ISOLATION OF STRAINS OF ACTINOMYCETES FROM JIAOZHOU BAY AND THEIR ACTIVITIES AGAINST PATHOGENIC MICROORGANISMS

CUI Hong-Xia[1,2], HOU Zhu-Mei[3] and QIN Song[1]

(1. Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071;
2. College of Environmental and Chemical Engineering, Yanshan University, Qinhuangdao 066004;
3. Hydrobiology and Engineering Department of Laiyang Agricultural College, Qingdao 266109)

Abstract: We isolated 224 actinomycetes strains from sea mud samples and 32 strains from seawater samples of the Jiaozhou Bay, China, by using the spread method. The isolates were grouped into 7 groups on basis of their morphological characteristics. Their antimicrobial activities were tested by using the cup-plate method, and the results showed that 7 % of the isolates were active against *Staphylococcus aureus*, 11 % against *Sarcine lutea*, 10 % against *Escherichia coli*, 11 % against *Bacillus pyocyaneus*, 2 % against *Candida albicans*, 5 % against *Cryptococcus*, 6 % against *Streptomyces viridochromogenes*, and 6 % against *Mucor miehei*. Twenty-two percent of isolates were active against at least one of the test organisms. Our data suggests that actinomycetes from the Jiaozhou Bay, China can produce various active metabolites against pathogenic microorganisms, which provids unique and plentiful sources to screen novel natural products for combating diseases in man.

Key words: Actinomycetes; Antimicrobial activity; Microorganisms; Metabolites
CLC number: Q93-331 **Document code:** A **Article ID:** 1000-3207(2007)05-0649-06

Marine microorganisms represent the greatest number of undescribed marine species[1]. These microorganisms have received increased attention over the past 20 years because of their physiological functions, exhibiting properties that possibly may provide a source for new secondary metabolites with potentially can be used as therapeutants[2—4]. Marine microorganisms have been screened for novel microbial products exhibiting antimicrobial, antiviral, anti-tumor, as well as anticoagulant and cardioactive properties[5—7]. Identified active compounds may function as model structures in the discovery and development of new drugs[8,9]. It has recently been shown that marine actinomycetes can produce useful diverse compounds with biological activities[10—12]. In this paper we describe the isolation of actinomycetes strains obtained from the Jiaozhou Bay, China and the subsequent screening for strains with activity against pathogenic microorganisms.

1 Materials and Methods

1.1 Media The Gause No. 1 medium was used for actinomycetes fermentation containing 20g soluble starch, 1g KNO_3, 0.5g K_2HPO_4, 0.5g $MgSO_4 \cdot 7H_2O$, and 0.01g $FeSO_4 \cdot 7H_2O$ dissolved in 500mL seawater and 500mL distilled water. The pH was adjusted to 7.2 before sterilization. Actinomycetes were isolated on solid Gause No. 1 including 0.003 % potassium dichromate medium. This was prepared by dissolving 15g agar and 0.03 g potassium dichromate in 1000 mL Gause No. 1 medium before autoclaving. 0.003 % potassium dichromate was used to suppress the growth of non-actinomycetes strains.

Received date: 2006-10-26; **Accepted date:** 2007-05-16
Foundation item: Hi-Tech Research and Development Program of China (2001AA624020); Key Innovative Project of the Chinese Academy of Science (KZCX3-SW-215)
Biography: Cui Hong-Xia (1977—), female, born in Shangzhi, Heilongjiang Province; Doctor, engaged in Bio-pharmaceutical. E-mail: cuihongxia2002@hotmail.com
Corresponding author: Qin Song, E-mail: sqin@ms.qdio.ac.cn

1.2 Sample collection Seawater and marine mud samples were collected by sterile methods from six different locations of the coast of the Jiaozhou Bay, Qingdao, East China. The samples were placed into sterile plastic tubes and sealed immediately by caps to avoid contamination and then taken to the laboratory where they were processed within 2h after collection.

1.3 Isolation of actinomycetes Portions (0.5mL) of seawater samples were transferred to 4.5mL of sterile seawater and subsequently diluted to 10 and 10^2. 0.1mL of serial dilutions (0, 10 and 10^2) and spread onto selective solid medium, Gause No. 1 plates including 0.003% potassium dichromate. Samples were incubated at 28 ℃. Single colonies were observed after 3—4 days, picked and transferred to Gause No. 1 agar plates and allowed to grow for one more week at 28 ℃.

One gram of each mud sample was resuspended in 9mL of sterile seawater. The mixtures were shaken vigorously for 30min and then allowed to settle for 1h. Portions (0.5mL) from the remaining suspensions (undiluted) were transferred to 4.5mL of sterile seawater and subsequently diluted in steps of 10, 10^2, and 10^3. Thereafter 0.1mL of the dilutions (10, $10^2$and 10^3) was spread onto a set of isolation plates as the seawater samples. Single colonies were observed after 3—4 days of incubation at 28 ℃and subsequently transferred onto Gause No. 1 agar plates to allow for further growth about 4—7 days.

1.4 Classification of actinomycetes The isolated strains were preliminarily identified according to the traditional morphological criteria, including the characteristics of colonies on the plates, substrate morphology, aerial hyphae and the pigments produced[13]. On basis of the morphology these strains were divided into several different groups.

1.5 Screening for antimicrobial activity of the isolates

1.5.1 Pathogenic microorganisms and culture conditions We selected 8 representative pathogenic microorganisms to study the antimicrobial activities of the isolates: two gram-positive strains (*Staphylococcus aureus* (CMCC 26003) and *Sarcine lutea* (isolated from the clinic)), two gram-negative strains (*Escherichia coli* (35218) and *Bacillus pyocyaneus* (CMCC 10104)), two yeast strains (*Candida albicans* (isolated from the clinic) and *Cryptococcus* (also isolated from the clinic)), one Streptomyces strain (*Streptomyces viridochromogenes* (Tü57)) and one fungus strain (*Mucor miehei* (Tü284)). The microorganisms used for antimicrobial assays and their culture conditions are shown in Tab. 1. *Streptomyces viridochromogenes* and *Mucor miehei* were made kindly available by the Department of Organic Chemistry, University of Göttingen, Göttingen, Germany D-37077. The other strains were provided by the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, P. R. China.

Tab. 1 Test-microorganisms used to assay the liquid cultures of actinomycetes and the respective culture conditions

Strains	Test-microorganisms	Media	Temperature	Components of media (1L)
G^+ bacteria	*Staphylococcus aureus* (CMCC 26003)	Nutrient-broth medium	37 ℃	5g beef extract, 10g peptone, 5g NaCl,
	Sarcine lutea (isolated from the clinic)			pH 7.0—7.2
G^-	*Escherichia coli* (35218)	-	-	-
bacteria	*Bacillus pyocyaneus* (CMCC 10104)			
Yeast	*Candida albicans* (isolated from the clinic)	Sabouraud's medium	28 ℃	10g peptone, 40g glucose, pH nature
	Cryptococcus (isolated from the clinic)			
Streptomyces	*Streptomyces viridochromogenes* (Tü57)	M_2^+ medium	28 ℃	10g malt, 4g yeast extract, 40g glucose, pH 7.4
fungus	*Mucor miehei* (Tü284)	-	-	-

Note: G^+ = gram positive bacteria; G^- = gram negative bacteria; "-" means as above

1.5.2 Sample preparation Pure cultures of isolates were inoculated in 20mL liquid Gause No. 1 medium

and grown in 100mL Erlenmeyer flasks were employed at 28 ℃for 4 days with shaking at 180r/min. All the fermented broth cultures were filtered with filter paper and the filtrates were used for antimicrobial testing.

1.5.3 Assay of antimicrobial activity The antimicrobial activity of the actinomycete isolates was tested using the cup-plate method (cylinder plate method)[14]. The pathogenic test bacteria and fungi were incubated in liquid media for 18—24h and 48h, respectively. The suspension was adjusted to 2—8 $\times 10^7$ cell per mL by adding sterile distilled water. A diluted suspension (5%, v/v) was placed into the respective solid medium which melted at about 45 ℃and then the suspension was mixed and poured onto Petri dish (9cm in diameter). Stainless steel cups (6mm in diameter) were placed on the surface of the assay plates and 300μL of the actinomycetes filtrate was pipetted into the cups. The assay plates were incubated at 37 ℃(bacteria) or 28 ℃(fungi) and inhibition zones (mm) around the cups were measured by ruler after 24h (bacteria) or 48h (fungi).

2 Results

2.1 Isolation of actinomycetes

Eighteen sea mud and 12 seawater samples were collected from the coast of Jiaozhou Bay, China. A total of 224 actinomycetes strains were isolated from sea mud samples, and 32 strains from seawater samples. Through combining the results of 16S rDNA sequencing (data not shown) with the morphological characteristics of the isolates, we found one strain as *Actinomadura* and 14 strains as *Streptomyces* spp of 15 isolates. These results suggest that *Streptomyces* is the most dominant actinomycetes species present in our sampling area.

As outlined in Tab. 2, all the isolates were divided into one of the following groups: White series, Gray series, Brown series, Pink series, Blue series, Green series and Yellow series. Assignment to a certain group was based on the color of the mature sporulated aerial mycelium, diffusible pigments and color of the substrate mycelium when grown on Gause No.1 medium. A total of 95 of isolates belonged to the White series, 121 to the Gray series, 6 to the Brown series, 5 to the Pink series, 8 to the Blue series, 5 to the Green series and 16 to the Yellow series. There were different colors of aerial hyphae and substrate hyphae in every series. Isolates belonging to either the White series or Gray series were the principal strains among the isolated actinomycetes from Jiaozhou Bay, China.

Tab.2 Characteristics by color of hyphae and substrate hyphae and generating diffusible pigment or not and number of the actinomycete groups isolated from the Jiaozhou Bay, China

Group	Color of aerial hyphae	Color of substrate hyphae	Diffusible pigment	Number of strains
White series	White	Colorless, yellow, pale yellow, orange, red, green, brown, blue	±	51
	Milky white	Colorless, yellow	-	13
	Yellowish white	Colorless, yellow, brown	±	19
	Unclear	Green, yellow, white, brick red, brown	±	12
Gray series	Pale gray	Colorless	-	25
	Gray	Colorless, pale yellow, yellow, green, blue	±	82
	Dark gray	Violet, yellow, pink, white	±	14
Brown series	Brownish orange	Yellow, green, brownish red	±	5
	Grayish brown	Colorless	-	1
Pink series	Pink	Pale yellow, orange, white, brown	±	5
Blue series	Pale blue	Blue, yellow, brown	±	4
	Blue	Colorless, yellowish brown, yellow, grayish blue	±	4
Green series	Green	Pale yellow, coffee, brownish red	±	5
Yellow series	Yellow	Yellow, pink, violet	±	9
	Dark yellow	Brown, yellowish brown, pale yellow, brownish red	±	7
Total				256

Note: "-" = can not generating diffusible pigment; "±" = can generating diffusible pigment or can not

2.2 Screening for antimicrobial activity

All the 256 isolated strains were fermented and assayed for antimicrobial activity against 8 representative test-microorganisms (see Fig. 1 for an example of actinomycetes possessing activity against to different pathogens). The percentage of active actinomycetes strains against Gram$^+$, Gram$^-$ bacteria, yeast, *Streptomyces*, and fungus was 13 %, 12 %, 7 %, 6 % and 6 %, respectively (see Tab. 3). The activity against the following strains, *Staphylococcus aureus*, *Sarcine lutea*, *Escherichia coli*, *Bacillus pyocyaneus*, *Candida albicans*, *Cryptococcus*, *Streptomyces viridochromogenes*, and *Mucor miehei* was 7 %, 11 %, 10 %, 11 %, 2 %, 5 %, 6 % and 6 %, respectively (see Tab. 3). Out of 256 strains, 57 isolates showed activity against at least one of the tested microorganisms. Thus the overall percentage of active strains is 22 % (Tab. 3). The activity of the different series (White series, Gray series, etc) was also investigated and the results are shown in Tab. 4.

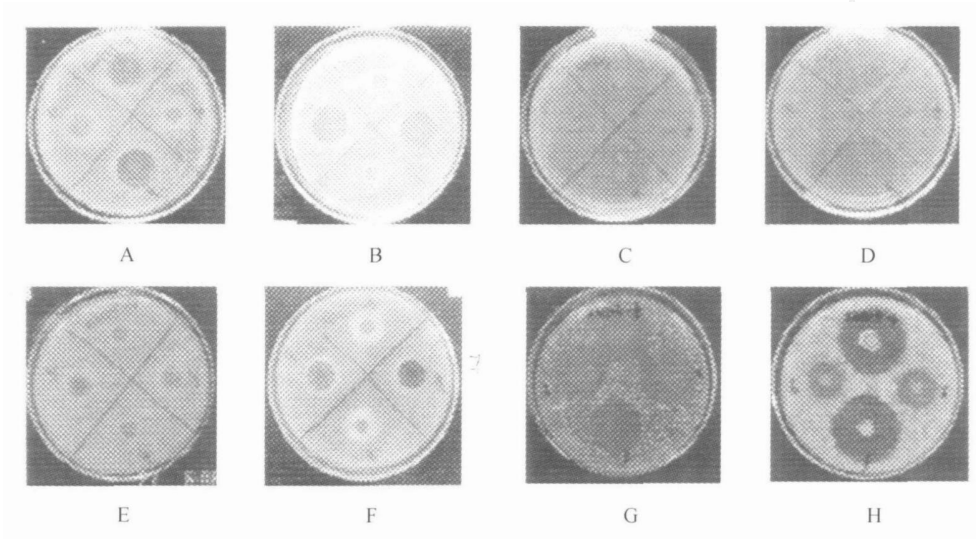

Fig. 1 The pictures of inhibition zone (transparent zone) of marine actinomycetes against *Staphylococcus aureus* (A), *Sarcine lutea* (B), *Escherichia coli* (C), *Bacillus pyocyaneus* (D), *Candida albicans* (E), *Cryptococcus* (F), *Streptomyces viridochromogenes* (G) and *Mucor miehei* (H). It showed that the actinomycetes had the antimicrobial activity in the pictures because the diameter of inhibition zone was more than 8 mm. Among them, the diameter of inhibition zone was high to 20mm in H picture

Tab. 3 Results of the screening tests for antimicrobial activities of actinomycetes isolated from the Jiaozhou Bay, China

Type of strains	Number of active actinomycetes	Activity ratio (%)	Test-microorganisms	Number of active actinomycetes	Activity ratio (%)
G$^+$ bacteria	34	13	*Staphylococcus aureus*	19	7
			Sarcine lutea	29	11
G$^-$ bacteria	30	12	*Escherichia coli*	25	10
			Bacillus pyocyaneus	27	11
Yeast	19	7	*Candida albicans*	5	2
			Cryptococcus	14	5
Streptomyces	16	6	*Streptomyces viridochromogenes*	16	6
fungus	16	6	*Mucor miehei*	16	6
Total	57	22	Total	57	22

Note: Activity ratio = Number of active actinomycetes against different test-microorganisms/ Number of actinomycetes isolated; Number of actinomycetes isolated = 256

Tab. 4　Antimicrobial activities of different group of actinomycetes

Group	Number of strains	Number of active strains	Activity ratio (%)
White series	95	21	22
Gray series	121	17	14
Brown series	6	2	33
Pink series	5	1	20
Blue series	8	3	38
Green series	5	2	40
Yellow series	16	11	69
Total	256	57	22

Note: Activity ratio = Number of active actinomycetes/ Number of actinomycetes

3　Discussion

Large-scale isolation and purification of microorganisms is the labor-consuming but pivotal step to screen potentially interesting antimicrobial compounds which might be of interest for the pharmaceutical industry, and the search for novel metabolites, especially from actinomycetes, requires a large number of isolates. As suggested by Oskay, *et al*.[15] this search will perhaps be more promising when plentiful diverse actinomycetes are sampled and screened. It was for this reason that, we isolated actinomycetes from marine samples. We observed that sea mud samples contain more strains than seawater samples. Perhaps actinomycetes adapt more easily to the sea mud environment. We also observed that the substrate colors and aerial mycelium were very different among seven groups, which show that the morphological diversities of these actinomycetes isolates are high. So we maybe probably obtain different kinds of metabolites of which some new produced by the isolated actinomycetes. The majority of the isolates were assigned to the White series or the Gray series. However, the activity ratio of isolates belonging to these series was lower than the activity ratio of isolated belonging to any other series (except pink series) (Tab. 4). This implies that the normal group of actinomycetes belong to the White and Gray series and that the strains possessing antimicrobial activity belong to the yellow and green series.

In this study, the cup-plate method was used to screen for strains with antimicrobial activity. The cup-plate method is a widely used method because it is highly sensitivity, simple, and cost-effective[16]. Moreover, it is easy and quick to operate because the liquid of the culture strains can be used directly for screening without further extraction. The results of cup-plate screening have good credibility. For example, isolate M048 has already been found to produce novel anticancer antibiotics[17], M095 can produce holomycin which has antifungal abilities[18], and M097 can generate the known compounds Lactoquinomycin and Feigrisolides A, B, C and D[19,20]. The further works for finding more new active compounds from isolates have been carrying on.

All actinomycetes strains isolated were tested for antimicrobial activity with the ultimate aim to find novel active compounds. Our results indicate that the percentage of actinomycetes with activity against *Candida albicans* was the lowest in comparison to the other strains tested (Tab. 3). It is perhaps worthwhile to intensify the screens for actinomycetes that possess antimicrobial activity against *Candida albicans* because it is a frequent yeast pathogen and isn't sensitive to the samples of actinomycetes in our study. Nevertheless, the activity of isolates against other test organisms is very strong (Fig. 1). A promising 22 % of actinomycetes isolates generated active metabolites against at least one of the test microorganisms. Our results indicate that the actinomycetes isolated from Jiaozhou Bay, China harbor a significant capacity to produce a variety of antimicrobial metabolites, which makes them interesting for programs screening novel natural products. Strains with antimicrobial activity offer ample opportunities to generate new types of active compounds.

4　Conclusion

Two hundred-and-fifty six (256) actinomycetes were isolated from sea mud and seawater samples. The ratio of actinomycetes with antimicrobial activity against one of the test strains (*Staphylococcus aureus*, *Sarcine lutea*, *Escherichia coli*, *Bacillus pyocyaneus*, *Candida albicans*, *Cryptococcus*, *Streptomyces viridochromogenes*, and *Mucor miehe*) was 22 %. The activity of the actinomycetes isolates is higher against bacteria than against the other test organisms. Isolates with antimicrobial activity could be a candidate source for new active antimicrobial com-

pounds and can be of interest for the pharmaceutical industry.

References:

[1] Colwell R R. Microbial biodiversity and biotechnology [A]. In Reanar-Kudla M E, Wilson E (Eds.), Biodiversity Ⅱ-Understanding and Protecting Our Biological Resources [C]. Washington D C: Joseph Henry Press. 1997, 279—287

[2] Fenical W and Jensen P R. Marine microorganisms: a new biomedical resource [A]. In Attaway D H, Zaborsky O R (Eds.), Marine Biotechnology Ⅰ-Pharmaceutical and Bioactive Natural Products [C]. New York: Plenum Press. 1993, 419—457

[3] Jensen P R and Fenical W. Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives [J]. *Annual Review Microbiology*, 1994, **48**: 559—584

[4] Fenical W. Chemical studies of marine bacteria: developing a new resource [J]. *Chemical Reviews*, 1993, **93**: 1673—1683

[5] Marderosian A D. Marine pharmaceuticals [J]. *Journal of Pharmaceutical Sciences*, 1969, **58**: 1—30

[6] Molinski T F. Developments in marine natural products. Receptor-specific bioactive compounds [J]. *Journal of Natural Products*, 1993, **56**: 1—8

[7] Austin B A. Novel pharmaceutical compounds from marine bacteria [J]. *Journal of Applied Bacteriology*, 1989, 67: 461—470

[8] Bernan V S, Greenstein M, Maiese W M. Marine microorganisms as a source of new natural products [J]. *Advances in Applied Microbiology*, 1997, **43**: 57—90

[9] Fenical W. New pharmaceuticals from marine organisms [J]. *Trends in Biotechnology*, 1997, **15**: 339—341

[10] Feling R H, Buchanan G O, Mincer T J, *et al*. Salinosporamide A: a highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus *Salinospora* [J]. *Angewandte Chemie International Edition English*, 2003, **42**: 355—357

[11] Mincer T J, Jensen P R, Kauffman C A, *et al*. Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments [J]. *Applied and Environmental Microbiology*, 2002, **68**: 5005—5011

[12] Rodriguez J C, Puentes J L F, Baz J P, *et al*. IB-00208, a new cytotoxic polycyclic xanthone produced by a marine-derived *Actinomadura* Ⅱ. Isolation, physico-chemical properties and structure determination [J]. *Journal of Antibiotics*, 2003, **56**: 318—321

[13] Goodfellow M, Cross T. Classification [A]. In Goodfellow M, Mordarski M, Williams S T (Eds.), The Biology of the Actinomycetes [C]. London: Academic Press. 1984, 7—164

[14] Abraham E P, Chain E, Fletcher C M, *et al*. Further observation on penicillin [J]. *Lancet*, 1941, **2**: 177—188

[15] Oskay M, Tamer A Ü, Azeri C. Antibacterial activity of some actinomycetes isolated from farming soils of Turkey [J]. *African Journal of Biotechnology*, 2004, **3**: 441—446

[16] Heatley N G. A method for the assay of penicillin [J]. *Biochemical Journal*, 1944, **38**: 61—65

[17] Maskey R P, Li F C, Qin S, *et al*. Chandrananimycins A-C: Production of novel anticancer antibiotics from a marine *Actinomadura* sp. Isolate M048 by variation of medium composition and growth conditions [J]. *The Journal of Antibiotics*, 2003, **56**: 622—629

[18] Cui H X, Li F C, Yan B L, *et al*. *Streptomyces* sp. M095 from Jiaozhou Bay produces inhibitory-fungal antibiotic, Holomycin [J]. *Chinese Journal of Marine Drugs*, 2006, **25**: 11—15

[19] Tang Y Q, Sattler I, Thiericke R, *et al*. Feigrisolides A, B, C and D, new lactones with antibacterial activities from *Streptomyces griseus* [J]. *Journal of Antibiotics*, 2000, **53**: 934—943

[20] Tanaka N, Okabe T, Isono F, *et al*. Lactoquinomycin, a novel anticancer antibiotic. I. Taxonomy, isolation, and biological activity [J]. *Journal of Antibiotics*, 1985, **38**: 1327—1332

胶州湾放线菌的分离及其抗病原微生物的活性检测

崔洪霞[1,2] 侯竹美[3] 秦 松[1]

(1. 中国科学院海洋研究所, 青岛 266071; 2. 燕山大学环化学院, 秦皇岛 066004; 3. 莱阳农学院水生生物与工程系, 青岛 266109)

摘要: 采用涂布法, 从胶州湾海泥样品中分离到 224 株放线菌菌株, 并且从海水样品中分离到 32 株放线菌菌株。根据形态学特性, 菌株被分成了 7 个组。同时利用杯碟法测定了它们的抗菌活性, 其中约 7 %对金黄色葡萄球菌有活性, 11 %对八叠球菌有效, 10 %对大肠杆菌有效, 11 %对绿脓杆菌有效, 2 %对白色念珠菌有效, 5 %对隐球菌有效, 6 %对绿色产色链霉菌有效和 6 %对米赫毛霉有效。分离到的 22 %放线菌对所测定的病原微生物有抗性作用。我们的结果表明胶州湾放线菌能够产生对病原微生物有抗性作用的不同代谢物, 这些代谢物可以作为筛选新颖天然产物的独特和丰富的资源。

关键词: 放线菌; 抗菌活性; 微生物; 代谢物