ISOLATION AND IDENTIFICATION OF A CHITINASE-PRODUCING AND BIOFILM-FORMING BACTERIUM *Aeromonas salmonicida* AND ITS POTENTIALS AS BIOCONTROL OF PHYTOPATHOGENIC FUNGI

Dinh Minh Tran[1*], Kazushi Suzuki[2]

[1] Institute of Biotechnology and Environment, Tay Nguyen University, Vietnam
[2] Graduate School of Science and Technology, Niigata University, Japan

SUMMARY

To develop a novel type of bacteria for biocontrol agents of plant pathogenic fungi, we are focusing on bacteria that possess high chitinase activity and more biofilm formation. In this study, a bacterial strain was isolated from chitin flakes placed into the water of a sand dune lake in Niigata, Japan and was identified as *Aeromonas salmonicida* based on morphology and phylogenetic analysis of the 16S rRNA gene sequencing. The train showed high chitinase activity and formed the highest biofilms compared to other isolates. Total activity of chitinases produced by the strain in the presence of chitin powder was lower than that of a reference, *Serratia marcescens* 2170. However, total protein concentration produced was lower than that of the reference, indicating the bacterium produces a considerable amount of chitinases in the presence of chitin. Zymogram analysis of chitinases demonstrated that the strains produced at least one chitinase into culture supernatant. In addition, the dialyzed culture supernatant containing chitinases produced by the bacterium showed antifungal activity against the growth of *Trichoderma reesei*. These results indicate that our isolate might a good candidate for further studies on biocontrol of plant pathogenic fungi. We plan to identify and characterize the chitinase molecules of the bacterium by applying gene cloning for the development of a biocontrol agent.

Keywords: Aeromonas salmonicida, chitinase activity, biofilm formation, antifungal activity, biocontrol.

INTRODUCTION

Chitin is an insoluble linear β-1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc) and is the second most abundant polysaccharide in nature next to cellulose. It is a common constituent of fungal cell walls, exoskeletons of insects... Chitinases (EC 3.2.1.14) are enzymes that degrade chitin by hydrolyzing β-1,4-glycosidic linkages. These enzymes occur in a variety of organisms and are classified into two different families of glycoside hydrolases, families 18 and 19 (Henrissat, 1991). Chitinase genes from various chitinolytic bacteria have been cloned, analyzed, and their biochemical properties have been examined in detail (Huang *et al*., 2012; Watanabe *et al*., 1997). A large number of studies have revealed that chitinases and chitinolytic bacteria play an important role in controlling mycelial extension of various plant-pathogenic fungi (Chernin *et al*., 1995; Huang *et al*., 2012). Therefore, bacterial chitinases play a critical role in digestion of chitin in fungal cell walls, and chitinolytic bacteria could be widely applied as environmentally friendly agents for biocontrol of phytopathogens. Most of chitinolytic bacteria and their chitinases that have been studied in term of phytopathogenic biocontrol have been isolated from soil and marine environments, while only a few of them have been isolated from freshwater environments (Huang *et al*., 2012). Therefore, studies on chitinolytic bacteria isolated from the freshwater environment and characterization of their chitinases are important for understanding their function against pathogenic fungi in agriculture. Microbial cells attach to biotic or abiotic surfaces and develop biofilms. A biofilm can be formed by a single bacterial species or can contain numerous species of bacteria, fungi, algae. Biofilms have been shown to protect microorganisms against environmental stresses (Singh *et al*., 2006). Recently, chitinolytic bacteria that form biofilms are thought to be more efficient at degrading chitin in fungal cell walls than bacteria with no or low ability to form biofilms. However, no studies on bacteria that form biofilms and produce chitinases and their applications in agricultural fungal control have been reported, so far. Against this background, in this study, we described screening of a chitinolytic bacterium that possesses high chitinase activity and forms the highest biofilms from a freshwater lake, Sakata and we analyzed chitinase and antifungal activities of the bacterium.

MATERIALS AND METHODS

Sample collection and isolation of chitinolytic bacteria

Sakata is a sand dune lake in Niigata, Japan (37°49′N, 138°52′E, 5 m above sea level, and 76 ha of the area). The lake water is mainly provided by groundwater running under the dunes. Sakata has only one small stream, along which the lake water flows into a river, which does not freeze in winter. Various aquatic species live in the lake, such as shrimp, crabs, and fishes. Because no rivers flow into the lake, the chitin sources accumulated in

Sakata are hardly altered by the action of flowing water; hence, Sakata is thought to be a promising place to isolate different types of chitinolytic bacteria.

To collect chitinolytic bacteria from the lake water, two nylon nets containing crab shell chitin flakes (Tokyo Chemical Industry, Tokyo, Japan) and two nylon nets containing shrimp shell chitin flakes (Sigma-Aldrich, USA), 10 g per bag, were placed in the lake water at several different positions. After one week, the chitin flakes were recovered and bacteria bound to them were isolated as previously described (Sato *et al*., 2009).

To isolate chitinolytic bacteria strongly adhering to the chitin flakes, 1.0 g of the flake was vigorously washed with 9.0 ml of sterile water, and bacterial cells from the water were inoculated and purified on the yeast extract-supplemented minimal (YEM) agar medium (w/v, 0.05% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.136% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, pH 8.5, 1.5% agar) containing 0.2% (w/v) colloidal chitin and incubated at 30°C for 2 days (Sato *et al*., 2009).

Chitinase activity of the isolates

Isolated chitinolytic bacteria were grown in YEM medium containing 0.2% colloidal chitin for 3 days (30°C, 150 rpm). Cells were separated by centrifugation (8,000*g*, 5 min, 4°C) and the supernatant was dialyzed against 20 mM sodium phosphate buffer (pH 6.0) overnight at 4°C. The dialyzed protein solution was used for measuring chitinase activity and protein concentration.

The chitinase activity assay was conducted in a reaction mixture (total volume, 600 µl) containing 0.1% colloidal chitin as the substrate and an appropriate volume of crude enzyme in 20 mM sodium phosphate buffer (pH 6.0) (Imoto and Yagishita, 1971). One unit of chitinase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar per min. Protein concentration in the culture supernatant was measured using a BCA Protein Assay Kit (Thermal Scientific, USA).

Biofilm formation of the isolates

Biofilm formation of the isolates was estimated using a 96-well microtiter plate as described previously (Jackson *et al*., 2002).

Phylogenetic analysis of the 16S rRNA gene

Genomic DNA from an overnight culture of the isolate was extracted by boiling for 5 min, followed by centrifugation (13,000 rpm, 2 min, 4°C) to remove debris and unbroken cells. The genomic DNA in the supernatant was used as a template for amplification by PCR using universal primers, 27f-YM (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492r (5'-TACCTTGTTACGACTT-3') and a KOD-Plus-Neo Kit (Toyobo Co., Ltd., Osaka, Japan) following the manufacturer's instructions. The amplified products were separated by electrophoresis on 1.0% agarose gel. The target band was cut out and purified using a Wizard SV Gel and Clean-Up Kit (Promega Co., USA). Sequencing reactions were conducted in a CEQ8000 Genetic Analysis System (Beckman Coulter Inc., USA) by using a CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc., USA) based on the supplier's instructions.

The nucleotide sequence of the 16S rRNA gene was compared with the known 16S rRNA gene sequences available in the DDBJ/Genbank/EMBL databases using BLAST to determine the taxonomic positions of the isolates. A phylogenetic tree was built using the MEGA 6.0 software. The tree was constructed using the neighbor-joining method and evolutionary distances were computed using the Kimura two-parameter method. A bootstrap analysis (1000 replications) was carried out to evaluate the topology of the resulting tree.

Chitinase production in culture supernatant by the isolate

In this study, *S. marcescens* 2170 was used as a reference strain to compare the production of chitinases of the isolate because *S. marcescens* is a well-known chitinase-producing bacterium (Vaaje-Kolstad *et al*., 2013). Each bacterium was aerobically grown in the YEM medium containing 0.5% chitin powder at 30°C and 150 rpm. At each time point, a portion of the culture was sampled. After centrifugation (8,000*g*, 5 min, 4°C) to remove the cells and debris, the supernatant was dialyzed against 20 mM sodium phosphate buffer (pH 6.0) at 4°C for overnight. The chitinase activity and protein concentration of the dialyzed solution were measured and the protein solution was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography.

SDS-PAGE was carried out as previously described (Laemmli, 1970) using 12.5% polyacrylamide gels. The renaturation of the enzymes in polyacrylamide gels after SDS-PAGE and the detection of chitinase activity were performed as previously described (Watanabe *et al*., 1990).

Antifungal activity assay

Crude proteins containing chitinases prepared from the culture supernatant of the isolate were also analyzed for their inhibition of the extension of *Trichoderma reesei* IFO mycelia following a previously described method

(Watanabe *et al*., 1999). The tests were conducted in triplicates. The radial growth of fungal mycelium was measured and the percentage of growth inhibition calculated as follows:

Rate of mycelium growth inhibition (%) = [(D1 – D2)]/D1] × 100, where D1 = diameter of the fungus mycelium grown on the control (cm) and D2 = diameter of the fungus mycelium grown on the test (cm).

RESULTS

Isolation of chitinolytic bacteria

More than 2,500 isolates formed clearing zones caused by colloidal chitin degradation by chitinases on the agar plates were carefully observed. Based on the size of the clearing zones and the morphological characteristics of their colonies, 13 isolates were selected for further examination. These isolates were circular or irregular, smooth or slotted, translucent, umbonal, and entire when grown on the plates containing colloidal chitin (data not shown).

Chitinase activity and biofilm formation

The chitinase activity in the culture supernatant when the isolates were grown in the presence of 0.2% colloidal chitin was examined, and biofilms formed in the polystyrene microtiter plate containing YEM medium were quantified. As shown in Figure 1, all strains exhibited high chitinase activity and formed biofilms in the polystyrene microtiter plate supplied LB medium. So far, to select bacterial candidates for biocontrol studies, almost works have been focused on high chitinase activity and no studies on bacteria that form more biofilms and produce chitinases have been reported. Therefore, in this study, a bacterial strain, SWSY-1.411 which formed the highest biofilms and showed high chitinase activity was selected for taxonomy determination based on the 16S rRNA gene sequencing.

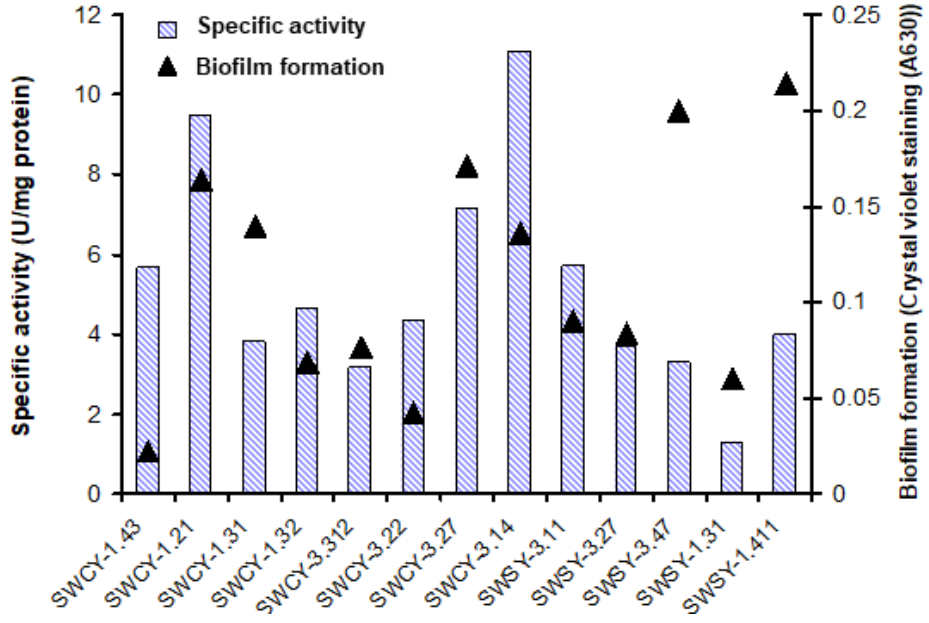

Figure 1. Chitinase activity and biofilm formation of the isolates. Chitinase activity of the isolates produced in the culture supernatants of the YEM medium in the presence of 0.2% colloidal chitin was measured by a modified version of the Schales' procedure. Biofilm formation of the isolates formed in the 96-well polystyrene microtiter plates supplied the LB medium was estimated by absorbance at 630 nm

Phylogenetic analysis of the isolates

The nucleotide sequence (1380 bp) of the 16S rRNA gene of strain SWSY-1.411 shows 100% identity to that of *Aeromonas salmonicida* YZ-4 (JX164205) and *A. salmonicida* A449 (CP000644). A phylogenetic analysis of the 16S rRNA gene (Figure 2) indicates that the strain SWSY-1.411 could be identified as *Aeromonas salmonicida* belonging to family *Aeromonadaceae*, class *Gammaproteobacteria*.

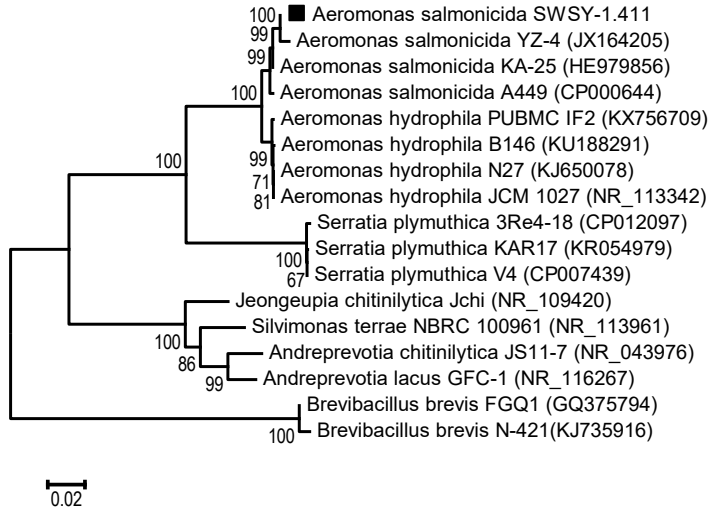

Figure 2. Phylogenetic analysis of the isolate (filled rectangle) based on the 16S rRNA gene sequences. Accession numbers for the available 16S rRNA gene sequences used are given in parentheses behind species and strain names. The phylogenetic tree was calculated and drawn by using the MEGA 6.0. The numbers at the branches are bootstrap confidence percentages (%) based on 1000 resampled data sets. Bar, 0.02 substitutions per nucleotide position

Chitinase production in the culture supernatants and chitinase detection by SDS-PAGE and zymography

As shown in Figure 3, strain SWSY-1.411 exhibited lower chitinase activity than that of *S. marcescens* 2170 used as a reference strain when grown in the YEM medium containing chitin powder (Figure 3A). However, the strain produced a low level of total protein in the culture medium compared with the reference (Figure 3B). Hence, the strain showed higher specific activity of chitinases than the reference. For example, on day 8 of cultivation, the specific activity of the chitinase produced by SWSY-1.411 was 2.1 (U/mg protein), while that from the reference was 0.8 (U/mg protein). This result indicated that the isolate produces a considerable amount of chitinases in the presence of chitin.

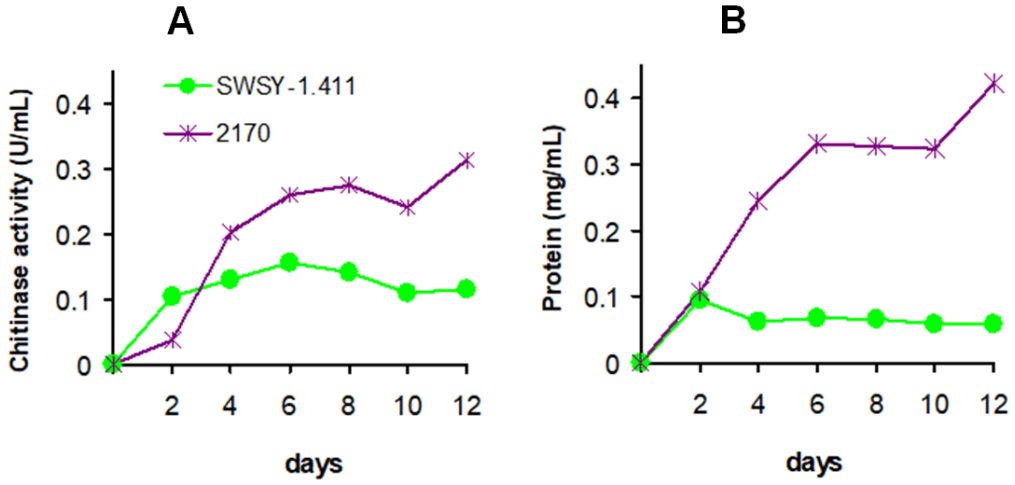

Figure 3. Time course of chitinase production in the liquid culture. The isolate and reference were cultured in the YEM medium containing 0.5% chitin powder at 30°C. At certain intervals, a portion of the culture medium was withdrawn, and chitinase activity in the culture supernatants was measured by a modified version of the Schales' procedure with GlcNAc as a standard. Protein concentration was measured using the BCA Protein Assay Kit. SWSY-1.411, *A. salmonicida* SWSY-1.411; 2170, *S. marcescens* 2170. *S. marcescens* 2170 was used as a reference

The production of chitinases was also detected in the agarose gel sheet containing glycol chitin as the substrate after SDS-PAGE (Figure 4A). The zymography analysis showed that SWSY-1.411 produced one active chitinase bands (Figure 4B, lane 2).

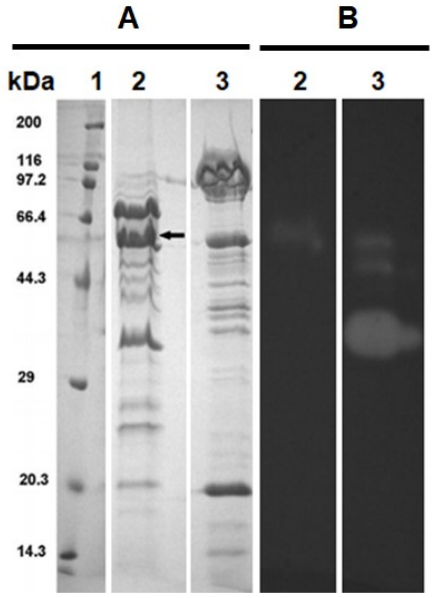

Figure 4. SDS-PAGE (A) and zymography (B) analyses of chitinase production in the culture supernatant. Strain SWSY-1.411 and the reference were grown in YEM medium containing 0.5% chitin powder at 30°C. The chitinase production was taken from the cultured supernatant at day 6 of cultivation and employed for SDS-PAGE and zymography analyses. (A) Protein staining with 0.2% coomassie brilliant blue R-250. (B) Chitinase activity detected on an agar replica of the SDS-polyacrylamide gel. Lane 1, size markers in kDa; lane 2, proteins secreted by *A. salmonicida* SWSY-1.411; lane 3, proteins secreted by *S. marcescens* 2170. kDa, kilodaltons; arrows, protein active bands

Antifungal activity

The crude proteins containing chitinases prepared from the culture supernatant of the bacterium were employed for antifungal activity by inhibiting the mycelial extension of *T. reesei*. As shown in Figure 5, the crude proteins containing chitinases prepared from the bacterium inhibited the growth of *T. reesei* with mycelium growth inhibition was 21.31%. On the other hand, the boiled crude proteins prepared from the bacterium did not inhibit the growth of fungus (Figure 5, well 2). These results suggest that the crude proteins containing chitinases prepared from *A. salmonicida* SWSY-1.411 can inhibit the growth of fungi.

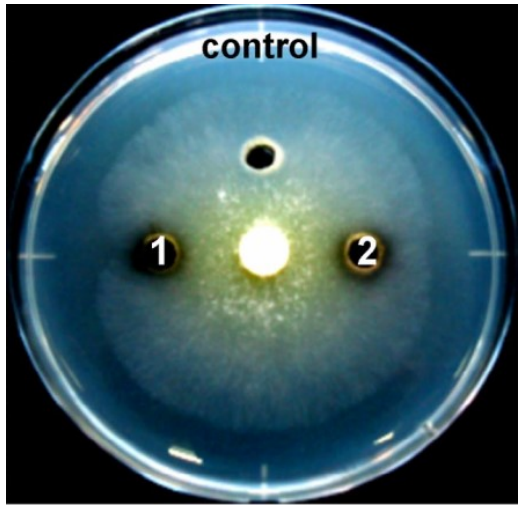

Figure 5. The inhibition of hyphal growth of *T. reesei* treated by crude proteins containing chitinases. A suspension of conidia of *T. reesei* was inoculated onto a paper disk. After 24 h of cultivation at 25°C, a solution containing 0.4 mg of proteins was applied to a well punched at a distance of 15 mm from the plate center. The plates were then incubated at 25°C for a further 21 h and the inhibition of hyphal growth was evaluated by visual inspection. Control, sterile water. Well 1, a protein solution containing crude chitinases; Well 2, a protein solution containing inactivated crude chitinases by boiling for 10 min

DISCUSSION

This study aimed to select chitinolytic bacteria that possess high chitinase activity and form more biofilms in order to develop a novel type of biological control agents. We assumed that a large number of chitinase-producing bacteria and biofilm-forming bacteria live in the lake. Therefore, chitin flakes were used and placed in the water to trap the biofilm-forming bacteria. Previously, Sato *et al*. also used this method to collect and isolate strong chitinolytic bacteria from the freshwater environment (Sato *et al*., 2010).

In this study, a chitinolytic bacterium which showed high chitinase activity and formed the highest biofilms was isolated from the chitin flakes placed into the lake water of Sakata, in Niigata, Japan and identified as *A. salmonicida* SWSY-1.411 based on the morphology and phylogenetic analysis of its 16S rRNA gene sequence. *A. salmonicida* SWSY-1.411 secreted one chitinases into the medium in the presence of chitin powder. Chitin-binding proteins that were previously classified into carbohydrate-binding modules (CBMs) in family 33 have been reclassified into the auxiliary activities family 10 (AA10) of lytic polysaccharide monooxygenases. The bacterial AA10 proteins are essential for the hydrolysis of insoluble chitin by an oxidative reaction mechanism (Vaaje-Kolstad *et al*., 2010). In this study, a partial 16S rRNA gene nucleotide (1380 bp) of *A. salmonicida* SWSY-1.411 showed 100% identity to those of *A. salmonicida* available in the CAZy database. In the CAZy database (http://www.cazy.org/b534.html), these strains possess at least one family 18 chitinase, two family 19 chitinases, and one AA10 protein. However, biofilm formation, chitinases, and AA10 protein of the *A. salmonicida* have not been characterized in detail, yet. Hence, further studies are necessary to classify the chitinases and AA10 proteins and to analyze the chitinases and antifungal activities of *A. salmonicida* SWSY-1.411.

CONCLUSION

Aeromonas salmonicida SWSY-1.411 has great potentials for further studies as development of a biocontrol agent and studies on chitinase system. The strain showed the high chitinase activity, formed the highest biofilms, and its crude proteins containing chitinases possessed antifungal activity against *T. reesei*. In addition, the chitinase system of the strain has been unreported yet. We are cloning and purifying chitinases and AA10 proteins of the strain and characterizing purified enzymes concerning chitinase and antifungal activities.

REFERENCES

Henrissat B (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* 280: 309-316.

Watanabe T, Kimura K, Sumiya T, Nikaidou N, Suzuki K, Suzuki M, Taiyoji M, Ferrer S, Regue M (1997). Genetic analysis of the chitinase system of *Serratia marcescens* 2170. *J Bacteriol* 179: 7111-7117.

Chernin L, Ismailov Z, Haran S, Chet I (1995). Chitinolytic *Enterobacter agglomerans* Antagonistic to Fungal Plant Pathogens. *Appl Environ Microbiol* 61: 1720-1726.

Sato K, Kato Y, Taguchi G, Nogawa M, Yokota A, Shimosaka M (2009). *Chitiniphilus shinanonensis* gen. nov., sp. nov., a novel chitin-degrading bacterium belonging to *Betaproteobacteria*. *J Gen Appl Microbiol* 55: 147-153.

Huang L, Garbulewska E, Sato K, Kato Y, Nogawa M, Taguchi G, Shimosaka M (2012). Isolation of genes coding for chitin-degrading enzymes in the novel chitinolytic bacterium, *Chitiniphilus shinanonensis*, and characterization of a gene coding for a family 19 chitinase. *J Biosci Bioeng* 113: 293-299.

Singh R, Paul D, Jain RK (2006). Biofilms: implications in bioremediation. *Trends Microbiol* 14: 389-397.

Imoto T, Yagishita K (1971). A simple activity measurement of lysozyme. *Agric Biol Chem* 35: 1154-1156.

Jackson DW, Suzuki K, Oakford L, Simecka JW, Hart ME, Romeo T (2002). Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. *J Bacteriol* 184: 290–301.

Vaaje-Kolstad G, Horn SJ, Sørlie M, Eijsink VG (2013). The chitinolytic machinery of *Serratia marcescens* - a model system for enzymatic degradation of recalcitrant polysaccharides. *FEBS J* 280: 3028–3049.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Watanabe T, Kanai R, Kawase T, Tanabe T, Mitsutomi M, Sakuda S, Miyashita K (1999). Family 19 chitinases of *Streptomyces* species: characterization and distribution. *Microbiology* 145: 3353-3363.

Vaaje-Kolstad G, Westereng B, Horn SJ, Liu Z, Zhai H, Sørlie M, Eijsink VG (2010). An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* 330: 219-222.

PHÂN LẬP VÀ NHẬN DIỆN VI KHUẨN SỞ HỮU CHITINASE VÀ TẠO MÀNG SINH HỌC *Aeromonas salmonicida* VÀ TIỀM NĂNG ỨNG DỤNG CHO KIỂM SOÁT NẤM BỆNH HẠI CÂY TRỒNG

Trần Minh Định[1*], Kazushi Suzuki[2]

[1] Viện Công nghệ Sinh học và Môi trường, Trường Đại học Tây Nguyên
[2] Khoa Sau Đại học về Khoa học và Công nghệ, Đại học Niigata, Nhật Bản

TÓM TẮT

Để phát triển một tác nhân kiểm soát sinh học mới ứng dụng trong nông nghiệp, chúng tôi đang tập trung nghiên cứu vi khuẩn có hoạt tính chitinase cao và tạo màng sinh học. Trong nghiên cứu này, một chủng vi khuẩn tiềm năng đã được phân lập từ giá thể chitin được ngâm ở hồ nước ngọt tại tỉnh Niigata, Nhật Bản và được nhận diện là *Aeromonas salmonicida* dựa vào phân tích phát sinh loài thông qua giải trình tự gen 16S rRNA. Kết quả xác định hoạt tính chitinase và tạo màng sinh học cho thấy, vi khuẩn của chúng tôi có hoạt tính chitinase khá cao và tạo màng sinh học nhiều nhất trong số các chủng được phân lập. Kết quả phân tích hoạt tính chitinase bằng kỹ thuật zymogram bước đầu cho thấy chủng vi khuẩn này sở hữu ít nhất một enzyme chitinase. Ngoài ra, các chitinase thô được sản xuất từ loài này có hoạt tính ức chế sinh trưởng của nấm *Trichoderma reesei*. Từ những kết quả trên, đây là chủng vi khuẩn có tiềm năng cho các nghiên cứu tiếp theo về kiểm soát sinh học nấm bệnh hại cây trồng. Các công việc kế tiếp sẽ tập trung vào nhận diện và nghiên cứu vai trò của các enzyme chitinase từ loài này bằng kỹ thuật tạo dòng và biểu hiện gen nhằm phát triển vi khuẩn này thành tác nhân kiểm soát sinh học.

Từ khóa: Aeromonas salmonicida, hoạt tính chitinase, màng sinh học, hoạt tính kháng nấm, kiểm soát sinh học.

[*] Author for correspondence: Tel: +84-943095079; Email: minhdinh_chhcm@yahoo.com