Screening of Sponge-associated Actinobacteria against Human Pathogenic *Candida albicans* in Kien Giang Sea, Vietnam

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Abstract**—** *From 198 actinomycetes isolated from sponges at the Kien Giang Sea in Vietnam, 42 actinomycete isolates were selected with the ability to resist Candida albicans, a species of yeast causes human diseases. There were eight isolates having strong resistance, 31 moderate, and three weak resistances. Eight best isolates were selected to identify by 16S rDNA gene analysis and sequencing. The result showed that six strains were characterized as Streptomyces spp., one strain belonged to genus Microbacterium, and one strain was genus Rhodococcus*.

***Keywords— Antimicrobial activity, Candida albicans, Kien Giang Sea, sponge, Streptomyces*.**

I. INTRODUCTION

Candida albicans is an opportunistic pathogenic yeast [1] that is a standard member of the human gut flora. It can also survive outside the human body [2]. It is detected in the gastrointestinal tract and mouth in 40–60% of healthy adults [3]. It is usually a commensal organism, but it can become human pathogenic in immunocompromised individuals under a variety of conditions [4]. It is one of the few species of the genus Candida that causes the human infection candidiasis, which results from an overgrowth of the fungus [5]. Candidiasis is, for example, often observed in HIV-infected patients [6]. Candidiasis is understood to cause gastrointestinal (GI) symptoms particularly in immunocompromised patients or those receiving steroids (e.g. to treat asthma) or antibiotics. Recently, there is an emerging literature that an overgrowth of fungus within the intestine of non-immunocompromised subjects may cause unexplained GI symptoms. *Candida albicans* is a species of yeast - a single-celled fungus, in fact – that lives naturally in the body. This yeast may be a normal part of the microbes that survive your skin and in your alimentary canal, but under some circumstances, it can multiply out of control. Small amounts of *Candida albicans* also sleep in various warm, moist areas throughout the body, including on the skin, within the mouth and gut, and also the rectum and vagina.

Elbendary [7] using ethyl acetate extraction method, the isolates culture's supernatants were tested by diffusion method against indicator microorganisms. These results indicated that actinobacteria isolated from Egypt farms could be sources of antimicrobial bioactive substances.

These identified isolates showed antimicrobial activity against the test organism, and the range of inhibition zones was between 2.5 and 10.2 mm. Out of 287 actinomycetes166 isolates were found antagonistic to *Candida albicans* isolated and selected a *Streptomyces* sp. [8]. Al-Dhabi [8] used the chromatogram of GC-MS analysis of this ethyl acetate extract (EA) had diverse chemical compounds namely benzene acetic acid (7.81%), acetic acid, methoxy-, and 2-phenylethyl ester (6.01%) were the major compounds. Minimum inhibitory concentrations (MIC) values were observed against Candida albicans and *Aspergillus niger* by (312 µg/ml).

This study aimed to selected and identify the Actinobacteria and discover potential sources of antimicrobial secondary metabolites to human pathogenic yeast, especially *Candida albicans*.

II. MATERIALS AND METHODS

2.1 Materials

One hundred and ninety-eight actinobacteria were isolated from sponges in the Kien Giang Sea, Vietnam, and *Candida albicans* (ATCC 10231) used for testing the agent of antibacterial isolates.

2.2 Screening assays for antibacterial activity

The liquid cultures were grown with shaking at 150 rpm for one day at 30°C. The broth was centrifuged at 5,000 rpm, 15 minutes. The supernatant was stored at 4°C. The *Candida albicans* test organisms were plated in the LB medium. The antimicrobial extract was added to the wells, the plates were incubated at 4°C for 2 hours for the diffusion of antimicrobial extract and observed for the zones of inhibition at 28°C for 48 hours.

2.3 The agar well diffusion method

The active isolates were cultured by the method given in the previous step. The supernatants were used for testing extracellular antimicrobial activity by the agar well diffusion method. By using a sterile cork borer, wells were punctured in the appropriate agar medium previously seeded with *Candida albicans*. One hundred microliters of the culture supernatants were added to each well. The plates were then incubated at 4^oC for at least 2 hours to allow the diffusion of crude extracts followed by incubation for 48 h at 28^oC for yeast. The diameters of inhibition zones were monitored and measured [9], and the positive control was nystatin.

Screening of isolated microorganisms had for inhibitory activity. The isolates screened for antibacterial metabolite production using the agar well diffusion method that inocula were prepared by growing the varied test organisms on separate agar plates. The colonies from plates were transferred with inoculating loop into 3 mL of normal saline in a test tube. The density of these suspensions adjusted to 0.5 McFarland standards.

By means of a sterile cork borer wells (8 mm in diameter) were made in the agar and filled with 0.2 ml of 72 hours culture of the isolated microorganism. Two replicates of the experiment were done, and the plates were incubated at 37°C for 18 hours. The diameters of the zone of growth-inhibition produced were measured, and the mean values calculated.

2.4 Genomic DNA extraction and 16S rDNA gene amplification and sequencing

Actinobacteria cells from these cultures were collected by centrifugation, and genomic DNA was extracted [10]. The PCR was performed in a final volume of 25 µl which was composed of about 50ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 ρM of Actinomycetes specific primers S-C-Act-0235-a-S-20 (5'-CGCGGCCTATCAGCTTGTTG-3') and S-C-Act-0878-a-A-19 (5'-CCGTACTCCCCAGGCGGGG-3') [11] and 1U of Taq polymerase with the appropriate reaction buffer under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 50s, annealing at 52°C for 50s, and 72°C for 90s. The amplified products were separated by gel electrophoresis in 1.2% agarose gels which were stained with Safeview dye.

2.5 Sequence analysis

The 16S rRNA gene sequences are compared with those from the type strains available in NCBI (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST).

For phylogenetic analysis, multiple sequence alignment performed using CLUSTALX, version 1.81. The Phylogenetic tree constructed using Mega 7.0. The consistency of the trees was verified by bootstrapping (1000 replicates) for the Neighbor-joining method.

2.6 Statistical analysis

The experimental results were analyzed as ANOVA with the isolates and with levels of diameters of inhibition zones. All analyses were conducted using the program MSTATC, Minitab 16. The data were considered significantly different at $P<0.01$. Duncan's test at $P = 0.01$ was used to differentiate between statistically.

III. RESULTS AND DISCUSSION

3.1 Screening assays for antibacterial activity

Total 198 isolates of endophytic actinomycetes were obtained from sponges collected from the Kien Giang Sea. However, there were 42/198 isolates against *Candida albicans* among 8 isolates strong resistance (7.1%), 31 moderate resistance (73.8%), and 3 weak resistance (19.1%) (Table 1, Figure 1).

TABLE 1
MICROBIAL ACTIVITY OF 42 ACTINOBACTERIAL ISOLATES ON *CANDIDA ALBICANS*

No	Actino-bacterial isolate	Diameter of Sterile ring (mm)	Evaluated of Galindo (2004)	No	Actino-bacterial isolate	Diameter of Sterile ring (mm)	Evaluated of Galindo (2004)
01	ND1.1a	23.0 c	+++	22	HD1.5c	6.0 t	++
02	ND1.3b	16.0 i	++	23	HD1.6a	18.0 g	++
03	ND1.5a	12.0 n	++	24	HD2.1a	18.0 g	++
04	ND1.5c	14.0 l	++	25	HD2.3a	16.0 i	++
05	ND1.7a	23.7 b	+++	26	HD2.3b	6.0 t	++
06	ND1.7b	25.7 a	+++	27	HD2.3c	26.0 a	+++
07	ND2.4	5.0 u	+	28	HD2.3d	3.0 v	+
08	ND2.6c	21.7 cd	+++	29	HD2.3e	11.7 o	++
09	ND2.7b	6.0 t	++	30	HD2.4a	17.0 h	++
10	ND2.7c	22.0 d	+++	31	HD2.5a	14.0 l	++
11	RL1c	6.0 t	++	32	HD2.5b	8.0 r	++
12	RN3c	6.0 t	++	33	HD2.5d	12.0 n	++
13	HD1.2a	5.0 u	+	34	HD2.6c	10.0 p	++
14	HD1.2c	26.0 a	+++	35	HD2.7d	15.0 k	++
15	HD1.3c	16.0 i	++	36	HD2.8l	14.0 l	++
16	HD1.3d	14.0 l	++	37	HD2.8p	14.0 l	++
17	HD1.3e	19.0 f	++	38	HD2.9a	14.0 l	++
18	HD1.3f	9.0 q	++	39	N1a	21.0 e	+++
19	HD1.4b	9.0 q	++	40	N4a	7.0 s	++
20	HD1.4d	6.0 t	++	41	N5c	13.7 m	++
21	HD1.5a	8.0 r	++	42	N10b	8.0 r	++
	CV (%) = 2.46				Positive control (tetracycline)	7.0 p	

Means within a column followed by the same letter/s are not significantly different at p < 0.01

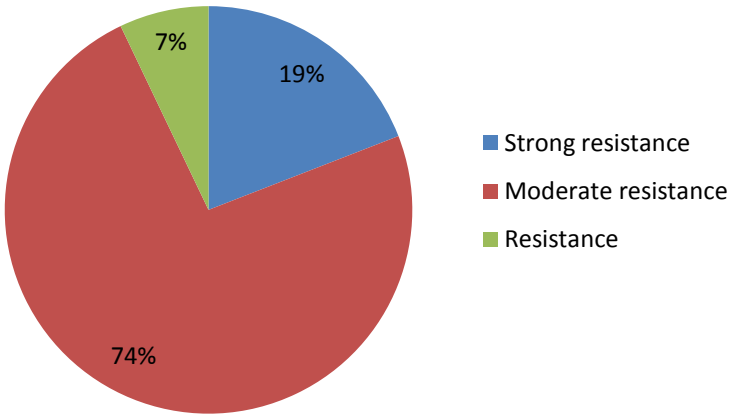

FIGURE 1: Microbial activity of 42 actinobacterial isolates to *Candida albicans* [9]

Based on evaluated of Galindo [9], the isolates as HD2.3c, HD1.2c, ND1.7b, ND1.7a, ND1.1a, ND2.7c, ND2.6c, and N1a were the best isolates with diameter >21 mm differed from the others statistically. They were chosen to identify by 16S-rDNA gene PCR technique and sequencing (Table 2).

3.2 Identify actinobacterial isolates

TABLE 2
PHYLOGENETIC AFFILIATION OF ISOLATES SUPPORTED 16S RRNA GENE SEQUENCES BY USING BLAST PROGRAM WITHIN THE GENBANK DATABASE SUPPORTED SEQUENCES SIMILARITY

No	Actinobacterial isolates	Cloest species relative	Similarity (%)
	Actinomycetaceae		
1	ND1.1a	*Streptomyces coelicolor* strain DSM 40233 (KY820720.1)	100
		Streptomyces sampsonii strain NRRL B12325 (KY820696.1)	100
2	ND1.7a	*Streptomyces tateyamensis* strain 18I (MG009024.1)	100
		Streptomyces chumphonensis strain HQA999 (MH041238.1)	100
3	ND1.7b	*Streptomyces ambofaciens* strain I (MK929479.1)	100
		Streptomyces olivaceus strain HQA933 (MH044533.1)	100
4	HD2.1c	*Streptomyces recifensis* strain WZS121 (MH497607.1)	100
		Brevibacterium sediminis strain YIM102079 (MN099340.1)	100
5	HD2.3c	*Streptomyces coelicolor* strain DSM 40233 (KY820720.1)	100
		Streptomyces sampsonii strain NRRL B12325 (KY820696.1)	100
6	N1a	*Streptomyces coelicolor* strain DSM 40233 (KY820720.1)	99.67
		Streptomyces sampsonii strain NRRL B12325 (KY820696.1)	99.67
	Microbacteriaceae		
7	ND2.7c	*Microbacterium tumbae* strain C3 (MG958700)	100
		Microbacterium kyungheense strain MK (MF373498)	100
	Nocardiaceae		
8	ND2.6c	*Rhodococcus hoagii* strain AL01 (MF928189)	100
		Rhodococcus equi strain TRB132 (KX981343)	100

A Neighbor-joining phylogenetic tree (Figure 2) of these isolates described the two clusters. Cluster A had five strains including *Streptomyces coelicolor* N1.1a, *Streptomyces coelicolor* HD2.3c with high similarity, both strains related with *Microbacterium tumbae* ND2.7c, and three had a high relationship closely with *Streptomyces coelicolor* N1a. All four had a relationship with strain *Rhodococcus hoagie* ND2.6 while cluster B had three strains: *Streptomyces recifensis* H1.2c, *Streptomyces tateyamensis* N1.7a, and *Streptomyces ambofaciens* N1.7b had a close relationship.

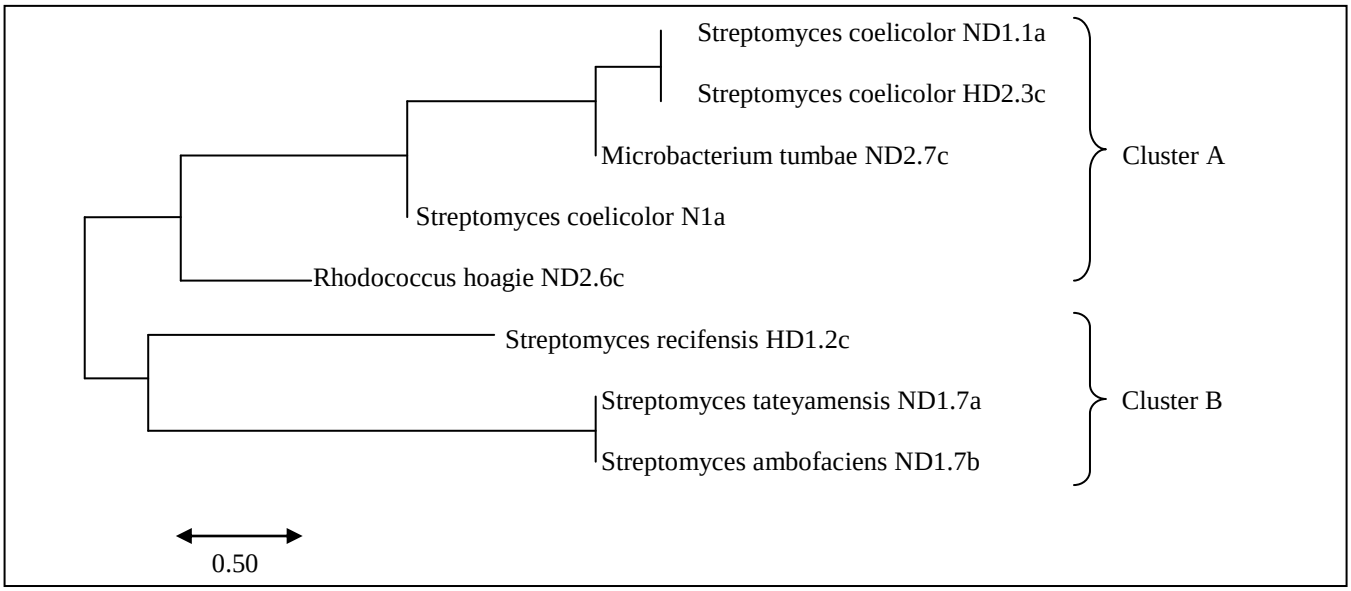

FIGURE 2: The Neighbor-joining phylogenetic tree of partial 16S rRNA gene sequences of actinobacteria isolated from sponges that closely related type strains. Numbers in the figure refer to percentage bootstrap values calculated for 1000 replicates. Bar, 0.02 was per nucleotide position.

Candida albicans is the most important fungal opportunistic pathogen of humans [12]. *Candida* species and other microorganisms are involved in this complicated fungal infection, but *Candida albicans* continue to be the most prevalent. In the past two decades, it has been observed that abnormal overgrowth in the gastrointestinal, urinary, and respiratory tracts, not only in immunocompromised patients but also related to nosocomial infections and even in healthy individuals. There is a wide variety of causal factors that contribute to yeast infection which means that candidiasis is a good example of a multifactorial syndrome [4]. The antibiotics for the treatment of infections are derived either directly from natural sources, semi synthesized from a natural product parent, or completely synthesized but modeled after a natural product lead compound [13]. It has been estimated that about two-thirds of the natural antibiotics have been isolated from actinobacteria, especially from the genus *Streptomyces* [14]. Belghit [15] found a strain of actinobacteria, designated G61, was isolated from Saharan soil and tested for its activity against these microorganisms. New antifungal antibiotics were active against *Candida albicans* and other pathogenic fungi. The analysis of G61 by PCR 16S rDNA gene and sequencing showed a similarity level of 100% with *Streptomyces mutabilis* NBRC 12800T and was determined NMR to be 2,4-Di-tert-butylphenol (2,4,DTBP). Palla [16] isolated the actinomycetes from Koringa mangrove soil samples near Kakinada, Andhra Pradesh, India. The scientists found the potent strain KMFA-1 having activity against dermatophytes *Candida albicans* and *Pectinotrichum llanense*. Based on physiological, morphological characteristics and 16s rRNA gene sequencing the isolated strain was identified as *Streptomyces hydrogenans*. The crude antifungal metabolite produced by the *Streptomyces* spp. isolate KMFA-1 is found to thermostable and the antifungal activity was not lost over a wide range of pH (2–10) indicating that it is active at various physiological pH. The antifungal activity of crude supernatant was not lost even after 24 months at 4°C, suggesting that the bioactive metabolite possess longer shelf life at refrigerated temperature and the specific nature of this bioactive metabolite produced by the selected isolate could be a diversified molecule and exerts unique mode of action to inhibit the growth of *C. albicans*.

Our results also discovered at least five actinobacterial strains (*Streptomyces* sp.) having the high antimicrobial activity to *Candida albicans* from originating from sponges in the Kien Giang Sea. Further investigations are necessary for isolation and chemical characterization of the compound by chromatographic and other spectral analysis as GC-MS.

IV. CONCLUSION

From 198 isolated actinomycetes, 42 isolates had the ability against *Candida albicans*, a species of yeast that causes human diseases. The six best isolates were chosen to identify by PCR 16S rRNA technique. They belonged to four *Streptomyces*, one *Microbacterium*, and one *Rhodococcus*. The present study showed that the potential bioactive compound from sponge-associated actinobacteria has not been exploited yet in the Kien Giang Sea, Vietnam. The other invertebrates as coral with endophytes containing many novel secondary metabolites especially, antibiotics, antimicrobial, antifungal, anti-cancer, and so on, need to study in the future.

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