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Production of antifungal compounds from chitin by *Bacillus subtilis*

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Abstract

Two strains of *Bacillus subtilis* were isolated from the soil. Crude fungicides obtained from the culture broth of these strains grown aerobically in a medium containing chitin of marine waste displayed antifungal activity on pathogenic *Fusarium oxysporum*. *B. subtilis* W113 and *B. subtilis* W118 exhibited the maximal antifungal activity, when grown in a medium with the supplemented chitin being 1.75 and 0.75%, respectively. The inhibitory effects of the crude fungicides produced by these two strains were not significantly influenced by variation of pH. These crude fungicides were remarkably thermostable, and the inhibitory activities were retained to some extent even after the crude fungicides were heated at 100 °C for 30 min. These characteristics were unique in comparison with other known bio-fungicides. The utilization of chitin of marine waste to produce chitinolytic enzymes/fungicides by *B. subtilis* is seen for the first time
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Keywords: Chitin; Marine wastes; Fungicide; *Bacillus subtilis*

1. Introduction

Synthetic chemical fungicides have long been used as active agents in reducing the incidence of plant diseases. However, they are costly, can cause environmental pollution, and may induce pathogen resistance. Considering the limitations of chemical fungicides, it seems appropriate to search for a supplemental control strategy. Biological control, or the use of microorganisms or their secretions to prevent plant diseases, offers an attractive alternative or supplement to pesticides and genetic resistance for the management of plant diseases without the negative impact of chemical control. Therefore, biological control tactics have become an important approach to facilitating sustainable agriculture.

Recent biochemical research on plant disease control focused on two prime objectives. They were to (i) select and identify microorganisms with antifungal activities, isolate and characterize the specific antifungal factors within these microorganisms; (ii) determine the operative mechanisms of these antifungal agents. In the past few years, numerous microorganisms with antifungal activities and their active factors have been identified [1–6]. The mechanisms by which these microorganisms inhibit the growth of potentially pathogenic fungi have been demonstrated [2,4,6–10].

Chitin, a homopolymer of *N*-acetyl-D-glucosamine (Glc-NAc) residues linked by β-1–4 bonds, is the most abundant renewable natural resource after cellulose [11]. The main source of chitin is crustacean wastes, it also occurs in fungi, insects, etc. [12]. Shrimp and crab processing waste containing chitin, protein, and calcium carbonate is preteated by size reduction, deproteination, and demineralization to yield a chitin material suitable for bioconversion or other uses [13,14]. Chitin and its derivatives hold great economic value because of their versatile biological activities and agrochemical applications [15–17]. It is estimated that the worldwide annual recovery of chitin from the processing of marine crustacean wastes is 37,300 metric tons [18]. Chitin bioconversion has been proposed as a waste treatment alternative to the disposal of shellfish waste [13,14]. Some pretreated chitin is used as substrate for microbial chitinase production [19–25]. To further enhance the utilization of chitin containing marine crustacean waste, we have recently investigated the bioconversion of shrimp and crab shell powder (SCSP) for bio-fungicide production. We have demonstrated that *Pseudomaonas aeruginosa* K-187 is an antifungal strain in the SCSP medium and it exhibits a broad range of antagonism toward fungal phytopathogens.

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The fungicide from *P. aeruginosa* K-187 was found to cause hyphae swelling and lysis in *Fusarium oxysporum* by microscopic observation. The antifungal characteristics of this fungicide were also evaluated as seed treatment to prevent damping off in alfalfa caused by *F. oxysporum* [26]. In the present work, we further found that two soil-borne strains of *Bacillus subtilis* exhibit excellent antifungal properties in the presence of pretreated chitin of shrimp and crab waste. Identification of the isolated strains, medium composition, pH, and thermal stability of the fungicide was described. The effects of the fungicides on the growth of pathogenic *F. oxysporum* were also investigated.

2. Materials and methods

2.1. Materials

Flake chitin and powder chitosan from crab shell were purchased from biotech Co. (Kau-shawn, Taiwan). Yeast extract, polypeptone, and potato dextrose agar (PDA) were purchased from DIFCO Laboratories, Michigan, USA. Beef extract was from Sigma. All other reagents used were of the highest grade available. Chinese herb residue (CHR) and *Gandoderma zucidum* residue (GZR) were kindly provided by Soon-Chung Co. (Nantou, Taiwan). Both were discarded wastes after the major active ingredients were extracted.

2.2. Microorganisms

The microorganisms used for comparison with that isolated in this study were *Pseudomaonas aeruginosa* K-187, *Stretomyces actuosus* A151, *Bacillus alvei*, *Bacillus sphaericus*, *Bacillus cereus*, *B. subtilus* CCRC 10029. These strains were purchased from the Culture Collection and Research Center (CCRC), Taiwan, or obtained from stock cultures in our laboratory [23–25].

2.3. Isolation and screening of fungicide producing strain

Microorganisms isolated from soils collected at different locations in northern Taiwan were screened on agar plates containing 0.2% flake chitin, 0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$, and 2% agar (pH 7). The plates were incubated at 30 °C for 2 days. Colonies that grew well under such conditions were isolated and retained for subsequent screening. Ninety-six bacteria were obtained from the first screening. Those organisms obtained from the first screening were subcultured in liquid media (containing 0.2% chitin, 0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$) in shaken flasks at 30 °C and 180 rpm. After incubation for 2 days, the culture broth was centrifuged (4 °C and 820 × *g* for 20 min) and the supernatants were collected for measurement of antifungal activity. Twelve strains out of 96 isolates showed fungicide activities in the culture broth. These strains were numbered as W111–W122.

2.4. Effects of chitin concentration on the antifungal activity

W111–W122 obtained from above were cultured in liquid media containing 0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$. The media was further supplemented with 0.75, 1.5, or 3% of chitin. After incubation at 30 °C and 180 rpm for 2 days, the culture broth was centrifuged (4 °C and 820 × *g* for 20 min) and the supernatants were collected for measurement of antifungal activity. Among the 12 strains tested, strains W113 and W118 showed the highest antifungal activities.

2.5. In vitro antifungal activity tests

The antifungal activity for the crude fungicide was estimated using a growth inhibition assay described earlier [2,6]. The fungal spores of pathogenic *F. oxysporum* were grown on petri plates filled with PDA. After 10 days incubation at 25 °C, the fungal colonies were removed with sterile water containing 0.1% (v/v) Tween 80. The resulting suspension was filtered aseptically through 0.45 μm pore size membrane filters. The filtrate was adjusted with sterile water to a concentration of 1×10^6 spores per ml, and stored at 4 °C. To test the antagonistic effect of the supernatants obtained above or the crude fungicide produced, petri plates were filled with molten PDA precooled to 45 °C, and divided into two groups (triplicate for each). To each plate in the experimental group (E), an appropriate amount of the supernatant or crude fungicide solution was added. To those of the control group (C) was added an equal amount of sterile water. After the plates were cooled, the fungal inoculum was then placed onto an agar surface. Both groups were incubated for 72 h at 25 °C. The diameters of the largest and smallest fungal colonies were recorded and the averages were calculated. The inhibition ratios were calculated with the following formula. If the inhibitory ratio was greater than 20%, the test strain would be considered inhibited and the minimal inhibitory concentration (MIC) for that strain was then determined.

$$\text{Inhibition ratio (\%)} = \frac{C - E}{C} \times 100\%$$

where, *C* is the average diameter of the largest and smallest colonies of the control groups; *E* is the average diameter of the largest and smallest colonies of the experimental groups.

2.6. Effect of culture condition

In the investigation of the culture condition, growth was carried out in a basal medium containing 0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7), and gradually supplemented with the various ingredients to be investigated. The major ingredients being investigated included chitin, carbon sources (glucose, carboxymethyl cellulose (CMC), CHR, GZR), nitrogen sources (beef extract, polypeptone, yeast extract, bacto-peptone, sodium glutamate, sodium nitrate,

ammonium nitrate), and inorganic salts (ferrous sulfate, zinc sulfate, copper sulfate, manganese sulfate, sodium chloride). They were added and investigated in one kind at a time fashion. A 100 ml of the resultant medium in a 250 ml Erlenmeyer flask was aerobically cultured at 30 °C for 48 h on a rotary shaker (180 rpm). After centrifugation (820 × *g*, 4 °C, 20 min, Beckmen J2-21 M/E), the supernatant was used for bioassay. Usually an effective prior condition was used as the basis for the latter experiment until the optimal culture composition was obtained. With the use of the optimal culture composition, the effects o f the initial pH, temperature, cultivation volume and cultivation time on the production of fungicide were investigated in the same fashion until the optimal condition was found.

2.7. Effect of temperature and initial pH

Growth was carried out in the optimal culture composition found above. The media were adjusted with NaOH and HCl to pH 3–11. The flasks were removed after 2 days of incubation at 25, 37, and 45 °C, respectively. The antifungal activities were assayed.

2.8. Effect of medium volume

Growth was carried out in the optimal culture composition found above. Flasks of 250 ml containing 50, 100, 150, and 200 ml of the culture media were incubated with reciprocal shaking at 30 °C. The flasks were removed after 2 days of incubation and the antifungal activities were assayed.

2.9. Time course of fungicide production

Growth was carried out in the optimal culture composition found above. The media were assayed for antifungal activities every 24 h for up to 10 days.

2.10. Preparation of crude fungicide solution

B. subtilis W113 and W118 were both cultured under their optimal culture conditions. After centrifugation (820 × *g*) at 4 °C for 20 min, the cell-free supernatants were subjected to ammonium sulfate precipitation. The resultant precipitate was collected and dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7), followed by dialysis against the same buffer overnight. The resultant dialyzate was filtered aseptically through 0.45 μm pore size membrane filters and used for the bioassays.

2.11. pH and thermal stability of the crude fungicide

The pH stability of the crude fungicide was determined by measuring the residual inhibitory activity at pH 7, as described above, after dialyzing the samples against a 50 mM buffer solution of various pH (pH 3–11) in seamless cellulose tubing (Sankyo, Japan). The buffer systems used were glycine–HCl (50 mM, pH 3), acetate (50 mM, pH 4.5), phosphate (50 mM, pH 6–8), Na_2CO_3–$NaHCO_3$ (50 mM, pH 9-11). The thermal stability of the crude fungicide was studied by heating the samples at 100 °C for various time periods. The residual inhibitory activity was measured as described above.

2.12. Hyphal morphology affected by the crude fungicide

Fungal spores (20 μl) were grown in test tubes (∅16 mm × 100 mm) containing 12 ml potato dextrose broth, 3 ml of sample solution of crude fungicide was added simultaneously or 24 h later so that the crude fungicide in test was regarded as 20% (v/v). The resultant solution was incubated at 25 °C, and the hyphae was observed using a light microscope at various intervals.

2.13. Other microorganisms used for comparison

The antifungal activities of microorganisms isolated in this study were compared with those of other strains of microorganisms known to produce chitinase or cellulase.. The microorganisms used for comparison with were *P. aeruginosa* K-187, *S. actuosus* A151, *B. alvei*, *B. sphaericus*, *B. cereus*, *B. subtilus* CCRC 10029. Among them, *S. actuosus* A151 is a cellulase/xylanse producing strain and the rest are known to produce chitinase. These strains were purchased from the Culture Collection and Research Center (CCRC), Taiwan, or obtained from stock cultures in our laboratory [23–25].

3. Results

3.1. Effects of chitin concentration on the antifungal activity

After the preliminary screening of organisms was performed, 12 strains numbered W111–W122 exhibited better results in antifungal activities. A series of experiments was carried out to study the effect of different initial chitin concentrations on fungicide production of these 12 strains. The cultural medium was supplemented with 0, 0.75, 1.5, and 3.0% of chitin, and the cultural broth was assayed for antifungal activity. The antagonistic activities against *F. oxysporum* were displayed as percent inhibition and were shown in Table 1. The results showed that chitin is an essential element for induction of antifungal activity. In the presence of chitin, all 12 strains exerted antifungal activity to some extent. However, high chitin concentration is not critical for higher inhibition. It is also seen that only W113 and W118 attained more than 60% of inhibition disregarding the chitin concentration used. Therefore, optimal conditions were sought for these two strains to exert highest antifungal activity.

Table 1
Effects of initial chitin concentration on the antifungal activity of 12 isolated strains (shown as percent inhibition)

Strains number	Chitin concentrations (%)			
	0	0.75	1.5	3.0
W111	0	58	50	58
W112	0	50	40	69
W113	0	64	65	63
W114	0	52	49	57
W115	0	56	48	50
W116	0	78	46	51
W117	0	50	55	56
W118	0	81	69	61
W119	0	77	37	42
W120	0	70	39	47
W121	0	73	33	40
W122	0	79	56	49

Table 2
The optimal operative conditions for *B. subtilis* W113 and *B. subtilis* W118 to produce fungicide

Optimal conditions	Strains	
	W113	W118
Chitin (%)	1.75	0.75
Medium volume (ml)	50	100
Cultivation time (day)	3	2
Cultivation temperature (°C)	25	30
Carbon source (CHR)[a] (%)	0.15	–[a]
Carbon source (GZR)[b] (%)	–[a]	0.05
pH	7	7

[a] CHR: Chinese herb residue, is a leftover after major active ingredients have been extracted.
[b] GZR: *Gandoderma zucidum* residue, is a leftover after major active ingredients have been extracted.

3.2. Identification of microorganisms

From the morphological observation and physiological characteristics, the microorganisms were identified according the description in Bergey's *Mannual of Determinative Bacteriology* [27]. W113 and W118 were both identified as strains of *B. subtilis*.

3.3. Effects of culture conditions

A series of experiments was carried out to study the effect of different initial chitin concentration on the fungicide production. The results showed that *B. subtilis* W113 and *B. subtilis* W118 exhibited the maximal antifungal activity when the supplemented chitin was 1.75 and 0.75%, respectively. To study the effect of carbon sources on the production of fungicide, growth was carried out in basal mediums as described above with or without of addition of carbon sources. The carbon sources used were glucose, CMC, CHR, GZR, respectively, which was adjusted to 0.1%. The production of fungicide by *B. subtilis* W113 was slightly enhanced by the addition of CHR into the medium, and *B. subtilis* W118 by GZR. When the concentration effects of these carbon sources were further studied, it was found that the most effective substrate and concentration for fungicide production was 0.15% CHR for strain W113 and 0.05% GZR for strain W118. Hence, the optimal concentration of carbon source was added to the culture for the next study. Among all the nitrogen sources and inorganic salts tested, none of them showed any significance in fungicide production.

To study the effect of initial pH on the production of fungicide, growth was carried out in the optimal medium at various initial pH and temperatures for 2 days. The results showed that the highest antifungal activity was obtained when the initial pH and temperature were 7, 25 °C for strain W113 and 7, 30 °C for strain W113, respectively. The effect of the oxygen supply on the fungicide production was investigated by altering the volume of the medium in a 250 ml Erlenmeyer flask. The best result was observed when the culture volume was 50 ml for W113 and 100 ml for W118. A typical time course study of antifungal activity revealed that the antagonistic activities against *F. oxysporum* reached the peak at 3 days and 2 days for W113 and W118, respectively. The optimal operative conditions for *B. subtilis* W113 and *B. subtilis* W118 to produce fungicide were summarized in Table 2.

3.4. pH and thermal stability of crude fungicides produced by B. subtilis W113 and B. subtilis W118

The effects of pH on the inhibitory activities of crude fungicides on *F. oxysporum* were studied at various pH. It was found that the inhibitory effects of fungicides produced by both *B. subtilis* W113 and *B. subtilis* W118 were not significantly influenced by variation of pH. The inhibitory activities were highly retained even at extreme pH. The fungicide of *B. subtilis* W113 showed more than 60% of inhibition even at extreme acidic (pH 3) or basic (pH 11), and the fungicide of *B. subtilis* W118 showed more than 50% of inhibition at these extreme pH. The maximal inhibitory activity for fungicide of strain W113 appeared at pH 8 with 80% of inhibition, and at pH 7 with 70% of inhibition for fungicide of strain W118. The thermal stability of the crude fungicide was studied by heating the samples at 100 °C for various time periods. The residual inhibitory activities on *F. oxysporum* were measured. It was found that the crude fungicides were remarkably thermostable. The inhibitory activities were retained to some extent even after the crude fungicides were heated at 100 °C for 30 min. The inhibitory activities after treatment were 75 and 50% for fungicides of *B. subtilis* W113 and *B. subtilis* W118, respectively. However, longer heating will decrease the inhibitory activities drastically.

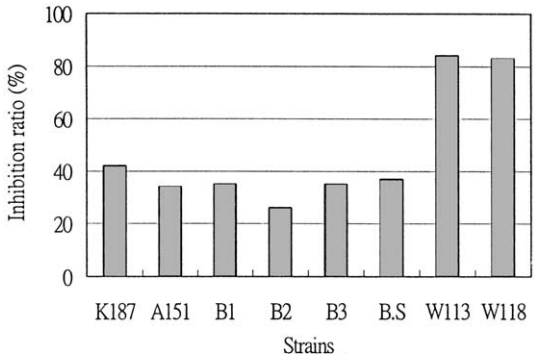

Fig. 1. Comparison of antifungal activities of various organisms. K-187: *P. aeruginosa* K-187; A-151: *S. actuosus* A-151; B1: *B. alvei*; B2: *B. sphaericus*; B3: *B. cereus*; W113: *B. subtilis* W113.

3.5. Comparison of antifungal activities of various organisms

Several microorganisms including *P. aeruginosa* K-187, *S. actuosus* A151, *B. alvei*, *B. sphaericus*, *B. cereus*, *B. subtilus* CCRC 10029 were cultured in the same optimal conditions used for *B. subtilis* W113 and *B. subtilis* W118 as described above. The inhibitory activities on *F. oxysporum* were measured and the results were shown in Fig. 1. It is apparent that the inhibitory activities of *B. subtilis* W113 and *B. subtilis* W118 were significantly higher than all the other microorganisms tested.

3.6. Concentration effects of fungicides produced by strains W113 and W118 on the inhibitory activities

In the study of the concentration effects on the inhibitory activities, various concentrations of fungicides produced by strains W113 and W118 were applied. The results were shown in Figs. 2 and 3. It was shown that the inhibitory activities of both fungicides gradually increase with the increase of concentrations. The MIC for *B. subtilis* W113 and *B. subtilis* W118 was 25 and 23%, respectively.

3.7. Hyphal morphology affected by crude fungicide

The effect of the crude fungicide on the hyphal growth was examined using a light microscope after the tested fungi were incubated with the fungicide (20%, v/v) at 25 °C. The effect of the crude fungicide from *B. subtilis* W113 on the morphology of *F. oxysporum* is shown in Fig. 4. It was observed that the hyphae of *F. oxysporum* growing in the absence of the crude fungicide did not show any obvious growth aberrations (Fig. 4A). However, an abnormal hyphal swelling was observed (Fig. 4B) when the crude fungicide was added to the culture broth of *F. oxysporum*. The *F. oxysporum* was preincubated for 24 h before crude fungicide was added, followed by incubation for an additional 24 h. The abnormal hyphae distinguished itself from that of the control. In some instances, extensive degradation of *F. oxysporum* hyphae, or lysis of the hyphal tips caused by the presence of the crude fungicide were also observed. In addition, we found that germination of *F. oxysporum* was hindered profoundly by the crude fungicide. When the *F. oxysporum* spores and crude fungicide were incubated simultaneously, the mycelial mass could scarcely be seen until 7 days after incubation. When the mycelial mass of *F. oxysporum* was compared before and after the crude fungicide was added, the mycelial mass decreased drastically 48 h after the addition of the fungicide. This evidence further suggested that the lysis of hyphae might have occurred. In addition to that of *B. subtilis* W113, similar swelling, degradation, and lysis were also observed on *F. oxysporum* when the fungicide of *B. subtilis* W118 was tested (data not shown).

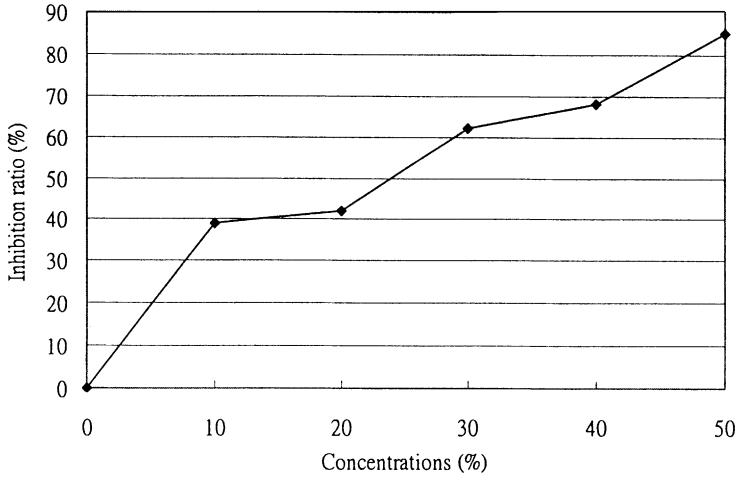

Fig. 2. Concentration effect of fungicide produced by *B. subtilis* W113 on the inhibitory activity.

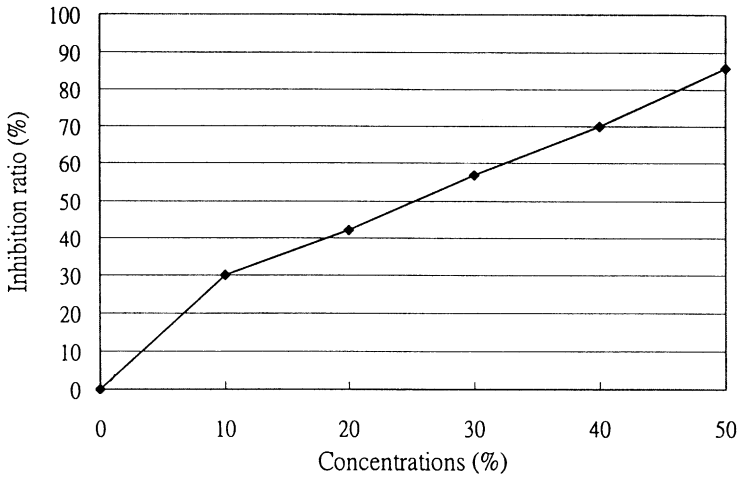

Fig. 3. Concentration effect of fungicide produced by *B. subtilis* W118 on the inhibitory activity.

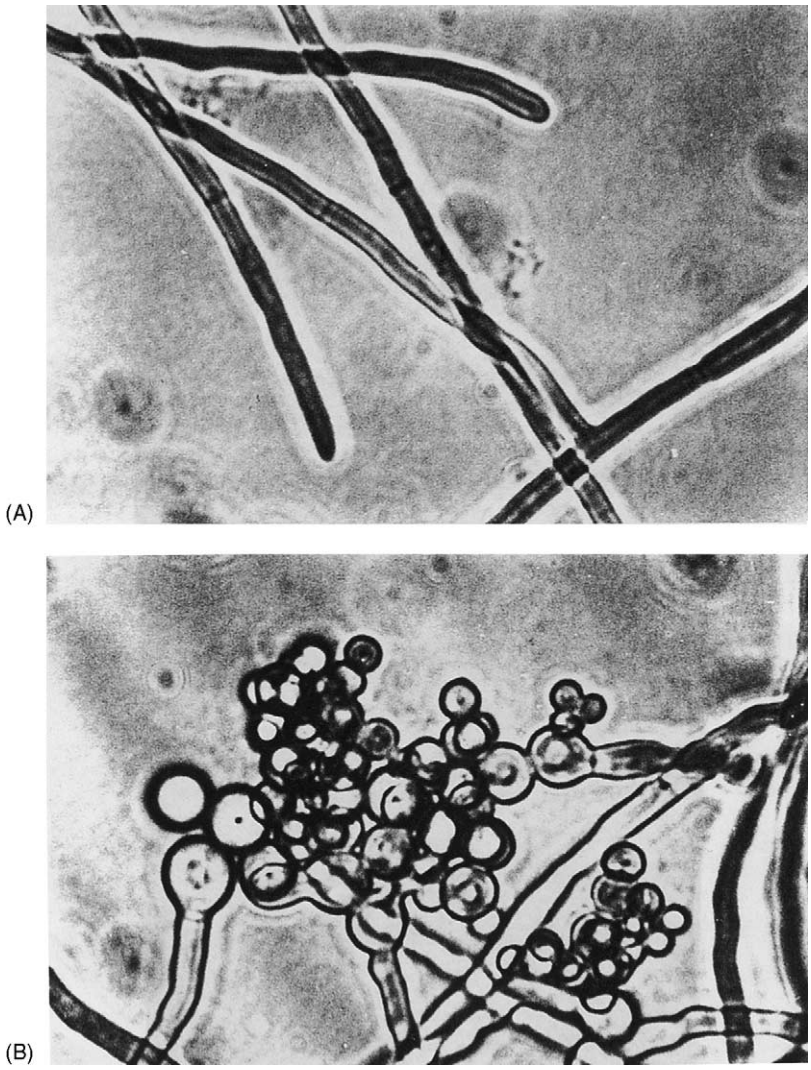

Fig. 4. Effect of the crude fungicide of *B. subtilis* W113 on morphology of *F. oxysporum*. (A) normal mycelia of *F. oxysporum*; (B) swelling of *F. oxysporum* hyphae in the presence of *B. subtilis* W113 crude fungicide (20%, v/v).

4. Discussion

In this study, two strains of *B. subtilis* were isolated from the soil of northern Taiwan. It was demonstrated that crude fungicides obtained from the culture broth of these strains grown aerobically in a medium containing chitin displayed antifungal activity on pathogenic *F. oxysporum*. The thermal stability of these fungicides is remarkable and the inhibitory activities are not significantly affected by variation of pH, even at extreme pH. Furthermore, it was thought to have a high molecular weight because it precipitated upon ammonium sulfate treatment and non-dialyzable. Many known antifungal compounds were small molecules and most are peptides [1] or proteins [4,28–31] in nature. Many of them were found unstable at extreme pH and temperatures. In contrast, fungicides found in this study were extremely thermostable and pH resistant. The evidences suggest that these fungicides are different from the antifungal compounds previously described.

Numerous microorganisms with antifungal activities have been identified [1–6], and many have been effective in field experiments [32,33]. So far gram-negative bacteria, especially *Pseudomonas* strains, have been intensively investigated as biological control agents. The gram-positive bacteria, like *Bacillus* spp., however, have been studied less intensively than gram-negative bacteria [33], although *B. subtilis* is considered to be a safe biological agent [34–38]. *B. subtilis* is an organism known for protease production [39,40], it is, however, rarely used for fungicide production. Previous reports showed that *B. subtilis* NB22 and *B. subtilis* RB14 produced antifungal peptide antibiotic-iturin A and surfactin in solid state fermentation using soybean curd residue (okara) [1]. Iturin A is a cyclolipopeptide containing seven residues of α-amino acids and one reside of a β-amino acid, it is a small molecules yet displays strong antifungal activity. The other lipopeptide, surfactin, in contrast has week antibiotic activity. The characteristics of these two compounds clearly distinguished them from the fungicide found in this study.

Although there are a few organisms using pretreated chitin of marine waste as substrate for microbial chitinase production and the chitinases thus produced were presumably responsible for antifungal activities for these organisms [19–25], our finding of the utilization of chitin of marine waste to produce fungicide by *B. subtilis* is seen for the first time. Although the crude fungicide found here showed partial chitinase activity (data not shown), there is little evidence suggested that chitinase was responsible for the present antifungal activity. In fact, judging from the difference in the physical properties between chitinase and the crude fungicide described here, such scenario is very unlikely. Besides, recent studies showed that chitinase produced by *P. aeruginosa* K-187, using SCSP as a carbon source, displayed no antifungal activity [16,25].

In addition to the utilization of marine waste, strains W113 and W118 in this study were able to utilize other waste resources as carbon sources for the production of fungicides. The production of fungicide by *B. subtilis* W113 was enhanced by the addition of CHR into the medium, and *B. subtilis* W118 by GZR. Although the compositions of wastes of these categories were not well defined, they nevertheless consist of large quantity of potential carbon sources such as cellulose. Annually, substantial amounts of these wastes were produced in Asia, but their proper utilization is rarely seen. Effective utilization of such wastes as well as marine wastes will not only solve environmental problems, but also promote the economic values of the marine and agricultural products. These attractive characteristics will further enhance the values of development of *B. subtilis* W113 and *B. subtilis* W118 as bio-control agents.

Cultured in the same medium, several local microorganisms including *P. aeruginosa* K-187, *S. actuosus* A151, *B. alvei*, *B. sphaericus*, *B. cereus*, *B. subtilus* CCRC 10029 were far less effective in inhibition than *B. subtilis* W113 and *B. subtilis* W118. This result substantiates the uniqueness of strains W113 and W118 with regarding to fungicide production by the utilization of marine and agricultural wastes.

To further characterize these new fungicides, the purification and characterization is necessary. The mechanisms by which antifungal factors inhibit growth of potentially pathogenic fungi are also critical. These are currently all under investigation. Although many works remain to be done before its application in the field, the results presented suggests that the development of *B. subtilis* W113 and *B. subtilis* W118 as bio-control agents is an environmentally benign alternative to current disease control strategy.

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