Maejo Int. J. Sci. Technol. **2012**, *6*(01), 95-104

Maejo International Journal of Science and Technology

ISSN 1905-7873
Available online at www.mijst.mju.ac.th

Full Paper

Chitinase production and antifungal potential of endophytic *Streptomyces* strain P4

Julaluk Tang-um and Hataichanoke Niamsup*

Department of Chemistry and Centre of Excellence for Innovation in Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

* Corresponding author, e-mail: hataichanoke.n@cmu.ac.th

Received: 2 August 2011 / Accepted: 11 February 2012 / Published: 29 February 2012

Abstract: The endophytic actinomycete P4 strain, previously isolated from sweet pea root, was identified as *Streptomyces* sp. by full 16S rRNA sequencing. It is mostly related to *Streptomyces griseoflavus* with a 99.7% identity score. The *Streptomyces* sp. P4 was tested for its hydrolytic activities by plate method. The result showed the presence of chitinase. The extent of chitinase activity was assessed by spectrophotometric method along with growth monitoring. Chitinase production was growth-associated and showed the highest activity on the fifth day. The dual culture method revealed that the strain was effective in restricting the radial growth of *Fusarium oxysporum* f.sp. *lycopersici*, an important phytopathogen of tomato. Scanning electronic microscopic analysis showed that the rupture of the *F. oxysporum* mycelial cell wall occurred at the area of interaction between *F. oxysporum* and *Streptomyces* sp. P4. This was possibly due to the chitinolytic activity of the P4. Thus, this actinomycete has the potential for being used as a biocontrol agent, thereby reducing the use of chemical fungicides.

Keywords: endophyte, streptomycete, chitinase, fusarium, wilt, antimicrobial activity

INTRODUCTION

As worldwide concern for the natural environment and human health has increased, so has interest in organic farming. A research institute of organic agriculture (FiBL) and the international federation of organic agriculture movements (IFOAM) reported that organic agricultural lands have expanded globally from 11.0 million hectares in 1999 to 37.2 million hectares in 2009, accounting for 0.85% of the total agricultural lands [1]. One of the criteria for organic farming is the avoidance of chemical usage. Soilborne plant diseases can be controlled through agronomic practices and microbial

Maejo Int. J. Sci. Technol. **2012**, *6*(01), 95-104

biocontrol agents instead [2]. Biological controlling agents can replace chemical agents in controlling pathogenic insects, microbials and weeds. Several biofungicides are based on antibiotic metabolites and hydrolytic enzymes. For example, *Streptomyces griseoviridis* strain K61, a soilborne fungal antagonist which produces aromatic antibiotics with characteristic 7-membered rings in the molecules was commercialised as Mycostop® by Verdera Oy, a Finnish company [3], and *Streptomyces* sp. Di-944 was formulated to suppress Rhizoctonia damping-off [4].

Streptomyces is a major genus of actinimycetes, the Gram-positive terrestrial or marine bacteria found in both colony and mycelium forms. Although *Streptomyces* species with a characteristic earthy smell may be thought of as pathogens, the antibiotics that they produce have been profitably exploited [5]. For example, *S. clavuligerus* produces the β-lactam cephamycin C and clavulanic acid, a β-lactamase inhibitor. A new stereoisomeric anthracyclin with anticancer activity was isolated from *Sreptomyces* sp. Eg23 [6]. Various hydrolytic enzymes, e.g. proteases/peptidases, chitinases/chitosanases, cellulases/endoglucanases, amylases, and pectate lyases, are produced by *S. coelicolor* [7].

In this study, the hydrolytic enzyme production of the endophytic actinomycete P4 strain, previously isolated from sweet pea root [8], is investigated. Additionally, the potential use of this endophyte as a biocontrol agent is studied by assessing its antagonism against pathogenic fungi.

MATERIALS AND METHODS

Microorganism Strains and Growth Conditions

The bacterial strain P4, generously provided by Asst. Prof. Dr. Ampan Bhromsiri (Department of Soil Science and Conservation, Chiang Mai University) was previously isolated from sweet pea root. The bacteria was maintained at 30°C on an IMA-2 agar medium consisting of 5 g glucose, 5 g soluble starch, 1 g beef extract, 1 g yeast extract, 2 g N-Z-case®, 2 g NaCl and 1 g $CaCO_3$ per litre [9]. For the production of chitinase, the culture was transferred into a colloidal chitin medium, 1 litre of which consisted of 20 g colloidal chitin, 0.5 g yeast extract, 1 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4 \cdot 7H_2O$ and 1.36 g KH_2PO_4, with an adjusted pH of 7.0 [10]. Colloidal chitin was prepared according to the method described by Souza et al. [11]. The liquid culture was incubated at 30°C with agitation at 160 revs/min.

The pathogenic fungi *Fusarium oxysporum f.*sp. *lycopersici*, *Corynespora cassiicola* and *Rhizoctonia solani* were obtained from the Department of Agriculture, the Ministry of Agriculture and Cooperatives (Thailand). They were maintained on potato-dextrose agar (PDA).

Identification by 16s rRNA Sequencing

An approximately 1.5-kb polymerase chain reaction (PCR) product was amplified from genomic DNA of the bacterial strain P4 using two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'), directed to the 16S rRNA region. The following conditions were used: an initial denaturation step at 94°C for 3 min., 25 cycles at 94°C (1 min.), 50°C (1 min.) and 72°C (2 min.), followed by a final extension at 72°C (3 min.). The purified PCR product was sequenced by an ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) on an ABI Prism® 3730XL DNA Sequence (Applied Biosystems, USA). Homology was analysed using the BLAST program from the GenBank database [12].

Maejo Int. J. Sci. Technol. **2012**, *6*(01), 95-104

Plate Screening of Hydrolytic Enzymes

The bacterial strain P4 (identified as *Streptomyces*) was screened for its capacity to produce hydrolytic enzymes using the plate method. The bacterium was allowed to grow at 30°C on nutrient agar plates supplemented with different substrates, i.e. colloidal chitin, gelatin, sodium carboxymethyl-cellulose and Tween20, for detection of chitinase, protease, cellulase and lipase respectively. The clear zone around the colonies observed after 7-14 days is an indication for enzyme production. In the cases of cellulase and protease, the plates were reacted with 0.2% Congo red and saturated $(NH_4)_2SO_4$ respectively prior to the observation of growth [13-14].

Quantification of Extracellular Chitinase Activity

The strain P4 was grown in a colloidal chitin medium broth at 30ºC with continuous shaking at 160 rpm. The supernatant fluid was harvested every two days for 17 days by filtration through Whatman no.1 filter. Chitinase activity in the supernatant was assayed using 0.6% colloidal chitin as a substrate and was based on a procedure by Taechowisan et al. [13]. The supernatant fluid (700 µl) was added with 2% colloidal chitin (300 µl) in 0.1M potassium phosphate buffer at pH 7.0, and the mixture was incubated in a water bath at 40ºC for 3 hr. One mL of Somogyi's reagent [15] was added and the reaction mixture was boiled at 100ºC for 10 min. and cooled to room temperature. Then Nelson's reagent (1 mL) was added and the mixture cooled to room temperature for 20 min. After centrifugation of the reaction mixture, the amount of N-acetyl glucosamine (GlcNAc) released in the supernatant was spectrophotometrically measured by the method of Somogyi-Nelson [15]. The method is based on the 520-nm absorbance given by a coloured complex formed between a copper-oxidised sugar and arsenomolybdate. One unit (U) of chitinase activity was defined as the amount of enzyme required to produce 1 µmol of reducing sugar per min. under the conditions of the experiment.

The cell growth was followed by measurement of the cells' dry weight. After removing the supernatant for chitinase assay, the cell pellet was dried in an oven at 80°C until a constant weight was obtained. All measurements were performed in triplicate.

In Vitro Antagonism Tests against Fungi

The identified *Streptomyces* P4 was evaluated for antagonistic activity towards three fungal phytopathogens, i.e. *F. oxysporum*, *C. cassiicola* and *R. solani*, by the dual culture method. An agar plug of 6 mm in diameter taken from a 7-day-old colony of each test fungus and that taken from a 14-day-old P4 bacterial strain were placed on PDA plates with 4-cm spacing (3 replicates each). The cultures were incubated at room temperature (30-32°C) and the diameters of the fungal colonies in the direction of the actinomycete were measured every 2 days for 14 days. The test fungi were grown alone to serve as control. Data were statistically analysed for significance ($p<0.05$) using the SPSS statistics 17.0 software.

Scanning Electronic Microscopic Analysis

A scanning electron microscope (SEM) (Model JSM-5910LV, Type LV, JEOL Ltd., Japan) was employed to monitor the effect of the *Streptomyces* P4 on the fungal cell walls. Fungal mycelia in a 14-day-old dual culture plate were taken from a free-growing area and a dual-growing area in the direction of the P4 strain. The two mycelial samples were pre-fixed with glutaraldehyde and post-fixed with

Maejo Int. J. Sci. Technol. **2012**, *6*(01), 95-104

osmium tetroxide. After dehydration in ethanol, the samples were dried to their critical points with carbon dioxide [16], mounted on slides and coated with gold for observation by SEM.

RESULTS AND DISCUSSION

Identification of Bacterial Strain

A sequence of 1438-bp in length was obtained from the 1.5-kb PCR product. The DNA sequence was then submitted to GenBank database under accession no. JN102356 and was blasted against non-redundant DNA sequences in the database [12]. The 16S ribosomal DNA sequence showed the highest similarity to *Streptomyces griseoflavus* gene (accession no. EU741217) with a 99.7% identity score, followed by 99.5% score when aligned with *S. variabilis* (DQ442551, AB184884, AB184763), *S. vinaceus* (AB184186) and *S. griseoincarnatus* (AB184207, AJ781321). The P4 strain could not be definitely identified down to species level. It is worth noting that the 16S ribosomal RNA sequences are not highly variable among *Streptomyces* species and that *Streptomyces* systematics are rather complex. Lanoot et al. [17] suggested that 16S-ITS RFLP fingerprinting had a higher taxonomic resolution than 16S rDNA sequencing. A transformation reaction of progesterone has also been proposed for *Streptomyces* taxonomic classification [18].

S. griseoflavus was reported to produce many potent secondary metabolites, e.g. hormaomycin, a peptide lactone and a bacterial signalling metabolite and narrow-spectrum antibiotic [19]; okilactomycin, a polyketide antibiotic against gram-positive bacteria [20]; bicozamycin, a cyclic peptide antibiotic [21]; desferrioxamine, a precursor of iron chelator [22]; and an alkaline protease inhibitor [23]. However, there has been no report on the hydrolytic enzyme production in *S. griseoflavus*.

Chitinase Activity

From a preliminary screening of enzymes by the plant method (Figure 1), a clear zone surrounding the bacterial colonies was observed in the plate containing colloidal chitin as shown in Figure 1(A), indicating that the *Streptomyces* sp. P4 produced chitinase, whose activity was assayed during cell growth. Figure 2 shows that the activity was at a maximum on the fifth day, followed by a decrease upon approaching a stationary phase of growth. The findings imply that chitinase production is growth-associated. It should be noted that the *Streptomyces* sp. P4 strain had been pre-cultured in colloidal chitin medium prior to this experiment. As a consequence, the lag phase of growth was not observed, while logarithmic phase continued until the 5^{th} day before reaching a stationary phase.

This result of chitinase production of *Streptomyces* sp. P4, induced in a colloidal chitin-containing environment as previously reported [10, 24] and indicating growth-associated behaviour, is similar to that obtained from the study on *S. hygroscopicus* [25]. However chitinase production in *S. hygroscopicus* occurred 1-2 days before cell growth, while in our case chitinase production of *Streptomyces* sp. P4 was closely associated with cell growth, which should be because the pre-cultured and pre-induced bacteria were used in our experiment. Closely paralleling growth, the chitinolytic enzyme production by *Streptomyces* may be for the purpose of hydrolysing chitin into monosaccharides to be used as carbon and nitrogen sources [26]. However, the chitinase activity of 0.00093 U/mL in *Streptomyces* sp. P4, which corresponds to a specific activity of 0.050 U/mg protein, was 4 times lower than that in *S. viridificans* (0.0038 U/mL) [10]. In nature chitinase is produced by actinomycetes in order to degrade complex nutrients from the soil. As fungal cell walls and insect structures largely contain chitin, chitinase produced from endophytes can be deleterious to pathogens and pests [27-28].

Maejo Int. J. Sci. Technol. **2012**, *6*(01), 95-104

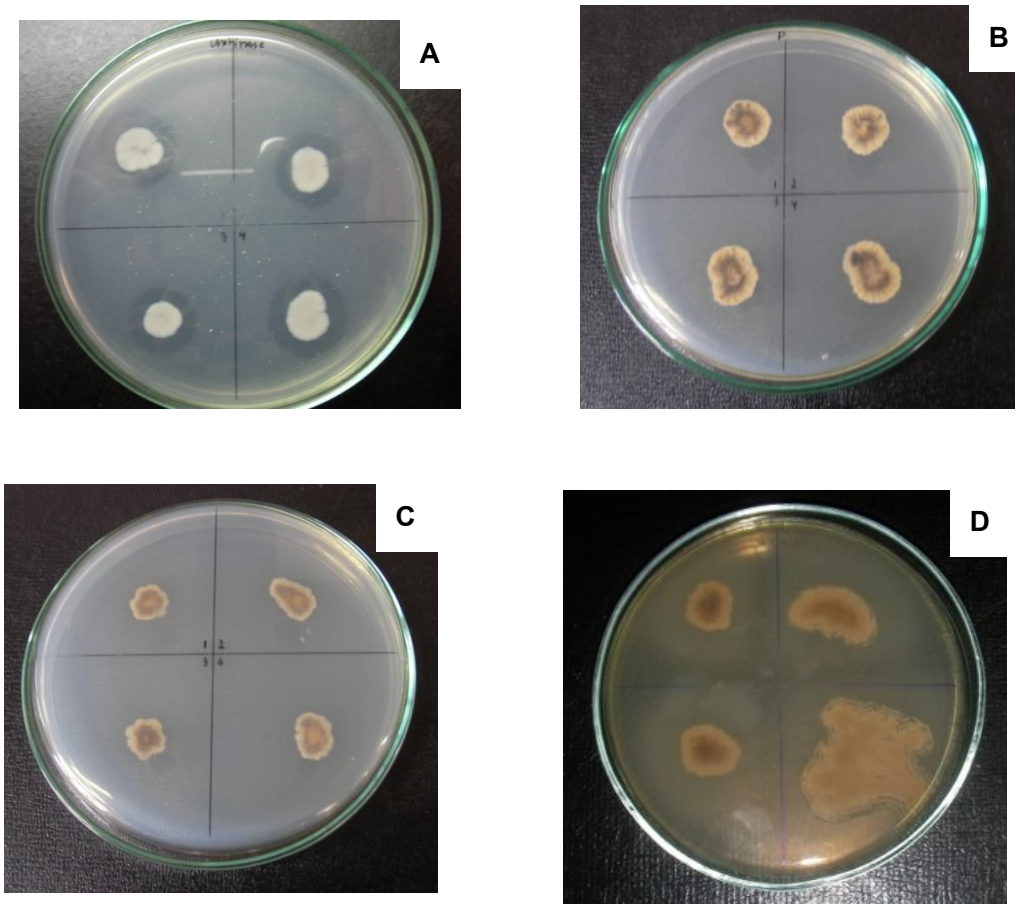

Figure 1. Plate screening tests for hydrolytic enzyme production of *Streptomyces* sp. P4. Agar plates contain the corresponding substrates for chitinase (A), protease (B), cellulase (C) and lipase (D). Each plate represents a duplicate experiment.

Antifungal Activity

The results in Figures 3-4 show that *Streptomyces* sp. P4 could effectively suppress the growth of *Fusarium oxysporum f.*sp. *lycopersici,* a fungus causing Fusarium wilt, a severe disease in tomato [29]. Maximal inhibition was observed on the 9th day with 12.50% inhibition (Figure 3). The radial growth diameter of *F. oxysporum* when grown on the same plate as *Streptomyces* sp. P4 (5.37±0.23 cm) was statistically smaller than that when cultured alone (6.13±0.38 cm). However, *Streptomyces* sp. P4 did not suppress the growth of the other two tested fungi, i.e. *Corynesopra cassiicola* (which causes leaf spot [30]) and *Rhizoctonia solani* (which causes root rot [31]) during the 14 days of observation (Figure 3). Anitha and Rabeeth [28] also reported the different responses of fungi to *S. griseus* chitinase. They suggested that the protein composition in the cell walls of different pathogenic fungi might make some fungal cell walls more resistant to chitinolytic degradation. Thus, only the co-culture containing *F. oxysporum* and *Streptomyces* sp. P4 was selected for SEM experiment.

Maejo Int. J. Sci. Technol. **2012**, *6*(01), 95-104

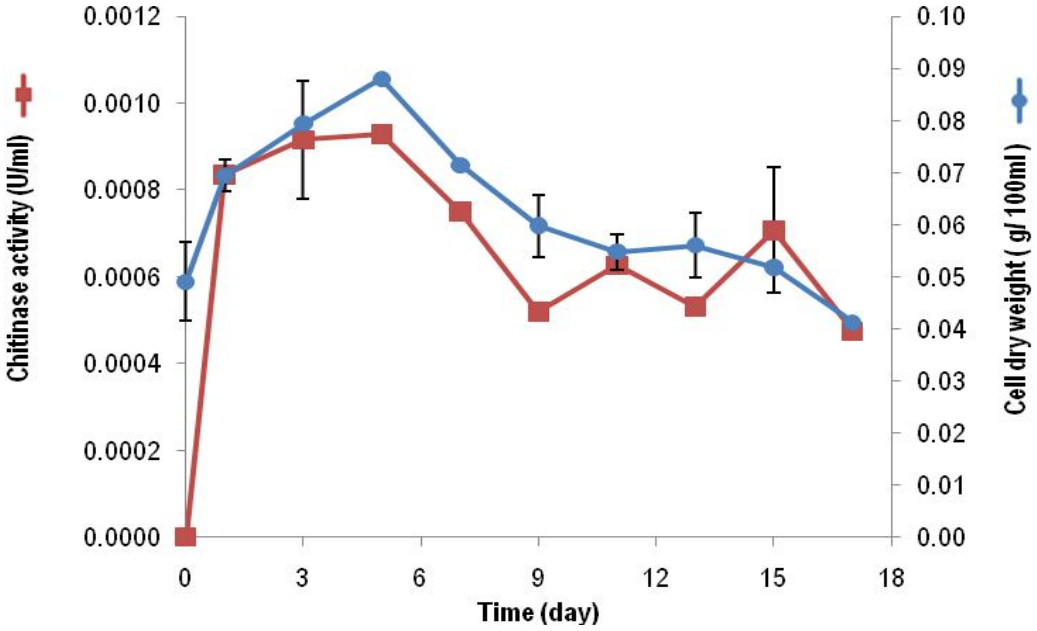

Figure 2. Chitinase activity and cell dry weight during the growth of *Streptomyces* sp. P4 . Error bars represent the standard deviation of 3 replicates.

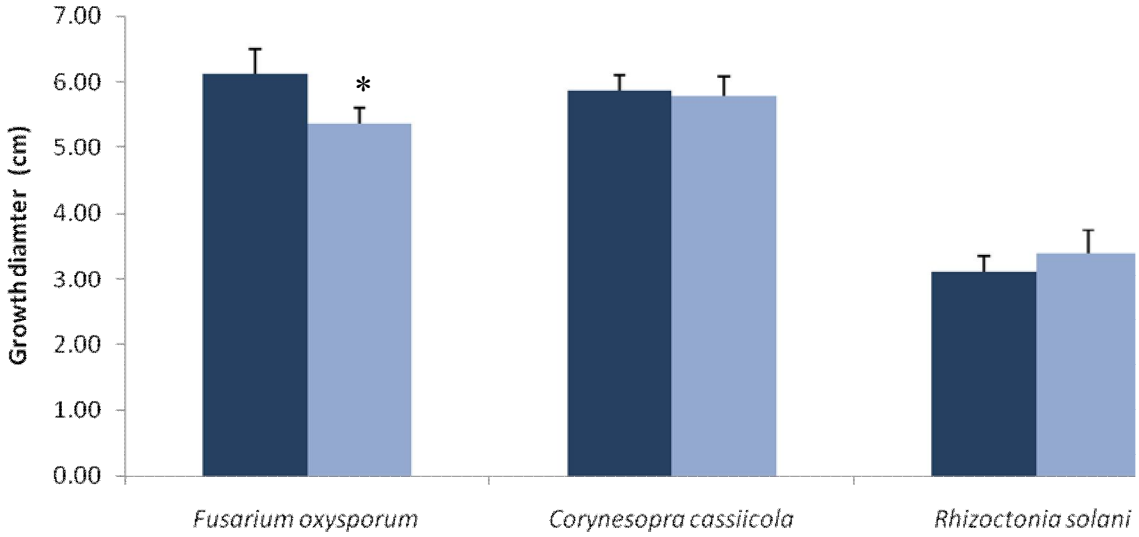

Figure 3. In vitro inhibitory activity of *Streptomyces* sp. P4 against three fungi: *Fusarium oxysporum f.*sp. *lycopersici*, *Corynesopra cassiicola* and *Rhizoctonia solani*. Dark blue and light blue bars correspond to radial growth diameters of the fungi cultured alone (control) and co-cultured with *Streptomyces* sp. P4 (dual culture) respectively on the 9th day of growth on PDA plates. Error bars represent standard deviation of 3 replicates. The asterisk indicates that the value differs significantly from control (p<0.05).

Maejo Int. J. Sci. Technol. **2012**, *6*(01), 95-104

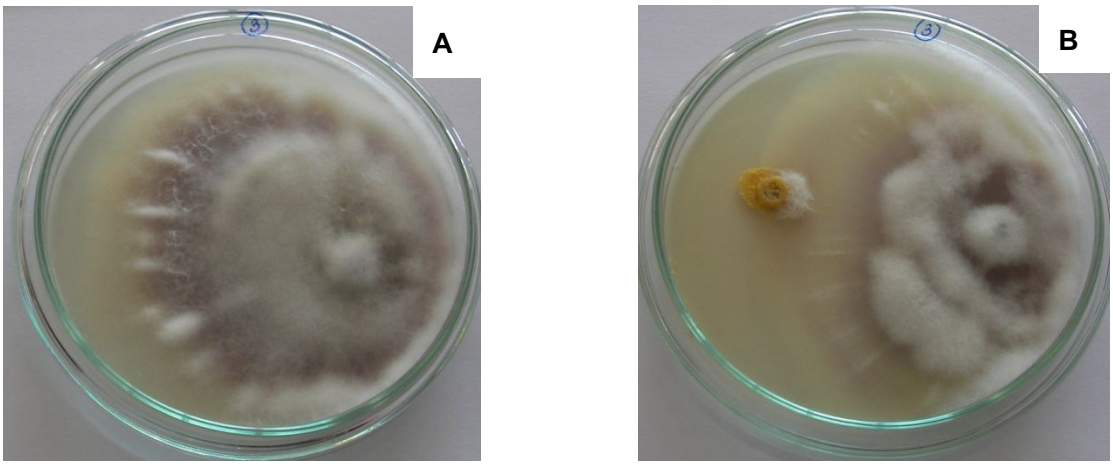

Figure 4. Growth of *Fusarium oxysporum f.*sp. *lycopersici* grown alone (A) and co-cultured with *Streptomyces* sp. P4 (B) on PDA for 14 days

Results obtained from SEM showed the breakage of the cell walls of *F. oxysporum* mycelia growing towards *Streptomyces* sp. P4 (Figure 5B) as compared to a control region (Figure 5A). The findings suggest that extracellular secondary metabolites and/or hydrolytic enzymes including chitinase play a crucial role in fungal growth inhibition. Prapagdee et al. [25] reported that the antifungal activity of *S. hygroscopicus* during exponential growth was mainly due to hydrolytic enzymes, while in the stationary phase it was due to secondary thermostable compound(s). In addition, there was a report on a positive correlation between chitinolytic and antagonistic activities of *Streptomyces* against the fungi *Collectotrichum sublineolum, Guignardia citricarpa, Rhizoctonia solani* and *Fusarium oxysporum*, but not in the oomycetes *Pythium* sp. and *Phytophthora parasitica*, which contain cellulose as a major cell wall component [16].

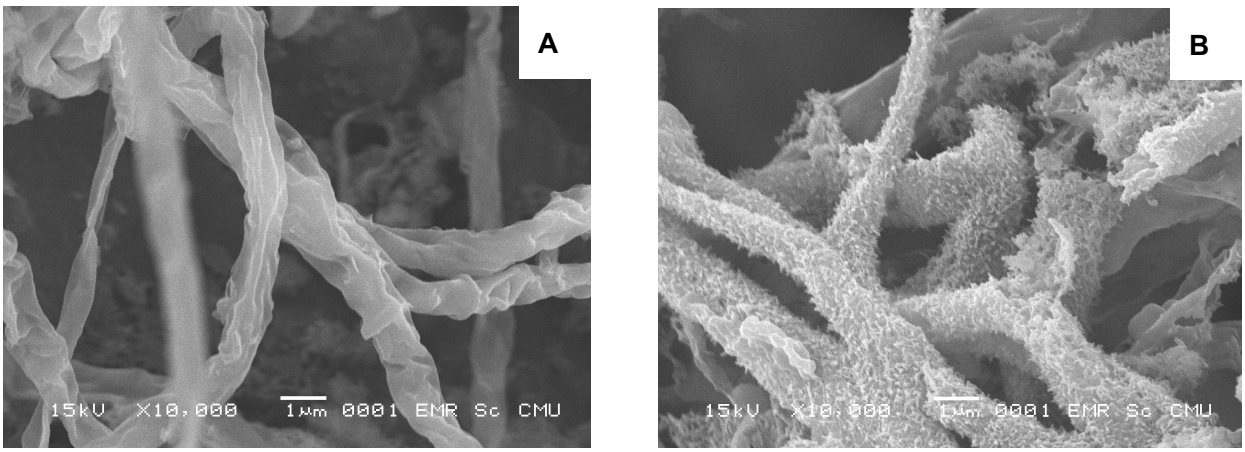

Figure 5. Scanning electron microscopic analysis of *Fusarium oxysporum f.*sp. *lycopersici* grown alone (A) and co-cultured with *Streptomyces* sp. P4 (B). Bars indicate 1 µm.

Maejo Int. J. Sci. Technol. **2012**, *6*(01), 95-104

CONCLUSIONS

The present study provides background information for the potential use of the endophytic *Streptomyces* sp. P4 strain as a biocontrol agent antagonistic to specific fungi. The chitinase production and its association with the growth of *Streptomyces* sp. P4 were demonstrated. The fungal growth inhibition of *F. oxysporum f.*sp. *lycopersici* by *Streptomyces* sp. P4 was observed and demonstrated to result from the disruption of the fungal cell walls.

ACKNOWLEDGEMENTS

This work was financially supported by the Centre of Excellence for Innovation in Chemistry (PERCH-CIC), Lanna Products Company and the Graduate School of Chiang Mai University. We thank S. Chantal E. Stieber (Department of Chemistry and Chemical Biology, Cornell University) for critical reading of the manuscript.

REFERENCES

1. H. Willer and L. Kilcher (Eds.), "The world of organic agriculture–statistics and emerging trends 2011", IFOAM and FiBL, **2011**, http://www.organic-world.net/ (Accessed: 1 August 2011).
2. M. Shoda, "Bacterial control of plant diseases", *J. Biosci. Bioeng.*, **2000**, *89*, 515-521.
3. D. R. Fravel, "Commercialization and implementation of biocontrol", *Annu. Rev. Phytopathol.*, **2005**, *43*, 337-359.
4. S. Sabaratnam and J. A. Traquair, "Formulation of a *Streptomyces* biocontrol agent for the suppression of Rhizoctonia damping-off in tomato transplants", *Biol. Control*, **2002**, *23*, 245-253.
5. G. Sykes and F. A. Skinner, "Actinomycetales: Characteristics and Practical Importance", Academic Press, London, **1973**, pp.64-75.
6. M. S. Abdelfattah, "Screening of terrestrial *Streptomyces* leading to identification of new stereoisomeric anthracyclines", *World J. Microbiol. Biotechnol.,* **2008**, *24*, 2619-2625.
7. P. Dyson, "*Streptomyces*", in "Encyclopedia of Microbiology" (Ed. M. Schaechter), 3rd Edn., Academic press, London, **2009**, pp.318-332.
8. P. Thapanapongworakul, "Characterization of endophytic actinomycetes capable of controlling sweet pea root rot diseases and effect on root nodule bacteria", MS Thesis, **2003**, Chiang Mai University, Thailand.
9. M. Shimizu, Y. Nakagawa, Y. Sato, T. Furumai, Y. Igarashi, H. Onaka, R. Yoshida and H. Kunoh, "Studies on endophytic Actinomycetes (I) *Streptomyces* sp. isolated from rhododendron and its antifungal activity", *J. Gen. Plant Pathol.*, **2000**, *66*, 360-366.
10. R. Gupta, R. K. Saxena, P. Chaturvedi and J. S. Virdi, "Chitinase production by *Streptomyces viridificans*: Its potential in fungal cell wall lysis", *J. Appl. Bacteriol.*, **1995**, *78*, 378-383.
11. C. P. Souza, E. M. Burbano-Rosero, B. C. Almeida, G. G. Martins, L. S. Albertini and I. N. G. Rivera, "Culture medium for isolating chitinolytic bacteria from seawater and plankton", *World J. Microbiol. Biotechnol.*, **2009**, *25*, 2079-2082.
12. D. A. Benson. I. Karsch-Mizrachi, D. J. Lipman, J. Ostell and E.W. Sayers, "GenBank", *Nucleic Acids Res.*, **2011**, *39* (Database issue), D32-37.

13. T. Taechowisan, J. F. Peberdy and S. Lumyong, "Chitinase production by endophytic *Streptomyces aureofaciens* CMU Ac 130 and its antagonism against phytopathogenic fungi", *Ann. Microbiol.*, **2003**, *53*, 447-461.
14. G. L. Maria, K. R. Sridhar and N. S. Raviraja, "Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India", *J. Agric. Technol.*, **2005**, *1*, 67-80.
15. F. Green, C. A. Clausen and T. L. Highley, "Adaptation of the Nelson-Somogyi reducing-sugar assay to a microassay using microtiter plates", *Anal. Biochem.,* **1989**, *182*, 197-199.
16. M. C. Quecine, W. L. Araujo, J. Marcon, C. S. Gai, J. L. Azevedo and A. A. Pizzirani-Kleiner, "Chitinolytic activity of endophytic *Streptomyces* and potential for biocontrol", *Lett. Appl. Microbiol.*, **2008**, *47*, 486-491.
17. B. Lanoot, M. Vancanneyt, B. Hoste, K. Vandemeulebroecke, M. C. Cnockaert, P. Dawyndt, Z. Liu, Y. Huang and J. Swings, "Grouping of *Streptomycetes* using 16S-ITS RFLP fingerprinting", *Res. Microbiol.*, **2005**, *156*, 755-762.
18. F. M. Atta and A. A. Zohri, "Transformation reactions of progesterone by different species of *Streptomyces*", *J. Basic Microbiol.,* **1995**, *35*, 3-7.
19. I. Höfer, M. Crüsemann, M. Radzom, B. Geers, D. Flachshaar, X. Cai, A. Zeeck and J. Piel, "Insights into the biosynthesis of hormaomycin, an exceptionally complex bacterial signaling metabolite", *Chem. Biol.*, **2011**, *18*, 381-391.
20. H. Imai, A. Nakagawa and S. Omura, "Biosynthesis of the antibiotic okilactomycin", *J. Antibiot.*, **1989**, *42*, 1321-1323.
21. K. Ochi, Y. Tsurumi, N. Shigematsu, M. Iwami, K. Umeharaand and M. Okuhara, "Physiological analysis of bicozamycin high-producing *Streptomyces griseoflavus* used at industrial level", *J. Antibiot.*, **1988**, *41*, 1106-1115.
22. R. Nazari, A. Akbarzadeh, D. Norouzian, B. Farahmand, J. Vaez, A. Sadegi, F. Hormozi, M. Kiani-Rad and B. Zarbaksh, "Applying intra-specific protoplast fusion in *Streptomyces griseoflavus* to increase the production of desferrioxamine B", *Curr. Sci.*, **2005**, *88*, 1815-1820.
23. A. Kuramoto, A. Lezhava, S. Taguchi, H. Momose and H. Kinashi, "The location and deletion of the genes which code for SSI-like protease inhibitors in *Streptomyces* species", *FEMS Microbiol. Lett.*, **1996**, *139*, 37-42.
24. B. Mahadevan and D. L. Crawford, "Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYEC108", *Enzyme Microb. Technol.*, **1997**, *20*, 489-493.
25. B. Prapagdee, C. Kuekulvong and S. Mongkolsuk, "Antifungal potential of extracellular metabolites produced by *Streptomyces hygroscopicus* against phytopathogenic fungi", *Int. J. Biol. Sci.*, **2008**, *4*, 330-337.
26. M. Charpentier and F. Percheron, "The chitin-degrading enzyme system of a *Streptomyces* species", *Int. J. Biochem.,* **1983**, *15*, 289-292.
27. N. Dahiya, R. Tewari and G. S. Hoondal, "Biotechnological aspects of chitinolytic enzyme: A review", *Appl. Microbiol. Biotechnol.*, **2006**, *71*, 773-782.
28. A. Anitha and M. Rabeeth, "Degradation of fungal cell walls of phytopathogenic fungi by lytic enzyme of *Streptomyces griseus*", *Afr. J. Plant Sci.*, **2010**, *4,* 61-66.
29. A. Anitha and M. Rabeeth, "Control of fusarium wilt of tomato by bioformulation of *Streptomyces griseus* in green house condition", *Afr. J. Basic Appl. Sci.,* **2009**, *1,* 9-10.

Maejo Int. J. Sci. Technol. **2012**, *6*(01), 95-104

30. S. Kurt, "Genetic variation in *Corynesopra cassiicola*, the target leaf spot pathogen", *Pakistan J. Biol. Sci.,* **2005**, *8,* 618-621.
31. K. L. Olmos, S. H. Delgado and N. M. Pérez, "AFLP fingerprinting for identification of anastomosis groups of *Rhizoctonia solani* Kühn from common bean (*Phaseolus vulgaris* L.) in Mexico", *Rev. Mexicana Fitopatol.*, **2005**, *23,* 147-151.