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Research Article

ANTIFUNGAL ACTIVITY OF *Bacillus* SPECIES ISOLATED FROM SOIL SAMPLES OF NEPAL AGAINST AFLATOXIN PRODUCING *Aspergillus* spp

Suprina Sharma[1], Dev Raj Joshi[1], Pramod Poudel[2], Supriya Sharma[1], Tika Bahadur Karki[3], Reshma Tuladhar[1,*]

[1]Central Department of Microbiology, Institute of Science and Technology, Tribhuvan University, Kirtipur, Kathmandu, Nepal
[2]Central Department of Biotechnology, Institute of Science and Technology, Tribhuvan University, Kirtipur, Kathmandu, Nepal
[3]Department of Biotechnology, School of Science, Kathmandu University, Dhulikhel, Nepal
**Correspondence: reshma.tuladhar@cdmi.tu.edu.np*

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ABSTRACT

In Nepal, improper storage of staple foods leads to contamination by aflatoxin-producing molds, posing serious health risks like liver cancer. Farmers, unaware of these dangers, often use unsafe chemicals for prevention, while biological control using *Bacillus* spp., a proven, safe, and multifunctional alternative, remains unexplored. This study aims to isolate native *Bacillus* species capable of inhibiting *Aspergillus flavus* growth and detoxifying aflatoxins, offering a sustainable solution. A total of 50 samples (40 soil samples, 5 compost and 5 food samples) were randomly collected from different places of Nepal. *Bacillus.* spp were selectively isolated by heating the samples at 80^0C for 10 minutes. The isolates were identified by standard microbiological techniques. Growth inhibition of *A. flavus* by *Bacillus.* spp was done by dual-culture technique on potato dextrose agar. Isolates showing high inhibitory effect against *A. flavus* were further identified by 16S rRNA gene sequencing. Altogether 51 isolates were presumptively identified as *Bacillus* species. Five isolates, *Bacillus subtilis, B. tequilensis, B. licheniformis, B. cereus, B. weidmannii* effectively inhibited the growth of aflatoxigenic *A. flavus*. These potent *Bacillus* strains will have implication in the commercial production of bioactive anti-aflatoxigenic compound to minimize the postharvest contamination of the crops.

Keywords: Aflatoxin, *Aspergillus flavus, Bacillus* species from soil, biocontrol, postharvest contamination

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by molds such as *Aspergillus*, *Fusarium*, and *Penicillium*, which frequently contaminate staple foods like maize before and after harvest (Awuchi et al., 2021; Wu et al., 2014). Among them, aflatoxins mainly AFB1, B2, G1, and G2 are produced by *A. flavus* and *A. parasiticus*. AFB1, the most potent, is a Group I carcinogen (Ostry et al., 2017) and is biosynthesized via a polyketide pathway under favorable environmental conditions. Consumption of aflatoxins leads to acute liver damage (aflatoxicosis), immune suppression, stunting, and liver cancer (WHO, 2023).

In Nepal, maize is the second most consumed cereal, particularly among low-income populations. A nationwide study reported that 78% of maize samples were contaminated with aflatoxins, and 24% exceeded the national safety limit of 20 µg/kg (Joshi et al., 2022). Co-contamination with multiple mycotoxins was also common, raising serious food safety concerns. Factors such as high humidity, poor postharvest handling, and traditional storage structures contribute to fungal proliferation and toxin accumulation.

Biological control using *Bacillus* spp. provides a promising alternative to chemical methods. These bacteria produce antifungal lipopeptides, enzymes, and other metabolites that inhibit mold growth and can degrade mycotoxins (Ahmad et al., 2022). In addition to food safety applications, *Bacillus*-derived compounds are also being explored for their potential in developing antibiotics, antifungals, and antivirals. However, their role in aflatoxin detoxification remains underexplored. This study aims to isolate native *Bacillus* spp. from different soil, compost and food sample in Nepal and evaluate their antagonistic activity against *Aspergillus flavus*, with the goal of identifying potential Bacillus species capable of inhibiting the growth of *A. flavus* for future use in biocontrol and food safety interventions.

MATERIALS AND METHODS

Study Design

A cross-sectional study was conducted from November 2022 to December 2024 for isolation of *Bacillus* spp. from soil, compost and food samples collected from different places of Nepal.

Study Site

A total of 50 samples (40 soil samples, 5 compost and 5 food samples) were collected from different places of Nepal, mainly from Terai and Hilly region (Kathmandu, Bhaktapur, Lalitpur, Hetauda, Kirtipur, Sankhu, Biratnagar, Chitwan, Surkhet, Dharan, Pokhara). The non-probability purposive sampling technique was used to collect the samples. Samples were collected both from humid and less humid areas of Nepal.

Sample Collection and Transportation

Sample was aseptically collected following standard microbiological techniques. Samples were collected from the cultivated areas of Nepal, mainly hilly and terai

regions. Food samples were collected from Kathmandu, Bhaktapur, Lalitpur, Chitwan and Biratnagar based on Khadka et al. and Mallick et al. The samples were collected in sterile zip-lock bags separately and stored at 4°C and were transported to CDMi, TU for further laboratory analysis. These samples were analyzed using the standard microbiological method. The ethical approval (Ref. No. 414) was obtained from the Ethical Review Board, NHRC.

Sample Processing

Twenty-five grams of each soil or compost sample was transferred separately to 250 mL of sterile normal saline and heated in a water bath at 80°C for 10 minutes with constant stirring. Tenfold serial dilution was done up to 10^{-6} for each sample, and then, 0.1 mL of an aliquot from the dilutions of 10^{-2}, 10^{-4}, and 10^{-6} were aseptically inoculated into sterile nutrient agar (NA) (Hi Media, India) by spread plate technique (Magar et al., 2022; Zhao et al., 2018). Each plate was incubated at 37^{0}C for 48 hours for the isolation of *Bacillus* spp (Hall et al., 2015).

Phenotypic identification of *Bacillus* spp.

henotypic identification of *Bacillus* isolates was performed based on colony morphology, Gram staining, spore staining, biochemical tests, and sugar fermentation profiles.

Antifungal activity of Bacillus spp against aflatoxin producing Aspergillus flavus

The isolated *Bacillus* species were tested for their antifungal activity against *A. flavus* (Farzaneh *et al.*, 2012). A pure culture of *Bacillus* spp. was streaked at the center of a Potato Dextrose Agar (PDA) plate. A suspension of *A. flavus* was spot inoculated on the same plate at a distance of approximately three centimeters from the *Bacillus* streak. The plate was incubated at 28°C, and the radial growth of the fungal colony was measured daily for up to 6 days. Any inhibition of fungal growth by *Bacillus* spp. was observed and recorded (Mannaa et al., 2017). The aflatoxin-producing *A. flavus* ATCC 26949 was used as the reference strain and aflatoxin production ability was checked in Aspergillus Differentiating Media (Davis et al.,1987).

The percentage inhibition was calculated by comparing the growth of *Aspergillus* in the presence of *Bacillus* (treatment plate) with the growth in its absence (control plate), using the formula:

$$\% \text{ Inhibition} = \frac{\text{Growth in control (C)} - \text{Growth in treatment (T)}}{\text{Growth in Control (C)}} \times 100$$

Identification of potent antifungal *Bacillus* spp by 16srRNA gene sequencing

Potent *Bacillus* isolates were identified by 16S rRNA gene sequencing. DNA was extracted by boiling bacterial colonies in Tris-EDTA buffer followed by centrifugation. The 16S rRNA gene was amplified using universal primers 8F (5'-AGA GTT TGA TCC TGC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') under specific PCR conditions. Briefly the amplification conditions were: initial denaturation at 98°C for 30 s followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were purified and sequenced bi-directionally by Sanger sequencing. Sequence analysis was performed with BioEdit software and compared to GenBank using NCBI BLAST. Multiple sequence alignment and phylogenetic tree construction used CLUSTAL W and MEGA 12 with neighbor-joining and bootstrap validation (Tamura et al., 2021).

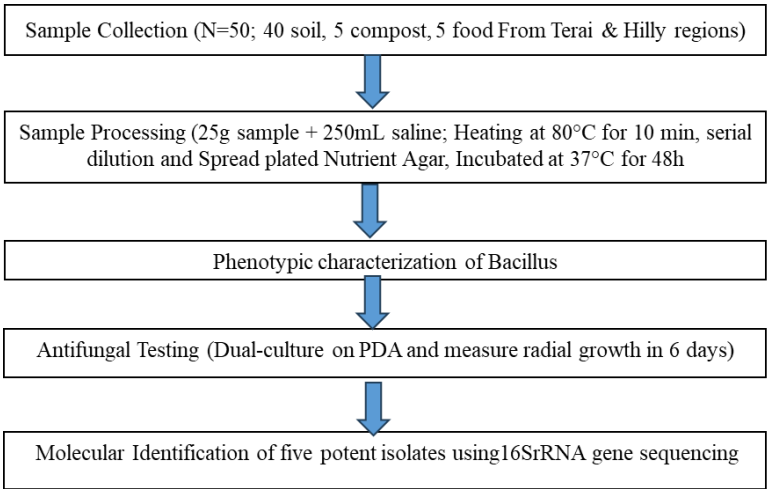

Figure 1. Simple flowchart representing the overall methodology of this study

RESULTS

From 150 randomly selected colonies, 51 Gram-positive, spore-forming isolates were confirmed as *Bacillus* spp. (34% isolation efficiency). Soil samples yielded higher *Bacillus* recovery compared to compost and food samples. (Table 1).

Table 1. Number of *Bacillus* species isolated from different samples

Type of sample	Number of samples processed	Number of *Bacillus* species isolated
Soil sample	40	49
Food samples	5	1
Compost samples	5	1

To explore the regional variation in *Bacillus* isolation, samples were collected from three distinct topographical regions. The Terai region yielded the highest number of isolates compared to hill and mountain areas (Table 2).

Table 2. Isolation of *Bacillus* species from different topographical regions

Region	No. of samples processed	No. of *Bacillus* species isolated
Hill	15	7
Terai	30	43
Mountain	5	1

The antifungal potential of the isolated *Bacillus* spp. was evaluated against aflatoxin-producing *Aspergillus flavus* using a dual culture technique. Several isolates, particularly those from soil samples, demonstrated strong inhibitory effects, as indicated by high percent inhibition values (Table 3).

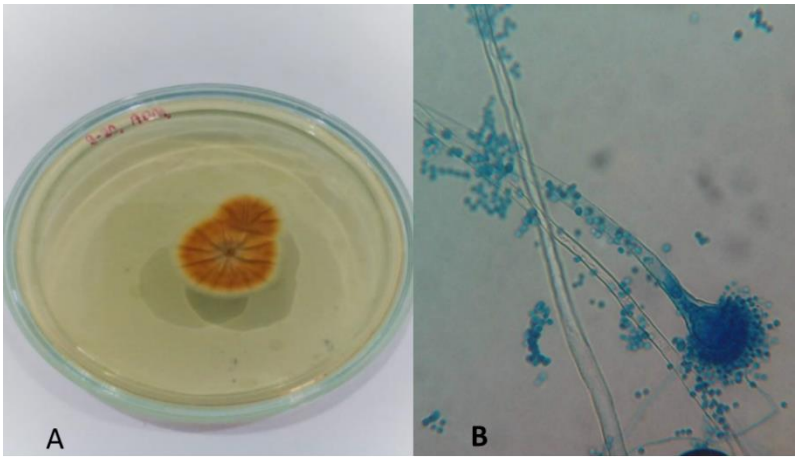

Figure 2. Colony color of *A. flavus* (aflatoxin producers show yellow pigmentation) in Aspergillus Differentiating Media and microscopic morphology of *A. flavus* under 100 X magnification.

Table 3. Antifungal activity of *Bacillus* spp against aflatoxin producing *Aspergillus flavus*

Code of *Bacillus* sp.	Sample type	% Inhibition
BS1	Soil	70.02%
BS2	Food	92.00%
BS3	Soil	90.00%
B1	Compost	51.00%
B2	Soil	48.88%

Table 3 shows the antifungal activity of five *Bacillus* isolates against *Aspergillus flavus*, measured by percentage inhibition of fungal growth. Isolates BS2 (from food) and BS3 (from soil) showed the highest inhibition, at 92.00% and 90.00%, respectively, indicating strong antifungal potential. BS1 (soil) showed moderate inhibition (70.02%), while B1 (compost) and B2 (soil) showed lower activity, with 51.00% and 48.88% inhibition. These results suggest that *Bacillus* isolates, especially BS2 and BS3, could be effective in controlling aflatoxin-producing fungi.

Figure 3. Growth inhibition by isolate BS2 against aflatoxin producing *A. flavus* on PDA media up to 6 days of incubation. A, represents growth inhibition by isolate BS2 and B, represents control plate without isolate BS2.

Table 4. Identification of *Bacillus* spp based on 16SrRNA gene sequences from the database

Bacterial isolates	Closely related strains in NCBI	% similarity based on 16S rRNA gene
BS1	*B. tequilensis*	99.80
BS2	*B. subtilis*	99.93
BS3	*B. licheniformis*	99.73
B1	*B. cereus*	99.77
B2	*B. weidmannii*	99.69

Table 4 summarizes the molecular identification of five bacterial isolates based on 16S rRNA gene sequencing. All isolates showed high similarity (above 99.6%) with known *Bacillus* species listed in the NCBI database. Isolate BS1 was closely related to *Bacillus tequilensis* with 99.80% similarity, while BS2 showed the highest match with *Bacillus subtilis* (99.93%). Similarly, BS3 matched with *Bacillus licheniformis* (99.73%), B1 with *Bacillus cereus* (99.77%), and B2 with *Bacillus wiedmannii* (99.69%). These results confirm that the isolates belong to the *Bacillus* genus with strong genetic similarity to reference strains.

Evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.133 is shown (Figure 4). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. The analytical procedure encompassed 30 nucleotide sequences. The pairwise deletion option was applied to all ambiguous positions for each sequence pair resulting in a final data set comprising 1,587 positions. Bootstrap values (expressed as percentages) are shown at the branch nodes and were generated from 1000 replicates. Evolutionary analyses were conducted in MEGA12.

DISCUSSION

This study successfully isolated 51 *Bacillus* species from soil, compost, and food samples across Nepal. Most isolates (49 out of 51) obtained from soil, confirming its role as a key habitat for these bacteria (Hong et al., 2009). The Terai region had the highest number of isolates (N=43), likely due to its warm, humid climate, which supports microbial growth better than cooler mountain areas (Rousk et al., 2009).

Only five isolates showed strong antifungal activity against *Aspergillus flavus*, with *Bacillus subtilis* BS2 being the most effective (92% inhibition). These results match previous studies where *Bacillus* species like *B. subtilis* and *B. licheniformis* were found to produce natural antifungal compounds (Ahmad et al., 2022; Farzaneh et al., 2012). 16S rRNA gene sequencing confirmed these strains were closely related (>99% match) to known *Bacillus* species used in biocontrol worldwide. However, this is the first report demonstrating the inhibitory effect of indigenous *Bacillus* species against aflatoxin-producing *Aspergillus flavus*.

The findings suggest that native *Bacillus* strains from Nepal could be used to fight aflatoxin contamination in crops. Since aflatoxins are a major health risk in Nepal (Joshi et al., 2022), these bacteria may offer a safe, natural alternative to chemical fungicides. However, more research is needed to test their effectiveness in real farm conditions and identify the exact compounds responsible for their antifungal effects.

This study supports the idea that native *Bacillus* isolates could help reduce aflatoxin levels in food, improving food safety in Nepal and similar regions. Future work should explore large-scale applications and possible commercial production of these biocontrol agents.

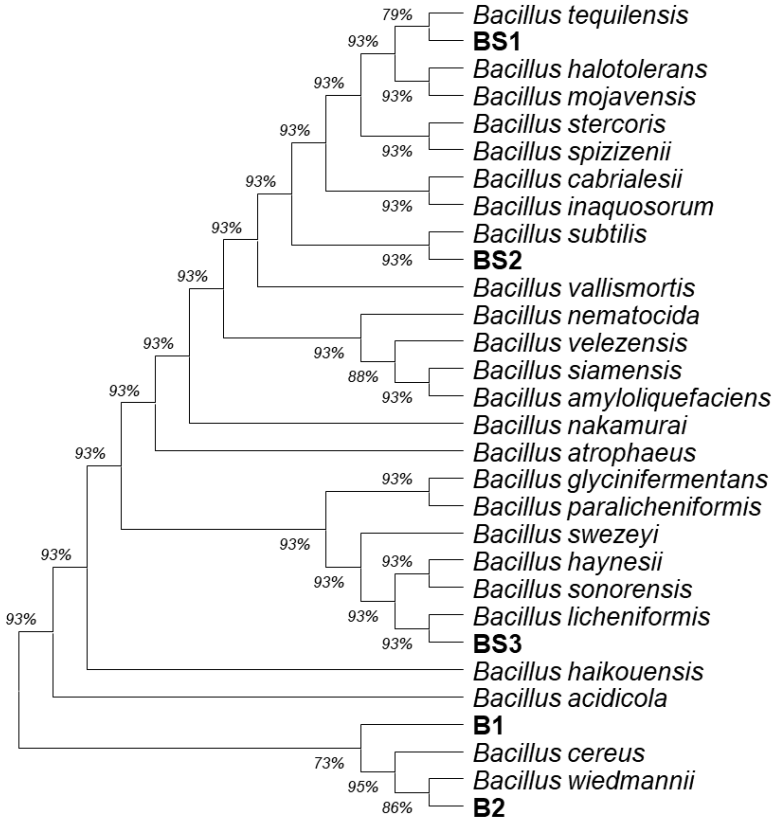

Figure 4. Phylogenetic tree of *Bacillus* spp. based on 16S rRNA gene sequencing.

CONCLUSIONS

This study identified five native *Bacillus* species (*B. tequilensis, B. subtilis, B. licheniformis, B. cereus*, and *B. weidmannii*) with potent antifungal activity against aflatoxigenic *Aspergillus flavus*, demonstrating their potential as sustainable biocontrol agents for mitigating aflatoxin contamination in Nepal's food supply. Further characterization of their antifungal metabolites and field validation are needed to develop effective agricultural applications.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest pertinent to this work.

AUTHOR CONTRIBUTIONS

SS: Designed the study, collected samples, performed laboratory work, data analysis, wrote the manuscript and finalized manuscript; RT, DRJ, and TBK: Supervised, analyzed data, reviewed and finalized manuscript; PP and SS: reviewed manuscript, analyzed data and finalized manuscript.

ETHICAL STATEMENT

The authors declare this is our original work and has not been previously published or submitted for publication elsewhere.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available from the corresponding author, upon reasonable request.

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