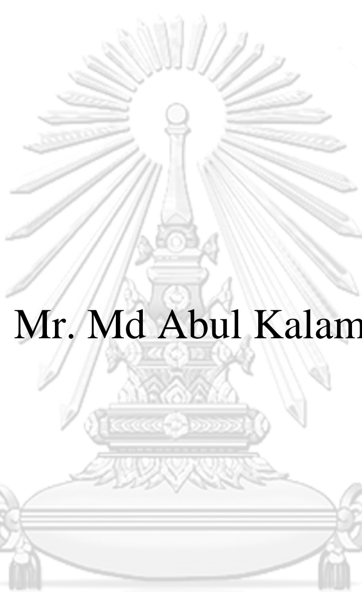
TAXONOMY AND BIOLOGICAL ACTIVITIES OF A NOVEL *SACCHAROTHRIX* SPECIES FROM SOIL OF SICHANG ISLAND



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อนุกรมวิธานและฤทธิ์ทางชีวภาพของเชื้อแซคคาโรทริกซ์ชนิดใหม่จากดินของเกาะสีชัง

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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แอคติโนแบคทีเรียเป็นแหล่งของผลิตภัณฑ์ธรรมชาติที่มีฤทธิ์ทางชีวภาพ แอคติโนแบคทีเรียหายากหมายถึงสกุลของแอคติโนแบคทีเรียที่มีความถี่น้อยที่จะถูกคัดแยกได้จากธรรมชาติ ระหว่างการศึกษาความหลากหลายทางชีวภาพของแอคติโนแบคทีเรียหายาก เชื้อไอโซเลต SC076^T ได้ถูกคัดแยกจากดินของเกาะสีชัง จังหวัดชลบุรี เชื้อดังกล่าวมีความคล้ายคลึงสูงสุดของยีนบริเวณ 16S rRNA กับเชื้อ *Saccharothrix australiensis* DSM 43800^T (98.58%) and *Saccharothrix espanaensis* DSM 44229^T (98.58%) สามารถสร้างสายสปอร์แบบสลับฟันปลาบนเส้นใยอากาศ มี *meso*-diaminopimelic acid ในเพบไทโดไกลแคน พบน้ำตาลแรมโนส ไรโบส แมนโนส กลูโคสและกาแลกโตสภายในเซลล์ ลิปิดมีขั้วของเซลล์ประกอบด้วยฟอสฟาทิดิลเอทาโนลามีน ไฮดรอกซีฟอสฟาทิดิลเอทาโนลามีน ไดฟอสฟาทิดิลกลีเซอรอล ฟอสฟาทิดิลกลีเซอรอล ฟอสฟาทิดิลอิโนซิทอลแมนโนซาย ฟอสฟาทิดิลอิโนซิทอล และยังพบ ไกลโคลิปิดที่ให้ผลบวกกับนินไฮดริน ไกลโคคลิปิดและลิปิดที่ไม่สามารถระบุชนิดได้ พบ MK-9(H_4) MK-9(H_8) MK-9(H_2) และ MK-9(H_0) ในระบบควิโนน และมีกรดไขมันหลักคือ iso-$C_{16:0}$ iso-$C_{15:0}$ และ anteiso-$C_{17:0}$ เมื่อเปรียบเทียบจีโนมของสายพันธุ์ SC076^T กับเชื้อสายพันธุ์ใกล้เคียงพบว่ามีค่า dDNA-DNA hybridization และ ค่า average nucleotide identity ที่ 77.7-82.5% และ 84.9-86.7% ตามลำดับ นอกจากนี้พบว่าไอโซเลต SC076^T สามารถแสดงฤทธิ์ยับยั้งแบคทีเรียก่อโรคแกรมบวกแต่ไม่มีฤทธิ์ยับยั้งเชื้อรา และจากการคัดกรองฤทธิ์ต้านมะเร็งด้วยวิธี MTT assay พบว่าสามารถยับยั้งเซลล์ non-small lung cancer H460 ได้และมีค่า IC_{50} เท่ากับ 126.668 μg/mL และพบความเป็นพิษต่อเซลล์เมื่อใช้ปริมาณมากขึ้น อย่างไรก็ตามเป็นที่น่าเสียดายว่าไม่พบฤทธิ์ต่อการเคลื่อนที่ของเซลล์และการรุกรานของเซลล์ นอกจากนี้ได้ศึกษาฤทธิ์ต้านสารอนุมูลอิสระและฤทธิ์ต้านกิจกรรมของเอนไซม์ acetylcholinesterase แต่เป็นที่น่าเสียดายว่าไม่พบฤทธิ์ทั้งสองชนิด อย่างไรก็ตามจากการศึกษาสารเมแทบอไลต์ทุติยภูมิในจีโนมพบว่ามีบางชนิดที่มีความเหมือน 100% กับสารในกลุ่มเทอร์ปีน และ PKS จากการศึกษาองค์ประกอบทางเคมีของสารสกัดหยาบ สารออกฤทธิ์ต้านแบคทีเรียในสารสกัดหยาบจะถูกคัดแยกและทำบริสุทธิ์ในการศึกษาต่อไป จากข้อมูลการศึกษาทางอนุกรมวิธานแสดงให้เห็นว่าเชื้อไอโซเลต SC076^T เป็นแบคทีเรียสปีชีส์ใหม่ของสกุล *Saccharothrix* และได้ตั้งชื่อว่า *Saccharothrix oblica* และมี type strain ของสปีชีส์นี้คือสายพันธุ์ SC076^T (=TBRC 14540^T =NBRC 115117^T) การศึกษานี้จึงสามารถสรุปได้ว่าเชื้อสายพันธุ์ SC076^T เป็นเชื้อสายพันธุ์ใหม่ของสกุล *Saccharothrix* ที่มีฤทธิ์ต้านแบคทีเรียและต้านเซลล์มะเร็ง โดยเชื้อแอคติโนแบคทีเรียที่คัดแยกได้จากตัวอย่างดินนั้นสามารถแสดงฤทธิ์ทางชีวภาพและสามารถเพื่อการค้นพบยาปฏิชีวนะต่อไป

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Md Abul Kalam Azad : TAXONOMY AND BIOLOGICAL ACTIVITIES OF A NOVEL *SACCHAROTHRIX* SPECIES FROM SOIL OF SICHANG ISLAND. Advisor: WONGSAKORN PHONGSOPITANUN, Ph.D. Co-advisor: Asst. Prof. PREEDAKORN CHUNHACHA, Ph.D.,Prof. SOMBOON TANASUPAWAT, Ph.D.

Actinobacteria are the sources of bioactive natural products. Rare actinobacteria are the less frequently isolated genera of actinobacteria. During an investigation of diversity of rare actinobacteria, actinobacteria isolate SC076^T was isolated from a soil sample collected from Sichang Island, Chonburi Province, Thailand. The strain showed the highest 16S rRNA gene similarity to *Saccharothrix australiensis* DSM 43800^T (98.58%) and *Saccharothrix espanaensis* DSM 44229^T (98.58%). The zigzag morphology of the spore chain was observed on the aerial mycelia. *meso*-diaminopimelic acid was detected in the peptidoglycan. Whole-cell sugars contained rhamnose, ribose, mannose glucose and galactose. Polar lipids are phosphatidylethanolamine, hydroxyphosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol mannoside, phosphatidyl inositol, unidentified ninhydrin positive glycolipid, unidentified glycolipid and four unidentified lipids. Menaquinones are MK-9(H_4), MK-9(H_8), MK-9(H_2) and MK-9(H_0). The predominant cellular fatty acids were iso-$C_{16:0}$, iso-$C_{15:0}$, and anteiso-$C_{17:0}$. The strain showed both dDNA-DNA hybridization value and average nucleotide identity of 77.7 – 82.5% and 84.9 – 86.7%, respectively. Moreover, the strain showed antimicrobial activity against gram positive pathogenic organisms but did not produce any antifungal activity. For the anticancer activity screening, MTT assay showed IC_{50} value 126.668 µg/mL against non-small lung cancer H460 cells. In the higher dose strain showed cytotoxic effect. Unfortunately, the strain did not have potential cell migration and cell invasion effects. Besides, antioxidant and anticholinease activity also tested but no activity represented from the strain. However, from the secondary metabolite profiling from the whole genome sequences it showed 100% similarity with Terpene, PKS compound. Besides chemical profiling of the crude extract determines the strain contains some antibiotic potential chemical compound that will be isolated and purified as further study. Based on the taxonomic evidence, strain SC076^T represents a novel species of the genus *Saccharothrix* for which the name *Saccharothrix oblica* sp. nov. is proposed. Type strain is SC076^T (=TBRC 14540^T =NBRC 115117^T). In conclusion, these finding ensured that strain SC076^T is the novel species of genus *Saccharothrix* and possesses antibacterial activity as well as cytotoxic effects. The *Actinobacteria* isolated from soil sample provided biological activity information in drug discovery as the good candidates for the development of antibiotics.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature ....
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		Co-advisor's Signature ....
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TABLE OF CONTENTS

Page

ABSTRACT (THAI) ... iii

ABSTRACT (ENGLISH) ... iv

ACKNOWLEDGEMENTS ... v

TABLE OF CONTENTS ... vi

LIST OF TABLES ... ix

LIST OF FIGURES ... x

CHAPTER I ... 12

INTRODUCTION ... 12

Hypothesis: ... 13

Objectives: ... 13

CHAPTER II ... 14

LITERATURE REVIEW ... 14

2.1 Rare Actinobacteria ... 14

2.2 The genus *Saccharothrix* ... 15

2.3 Isolation strategies of rare actinobacteria ... 16

2.4 Biological activities and novel bioactive compounds of *Saccharothrix*: ... 16

CHAPTER III ... 18

RESEARCH METHODOLOGY ... 18

3.1 Microorganism and preservation ... 18

3.2 Phenotypic characteristics ... 18

3.2.1 Morphological and cultural characteristics ... 18

3.2.2 Nitrate reduction ... 18

3.2.3 Starch Hydrolysis ... 18

3.2.4 Skim milk peptonisation ... 19

3.2.5 Liquify gelatine ... 19

3.2.6 Carbone Utilization 19
3.3 Chemotaxonomic Characteristics 19
3.3.1 Diaminopimelic acid (DAP) isomer analysis 19
3.3.2 Whole cell sugar analysis 20
3.3.3 Mycolic acid analysis 20
3.3.4 Menaquinone analysis 21
3.3.5 Cellular fatty acids analysis 21
3.3.6 Polar lipids analysis 21
3.4 Genotypic Characteristics 22
3.4.1 DNA Extraction 22
3.4.2 16S rRNA gene analysis: 23
3.4.3 Whole genome sequence analysis 23
3.4.4 Phylogenetic trees and bioinformatics: 24
3.5 Biological activity screening 25
3.5.1 Fermentation and Extraction 25
3.5.2 Antibacterial activity screening 25
3.5.3 Antifungal activities 25
3.5.4 Anticancer activity screening 26
3.5.4.1 Wound-healing assay 27
3.5.4.1 Cell invasion assay 27
3.5.5 Antioxidant activity assays 28
3.5.6 Acetylcholinesterase inhibitory activity 28
3.5.7 Secondary metabolite profiling of the crude extract 29
CHAPTER IV 30
RESULT AND DISCUSSION 30
4.1 Taxonomic studies of novel *Saccharothrix* species: 30
4.1.2 Chemotaxonomic characteristics: 33
4.1.3 16S rRNA gene sequence analysis 34
4.1.4 Genome of strain SC076^T 35

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.2 Biological activity of *Saccharothrix oblica* sp. nov.: 40

4.2.1 Antimicrobial Activity screening: 40

4.2.2 Anticancer activity: 40

4.2.3 Antioxidant activity assay: 42

4.3 Secondary Metabolite or chemical profiling of the crude extract: 43

CHAPTER V 45

CONCLUSION 45

REFERENCES 46

VITA 51

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLES

Page

Table 1 Cultural characteristics of strain SC076^T and related Saccharothrix type strain: 31

Table 2 Differential characteristics between strain SC076^T and related Saccharothrix type strains: All data were examined in this study except for * which was obtained from Otoguro et al -, Not present. nd, no data. 32

Table 3 Cellular fatty acid composition of strain SC076^T and closely related Saccharothrix species: 33

Table 4 BLAST result of strain SC076^T based on EzbioCloud database. 34

Table 5 Specialty Genes Found in the genome of strain SC076^T 36

Table 6 Antimicrobial Resistance Genes found in the genome of strain SCO76^T : 37

Table 7 Identified secondary metabolite regions using strictness “relaxed” 38

Table 8 ANIb and ANIm values (%) and the digital DNA-DNA hybridization (dDDH) values between the draft genomes of strain SC076^T and its closest related type strains 40

Table 9 Antimicrobial activity of the crude ethyl acetate extract of strain SC076^T 40

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF FIGURES

Page

Figure 1 Scanning electron microscopy of strain SC076^T (a) aerial mycelia which differentiated to form the spores (b) zigzag morphology of the aerial mycelia...........29

Figure 2 Neighbor-joining tree of the 16S rRNA gene sequences of strain SC076^T and type strains of the genus Saccharothrix. Numbers at branch nodes indicate the bootstrap values obtained from 1000 replication. Only the values over 50 are shown. Bar, 0.005 substitutions per nucleotide position. 33

Figure 3 Phylogenetic tree based on maximum-parsimony of the 16S rRNA gene sequences of strain SC076^T and type strains of the genus Saccharothrix. Umezawaea tangerine MK27-97F2T was used as the out group. Numbers at branch nodes indicate the bootstrap values obtained from 1000 replication. Only the values over 50 are shown. 34

Figure 4 An overview of the subsystems for this genome...35

Figure 5 The phylogenomic tree of strain SC076^T and related type strains calculated by TYGS server. Streptomyces albus NRRL B-1811^T was used as the outgroup. The numbers at the branch node indicate GBDP pseudo-bootstrap support values > 60% obtained from 100 replications. 38

Figure 6 (a) and (b) MTT assay represents percentage of cell viability under the different concentration where IC_{50} value 126.4568 μg/mL. 40

Figure 7 Cell migration effect on non-small lung cancer H460 cells after 24 h incubation..40

Figure 8 Blue fluorescent of Hoechst 33342 and red fluorescence of propidium iodine (a) in the several doses represented as cellular apoptosis (b) or necrosis (c) on H460 cell line. 41

Figure 9 Chemical profiling: TLC chromatogram will be visualized by using various spraying reagent including Dragendorff's, ninhydrin, anisaldehyde and sulfuric acid. 42

Figure 10 HPLC chromatogram of ethyl acetate crude extract of Saccharothrix oblica species. 43

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER I

INTRODUCTION

Actinobacteria is the phylum of Gram stain-positive bacteria with high guanine and cytosine content. The best-known genus of actinobacteria is *Streptomyces* which produces various commercially bioactive metabolites (1). Recently, rare actinobacteria, the genera other than *Streptomyces* (or so-called non-*Streptomyces* actinobacteria), have attracted attention and have been considered as a promising source of novel bioactive compounds (2). More than 2,500 bioactive compounds have been reported from the rare actinobacteria (3).

Saccharothrix, belonging to the family *Pseudonocardiaceae*, is a rare actinobacterium first described by Lebeda et al. (4). *Saccharothrix* spp. are distributed in the soil as well as marine habitats. To date, the genus *Saccharothrix* has 21 species with validated published names. Several novel bioactive compounds have been isolated from *Saccharothrix* species. Cyanogrisides I-J, a new cyanogriside antibiotic, were isolated from *Saccharothrix Xinjiangensis* (5). Iso-hexanoyl-pyrrothine, a dithiolopyrrolone antibiotic, was isolated from *Saccharothrix algeriensis* NRRL B-24137 (6). This compound showed potent antibacterial and antifungal activities. In addition, the marine mollusk-derived *Saccharothrix espanaensis* An 113 could produce two new angucycline, saccharothrixmicines A-B with antifungal activity (7).

According to the taxonomy and hypothetical history of analysis from the last decades by the evolutionary relationship between the bacteria and fungi *Actinobacteria* produces secondary metabolites and several enzymes where *Saccharothrix* species produce novel class of antibiotics that are active in both in-vivo and in-vitro against bacteria and yeast that have highly molecular mass of toxic chemical compounds that are used in both antimicrobial as well as antitumor antibiotics. Most of the drug compounds that were invented in the last few years many antibiotics are resistant due to the replication or change in response to use of such medicines.

Antibiotic resistance is rising enormously in all parts of the world. Consequently, new resistance mechanisms have come into view globally which continuously emerged and spread everywhere that might be threatening to treat common diseases. Some listed infections such as tuberculosis, pneumonia, gonorrhea, blood poisoning and foodborne diseases, etc. are becoming out of control, even impossible to treat. Usual antibiotics are becoming less effective day by day. Therefore, an attempt has been made to isolate the novel rare *Actinobacteria* like *Saccharothrix* species in order to find novel species. As soil is the primary source of most productive *Actinobacteria* this possesses me to the research has been turned into the more exotic environment so that the isolated sample were collected from Sichang island.

It is necessary to discover biologically active compound in order to get antibiotics and other beneficiary chemical metabolites for saving human life from

organisms or treating the diseases. So, emphasizing focus on the pathogenic organism's role and safety, a complete taxonomic study is used to isolate and identify the novel species and biological activity of the active producers where the promising active compound is contributed to the drug development.

Hypothesis:

It is hypothesized that the attempt of finding novel actinobacterial species made an effective concern to get naturally diverse bioactive metabolites or compounds that proved the optimistic advantage to the drug development. Strategy for supporting lives by finding novel species was contributed to the benefits of ecosystems on which people depend. Very often, due to climate change there were some keystone organisms, tremendous plants or species that was playing momentous preamble coadjutor mounting as maintaining ecological balance by increasing soil fertility, preventing incompatible things from the environment. Moreover, it is difficult to isolate such organisms without using the appropriate method. Therefore, the possibility of finding such species in the environment is the perceptible issue and preserving new *Actinobacterial* species like *Saccharothrix* genus and their bioactive novel compounds have a direct positive impact on human life. Consequently, this research has been successful because of applying rational method and soil sample source is very authentic in nature, Sichang Island in Thailand. In this study, the polyphasic taxonomy of the novel *Saccharothrix* strain SC076^{T}, were investigated by phylogenetic analysis, chemotaxonomy, phenotypic and genome properties, and biological activities.

Objectives:

1. To identify and complete taxonomic study of a new *Saccharothrix* sp. strain SC-076^{T} collected from the terrestrial soil sample in Sichang island, Thailand.
2. To screen the biological activities of the new *Saccharothrix* strain SC076^{T}

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CHAPTER II

LITERATURE REVIEW

The phylum *Actinobacteria* is one of the largest taxonomic groups of aerobic, spore-forming filamentous Gram-positive bacteria. These bacteria contain high guanosine and cytosine in chromosomal DNA ratio range from 50-70 % (8). According to the origin of such type's bacteria having inference of both bacteria and fungus recognized as prokaryotic microorganisms but their taxonomic characters determined it into bacterial Domain (9). These bacteria are ubiquitously distributed worldwide, especially in the natural environment such as terrestrial locations, including all layers of soil, desert soil, plants, freshwater systems, rhizosphere and marine environments (10). In analytical bioprospecting research for the investigation of bioactive secondary metabolites, *Actinobacteria* is the potential producer of bioactive compounds, specifically *Streptomyces, Micromonospora, Actinoplanes, Amycolatopsis, and Saccharopolyspora* (11). They are the producer of the heterotrophic chemical substance having complex polymers and diverse energy. *Actinobacteria* is classified into two groups: *Streptomyces* genus and non-*Streptomyces* genus, which is entitled as rare *Actinobacteria.*

2.1 Rare Actinobacteria

Certain *Actinobacteria* that are difficult to isolate from the natural environment, this nominal actinobacterial isolates are represented as rare *Actinobacteria*. These *Actinobacteria* are comparatively huge in natural habitats and according to the acumen observation of actinobacterial molecular insights such bacteria can be rescued by researchers on well isolated techniques. Soil, plants and insects are the foremost sources of rare actinomycetes. Beyond this, Marine environments are also a promising source of rare *Actinobacteria*. The investigation by biological diversity of culture-independent studies has demonstrated that sea site area till now contains a high diversity of rare actinomycetes that is emerging source of potential drug development (12). Recently, researchers found structurally diverse metabolites from the origin of microorganisms. Following viewpoint of Bérdy (3) and Subramani (13), bioactive metabolites more than 22,000 metabolites where 70% of metabolites come from actinomycetes and more than 10,000 is known and from Bérdy analysis 26% is rare *Actinobacteria* like *Saccharothrix* among thence 11% shows rare actions. Consequently, scientist research is histrionically glittering within a look for novel bioactive compounds which might remain active pharmacological activities compared to another genus of *Actinobacteria* that indicated rare *Actinobacteria.*

Whatever in a review of Amin et al. (14) and Ding et al. (15) such genera discovered as the rare *Actinobacteria* such as *Actinoplanes, Actinomadura, Amycolatopsis, Actinosynnema, Actinokineospora, Actinoalloteichus, Acrocarpospora, Cryptosporangium, Catenuloplanes, Dietzia, Dactylosporangium, Kineosporia, Kibdelosporangium, Kribbella, Kutzneria, Lechevalieria, Microbiospora,*

Microtetraspora, Micromonospora, Nocardia, Nonomuraea, Planobispora Planomonospora, Pseudonocardia, Streptosporangium, Saccharothrix, Saccharomonospora, Spirilliplanes, Saccharopolyspora, Thermobifida, Thermomonospora and *Virgosporangium* as well as some uncommon *Streptomyces* species.

However, the isolation of novel bacterial metabolites in uncommon ecosystems such as mangroves is becoming popular recently for the natural products as well (16). Accordingly, novel compounds coming from the novel rare *Actinobacteria* thought to be producer of potential new drugs in drug discovery areas in pharmaceutical and medical sectors such as enzymes, enzyme inhibitors, antibiotics, antimicrobials, antifungals, anti-Alzheimer, antitumor and anticancer etcetera. In this review, such typical so-called bacterial species emphasizes the latest experiment on discovering the natural bioactive compounds from the novel rare Actinobacteria, which has been taken steps insight into the biological research domain.

2.2 The genus *Saccharothrix*

Saccharothrix is classified in the family *Pseudomonadaceae* (17). The members of this genus are ecologically important for the production of bioactive compounds. *Saccharothrix* was characterized by a fragmented part of vegetative and aerial mycelia as oval shaped. Several *Saccharothrix* species have been described in the past few years. *Saccharothrix stipae* (18) and *Saccharothrix deserti* (19) isolated from the soil collected from northwest China where the study revealed that the strain is aerobic, Gram-positive mesophilic actinobacteria that is identified by 16S rRNA and the *S. stipae* strain contains major *meso*-diaminopimelic acid and major amino acids are alanine, glycine, glutamic acid, and mannose, rhamnose and galactose were specified by analysis of whole-cell sugars. *S. deserti* strain cell wall hydrolysates contained *meso*-diaminopimelic acid, galactose, and mannose.

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The cell chemistry of *S. australiensis* where cell wall contains *meso*-diaminopimelic acid, galactose and rhamnose as whole cell sugar, 73% cytosine plus guanosine ratio in DNA and absence of mycolic acid and phospholipids in their cell wall. Study demonstrated from *Saccharothrix* SA198 contained *meso*-diaminopimelic acid without glycine, whole-cell hydrolysates contained galactose, rhamnose, ribose and traces of mannose, which have type IIIE cell-wall and type PII phospholipids were detected as phosphatidyl-ethanolamine and phosphatidyl-hydroxy-ethanolamine (4), (20). Furthermore, by the combination of morphological, cultural characteristics and biochemical properties where the strain produced melanoid pigments on ISP7 medium and can degraded gelatin, casein esculin, Tween 80, hypoxanthine, tyrosine and have capability to utilize alanine, proline as nitrogen sources and acetate, cellobiose, fructose, glucose, propionate, pyruvate ribose, sucrose, succinate was utilized as carbon sources. However, result from phylogenetic analysis, DNA-DNA relatedness value represented this strain was closely related to *Saccharothrix australiensis*. Accordingly, chemical profiling and new active compound isolated by thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), column chromatography, mass, infrared

(IR), nuclear magnetic resonance (NMR) spectra. Consequently, based on these genotypic, phenotypic, and chemotaxonomic data were used to identify a novel strain of the genus *Saccharothrix.*

2.3 Isolation strategies of rare actinobacteria

Isolation of rare *Actinobacteria* is not a common matter but needs an established procedure or technique to isolate, preserve and cultivate because these typical organisms are masquerade by other organisms like bacteria, fungi and common actinomycetes. The isolation technique of rare *Actinobacteria* could increases the chances of discovering novel potent compounds. Therefore, it will be proposed to take better knowledge in order to facilitate the isolation of these strains by using kinky approach to get it in conformity with their diversity as well as distribution in the natural environment.

However, according to the review of Amin et al. (14) and Monisha et al. (21) established develop methodologies such as physical and chemical parameters, pretreatments for example sucrose gradient centrifugation, sodium dodecyl sulfate treatment, dry heat and phenol treatments, selective media containing macromolecules such as casein, chitin, and humic acid are preferred to remove the non-filamentous bacterial contamination from the samples and suppress fungal growth thus helps the growth of slow growing rare *Actinobacteria*. By this isolation medium and techniques carried out the most selective rare *Actinobacteria* from the sample.

2.4 Biological activities and novel bioactive compounds of *Saccharothrix*:

Saccharothrix is the biologically active *Actinobacteria* that are prolific producers of novel compounds. From the comprehensive review of the past two decades greater portion of bioactive compounds comes from the bacteria belonging to *Actinomycetales*. Following the study of Abdelhadi et al. (5) has demonstrated that they found a novel bipyridine antimicrobial compound from *Saccharothrix* species where MIC of antibiotics determined against pathogenic organisms and is the first report that produced cyanogrisides and caerulomycins compounds from the member of *Saccharothrix* genus.

The novel antitumor agents fluroindolocarbazoles and rebeccamycin antibiotics have been discovered from *Saccharothrix aerocolonigenes* ATCC 39243 (22). In the study of Babadi et al. (23) structurally diverse metabolites from *Saccharothrix Xinjiangensis*, researchers have isolated three new compounds caerulomycin M, saccharopyrone, saccharonic acid and a known compound, caerulomycin A where the structures were elucidated by high resolution electrospray ionization mass spectroscopy and nuclear magnetic resonance spectroscopy (NMR) data analysis. Furthermore, saccharopyrone compound shows cytotoxic activity against human cervix carcinoma cell with IC_{50} value of 5.4 µM. *Saccharothrix* PAL54 an actinomycete strain which produced chloramphenicol and the strain isolated from Saharan soil (24). In deep Study of biocontrol activity demonstrated that the strain showed strong activity against Gram-positive bacteria and Gram-negative bacteria and this antibiotics compound was

elucidated by nuclear magnetic resonance (NMR), mass and ultra-violate (UV) visible spectroscopy.

The taxonomic position of most *Saccharothrix* species isolated from soil sample, for example, diversely explored bioactive metabolite produced by *Saccharothrix flava* VSM-3 (25). This strain showed significant broad-spectrum activity against plant pathogens in addition where identified several compounds such as 7-deazaadenosine, 5-hydroxy-9-methylstreptimidone, amiclenomycin, epopromycin A, dihydroabikoviromycin, etc. by liquid chromatography and mass spectroscopy using ethyl acetate extract. It has been confirmed that novel compound heptadecaglycoside antibiotics saccharomycins A, B actively work against Gram-positive and Gram-negative bacteria produced by *Saccharothrix espanaensis* where proposed antibiotics saved mice from lethal challenges by *Staphylococci* (26).

In the taxonomic study of Djinni et al. (12) they isolated some novel species like *Saccharothrix australiensis* (4), *Saccharothrix isguenensis* (27), *Saccharothrix ghardaiensis* (28), *Saccharothrix tamanrassetensis* (29), *Saccharothrix saharensis* (Boubetra et al., 2013), *Saccharothrix hoggarensis* (30) and these typical strains produced new antibiotic compound that have antimicrobial activity against fungi, Gram-positive and Gram-negative bacteria, even antitumor activity as well where the strains were isolated from the Saharan soil. An antioxidant and enzyme inhibitory metabolites have been discovered from a novel *Saccharothrix ecbatanensis* strain (31).

Beside this there are some enlisted typical *Saccharothrix* strains which has been identified as novel species such as *Saccharothrix albidocapillata* (32), *Saccharothrix carnea* (33), *Saccharothrix coeruleoviolacea* (34), *Saccharothrix cryophilis* (35), *Saccharothrix ecbatanensis* (36), *Saccharothrix ghardaiensis* (28), *Saccharothrix lopnurensis* (37), *Saccharothrix mutabilis* (38), *Saccharothrix syringae* (39), *Saccharothrix tangerinus* (40), *Saccharothrix texasensis* (41), *Saccharothrix variisporea* (42), *Saccharothrix violacea* (32), *Saccharothrix waywayandensis* (41) etcetra. Those typical strains future study will need to do for the determination of secondary metabolites and their activity.

The overall study concludes that *Saccharothrix species* play a significant role in drug development and production of the percentage of novel bioactive secondary metabolites discovered from these rare *Actinobacteria* like *Saccharothrix.*

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CHAPTER III
RESEARCH METHODOLOGY

3.1 Microorganism and preservation

The *Saccharothrix* strain SC076^T, isolated from soil sample collected from Sichang Island, Chonburi Province, Thailand, was obtained from the culture collection of the faculty of Pharmaceutical Sciences, Chulalongkorn University. The strain was preserved by the lyophilization method for long-term preservation.

3.2 Phenotypic characteristics

Microscopic morphology, cultural characteristics and biochemical tests were used for the phenotypic studies. The inoculum was obtained from the culture cultivated in yeast-malt extract broth under shaking conditions at 180 rpm, 28±2°C, 4-7 days (43). The cells were washed three times with 0.85% (v/v) NaCl solution to remove the media and use it for phenotypic study. For the phenotypical test, two ways was followed to identify bacterial isolates. From where one was identified by following morphological and cultural characteristics and another one was checked by several biochemical tests like nitrate reduction, starch hydrolysis, skim milk peptonisation, liquify of gelatine and carbon source utilization.

3.2.1 Morphological and cultural characteristics

Morphological characteristics were observed after culturing the strain on ISP2 agar for 14-21 days at 28±2°C by using scanning electron microscopy (JEOL, JSM-6610V, Tokyo, Japan). Cultural characteristics was ascertained from the incubation period 14 days at 28±2°C on various International *Streptomyces* Project media (43), including yeast extract- malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salt-starch agar (ISP4), glycerol-asparagine agar (ISP5), peptone-yeast extract iron agar (ISP6) and tyrosine agar (ISP7). The names and designation of the colony colours and soluble pigments was assessed by using the colour harmony manual (44). Moreover, the culture was characterized by checking the morphology of those strain such as aerial mycelium, substrate mycelium, soluble pigment, and growth of bacteria.

3.2.2 Nitrate reduction

The strain was newly prepared by using peptone KNO_3 broth and incubated for 14 days at room temperature. Then, the result was checked by the addition of alpha naphthylamine and sulfanillic acid. If the colour change to red: it means positive, and if no change in colour it means negative. In the case of a negative result, the zinc powder was added. If the colour change to red, it means negative and if no change in colour it means positive result.

3.2.3 Starch Hydrolysis

Inoculate the bacteria to the newly prepared ISP4 medium by suspending 22 g ISP4 medium with 1,000 ml distilled water; pH 7.0 and incubate it for 14 days. After

that, the iodine was added and finally check the result. If the zone is present inside the colony, it means a positive result; otherwise, a negative result.

3.2.4 Skim milk peptonisation

Inoculate the bacteria to the newly prepared 10% (v/v) skim milk agar medium and incubated it for 14 days. If this strain shows the digestion zone around the colony, it means a positive result otherwise negative result.

3.2.5 Liquify gelatine

Inoculate the bacteria to the newly prepared medium Bouillon-gelatine medium or broth incubated it for 14 days. After that, the broth was kept in the refrigerator at 4°C for 5 to 10 min with control. Checked the result by comparing the broth with control if the broth gelatine was not coagulated then it means a positive otherwise negative result.

3.2.6 Carbone Utilization

Carbohydrate utilization as the main carbon source was determined by inoculating the bacteria to the newly prepared ISP9 basal medium supplemented with 1% (v/v) carbon source such as glucose, sucrose, galactose, mannitol, cellulose, xylose, glycerol, raffinose, mannose, *myo*-inositol etc. and incubate it for 14 days. After that, the result was checked by comparing to the negative control and normal growth in glucose medium. If the bacterial isolate shows the growth rate similar to the glucose its positive (+). If the growth of the bacterial isolate shows growth rate higher than the glucose it indicates positive (++). When the growth of the bacterial isolate shows growth rate lower or similar to the negative control, it will indicate negative (-) result.

3.3 Chemotaxonomic Characteristics

Biological chemotaxonomic characteristics was determined by using biomass obtained from yeast extract-malt extract broth cultures grown under shaking condition at 180 rpm, 30 °C for 7 days and the cell was separated by using centrifuge and finally freeze dried the cell.

3.3.1 Diaminopimelic acid (DAP) isomer analysis

Diaminopimelic acid was analyzed by using thin layer chromatography (TLC) (45). Approximately 10 mg of dried cells placed into a small screw capped tube with 1 ml of 6 N hydrochloric acid and heated for 18 hours at 100°C. After cooling, the whole cell hydrolysates were filtered through filtered paper (Whatman no.1) and evaporate to dryness. The dried filtrates dissolved with 0.3 ml of distilled water and applied on the base line of cellulose TLC sheet (20 cm x 20 cm) with 1 µl 0.01 M DAP standard. Ascending the TLC was developed twice with the mobile phase, methanol-water- 6 N HCl-pyridine (80:26:4:10, v/v). Around 2-3 hour need to application and develop the process on TLC. After developing TLC, air dry the chromatogram. Then the chromatograms spots were easily visualized by spraying with 0.2% ninhydrin solution and heat at 100°C for 3-5 minutes. DAP isomers appeared as gray-green fading to yellow spots on TLC sheet as comparison with DAP

standard solution where L-isomer was moving forward to the meso isomer that contained both *meso*-DAP and LL-DAP. Beside this hydrolysates spots of amino acid have to be appeared as purple or red color and migrated forward the DAP speck color.

3.3.2 Whole cell sugar analysis

The analysis of carbohydrates was identified by TLC method (45) following Lechevalier article demonstrated approximately 50 mg of freeze-dried cell was hydrolyzed with 1 ml of 1 N H_2SO_4 at 100°C for 2 h in a screw capped tube. After cooling the hydrolysate adjusted pH to 5.2-5.5 by dropwise adding saturated barium hydroxide [$Ba(OH)_2$] solution and pH paper. The precipitate was removed from the tube by centrifugation at 4,500 rpm for 10 minutes. The supernatant was evaporated in a 50 ml beaker in a stream of air and residue will dissolve again in 0.3 ml distilled water. If there is any insoluble martial present then need to centrifuge again for the removal of those substance. Afterwards 1 µl of hydrolysates applied to the baseline of TLC sheet and 1 µl of each of standard solutions standard-1 contained galactose, arabinose and xylose, standard-2 contained rhamnose, mannose, glucose and ribose where each sugar at 1% concentrations. Thin layer chromatogram mounting was performed with the solvent system n-butanol-distilled water-pyridine-toluene (10:6:6:1, v/v). Sugar speck was detected by spraying over the dry TLC with acid aniline phthalate (phthalic acid 3.25 g, water saturated butanol 100 ml plus aniline 2 ml) and finally heat under 100°C for 4 minutes. The carbohydrates were identified by the migration of the following order slow moving component to fast moving component sequentially galactose, glucose, arabinose, mannose, xylose, ribose, and rhamnose comparing with standard. Thereby existence of 3-o-methyl-p-galactose or madurose showed identical distance as xylose and was recognized by its yellow color. On the other hand, hexose sugar spots were showed yellow as well as pentose sugar was showed maroon color after heating.

3.3.3 Mycolic acid analysis

The mycolic acid was analyzed by following the method of Tomiyasu et al. (46). Approximately 50-200 mg of biomass placed into a screw-capped tube and hydrolyzed with 2 ml of 10% KOH-methanol (2:1, v/v) at 100°C for hours. Then, 0.6 ml 6 N HCl and 2 ml n-hexane added into the residue and shaking well to extract the lipids. After shaking, the extract centrifuged at 3000 rpm for 10 minutes and the upper layer transferred into a new tube. The lower layer was extracted again with 2 ml of n-hexane and collected the upper layer. The upper layer was dried with nitrogen gas, 2 ml of benzene-methanol-H_2SO_4 (10:20:1, v/v) added to the residue and heated for 2 hours at 100°C. After cooling, the residue was added with 2 ml of distilled water and hexane and vortexed for 5 minutes, and then centrifuged at 3000 rpm for 10 minutes. Then, the upper layer was transferred to a new tube and the lower layer extracted again with 2 ml of hexane. The upper layer was evaporated till to dryness, then dissolved with a small amount of n-hexane and applied on the silica TLC plate (10cm x 10cm). The lipids extract of *Nocardia nova* JCM 4044^T was used as positive control of mycolic acids. The TLC plate was developed with the solvent system of hexane-

diethyl ether (4:1, v/v) and visualized the chromatogram by iodine vapor. Based on the system, mycolic acids will appear approximately R_f 0.47.

3.3.4 Menaquinone analysis

The menaquinones was extracted by following the method of Collins et al. (47). The 300 mg of freeze-dried cells was extracted with 20 ml of chloroform-methanol (2:1, v/v) and stirred continuously overnight. Then the residue was removed by filtration and the extract evaporated to dryness using rotatory evaporator at low temperature (>37°C). The extract was applied on a preparative silica gel TLC plate (5 cm x 20 cm). Ascending the TLC plate was performed with benzene. The chromatogram was visualized under UV light at 254 nm, scrap off and extracted with acetone (HPLC grade). The acetone extract was filtered through 0.5 µm nylon membrane filter and analyzed by HPLC (Agilent 1100, Santa Clara, Ca, USA) and mass spectrometer (JEOL JMS-T, 100 LP) have to equipped with a Pegasil ODS column (Aenshu, Tokyo, Japan), according to Tamaoka et al.(48).

3.3.5 Cellular fatty acids analysis

The cellular fatty acid methyl esters were extracted according to the standard method (49) with slight modification. Four steps to prepare the cellular fatty acid methyl ester was described as following; a) Saponification: 1.0 ml of reagent-1(45 g of NaOH dissolved in 150 ml each of methanol and distilled water) added to screw-capped tube bearing 40 mg of freeze-dried cells. Then vortexed well and heated at 100°C for 5 minutes. After heating the tube was vigorously vortexed for 5-10 seconds, heat again at 100°C for 25 minutes and immediately cool. b) Methylation: The cooled tube added with 2 ml of reagent-2 (325 ml of 6 N HCl and 275 ml of methanol) and vortexed briefly. The tube was heated for 10 minutes at 80±1°C (This step was critical in time and temperature).c) Extraction: 1.25 ml of reagent-3(hexane and methyl tret-butyl ether, 1:1, v/v) added to the cooled tube and vortex for 5 minutes and the tube centrifuged for 4,500 rpm for 10 minutes. Then the upper layer was transferred to a new tube and the lower layer was discarded. d) Base wash: 3.0 ml of reagent-4 (10.8 g of NaOH dissolved in 900 ml distilled water) added to the upper layer, vortexed for 5 minutes. The tube was centrifuged for 4500 rpm for 10 minutes, 2/3 of the upper phase was collected to a vial for gas chromatography analysis. The cellular fatty acid methyl esters were analyzed by gas chromatography following the instruction of the microbial identification system (MIDI) Sherlock system version 6.0.

3.3.6 Polar lipids analysis

The polar lipids was extracted by the method of Minnikin et al. (50). Approximately 150 mg of freeze-dried cells were taken into a test tube with a screw cap and added 3 ml of methanol 0.3% (w/v) NaCl (100:10) as well as 3 ml of petroleum ether. After that, mixed well for 10 min of centrifugation and removed the upper layer. Added 1 ml of petroleum ether to the lower layer and mixed well again. Then removed the upper layer. The lower layer was heated at 100°C for 5 min and it was immediately cooled at 37°C for 5 min or tap water. The residue was added with 2.3 ml of chloroform-methanol-water (90:100:30, v/v) and mixed well for 10-15 min

in vortex, centrifuge (3,000 rpm, 2-3 min) and transferred the supernatant in another tube-1. The lower layer was extracted twice time with 2.3 ml of chloroform-methanol-water (50:100:40, v/v) and mixed well for 10 min, centrifuged (3,000 rpm, 2-3 min) and was transferred the supernatant to the tube-1. Finally, added 1.3 ml each of chloroform and water into the supernatant fraction, mixed well, centrifuged and removed the upper layer. Afterwards dried up the upper layer with N_2 gas (<37°C).

The polar lipids were analyzed by two-dimensional thin layer chromatography. The extract was dissolved with 200 µl of chloroform-methanol (2:1) and applied 10 µl of the solution to two-dimensional silica gel TLC (10cm x 10cm) described by Minnikin et al. (51).The first dimension of the TLC developing was performed in the solvent system of chloroform-methanol-water (65:25:4, v/v). The 2nd dimension was developed in solvent system of chloroform-acetic acid-methanol-water (40:7.5:6:2, v/v). Step-2, detection: It was visualized and compared the chromatogram patterns each of TLC sheet was sprayed with five specific following reagents. (a) ninhydrin reagent (0.4% ninhydrin in water saturated n-butanol): Heated at 110°C for 10 min after spraying for phosphatidyl-ethanolamine (PE) and its derivatives (*lyso*-PE, OH-PE, methyl-PE). (b) Dittmer and Lester reagent: solution A- dissolve 4.011 g of MoO_3 in 100 ml of 25 N H_2SO_4 with heating. Solution B: added 0.178 g of molybdenum powder into 50 ml of solution A, and boiled it for 15 min. After cooling, the precipitation was removed by decantation. Then, solution A (50 ml), solution B (50 ml) plus water 100 ml was mixed carefully before spraying. This reagent was used for the detection of phospholipids. (c) Phosphomolybdic acid in 5% EtOH was applied for the detection of lipids, steroids, lactones, keto acids, hydroxy acids, unsaturated fatty acids, and phenolic compounds. (d) Dragendorff's reagent: solution A- dissolved 1.7 g of basic bismuth nitrate in 20 ml of acetic acid and 80 ml of water. Solution B- dissolved 40 g of KI in 100 ml of water. After that, the solution A (10 ml), solution B (10 ml) and acetic acid (10 ml) was mixed before spraying. Thus, it was used for the detection of choline containing phospholipids (phosphatidylcholine). (e)Anisaldehyde reagent: (composition: ethanol-90 ml, H_2SO_4-5.0 ml, p-Anisaldehyde-5.0 ml, acetic acid-1.0 ml) Reagent was heated at 110°C for 10 min after spraying for glycolipids was showed green, yellow spots and other lipids was showed blue spots.

3.4 Genotypic Characteristics

3.4.1 DNA Extraction

Strain SC076^T was cultured in YD broth or ISP2 broth under the shaking conditions at 180 rpm in room temperature for 3 days. The selective 1,000 µl sample was taken in eppendorf tube and centrifuged at 12,000 to 14,000 rpm for two minutes. The sample was washed by 500 µl sterilize water 2-3 times. Then added 300 µl TE buffer and aluminium oxide (Al_2O_3) (1:1) and mixed by micromixer for 90 sec for cell lysis. After that, 300 µl of the phenol-chloroform (1:1) was added and mixed for 30 sec. Then it was centrifuged 14,000 rpm for 15 minutes. After centrifugation there were four phases found that was aqueous phase: DNA, interphase: proteins, organic phase: RNA, lipid, chloroform and aluminum oxide. Keep supernatant DNA in a new

eppendorf tube by pipette 100 µl each time. Added 3 M sodium acetate (1/10 volume) to interpret hydrogen bonding between water and phosphate on DNA and cold ethanol 95% (2 volume). Afterwards, centrifuged 14,000 rpm for 5 minutes and discarded the supernatant from the tube. Let, washed the DNA tube by ethanol 2-3 times and kept it 1-2 hr. for air drying. Finally, added 50 -100 µl sterilize water to dissolve the DNA and kept it in the refrigerator at 4°C.

3.4.2 16S rRNA gene analysis:

Amplification of 16S rRNA by PCR: A standard polymerase chain reaction consists of four steps. Firstly, added required reagents or master mix (50 µl) :27F primer (10 µM): 2 µl, 1492 R primer (10 µM): 2 µl, $MgCl_2$ 25 mM: 10 µl, *Taq* buffer 10x: 5 µl, DNTP 10 mM: 1 µl, *Taq* polymerase 5 unit/ µl: 2 µl, ultrapure water: 30.75 µl, sample: 5 µl. Secondly, added template to PCR tubes and mixed by vortex. Thirdly, amplification of the DNA per thermo cycler that were initial denaturation 94°C for 2 min, denaturation 94°C for 1 min, annealing 55°C for 1 min and extension 72°C & final extension 72°C for 5 min and with universal primers forward 27F= (5'-A GAGTTTGATCCTGGCTCAG-3') and reverse primer 1492*R*= (5'-GGTTACCTTGTTACGAC TT-3'). Finally, evaluate amplified DNA or PCR product was detected by 1% (w/v) agarose gel electrophoresis followed by ethidium bromide staining.

Purification of DNA: There were four steps followed for the purification of DNA**.** Firstly, preparation of sample: 100 µl reaction product was transferred to a 1.5 ml microcentrifuge tube. If the sample lower than 50 µl, it was adjusted the volume by distilled water. Then, 5 volume of Gel PCR buffer was added to 1 volume of the sample and mixed by vortexes. If the mixer colour turned to purple 3 M sodium acetate (pH 5.0) was added & mixed thoroughly. Secondly, bind DNA by the placement of a DFH column in 2ml collection tube. The mixer was transferred to the DFH column and centrifuged at 14-16,000 rpm for 30 sec. Then discarded the flow–through and was placed the DFH column back to 2 ml collection tube**.** Thirdly, Washed the DNA by the addition of 600 µl of wash buffer into centre of the DFH column. Let, it was kept for 1 minute at room temperature and centrifuge at 14,000 rpm for 30 sec. Again, discarded the flow–through and placed the DFH column back in the 2 ml collection tube. After that, centrifuged it at 14,000 rpm for 3 minutes to dry the column. DNA was eluted by transferring the DFH column into a new (1.5 ml) microcentrifuge tube. Then, 20-50 µl pre-heated (60-70°C) eluting buffer or TE was added to the centre of the column matrix. To ensure adsorption column was stand at least 2 minutes. Eventually, column tube was centrifuged for 2 min at 14,000 rpm to elute the purified DNA.

3.4.3 Whole genome sequence analysis

The DNA for genome sequencing was extracted by using PureLink™ Genomic DNA extraction Kit.

DNA extraction and purification: At first, the cell was washed by 500 µl sterilize water by three times and centrifuged it 14,000 rpm/min for 3 minutes. Then added

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180 µl lysozyme buffer (0.015 µg lysozyme + 400 µl EDTA + 4 µl triton x 100). Tube was briefly vortex by vortex machine and was incubated at 37°C into water bath. After that added 20 µl RNase A, 20 µl protease K and was incubated at the room temperature for 2 minutes. Afterwards, 200 µl genomic lysis reagent or binding buffer was added and mix well by brief vortex and was incubated at 55°C for 30 min. Finally, added 200 µl 96-100% ethanol to the lysate and mixed well by 5 seconds brief vortex and proceed to binding DNA. Binding DNA: the spin column was removed and the column centrifuged at 14,000 rpm for 1 minutes. Then, the collection tube was discarded and placed the spin column into a new collection tube. Afterwards, DNA was washed by 500 µl prepared genomic wash buffer-1 with ethanol and was centrifuge for one min**.** Again, the collection tube was discarded and taken the DNA cell in a new collection tube. After that, washed DNA by 500 µl prepared genomic wash buffer-2 with ethanol and centrifuged for 3 minutes at maximum speed. Finally, the collection tube was discarded and proceed to eluting DNA. Eluting DNA: The spin column was placed in a sterilize (1.5 ml) microcentrifuge tube. Then added 100 µl genomic eluting buffer and was incubated at room temperature for 3 min. Then it was centrifuged 14,000 rpm at room temperature for 1 min. Collection of DNA from the column: DNA was transferred by micropipette to the eppendorf tube. DNA quality like purity and concentrations was checked by using Nanodrop ND-2,000 Ultraviolet-visible (UV-Vis) spectrophotometer. Pure DNA normal yield in absorbance 260/280 ratio of 1.8-2.0 and 260/230 in range of 1.8-2.2 as well as gel electrophoresis. After that, proceed the DNA sample for whole genome data have to sequenced by omics science and bioinformatics centre by following sequencing method: 100 ng of gDNA was subjected to DNA sequencing library preparation using QIAGEN FX kit (Qiagen, USA). Briefly, gDNA was fragment by using enzymatic reaction and was cleaned with magnetic beads. An adaptor index was ligated to the fragmented DNA. Quality and quantity of the indexed libraries was measured by using Agilent 2,100 Bioanalyzer and Denovix fluorometer and pool in equimolar quantity. Cluster generation and paired-end 2×150 nucleotide read sequencing was performed on Illumina HiSeq X ten sequencer.

3.4.4 Phylogenetic trees and bioinformatics:

The 16S rRNA partial gene sequence was subject to BLAST (Basic Local Alignment Search Tools) search in the EzBioCloud data bases (www.ezbiocloud.net/) (52). Where for the phylogenic tree construction need to collect the FASTA format data and was aligned it in BioEdit software with the selection of an outgroup (53). After that, the phylogenetic tree was constructed with MEGA X. Phylogenic trees were represented the inferred evolutionary relationships where the sequence was separated by shorter evolutionary distance respected to most similar to one another. Similar tree topologies were monitored in the phylogeny was constructed through maximum parsimony (54) and maximum likelihood (55). Then sequences were evaluated in a longer evolutionary distances based on time calibration between nodes value and length of the branches. Then selected this references strain with identical accession number and was searched in LPSN.dsmz.de (https://bacterio.net/) type

strain for checking the whole genome sequence data. By this way the type strain was selected and after that DNA-DNA compared genome sequence was identified whether it was known species or the new strain.

For the whole genome sequence analysis, raw reads quality was checked by using FASTQC software. Adaptors and poor-quality reads were removed by using Fastp, and the filtered reads used as an input for Unicycler, genome assembly program. Annotation of assembled genome performed by using the PATRIC RASTtk-enabled Genome Annotation Service. In addition, the average nucleotide identity (ANI) was calculated and compared using JSpeciesWS, web server tool. Further study conducted for the genome assembly was analyzed by the antiSMASH, server for predicting secondary metabolites and was constructed the genome trees by using type strain genome (https://tygs.dsmz.de/) server.

3.5 Biological activity screening

3.5.1 Fermentation and Extraction

Isolated pure barterial sample (1 or 2 loop) was transferred to the 10 ml seed medium for example YD, ISP2 broth tube and cultured it 160 rpm on shaker for 3 days at 28±2°C. After that cultured medium containing bacteria (2 ml) was transferred to the 150 ml production medium such as ISP2, 54 media in conical flask according to the required volume of expected crude extract and cultured it 160 rpm on shaker for 7-14 days. Then added ethyl acetate (2:1) ratio where 200 ml ethyl acetate with 100 ml extract and was separated the solvent by using separatory funnel. Eventually, extracted the crude metabolites by using rotatory evaporator and final concentration of crude extract was dissolved in methanol and chloroform. Then it kept in a known volume amber vial, vial mouth covered with small pricking foil paper for the evaporation of solvent. Finally, keep the residue crude extract was preserved in desiccator or refrigerator -20°C. At last, concentration 100 mg/ml was determined for the antimicrobial or antifungal activities 20 µl/ 6mm disc by following disc diffusion method.

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3.5.2 Antibacterial activity screening

One loop pure bacterial sample was transferred to the 10 ml seed medium YD broth and kept it 160 rpm on shaker for 3 days. After that, culture medium containing bacteria (1 ml) was transferred to the 10 ml production medium tube both ISP2 and 54 media and cultured it 160 rpm on shaker for 14 days. Added 10 ml 95% ethanol each of the production medium tube and broth supernatant was transferred to the 10 ml conical tube and kept it at -20 °C in the refrigerator. Finally check the antimicrobial activities by following disc diffusion method (56) using several pathogenic organisms where I used vancomycin as the positive control for gram-positive bacteria and gentamicin for some gram-negative bacteria.

3.5.3 Antifungal activities

To the assessment of broad-spectrum antifungal activity seven *Candida* species was selected for *Saccharothrix*. They were *Candida guilliermondii, Candida pseudotropicalis, Candida albicans, Candida tropicalis, Candida krusei, Candida*

glabrata, Candida parapsilosis. Moreover, crude extracts of *Saccharothrix* species also used to detect the antifungal activities against this selected seven phytopathogenic fungi. The antifungal activities were checked by using sterilize cotton bat spread all the *Candida* strains to the SDA medium. Inoculum was prepared by (0.9% NaCl solution) sterilize saline water where one loop of bacterial inoculum was transferred from the activated plate to 3 ml sterilize saline water tube and compared the turbidity by McFarland standard (10^6 cfu/ml of 0.2 to 0.5 McFarland standard) solution. After that, the crude extract sample containing 6 mm disk putted on the spreading *Candida* plate by following disc diffusion method where I used amphotericin B or nystatin as a positive control. Finally, it was checked the result on the next day by checking zone of inhibition. If the strains were showed clear zone on to the plate it means positive otherwise negative result and measured the zone of inhibition (mm) by slide callipers.

3.5.4 Anticancer activity screening

The anti-cancer activity of the crude extract was determined by using MTT assay. There are three steps were followed for the for checking cell viability. Step-1, seed the cells (10×10^4 cells/well) on 96 well plate: (A) Harvest cells: The human non-small cell lung cancer cell line NCI-H460 American Type Culture Collection (ATCC HTB-177) cancer cells was taken from the stock by following thaw methods: centrifuge the freezing stock H460 lung cancer cells and removed the stocked cell suspended medium. Afterward added 1 ml growth factor (10-20%) fetal bovine serum (FBS) with 6 ml complete RPMI -1640 medium. Then the cells were cultured on a small 3D Petri dish for 3 days with 5% CO_2 at 37 °C. After 3 days incubation cell growth with 100% confluency was prepared the cells for seeding on 96 well plate. (B) Separate the pure cell suspension in a plastic conical tube: The pure cultured (H460 lung cancer cells in a small 3D petri dish) plate with 100% confluency was taken and removed the medium from the plate. Then washed the cells with 500 µl 10% PBS. Added 200 µl trypsin for the detachment of cells from the plate for 1 minute and checked the cell detachment under microscope. Afterwards, trypsin was removed from the plate carefully. Then added 1,000 µl of RPMI into the plate and triturated the cells suspension or use physical force by pipette for completely detachment. After that it was pipetting the pure H460 cells in 20 ml conical tube by sterilize tip. Lastly, the cells were diluted by adding 2 ml of complete RPMI and was shaking these 3 ml cells by hand. (C) Cell counting: Cell was counted by hemocytometer and prepared hemocytometer with cover slip. Shake the cell suspension and gently put 10 µl of cell suspension to the cell counting chamber. Once the total cell count was finished, cell concentration was calculated by the following formula:

$$\text{Total cells/ml} = \text{Total cell counted} \times \frac{\text{dilution factor}}{\text{\# of squares}} \times 10{,}000 \text{ cells/ml}$$

Viable cell was counted and measured as manner of 10×10^4 cells/well with 100 µl volume per well following formula; $V_1 S_1 = V_2 S_2$ where, v_1 = stock volume needed, v_2 = dilution volume, S_1= stock concentration, S_2= dilution concentration. (D)

Mix and seed: It was mixed the stocked H460 cell suspension with RPMI and eventually cells were ready to seed on the 96 well plates 100 µl per well and incubated at 37°C with 5% co_2 for one day. Step-2, treat with crude extract: At first, the crude extract of *Saccharothrix* species was taken and ensured the exact amount of extract. After that, 50 mg/ml of DMSO was added with RPMI medium where DMSO should be <0.5%. For the RPMI with 0.32% DMSO results (996.8 µl + 3.2 µg/ml) and concentration was serially diluted with 4 times dilution (750 µl+250=950 µl) each of 160 µg/ml, 40 µg/ml, 10 µg/ml, 2.5 µg/ml, 0.625 µg/ml along with a control (0 µg/ml) concentration respectively. Then the medium was removed from the seeding 96 wells plate and added prepared 100 µl crude *Saccharothrix* extract each of 160 µg/ml, 40 µg/ml, 10 µg/ml, 2.5 µg/ml, 0.625 µg/ml along blank 4 times was treated with the seeding H460 cells. Then incubated it for 24 hours. Step-3, addition of MTT reagent (4mg/ml): After 24 h incubation cells were incubated with fresh medium containing 0.5% thiazolyl blue tetrazolium bromide (MTT) 100 µl reagent (Sigma, M2128, Irvine, UK) for 4 h in the dark condition. So, 4 mg of MTT reagent was dissolved in 1 ml of PBS solution. The resulting solution was diluted with 9 ml of medium. Then removed the medium from each 96 well plate and wash with 10% PBS 100 µl solution. Afterward, added 100 µl MTT reagent and was incubated for 4 hours at incubator. After incubation, MTT reagent was removed and added 100 µl dimethyl sulfoxide (DMSO) for dissolving the formazan crystal. Finally, it was measured absorbances at 570 nm (Microplate reader) and observed the cell condition on the basis of cell proliferation and viability. Cytotoxicity of each sample must express as an IC_{50} value was the concentrations of the test sample that causes 50% of cell growth average from three replicate experiments.

3.5.4.1 Wound-healing assay

The migratory potential of cancer cells was analyzed by using a wound-healing assay following method (57). Briefly, the confluent monolayer of cancer cells was scratched using a tip. Each well was washed with PBS to remove nonadherent cells. Then, cells were treated with crude extracts compound of *Saccharothrix* species at 0.625, 2.5, 10, 40, 120 & 160 µM and it was incubated at 37°C for up to 24 h. The central perimeter of cell-free gap was ensured under an optical microscope (Olympus).

3.5.4.1 Cell invasion assay

Cell invasion assay was performed by following the methodology of Lee et al. (57) where cell invasion was examined by using trans-well chamber insert with a pore size of 8.0 µM. Lower and upper sides of the polycarbonate filter was coated with10 µL of gelatin (1 mg/mL) and Matrigel (3 mg/ml), respectively. Cancer cells were seeded into the upper chamber of the filter while extract of *Saccharothrix* at 0.625, 2.5, 10, 40, 120 and 160 µM added to the lower chamber filled with complete RPMI medium and incubated at 37°C for 24 hours. Then, cells were settled with methanol and stain by hematoxylin or eosin. Number of cells that invaded inside the filter lower chamber, it was counted by utilizing an optical microscope. Finally, the cell invasion assay was determined on the basis of triplets.

3.5.5 Antioxidant activity assays

The analysis of antioxidant activity of the crude extracts was analyzed by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging test on rapid TLC screening method described by Fatemeh et al. (31). To perform the DPPH radical scavenging activity assay, crude extracts antioxidant effects was measured in a 96-well microtiter plate format (58). Concentrations 10µg/ml of crude extracts was added with 190 µl of a 100 µM DPPH solution into methanol. After 30 min incubation at 37°C in dark environment each solution absorbance will determine at 492 nm by using a microplate reader. Thereby, BHT and ascorbic acid will be used as positive controls as the same concentration compare with extract's concentration whereas extract of non-inoculated fermentation medium was used as a negative control. The wells without extract reckoned as blank samples. Finally, the ability to scavenge the DPPH radical was computed by consequent upon a formula, Radical scavenging activity (SCA %) = [($A_{Blank} - A_{Sample} / A_{blank}$) × 100], where A_{Blank} was considered as the absorbance of the control and A_{Sample} represents the absorbance of the sample. Consequently, the percentage of DPPH was plotted against the sample per standard concentration to get the exact amount of antioxidant necessary to minimize the primary concentration by 50% (EC_{50}) seed extract concentrations µg/ml of DPPH was averaged from reaction medium by three replicate experiments.

3.5.6 Acetylcholinesterase inhibitory activity

The anticholinesterase activity of the crude extracts was analyzed by following method Cardoso-Lopes et al. (59). The activity was measured by using the 96-well microplate reader based on the method Ellman et al. (60) where the enzyme was hydrolyzed with the substrate acetylthiocholine which produced thiocholine. Thus, thiocholine was reacted with 5,5'-di-thiobis,2-nitrobenzoic acid (DTNB) to produce 5-thio-2-nitrobenzoate and 2-nitrobenzoate-5-mercaptothiocholine which was detected by absorbance light at 405nm. 25 µl of crude extract sample (5 mg/ml in methanol was diluted 10 times with 50 mM Tris-hydrogen chloride buffer to make a concentration of 0.5 mg/ml), 25 µl (15 mM acetylthiocholine iodide in water), 50 µl (50 mM Tris-HCl buffer containing 0.1% BSA), 125 µl (3 mM DTNB in 50 mM Tris-HCl buffer containing 0.1 M NaCl) and 0.02 M $MgCl_2 \cdot 6H_2O$ was added in the 96-well plate. Thus, the absorbance was measured at 405 nm each 30 seconds for 3 times. Then added 25 µl of 0.22 U/ml of acetylcholinesterase from electric eel. Again, the absorbance was read every 45 seconds for 8 times. However, comparing with this crude bacterial natural product or extract I used galantamine hydrobromide used as a positive control. Thereby, sometimes rising absorbance because of the rapid hydrolysis of the substrate was settled by minimizing the rate of the reaction before run-on the enzyme from the value of enzyme reaction. Finally, the inhibition rate was determined by comparing with the rates of sample to a blank.

3.5.7 Secondary metabolite profiling of the crude extract

The chemical profile of the crude extract of strain SC076^T was analyzed by using thin layer chromatography (TLC) and the high-performance liquid chromatography (HPLC) (61). The TLC chromatogram was visualized by using various spraying reagent including Dragendorff's, ninhydrin, anisaldehyde and sulfuric acid.

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CHAPTER IV

RESULT AND DISCUSSION

4.1 Taxonomic studies of novel *Saccharothrix* species:

In this study, phylogenetic analysis, chemotaxonomy, phenotypic and genome properties of strain SC076^{T} were investigated.

4.1.1 Phenotypic properties

Strain SC076^{T} grew well on all media used in this study. The pale-yellow aerial masses could be observed on the agar media while light to greenish yellow was observed for the substrate mycelia. A grayish-greenish yellow pigment was observed on ISP2, ISP3, ISP4, ISP5 and ISP6 while a brownish-black pigment was observed on the ISP7 agar. The strain SC076^{T} produced a long chain cocci shape of the smooth spores on ISP2 plate. The optimum growth temperature and pH were 30-37° C and pH 6-10, respectively. The cultural characteristics of strain SC076^{T} were significantly different from its closely related *Saccharothrix* type strains as shown in Table 1. The aerial masses of strain SC076^{T} showed a zigzag pattern (Figure 1)- a characteristic typically observed in most *Saccharothrix* species. Spores with smooth surface were observed on the aerial mycelia. Strain SC076^{T} tolerated up to 7% of NaCl while the other type strain were unable to grow in this condition. Strain SC076^{T} showed positive results for nitrate reduction. This property differentiated strain SC076^{T} from *S. tamanrassetensis* DSM 45947^{T} and *S. algeriensis* JCM 13242^{T}. Strain SC076^{T} showed a negative result for starch hydrolysis, whereas *S. variesporea* JCM 3273^{T}, *S. espanaensis* JCM 9112^{T} and *S. tamanrassetensis* DSM 45947^{T} showed positive results. Strain SC076^{T} could utilize all sugars used in this study which was similar to the most closely related *Saccharothrix* type strain. However, the carbon utilization was clearly different between strain SC076^{T} and *S. espanaensis* JCM 9112^{T}. Details for others phenotypic characters are shown in Table 1 and 2.

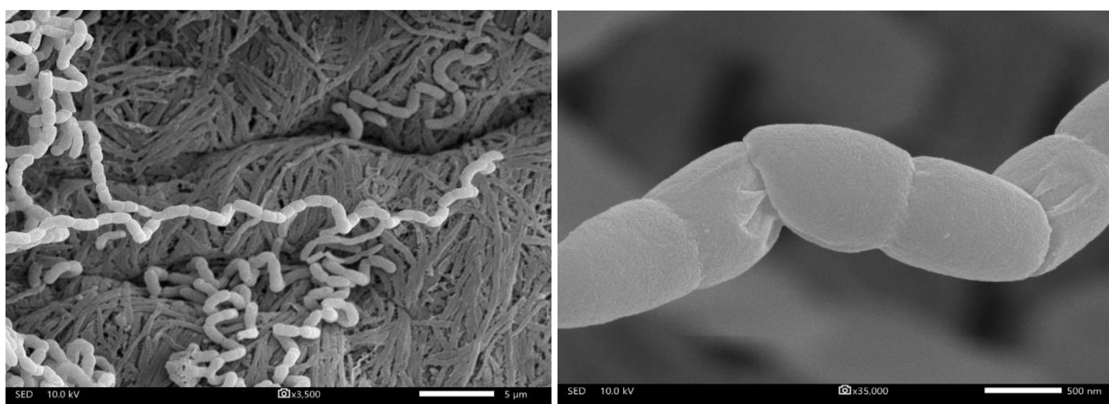

Figure 1 Scanning electron microscopy of strain SC076^{T} (a) aerial mycelia which differentiated to form the spores (b) zigzag morphology of the aerial mycelia.

Table 1 Cultural characteristics of strain SC076^T and related *Saccharothrix* type strain:

Medium/ Growth	SC076^T	*S. violaceurobra* JCM 16955^T	*S. variiesporea* JCM 3273^T	*S. australiensis* JCM 3370^T	*S. espanaensis* JCM 9112^T	*S. tamanrassetensis* DSM 45947^T	*S. algeriensis* JCM 13242^T
ISP2/Good							
Aerial mycelia	Pale yellow	Strong reddish orange	Pale greenish yellow	Light greenish gray	Deep yellow	Brilliant yellow	Moderate yellowish brown
Colony color	Grayish yellow	Light orange yellow	Light yellow green	Brownish black	Light yellow	Pale yellow	Grayish brown
Substrate mycelia	Light yellow	Brilliant orange	Light yellow	Light yellow	Moderate yellow	Brilliant yellow	Dark yellowish brown
Soluble pigment	Grayish greenish yellow	Grayish greenish yellow	Grayish greenish yellow	Pale greenish yellow	Grayish greenish yellow	Grayish greenish yellow	Dark yellowish brown
ISP3/Good							
Aerial mycelia	Pale yellow	Light yellow	Pale greenish yellow	White	Light yellow	Pale yellow	Strong greenish yellow
Colony color	Grayish yellow	No color	Yellowish white	Dark greenish yellow	Light yellow	Pale yellow	Moderate greenish yellow
Substrate mycelia	Pale greenish yellow	Pale greenish yellow	Light yellow	Pale yellowish green	Pale yellow	Dark yellow	Brilliant yellow
Soluble pigment	Grayish greenish yellow	Pale greenish yellow	Pale yellow	Dark greenish yellow	Pale greenish yellow	Grayish olive	Deep greenish yellow
ISP4/Good							
Aerial mycelia	Pale greenish yellow	Light yellow	Pale greenish yellow	Grayish white	Brilliant yellow	Light yellow	Pale greenish yellow
Colony color	Yellowish white	Light yellow	Pale greenish yellow	Moderate olive	Light yellow	Light yellow	Pale greenish yellow
Substrate mycelia	Pale greenish yellow	Light yellow	Light yellow green	Grayish greenish yellow	Light yellow	Light yellow	Light yellow
Soluble pigment	Grayish greenish yellow	Pale greenish yellow	Grayish greenish yellow	Light greenish gray	Grayish greenish yellow	Pale greenish yellow	Yellowish white
ISP5/Good							
Aerial mycelia	Yellowish white	Pale yellowish green	Brilliant orange yellow	Grayish white	Deep yellow	Pale yellow	Pale greenish yellow
Colony color	Dark grayish yellow	Yellowish white	Pale greenish yellow	Moderate olive	Brilliant orange yellow	Light yellow	Grayish greenish yellow
Substrate mycelia	Pale yellow	Vivid yellow	Brilliant orange yellow	Dark yellow	Light yellow	Light yellow	Yellowish white
Soluble pigment	Grayish greenish yellow	Pale greenish yellow	Deep yellow	Grayish olive green	Grayish greenish yellow	Grayish greenish yellow	Yellowish white
ISP6/Good							
Aerial mycelia	Yellowish white	Light yellow	Light yellow green	Grayish white	Yellowish white	Light orange yellow	Greenish white
Colony color	Light yellow	Light yellow	Pale greenish yellow	Strong greenish yellow	Deep greenish yellow	Pale greenish yellow	Pale greenish yellow
Substrate mycelia	Brilliant yellow	Brilliant yellow	Brilliant yellow	Brilliant orange yellow	Brilliant yellow	Brilliant orange yellow	Vivid yellow
Soluble pigment	Strong greenish yellow	Brilliant orange yellow	Deep yellow	Moderate olive	Strong greenish yellow	Deep yellow	Deep yellow
ISP7/Good							
Aerial mycelia	Dark grayish yellowish brown	Light yellow	Strong orange yellow	Very pale green	Brilliant orange yellow	Light yellow	Yellowish gray
Colony color	Dark grayish yellow brown	Pale greenish yellow	Light orange yellow	Moderate olive brown	Deep yellow	Pale yellow	Yellowish gray
Substrate mycelia	Dark yellowish brown	Pale greenish yellow	Vivid yellow	Deep yellowish brown	Grayish yellow	Brilliant yellow	Pale yellow
Soluble pigment	Brownish black	Pale greenish yellow	Dark orange yellow	Dark yellowish brown	Deep greenish yellow	Grayish greenish yellow	Light greenish olive

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Table 2 Differential characteristics between strain SC076T and related *Saccharothrix* type strains: All data were examined in this study except for * which was obtained from Otoguro et al -, Not present. nd, no data.
Strain: 1, SC076T; 2, *S. variiesporea* JCM 3273T; 3, *S. australiensis* JCM 3370T; 4, *S. espanaensis* JCM 9112T; 5, *S. tamanrassetensis* DSM 45947T; 6, *S. algeriensis* JCM 13242T; 7, *S. violaceirubra* NBRC 102064T.

Characters	1	2	3	4	5	6	7*
Maximum NaCl tolerance (%)	7	5	5	5	4	4	4
Growth pH	6-10	6-10	7-10	6-9	6-10	6-10	6-11
Gelatin liquefaction	+	+	+	-	+	-	-
Nitrate reduction	+	+	+	+	-	-	+
Starch hydrolysis	-	+	-	+	+	-	-
Utilization of Carbon sources							
Mannitol	+	++	+	-	+	+	-
Melibiose	+	++	+	-	+	++	-
D-xylose	+	-	+	-	+	+	-
Sucrose	++	++	+	-	+	++	+
Melezitose	+	+	+	-	+	+	Nd
Glycerol	+	++	+	+	+	++	Nd
Fructose	++	++	++	+	+	++	Nd
Maltose	+	++	+	+	W	+	+
Rhamnose	+	++	+	-	+	+	-
Cellobiose	+	++	+	-	+	+	Nd
Galactose	+	++	+	-	+	+	Nd
Raffinose	+	++	+	-	+	+	-
Lactose	+	++	+	-	+	+	-
Inositol	+	++	+	-	+	+	-
Enzyme activities							
Alkaline phosphatase	W	+	-	W	+	-	Nd
Esterase (C4)	-	+	+	+	+	+	Nd
Esterase lipase (C8)	-	+	+	W	+	W	Nd
Lipase (C14)	-	-	W	-	W	-	Nd
Leucine arylamidase	+	+	+	+	+	+	Nd
Valine arylamidase	+	+	+	+	+	+	Nd
Valine arylamidase	W	+	W	W	+	-	Nd
Cystine arylamidase	+	+	+	+	+	W	Nd
Trypsine	+	+	+	+	+	+	Nd
α-chymotrypsine	+	+	+	+	+	W	Nd
Acid phosphatase	W	+	+	-	-	+	Nd
Naphthol-AS-BI-phosphohydrolase	-	+	-	-	-	-	Nd
α-galactosidase	-	+	-	-	-	-	Nd
B-galactosidase	-	-	-	-	-	-	Nd
α-glucosidase	+	+	+	+	+	W	Nd
B-glucosidase	+	+	-	W	W	-	Nd
N-acetyl-β-glucosaminidase	-	+	+	+	+	-	Nd
α-mannosidase	W	+	-	+	+	W	Nd
α-fucosidase	-	-	-	-	-	-	Nd

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4.1.2 Chemotaxonomic characteristics:

Cell wall peptidoglycan contains *meso*-diaminopimelic acid. Rhamnose, ribose, mannose glucose and galactose are detected as the whole-cell sugars. Polar lipids are phosphatidylethanolamine, hydroxy-phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol mannoside, phosphatidyl inositol, unidentified ninhydrin positive glycolipid, unidentified glycolipid and four unidentified lipids (Appendix C). Menaquinones were MK-9(H_4) (77.5%), MK-9(H_8) (10.03%), MK-9(H_2) (6.56%) and MK-9(H_0) (5.87%). The predominant fatty acids are iso-$C_{16:0}$, iso-$C_{15:0}$, and anteiso-$C_{17:0}$ (Table 3). It contained mycolic acids in their cell mass.

Table 3 *C*ellular fatty acid composition of strain SC076^T and closely related *Saccharothrix* species:
Strain: 1, SC076^T; 2, *S. variiesporea* JCM 3273^T; 3, *S. australiensis* JCM 3370^T; 4, *S. espanaensis* JCM 9112^T; 5, *S. tamanrassetensis* DSM 45947^T; 6, *S. algeriensis* JCM 13242^T; 7, *S. violaceirubra* NBRC 102064^T.
All data were examined in this study except * which was obtained from Otoguro et al.
- Not present. Fatty acids comprising less than 0.9 % in all strains are omitted.

Fatty acid	Strain						
	1	2	3	4	5	6	7*
Saturated fatty acids							
$C_{16:0}$	2.7	3.24	1.7	3.6	3.5	6.3	-
$C_{17:0}$ 10-methyl	1.8	-	-	-	-	-	-
$C_{17:0}$	-	1.7	1.0	2.1	2.1	6.3	-
$C_{18:0}$ 3-OH	1.7		-	-	-	-	-
$C_{18:0}$	3.4	1.3	3.0	1.6	1.7	3.7	-
Unsaturated fatty acids							
$C_{15:1}\omega6c$	-	1.0	-	-	1.6	1.7	-
$C_{17:1}\omega6c$	-	-	-	-	-	-	7.2
$C_{17:1}\omega8c$	-	1.4	-	2.6	4.5	5.5	-
$C_{18:1}\omega9c$	-	-	-	0.9	-	2.0	-
Branched fatty acids							
iso-$C_{14:0}$	1.2	-	-	1.4	1.0	2.1	7.1
iso-$C_{15:0}$ G	-	-	-	-	-	-	1.3
iso-$C_{15:0}$	15.8	16.5	24.6	30.9	28.3	16.7	10.4
anteiso-$C_{15:0}$	3.9	6.5	4.1	7.7	5.3	7.2	17.9
iso-$C_{16:0}$	22.5	21.6	16.0	12.2	17.6	20.3	23.0
iso-$C_{17:0}$	9.0	7.7	13.2	10.7	6.0	4.7	-
anteiso-$C_{17:0}$	12.1	25.6	16.8	11.5	9.2	10.5	8.9
Unsaturated branched fatty acids							
iso-$C_{15:1}$ G	-	-	-	2.2	2.7	-	-
iso-$C_{16:1}$ G	-	-	-	-	-	1.7	-
iso-$C_{16:1}$ H	1.5	2.3	1.1	1.3	2.2	-	12.7
iso-$C_{17:1}\omega9c$	-	-	-	-	-	-	1.6
anteiso- $C_{17:1}\omega9c$	-	-	-	-	-	-	1.6
Summed in feature 3	6.1	3.7	2.7	4.5	6.8	6.7	-
Summed in feature 8	1.1	-	-	-	-	-	-
Summed in feature 9	11.4	4.0	7.1	4.0	4.4	0.9	-

Summed feature 3 comprised C16:1 ω7c and/or C16:1 ω6c.
Summed feature 8 comprised c18:1 ω7c or c18:1 ω6c.
Summed feature 9 comprised c16:0 10-methyl or iso-C17:1 ω9c.

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4.1.3 16S rRNA gene sequence analysis

Based on the 16S rRNA gene sequence analysis, it was identified as more than 98.0% similarity with the *Saccharothrix* genera. The type strains which exhibited the highest similarities of 16S rRNA gene sequence with the major isolates are showed in the table 4. The phylogenetic tree showed that strain SC076^T shared the node with *Saccharothrix violaceirubra* NBRC 102064^T. This node was also recovered in the NJ and MP with a bootstrap value of over 50 (Figures 2 and 3).

Table 4 BLAST result of strain SC076^T based on EzbioCloud database.

Sample Name	References Strain name	Accession No	Blast similarity
SC076	*Saccharothrix australiensis*	RBXO01000001	98.53%
	Saccharothrix espanaensis	HE804045	98.53%
	Saccharothrix variisporea	RBXR01000001	98.46%

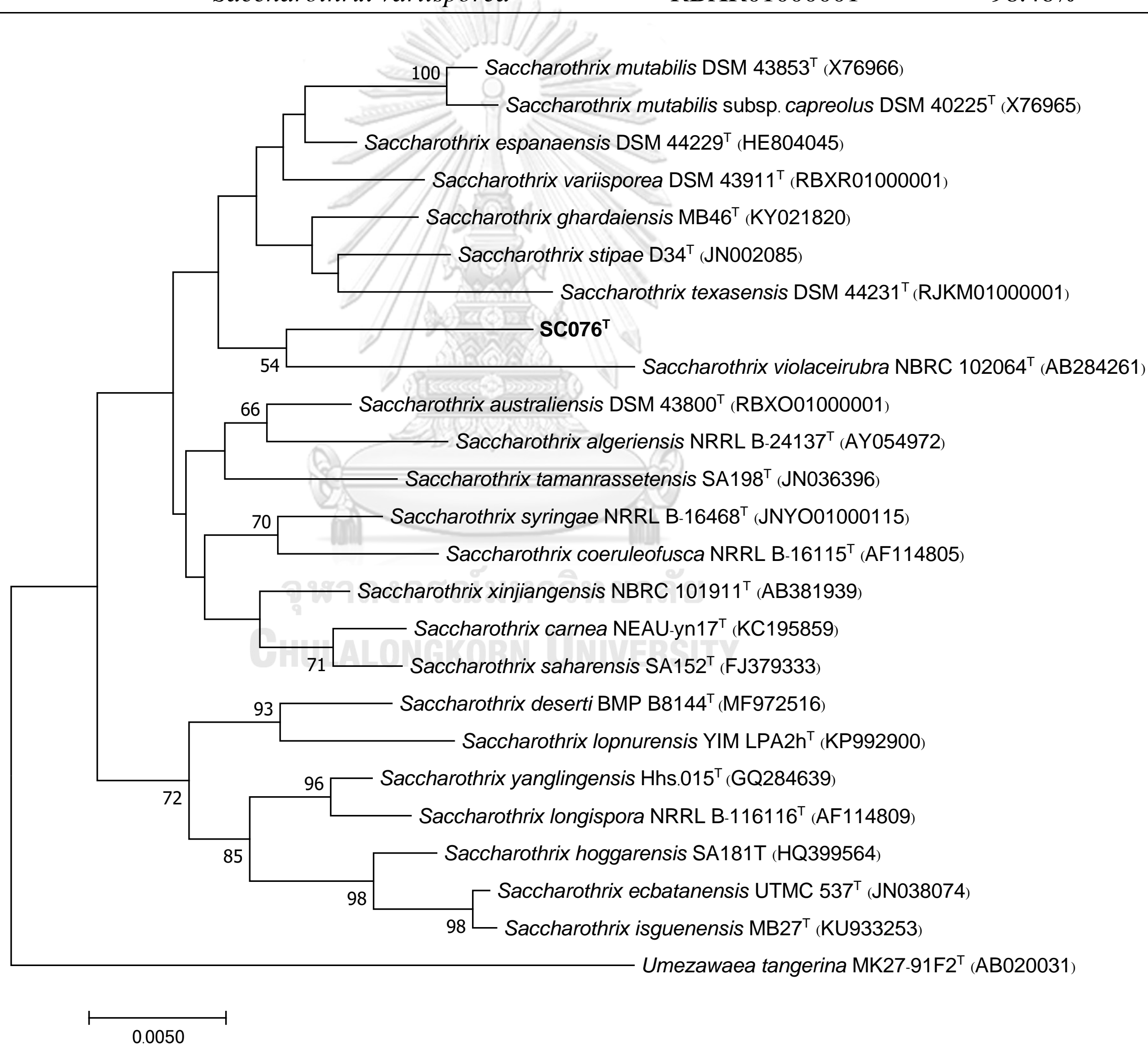

Figure 2 Neighbor-joining tree of the 16S rRNA gene sequences of strain SC076^T and type strains of the genus *Saccharothrix*. Numbers at branch nodes indicate the bootstrap values obtained from 1000 replication. Only the values over 50 are shown. Bar, 0.005 substitutions per nucleotide position.

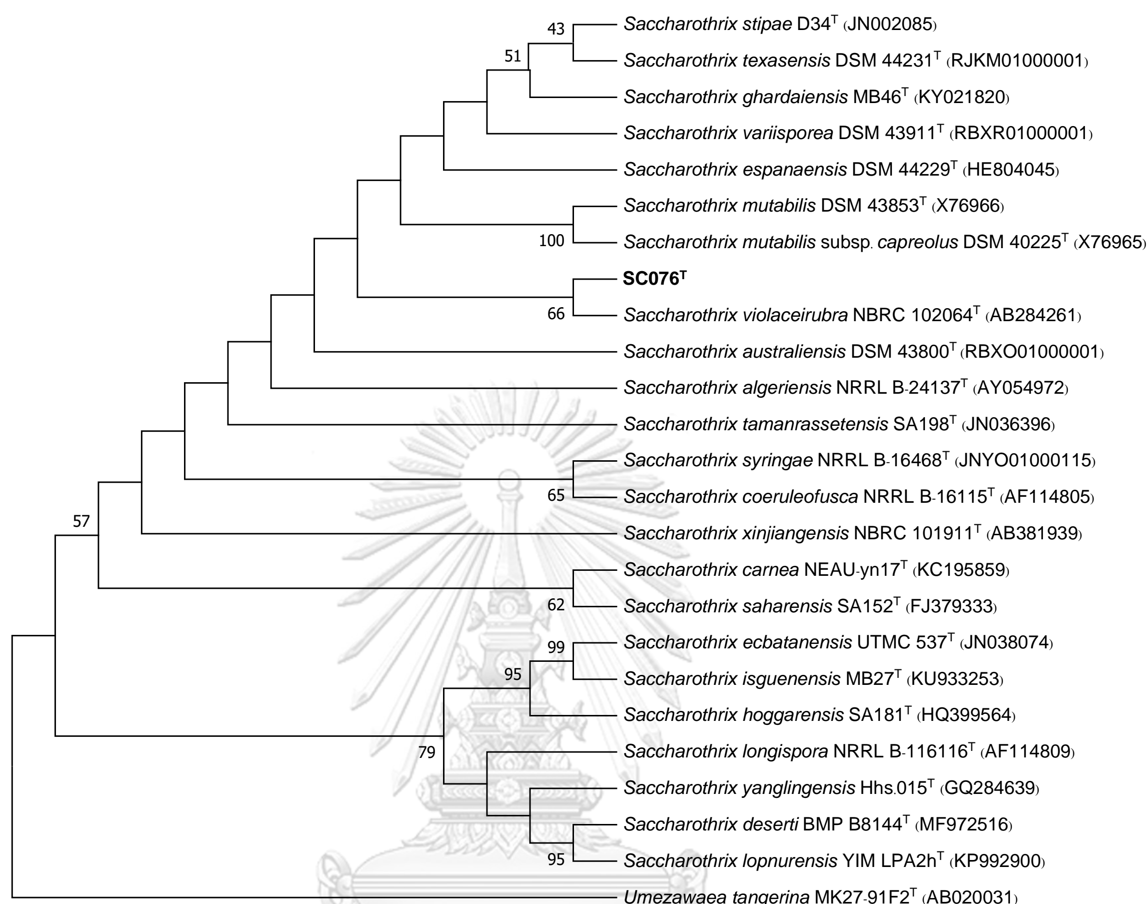

Figure 3 Phylogenetic tree based on maximum-parsimony of the 16S rRNA gene sequences of strain SC076^T and type strains of the genus *Saccharothrix. Umezawaea tangerine* MK27-97F2T was used as the out group. Numbers at branch nodes indicate the bootstrap values obtained from 1000 replication. Only the values over 50 are shown.

4.1.4 Genome of strain SC076^T

Genome of strain SC076^T had 50 contigs with a total bases of 8,040,245 nucleotides. The G+C content was 72.53%. The genome was in the super kingdom Bacteria and was annotated using genetic code 11. The taxonomy of this genome was: cellular organisms > *Bacteria* > *Terrabacteria* group > *Actinobacteria* > *Actinobacteria* >*Pseudonocardiales* > *Pseudonocardiaceae* > *Saccharothrix*. This genome has 7,502 protein coding sequences (CDS), 48 transfer RNA (tRNA) genes, and 2 ribosomal RNA (rRNA) genes. The genome included 3,265 hypothetical proteins and 4,237 proteins with functional assignments (Table 11). The proteins with functional assignments included 1,268 proteins with Enzyme Commission (EC) numbers (62) 1,064 with Gene Ontology (GO) assignments (Ashburner et al., 2000) and 972 proteins that were mapped to KEGG pathways (63). PATRIC annotation includes two types of protein families (64) and this genome has 5,102 proteins that

belong to the genus-specific protein families (PLFams) for, and 5,710 proteins that belong to the cross-genus protein families (PGFams).

An assembled genome for *Saccharothrix* SC076^T was submitted to the comprehensive genome analysis service at PATRIC (65). Based on the annotation statistics and a comparison to other genomes in PATRIC within this same species, this genome appears to be of good quality. Details of the analysis, including genes of interest (specialty Genes), a functional categorization (subsystems), and a phylogenetic tree (phylogenetic analysis) are provided in Table 5 and Figure 4.

A circular graphical display of the distribution of the genome annotations is provided. This includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to know virulence factors, GC content and GC skew. The colors of the CDS on the forward and reverse strand indicate the subsystem that these genes belong to (Appendix B).

Table 5 Specialty Genes Found in the genome of strain SC076^T

	Source	Genes
Antibiotic Resistance	CARD	4
Antibiotic Resistance	NDARO	1
Antibiotic Resistance	PATRIC	46
Drug Target	Drug bank	3
Drug Target	TTD	2
Transporter	TCDB	2
Virulence Factor	PATRIC_VF	6
Virulence Factor	Victors	4

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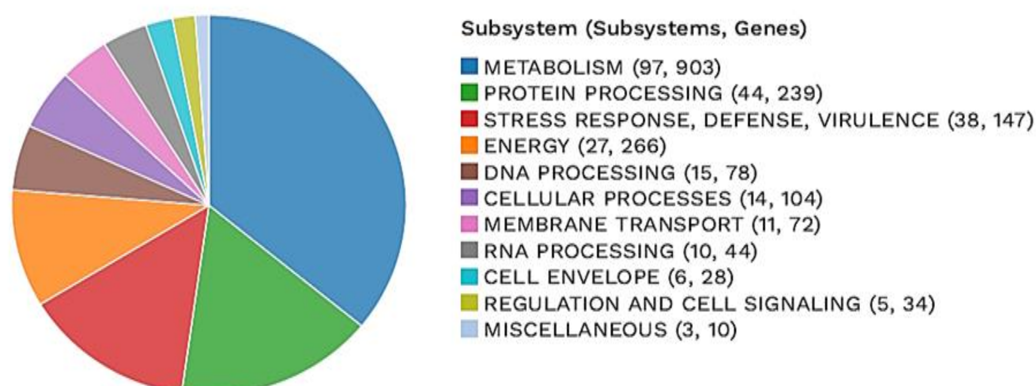

Figure 4 An overview of the subsystems for this genome

Many of the genes annotated in have homology to known transporters (66) virulence factors (67), (68) drug targets (69), (70) and antibiotic resistance (71) genes. The number of genes and the specific source database where homology was found is

provided (Table 11). The Genome Annotation Service in PATRIC uses k-mer-based AMR genes detection method, which utilizes PATRIC's curated collection of representative AMR gene sequence variants (65) and assigns to each AMR gene functional annotation, broad mechanism of antibiotic resistance, drug class and, in some cases, specific antibiotic it confers resistance to. The presence of AMR-related genes (even full length) in a given genome does not directly imply antibiotic resistant phenotype. It is important to consider specific AMR mechanisms and especially the absence/presence of SNP mutations conveying resistance. A summary of the AMR genes annotated in this genome and corresponding AMR mechanism is provided in the Table-6. Based on AntiSMASH, several biosynthetic gene clusters of secondary metabolites including non-robosomal peptide synthase, polyketide and terpene were detected in the genome of strain SC076^T (Table 7). The results demonstrated that the 100% similarity with terpene (Geosmin) and PKS-1 (E-837).

Table 6 Antimicrobial Resistance Genes found in the genome of strain SCO76^T :

AMR Mechanism	Genes
Antibiotic inactivation enzyme	KatG
Antibiotic inactivation enzyme	AAC (2')-1, APH (6)-la/APH (6)-lb, APH (7'')-1
Antibiotic target in susceptible species	Alr, Ddl, dxl, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabl, Iso-tRNA, KasA, MurA, rho, rpoB, rpoC, S10p, S12p.
Antibiotic target protection protein	Qnr family
Antibiotic target replacement protein	FabG
Gene conferring resistance via absence	gidB
Protein altering cell wall charge conferring antibiotic resistance	GdpD, PgsA
Protein altering cell wall structure conferring antibiotic resistance	VanH, Vanl, VanX
Regulator modulating expression of antibiotic resistance genes	LpqB, MtrA, MtrB, Van-O type

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Table 7 Identified secondary metabolite regions using strictness "relaxed"

Region	Type	From	To	Most similar Known cluster		Similarity
1.1	Oligosaccharide	686,548	733,253	calicheamicin	Polyketide	4%
1.2	Lassopeptide	758,949	781,278	Warkmycin CS1/warkmycin CS2	Polyketide + saccharide hybrid/ tailoring	5%
2.1	T1PKS	1	31,856	Streptazone E	Polyketide	58%
2.2	NPRS	147,667	217,502	Myxochelin A/myxochelin B	NRP	50%
2.3	Betalactone	564,713	588,218	Belactocin A/belactocin C	other	12%
2.4	RiPP- like	859,630	870,421			
3.1	Lanthipeptide-class iv	1	21,159	Xiamycin A	Terpene	18%
3.2	Indole	73,086	94,213	Frankiamycin	Polyketide	14%
3.3	NRPS	222,007	294,321	Cysteoamide	NRP	18%
3.4	Thaiopeptide, LAP	323,522	360,178			
3.5	T1PKS, NRPS-like	378,762	477,163	Meoabyssomycin/ abyssomycin	NRP	50%
3.6	NRPS	548,285	608,123	Lysocin	NRP	9%
3.7	Lassopeptide, RRE-containing	804,584	827,161	Anantin C	RiPP	75%
3.8	Terpene	867,146	886,686	Isorenieratene	Terpene	71%
4.1	T2PKS, NRPS, T1PKS, Lassopeptide	90,209	195,320	Lugdunomycin	Polyketide	48%
4.2	RRE-containing	198,589	218,849			
4.3	Lassopeptide	226,342	248,634	Natamycin	Polyketide	9%
4.4	Melanin	464,593	475,153			
4.5	NPRS	665,964	758,755	Crochelin A	NRP+ Polyketide	11%
5.1	Ranthipeptide	339,626	361,006	Lankacidin C	NRP+ Polyketide	26%
7.1	Terpene, lassopeptide	145,873	170,706	Anantin C	RiPP	50%
8.1	NRPS-like	59,630	99,604	Thiocoraline	NRP, cyclic depsipeptide	5%
8.2	Free-Containing, transAT-PKS, NRPS, PKS-like	103,453	214,355	Leinamycin	NRP+ Polyketide: Modular type 1+Polyketide trans AT- type1	15%
9.1	Terpene	76,327	98,528	Geosmin	Terpene	100%
10.1	Terpene	1	12,159	SF2575	Polyketide: Type II + Saccharide Hybrid/ tailoring	6%
10.2	Lanthipeptide class-ii	168,381	191,293	Rishirilide B/ Rishirilide A	Polyketide: Type II	10%
11.1	T1PKS, PKS-like	47,049	128,421	Meridamycin	NRP+ Polyketide	60%
12.1	RiPP-like	109,651	120,589			
12.2	T1PKS, PKS-like	142,033	206,862	E-837	Polyketide	100%
12.3	Butyrolactone, Furan	211,094	237,346	Methylenomycin A	Other	19%
13.1	Terpene	25,264	46,313	Isorenieratene	Terpene	42%
13.2	Lanthipeptide class-iii	103,256	125,781	Ery-9/ Ery-6/ Ery-8/ Ery-7/ Ery-5/ Ery-4/ Ery-3	RiPP:Lanthipeptide	75%
14.1	T1PKS, arylpolyene, NRPS, Other, Ectoine	9,204	137,614	Kedarcidin	Polyketide: Iterative type I+ Polyketide Enediyene type I	74%
15.1	T1PKS	1	28,385	Tiacumicin B	Polyketide: modular type I	12%
17.1	Other, NAPAA, T1PKS, Amglyccycl	27,640	91,497	Cetoniacytone A	Other:Cyclitol	41%
19.1	Lanthipeptide-class-i	1	22,240			
20.1	T1PKS	26,986	52,155	Microansamycin	Polyketide	14%
23.1	T1PKS	13,590	40,373	Dynemycin	Polyketide	8%
25.1	T1PKS	1	5,946	griseochelin	Polyketide	53%

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Based on the phylogenetic tree constructed from the genome sequences, strain SC076^T shared the relative with *S. espanaensis, S. algeriensis, S. tamanrassetensis and S. australiensis* (Figure 5). The comparative genome analysis revealed that strain SC076^T had districted genome from the relative type strain. The DNA-DNA hybridization values between strain SC076^T and other related type strain ranged from 23.6 – 32.8%. In addition, the average nucleotide identity values among these strains ranged from 77.7-86.7% (Table 8). Both dDDH and ANI values of strain SC076^T were much lower than the threshold 70% of dDDH and 95% of ANI used for separating the strains in the same species.

Eventually, both genotypic and phenotypic data clearly demonstrate that strain SC076^T is in the genus *Saccharothrix*. And from the above discussion of the phylogenetic position and chemotaxonomic profile of the typical members are distinguished as the different species. Moreover, taxonomic data convincingly that the strain forms a new position of the taxonomic variation within *Saccharothrix* genus.

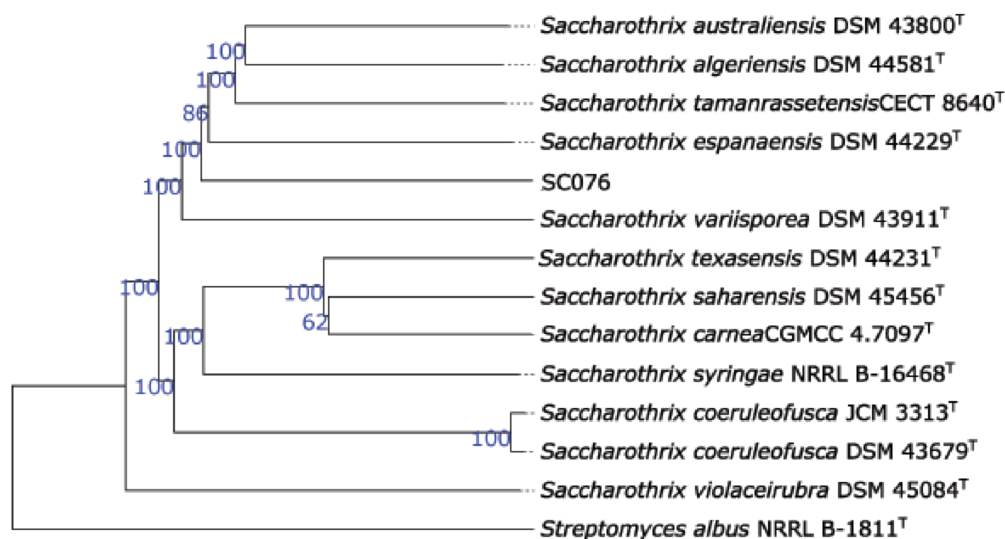

Figure 5 The phylogenomic tree of strain SC076^T and related type strains calculated by TYGS server. *Streptomyces albus* NRRL B-1811^T was used as the outgroup. The numbers at the branch node indicate GBDP pseudo-bootstrap support values > 60% obtained from 100 replications.

Table 8 ANIb and ANIm values (%) and the digital DNA-DNA hybridization (dDDH) values between the draft genomes of strain SC076^T and its closest related type strains

Strain	Genome size (bp)	Accession number	% GC content	% dDDH	ANIb	ANIm
SC076^T	8,040,245			-	-	-
S. algeriensis DSM 44581^T	6,878,582	GCA_016907655	74.1	32.1	82.5	86.7
S. australiensis DSM 43800^T	7,861,373	GCA_003634935	73.5	32.8	82.1	86.8
S. tamanrassetensis CETC 8640^T	8,053,586	GCA_014203665	71.4	31.9	81.9	86.5
S. variisporea DSM 43911^T	9,408,895	GCA_003634995	71.7	26.1	80.1	85.9
S. espanaensis DSM 44229^T	9,360,653	GCA_000328705	72.2	31.9	82.5	86.6
S. violaceirubra DSM 45084^T	7,354,386	GCA_014203755	71.3	23.6	77.7	84.9

4.2 Biological activity of *Saccharothrix oblica* sp. nov.:

4.2.1 Antimicrobial Activity screening:

Strain SC076^T was cultured in ISP2 broth at 30ºC at 180 rpm for 14 days. The culture broth was partitioned three times with ethyl acetate. The ethyl acetate layers were collected and evaporated to dryness. The antimicrobial activity of the crude extract was performed using the agar disc diffusion method against *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231. The crude extract exhibited antimicrobial activity against Gram-positive bacteria including *K. rhizophila* ATCC 9341, *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 but no activity was observed against Gram-negative bacteria and yeast (Table 9).

Table 9 Antimicrobial activity of the crude ethyl acetate extract of strain SC076^T

Tested microorganisms	Inhibition zone (mm)
B. subtillis	11.35
S. aureus	20.05
M. luteus	20.35
E. coli	-
P. aeruginosa	-
C. albicans	-

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4.2.2 Anticancer activity:

In vitro growth inhibitory activities of strain SC076^T with non-small lung cancer cell lines. To determine the non-toxic dose for further evaluation of detachment induced cell death, MTT assay was performed after treatment of adherent lung cancer cells with crude extract of SC076 strain (0-160 µg/mL). For the strain cytotoxicity profile did not cause significant cause of cell viability of human lung cancer cells. PI staining is performed to confirm the cell death. Respectively, in H460 cells it was treated with (0, 0.625, 2.5, 10, 40, 120, 160 µg/mL) concentrations for 24h incubation at 37ºC. In migration assay cell was not migrated effectively on H460 cell line in the lower dose but in the higher dose it showed would is relatively come closure after 24-hour incubation. However, there was no prominent detectable apoptosis or necrosis represented by blue fluorescent of Hoechst 33342 and red

fluorescence of propidium iodine in the lower dose. Thereby, it was determined that in higher dose like (40, 120, 160 µg/mL) it showed prominent cell death and apoptosis (Figure 6, 7 and 8). Values are means of the independent triplicate experiment ± SD. *p < 0.05 versus non-treated controls.

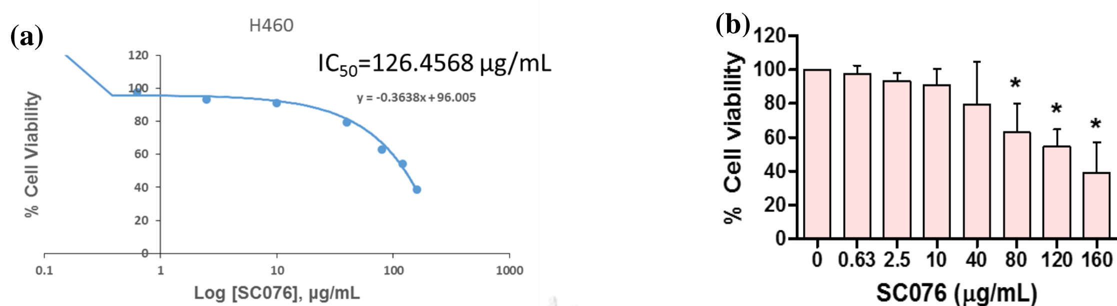

Figure 6 (a) and (b) MTT assay represents percentage of cell viability under the different concentration where IC_{50} value 126.4568 µg/mL.

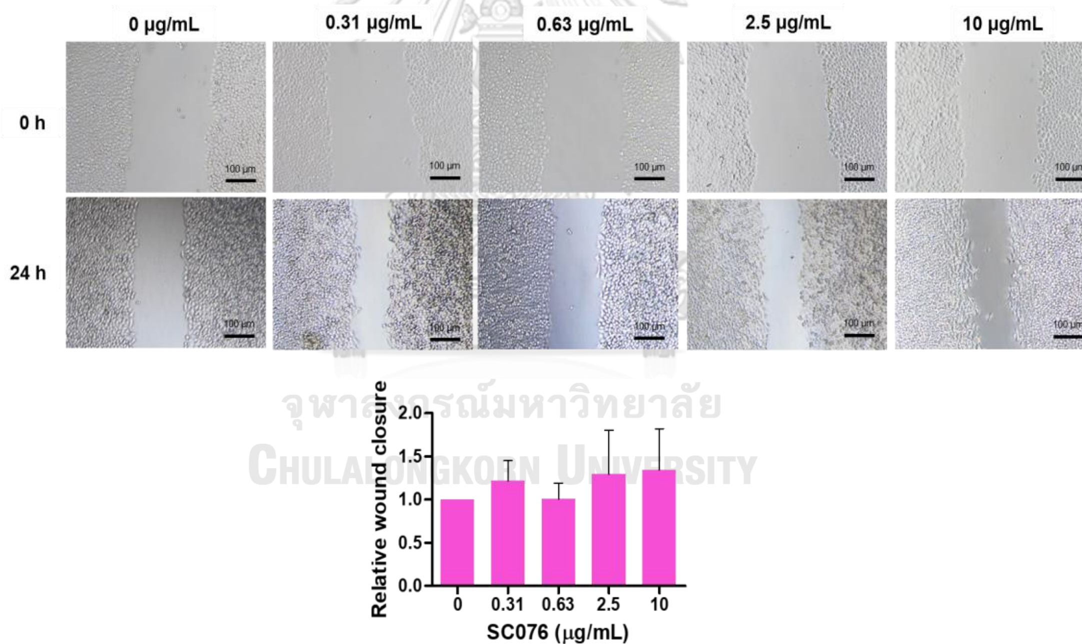

Figure 7 Cell migration effect on non-small lung cancer H460 cells after 24 h incubation.

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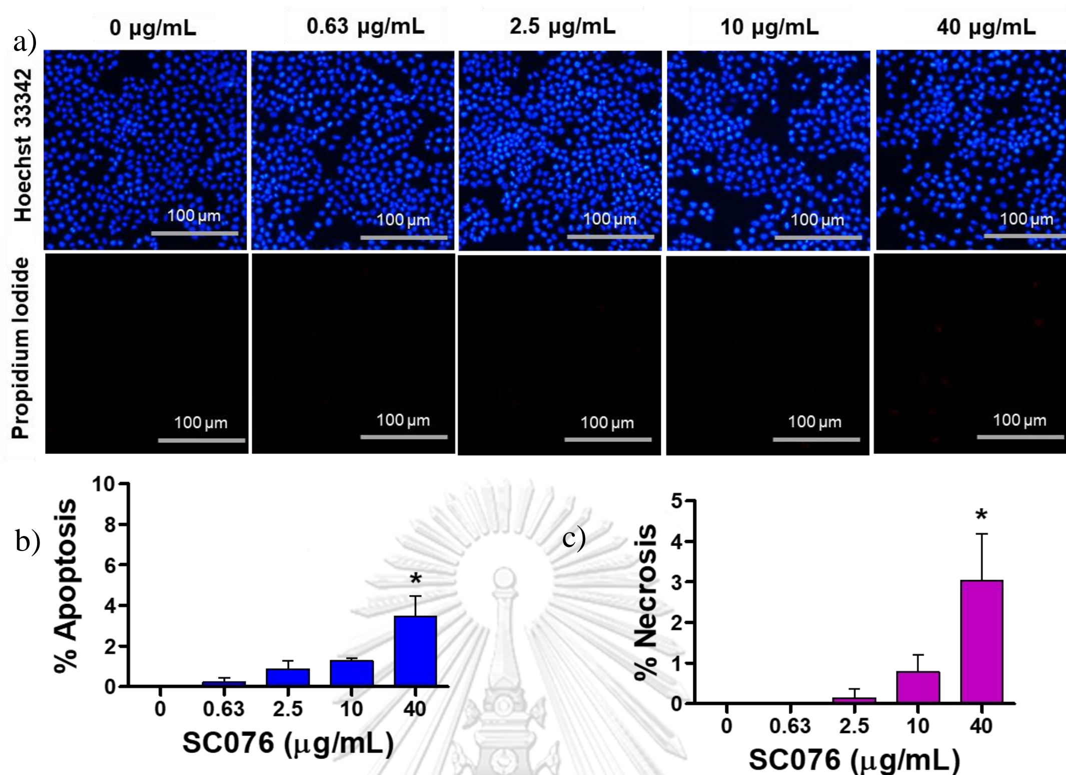

Figure 8 Blue fluorescent of Hoechst 33342 and red fluorescence of propidium iodine (a) in the several doses represented as cellular apoptosis (b) or necrosis (c) on H460 cell line.

4.2.3 Antioxidant activity assay:

Antioxidant activity assay was considered as an important factor in many human diseases and health impairments. Antioxidant activity assay was shown by radical scavenging method, described by intense violet due to the free radical or electrons. From the assay results revealed that strain SC076^T has no scavenging activity or antioxidant effects. Moreover, the inhibitory result provided on Table-10 and Figure 18 in the Appendix D.

4.2.4 Anti-cholinersase activity: Following the assay results in different concentrations, Strain SC076^T have no anti-cholinerase activity. Additionally, the assay result provided on Table 11 and Figure 19 in the Appendix D.

4.3 Secondary Metabolite or chemical profiling of the crude extract:

The chemical profiling by using TLC was observed and analyzed where the ethyl acetate crude extract of the strain SC076^{T} contained some chemical compound. More precisely, TLC band was detected by the several reagents under UV light at 254 nm. In this assay, it would not identify the specific chemical compound but from the TLC screening it was clearly predicted that the strain contains some chemical substances that was observed by the TLC marked band under ultraviolet light at 254 nm.

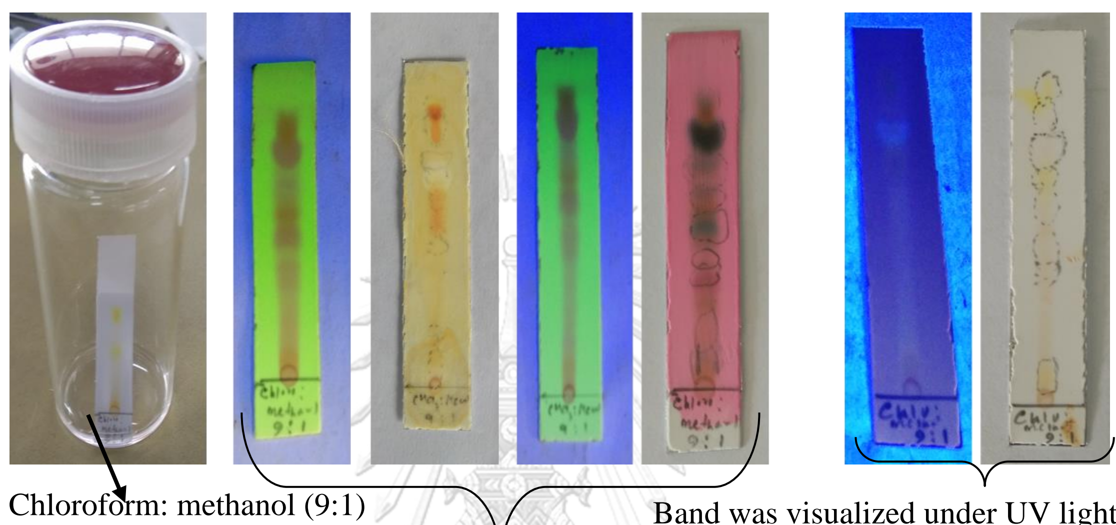

Figure 9 Chemical profiling: TLC chromatogram will be visualized by using various spraying reagent including Dragendorff’s, ninhydrin, anisaldehyde and sulfuric acid.

Comparative study revealed that the pure crude extract contains potential secondary metabolites. Besides, the ethyl acetate crude extract was also analyzed by HPLC chromatogram peak under two different UV length 254 nm and 210 nm. However, it was observed from the chromatogram peak that the crude extract detected peak at different retention time 7.193, 7.362, 12.926, 14.717 and 15.262 respectively that represented on the Figure 10. However, further study will need to identify the chemical metabolites.

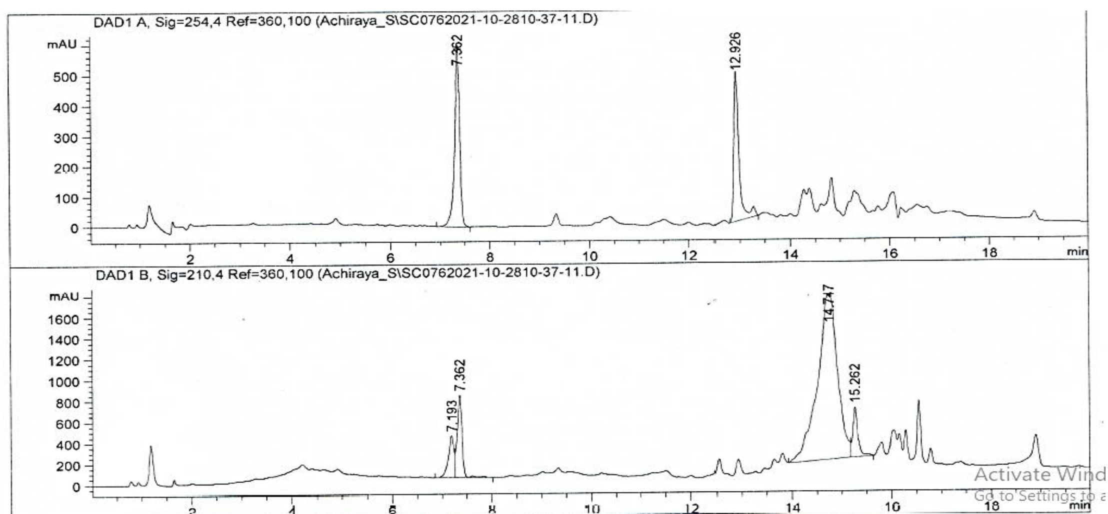

Figure 10 HPLC chromatogram of ethyl acetate crude extract of *Saccharothrix oblica* species.
HPLC condition (Column: Purospher column RP-18 from Merck), (Mobile Phase: 0-100% Acetonitrile in water) and (Flow rate: 0.5 ml/ min)

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CHAPTER V
CONCLUSION

It is necessary to discover biologically active compound in order to get antibiotics and other beneficiary chemical metabolites for saving human life from organisms or treating the diseases. However, Thailand has been considered to have great biodiversity of tropical region of numerous species has titled studies on isolation of soil derived *Actinobacteria*. In this study mainly focused on taxonomic study of novel rare *Actinobacteria Saccharothrix oblica* sp. nov. isolated from Sichang Island.

So, emphasizing focus on the pathogenic organism's role and safety, a complete taxonomic study was used to isolate and identify the novel species and biological activity like antimicrobial activity revealed that the strain exhibited antimicrobial activity against some gram-positive pathogenic bacteria. The crude extract exhibited antimicrobial activity against Gram-positive bacteria including *K. rhizophila* ATCC 9341, *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 but no activity was observed against Gram-negative bacteria and yeast.

Besides, in this study anticancer activity screening also checked where higher dose 40-160 µg/mL showed cytotoxic effect through IC_{50} value 126.4568 µg/mL. But result revealed from the migration test that strain have no prominent chemotactic migratory effect on non-small lung cancer cells. Meanwhile, PI staining represents higher dose produced cell death or cytotoxicity. Thereby, strain has no antioxidant and anticholinerase activity. However, secondary metabolites profiling demonstrates some chemical compounds that would be promising active compound. However, further study would be isolated from the crude extract and pure active compound will be contributed to the drug development.

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