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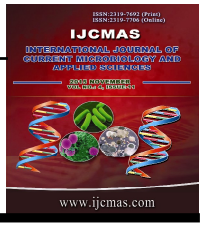


**Original Research Article**

# Screening and Isolation of Antibiotic Producing *Bacillus* Species Antagonistic to Fungal Pathogens

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**A B S T R A C T**

Aflatoxins are extremely toxic chemicals which can cause liver cancer in animals and humans when contaminated feed or food is consumed. Identification of new antifungal peptides with activity against *A. flavus* could lead to the development of biotechnological strategies for controlling Aflatoxin contamination. So, in the present study, attempts were made to produce, purify and characterize the antifungal substance produced by *B. subtilis* from the culture filtrate, which is a member of the iturin family along with mycosubtilin and iturin A, exhibit strong antifungal and hemolytic activities and limited antibacterial activity. Also attempts were made to assess the antifungal spectrum of the purified antifungal compound and seen that the substance have highest antifungal activity against *A. flavus* and moderately against *C. tropicalis*, and *C. parapsilosis*, which means the substance have moderate antifungal activity.

## Introduction

Aflatoxins are extremely toxic chemicals which can cause liver cancer in animals and humans when contaminated feed or food is consumed. Aflatoxin production occurs when *A. flavus* or *A. parasiticus* invades peanuts, cotton seed, corn and certain nuts under favourable conditions of temperature and humidity. *Aspergillus* spp. 2 are also involved in respiratory infection in immunodeficient patients (Lotholary *et al.*, 1993). *Fusarium oxysporum var cucumerinumis* the primary cause of vascular wilt in cucumber plants, and it causes substantially reduced cucumber yields in China (Minuto *et al.*, 2006; Moyne *et al.*, 2001). The symptoms of the disease begin with the development of necrotic lesions, followed by foliar wilting as the pathogen invades the vascular system of the plant, and eventually, plant death (Zhang *et al.*, 2008). The current treatments to defeat fungal infections are limited to some antifungal agents such as amphotericin B, nystatin and azole derivatives. However,

most of these compounds are synthetic derivatives with known serious side effects and toxicity. In addition, their failure has increased because of a rapid emergence of resistant fungal pathogens (Onishi *et al.*, 2000). Identification of new antifungal peptides with activity against *A. flavus* could lead to the development of biotechnological strategies for controlling Aflatoxin contamination and, for example, increase plant resistance to fungal invasion through genetic engineering. Several natural lipopeptides produced by microorganisms have been developed as new therapeutics (Pirri *et al.*, 2009).

The lipopeptides have a hydrophilic peptide portion and a hydrophobic fatty acid portion (Ramarathnam *et al.*, 2007). The member of the iturin family exhibit strong antifungal and hemolytic activities and limited antibacterial activity (Maget-Dana and Peypoux, 1994).

Among the most promising candidates are several species of the genus *Bacillus*, which are ubiquitously occurring safe microorganisms with proven excellent colonization aptitudes, and versatility in effectively protecting plants from pathogens. The potential of *Bacillus* spp. is to synthesize a wide variety of metabolites with antibacterial and/or antifungal activity has been intensively exploited in medicine and agricultural industry, and is one determinant of their ability to control plant diseases when applied as a biological control agent (Moyne *et al.*, 2001). *Bacillus* species especially *Bacillus subtilis*, *Bacillus cereus* and *Bacillus amyloliquefaciens* are effective for the control of plant diseases caused by soil borne, foliar and post harvest fungal pathogens (Ramarathnam *et al.*, 2007).

The purpose of this study was to isolate, purify and characterize the antifungal substance produced by *B. subtilis* from the culture filtrate, and to assess the antifungal spectrum of the purified antifungal compound.

## Material and Methods

**Collection of soil samples:** The soil samples are collected from Botanical garden, Yeshwant Mahavidyalaya, Nanded. The soil samples are collected in sterile plastic zipper (polythene) bags by digging 5–10 cm deep from different sites in the garden & rhizospheric soil of some inhabitant plants also collected with sterile spatula (Joshi *et al.*, 2007). Soil samples are kept in an icepack cabinet maintained at temperature below $10^{o}$C.

**Isolation and identification of bacterial species:** The collected soil samples from Botanical garden, Yeshwant Mahavidyalaya, Nanded were serially diluted using sterile distilled water. Then diluted soil samples were added in nutrient broth separately within 6 hrs of sampling and enriched by incubating them at optimum temperature for 18 to 24 hrs in a rotary shaker at 120 rpm at $30^{o}$C at Research lab, Department of Microbiology, Yeshwant Mahavidyalaya, Nanded, Maharashtra, India. After incubation the enriched samples were streaked on nutrient agar plate, Hichrome Bacillus agar and Hichrome UTI agar and incubated at optimum temperature i.e. $30^{o}$C for 18 to 24 hrs. The isolated colonies observed after incubation and colony characters were recorded. Then colonies were sub-cultured on to respective media slants. The sub-cultured cultures on slants were used for identification of cultures using Bergey's manual of systematic bacteriology $9^{th}$ edition (Bergey *et al.*, 1994). The identified isolates were used for further study.

**Production of antifungal compound:** The five isolates were inoculated in to 5 ml Luria-Bartani (LB) medium separately and cultured overnight at 27$^{o}$C with constant shaking at 140 rpm. For the production of antifungal compound 250ml flasks each containing 100 ml of LB medium were inoculated with this overnight culture and incubated at 27$^{o}$C with constant shaking at 80 rpm. Every day samples 5 ml were removed and centrifuged (10000 rpm for 10 min). The supernatant fluids were filter and used as crude antifungal compound (Moyne *et al.,* 2001).

**Partial purification of antifungal compound:** After incubation, the culture medium was centrifuged at 10000 rpm for 30 min at 4°C to remove the bacteria. The culture supernatant fluid was subjected to sequential ammonium sulphate precipitation to achieve 20, 40, 60 and 80% saturation at 0°C with constant and gentle stirring. The precipitated proteins were pelleted by centrifugation at 10000 rpm for 20 min. The protein pellet was dissolved in 0.02 mol/lit sodium phosphate buffer, pH 7.0 and this crude preparation was then stored at 4°C for further analysis (Moyne *et al.,* 2001).

**Bioassay of antifungal compound:** The crude preparation of antifungal compound was used for bioassay. The cultures of test organisms, *Aspergillus flavus, Candida tropicalis*, and *Candida parapsilosis* were streaked on sterile PDA with sterile swabs. Then wells were made on PDA. The wells were filled with 100 µl of crude antifungal compound. The plates were kept in refrigerator for diffusion of compound. The plates were then incubated at 27±0.5°C for 24 hours and then diameter of zone of inhibition was noted and antifungal activity of the compound was evaluated by the presence of zone of inhibition (Moyne *et al.,* 2001).

## Results and Discussion

The lipopeptide compounds are synthesized non-ribosomally by a large modular multienzyme templates designated as peptide synthetases. The ability of *Bacillus* species to synthesize a wide variety of lipopeptide antibiotics has been extensively exploited in medicine and agriculture (Moyne *et al.,* 2001).

In a search for an antifungal compound with a high activity against *Aspergillus flavus, Candida tropicalis*, and *Candida parapsilosis*, the soil samples were collected and twenty five isolates of *Bacillus species* were isolated from Botanical garden, Yeshwant Mahavidyalaya, Nanded. Five isolates of the twenty five isolates were identified as *Bacillus subtilis*, using Bergy's manual of systematic bacteriology 9$^{th}$ edition as BS 01,10,14,16,19 (Table 1) (Bergey *et al.,* 1994).

In present study, the identified isolates were used for production of antifungal compound. After production of antifungal compound the compound was assayed for antifungal activity against *Aspergillus flavus, Candida tropicalis*, and *Candida parapsilosis*. The antifungal compound was partially purified by using ammonium sulphate fractionation method. After partial purification, the antifungal activity of the compound was assyed against test organisms *Aspergillus flavus, Candida tropicalis*, and *Candida parapsilosis* (Table 2; Graph 1), and observed that the compound is having high antifungal activity against *Aspergillus flavus,* as compared to *Candida tropicalis*, and *Candida parapsilosis*, but the compound is having moderate activity with *Candida tropicalis,* and *Candida parapsilosis* also. So, from the study conducted we can conclude that the compound is having antifungal activity.

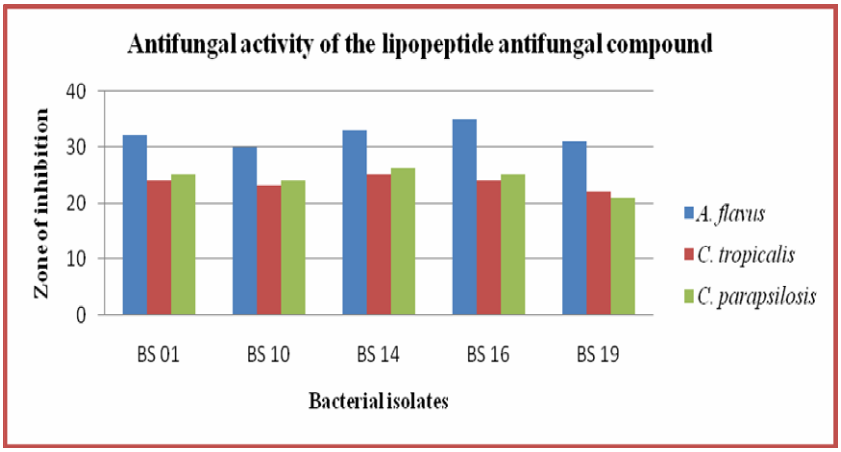
**Table.1** Identification of the bacterial isolates

| Sr. No. | Test | Bacterial isolates | | | | |
|---|---|---|---|---|---|---|
| | | **BS01** | **BS10** | **BS14** | **BS16** | **BS19** |
| 1 | Endospore | Central | Central | Central | Central | Central |
| 2 | Gram nature | + | + | + | + | + |
| 3 | Catalase | + | + | + | + | + |
| 4 | Oxidase | + | + | + | + | + |
| 5 | Amylase | + | + | + | + | + |
| 6 | Gelatinase | + | + | + | + | + |
| 7 | Urease | + | - | + | - | + |
| 8 | Indole | - | - | - | - | - |
| 9 | Methyl Red | + | + | + | + | + |
| 10 | VP | - | - | - | - | - |
| 11 | Citrate | + | + | + | + | + |
| 12 | Glucose | + | + | + | + | + |
| 13 | Xylose | + | + | + | + | + |
| 14 | Mannitol | + | + | + | + | + |
| 15 | Lecithinase | - | - | - | - | - |
| 16 | Growth at 45$^{o}$C | + | + | + | + | + |
| 17 | Growth at 65$^{o}$C | - | - | - | - | - |

**Table.2** Antifungal activity of the lipopeptide antifungal compound

| Bacterial Isolates | Antimicrobial activity of lipopeptide antifungal compound (in mm) | | |
|---|---|---|---|
| | *Aspergillus flavus* | *Candida tropicalis* | *Candida parapsilosis* |
| BS 01 | 32 | 24 | 25 |
| BS 10 | 30 | 23 | 24 |
| BS 14 | 33 | 25 | 26 |
| BS 16 | 35 | 24 | 25 |
| BS 19 | 31 | 22 | 21 |

**Graph.1** Antifungal activity of the lipopeptide antifungal compound



Among lipopeptide compounds, members of the iturin family comprising Bacillomycin D, iturin and mycosubtilin are potent antifungal agents and display hemolytic and limited antibacterial activities (Maget-Dana and Peypoux, 1994); Fengycin is endowed with a specific antifungal activity against filamentous fungi and inhibits phospholipase A2 (Nishikori *et al.,* 1986); surfactin was revealed to be an interesting peptide for clinical applications, displaying both antiviral and antimycoplasma activities beside its antifungal and antibacterial properties (Vollenbroich *et al.*, 1997a, b). In the antifungal assay reported here, the compound produced is effective in controlling *A. flavus* growth.

Antibiotics of the iturin group were found to act upon the sterol present in the cytoplasmic membrane of the organism (Quentin *et al.,* 1982). *A. flavus* conidia membranes contain ergosterol and cholesterol (DeLucca *et al.,* 1997) which could be the target of the antifungal compound. Further research is being conducted to investigate the mechanism of action of the antifungal compound produced against *A. flavus*.

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