, Phylogenetic analysis.

INTRODUCTION

A. flavus is a filamentous fungus which produces carcinogenic secondary metabolites called Aflatoxins B_1 and B_2 (Wogan, 1999). When young, the conidia of *A. flavus* appear yellow green in color. As the fungus ages the spores turn a darker green. *A. flavus* can also be pathogenic on several plant and animal species, including humans and domestic animals (Hedayati *et al.,* 2007). The fungus can infect seeds of corn, peanuts, cotton, and nut trees. The fungus can often be seen sporulating on injured seeds such as in maize kernels (Abbas *et al.*, 2006; Cotty 1989; Bayman *et*

al., 2002). Most foods are susceptible to aflatoxigenic fungi (Palmgren and Hayes, 1987) at some stage of production, processing, transportation, and storage. Often, only a few kernels will be visibly infected. (Dorner, 2004; Dorner, 2008; Palmgren and Hayes 1987).

The outbreak of aflatoxicosis (famous as Turkey "X" disease) in England in 1960 caused the death of a large population of livestock (Blount, 1961; Vander Zijden *et al*, 1962) and led to the discovery of aflatoxin in groundnut meal contaminated by *A. flavus* (Hesseltine, 1979). Subsequently, aflatoxins were found in other feeds, especially maize (Shotwell, 1977; Chakrabarty, 1981) and cottonseed meal (Lillehoj *et al.*, 1979; Sharma et al., 1994).

Growth of the fungus on foods leads to contamination with aflatoxin. *A. flavus* is also the second leading cause of aspergillosis in humans. Patients infected with *A. flavus* often have reduced or compromised immune systems.

MATERIALS AND METHODS

Microorganisms

The microorganisms used in the present study were *Aspergillus flavus, A. flavus* (CMI 102566) toxigenic strain produces aflatoxins, whereas *A. flavus* (CMI 93803) non-toxigenic strain does not produce aflatoxins. Both these strains of *A. flavus* were provided by Prof. J.E. Smith, of Applied Microbiology Division, University of Strathclyde, Glasgow, U.K.

Two fungi *Trichoderma reesei* and *Talaromyces flavus* were isolated from soil of Garhwal, Himalaya and two bacteria *Bacillus licheniformis* and *Bacillus cereus*, isolated from garden soil. Bacterial strain which was used for this study is *Rugerria sp*. (Arora M, et al 2012).

Furthermore microorganisms were isolated from soil sample collected from Hindu College, Yamuna river water, Hot stream water, Delhi University garden, Road side plant rhizospheric soil.

Media Used in the study

Commercially available media were used Potato Dextrose Agar Medium (PDA), Nutrient Agar Medium (NA) and Czapek Dox Agar Medium (CDA).

Sterilization

The parameters for sterilization with an autoclave are 121°C at 15 psi for 15 minutes.

Serial Dilution of Samples

Samples collected from various places were subjected to serial dilution, which involves repeatedly mixing of 1 gm/1ml of sample with 9 ml of sterilized saline, and further dilutions were made accordingly.

Preparation of Pure Culture

With the help of streaking we obtained the pure cultures of the isolated microbes.

Duel Culture assay

Duel Culture Assay (Huang and Huang, 1976) was performed in sterilized petri plates containing autoclaved and solidified PDA and CDA medium. Each of the known organisms i.e., *Trichoderma viridae and Talaromyces flavus, Rugerris sp.* and the unknown isolated organisms were also point inoculated at a distance of approximately 1 cm distance along with *Aspergillus flavus* toxigenic strain. Presence of any inhibition zone in the Petriplates was observed after every 24 hr.

Weight loss study

Growth inhibition by bacteria was also be recorded by weight loss method. *A. flavus* was grown in liquid medium and kept in incubator shaker at 30° C and rotation of 200 rpm after 24h of fungal growth 20ml of 24 hr grown bacterial suspension was added. This culture media were kept in incubator shaker for 7 days at 30°C. After then, the dry weight of mycelia was noted.

Molecular identification of isolated bacteria using 16S rRNA gene sequences analysis

DNA was isolated from the bacterial pure culture. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1331bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.

RESULTS AND DISCUSSION

Here are the pictures of Serial Diluted plates

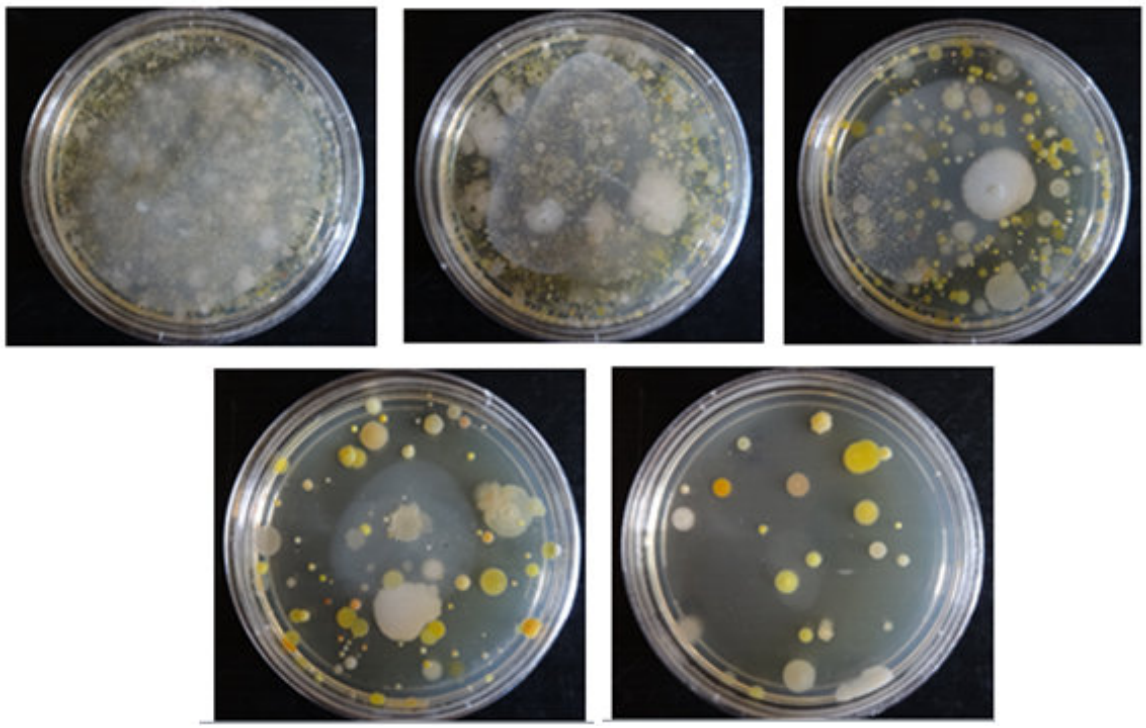

Fig. 1: Serial Dilution and plating on LBA medium from soil sample (After 24 hr incubation at 37°C). Plates are arranged by 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilution.

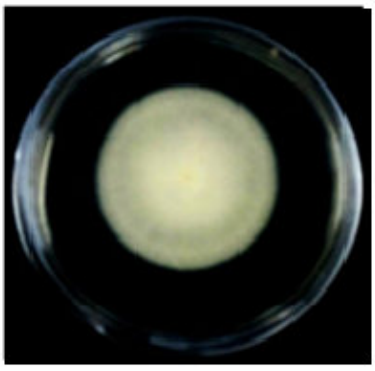

Fig. - 2. Control plate *A. flavus*

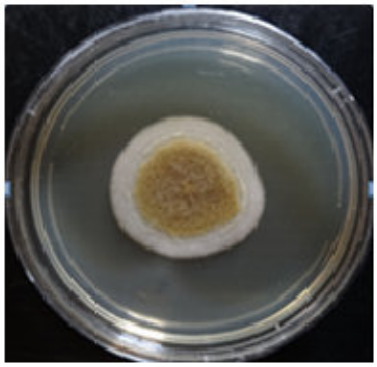

Fig. - 3. Control plate *A. flavus*

Fig. - 4. Duel culture (*A. flavus and P. fluorescens*)

Fig. - 5. Duel culture (*A. flavus and Bacillus subtilis*)

Table 1: Colony radius of Duel Culture and Control on CDA and PDA medium after incubation for 48 hrs. at 30°C. All of the experiments were done 3 times and their mean value was taken.

Serial No.	Microbe Used	Control Diameter of *A. flavus* (cm.)	Diameter in Duel (cm.)	Percentage of inhibition
1.	*P. fluorescens*	2.1	0.7	66.66%
2.	*Bacillus subtilis*)	1.5	0.3	83%

Table 2: Comparative chart of dry weight of Duel Culture and Control organism grown on LB for 7 days at 30°C. All of the experiments were done 3 times and their mean value was taken.

Serial No.	Organism Used	Control Weight *A. flavus* (gm.)	Dual culture Weight (gm.)	% Weight loss
1.	*P. fluorescens*	1.85	0.56	69.72
2.	*Bacillus subtilis*	3.80	1.08	72.68

Percentage inhibition of *A. flavus*

Data were obtained for the percentage inhibition of radial growth [100 x (R1 - R2)/R1], where R1 = radial growth of the pathogen in control and R2 = radial growth of the pathogen in dual culture with antagonist (Mohsin T, et al 2010). Results are means of three replicates.

Weight Loss of *A. flavus*

Weight loss study was also done with cell extract of *pseudomonas fluorescens* and *Bacillus subtilis.*

Morphological Identification:

Normal morphological identification was done for the bacterium. *P. fluorescens* appears pale yellow in young and reddish yellow in old while grown in NA medium. It is a rod shaped Gram (-)ve bacteria. Other *Bacillus sp.* were appeared off-white on LB medium.

16s rRNA sequencing and identification:

One antagonist organism which named as HGA was found to be closely related to *Pseudomonas fluorescens* and other three are believed to be *Bacillus sp.* based on the basis of nucleotide homology and phylogenetic analysis.

Gel Image of 16SrDNA amplicon (Sample: HGA)

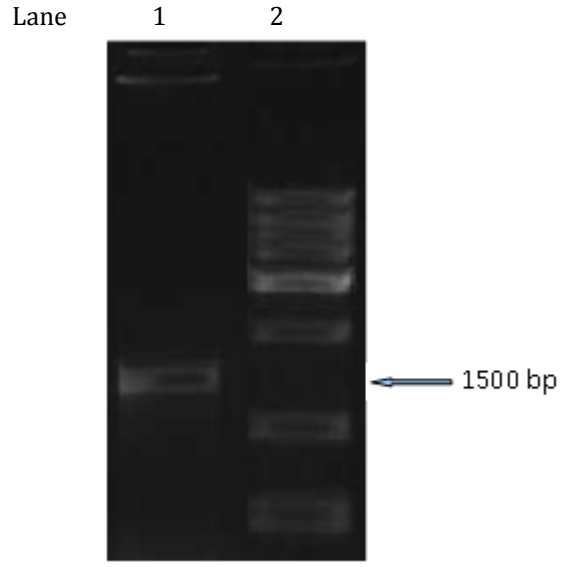

Fig. 6: Agarose Gel Image (Lane 1:16S rDNA amplicon band and Lane 2: DNA marker)

Analysis of Phylogenetic tree:

The culture, which was labeled as *Pseudomonas fluorescens HGA* was found to be closely related (99% similarity) to ***Pseudomonas fluorescens strain R15*** based on nucleotide homology and phylogenetic analysis.

Information about other close homologs for the microbe is shown in the Alignment View table.

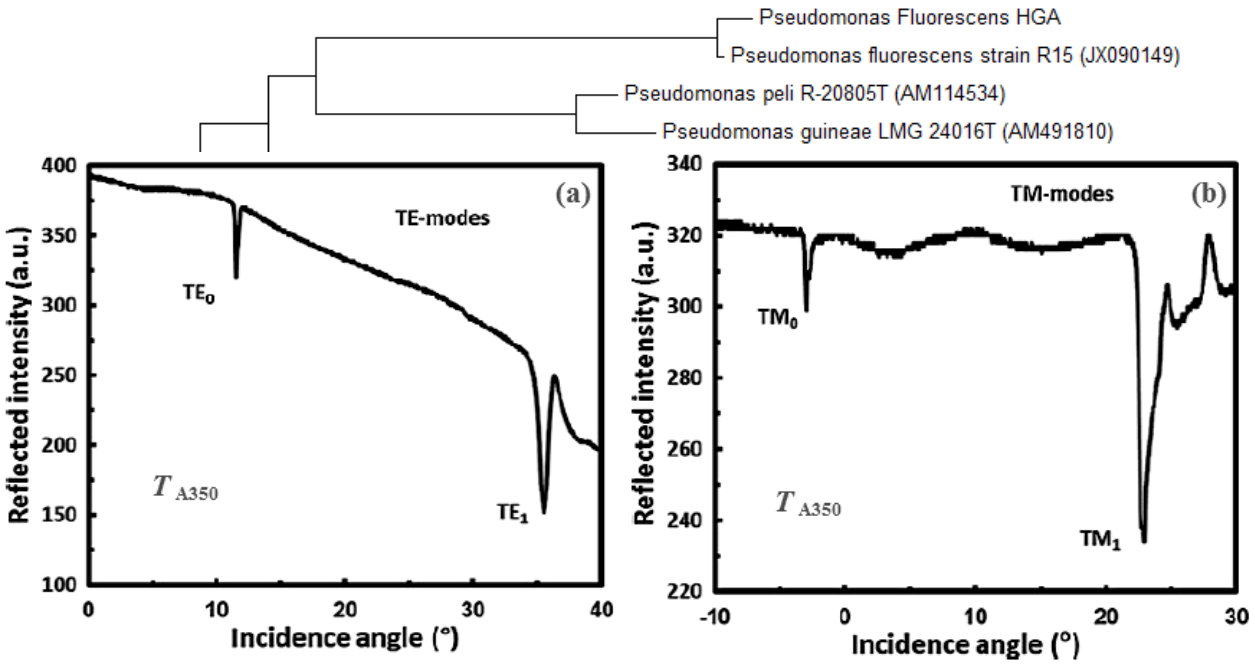

Fig. 7: Phylogenetic Tree (Neighbor Joining) ***Pseudomonas fluorescens strain R15***

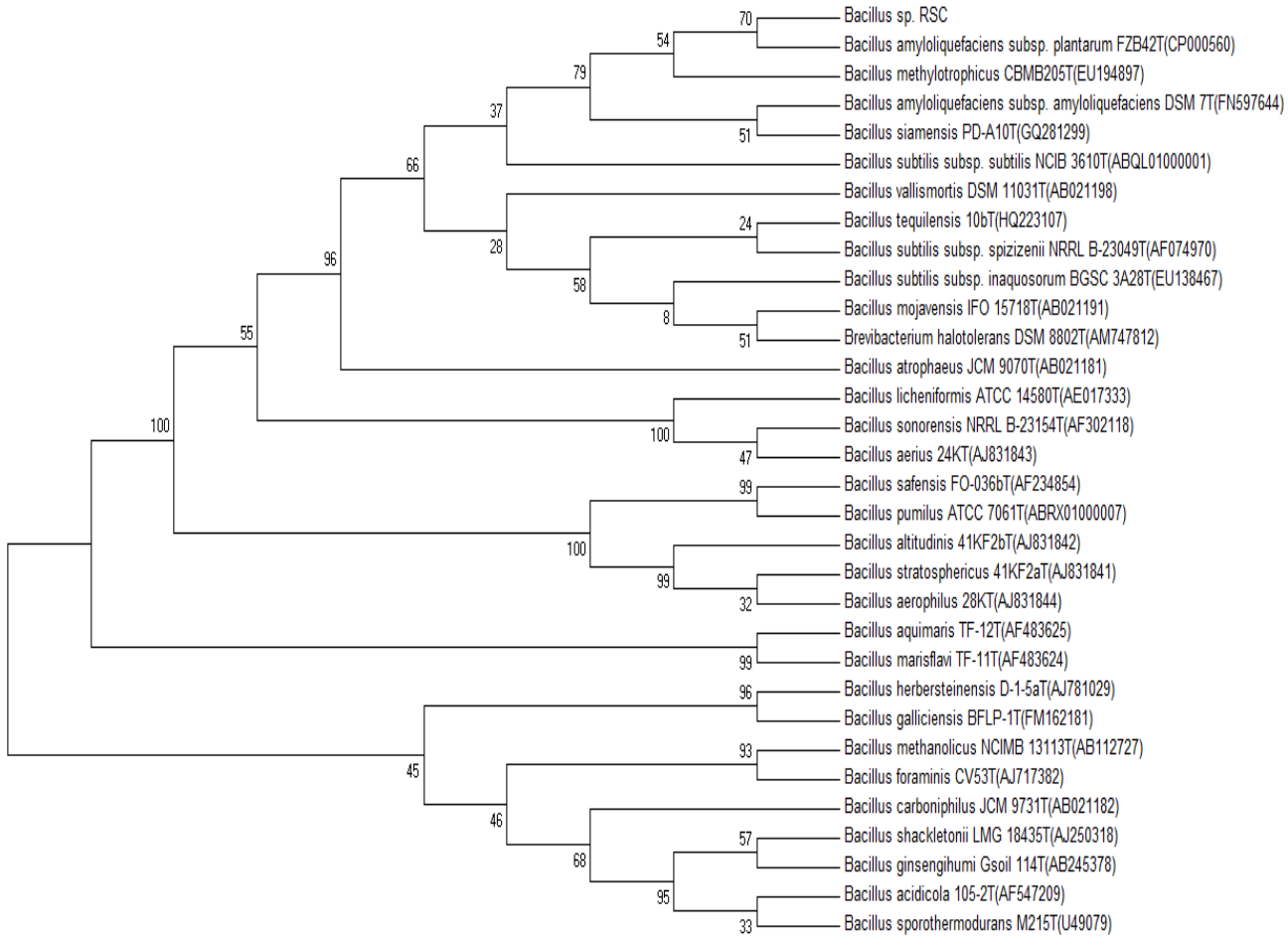

Fig. 8: Phylogenetic Tree (Neighbor Joining) ***Bacillus subtilis***

The two microbes were isolated from the soil and identified as *Pseudomonas fluorescens* and *Bacillus subtilis.* Both the microbe showed significant inhibition and weight loss of the A. flavus toxigenic strain. These two microbes can be utilized as bio-control agents.

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