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ANTIMICROBIAL POTENTIAL OF *STREPTOMYCES* ISOLATES FROM CON DAO NATIONAL FOREST

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SUMMARY

Streptomyces are considered the most potential microbes with the ability to produce antimicrobial and anticancer agents. These bacteria are found mostly in soil across the globe and play a pivotal role in material recycling processes. Isolation of *Streptomyces* in Vietnam has been conducted for years. However, there are few investigations on protected regions which have very little exchanging activities. This project aims to explore the resource for antimicrobial activity of *Streptomyces* isolated from Con Son Island (Con Dao). Eighteen soil samples were collected in different surface lands in Con Dao and cleaned prior to culturing and isolation. Twenty-five isolates (named from C1 to C25) were obtained from ISP4 agar plates and stored in glycerol 30% at -80^{o}C. Liquid cultures were established in 3 different media (ISP4, Gause I and TSB) for all isolates. Broth collected from cultures at stationary phase was extracted with ethyl acetate at the ratio 1:1 (v/v) followed with antimicrobial tests against three bacterial and two fungal microorganisms (*S. aureus, B. subtilis, E. coli, C. albicans, A. parasiticus*). Sixty percent of isolates show activity against at least one microbe. The isolates C13, C22 and C24 showed the ability to inhibit both bacteria and fungi tested. Results from C13 and C22 express remarkable activity to prevent the growth of *B. subtilis* and *C. albicans*, respectively. This study suggests the potential of *Streptomyces* from the investigated area and recommends more optimization of the culture condition as well as extractions with other solvents to get better antimicrobial activities.

Keywords: antimicrobial, antibacterial, antifungal, Streptomyces

INTRODUCTION

Antimicrobials are certainly one of the most successful forms of chemotherapy in the history of medicine. It is obvious that antibiotics have saved and significantly contributed to the control of infectious diseases that were the leading causes of human fatalities (Aminov, 2010). However, over the past decades, human is facing the potential drug resistance of bacteria and fungi with increasing frequency (Tor, Fair, 2014). New surveillance data released today by the World Health Organization (WHO) reveals widespread, and in some cases, high levels of antibiotic resistance across the globe in the most common bacterial infections. Despite the fact that number of antibiotics is large, searching for the effective compounds and controlling the spread of infections are still continuing, in which the need for new, safe and more effective antimicrobial agents is greater than ever in the pharmaceutical industry (Hopwood, 2007).

Streptomyces constitute 50% of the total

Actinomycetes population found in soils and play a key role in the material recycling (Mellouli *et al.*, 2003). This genus is known as the largest group of antibiotic producers with more than 75% of total antibiotic yield (Miyadoh, 1993). It is estimated that *Streptomyces* might produces at least 1 million new compounds of biological interest (Watve *et al.*, 2001). Over 500 species of *Streptomyces* strains have been described and the industrial significance of the genus as the producer of a wide variety of bioactive compounds has been well documented (Kim *et al.*, 2012). Secondary metabolites from *Streptomyces* have important applications in human medicine as antibacterial and antifungal agents.

Research on *Streptomyces* and antibiotic in Vietnam has brought some achievement over the last decades. Species from this genus have been found in almost every ecological area across the country. In 2006, Bui reported a collection of more than 500 isolates of *Streptomyces* from soils in some regions, in which some strains showed remarkably high antibacterial and antifungal activities (Bui, 2006). Strains found in Vietnam are considered promising for studying of antibiotics and further applications.

To explore other resources for *Streptomyces*, we investigated Con Dao Island whose the primitive forest is well protected or having very little exchanging activity. Using standard isolation methods, several isolates are classified by morphology and chemical activity. Preliminary antimicrobial activities were obtained from ethyl acetate extract of broth cultures in laboratory condition against common microbes. Most of the isolates showed the activity on at least one tested strain promising that the potential of these bacteria could be explored with more optimization of culture condition and/or extraction with other solvents.

MATERIALS AND METHODS

Soil sampling and bacterial characterization

Soil samples were taken from surface and from 10-centimeter depth, in variety of locations around Con Dao primitive forest. The samples were then air dried and transported to the laboratory. Prior to isolation, soil was sieved to eliminate dirty, death parts of animals and plant, and other non-biodegraded substances. NaCl 0.9% solution was used to prepare soil suspension for spreading. Single colonies with *Streptomyces* characteristics such as hard surfaces with smooth, clear and determined border, weft of aerial mycelium that appeared floccose, granular, and powdery or velvet were obtained and sub - cultured until purity. Strains were then selectively sent for 16S RNA sequencing for identification.

Culture media and culture conditions

ISP4 was used according to The International *Streptomyces* Project as good source for inorganic salts (Shirling, Gottlieb, 1966), while Gauze I and TSB (Tryptic Soy Broth) are rich in organic nitrogenous substances such as amino acids and proteins. These media were selected to explore the potential of isolates in producing active agents. For each isolate, culture in selected medium was prepared with 3 replicates.

Streptomyces cultures were prepared in either Gauze I, ISP4 or TSB liquid media. Single colony from each isolate was submerged in 50 ml liquid medium and incubated at 28ºC with shaking continuously for 4 days in TSB or ISP 4, and 7 days for those grew in Gauze I medium (Yong *et al.*, 2017). Growth curves were also built to determine that collection time is in the stationary phase where antimicrobial compounds were produced the most.

To prepare for the growth profile of the isolates, a single colony was cultured in liquid medium at 28ºC with shaking condition as described above. Samples were taken every 12 hours to check for optical density at 600 nm wavelength with sterile medium used as blank. All readings were plotted on the graph with time versus OD values.

Solvent extraction

In order to have the primary screening for the active fraction, broth from all cultures were

extracted with absolute ethyl acetate at the ratio 1:1 (v/v) by gently inversion for one hour. Organic fractions were collected and let air dried until no solvent trace remained. Decant after solvent evaporation from each culture was dissolved in 500 µl of distilled water and kept at -20^{o}C until used for antimicrobial test. Controls were also prepared by incubating culture media at the same conditions without microorganism.

Antimicrobial test

Two Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633), one Gram-negative bacterium (*Escherichia coli* ATCC 25922), one yeast (*Candida albicans* ATCC 10231), and one mycelial mold (*Aspergillus parasiticus*) were selected as indicators for antimicrobial tests. Forty microliters of above mentioned ethyl acetate extract from each culture were added into well diffusion tests, prepared in LB agar plates (for antibacterial tests) or malt extract agar (for antifungal tests). Each plate containing 10^6 CFU of indicator bacteria or fungi spread smoothly (Ogidi *et al.*, 2015; Balouiri *et al.*, 2016). Negative control (sterilized medium) and positive control for bacteria (ciprofloxacin 128 µg/ mL) and for fungi (itraconazole 180 µg/ mL) were also included in each test. All plates were maintained at 4°C for 30 min allowing perfusion of well content and then incubated at 37°C for 24 hours (Hadi *et al.*, 2013). Inhibition zone was determined as the length of clear zone diameter generated around the well with culture extract. Negative results indicated no inhibition zone formed.

Data analysis

Data were analyzed by One-way ANOVA and student t- test in SPSS software. The isolates were considered high activity if inhibition zone generated from the culture was higher significantly than the others with p value <0.05.

RESULTS AND DISCUSSION

Isolation of *Streptomyces*

From 18 soil samples, 25 strains of *Streptomyces* named from C1-C25 were isolated in ISP4 agar medium. These strains were observed and described based on morphological categories such as growth time, colony features, mycelial characteristics …

Colonies of *Streptomyces* appeared at different culture times. With continuous observing for one week, almost *Streptomyces* isolates came at almost 72 hours. While the average time was from 2 and a half to 3 days, isolate C3 showed its best enlargement after 48 hours and C20 grown the latest with the average time is around 84 hours (Figure 1). After 5 days from the emerging time, most of colonies gradually appeared aging characteristics as turning dark, forming spores along with the diminishing of the culture medium. All isolates were determined as medium to slow growing (Kunova *et al.*, 2016), aerobic, glabrous or chalky, folded and with aerial and substrate mycelia of different colors. These special strains have complex substrate mycelium that can plug deeply in the agar to collect the organic compound for development. Aerial mycelium, with the ability to produce spore, grow above the agar. Two mycelia combine in almost *Streptomyces spp.*, which help them to suffer in extremely poor condition. All isolates were grown in aerobic and mesophilic temperature (25 to 30^{o}C) in which most of the strains had the same white color for aerial mycelium, while substrate mycelium color was more diverse including red, white, yellow and brown. Several bioactive compounds from *Streptomyces* have color and are secreted to the medium, forming colorful colonies when these microbes go to the stationary phase. Pigment formation is therefore one of the characteristics to predict the potential of the *Streptomyes* to produce active compounds (Bibb, 1996). In total 25 isolates observed, white colony had the highest ratio compared with the others. There were 11 isolates having this dominant color, while yellow and brown color had 7 and 6 isolates, respectively. Only C14 had red colony. White color colonies were also obtained as the majority from all isolates in the investigations by An in Long An farm (An, 2014), or by Le in various regions across Viet Nam (Le *et al.*, 2014). Figure 2 describes the

number of isolates based on color morphology of substrate mycelium.

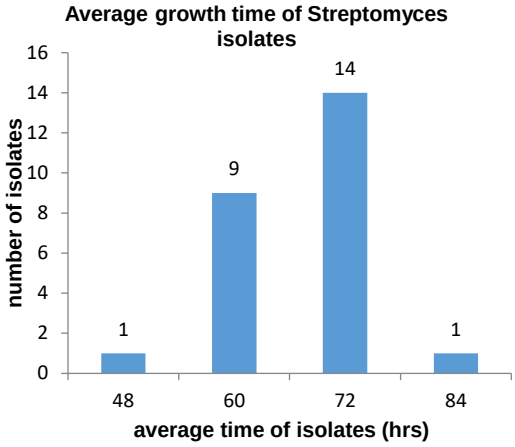

Figure 1. Average growth time of *Streptomyces* isolates.

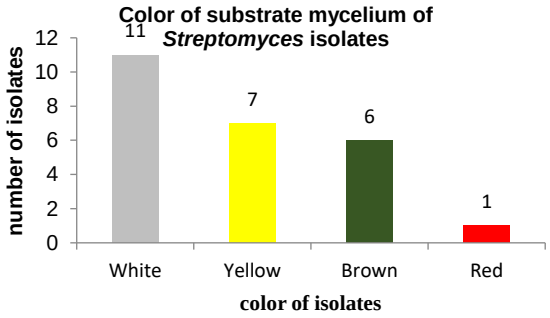

Figure 2. Color of substrate mycelium of *Streptomyces* isolates.

Evaluating the antimicrobial potential of *Streptomyces* isolates

The antimicrobial compounds production of microorganism is generally related to the nature of the habitat and the composition of the substrates. Antibiotic production in *Streptomyces* is considered sensitive, responding to the environmental conditions changed, especially carbon, nitrogen source and sensing compounds by activating different biosynthetic pathways to produce diverse classes of chemical substances. In this study, we used three different liquid media (ISP 4, Gause I and TSB), in which ISP 4 is purely inorganic while Gause I and TSB contain source of organic nitrogen and sugar. Each isolate was cultured in one medium then another. To investigate the secreted compounds from these isolates to the medium, culture broths from all isolates were collected by centrifugation, extracted with ethyl acetate and then tested against five chosen microorganisms. Variation in antimicrobial activity of *Streptomyces* against several pathogens has also been previously reported (Thumar *et al.*, 2010). In the first round of screening for antimicrobial activity, we roughly applied general extraction with mild solvent and tested on diverse groups of microorganisms such as Gram positive and negative bacteria, yeast and mold. In total number of twenty-five isolated *Streptomyces* subjected for the primary screening, fifteen isolates (60%) showed antimicrobial activity against at least one of the tested microorganisms (Table 1). The majority of the result showed the ability to inhibit Gram positive bacteria *S. aureus* (10 isolates), and *B. subtilis* (8 isolates). Followings are 7 isolates against *E. coli*, 3 isolates against *C. albicans* and 2 isolates against *A. parasiticus*. The results confidently indicate that, extraction with ethyl acetate can recover the secreted compounds from broth culture of all isolates in selected media, however, activities of these isolates are variable, and they mainly prevented the growth of bacteria under experimental conditions. Investigations by Bui and An on samples collected from different regions across Vietnam also indicated the majority of strains that inhibit Gram positive bacteria, and small percentage of isolates showing activity on both bacteria and fungi (Bui, 2006; An, 2014). Using the same procedure for culture and extraction, C3, C13, C22 and C24 strains can inhibit both bacteria and fungi, in which Streptomyces isolates C22, C24, C13 were comparatively more active than other isolates against Gram-positive bacterial and fungal microorganisms. These strains are considered for further optimization and genetic studies.

Data from the first screening proved that the signal from the ethyl acetate extracts of all isolates apparently have ability against tested microorganisms. We used ethyl acetate as first

choice for extraction because of its safety properties as low toxicity to human and animal cells. This low polarity and volatile solvent is expected to capture most of components in culture broth as crude extract, and be able to fast vaporize to collect fraction in extraction process. This is the reason why this solvent is always chosen firstly to screen for the activity of isolated microorganisms prior to more specific extractions and characterization of compounds (Thakur *et al.*, 2007; Le *et al.*, 2014; An, 2014). Compared to results of the experiment with simple filtering the culture broth through filter paper which had no antimicrobial activity (data not shown), the ethyl acetate extract could bring some signal of the production of secreted antimicrobial compounds by *Streptomyces* isolates.

Table 1. Antimicrobial activity of isolates against test microbes.

Inhibition zone diameter (mm)						
Isolate ID	**Medium**	***S. aureus***	***E. coli***	***B. subitilis***	***C. albicans***	***A. parasiticus***
Positive control		24 ± 0.0^{++}	24 ± 0.0**	30 ± 0.0	23 ± 0.0^{+}	11 ± 0.0^{+}
C2	Gause I	-	12 ± 0.0*	-	-	-
C3	ISP 4	-	-	-	**16 ± 0.0***	**12 ± 0.0***
C6	Gause I	-	13 ± 0.0^{+}	-	-	-
C7	Gause I	**21.9 ± 0.1**$^{++}$	**15.6 ± 0.6**$^{++}$	-	-	-
	ISP 4	15.8 ± 0.3^{-}	-	-	-	-
C9	Gause I	12.3 ± 0.5*	-	-	-	-
	ISP 4	**23.6 ± 0.6**$^{++}$	-	-	-	-
C10	Gause I	-	12 ± 0.0*	-	-	-
C11	Gause I	**14 ± 0.0**$^{+}$	**14.9 ± 0.1**$^{\#}$	-	-	-
	TSB	17 ± 0.0$^{\#}$	-	14 ± 0.0^{+}	-	-
	ISP 4	17 ± 0.0$^{\#}$	-	17 ± 0.0$^{\#}$	-	-
C12	Gause I	11 ± 0.0*	13 ± 0.0^{+}	-	-	-
C13	Gause I	-	**14 ± 0.0**$^{-}$	-	-	-
	TSB	-	-	**25.6 ± 0.6**$^{\#\#}$	**-**	**11.3 ± 0.3**$^{+}$
	ISP 4	-	-	18 ± 0.0^{++}	-	-
C14	Gause I	12 ± 0.0*	-	13 ± 0.0*	-	-
C15	TSB	12 ± 0.0*	-	15 ± 0.0^{-}	-	-
C19	Gause I	11.3 ± 0.6*	-	-	-	-
	ISP 4	**22.3 ± 0.6**$^{++}$	**-**	**14 ± 0.0**$^{+}$	-	-
C21	Gause I	**22.8 ± 0.3**$^{++}$	**-**	**26 ± 0.0**$^{\#\#}$	-	-
	TSB	14 ± 0.0^{+}	-	15 ± 0.0^{-}	-	-
	ISP 4	22.2 ± 0.3^{++}	-	22 ± 0.0**	-	-
C22	Gause I	17 ± 0.0$^{\#}$	-	18 ± 0.0^{++}	-	-
	ISP 4	**18.25 ± 0.35****	-	-	**23 ± 0.0**$^{+}$	-
C24	Gause I	14.1 ± 0.3^{+}	-	-	-	-
	TSB	14.3 ± 0.6^{+}	-	-	-	-
	ISP 4	**11.3 ± 0.6***	**-**	**13 ± 0.0***	**15 ± 0.0***	-

The numbers appeared in table indicate the diameters of the inhibition zones in millimeter (mm). Strain with no activity were labeled with minus symbol (-). Value noted with the same symbol indicate no significant differences. Cultures grew in TSB or ISP 4 media were collected after 4 days of incubation and 7 days for those grew in Gause I medium (Yong *et al.*, 2017).

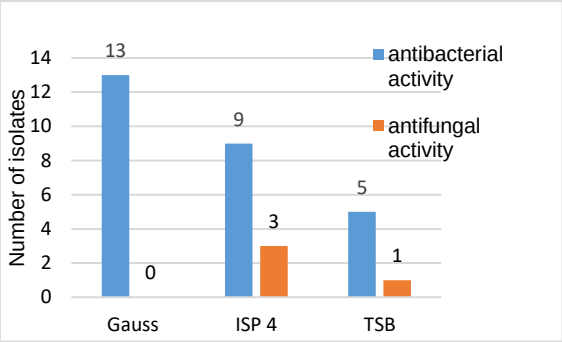

Figure 3. Number of isolates showed antimicrobial activity in different media.

From the primary screening, we repeated the experiments with isolates having good antimicrobial activity significantly compared with others, in which collection times were adjusted. Of those to be re-examined, C3, C9, C19, C22 and C24 were cultured in ISP4 liquid medium. Isolates C7, C11, C13, C21 were cultured in Gause I, and C13 in TSB. Growth curve of each isolate established in the indicated medium was used to determine the stationary phase where antimicrobial compounds are expectedly produced. The following Table 2 suggested the harvested time of isolates when cultured in above mentioned media.

The collected broth from all 9 cultures were subjected for ethyl acetate extraction as described above, and total organic extracts were tested again to 5 microorganisms. While these extracts remained the activity against the same microbes tested before, some isolates seem to have better activity when harvested times were adjusted to the middle of stationary phase. For example, C9 showed the inhibition zone of 27 ± 0.5 mm against *S. aureus* compared with 23.6 ± 0.6 mm in the first screening. C19 and C24 created the larger inhibition zones against both *S. aureus* and *B. subtilis* in the second experiments. All data are significantly different in student t test with p value <0.05 (Figure 4).

Even though the data is primarily brief, it still exhibits the ability to change the production as well as the antimicrobial bioactivity if we could do more on optimization. We recommend to extent the investigation on collection times, media components as well as variable solvent extractions in order to have better understanding about the antimicrobial activity of the isolates from Con Dao Island.

Table 2. Growth and phase time of nine selected *Streptomyces* strains. Collected time was determined when the culture is at the middle stationary phase of each isolate.

Isolate ID	Medium	Log phase approximately start at (hr)	Stationary approximately start at (hr)	Collection time (hr)
C3	ISP 4	12	84	204
C9	ISP 4	84	108	204
C19	ISP 4	24	144	204
C22	ISP 4	24	84	132
C24	ISP 4	12	96	156
C7	Gause I	96	120	180
C11	Gause I	180	264	312
C13	Gause I	144	192	228
C21	Gause I	24	192	312
C13	TSB	12	60	120

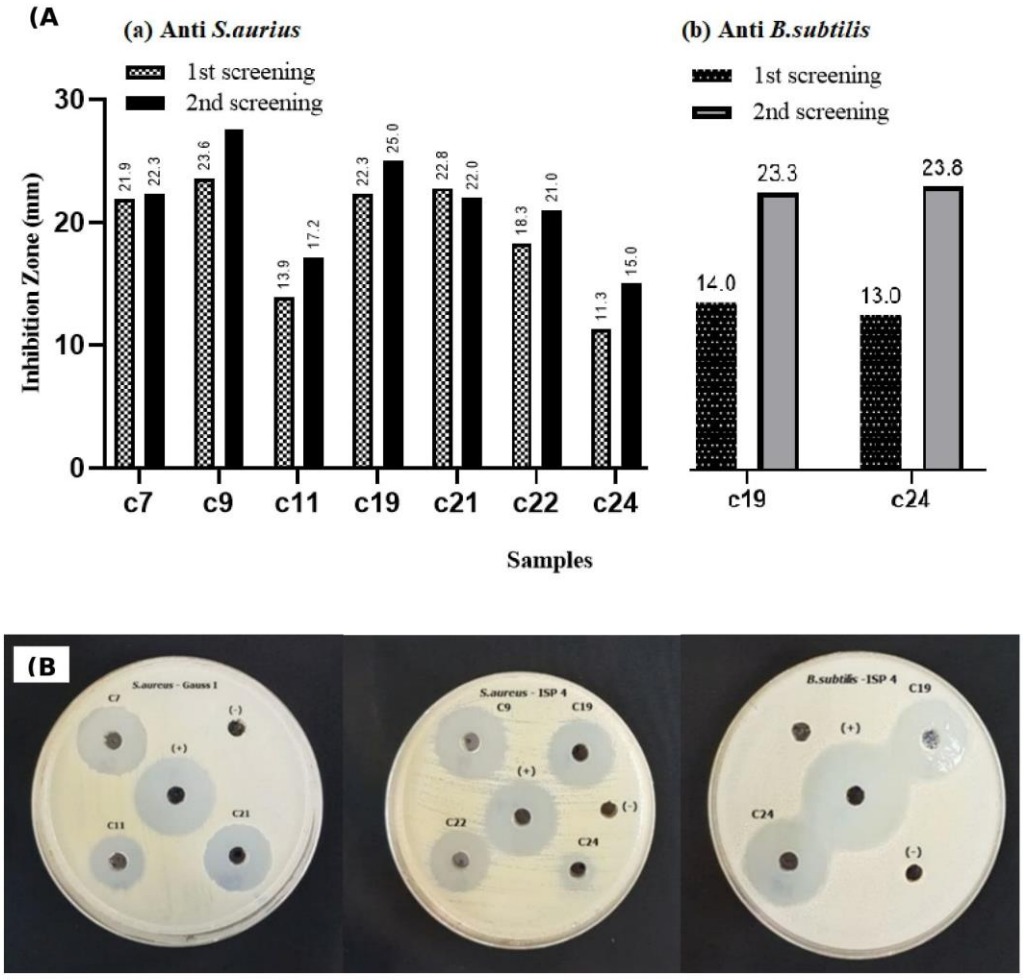

Figure 4. Antimicrobial activity of selected *Streptomyces* isolates after adjustment. (A) Inhibition zone of *Streptomyces* isolates measured on *S. aureus* (a) and *B. subtilis* (b) agar plates from first and second screening. (B) Agar plates showing the activity of C7, C9, C11, C19, C22, C24 against *S. aureus* and C19, C24 against *B. subtilis* in second screening. Positive control for both microbes is 100 µl of ciprofloxacin (128 µg/mL).

CONCLUSION

From soil samples of Con Dao Island, 25 *Streptomyces* isolates were obtained in ISP4 with medium growing and spore forming characteristics. These isolates almost showed the antimicrobial activity in ethyl acetate extracts against at least one of the tested microbes including *S. aureus, E. coli, B. subtilis, C. albicans* and *A. parasiticus*. The antimicrobial activity of these isolates is apparently high against Gram positive bacteria and could promisingly be improved with more optimizations on culture as well as extraction.

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