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PIGMENTED *ACTINOMYCETES* FROM COASTAL AREAS AND THEIR BIOACTIVE SECONDARY METABOLITES.

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ABSTRACT

Forty soil samples were collected from coastal areas of Chonburi, Rayong, and Chanthaburi provinces. These samples were pretreated at 55 °C for 15 min and 100 °C for 1 h, respectively. They were subsequently made a series of 10-fold-dilutions and inoculated onto starch casein agar and humic acid vitamin agar plates to look for *Actinomycetes* that having some of bioactive secondary metabolites. Various strains of *Actinomycetes* grown on both agar plates were selected after 7-14 days of incubation at 32°C. The purification of selected grain was made to obtain pure isolates. One hundred and seventy nine isolates were screened for antimicrobial activity and 122 isolates were found to produce active products against gram-positive and/or gram-negative bacteria, including yeasts. *Micrococcus luteus* TISTR 884, *Staphylococcus aureus* TISTR 885, and *Staphylococcus aureus* 815(MRSA 815, the methicillin resistant strain) were used as representative strains of gram-positive bacteria in the antimicrobial activity test. They were inhibited by most *Actinomycetes* antimicrobial producing strains, while gram-negative bacteria, *Pseudomonas aeruginosa* TISTR 781, was inhibited by 11 isolates of *Actinomycetes*. Most antimicrobial producing isolates could inhibit against both bacteria and yeasts, and 29 isolates could inhibit only yeasts. *Candida albicans* TISTR 5239, *Candida tropicalis* TISTR 5045, *Debaryomyces hansenii* TISTR5265, *Pichia kluyveri* TISTR 5150, and a hospital strain of *Candida* sp. were used as test strains. Most isolates, both antimicrobial producers and non-antimicrobial producers, were able to produce colorful colonies as well as soluble pigments in some strains.

Keywords: Pigmented *Actinomycetes*, antimicrobial substances.

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INTRODUCTION

Actinomycetes are found mainly in soil. More than one million *Actinomycetes* can be found in one gram of fertile soil. The most important thing is that *Actinomycetes* are commomly known as producers of bioactive compounds for medicinal, agricultural, ecological and industrial uses (Williams et al., 1989; Miyadoh et al., 1979; Naidenova and Vladimirova, 2002; Dastager et al., 2006). A literature survey of the Antibiotic Literature database (ABL) and from the Bioresearch Italia database revealed more than twenty-three thousand microbial products possessing some biological activity, i.e., antifungal, antibacterial, antiviral, anti-tumor, cytotoxic and immunosuppressive activity. Forty-six percent out of more than eight thousand antimicrobial products described in the ABL database are produced by *Streptomyces*, 21.5% are produced by fungi. Other bacteria produce 16.9% and another 16% produced by rare genera of *Actinomycetes* (Lazzarini et al., 2000).

At the same time, many genera of *Actinomycetes* produce various kinds of pigments: melanoid, carotenoid, violacein, prodigiosin, anthocyanin, caerulomycin, etc., and some species can produce various colors of diffusible pigments. *Streptomycetes* produce anthracyclinglycoside, diazindophenol, naphthoquinone, phenoxazinone, and prodigiosin pigments , but more extensive studies are needed before the value of pigment type can be objectively assessed (Williams et al., 1989). Some antibiotics produced from *Actinomycetes* are colorful; i.e., rifampicin, erythromycin, violacein, prodigiosin, hygromycin A, hygromycin B and caerulomycin, that were discovered many decades ago and still play an important role in medical and pharmaceutical works. The main purposes of this present study were to search for new antimicrobial antibiotics from *Actinomycetes* isolated from some coastal areas in the east coast of Thailand, and to search for useful dyes from these colorful microorganisms.

MATERIALS AND METHODS

Isolation of Actinomycetes

Forty soil samples were collected from various locations along the coastal areas in the east coast of the Gulf of Thailand, i.e., Chonburi, Rayong, and Chanthaburi provinces. The humidity, pH, and humic acid of each soil sample were recorded. Ten grams of each soil sample were pre-treated at 100° C for 60 min to eliminate other soil bacteria and also to activate the dormant bacterial spores (Ruan, 1994). They were subsequently suspended in 100 ml peptone water solution (Athalye et al., 1981), mixed, diluted and spread on starch casein agar and humic acid vitamin agar plates. The plates were incubated at 32°C and examined weekly for four weeks. Pigmented colonies of suspected *Actinomycetes* were selected, purified and kept for morphological and biochemical studies according to methods described by others (Williams et al., 1989; Ruan, 1994). All selected isolates were used to screen for antimicrobial-producing strains.

Morphological characteristics

cover slip technique was used for observing morphology of *Actinomycetes*-like microorganism. Agar plate was inoculated with cover slips inserted at an outside angle. The cover slips were withdrawn after 3-7 days of incubation, mounted on micro-slides and examined for spore chains and hyphae of both aerial- and substrate-mycelium under light microscope (Williams et al., 1989). Some of promising strains were further examined by scanning electron microscopy.

Test organisms and antibiotic bioassay

Antibacterial and antifungal activities were tested against *Micrococcus luteus* TISTR 884, *Staphylococcus aureus* TISTR 885, *S. aureus* TISTR 517(ATCC25923), *S. aureus* (MRSA 815, a methicillin resistant strain), *Pseudomonas aeruginosa* TISTR 781, *Candida albicans* TISTR 5239, *C. tropicalis* TISTR 5045, a hospital strain of *Candida* sp., *Debaryomyces hansenii* TISTR5265, and *Pichia kluyveri* TISTR 5150. Antibacterial and antifungal activities were initially screened by using the cross-streak method.

Each *Actinomycetes* isolate was streaked on Mueller Hinton(MH) and Potato Dextrose Agar (PDA) plates for 3 days, incubated at 30°C. The test organism was perpendicularly streaked across the given known fungus and incubated for 1-3 more days. Those promising isolates were sub-cultured in broth medium and shaked at 110 rpm reciprocally, at 30° C for 7 days. The antimicrobial products were extracted with ethyl acetate. Ten µl of the crude extract from each strain was applied to 6 mm sterile paper disc and placed on MH or PDA plates seeded with test strains. The antibacterial activity was examined and recorded after incubated for 24-48 hours.

Chemical characteristics of antimicrobial producing strains.

Only those of pigmented *Actinomycetes* that having broad-spectrum antimicrobial substances were selected for the study of the composition of cell wall by determining diaminopimelic acid and sugar pattern derived from whole-cell hydrolysates. The isolates were grown in glucose-yeast extract medium shaken at 110 rpm for 4-5 days. The mycelia were then collected by centrifugation at 8,000 rpm, washed twice with distilled water, and air dried at room temperature. Diaminopimelic acid and sugars of whole-cell hydrolysates were determined according to Ruan (1994).

RESULTS

It was found that 122 out of 179 isolates of *Actinomycetes* were antimicrobial-producing strains detected by using antibiotic bioassay plates. It appeared that 55 out of 122 isolates, which consisting of 52 isolates collected in Chonburi, 31 isolates collected in Rayong, and 39 isolates collected in Chanthaburi, were able to inhibit the growth of both pseudomycelia and vegetative cells of some *Candida, Pichia,* and *Debaryomyces.* Moreover, 29 of these 55 isolates produced only antifungal yeast substances, whereas only 11 isolates produced antibacterial product against gram-negative bacteria, *Pseudomonas aeruginosa* TISTR 781. The strains that have antimicrobial activity showed various levels of inhibitory effect are summarized in Figure 1. Most of antimicrobial producing strains had appearance in gray, white, brown, reddish orange spore mass with yellowish brown or light brown, yellow, or light yellow pink or reddish orange substrate mycelium as shown in Figure 2.

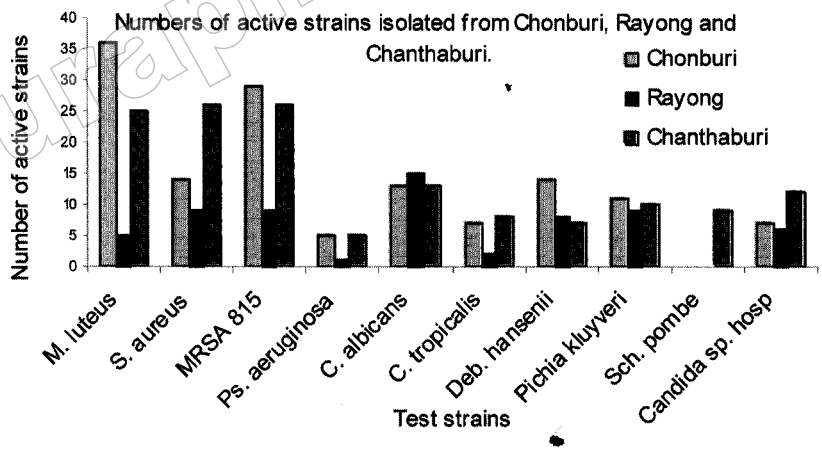

Figure 1. Antimicrobial producing strains isolated from samples collected in Chonburi, Rayong, and Chanthaburi provinces. They produced antimicrobial substances against some of test microorganisms, *B. subtilis* TISTR 008, *S. aureus* TISTR 885, *S. aureus* TISTR 517 (ATCC25923), *M. luteus* TISTR884, a methicillin resistant strain of *Staphylococcus aureus* 815 (MRSA 815), *Pseudomonas aeruginosa* TISTR 781, *Debaryomyces hansennii* TISTR 526, *Pichia kluyveri* TISTR 5150, *Schizosaccharomyces pombe* TISTR 5205, and a hospital strain of *Candida* sp.

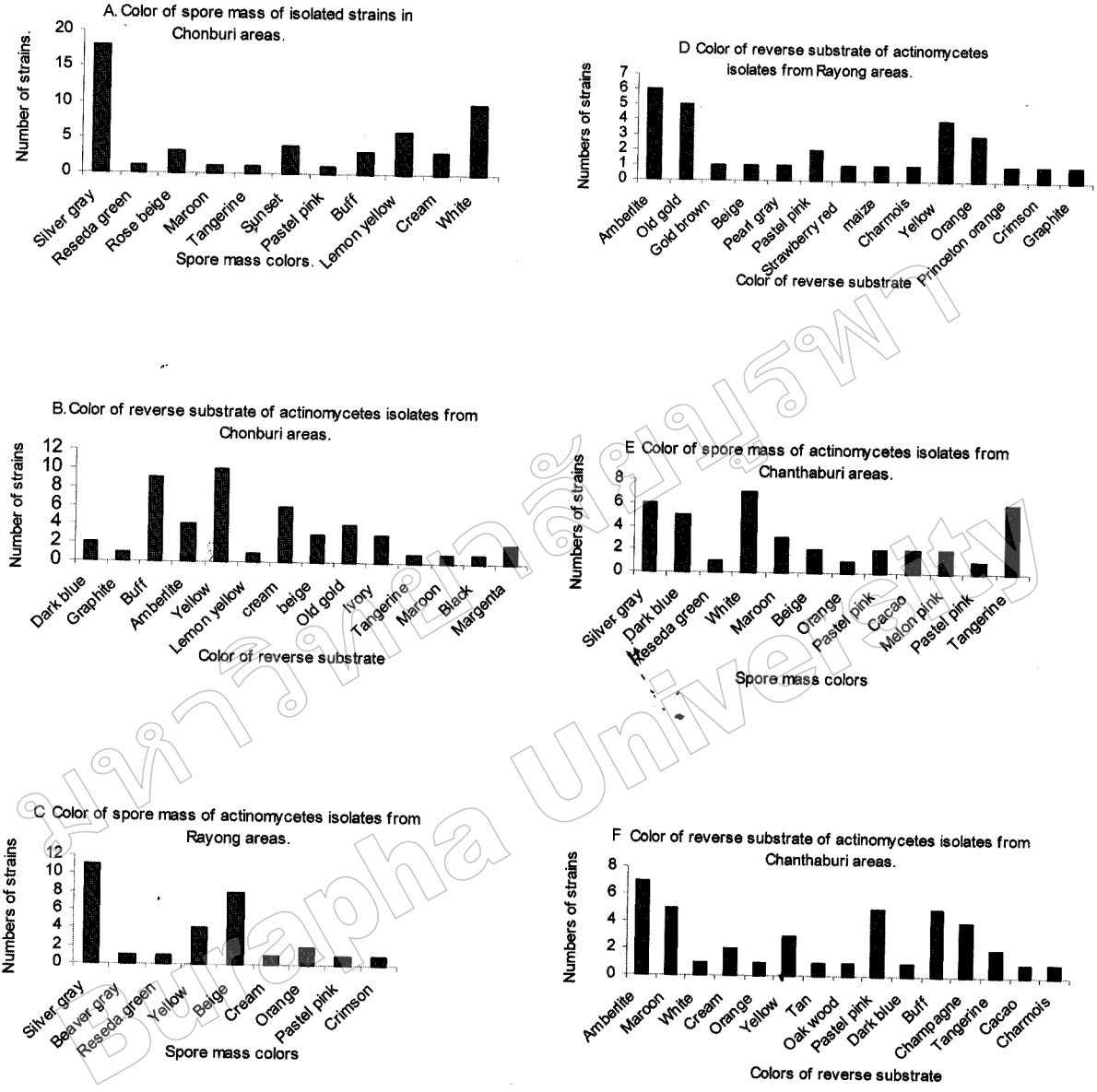

Figure 2. A, B, numbers of antimicrobial producing strains that produced color of spore mass and pigment in reverse substrate, which were found in Chonburi areas; C, D, E, and F are those found in Rayong and Chanthaburi areas, respectively. The color of spore mass and of pigment in reverse side of the colony was given according to the standard color card of America and U.S. Army Color Card.

Table 1. Some morphological and chemical characteristics of isolates which were able to inhibit growth both pseudomycelia and vegetative cells of some yeasts.

Strain no	DAP	Sugar pattern	Spore chain type	Genus	Strain no	DAP	Sugar pattern	Spore chain type	Genus
Chonburi					R76-8	*Meso*-DAP	gal,mad	S	Acd
C11-4	*L*-DAP	-	h, l	Strep	R76-9	*Meso*-DAP	gal,mad	S	Acd
C11-6	*Meso*-DAP	xyl,ara	mono	Min	R77-3	*L*-DAP	-	S	Strep
C11-9	*Meso*-DAP	xyl, ara	momo	Min	R77-5	*Meso*-DAP	gal,mad	S	Mad
C11-10	*Meso*-DAP	rib,gal, ara	zigzag	Pseu	R77-6	*Meso*-DAP	gal,mad	Rec	Mad
C11-12	*Meso*-DAP	gal, mad	h, spgm l,s,	Stpsp	R77-19	*L*-DAP	-	S,l	Strep
C11-16	*Meso*-DAP	gal, ara	irs	Noc	R79-16	*L*-DAP	-	S,h	Strep
*C11-17	*Meso*-DAP	rib,gal	mono	-	R79-17	*Meso*-DAP	gal,mad	s	Acd
C11-18	*Meso*-DAP	gal,xyl	-	Min					
*C11-20	*Meso*-DAP	rib,man,gal	irs	-	Chanthaburi				
C12-14	*Meso*-DAP	gal,mad	s	Stpsp	CH51-2	*Meso*-DAP	mad	Rect,l	Acd
C12-18	*L*-DAP	-	s	Strep	CH51-12	*L*-DAP	-	l,s	Strep
C12-22	*L*-DAP	-	h,l	Strep	CH52-4	*Meso*-DAP	mad	s,h	Acd
C13-3	*L*-DAP	-	rect	Strep	CH52-10	*L*-DAP	-	l,rect	Strep
*C13-7	*Meso*-DAP	mad,rhm,gal	s,h	-	CH52-7	*L*-DAP	-	l,rect,s	Strep
C13-8	*L*-DAP	-	s	Strep	CH53-3	*Meso*-DAP	man,rhm,gal	s	Stpallo
*C14-1	*Meso*-DAP	mad,rhm,gal	l,h	-	CH53-4	*L*-DAP	-	s	Strep
*C14-2	*Meso*-DAP	mad,rhm,gal	l,rect	-	CH54-1	*L*-DAP	-	rect,l	Strep
C14-10	*L*-DAP	-	l	Strep	CH54-4	*L*-DAP	-	rect,l	Strep
C14-12	*Meso*-DAP	gal,mad	l,rect,h	Acd	CH54-8	*L*-DAP	-	s	Strep
C15-1	*Meso*-DAP	gal,mad		Acd	CH54-12	*L*-DAP	-	-	Strep
					CH55-2	*L*-DAP	-	short	Strep
Rayong					CH56-5	*Meso*-DAP	mad	-spgm	Stpsp
R71-12	*L*-DAP	-	s,rect	Strep	CH57-11	*Meso*-DAP	man,rhm,gal	rect,spgm	Stpallo
R72-5	*Meso*-DAP	gal,mad	l,h	Acd	CH60-4	*L*-DAP	-	short	Strep
R73-12	*Meso*-DAP	gal,mad	s,l	Acd	CH60-5	*Meso*-DAP	gal,mad	rect	Acd
R74-12	*Meso*-DAP	gal,mad	rect	Acd	CH60-12	*L*-DAP	-	s,l	Strep
R74-26	*Meso*-DAP	gal,mad	s	Acd	CH62-4	*Meso*-DAP	man,rhm,gal	rect,spgm	Stpallo
R75-8	*L*-DAP	-	s	Strep	CH62-5	*Meso*-DAP	-	curl,zigzag	Nocop
R76-6	*Meso*-DAP	gal,man,rhm	s,spgm	Stpallo	CH62-9	*Meso*-DAP	-	curl,zigzag	Nocop

Symbol: * = unidentified

Abbreviations: ara = arabinose; gal = galactose; mad = madurose; man = mannose; rhm = rhamnose; rib = ribose; mono =1 spore; h = hook; irs = irregular spiral, l = loop or retinaculiaperti; rect = rectiflexibile, s = spiral; spgm = sporangium; Acd = *Actinomadura;* Min = *Micromonospora;* Noc = *Nocardia;* Nocop = *Nocardiopsis;* Pseu = *Pseudonocardia;* Strep = *Streptomyces;* Stpallo = *Streptoalloteichus;* Stpsp = *Streptosporangium.*

The ethyl acetate crude extracts of some promising isolates were confirmed and determined for the stability of antimicrobial substances by using disc diffusion method on Mueller Hinton Agar. Results of clear inhibition zone are shown in Figure 3. The chemical compositions of cell wall and of whole-cell hydrolysates as well as morphological study (see Table 1) revealed that most pigmented antimicrobial producing strains had wall chemotype I with spirale, hook and loop (or retinaculiaperti) spore chain type of *Streptomyces*. Some of other antimicrobial producers were *Actinomadura*, *Micromonospora*, *Streptoalloteichus* and *Streptosporangium*.

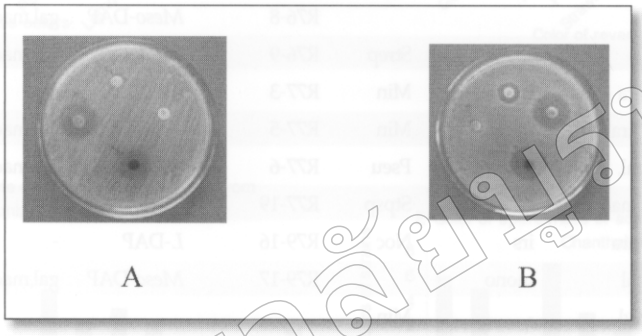

Figure 3. Examples of pigmented producing strains detected by Disc diffusion bioassay. They are red, yellow, and light yellow pigments. A = the inhibitory effect to *Candida albicans* TISTR 5426 and B = to *Candida albicans* MRSA 815.

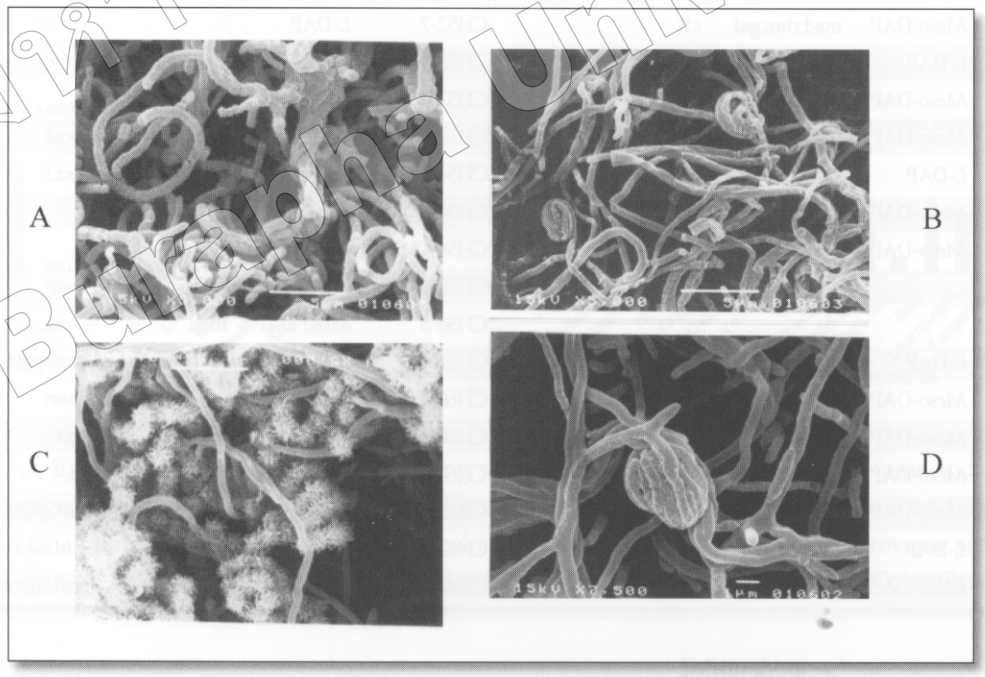

Figure 4. Examples of the electron micrographs of some pigmented *Actinomycetes* antimicrobial producing strains (A, B, C, and D), i.e., *Streptomyces* 15-1, *Streptomyces* 54-4, *Streptomyces* 9-8, and *Streptomyces* 54-4. The pictures taken at the magnification of 5,000X, 2,000X, 5,000X and 7,500X, respectively.

DISCUSSION

Results of chemical composition analysis of cell wall, the sugar pattern hydrolysates derived from whole- cell extracts, the morphological examination, and the color of pigments produced by the isolates with broad spectrum antimicrobial activity suggested that they were *Streptomyces*. Some of antimicrobial producing agents that are of interest were *Actinomadura, Micromonospora, Streptoalloteichus,* and few of *Streptosporangium,* which are rare *Actinomycetes*. In general, *Actinomycetes* can produce different kinds of pigments, i.e., gray brown, yellow, orange, red, purple, pink, green and black, excluding melanoid and some other soluble pigments. In this study, even though the isolates appeared to be less diverse in genera, the diversity of their spore mass colors or pigments produced in reverse side of colony including the studies of morphology and chemical composition analysis revealed that they were probably consisting of a variety of species or strains. Besides the special characteristics of branching hyphae and spore-bearing structure of *Actinomycetes* that draw attentions from biotechnologists and geneticists, the production of antimicrobial substances and pigments by these microorganisms are of interest (Williams et al., 1989; Al-Musallam et al., 2003; Bibb, 2005). Another characteristic of *Actinomycetes* is a diversity of metabolites that is advanced metabolic differentiation. To date, researchers have discovered approximately 10,000 biologically active compounds of microbial origin and roughly two-thirds of these are *Actinomycetes* products (Miyadoh et al., 1979). That is why researchers who work with *Actinomycetes* are still searching for some other resources to look for novel species and novel bioactive compounds (Meyers et al., 2003; Naidenova and Vladimirova, 2002; Stach et al., 2003; Zitouni et al., 2004; Sahin et. al., 2002). There were continual reports indicating that rare *Actinomycetes* or even in *Streptomyces* in which antibiotics naturally produced, some were novel species (Wu and Chen, 1995; Goodfellow et al., 2002; Sahin et al, 2002; Magarvey et al., 2004).

Preliminary tests for antimicrobial activity of the isolated pigmented strains obtained from three provinces clearly demonstrated that high percentage of many genera have the ability of producing antimicrobial compounds. It shows that the fertility of sample soils in coastal areas, one of the microbial resources for antimicrobial search is remained. Most of the antimicrobial producing strains could inhibit bacteria and yeasts, and some could inhibit gram-negative bacteria. Even the *Candida* sp. hospital strain that normally resisted most antibiotics was inhibited by compound obtained from many isolates. Some promising strains of *Streptomyces* in Figure 4 that produced pigmented antimicrobial compounds had spore chain types and spore surface that were rarely to be found and might be ones that would be useful to study further. In general, the isolated strains that have some morphological differences from known species have high tendency to be novel species or novel strains.

In our opinion, these results showed that *Actinomycetes* are awesome microorganisms which produce various kinds of precious secondary metabolites, not only antibiotic substances that are worthy for pharmaceutical companies, but also some other pigments which take important roles in many industrial sections. Furthermore, *Actinomycetes* may be an important gene pool for genes that encoding for pigments, and in the near future by genetic manipulation technique, agricultural researchers might have found new hybrids of orchids having various beautiful color shade of the flower petals.

Future work with the identification of antimicrobial producing *Actinomycetes* strains and their antimicrobial substances, including pigments and dyes are the aims of further studies.

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