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Production of Bioactive Compounds by *Bacillus subtilis* against *Sclerotium rolfsii*

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ABSTRACT

This study aims to investigate the characteristic of bioactive compound produced by *Bacillus subtilis* against *Sclerotium rolfsii* and the influence of additive supplements on the antagonistic activity of *B. subtilis*. The fact that *B. subtilis* produced an antifungal substance which has inhibitory effect on wide range of fungi, including *S. rolfsii*, is well known. To learn the effect of pH, temperature and light condition on the production of antifungal compound, *B. subtilis* was inoculated in Potato Dextrose Broth at various initial pH, temperatures and light conditions, respectively. This antagonist was found to produce antifungal compound that stable at 80^{o}C with 58.3 % inhibition on *S. rolfsii*. The activity was constant within a wide range of pH (3–11). However, treatment with pH11 lead to higher antifungal activity (31.57 % inhibition) and it was also found to produce substance that can endure dark condition (46.24 % inhibition) with fungicidal effect on *S. rolfsii*. A series of experiments also been carried out to enhance the antifungal production by supplementing different carbon source preparation into bacterial liquid culture. *B. subtilis* were grown in minimal medium containing 1 % of oil palm root, *Ganoderma lucidum* or chitin, respectively prior to bioassay. Crude culture from oil palm root supplemented culture shown significantly reduction in *S. rolfsii* growth compared to other carbon source crude culture or the antagonism alone, suggesting that this approach may provide improved biocontrol efficiency.

Keyword: *Bacillus subtilis* , *Sclerotium rolfsii*, antifungal activity, carbon source

INTRODUCTION

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 (Souto *et al*., 2004). There were several reports on *Bacillus subtilis* potential as biological control agent against plant pathogenic fungi and bacteria (Ferreira *et al*., 1991). In other previous work, *B. subtilis* was able to reduce the incidence of bean rust caused by *Uromyces appendiculatus* up to 74 % under field conditions (Baker and Cook, 1982). It has also been reported that some *Bacillus* strains showed significant inhibition activity against *Botrytis cinerea* (Walker *et al.,* 1998), *Puccinia pelargonii-zonalis* germination (Rytter *et al.,* 1989) and *Fusarium oxysporum* (Lang *et al*., 2002) under greenhouse experiments.

The proposed mechanisms resulting in biocontrol are competition for substrate (Sivan and Chet, 1989), ability to colonize the niche favored by the pathogen, antagonism by antibiotics, antibiosis (McKeen *et al*., 1986) and action of cell-wall degrading enzymes (Chet *et al*., 1998). Fiddaman and Rossall (1993) had suggested that another potential mode of action may lie with the production of antifungal metabolites. In this study, *B. subtilis* was discovered to produce antifungal compound which was antagonistic to the growth of *S. rolfsii*. Therefore this study aims to investigate the characteristic of bioactive compound produced by *Bacillus subtilis* against *Sclerotium rolfsii* and the influence of additive supplements on the antagonistic activity of *B. subtilis*.

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MATERIALS AND METHOD

Microorganisms and Culture Condition

For these studies *Bacillus subtilis* obtained from Department of Biology Culture Collection, Universiti Putra Malaysia and *Sclerotium rolfsii* from Agriculture Faculty, Universiti Putra Malaysia were used. Both *B. subtilis* and *S. rolfsii* were maintained on nutrient Agar (NA) and Potato Dextrose Agar (PDA) slants, respectively and stored at room temperature (28^{o}C ± 2).

Carbon Source Preparation

Oil palm root and basidiocarp of *Ganoderma lucidum* were collected from Taman Pertanian Universiti, UPM. Samples were oven dried (60^{o}C) before grinded to powder form and kept for further use in air tight container. Chitin powder was purchased from Sigma-Aldwich.

Minimal Media Preparation

Forty flasks each containing 25 ml of Minimal Media (0.1 % (w/v) KH_2PO_4 and 0.05 % (w/v) $MgSO_4.7H_2O$) were then added with 1 % (w/v) palm oil root, *Ganoderma lucidum* or chitin respectively, before been autoclaved at 121^{o}C for 15 minutes and added with 1x10^{7} cell/ml of *B. subtilis* stock. The cultures were incubated for 96 h and agitated on rotary shaker at 140 rpm at 28^{o}C. Samples were harvested every 12 h, filtered through Whatman

Paper No.1 before re-filtered using 0.2µm cellulose acetate filter and freeze-dried for further use.

Bioassay Test

Tests were conducted to distinguish the efficiency of the bacterial isolates in inhibiting growth of *Sclerotium rolfsii*. For this, dual culture system was carried out as described by Kobayashi *et al*. (2000). Antagonistic activities were assessed every 12 h for 4 days after incubation by measuring the radius of the pathogen colony in direction of the bacterial colony and the radius of the pathogen colony in the control plate. The readings were then transformed into percentage, where higher percentage indicates greater ability of the bacteria to inhibit the growth of the pathogen.

Stability Test

Stability test of a range of pH (3, 5, 7, 9, 11), temperature (-20, 4, 28, 80, 100^{o}C) and light condition (light, dark, UV) were carried out as suggested by Yu *et al.* (2002).

Agar Diffusion Assay

Bacillus crude culture used in this assay was prepared using minimal media as describe previously. The concentrated preparation of metabolites was tested for antifungal activity. Method by Walker *et al*. (1998) was used in order to assess antifungal activity for different carbon source treatments by measuring the radius of the zones of inhibition. In each well, crude culture was applied randomly and the plates were incubated at room temperature (28^{o}C ± 2) for 4 days.

RESULTS AND DISCUSSION

Two separate bioassay tests (application of *B. subtilis* alone and combined with carbon source) were conducted to assess antifungal activity against pathogen. However, the degree of inhibition varies between the first and the second experiment. The readings obtained were then transformed into percentage to indicate the ability of the bacteria to inhibit the growth of the pathogen. *Bacillus* was found to subdue *S. rolfsii* growth, up to 13.05 %, based on the percentage of growth analysis; thus indicating that *S. rolfsii* was sensitive towards *Bacillus* secretions. Summary of the results were shown Table 1. This finding was similar to the test conducted by Souto *et al*. (2004), where mycelia growth of *Sclerotium* sp. was inhibited by application of *Bacillus* sp. using the dual culture technique. When *Sclerotium* sp. was challenged with the *Bacillus* sp. culture, growth inhibition of mycelia occurred accompanied by a decreased sclerotia production. Application of other biological control agent such as *Trichoderma koningii* (Tsahouridou and Thanassoulopoulos, 2002), *T. harzianum* (Lima *et al*., 1999) and *Serratia marcescen* (Ordentlich *et al*., 1987) were also found to be effective in controlling *S. rolfsii* via secretion of chitinase.

Table 1: Inhibition (%) of *Sclerotium rolfsii* after tested against *Bacillus subtilis*.

Incubation period (h)	Growth without *Bacillus subtilis* (cm)	Growth with *Bacillus subtilis* (cm)	Percentage of inhibition (%)
12	0.8	0.8	0
24	2.384 ± 0.084	2.269 ± 0.1	4.82 ± 0.13
36	3.704 ± 0.059	3.431 ± 0.06	7.36 ± 1.52
48	5.060 ± 0.041	4.636 ± 0.08	8.38 ± 1.57
60	6.540 ± 0.086	6.001 ± 0.07	8.24 ± 1.76
72	7.556 ± 0.054	6.570 ± 0.09	13.05 ± 1.09
84	7.684 ± 0.010	7.025 ± 0.01	8.58 ± 0.98

Note:Values are percentage of inhibition ± SD (n=7).Means in a column with the same superscript are not significantly different (p>0.05). nd – Not detected

When culture filtrates were given direct sunlight and dark treatments, reduction of diameter as high as 38.36 % and 46.24 %, respectively, can be seen (Table 2). However, when exposed to UV condition the inhibition is vaguely lower (25.65 %). The reduction in the inhibition activity caused by the radiation emitted could have caused alteration in the antifungal compounds structure thus prevent *B. subtilis* from performing at their optimal performance (Tortora *et al.,* 1997). This were consistent with the study by Papavizas and Lewis (1983) where *Trichoderma* spp. induced by UV showed different ability to suppress damping off caused by *P. ultimum* and *R. solani* and therefore suggested long exposure to UV radiation to induce new and stable *Trichoderma* spp.. Neither direct light nor UV had altered the antifungal activity of each culture filtrates as microorganisms are able to repair damage and alterations induced to their DNA by UV treatment following the administration of a sub-lethal dose (Hassen *et al*., 2000). Hassen *et al*. (2000) also reported some classified microorganisms and their propagules in the following increasing order of resistance to UV treatment: bacteria < viruses <fungi < spores <cysts.

The antifungal compounds were also remarkably thermo stable. Heat/temperature treatment showed no alteration in the inhibition activity of the culture filtrates even after treated at 100^{o}C (46.53 %). The antifungal activity can be detected throughout the study and this showed that different treatment of heat and temperatures would not completely destroy the antifungal compound. More than 50 % of inhibition was detected when crude culture was place at 80^{o}C. This finding was similar to the report by Shoda (2000) on the special characteristics of *Bacillus* which is high thermal tolerance, rapid growth in liquid culture and ready formation of resistant spores. Cléry-Barraud *et al*. (2004) in their study on inactivation of *Bacillus anthracis* spore using heat and pressure, also proved that spores of *B. anthracis* are extremely resistant

Table 2: Inhibition (%) of *Sclerotium rolfsii* in the presence of *Bacillus subtilis* culture filtrate after treated with different heat, pH and light conditions.

Treatment		Hour					
		24	36	48	60	72	84
Heat (^oC)	-20	57.16 ± 1.13^a	52.02 ± 0.46^a	31.89 ± 1.12^b	26.08 ± 1.06^b	21.88 ± 0.71^a	7.96 ± 0.43^a
	4	21.65 ± 1.82^c	-3.66 ± 2.55^d	-7.13 ± 2.07^e	4.28 ± 1.23^d	nd	nd
	28	4.82 ± 2.08^d	7.36 ± 3.27^c	8.38 ± 2.47^d	8.24 ± 2.58^c	13.05 ± 1.94^b	7.68 ± 0.04^a
	80	58.30 ± 2.09^a	52.24 ± 3.33^a	38.19 ± 2.52^a	30.54 ± 2.55^a	20.82 ± 1.87^a	nd
	100	46.53 ± 3.72^b	23.00 ± 3.14^b	12.18 ± 1.33^c	9.70 ±c 1.53^c	13.60 ± 0.86^b	7.68 ± 0.03^a
pH	3	-15.88 ± 3.51^i	-20.15 ± 2.12^h	-13.85 ± 1.53^i	2.76 ± 1.36^i	7.85 ± 0.65^{h,i}	nd
	5	28.33 ± 2.98^f	7.92 ± 2.13^g	15.15 ± 1.52^f	16.09 ± 0.94^f	17.46 ± 1.01^f	8.02 ± 0.73
	7	-4.66 ± 2.91^h	-19.30 ± 1.67^h	-12.91 ± 1.86^i	3.14 ± 1.44^i	9.85 ± 0.93^g	nd
	9	-3.34 ± 4.11^h	-18.23 ± 2.28^h	-6.21 ± 1.93^h	6.37 ± 1.17^h	8.60 ± 1.07^h	nd
	11	10.96 ± 2.63^g	31.57± 1.88^f	11.09 ± 1.13^g	14.32 ± 1.72^g	7.09 ± 0.55^i	nd
Light	Light	38.36 ± 3.41^l	35.75 ± 1.84^k	28.75 ± 2.33^k	25.54 ± 1.74^k	21.58 ± 1.07^k	8.21 ± 0.72^k
	Dark	46.24 ± 2.53^k	37.71 ± 2.76^k	28.62 ± 2.03^k	24.67 ± 0.62^k	13.91 ± 0.93^l	nd
	UV	25.65 ± 2.74^m	20.87 ± 3.17^l	25.54 ± 3.28^l	24.62 ± 1.34^k	22.10 ± 0.84^k	8.02 ± 0.44^k

Note: Values are percentage of inhibition ± SD (n=7). Means in a column with the same superscript are not significantly different (p>0.05). nd – Not detected

to heat treatment, irradiation, desiccation, and disinfectants. Furthermore, it have been suggested 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 (Hecker *et al*., 1996).

B. subtilis culture filtrates are also not influenced by variation of pH and had shown tolerance to wide pH range in the initial pH of the culture medium. Treatment with various pH showed different level of antifungal activity, with the lowest inhibition recorded when treated with pH3 (2.76 %) and highest when treated with pH11 (31.57 %). Though pH treatment managed to alter the production of *Bacillus* antifungal activity against pathogen (Wang *et al*., 2002), Silo-Suh *et al*. (1994) proved that *Bacillus cereus* UW85 managed to subdue damping-off of alfalfa caused by *Phytophthora medicaginis* at pH7. Therefore, the antifungal activity produced in this study can be classified as different from the one being described by Wang *et al*. (2002) as the activities were not totally destroyed by very acidic treatments. Nicholson *et al*. (2000) suggest that in order to maintain the potential for spore viability, the spore must either (i) Prevent damage which would inactivate critical cellular components needed for successful germination and resumption of growth or (ii) Repair or replace those damaged critical components during germination, before their inactivation results in cell death. Since the treatment given can damage proteins as well as DNA, it is possible that repair of protein damage during spore germination and outgrowth plays a role in resistance of spores (Setlow, 1995).

Bacillus was able to grow on all carbon sources tested with significant different antifungal production as in Table 3 (ANOVA, $p<0.05$). Among several carbon sources, the maximum growth of bacterium and β-glucanase production was obtained with lactose as sole carbon source (Usama, 2003). When 1 % palm oil root was used as carbon source, inhibition zone reached it maximum of 2.93 cm in diameter after 36 h; meanwhile by using *G. lucidum,* the maximum inhibition zone reached after 12 h was 2.84 cm. Moreover, additional of 1 % chitin revealed inhibition area of 2.85 cm more compared to the bioassay test conducted earlier after 12 h incubation. Results (Figure 1) showed antifungal compound was more induced after 36 h (32.38 %) when cells were grown in media containing oil palm root preparation.

The enhancement could have been due to nutritional factors acting as inducers, since additional carbon source led to an increased hydrolytic enzyme production which have been known to degrade fungal cell wall (Lang *et al*., 2002). Secondary products from higher plants represent an enormous diversity of biologically active compounds that can be exploited as pesticides, when plant residues

decomposed are incorporated as green manure. These metabolites are usually produced inside plant tissue and can be released when plant debris is incorporated into the soil and subjected to microbial degradation (Gamliel *et al*., 2000). In addition, application of organic soil amendment may induce chemical and physical changes that also affect soil microflora. The presence of organic substances rich in cellulose was also shown to stimulate growth of organisms with cellulose activity (Elad *et al*., 1985).

Table 3: Inhibition (cm) of *Sclerotium rolfsii* in the presence of *Bacillus subtilis* culture filtrate supplemented with 1 % (w/v) carbon source.

Incubation (h)	Carbon Source		
	Oil palm root	*Ganoderma lucidum*	Chitin
12	$2.43 \pm 0.07^{c,d}$	2.84 ± 0.07^{e}	2.85 ± 0.06^{f}
24	$2.55 \pm 0.09^{d,e}$	2.80 ± 0.05^{e}	2.54 ± 0.06^{e}
36	2.93 ± 0.07^{f}	2.28 ± 0.05^{b}	2.39 ± 0.04^{d}
48	$2.56 \pm 0.04^{d,e}$	2.58 ± 0.08^{d}	2.27 ± 0.05^{c}
60	$2.66 \pm 0.08^{d,e,f}$	2.13 ± 0.06^{a}	2.41 ± 0.05^{d}
72	$2.42 \pm 0.05^{c,d}$	2.48 ± 0.05^{c}	1.82 ± 0.09^{a}
84	$2.82 \pm 0.05^{e,f}$	2.18 ± 0.03^{a}	$1.86 \pm 0.09^{a,b}$

Note: Values are mean of inhibition ± SD (n=7). Means in a column with the same superscript are not significantly different (p>0.05).

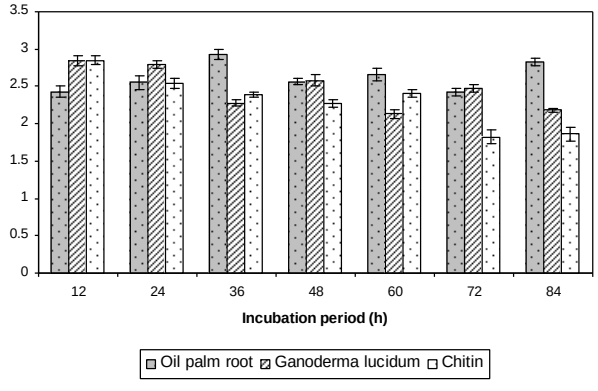

Figure 1: Diameter of inhibition (cm) of *Sclerotium rolfsii* after treated with *Bacillus subtilis* supplemented with 1% (w/v) of various types of carbon sources.

CONCLUSION

Bacillus subtilis produced bioactive compound active against the plant pathogenic fungi, *Sclerotium rolfsii*. The metabolite excreted by *B. subtilis* remained active after treated with heat, resistant over a wide pH range and UV. Application of additional carbon source did enhance the antifungal activity; with grinded oil palm root supplement showed highest antifungal activity (up to 40 % inhibition).

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