Full Length Research Paper

Screening for actinomyces isolated from soil with the ability to inhibit *Xanthomonas oryzae* pv. *oryzae* causing rice bacterial blight disease in Vietnam

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Bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the major diseases in rice culture of Northen Vietnam, as well as other rice-growing regions of the world. In this study, we isolated and screened for actinomycete strains from Vietnam with the ability to inhibit *Xoo* isolates from northern Vietnam. From 90 actinomycete strains taken from soil in northern Vietnam in 2010, we screened for their antagonistic activity against 10 races of *Xoo* causing rice bacterial blight disease. Three actinomycete strains were found to inhibit all 10 *Xoo* races. Among the three strains, a strain namely VN10-A-44 was shown not to have the ability to produce toxic compounds and was selected for further study. The strain was identified as *Streptomyces virginiae* by 16S ribosomal RNA gene sequencing. We replaced soybean meal with tofu waste in antibiotic producing medium to improve antagonistic activity of VN10-A-44 against the *Xoo* pathogen and to make use of tofu waste for large-scale fermentation of VN10-A-44. We found that replacing soybean meal with 20 and 30 g of tofu waste/litter in the antibiotic producing medium gave the largest inhibition zone against the *Xoo* pathogen.

Key words: *Xanthomonas oryzae* pv. *oryzae*, rice bacterial blight disease, *Streptomyces virginiae,* Vietnam.

INTRODUCTION

Rice is one of the most important food crops of the world. However, its yield is constantly affected by several major diseases such as bacterial blight (BB), blast and tungro (Dai et al., 2007). Among these diseases, BB caused by *Xanthomonas oryzae* pv. o*ryzae* (*Xoo*) is one of the major diseases in Vietnam as well as other rice-growing regions of the world. Bacterial blight disease in rice has reduced Asia's annual rice production by as much as 60% (Dai et al., 2007). There are many methods and strategies which aim at reducing losses in rice yield and avoiding outbreaks of disease (such as rice breeding

programs and developing BB-resistant rice cultivars, and using chemicals to treat the bacterial pathogen). Chemical control of rice bacterial blight was evidently an ineffective method (Gnanamanickam, 2009; Hastuti et al., 2012). In addition, breeding for BB-resistance, which uses a single major gene, has proved unsuccessful. This is due to rapid evolution of sub-populations overcoming the resistance genes of rice (Gnanamanickam, 2009).

Research by Gnanamanickam (2009) in India and the Philippines have shown that *Pseudomonas fluorescens* (Velusamy et al., 2006) and some *Bacillus (Bacillus* spp., *Bacillus lentus, Bacillus cereus* và *Bacillus circulans*) (Velusamy, 2002; Velusamy and Gnanamanickam, 2003) strains isolated from rice rhizo-shere samples, inhibited the growth of *Xoo* in the laboratory. From *Bacillus* strains, Gnanamanickam (2009) and Vasudevan (2002) showed

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Table 1. List of races of *Xoo* used for antagonistic testing.

S/N	*Xoo* race	Isolated from rice cultivar	Collected from province
1	R1	Khang dan, Bac thom 7	Bac Ninh, Ha Noi
2	R2	Nep tan, HT1	Son La, Hai Duong
3	R3	Nhi uu 838, Huong com	Nghe An, Thai Binh
4	R4	Thuc Hung	Thai Binh
5	R5	Khang dan, Bac thom	Nghe An, Hai Duong
6	R6	Nhi uu 383	Yen Bai
7	R7	Q5, Nang huong	Hai Duong, Thai Binh
8	R8	Nep thom	Hai Duong
9	R9	Q5	Hai Duong (appeared in Japan)
10	R10	Hybrid rice	Yen Bai (appeared in Japan)

that they can reduce BB in rice from 21 to 59% (as compared to untreated plots). In 2008, Ji and his colleagues (2008) reported that *Lysobacter antibioticus* isolated from the rhizoshere of rice in the Yunnan province of China can significantly inhibit the growth of various phytopathogenic fungi and bacteria including *Xoo*. This strain is able to inhibit *Xoo* growth, with disease suppression efficiency up to 69.7%. Disease suppression efficiency by *L. antibioticus* 13-1 on *Xoo* varied significantly among various ice cultivars. Additionally, this efficiency of bio-control is affected by different pathogens of *Xoo* isolates. These results suggest that antibiotics and density of *L. antibioticus* 13-1 colonization on rice leaves may be involved in biocontrol of rice BB (Ji et al., 2008).

Recently, research has shown that some species of the genus *Streptomyces* fight against some plant pathogenic microorganisms, including *Xoo* (Cazorla et al., 2007; Chithrashree et al., 2011; Ndonde and Semu, 2000; Park et al., 2011; Rizk et al., 2007). For examples, bottromycin A2 and dunaimycin D3S from *Streptomyces bottropensis* suppressed rice bacterial blight (Park et al., 2011). Applying selected actinomycete strains and their bio-active compounds for biological control (using a balance of microorganisms and their natural components to suppress pathogens and to favour plants) of bacterial blight in rice, therefore, appears to be cost-effective and ecologically conducive strategy (Gnanamanickam, 2009).

In this study, we aimed to isolate and screen for action-mycete strains that can inhibit races of *Xoo* from northern Vietnam and those less harmful to other microorganisms. Additionally, we tested the possibility of replacing soybean meal in antibiotic-producing medium with tofu waste to improve inhibition ability of the actinomycete strain and to make use of the waste byproduct excreted in tofu production.

MATERIALS AND METHODS

Isolation of actinomycete strains

In 2010, 12 soil samples were collected from Lang Biang mountain, Da Lat City and Cat Tien National Park, near Ho Chi Minh City, and a plant root from the Vietnam National University, Hanoi City, Vietnam. We isolated actinomycete strains by using the sodium dodecyl sulfate-yeast extract dilution method and the rehydration-centrifugation method (Hayakawa et al., 1989; Hayakawa et al., 2000; Hop et al., 2011).

There were a total of 90-actinomycete strains isolated. These strains were preserved at the Vietnam Type Culture Collection in Vietnam as well as NITE in Japan. They were used to screen for actinomycete strains that can inhibit races of *Xoo* causing rice bacterial blight disease in Vietnam.

Xoo races

A total of 10 races of *Xoo*, namely R1, R2, R3, R4, R5, R6, R7, R8, R9 and R10, that were provided by Prof. Phan Huu Ton from Hanoi University of Agriculture, were used as the test microogarnisms. The 10 races were isolated from rice leaves in northern Vietnam. Distribution of the races are listed in Table 1 and Figure 1. It was reported that pathovars R2 and R3 were the most widely distributed in many provinces in northern Vietnam. R5 has caused the greatest loss in rice production; and R9 and R10 were isolated in Vietnam and appeared in Japan (Unpublished data).

Media

Five media were used in this study. For culture and preservation of the 10 races of *Xoo*, Wakimoto medium was used with the following contents: potato, 300 g; $NaHPO_4.12H_2O$, 2 g; $Ca(NO_3)_2.4H_2O$, 0.5 g; peptone, 5 g; saccarose, 15 g; agar, 15 g; distilled water, 1 L and pH, 7.0 while yeast extract–soluble starch medium (YS medium) (soluble starch, 10 g; yeast extract, 2 g; agar, 15 g; distilled water, 1 L and pH 7.3) and antibiotic producing medium (APM) (starch, 10 g; glucose, 10 g; soybean meal, 10 g; $CaCO_3$, 3 g; peptone, 10 g; agar, 20 g; Tween 80, one drop; distilled water, 1 L; pH, 7.0) were used for the actinomycete strains. Yeast-extract malt medium (YM medium) (malt extract, 3 g; yeast extract, 3 g; glucose, 10 g; peptone, 5 g; agar, 17 g; distilled water, 1 L; pH, 7.0) was used for culture of *Candida albicans* and Muller Hinton agar (MHA) was used for culture of *Micrococcus luteus* and *Escherichia coli* in antagonism and toxic-testing assays. All the chemicals and media were purchased from the companies of Sigma and BD.

Assay for antagonism to Xoo races

Antimicrobial activity of actinomycete strains was carried out by the agar-piece method (φ 6 mm) on Wakimoto medium. A total of 90
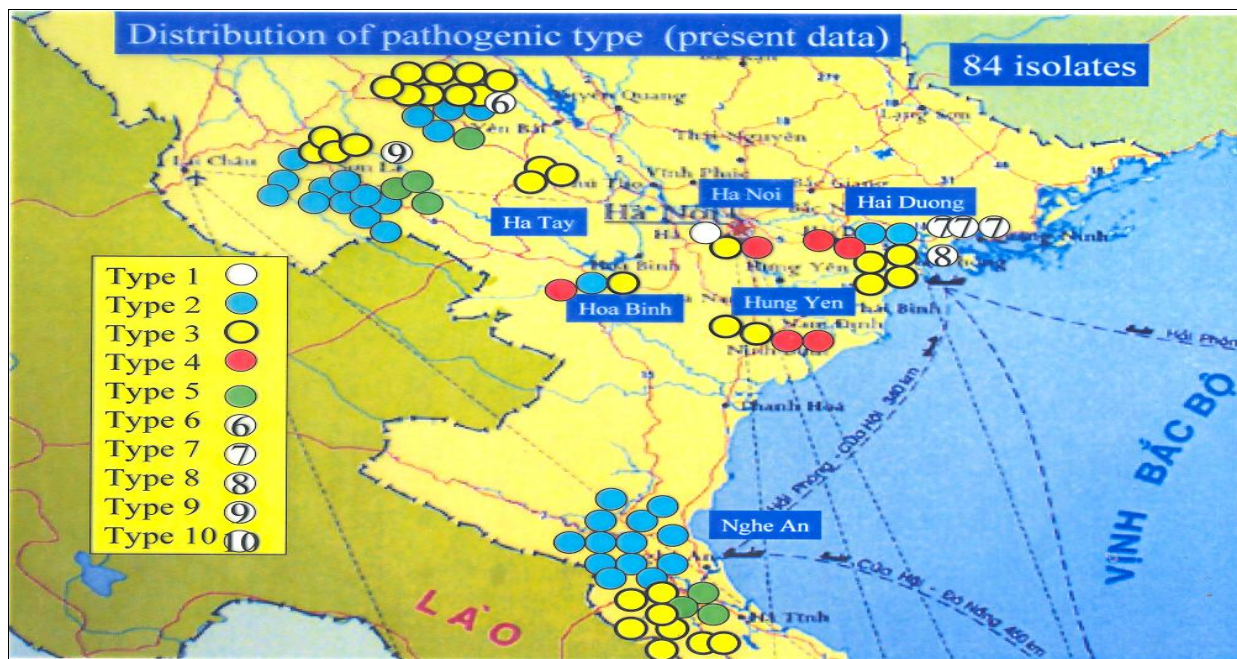
Figure 1. Distribution of the 10 *Xoo* races in north Vietnam. Pathogen types: 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 are races of *Xoo*: R1, R2, R3, R4, R5, R6, R7, R8, R9 and R10, respectively.

actinomycete strains were screened for their antogonism to races of *Xoo*. Pure cultures of actinomycete isolates were inoculated on YS medium at room temperature (25 to 30°C) for 7 to 14 days. After adequate growth of the isolates, the pieces of agar (φ 6 mm) that contained actively-growing actinomycete isolates were cut and placed onto the test plates culturing races of *Xoo*. The plates were incubated at 28 ± 1°C for 2 to 4 days and the inhibitory effect of the actinomycete isolates on *Xoo* growth was evaluated by measuring inhibition zones (cm).

To prepare the test plates, races of *Xoo* were cultured in Wakimoto broth to a concentration of ca 10^8 CFU/ml. The 150 ml of Wakimoto agar was autoclaved and cooled down to 45 to 50°C and then mixed with 10 ml of the *Xoo* cultures. This mixture was poured into Petri disks and used as the test plates.

Since race R2 and R3 were the most abundant races causing rice bacterial blight disease in northern Vietnam, their inhibition by actinomycete isolates was first tested and measured (Unpublished data). Subsequently, the actinomycete isolates inhibiting both the 2 races of R2 and R3 were tested continuously for their inhibition to the remaining 8 races of pathogens. Isolates that were able to inhibit all the 10 races of *Xoo* were selected for futher study.

Primary test for toxicity producer

To understand whether the actinomycete isolates inhibiting all the 10 races of pathogen produced toxic coumponds or not, we checked their inhibition of four microogarnisms: *M. luteus* (NBRC 13867), *E. coli* (NBRC 14237)*, Bacillus subtilis* (NBRC 3134) and *Saccharomyces cerevisiae* (NBRC 10217) (these micro-organisms were kindly provided by NITE, Japan) by using the agar-piece method, as mentioned above*.* Once the isolate inhibited all the four test microorganisms, it indicated the possibility of producing toxic compounds (Blackburn and Avery, 2003; Fredrickson and Elliott, 1985). Following the toxicity production test, we found that a specific strain, namely VN10-A-44, inhibited *B. subtilis* only and it

was therefore selected for further study.

Replacing soybean meal with tofu waste in antibiotic producing medium

Tofu waste was replaced with soybean meal in the original antibiotic producing medium (APM) at the same rate (1:1). VN10-A-44 was cultured on MAPM broth for 7 days at 30°C with shaking for 150 cycles/minute. The cultured broth (100 μl per well) was placed on the test medium seeded with R2 with the method mentioned previously in the materials and methods. After incubation for 24 to 72 h, the results were quantified by the diameter of the inhibition zone (cm).

Preliminary extraction tests of antibiotic produced by VN 10-A44

VN10-A44 was cultured in 50 ml of APM broth at 28 to 30°C using a shaker with 120 rpm rotation for 7 days. The culture was centrifuged at 4°C, 8000 rpm for 15 min to separate the cells and the supernatant. First, we examined whether there were antibiotic remains in the cells or were excreted to culture broth. 100 μl of the supernatant and only cells were placed in wells of the test plates seeded with the R2 and R3 pathogen. We found that the active compound remaining in cells and the supernatant failed to inhibit any of the race pathogens. Therefore, cells of VN10-A-44 were extracted by 50% acetone. Thereafter, preliminary tests such as dilution assay, stability test and a solvent extraction test were performed to examine the following from the cell-extracted solution:

Dilution assay of the cell extract

The cell extract was diluted 2 and 4 times and 100 μl of original cell extract and the diluted treatments were placed on test plates seeded

Table 2. Abilities of actinomycete strains to inhibit races of *Xoo*.

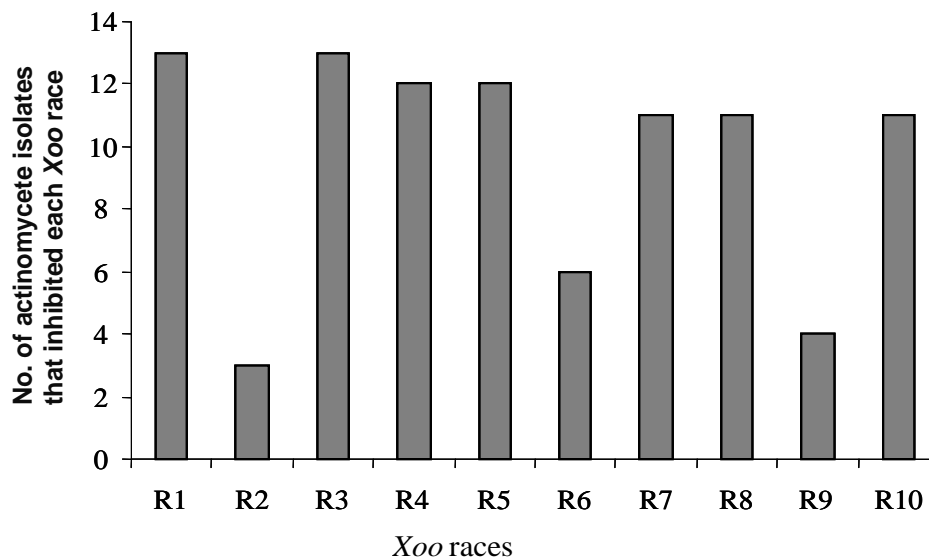| Number of *Xoo* races	0	1	2	3	4	5	6	7	8	9	10
Number of actinomycete isolates	77 (85.6%)	0 (0%)	1 (1.1%)	0 (0%)	1 (1.1%)	0 (0%)	0 (0%)	5 (5.6%)	2 (2.2%)	1 (1.1%)	3 (3.3%)

Figure 2. Inhibition of *Xoo* races by actinomycete isolates.

by R2 race in triplicate. After incubation for 24 to 72 h, results were read by the diameter of the inhibition zone.

pH and heat stability test

A volume of 1 ml cell extract/1.5 ml-Eppendorf tube was boiled at 100°C for 10 min. On the other hand, the cell extract solution was adjusted to pH 2, 7 and 10. 100 µl of the treated solutions were used for antagonistic activity assays in the R2 race.

Solvent extraction test

The cell extract by acetone was extracted again by ethyl-acetate and *n*-butanol at pH 2 and 10. 100 µl of the treated solution (top and bottom of ethyl acetate extract at pH 2 and 10; top and bottom of *n*-butanol extract at pH 2 and 10) were used for antagonistic activity assays in the R2 race.

Identification of VN 10-A44

Identification of VN-A-44 was based on morphological, chemical and molecular approaches (Sakiyama et al., 2009). The VN10-A-44 was identified by 16S rRNA gene sequencing. Genomic DNA extraction was carried out using a Promega (Madison,WI, USA) extraction kit according to the manufacturer's protocol. The 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using TaKaRa Ex Taq (Takara Bio, Otsu City, Shiga, Japan) with the primers, 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3'), or occasionally 1510R (5'-

GGCTACCTTGTTACGA-3'). Almost all the entire sequence of the 16S rRNA gene (1300 to 1400 bp) was amplified by PCR as reported by Tamura et al. (1999) and directly sequenced using an ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI Model 3730 automatic DNA sequencer. The 16S rRNA gene sequence was compared with other sequences in the EMBL/GenBank/DDBJ database using BLAST searches and in the EzTaxon16 database, which includes only type strain sequences.

RESULTS

Screening for the actinomycete strains capable of inhibiting *Xoo* races

Results are summarized in Table 2 and Figure 2. We found that 13 among 90 isolates (14.4%) investigated were able to inhibit at least two races of *Xoo*. Three (3.3%) isolates were able to inhibit all the 10 races of pathogen (Tables 2 and 3).

All the 13 (100%) selected strains were able to inhibit pathogen races R1 and R3; whereas 12/13 (92.3%) inhibited R4 and R5; and 11/13 (84.6%) inhibited R7 and R8. Six among 13 selected strains inhibited R6 and four inhibited R9 (Figure 2). Only 3/13 actinomycete strains could inhibit the pathogen race R2. The actinomycete strains that inhibited race R2 (VN10-A-23, VN10-A-44and VN10-A-54) were also able to inhibit race R9 (Table 3).

Table 3. Antagonistic activities of actinomycete-tested isolates.

Actinomycete strain	Inhibition zones against to a *Xoo* race (D-d) cm									
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
VN10-A-15	2.0	-	1.2	2.8	3.5	-	2.0	2.6	-	2.0
VN10-A-16	0.9	-	1.5	1.2	1.9	0.2	0.3	1.2	0.1	0.1
VN10-A-19	0.9	-	1.1	0.4	0.3	-	-	-	-	-
VN10-A-23	0.9	0.3	2.3	1.1	1.8	0.1	0.9	1.2	0.1	0.2
VN10-A-24	2.4	-	2.7	2.5	1.7	0.1	1.0	1.5	-	2.0
VN10-A-30	1.1	-	1.2	1.1	1.5	-	0.7	0.5	-	1.0
VN10-A-38	1.8	-	1.8	1.5	1.7	-	1.3	1.3	-	1.3
VN10-A-39	1.1	-	0.7	0.7	1.3	-	0.8	0.8	-	+
VN10-A-44	2.5	0.1	2.5	1.5	2.5	0.2	1.9	1.9	0.3	2.0
VN10-A-58	2.6	0.1	0.1	1.8	1.8	0.5	1.7	1.3	0.1	0.1
VN10-A-74	0.5	-	1.8	-	-	-	-	-	-	-
VN10-A-76	3.5	-	0.8	1.1	0.9	0.1	1.5	0.8	-	0.9
VN10-A-77	2.0	-	1.1	1.8	2.3	-	1.1	1.1	-	+

The inhibition zones were measured by (D-d) cm; the plus denotes that actinomycete strains have positive results but these were not large enough to be measured; the minus denotes that actinomycete strains did not inhibit the pathogen races.

Table 4. Antagonistic activities of the selected strains.

Microorganism	Actinomycete strain		
	VN10-A-44 (D-d) cm	VN10-A-23 (D-d) cm	VN10-A-58 (D-d) cm
B. subtilis	1.4 ± 0.2	1.3 ± 0.4	2.1 ± 0.1
E. coli	-	+	+
S. cerevisiae	-	4.7 ± 0.3	0.9 ± 0.1
M. luteus	-	+	3.0 ± 0.2

The inhibition zones were measured by (D-d) cm; the plus denotes that actinomycete strains have positive results but these were not large enough to be measured; the minus denotes that actinomycete strains did not inhibit the microorganisms; ±, indicates standard deviation.

Primary tests for toxicity producer

We selected 3 strains that inhibited all the 10 races of *Xoo* for primary toxicity production test. The 3 strains were tested for their inhibition of 4 indicator micro-organisms (*B. subtilis*, *M. luteus*, *E. coli* and *S. cerevisiae*). We found that VN10-A-23 and VN10-A-58 could inhibit all the 4 tested microorganisms; whereas VN10-A-44 inhibited *B. subtilis* only and was therefore chosen for further study (Table 4).

Enhancing antibiotic production of VN10-A-44 by replacing soybean meal with waste of tofu in antibiotic producing medium

VN10-A-44 inhibits all the 10 pathogen races; however, the inhibition zone for R2 was very small (Figure 3). Tofu waste is abundant in Vietnam and some other countries in Asia. The APM with tofu waste is called modified APM recycled for producing antibiotics of VN10-A-44. If so,

tofu waste may be used for large-scale production of the antibiotics.

Result shows that the inhibition zone was improved (Table 5) when VN10-A-44 was cultured in broth APM (Figure 3B), and largest in broth MAPM (Figure 3C). The amount of tofu waste for replacing soybean meal was determined (Table 6). We found that 20 and 30 g of tofu waste/litter gave the highest inhibition zones for R2. On a daily basis, large amounts of tofu waste were excreted at tofu production sites in Vietnam. We examined the influence of the life-time of tofu waste on the results of R2 inhibition. The results are recorded in Figure 4. We found that 4-day old tofu waste was the best to produce antibiotics against race R2.

Research on preliminary extraction tests of antibiotics produced by VN 10-A-44 showed that the active compound was in n-butanol cells and a pH of 2 was the best conditions for extract. The active compound lost its Inhibition activity against the pathogen when being treated at 100°C.
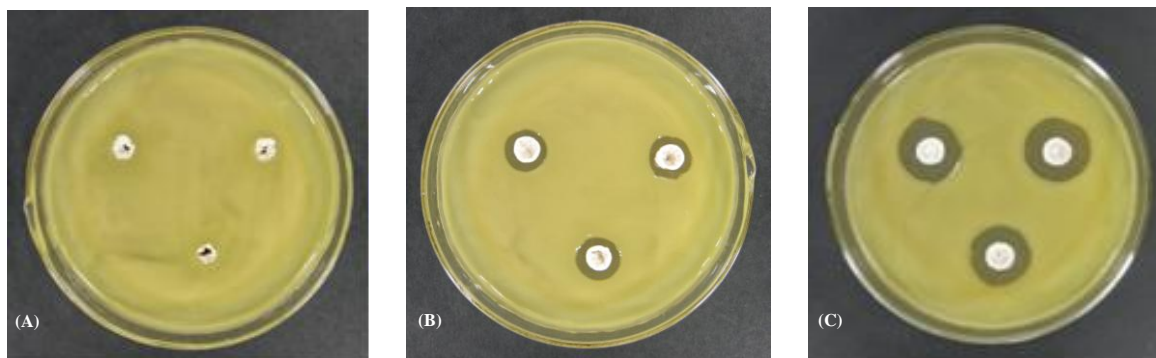
Identification of VN-A-44 by analyzing the 16S- DNA

Figure 3. The inhibition of *Xoo* R2 by VN10-A-44. A, Broth YS medium; B, broth APM; C, broth MAPM.

Table 5. Inhibition zones (D-d) cm of VN10-A44 against 10 *Xoo* races in YS, APM and MAPM.

Medium	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
YS	2.5	0.1	2.5	1.5	2.5	0.2	1.9	1.9	0.3	2.0
APM	1.9	0.6	1.5	1.8	2.2	1.0	1.3	1.7	1.0	1.7
MAPM	2.5	1.4	2.2	2.0	2.5	1.0	2.0	2.0	1.2	2.0

YS, Yeast-extract medium; APM, antibiotic-producing medium; MAPM, modified antibiotic producing medium.

Table 6. Antagonistic activities of VN10-A-44 against *Xoo* R2 with different amounts of tofu waste in broth MAPM.

Medium and different amounts of tofu waste	Inhibition zone (D-d) cm			
	Test 1	Test 2	Test 3	Average
YS	0.1	0.1	0.1	0.1
APM (10 g/ littler or 1% soybean meal)	0.7	0.8	0.8	0.8
MAPM (10 g/ littler or 1% tofu waste)	1.3	1.3	1.4	1.3
MAPM (20 g/ littler or 2% tofu waste)	1.3	1.4	1.4	1.4
MAPM (30 g/ littler or 3% tofu waste)	1.3	1.4	1.5	1.4
MAPM (40 g/ littler or 4% tofu waste)	1.4	1.4	1.4	1.4
MAPM (50 g/ littler or 5% tofu waste)	1.2	1.3	1.4	1.3
MAPM (60 g/ littler or 6% tofu waste)	1.2	1.2	1.3	1.2
MAPM (70 g/ littler or 7% tofu waste)	1.2	1.3	1.3	1.3
MAPM (80 g/ littler or 8% tofu waste)	1.2	1.2	1.2	1.2
MAPM (90 g/ littler or 9% tofu waste)	1.4	1.3	1.3	1.3
MAPM (100 g/ littler or 10% tofu waste)	0.9	0.9	1.0	0.95
MAPM (130 g/ littler or 13% tofu waste)	0.9	0.9	1.2	1.0
MAPM (150 g/ littler or 15% tofu waste)	1.0	1.0	1.0	1.0
MAPM (200 g/ littler or 20% tofu waste)	0.9	0.9	1.0	0.9

(MAPM) and it is used to test whether tofu waste can be sequence showed that the VN10-A-44 strain was closest to *Streptomyces virginiae* (100%, 1464/1464 bp, identity to accession number AB184175) (Figure 6). Morphology of VN10-A-44 was recorded and is shown in Figure 5. It belongs to *Streptomyces* with spore chains.

DISCUSSION

Biocontrol of *Xoo* was recently reported in Asia (Gnanamanickam 2009; Ji et al., 2008; Park et al., 2011). With an attempt to select a biological agent from strains of actinomycete, in this study, we screened for
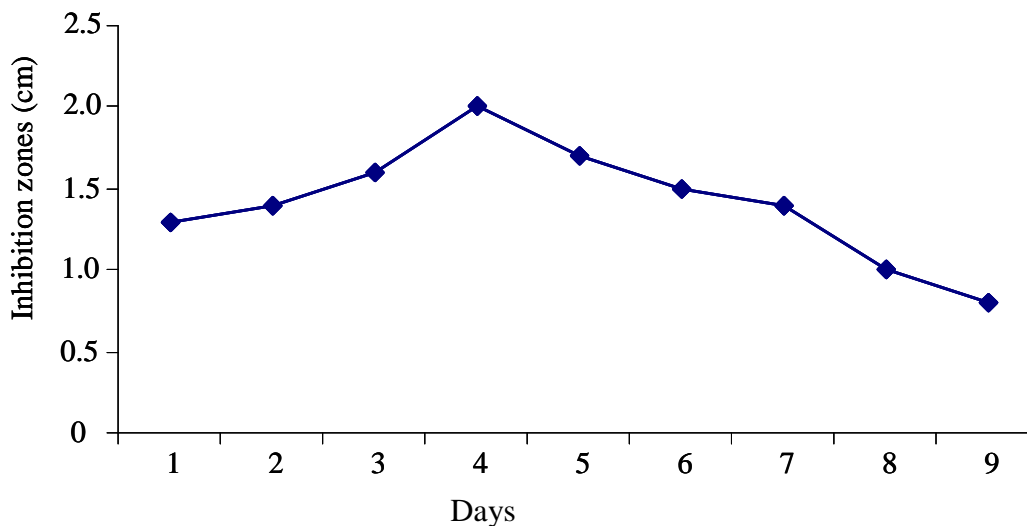
Figure 4. Antagonistic actives of VN10-A-44 depending on life-time points of tofu waste after production.

Figure 5. Morphology of VN10-A-44. a, Colony; b, spore.

actinomyce strains inhibiting all the 10 races of *Xoo*, whereas at the same time selecting for strains that were not harmful to other micro-biota and humans. Among 90-tested strains, we selected 13 actinomyces isolates that were able to inhibit from one to 10 races of *Xoo*. This number of actinomyces strains inhibiting races of *Xoo* (14.4%) was not high, and this result is in agreement with another study showing lower numbers of *Streptomyces* strains capable of inhibiting *Xoo*; whereas most of streptomyces isolates inhibited *Xanthomonas vascatoria* and *Clavibacter michiganensis* ssp. *michiganensis* (Ndonle and Semu, 2000). In the present study, we found that the R1 and R3 races of *Xoo* were inhibited by 13 actinomyces strains; whereas R2 and R9 were the most difficult to inhibit. Most of the actinomyces strains can inhibit at least 2 races of *Xoo* (R1 and R3), however only 3 strains (VN10-A-23; VN10-A-44; VN10-A-58) were able

to inhibit all the 10 races.

Antibiotic production of actinomycete-inoculated soils was demonstrated in soils supplemented with a suitable organic source. Stevenson (1959) found that fresh grass (3%), clover (3%) and soybean meal (2%) were suitable supplements for antibiotic production by the majority of the actinomycetes. In this study, we showed that replacing soybean meal in APM with tofu waste (from 2 to 4%) gave the largest inhibition zones (1.4 cm) against race R2; especially the 4-day old tofu waste (inhibition zones up to 2.0 cm). This could be due to the fact that the nutrient values obtained from fresh soybean found in tofu waste were much higher than those of soybean meal. To make soybean meal, soybean was dried by heating and then made into powder. This process reduced many vitamins and some essential amino acids which are very important for the development of actinomycetes from
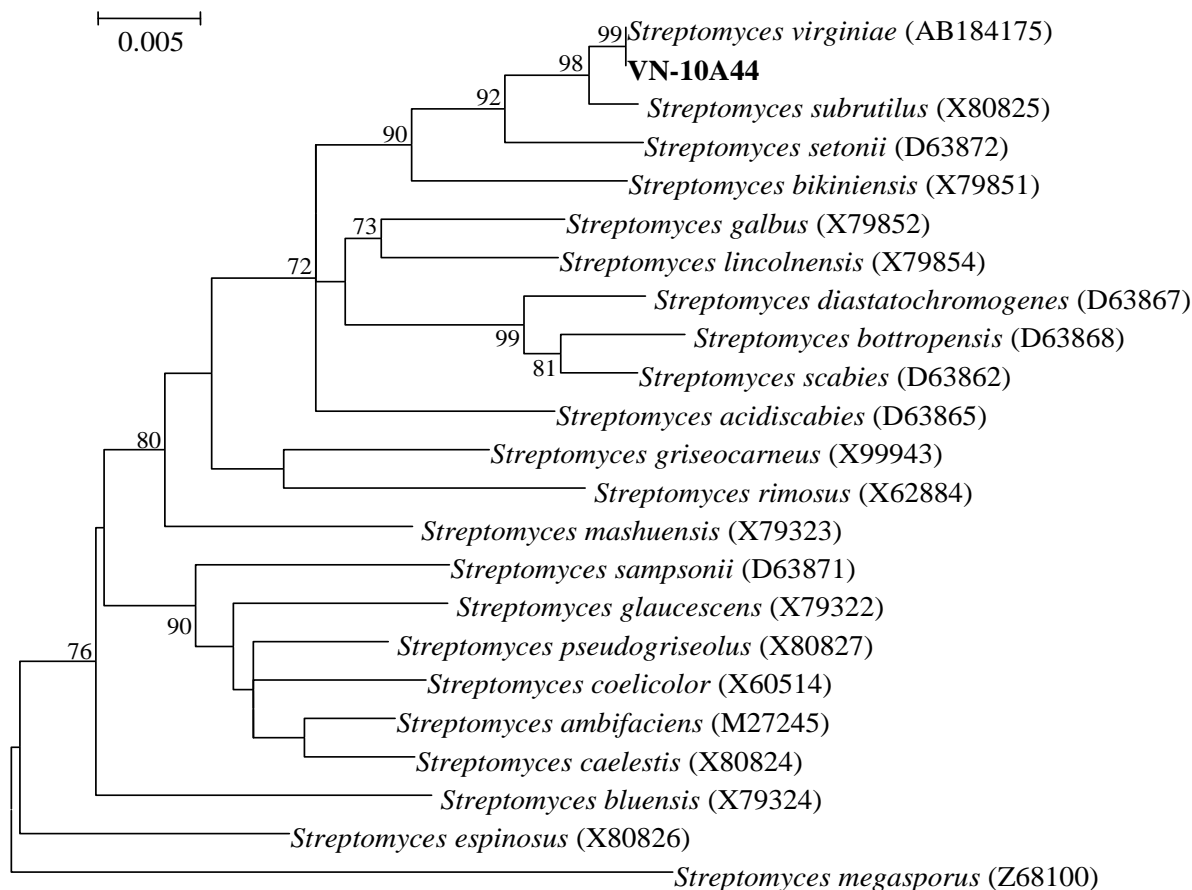
Figure 6. Phylogeny tree of VN10-A-44 and its relationship with some *Streptomyces* species.

fresh soybean. If tofu waste is used for fermentation of VN10-A-44, it may reduce the cost of large-scale antibiotic production and help recycle tofu waste.

Identification of VN10-A-44 by 16S-rDNA sequencing showed that VN10-A-44 is identical to *S. virginiae*. This species is reported to produce VirginiamycinM1 (Suzuki et al., 1998) and it has a high activity *in vitro* against Gram-positive and negative bacteria, yeast and fungi (Rifaat and Kansoh, 2005).

ACKNOWLEDGEMENTS

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