การคัดแยกแอคติโนมัยสีทจากสมุนไพรทองพันชั่งและว่านมหากาฬ และประสิทธิภาพในการยับยั้งจุลินทรีย์

Isolation of actinomycetes from *Rhinacanthus nasutus* (Linn.) Kurz and *Gynura pseudo-chian DC. var. hispida* Thv. and their antimicrobial activities

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บทคัดย่อ

คัดแยกแอคติโนมัยสีท 54 สายพันธุ์ จากดินรอบรากและรากพืชสมุนไพรทองพันชั่งและว่านมหากาฬที่เก็บจากจังหวัดกรุงเทพและเชียงใหม่ ในจำนวนนี้ 52 สายพันธุ์แยกได้จากดินรอบราก อีก 2 สายพันธุ์แยกได้จากรากสมุนไพรว่านมหากาฬ แอคติโนมัยสีทที่แยกได้ทั้งหมดมี LL-isomer diaminopimelic acid เป็นองค์ประกอบยกเว้นสายพันธุ์ KKD096 เมื่อทดสอบประสิทธิภาพในการยับยั้งจุลินทรีย์ของแอคติโนมัยสีทที่คัดแยกได้กับ *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923 และ *Candida utilis* พบว่า 58% ของแอคติโนมัยสีทที่แยกได้สามารถยับยั้งจุลินทรีย์ทดสอบอย่างน้อย 1 ชนิด และมี 5 สายพันธุ์ที่มีประสิทธิภาพยับยั้งจุลินทรีย์ทดสอบทุกชนิด จากการจำแนกชนิดของสายพันธุ์ที่มีประสิทธิภาพยับยั้งจุลินทรีย์ทดสอบเหล่านี้ โดยศึกษาลำดับยีนชนิด 16SrRNA พบว่าทุกสายพันธุ์อยู่ในจีนัส *Streptomyces* นอกจากนี้สายพันธุ์ KKD096 และ KKD098 ซึ่งเป็นเอนโดไฟต์ที่แยกจากรากว่านมหากาฬพบว่าเป็นสมาชิกของจีนัส *Kineococcus* และจีนัส *Streptomyces* ตามลำดับ

ABSTRACT

Fifty-four strains of actinomycetes were isolated from rhizosperic soil and root of Thai medicinal plants (*Rhinacanthus nasutus* (Linn.) Kurz and *Gynura pseudo-chian DC. var. hispida* Thv.) collected from Bangkok and Chiang Mai. Fifty two strains were recovered from rhizospheric soil and the remaining 2 strains were isolated from *Gynura pseudo-chia DC. var. hispida* Thv. root. Most isolates were found to contain LL-isomer of diaminopimelic acid except strain KKD096. Antimicrobial activity of all isolates was determined against *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923 and *Candida utilis*. It was found that 58 % of the isolates could inhibit at least one of the test organisms and five strains showed an effective activity against all test organisms. The isolates with ability to inhibit all test organisms were identified by 16SrRNA gene sequence analysis. The result showed that they belong to genus *Streptomyces*. In addition, two endophytic strains KKD096 and KKD098 which were isolated from *Gynura pseudo-chian DC. var. hispida* Thv. root were found to be members of the genus *Kineococcus* and *Streptomyces* respectively.

Key words: actinomycetes, antimicrobial, endophyte, medicinal plant, rhizosphere,

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Introduction

Actinomycetes are Gram positive bacteria with high G+C content in their genomes. In general actinomycetes, particularly streptomycetes are primarily saprophytic bacteria which responsible for the turnover of complex biopolymers. They are also well known as the majority sources of secondary metabolites and constitute potentially important sources of antibiotics. The increasing emergence of multi-resistant bacteria throughout the world (Livermore, 2004), has lad to a worldwide search for antibiotics to fight with these pathogenic agents from various sources and geographic regions (Basilio *et al*., 2003; Fiedler *et al*., 2005).

Rhizosphere is an ecological niche of microbial communities. Distribution of rhizospheric actinomycetes that produce active antimicrobial substances has been reported in many studied (Basilio *et al*., 2003; Gesheva, 2002). Some actinomycetes are known to form associations with plants and colonize in their internal tissue. The endophytic *Streptomyces* sp. has been found to produce new bioactive compound such as naphthoquinone (Bieber *et al*., 1998).

Thailand located in the tropical area with diverse plants species that being used as self-medication for skin diseases. *Rhinacanthus nasutus* (Linn.) Kurz and *Gynura pseudo-chian DC. var. hispida* Thv. or commonly known as Thong-Pan-Chung and Waan Maha Karn, respectively are two examples of these medicinal plants. Waan Maha Karn is used for the treatment of eczema. Thong-Pan-Chung has substances that relieve many diseases including skin disease. (Wu *et al*., 1998). The aims of this study were to isolate actinomycetes from roots and rhizospheric soils of two Thai medicinal plants (Thong-Pan-Chung and Waan Maha Karn) and screen the isolates for their ability to produce antimicrobial metabolites. The identification of the active strains based on the combination of genotypic and phenotypic data were also reported.

Materials and methods

Samples and microbial strains

The roots and rhizospheric soil samples of healthy Thong-Pan-Chung and Waan Maha Karn plants were collected from 4 difference places in Bangkok and Chiang Mai during December 2005. The plants were pulled out from the soil, placed in a sterile plastic bag and transported to the laboratory. The pH values of soil samples were determined according to the method described by Reed and Cummings (1945).

The test microorganisms for screening of antimicrobial activity were obtained from the American Type Culture Collection (ATCC): *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 8739 *Staphylococcus aureus* ATCC 25923 and *Candida utilis*. All strains were maintained on nutrient agar at 28°C except *Candida utilis* was maintained on yeast extract-malt extract agar.

Actinomycetes isolation methods

Root associated actinomycetes

Plant roots were washed in running water to remove soil particles and sterilized by shaking in 70% ethanol for 5 minutes following by a solution of 1.0 % sodium hypochlorite for 10 minutes. Samples were then washed three times in sterile water to remove surface sterilization agent. The root was then crushed with sterile glass rod and diluted with ¼ strength Ringer's solution before spreading on starch casein agar (Küster and Williams, 1964) supplemented with antifungal and antibacterial antibiotics and incubated at room temperature. The effectiveness of surface sterilization was tested by soaking washed sample in 5 ml sterile water and stirred for 1 minute. An aliquot of 0.2 ml suspension were inoculated on starch casein agar plates and incubated to examine microbial growth.

Rhizospheric soil actinomycetes

One gram of each air-dried rhizospheric soil was added to 9 ml of ¼ strength Ringer's solution and the resultant 10^{-1} dilution was heated at 55°C for 6 minutes; these preparations were serially diluted down to 10^{-5} using ¼ strength Ringer's solution. Aliquots of 0.1 ml of each dilution were spread over the surfaces of starch casein agar plates which had been dried for 15 minutes prior to inoculation. The inoculated plates were incubated at room temperature for 14 days. Colonies of actinomycetes were expressed as the mean number of colony forming units (cfu) per gram dried soil. The colonies were collected, purified and preserved in 20% (v/v) glycerol at –20°C.

Morphological and chemotaxonomic characterization

The isolates were inoculated onto oatmeal plates and incubated up to 14 days. The plates were examined for the aerial spore mass colour, substrate mycelium pigmentation and colour of soluble pigments. Spore chain morphology was studied under light microscope. The isolates were then assigned to colour groups based on the recorded properties.

The isomeric form of diaminopimelic acid (A_2pm) of each isolates was determined by paper chromatography of whole-organism hydrolysates using established method (Hasegawa *et al*., 1983).

Detection of antimicrobial activity

Antimicrobial activity of actinomycete isolates was determined according to the method of Widdick *et al*. (2003) with slight modifications. Each isolate was spot on ISP2 agar plate (Shirling and Gottlieb, 1966) and incubated for 7 days at room temperature. A total of 0.5 ml of an overnight culture of each test microorganism (OD_{600}=0.2) was added to 5 ml of warm melted soft nutrient agar (0.6 % agar). The mixture was immediately poured over the ISP2 agar plate containing the isolate. The assay plates were incubated at 37°C overnight to observe a zone of inhibition. The antimicrobial activity was expressed as the ratio of the diameters of inhibition zone to the colony diameter (Z/C) as described by Dhar and Bose (1968).

Molecular identification of active actinomycete strains

The endophytic isolates (KKD096, KKD098) and strains that shown broad antimicrobial activities against all test microorganisms were identified by sequencing of a fragment of the 16SrRNA gene. Chromosomal DNA of each strain was extract following the method as described by Kieser *et al*. (2000). PCR and sequencing of 16SrRNA genes including the phylogenetic tree construction was carried out according to Duangmal *et al*. (2005).

Results and discussions

Presumptive actinomycete colonies were distinguished from other bacterial colonies growing on isolation plates on the basis of their morphological characteristics (Shirling and Gottlieb, 1966). The total number of actinomycetes obtained from each sample was shown in Table 1. The total count of actinomycetes were ranged from 1.2-8.1×10^5 cfu·g^{-1} dried soil. Actinomycete colonies from each soil sample were streaked on glucose yeast extract agar and oatmeal agar to receive pure culture for further detailed characterizations. The presence of a high number of actinomycetes in soil was in agreement with previous reports which showed that soil is the principal reservoir of actinomycetes (Crawford *et al*., 1993). The soil pH were between 4.6-7.9 which yielded about the same number of actinomycetes as reported by Shirokikh *et al*. (2002).

Table 1 Total count of actinomycetes recorded for rhizospheric soil samples.

Sample*	Source	pH	Number of actinomycetes (× 10^5 cfu ·g^{-1} dry soil)	Number of isolates
1	Thong-Pan-Chung	5.65	8.1±4.2	11
2	Thong-Pan-Chung	7.90	1.2±1.4	2
3	Thong-Pan-Chung	7.33	1.6±0.7	5
4	Thong-Pan-Chung	6.87	1.4±2.1	7
5	Waan Maha Karn	6.53	3.8±5.6	5
6	Waan Maha Karn	7.19	3.7±3.5	4
7	Waan Maha Karn	4.61	4.8±2.8	11
8	Waan Maha Karn	5.46	4.0±1.4	7
Total				**52**

* All samples were collected from Bangkok except for sample 4 which was from Chiang Mai.

For the isolation of endophytic actinomycetes from these two medicinal plants, water washing of each surface-sterilized root samples was spread on to media to check the surface sterilization of root. There was no microbial growth on starch casein agar plates after 14 days of incubation. The result which indicated that the obtained actinomycetes were endophytic actinomycetes. Almost all of the isolation plates showed no growth of actinomycetes after 4 weeks of incubation except sample 6 (root of Waan Maha Karn) which yielded 2 actinomycetes strains designated as KKD096 and KKD098.

Colour grouping and chemotaxonomy

Fifty two rhizospheric isolates were assigned into 5 multi-membered colour groups based on their cultural characteristics on oatmeal agar (Table 2). The strains with grey spore (group 1 and 2) were dominated (42 strains), six strains were in white and one in pink. However, each group contained members with different colour of substrate mycelium and soluble pigment.

Analysis of whole-organism hydrolysates showed that all strains were rich in the LL-isomer of diaminopimelic acid, a result consistent with their assignment to the genus *Streptomyces*. The only exception was strain KKD096 which was assigned to non-streptomycete as it was rich in *meso*-form of diaminopimelic acid. This result is in agreement with previous reports that actinomycetes distributed in rhizosphere soils were mainly members of the genus *Streptomyces* (Gesheva, 2002; Xu *et al*.,1996).

Table 2. Colour group of streptomycete isolates on oatmeal agar

Group	Spore colour	Substrate colour	Soluble pigment colour	Isolates
1	dark grey	yellow	yellow	M2.1*, M4.2, M4.5, T2.1, T4.1,T4.3
		yellow	not produce	M1.4, M3.2, T1.2, T3.9
		pink	yellow	M1.9
		pink	not produce	M1.1, M1.5, M1.6, M3.1, T3.1, T3.2, T3.3, T3.6, T4.6
2	grey	grey	not produce	M1.2, M1.3, M4.3*, M4.4, T1.1
		yellow	yellow	M1.8, M2.2, M3.5, T1.3, T1.4, T2.4, T3.4, T4.7
		yellow	not produce	M1.7, M1.10, M3.4, M4.7, T2.2, T2.3, T3.5*, T4.4
		pink	not produce	T3.7, T3.8, T3.10, T3.11
3	white	yellow	not produce	M1.11, M4.6, T1.5, T4.5
		yellow	brown	M4.1*
		black	dark brown	M3.3
4	pink	purple	yellow	T4.2*

* The strains which were selected for 16SrRNA gene sequencing.

Antimicrobial activities

Screening of antimicrobial activities of all actinomycete isolates (54 strains) showed that 5 strains could inhibit all test bacteria and yeast. Thirty one strains (58%) were active against at least one of the test organism and 12 strains (22%) of the total isolates showed activities against *C. albicans*. Twenty nine, seventeen and twenty one strains showed activities to inhibit *B. cereus, E. coli and S.aureus*, respectively. The remaining 23 strains did not show any inhibition against any test organisms. The result was in agreement with report of Basilio *et al*. (2003) that streptomycete isolates showed inhibition to Gram-positive test bacteria better than both Gram negative bacteria and yeast.

Antimicrobial activities of isolates M2.1, M4.1, M4.3, T3.5 and T4.2 that inhibited the growth of all test organisms was presented in Table 3. These strains were isolated from the soil sample numbers 3, 4, 6 and 8. Ratios of diameter of inhibition zone/colony diameter are in range of 1.2 to 6.0. Strains M4.1 and M 4.3 showed the highest inhibition activities against *E. coli* and *B. cereus*, respectively. However, the ratio of the strains producing antimicrobial substances may be higher if the cultivation conditions were optimized.

Table 3. Antimicrobial activity of representative isolates against test microorganisms.

Isolates	Diameter ratio of inhibition zone/colony diameter			
	Candida utilis	*Bacillus cereus*	*Escherichia coli*	*Staphylococcus aureus*
M2.1	1.3	2.4	1.4	1.2
M4.1	1.2	3.9	2.3	1.2
M4.3	1.2	6.0	1.2	1.2
T3.5	2.3	1.8	1.2	1.2
T4.2	2.7	2.9	1.4	1.4

Screening of antimicrobial activities from endophytic actinomycetes showed that strain KKD098 could inhibit the growth of *B. cereus* and *E. coli* whereas strain KKD096 did not inhibit any test organism.

Molecular identification

More than 700 bp of the 16SrRNA genes of strains M2.1, M4.1, M4.3, T3.5 and T4.2 in the gamma region were sequenced. Analysis of these sequences by BLASTN indicated that these strains were members of the genus *Streptomyces*. It is evident from Figure 1 that all 5 selected isolates are in the genus *Streptomyces*. These strains showed 99 to 100% similarity with their nearest neighbours, values equivalent to 0-2 nucleotide differences.

The endophytic strains KKD096 and KKD098 were found to be closely related to genus *Kineococcus* and *Streptomyces*, respectively. The strain KKD098 shared 97% similarity to *S. kunmingensis* NBRC 14463^{T} with 18 bases differences from total 740 bases within the gamma region. The strain KKD096 which showed low similarity to its nearest neighbour from BLASTN search was subjected to the sequencing of full length 16SrRNA gene. It was found that isolate KKD096 shared 97.11 % similarity to *Kineococcus radiotolerans* DSM 14245^{T}, a value which corresponds to 38 nucleotides differences. This finding suggested that isolate KKD096 may represent a novel species within the genus *Kineococcus*. This is the first report on the isolation of members of the genus *Kineococcus* from plant root. The taxonomic description of this putative novel species will be published elsewhere.

Conclusion

The result of screening actinomycetes from the rhizospheric and root associated of Thai medicinal plant showed that the genus *Streptomyces* appears to be abundant in rhizospheric soil.

Fifty eight percent of the isolates were active against at least one of the test organisms. These findings provided evidence that rhizosphere of medicinal plant is a potential source for isolation of bioactive producing actinomycetes. The presence of putative novel endophytic actinomycetes also supported the view that plant associated habitat is still a poorly studied environment which waiting to be explored.

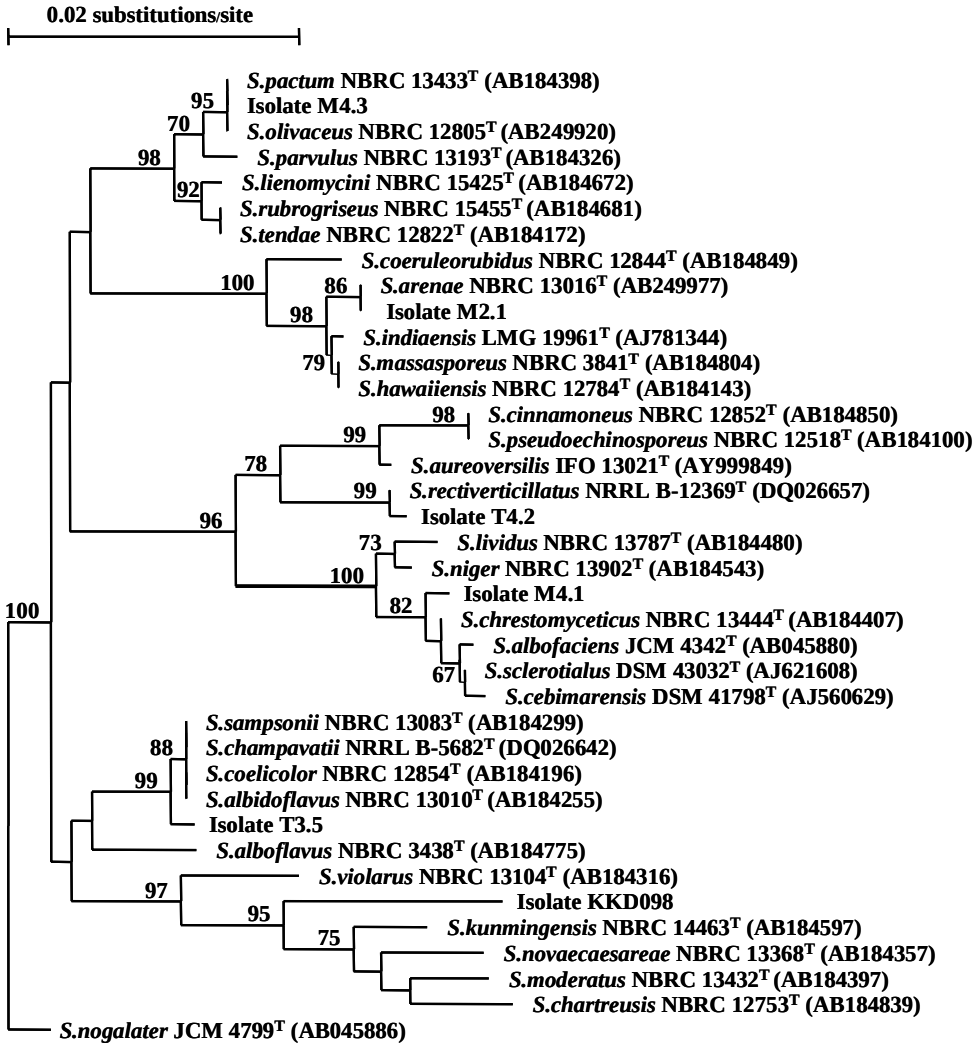

Figure 1 Neighbour-joining tree based on 740 bases of 16SrRNA gene sequences showing relationships between the isolates and marker strains of the genus *Streptomyces*. The numbers at the nodes indicate the level of bootstrap support (%) based on a neighbour joining analysis of 1000 resampled datasets.

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