เมแทบอไลต์ทุติยภูมิผลิตโดย *Streptomyces parvulus* สายพันธุ์ J6.2

นายณัฐชัย เก่งพิพัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

SECONDARY METABOLITES PRODUCED BY *Streptomyces parvulus* STRAIN J6.2

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2012

Thesis Title	SECONDARY METABOLITES PRODUCED BY *Streptomyces parvulus* STRAIN J6.2
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Field of Study	Biotechnology
Thesis Advisor	Associate Professor Prakitsin Sihanonth, Ph.D.

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ณัฐชัย เก่งพิพัฒน์ : เมแทบอไลท์ทุติยภูมิผลิตโดย *Streptomyces parvulus* สายพันธุ์ J6.2. (SECONDARY METABOLITES PRODUCED BY *Streptomyces parvulus* STRAIN J6.2) อ. ที่ปรึกษา : รศ.ดร.ประกิตติ์สิน สีหนนทน์ , 107 หน้า

Streptomyces parvulus J6.2 แยกได้จากดินบริเวณป่าชายเลน ในจังหวัดจันทบุรี ประเทศไทย จากการศึกษาลักษณะทางสัญฐานวิทยา และตรวจสอบลำดับเบสของยีนประมวลรหัส 16S rRNA สามารถจัดจำแนกชนิดเป็น *S. parvulus* และจากความสามารถในการใช้แหล่งคาร์บอน พบว่าเป็น *S. parvulus* สายพันธุ์ใหม่ จากการเปรียบเทียบยีนประมวลรหัส 16S rRNA พบว่ามีความสัมพันธ์ใกล้เคียงกับ *Streptomyces* spp. ที่ผลิตแอกติโนมัยซินดี เมื่อนำสารสกัดเอทิลอะซิเตตมาแยกด้วยวิธีคอลัมน์โครมาโทกราฟี และ HPLC ชนิดกลับเฟส โดยใช้เมทานอลในน้ำ 30% เป็นตัวทำละลายเคลื่อนที่ สามารถแยกแอกติโนมัยซินดี ซึ่งเป็นสารองค์ประกอบหลักที่ออกฤทธิ์ทางชีวภาพ โดยตรวจสอบโครงสร้างโมเลกุลของแอกติโนมัยซินดี ด้วยวิธี 2D-NMR ในการศึกษาอาหารเลี้ยงเชื้อที่เหมาะในการผลิตแอกติโนมัยซินดี พบว่าผลิตได้สูงสุด 378.84 กรัม ต่ออาหารเลี้ยงเชื้อ 1 ลิตร เมื่อเลี้ยงในอาหารเหลวโซเดียมเคซีเนต ที่เติมสารสกัดมอลต์ 20 กรัมต่อลิตร แป้งและสารสกัดมอลต์ที่เติมลงในอาหารเลี้ยงเชื้อมีผลทำให้ปริมาณสารเมแทบอไลต์เพิ่มขึ้น และมีผลทำให้ผลิตแอกติโนมัยซินดีเพิ่มขึ้นด้วย ความเข้มข้นของเกลือ ในอาหารเลี้ยงเชื้อมีผลยับยั้งการผลิตเมแทบอไลต์ที่เป็นไตรกลีเซอไรด์ ทำให้สามารถผลิตแอกติโนมัยซินดีแบบจำเพาะได้มากขึ้น

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

5073942623 : MAJOR BIOTECHNOLOGY

KEYWORDS : *Streptomyces parvulus* / ACTINOMYCIN-D / SECONDARY METABOLITE / OPTIMIZATION

NATTACHAI KENGPIPAT : SECONDARY METABOLITES PRODUCED BY *Streptomyces parvulus* STRAIN J6.2. ADVISOR : ASSOC. PROF. PRAKITSIN SIHANONTH, Ph.D., 107 pp.

Streptomyces parvulus J6.2 was isolated from soil in a mangrove area and studied its metabolites. The morphology and 16S rDNA sequences were used to identify it as belonging to *S. parvulus.* Comparison of 16S rDNA sequences showed that *S. parvulus* J6.2 had a close relationship to the actinomycin-D producing *Streptomyces* species. Carbon utilization indicated that *S. parvulus* J6.2 was a novel strain. Actinomycin-D, a major bioactive component, was isolated from ethyl acetate crude extracts by reverse phase HPLC using isocratic of 30% methanol in water as the mobile phase with a 3 ml min^{-1} flow rate. The molecular structure of actinomycin-D was confirmed by 2D-NMR. Medium optimization gave the highest yield of 378.84 mg/l actinomycin-D when grown in a sodium caseinate broth supplemented with 20 g/l malt extract as the culture medium. Starch and malt extract supplemented culture medium produced high crude metabolite yield therefore increasing actinomycin-D production. High concentration of NaCl in culture medium suppressed production of triglyceride metabolites resulting in more specific production of actinomycin-D.

Field of Study : Biotechnology Student's Signature .....

Academic Year : 2012 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my gratitude to my thesis advisor Associated Prof. Dr. Prakitsin Sihanonth. I am gratitude Associated Prof. Dr. Surachai Pornpakakul for providing excellent scientific advices and always supported me for valuable comments and discussions. I would like to thank Dr. Nuttika Suwannasai and Assistant Prof. Dr. Jittra Piapukiew for the molecular genetics identification of *Streptomyces*. Special thanks to Dr. Geoff Robson, Faculty of Life Science, The University of Manchester for very warm support me when I lived in Manchester.

I would like to thank Associated Prof. Dr. Suthep Thaneyiwan, Assistant Prof. Dr. Suchada Chanprateep Napathorn and Assistant Prof. Dr. Ek Sangvichien for some advice of this thesis.

I would like to thank The Royal Golden Jubilee Ph.D. Program, Thailand research fund and Chulalongkorn University for financial support. I also wish to thank Program in Biotechnology and Department of Microbiology, Faculty of Science, Chulalongkorn University for welcome support.

I would like to thank Mr. Jatupon Lengsakul, Mr. Suphongphan Srisurichan, Mr. Sonchai Wannatess for their assistant and comments.

Finally, I am extremely grateful to my family for the lasting support thoughout my studies.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
bp	base pair
°C	degree Celsius
cm	centimeter
^{13}C – NMR	csrbon – 13 nuclear magnetic resonance
COSY	Correlated Spectroscopy
DO	Dissolved Oxygen
ESI – TOF	Electrospary Ionization – Time of flight
g	gram
h	hour
HMBC	Heteronuclear Multiple Bond Cerrelation
^{1}H – NMR	proton nuclear magnetic resonance
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
ISP	International *Streptomyces* Project
M^{+}	molecular ion
MHz	megahertz
mg	milligram
min	minute
mL	milliliter (s)
mm	millimeter (s)
MS	Mass Spectroscopy
m/z	mass to change ratio
No.	number
ppm	part per million
Rf	rate of flow in chromatography
rpm	round per minute
sp.	species
TLC	Thin Layer Chromatography
UV	ultraviolet

µg	microgram
µL	microliter
$[\alpha]^{20}_{D}$	specific rotation at 20 °C and sodium D line (569 nm)
δ	chemical shift
λ_{max}	wavelength at maximum absorbtion (UV)

CHAPTER 1
INTRODUCTION

1.1 Background and Rationale

Streptomyces is a group of filamentous aerobic microorganism classified in actinobacteria group. It is capable of producing aerial mycelium bearing chains of naked and nonmotile spores. *Streptomyces* is a free-living saprophyte living primarily in soil although some are pathogenic. It plays an important role in mineralization of organic matter owing to its ability to degrade complex organic compounds.

Apart from its crucial ecological role, *Streptomyces* are best known as antibiotic producing bacteria (Běhal, 2000). Some antibiotics produced by *Streptomyces* exhibit antifungal or antitumor activity. Other bioactive metabolites synthesized by *Streptomyces* include herbicides, insecticides, antiparasitic agents, immunosuppressantsand hypoglycemic/antidiabetic agents (Deshpandes *et al*. 1988; Pritchard, 2005).

The fact that mangrove swamp is an area of high biodiversity suggests its enormous potential for the isolation of new *Streptomyces* species or strains capable of either producing novel bioactive compounds or being a more productive system in the production of known bioactive compounds. Kengpipat (2007) isolated antibiotic-producing actinomycetes from 34 soil and sand samples collected from Thailand's beach and mangrove areas and found that among 77 isolates obtained, 1 isolate showed only broad spectrum antimicrobial activity as tested by cross-streak method. Eleven isolates show only specific antitumor activity as tested by MTT assay and 3 isolates show both antimicrobial and antitumor activities. Isolate J6.2 which is one of the isolates exhibiting both antimicrobial and antitumor activities was identified using morphological study, culture characteristics, biochemical propertiesand 16S rDNA homology as *Streptomyces parvulus*.

Bioactive compounds produced by *S. parvulus* were used in various applications. Actinomycin-D is an antitumor agent (Chu and DeVita, 2009) while borrelidin exhibits angiogenesis inhibition activity (Kawamura *et al*. 2003). Oleficin is an antibiotic against Gram-positive bacteria and tumor (Gyimesi *et al.*, 1971). Manumycin provided the activities to inhibit growth and invasive of human pancreatic cancer cells caused by the inhibition enzyme farnesyl transferase (Kainuma *et al*. 1997). In this study, crude extract of *S. parvulus* isolate J6.2 culture were examined for major bioactive compounds. Effects of culture conditions on productivity of the major bioactive compounds were also investigated.

1.2 Objectives

1.2.1 To study effects of culture media compositions on secondary metabolites production by *S. parvulus* isolate J6.2,

1.2.2 To purify and characterize the structure of secondary metabolites produced by *S. parvulus* isolate J6.2, and

1.2.3 To test biological activity of the purified compounds.

CHAPTER 2
LITERATURE REVIEW

2.1. General information of *Streptomyces*

2.1.1. Introduction to the taxonomic genus *Streptomyces*

As the largest genus in the Actinobacteria (high G+C Gram-positive *Bacteria*; actinomycetes) which includes over 580 species (Garrity, 2007), *Streptomyces* is a group of filamentous and aerobic Gram positive bacteria characterized by (1) the unfragmented substrate hyphae of indefinite length, (2) the present of *L*-Diaminopimelic acid and glycine in cell wall with no characteristic sugar and (3) naked, nonmotile and heat-intolerant chains of exospores on sporophores (aerial spore-producing hyphae). Based on 16S rRNA sequence, Figure 2.1 shows the genetic relation of *Streptomyces* with other genera of actinomycetes.

Actinomycetes typically form long, fragmented or unfragmented, filaments (0.5–2.0 µm in diameter) called hyphae. As the hyphal tips grow, they often branch to produce a compact, convoluted network called mycelium which subsequently results in a colony. Actinomycetes mycelia are analogous to mycelia formed by filamentous fungi. The key differences between them are the fact that actinomycetes are prokaryotic organisms lacking nuclear membranes and mitochondria (Cross and Goodfellow, 1973) and that actinomycetes hyphae are smaller with cell walls containing 2,6-diaminopimelic acid, alanine, glycine, glutamic acid and mucopeptide (*N*-acetyl glucosamine linked with *N*-acetyl muramic acid) (David, 1959).

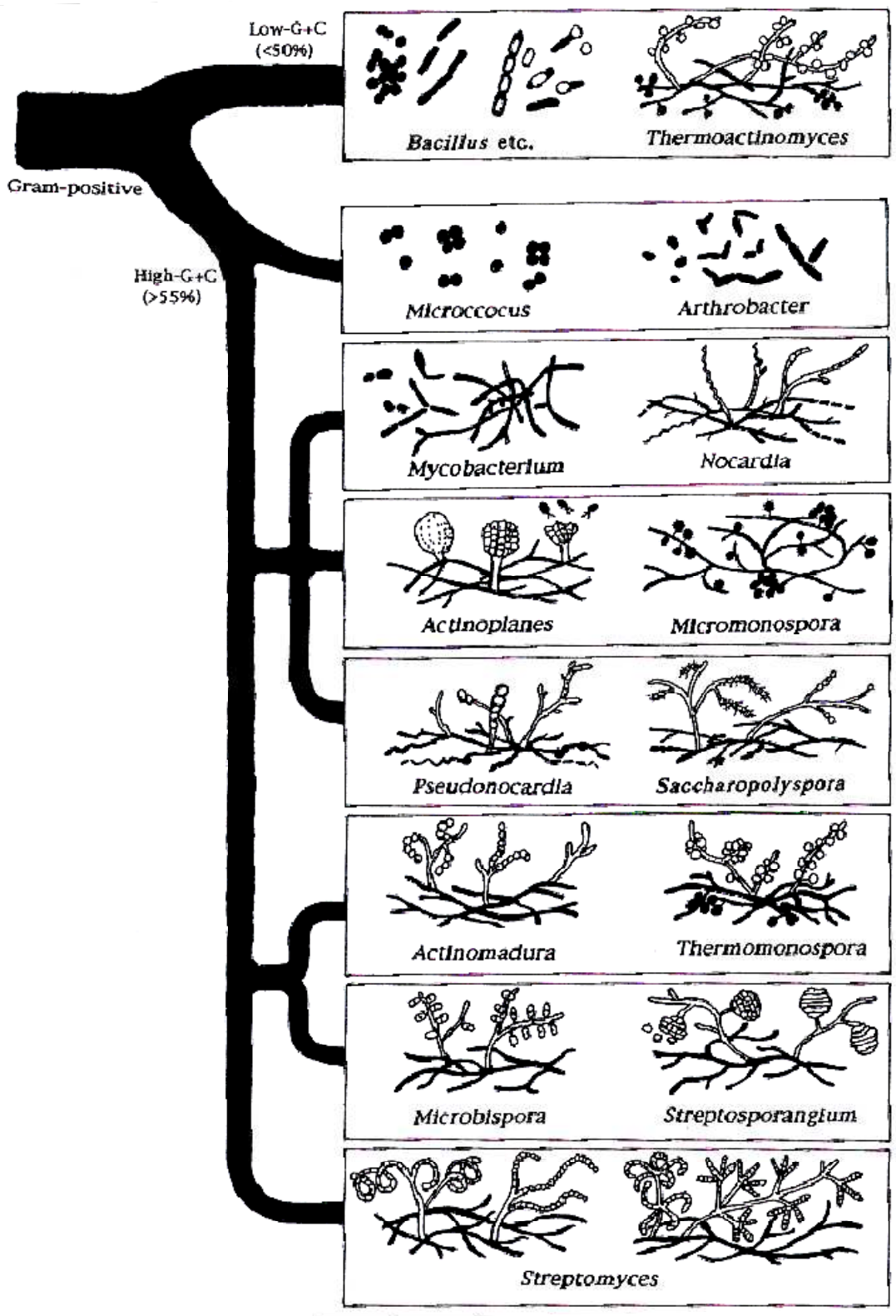


Figure 2.1 Phylogenetic relation among Actinobacteria constructed based on 16S rRNA sequence (Miyadoh, 1997).

2.1.2. Morphology of *Streptomyces* colony

A mature *Streptomyces* colony consists of substrate mycelium and aerial mycelium which grows and projects above the substrate mycelium (Figure 2.2). Typical hyphal internal structure of *Streptomyces* is given in Figure 2.3. *Streptomyces* colonies are compact with a dusty appearance of various colors owing to the pigmented conidia and sporophores (Figure 2.4 and Figure 2.5). Colony diameter ranges from approx. 1 mm to 2–3 cm.

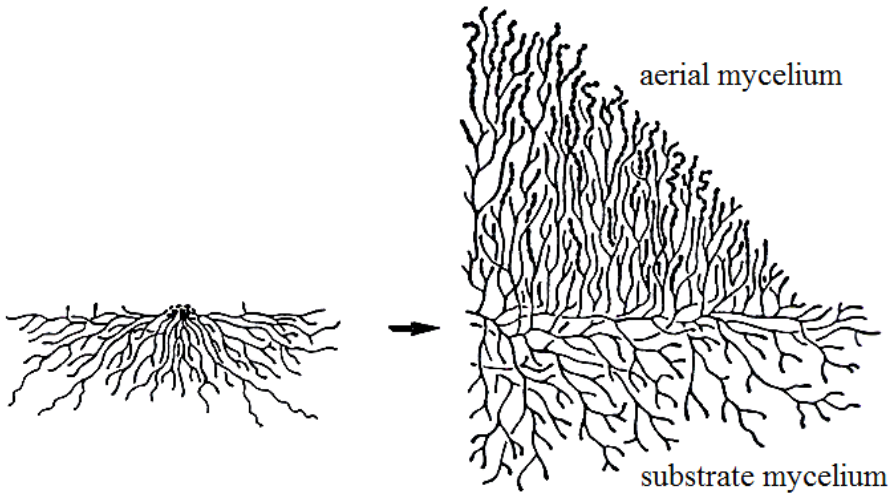


Figure 2.2 Two phase of the development of *Streptomyces* colony. (Adapted from Vobis, 1997)

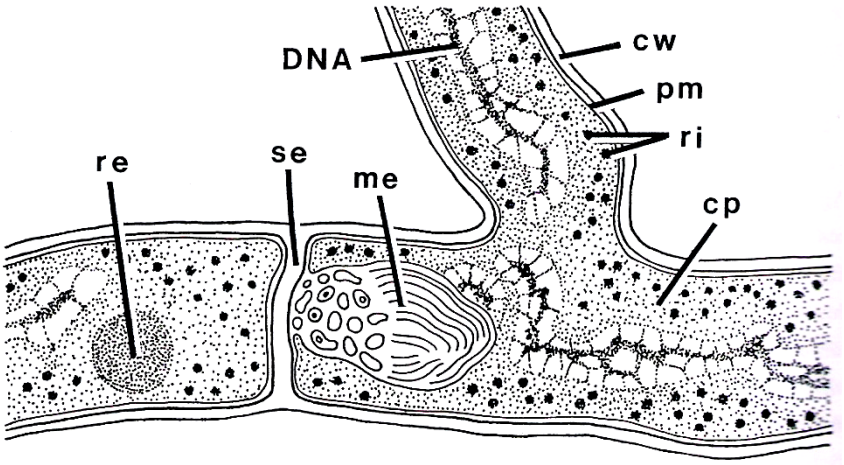


Figure 2.3 Typical cross section of *Streptomyces* hyphae; cp: cytoplasm, cw: cell wall, pm: plasma membrane, ri: ribosome, re: reserve material, and se: septum, me: mesosome (Vobis, 1997).

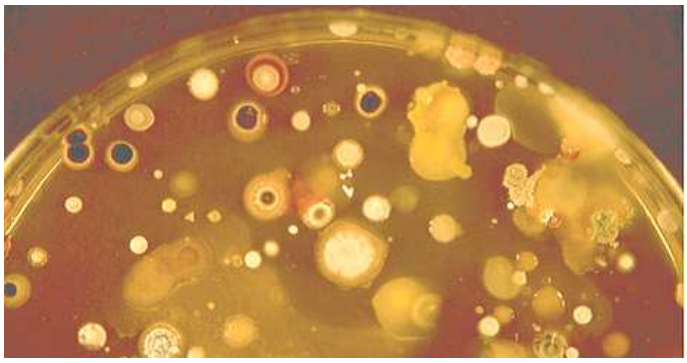
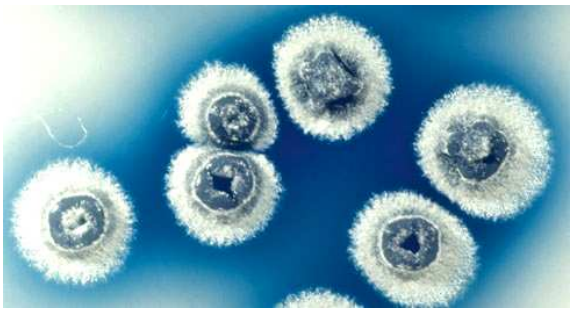


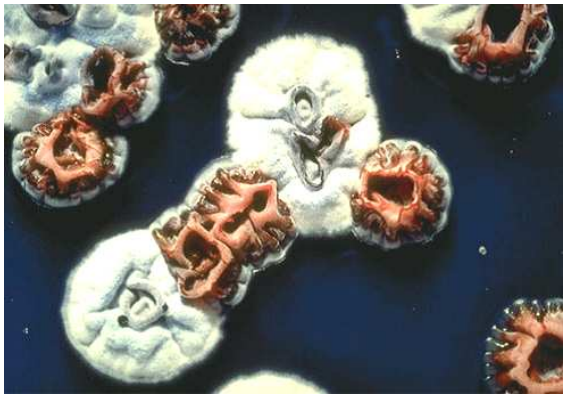
Figure 2.4 *Streptomyces* and other soil bacteria grew on casein–starch agar plate. These *Streptomyces* showed various colony colors which could easily be identified by their opaque, rough, nonspreading morphology. (Madigan *et al*., 2012).

2.1.3. Life cycle of *Streptomyces*

Life cycle of the common *Streptomyces coeliocolor*A3(2) (Figure 2.5) is presented in Figure 2.6. Upon the germination of a spore, substrate mycelium lacking of cross-walls (septa) first grows into the culture medium or other solid substrata (Figure 2.7a) forming a young colony. As the colony ages, the upward growing aerial mycelium begins to formed above the substratum and at this time *Streptomyces* begins to synthesize useful secondary compounds. Apical aerial spore-producing hyphae, called sporophores, grow out from the tips of aerial mycelium and differentiate to form spores. Sporophores are multinucleate producing spores, called conidia, by the formation of sporulation-specific septa (Figure 2.7b). Despite *Streptomyces* complex life cycle, theirs conidia are simply formed by the separation of the individual cells directly into spores (Figure 2.8) without elaborate differentiation that found in the formation of *Bacillus, Clostridium* or *Thermoactinomyces* endospores. The sporophores later become conidia-supporting structures that project them above *Streptomyces* colonies. The dispersion of mature spores begins the next *Streptomyces* life cycle.



(a)



(b)

Figure 2.5 Close-up photo of colonies of *Streptomyces coelicolor*.The antibiotic undecylprodigiosin is excreted in the red regions of the colony in (b). (Madigan *et al*., 2012)

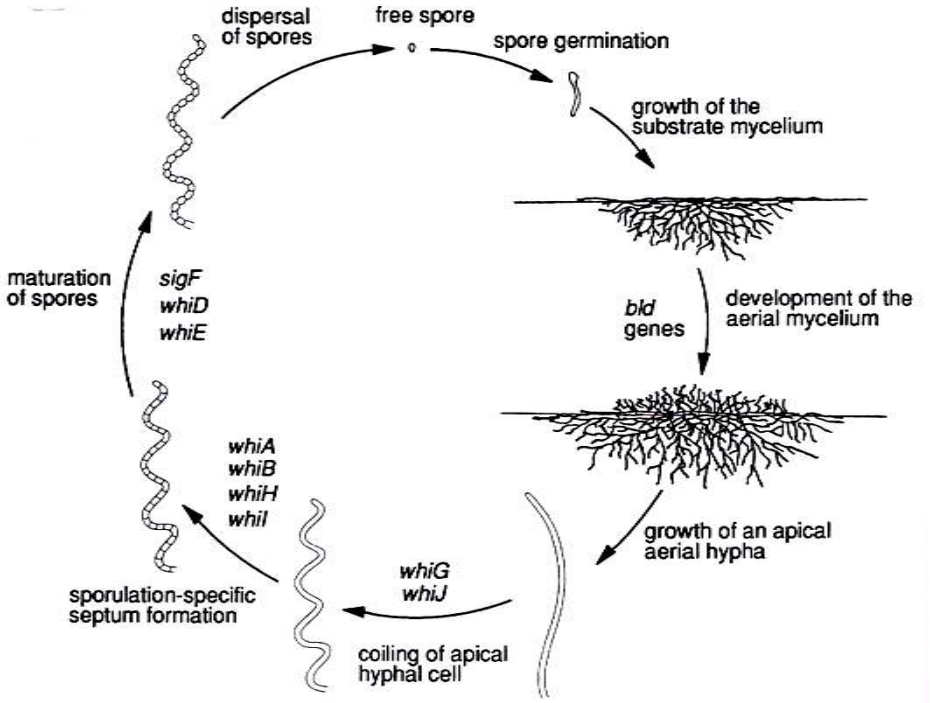
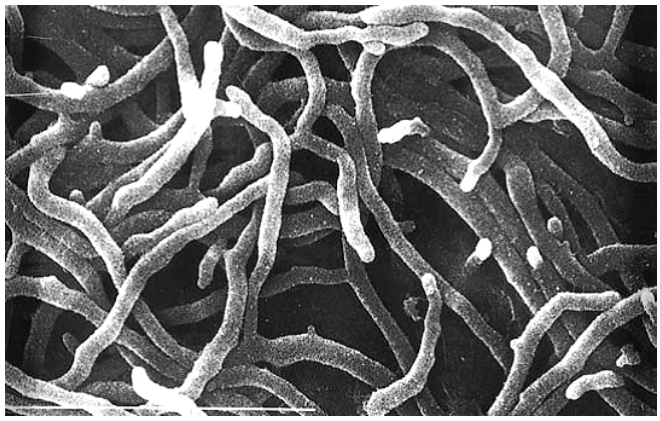
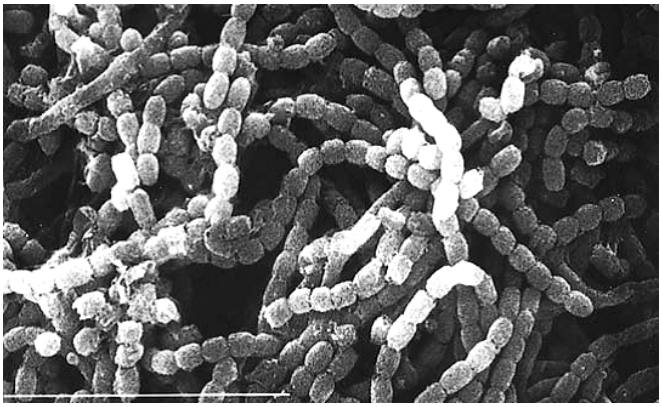


Figure 2.6 Life cycle of *Streptomyces coelicolor*A3(2) (Chater, 1997).



(a)



(b)

Figure 2.7 SEM images of *Streptomyces coelicolor*: (a) Substrate hyphae that are straight which branches and (b) Chains of spores that will eventually pinch off and dispersed (Willey, 2008).

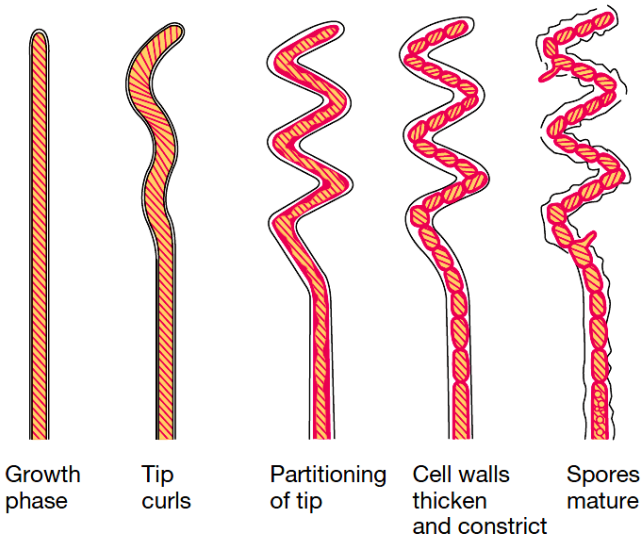


Figure 2.8 Diagram of stages in the conversion of an aerial hypha (sporophore) into spores (conidia) (Madigan, 2012).

2.1.4. Ecology and isolation of *Streptomyces*

Streptomyces are free-living and primarily soil inhabitants. However, they can live in water and inside plant tissue (Weete *et al.*, 1979; Benimeli *et al.*, 2003; Cao *et al*., 2005; Taechowisan *et al.*, 2006). Soil *Streptomyces* are responsible for the odor of moist earth which mainly consists of volatile substances geosmin (Gerber and Lechevalier, 1965). They usually assume the role of saprophytes in soil ecosystem and are extremely important in the mineralization of organic matter due to theirs nutritional flexibility enable them to aerobically degrade complex resistant organic compounds, such as lignin, pectin, agar, chitin, keratin, latex and aromatic compounds (Ruttimann *et al.*, 1987; Mahadevan and Crawford, 1997; Nishimura *et al.*, 2006; Meanwell and Sharma, 2008; Jacob *et al.*, 2008).

However, certain species of them can be a pathogen in plants and animals. Phytopathogenic *Streptomyces* spp. cause diseases mainly by attacking underground structure of diverse plant species (Loria *et al.*, 1997). Some common phytopathogenic *Streptomyces* causing significant economic loss are *S. scabies* causing common potato scab (Lambert and Loria, 1989) which makes an unpleasant appearance and devalues the potato but not harm inside and *S. ipomoeae* causing sweet potato soil rot (Person and Martin, 1940) which reduces yield as well as marketability of storage root. In human, *S. lanatus* inflection results in *Streptomyces* pneumonia (Kofteridis *et al.*, 2007) while *S. somaliensis* infection is the common cause of actinomycetoma lesions (El Hassan, 2001).

Alkaline to neutral pH are more favorable to the development of *Streptomyces*. Well-drained soils are preferred by *Streptomyces* to water logged soils that quickly become anoxic although *Streptomyces* are reported to be isolated from mud (Sponga *et al.*, 1999). To isolate *Streptomyces* from soil, a suspension of soil in sterile water is diluted and spread on selective agar medium, and the plate is incubated aerobically at 25°C for 5–7 days. Common selective agents used in *Streptomyces* isolation are antibiotics such as cyclohexamide, kanamycin, leucomycin, nalidixic acid, nivobiocin, norfloxacin, nystatin and tunicamycin (Hayakawa *et al.*, 1996; Suzuki *et al.*,

1999; Boudjella *et al.*, 2006). Apart from antibiotics, selective agents for *Streptomyces* are usually polymeric organic nutrients such as starch, cellulose, hemicellulose, lignin, tannin, chitin and casein. Pretreatment of soil samples may be required to reduce the number of undesired microorganism. To this end, incubation at high temperature or heat or viral infection can be employed (Kurtboke *et al.*, 1992; Suzuki *et al.*, 1999).

2.1.5. Strain identification

2.1.5.1. Classification of actinobacteria based on morphology versus molecular genetic data

There is marked discrepancy in the classification of actinobacteria based on morphology in *Bergey's manual of determinative bacteriology, 9th edition.* And that in *Bergey's manual of systematic bacteriology, 2nd edition, Vol. 5* that is largely phylogenetic (Holt *et al*., 1994; Whitman *et al*., 2012). The hyphae-producing *Thermoactinomyces* that were not classified as actinomycetes due to the fact that they produce mycelium were found to relate genetically more to *Bacillus* than to other hyphae-producing actinomycetes. On the other hand, *Micrococcus* was classified as actinobacteria based on sequences of rRNA, DNA and proteins despite its lacking of the ability to produce hyphae.

2.1.5.2. Classification of *Streptomyces* species

Both morphological and physiological characteristics are used to determine *Streptomyces* species: Color of the aerial and substrate mycelia, shape and arrangement of aerial hyphae and spore-bearing structures, surface features of individual spores, spore heat resistance, cell wall composition, phospholipid composition of plasma membranes, carbohydrate use, antibiotic production, melanin synthesis, nitrate reduction and the hydrolysis of urea and hippuric acid. Morphologies of spore-bearing structures of various streptomycetes are given in Figure 2.9. Streptomycetes spores can be smooth, hairy, or spiny in texture as shown in Figure 2.10.

Four major cell wall types can be distinguished according to three features of peptidoglycan composition and structure: the amino acid in tetrapeptide side chain position 3, the presence of glycine in interpeptide bridges, and peptidoglycan sugar content (Table 2.1). Cell wall type determined by the analysis of whole cell extracts indicates that of *Streptomyces* have cell wall types I characterized by the present of *L*-isomer of diaminopimelic acid and the amino acid glycine in interpeptide bridge. There is no characteristic sugar in cell wall (Type C) of *Streptomyces*.

Recently, molecular genetic methods were employed to speed up the process of *Streptomyces* species identification. In 2001, Rintala *et al*. presented a *Streptomyces* phylogenetic tree constructed based on 16S-rRNA sequences that were PCR-amplified using 3 primer pairs. In the application of 16S-ITS RFLP finger printing to identify streptomycetes, Lanoot *et al*. (2005) can distinguish 463 *Streptomyces* isolates into 59 distinct groups.

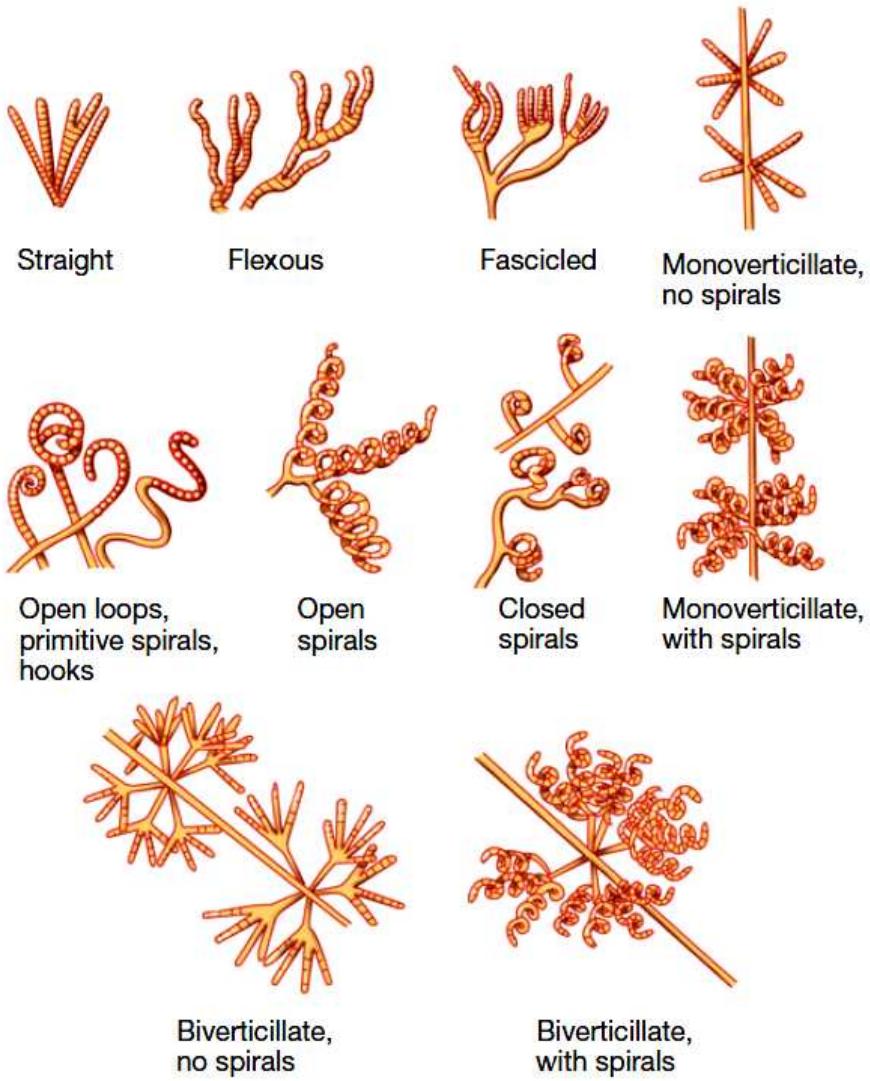
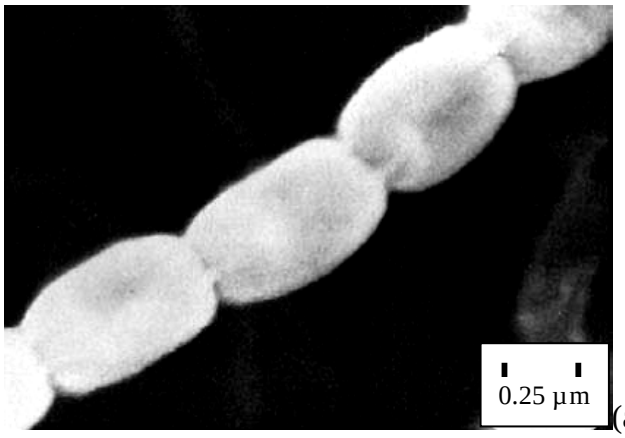
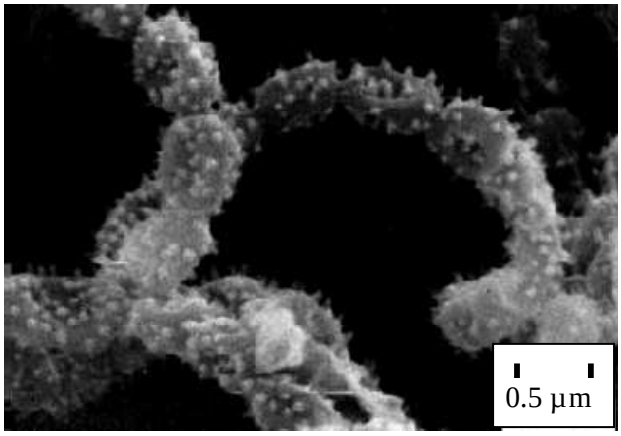


Figure 2.9 Morphologies of spore-bearing structures in the *Streptomyces* (Madigan *et al*., 2012).



(a)



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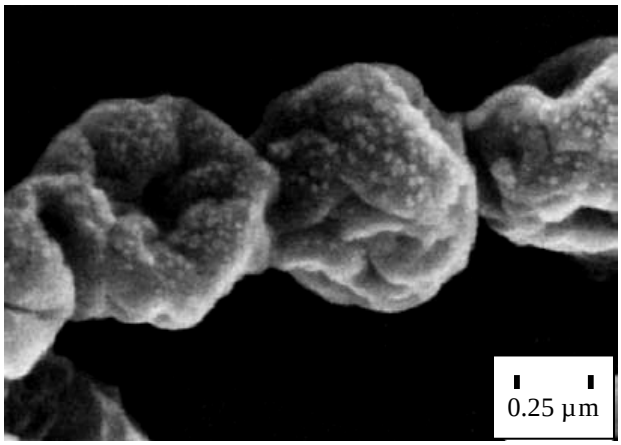


(c)

Figure 2.10 SEM images of **(a)** Smooth spores of *Streptomyces niveus,*
(b) Spiny spores of *S. viridochromogenes*, and
(c) Warty spores of *S. pulcher* (Willey, 2008).

Table 2.1 Actinomycetes cell wall types and whole cell sugar pattern

Peptidoglycan composition and structure	**Amino acid type in cell wall**			
	I	**II**	**III**	**IV***
- Optical isomer of di-aminopymeric acid	L, L	*meso*	*meso*	*meso*
- Glycine linkage between tetrapeptide	+	+	-	-
Sugar in whole cell	**Sugar pattern in whole cell**			
	A	**B**	**C**	**D**
- Arabinose	+	-	-	+
- Galactose	+	-	-	-
- Xylose	-	-	-	+
- Madulose	-	+	-	-

\+ present

− absent

* Cell Wall Type IV always exhibit sugar pattern A.

2.1.6. Genome of *Streptomyces*

The 9.1Mbp of *Streptomyces coelicolor* linear chromosome was sequenced in 2002 and found to contain 8,153 open reading frames (Zhou, 2012). It is one of the largest prokaryotic genomes reflecting the number of proteins required to undergo a complex life cycle. The core genome as analyzed using Ortho MCL comparative analysis method makes up approximately 40% of the genome. It contains important genes for *Streptomyces* biology including those involved in complex gene regulation network, powerful secretion system, secondary metabolism and morphological differentiation (Zhou, 2012). Approximately 23 clusters of genes accounting for 4.5% of the genome have been assigned to the production of secondary metabolites (Marinelli, 2009). Apart from genes that encode for its 4 antibiotics, genes were discovered that are thought to encode an additional 18 secondary metabolites (Willey, 2008).

2.1.7. Horizontal gene transfer involving *Streptomyces*

Gene duplication and lateral gene transfer are believed to be major evolutionary forces driving the genome evolution in bacteria. Among filamentous bacteria, natural transformation has been discovered only in *Thermoactinomyces*. By means of conjugation of its plasmid, however, *Streptomyces* is the common source of antibiotic-producing phenotype. By the construction of phylogenetic trees based on *trpB*, a housekeeping gene involved in tryptophan biosynthesis, and streptomycin biosynthetic cluster (*strB1*), Egan *et al*. (2001) reported the phylogenetic incongruency between *trpB* and *strB1* trees. The author suggested that 2 isolates of *Streptomyces* were recipients in an intra-genera lateral gene transfer event. One strain had acquired the entire streptomycin biosynthetic cluster while the other contained only certain parts of the cluster. Novel rhodostreptomycin A and B were isolated from *Rhodococcus* strain (307CO) emerged from the competitive co-cultures between a strain of *Rhodococcus fascians* that is resistant to multiple antibiotics and a strain of *Streptomyces padanus* that is a highly stable actinomycin producer (Kurosawa, 2008; Kurosawa *et al.,* 2010). Since the parent *Rhodococcus fascians* strain does not synthesized any antibiotic, analysis of *Rhodococcus* 307CO genome was carried out and found a large segment of DNA derived from the *Streptomyces pandanus*. This is an example lateral gene transfer involving *Streptomyces* and other genetically unrelated bacteria.

2.2 Bioactive compounds produced by *Streptomyces*

2.2.1 Secondary compounds produced by *Streptomyces*

It has been estimated that almost 12,000 bioactive secondary metabolites were discovered during 1940s – 1960s. Among them, 55% were produced by *Streptomyces* sp., 11% from other actinomycetes, 12% from non-filamentous bacteria and 22% from filamentous fungi (Marienlli, 2009). Apart from being employed as drug precursors, 2 mains applications of secondary metabolites produced by *Streptomyces* are as antibiotics and as cancer chemotherapeutic agents. Bioactive compounds with percentages above 1%

produced by *Streptomyces* isolated at Basic Research Center of Merck Sharp & Dohme in Spain are shown in Figure 2.11.

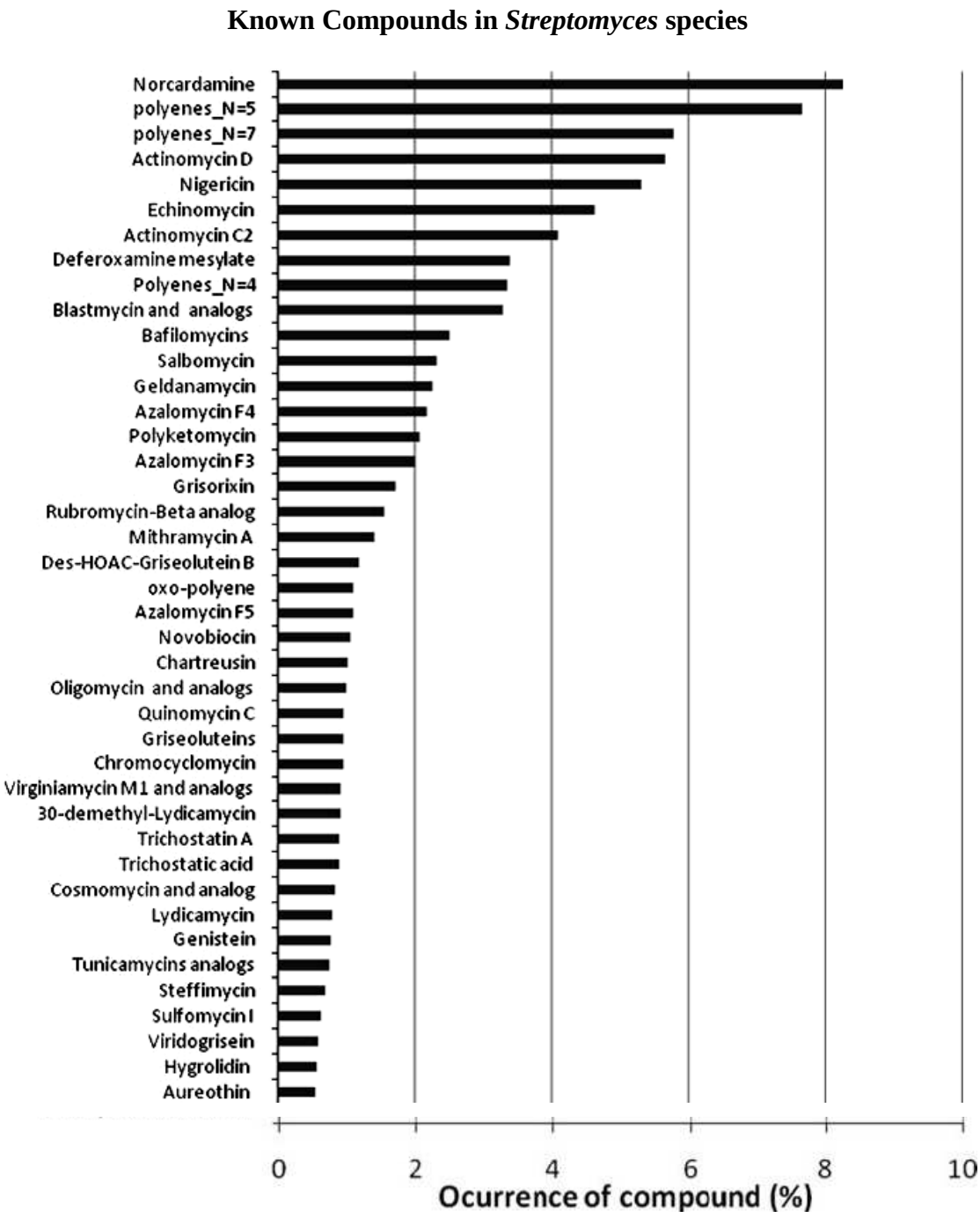


Figure 2.11 Bioactive compounds produced by *Streptomyces* isolated at Basic Research Center of Merck Sharp &Dohme (Genilloud *et al.*, 2011). Note that only those with percentages above 1% are shown.

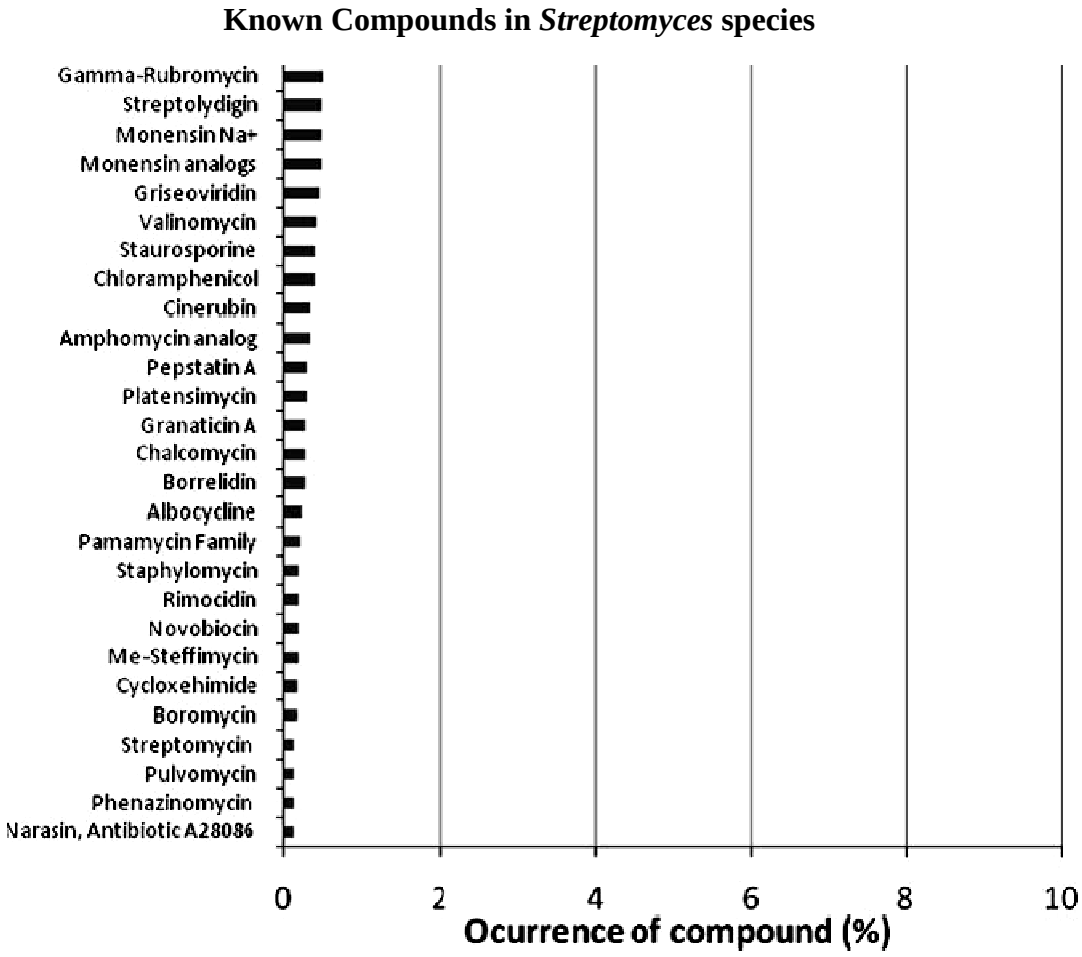


Figure 2.11 (*Continued*) Bioactive compounds produced by *Streptomyces* isolated at Basic Research Center of Merck Sharp &Dohme (Genilloud *et al.*, 2011). Note that only those with percentages above 1% are shown.

2.2.2 Antibiotics produced by *Streptomyces*

Approximately 50% of all *Streptomyces* isolated so far had been found to be antibiotic producer, some of which could produce more than one antibiotic that are often chemically unrelated (Madigan *et al.*, 2012). Lee and Hwang (2002) reported that more than half of *Streptomyces* isolated from soil sample collected from west parts of South Korea can inhibit fungal growth. Over 500 distinct antibiotics were produced by *Streptomyces* and many more are suspected (Madigan *et al.*, 2012). Approximately 3,000 antibiotics are isolated from Streptomycetes during 1947 – 1997 (Watve *et al.*, 2001) showed in Figure 2.12. From the antibiotic stand point, *Streptomyces* is the most

productive genus of microorganisms. Three out four members of the initial quartet of “wonder drugs” which are broad-spectrum antibiotics, i.e. streptomycin, chloramphenicol and tetracycline, are synthesized by *Streptomyces* spp. (Pommerville, 2011) (The other wonder drug is penicillin synthesized by *Penicillium* spp.). Some common antibiotics produced by *Streptomyces* are given in Table 2.2.

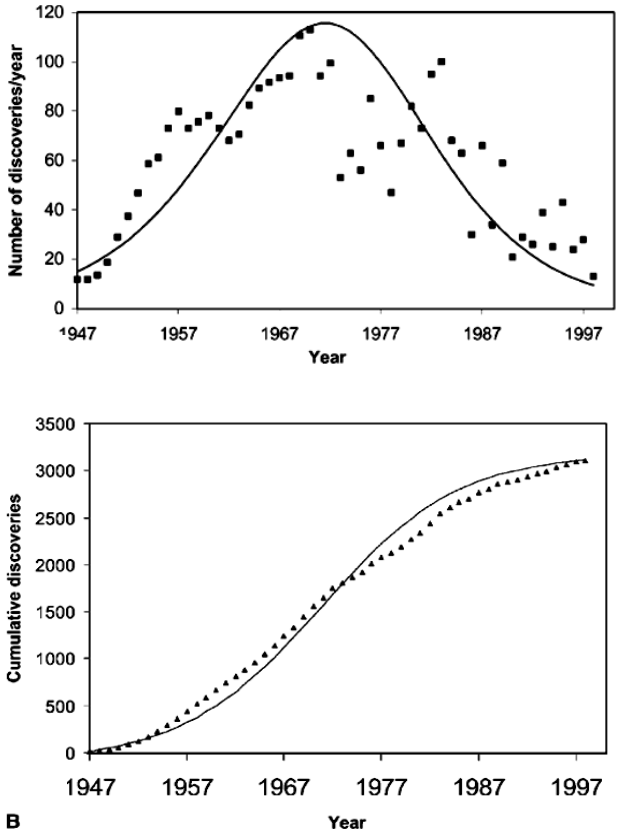


Figure 2.12 Number of antibiotics (marked by ▪ or ▲) isolated from *Streptomyces* during 1947 – 1997. The line denotes the fitted logistic curve.

Table 2.2 Some common antibiotics synthesized by *Streptomyces* spp.

Chemical class (MoA[a]/Effect)	**Common name**	**Source**	**Active against[b]**
Aminoglycosides (iPS-30S/Cidal)	Streptomycin	*Streptomyces griseus*	Most Gram negative bacteria
	Spectinomycin	*Streptomyces* spp.	*Mycobacterium tuberculosis*, penicillinase-producing *Neisseria gonorrhoeae*
	Neomycin	*Streptomyces fradiae*	Broad spectrum, usually used in topical applications because of toxicity
Tetracyclines (iPS-30S/Static)	Tetracycline	*Streptomyces aureofaciens*	Broad spectrum, Gram positive and Gram negative bacteria, rickettsias and chlamydias, *Mycoplasma*
	Chlorotetracycline	*Streptomyces aureofaciens*	As for tetracycline
Macrolides (iPS-50S/Static)	Clindamycin	*Streptomyces lincolnensis*	Effective against obligate anaerobes, especially *Bacteroides fragilis*, the major cause of anaerobic peritoneal infections
Polyenes	Nystatin	*Streptomyces noursei*	Fungi, especially *Candida* infections
	Amphotericin B	*Streptomyces nodosus*	Fungi
Other	Chloramphenicol (iPS-50S/Static)	*Streptomyces venezuelae*	Broad spectrum; drug of choice for typhoid fever

[a] Mechanism of action:

iPS-30S Inhibition of protein synthesis via 30S ribosomal subunit

iPS-50S Inhibition of protein synthesis via 50S ribosomal subunit

[b]Most antibiotics are effective against several different Bacteria. The entries in this column refer to the common clinical application of a given antibiotic.

Source: Adapted from Madigan (2012)

Recently, 2 new broad-spectrum antibiotics, platensimycin and platencin were discovered in strains of *S. platensis* isolated from soil sample collected from South African and Spain, respectively. Platensimycin targets FabF condensing enzymes (Wang *et al.*, 2006; Genilloud, 2011) while platencin targets both FabH and FabF condensing enzymes (Wang *et al.*, 2007; Genilloud, 2011). They, therefore, selectively inhibits cellular fatty acid biosynthesis. Such unique mechanism of action along with theirs novel structures provides a great opportunity to develop a new class of antibiotics. Furthermore, a pharmacological study using rats reported the potential of applying plantensimycin or derivatives as anti diabetic agent in treatment of diabetes and related metabolic disorders (Wu *et al.*, 2011).

2.2.3 Cancer chemotherapeutic agents. produced by *Streptomyces*

Physicians' Cancer Chemotherapy Drug Manual 2010 lists 9 cancer chemotherapeutic agents produced by *Streptomyces* spp. which are summarized in Table 2.3.

Table 2.3 Cancer chemotherapeutic drug produced by *Streptomyces* spp. in current use.

I Antitumor Antibitics		
1.	**Name/Trade Name**	Bleomycin, Blenoxane
	Source	*S.verticillus*
	Mechanism of Action	Formation of reactive oxygen species resulting breakage in DNA strand.
	Indication	1. Hodgkin's and non-Hodgkin's lymphoma. 2. Germ cell tumors, 3. Head and neck cancer. 4. Squamous cell carcinomas of the skin, cervix and vulva. 5. Sclerosing agent for malignant pleural effusion and ascites.

2.	**Name/Trade Name**	Dactinomycin, Actinomycin-D, Cosmegen
	Source	*Streptomyces* sp.
	Mechanism of Action	Chromophore intercalates preferentially between G-C basepairs and thus inhibits DNA synthesis and function; Can also bind single-stranded DNA; Formation of reactive oxygen species resulting breakage in DNA strand.
	Indication	1.Wilms' tumor. 2.Rhabdomyosarcoma. 3.Germ cell tumors. 4.Gestational trophoblastic disease. 5. Ewing's sarcoma.
3.	**Name/Trade Name**	Daunorubicin, Daunomycin, Cerubidine, Rubidomycin
	Source	*S. peucetius, S. insignis, S. coeruleorubidus*
	Mechanism of Action	Intercalate into DNA resulting in the inhibition DNA synthesis and function; Inhibits DNA-dependent RNA Polymerase and thus transcription; Formation of a cleavable complex with DNA and topoisomerase II leading to DNA breaks; Formation of reactive oxygen species resulting breakage in DNA strand.
	Indication	1. Acute myelogenousleukemia—Remission induction and relapse. 2. Acute lymphoblastic leukemia—Remission induction and relapse. Daunorubicin Liposome: HIV-associated, advanced Kaposi's sarcoma—First-line therapy
4.	**Name/Trade Name**	Doxorubicin, Adriamycin, Hydroxydaunorubicin
	Source	*S. peucetius*
	Mechanism of Action	Intercalate into DNA resulting in the inhibition DNA synthesis and function; Inhibits DNA-dependent RNA

		Polymerase and thus transcription; Formation of a cleavable complex with DNA and topoisomerase II leading to DNA breaks; Formation of reactive oxygen species resulting breakage in DNA strand.
	Indication	1. Breast cancer. 2. Hodgkin's and non-Hodgkin's lymphoma. 3. Soft tissue sarcoma. 4. Ovarian cancer. 5. Non–small cell and small cell lung cancer. 6. Bladder cancer. 7. Thyroid cancer. 8. Hepatoma. 9. Gastric cancer. 10. Wilms' tumor. 11. Neuroblastoma. 12. Acute lymphoblastic leukemia. Doxorubicin Liposome: 1. AIDS-related Kaposi's sarcoma—Used in patients with disease that has progressed on prior combination chemotherapy and/or in patients who are intolerant to such therapy. 2. Ovarian cancer—Metastatic disease refractory to both paclitaxel and platinum-based chemotherapy regimens. 3. Multiple myeloma—FDA approved in combination with bortezomib in patients who have not previously received bortezomib and who have received at least one prior therapy.
5.	**Name/Trade Name**	Epirubicin, 4 Epi-doxorubicin, Ellence
	Source	Semisynthetic derivative of Doxorubicin
	Mechanism of Action	Intercalate into DNA resulting in the inhibition DNA

		synthesis and function; Formation of a cleavable complex with DNA and topoisomerase II leading to DNA breaks; Formation of reactive oxygen species resulting breakage in DNA strand.
	Indication	1. Breast cancer—FDA-approved as a component of adjuvant therapy in women with axillary node involvement following resection of primary breast cancer. 2. Metastatic breast cancer. 3. Gastric cancer.
6.	**Name/Trade Name**	Idarubicin, Idamycin, 4-Demethoxydaunorubicin
	Source	Semisynthetic derivative of Daunorubicin
	Mechanism of Action	Intercalate into DNA resulting in the inhibition DNA synthesis and function; Formation of a cleavable complex with DNA and topoisomerase II leading to DNA breaks; Formation of reactive oxygen species resulting breakage in DNA strand. * Specificity, in part, for the late S- and G2-phases of the cell cycle.
	Indication	1. Acute myelogenousleukemia. 2. Acute lymphoblastic leukemia. 3. Chronic myelogenousleukemia in blast crisis. 4. Myelodysplastic syndromes.
7.	**Name/Trade Name**	Mitomycin-C, Mutamycin, Mitomycin (Also an alkylating agent)
	Source	*S. caespitosus*
	Mechanism of Action	Inhibits DNA-dependent RNA Polymerase and thus transcription; Reduced derivatives by cellular enzymes inhibit DNA synthesis and function; Cross-links of DNA strand by alkylation results in inhibition of DNA synthesis and function.

		Preferential activation of mitomycin-C in hypoxic tumor cells.
	Indication	1. Gastric cancer. 2. Pancreatic cancer. 3. Breast cancer. 4. Non–small cell lung cancer. 5. Cervical cancer. 6. Head and neck cancer (in combination with radiation therapy). 7. Superficial bladder cancer.
II Anti-metabolites		
8.	**Name/Trade Name**	Pentostatin, 2'-Deoxycoformycin, Nipent, dCF
	Source	*S. antibioticus*
	Mechanism of Action	Inhibition of the enzyme adenosine deaminase results in accumulation of dATP which is cytotoxic to lymphocytes; dATP also inhibits ribonucleotidereductase resulting in inhibition of DNA synthesis and function; Inhibition of S-adenosyl-L-homocysteine hydrolase resulting in inhibition of one-carbon dependent methylation reactions.
	Indication	1. Hairy cell leukemia. 2. Chronic lymphocytic leukemia. 3. Cutaneous T-cell lymphoma. 4. Acute lymphoblastic leukemia.
III Alkylating Agents		
9.	**Name/Trade Name**	Streptozocin, Streptozoticin, Zanosar
	Source	*Streptomyces* sp.
	Mechanism of Action	Intrastrand cross-links of DNA results by alkylation results in inhibition of DNA synthesis and function; * Selectively targets pancreatic β cells, possibly due to the presence of glucose moiety on the compound.

		** In contrast with other nitrosoureaanalogs, no effect on RNA or protein synthesis.
	Indication	1. Pancreatic islet cell cancer. 2. Carcinoid tumors.
Source: Chu and De Vita, Jr., 2010.		

Some *Streptomyces* secondary metabolites are in clinical trial and evaluated for potential of application as cancer chemotherapeutic drugs. Geldanamycin, a benzoquinoidansamycin antibiotic,was first isolated in 1970 from *Streptomyces hygroscopicus* var. geldanus var. nova. It inhibits the chaperone function of Hsp90 and is broadly cytotoxic in the NCI-60 cell-line screen. Its semisynthetic derivatives, 17-allylamino-17-demethoxy geldanamycin, is less toxic in rats and caused growth inhibition in breast, melanoma and ovarian mouse xenograft models (Necker, 2008; Airley, 2009). Enediyne antibiotics are distinguished structurally by its 9- or 10-membered unsaturated macrocyclic core with two acetylenic groups conjugated to a double bond. As a prototype representing the 9-membered enediynes, C-1027 synthesized by *S. globisporus* was studied extensively and reported to show high cytoxicity to cancer cells. Its mechanism of action was demonstrated to include the induction of oxygen-independent interstrand DNA crosslinks in addition to the oxygen-dependent DNA strand breaks typically generated by other enediynes. This unique oxygen-independent mechanism suggests that C-1027 may be effective against hypoxic tumor cells (Shao and Zhen, 2008). It is currently undergoing phase II clinical trials (Chen *et al.*, 2011).

2.2.4 Some other bioactive compounds produced by *Streptomyces*

Some common bioactive compounds other than antibiotics and cancer chemotherapeutic drugs that are produced by *Streptomyces* are summarized in Table 2.4.

Table 2.4 Some Non-Antibiotics Bioactive Compounds Synthesized by *Streptomyces*

Use	Source	Specific Product	References
Antiprotozoal Agent (Polyethers); Coccidiostat (coccidinia parasite), rumenal growth promoter	*Streptomyces cinnamonensis*	Monensin	Butaye *et al*., 2003
	Streptomyces lasaliensis	Lasalocid	Berger *et al*., 1951
	Streptomyces albus	Salinomycin	Blazsek and Kubis, 2005
Antihelminthic agents; Helminths and arthropods	*Streptomyces avermitilis*	Avermectins	Omura and Shiomi, 2007
Enzyme inhibitors; Penicillinase inhibitor	*Streptomyces clavaligerus*	Clavulanic acid	Hirakata *et al*., 2009
Bioherbicides	*Streptomyces hygroscopicus*	Bialaphos	Muramaki *et al.*, 1986
Immunosuppressants; Organ transplants	*Streptomyces tsukabaensis*	FK-506/ tacrolimus	Pritchard, 2005
	Streptomyces hygroscopicus	Rapamycin	Pritchard, 2005

2.2.5 *Streptomyces* as Biological Control Agent

Besides being used in the manufacture of antibiotics, certain *Streptomyces* spp. are also applied as a biological control agent. Xiao *et al.* (2002) reported the use of *Streptomyces* to control root rots on alfalfa and soybean caused by a fungus *Phytophthora*.

Antagonistic *Streptomyces* (*S. diastatochromogenes* strain PonSSII and *S. scabies* strain PonR) capable of controlling potato scab were also reported (Liu *et al.*, 1995).

2.3 Actinomycin-D produced by *Streptomyces parvulus*

2.3.1 Bioactive compounds produced by *S.parvulus*

S. parvulus was first identified from soil sample in 1940 by Waksman who named the bacterium with 2 L's, i.e. *S. parvullus*, that was later corrected to *S. parvulus* by Hill *et al.* (1984). Bioactive compounds reported to be synthesized by *S. parvulus* is summarized in Table 2.5.

Table 2.5 Selected bioactive compounds produced by *S. parvulus*

No.	Compounds	Action	References
1.	Actinomycin-D	Antitumor antibiotics	Waksman and Woodurff, 1940
2.	Borrelidin	Angiogenesis inhibitor	Olano *et al*., 2004
3.	Manumycin A, B and C	Antineoplastic agent; Antibiotic against Gram positive bacteria	Zeeck *et al*., 1987; Thiericke *et al*., 1990
4.	Oleficin	Antibiotic against Gram positive bacteria; Antitumor	Gyimesi *et al.*, 1971
5.	Pyridindolol	Antibacterial agent; *β*- galactosidase inhibitor	Hagmann *et al*., 1988

2.3.2 Structure and uses of actinomycin-D

Actinomycin-D (Dactinomycin, Cosmogen; Figure 2.13) was first isolated from *Streptomyces parvulus* in 1940 (Waksman and Woodurff, 1940). It consists of a non-symmetric tricyclic phenoxazonechromophore called actinocin, which is linked to two short, identical cyclic pentapeptides. It is a potent inhibitor of DNA-dependent RNA synthesis. The chromophore moiety allows actinomycin-D to intercalate itself to guanine-cytidine base pairs resulting inthe inhibition of DNA synthesis and function. Actinomycins discovered in 1940 by Waksman and colleagues are so toxic to higher organisms (White, 2012) that it was suggested they be used as rat poison (Willey, 2008). Today, apart from being used in the cancer chemotherapy, actinomycin-D is a standard tool used in research to specifically block DNA-dependent RNA synthesis.

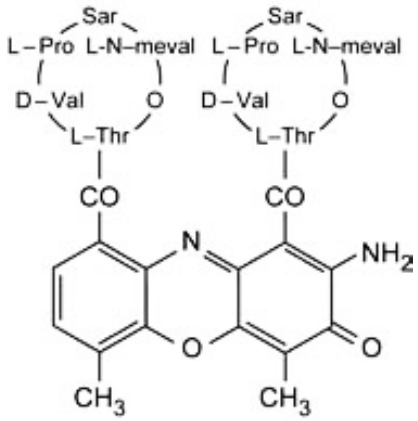


Figure 2.13 Structure of Actinomycin-D.

Although the pentapeptide rings are identical, they can be differentiated based on the non-symmetrical nature of the chromophore: α-chain peptide on benzenoid ring and β-chain peptide on quinoid ring. Various types of actinomycins antibiotics are differed in the amino acid sequences of the α- and/or β-chain (Table 2.6). If the pentapeptide rings are identical such as in actinomycin-D, the compound is said to be isoactinomycin, otherwise it is called *an*isoactinomycin.

Table 2.6 Amino Acid Sequences in Different Actinomycins

(Hollstein1974; Bitzer et al. 2006)

Actinomycin	Amino acid					
	Chain	1	2	3	4	5
C_1 (IV, D, I_1, X_1)	*α*	Thr	Val	Pro	Sar	MeVal
	β	Thr	Val	Pro	Sar	MeVal
C_2 (VI, I_2)	*α*	Thr	Val	Pro	Sar	MeVal
	β	Thr	α-Ile	Pro	Sar	MeVal
C_{2a} (i-C_2)	*α*	Thr	α-Ile	Pro	Sar	MeVal
	β	Thr	Val	Pro	Sar	MeVal
C_3 (VII, I_3)	*α*	Thr	α-Ile	Pro	Sar	MeVal
	β	Thr	α-Ile	Pro	Sar	MeVal
E_1	*α*	Thr	α-Ile	Pro	Sar	MeVal / Me-Ile
	β	Thr	α-Ile	Pro	Sar	
E_2	*α*	Thr	α-Ile	Pro	Sar	Me-Ile
	β	Thr	α-Ile	Pro	Sar	Me-Ile
F_1	*α*	Thr	Val / α-Ile	Sar	Sar	MeVal
	β	Thr		Sar	Sar	MeVal
F_2	*α*	Thr	Val / α-Ile	Pro / Sar	Sar	MeVal
	β	Thr			Sar	MeVal
Z_1	*α*	Thr	Val	HMPro	Sar	MeVal
	β	HThr	Val	MOPro	Sar	MeAla
Z_2	*α*	Thr	Val	HMPro	Sar	MeVal
	β	Thr	Val	MOPro	Sar	MeAla
Z_3	*α*	Thr	Val	HMPro	Sar	MeVal
	β	ClThr	Val	MOPro	Sar	MeAla
Z_4	*α*	Thr	Val	MePro	Sar	MeVal
	β	Thr	Val	MOPro	Sar	MeAla
Z_5	*α*	ClThr	Val	MePro	Sar	MeVal
	β	Thr	Val	MOPro	Sar	MeAla
G_1	*α*	Thr	Val	Pro	Sar	MeVal
	β	HThr	Val	HMPro	Sar	MeAla
G_2	*α*	Thr	Val	HMPro	Sar	Me-Ile
	β	ClThr	Val	Pro	Sar	MeAla
G_3	*α*	Thr	Val	HMPro	Sar	MeVal
	β	HThr	Val	Pro	Sar	MeAla
G_4	*α*	Thr	Val	HMPro	Sar	MeVal
	β	Thr	Val	Pro	Sar	MeAla
G_5	*α*	Thr	Val	HMPro	Sar	MeVal
	β	HThr	Val	Pro	Sar	MeAla

MeVal = methyl valine, MeIle = methyl isoleucine, MeAla = methyl alanine,
MePro = methyl proline, HMPro = hydroxy-methyl proline,

MOPro = methyl-oxoproline, ClThr = chlorothreonine, HThr = hydroxythreonine

2.3.3 Sources of actinomycin-D

Actinomymins are synthesized by various species of *Streptomyces* (Table 2.7). Some species produces more than one type of actinomycins making purification of actinomycin-D a complicated process due to structural similarity. One advantage of the production of actinomycin-D in *S. parvulus* is that no other actinomycins are produced by the organism (Inbar and Lapidot, 1988).

Table 2.7 Sources of actinomycin-D.

Produced by	**Source**	**Reference**
Streptomyces chrysomallus		Brockmann *et al*.,1960
S. antibioticus		Hollstein, 1974
S. pandanus	Soil from flowerpot	Kurosawa *et al*., 2006
Streptomyces sp. Tc022	Endophyte in *Alpinia galanga*	Taechowisan *et al*., 2006
S. sindenensis	Soil from steel plant effluent sediment	Praveen *et al*., 2008 (a)
S.halstedii and S.anulatus	soil	Praveen *et al*., 2008 (b)
S.griseoruber	Soil associated with roots of a medical plant, *Azadirachta indica*	Praveen and Tripathi, 2009
S. avermitilis	Sediment from sea	Chen *et al*., 2012
S. plicatus		Lam *et al*., 2002

2.3.4 Biosynthesis of actinomycin-D

2.3.4.1 Switching to antibiotic production

Antibiotic production in *Streptomyces* is developmental-phase dependent. In liquid culture, the production of the antifungal candicid in by *Streptomyces griseus* occurs after vegetative growth has finished (Martin and McDaniel, 1975). Similarly, on agar surface, the onset of oleandomycin production by *S. antibioticus* coincides with beginning aerial mycelium development (Méndez *et al*., 1985). Antibiotic biosynthetic genes occur in clusters. The tight clustering of such genes was first demonstrated the fact that *S. parvulus* can synthesize the blue-pigmented polyketide antibiotic actinorhodin right after being transformed with a 35 kb fragment of *S.coelicolor* DNA and it is not known to make any structurally related compounds (Malpartida and Hopwood, 1984). Transcriptional activators encoded in regulatory genes of many antibiotic-producing-gene clusters control antibiotic production. Many transcriptional activators are pathway-specific and likely to determine the onset antibiotic production by a specific gene cluster while some has pleiotropic effects and likely to play regulatory roles in antibiotic production (Bibb, 1996). Factors appear to influence the onset of antibiotic production in streptomycetes are summarized graphically in Figure 2.14.

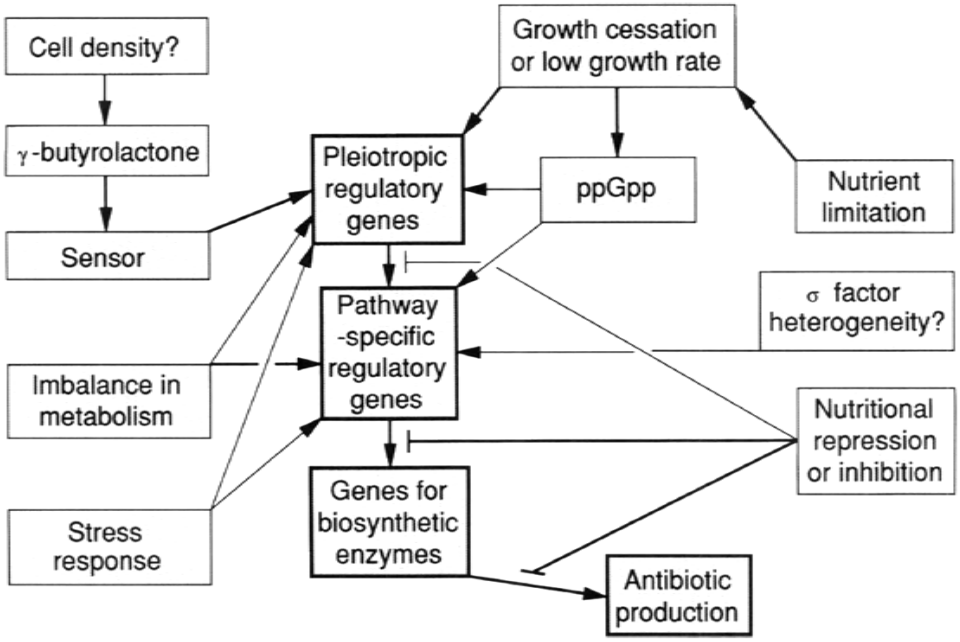


Figure 2.14 Factors potentially determining the onset of antibiotic production in *Streptomyces* sp. (Thinner lines represent plausible interactions for which there is currently no direct evident.)

2.3.4.2 Metabolic regulation of actinomycin-D biosynthesis

Biosynthetic pathway of actinomycinchromophore (actinocin) from tryptophanis summarized in Figure 2.15. On the study of regulation of tryptophan dioxygenase (tryptophan 2,3-dioxygenase; *L*-tryptophan:oxygen 2,3-oxidoreductase; EC 1.13.11.11) in *S. parvulus* cultured in a medium containing glutamate and fructose, Foster and Katz (1981) found that activity of the enzyme increased before the onset of actinomycin-D synthesis. Inbar and Lapodot (1988) put forward that actinomycin-D synthesis cannot start before a release from L-glutamate catabolite repression. L-Glutamate can provide both the nitrogen and the carbon requirements of *Streptomyces parvulus* cells. In the catabolism of a compound that can provide both the nitrogen and carbon requirements of a cell, carbon catabolite repression of the synthesis of enzymes catalysing the utilization of nitrogenous carbon compounds is modulated by the state of the nitrogen supply.

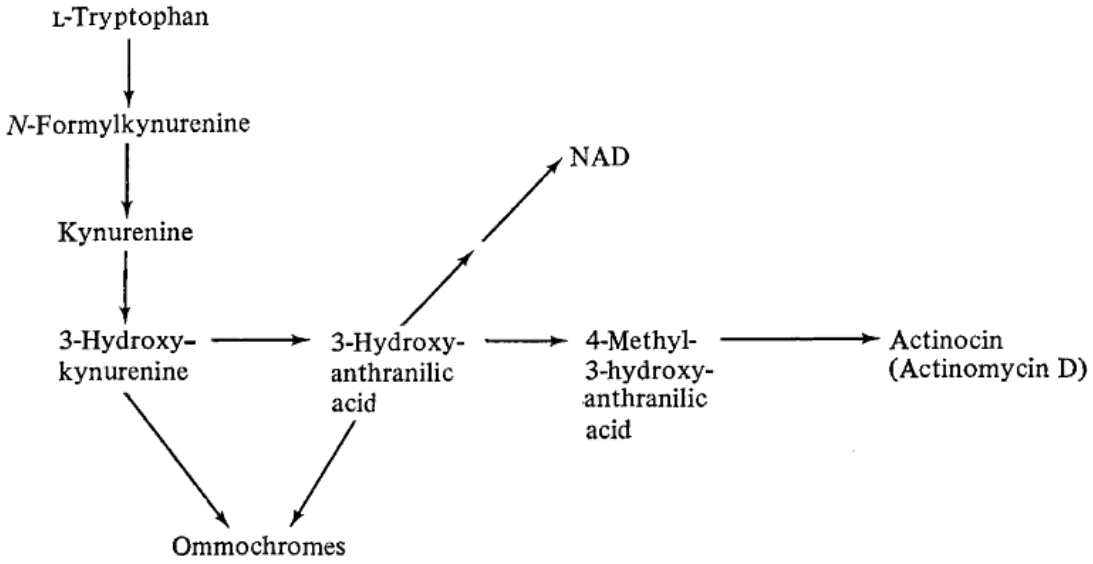


Figure 2.15 Pathways of tryptophan metabolism leading to actinocin biosynthesis (Troost and Katz, 1978).

Inbar and Lapodot (1988) went further by suggesting the regulation mechanism of actinomycin-D synthesis in *Streptomyces parvulus*: After extracellular glutamate is uptaken and completely consumed, catabolite repression of enzymes catalysing the utilization a carbohydrate source is overridden and released. Upon the take-up of extra cellular *D*-fructose the new intracellular pool of glutamate builds up and are followed by the synthesis of actinomycin-D. Based on radioactive study using ^{13}C, Inbar and Lapodot (1988) show that that D-fructose is the only precursor of sarcosine and methyl-valine despite the medium enrichment with the 5 amino acids found in actinomycin-D. *L*-glutamate mainly contributes its carbons to *L*-proline and *L*-threonine before the synthesis of actinomycin-D begins. The *N*-methyl groups of sarcosine and *N*-methyl-valine, and the methyl groups of the chromophore, arose from *D*-fructose catabolism.

Methyltransferase activity may accounts for the presence of *N*-methyl-amino acids in actinomycin-D. Recently, a methyltransferase activity which catalyzes the transfer of amethyl group from S-[^{14}C]adenosylmethionine to 3-hydroxyanthranilic acid was demonstrated in actinomycin-producing *S. antibioticus* (Jones, 1987).

Inbar and Lapidot (1988) also found that, *in S. parvulus*, the methyl group of exogenous methionine is extensively incorporated into the *N*-trimethyl group of choline and subsequently into the *N*-methyl-amino acids and the methyl groups of the chromophore. The fact that choline accumulated and is located in the membrane during actinomycin-D synthesis leads Inbar and Lapidor to suggested that the site of actinomycin-D synthesis is associated with the membrane where transmethylation occurs.

2.3.4.3 Optimization of culture condition to increase *Streptomyces parvulus* actinomycin-D production

The condition of actinomycin-D production in *S.parvulus* are summarized in Table 2.8.

Table 2.8 Selected culture conditions optimization for higher actinomycin-D production by *S. parvulus*

Strain	**Culture media**	**Condition**	**Yield (mg/l)**	**Reference**
ATCC 12434	D-fructose, L-glutamic acid, L-histidine, K_2HPO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ (Mg^{2+} 4.88 mg/l), $ZnSO_4 \cdot 7H_2O$ (Zn^{2+} 4.6 – 13.7 mg/l), $CaCl_2 \cdot 2H_2O$ (Ca^{2+} 7.5 – 14.6 mg/l), $FeSO_4 \cdot 7H_2O$ ($Fe2^{+}$ 8 – 10 mg/l), $CoCl_2 \cdot 6H_2O$ (Co^{2+} 0.003 – 0.006 mg/l)	Shaking flask 250 rpm, 30°C, 48 hr	500 - 600	Williams and Katz, 1977
ATCC 12434	same as Williams and Katz. 1977	Immobilized cell, same as Williams and Katz. 1977	50 *(discontinuous)* 73*(fed batch)* 80 *(continuous)*	Dalili and Chou, 1988)
DAUFPE 3124	Soy milk 30 g/l, glucose 20 g/l, $CaCO_3$ 2 g/l	Shaking flask 250 rpm, 30°C, 144 hr	530	Sousa *et al*., 1997
DAUFPE 3124	D-fructose 20 g/l, L-threonine 3.57 g/l, K_2HPO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 25 mg/l, $ZnSO_4 \cdot 7H_2O$ 25 mg/l, $CaCl_2 \cdot 2H_2O$ 25 mg/l, $FeSO_4 \cdot 7H_2O$ 25 mg/l	Shaking flask 250 rpm, 30°C, 144 hr	133	Sousa *et al*., 2001

DAUFPE 3124	Soy milk 30 g/l, fructose 20 g/l, $CaCO_3$ 2 g/l	Cultured in 30 °C bioreactor 144 hr, aeration 1.5 vvm, stirring speed 500 rpm	1530	Sousa *et al*., 2002
MITKK 103	Soluble starch 10 g/l, Glucose 20 g/l, Soytone 25 g/l, Yeast extract4 g/l, Malt extract 1 g/l, K_2HPO_4 0.05 g/l NaCl 2 g/l	Shaking flask 250 rpm, 30°C, 120 hr	30	Kurosawa *et al.*, 2006
MITKK 103	ISP-2 : Yeast extract 4 g/l, Malt extract 10 g/l, Glucose 4 g/l	Shaking flask 250 rpm, 30°C, 120 hr	50	Kurosawa *et al.*, 2006

CHAPTER 3
METHODOLOGY

3.1 Isolation, cultivation and maintenance of *Streptomyces parvulus* J6.2

Soil sample was collected from mangrove area in Chantaburi province, Thailand and 5g of sample was suspended in sterile 0.85% NaCl 45 mL to be diluted 10^{-1} fold. The soil suspension was shaken 200 rpm at 40 °C for overnight to decrease others bacteria. The sample was serial tenfold diluted and spread on starch-casein agar plates supplemented with 50 µg/mL cyclohexamide and 50 µg/mL nystatin for inhibit unexpected fungi. Culture plates were incubated at 30 °C for 14 days. The colonies of actinomycetes – showed compact, dusty appearances of various colors were selected under stereo microscrope. The selected colonies were streaked on sodium caseinate agar to isolate single colonies. The single colonies were sub-culture into sodium caseinate agar (SCA).

S*treptomyces* sp. isolated J6.2 can be cultured on general media as nutrient agar (NA) and malt extract agar (MEA). It can be also cultured on the international *Streptomyces* project media (ISP) including Tryptone- yeast extract agar (TYEA), ISP2- Yeast- malt extract agar (YMEA), ISP3- Oat meal agar (OA), ISP4- Inorganic salts starch agar (ISSA), ISP5- Glycerol asparagine agar (GAA) and ISP6- Peptone- yeast extract agar (PYEA).

Sodium caseinate which selective for *Streptomyces* growth was used to maintenance their colony for prevent contamination by other microorganisms. The colony on sodium caseinate agar can be kept at 4 °C for 4 – 6 months.

Glycerol strock was prepared by loop transfer spore into 20% sterile glycerol, the stock can be kept at -20 °C for 2 – 3 years.

3.2 Strain identification

3.2.1 Morphological study

Slide culture

Slide culture was used to study the isolated J6.2 morphology. The isolated J6.2 was cultured on sodium caseinate agar which is stabbed with cover slip and incubated at 30 °C until mycelium was colonized onto the cover slip and spores were formed. Slide was stained with lacto phenol cotton blue. The cover slip which shows hypha growth used to observe under light microscrope.

Scanning electron microscopic study

The isolated J6.2 was cultured on sodium caseinate agar until conidia were formed. Culture sample was fixed by osmium tetaoxide vapour (OsO_4) and then incubated under moist condition at 20 °C for 1 – 2 hr. The sample was dehydrated by soaking with 30%, 50%, 70% and 90% ethanol for 10 – 20 min each concentration and then soaked 3 times with absolute ethanol for 10 min. The sample was dried by critical point drying and coated with goal particle 20 nm think by ion sputter coater. The sample was studied it morphology by scanning electron microscope (SEM).

3.2.2 16S rDNA sequencing

3.2.2.1 DNA extraction of total DNA

The isolated J6.2 was cultured in NA for 3 – 5 days. Cell 20 – 50 mg was grinded in a mortar. Washing buffer 1,000 µl was added in the cell and transferred to 1.5 ml micro tube. The micro tube was centrifuged 15,000 rpm for 3 min and discharge supernatant to remove polysaccharide. The cell was washed again with washing buffer for 2 – 3 times or until the supernatant clear.

Cell was broken by a procedure described by Zhou *et al*. (1999). The cell was added with 2X CTAB lysis buffer for 700 µl and incubated at 65 °C for 1 hr. The sample was added 24 : 1 chloroform : isoamylalcohol solution for 700 µl and then mixed with vortex. The sample was centrifuged 15,000 rpm at room temperature for 8 min. Supernatant was kept into new micro tube.

The cell residual was extracted again. The supernatant was added with iso-propanol for 700 µl and kept in ice bath for 30 min. The sample was centrifuged 8,000 rpm at 4 °C for 10 min in order to precipitating DNA. The DNA precipitate was washed by added 70% ethanol for 500 µl and centrifuge 8,000 rpm at 4 °C for 5 min and then remove supernatant. The DNA precipitate was dried at room temperature and then dissolved with TE buffer for 100 µl.

For pretreatment of DNA template, the DNA solution which is dissolved in TE buffer was added 10 mg/ml RNase for 1 µl and then incubated at room temperature for 30 min. The sample was added with 60 µl PEG solution and kept in ice bath for 20 min. The sample was centrifuged 15,000 rpm at 4 °C for 10 min and then supernatant was removed. The DNA precipitate was washed with 70% ethanol for 500 µl and then centrifuged 15,000 rpm at 4 °C for 5 min and then ethanol was discharge. The DNA precipitate was dried at room temperature and then dissolved with TE buffer for 100 µl. The DNA solution was stored at -20 °C until use.

3.2.2.2 Amplification 16S rDNA of isolated J6.2

Amplification by PCR technique

PCR amplification of the 16S rDNA was performed using the DNA in step 3.3.2.1 as template. Primer was designed according to Lanoot *et al*. (2005) and Rintala *et al*. (2001**).**

- *pA*(5’AGAGTTTGATCCTGGCTCAG3’)
  (attracted to base pair 8 – 27)
- *pH*(5’AAGGAGGTGATCCAGCCGCA3’)
  (attracted to base pair 1541 – 1522)
- *StrepB*(5’ACAAGCCCTGGAAACGGGGT3’)
  (attracted to base pair 139 – 158)
- *StrepE*(5’CACCAGGAATTCCGATCT3’)
  (attracted to base pair 640 – 657)
- *StrepF*(5’ACGTGTGCAGCCCAAGACA3’)
  (attracted to base pair 1194 – 1212)

PCR mixture 10 µl consisted of

- 10x buffer 1 µl
- 2mM dNTP mix 1 µl
- 25mM $MgCl_2$ 0.6 µl
- forward primer (20 µM) 0.5 µl
- reverse primer (20 µM) 0.5 µl
- Taq DNA polymerase (5U) 0.1 µl
- sterile water 5.3 µl
- DNA template 1 µl

Amplification profile was followed by

	initial denaturation	at 95 °C for 5 minute
⎛	denaturation	at 95 °C for 1 minute
⎜	annealing	at 53 °C for 1 minute
⎝	extension	at 72 °C for 1 minute
	final extension	at 72 °C for 15 minute
	Hold (store)	at 4 °C until use

Reaction in step denaturation, annealing and extension were repeated 35 cycles.

Check PCR product by gel electrophoresis

PCR product was checked its size and purity by gel electrophoresis. Agarose gel was set and placed in electrophoresis chamber containing with 1X TAE buffer. The PCR product was mixed with loading dye ratio 5 : 2 and then loaded into the agarose gel. The gel was run under voltage 100 Volt for 20 – 30 min. DNA was detected by straining the gel with ethidium bromide and then detected the fluorescent under UV radiation. DNA size was measured by compared with standard DNA marker 100 bp + 1.5 kb DNA ladder.

3.2.2.3 Sequencing 16S rDNA and blasting compared with database

PCR products, in step 3.3.2.2, were purified, sequenced and aligned with selected sequences obtained from the GenBank/NCBI databases using CLUSTAL X version 1.83. The alignment was constructed for phylogenetic tree using the neighbor-joining method.

3.2.3 Physiological characterization

3.2.3.1 Physiological properties

Melanin production ability was tested by cultured the isolated J6.2 on peptone yeast extract iron agar (ISP6) and tyrosine agar. The culture was incubated at 30 °C for 4 days. Black or dark brown color on agar was observed if the culture had ability to produce melanin.

Hydrogen sulfide production ability was tested by stabbed the isolated J6.2 into ISP6 agar medium in 16 x 150 ml test tube. The culture was incubated at 30 °C for 1 day. Bluish-black color in agar medium was observed if the culture had ability to produce hydrogen sulfide compared with uninoculated control.

Nitrate reducing ability was tested by stabbed the isolated J6.2 into nitrate agar and incubated at 30 °C for 14 days. Nitrate reducing was detected by dropped 1 – 2 drop of solution A and solution B. The solutions consisting of

solution A sulfanilic acid 0.8 g dissolved in 100 ml of 5 normal acetic acid

solution B alphanaphthylamine 0.5 g dissolved in 100 ml of 5 normal acetic acid

If nitrate was reduced to be nitrite, the tested solution will be turned to red color. Negative false might be observed when nitrite converted to ammonia. Zinc power was added to the solution to detect nitrite remained if the red color appeared (Shirling and Gottlieb, 1996)

An ability to grow on pH 4.3 was tested by streaking the isolated J6.2 on Bennett's agar pH 4.3. The culture was incubated at 30 °C for 7 – 14 days and observed its growth.

An ability to grow on various temperatures were tested by streaking the isolated J6.2 on modified Bennett's agar and incubated at 37 and 45 °C for 7 – 14 days and incubated at 4 °C for 2 – 4 weeks and observed its growth.

3.2.3.2 Degradation activities

Isolated J6.2 was streaked on modified Bennett's agar adding various test degradation sources including 0.5% adenine, 0.5% tyrosine, 0.4% xylan, 0.4% xanthine, 0.1% casein, 0.4% gelatin and 0.1% starch. Degradation activity was detected by observed clear zone around culture colony. Gelatin and starch degradation activities were observed after pouring respectively with acidified mercuric chloride ($HgCl_2$) and iodine onto culture media.

3.2.3.3 Nitrogen utilization

Nitrogen utilization was tested by using 0.1% arginine, 0.1% histidine, 0.1% methionine, 0.1% KNO_3, 0.1% phenylalanine, 0.1% serine, 0.1% theonine and 0.1% valine as N – sources. Isolated J6.2 was streaked on basal medium agar consisting of various N – sources described above and incubated at 30 °C for 15 days. Nitrogen utilization was detected by compared their growth with basal medium without N – source. Basal medium supplemented with 0.1% asparagine or 0.1% proline was used as positive control.

3.2.3.4 Carbon utilization

Carbon utilization was tested by using 1% *L*-arabinose, 1% dextran, 1% *D*-fructose, 1% *D*-galactose, 1% *meso*-inositol, 1% *D*-lactose, 1% *D*-manitol, 1% *D*-mannose, 1% *L*-rhamnose, 1% sucrose, 1% theharose, 1% xylose, 1% sodium acetate, 1% sodium citrate and 1% sodium malonate. Isolated J6.2 was streaked on carbon utilization agar consisting of various C – sources described above and incubated at 30 °C for 5 days. Carbon utilization was detected by compared their growth with basal medium without C – source. The medium supplemented with 1% glucose was used as positive control.

3.3 Study of cultivation condition for secondary metabolite production

3.3.1 Effect of culture media

Spore suspension of *Streptomyces parvulus* strain J6.2 was prepared in sterile 0.85% NaCl and pipetted 100 µl into each 120 ml of difference culture media and culture condition shown in Table 3.1

Table 3.1 Culture media and cultivation condition used to study secondary metabolites

Test	Media	Condition
1	sodium caseinate broth (SCB)	Static cultivation
2	sodium caseinate broth	Static occasionally stirred
3	sodium caseinate broth	Shaken flask
4	sodium caseinate broth	Shaken flask with spring coil*
5	glucose yeast malt extract broth (GYM)	Shaken flask
6	GYM and SCB	Shaken flask
7	starch casein broth	Shaken flask

* stainless steel spring coil for increase DO level in broth

The cultures were incubated by shaken 200 rpm at 30 °C for 3 weeks. After incubation, the cultures were filled through filter paper no.4. The cell in each culture was dried in hot air oven, the sample was cooled down in desiccator. Broth was extracted with ethyl acetate and evaporated to be dryness under low pressure. The crude extracts were measured their weight and detected for their TLC and NMR pattern. Cell cultures were filled and dried in hot air oven and then measured their cell mass.

3.3.2 Effect of C and N source supplemented in sodium caseinate broth

Spore suspension was added into sodium caseinate broth supplemented with 0.5% C and N source. Glucose, galactose and starch were used as supplemented C – source. Yeast extract, malt extract and peptone were used as supplemented N – source described in Table 3.2

Table 3.2 C and N sources supplemented in sodium caseinate broth

Test	C - source	N - source
1	glucose	yeast extract
2	glucose	malt extract
3	glucose	peptone
4	galactose	yeast extract
5	galactose	malt extract
6	galactose	peptone
7	starch	yeast extract
8	starch	malt extract
9	starch	peptone

The cultures were incubated at 30 °C for 2 weeks. After incubation, the cultures were extracted with ethyl acetate and evaporated to be dryness under low pressure. The crude extracts were measured their weight and detected for their TLC and NMR pattern. Cell cultures filled and dried in hot air oven and then measured their cell mass.

3.3.3 Effect of starch and malt extract content supplemented in sodium caseinate broth

Spore suspension was added into sodium caseinate broth supplemented with various contents of supplemented starch and malt extract. Starch and malt extract 0.1%, 0.2%, 0.5%, 1.0% and 1.5% were applied into culture media. The cultures were incubated by shaken 200 rpm at 30 °C for 2 weeks. After incubation, the cultures were extracted with ethyl acetate and evaporated to be dryness under low pressure. The crude extracts were measured their weight and detected for their TLC and NMR pattern. Cell cultures were filled and dried over night in 50 °C hot air oven and then measured their cell mass.

3.3.4 Growth curve on metabolite production

Spore suspension was added into sodium caseinate broth supplemented with 1% starch and 1.5% malt extract. The cultures were incubated by shaken 200 rpm at 30 °C for 5 weeks. The samples were collected every week and extracted with ethyl acetate and evaporated to be dryness under low pressure. The crude extracts were measured their weight. Cell cultures were filled and dried in 50 °C hot air oven and then measured their cell mass.

3.4 Purification and characterization of actinomycin-D

3.4.1 Actinomycin-D purification

Compounds produced by *Streptomyces parvulus* strain J6.2, both mycelium and fermentation broth, were extracted by ethyl acetate twice. The solution was evaporated to dryness. The crude extract was purified by silica column chromatography using the ratio of hexane, di-chloromethane and methanol as mobile phase. Fractions which collected from column chromatography were detected their components by thin layer chromatography (TLC) using 9: 1, di-chloromethane: methanol as mobile phase. UV wavelength of 254 and 365 nm were used to detection. Fraction consisted of major compound which was demonstrated by TLC was continue to purify by reverse phase preparative high performance liquid chromatography (HPLC) using column Thermo® ODS Hypersil C18 25 cm x 10 mm, 5 μm and

stepwise gradient of methanol in water as mobile phase using 70% for 0 – 20 min, 75% for 25 – 45 min and 100% methanol for 50 – 60 min, 3 ml min^{-1} flow rate. UV wavelength of 220, 254 and 365 nm were used to detection. Sample was filled through 0.45 µm syringe filter and injected 100 µl each injection.

3.4.2 Actinomycin-D characterization

The actinomycin-D was characterized using electro spay ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) techniques.

3.5 Optimization for actinomycin-D production

3.5.1 Quantitative analysis

Quantitative analysis of actinomycin-D in each crude extracts was compared with calibration curve using standard actinomycin-D (Sigma) as a standard. Actinomycin-D was analyzed by reverse phase HPLC Thermo scientific®, BDS Hypersil C8 250 x 4.6 mm, particle size 5 µm. Isocratic of 70% methanol in water was used as mobile phase, 1 ml min^{-1} flow rate. UV 244 nm was used to detection. Under such condition, actinomycin-D was eluted at 12.5 min.

3.5.2 Medium optimization

Actinomycin-D production was optimized in seven different media. Sodium caseinate broth was used for actinomycin-D production compared with Sousa *et al*. (1997).

- soytone 30 g/l, glucose 20 g/l, $CaCO_3$ 2 g/l
- SCB, starch 10 g/l, malt extract 15 g/l
- SCB, malt extract 15 g/l
- SCB, malt extract 20 g/l
- SCB, malt extract 20 g/l, NaCl 10 g/l
- SCB, malt extract 20 g/l, NaCl 20 g/l
- SCB, malt extract 20 g/l, NaCl 30 g/l

The cultures were cultivated into 150 ml of the media in 250 ml Erlenmeyer flask by shaking 200 rpm at 30 °C for 2 weeks. The effect of starch, malt extract and concentration of NaCl on actinomycin-D production yields were investigated by HPLC.

3.6 Bioactive activity tests

3.6.1 Anti micromicrobial activity

The fraction elucidations from ethyl acetate crude extract were tested for antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa and Candida albicans* by agar well technique (Murray and Bacon, 1999). The test microorganisms 0.5 Mc Farland turbidity were spreaded on agar medium using Mueller – Hinton agar for bacteria and Sabouraud agar for yeast. The tested agars were made 6 mm diameter holes by cork borer and removed a piece of agar by sterile needle. Test samples were diluted with methanol for concentration 100 µg/ml and inoculated for 20 µl in each well. The test bacteria were incubated at 37 °C for 18 – 24 h and test yeast were incubated at 30 °C for 48 h. The diameter of inhibition zone was measured for 3 replicates. Streptomycin 100 µg/ml and methanol were used as positive and negative control, respectively.

The 7 ethyl acetate extracts were tested for antimicrobial activities against *Staphylococcus epidermidis, Mycobacterium smegmaltis, Escherichia coli, Salmonella typhimurium and Candida albicans.* The MIC value against *S. epidermidis, M. smegmaltis* and *C. albicans* was determined for each sample with chloramphenecol and nystatin used as positive controls for antibacterial and antifungal activities respectively. The samples were dissolved in methanol and the antimicrobial activities determined in triplicates in 96 wells plates by absorbance 550 nm. The percentage of inhibition was calculated by

Absorbance of $\frac{(\text{negative control} - \text{test})}{(\text{negative control})} \times 100$

3.6.2 Antioxidant assay

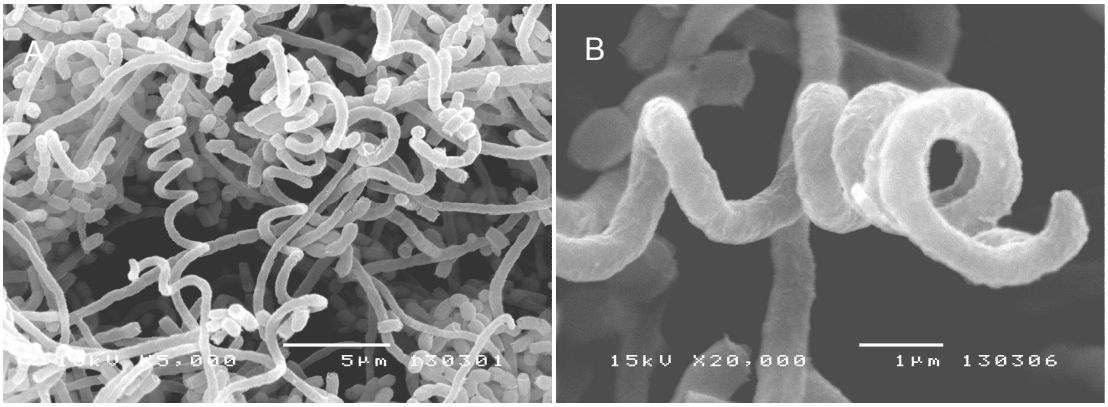
DPPH (radical 2,2-diphenylpicryhydrazyl, Fluka Chemical), solution will be freshly prepared at the concentration of 200 µM in absolute ethanol and kept in the dark by covering with aluminum foil until use. Each isolated compound will be dissolved in DPPH solution to give total concentration of 12.5, 25, 50, 100, 250, 500, 1000 and 2000 µg/ ml and the solution will be then incubated in the dark chamber at 37 ^{o}C for 30 minutes. The solution (200 µl) will be placed in 96 well microtiter plates. The absorbance will be measured at 517 nm against 200 µM DPPH solution, and 1 mg of tocopherol as a position control. All tests will be run in microtiterplate triplicate and averaged. The percent inhibition (IC_{50}) of each sample will be determined by comparison with DPPH solution (Brand-Williums, 1995).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Identification of *Streptomyces parvulus* J6.2

The isolated J6.2 was isolated from mangrove soil in Chantaburi province, Thailand as described by Kengpipat (2007). It had white mycelium and grey spore. The spiral spore chain indicated that this isolated belonging to the genus *Streptomyces*. The spore surface was smooth and arranged to be long chain. The spore chain had more than 50 spores per chain can be determined as *Streptomyces parvulus* (Shirling and Gottlieb, 1968). Electron micrographs of strain J6.2 are shown in Figure 4.1. *Streptomyces parvulus* had been found in mangrove soil (Raghavendrudu and Kondalarao, 2007; Usha *et al*., 2010) and also found as an endophyte (Taechowisan *et al*., 2006). The reports showed that *S. parvulus* are cosmopolitan distribution.

Figure 4.1 Scanning electron micrographs of isolate J6.2.



(A) The spiral conidia chains developed on mycelium.

(B) High magnification electron micrograph showed smooth surface of spiral conidia chain.

The morphological characteristics of isolated J6.2 were studied on various Intrenational *Streptomyces* media (ISP media). Growth, spore formation, spore color, substrate mycelium, aerial mycelium and soluble pigment were varied as showed in Table 4.1.

Table 4.1 Morphological characteristics of isolate J6.2 on the various ISP media

	ISP-1	ISP-2	ISP-3	ISP-4	ISP-5	ISP-6
Growth	very good	very good	good	good	very good	poor
Spore formation	fine	very good	good	very good	very good	poor
Spore color	grey	white	grey	grey	grey	white
Substrate mycelium	white	brown	bright yellow	yellow	bright yellow	yellow
Aerial mycelium	white	white	white	white	white	yellow
Soluble pigment	none	yellow	yellow	yellow	yellow	yellow

ISP-1 tryptone yeast extract agar, ISP-2 yeast malt extract agar, ISP-3 oat meal agar, ISP-4 inorganic salt starch agar, ISP-5 glycerol asparagine agar, ISP-6 peptone yeast extract iron agar.

The isolated J6.2 could neither be able to produce melanin and H_2S nor reduce nitrate. It could grow at 37 ºC but not at 4 ºC, 45 ºC and pH 4.3. The isolated J6.2 could degrade adenine, casein, gelatin and starch. The isolated J6.2 used arginine, histidine, methionine, phenylalanine, serine, theonine, valine and KNO_3 as N – sources. It used dextran, galactose, inositol, lactose, manitol, rhamnose, thehalose and xylan as N – sources. Physiological characteristics of isolated J6.2 were showed in Table 4.2.

Table 4.2 Physiological characteristics of isolate J6.2.

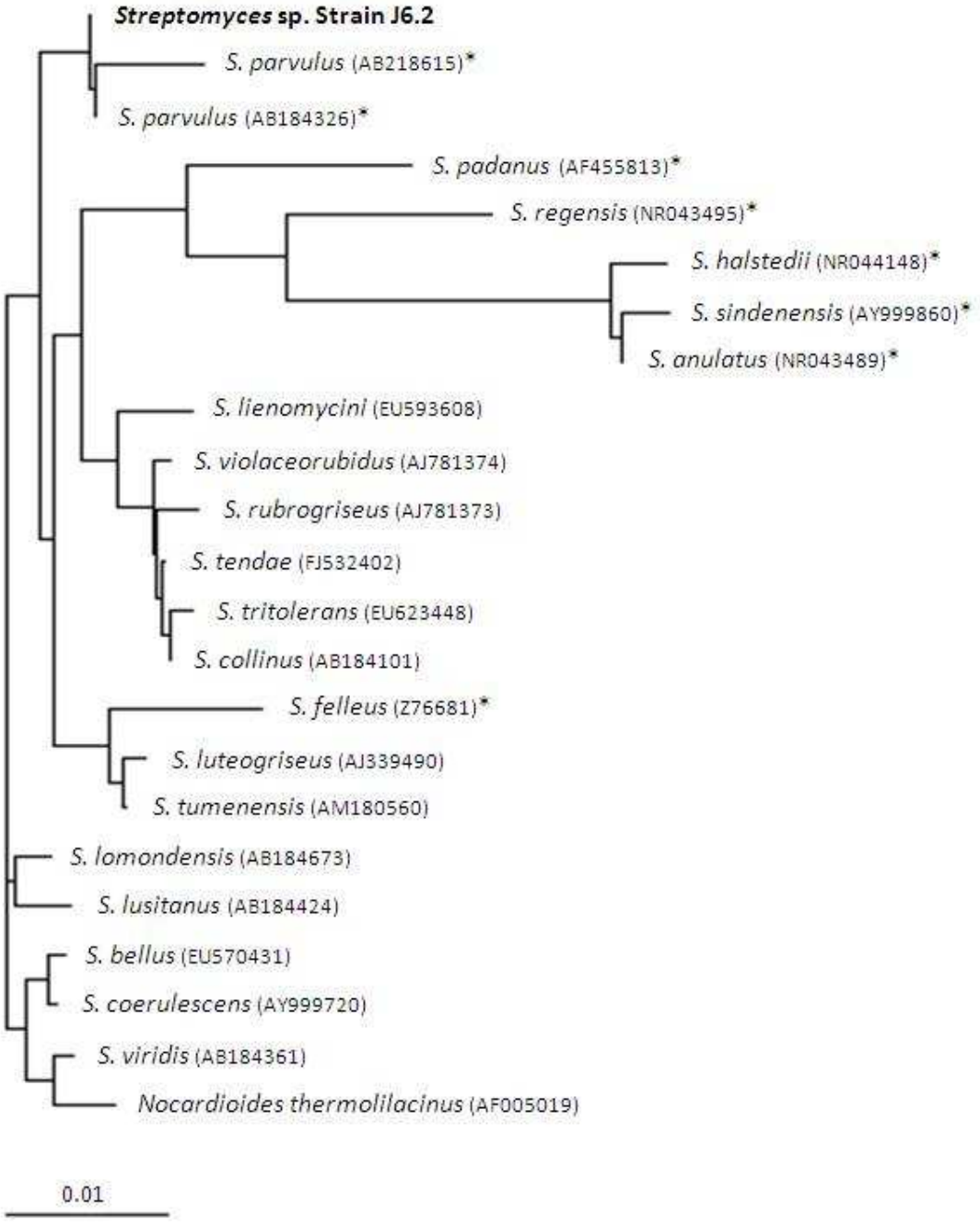
Physiological properties		Degradation activities		Nitrogen sources		Carbon sources	
Melanin production	-	0.5% Adenine	+	Arginene	+	Arabinose	-
H_2S production	-	0.5% Tyrosine	-	Histidine	+	Dextran	+
Nitrate reducing	-	0.4% Xanthine	-	Methionine	+	Fructose	-
Growth on pH 4.3	-	0.1% Casein	+	Phenylalanine	+	Galactose	+
Growth on 4 °C	-	0.4% Gelatin	+	Serine	+	Inositol	+
Growth on 37 °C	+	0.1% Starch	+	Theonine	+	Lactose	+
Growth on 45 °C	-			Valine	+	Manitol	+
				KNO_3	+	Mannose	-
						Rhamnose	+
						Sodium acetate	-
						Sodium citrate	-
						Sucrose	-
						Thehalose	+
						Xylose	-
						Xylan	+

Melanin and H_2S production (+) produce, (-) not produce; Activity of nitrate reducing (+) reduce, (-) not reduce; Growth on pH 4.3 and various temperature (+) growth, (-) no growth; Abilities to degrade adenine, tyrosine, xanthine, casein, gelatin and starch and abilities to use nitrogen and carbon source (+) growth, (-) no growth

The partial sequence of 16S rDNA was analyzed to confirm species identification. The amplification fragment of strain J6.2 of 1,401 bp was sequenced (GenBank accession no. FJ999671) Comparison of the sequence with GenBank database was done by BLASTN analysis. Result showed the strain J6.2 had a similarity level of 100% with *Streptomyces parvulus.*

The phylogenetic tree was constructed compared with related species and some species of actinomycin-D producing *Streptomyces* spp. from GenBank database shown in Figure2. The comparison of 16S rDNA in the genus *Streptomyces* showed a close relationship between actinomycin-D producing species and strain J6.2.

Figure 4.2 Neighbour-joining phylogenetic tree of related species based on almost complete 16SrRNA gene sequences.



Showing the relationships among *Streptomyces* sp. strain J6.2 and others species of *Streptomyces* spp. *Nocardioides thermolilacinus* was used as an outgroup. Bar, 0.01 represents nucleotide substitutions per position. GenBank accession number was shown in parenthesis. Asterisks indicate actinomycin-D producing *Streptomyces*.

Many approaches have been considered to identify *Streptomyces* into genus, species and strain level. Genetic methods based on 16S rDNA sequence were used certainly as a tool for identification (Awad *et al*., 2009). The 16S rDNA sequences showed very close similarity with two isolated *Streptomyces parvulus*, AB218615 from Thailand (Taechowisan *et al*., 2006) and AB184326 from Japan (unpublished). The morphological and physiological characteristics of isolated strain J6.2 was compared with *S. parvulus* of Shirling and Gottlieb (1968). The results showed some different physiological characteristics between the two strains (Table 4.3). Both two strains did not produce melanin in peptone – yeast iron agar and tyrosine agar. Carbon utilization was tested with various C – sources using basal medium as negative control, the procedure described by Pridham and Gottlieb (1948). The result was showed that *S. parvulus* J6.2 was a new strain of *S. parvulus* different to previous reported.

Table 4.3 Comparison of carbon utilization between *Streptomyces parvulus* J6.2 and the *S. parvulus* original strain reported by Shirling and Gottlieb (1968)

carbon utilization	Shirling and Gottlieb (1968)	Strain J6.2
glucose	+	+
L-arabinose	+	-
sucrose	+	-
D-xylose	+	-
inositol	+	N/D
D-mannitol	+	+
D-fructose	+	-
rhamnose	+/-	+

+ stand for the growth was more than in basal medium, - stand for the growth was equal or less than in basal medium, + / - stand for variable on growth, N/D stand for not detected.

4.2 Secondary metabolite production of strain J6.2

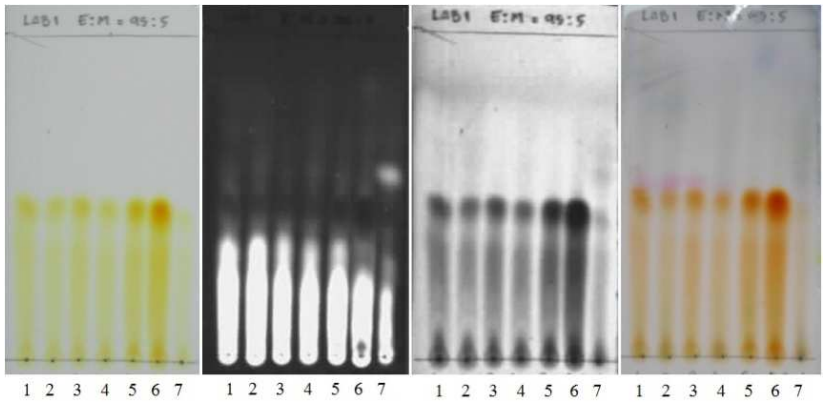
4.2.1 Effect of culture medium

Streptomyces parvulus J6.2 was cultured in various conditions. Cell weight and compound weight were measured after incubated at 30 ºC for 3 weeks. The results were showed in Table 4.4. The ethyl acetate crude extract of each medium was analyzed its chemical components by TLC, the results were showed in Figure 4.3.

Table 4.4 Effect of culture medium on cell growth and metabolite production of *S. parvulus* J6.2

Culture medium	Cell weight (g)	Weight of crude extract (mg)
1) SCB, static	0.10	3.5
2) SCB, static occasionally stir	0.09	3.6
3) SCB, shaken	0.09	6.3
4) SCB, shaken with spring coil	0.04	4.2
5) GYM, shaken	0.64	29.8
6) GYM and SCB added, shaken	1.04	53.9
7) Starch casein, shaken	0.08	1.3

Figure 4.3 TLC chromatogram of ethyl acetate crude extracts produced by *S. parvulus* J6.2 in various cultivation conditions using 5% methanol in ethyl acetate as mobile phase



(A) (B) (C) (D)

(A) eye observation, (B) detection by UV 365, (C) detection by UV 254, (D) detection by vanillin; 1: SCB, static, 2: SCB, static occasionally stir, 3: SCB, shaken, 4: SCB, shaken with spring coil, 5: GYM, shaken, 6: GYM and SCB added, shaken, 7: starch casein, shaken.

The results showed that shaken condition with no spring coil provided the most metabolites. The shaken condition gave more crude metabolite yield than static condition because of oxygen needed of *S. parvulus* J6.2. The spring coil placed in fermentation flask neither activate cell growth nor metabolite production. It may cause by bubbles, which produced from broth strike against spring coil during cultivation, locked oxygen then it could not pass through the fermentation broth. GYM – Glucose Yeast Malt extract medium composed a lot of enrich C and N source could activate both cell growth and metabolite production. GYM together with SCB in shaken condition was the best medium to activate cell growth and metabolite production.

SCB and GYM gave the similar TLC patterns but there was a little metabolite when used starch casein broth as culture medium.

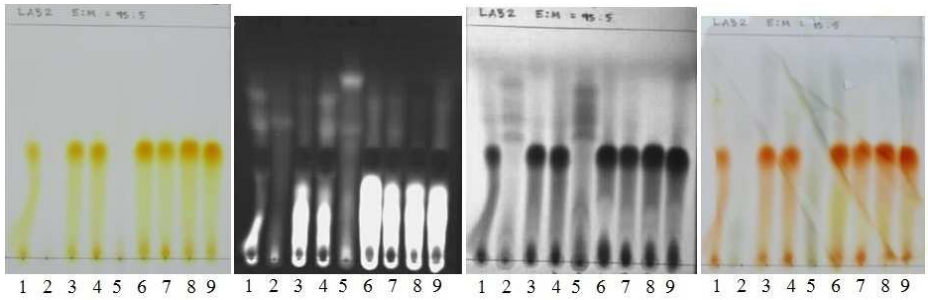
4.2.2 Effect of C and N source supplemented in sodium caseinate broth

Streptomyces parvulus J6.2 was cultured in SCB supplemented with glucose, galactose, starch, yeast extract, peptone and malt extract, described in 3.4.1. Cell weights and compound weights after incubated at 30 °C for 2 weeks were detected. The results were showed in Table 4.5. TLC chromatograms and ^{1}H-NMR spectra of ethyl acetate crude extracts from various media were showed in Figure 4.4 and 4.5, respectively.

Table 4.5 Effect of C and N sources supplemented in SCB on cell growth and metabolite production of *S. parvulus* J6.2

supplements	Cell weight (g)	Weight of crude extract (mg)
1) glucose, yeast extract	0.41	4.2
2) glucose, malt extract	0.51	0.8
3) glucose, peptone	0.30	6.6
4) galactose, yeast extract	0.42	5.4
5) galactose, malt extract	0.41	1.5
6) galactose, peptone	0.31	11.2
7) starch, yeast extract	0.30	11.1
8) starch, malt extract	0.48	71.3
9) starch, peptone	0.45	7.8

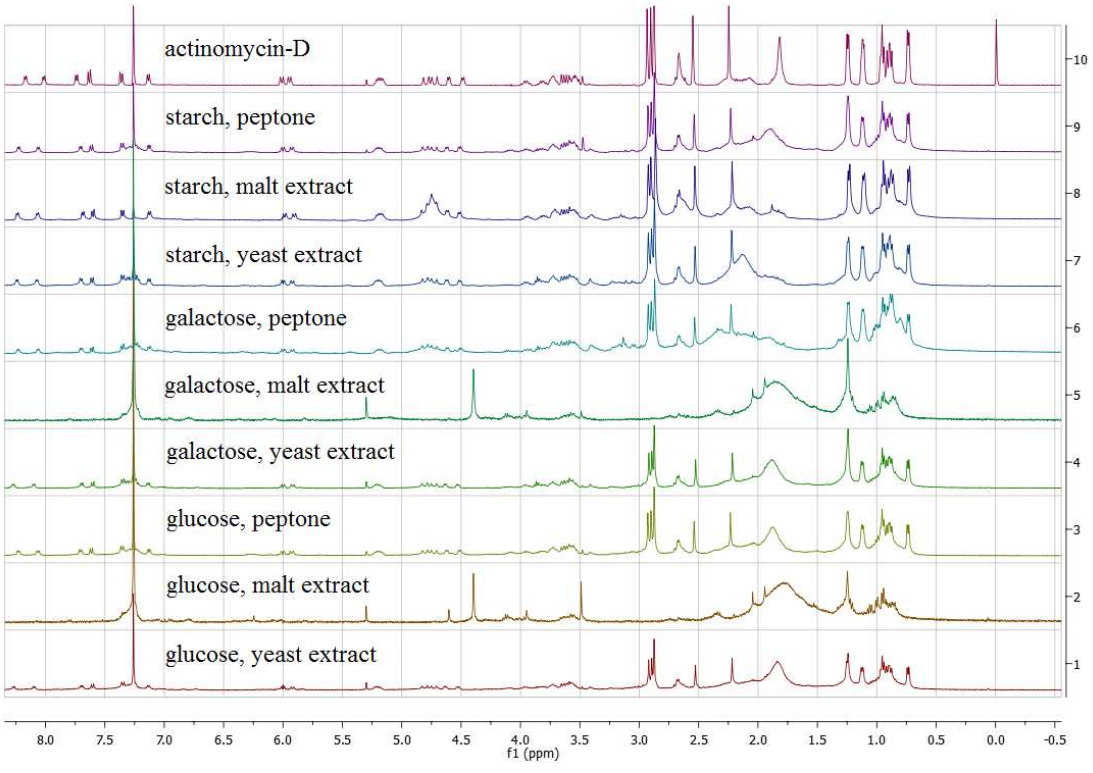
Figure 4.4 TLC chromatogram of ethyl acetate crude extract of *S. parvulus* cultured in SCB supplemented with various C and N sources.



(A) (B) (C) (D)

(A) eye observation, (B) detection by UV 365, (C) detection by UV 254, (D) detection by vanillin; 1: glucose, yeast extract, 2: glucose, malt extract, 3: glucose, peptone, 4: galactose, yeast extract, 5: galactose, malt extract, 6: galactose, peptone, 7: starch, yeast extract, 8: starch, malt extract, 9: starch, peptone.

Figure 4.5 ^{1}H – NMR spectra of ethyl acetate crude extract of *S. parvulus* cultured in SCB supplemented with various C and N sources.



The result showed effect of medium composition on metabolite production of *S. parvulus* J6.2. Glucose and galactose provided same patterns of TLC profile but starch provided dominant compound with less impurity. Starch and malt extract supplemented in SCB which provided the most metabolite was chosen to study optimum contents to obtain highest yield of metabolite. ^{1}H – NMR spectra of ethyl acetate crude extract showed that *S. parvulus* J6.2 produced different metabolites when cultured in different medium. However, a major compound composed in ethyl acetate crude extract was actinomycin-D.

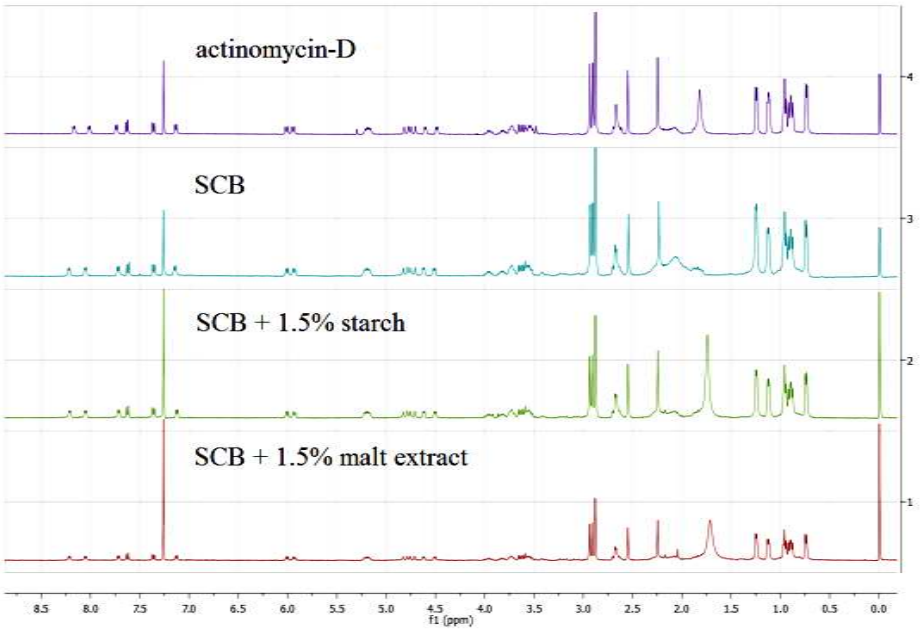
4.2.3 Effect of starch and malt extract content supplemented in sodium caseinate broth

Starch 1% and malt extract 1.5% supplemented in SCB were the most activated *S. parvulus* J6.2 growth and metabolite production (Table 4.6). ^{1}H – NMR spectra showed the most component in ethyl acetate crude extract was actinomycin-D (Figure 4.6).

Table 4.6 Effect of starch and malt extract content supplemented in SCB on cell growth and metabolite production of *S. parvulus* J6.2

Supplements	Cell dry weight (g)	Weight of crude extract (mg)
1) no supplemented	0.0931	7.9
2) 0.1 % starch	0.1209	16.9
3) 0.2 % starch	0.1813	18.4
4) 0.5 % starch	0.3514	45.1
5) 1.0 % starch	0.7689	54.5
6) 1.5 % starch	0.1588	53.5
7) 0.1 % malt extract	0.1235	17.5
8) 0.2 % malt extract	0.1939	39.9
9) 0.5 % malt extract	0.3796	25.0
10) 1.0 % malt extract	0.5568	42.3
11) 1.5 % malt extract	0.8611	82.0

Figure 4.6 ^{1}H – NMR spectra of ethyl acetate crude extract of *S. parvulus* cultured in SCB supplemented with starch and malt extract.



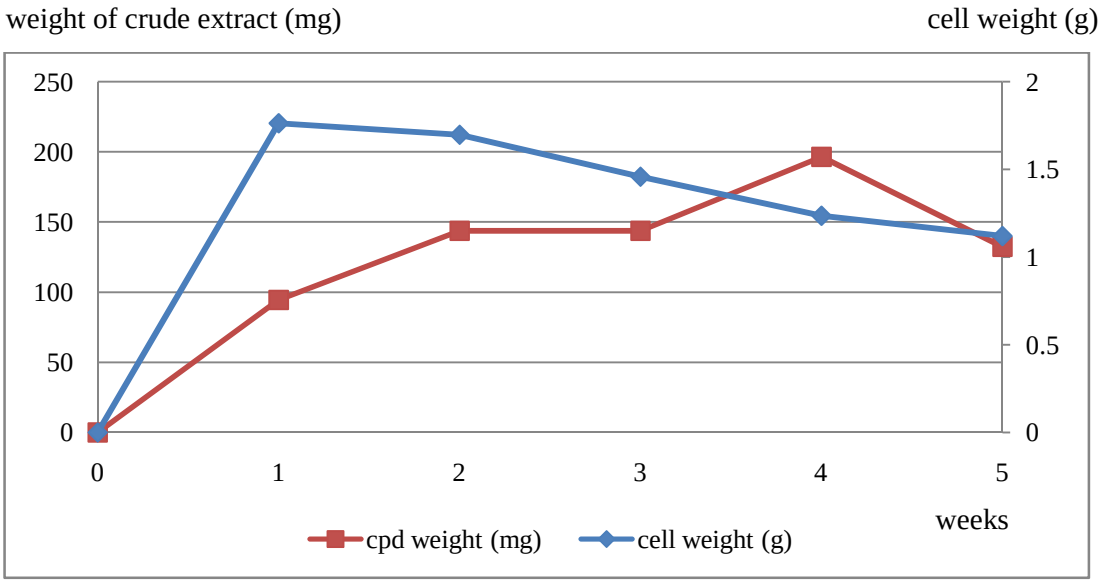
4.2.4 Growth curve on metabolite production

Cell growth was maximum in 1 week, after that the cell was in stationary and death phase. Metabolite was increase until 2 weeks, after that it was remained. The metabolite was increase again only in fourth week and it decrease in fifth week, it may effect by metabolite production from cell culture or it caused by manual error of extraction process. However, S. *parvulus* J6.2 would be cultured in 2 weeks because the maximum growth to produce secondary metabolite and would not use too long time.

Table 4.7 Cell growth and metabolite production of *S. parvulus* J6.2 in 5 weeks

Time (week)	Cell dry weight (g)	Weight of crude extract (mg)
1	1.7624	94.4
2	1.6971	143.7
3	1.4582	143.8
4	1.2354	196.4
5	1.1194	132.4

Figure 4.7 Growth curve and metabolite production of *S. parvulus* J6.2 in 5 weeks

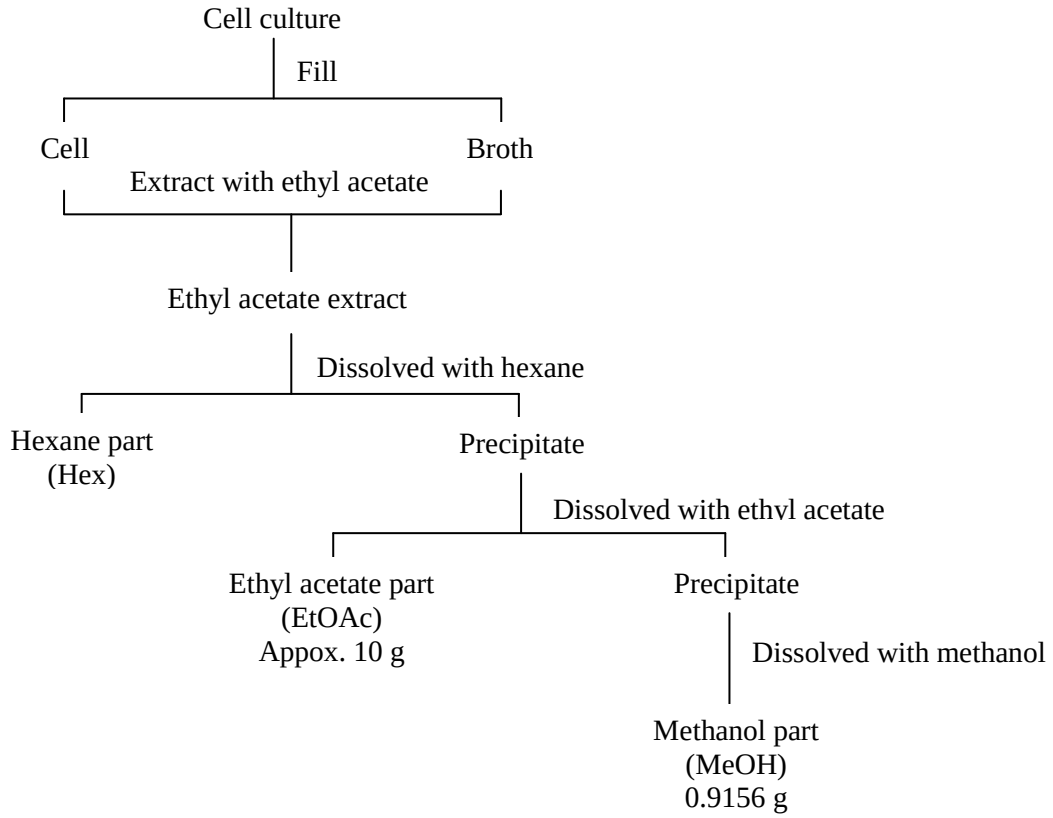


4.3 Purification and characterization of actinomycin-D

4.3.1 Actinomycin-D purification

Cell culture of *Streptomyces parvulus* J6.2 was filled. Broth and cell were extracted with ethyl acetate separately. Ethyl acetate extract of cell culture and fermentation broth were detected their chemical components by TLC and NMR showed the same patterns of composition then they were combined together. Oil composed in ethyl acetate crude extract was removed by dissolved with hexane. The precipitate which could not dissolved in hexane was dissolved again with ethyl acetate. The solution in ethyl acetate part was dried under reduced pressure, it had approximately 10 g. The residue which could not dissolved in ethyl acetate could be dissolved in methanol; its part had 0.915 g (Figure 4.8).

Figure 4.8 Diagram of *S. parvulus* J6.2 culture extraction



Ethyl acetate crude extract was purified by silica column chromatography (Figure 4.9) The silica column (normal phase) used Di-chloromethane in methanol as mobile phase and the reverse phase silica column used methanol in water as mobile phase as showed in Table 4.8. The fractions passing though each column was detected their chemical components by TLC.

Figure 4.9 Purification diagram of ethyl acetate crude extract by produced by *S. parvulus* J6.2

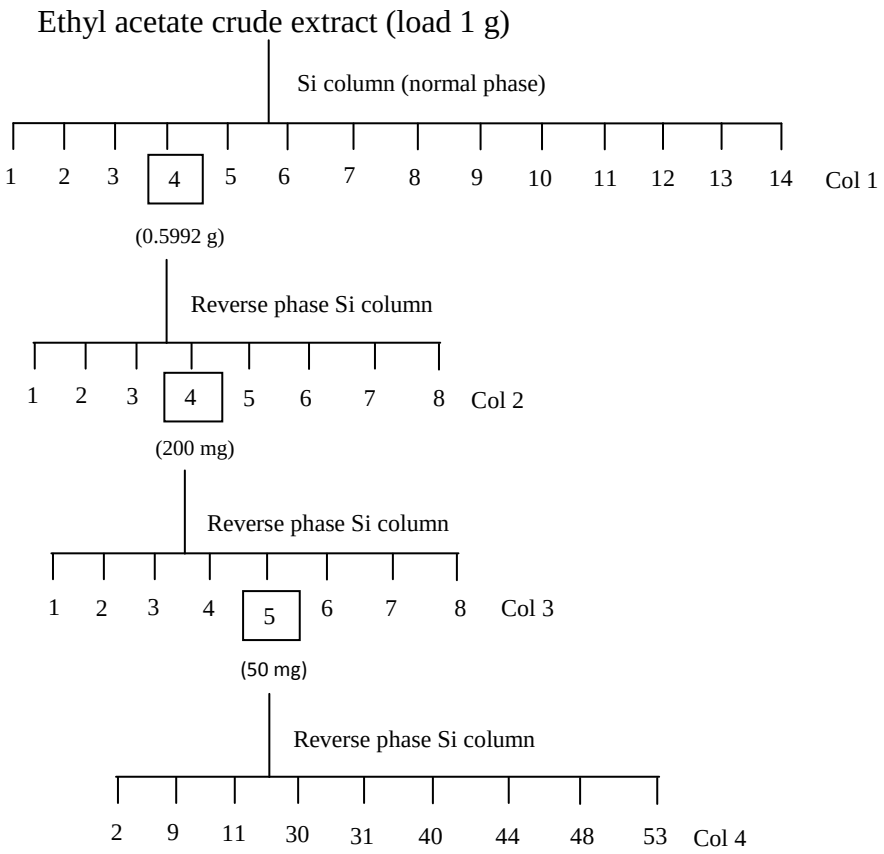
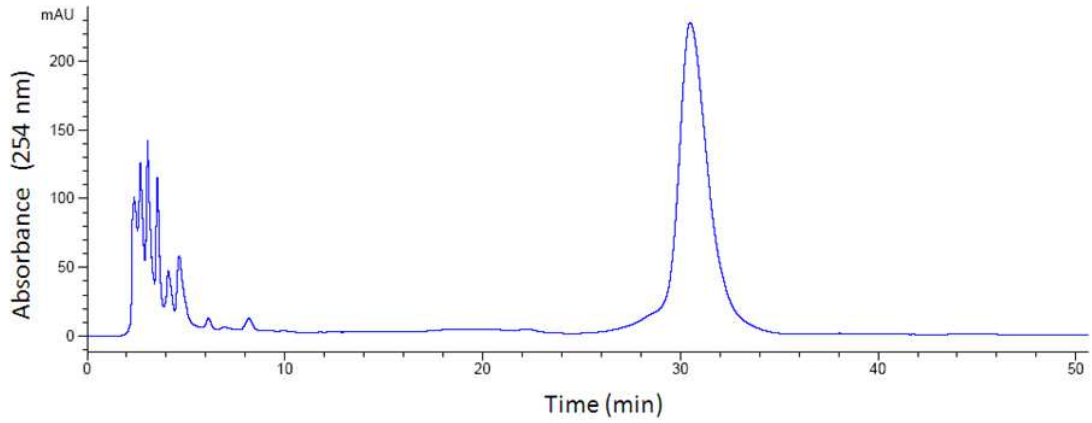


Table 4.8 Solvent system used in column chromatography of ethyl acetate crude extract

Column 1: sample E2 1 g, silica column normal phase, di-chloromethane in methanol as mobile phase		
Fraction	Sub fraction	Condition
1 col 1	1 – 50	0 – 3% methanol
2 col 1	51 – 70	3 – 5% methanol
3 col 1	71 – 75	5% methanol
4 col 1	76 – 102	5% methanol
5 col 1	103 – 113	5 – 7% methanol
6 col 1	114 – 119	7% methanol
7 col 1	120 – 131	10% methanol
8 col 1	132 – 137	10% methanol
9 col 1	138 – 139	10% methanol
10 col 1	140 – 152	10 – 20% methanol
11 col 1	153 – 159	30% methanol
12 col 1	160 – 167	50% methanol
13 col 1	168	100% methanol
14 col 1	169	100% methanol
Column 2: sample 4col1 0.5992 g, reverse phase, methanol in water as mobile phase		
Fraction	Sub fraction	Condition
1 col 2	1	20% water
2 col 2	2 - 7	20% water
3 col 2	8 – 9	20% water
4 col 2	10 – 20	20% water
5 col 2	21 – 28	20% water
6 col 2	29 – 37	20 – 10% water
7 col 2	38 – 41	10% water
8 col 2	42 – 43	0% water
Column 3: sample 4col2 200 mg, reverse phase, methanol in water as mobile phase		
Fraction	Sub fraction	Condition
1 col 3	1 – 7	40 – 35% water
2 col 3	8 – 12	35% water
3 col 3	13 – 14	35% water
4 col 3	15 – 27	35 – 30% water
5 col 3	34 – 41	30% water
6 col 3	42 – 48	30 – 25% water
7 col 3	49 – 54	25 – 20% water
8 col 3	55 - 58	10% water

The ethyl acetate crude extract was purified by semi-preparative HPLC column Thermo® ODS Hypersil C18 25 cm x 10 mm, 5 µm and stepwise gradient of methanol in water as mobile phase using 70% for 0 – 20 min, 75% for 25 – 45 min and 100% methanol for 50 – 60 min, 3 ml min^{-1} flow rate. Actinomycin-D was eluted at retention time 28 – 34 min. The HPLC chromatogram was showed in Figure 4.10

Figure 4.10 HPLC chromatogram of crude sample from *S. parvulus* strain J6.2.



4.3.2 Actinomycin-D characterization

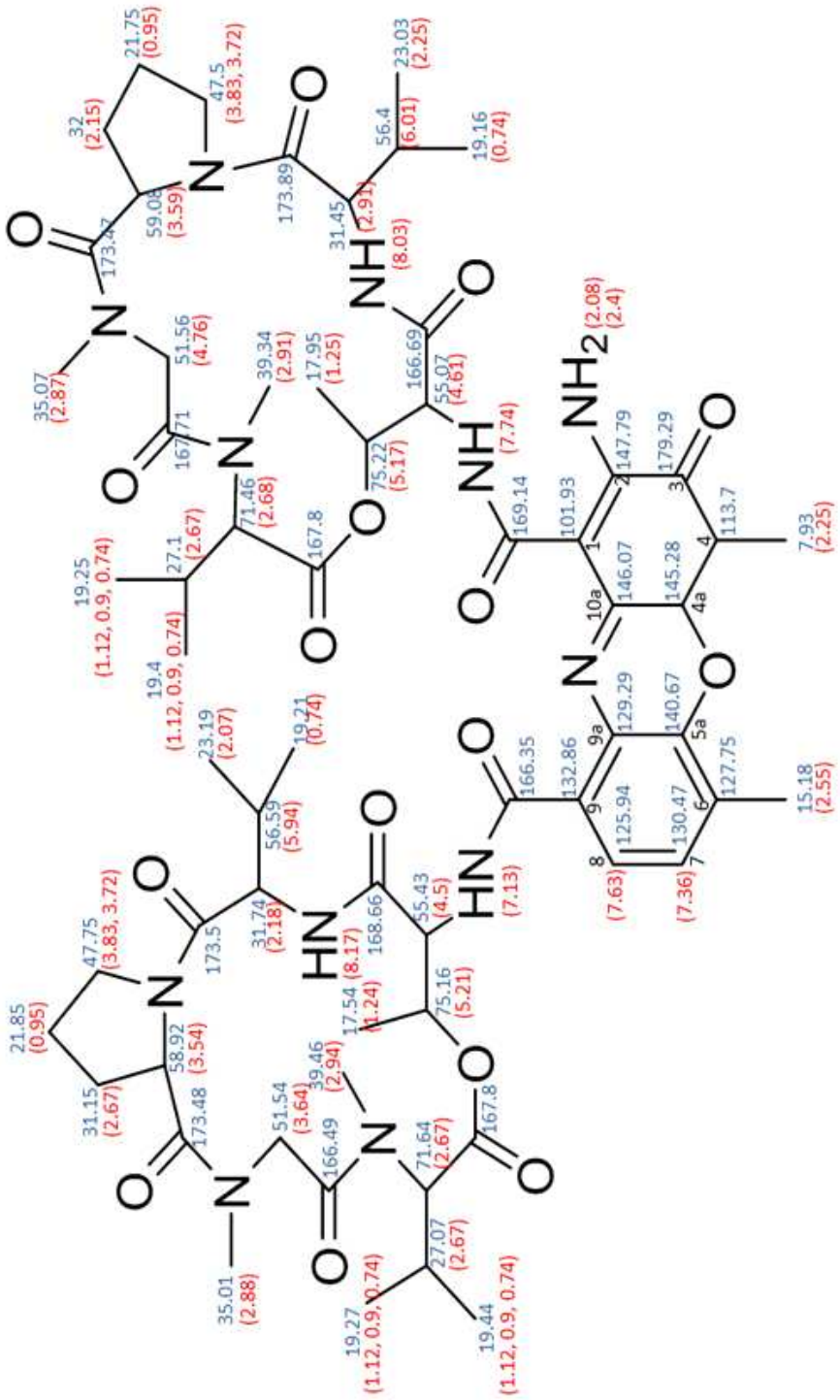
A major pure compound isolated from column chromatography and HPLC was red – orange color. The NMR data confirmed that the major pure compound was actinomycin-D. The molecular structure of actinomycin-D was characterized by 1D and 2D-NMR. The ^{1}H and ^{13}C chemical shifts of isolated actinomycin-D were shown in Table 4.9 and Figure 4.11 The molecular weight of actinomycin-D produced by *S. parvulus* J6.2 measured by ESI-MS was 1255.6149 *m/z* $[M+H]^+$ closely related to standard actinomycin-D (exact mass $[M+H]^+$ 1255.636324 *m/z*). Optical rotation $[\alpha^{20}_{D}]$ was -381° (c0.11) in methanol (the standard was -314.5, c0.25 in methanol). The results defined molecular formula as $C_{62}H_{86}N_{12}O_{16}$.

Table 4.9 ^{1}H and ^{13}C-NMR chemical shifts and assignments of the actinomycin-D produced by *Streptomyces parvulus* strain J6.2

Group	^{13}C (δ) ppm	^{1}H shifts (δ) ppm
Phenoxazone ring		
C4 (CH_3)	7.93	2.25
C6 (CH_3)	15.18	2.55
C7	130.47	7.36
C8	125.94	7.63
C3 (CO)	179.29	
C9 (CO)	166.35	
C1 (CO)	169.14	
NH_2		2.08; 2.4
Hetero		
C2 (C)	147.79	
$C10_a$ (C)	146.07	
$C4_a$ (C)	145.28	
$C5_a$ (C)	140.67	
$C9_a$ (C)	129.29	
C9 (C)	132.86	
C6 (C)	127.75	
C4 (C)	113.7	
C1 (C)	101.93	
Threonine		
***α*-ring**		
NH		7.13
$C_α$ (CH)	55.43	4.5
$C_β$ (CH)	75.16	5.21
$C_γ$ (CH)	17.54	1.24
CO	168.66	
***β*-ring**		
NH		7.74
$C_α$ (CH)	55.07	4.61
$C_β$ (CH)	75.22	5.17
$C_γ$ (CH)	17.95	1.25
CO	166.69	
Valine		
***α*-ring**		
NH		8.17
$C_α$ (CH)	31.47	2.18
$C_β$ (CH)	56.59	5.94
$C_γ$ (CH_3)	23.19	2.07
	19.21	0.74
CO	173.5	
***β*-ring**		
NH		8.03
$C_α$ (CH)	31.45	2.91
$C_β$ (CH)	56.4	6.01
$C_γ$ (CH_3)	23.03	2.25
	19.16	0.74
CO	173.89	

Group	^{13}C (δ) ppm	^{1}H shifts (δ) ppm
Proline		
***α*-ring**		
$C_α$ (CH)	58.92	3.54
$C_β$ (CH_2)	31.74	2.18
$C_γ$ (CH_2)	21.85	0.95
$C_δ$ (CH_2)	47.75	3.83; 3.72
CO	173.48	
***β*-ring**		
$C_α$ (CH)	59.08	3.59
$C_β$ (CH_2)	32	2.15
$C_γ$ (CH_2)	21.75	0.95
$C_δ$ (CH_2)	47.5	3.96; 3.72
CO	173.47	
Sarcosine		
***α*-ring**		
$C_α$ (CH)	51.54	3.64
NCH_3	35.01	2.88
CO	166.49	
***β*-ring**		
$C_α$ (CH)	51.56	4.76
NCH_3	35.07	2.87
CO	167.71	
Methyl-Valine		
***α*-ring**		
$C_α$ (CH)	71.64	2.67
$C_β$ (CH)	27.07	2.67
$C_γ$ (CH_3)	19.44	1.12; 0.90, 0.74
	19.27	1.12; 0.90, 0.74
NCH_3	39.46	2.94
CO	167.8	
***β*-ring**		
$C_α$ (CH)	71.46	2.68
$C_β$ (CH)	27.1	2.67
$C_γ$ (CH_3)	19.4	1.12; 0.90, 0.74
	19.25	1.12; 0.90, 0.74
NCH_3	39.34	2.91
CO	167.8	

Figure 4.11 ^{1}H and ^{13}C-NMR chemical shifts in molecular structure of actinomycin-D. The blue number were ^{13}C chemical shifts, the red number in parenthesis were ^{1}H chemical shifts.

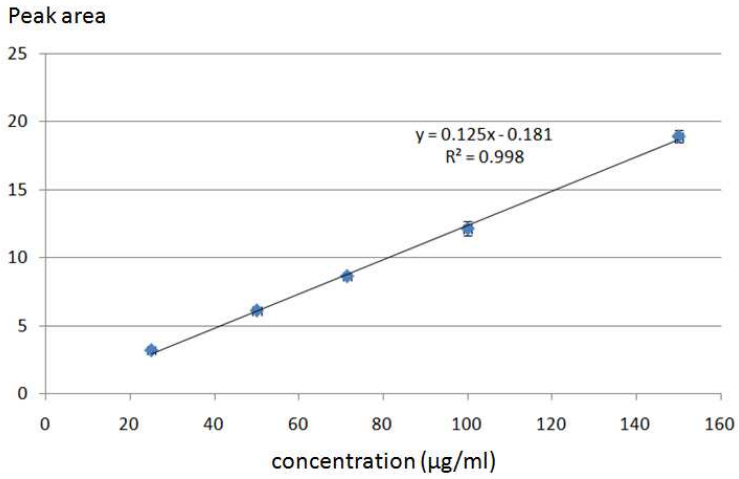


4.4 Optimization of actinomycin-D production

4.4.1 Quantitative analysis of actinomycin-D

Quantitative analysis of actinomycin-D was compared with calibration curve using standard actinomycin-D (Sigma) as a standard and analyzed by HPLC. Actinomycin-D was eluted at 12.5 min. Peak area was plotted against various concentrations actinomycin-D in range 25 – 150 µg/ml providing linear regression. Each sample was analyzed for 3 replicates and error bars were applied (Figure 4.12).

Figure 4.12 Calibration curve of standard actinomycin-D



4.4.2 Medium optimization of actinomycin-D production

Streptomyces parvulus J6.2 was cultured in various culture media described in 3.5. Ethyl acetate crude extract from each culture medium (broth and whole cell extract) was dissolved and filled pass though 0.45 µm HPLC syringe filter. The samples were diluted with methanol to be 100 µg/ml and injected for 20 µl. Actinomycin-D productions cultivated in seven different media were investigated compared with calibration curve of standard actinomycin-D. The results showed in Table 4.10

Table 4.10 Actinomycin-D yield produced by *Streptomyces parvulus* J6.2 in difference culture media 1 liter

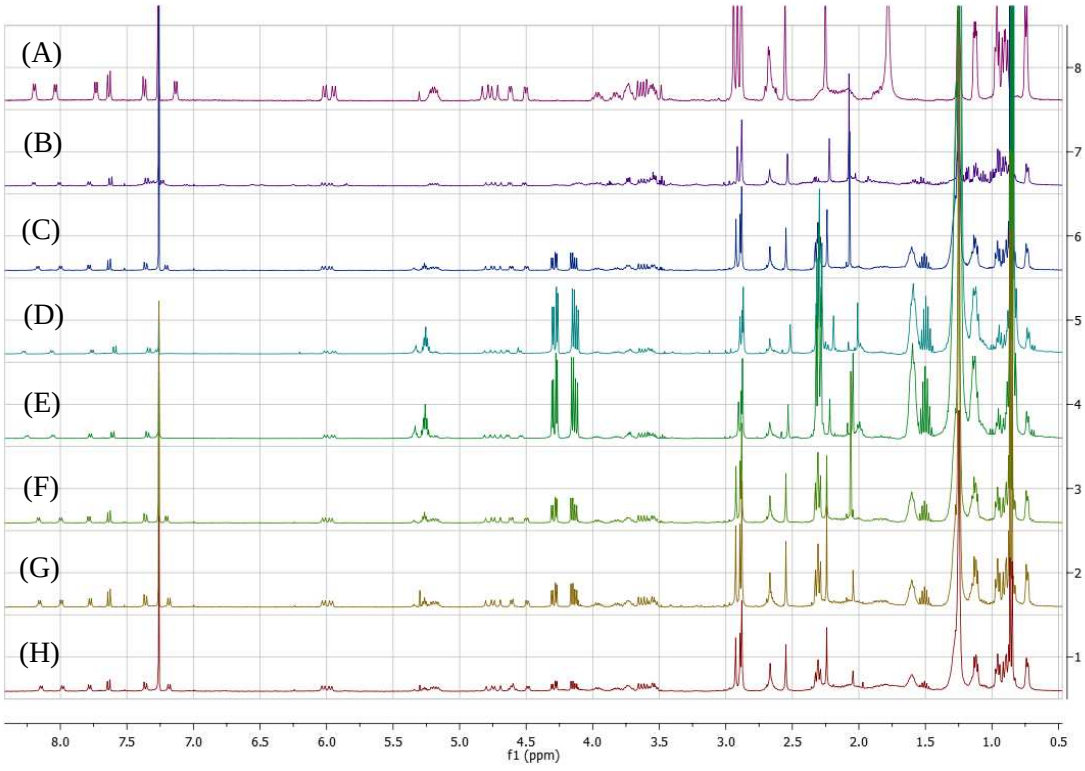
Culture media	Weight of crude extract (mg)	Percentage of act-D (%)	act-D yield (mg)
soytone 30 g/l, glucose 20 g/l, $CaCO_3$ 2 g/l	341.1	18.60	63.45
SCB, starch 10 g/l, malt extract 15 g/l	2424.9	13.81	334.78
SCB, malt extract 15 g/l	651.2	39.04	254.22
SCB, malt extract 20 g/l	1920.7	19.72	378.84
SCB, malt extract 20 g/l, NaCl 10 g/l	797.5	26.48	211.18
SCB, malt extract 20 g/l, NaCl 20 g/l	542.7	32.22	174.87
SCB, malt extract 20 g/l, NaCl 30 g/l	288.6	50.86	146.78

Streptomyces parvulus J6.2 produced actinomycin-D as bioactive compound dominantly. The molecular structure was confirmed by 2D NMR. *S. parvulus* was reported its ability to produce only one kind of actinomycin, actinomycin-D (Sousa *et al*., 2002). A chemically defined medium was optimized for actinomycin-D production consisting of *D*-fructose, *L*-glutamic acid, *L*-histidine and other trace minerals, yielding 500 – 600 mg/l of actinomycin-D (Williams and Katz, 1977). Bioprocess for actinomycin-D production was developed by using different concentrations of fructose, degrees of aeration and stirring speeds giving a maximum yield of 1530 mg/l in a bioreactor (Sousa *et al*., 2002). Identification of actinomycin-D producing *Streptomyces* strains from natural sources coupled with optimization can lead to improvements in yields (Preveen *et al*., 2008).

Sodium caseinate broth supplemented with starch and malt extract was an optimum medium for *S. parvulus* J6.2 growth and actinomycin-D production. Actinomycin-D production is depended on amino acids precursor essential in its biosynthesis pathway (Polsinelli *et al*., 1965). Sousa *et al*. (2001) reported that threonine is the most important amino acid required to

produce actinomycin-D by *S. parvulus*. Table 4.10 showed malt extract content would activate not only actinomycin-D yield but also activate other metabolites. Malt extract may be used as C and N – sources for cell growth and also a precursor for actinomycin-D production. Starch supplemented in culture medium obtained high crude metabolite therefore increased actinomycin-D production. Weight of crude extract increased for 3.7 times when 10 g/l starch was supplemented but it gave low percentage of actinomycin-D. NMR data showed high level of triglyceride composed in crude extract when starch was used in culture medium (Figure 4.13). Starch should effect to cell growth and triglyceride would be produced for cellular composition.

Figure 4.13 ^{1}H NMR spectra of ethyl acetate crude extract from various media cultured by *S. parvulus* J6.2.



(A) standard actinomycin-D; (B) soytone 30 g/l, glucose 20 g/l, $CaCO_3$ 2 g/l; (C) SCB, starch 10 g/l, malt extract 15 g/l; (D) SCB, malt extract 15 g/l; (E) SCB, malt extract 20 g/l; (F) SCB, malt extract 20 g/l, NaCl 10 g/l; (G) SCB, malt extract 20 g/l, NaCl 20 g/l; (H) SCB, malt extract 20 g/l, NaCl 30 g/l

Broth extracts of *S. parvulus* J6.2 cultured in various media were investigated actinomycin-D production including of percentage and yield. Actinomycin-D was analyzed by HPLC described in 3.5.1 compared with calibration curve. The effect of culture medium on actinomycin-D production was showed in Table 4.11. The effect of starch and malt extract content on actinomycin-D production was showed in Table 4.12. The results showed that SCB with GYM (consisting of glucose, yeast extract and malt extract) provided the highest crude metabolite, percentage of actinomycin-D and actinomycin-D yield. However, when starch was supplemented in SCB instead of glucose, percentage of actinomycin-D extracted from broth was more than 77%. Malt extract effected to increase both crude metabolite and actinomycin-D yield.

Table 4.11 The effect of culture medium on actinomycin-D production

Culture media	cell weight (g/l)	crude extract (mg/l)	percentage of act-D (%)	act-D yield (mg/l)	Error (+/-)
SCB	0.25	17.5	20	3.50	2.65
GYM	1.78	82.78	25	20.39	0.68
SCB + GYM	2.89	149.72	61	90.83	0.38
starch casein	0.22	3.61	7	0.27	0.05

Table 4.12 The effect of starch and malt extract content on actinomycin-D production

Culture media	cell weight (g/l)	crude extract (mg/l)	percentage of act-D (%)	act-D yield (mg/l)	Error (+/-)
SCB	0.31	26.33	46	12.05	0.7
SCB + 0.1% starch	0.4	56.33	98	55.00	1.1
SCB + 1% starch	2.56	181.67	77	140.74	0.29
SCB + 1.5 % starch	3.86	178.33	100	178.33	3.15
SCB + 0.1% malt	0.41	58.33	75	43.61	2.3
SCB + 1% malt	1.86	141	78	110.29	0.35
SCB + 1.5 % malt	2.87	273.33	83	225.74	0.48

Figure 4.14 The effect of culture medium on actinomycin-D production

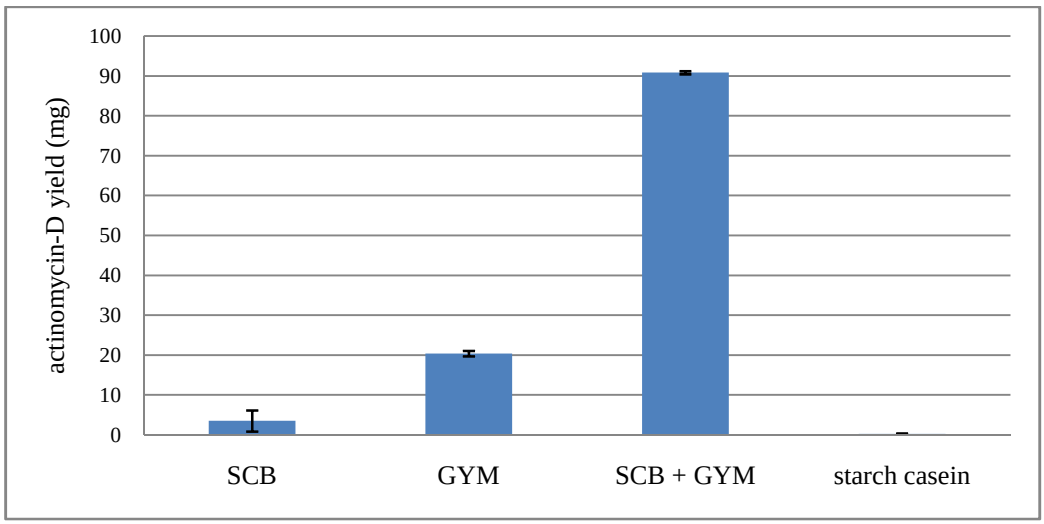
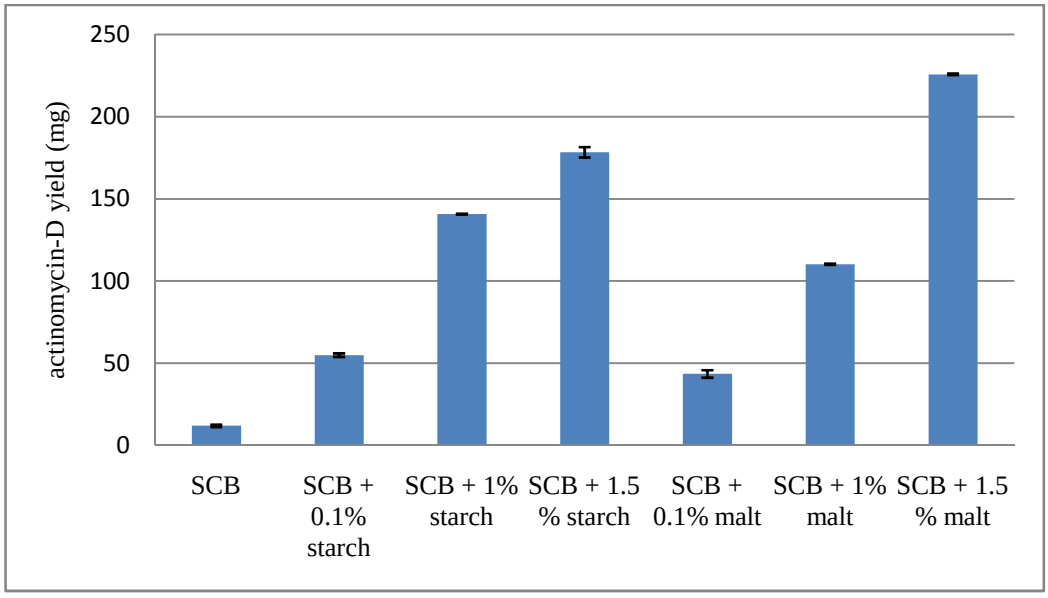


Figure 4.15 The effect of starch and malt extract content on actinomycin-D production



4.5 Bioactive activity test

4.5.1 Antimicrobial activity test of column chromatography fractions

Fractions from column chromatography were tested for antimicrobial activity against test microorganisms by agar well (Table 4.13). Major bioactive compound consisting in ethyl acetate crude extract could inhibit Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, and yeast, *Candida albicans* but no active against Gram-negative bacteria, *Escherichia coli* and *Pseudimonas aeruginosa*. The fraction 4col1 – consisting of actinomycin-D gave high activity against Gram-positive bacteria but no against yeast. Fractions 4col1, 6col1, 4col2, 4col3, 5col3 which provided high activity against Gram-positive should have the same major component. The difference of purity effected to their activities. Fraction 9col1 should consist of a different component because it inhibited only *B.subtilis*. Fraction sub48col4 had ability to inhibit Gram-positive bacteria should be a different bioactive metabolite. Fraction 3col1 could inhibit yeast; it suggested that a low polar component had anti-yeast activity. Many secondary metabolites had no antimicrobial activities such as 11col1, 2col3, 8col3, sub58col3, sub61col3 and sub63col3.

Table 4.13 Diameter of inhibition zone of fraction elucidation from ethyl acetate crude extract against test microorganisms

Sample	Test Microorganisms				
	E.coli	*P.aeruginosa*	*B.subtilis*	*S.aureus*	*C.albicans*
Streptomycin	8.5	8.5	14	10	-
E2 crude extract	-	-	17	18	8
3 col 1	-	-	16	16.5	8.5
4 col 1	-	-	19.5	15.5	-
6 col 1	-	-	18.5	16	-
9 col 1	-	-	11.5	-	-
11 col 1	-	-	-	-	-
4 col 2	-	-	21	20	-
2 col3	-	-	-	-	-
4 col3	-	-	22	20	-
5 col3	-	-	18.5	18.5	-
8 col3	-	-	-	-	-
sub 58 col3	-	-	-	-	-
sub 61 col3	-	-	-	-	-
sub 63 col3	-	-	-	-	-
sub 48 col4	7	7	21	19	-

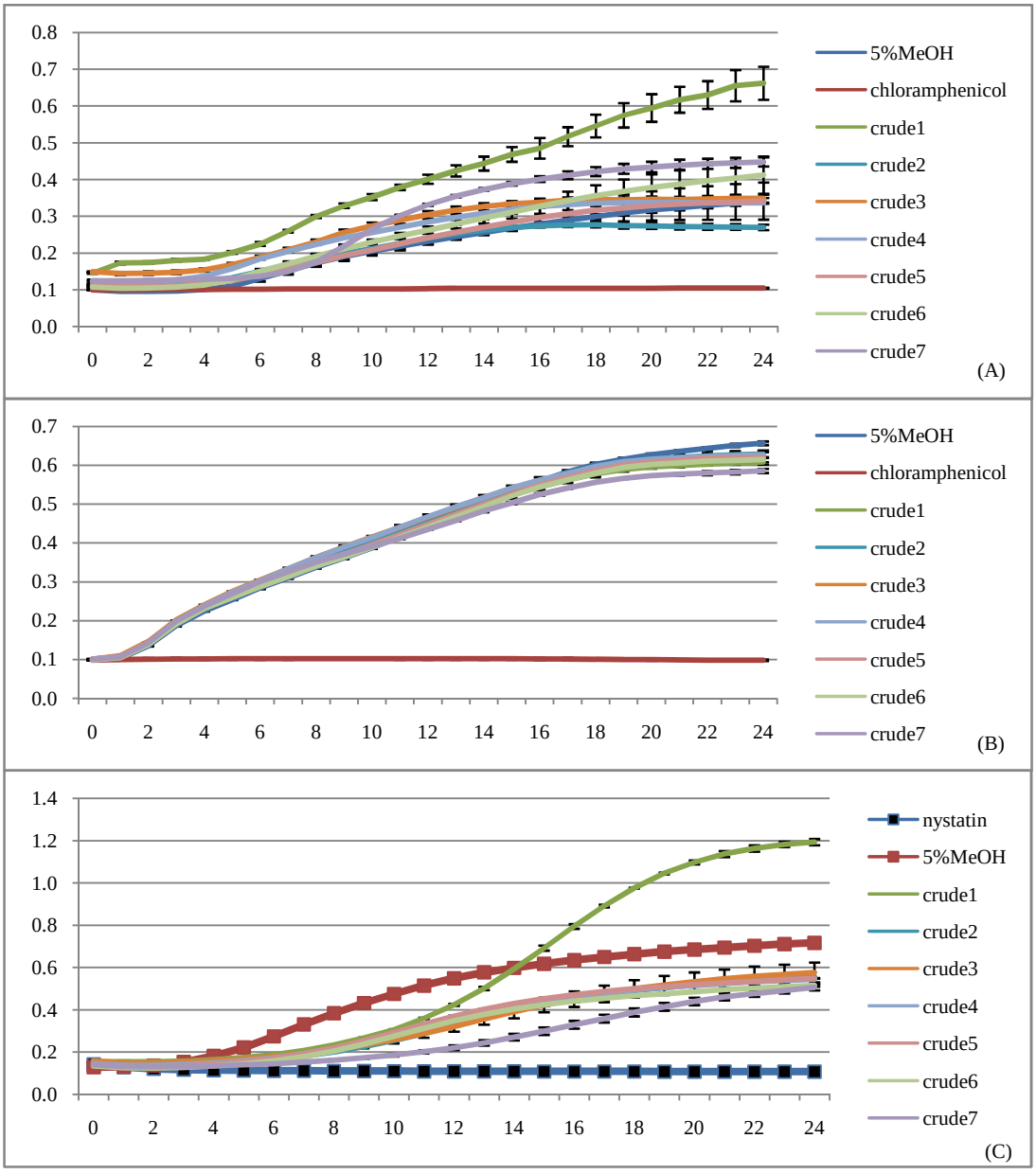
4.5.2 Antimicrobial activity test of ethyl acetate crude extracts of *S. parvulus* J6.2 cultured in various media

Crude extract of *S. parvulus* J6.2 cultured in various media according to 3.5.2 were tested against test microorganisms (Figure 4.16). The results showed that all crude extract had no activity against *Salmonella typhimurium* at 500 ppm. Crude1 could not against any test microorganisms, *E. coli*, *S. typhimurium* and *Candida albicans*. Crude2 – crude7 had very low activity against *E. coli* and *C. albicans*.

However, all crude extracts could inhibit Gram-positive bacteria against *Staphylococcus epidermidis* and *Mycobacterium smegmaltis*. A major compound consisting in all crude extracts were isolated with preparative reverse phase HPLC, column Gemini C18 110A, 5 μm (250 x 10 mm) and isocratic of 80% methanol in water as mobile phase, 5 ml min^{-1} flow rate. The major compounds in different media were collected at a retention time between 8 – 10 min. They displayed different antimicrobial activities as shown in Figure 4.17. MIC values of the major compounds against *S. epidermidis*, *M. smegmaltis* and *C. albicans* were 2, 2 and 10 ppm, respectively.

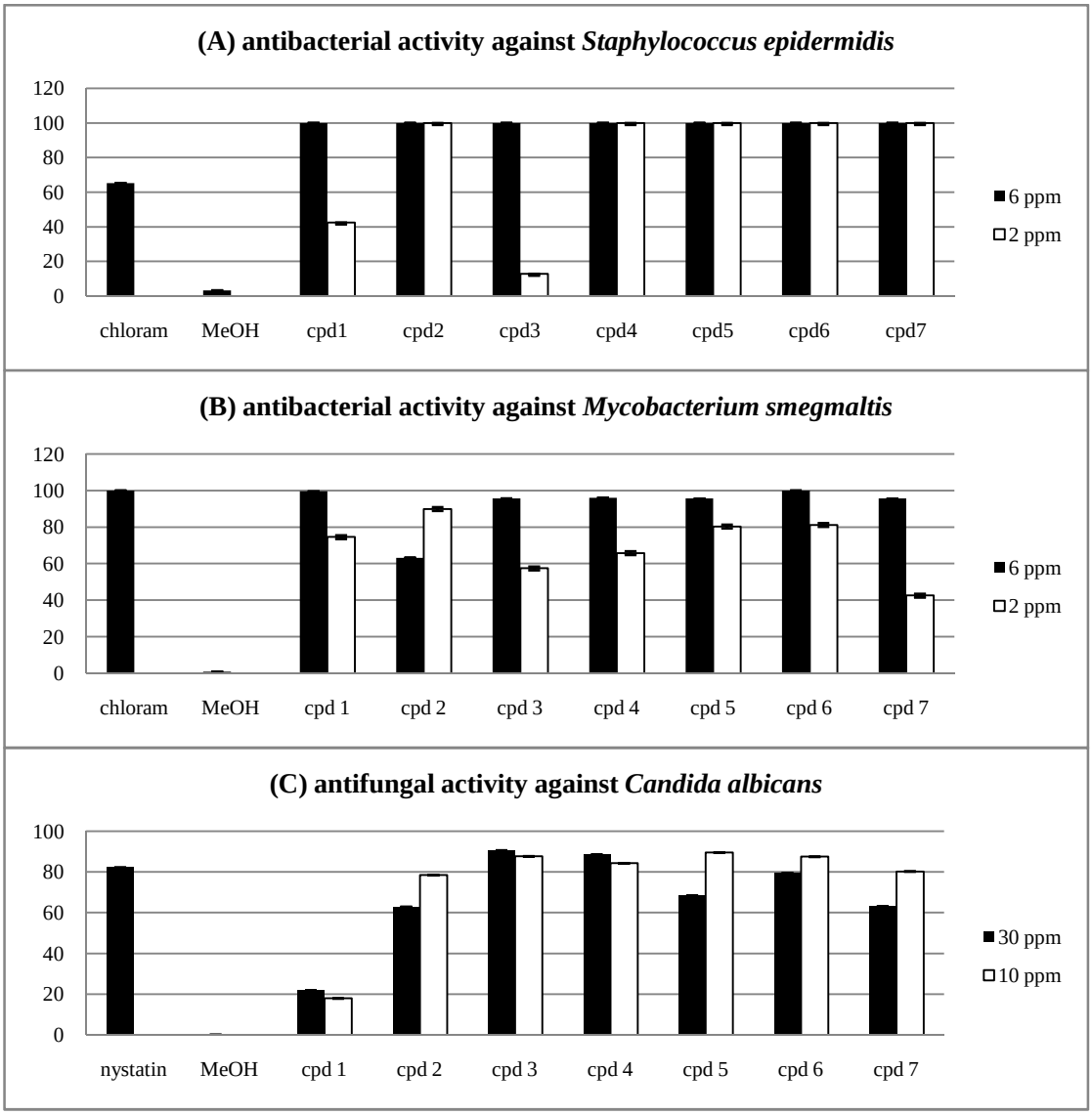
The results concluded that the secondary metabolites produced by *S. parvulus* in various media dominantly inhibited Gram-positive but not inhibited Gram-negative bacteria, they could also inhibit yeast when high concentration was used. The major bioactive component in crude extract was actinomycin-D.

Figure 4.16 Growth curve of test microorganisms against ethyl acetate crude extracts of *S. parvulus* J6.2 cultured in various media



(A) *E. coli*; (B) *Salmonella typhimurium*; (C) *Candida albicans*. Sample concentration was 500 ppm. Crude1 was extracted from soytone 30 g/l, glucose 20 g/l, $CaCO_3$ 2 g/l; crude2 from SCB, starch 10 g/l, malt extract 15 g/l; crude3 from SCB, malt extract 15 g/l; crude4 from SCB, malt extract 20 g/l; crude5 from SCB, malt extract 20 g/l, NaCl 10 g/l; crude6 from SCB, malt extract 20 g/l, NaCl 20 g/l; crude7 from SCB, malt extract 20 g/l, NaCl 30 g/l. Error bars represent for standard deviations from triplicate analyses.

Figure 4.17 Percentage of inhibition of actinomycin-D isolated from *Streptomyces parvulus* J6.2 cultured in various medium composition



cpd 1 – 7 were purified by HPLC from ethyl acetate crude extracts cultured in various media. cpd1: soytone 30 g/l, glucose 20 g/l, $CaCO_3$ 2 g/l; cpd2: SCB, starch 10 g/l, malt extract 15 g/l; cpd3: SCB, malt extract 15 g/l; cpd4: SCB, malt extract 20 g/l; cpd: 5 SCB, malt extract 20 g/l, NaCl 10 g/l; cpd6: SCB, malt extract 20 g/l, NaCl 20 g/l; cpd7: SCB, malt extract 20 g/l, NaCl 30 g/l. 50 µg/ml chloramphenicol was used as positive control to inhibit *S.epidermidis* and *M.smegmaltis*. 100 µg/ml nystatin was used as positive control to inhibit *C.albicans*. Error bars represent for standard deviations from triplicate analyses.

4.5.3 Antioxidant activity

Fractions of ethyl acetate crude extracts passing through HPLC were tested antioxidant activity (Table 4.14). Column Thermo® ODS Hypersil C18 25 cm x 10 mm, 5 μm was used to purify and stepwise gradient of methanol in water as mobile phase using 70% for 0 – 20 min, 75% for 25 – 45 min and 100% methanol for 50 – 60 min, 3 ml min^{-1} flow rate.

Secondary metabolites produced by *Steptomyces parvulus* J6.2 had very low antioxidant activity. Actinomycin-D, eluting at retention time 33 – 36 min, had no antioxidant activity but it activated oxidation reaction. The fraction at retention time 29 -32 min provided the most antioxidant activity as 14.759 % inhibition at 80 ppm.

Table 4.14 Antioxidant activity of fractions pass through HPLC

Sample	% inhibition	Sample	% inhibition
VitE, 200	87.172	E2, 200	0.046
VitE, 100	86.989	E2, 50	-2.207
VitE, 50	91.264	9-10.2 HPLC	1.195*
VitE, 25	61.287	10.2-12 HPLC	5.103
VitE, 20	63.218	12.5-16 HPLC	6.391
VitE, 10	41.241	16-20 HPLC	4.966**
VitE, 5	29.379	20-29 HPLC	5.931
VitE, 1	5.287	29-32 HPLC	14.759**
VitE, 0	1.103	29.3-33 HPLC	1.011
*%inhibition at concentration 120 ppm		ActD HPLC	-5.287
**%inhibition at concentration 80 ppm		36-45 HPLC	2.805
		45-60 HPLC	2.299

CHAPTER 5
CONCLUSIONS

Streptomyces parvulus J6.2, isolated from soil in mangrove area, had smooth surface, spiral spore chain. The physiology and 16S rDNA sequence were analyzed to identification. Phylogenetic tree was constructed by comparison of the 16S rDNA and showed close relationship between *S. parvulus* J6.2 and actinomycin-D producing *Streptomyces* spp. Carbon utilization indicated that *S. parvulus* J6.2 was a novel strain different to the original strain found before.

The secondary metabolite production of *S. parvulus* J6.2was examined when cultured in various media and conditions. Sodium caseinate broth supplemented with starch 10 g/l and malt extract 15 g/l gave the highest yield of secondary metabolite, therefore it used for mass production. Secondary metabolite of *S. parvulus* J6.2 ethyl acetate crude extract was purified by column chromatography and HPLC. Actinomycin-D, a major bioactive component, was isolated by reverse phase HPLC using column Thermo® ODS Hypersil C18 25 cm x 10 mm, 5 μm and stepwise gradient of methanol in water as mobile phase using 70% for 0 – 20 min, 75% for 25 – 45 min and 100% methanol for 50 – 60 min, 3 ml min^{-1} flow rate. Actinomycin-D was eluted at retention time 33 – 38 min. The molecular structure of actinomycin-D was confirmed by 2D-NMR.

Quantitative analysis of actinomycin-D was compared with calibration curve using standard actinomycin-D (Sigma) as a standard and analyzed by HPLC. Actinomycin-D was eluted at 12.5 min. Peak area was plotted against various concentrations actinomycin-D in range 25 – 150 μg/ml providing linear regression. Medium optimization gave the highest yield of 378.84 mg/l actinomycin-D when grown in a sodium caseinate broth supplemented with 20 g/l malt extract as the culture medium. Starch and malt extract supplemented culture medium produced high crude metabolite yield therefore increasing actinomycin-D production. High concentration of NaCl in culture medium suppressed production of triglyceride metabolites resulting in more specific production of actinomycin-D.

Fractions from column chromatography were tested for antimicrobial activity. Major bioactive compounds consisting in ethyl acetate crude extract could inhibit only Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus* but no active against *Escherichia coli*, *Pseudimonas aeruginosa* and *Candida albicans*. However, fraction sub48col4 gave activity against Gram-negative bacteria and 3col1 could inhibit against yeast. The secondary metabolites produced by *S.parvulus* in various media dominantly inhibited Gram-positive but not inhibited Gram-negative bacteria, they could also inhibit yeast when high concentration was used. The major bioactive component in crude extract was actinomycin-D. Secondary metabolites produced by *Steptomyces parvulus* J6.2 had very low antioxidant activity. Actinomycin-D had also no antioxidant activity but it activated oxidation reaction. The fraction at retention time 29 -32 min provided the most antioxidant activity as 14.759 % inhibition at 80 ppm.

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APPENDICES

Appendix A
Culture media and chemical solution

All standard media were dissolved in distilled water to final volume 1 liter and sterile in autoclave at 121 °C for 15 min. The pH was adjusted with NaOH and HCl before adding agar and sterilization. The broth media did not add agar in the formula.

1. Starch Casein Agar

Starch	10	g
Casein (vitamin free)	0.3	g
$CaCO_3$	0.02	g
$FeSO_4.7H_2O$	0.01	g
KNO_3	2	g
KH_2PO_4	2	g
$MgSO_4.7H_2O$	0.05	g
NaCl	2	g
Agar	18	g
Water	1000	mL

The medium was steriled in autoclave. Sterile cyclohexamide and nystatin were prepared by filled through 0.45 μm syringe filter and then added to sterile medium for final concentration 50 μg/mL.

2. PDA (Potato dextrose agar)

Potato	200.0	g
Glucose	20.0	g
Agar	20.0	g
Water	1,000	mL

3. TSA (Tryptic Soy Agar)

Bacto tryptone	17.0	g
Bacto soytone	3.0	g
NaCl	5.0	g
K_2HPO_4	2.5	g
Glucose	2.5	g
Agar	15.0	g
Water	1,000	mL

4. SCA (Sodium Caseinate Agar)

Sodium Caseinate	2.0	g
K_2HPO_4	0.5	g
$MgSO_4$	0.2	g
$FeCl_3$	0.01	g
Agar	18.0	g
Water	1,000	mL

5. NA (Nutrient Agar)

Beef extract	1.0	g
Peptone	5.0	g
NaCl	8.0	g
Agar	18.0	g
Water	1,000	mL

6. MEA (Malt Extract Agar)

Malt extract	2.0	g
Peptone	1.0	g
Glucose	20.0	g
Agar	18.0	g
Water	1,000	mL

7. GYM (Glucose Yeast Malt Extract)

Glucose	4.0	g
Yeast extract	4.0	g
Malt extract	10.0	g
Calcium carbonate	2.0	g
Water	1,000	mL

8. Tryptone Yeast Extract Agar (ISP1)

Tryptone	5.0	g
Yeast Extract	3.0	g
Agar	18.0	g
Water	1,000	mL

9. Yeast Malt Extract Agar (ISP2)

Malt extract	10.0	g
Yeast extract	4.0	g
Glucose	4.0	g
Agar	18.0	g
Water	1,000	mL

10. Oat Meal Agar (ISP3)

Oat meal	20	g
Trace salts solution	1.0	mL (Appendix A.20)
Agar	18.0	g
Water	1,000	mL

Oat meal was boiled in distilled water for 20 min and then filled to discharge oat meal. Trace salts solution was added and adjusting to final volume 1,000 mL.

11. Inorganic Salts Starch Agar (ISP4)

Soluble starch	10.0	g
K_2HPO_4	1.0	g
$MgSO_4 \cdot 7H_2O$	1.0	g
NaCl	1.0	g
$(NH_4)_2SO_4$	2.0	g
$CaCO_3$	2.0	g
Trace salts solution	1.0	mL (Appendix A. 20)
Agar	20.0	g
Water	1,000	mL

12. Glycerol Asparagine Agar (ISP5)

Glycerol	10.0	g
L- Asparagine	1.0	g
K_2HPO_4	1.0	g
Trace salts solution	1.0	mL (Appendix A.20)
Agar	20.0	g
Water	1,000	mL

13. Peptone Yeast Extract Iron Agar (ISP6)

Bacto peptone	15.0	g
Proteose peptone	5.0	g
Ferric ammonium citrate	0.5	g
K_2HPO_4	1.0	g
Na_2SO_4	0.08	g
Yeast extract	1.0	g
Agar	18.0	g
Water	1,000	mL

14. Nitrate Agar

Beef extract	3.0	g
Bacto peptone	5.0	g
KNO_3	2.0	g
Agar	5.0	g
Water	1,000	mL

15. Tyrosine Agar

Glycerol	15.0	g
L- Tyrosine	0.5	g
L- Asparagine	1.0	g
K_2HPO_4	0.5	g
$MgSO_4 \cdot 7H_2O$	1.0	g
$FeSO_4 \cdot 7H_2O$	0.1	g
Trace salts solution	1.0	mL (Appendix A.20)
Agar	20.0	g
Water	1,000	mL

Sterile in autoclave at 110 °C, 10 psi for 10 min.

16. Modified Bennett's Agar

Beef extract	1.0	g
Glycerol	10.0	g
Bacto peptone	2.0	g
Yeast extract	1.0	g
Agar	15.0	g
Water	1,000	mL

Sterile in autoclave at 110 °C, 10 psi for 10 min.

17. Bennett's Agar

Glucose	10.0	g
Yeast extract	1.0	g
Bacto peptone	2.0	g
Beef extract	1.0	g
Agar	18.0	g
Water	1,000	mL

18. Basal Medium Agar

$MgSO_4 \cdot 7H_2O$	0.5	g
NaCl	0.5	g
$FeSO_4 \cdot 7H_2O$	0.01	g
K_2HPO_4	1.0	g
Agar	12.0	g
Water	1,000	mL

Sterile in autoclave at 110 °C, 10 psi for 10 min.

19. Carbon Utilization Agar

$(NH_4)_2SO_4$	2.64	g
KH_2PO_4	2.38	g
K_2HPO_4	1.0	g
$MgSO_4 \cdot 7H_2O$	1.0	g
NaCl	1.0	g
$MgSO_4 \cdot 7H_2O$	0.5	g
Pridham and Gottlieb trace salts solution	1.0	mL (Appendix A.21)
Agar	12.0	g
Water	1,000	mL

Sterile in autoclave at 110 °C, 10 psi for 10 min.

20. Trace Salts Solution

$FeSO_4 \cdot 7H_2O$	0.1	g
$MnCl_2$	0.1	g
$ZnSO_4 \cdot 7H_2O$	0.1	g
Water	1,000	mL

Sterile by filling through 0.45 μm syringe filter and then adding to culture medium at temperature 45 – 50 °C.

21. Pridham and Gottlieb trace salts solution

$CuSO_4 \cdot 7H_2O$	0.64	g
$FeSO_4 \cdot 7H_2O$	0.11	g
$MnCl_2$	0.79	g
$ZnSO_4 \cdot 7H_2O$	0.15	g
Water	1,000	mL

Sterile by filling through 0.45 μm syringe filter and then adding to culture medium at temperature 45 – 50 °C.

22. 0.5 McFarland Standard

$BaCl_3$	0.048	M, 0.5 mL
H_2SO_4	0.36	M, 99.5 mL

Adjust A625 nm to be 0.08 – 0.10.

23. Vanillin solution

Vanillin	4.5	g
95% ethanol	95	mL
Sulfuric acid	0.5	mL

Mix together to homogenous solution and kept in dark brown bottle until use.

Appendix B

Chemicals for molecular assay

1. 1 M Tris-HCl (pH 8)

Tris base	121	g
Distilled water	800	mL

Dissolved Tris base in distilled water homogeneously and adjusted pH for 8 and then added water to final volume 1 liter. The solution was sterile in autoclave at 121 °C, 15 psi for 15 min and keep at 4 °C.

2. 0.5 M EDTA (Ethylenediamine tetra acetic acid)

EDTA	186.10	g
Distilled water	800	mL

Dissolved EDTA in distilled water homogeneously and adjusted pH for 8 and then added water to final volume 1 liter. The solution was sterile in autoclave at 121 °C, 15 psi for 15 min and keep at 4 °C.

3. Washing buffer (for 200 mL)

PVP (Polyvinylpyrrolidone)	2	g
Ascorbic acid	1.76	g
1 M Tris-HCl (pH 8.0)	20	mL (Appendix B.1)
2-mercaptoethanol	4	mL

Dissolved all mixtures in distilled water homogeneously and adjusted water to final volume 200 mL and keep at 4 °C.

4. 2X CTAB lysis buffer (for 200 mL)

CTAB	4	g
1 M Tris-HCl (pH 8.0)	20	mL (Appendix B.1)
0.5 M EDTA (pH 8.0)	8	mL (Appendix B.2)
NaCl	16.36	g
2-mercaptoethanol	1	mL

Dissolved all mixtures in distilled water homogeneously and adjusted water to final volume 200 mL and keep at room temperature.

5. Choloroform/isoamyl alcohol (24: 1 v/v) (for 200 mL)

Choloroform	192	mL
Isoamyl alcohol	8	mL

6. 20 % Polyethylene glycol 6000 (PEG) (for 200 mL)

Polyethylene glycol 6000	20	g
NaCl	14.61	g

Dissolved in distilled water homogeneously to final volume 200 mL and keep at room temperature.

7. Tris-EDTA buffer (TE buffer)

1 M Tris-HCl (pH 8)	10	mL (Appendix B.1)
0.5 M EDTA (pH 8)	2	mL (Appendix B.2)

Dissolved in distilled water homogeneously and adjusted water to final volume 1 liter. The buffer was sterile in autoclave at 121 °C, 15 psi for 15 min and keep at room temperature.

8. Ethidium bromide, 10 mg/mL (for 20 mL)

Ethidium bromide	0.2	g
Distilled water	20	mL

Mixed homogeneously and keep at 4 °C in dark brown bottle until use.

9. 1.5% Agarose gel (w/w)

Agarose	1.65	g
0.5 X TBE (pH 8)	110	mL (Appendix B.2)
Ethidium bromide	4	µL

Melted and mixed homogenously and keeped at room temperature.

Appendix C

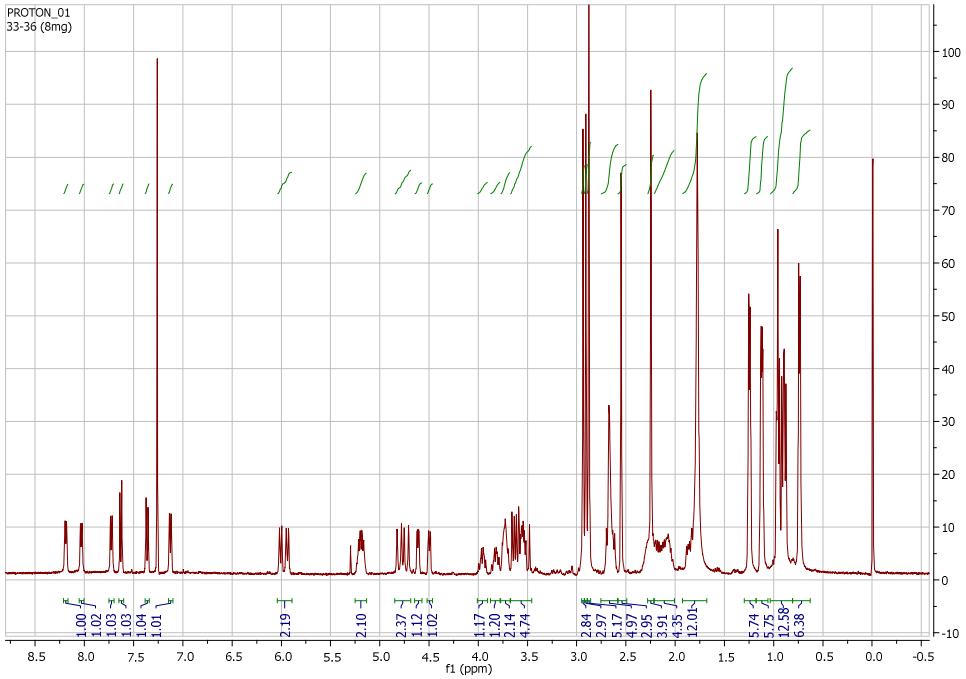
Alignment data

Accession	Description	Max score	Total score	Query coverage	Max ident
NR_041119.1	*Streptomyces parvulus* strain NBRC 13193 16S ribosomal RNA, partial sequence	2588	2588	100%	100%
NR_041423.1	*Streptomyces malachitospinus* strain NBRC 101004 16S ribosomal RNA, partial sequence	2543	2543	100%	99%
NR_041202.1	*Streptomyces olivaceus* strain NBRC 3200 16S ribosomal RNA, partial sequence	2529	2529	100%	99%
NR_041185.1	*Streptomyces lomondensis* strain NBRC 15426 16S ribosomal RNA, partial sequence	2529	2529	100%	99%
NR_041134.1	*Streptomyces pactum* strain NBRC 13433 16S ribosomal RNA, partial sequence	2529	2529	100%	99%
NR_025871.1	*Streptomyces tendae* strain ATCC 19812 16S ribosomal RNA, complete sequence	2527	2527	100%	99%
NR_042309.1	*Streptomyces violaceorubidus* strain :LMG 20319 16S ribosomal RNA, complete sequence	2523	2523	100%	99%
NR_041231.1	*Streptomyces iakyrus* strain NBRC 13401 16S ribosomal RNA, partial sequence	2521	2521	100%	99%
NR_041128.1	*Streptomyces luteogriseus* strain NBRC 13402 16S ribosomal RNA, partial sequence	2521	2521	100%	99%
NR_041222.1	*Streptomyces coeruleorubidus* strain NBRC 12844 16S ribosomal RNA, partial sequence	2512	2512	100%	99%
NR_043337.1	*Streptomyces coerulescens* strain ISP 5146 16S ribosomal RNA, partial sequence	2512	2512	100%	99%
NR_043745.1	*Streptomyces tritolerans* strain DAS 165 16S ribosomal RNA, complete sequence	2510	2510	100%	99%
NR_041143.1	*Streptomyces lusitanus* strain NBRC 13464 16S ribosomal RNA, partial sequence	2510	2510	100%	99%
NR_042760.1	*Streptomyces* sp. 40003 strain 40003 16S ribosomal RNA, partial sequence	2510	2510	100%	99%
NR_041224.1	*Streptomyces purpurascens* strain NBRC 12879 16S ribosomal RNA, partial sequence	2505	2505	100%	99%
NR_041188.1	*Streptomyces rubrogriseus* strain NBRC 15455 16S ribosomal RNA, partial sequence	2505	2505	100%	99%
NR_041115.1	*Streptomyces violaceus* strain NBRC 13103 16S ribosomal RNA, partial sequence	2505	2505	100%	99%
NR_042302.1	*Streptomyces lienomycini* strain :LMG 20091 16S ribosomal RNA, complete sequence	2505	2505	100%	99%
NR_025493.1	*Streptomyces roseoviolaceus* strain ISP 5277 16S ribosomal RNA, partial sequence	2505	2505	100%	99%
NR_041147.1	*Streptomyces longispororuber* strain NBRC 13488 16S ribosomal RNA, partial sequence	2501	2501	100%	99%
NR_041105.1	*Streptomyces malachitofuscus* strain NBRC 13059 16S ribosomal RNA, partial sequence	2501	2501	100%	99%
NR_042101.1	*Streptomyces* sp. strain ISP 5310 16S ribosomal RNA, partial sequence	2501	2501	100%	99%
NR_041178.1	*Streptomyces djakartensis* strain NBRC 15409 16S ribosomal RNA, partial sequence	2499	2499	100%	99%
NR_041190.1	*Streptomyces tuirus* strain NBRC 15617 16S ribosomal RNA, partial sequence	2499	2499	100%	99%
NR_042829.1	*Streptomyces* sp. 40002 strain 40002 16S ribosomal RNA, partial sequence	2499	2499	100%	99%
NR_041159.1	*Streptomyces spinoverrucosus* strain NBRC 14228 16S ribosomal RNA, partial sequence	2495	2495	100%	99%

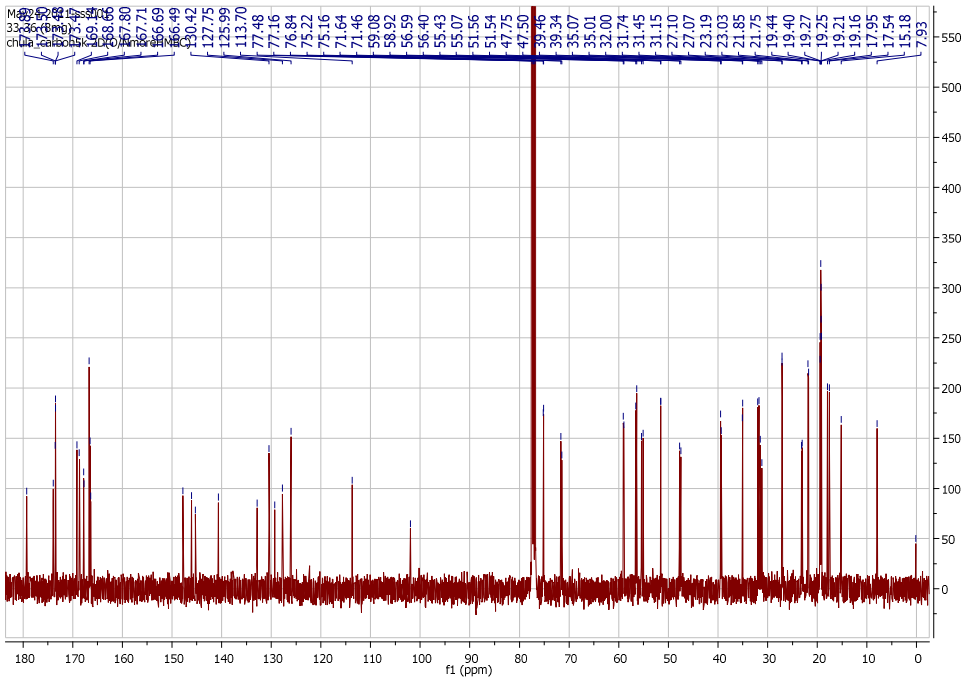
Accession	Description	Max score	Total score	Query coverage	Max ident
NR_041189.1	*Streptomyces tricolor* strain NBRC 15461 16S ribosomal RNA, partial sequence	2494	2494	100%	99%
NR_041168.1	*Streptomyces anthocyanicus* strain NBRC 14892 16S ribosomal RNA, partial sequence	2494	2494	100%	99%
NR_041155.1	*Streptomyces indiaensis* strain NBRC 13964 16S ribosomal RNA, partial sequence	2494	2494	100%	99%
NR_027222.1	*Streptomyces coelescens* strain AS 4.1594 16S ribosomal RNA, partial sequence	2494	2494	100%	99%
NR_041914.1	*Streptomyces violaceoruber* strain DSM 40049 16S ribosomal RNA, partial sequence	2494	2494	100%	99%
NR_044149.1	*Streptomyces minutiscleroticus* strain NRRL B-12202 16S ribosomal RNA, partial sequence	2488	2488	100%	99%
NR_043818.1	*Streptomyces geysiriensis* strain NRRL B-12102 16S ribosomal RNA, partial sequence	2488	2488	100%	99%
NR_041218.1	*Streptomyces flavoviridis* strain NBRC 12772 16S ribosomal RNA, partial sequence	2488	2488	100%	99%
NR_041073.1	*Streptomyces pilosus* strain NBRC 12807 16S ribosomal RNA, partial sequence	2488	2488	100%	99%
NR_041068.1	*Streptomyces hawaiiensis* strain NBRC 12784 16S ribosomal RNA, partial sequence	2488	2488	100%	99%
NR_027223.1	*Streptomyces violaceolatus* strain DSM 40438 16S ribosomal RNA, partial sequence	2488	2488	100%	99%
NR_025250.1	*Streptomyces humiferus* strain DSM 43030 16S ribosomal RNA, partial sequence	2488	2488	100%	99%
NR_043336.1	*Streptomyces coeruleorubidus* strain ISP 5145 16S ribosomal RNA, partial sequence	2488	2488	100%	99%
NR_044139.1	*Streptomyces mutabilis* strain NRRL ISP-5169 16S ribosomal RNA, partial sequence	2484	2484	100%	99%
NR_025212.1	*Streptomyces speibonae* strain PK-Blue 16S ribosomal RNA, partial sequence	2484	2484	100%	99%
NR_041091.1	*Streptomyces rochei* strain NBRC 12908 16S ribosomal RNA, partial sequence	2483	2483	100%	99%
NR_041088.1	*Streptomyces matensis* strain NBRC 12889 16S ribosomal RNA, partial sequence	2483	2483	100%	99%
NR_041066.1	*Streptomyces griseorubens* strain NBRC 12780 16S ribosomal RNA, partial sequence	2483	2483	100%	99%
NR_041063.1	*Streptomyces collinus* strain NBRC 12759 16S ribosomal RNA, partial sequence	2483	2483	100%	99%
NR_041082.1	*Streptomyces chromofuscus* strain NBRC 12851 16S ribosomal RNA, partial sequence	2481	2481	100%	99%
NR_043843.1	*Streptomyces viridochromo*genes strain NRRL B-1511 16S ribosomal RNA, partial sequence	2477	2477	100%	99%
NR_041206.1	*Streptomyces flaveolus* strain NBRC 3408 16S ribosomal RNA, partial sequence	2477	2477	100%	99%
NR_041167.1	*Streptomyces paradoxus* strain NBRC 14887 16S ribosomal RNA, partial sequence	2477	2477	100%	99%
NR_041184.1	*Streptomyces levis* strain NBRC 15423 16S ribosomal RNA, partial sequence	2475	2475	100%	99%
NR_043381.1	*Streptomyces fragilis* strain NRRL 2424 16S ribosomal RNA, partial sequence	2475	2475	100%	99%
NR_041175.1	*Streptomyces coelicoflavus* strain NBRC 15399 16S ribosomal RNA, partial sequence	2473	2473	100%	99%
NR_043366.1	*Streptomyces ghanaensis* strain KCTC 9882 16S ribosomal RNA, partial sequence	2473	2473	99%	99%
NR_041219.1	*Streptomyces glaucescens* strain NBRC 12774 16S ribosomal RNA, partial sequence	2471	2471	100%	99%

Appendix D

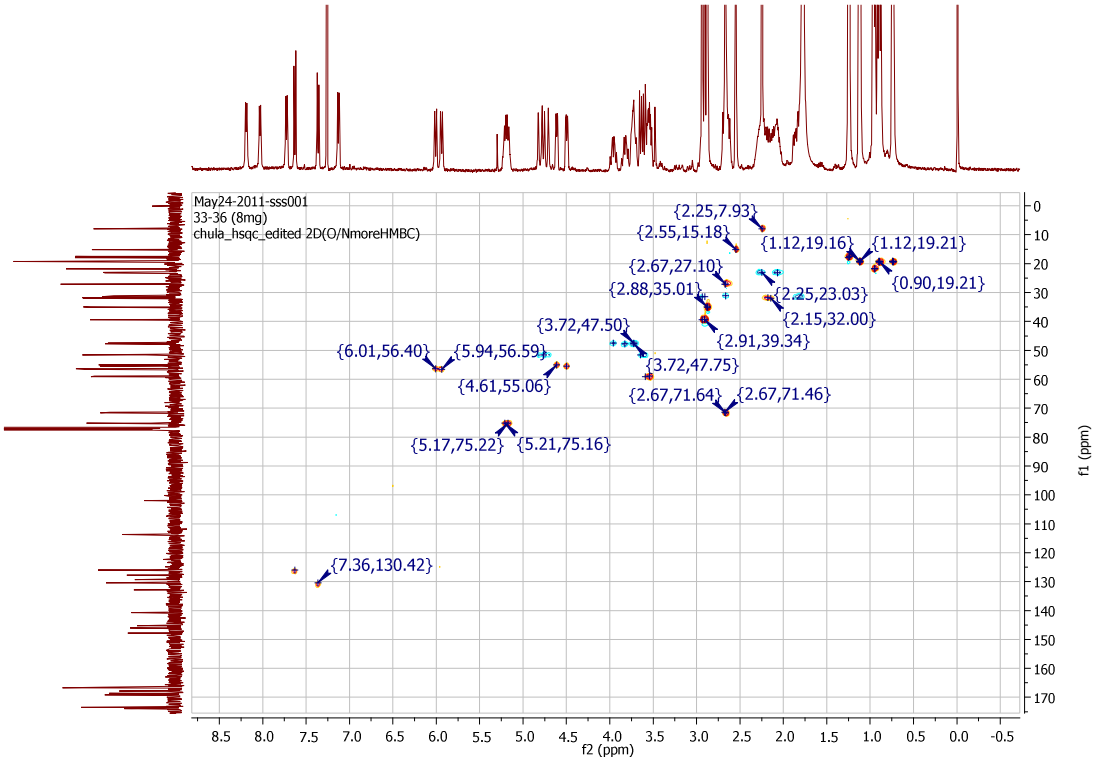
Spectroscopy data



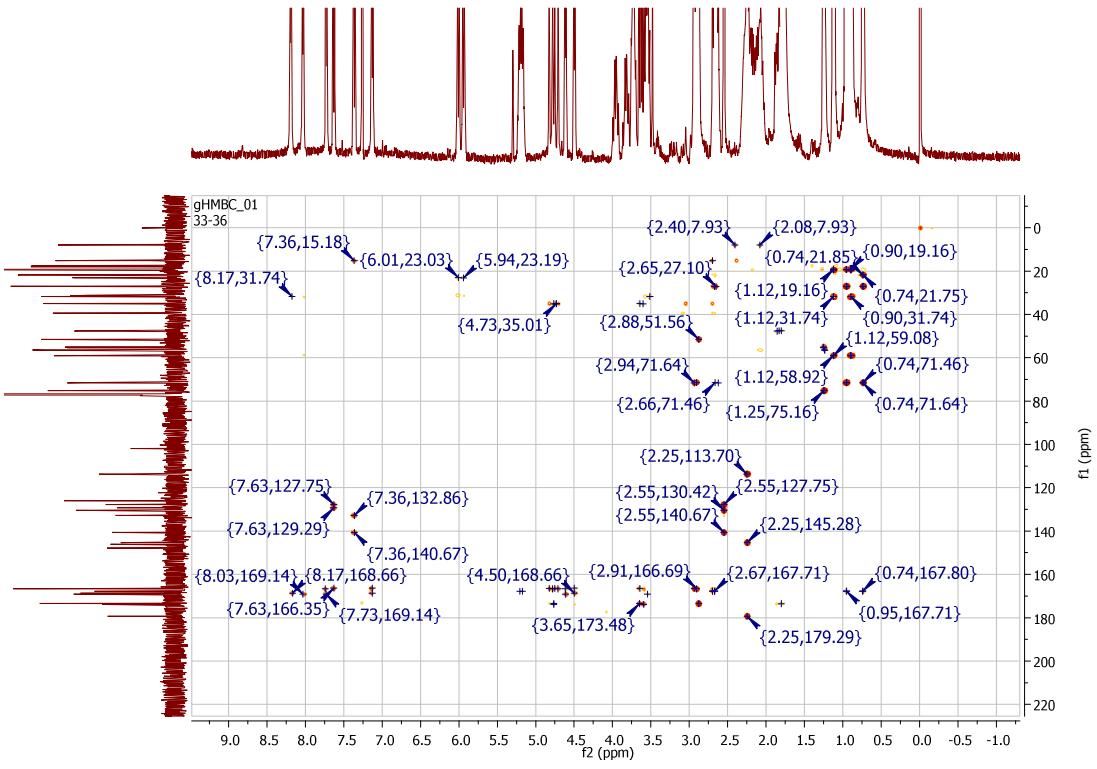
^{1}H – NMR spectrum of actinomycin-D



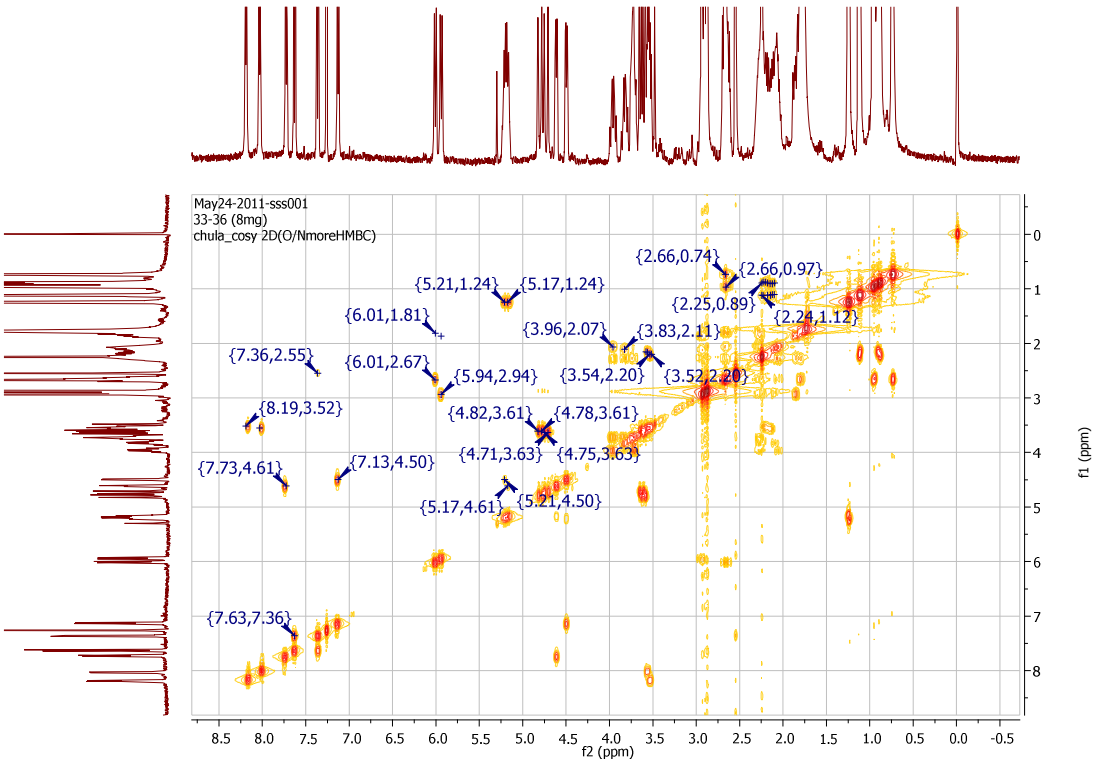
^{13}C – NMR spectrum of actinomycin-D



HSQC of actinomycin-D



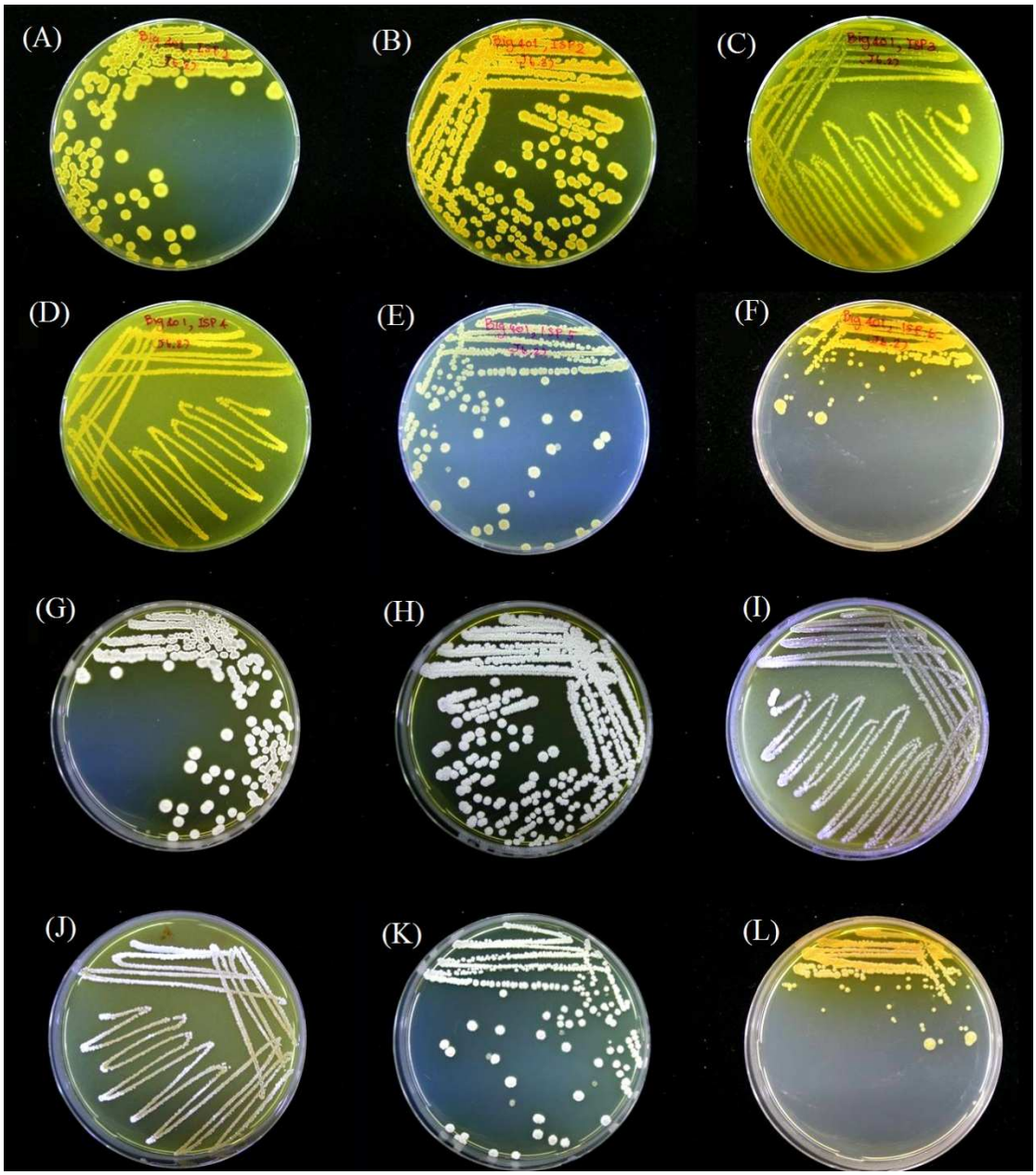
HMBC of actinomycin-D



COSY of actinomycin-D

Appendix E

Streptomyces parvulus J6.2 colony on ISP media



Streptomyces parvulus J6.2 colonies on ISP media; A – F were substrate mycelia grew on ISP1 – ISP6 media, respectively; G – L were aerial mycelia on ISP1 – ISP6 media, respectively.

BIOGRAPHY

Mr. Nattachai Kengpipat was born on December 31st, 1982 in Bangyai, Nonthaburi, Thailand. He was a son of Napon and Suraksa Kengpipat. He graduated high school at Bangkok Christian Collage in 2001, Bachelor degree on Microbiology at Chulalongkorn University in 2005 and Master degree on Biotechnology at Chulalongkorn University in 2007. In 2011, he used to do a research project according to his doctoral thesis as an honorary staff at The University of Manchester, United Kingdom.

Academic works

Year 2000 "Investigation the denitrification method for reduces TKN in effluent to be less than 5 mg/l". student trainee project at Ajinomoto Co., (Thailand) Ltd. Pathumthani factory.

Year 2006 "Screening for antimicrobial and antitumor activity of actinomycetes isolated from beaches and mangroves in Thailand". poster presentation of The 18th Annual Meeting of the Thai Society for Biotechnology.

Year 2010 "Monitoring survival of phenanthrene-utilizing *Sphingobium* sp. P2 in soil microcosms using green fluorescent protein as a marker". *Sciences Asia* 36: 76-80.

Scholarships

Year 2006 Research Scholarship, Program in Biotechnology, Chulalongkorn University.

Year 2007 Teacher Assistant Scholarship, Program in Biotechnology, Chulalongkorn University.

Year 2008 The Royal Golden Jubilee Ph.D. Program Scholarship, Thailand Research Fund.