IDENTIFICATION AND ANTIMICROBIAL ACTIVITIES OF *STREPTOMYCES* STRAINS ISOLATED FROM SOILS

Sirinin Chatujinda[1] Somboon Tanasupawat[2,*]
Surattana Amnuoypol[1] Chaiyo Chaichantipyuth[1]
[1]Department of Pharmacognosy, [2]Department of Microbiology,
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT: Thirty five strains of *Streptomyces* from 24 soil samples collected in Krabi, Chaiyaphoom, Chanthaburi, Chonburi, Lampang, Kanchanaburi, Nongkhai, Ratchaburi, Chachoengsao, and Srakaew provinces in Thailand were screened for antimicrobial activities. Thirty strains exhibited antibacterial activity against *Bacillus subtilis* ATCC 6633, 15 strains inhibited *Staphylococcus aureus* ATCC 25933, 3 strains inhibited *Escherichia coli* ATCC 25922 and only a strain CB5-3 showed antifungal activity against *C. albicans* ATCC 10231. The potent strain CB5-3, was selected for further antimicrobial activity tests and fermentation. On the basis of the morphological, cultural, physiological and biochemical characteristics including the 16S rDNA sequence similarity (99.9%) of strain CB5-3 was closely related to *Streptomyces hygroscopicus* NBRC 13868^T, therefore, it was identified as *Streptomyces hygroscopicus*. In addition, the antibiotic fermentation of this CB5-3 strain was carried out in peptone-yeast extract (PY) medium at 28 $^{\circ}$C for 14 days. The isobutanol extract of fermentation broth was tested by bioautographic method and found that the active spot was shown at R_f 0.7 value. This isolated extract was shown to be active against *Candida albicans* ATCC 10231.

Key words: Actinomycetes, antimicrobial activity, soil, *Streptomyces hygroscopicus*

INTRODUCTION: Actinomycetes have been described as the greatest source of antibiotic producers. They produced antibiotics approximately 53 % whereas 29 % produced by fungi and a few (18%) by bacteria[1]. The actinomycetes especially the strains of *Streptomyces* are the most common antibiotic-producing microorganisms found in soil. The *Streptomyces* strains provided 6,550 antibiotics and the rare actinomycetes produced 2250 of naturally occurring antibiotics discovered, including many of those important in medicine, such as aminoglycoside, anthracyclines, chloramphenicol, ß-lactams, macrolides and tetracyclines[1,2]. In Thailand, *Streptomyces* strains isolated from soil were reported to express antimicrobial activities[3-5]. Furthermore, the biological activity and chemical structures of streptothricin and geldanamycin were also reported[3,6,7]. This paper deals with the screening and identification of *Streptomyces* strains isolated from soil including the assay for antibacterial and antifungal activities and the extraction of substances.

MATERIALS AND METHODS:

Screening and identification of strains

Soil samples were collected from Krabi, Chaiyaphoom, Chanthaburi, Chon Buri, Lampang, Kanchanaburi, Nong Khai, Ratchaburi, Chachoengsao, and Srakaew provinces in Thailand. Each dried soil sample (0.5 g) was mixed with 1.5 ml of steriled distilled water and was kept at 55 ºC for 6 minutes. The diluted soil suspension (0.1 ml) was spreaded on potato carrot agar (PCA, 30 g, potato, 25 g, carrot and 1,000 ml tap water and was adjusted to pH 7.0) and sodium casienate agar (SCA) with steriled glass rod, and the plates were incubated at 30 ºC for one to two weeks[4]. *Streptomyces* colonies were picked up and purified on yeast extract and malt extract (YM) agar[4]. *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and

*To whom correspondence should be addressed.
E-mail: Somboon.T@chula.ac.th, Tel. 0 2218 8376; fax. 0 2254 5195

Candida albicans ATCC 10231 were used as test organisms for primary screening[4]. The other Gram-positive and Gram-negative bacteria including fungi were used for secondary screening. Morphological, cultural, physiological and biochemical characteristics of strains were determined as described by Shirling and Gottlieb[8] and Arai *et al*[9]. Scanning electron microscope was used for determining the morphology of strain CB5-3 grew on diluted 10 % PCA.

DNA of the strain was isolated from cells grown in Yeast extract-Malt extract broth (YMB) with 0.2% of glycine[10] and purified as described by Saito and Miura[11] and Tamaoka and Komagata[12]. The amplified 16S rDNA was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and analyzed by AB1377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in DNA Thermal Cycler (Gene Amp PCR System 2400; Perkin Elmer) by using primers, 8-27f (5'-AGAGTTTGATC (A/C)TGGCTCAG-3'), 530f (5'-GTGCCAGC(A/C)GCCGCGG-3') and 111 4f (5'GCAACGAGCGCAACCC-3'). Homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server http://www.ncbi.nlm.nih.gov/BLAST/ against previously reported sequence at the GenBank/EMBL/ DDBJ database. The sequence was multiply aligned with selected sequences obtained from Genbank/ EMBL/DDBJ by using the CLUSTAL W version 1.81. The alignment was manually verified and adjusted prior to the construction of phylogenetic tree. The phylogenetic tree was constructed by using neighbor-joining[13] in the MEGA program version 2.1[14]. The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses[15] based on 1000 resamplings. The values for sequences similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL X program[16]. Gaps and ambiguous nucleotides were eliminated from calculations. The 16S rDNA sequence of CB5-3 was under the accession number EU016370.

Fermentation and antimicrobial activity assay

The potent strain CB5-3 was selected for fermentation of antibiotic in seed medium (GBP) and production medium (PY) incubating on rotary shaker (200 rpm) at 28 ºC for 7 to 14 days. The GBP medium composed of 15 g glucose, 0.6 g peptone, 0.3 g beef extract, 0.3 g yeast extract, 0.5 g NaCl, 0.25 g $MgSO_4.7H_2O$, and 1,000 distilled water and was adjusted to pH 7.0. PY medium contained 20 g glucose, 10 g soluble starch, 3 g yeast extract, 5 g peptone, 5 g beef extract, 3 g $CaCO_3$, 5 g NaCl, and 1,000 ml distilled water, and was adjusted to pH 7.0. The agar disc diffusion method (6 mm diameter, paper disc) was used for assay of the active fractions[17-18].

Isolation and extraction of antimicrobial substance

The fermentation broth (20 l) of strain CB5-3 was collected by centrifugation. One liter of fermentation broth was partitioned with 250 ml isobutanol 2-3 times. The isobutanol layer was evaporated under reduced pressure and was fractionated by Silica gel flash column chromatography (1.5 x 90 cm) eluted with ethyl acetate: methanol: water (6: 4: 1). The eluates were checked by TLC using the same solvent system (ethyl acetate: methanol: water, 6: 4: 1) as developing solvent and combined eluates which gave similar chromatographic pattern and the antibiotic activity was checked by agar disc diffusion method. Only the active fractions were selected for column chromatography. Gel filtration Chromatography (2.5 x 80 cm) using Sephadex LH-20 as the adsorbent was used for fractionation using methanol as eluent. The eluate was concentrated and applied to a column of Polystyrene (MC-I gel, 1.5x3 cm) which was developed with ethyl acetate: methanol (1:1). After concentrating and drying, the combined fractions were obtained.

RESULTS AND DISCUSSION: Thirty-five strains of actinomycetes were isolated from 24 soil samples collected in different provinces in Thailand (Table 1). Thirty-strains exhibited antibacterial activity against *B. subtilis* ATCC 6633, 15 strains could inhibit *S. aureus* ATCC 25933, 3 strains could inhibit *E. coli* ATCC 25922 and only a strain CB5-3 showed antifungal activity against *C. albicans* ATCC 10231 (Table 2).

The isolated colonies are white, cream, yellow, gray, brown and black on YM agar. Most of the isolated strains produced aerial mycelium with long chains of spores on YM agar. The spore shapes are oval, circular, cylindrical and rod shape and they formed spiral, rectus, flexous or retinaculiaperti spore chains. All of them were included in the genus *Streptomyces*[19]. Only a strain CB5-3 could not produce spores on YM agar. PCA (diluted 10 %) was used for sporulation and the spore morphology was determined by scanning electron microscope as shown in Fig. 1 (a and b). A strain CB5-3 showed moderate and good growth but did not produce soluble pigment on various media (Table 3).

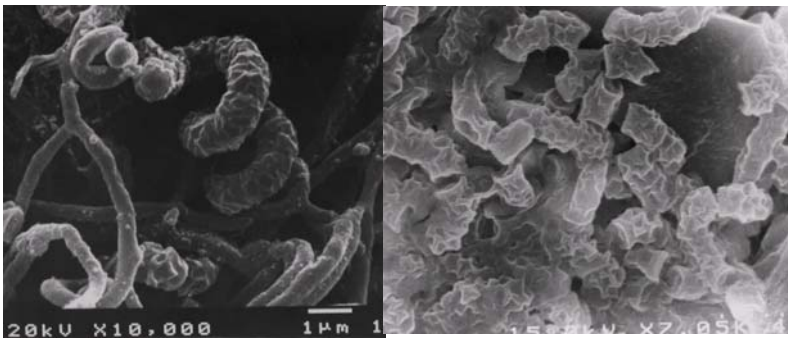
Table 1 Isolation source and strain number of *Streptomyces* strains

Sample no.	Province	Date of isolation	Isolate no.
A3	Krabi	28 June 1995	A3-2
A5, C3, C10, C11	Chaiyaphoom	14 July 1995	A5-1, C3-1, C10-1, C11-1
CB5, CB12	Chanthaburi	17 May 1996	CB5-3, CB5-6, CB12-1, CB12-2, CB12-4, CB12-8
CPM3	Chonburi	17 May 1996	CPM3-1, CPM3-2
L1	Lampang	1 May 1996	L1-2
L4, L5	Lampang	24 May 1996	L4-1, L5-2, L5-4
KB1	Kanchanaburi	17 May 1996	KB1-1
NK3	Nongkhai	1 April 1996	NK3-1
R1	Ratchaburi	1 May 1996	R1-3
R4, R6, R7, R8	Ratchaburi	10 May 1996	R4-1, R4-3, R6-2, R7-1, R7-2, R8-4
019, 020, 024, 029	Chonburi	17 May 1996	019-2C, 019-3C, 020-5P, 024-P, 029-P
CS1	Chachoengsao	14 June 1997	CS1-1, CS1-2
SK1	Srakaew	14 June 1997	SK1-1, SK1-2

Table 2 Primary screening antimicrobial activities of *Streptomyces* strains

Strain no.	Inhibition zone (mm)			
	B. subtilis **ATCC 6633**	***C. albicans*** **ATCC 10231**	***E. coli*** **ATCC 25922**	***S. aureus*** **ATCC 25923**
A3-2, C10-1, C11-1, CB12-4, NK3-1, R1-3, R4-1, R7-1, CS1-2, SK1-2	4.7-16.5	0	0	5.7-34.1
A5-1, C3-1, CB12-2, CB12-8, CPM3-1, CPM3-2, L4-1, L5-2, KB1-1, R4-3, R6-2, R7-2, R8-4, 019-3C, 024-P, 029-P, CS1-1	5.0-33.3	0	0	0
CB5-3	17.5	21.3	0	24.2
CB5-6, CB12-1	0	0	0	4.3-6.8
L1-2	11.9	0	7.1	0
019-2C	0	0	15.7	12.5
020-5P	17.6	0	4.6	4.3

Strains L5-4 and SK1-1 showed no antimicrobial activities to all the indicator strains.



a b

Figure 1 Scanning electron micrograph of CB5-3 on PCA (diluted 10 %): a, a spiral of spore-chain (x 10,000); and b, spores (x 7,000)

Table 3 Cultural characteristics of *S. hygroscopicus* CB5-3 on various media

Medium	Growth	Substrate mycelium	Upper colony color	Lower colony color	Soluble pigment
Potato carrot agar(diluted 10 %)	Good	White	White	Cream	-
Glucose asparagine agar	Good	Dark grayish yellow	Cream	Grayish yellow	-
Inorganic salt starch agar	Good	Brownish yellow	White	Yellow	-
Nutrient agar	Moderate	Light yellow	White	Light yellow	-
Oatmeal agar	Good	Olive brown	Light greenish yellow	Light olive Brown	-
Sucrose nitrate agar	Moderate	Yellow	Yellow	Yellow	-
Yeast starch agar	Moderate	Yellow	White	Yellow	-

-, negative reaction

Table 4 Physiological and biochemical characteristics of *S. hygroscopicus* CB5-3

Characteristics	Reaction
Growth on 4% NaCl	+
Growth on 7% NaCl	-
Growth at 30-37°C	+
Growth at 42°C	w
Litmus milk: Peptonization	+
Nitrate reduction	-
Hydrolysis of gelatin, starch	+
Melanoid pigment (ISP 7)	+
Utilization of:	
Cellobiose, D-fructose, D-glucose, D-galactose, maltose, mannitol, D-mannose , melibiose, rhamnose , D-Ribose , starch, D-trehalose	+
L-Arabinose, D-xylose	w
Raffinose, L-sorbose, sucrose	-

+, positive reaction; w, weak reaction; -, negative reaction

On PCA (diluted 10%), it produced white mycelium with a black masses of spores and spiral spore-chains. The spore surface is rugose. This strain could grow in ISP 2 medium with 4 % NaCl and at 30-37 ºC. It hydrolysed starch and gelatin, liquefied litmus milk and utilized several kinds of carbon sources. Variable characteristics are shown in Table 4. The 16S rDNA sequencing (99.9% similarity) and phylo-genetic analysis of the strain CB5-3, revealed that it was closely related to *S. hygroscopicus* NBRC 13868^T (Fig.2). Therefore, this strain was identified as *Streptomyces hygroscopicus* [20-21].

The strain CB5-3 gave good activity when fermented on PY medium adjusted to pH 6.5 to 7.0 and incubated at 28ºC on rotary shaker. The crude extract of this strain exhibited antibacterial activities against Gram-positive bacteria and antifungal activities against *Candida albicans* ATCC 10231 and

Microsporum gypseum PCU strain (Table 5). The crude isobutanol extract (16.55 g) was detected for active spot by bioautographic technique and showed active spot with R_f value 0.7 (ethyl acetate: methanol: water, 6:4:1). The isobutanol extract was fractionated by Silica gel flash column chromatography to give fractions A1/1, A1/2, A1/3, A1/4, and A1/5. Fractions A1/3 was further purified by Sephadex LH-20 to give fractions B1/1, B1/2, B1/3, B1/4, B1/5, and B1/6. Fraction B1/3 was separated to fractions C1/1, C1/2, C1/3, C1/4, and C1/5 by using Polystyrene (MC-I) as adsorbent, and eluted with ethyl acetate: methanol (1:1). All fractions were monitored for antifungal activity by agar disc diffusion method. The fractions C1/2 (102.7 g) and C1/3 (138.2 g) showed antifungal activity with its R_f value 0.7 (ethyl acetate : methanol : water, 6:4:1). The inhibition zone of active fractions activity against *C. albicans* ATCC 10231 ranged from 12.5-17.5 mm. The strains of *Streptomyces hygroscopicus* were reported to produce a number of antimicrobial agents [22]. The *S. hygroscopicus* CB5-3 isolated from soil in Thailand, may be a new source for antifungal agent production. The structure elucidation of antibiotic of this strain is required for further study.

ACKNOWLEDGEMENTS: We wish to thank the Department of Pharmacognosy and Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand for providing facilities during this study.

REFERENCES:

1. Bérdy J. 2005. Bioactive microbial metabolites. J Antibiot 58: 1-26.

2. Goodfellow M., Williams ST. and Mordarski M. 1988. Actinomycetes in Biotechnology, Academic Press Inc, London, p. 1-88.

3. Keeratipibul S., Sugiyama M., Nimi O. and Nomi R. 1984. Streptothricin production by a new isolate of Streptomyces from Thailand soil. J Ferment Technol 62: 19-31.

4. Virochsaengaroon N., Naenna P. and Srimaha-songcram S. 1984. Isolation of antibiotics-producing Streptomyces from soil in Thailand, Annual Report, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, p. 1-55.

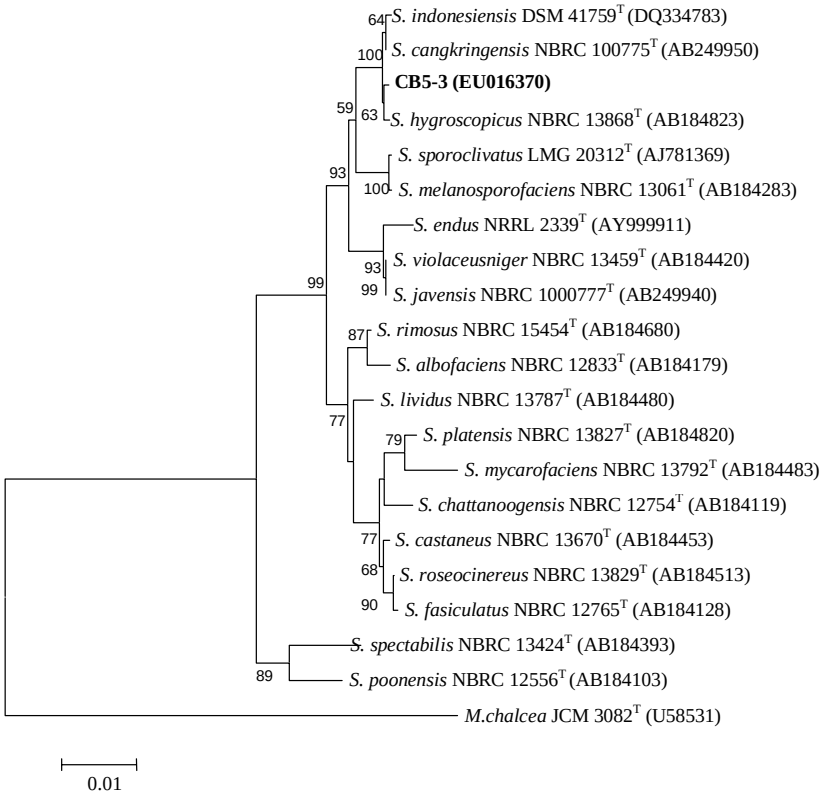


Figure 2 A neighbour-joining tree of strain CB5-3 and related *Streptomyces* species based on 16S rDNA sequence. Bootstrap percentages above 59%, based on 1000 replications are shown at the nodes. Bar, 1 substitutions per 100 nucleotide positions.

5. Naenna P., Virochsaengaroon N. and Tanasupawat S. 1994. Scanning electron micrograph and antimicrobial activity of Streptomyces species N28-1 and N97-1. Thai J Pharm Sci 18: 47-51.

6. Jongrungruangchok S., Tanasupawat S., Kittakoop P., Bavovada R., Kobayashi H. and Kudo T. 2006. Identification of Streptomyces and Kitasatospora strains from Thai soils with geldanamycin production strain. Actinomycetologica 20: 10-14.

7. Anansiriwattana W., Tanasupawat S., Amnuoypol S. and Suwanborirux K. 2006. Identification and antimicrobial activities of actinomycetes from soils in Samed island, and gedanamycin from strain PC4-3. Thai J Pharm Sci 30: 49-56.

8. Shirling EB. and Gottlieb D. 1966. Methods for characterization of Streptomyces species. Int J Syst Bacteriol 16: 313-340.

9. Arai T., Tamotsu F., Masa H., Akihiro M., Yuzuru M. Akio, S., *et al.* 1976. Culture media for Actinomycetes, The Society for Actinomycetes, Japan, p.1-31.

10. Yamada K. and Komagata K. 1970. Taxonomic studies on coryneform bacteria. III. DNA base composition of coryneform bacteria. J Gen Appl Microbiol 16: 215-224.

11. Saito H. and Miura K. 1963. Preparation of transforming deoxyribonucleic acid by phenoltreatment. Biochim Biophys Acta 72: 619-629.

12. Tamaoka J. and Komagata K. 1984. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol Lett 25: 25-128.

13. Saitou N. and Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.

14. Kumar S., Tamura K., Jakobson IB. and Nei M. 2001. MEGA 2: Molecular Evolution Analysis software. Bioinformatics 17: 1244-1245.

15. Felsenstien J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

16. Thompson JD., Gibson TJ., Plewniak F., Jeanmougin F. and Higgins DG. 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876-4882.

17. Murray RP. 1995. Manual of Clinical Microbiology. 6nd ed., ASM Press, Washington, D.C., p. 238-239.

18. Lorian V. 1991. Antibiotics in Laboratory Medicine, The Williams and Wilkins Company, Baltimore, p. 1-51.

19. Holt JG. 1989. Bergey's Manual of Systemic Bacteriology. vol. IV, The Williams and Wilkins Company, Baltimore, p. 2333-2505.

20. Shirling EB. and Gottlieb D. 1972. Cooperative description of type strains of Streptomyces. V. additional descriptions. Int J Syst Bacteriol 22: 265.

21. Lanoot B., Vancanneyt M., Hoste B., Vandemeulebroecke K., Cnockaert MC., Dawyndt P., *et al.* 2005. Grouping of Streptomycetes using 16S- ITS RFLP fingerprinting. Res Microbiol 156: 755-762.

22. Glasby SJ. 1993. Encyclopedia of Antibiotics. 3rd ed. John Wiley & Sons, London, p. 1-507.

การพิสูจน์เอกลักษณ์และฤทธิ์ต้านจุลชีพของสเตรปโตมัยซีสที่แยกได้จากดิน

สิรินินทร์ จตุจินดา[1] สมบูรณ์ ธนาศุภวัฒน์[2,*] สุรัตนา อำนวยผล[1] ชัยโย ชัยชาญทิพยุทธ[1]

[1]ภาควิชาเภสัชเวท [2]ภาควิชาจุลชีววิทยา คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

บทคัดย่อ: การศึกษาแบคทีเรียสเตรปโตมัยซีสจำนวน 35 สายพันธุ์ที่แยกจากดิน 24 ตัวอย่างที่เก็บจากจังหวัดกระบี่ ชัยภูมิ จันทบุรี ชลบุรี ลำปาง กาญจนบุรี หนองคาย ราชบุรี ฉะเชิงเทรา และ สระแก้วเพื่อคัดกรองฤทธิ์ต้านการเจริญของจุลินทรีย์ พบว่าแบคทีเรียจำนวน 30 สายพันธุ์สามารถสารสร้างที่มีฤทธิ์ต้านการเจริญของแบคทีเรีย *Bacillus subtilis* ATCC 6633 จำนวน 15 สายพันธุ์สามารถต้านการเจริญของ *Staphylococcus aureus* ATCC 25923 และแบคทีเรีย 3 สายพันธุ์สามารถต้านการเจริญของ *Escherichia coli* ATCC 25922 และพบว่ามีเพียงสายพันธุ์ CB5-3 เท่านั้นที่สามารถต้านการเจริญของ *Candida albicans* ATCC 10231 สายพันธุ์ CB5-3 ที่มีศักยภาพจึงถูกคัดเลือกเพื่อนำไปทดสอบฤทธิ์ต้านการเจริญของจุลินทรีย์และใช้ในการหมักสาร จากผลการศึกษาลักษณะทางสัณฐานวิทยา การเจริญ สรีรวิทยา และชีวเคมี รวมทั้งความคล้ายคลึงของลำดับเบสในช่วง 16S rDNA (99.9%) กับ *Streptomyces hygroscopicus* NBRC 13868^T ดังนั้นจึงพิสูจน์เอกลักษณ์แบคทีเรียสายพันธุ์นี้ได้เป็น *Streptomyces hygroscopicus* นอกจากนี้ได้ทำการหมักสารปฏิชีวนะของ CB5-3 ในอาหาร peptone-yeast extract (PY) โดยเลี้ยงที่ 28 $^{\circ}$ซ เป็นเวลา 14 วัน เมื่อทำการทดสอบด้วยวิธี bioautographic ของส่วนสกัดที่เป็น isobutanol ของน้ำหมัก พบจุดที่แสดงฤทธิ์มีค่า R_f 0.7 และพบว่าส่วนสกัดที่แยกได้นี้มีฤทธิ์ต้านการเจริญของ *Candida albicans* ATCC 10231

คำสำคัญ: แอคติโนมัยซีทส์ ฤทธิ์ต้านจุลชีพ ดิน *Streptomyces hygroscopicus*

*ติดต่อได้ที่ Somboon.T@chula.ac.th โทรศัพท์ 0 2218 8376 โทรสาร 0 2254 5195