Degradation of Aflatoxin B1 by *Bacillus subtilis*

SHT 1 from Sheep Hide Tanning Site

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**Abstract:** The soil from sheep hide tanning site in Tiruchengode region was collected and analysed for aflatoxin degrading bacteria. The bacterial isolate which utilize coumarin as its sole carbon source was selected as the coumarin ring forms the basic structure of Aflatoxin B1 (AFB1). Then aflatoxin degrading activity of the isolate was studied at different concentrations of standard AFB1. Both cell free supernatant and pellets were analysed for aflatoxin estimation using HPTLC. The results indicated 100% degrading activity of bacterial isolate at 400 ppm causing concentration at 37°C within 72 hours at the pH of 8. The 16 s rRNA of the isolate was amplified and the PCR product was sequenced. Using U BLAST tool, the phylogenetic tree was constructed. The isolate showed 100% sequence similarity with *Bacillus subtilis*. Thus a bacterial isolate, *Bacillus subtilis* SHT 1, showing 100% AFB1 degrading activity was reported for the first time. Owing to the efficiency of the enzyme in degrading AFB1, the study suggests the promising application of the same in the control of aflatoxins during food and feed processing and storage.

**Key words:** *Bacillus subtilis*, Aflatoxin B1, 16s rRNA, AFB1 degradation

**Introduction**

Aflatoxins, produced by certain strains of the *Aspergillus* sp., are cancerogenic compounds. In order to circumvent the health risk posed by aflatoxins, it is essential to prevent the contamination of food...
and feed. Unfortunately, these contaminants can not be completely removed, and on that account, many studies have been carried out to explore an effective process of their detoxification to a threshold level. Aflatoxins are a group of structurally related difuranocoumarin derivatives produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* [1]. *A. flavus* and closely related subspecies *parasiticus* have a worldwide distribution and normally occur as saprophytes in soil and on many kinds of decaying organic matter. These fungi readily colonize several important crops such as corn, cottonseed, peanuts and tree nuts [2]. Aflatoxins mainly inhibit protein and DNA synthesis in bacteria and animal cells [3]. Aflatoxin B1 (AFB1), one of the most hazardous mycotoxins, has been reported to be extremely toxic, mutagenic and carcinogenic [4,5]. It poses serious threat to both livestock productivity and human health thus bringing huge worldwide economic losses each year [6]. Biological decontamination seems to be attractive because it works under mild and environment friendly conditions. Microorganisms such as soil or water borne bacteria, fungi, and protozoa and specific enzymes isolated from microbial systems can degrade members of aflatoxin group with varied efficiency to yield less- or nontoxic products. Yeasts and lactic acid bacteria work as biological adsorbents that prevent aflatoxin’s transfer to the intestinal tract of humans and animals. Aflatoxin B1 absorbed into the organism could be metabolized significantly by different pathways [7].

Various physical and chemical methods have been developed and tested for controlling AFB1. However, disadvantages of these methods, such as nutritional loss, sensory quality reduction and high cost of equipment, have limited their practical applications [8,9]. Aflatoxins are a group of bisfuranocoumarin derivatives and the lactone ring in the common coumarin structure plays an important role in their toxicity and mutagenicity [10].

As Aflatoxin B1 (AFB1) is regarded one of the most harmful agent in animal production and food industry, a safe, effective and environmentally sound detoxification method is needed for controlling this toxin. Recently, here has been a great interest in biological detoxification of AFB1. Several fungal species have been shown to transform AFB1 into less toxic metabolites. The degradation activities of these fungi were mainly due to their cell extracts. However, practical applications of these fungi may be limited by factors such as long incubation time (more than 120 h, required for the detoxification) and complicated procedures needed for obtaining the active extracts. Reduction of AFB1 by bacteria has also been reported. However, the AFB1 reduction by the bacteria was proven to be mainly by cell binding rather than metabolism or degradation. Most importantly, this kind of binding seems to be reversible, which means that AFB1 can hardly be removed completely from contaminated media [11]. AFB1 degrading activity by bacteria such as *Propionibacterium*, *Lactococcus*, *Rhodococcus erythropolis*, *Mycobacterium fluoranthenum* and *Nocardia corynebacterioides* had already been reported several researchers [12-16].

The current research was aimed at finding a new AFB1 degrading bacterium from sheep hide tanning site which could use coumarin as carbon source.
Materials and Methods

Sample collection

Ten gram of soil sample was collected aseptically from the four corners and the centre of a sheep hide tanning site in Tiruchengode region located in Tamilnadu state, India and brought to the laboratory under aseptic conditions for the isolation of aflatoxin degrading bacteria. All the samples were mixed properly and 1 gram of soil sample was serially diluted and total heterotrophic bacteria were enumerated by spread plate technique [17].

Identification of Aflatoxin Degrading Bacteria

Isolated pure colonies were streaked on the coumarin medium (CM) plates. The medium contains Coumarin (10.0 g/lit), KH₂PO₄ (0.25 g), NH₄NO₃ (1.0 g), CaCl₂ (1.0 g), MgSO₄.7H₂O (0.25 g), FeSO₄ (1.0 mg), Agar (15.0 g), pH - 7.0. The plates were incubated at 37°C for 3-7 days until visible colonies appeared. Single colonies were isolated and subsequently transferred to fresh CM plates for three times. Colonies that were able to grow on the medium were selected and preserved as pure isolates on nutrient agar, and tested for AFB1 degradation [11]. The bacterial isolate which was able to utilize coumarin was subjected to morphological and physiological characterization according to Bergey’s Manual of Determinative Bacteriology (8th edition). Further to tests such as gram staining, spore staining, motility test, Indole test, Methylred test, Voges Proskauer (VP) test, Catalase test and Starch hydrolysis the organism was identified up to genus level.

AFB1 Degradation by Bacterial Isolates

Degradation of aflatoxin B1 by the selected isolates was carried out in liquid culture using the procedure described by Guan et al. [11]. The bacterial isolates were cultured in nutrient broth. For inoculation, 2.5 ml of 12 hours culture was transferred to 50 ml broth in 250 ml conical flask. The bacteria were grown at 37°C under shaking for 24 hours. AFB1 standard (Sigma Chemicals) solution was diluted with Benzene:Acetonitrile (99:1) to prepare the stock solution of 2.5 ppm/µl. One ml of microbial culture were taken in 4 microfuge tubes and 40 µl (100ppm), 80 µl (200ppm). 120 µl (300ppm) and 160 µl (400ppm) of standard solution were added respectively. The degradation test was conducted in the dark at 37°C without shaking for 72 hours. After incubation, the bacterial cells were removed by centrifugation at 10,000 rpm for 10 minutes. The supernatant and pellet were taken for analysis by HPTLC (High Performance Thin Layer Chromatography).

For AFB1 analysis, the HPTLC procedure prescribed by Association of Official Analytical Chemist [18] (AOAC, 2005), was used. TLC plate (Merck) was coated with silica gel and CAMAG Linomat 5 – spotter was used for spotting the sample on the plate. Ten µl of supernatant was spotted on the plate. Two hundred µl of chloroform was added to the pellet and mixed thoroughly and from this 10 µl aliquot was taken for analysis. Ten µl (1ppm/1 µl) of AFB1 standard was also spotted on the plate. Chloroform:Acetone (9:1) served as a solvent. After spotting, the plate was kept in the solvent beaker
and was viewed under UV chamber. The bands formed by samples were compared with standard bands. The TLC plates were scanned using CAMAG TLC scanner -3 for quantification of aflatoxin B1. Wincats software was used for generating chromatogram.

**Molecular Identification of Aflatoxin degrading Bacterium**

**Isolation of Genomic DNA**

Fifteen ml of overnight bacterial culture was prepared and the cells were harvested and collected in a microfuge tube. The pellet was re-suspended with 300 µl sucrose-Tris-EDTA buffer and to this 30 µl RNase/lysozyme mixture was added and kept in boiling water for 1 minute and 15 seconds. The tube was spun for 15 min and the supernatant was separated. To this 500 µl phenol was added and centrifuged. To the supernatant 1/10 volume 4 M lithium chloride (autoclaved) was added and kept in ice for 5-10 min. Then equal volume of isopropanol was added and kept at room temperature for 5 min. DNA which remained stuck to side all the way up the tube.

**16S rRNA Gene Amplification and Sequencing**

16SrRNA gene was amplified and sequenced using the procedure described by Trotha and Konig [19]. The reaction mixture containing template DNA (20 ng/1µl), master mix 10µl, forward primer 50 pmol/1µl, reverse primer 50 pmol/1µl and 1µl of Taq polymerase (0.5U) was taken in a microfuge tube to which 10 µl of sterile nuclease free water was added. The vials were kept in PCR machine and the program was set for 35 cycles. The following primers were used for this purpose:

- Forward primer- 16SrRNA F- 5' -TGA GGA AGA TAA TGA CGG -3'
- Reverse primer - 16SrRNA R - 5' -CCT CTA TCC TCT TTC CAA -3'

Using the PCR product directly as sequencing template, the primer sequenced nearly complete 16 s rRNA genes. As the PCR products were to be sequenced by a highly sensitive method, in order to avoid any impurities it was separated by electrophoresis on a 2% agarose gel in TBE buffer and DNA fragments were extracted from the gel using the QIAquick Gel Extraction Kit. After purification, the amplified product was separated by automated chip electrophoresis [20]. All the sequencing reactions were carried out using an automated DNA sequencer.

**Molecular Taxonomic Determination**

The 16 s rRNA gene sequences obtained by PCR amplification was considered as the query sequence. In order to identify the taxonomy of query sequence, similarity search (BLAST) was performed and several hits were obtained. Phylogenetic tree was constructed for the identification of the bacterium up to the species level.
Results

The inoculation and incubation soil sample of sheep hide tanning site on nutrient agar medium yielded ten bacterial isolates. Further screened of these isolates on coumarin medium indicated the growth of one bacterial isolate which used coumarin as carbon source (Fig.1). While it was cream coloured, flat and circular with undulate margins on nutrient media it formed white coloured colonies on coumarin media. Grams staining indicated the presence gram positive bacilli (Fig.2).

The spore staining, motility and other biochemical tests (Table 1) confirmed the isolate to belong to the genus *Bacillus* and it was designated as *Bacillus subtilis* SHT1.

Different concentrations of Aflatoxin B1 (AFB1) were prepared and first sample was spiked with 200ppm (2.5ppm/µl=80µl of STD), second sample with 300ppm (2.5ppm/µl=120 µl of STD), and finally third sample with 400ppm (2.5ppm/µl=80 µl of STD). Keeping the set up for 72 h at room temperature, it was centrifuged. Ten µl each of the supernatant from all the three samples was taken and spotted on HPTLC plate. Also 10 µl of standard AFB1 with the concentration of 1ppm/1 µl was spotted on the plate.

As the aflatoxins tend to bind on bacterium, the pellet was washed with 200 µl of chloroform and from which 10 µl was taken and spotted. Similar procedure was done for all the three pellets. It was confirmed by viewing under UV chamber that the aflatoxins did not bind to bacterial cells because their spots did not give fluorescence. Since only the spots of the supernatants gave fluorescence, it was confirmed that only the cell free extract had aflatoxins (but not the pellet) owing to their ability of spiking aflatoxin. The isolated bacterium had the ability to degrade AFB1 upto 400ppm. Fig. 3 shows the detection of aflatoxin using HPTLC plate under UV chamber, in that only AFB1 standard spots gives the bands.
Table 1. Biochemical identification of bacterial isolate

<table>
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<th>Results</th>
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<tr>
<td>Spore staining</td>
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</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
</tr>
<tr>
<td>Indole production</td>
<td>-</td>
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<tr>
<td>Methyl red</td>
<td>-</td>
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<tr>
<td>Voges proskauer</td>
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<td>Sucrose utilization</td>
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After separation the HPTLC plate was scanned for making chromatogram using Wincats software for the first three spots. Figures 4a and 4b show chromatogram generated by Wincats software showing no hike in supernatant spot but hike was reported in standard AFB1 spot. So it indicates complete degradation of AFB1 by the isolated organism.

[Spots 1 - 200ppm supernatant; 2 - 300ppm supernant; 3 - 400ppm supernatant; 4 - (1ppm/1ml) 10ml - AFB1 standard; 5 - (1ppm/1ml) 10ml - AFB1 standard; 6 - 200ppm pellet; 7 - 300ppm pellet; 8 - 400ppm pellet]

Fig. 3 Detection of Aflatoxin in HPTLC plate under UV chamber
Degradation of Aflatoxin B1 by Bacillus subtilis

Fig. 4a. HPTLC Chromatogram generated by Wincats software.

[No hikes in graph KSR1 S1 (for 200ppm) and KSR2 S2 (for 300ppm) indicating absence of AFB1 i.e., degradation of spiked standard AFB1]
Evaluation results

<table>
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[No hike in graph KSR S3 (for 400ppm) indicating 100% degradation of AFB1. Presence of hike in graph STD (1ppm/µl) indicates the presence of AFB1]

Fig. 4b. HPTLC Chromatogram generated by Wincats software
Lane 1-Marker DNA; Lane 2-Amplified product (unpurified); Lane 3-Amplified product (purified) - 1500 bp size

**Fig. 5.** 16S rRNA gene amplified product of *Bacillus subtilis* SHT 1

The DNA was isolated from the bacterium and then 16S rRNA gene was amplified for identification. The amplified 16S rRNA gene sequence was then purified and subjected to chip based electrophoresis. The template DNA was separated near the well owing to its high molecular weight. The second and third wells were loaded with unpurified purified PCR product. The results indicated that amplified product to possess a size of approximately 1500bp (Fig. 5).

The 16S rRNA gene sequence (query sequence) thus obtained was subjected to similarity search with the help of BLAST tool. Nucleotide blast search was performed against non-redundant database and several hits were obtained. Among them the first hit was observed to be identical to the query sequence with 100% similarity and the expected value (E) = 0. The results indicated the maximum degree of similarity between hit and query sequences, which confirmed the identity of the bacterium
as *Bacillus subtilis* (Fig 6). Then phylogenetic analysis was carried out again employing BLAST and Figure 7 shows the taxonomic determination of the query sequence with related taxa.

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TACATCGTCTCGAGGAGGCACATGGAGGCTTTCCTGTAGTTAGCGGCGGGAAGGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAGACCGGGGCTAATACCGGATGGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCCGGCGCATTAGCTAGTTGGTGAGGTAAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAAAGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACCTCCGCCTGGGGAGTACGGTCGCAGGACTGAAACTCAAGGAATGAC
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**Fig. 6** Primary 16SrRNA sequence of *B.subtilis* SHT 1
Degradation of Aflatoxin B1 by Bacillus subtilis

Fig. 7 Taxonomic determination of B. subtilis SHT 1 by Phylogenetic analysis
Discussion
In the present investigation ten isolates were obtained were screened on coumarin media in order to
determine their AFB1 degrading ability. Out of ten isolates only one was able to grow on the coumarin
plate thus indicating its utilization of coumarin as sole carbon source. As coumarin consists of the
basic structure similar to that of aflatoxins [21], the bacteria which utilize coumarin, as their carbon
source might also use aflatoxins. Guan [11] applied this principle to screen a vast number of microbial
populations of various sources.

The bacterial isolate of the present study which could degrade aflatoxin B1 was confirmed to be
*Bacillus subtilis*, a common soil inhabitant. Similarly, Kimura and Hirano [22] also had isolated *Bacillus
subtilis* from Japanese soil which inhibited the growth of *A.parasiticus* on PDA plate.

The isolated organism (culture supernatant and pellet) was incubated in the liquid medium at
37°C for 72 h and pH 8 along with AFB1 and further analyzed for its AFB1 degrading ability by HPTLC.
The result showed that *B.subtilis* SHS 1 exhibit 100% aflatoxin degrading activity up to 400ppm. Guan
[11] reported that *Stenotrophomonas maltophilia*, obtained from tapir feces could degrade aflatoxin
to the extent of 82.5% after incubation in the liquid medium at 37 °C for 72 h. The achievement of higher
rate of degradation of aflatoxin in the present study may be due to the maximal enzyme activity in the
supernatant at pH 8. While *B. subtilis* SHS 1 showed 100% degrading ability at pH 8, the same of *S.
maltophilia* was only 84.4% at the same pH. It may be due to the production of some extra cellular
enzymes by the isolate during the incubation period. Other studies with *B. licheniformis* isolated from
fresh fermented soybeen [23] and *Flavobacterium aurantiacum* [24] had been reported to show 74%
and 94.5% of AFB1 degrading ability respectively. Marisa et al. [25] reported the production of an
extra cellular enzyme by the edible mushroom *Pleurotus ostreatus* and attributed it for aflatoxin degrading
activity. The extracellular enzyme in the supernatant may play an important role to cleave the lactone
ring of aflatoxin [16]. The significant inhibitory effect on *Aspergillus* species by the bacteria of the
genus *Bacillus* such as *B. subtilis*, isolated from ground nut and *B. pumilus* had been reported [26].
Zhao [27] had demonstrated the occurrence of a 32kDa aflatoxin degrading enzyme in the supernatant
of *Myxococcus fulvus*

The 16S rRNA gene of the bacterial isolate of the present study when subjected BLAST and
phylogenetic analysis the bacterium isolated in the present study was identified as *Bacillus subtilis*.
Similar procedure was adopted by Trotha and Koning [18] for the sequencing of some medically
important bacteria.

*B. subtilis* metabolises a wide variety of carbon sources and synthesises large quantities of
industrially important enzymes and is not considered a human pathogen. In addition, as it is highly
amenable to genetic manipulation, it has therefore become a model organism being widely adopted for
many laboratory studies. The bacterial isolate of the present study showing higher aflatoxin degrading
activity could be well explored for promising applications, especially for the control of aflatoxins
during processing and storage of food and feed.
Degradation of Aflatoxin B1 by Bacillus subtilis

References


