ORIGINAL ARTICLE

Bioperspective of actinomycetes isolates from coastal soils: A new source of antimicrobial producers

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Abstract

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Bioperspective of actinomycetes isolates from coastal soils: A new source of antimicrobial producers

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Forty five soil samples were collected from four coastal islands on the east coast of Thailand: Chang, Hwai, Lao-yanai in Trat Province and Pai Islands in Chonburi Province. On 3 isolating media, Actinomycetes Isolation Agar, Starch Casein Agar and Glucose Asparagine Agar, 495 isolates of actinomycetes were found. Preliminary test to search for antimicrobial activity was done with *Bacillus subtilis* TISTR 008, *Staphylococcus aureus* TISTR 885, *Staphylococus aureus* TISTR 517 (ATCC 25923), *Micrococcus luteus* TISTR 884 and *Pseudomonas aeruginosa* TISTR 781 and *Escherichia coli* TISTR 887 (ATCC 25922). Fifty-eight actinomycetes were found to be antimicrobial-producing strains. From the morphological determination, cell wall diaminopimelic acid and sugars in whole-cell hydrolysate studies, among the 58 strains, *Streptomyces* sp. and *Actinomadura* sp. were the predominant genera. The other antibiotic active strains were *Micromonospora* sp., *Microbispora* sp., *Nocardia* sp., *Pseudonocardia* sp., *Saccharomonospora* sp., *Streptoalloteichus* sp. and *Streptoverticillium* sp. Most of them could inhibit gram-positive bacteria, especially *M. luteus* TISTR 884, and 8 strains (4 strains of *Actinomadura*, 2 strains of *Micromonospora*, 1 strain of *Microbispora*, and 1 strain of *Streptomyces*) could inhibit both gram-positive and gram-negative bacteria.

Key words : actinomycetes, antibiotic, *Streptomyces*, *Actinomadura*

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ส่วนสัดชีวภาพของแอคติโนมัยซีทที่แยกได้จากดินชายฝั่ง: แหล่งใหม่ของแอคติโนมัยซีทที่สามารถสร้างสารต้านจุลินทรีย์

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ได้เก็บตัวอย่างดิน จำนวน 40 ตัวอย่าง จากบริเวณเกาะชายฝั่งภาคตะวันออกของไทย คือ เกาะช้าง เกาะหวาย เกาะเหลายาใน ในจังหวัดตราด และเกาะไผ่ ในจังหวัดชลบุรี โดยใช้อาหารเลี้ยงเชื้อ 3 ชนิดในการแยกเชื้อ คือ อาหาร Actinomycetes Isolation Agar, Starch Casein Agar และ Glucose Asparagine Agar พบแอคติโนมัยซีททั้งหมด 495 ไอโซเลต และนำมาทดสอบเบื้องต้นเพื่อค้นหาแอคติโนมัยซีทที่สามารถในการสร้างสารยับยั้งแบคทีเรีย โดยใช้ *Bacillus subtilis* TISTR 008, *Staphylococcus aureus* TISTR 885, *Staphylococcus aureus* TISTR 517 (ATCC 25923) *Micrococcus luteus* TISTR 884, *Pseudomonas aeruginosa* TISTR 781 และ *Escherichia coli* TISTR 887 (ATCC 25922) เป็นแบคทีเรียทดสอบ พบว่ามีแอคติโนมัยซีทจำนวน 58 สายพันธุ์ สามารถสร้างสารแอนติ-ไบโอติกยับยั้งแบคทีเรียทดสอบได้ จากการตรวจสอบรูปร่างลักษณะทางสัณฐานวิทยา การศึกษาชนิดของกรดไดอะมิโนไพมิลิคที่พนังเซลล์และองค์ประกอบของน้ำตาลจากการย่อยสลายเซลล์ พบว่าใน 58 สายพันธุ์นั้นส่วนใหญ่เป็น *Streptomyces* sp. และ *Actinomadura* sp. นอกนั้นเป็น *Micromonospora* sp., *Microbispora* sp., *Nocardia* sp., *Pseudonocardia* sp., *Saccharomonospora* sp., *Streptoalloteichus* sp. and *Streptoverticillium* sp. และแอคติโนมัยซีทที่สามารถสร้างสารยับยั้งได้จะยับยั้งแบคทีเรียแกรมบวกเป็นส่วนใหญ่ โดยเฉพาะอย่างยิ่งสามารถยับยั้ง *Micrococcus luteus* TISTR 884 แต่ก็พบ 8 สายพันธุ์ คือ *Actinomadura* sp 4 สายพันธุ์ *Micromonospora* sp. 2 สายพันธุ์ *Microbispora* sp. 1 สายพันธุ์ และ *Streptomyces* sp. 1 สายพันธุ์ ที่สามารถสร้างสารยับยั้งได้ทั้งแบคทีเรียแกรมบวกและแกรมลบ

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Actinomycetes are gram-positive bacteria with high mole percent guanine plus cytocine content. They are found mainly in soil and more than one million actinomycetes can be found in one gram of soil (Miyado *et al*., 1997). Some genera are widely distributed such as *Streptomyces* and *Actinoadura* which can be isolated from soils of different regions (Williams *et al*., 1989). Among the gram-positive bacteria, actinomycetes exhibit the greatest morphological differentiation with braching hyphae and specialized spore-bearing structures (Vobis, 1997).

Actinomycetes are a particularly interesting group, as they are responsible for a substantial fraction of commercially important bioactive microbial products. A literature survey of the Antibiotic Literature Database (ABL), from the Bioresearch Italia database, on more than twenty-three thousand microbial products possessing some biological activity, i.e. antifungal, antibacterial, antiviral, antitumor, cytotoxic and immuno-suppressive, shows that the producing strains are mainly from the fungal kingdom and followed by strains belonging to the genus *Streptomyces* (32%). More actinomycetes produce 15.1% of the microbial products reported in the database and rare actinomycetes about 10.6% (Lazzarini *et al*., 2000).

Out of more than 8000 antimicrobial products described in the ABL database, 45.6% are produced by streptomycetes. Only 21.5% are produced by fungi, as many of the fungal products are either plant toxins or mammalian enzyme inhibitors. Other bacteria produced 16.9% of anti-infectives with another 16% produced by strains belonging to rare genera of actinomycetes

(Lazzarini *et al*., 2000).

Since the discovery of streptomycin, an aminoglycoside antibiotic, from *Streptomyces*, a large number of antibiotics and major therapeutic agents such as chloramphenicol, tetracyclines, macrolides and β-lactm cephamycin group have been obtained from many strains of *Streptomyces* and *Streptoverticillium* (Miyado *et al*., 1997).

In 1989, Matson *et al*. (1989) found novel antitumor antibiotic compounds, AT2433-A1, AT2433-A2, AT2433-B1 and AT2433-B2 produced by *Actinomadura melliaura*. A1, A2, B1 and B2 were active against gram-positive bacteria: *Staphylococcus aureus* ATCC 9537, *Streptococcus faecalis* ATCC20688, *Streptococcus faecium* ATCC 9790, *Micrococcus luteus* ATCC 9341, *Bacillus subtilis* ATCC 6633. A1 and B1 were active against P338 leukemia in mice. And Schroeder *et al*. (1995) found the compound BMY46164 from pure culture of *Actinomadura* Q473-8.

However, one result of intensive screening programs carried out over the past several decades indicated that there is a problem of rediscovery of already known bioactive compounds. One approach to solve this problem is to search for new sources of actinomycetes other than terrestrial soils. Coastal soils and the oceans could be another precious resource of both novel actinomycetes taxa and novel bioactive compounds. Goodfellow and Haynes (1984) reviewed the literature on isolation of actinomycetes from marine sediments and suggested that this source may be valuable for the isolation of novel actinomycetes with the potential to yield useful new products, and the taxonomic work was used to direct studies for natural product discovery.

In this study some rare actinomycetes were isolated from island soils where the ecological habitat has both terrestrial and sea water connections and a preliminary search for actinomycetes antimicrobial producing strains was carried out.

Materials and Methods

Isolation of actinomycetes

Forty five sample soils were collected from various locations along the coastal areas of Chang, Hwai, Lao-yanai in Trat Province and Pai Islands, Chonburi Province, in the Gulf of Thailand. Humidity, pH and humic acid of each soil sample were checked. Ten grams of each soil sample were pre-treated at 55ºC for 15 min and at 100ºC for 60 min to eliminate other soil bacteria and to activate the dormant spores (Nonomura and Hayakawa, 1988 cited by Ruan, 1994). They were then suspended in 100 ml peptone water solution (Athalye *et al*., 1981), shaken, diluted and spread on Actinomycetes Isolation Agar, Starch Casein and Glucose Asparagine Agar plates. The plates were incubated at 32ºC and examined weekly for four weeks. The actinomycetes colonies were selected, purified and kept for morphological and chemical studies (Williams *et al*., 1989; Ruan, 1994). All selected isolates were screened for antimicrobial-producing strains.

Morphological characteristics

Inclined coverslip technique was used for observing actinomycete morphology. Agar plate was inoculated with coverslips inserted at an angle. After 3-7 days incubation, the coverslips were withdrawn, mounted on microslides and the morphology of spore chains and hyphae, of both aerial and substrate mycelium, was examined under light microscope (Williams *et al*., 1989).

Chemical characteristics

Actinomycete cultures in Glucose-Yeast Extract medium were 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).

Test organisms and antibiotic bioassay

Antimicrobial activity was tested against *Bacillus subtilis* TISTR 008, *Staphylococcus aureus* TISTR 885, *Staphylococcus aureus* TISTR 517, *Micrococcus luteus* TISTR 884, *Escherichia coli* TISTR 887 and *Pseudomonas aeruginosa*

TISTR 781 obtained from the Thailand Institute of Scientific and Technological Research, TISTR. Actinomycetes in Glucose-Yeast Extract medium were incubated at 32ºC for 4-5 days before use and test organisms were cultured and swabbed on Mueller-Hinton Agar plates and incubated overnight at 32ºC. Twenty microlitres of filtrate broth from each isolate was then applied to sterile paper disc (Whatman) of 6 mm diameter and placed on the test strains and incubated at 32ºC for 12-24 hours (Coyle *et al*., 1984).

Results

Out of 495 isolates of actinomycetes, fifty-eight isolates were found to be antibiotic-producing strains on antibiotic bioassay plates. They were 22 *Streptomyces* spp., 13 *Actinomadura* spp., 5 *Nocardia* spp., 5 *Pseudonocardia* spp., 4 *Micromonospora* spp., 3 *Microbispora* spp.,3 *Streptoalloteichus* spp., 1 *Saccharomonospora* sp. and 1 *Streptoverticillium* sp. The results are summarized in Table 1. Most antimicrobial producing strains were pale grayish or grayish colonies with yellowish brown substrate mycelium, some were pale pinkish, whitish or whitish and changed into greyish-green, grey or greyish brown and some were black or maroon or orange. The strains having antimicrobial activity showed various levels of inhibitory effect against Gram-positive bacteria especially *Micrococcus luteus* TISTR 884. However, some strains which inhibited both Gram-positve and Gram-negative bacteria were found in all island soils except those from Lao-yanai island which had no inhibitory effect against Gram-negative bacteria.

Discussion

The active strains found belonged to both *Streptomyces* and rare species in moderately high proportion (11.7%). This rather low estimate of

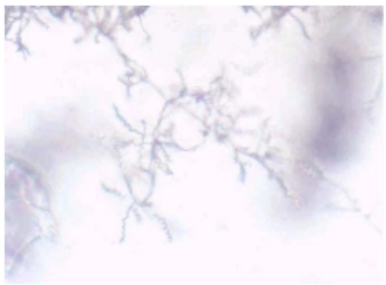

Figure 1. *Streptomyces* active strain, 7 day old on Starch Casein Agar at 32ºC, with spore mass in gray and substrate mycelium in yellow-brown.

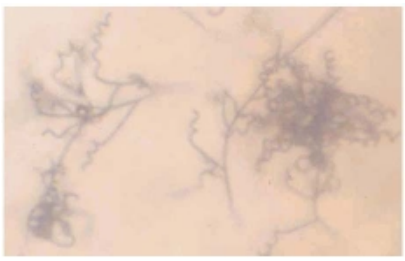

Figure 2. *Streptomyces* active strain, 7 day old on Starch Casein Agar at 32ºC, with spore mass in gray and substrate mycelium in maroon.

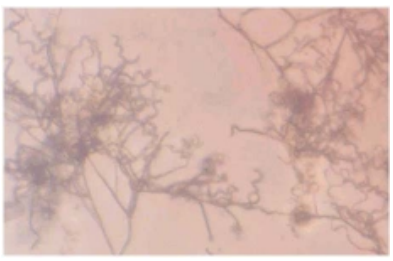

Figure 3. *Streptomyces* active strain, 7 day old on Starch Casein Agar at 32ºC, with spore mass in gray and substrate mycelium in orange.

Table1. Antimicrobial activity of active actinomycetes strains isolated from the four islands, against *B. subtilis* TISTR 008, *S. aureus* TISTR 885, *S. aureus* TISTR 517 (ATCC25923) *M. luteus* TISTR 884 *E.coli* TISTR 887 (ATCC 25922) and *Pseudomonas aeruginosa* TISTR 781.

No of strains	Activity against					
	B. subtilis **TISTR 008**	***S. aureus*** **TISTR885**	***S. aureus*** **TISTR 517**	***M. luteus*** **TISTR 884**	***Ps. Aeruginosa*** **TISTR 781**	***E. coli*** **TISTR 887**
Chang island						
Actinomadura sp.(4)	-	-	-	+	-	-
Actinomadura sp.(1)	-	-	-	+	+	-
Nocardia sp.(1)	-	-	-	+	-	-
Nocardia sp.(1)	-	+	+	+	-	-
Pseudonocardia sp.(1)	-	-	-	+	-	-
Pseudonocardia sp.(1)	+	+	+	+	-	-
Streptomyces sp.(13)	-	-	-	+	-	-
Streptomyces sp.(2)	-	+	+	+	-	-
Streptoalloteichus sp.(2)	-	-	-	+	-	-
Streptoverticillium sp.(1)	-	-	-	+	-	-
Lao-yanai island						
Actinomadura sp. (2)	+	+	+	+	-	-
Actinomadura sp. (1)	+	-	-	+	-	-
Micromonospora sp. (1)	+	+	+	+	-	-
Pseudonocardia sp.(1)	-	-	-	+	-	-
Pseudonocardia sp.(1)	+	+	+	+	-	-
Streptomyces sp.(1)	+	+	+	+	-	-
Streptoalloteichus sp.(1)	+	-	-	+	-	-
Hwai island						
Actinomadura sp.(2)	-	-	-	+	-	-
Micromonospora sp.(1)	-	-	-	-	+	-
Nocardia sp.(3)	-	-	-	+	-	-
Streptomyces sp.(4)	-	-	-	+	-	-
Pai Island						
Actinomadura sp.(2)	-	-	-	-	+	-
Actinomadura sp.(1)	-	-	-	+	+	+
Micromonospora sp.(1)	-	-	-	+	-	-
Micromonospora sp.(1)	-	-	-	-	+	+
Micromonospora sp.(1)	+	+	+	+	-	-
Microbispora sp. (2)	-	-	-	+	-	-
Microbispora sp. (1)	+	-	-	+	+	-
Pseudonocardia sp.(1)	-	-	-	+	-	-
Saccharomononspora sp.(1)	-	-	-	+	-	-
Streptomyces sp.(1)	+	-	-	-	-	-
Streptomyces sp.(1)	+	+	+	+	+	-

+ = positive activity, - = negative activity

the proportion of active strains may be due to the method of preliminary screening used. The small percentage of active strains might not indicate that the resource is not a good one, but rather that our method of screening was such that all isolates of actinomycetes could not grow well at the same incubation time. Moreover, there are so many factors which affect actinomycetes growth and antimicrobial compounds, including the chemical and biological environment. Hence, different specific antimicrobial-producing strains of actinomycetes need different kinds of media for producing substances. However, it is believed that the results shown in the table reveal that coastal soils from the islands are good sources for isolating actinomycetes. High or low numbers of active strains found depends on many factors: the sampling size, the probaility of right sample to be inoculated, the medium and methods of screening.

From many reports, it was found that novel actinomycetes could be found when a comparative taxonomic investigation was designed to establish the taxonomic relationship. Kim *et al*. (1998) found novel *Streptomyces*, *S. thermocarboxydovorans* and *S. thermocarboxydus* by comparing the 16S rRNA genes and phylogenetic tree of the four test strains: AT50, AT51, AT52 and AT37. Magarvey *et al*. (2004) reported two new genera within the family *Micromonosporaceae* of marine actinobacteria and interestingly, many of them had activities against multidrug-resistant gram-positive pathogens, malignant cells and vaccinia virus replication.

Generally, when the disc diffusion method was used for testing antimicrobial activity, the antimicrobial agent would diffuse into the medium around the paper disc, inhibiting the growth of the test organism if the concentration of substance was high enough. Normally, a large zone indicates more effective antimicrobial activity or greater diffusibility of the substance or both and no zone indicates complete resistance. On the other hand, a narrow zone might not indicate that the substance is not potent enough, but that it could not diffuse well into the medium because it was a non-polar substance or composed of rather non-polar components. The challenge now is to find more resources of actinomycetes screening from marine environments for future medicines.

Although most of the actinomycetes isolates could inhibit only Gram-positive bacteria, some of them were rare actinomycetes from which novel antimicrobial substances might be expected to be found. However, there were 8 strains in the genera *Actinomadura*, *Micromonospora*, *Microbispora* and *Streptomyces*, especially the strains from Pai Island, which produced antimicrobial substances inhibiting both Gram-positive and Gram-negative bacteria.

These data clearly show that coastal soils from the islands would be one of the valuable resources of novel antibiotic compounds, especially from rare actinomycetes apart from the sediments or in the oceans. Preliminary tests for antimicrobial activity of the isolated bacterial strains clearly demonstrated the ability of many genera to produce antimicrobial compounds. Generally, one active strain of actinomycetes might produce more than one active compound. The Trip Report by Dwight Baker, (Baker, 2004) referred to Goodfellow's premise that generally novel strains lead to the identification of new metabolites, which in turn lead to commercial successes.

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