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Screening the Antibacterial Activities of *Streptomyces* Extracts against Phytopathogens *Xanthomonas oryzae* pathovar *oryzae, Xanthomonas campestris* pathovar *vesicatoria,* and *Pectobacterium carotovorum* pathovar *carotovorum*

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Abstract *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), *X. campestris* pv. *vesicatoria* (*Xcv*), and *Pectobacterium carotovorum* pv. *carotovorum* (*Pcc*) are the causative agents of bacterial blight in rice, bacterial spot in pepper, and bacterial soft rot in carrot and cabbage, respectively. To isolate novel microbial extracts with antimicrobial activities against these bacteria, approximately 5,300 different *Streptomyces* extracts were prepared and tested. Microbial cultures from various *Streptomyces* strains isolated from the Jeju Island, Baekam, Mankyoung river, Jiri mountain etc. in Korea were extracted into three different factions -secreted hydrophobic, secreted hydrophilic, and mycelia- using ethyl acetate, water, and methanol. Initially, 34, 29, and 10 extracts were selected as having antibacterial activities against *Xoo*, *Xcv*, and *Pcc*, respectively. Extracts 1169G4, 1172E9, and 1172E10 had the highest growth inhibition activities against both *Xoo* and *Xcv*, and extracts 1151H7 and 1152H7 showed the highest growth inhibition activities against *Pcc*.

Keywords antimicrobial activity · *Pectobacterium carotovorum* pathovar *carotovorum* · *Streptomyces* · *Xanthomonas campestris* pathovar *vesicatoria* · *Xanthomonas oryzae* pathovar *oryzae*

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Introduction

Filamentous bacteria belonging to the genus *Streptomyces* produce over 50% of the antibiotics discovered to date (Taddei et al., 2006; Jayapal et al., 2007). *Streptomyces* bacteria and other Actinomycetes produce a diverse array of antibiotics, including aminoglycosides, anthracyclins, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers, and tetracyclines (Mellouli et al., 2003).

Rice, pepper, and cabbage are the first, second, and third most cultivated crops, respectively, in Korea. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *X. campestris* pv. *vesicatoria* (*Xcv*) are the causal agents of bacterial blight in rice and bacterial spot in pepper, respectively (Yang et al., 2005; Adhikari et al., 2010). *Pectobacterium carotovorum* pv. *carotovorum* (*Pcc*) causes soft rot in a wide range of plants, including cabbage (Mole et al., 2010). Because these bacteria cause severe crop damage and reduce yield, prevention and treatment of these diseases have been the focus of intense research in both the academy and industry (Khush, 1997). In 2005, the whole genomes of *Xoo* and *Xcv* were sequenced (Frank Thieme, 2005; Lee et al., 2005). The emergence of resistance against *Pcc* was reported in 2007 (T. Luzzatto, 2007).

Chemical pesticides are routinely used to control plant pathogens in agricultural fields. However, recently, indiscriminate usage of

chemical pesticides has raised many concerns, including environmental toxicity (Vivek K. Bajpai, 2010) and the possibility of generating antibiotic-resistant microbes. Therefore, biopesticides made from secondary metabolites from microbes or plants are being preferred over chemical pesticides.

The main objective of this study was to isolate novel antimicrobial *Streptomyces* extracts against the phytopathogens mentioned. *Streptomyces* species are ubiquitous in the soil, and play an important ecological role in the turnover of organic material. Furthermore, the diverse secondary metabolites produced by *Streptomyces* species are good sources of novel bioactive compounds. To date, more than half of the world's antibiotics (over 50 different antibiotics) have been isolated from *Streptomyces*. In this study, we prepared and screened 5,328 *Streptomyces* extracts, and tested their antimicrobial activities against *Xoo*, *Xcv*, and *Pcc*.

Materials and Methods

Strains and Culture Conditions. *X. oryzae* pv. *oryzae* KACC10331 (*Xoo*), *X. campestris* pv. *vesicatoria* KACC11127 (*Xcv*) and *P. carotovorum* pv. *carotovorum* KACC10227 (*Pcc*) were provided by the Korea Agriculture Culture Collection (KACC). *Xoo*, *Xcv*, and *Pcc* were grown in YPD (1.0% yeast extract, 2.0% peptone, and 2.0% dextrose) rich media at 28°C. *Streptomyces* strains, collected from various regions of the Republic of Korea, including Jeju island, Baekam, Mankyoung river, Jiri mountain etc. (S-Table 1), were maintained at the Extract Collection of Useful Microorganisms library at the Myoungji University, and grown at 28°C on GSS medium (1.0% soluble starch, 2.0% glucose, 0.1% beef extract, 0.4% yeast extract, 0.2% NaCl, 0.025% K_2HPO_4, and 0.2% $CaCO_3$ at pH 7.2), Bennett's medium (1.0% glucose, 0.1% yeast extract, 0.2% bacto-peptone, and 0.1% beef extract), or DYC medium (2.5% dextrin, 1.2% dry yeast, 2.0% corn starch liqueur, 0.1% NaBr, and 0.1% $CoCl_2$ at pH 7.0).

Preparation of the *Streptomyces* extracts. For preparation of *Streptomyces* extracts library, one gram of soil were suspended in 10 milliliter of 0.85% NaCl solution and serially diluted. The diluted soil suspension were spread on Humic acid- vitamin (HV) agar medium (0.1% humic acid, 0.05% Na_2HPO_4, 0.071% KCl, 0.005% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.002% $CaCO_3$, 0.2% B-Vitamin solution, 1.8% agar at pH 7.2) and incubated at 28°C for 14 days. The colony with representative actinomycetes were selected and identified by 16S rDNA sequence. Then *Streptomyces* strains were cultured in three different media: GSS medium for nutrient-rich condition, Bennett's medium for moderate-nutrition condition, and DYC medium for nutrient-poor condition. Three extract fractions were prepared from each *Streptomyces* culture broth. 30 mL of the culture broth were centrifuged at $5,000 \times g$ for 10 min. Mycelia were collected as a pellet, and extracted with 3 mL of methanol. This fraction was called "mycelia isolated from supernatant (MIS)". After pelleting the mycelia, the supernatant was further extracted with 30 mL of ethyl acetate and dried. The resulting dried extract was dissolved in 3 mL of methanol. This was called the "secreted hydrophobic (SHB) fraction". The remaining ethyl acetate-insoluble fraction was called the "secreted hydrophilic (SHL) fraction". Thirty microliter of each fraction was distributed into 96-well plate and dried completely before further use. The dry weights were measured to enable accurate calculation of GI50 (dose required to cause 50% growth inhibition).

Naming the *Streptomyces* extracts. All extracts were represented as "0000X0." The four digits, preceding the alphabet "X," refer to the plate number, which is named according to the region from where the sample was collected. The alphabet X refers the row in which the sample was present (numbered from top to bottom) and the last digit represents the column (numbered from left to right). For instance, sample 1172H2 is located in the second column from the left and at the bottom row of the 96-well plate numbered 1172. In addition, the last digit of the extract's name represents the extract-type: 2, 5, and 8 represent SHL; 3, 6, and 9 represent MIS; and 4, 7, and 10 represent SHB.

Antimicrobial activity test. The antimicrobial activities of the extracts were estimated by the growth inhibition test using a single dose in two steps. In the first round of screening, all the extracts were stored in columns 2–10 of the 96-well plate. Columns 1, 11, and 12 contained the control (medium only), bacterial culture, and

Table 1 Antibacterial extracts screened in the first and second round against *Xoo*, *Xcv*, and *Pcc*

		Xoo	*Xcv*	*Pcc*
1st	SHL	1119D8, 1117E2	1128D2, 1128B5 1128B2	1158H5, 1158G2
	SHB	1176A4, 1175D7, 1175A4, 1173E4, 1172E10, 1169H4, 1169G4, 1159B10, 1149F4, 1139G10, 1136G7, 1124A7, 1120F10, 1119F10, 1119C10, 1117E4	1175A4, 1169G4, 1169E10, 1144H7, 1144A7, 1141H7, 1141A10, 1133H10, 1132H7, 1131H4, 1128H4, 1128C4, 1128B4, 1121F10, 1120F10, 1172E10	1153E7, 1152H7, 1151H7, 1158G7
	MIS	1175A3, 1173E3, 1172E9, 1169G3, 1164E6, 1161F3, 1161E3, 1160D3, 1159B9, 1158A6, 1149F3, 1144B6, 1135H3, 1120F9, 1119C6, 1117E3	1172E9, 1152H3, 1144A6, 1141H6, 1132H6, 1131H3, 1128H6, 1128D3, 1128C6, 1128A3	1158H6, 1158G6, 1157A6, 1155E9
2nd	SHL	-	-	-
	SHB	1172E10, 1169G4	1172E10, 1169G4	1152H7, 1151H7
	MIS	1172E9	1172E9	-

bacterial culture with 6% dimethylsulfoxide (DMSO), respectively. The *Streptomyces* extracts were dissolved in 40 μL of 50% DMSO, and further diluted in 100 μL of YPD medium. *Xoo*, *Xcv*, and *Pcc* were grown to the early exponential growth stage (absorbance at 600 nm [A_{600}]=0.2), and 160 μL of each bacterial culture was dispensed into separate 96-well micro-plates (Nunc¢,, US). Then, 40 μL of previously diluted *Streptomyces* extract was added to each well. The cultures were grown at 28°C with continuous shaking at 700 rpm. The A_{600} values of the wells in the culture plates were measured every 4 h for 20 h using a multimode micro-plate reader (Infinite® M200 PRO, TECAN, Switzerland). To measure the antibacterial activity of each extract, we calculated the growth inhibition rate (GIR) at 20 h for *Xoo* and *Xcv* and at 12 h for *Pcc* using the following formula:

$$GIR=(1[CB]/[AB])\times100$$

where *A* was the A_{600} value of the bacterial culture with 6% DMSO; *B*=0.2, which was the A_{600} value of the bacterial culture when the extract was added; and *C* was the A_{600} value of the bacterial culture after treatment with the extract. For the second growth inhibition screen, the extracts selected from the first assay were tested three times using the same procedure.

The amount of growth inhibition induced by each extract was measured at five different dosages (1, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}
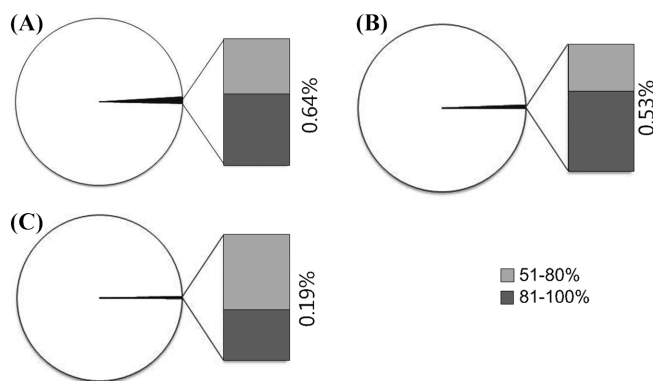

Fig. 1 A bar pie chart depicting the results of the first round of screening of the *Streptomyces* extracts for antimicrobial activity against (A) *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), (B) *X. campestris* pv. *vesicatoria* (*Xcv*), and (C) *Pectobacterium carotovorum* pv. *carotovorum* (*Pcc*). The bar lists screened antimicrobial extracts that showed more than 50% growth inhibition. The percentages of screened extracts are indicated. Extracts showing growth inhibition of 51–80 and 81–100% are indicated separately in the enlarged bar. The *Streptomyces* extracts were prepared as described in the Materials and Methods.

dilutions) and compared with the controls. From this, the GI50 was calculated. All the tests were carried out in triplicate.
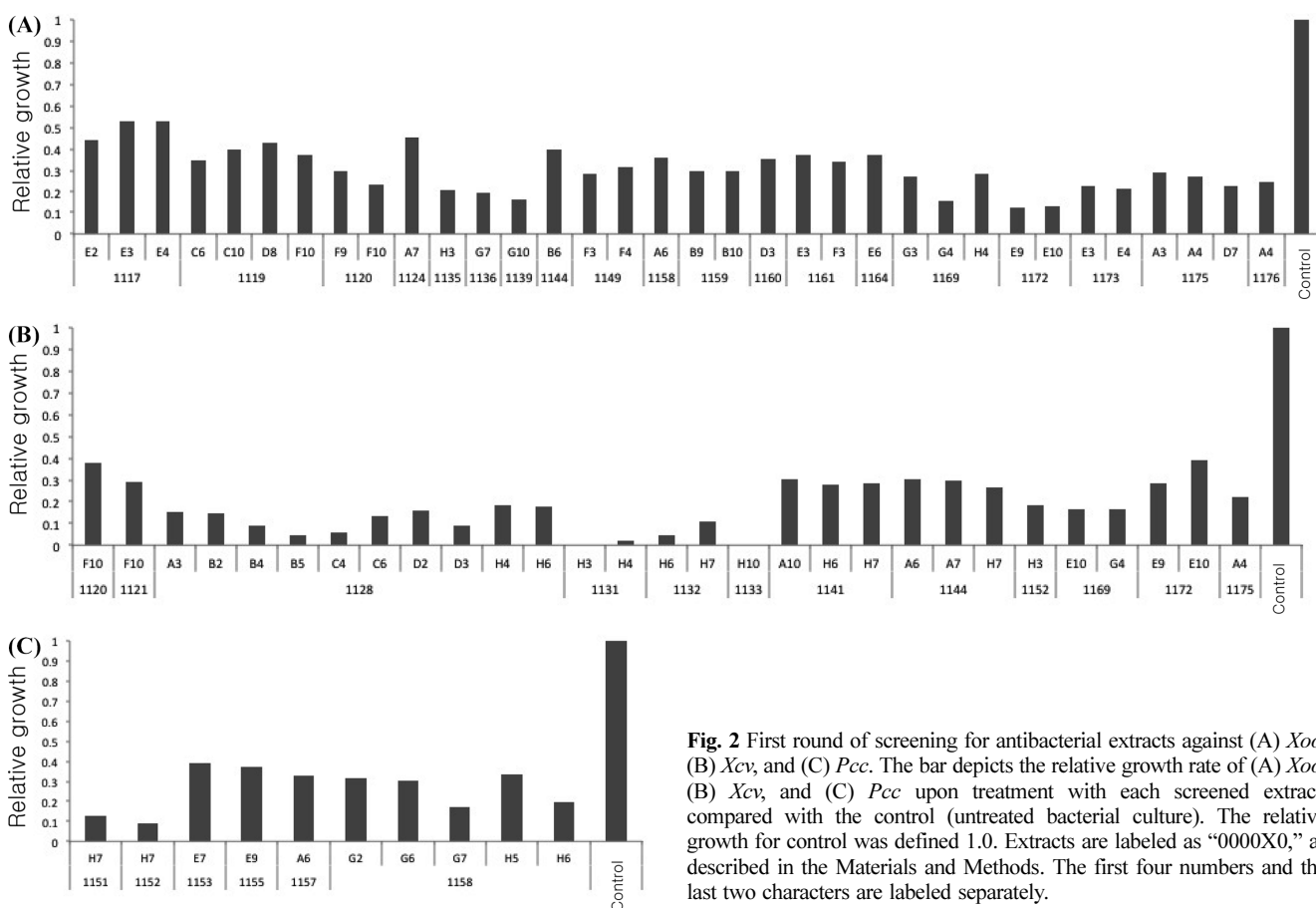

Fig. 2 First round of screening for antibacterial extracts against (A) *Xoo*, (B) *Xcv*, and (C) *Pcc*. The bar depicts the relative growth rate of (A) *Xoo*, (B) *Xcv*, and (C) *Pcc* upon treatment with each screened extract, compared with the control (untreated bacterial culture). The relative growth for control was defined 1.0. Extracts are labeled as "0000X0," as described in the Materials and Methods. The first four numbers and the last two characters are labeled separately.
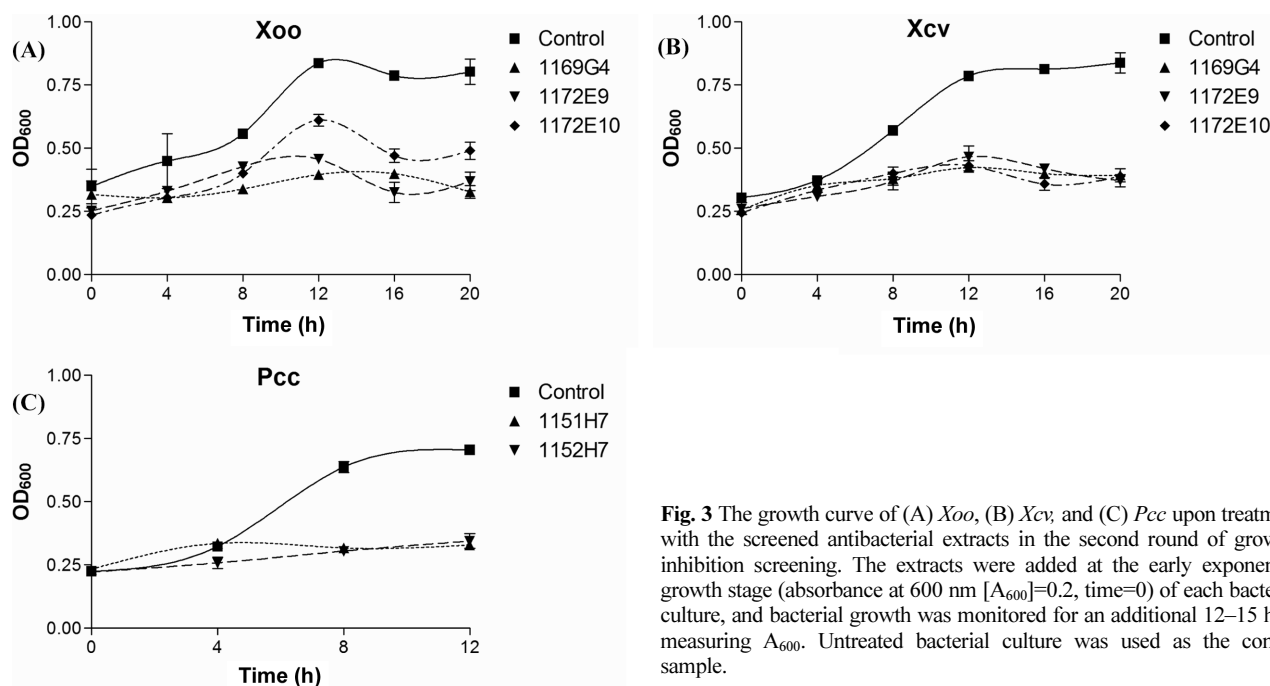
Fig. 3 The growth curve of (A) *Xoo*, (B) *Xcv*, and (C) *Pcc* upon treatment with the screened antibacterial extracts in the second round of growth-inhibition screening. The extracts were added at the early exponential growth stage (absorbance at 600 nm [A_{600}]=0.2, time=0) of each bacterial culture, and bacterial growth was monitored for an additional 12–15 h by measuring A_{600}. Untreated bacterial culture was used as the control sample.

Results

Preparation of extracts from *Streptomyces*. *Streptomyces* strains produce diverse physiological secondary metabolites depending on the culture conditions. To generate various secondary metabolites from each *Streptomyces* strain, three culture media with different nutritional levels were used to grow the *Streptomyces* cells. In addition, three extract fractions were prepared for each *Streptomyces* culture. The MIS was separated from the supernatant; SHBs were extracted from the supernatant fraction using ethyl acetate; and SHLs were obtained from the residual water layer. In total, 5,328 extracts were prepared and screened for their antimicrobial activities against the three plant pathogens, *Xoo*, *Xcv*, and *Pcc*.

First round of screening for antimicrobial activity. Extracts showing more than 50% cell growth inhibition activity were considered antimicrobial extracts; extracts 34, 29, and 10 were selected because they exhibited antimicrobial activity against *Xoo*, *Xcv*, and *Pcc*, respectively. These three extracts constitute 0.64, 0.53, and 0.19% of all the extracts tested in the study (Fig. 1 and Table 1).

Extract 1172H9, the most effective antimicrobial extract against *Xoo*, inhibited 90% of *Xoo* cell growth. Of the top 34 extracts against *Xoo*, two were SHLs, 16 were MISs, and 16 were SHBs (Fig. 2A). Extract 1131H3 exhibited approximately 100% growth-inhibition effect against *Xcv*, and was the most effective antimicrobial against *Xcv*. Of the top 29 extracts exhibiting growth-inhibition effect against *Xcv*, three were SHLs, 10 were MISs, and 16 were SHBs (Fig. 2B). Extract 1152H7 showed 90% growth-inhibition activity against *Pcc*, and was the most effective antimicrobial extract against *Pcc*. Of the top 10 extracts showing growth-inhibition effect against *Pcc*, two were SHLs, four were MISs, and

the remaining four were SHBs (Fig. 2C).

Second round of screening for antimicrobial activity. Extracts showing high growth-inhibition activity in the first round of screening were tested three times to confirm their antimicrobial activities. 1169G4 (SHB), 1172E9 (MIS), and 1172E10 (SHB) had the highest growth-inhibition activities against both *Xoo* and *Xcv* (Fig. 3A and 3B). 1172E9 (MIS) and 1172E10 (SHB) are from the same *Streptomyces* strain from Jeju island. 1169G4 (SHB) is also from Jeju island (S-Table 1). 1152H7 (SHB) and 1151H7 (SHB) showed the highest growth-inhibition activity against *Pcc* (Fig. 3C). 1152H7 (SHB) and 1151H7 (SHB) are from Baekam, Gyoungsangbukdo (S-Table 1). 1152H7 (SHB) showed slightly higher antibacterial activity than 1151H7 (SHB). In total, five different extracts -1169G4 (SHB), 1172E9 (MIS), 1172E10 (SHB), 1152H7 (SHB), and 1151H7 (SHB)- were found to possess antibacterial activities against *Xoo*, *Xcv*, and *Pcc*. Interestingly, all the extracts, except 1172E9 (MIS), were SHBs.

The GI50s of the five antimicrobial extracts were measured by serial dilution of the extracts (Table 2). 1172E9 (MIS) and 1172E10 (SHB) showed the highest antibacterial activities against *Xoo* and *Xcv* (Fig. 4 and 5). In particular, 1172E10 (SHB) showed a GI50 value of less than μg·mL^{-1}. 1152H7 (SHB) had a slightly

Table 2 Doses of screened antibacterial extracts required to cause 50% inhibition (GI50s)   (Unit: μg·mL^{-1})

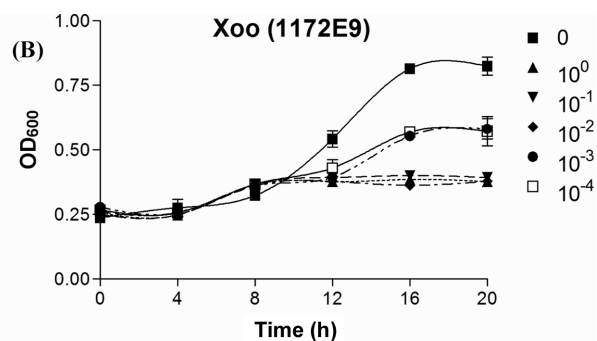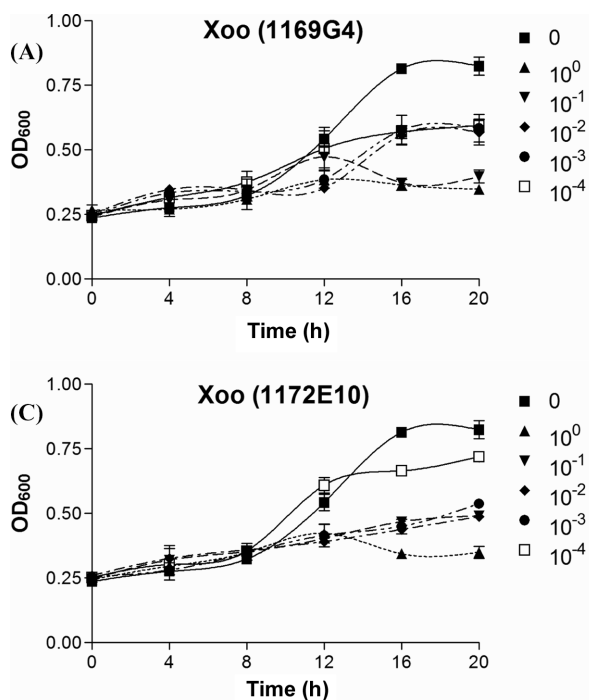|	Xoo	Xcv	Pcc
1169G4	4.1	1.7	-
1172E9	<1.7	<1.7	-
1172E10	0.3	0.4	-
1151H7	-	-	2.2
1152H7	-	-	0.5

Fig. 4 The growth curves of *Xoo* treated with (A) 1169G4, (B) 1172E9, and (C) 1172E10.
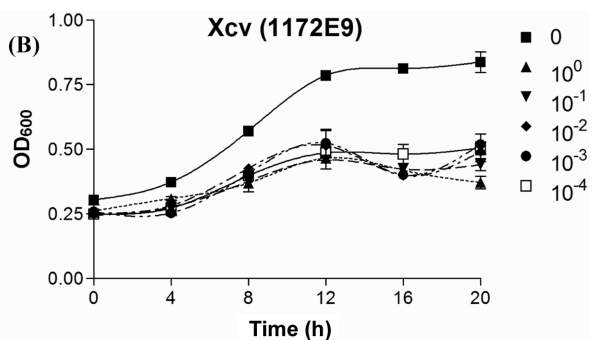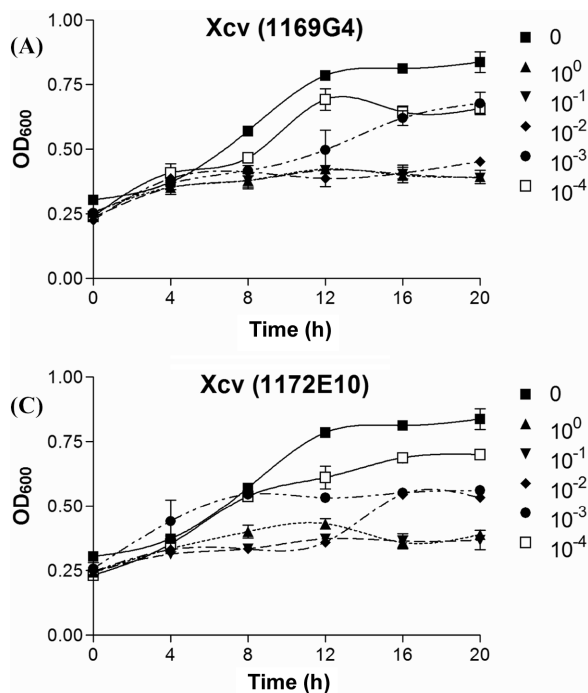

Fig. 5 The growth curves of *Xcv* treated with (A) 1169G4, (B) 1172E9, and (C) 1172E10.

higher antibacterial activity than 1151H7 (SHB) against *Pcc* (Fig. 6). The antimicrobial activities of all five extracts were less than μg·mL^{-1}. 1172E10 (SHB: MJM strain No. 10146GE) had the highest antimicrobial activity against *Xoo* and *Xcv* with GI50s of 0.3 and 0.4 μg·mL^{-1}, respectively. 1152H7 (SHB: MJM strain No. 9973DE) had the highest antimicrobial activity against *Pcc*, with a GI50 of 0.5 μg·mL^{-1}.

Discussion

Antibiotics are secondary metabolites that inhibit the growth of other microbial organisms. Growing incidences antibiotic-resistance among deadly pathogens has increased the need to discover novel antibiotics. Over the last few decades, new technologies for drug discovery have been developed, such as high throughput screening
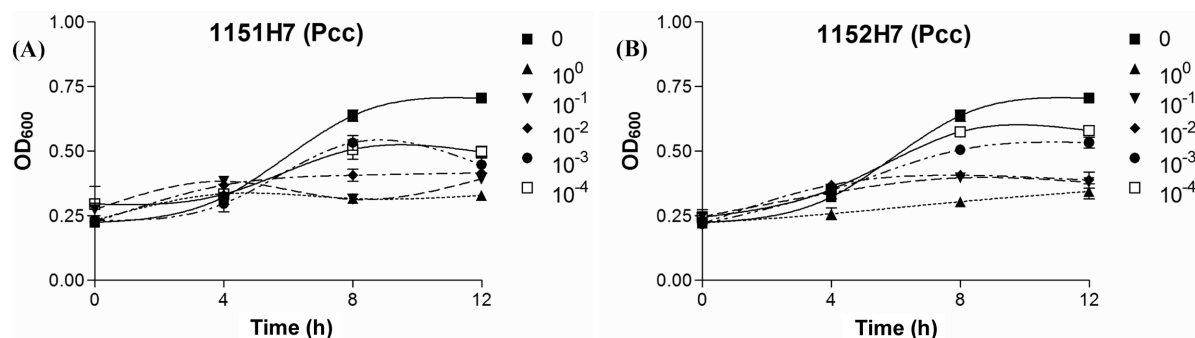
Fig. 6 The growth curves of *Pcc* treated with (A) 1151H7 and (B) 1152H7.

and combinatorial chemistry. Moreover, sequencing of pathogen genomes has led to the identification of new drug targets, and many chemical compounds have been synthesized by combinatorial chemistry and screened using cell-free assay systems and high-throughput screening systems. However, most screened compounds do not penetrate the pathogen to reach the target, or are easily degraded *in vivo*. Despite these technological advances, only five new classes of antibiotics have been introduced for human use (Talbot et al., 2006). Therefore, many studies are now focusing on screening secondary metabolites from microbes, especially *Streptomyces*, to identify novel antimicrobials. *Streptomyces* bacteria produce numerous secondary metabolites, and approximately 55% of currently used antibiotics are produced by the genus *Streptomyces*.

Five *Streptomyces* extracts showing high antimicrobial activity against *Xoo*, *Xcv*, and *Pcc* were screened. Of the five extracts, four (1152H7, 1151H7, 1169G4, and 1172E10) were secreted and hydrophobic, and only one extract (1172E9) was non-secreted. The secretory and hydrophobic properties of the extracts aid in the penetration of bacterial cell walls (Nikaido H, 1976; Savage et al., 2002). In particular, 1169G4, 1172E9, and 1172E10 were effective against both *Xcv* and *Xoo*. The genus *Xanthomonas* belongs to Proteobacteria, and it causes a variety of diseases in numerous crops. The extracts identified in this study may have applications as a novel antimicrobial drug against *Xanthomonas,* including *Xoo* and *Xcv*.

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