**RESEARCH ARTICLE**

# Production of Bioactive Compound by *Bacillus subtilis* and its antagonistic activity against *Sclerotium rolfsii*

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## ABSTRACT

*Bacillus subtilis* has the potential to produce bioactive compound antagonistic against plant pathogenic fungi *Sclerotium rolfsii*. Bioassay test as well as agar well diffusion tests were performed which showed that *Bacillus subtilis* has produced the antifungal compound which was inhibitory against *Sclerotium rolfsii*. Chitin as a carbon source was found to be effective supplement for the effective production of bioactive compound. Another finding was that the bioactive compound produced by *B. subtilis* remained effective even after treated with different temperature and a wide pH range. The stability test showed that at 72 hours of incubation, at $37^0$C and at pH 7, the maximum antagonistic activity (27mm and 26 mm respectively) was observed against *S. rolfsii*. The results obtained from the current study concluded that *Bacillus subtilis* and its bioactive compound can be used as a biological control agent for inhibition of phytopathogenic fungi *Sclerotium rolfsii.*

**Keywords:** *Bacillus subtilis*, Bioactive Compound, Chitin, *Sclerotium rolfsii*

## INTRODUCTION

The bioactive compounds include secondary metabolites such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors and phenolic compounds (Holker *et al*., 2004; Kris-Etherton *et al*., 2002; Nigam, 2009). In the last few years, great attention has been paid to the bioactive compounds due to their ability to promote benefits for human health, such as the reduction in the incidence of some degenerative diseases like cancer and diabetes (Conforti *et al*., 2009; Kim *et al*., 2009), reduction in risk factors of cardiovascular diseases (Jimenez *et al*., 2008), antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory and anti-microbial effects (Balasundram *et al*., 2006; Ham *et al*., 2009; Parvathy *et al*., 2009).

Diseases of plants should be controlled for the maintenance of quality and abundance of food, feed and fiber. Farmers often depends on

chemical fertilizers and pesticides for controlling the plant diseases which resulted into environmental pollution. Biological control using microorganisms is largely based on the negative interactions among the microbial population. Biological control offers a specific activity against the targeted pathogens combined with the ability to stay as the non-dominant species while maintaining its effectiveness in an ecosystem with low cost of mass production (Shoda, 2000). Exposure to chemicals may leads to the development of resistant phytopathogenic fungal strains. Biological control of plant pathogens is the good alternative of modern agriculture on chemical fungicides. *Sclerotium rolfsii,* an omnivorous, soilborne fungal pathogen, causes disease on a wide range of agricultural and horticultural crops (Aycock, 1966). Chitin is the main component of fungal cell wall and is a long-chain polymer of an N-acetyl glucosamine (Shahidi *et al*., 1999). There were several reports on *Bacillus subtilis* potential as biological control agent against plant pathogenic fungi and bacteria (Ferreira *et al*., 1991).

Increased public health concern about the accumulation of pesticide residues in the biosphere and the development of resistance among pathogens against conventional antibiotics has led scientists towards the development of alternative strategies for plant diseases suppression. Many countries in the world today are considering biological control of plant diseases as the best alternative to chemicals (Souto *et al*., 2004). Recognizing the hazards of fungicides and pesticides to man and the environment, many countries in the world today is considering biological control as the best alternative to chemical control of plant diseases and pests. In this study, *B. subtilis* was used to produce antifungal compound which was antagonistic to the growth of *S. rolfsii*. Therefore aim of the present study was to investigate the characteristic of antifungal compound produced by *Bacillus subtilis* against *Sclerotium rolfsii* and the influence of additive supplement chitin on the antagonistic activity of *Bacillus subtilis*.

## MATERIALS AND METHODS

### *Collection of Microorganisms:*

*Bacillus subtilis* NCIM 2010 and *Sclerotium rolfsii* NCIM 1084 were obtained from National Chemical Laboratory (NCL), Pune. Where, NCIM= National Collection of Industrial Microorganisms.

### *Minimal Media Preparation:*

The conical flask containing 25 ml of Minimal Media such as (0.1% (w/v) $KH_2PO_4$ and 0.05 % (w/v) $MgSO_4.7H_2O$ was prepared with the addition of 1 % (w/v) chitin as a carbon source. The medium was autoclaved at $121^0$C for 15 minutes (Nalisha *et al*., 2006).

### Qualitative Assay for Chitinase Production:

The qualitative assay for chitinase production was performed following the method of Dunne *et al*. (1997). *Bacillus subtilis* was inoculated by spotting on the plate having chitin minimal medium (CMM) as sole source of carbon and incubated at $28^0$C for 7 days. These plates were examined for development of clear zones around *B. subtilis* spot. For the visualization of the chitinolytic activity the agar plate was flooded with 0.1% congo red solution for 15-30 minutes. The visualization was further enhanced by washing the unbound congo red by 1% NaCl solution (Devkota *et al*., 2011).

### Bioassay Test:

The bioassay test was performed by dual culture method demonstrated by Kobayashi *et al*. (2000). *Bacillus subtilis* was tested for its antifungal activity against *Sclerotium rolfsii* on agar medium containing 1:1 ratio of potato dextrose agar and Nutrient agar. *Bacillus subtilis* was spot inoculated on the surface of agar plate 2 cm away from fungal disc. Antagonistic activity was observed after incubation at 28 $^0$C up to 4 days. Petri plate without bacterial isolate served as control. Antagonistic activity was assessed every 12 hours for 4 days after incubation by measuring the diameter of the zone of inhibition around the spots of *B. subtilis.* The readings were then taken where higher zone of inhibition indicates greater ability of the bacteria to inhibit the growth of the pathogenic fungi *S. rolfsii*.

### *Antagonism Using Cell-free Culture Filtrate*

After preparing minimal media it was inoculated with the culture of *Bacillus subtilis*. The culture was incubated upto 84 hours and agitated on rotary shaker incubator at 140 rpm at $28^0$C. Samples were harvested every 12 hours. For the secondary screening process, the culture broths after fermentation were centrifuged at 10,000 rpm for 15 minutes at $4^0$C in sterile condition. The culture supernatants were further made cell free by

passing through the Whatman Paper No. 1. Then the culture filtrate was used to assay the antifungal activity against the test phytopathogen, *Sclerotium rolfsii*. Nutrient agar and Potato Dextrose Agar (1:1) plates were prepared and an actively growing mycelia disc (5 day old) of *Sclerotium rolfsii* was kept separately in the center of each plate. Cell-free culture filtrate (15 µl) was added into wells (6 mm) made on modified plates 2 cm away from fungal disc and incubated at 28$^0$C for 4 days. Zone of inhibition of colony growth of the fungus was recorded (Kumar *et al*., 2012; Walker *et al*., 1998).

***Stability Test:***

Stability test was performed in the range of pH (5, 7, and 11), temperature (28$^0$C, 37$^0$C and 45$^0$C) for 4 days (Yu *et al*., 2002).

## RESULTS AND DISCUSSION

For the production of bioactive compound *B. subtilis* NCIM (2010) was used against *S. rolfsii*. Chitinase production was carried out using the *Bacillus subtilis in the* chitin minimal medium. Chitinase was produced after 24 hours onwards but the optimum production was after 72 hours. After 84 hours the chitinase production was decreased. The production of chitinase was shown by the zone of hydrolysis at different incubation period of the fermentation at 37$^0$C (Table 1) (Figure 1). These results were correlated with that of the Devkota *et al*. (2011). It was revealed from the present investigation that *Bacillus subtilis* has the potential to produce chitinase which resulted into the zone of hydrolysis around the *Bacillus subtilis*.

**Table 1:** Zone of Hydrolysis by *Bacillus subtilis* in Chitin Minimal Medium.

| **Incubation Time (hours)** | 12 | 24 | 48 | 60 | 72 | 84 |
|---|---|---|---|---|---|---|
| **Zone of Hydrolysis (mm)** | NZ | 9 mm | 11 mm | 12 mm | 16 mm | 13 mm |

**Table 2: :** Inhibition (mm) of *Sclerotium rolfsii* by *Bacillus subtilis* (Dual Culture Method)

| **Incubation Time (hours)** | 12 | 24 | 48 | 60 | 72 | 84 |
|---|---|---|---|---|---|---|
| **Zone of Inhibition (mm)** | NZ | 10mm | 14mm | 19mm | 22mm | 14mm |

**Table 3:** Inhibition (mm) of *Sclerotium rolfsii* by *Bacillus subtilis* Culture Filtrate

| **Incubation Time (hours)** | 12 | 24 | 48 | 60 | 72 | 84 |
|---|---|---|---|---|---|---|
| **Zone of Inhibition (mm)** | NZ | 10mm | 20mm | 24mm | 26mm | 14mm |

**Table 4:** Inhibition (mm) of *Sclerotium rolfsii* in presence of *Bacillus subtilis* Culture filtrate at different temperatures

| **Temperature** | **Incubation Time (in hours)** | | | | | |
|---|---|---|---|---|---|---|
| | **12 hrs** | **24 hrs** | **48 hrs** | **60 hrs** | **72hrs** | **84hrs** |
| 28$^0$C | NZ | 10mm | 20mm | 24mm | 26mm | 14mm |
| 37$^0$C | 10mm | 10mm | 21mm | 24mm | 27mm | 17mm |
| 45$^0$C | NZ | 9 mm | 17 mm | 20 mm | 22 mm | 16 mm |

**Table 5:** Inhibition (mm) of *Sclerotium rolfsii* in Presence of *Bacillus subtilis* Culture Filtrate at Different pH

| **pH** | **Incubation Time (in hours)** | | | | | |
|---|---|---|---|---|---|---|
| | **12 hrs** | **24 hrs** | **48 hrs** | **60 hrs** | **72hrs** | **84hrs** |
| 5 | NZ | 9 mm | 10 mm | 12 mm | 14 mm | 12 mm |
| 7 | NZ | 10mm | 20mm | 24mm | 26mm | 14mm |
| 11 | NZ | 10 mm | 10 mm | 14 mm | 15 mm | NZ |

The bioassay test was performed by dual culture method demonstrated by Kobayashi *et al*. (2000). *Bacillus subtilis* was tested for its antifungal activity against *Sclerotium rolfsii.* Antifungal activity was demonstrated by the formation of zone of inhibition around the spots of *Bacillus subtilis.* It was observed that in varying incubation time the antifungal activity was also varied in the form of zone of inhibition. Zone of inhibition was in increasing order upto 72 hours (22 mm) of incubation period and then it was found to be declined (Table 2) (Figure 2). This result revealed that the inhibition activity was due to the chitinolytic potential of *Bacillus subtilis*. *Sclerotium rolfsii* contain chitin as the major cell wall component. Chitinase production by *Bacillus subtilis* has the ability to degrade fungal chitin, thus showing the zone of inhibition. This result was in correlation with the result obtained by Chen *et al*. *(*2004). Similar findings were also observed by the study conducted by Souto *et al*. (2004) where mycelia growth of *Sclerotium* spp. was inhibited by application of *Bacillus* spp. using the dual culture technique. According to their study when *Sclerotium* spp. was challenged with the *Bacillus* spp., the fungal growth was inhibited by accompanying the decreased sclerotia production. This might be the reason for inhibition of *Sclerotium* spp. According to Sivan and Chet, (1989), the mechanism of biocontrol may be the competition for substrate. According to McKeen *et al*. (1986), the ability to colonize the niche favored by the pathogen and antagonism by antibiotics may also be responsible for inhibitory action. The cell wall degrading enzymes had been suggested by Chet *et al*. (1998) for the inhibition. Apart from this, Fiddaman and Rossall, (1995) had suggested that there was another potential mode of action for the production of antifungal metabolites.

**Figure 1:** Zone of Hydrolysis by *Bacillus subtilis* in Chitin Minimal Medium

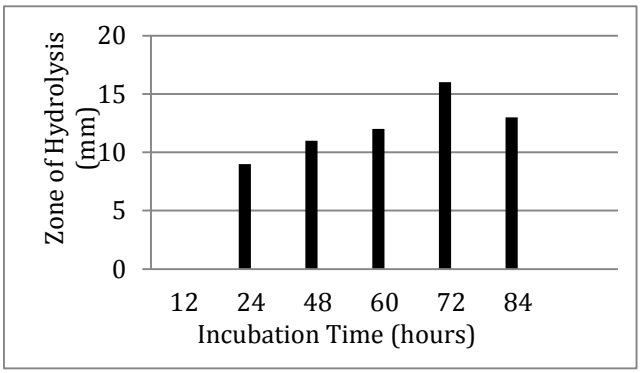



**Figure 2:** Inhibition (mm) of *Sclerotium rolfsii* by *Bacillus subtilis* (Dual Culture Method)

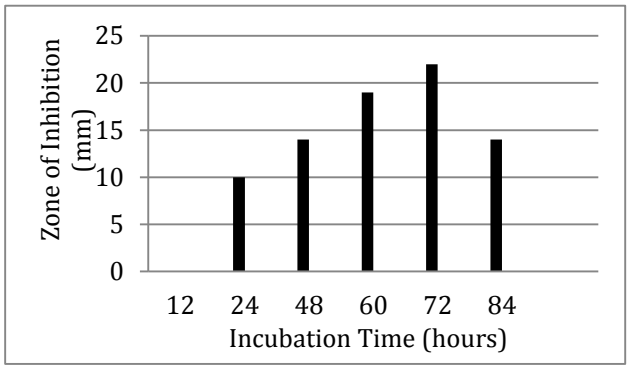
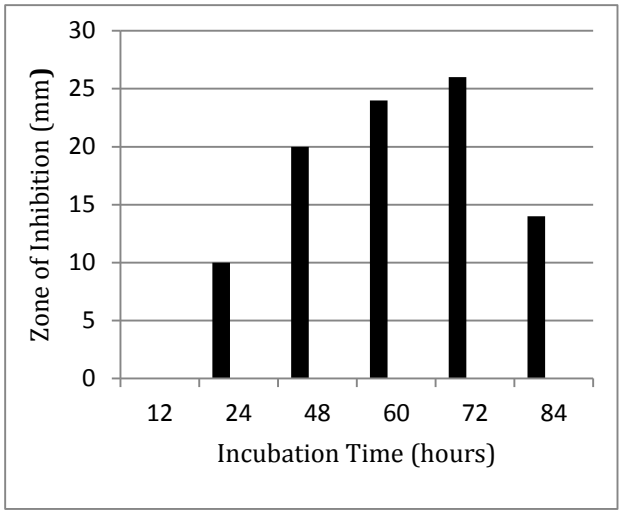


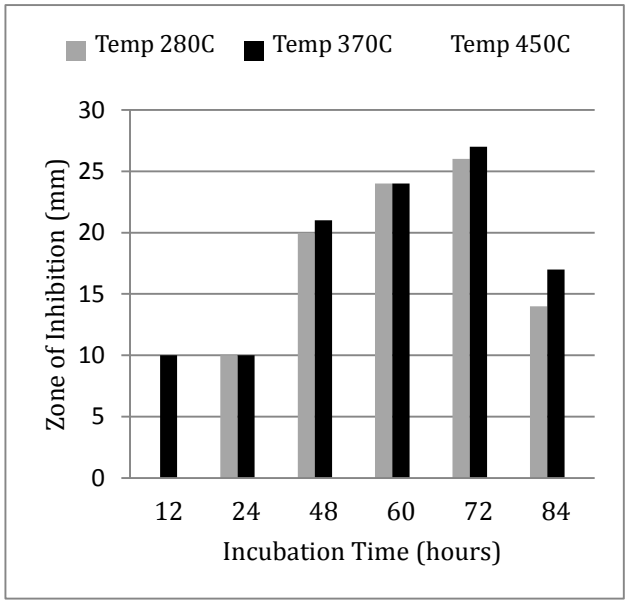

**Figure 3: Inhibition (mm) of *Sclerotium rolfsii* by *Bacillus subtilis* Culture Filtrate**




Antagonism in cell free culture filtrate showed that incorporation of chitin in the minimal medium was beneficial for more antifungal activity. Zones of inhibition were in increased form than that of the obtained in dual culture method. Incubation period of 72 hours was the optimum (26 mm) for bioactive compound production (Table 3) (Figure 3). The similar type of findings was reported by Nalisha *et al*. (2006). According to their study, incorporation of chitin in minimal media was found to be effective for inhibition of *S. rolfsii* at different incubation time. Another reason may be the presence of chitinase enzyme produced by *Bacillus subtilis* which can inhibit the *Sclerotium rolfsii*. In some other studies the application of other biological control agents such as *Trichoderma koningii, T. harzianum* and *Serratia marcescens* were also found to be effective in controlling *S. rolfsii* via secretion of chitinase and was demonstrated by Lima *et al*. (1999); Ordentlich *et al*. (1987); Tsahouridou and Thanassoulopoulos, (2002).

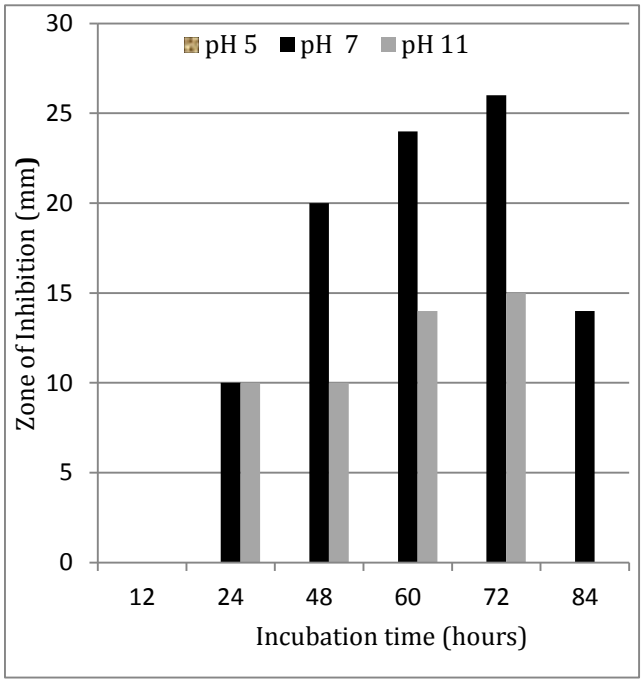
By performing stability test it was found that with various temperature treatments (28$^0$C, 37$^0$C, 45$^0$C) there was variation in inhibition activity of the culture filtrates of *B. subtilis*. According to the present study it was found that at 37$^0$C temperature, the zones of inhibition were maximum compared with that at 28$^0$C and 45$^0$C. It was also observed that at 37$^0$C temperature the highest zone of inhibition was recorded (27mm) after 72 hours incubation (Table 4) (Figure 4). The study of Kajimura *et al*. (1995) reported that *B. subtilis* produced lipopeptide antibiotic and bacillopeptide as antifungal antibiotic. Chang *et al*. (2007) demonstrated that decreases in the degree of growth inhibition were associated with the increases in the incubation period of the fungal culture. Thasana *et al*. (2010) reported that *B. subtilis* culture filtrate inhibited the growth of phytopathogenic fungi *Sclerotium rolfsii*. The bioactive compounds that exhibit antifungal activities in the culture filtrate of *B. subtilis* were cyclic lipopeptide such as subtulene A and iturin A. Iturins have generally been shown to display strong antifungal toxicity.

**Figure 4:** Inhibition (mm) of *Sclerotium rolfsii* in Presence of *Bacillus subtilis* Culture Filtrate at Different Temperatures




According to Raaijmakers *et al*. (2002); McKeen *et al*. (1986), antibiotics produced by bacterial antagonists have a broad spectrum activity against fungi. The antifungal compounds were also remarkably thermostable. Heat/temperature treatment showed some alteration in the inhibition activity of the culture filtrates. The antifungal activity can be detected throughout the present study and this showed that effect of different temperatures would not completely destroy the antifungal compound. The special characteristics of *Bacillus* having high thermal tolerance, rapid growth in liquid culture and ready formation of resistant spores was responsible for its stable nature Clery-Barraud *et al*. (2004); Shoda, (2000).

**Figure 5:** Inhibition (mm) of *Sclerotium rolfsii* in presence of *Bacillus subtilis* culture filtrate at different pH




Similarly *B. subtilis* culture filtrates were also tested for variation of pH. By performing stability test it was found that treatment with various pH showed different level of antifungal activity. At pH 5 and pH 11, the decreased zones of inhibition were recorded at varying incubation period. The optimum pH for the greater antifungal activity was demonstrated at pH 7. It was revealed that at pH 7 the maximum zone of inhibition (26 mm) was recorded at 72 hours of incubation (Table 5) (Figure 5). The result obtained from the present study has shown that the *B. subtilis* was active against *Sclerotium rolfsii.* However, fungal growth may be controlled due to the presence of extracellular antifungal compounds in the cuture filtrate of *B. subtilis*. The similar findings were found by Devkota *et al*. (2011); Nalisha *et al*. (2006).

According to the present findings, *B. subtilis* reduced disease incidence and severity of *S. rolfsii*. Similar findings had been reported by De-Curtis *et al*. (2010) when two commercial biofungicides based on *B. subtilis*

(BSF4) and *Trichoderma asperellum* were applied to the soil were evaluated on tomato plants. These bacteria significantly reduced both incidence and severity of the diseases caused by *Sclerotium rolfsii* or *Rizoctonia solani*. Furthermore, it have been suggested by Hecker *et al*. (1996) that the resistance of growing cells to heat stress was mainly caused by heat shock protein which plays a major role to form a heat resistance spore. *Bacillus subtilis* was found to be effective as the biocontrol agent. According to Monteiro *et al*. (2005) endospore forming *Bacillus subtilis* have the ability of producing peptide antibiotics which contributes for its utilization in biocontrol formulations of several plant diseases.

## CONCLUSION

Many countries in the world today are considering biological control of plant diseases as the best alternative to chemicals. Exposure to chemicals may leads to the development of resistant phytopathogenic fungal strains. Recognizing the hazards of fungicides and pesticides to man and the environment, many countries in the world today is considering biological control as the best alternative to chemical control of plant diseases. *Bacillus subtilis* was found to be the effective biocontrol agent against *Sclerotium rolfsii*. Owing to this fact, in the current investigation it was made to find out that the production of bioactive compound was effective as an antifungal compound against phytopathogenic fungi *Sclerotium rolfsii*. Chitin as a carbon source was found to be effective supplement for the effective production of bioactive compound. In the current study *Bacillus subtilis* has produced the antifungal compound which was inhibitory against *Sclerotium rolfsii.* Another finding was that the bioactive compound produced by *B. subtilis* remained effective even after treated with different temperature and a wide pH range. The results obtained from the current study concluded that *Bacillus subtilis* and its bioactive compound can be used as a biological control agent for inhibition of phytopathogenic fungi *Sclerotium rolfsii*.

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