

# Screening and Potency Evaluation of Antifungal from Soil Isolates of *Bacillus subtilis* on Selected Fungi

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## Abstract

Soil samples from different crop fields of paddy were used to isolate *Bacillus* species. As per Bergey's manual of determinative bacteriology the isolates were identified as *Bacillus subtilis*. Culture broth of the identified bacterial strain was tested for antifungal potential by disc diffusion technique against selected fungi such as *Candida albicans*, *Aspergillus niger*, *Rhizoctonia solani* and *Fusarium oxysporum*. The bacterial isolate was found to inhibit the growth of tested fungi hence further used for production, purification of antifungal compound. The crystalline extract was used to detect the minimum inhibitory concentration of tested fungi and evaluate its potency.

Key words: *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger*, *Rhizoctonia solani*, *Fusarium oxysporum*, antifungal compound.

## Introduction

Increase in population made man to improve crop yield to produce sufficient food for consumption. Drastic decrease or complete prevention of plant diseases can be important and interesting fact that is encountered by enhanced applications of synthetic chemicals against the diseases that attack crops by different plant pathogens. Fungi attack a crop during the different stages like development, harvest, storage, transport and processing (Abdel-Mallek *et al.* 1995). Plant pathogenic fungi were reported to resist more than one hundred fungicides in green house and field level studies (Ogawa *et al.* 1983). Biological control could be potent means to diminish the pathogen inoculum or its disease generating ability in the target host plant. The specific activities against pathogen will effectively maintain the ecosystem safely within low cost (Lang *et al.* 2002). Microbial antagonism help in the biocontrol of pathogens by means antibiotic production (Schwin 1982).

Currently microorganisms of genera *Penicillium*, *Streptomyces*, *Cephalosporium*, *Micromonospora* and *Bacillus* are known to produce more than 5000 different antibiotics (Todar 2002). Antibiotic producing microbes are abundant in soil, water, sewage and compost that serve source for isolation. Gram positive bacteria were less seriously studied compared to gram negative bacteria (Loeffler *et al.* 1986). Among the gram positive bacteria *Bacillus* spp that are rod shaped, mostly saprophytes, aerobic / facultative anaerobes, grow rapidly in liquid conditions and spore formers that resist heat, cold, desiccation, radiation and disinfectants are among the potential agents of biological control. Spore forming ability of *Bacillus* spp make them to flourish in thermophilic, psychrophilic, acidophilic, halotolerant / halophilic

environments (Shoda 2000). Antagonistic activity of *Bacillus* spp is due to their antifungal producing ability that can control phytopathogens and post harvest crop problems. *Bacillus cereus*, *B. mycoides*, *B. megaterium*, *B. pumilus*, *B. subtilis* and *B. polymyxa* were among *Bacillus* spp reported to suppress plant pathogenic fungi such as *Sclerotinia*, *Pythium*, *Fusarium*, *Gaeummanomyces*, *Phytophthora*, *Nectria* and *Rhizoctonia* (Fiddman & Rossall 1994).

Antifungals -Mycobacillin, subtilin, lipopeptides, bacillomycin, bacilysin, fengimysin, levansucrase and 2, 3-dihydroxy benzoyl glycine were reported to be produced by *Bacillus* spp (Valbuzzi *et al.* 1999). These antifungals were mostly cyclic peptides with amino acids, some enzymes like chitinases, volatiles and other compounds that improve plant resistance mechanisms (Kehlenbeck *et al.* 1994).

In the present study soil samples from different crop fields of paddy were used to isolate *Bacillus* species. *Bacillus subtilis* culture was tested for antifungal potential against selected fungi such as *Candida albicans*, *Aspergillus niger*, *Rhizoctonia solani* and *Fusarium oxysporum*. *Bacillus subtilis* culture isolate was used for production and purification of antifungal compound. The crystalline antifungal extract was used to detect the minimum inhibitory concentration of tested fungi and evaluate its potency.

## Material and methods

Soil samples were collected from different crop fields of paddy from Nizamabad district, Andhra Pradesh state, India. The soil samples from different sites were mixed to make uniform composite soil. 1 gm of

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composite sample was used to get 1: 100 dilutions. The soil suspension is allowed sediment and then dilutions 1:1000, 1:10000 were prepared. The soil dilutions were inoculated in the sterile petriplates by pour plate technique and incubated at 25°C for 4 days. The plates were checked for appearance of well isolated bacterial colonies.

Isolates were selected to identify the bacterial strain based on morphological (shape, gram's nature, spore formation and motility) and biochemical characteristics (IMViC, oxidase, starch hydrolysis, catalase, casein hydrolysis test, nitrate reduction and glucose fermentation tests). Cultured broth of identified bacterial strains was tested by disc diffusion technique on the fungi such as *Candida albicans*, *Aspergillus niger*, *Rhizoctonia solani* and *Fusarium oxysporum*. Based on zone of inhibition the strains were selected for the further study. Loopful of bacterial culture was inoculated in the nutrient broth and incubated at 30°C for 48 hrs on rotatory shaker at 120 rpm. Composition of production medium was 10 gm glucose, 0.5 gm L-glutamic acid, 0.1 gm potassium dihydrogen phosphate, 0.2 gm magnesium sulphate, 0.1 gm magnous sulphate, 0.01 gm ferrous sulphate, 1000 ml distil water and pH adjusted to 7.0.

100 ml of production medium was prepared in the triplicates in conical flasks and sterilized by autoclaving at 10 lbs for 40 min. 0.1 ml of inoculum was added to the medium. The inoculated flasks were incubated at 30°C for 72 hrs. Culture broth was centrifuged at 10000 rpm for 20 mins at 4°C to get cell free supernatant. The pellet was discarded and supernatant was used for antifungal recovery. Supernatant was chilled at 5°C for 15 mins and filtered by using whatman filter paper. The filtrate was acidified to pH 2.5 with dilute sulphuric acid. Then equal volumes of butyl acetate and acidified filtrate were shaken for 20 mins in separating funnel. Top layer is discarded and butyl acetate step was repeated. The precipitate was mixed with 80 % potassium acetate by constant and gentle shaking. The extract was crystallized in evaporating dish as potassium salt. The crystals were recovered from the dish and used for antifungal activity.

The crystalline extract was dissolved in sterile distill water to obtain the ten dilutions, 0.58, 1.17, 2.3, 4.68, 9.37, 18.75, 37.5, 75, 150 and 300 µg / ml. Sterile potato dextrose agar plates were spreaded with 0.1 ml of test cultures (*A. niger*, *R. solani*, *C. albicans* and *F. oxysporum*). Plates were prepared in triplicates and the paper discs impregnated in the different concentrations of extract. The paper discs were placed in the centre of the marked respective plates and incubated for 48 hrs at 25°C. Clear zone of inhibition for different concentrations were measured.

## Results and Discussion

Isolates of *Bacillus* spp. from soil were identified based on the morphological and biochemical tests as per Bergey's manual of Determinative Bacteriology and to be *Bacillus subtilis* (Table 1). From the earlier reports *B. subtilis* was potential agent in biological control due to its antagonistic action. This activity was found to reduce the incidence of bean rust caused by *Uromyces* spp., inhibited the activity of *Botrytis* spp., germination of *Puccinia* spp. and *Fusarium oxysporum* in green house experiments. *B. subtilis* is well popular to produce several kinds of antifungals such as mycosubtilin, mycobacillin and subtilisin. Hence in this present study *B. subtilis* strain was selected and the antifungal ability against the fungi i.e. filamentous, yeasts was evaluated. Initially

Characteristics	Result
Morphological	
Shape	Rod shaped
Gram's nature	positive
Motility	+
Spore formation	+
Biochemical	
Indole	-
Methyl red	-
Voges Proskauer	+
Citrate utilization	+
Catalase	+
Starch hydrolysis	+
Casein hydrolysis	+
Nitrate reduction	+
Glucose fermentation	-

Table - 1: Morphological and Biochemical characteristics of *Bacillus Isolates*

the strains of *B. subtilis* isolated from the cultivated soil samples was checked for the growth inhibition of test fungi such as *Candida albicans*, *Aspergillus niger*, *Rhizoctonia solani* and *Fusarium oxysporum*.

Based on the inhibition zones against these fungi the highly potential strain was selected to produce antifungal compound.

Under the optimum conditions like incubation period, pH of medium and glucose concentration antifungal compound was produced and extracted in pure form. The pure crystalline extract was used to make aqueous concentrations of antifungal compound and disc diffusion was carried on the test fungi i.e. one yeast (*Candida albicans*), three filamentous fungi (*Aspergillus niger*, *Rhizoctonia solani* and *Fusarium oxysporum*). Zone of inhibition was used to evaluate the potency of antifungal compound. All the fungi tested growth was sensitive to pure antifungal compound. 300 µg/ml of antifungal was the high concentration tested against the fungi and found to give inhibition zones of large size in all the test organisms. The inhibition zones were in the range of 23 – 29mm in diameter for test fungi (Table 2, Figures 1, 2, 3 & 4).

Conc. of Antifungal extract (µg/ml)	Test organism			
	A. Niger	C. Albicans	F. Oxysporum	R. Solani
0.58	4.8 ± 0.55	4 ± 0.35	3 ± 0.19	5 ± 0.15
1.17	6.1 ± 1.2	7.5 ± 0.65	5 ± 0.26	7 ± 0.22
2.3	8.9 ± 1.00	9 ± 1.05	7.9 ± 0.25	9 ± 0.27
4.68	10.5 ± 0.45	13 ± 0.56	12 ± 0.39	13 ± 0.35
9.37	11.9 ± 1.01	15 ± 0.87	17 ± 0.45	16 ± 0.41
18.75	12.2 ± 0.75	19 ± 0.95	19 ± 0.57	19 ± 0.46
37.5	13.7 ± 1.55	20 ± 0.69	21 ± 0.65	22 ± 0.55
75	14 ± 0.95	21 ± 0.83	23 ± 0.75	24 ± 0.66
150	15.9 ± 1.26	22 ± 0.58	25 ± 0.95	27 ± 0.70
300	23.7 ± 1.25	25 ± 0.49	28 ± 1.01	29 ± 0.95

Table - 2: Effect of Antifungal extract on the growth of test fungi

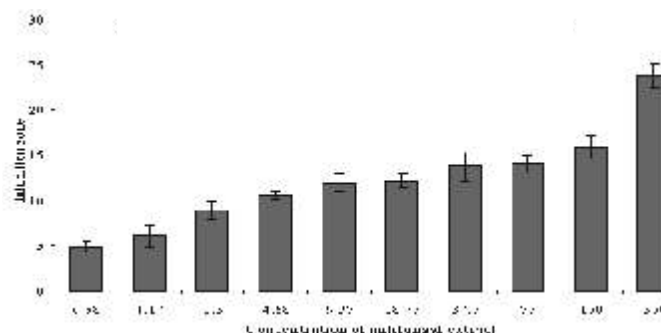


Figure 1. Effect of Antifungal extract from *B. subtilis* against *A. niger*

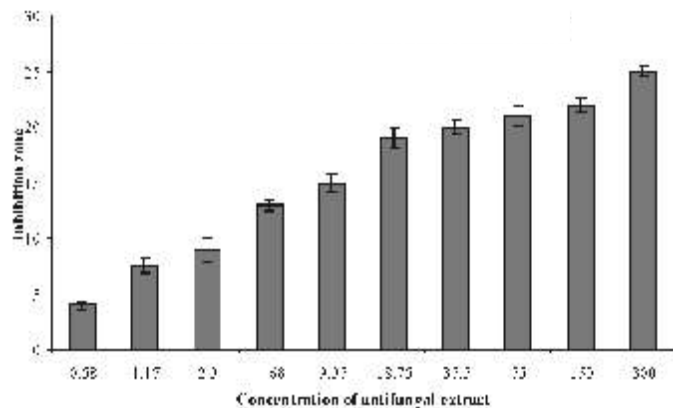


Figure 2. Effect of Antifungal extract from *B.subtilis* on *C. albicans*

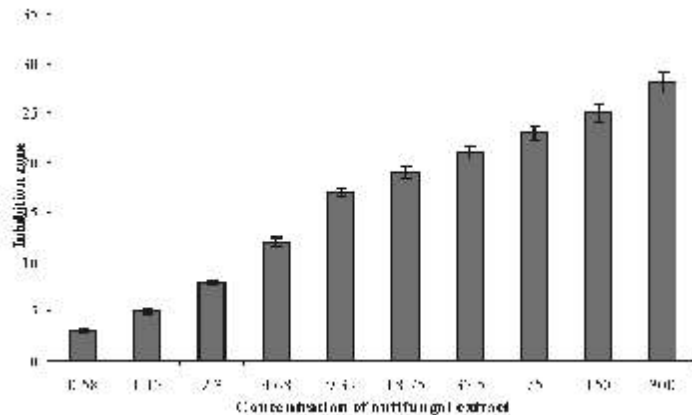


Figure 3. Effect of Antifungal extract from *B.subtilis* on *Foxyosporum*

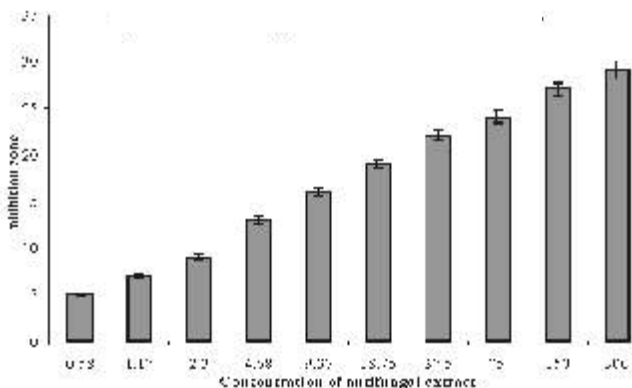


Figure 3. Effect of Antifungal extract from *B.subtilis* on *R. solani*

Among the test fungi *R. solani* was comparatively more sensitive to antifungal compound produced from soil isolate of *B. subtilis* (Figure 5). *R. solani* is common plant pathogen causing severe loss in the potato crop. Several synthetic fungicides are commonly employed to control the diseases of this fungus. From this study it can be concluded that the plant pathogenic fungi can be control by using the *B. subtilis* as potential agent of biological control.

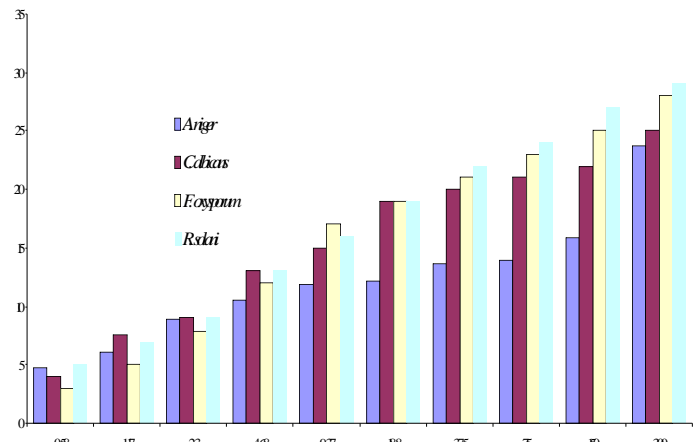


Figure 5. Comparative effect of antifungal extract from *B.subtilis* on pathogenic fungi

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