ANTAGONISTIC ACTIVITY OF SELECTED MARINE-DERIVED *STREPTOMYCES* SPP. AGAINST *PLESIOMONAS SHIGELLOIDES*

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DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

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2010

❖ ABSTRACT

In this study, primary and secondary screenings were conducted to evaluate the antibacterial and anti-yeast potential of the eleven selected marine-derived *Streptomyces* spp., particularly against *P. shigelloides*. The strains were categorized into three colour groups for morphological grouping according to the colour of aerial mycelium; namely grey, yellow green, and white colour groups. Diffusible pigments were produced by eight of the strains tested. Strain(s) from grey and white with red diffusible pigments colour groups displayed strong antibacterial activity. The yeasts tested were resistant to the bioactive metabolite(s) produced by some of the *Streptomyces* spp. which had shown high activity against the bacteria tested. In primary screening, there was no inhibition of growth of yeasts tested; *C. albicans* and *C. parasilopsis*. Of the streptomycete strains tested, eight strains were active against at least one bacteria tested. In secondary screening, four strains inhibited all the yeasts tested. Meanwhile, all strains were active against at least one bacteria tested. *Streptomyces* strain T15 displayed the best antibacterial activity against *B. subtilis, P. aeruginosa,* and *P. shigelloides.* Of the streptomycetes tested, three strains were found to display strong inhibition against at least one pathogen tested in both primary and secondary screenings. Meanwhile, two and three strains displayed moderate inhibition against at least one pathogen tested in primary and secondary screenings, respectively. Furthermore, six and all the strains displayed weak inhibition against at least one pathogen tested in primary and secondary screenings, respectively. The antibacterial and anti-yeast activities were prominent in secondary screening, where five of the strains exhibited broad spectrum activity.

Streptomyces strain T15 was selected for further studies. Culture conditions (media and agitation or static) were optimised to enhance the production of bioactive metabolite(s) in submerged fermentation. It was noted that fermentation of strain T15 in ISP2 medium at an incubation period of nine days at 28ºC ± 2ºC, pH 6.10 ± 1.70, and shaking at a speed of 120 rpm was the most ideal for growth and bioactive metabolite(s) production antagonistic to *P. shigelloides* at laboratory conditions. During the twelve days fermentation, the highest bioactive metabolite(s) production of strain T15 was noted on day nine in all the fermentation media used in this study. The highest bioactive metabolite(s) production by nine-day old strain T15 was achieved in ISP2 medium; however, the bioactive metabolite(s) production started on day six. The nature of culture condition that is, either agitation or static had an effect on the production of bioactive metabolite(s). For agitation culture condition, ISP2 medium was considered as the best fermentation medium for strain T15 based on the higher production of bioactive metabolite(s) that it supported compared to SA medium. Meanwhile, for static culture condition, ISP4 medium was the only fermentation medium that supported the bioactive metabolite(s) production by strain T15.

❖ ABSTRAK

Dalam kajian ini, penskrinan primer dan sekunder dijalankan ke atas sebelas *Streptomyces* spp. marin bagi menilai aktiviti antibakteria dan anti-yis terutamanya ke atas *P. shigelloides.* Strain-strain dikategorikan kepada tiga kumpulan warna berdasarkan warna miselium "aerial"; iaitu kelabu, kuning kehijauan, dan putih. Sebanyak lapan strain menghasilkan pigmen difusi. Strain dari kumpulan warna kelabu dan putih dengan pigmen difusi merah menunjukkan aktiviti antibakteria yang kuat. Yis yang dikaji adalah resistan terhadap *Streptomyces* spp. yang mempunyai aktiviti antibakteria yang kuat. Dalam penskrinan primer, tiada perencatan pertumbuhan yis; *C. albicans* dan *C. parasilopsis*. Sebanyak lapan strain aktif terhadap sekurang-kurangnya satu bakteria yang dikaji. Dalam penskrinan sekunder, empat strain merencatkan pertumbuhan semua yis yang dikaji. Manakala, semua strain adalah aktif terhadap sekurang-kurangnya satu bakteria yang dikaji. *Streptomyces* strain T15 adalah agen antibakteria paling kuat bagi *B. subtilis, P. aeruginosa,* dan *P. shigelloides.* Sebanyak tiga strain menunjukkan inhibisi yang kuat terhadap sekurang-kurangnya satu patogen dalam penskrinan primer dan sekunder. Manakala, dua dan tiga strain masing-masing menunjukkan inhibisi yang sederhana terhadap sekurang-kurangnya satu patogen dalam penskrinan primer dan sekunder. Sebanyak enam dan semua strain masing-masing menunjukkan inhibisi yang lemah terhadap sekurang-kurangnya satu patogen dalam penskrinan primer dan sekunder. Aktiviti antibakteria dan anti-yis adalah lebih menyerlah dalam penskrinan sekunder di mana lima strain tersebut mempunyai aktiviti berspektrum luas.

Streptomyces strain T15 dipilih untuk kajian selanjutnya. Optimisasi kondisi kultur iaitu media dan agitasi atau statik dilakukan untuk meningkatkan penghasilan

metabolit bioaktif melalui fermentasi tenggelam ("submerged fermentation"). Fermentasi strain T15 dalam medium ISP2 selama sembilan hari pada suhu 28ºC ± 2ºC, pH 6.10 ± 1.70, dan kelajuan putaran 120 rpm adalah paling sesuai untuk pertumbuhan dan penghasilan metabolit bioaktif yang antagonistik terhadap *P. shigelloides* dalam kondisi makmal. Penghasilan metabolit bioaktif tertinggi dicatatkan pada hari kesembilan bagi semua media yang digunakan dalam fermentasi sepanjang dua belas hari tersebut. Penghasilan metabolit bioaktif tertinggi diperolehi dalam medium ISP2 walaupun penghasilan metabolit bioaktif tersebut bermula pada hari keenam. Kondisi kultur iaitu sama ada agitasi atau statik mempunyai kesan ke atas penghasilan metabolit bioaktif. Medium ISP2 berbanding medium SA adalah medium fermentasi terbaik bagi penghasilan metabolit bioaktif dalam kondisi agitasi. Manakala, bagi kondisi statik, medium ISP4 adalah satu-satunya medium fermentasi yang dapat menyokong penghasilan metabolit bioaktif oleh strain T15.

❖ ACKNOWLEDGEMENTS

First and foremost, I wish to express my deepest appreciation to my project supervisor; Prof. Dr. Vikineswary Sabaratnam and co-supervisor; Dr. G. Y. Annie Tan for their guidance, encouragement, and invaluable advice throughout the period of experimental phase and preparation of this dissertation. I am also grateful to Prof. Ng Kim Peng and Prof. Thong Kwai Lin for providing the bacterial and fungal strains.

I would like to extend my heartfelt appreciation and gratitude to my ever loving parents; Mr. Nelson Kloni and Madam Florence Awie for giving me the opportunity and strength to withstand the challenges in completing my masters degree in University of Malaya.

I wish to express my love and sincere gratitude to my husband; Mr. Thomas Chaong for constantly supporting and engineering me towards my road to success.

I would also like to extend my thanks to the academic and support staffs of Institute of Biology, Faculty of Science, and Institute of Postgraduate Studies who have given their fullest co-operation and assisting me in many ways during this project.

Last but not least, I am thankful to Madam Chang May Hing, friends, and labmates; Norashimawati Ibrahim, Nur Rahimahtul Hayati Abdul Rahman, Christabel Loni, Murugan Kalimutho, Wong Kah Hui, Sujatha Ramasamy, Agnes Chan, Umaiyal Munusamy, Jaime Low, Kho Yong Sien, Mumtaz Hidayahtullah Yatau, Nurulaina Saidin, and Nur Azura Ahmad Badrul for their support, guidance, assistance, and suggestions during the course of this research.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

m	: metre
µm	: micrometer
mm	: millimetre
µl	: micro litre
ml	: millilitre
g	: gram
µg	: microgram
mg	: milligram
mg/ml	: milligram per millilitre
MDa	: mega Dalton
kHz	: kilo Hertz
ISP2	: yeast extract-malt extract agar
ISP4	: inorganic salts-starch agar
NA	: nutrient agar
SA	: sporulation agar
SDA	: Sabouraud dextrose agar
NaOH	: sodium hydroxide
DMSO	: dimethyl sulfoxide
MeOH	: methanol
mM	: millimolar
vvm	: volume per volume per minute
%	: percentage
^{o}C	: degree Celsius

psi : pounds per square inch

spp. : species

rpm : rotation per minute

min : minute

h : hour

ANOVA : analysis of variance

MRSA : methicillin-resistant *Staphylococcus aureus*

VRSA : vancomycin-resistant *Staphylococcus aureus*

ATP : adenosine triphosphate

β : beta

γ : gamma

λ : lambda

$\pm$: plus-minus

$>$: more than

$<$: less than

CHAPTER 1

1.0 INTRODUCTION

Plesiomonas shigelloides is an indigenous inhabitant of freshwater, sediment, and the intestinal tract of cold-blooded aquatic animals (Dulger, 2004). It is not only found in the aquatic environment in the tropical and subtropical regions, but also in cold climates (Lukasiewicz *et al.*, 2006). Based on previous reports, Dulger (2004) concluded that the highest isolation rates of these bacteria in surface waters were during the summer months, and no, or very few isolations in winter. According to González-Rey (2003), this bacterium is called the "Asian" bacteria because of the high incidence of isolations in countries such as Japan and Thailand. Macro-morphologically, *P. shigelloides* has different colonial appearance depending on the selective or differential agar used. Colonies vary from flat, round, and 1-2 mm size with smooth edges on blood agar to flat, irregular edge and shape, and around 1 mm size when plesiomonads were cultured on deoxycholate agar. Micro-morphologically, *P. shigelloides* is Gram-negative, motile, capsulated, flagellated, and non-spore-forming bacilli.

Plesiomonas shigelloides is an opportunistic pathogen (Jeppesen, 1995). Clark and Janda (1991) mentioned that it was unclear whether all *P. shigelloides* were equally virulent, or whether strain-to-strain variation occurred. Over the years, there have been a number of reports on the isolation of *P. shigelloides* from an assortment of clinical specimens; including cerebrospinal fluid, wounds, and respiratory tract (Niedziela *et al.*, 2002). Diarrhoea is one of the leading causes of morbidity and mortality in populations in developing countries, and is a substantial health issue throughout the world. Recent epidemiological evidence has strongly implicated *P. shigelloides* as a significant cause of diarrhoeal disease (Theodoropoulos *et al.*, 2001), where it has been implicated as an aetiological agent in sporadic cases and outbreaks of diarrhoea in

various parts of the world, and the causative agent of gastroenteritis as well as of extraintestinal infections (Rager *et al.*, 2000; Wong *et al.*, 2000). Recent attention has been drawn to this organism since it also has been implicated as an emerging pathogen (Krovacek *et al*., 2000; Theodoropoulos *et al*., 2001; Gonzalez-Rey *et al.*, 2004), and a causative agent of extraintestinal infections, which include meningitis, sepsis, arthritis, cholecystitis, and endophthalmitis (Wiegand and Burak, 2004). It is responsible for various extraintestinal infections of gastrointestinal origin, particularly in neonates and immunosuppressed adults, or people with an underlying disease (Lukasiewicz *et al.*, 2006). It also causes localized infections originating from infected wounds, which can disseminate to other parts of the body (Niedziela *et al.*, 2002).

Treatment with antibiotics is seldom required. However, appropriate antibiotic therapy is necessary for severe infections. Most frequently described severe infections are cases of septicaemia and meningitis, occurring mainly in patients with underlying health disorders and in immunocompromised patients (Wiegand and Burak, 2004). Many of the patients with extraintestinal disease died as a direct result of their plesiomonad infections (Clark *et al*., 1990). Abbott *et al.* (1991) and Groves (1996) highlighted that extraintestinal infections due to *P. shigelloides* were severe, but rare, with a reported fatality rate of 80% in the case of neonatal meningitis. Groves (1996) reported that most strains of *P. shigelloides* were resistant to penicillin, but susceptible to penicillin combined with a *β*-lactamase inhibitor, chloramphenicol, trimethoprim-sulfamethoxazole, quinolones, cephalosporins, and imipenem. However, susceptibility varies greatly from strain to strain. The variability of its susceptibility was proven when Wong *et al.* (2000) reported that most *P. shigelloides* strains produced *β*-lactamases, and were resistant or partial resistant to ampicillin, tetracycline, co-trimoxazole, and chloramphenicol. In addition, according to Obi *et al.* (2007), *P. shigelloides* were

resistant to neomycin and chloramphenicol, but were susceptible to ciprofloxacin, ofloxacin, amikacin, meropenem, imipenem, and co-trimoxazole. There were contradiction in the reports by Groves (1996), Wong *et al.* (2000), and Obi *et al.* (2007), where Groves (1996) reported susceptibility to chloramphenicol, whereas Wong *et al.* (2000) and Obi *et al.* (2007) reported resistance to it. *Plesiomonas shigelloides* have become a major health problem since they acquired resistance to multiple antibiotics (Stock and Wiedemann, 2001).

Multiple antibiotic resistances are becoming increasingly widespread, and therefore antimicrobial agents are becoming less and less effective (Obi *et al.*, 2007). An increase in the resistance of human pathogen populations to currently available antibiotics is of primary concern to the medical community and pharmaceutical industry (Saadoun and Gharaibeh, 2003). The continuous emergence of pathogens that are resistant to multiple antibiotics has complicated the therapy of many otherwise simple infections, which necessitates the continuous search for the development of new antibiotics (Saadoun *et al*., 2008). To combat the increasing emergence of resistant pathogens, the availability of new lead structures and novel bioactive compounds is one of the most important requirements for a continuing source of therapeutics (Vikineswary, 2004). Microbial natural products are the origin of most of the antibiotics on the market today (Pelàez, 2006). Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years by scientists (Oskay *et al*., 2004). New antimicrobial agents with a broad spectrum of activity against these multi-resistant pathogens are urgently sought, and one of the approaches is to expand the screening activity of the actinomycetes as it harbors great numbers of antibiotic producers (Saadoun *et al*., 2008).

Actinomycetes are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics (Ceylan *et al*., 2008). One of the theories that may explain antibiotic production is that antimicrobial compounds help the actinomycetes compete with other organisms in the relatively nutrient-depleted environment by reducing competition (Zheng *et al*., 2000). Actinomycetes have been the focus of aggressive research efforts since the discovery of actinomycin in 1940 from *Actinomyces antibioticus* by Selman Waksman (Mincer *et al*., 2002). It has been estimated that approximately two-third of the thousands of naturally occurring antibiotics have been isolated from actinomycetes. Screening projects have focused on species of *Streptomyces* as a source of antibiotics for many years (Saadoun and Gharaibeh, 2002). *Streptomyces* spp. are the producer of about 75% of commercially and medically useful antibiotics (Peela *et al*., 2005).

Actinomycetes are Gram-positive bacteria with branched filaments, which have been considered as a group well separated from common bacteria because of their well-developed morphological and cultural characteristics (Das *et al*., 2006). Actinomycetes reproduce either by fission or by special spores or conidia. They usually form a mycelium which may be of a single kind, designated as substrate (vegetative), or of two kinds, substrate (vegetative) and aerial (in part sporogenous) (Marwick *et al*., 1999). Actinomycete strains can be divided into two major groups, based on the colour of aerial/substrate mycelium: streptomycete-like such as *Streptomyces* spp. and non-streptomycete-like such as *Micromonospora* spp. (Tan *et al*., 2004). They occur in a multiplicity of natural and man-made environments (Augustine *et al*., 2005b). Most species are aerobic, saprophytic, and mesophilic forms whose natural habitat is soil, where they contribute significantly to the turnover of complex biopolymers such as

lignocellulose, hemicellulose, pectin, keratin, and chitin (Mincer *et al.*, 2002). Actinomycetes may also be isolated from terrestrial and aquatic habitats, and recovered from the air, where they temporarily exist as spores or mycelial fragments (Demain, 1992). Actinomycetes are also present in many free-swimming marine vertebrates and invertebrates, as well as in sessile ones (Ward and Bora, 2006).

The discovery of new antibiotics reached a peak in the 1970s then declined in the late 1980s and 1990s due to a decrease in screening efforts rather than an exhaustion of compounds (Watve *et al.*, 2001). The screening and isolation of promising strains of actinomycete with potential antibiotics is still a thrust area of research. Shiburaj (2003) suggested that explorations of materials from new areas and habitats were needed urgently in the search for new microbes and novel metabolites. It should be noted that for antibiotic production, the number of marine microorganisms with antimicrobial activity might be higher than that of terrestrial ones. Therefore, marine microorganisms need to be explored and exploited for new biological products (Imada, 2005). Actinomycetes have been isolated from the marine environment, largely from sediment samples from the continental shelf, or from brackish environments such as salt marshes (Labeda and Shearer, 1990). Their populations are denser in shallow sea than in deep, particularly at the muddy surface of the bottom of shallow seas (Weyland and Helmke, 1988). Tan *et al*. (2004) demonstrated that 16% actinomycetes isolated from marine microorganisms collected offshore had moderate to good activity against both test fungi and bacteria. Grein and Meyer (1958); and Okazaki and Okami (1976) reported respectively that 50% and 27% of *Streptomyces* isolated from the marine environment showed antimicrobial activity, and these percentages were increased when the tests were conducted in the presence of seawater. The exploitation of marine actinomycetes has recently surpassed that of their terrestrial counterparts although

limited screening efforts have been dedicated to date to marine actinomycetes (Lam, 2006).

Most of the clinically important antibacterial agents such as streptomycin, chloramphenicol, chlortetracycline, neomycin, oxytetracycline, erythromycin, leucomycin, oleandomycin, cycloserine, kanamycin, and rifamycin were discovered as actinomycete products by the late 1950s. Several antifungal agents from actinomycetes such as cephalosporin C, variotin, and siccanin were also clinically introduced (Imada and Hotta, 1992). Gorajana *et al.* (2005) reported that 1-hydroxy-1-norresistomycin from *Streptomyces chibaensis* AUBN1/7 possessed antibacterial activities against Gram-positive and Gram-negative bacteria. *Streptomyces venezuelae* ISP5230 produced a group of polyketide-derived angucycline antibiotics, jadomycins, with broad-spectrum cytotoxic activities (Zheng *et al*., 2007). Tobramycin, produced by *Streptomyces tenebrarius*, is important for its activity on *Pseudomonas aeruginosa* and other difficult pathogens (Lancini and Lorenzetti, 1993). Marine actinomycetes may provide an alternative source of potential bioactive compounds against bacterial and fungal pathogens. Marine actinomycetes also seem to be a promising source as producers of drug candidates such as new anticancer drugs salinosporamide A produced by *Salinispora tropica* (Lam, 2006) and thiocoraline produced by a marine *Micromonospora* spp. which are under preclinical assessment, or the antiviral drug cyclomarin A produced by a marine *Streptomyces* spp. (Fiedler *et al*., 2005). Vikineswary *et al.* (2005) reported that 38% and 64% sponges-derived actinomycetes exhibited a broad spectrum of antibacterial and antifungal activities in primary and secondary screenings.

The discovery of new antibiotics and other bioactive microbial metabolites continues to be an important objective in new drug research. Extensive screening has led to the discovery of thousands of bioactive microbial molecules, therefore new approaches must be taken in order to reduce the probability of rediscovering known compounds (Busti *et al*., 2006). In recent years, the search for novel antimicrobial substances has included actinomycetes obtained from sources other than soil such as marine environments (Pisano *et al*., 1992). Novel secondary metabolites including antibiotics from marine bacteria are attracting attention because of the growing demand for new antibiotics (Marwick *et al*., 1999). As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have characteristics different from those of terrestrial actinomycetes, and therefore may produce different types of bioactive compounds (Imada, 2005). Screening and isolation of promising strains of actinomycete with potential antibiotics is urgent to counter the threats posed by the fast emerging phenomenon of antibiotic resistance (Shiburaj, 2003).

Objectives of study

The objectives of this study were to :

a) culture and screen selected strains of indigenous actinomycetes from marine ecosystem for antibacterial and antifungal activity,
b) select the strain that best inhibited *Plesiomonas shigelloides* and
c) optimize selected parameters for production of bioactive compound(s) from the actinomycete strain in submerged fermentation.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Human Pathogens

In the present study, three Gram-positive test bacteria namely *Bacillus subtilis*, *Enterococcus faecalis*, and *Staphylococcus aureus*, two Gram-negative test bacteria; *Plesiomonas shigelloides* and *Pseudomonas aeruginosa*, and two yeasts; *Candida albicans* and *C. parasilopsis* were used to investigate the antagonistic bioactivity of the selected marine actinomycete strains. The test pathogens selected in this study displayed at least one of the criteria of target organisms for antimicrobial research and development. As described by Thompson *et al.* (2004), the criteria for antimicrobial research and development include :

a) organism of sufficient prevalence in population with disease under study,

b) organism causes serious and severe disease,

c) drug to which organism is resistant is commonly used in disease under study,

d) limited available therapies as a results of multidrug-resistance,

e) drug used to control spread of disease in population, and

f) clinical correlation of *in vitro* resistance with poor clinical outcome.

The test pathogens showed resistance to a number of front line antibiotics. These resistances have hindered antibiotic therapy, and caused reduction in key therapeutic options (Levy, 2005). Resistance continues to compromise the use of old and new antimicrobials alike. The clinical impact of resistance often because of inappropriate initial antimicrobial therapy is characterized by increased cost, length of hospital stay, and mortality (Poole, 2005). Therefore, the discovery of new antibiotics is crucial to combat these emerging phenomenons of antibiotic resistances.

2.2 Pathogenic Bacteria

2.2.1 *Plesiomonas shigelloides*

2.2.1.1 Introduction on *Plesiomonas shigelloides*

The genus *Plesiomonas* appears to occupy a position between the families *Enterobacteriaceae* and *Vibrionaceae* in the gamma group of Proteobacteria (Rager *et al.*, 2000). Initially, *Plesiomonas shigelloides* resided in the family *Vibrionaceae*, and was classified based on phenotypic characteristics such as polar flagella, oxidase production, and fermentation properties. However, phylogenetic analysis and assessment deducted from analysis of 16S rRNA gene sequences indicated a closer relationship to the family *Enterobacteriaceae* (Niedziela *et al.*, 2002; Woo *et al.*, 2004). Thus, the genus *Plesiomonas* was proposed to be moved to the family *Enterobacteriaceae*, either as a member of the genus *Proteus*, or as the genus *Plesiomonas*. The species name "shigelloides" was derived from the fact that many strains cross-reacted antigenically with *Shigella*, particularly *Shigella sonnei* (Horneman and Morris, 2007). Since 2001, *P. shigelloides* belongs to the family *Enterobacteriaceae* (González-Rey, 2003). The organism is the only species in the genus *Plesiomonas* (Theodoropoulos *et al*., 2001; Perales, 2003; Lukasiewicz *et al.*, 2006).

Plesiomonas shigelloides was first isolated in 1947 by Ferguson and Henderson from a human stool sample without evidence of intestinal disease. This organism was described as a motile organism possessing the major somatic antigen of *Shigella sonnei* phase I, and was named Paracolon C27. Since then, this microorganism had been renamed several times. It had been variously known as *Pseudomonas michigani*,

Aeromonas shigelloides, Fergusonia shigelloides, and *Vibrio shigelloides* (Ingram *et al*., 1987; Oviasogie and Ekhaise, 2006).

Plesiomonas shigelloides is a Gram-negative, capsulated and flagellated rod with rounded ends, motile, and non-spore forming. The size of a single cell is 0.7-1 µm x 2.1-3 µm. It is a facultative anaerobic bacterium which catabolises carbohydrates with acid production without gas, and produces cytochrome oxidase and catalase, but no diastase, lipase, or DNase. The G+C content of its DNA is 51 mol% (Chou *et al*., 1991; González-Rey, 2003; Lukasiewicz *et al.*, 2006). According to Stock and Wiedemann (2001), *P. shigelloides* is thermotolerant and pleomorphic. It does not grow at temperatures below 8ºC, but grows well at 35ºC, and produce non-haemolytic colonies within 24 hours (Groves, 1996; Wong *et al.*, 2000). The primary reservoirs of *P. shigelloides* are freshwater and estuarine water in temperate climates throughout the world, while fish and different kinds of seafood act as secondary reservoirs in these environments. This bacterium has also been isolated from humans as well as domestic animals such as dogs, cats, goats, sheep, and cows (Krovacek *et al*., 2000). The bacterium is not a part of the normal human faecal flora (Niedziela *et al.,* 2002). Human infections with *P. shigelloides* are mostly related to drinking untreated water, eating uncooked shellfish, and visiting countries with low sanitary standards. There have been reports of outbreaks attributed to contaminated water in Japan, consumption of freshwater fish in Zaire, and contaminated raw oysters and shellfish in the United States. In a case-control study from the United States in the 1980s, two factors that were strongly associated with infection with *P. shigelloides* were foreign travel and the consumption of raw oysters (Wong *et al.,* 2000). Recent studies implicated *P. shigelloides* as an opportunistic pathogen in immunocompromised hosts, and especially neonates (Niedziela *et al.,* 2002).

2.2.1.2 Pathogenesis and Virulence Factors of *Plesiomonas shigelloides* Infection

Plesiomonas shigelloides has been indicated as being an enteric pathogen in various clinical and epidemiological studies. However, an understanding of the mechanism of pathogenesis has been elusive because information on the virulence factors is still scarce. Furthermore, previous *in vitro* studies of its pathogenesis have been inconclusive (Kain and Kelly, 1989; González-Rey, 2003). Several virulence-associated factors have been described in strains of *P. shigelloides,* but most have the potential to cause infection even in the absence of such virulence factors (Avison *et al*., 2000). Vitovec *et al.* (2001) and Okawa *et al*. (2004) described that this bacteria possessed several seemingly pathogenic properties such as the production of heat-stable and heat-labile exotoxins, cytotoxins, haemolysin, hemaglutinin, and other potentially virulence factors. The natural resistance is likely to be attributed to the *Plesiomonas* outer membrane, which might prevent the entry of these antibiotics into the cell (Stock and Wiedemann, 2001). Endotoxin, the main surface antigen of Gram-negative bacteria, was found as a constituent of cytotoxin complex. *Plesiomonas shigelloides* cytotoxin is a new type of cytolytic enterotoxin distinct from cholera toxin. The cytotoxin of *P. shigelloides* is a complex of lipopolysaccharide (LPS) and anti-cholera toxin-reactive proteins (ACRP). LPS plays an important role for the effective barrier properties of the outer membrane. It constitutes a 'pathogen-associated molecular pattern' for host infection, and is one of the most powerful natural activators of the innate immune system (Lukasiewicz *et al.*, 2006).

The pathogenicity of a bacterial strain depends on both inherent bacterial and host factors. *Plesiomonas shigelloides*, like *Escherichia coli*, might consist of both

pathogenic and non-pathogenic strains. Pathogenicity might also depend on such factors in the host such as age and immunological status (Olsvik *et al*., 1990). The route of entry into the human and animal gastrointestinal tract seems to be the ingestion of *P. shigelloides*-contaminated food or water. Two features thought to play key roles in the regulation of gastrointestinal infection by enteropathogenic bacteria are gastric acidity and the presence of resident microbial flora in the lower gut. Gastric juices are extremely acidic in the healthy adult, with a pH of 2 or less. This acidic environment is surmised to act as an obstacle to enteric pathogens since bacteria are rapidly killed in the presence of hydrochloric acid (Janda, 1987). Lukasiewicz *et al.* (2006) reported that *P. shigelloides* entered the human intestinal Caco-2 cells *in vitro* through a phagocytic-like process. Moreover, it was found that live bacteria escaped from cytoplasmic vacuoles, and induced apoptotic cell death. According to Abbott *et al.* (1991), *P. shigelloides* possession of a large plasmid (approximately 200 MDa) might facilitate its uptake, or invasion in the gastrointestinal tract.

2.2.1.3 Clinical Manifestations of *Plesiomonas shigelloides* Infection

Plesiomonas shigelloides is known to cause either secretory or invasive diarrhoea, which is defined as the discharge of three or more loose stools in a 24-hour period. In healthy individuals, the diarrhoea is usually mild and self-limiting. However, the diarrhoea may cause a cholera-like illness, with numerous bowel movements occurring during the peak of the illness. Most patients recover spontaneously within four weeks of the onset of symptoms, but up to 32% may remain chronically symptomatic (Wong *et al.*, 2000). *Plesiomonas shigelloides* can cause three major types of gastroenteritis: (1) a secretory, watery form; (2) an invasive, dysentery-like form; and (3) a subacute or chronic form lasting between two weeks and three months

(Vitovec *et al*., 2001). Symptoms associated with gastroenteritis caused by *P. shigelloides* are diarrhoea, abdominal pain, nausea, chills, headache, fever, and vomiting. Although there are reports of bloody stools, most of the stools from patients with diarrhoea are described as watery. Incubation time varies from 24 to 50 hours, and symptoms generally last for one to nine days, although a more invasive *Shigella*-like type can last from two weeks to three months (González-Rey, 2003).

The outbreaks of travellers' diarrhoea in Japan and China are likely to be predominantly associated with *Plesiomonas* as it is ranked third among the aetiological agents (Lukasiewicz *et al.*, 2006). Traveller's diarrhoea is a syndrome that occurs when people cross international borders from the developed to tropical or semitropical developing countries. It is usually defined as the passage of at least three unformed stools within a 24-hour period; in association with at least one symptom of gastrointestinal disease such as nausea, vomiting, fever, abdominal pain or cramps, tenesmus, faecal urgency, or the passage of bloody or mucoid stools (Gomi *et al*., 2001; Diemert, 2006). Typically, symptoms develop within the first week of travel, and more than 90% of cases occur within the first two weeks. Normally, the symptom is between four and five loose or watery stools a day with little to no fever, without treatment; the diarrhoea usually lasts for only three to four days before resolving spontaneously in most cases. However, traveller's diarrhoea can result in disruption to an individual's trip. Severe diarrhoea can cause water and electrolyte losses, leading to significant dehydration, electrolyte imbalances, and even impairment of renal function. In most cases, traveller's diarrhea is neither life threatening nor severe. Therefore, treatment is for minimising the symptoms and duration of illness (Diemert, 2006).

Plesiomonas shigelloides has occasionally caused extraintestinal diseases in both immunocompromised and immunocompetent patients. Such infections include septicaemia, meningitis in neonates, cellulitis, septic arthritis, endophthalmitis, and acute cholecystitis. Approximately 70% of patients with plesiomonal diarrhoea have either an underlying disease such as cancer or cirrhosis, or an identifiable risk factor such as foreign travel, or the consumption of seafood or uncooked food. Patients with underlying carcinoma of the bowel had been reported to be susceptible to infection with *P. shigelloides* (Wong *et al.,* 2000). Obi and Bessong (2002) reported that *P. shigelloides* was one of the main bacterial pathogens that caused chronic diarrhoea in HIV-positive patients. Bacteraemia and pseudoappendicitis are also associated with its infection (Henderson *et al.,* 2001). The majority of bacteraemia cases that had been reported so far involved immunocompromised states either because of prematurity or because of an underlying disease such as Hodgkin's disease, sickle cell disease, Felty's syndrome, or alcoholic liver disease (Paul *et al*., 1990; Wong *et al.,* 2000). Table 2.1 below shows *P. shigelloides*-associated infections.

Table 2.1 *Plesiomonas shigelloides*-associated infections

Type	Reference
Acute secretory gastroenteritis	Henderson *et al.* (2001)
Invasive shigellosis-like disease	
Cholera-like illness	
Pancreatic abscess	Woo *et al*. (2004)
Spontaneous bacterial peritonitis	
Sepsis	Stock and Wiedemann (2001)
Pseudoappendicitis	Henderson *et al.* (2001); Okawa *et al.* (2004)
Cholecystitis	Wong *et al*. (2000); Woo *et al.* (2004)
Osteomyelitis	Stock and Wiedemann (2001); Woo *et al*. (2004)
Septic arthritis	Wong *et al*. (2000); Okawa *et al.* (2004); Woo *et al*. (2004)
Cellulitis	
Endophthalmitis	Wong *et al*. (2000); Woo *et al.* (2004); Lukasiewicz *et al.* (2006)
Bacteraemia	Henderson *et al.* (2001); Woo *et al.* (2004); Lukasiewicz *et al.* (2006)
Septicaemia	Wong *et al*. (2000); Okawa *et al*. (2004); Wiegand and Burak (2004); Lukasiewicz *et al.* (2006)
Meningitis	Wong *et al*. (2000); Henderson *et al.* (2001); Stock and Wiedemann (2001); Okawa *et al.* (2004); Woo *et al*. (2004); Lukasiewicz *et al.* (2006)

2.2.1.4 Antibiotic Susceptibility and Resistance of *Plesiomonas shigelloides*

Antibiotic therapy is not usually necessary to manage infection with *P. shigelloides* because of the self-limiting nature of the illness in the majority of patients. Nevertheless, the infection responds well to antibiotic therapy, which leads to a shorter illness compared with that in untreated patients. Patients with severe and protracted

symptoms, extra-intestinal infection, or a serious underlying disease may benefit from receiving the antibiotic therapy (Wong *et al.,* 2000). Groves (1996) reported that the susceptibilities of *P. shigelloides* vary greatly from strain to strain. Summarisation on the susceptibilities of *P. shigelloides* had been made based on *in vitro* studies reported by Wong *et al.* (2000), Stock and Wiedemann (2001), and Woo *et al.* (2004). It is shown that *P. shigelloides* strains were naturally susceptible or naturally susceptible and intermediate susceptible to quinolones, several aminoglycosides, imipenem, carbapenems, aztreonam, trimethoprim, sulfamethoxazole, azithromycin, nitrofurantoin, fosfomycin, cephalothin, cefuroxime, gentamicin, ciprofloxacin, and aminopenicillins in combination with β-lactamase inhibitors, and all selected cephalosporins such as cefotaxime and ceftriaxone, but except cefoperazone and cefepime. These authors also described that *P. shigelloides* strains were sensitive to ofloxacin or levofloxacin and ceftriaxone.

Medical microbiologists confine the use of the term resistance to changes in the susceptibility of a previously susceptible organism to such an extent that it no longer responds to treatment (Gilbert *et al.*, 2002). Persistent circulation of resistant bacteria strains in the environment, and the possible contamination of water and food have caused antimicrobial resistance. Several authors suggested that administration of antibiotics to food-producing animals for therapeutic purposes, or as growth promoters might be a primary factor in selecting for antimicrobial-resistant bacterial pathogens (Normanno *et al.*, 2007). In the South East Asian study, as reported by Reilly and Kaferstein (1997), it was found that the strains isolated were resistant to tetracycline caused by the attribution to the presence of antibiotic supplements in the poultry feed. On a cautionary note, tetracycline is one of the effective antibiotics to treat severe human illness due to *P. shigelloides.*

Antimicrobial therapy is indicated for moderate to severe disease to reduce the duration of illness. Traditionally, ampicillin, trimethoprim, or sulfamethoxazole and doxycycline had been used for the treatment of traveller's diarrhoea, while more recently fluoroquinolones had been recommended as the drugs of choice. Resistance to commonly used antimicrobial agents among enteric bacterial pathogens was an increasing problem, and had been reported worldwide (Gomi *et al*., 2001). According to Wong *et al*. (2000), most *P. shigelloides* strains produced *β*-lactamases, and were resistant or partial resistant to ampicillin, tetracycline, co-trimoxazole, and chloramphenicol. Stock and Wiedemann (2001) reported that *Plesiomonas* strains were naturally resistant and intermediate to streptomycin, erythromycin, and rifampicin. It was shown that *P. shigelloides* was naturally resistant to *β*-lactam antibiotics, namely benzylpenicillin, oxacillin, amoxicillin, acylaminopenicillins (piperacillin, mezlocillin, azlocillin), and ticarcillin. Whereas, *P. shigelloides* was naturally resistant to non-*β*-lactams such as most macrolides, lincosamides, streptogramins, glycopeptides, and fusidic acid. According to Gonzalez-Rey *et al*. (2004), vancomycin-resistant plesiomonads were also reported.

2.2.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa has been one the major pathogens responsible for a wide variety of infections and illness. It is particularly problematic for patients in intensive care units, where ventilator-associated pneumonia remains a serious complication of therapy of those with an underlying immunocompromised state such as arises from severe burns, human immunodefiency virus (HIV) infection, or chemotherapy for cancer, for patients with indwelling urinary catheters and venous-access catheters or other artificial medical devices (Pier, 2003). *Pseudomonas*

aeruginosa is mostly a nosocomial pathogen that is becoming increasingly multidrug resistant, commonly to β-lactams antibiotics such as imipenem (Hanberger *et al*., 2001; Corvec *et al*., 2008). It causes a wide array of community acquired clinical infections, including endocarditis, otitis externa, osteomyelitis, folliculitis (Foca, 2002), and eye infections resulting in the loss of sight (Hugo and Russell, 1992). *Pseudomonas* is a major cause of nosocomial pneumonia, urinary tract infections, and bloodstream infections or sepsis (Foca, 2002). Human carriage of the *P. aeruginosa* is uncommon as part of the normal microbial flora unless the person is hospitalised or an immunocompromised host. In these persons, the most frequent site of colonisation is the gastrointestinal tract followed by other moist body sites, including throat, nasal mucosa, axillae, and perineum (Chakraborty, 2004). *Pseudomonas aeruginosa* is an important endobronchial pathogen associated with morbidity and mortality in patients with cystic fibrosis (Morlin *et al*., 1994). It is a secondary infector of wounds, especially burns, but is not necessarily pathogenic. With the advent of immunosuppressive therapy following organ transplant, systemic infections including pneumonia have resulted from infection by this organism (Hugo and Russell, 1992).

2.2.3 *Bacillus subtilis*

Bacillus spp. are more usually associated with food poisoning, or they are dismissed as contaminants in clinical samples. Serious infections may occur, including meningitis, endocarditis, endophthalmitis, respiratory infection (pneumonia, abscess, and pleuritis), surgical wound infections, and severe bacteraemia in cancer patients (Ozkocaman *et al*., 2006). Beebe and Koneman (1995) cited a case of *B. subtilis* sepsis in a patient with acute myeloblastic leukaemia. *Bacillus* spp. other than *B. anthracis* caused serious infections, often in trauma, post surgical, and burn cases, and with

predisposing conditions that included alcoholism, diabetes, sickle-cell trait, and cancer. Mortality associated with disseminated *Bacillus* infections is high. Severe neutropenia is also associated in leukaemia cases with bacteraemia. Pneumonia commonly occurs because of haematogenous dissemination of the organism. *Bacillus subtilis* can cause food-borne gastroenteritis, septicaemia, peritonis, ophthalmitis, and catheter-related bloodstream infection (CRBSI) (Ozkocaman *et al*., 2006).

2.2.4 *Enterococcus faecalis*

Enterococcus faecalis is an indigenous flora in the human bowel. Although these pathogens are rarely associated with primary infections in the noncompromised host, they commonly cause nosocomial infections in hospitalised or immunocompromised patients (Takahashi *et al*., 1999; Elsner *et al*., 2000). It is an opportunistic pathogen that can cause urinary tract infections and endocarditis (Willey *et al*., 2006). Enterococcal endocarditis tends to be more subacute than endocarditis caused by other microbial agents. *Enterococcus faecalis* can also cause pneumonia, or bacteraemia (Sijpkens *et al*., 1995). Aminoglycoside resistance is seen among *E. faecalis*, where there are increasing reports of resistance towards gentamicin and vancomycin (Hanberger *et al*., 2001; Inglis, 2003).

2.2.5 *Staphylococcus aureus*

Staphylococcus aureus is a food-borne pathogen. Staphylococcal food poisoning (SFP) is one of the leading causes of food-borne diseases (Normanno *et al*., 2007). *Staphylococcus aureus* usually grows on the nasal membranes and skin, and is also found in the gastrointestinal and urinary tracts of warm-blooded animals. It can

cause boils, abscesses, wound infections, pneumonia, toxic shock syndrome (Willey *et al*., 2006), and is also the most common bacterial cause of conjunctivitis, orbital cellulites, septic arthritis, omphalitis, and haematogenous osteomyelitis (Inglis, 2003). Todar (2006) added that some strains could also cause pimples, impetigo contagiosa, staphylococcal scalded skin syndrome (SSSS), and septicaemia. According to Singh *et al*. (2000), *Staphylococcus* spp. were multi-resistant to antibiotics like *β*-lactams, tetracyclines, streptomycin, tobramycin, chloramphenicol, quinolones, and rifampin. Gravet *et al.* (1999) reported that meticillin- and gentamicin-resistant *S. aureus* strains were recognized as the causes of outbreaks of enteritis in hospitalised patients who were treated with extended-spectrum antibiotics, and in whom mild to fatal illnesses were observed. Infections caused by vancomycin-resistant *S. aureus* generally cannot be treated by antibiotic therapy because vancomycin is considered the "drug of last resort" (Willey *et al*., 2006). *Staphylococcus aureus* is also known as the leading cause of hospital-acquired nosocomial infections. It is feared as the causative agent for post-operative wound infections (Verhoef and Fluit, 2006).

2.3 Pathogenic Yeasts

Candida spp. have become a common cause of nosocomial bloodstream infection (Branchini *et al*., 1994). Septic arthritis (Inglis, 2003), oral candidiasis in newborns, paronychia, onychomycosis, candidal vaginitis, balanitis, AIDS arthritis, endophthalmitis, meningitis, myocarditis, myositis, and peritonitis are associated with *Candida* infections (Willey *et al*., 2006). Candidiasis is the most common mycosis, especially in hospitalised patients (Wroblewska *et al*., 2002) such as immunocompromised AIDS patients, and after prolonged antibiotic therapy and invasive surgery (Chakraborty, 2004). The dimorphic fungus, *C. albicans* is one of the

most frequent etiologic agents causing candidiasis that can be fatal when progressing to systemic dissemination (Villamón *et al*., 2004). *Candida albicans* is a part of the normal microbial flora that colonizes mucocutanous surfaces of the oral cavity, gastro-intestinal tract, and vagina. *Candida albicans* can cause life-threatening systemic disease in immunocompromised host who have congenital, induced, or disease-related immune dysfunction such as post surgical, burn, leukaemia, organ-transplanted, diabetic, and HIV-infected patients, or low-birth-weight infants, or patients with a congenital defect in neutrophil function (Buluc *et al*., 2005). Nasser *et al*. (2003) reported that *C. albicans* had been recovered from burn wounds. *Candida* spp. are harmless saprophytes as long as they colonize the burn wound, but as invaders of viable subeschar tissue, or the blood stream, they are dangerous pathogens with a mortality rate exceeding 90%. *Candida parasilopsis* is a nosocomial pathogen, which causes the rare prosthetic valve endocarditis (Tan *et al*., 2004), and is well known as a cause of fungaemia and invasive candidiasis associated with parenteral hyperalimentation, intravascular devices, and contaminated ophthalmic solutions (Branchini *et al*., 1994). According to Walsh (1992), The National Nosocomial Infections Survey from the Centers for Disease Control estimated that the frequency of deeply invasive candidiasis had increased nearly tenfold during the past decade. The limited available antifungal antibiotics contrast greatly with the successful discovery of antibacterial antibiotics due to the following reasons; (a) selective toxicity is less likely to occur with antifungal than with antibacterial agents because fungi are eukaryotes, as are animal and human cells, (b) methods in antifungal screening have not progressed rapidly, including the lack of standard methods for *in vitro* evaluation of antifungal activity, reliable animal models for evaluation of *in vitro* efficacy, and sensitive and selective techniques for diagnosis of fungal infections, (c) antifungal drugs picked up to date by *in vitro* screening show serious cytotoxicity, and (d) the host defence system and drug action

are the two major factors in successful antimicrobial chemotherapy. However, the host defence system is not expected to function well in fungal diseases because fungal infection is mostly associated with depression in host immune activity (Tanaka, 1992).

2.4 General Overview on Actinomycetes

The actinomycetes are high G+C Gram-positive bacteria that belong to the order *Actinomycetales* of class *Actinobacteria*. The actinomycete colonies can easily be distinguished on the plate from fungi and bacteria. They are recognized by their characteristic tough, often leathery, giving a conical appearance, dry surface, branched vegetative mycelia, and when present, aerial mycelia and spore formation (Jensen *et al*., 1991; Marwick *et al*., 1999). They are diverse group of heterotrophic prokaryotes forming hyphae at some stage of their growth, hence referred as filamentous prokaryotes (Augustine *et al*., 2005b). The filamentous growth and true branching of the actinomycetes differentiate these organisms from the true bacteria (Arai, 1976). When growing on solid substratum such as soil or agar, the actinomycetes develop a branching network of hyphae. The hyphae grow both on the surface of the substratum and into it to form a dense mat of hyphae, termed a substrate mycelium. Septae usually divide the hyphae into long cells (20 μm and longer), containing several nucleoids. In many actinomycetes, substrate hyphae differentiate into upwardly growing hyphae to form an aerial mycelium that extends above the substratum. Medically useful compounds, often called secondary metabolites are formed at this time. The aerial hyphae form thin-walled exospores upon septation. Like spore formation in other bacteria, actinomycete sporulation is usually in response to nutrient deprivation (Wiley *et al*., 2006).

Actinomycetes are present in all types of soil, fresh and marine waters, and plant debris (Rabeh *et al.*, 2007). They are found in the mud or the bottom of ponds, lakes, streams, and rivers. In freshwater environments, the numbers observed are extremely low, most likely reflection of contamination of water with soil or mud. Thermophilic actinomycetes are found in silage, dung, and other thermal environments (Labeda and Shearer, 1990). Actinomycetes play both detrimental and beneficial roles in nature. Among their negative attributes are their opportunistic pathogenic natures in diseases of animals, human, forestry, and plants such as "farmers' lung", hypersensitivity pneumonitis, in water pollution, formation of scums and foams in sewage treatment plants, and destroying valuable materials through biodeterioration. However, biodegradation by these organisms is useful in waste removal, and as an integral part of recycling materials in nature. Other beneficial roles are their activity as biological control agents of fungal disease. In addition, they also enhance plant growth by unknown mechanisms (Demain, 1992). As cited by Ismet (2003), filtrates and eluates of actinomycetes cultures have stimulated growth of maize similarly to auxins and gibberellins. The utmost importance of actinomycetes is the production of antibiotics and antitumour agents. Furthermore, they are prolific producers of enzyme inhibitors and immunomodifiers (Demain, 1992).

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics, antitumour agents, immunosuppressive agents, and enzymes (Lam, 2006). Actinomycetes are a source of structurally diverse natural products, possessing broad ranges of biological activities such as antibiotic (erythromycin and tetracycline), anticancer (mitomycin and daunomycin), immunosuppressant (rapamycin and FK506), and veterinary

(thiostrepton and monensin) agents (Das *et al.*, 2006). Actinomycetes have been famous as producers of antibiotics and other "secondary metabolites" with biological activity since the discovery of actinomycin in Selman Waksman's laboratory at Rutgers University in 1940, followed in 1943 by streptomycin, the first effective drug to treat tuberculosis. During the Golden Age of antibiotic discovery, in the 50s' and 60s' of the 20th century, antibacterial agents such as tetracycline, erythromycin, and kanamycin; and antifungal agents like candicidin and nystatin were discovered (Challis and Hopwood, 2003). In the 60s' and 70s', 75% to 80% of all discovered antibiotics derived from the order *Actinomycetales*, mainly from *Streptomyces* spp. In the 70s' and 80s', the ratio and significance of the other non-streptomycete actinomycetes (so called rare actinomycetes) increased up to 20% of all microbial antibiotics, and 30% to 35% of *Actinomycetales* species (Moncheva *et al.*, 2000-2002). Commercially important products such as antibiotics and enzymes are produced by a range of different genera (Labeda and Shearer, 1990). As highlighted by Jensen *et al.* (2005), actinomycetes accounted for approximately 7000 of the compounds reported in the Dictionary of Natural Products. In the past two decades, there has been a decline in the discovery of new lead compounds from common soil-derived actinomycetes as culture extracts yield unacceptably high numbers of previously described metabolites. For this reason, the cultivation of rare or novel actinomycete taxa has become a major focus in the search for the next generation of pharmaceutical agents (Mincer *et al*., 2002).

2.5 The Quest for Marine Actinomycetes

Actinomycetes in marine and estuarine sediments have not been well documented (Imada, 2005). Initially, according to review by Jensen *et al.* (1991), the origin of actinomycetes in marine habitats and to what extent these bacteria represent a

physiologically active component of the marine microbial community was unknown. Although the ecological roles of marine actinomycetes remain undefined, it is possible that, like their terrestrial counterparts, they are involved in the decomposition of recalcitrant organic materials such as chitin, a biopolymer that is particularly abundant in the sea (Jensen *et al*., 2005). Scepticism about the existence of indigenous populations of marine actinomycetes arising from the fact that the terrestrial bacteria produced resistant spores that were transported from land into sea, where they remained available but dormant for many years, or where the bacteria are exposed to water with salt concentrations and temperatures that differ from those of the terrestrial environment. As a result, some metabolic changes may occur in the organisms. Thus, it is generally assumed that actinomycetes isolated from marine samples are of terrestrial origin (Imada, 2005; Lam, 2006). According to Jensen *et al*. (1991), this conclusion was based on the findings that actinomycetes were more abundant in terrestrial soils relative to marine sediments, showed varying degrees of salt tolerance, and produced spores that were undoubtedly washed in large numbers from shore into the sea. Besides that, studies had shown that actinomycetes could grow in a seawater-based medium with increased hydrostatic pressures. The occurrence of *Micromonospora* increased with the increasing depth in deep-sea sediments, however due to the common observation that actinomycetes decreased in number as distances from shore increased, and a lack of experimental evidence of the distribution and metabolic activity of these bacteria in marine habitats, have led the authors to such conclusion.

This view is now changing with the discovery of bona fide marine actinomycetes (Imada, 2005). Reports by Mincer *et al*. (2002) showed the first evidence for the existence of widespread populations of obligate marine actinomycete, where about 99% actinomycete strains displayed an obligate requirement of seawater

for growth. More evidence is growing to support the view that the bona fide indigenous marine actinomycetes indeed exist in the oceans. These include members of the genera *Dietzia, Rhodococcus, Streptomyces, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium, Williamsia,* and *Verrucosispora*. Among these, the most exciting finding is the discovery of the first obligate new marine actinomycete genus, *Salinispora* that is formerly known as *Salinospora* (Mincer *et al*., 2005; Lam, 2006).

Recent findings as reported by Lam (2006) confirmed the presence of indigenous marine actinomycetes in the oceans, and indicated that they were widely distributed in different marine environments and habitats. Both culture-dependent and culture-independent methods demonstrated that novel actinomycetes can be found everywhere in the oceans namely deep sea floor, coral reef, sediments, invertebrates, and plants. Actinomycetes are present over the complete depth range found in the ocean realms, from the surface of the oceans such as in the near-shore and inter-tidal environments in French Guiana and Korean tidal flats, right down to the deepest abyss, such as below sub-floor sediments. Although many actinomycetes from shallow sea resemble those of terrestrial habitat in terms of their morphology and features, they have high salt-tolerance compared to those of terrestrial origin (Vikineswary *et al*., 1997). In actinomycetes isolation, marine organisms yielded an average of ten morphologically different strains per single source: 31% being streptomycetes, and 69% belonging to rare genera mainly represented by micromonosporas, followed by nocardioforms and actinomaduras: microtetrasporas. Therefore, in terms of abundance of both types of microbial communities, sponges could be considered a more suitable source than sediments. The abundance of microorganisms isolated is different in the different species of sponges (Sponga *et al*., 1999). Novel actinomycete groups have

been found in the sponges such as *Rhopaloeides odorabile*, *Pseudoceratina clavata*, *Candidaspongia flabellate*, *Aplysina aerophoba*, and *Theonella swinhoei* (Bull *et al*., 2000).

2.6 Marine and Marine-Derived Actinomycetes as a Source for Novel Metabolites

In the past fifty years, a significant amount of effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs. However, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased (Lam, 2006). The decreasing rate of discovery of novel drugs from established terrestrial sources has motivated the evaluation of new sources of chemically diverse bioactive compounds (Magarvey *et al.,* 2004). Strains isolated from the marine environment represent a relatively unexplored frontier for the discovery of new actinomycete biodiversity, and a resource for novel secondary metabolites (Mincer *et al*., 2005). As marine environment become a prime resource in search and discovery for novel natural products and biological diversity, marine actinomycetes turn out to be important contributors (Ward and Bora, 2006).

The oceans that covered more than 70% of the surface of the earth represent an underexplored environment for microbial discovery. Although new methods are under development, relatively few have been applied to reveal the microbial diversity of the ocean environment (Magarvey *et al.,* 2004). Lam (2006) stated that the distribution of actinomycetes in the sea was largely unexplored, and the presence of indigenous marine actinomycetes in the oceans remained elusive. Actinomycetes comprise about 10% of bacteria colonizing marine aggregates, and can be isolated from marine

sediments, including those obtained at depths of 10,898 m from the deepest part of the Mariana Trench. Many actinomycete strains from this deep-ocean source contain NRPS and PKS pathways, the hallmarks of secondary metabolite production (Baltz, 2007). Lam (2006) described that as marine environmental conditions were extremely different from terrestrial ones, it was surmised that marine actinomycetes had different characteristics, and might produced different types of bioactive compounds from those of terrestrial counterparts.

The importance of marine sources for the discovery of novel natural products with a pharmaceutical potential has been proved during the last decade and was emphasized in various review articles (Fiedler *et al.*, 2005; Gorajana *et al*., 2005). As reported by Pisano *et al.* (1987), several studies demonstrated that actinomycetes of the marine origin produced novel bioactive substances. Actinomycetes that produce bioactive secondary metabolites are common within the complex bacterial communities of prolific producer of novel metabolites sponges (Ward and Bora, 2006). Furthermore, Jensen *et al*. (2005) suggested that taxonomically unique population of marine actinomycetes had added an important new dimension to microbial natural product research due to the recent discovery of novel secondary metabolites from these bacteria. Examples of natural products produced by marine-derived actinomycetes are shown in Figure 2.1. Lam (2006) also reported that numerous novel metabolites had been isolated from actinomycetes that evolved from and adapted to the unique marine ecosystems even though the exploitation of marine actinomycetes as a prolific source for discovery of novel secondary metabolites was at an early stage. Examples of novel secondary metabolites isolated from marine actinomycetes from 2003 to 2005 are shown in Table 2.2.

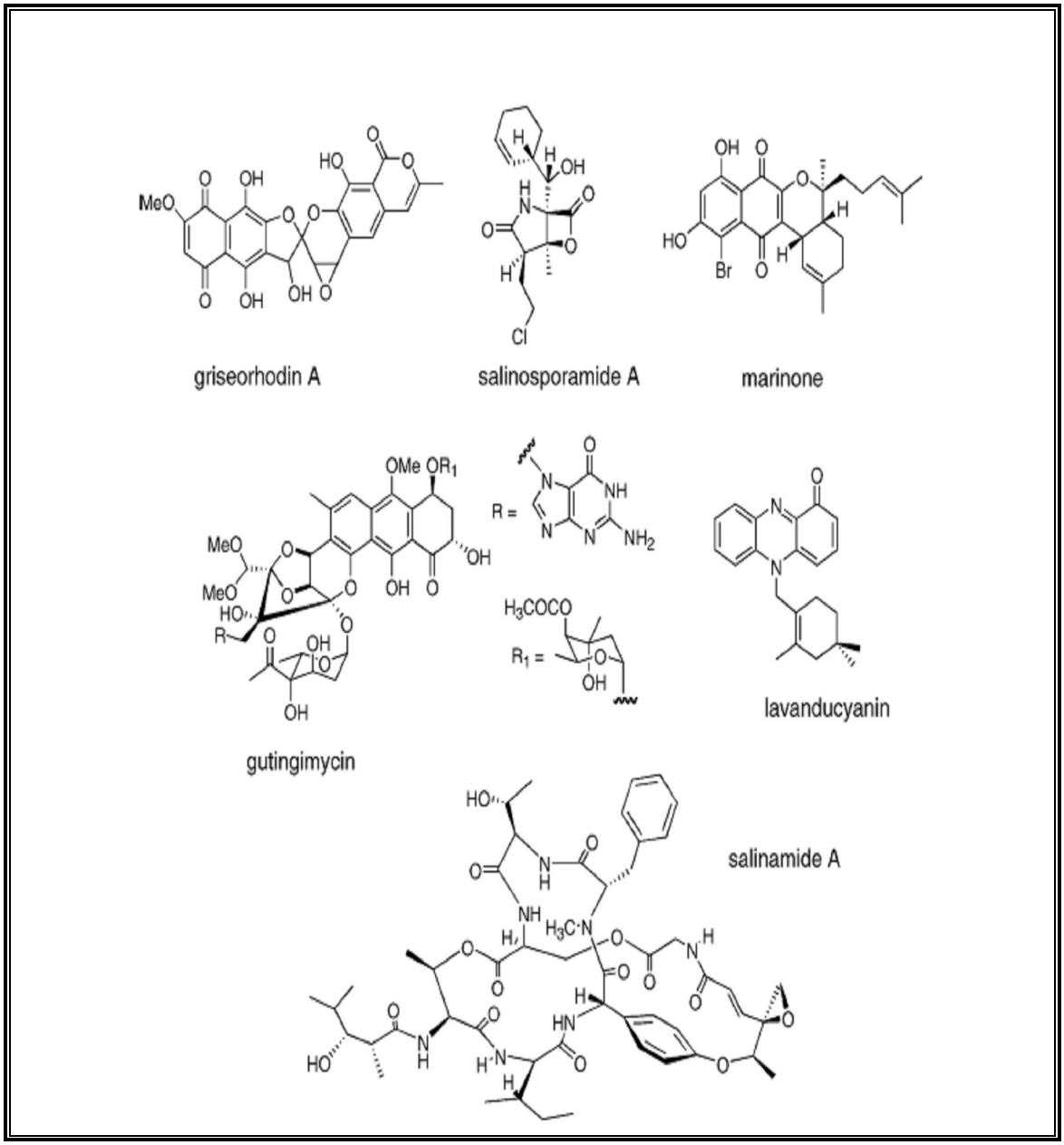


Figure 2.1 A selection of natural products produced by marine-derived actinomycetes. Griseorhodin A from an ascidian-derived actinomycete, salinosporamide A from *Salinospora* spp., marinone and lavanducyanin from *Streptomyces* spp. CNH-099, gutingimycin from *Streptomyces* spp. B8652, and salinamide A from *Streptomyces* spp. CNB-099 (Moore *et al.*, 2005)

Table 2.2 Novel metabolites produced by marine actinomycetes during the period 2003-2005

Compound	**Source**	**Activity**
Abyssomicins	*Verrucosispora* spp.	Antibacterial
Aureoverticillactam	*Streptomyces aureoverticillatus*	Anticancer
Boractin	*Streptomyces* spp.	Antibacterial; antifungal
Caprolactones	*Streptomyces* spp.	Anticancer
Chinikomycins	*Streptomyces* spp.	Anticancer
Chloro-dihydroquinones	Novel actinomycete	Antibacterial; anticancer
Diazepinomicin (ECO-4601)	*Micromonospora* spp.	Antibacterial; anticancer; anti-inflammatory
Frigocyclinone	*Streptomyces griseus*	Antibacterial
Glaciapyrroles	*Streptomyces* spp.	Antibacterial
Gutingimycin	*Streptomyces* spp.	Antibacterial
Helquinoline	*Janibacter limosus*	Antibacterial
Himalomycins	*Streptomyces* spp.	Antibacterial
IB-00208	*Actinomadura* spp.	Anticancer
Komodoquinone A	*Streptomyces* spp.	Neuritogenic activity
Lajollamycin	*Streptomyces nodosus*	Antibacterial
Marinomycins	*Marinispora* spp.	Antibacterial; anticancer
Mechercharmycins	*Thermoactinomyces* spp.	Anticancer
Salinosporamide A (NPI-0052)	*Salinispora tropica*	Anticancer
Trioxacarcins	*Streptomyces* spp.	Antibacterial; anticancer; antimalarial

Source : Lam (2006)

Fiedler *et al*. (2005), Lam (2006), and Baltz (2007) described a few examples of recently isolated novel secondary metabolites that included abyssomicin C and diazepinomicin. Abyssomicin C was produced by a *Verrucosispora* spp., and being evaluated as candidates for treating drug-resistant Gram-positive pathogens, amongst them clinical isolates of multiresistant and vancomycin-resistant *Staphylococcus aureus* strains. Diazepinomicin was produced by a *Micromonospora* spp., and possessed antibacterial, anti-inflammatory, and antitumour activity. Balagurunathan and Subramanian (1994) added that some of the antibiotics such as neomycin A and B, aplasmomycin, istamycin A and B, altemicidin, and tetrozomine were all isolated from marine actinomycetes.

2.7 The Genus *Streptomyces*

Streptomycetes, which belong to the order *Actinomycetales* and family *Streptomycetaceae* are Gram-positive, filamentous bacteria that are ubiquitous in soil (Davelos *et al*., 2004). *Streptomyces* are the most widely studied and well known genus of the actinomycetes (Aghighi *et al*., 2004). *Streptomyces* is comprised of around 150 species (Willey *et al*., 2006). The *Streptomyces* is not an acid-alcohol-fast bacterium, and have an oxidative type of metabolism. The genus *Streptomyces* is catalase positive, and generally reduces nitrates to nitrites and degrades adenine, esculin, casein, gelatin, hypoxanthine, starch, and L-tyrosine. The cell wall peptidoglycan contains major amounts of L-diaminopimelic acid (L-DAP), and the vegetative hyphae (0.5-2.0 μm in diameter) produce an extensively branched mycelium that rarely fragments (Holt *et al*., 1994). Willey *et al*. (2006) described that *Streptomyces* spp. were determined by means of a mixture of morphological and physiological characteristics, including the following: the colour of the aerial and substrate mycelia, spore arrangement, surface

features of individual spores, carbohydrate use, antibiotic production, melanin synthesis, nitrate reduction, and the hydrolysis of urea and hippuric acid. According to Holt *et al*. (1994), the aerial mycelium at maturity forms chains of three to many spores. A few species bear short chains of spores on the substrate mycelium. Some species may form sclerotia-, pycnidial-, sporangia-, and synnemata-like structures. The spores are non-motile. The *Streptomyces* form colonies that are discrete and lichenoid, leathery or butyrous. Initially, colonies are relatively smooth surfaced, but later they develop a weft of aerial mycelium that may appear floccose, granular, powdery, or velvety. They produce a wide variety of pigments responsible for the colour of the vegetative and aerial mycelia. Coloured diffusible pigments may also be formed. Organic compounds are their sole sources of carbon for energy and growth. Their optimal growth temperature is 25ºC to 35ºC; some species are psychrophilic and thermophilic. Meanwhile, optimum pH for their growth ranges from 6.5 to 8.0.

According to Challis and Hopwood (2003), the classical habitat of *Streptomyces* spp. was as free-living saprophytes in terrestrial soils. In soils, they are important decomposer (Aghighi *et al*., 2004). The numerical predominance in soils explains why the majority of metabolites from actinomycetes discovered and developed in the 1950s and 1960s were secondary metabolites from *Streptomyces* (Labeda and Shearer, 1990). The ecology of streptomycetes is of considerable interest for search and discovery of natural products. Currently novel products are sought from organisms isolated from extreme or novel environments. There was also good evidence for the growth of streptomycetes in marine soils. *Streptomyces* spp. contributed an average of nearly 4% to the bacterial community of in-shore sediments, and concluded that the wash-in of spores of terrestrial species was not the source of these populations (Bull *et al*., 2000).

The ability of *Streptomyces* spp. to produce metabolites capable of inhibiting growth and development of pathogenic microorganisms has continued to manifest itself. Recent reports showed that the *Streptomyces* still remains as prolific antibiotic producers (Ndonde and Semu, 2000). Streptomycetes are non-motile, so stresses cannot be avoided, but have to be met. Antibiotics are typically produced in small amounts at the transition phase in colonial development when the growth of the vegetative mycelium is slowing as a result of nutrient exhaustion, and the aerial mycelium is about to develop at the expense of nutrients released by breakdown of the vegetative hyphae. Such antibiotics are proposed to defend the food source when other microorganisms threaten it (Challis and Hopwood, 2003). Davelos *et al*. (2004) and Zheng *et al*. (2007) described that streptomycetes were prolific producers of extracellular enzymes, and these microbes require specific nutritional and environmental conditions to express a series of enzymes performing coordinated functions to process various precursor building blocks into secondary metabolites such as antibiotics.

Antonova-Nikolova *et al*. (2004) and Jensen *et al*. (2007) highlighted that the genus *Streptomyces* was the source of the vast majority of actinomycete secondary metabolites that had been discovered to date, within which antibiotics were of commercial relevance. Peela *et al*. (2005); and Hugo and Russell (1992) reported that *Streptomyces* spp. produced about 75% of commercially and medically useful antibiotics. The productivity of *Streptomyces* spp. as antibiotic producers remains unique amongst *Actinomycetales* strains (Moncheva *et al*., 2000-2002). According to Holt *et al*. (1994) and Willey *et al*. (2006), many strains could produce one or more antibiotics. Antibiotic-producing streptomycetes can inhibit a broad range of soil borne microbes, including Gram-positive and Gram-negative bacteria, fungi, and nematodes. As shown in Figure 2.2, a large selection of antibiotics such as actinomycins,

echinomycin, antimycin A, bafilomycin, filipin, lagosin, lipomycin, tetracenomycin D, and chromomycin A3 that were originally isolated from terrestrial streptomycetes were also found in marine-derived *Streptomyces* spp.

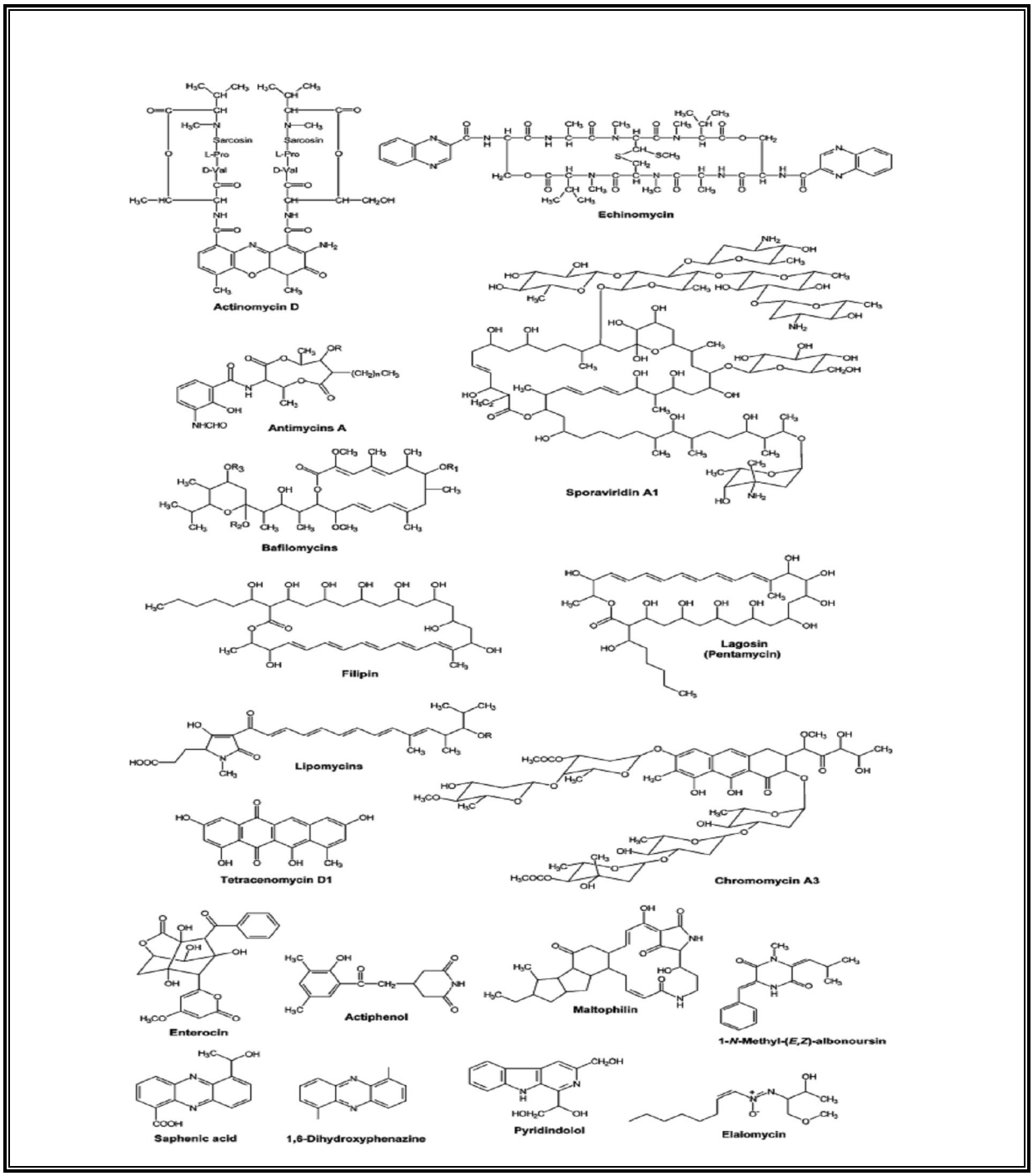


Figure 2.2 Known antibiotics from terrestrial streptomycetes produced by marine *Streptomyces* spp. (Fiedler *et al*., 2005)

2.8 Research on Actinomycetes in Malaysia

There are a number of reports on the research of actinomycetes in Malaysia, where actinomycetes have been isolated from a wide variety of sources such as soil (Al-Tai *et al*., 1999; Tan *et al*., 2001), marine organisms (Tan *et al*., 2004; Tan, 2007; Nor Ainy, 2008), plants (Becker, 1983; Zin *et al*., 2007; Ghadin *et al*., 2008), agriculture soils (Jeffrey, 2008), tropical rainforests soil (Numata and Nimura, 2003), and primary dipterocarp forest soil (Ho *et al*., 2000; Nakajima *et al*., 2003). Research on rare actinomycetes was also conducted in the search of novel antibiotics, where rare actinomycetes isolated from mangrove soils and leaf litter was investigated by Vikineswary *et al*. (2003).

The actinomycetes from mangrove ecosystem in Malaysia have been shown to have a range of bioactivity, thus they are recognized as a potential source of new and novel secondary metabolites and unique lead structures (Vikineswary *et al*., 1997). Ismet *et al*. (1999; 2002) and Ismet (2003) investigated the diversity, biological, molecular, and chemical characteristics of *Micromonospora* spp. isolated from the mangrove rhizosphere ecosystem. In addition, research on production and chemical characterization of antifungal metabolites from *Micromonospora* spp. from mangrove rhizosphere soil was carried out by Ismet *et al*. (2004). Ho *et al*. (2000) isolated, characterized, and screened the bioactivity of actinomycetes from dipterocarp rain forest soils. The antifungal activities of *Streptomyces* spp. against selected plant pathogenic fungi were evaluated by Getha *et al*. (2004; 2005). The utilization of the actinomycetes for agriculture industry is the current research by Jeffrey (2008).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Actinomycete Strains

Eleven strains of marine-derived actinomycete from culture collection at Plant Pathology and Mycology Lab, Institute of Postgraduate Studies, University of Malaya, classified in the genus *Streptomyces* were investigated in this study. The selection of these eleven strains was based on preliminary antibacterial and anti-yeast screenings conducted on one hundred thirty six actinomycetes that were isolated from marine sponges of Tioman Island. The selected *Streptomyces* spp. were culturally and morphologically grouped into three colour groups according to the colour of their mature sporulating aerial mycelium estimated by using a colour chart (Tan *et al.*, 2004; Kavithambigai, 2006). Stock cultures, agar plugs of live cultures of the streptomycetes were preserved in glycerol solution (Appendix A6), and were revived prior to primary screening. Plate cultures in triplicates were prepared on sporulation agar (SA) (Appendix A3) except strains T3 and T4, which were prepared on their best growth solid medium; inorganic salts-starch agar (ISP4) (Appendix A2), by incubation at 28ºC ± 2ºC for 2 weeks.

3.2 Bacterial and Fungal Pathogens

Gram-positive (*Bacillus subtilis, Enterococcus faecalis,* and *Staphylococcus aureus*) bacteria and Gram-negative (*Plesiomonas shigelloides* and *Pseudomonas aeruginosa*) bacteria were obtained from Prof. Thong Kwai Lin, Institute of Biological Sciences, Faculty of Science, University of Malaya. The bacterial cultures were incubated at 37ºC ± 2ºC for 48 h, and maintained on nutrient agar (NA) (Appendix A5) plates. *Candida albicans* and *C. parasilopsis* were obtained from Prof. Ng Kee Peng,

Faculty of Medicine, University of Malaya. The fungal cultures were incubated at 37ºC ± 2ºC for 48 h, and maintained on Sabouraud dextrose agar (SDA) (Appendix A4) plates.

3.3 Primary Screening of Antagonistic Activities

3.3.1 Antibacterial assay

The *Streptomyces* spp. were subjected to primary screening by cross streak method against the bacterial pathogens (Kavithambigai, 2006). Primary screening was to detect the presence of extracellular metabolite(s) from streptomycetes. In the antibacterial assay, all *Streptomyces* spp. were lawned on one third of the SA plates (antibacterial assay was conducted on SA medium as it supported good growth of *Streptomyces* spp. and all the bacteria tested), and incubated at 28ºC ± 2ºC for 2 weeks. The bacteria test species were cross-streaked vertically to the border of the one-third streptomycetes-lawned SA plates. Then, the cross-streaked plates were reincubated for 48 h at 37ºC ± 2ºC. These test plates were examined for any sign of inhibition. Inhibition, if any was recorded as strong, moderate, or weak, and represented by the "+++", "++", or "+" symbols, respectively. As indicated in Figure 3.1, strong, moderate, or weak inhibitions were defined as complete, half, or one-third bacterial growth inhibition lengths, respectively. No inhibition was represented by the "-" symbol. Plates streaked with bacterial pathogens with the absence of streptomycetes were used as control plates. Plate 3.1 shows a plate cross-streaked with test bacteria and streptomycete.

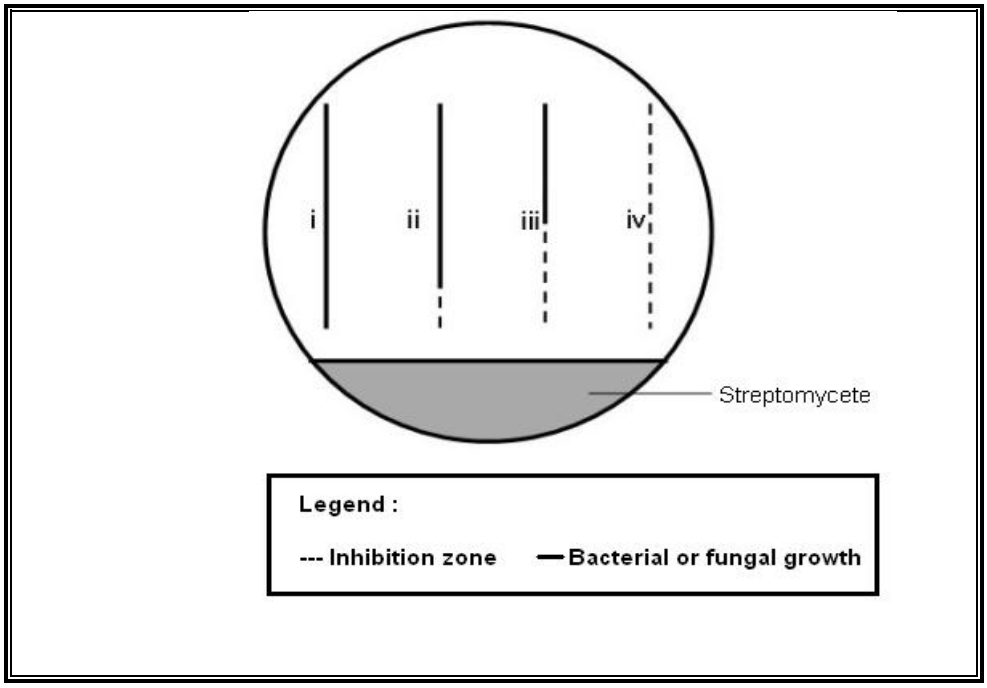


Figure 3.1 Quantification of bacterial or fungal growth inhibition based on inhibition lengths (i : no inhibition, ii : weak inhibition, iii : moderate inhibition, and iv : strong inhibition)

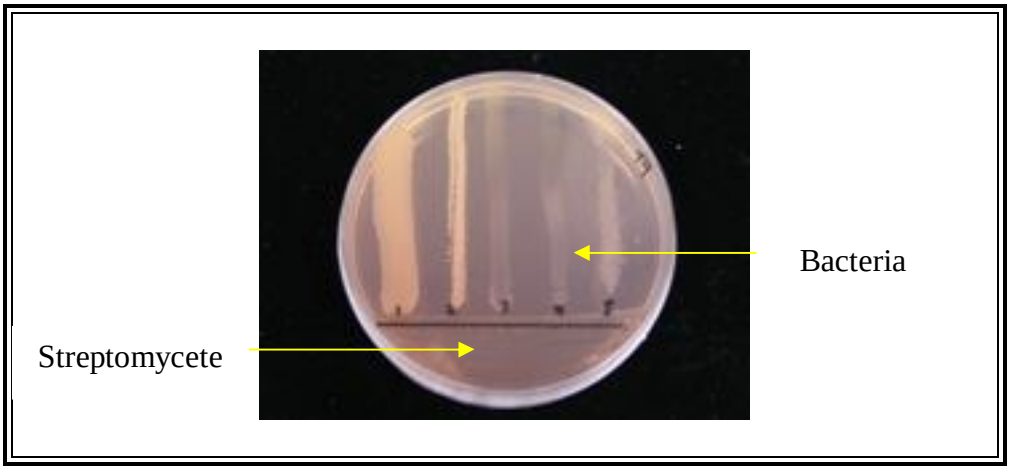


Plate 3.1 Cross streak method: Bacteria and streptomycete (Bacteria were streaked on SA plate which had been pre-inoculated with streptomycete, and further incubated at 37ºC ± 2ºC for 48 h)

3.3.2 Anti-yeast assay

The streptomycetes were primarily screened by cross streak method against the fungal pathogens (Kavithambigai, 2006). In anti-yeast assay, all *Streptomyces* spp. were lawned superficially on one third of the SA plates (anti-yeast assay was conducted on SA medium as it supported good growth of *Streptomyces* spp. and all the yeasts tested), and incubated at 28ºC ± 2ºC for 2 weeks. The test yeasts were cross-streaked vertically to the border of the one-third streptomycetes-lawned SA plates. Then, the cross-streaked plates were reincubated for 48 h at 37ºC ± 2ºC. These test plates were examined for any strong, moderate, or weak signs of inhibition, which were represented by the "+++", "++" or "+" symbols, respectively. As indicated in Figure 3.1, strong, moderate, or weak inhibitions were defined as complete, half, or one-third yeast growth inhibition lengths, respectively. No inhibition was represented by the "-" symbol. Plates streaked with yeasts without the presence of streptomycetes were used as control plates. Plate 3.2 shows a plate cross-streaked with test yeasts and streptomycete.

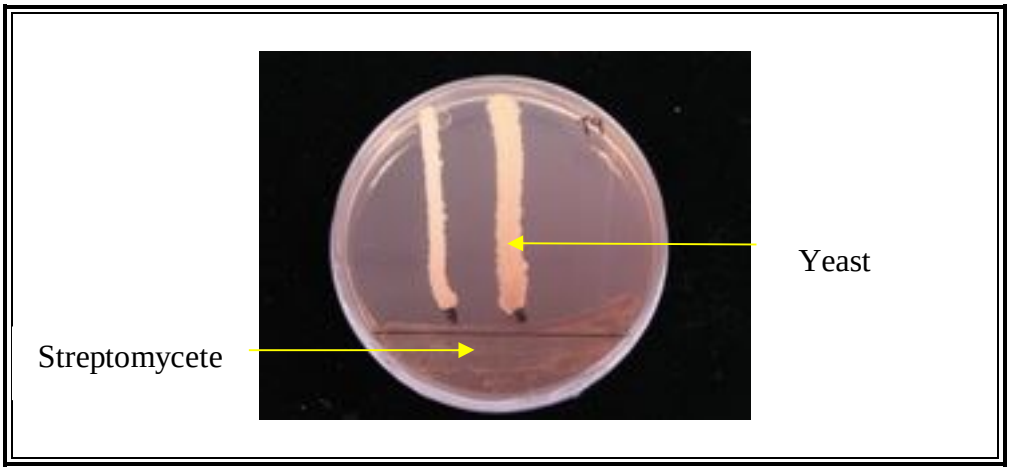


Plate 3.2 Cross streak method: Yeasts and streptomycete (Yeasts were streaked on SA plate which had been pre-inoculated with streptomycete, and further incubated at 37ºC ± 2ºC for 48 h)

3.4 Secondary Screening of Antagonistic Activities

3.4.1 Preparation of crude extracts of actinomycetes

The crude extracts were prepared by extraction with methanol: dichloromethane (1:1) solvent system (Tan *et al.*, 2004; Kavithambigai, 2006). Each full-grown plate cultures of the *Streptomyces* spp. was transferred as 10 ml inoculum suspension in sterile distilled water into 250 ml Erlenmeyer flasks containing sterile 90 ml of their respective liquid growth media (ISP4 or SA). The liquid culture was set up in triplicates. The submerged cultures were incubated for two weeks on a rotary shaker (Environ-Shaker 3597-1PR) at 120 rpm and 28ºC ± 2ºC. Then, the culture broth from each flask was freeze-dried for 24 h. Freeze-dried material was weighed and soaked in 100 ml methanol: dichloromethane (1:1) prior to overnight shaking on rotary shaker at 120 rpm at room temperature, and ultrasonication (Branson 3510) for 30 min at 40 kHz. The culture extracts were filtered and rotor-evaporated (Buchi Rotavapor R-114) at 55ºC ± 2ºC to obtain the crude extracts, which were redissolved in methanol to prepare 25 mg/ml of crude extracts.

3.4.2 Antagonistic bioassays

The eleven streptomycetes were then subjected to secondary screening to assess the presence of intracellular metabolite(s). Two pathogenic yeasts *C. albicans* and *C. parasilopsis* were test species for the detection of anti-yeast activity whereas *B. subtilis, S. aureus, E. faecalis, P. shigelloides,* and *P. aeruginosa* were test species for the detection of antibacterial activity. Bioactivity assays against fungal and bacterial pathogens were performed using crude extracts of the streptomycetes. The crude extracts were assessed for their bioactivity against the bacteria and yeasts by disc

diffusion method (Kavithambigai, 2006). Single colonies of bacteria and yeasts cultured for 48 h were lawned, respectively on NA and SDA plates before placing the paper discs. Each 6 mm sterile Whatman paper disc on the lawned plates was impregnated with 15 µl (equivalent to 0.375 mg of extract) of each extract. Sterile 6 mm Whatman paper disc impregnated with 15 µl methanol (MeOH) was used as negative control. Commercial nystatin discs (100 units per disc) were used as positive control for anti-yeast assay. Meanwhile, commercial novobiocin (5 µg per disc) and streptomycin (10 µg per disc) discs were used as positive control for antibacterial assay. The antimicrobial activity was observed after 48 h incubation at 37ºC ± 2ºC. The test plates were examined for clear zones around the paper discs where growth of pathogens was inhibited. Diameter of the inhibition zone of growth, if any, for each extract against test pathogens was recorded. The actual clear zone from the extracts was deducted from the methanol clear zone. The results of antibacterial and anti-yeast assays were recorded as strong ("+++"), moderate ("++"), or weak ("+") inhibitions. No inhibition was represented by the "-" symbol. Strong inhibition was defined as greater than 15 mm (> 15 mm) diameter of the inhibition zone of growth, while moderate and weak inhibitions were defined as 10 to 15 mm (10-15 mm) and less than 10 mm (< 10 mm), respectively.

3.5 Optimisation of Culture Conditions for Bioactive Metabolite(s) Production by *Streptomyces* Strain T15 against *Plesiomonas shigelloides*

Streptomyces strain T15 was chosen for further studies as it best inhibited *P. shigelloides* in both primary and secondary screenings. Three media (ISP2, ISP4, and SA) and two culture conditions (agitation and static) were selected for optimisation studies. Strain T15 was cultivated in triplicates on ISP2, ISP4, and SA plates. Full-

grown cultures were transferred as 10 ml inoculum suspension in sterile distilled water into 250 ml Erlenmeyer flasks containing sterile 90 ml of liquid fermentation media (ISP2, ISP4, and SA). Each medium was set up in triplicates. The inoculated cultures were incubated at 28ºC ± 2ºC at 120 rpm for 3 to 12 days on a shaking rotary shaker. A set of inoculated cultures were also incubated at 28ºC ± 2ºC for 3 to 12 days in a static condition. The parameters monitored were pH, weight of crude extracts, and antagonistic activities. The profile of bioactive metabolite(s) production for every three days throughout fermentation was evaluated by disc diffusion method (Kavithambigai, 2006) using *P. shigelloides* as the target test bacteria. The liquid cultures were harvested every three days for twelve days. The pH of the fermentation media was noted after harvesting. Then, the culture broth from each flask was freeze-dried for 24 h. Freeze-dried material was weighed, and subsequently extracted using 100 ml dichloromethane and methanol solvents in 1:1 ratio as described in secondary screening. The mixture was subjected to 30 min of ultrasonication at 40 kHz, followed by filtering and rotor-evaporating at 55ºC ± 2ºC to obtain crude extracts. The crude extracts were weighed. Then, the crude extracts were redissolved in dimethyl sulfoxide (DMSO) to prepare 25 mg/ml of crude extract. Finally, 15 µl (equivalent to 0.375 mg of extract) of each extract was loaded onto sterile 6mm Whatman paper discs that were placed on the nutrient agar (NA) plates lawned with single colony of *P. shigelloides* cultured for 48 h. The antibacterial activity was observed after 48 h incubation at 37ºC ± 2ºC. The test plates were examined for clear zones around paper disc indicating growth was inhibited. Diameter of the inhibition zone of growth, if any, was measured. Chloramphenicol (30 µg per disc), novobiocin (5 µg per disc), and streptomycin (10 µg per disc) served as positive controls for antibiotic susceptibility testing against *P. shigelloides.* Meanwhile, DMSO served as negative control. DMSO was selected as negative control as it was less toxic and did not inhibit the growth of *P. shigelloides.*

CHAPTER 4

4.0 RESULTS AND DISCUSSIONS

4.1 Actinomycete Strains

The streptomycetes tested in this study were categorized culturally and morphologically into four series according to the colour of their mature sporulating aerial mycelium (Plate 4.1). Two out of eleven strains of streptomycete tested were grouped in the grey colour group; namely strains T3 and T4. Strain T6 was the only strain that belonged to the yellow green colour group. Four strains; T9, T12, T13, and T15 were assigned to the white with red diffusible pigments colour group. Another four strains; T16, T20, T52, and T53 belonged to the white with brown diffusible pigments colour group.

4.2 Antagonistic Activity of Selected Marine-Derived Actinomycetes in Primary and Secondary Screenings

4.2.1 Bioactivity of different colour groups of *Streptomyces* spp. in primary and secondary screenings

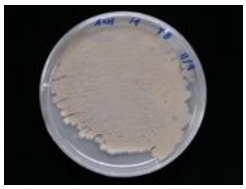
In primary screening, there was no inhibition of growth of *C. albicans* and *C. parasilopsis* by all the *Streptomyces* spp. tested. The *Streptomyces* spp. from different colour groups displayed varying degree of inhibition of bacteria tested. As shown in Table 4.1 and Plate 4.2, one out of two strains (T3), belonging to the grey colour group exhibited strong antibacterial activity against *E. faecalis* and *S. aureus*. The only strain in yellow green group, strain T6 was moderately active against *E. faecalis* but weakly inhibited *P. aeruginosa*. All the strains from the white with red diffusible pigments colour group were active against all the bacteria tested. Strain T15 strongly inhibited all the bacteria tested except *S. aureus* which was strongly inhibited by strain T9. Strain

T12 moderately inhibited all the bacteria tested. Strains T9 and T13 weakly inhibited all the bacteria tested except *S. aureus* which was weakly inhibited by strains T13 and T15. Two of the strains (T52 and T53) from the white with brown diffusible pigments colour group were active against at least two bacteria tested. Strain T52 posed weak inhibition of all the bacteria tested, while strain T53 weakly suppressed the growth of all the Gram-negative bacteria tested; *P. aeruginosa* and *P. shigelloides.*

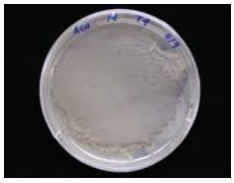
Table 4.1 Antibacterial activity of *Streptomyces* spp. in primary screening assessed via cross streak method

Strain	**Colour group**	**Test bacteria**				
		Gram-positive			**Gram-negative**	
		B. subtilis	***E. faecalis***	***S. aureus***	***P. aeruginosa***	***P. shigelloides***
T3	**Grey**	-	+++	+++	-	-
T6	**Yellow green**	-	++	-	+	-
T9	**White/Red diffusible pigments**	+	+	+++	+	+
T12		++	++	++	++	++
T13		+	+	+	+	+
T15		+++	+++	+	+++	+++
T52	**White/Brown Diffusible pigments**	+	+	+	+	+
T53		-	-	-	+	+

* Test bacteria were streaked on SA plates and incubated at 37ºC ± 2ºC for 48 h for the cross streak antibacterial assay. Growth inhibition was defined as +++: strong inhibition (complete bacterial growth inhibition length), ++: moderate inhibition (half bacterial growth inhibition length), +: weak inhibition (one-third bacterial growth inhibition length), and -: no inhibition



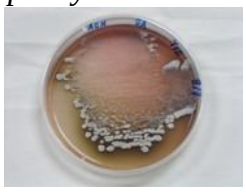
Streptomyces strain T3 *



Streptomyces strain T4 *

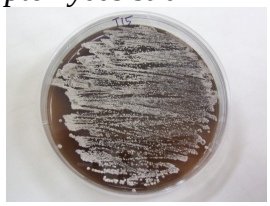
Streptomyces strain T6 **

Streptomyces strain T9 ***

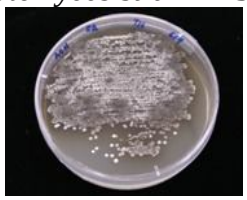


Streptomyces strain T12 ***

Streptomyces strain T13 ***



Streptomyces strain T15 ***



Streptomyces strain T16 ****

Streptomyces strain T20 ****

Streptomyces strain T52 ****

Streptomyces strain T53 ****

Plate 4.1 Cultures of the *Streptomyces* spp. that were lawned on SA plates except strains T3 and T4, which were prepared on ISP4 plates and incubation at 28ºC ± 2ºC for 2 weeks (*: grey colour group, **: yellow green colour group, ***: white/ red diffusible pigments colour group, ****: white/ brown diffusible pigments colour group)

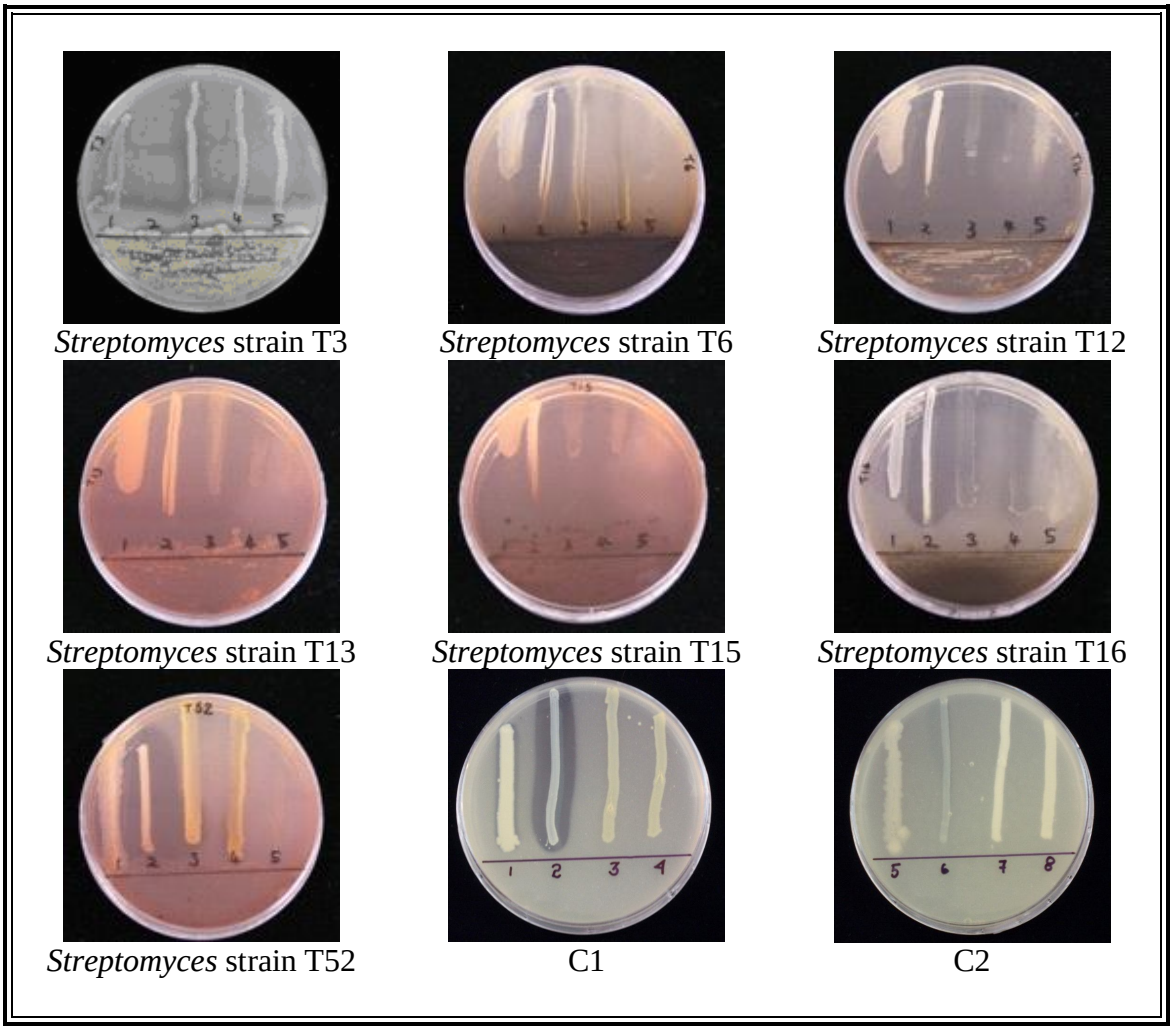


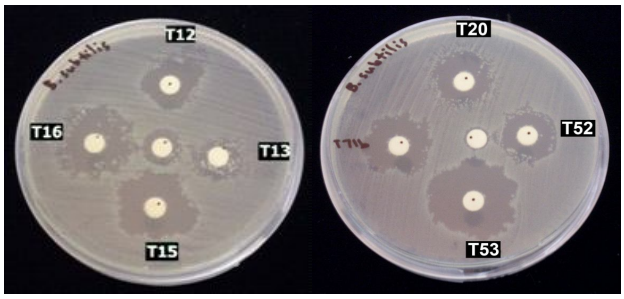
Plate 4.2 Test plates of SA one-third-lawned with streptomycetes and streaked with test bacteria were incubated at 37ºC ± 2ºC for 48 h for the cross streak antibacterial assay in primary screening. Control SA plates, C1 and C2 streaked with test bacteria (1: *E. faecalis*; 2: *S. aureus*; 3: *P. shigelloides*; 4: *B. subtilis*; 5: *P. aeruginosa*) and yeasts (7: *Candida parasilopsis*; 8: *C. albicans*), respectively without the presence of streptomycetes were incubated at 37ºC ± 2ºC for 48 h

In secondary screening, as indicated in Table 4.2 and Plate 4.3, all the strains from the grey colour group had weak inhibition of all the bacteria tested, except *P. aeruginosa*. Moreover, only strain T4 had weak inhibition of both yeasts. Strain T6 from the yellow green colour group weakly inhibited *E. faecalis*, *S. aureus*, and *P. shigelloides*. All the strains from the white with red diffusible pigments colour group were active against *E. faecalis*, *S. aureus*, and *P. shigelloides*. Three strains (T12, T13, and T15) were active against *B. subtilis*, while two strains each inhibited *P. aeruginosa* (T9 and T12) and *C. parasilopsis* (T13 and T15), and only one strain (T15) inhibited *C. albicans*. Strong inhibition against *P. shigelloides* was displayed by strain T15. Strain T13 and strain T15 moderately inhibited *P. shigelloides* and *B. subtilis*, respectively. However, all the strains weakly inhibited *E. faecalis* and *S. aureus*. Strains T9 and T12 weakly inhibited both Gram-negative bacteria tested, while strains T12 and T13 weakly inhibited *B. subtilis*. Strain T15 weakly inhibited both yeasts, while strain T13 only inhibited *C. parasilopsis*. All the strains from the white with brown diffusible pigments colour group were inhibitory towards *B. subtilis*, *E. faecalis*, and *S. aureus*, while three strains (T16, T52, and T53) were active against *P. shigelloides*. Activity against *P. aeruginosa* was attributed to two strains (T52 and T53). Strain T16 and strain T53 strongly inhibited *P. shigelloides* and *E. faecalis*, respectively. Strain T53 had moderate inhibition of all the bacteria tested except *E. faecalis*. Strains T16, T20, and T52 had weak inhibition of all the Gram-positive bacteria tested, while only strain T52 weakly inhibited all the Gram-negative bacteria tested. Furthermore, strains T16 and T53 weakly suppressed the growth of both yeasts. The control plates are shown in Plate 4.4.

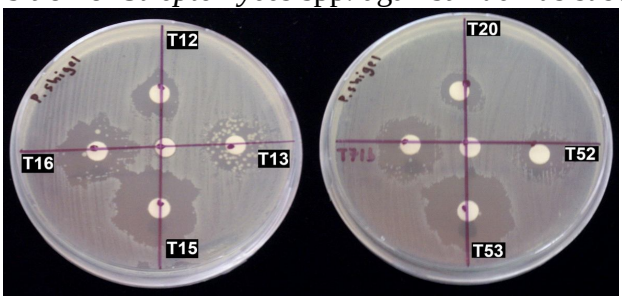
Table 4.2 Inhibition spectrum (mm) of *Streptomyces* spp. against the test pathogens in secondary screening assessed via disc diffusion method

Strain	Colour group	Diameter of inhibition zone (mm)						
		Gram-positive bacteria			Gram-negative bacteria		Yeast	
		B. subtilis	*E. faecalis*	*S. aureus*	*P. aeruginosa*	*P. shigelloides*	*C. albicans*	*C. parasilopsis*
T3	Grey	4.0 ± 1.9	1.7 ± 0.5	1.7 ± 0.5	-	4.7 ± 0.4	-	-
T4		4.0 ± 0.0	6.0 ± 1.0	4.7 ± 1.0	-	2.7 ± 0.4	5.4 ± 1.7	6.3 ± 1.4
T6	Yellow green	-	1.0 ± 0.0	2.4 ± 0.3	-	4.0 ± 1.0	-	-
T9	White/Red diffusible pigments	-	4.0 ± 2.6	6.4 ± 0.9	2.7 ± 0.6	6.0 ± 1.0	-	-
T12		1.0 ± 3.6	6.3 ± 0.5	2.4 ± 0.8	5.4 ± 1.9	5.0 ± 1.0	-	-
T13		4.0 ± 1.9	2.0 ± 1.0	4.7 ± 1.5	-	12.0 ± 1.0	-	1.6 ± 1.7
T15		12.0 ± 1.7	7.7 ± 0.5	6.4 ± 0.9	-	16.3 ± 0.4	5.7 ± 1.1	5.0 ± 0.9
T16	White/Brown Diffusible pigments	9.4 ± 0.6	6.3 ± 2.8	5.0 ± 0.9	-	15.7 ± 1.1	5.0 ± 0.9	4.6 ± 0.0
T20		8.0 ± 0.0	8.3 ± 0.4	8.0 ± 0.0	-	-	-	-
T52		8.4 ± 0.0	9.3 ± 0.4	8.4 ± 0.9	9.0 ± 0.9	8.3 ± 0.4	-	-
T53		13.7 ± 0.6	16.0 ± 0.7	14.0 ± 0.0	13.0 ± 0.0	12.0 ± 1.0	6.7 ± 0.6	5.3 ± 1.4

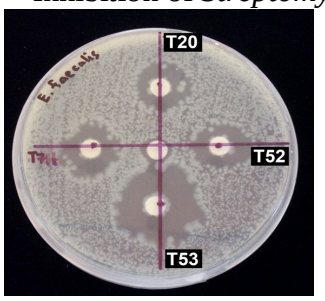
* Mean of three readings with standard deviation, test bacteria and yeasts were lawned on NA and SDA plates, respectively, and incubated at 37ºC ± 2ºC for 48 h for the disc diffusion antibacterial and anti-yeast assays. Cultures of the *Streptomyces* spp. were prepared in SA or ISP4 (T3 and T4) liquid media and were incubated at 28ºC ± 2ºC on a rotary shaker at 120 rpm for two weeks. Extracts were prepared by extraction with methanol: dichloromethane (1:1). Strong inhibition was defined as greater than 15 mm (> 15 mm) diameter of the inhibition zone of growth, while moderate and weak inhibitions were defined as 10 to 15 mm (10-15 mm) and less than 10 mm (< 10 mm), respectively



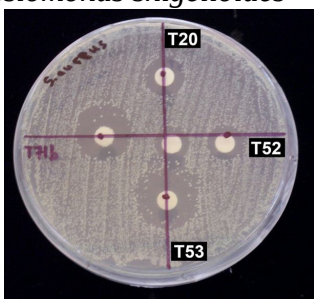
Inhibition of *Streptomyces* spp. against *Bacillus subtilis*



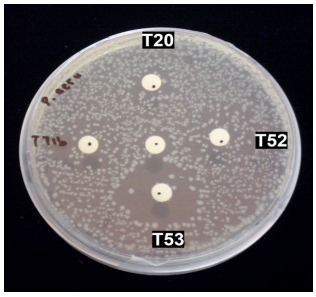
Inhibition of *Streptomyces* spp. against *Plesiomonas shigelloides*



Inhibition of *Streptomyces* spp. against *Enterococcus faecalis*



Inhibition of *Streptomyces* spp. against *Staphylococcus aureus*



Inhibition of *Streptomyces* spp. against *Pseudomonas aeruginosa*

Plate 4.3 Test plates of NA lawned with test bacteria displayed antagonistic activities by *Streptomyces* spp. (Test bacteria were incubated at 37ºC ± 2ºC for 48 h; cultures of the *Streptomyces* spp. were prepared in SA or ISP4 (T3 and T4) liquid media and were incubated at 28ºC ± 2ºC on a rotary shaker at 120 rpm for two weeks. Extracts were prepared by extraction with methanol: dichloromethane (1:1); paper discs seeded in the centre of test plates were impregnated with methanol that served as negative control)

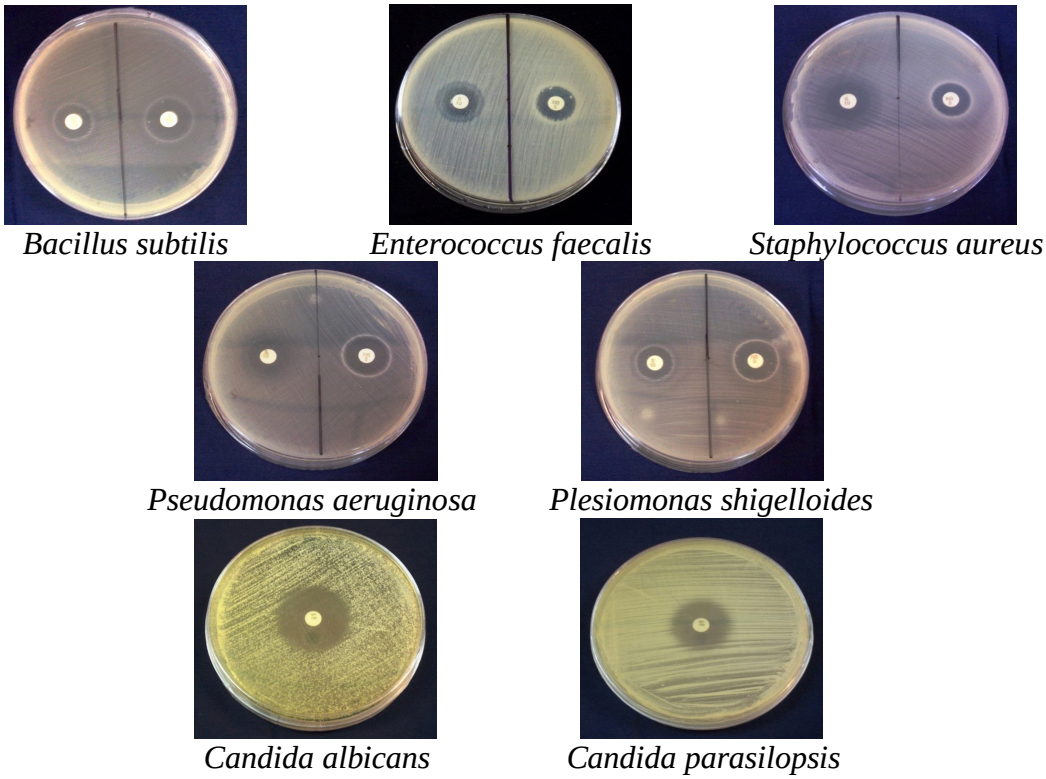


Plate 4.4 Inhibition zones on control plates lawned with test bacteria and yeasts in secondary screening assessed via disc diffusion method. Standard antibiotic discs of 10 µg streptomycin and 5 µg novobiocin were seeded on the left and right of the NA plates, respectively. Standard antibiotic discs of nystatin were seeded in the centre of the SDA plates (Test bacteria and yeasts were incubated at 37ºC ± 2ºC for 48 h)

Following incubation in a disc diffusion method, the zones of inhibition of growth were measured; the more active the compound, the larger the zone. Therefore, some idea of the potency of the bioactive compound(s) was gained from the zone size. With poorly soluble compounds which diffused insufficiently in the agar, the zone of the inhibition might not reflect the true antimicrobial potential of the compounds (Barry and Thornsberry, 1985).

The antagonistic activity of strains from different colour groups in primary and secondary screenings was compared. In primary screening, half of the strains from the grey and white with brown diffusible pigments colour groups displayed antagonistic bioactivity against at least one of the pathogens tested compared to antagonism by all the strains in secondary screening. Contrast results were obtained by Kavithambigai (2006), where prominent bioactivity of strains from the grey and white colour groups was observed in primary screening. Antagonism by all the strain(s) from the yellow green and white with red diffusible pigments colour groups against at least one of the pathogens tested were detected in both primary and secondary screenings.

As indicated in Table 4.3, strains from the grey, white with red diffusible pigments, and white with brown diffusible pigments colour groups showed improved activity in secondary screening. In primary screening, one out of two strains from the grey colour group was active against at least one Gram-positive bacteria tested, while both strains were active in secondary screening. Antimicrobial activity by strains from the grey colour group against Gram-negative bacteria and yeasts tested was not detected in primary screening. However, in secondary screening, all of the strains were active against at least one Gram-positive and Gram-negative bacteria tested, while half of the strains were active against at least one of the yeasts. This result was in good agreement with findings by Sujatha *et al.* (2005) in which a marine-derived *Streptomyces* strain BT-408 from grey colour group showed a broad antimicrobial spectrum against Gram-positive and Gram-negative bacteria, fungi, and yeast. In secondary screening, all strains from the grey colour group posed weak inhibition towards all the bacteria tested, except *P. aeruginosa*.

Table 4.3 Comparison of antagonistic activity of *Streptomyces* spp. from different colour groups against the test pathogens in primary and secondary screenings

Colour group	Primary screening			Secondary screening		
	Gram-positive bacteria	Gram-negative bacteria	Yeast	Gram-positive bacteria	Gram-negative bacteria	Yeast
Grey (T3, T4)	1	-	-	2	2	1
Yellow green (T6)	1	1	-	1	1	-
White/ Red diffusible pigments (T9, T12, T13, T15)	4	4	-	4	4	2
White/ Brown diffusible pigments (T16, T20, T52, T53)	1	2	-	4	3	2

* Primary screening: Test bacteria were streaked on SA plates and incubated at 37ºC ± 2ºC for 48 h for the cross streak antibacterial assay. Secondary screening: Test bacteria and yeasts were lawned on NA and SDA plates, respectively, and incubated at 37ºC ± 2ºC for 48 h for the disc diffusion assay.

Dhanasekaran *et al*. (2005) reported a marine *Streptomyces* sp. strain DPTD 14 from the grey colour group that weakly inhibited against *S. aureus*. However, contrary to the present results, marine *Streptomyces* sp. isolate B6921 from the white to reddish grey colour group (dependent on the medium) exhibited high antagonistic activity against *S. aureus* and *B. subtilis* (Maskey *et al*., 2003). In addition, soil *Streptomyces halstedii* from the grey colour group had strong inhibition towards *S. aureus* and *B. subtilis* while *S. lydicus* exhibited moderate inhibition against *B. subtilis* (Oskay *et al*., 2004). Kock *et al*. (2005) reported that a marine *Streptomyces* isolate B8005 from the grey colour group showed inhibitory activity against *S. aureus* and *C. albicans*. In accordance to the findings by Kock *et al*. (2005), bioactivity of one of the strains from the grey colour group towards *C. albicans* was also observed in the present study.

As shown in Table 4.3, the single strain from the yellow green colour group (T6) displayed similar pattern of antagonism in both primary and secondary screenings. The strain was active against at least one Gram-positive and Gram-negative bacteria tested but was inactive against the yeasts. These results led to the speculation that the same antibacterial bioactive metabolite(s) was produced extracellularly and intracellularly. Strain T6 was inactive against *B. subtilis* in both primary and secondary screenings. The bioactivity of strain of the yellow green colour group in the present study was compared to that of the green and yellow colour groups in the study by Kavithambigai (2006), in which similar pattern of antagonism in both primary and secondary screenings was also displayed. Kavithambigai (2006) demonstrated that all strains of the green and yellow colour groups showed antibiosis against the test microorganisms in both primary and secondary screenings.

All the strains of the white with red diffusible pigments colour group showed antibacterial activity against at least one Gram-positive and Gram-negative bacteria tested in both primary and secondary screenings (Table 4.3). Moreover, in secondary screening, half of the strains were active against at least one of the yeasts, but none was active in primary screening. One out of four strains from the white with brown diffusible pigments colour group was active against Gram-positive bacteria tested in primary screening, while in secondary screening, all strains were active. Two strains in primary screening compared to three strains in secondary screening were antagonistic towards Gram-negative bacteria tested. Furthermore, anti-yeast activity was displayed by two strains in secondary screening, whereas no anti-yeast activity was detected among the strains from the white with brown diffusible pigments colour group in primary screening. These results were contrary to the results observed by

Kavithambigai (2006), where anti-yeast activity was produced by strains from the white colour group only in primary screening.

Overall, strain(s) from the grey and white with red diffusible pigments colour groups displayed strong antibacterial activity in primary screening. Tan (2007) reported that all marine-derived actinomycetes from the grey and yellow green colour groups possessed antibacterial activity. In secondary screening, the *Streptomyces* spp. belonging to the white colour group were the most active, where the most prominent were strains of white with brown diffusible pigments colour group, followed by strains of white with red diffusible pigments colour group. These results were in accordance with the findings by Saadoun *et al.* (2008) that indicated the most active *Streptomyces* had colonies of white or grey aerial mycelium.

4.2.2 Bioactivity of strains in primary and secondary screenings

In primary screening as indicated in Table 4.1 and Plate 4.2, three strains (T4, T16, and T20) showed no antagonistic activity against all the pathogens tested. All the strains did not inhibit the yeasts. Eight strains were active against at least one bacteria tested. In addition, seven strains were antagonistic against at least one Gram-positive and Gram-negative bacteria tested. Five strains (T9, T12, T13, T15, and T52) were active against all the Gram-positive bacteria tested; *B. subtilis*, *E. faecalis*, and *S. aureus*. Meanwhile, six strains (T9, T12, T13, T15, T52, and T53) were active against all the Gram-negative bacteria tested; *P. aeruginosa* and *P. shigelloides*. Overall, five strains were active against all the bacteria tested. As shown in Table 4.1, three, two, and six strains displayed strong, moderate, and weak inhibitions, respectively. Strain T15 exhibited strong inhibition against most of the bacteria tested, namely *E. faecalis*, *P.*

shigelloides, *B. subtilis*, and *P. aeruginosa*. However, it displayed weak inhibition against *S. aureus*. Strain T9 displayed contrast antagonistic activity from strain T15. It exhibited strong inhibition only against *S. aureus*, while weakly inhibited the remaining bacteria tested. Strain T3 exhibited strong antibacterial activity against *E. faecalis* and *S. aureus*, but did not inhibit the rest of the bacteria tested. Strain T12 showed moderate inhibition of all the bacteria tested. Strain T6 only showed moderate and weak inhibitions against *E. faecalis* and *P. aeruginosa*, respectively. Strains T13 and T52 showed weak inhibition of all the bacteria tested, while strain T53 weakly inhibited all the Gram-negative bacteria tested only.

In secondary screening, preliminary data as shown in Table 4.2 and Figure 4.1 showed that all the strains tested were inhibitory to at least one Gram-positive bacteria tested, where nine out of eleven strains inhibited all the Gram-positive bacteria tested. Meanwhile, ten strains displayed inhibition towards at least one of the Gram-negative bacteria tested, where four strains inhibited all the Gram-negative bacteria tested. On the other hand, five strains inhibited at least one of the yeasts; *C. albicans* or *C. parasilopsis*, where four strains inhibited both yeasts. Three out of eleven strains exhibited strong and moderate inhibitions, while all the strains tested exhibited weak inhibition against at least one pathogen tested. Strain T53 exhibited strong inhibition against *E. faecalis*, while moderately inhibited *B. subtilis*, *S. aureus*, and all the Gram-negative bacteria tested, but weakly inhibited all the yeasts. Strain T15 strongly inhibited *P. shigelloides*, moderately inhibited *B.* subtilis, but weakly inhibited *E. faecalis*, *S. aureus*, and all the yeasts. Strain T16 displayed strong inhibition against *P. shigelloides*, but weakly inhibited all the Gram-positive bacteria and yeasts tested. Strain T13 moderately inhibited *P. shigelloides* but weakly inhibited all the Gram-positive bacteria tested and one of the yeasts; *C. parasilopsis*. Strain T4 weakly

inhibited all the pathogens tested except *P. aeruginosa*. Strains T12 and T52 weakly suppressed the growth of all the bacteria tested. Strain T3 and strain T9 exhibited weak inhibition of all the bacteria tested except *P. aeruginosa* and *B. subtilis*, respectively. Strain T6 weakly inhibited *E. faecalis*, *S. aureus*, and *P. shigelloides*. Meanwhile, strain T20 had minimal activity against all the Gram-positive bacteria tested.

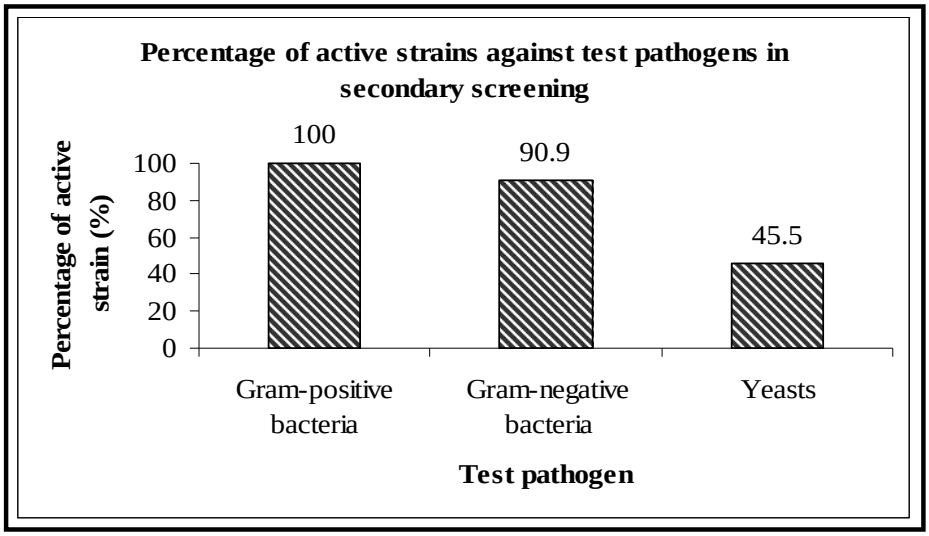


Figure 4.1 Percentage of active strains out of eleven strains against at least one test pathogen in secondary screening

Streptomyces spp. investigated in this study showed prominent antibacterial and anti-yeast activities in secondary screening compared to primary screening. Table 4.4 shows the percentage of active streptomycetes in primary and secondary screenings. Strains in secondary screening exhibited greater growth inhibition against all the pathogens tested except *P. aeruginosa*, where four strains were active against it compared to seven strains in primary screening. The production of low concentration of bioactive metabolite(s) against *P. aeruginosa* in liquid culture could be the main factor leading to this observation (Tan *et al*., 2004).

Table 4.4 Comparison of percentage of active *Streptomyces* spp. against the test pathogens in primary and secondary screenings

Test pathogen		Percentage of *Streptomyces* spp. (%)	
		Primary Screening	Secondary Screening
Gram-positive bacteria	***Bacillus subtilis***	45.5	81.8
	Enterococcus faecalis	63.6	100
	Staphylococcus aureus	54.5	100
Gram-negative bacteria	***Pseudomonas aeruginosa***	63.6	36.4
	Plesiomonas shigelloides	54.5	90.9
Yeast	***Candida albicans***	-	36.4
	Candida parasilopsis	-	45.5

* Primary screening: Test bacteria were streaked on SA plates and incubated at 37ºC ± 2ºC for 48 h for the cross streak antibacterial assay. Secondary screening: Test bacteria and yeasts were lawned on NA and SDA plates, respectively, and incubated at 37ºC ± 2ºC for 48 h for the disc diffusion antibacterial and anti-yeast assays

In primary screening, eight strains were active against at least one bacteria tested, where seven strains each were antagonistic against at least one Gram-positive and Gram-negative bacteria tested. Five strains were active against all the Gram-positive bacteria tested, while six strains were active against all the Gram-negative bacteria tested. Several authors have reported antibacterial potential of *Streptomyces* spp. Based on Tan's (2007) investigation, 28.2% marine actinomycetes displayed moderate antibacterial activity assessed by cross plug method, where 27.1% and 10.6% strains had inhibitory effect against at least one Gram-positive and Gram-negative bacteria tested, respectively. Higher antibacterial activity in the present study might be caused by the diffusion of higher concentration of bioactive metabolite(s) contained in

the one-third actinomycete-lawned plate compared to the actinomycete plug in the cross plug method.

The previous findings by Pisano *et al*. (1986) and Pisano *et al*. (1989) had outdone the present findings in terms of inhibition spectrum, where 15.8% and 40.0% strains obtained from marine sediments displayed antimicrobial activity against all the microorganisms tested (Gram-positive and Gram-negative bacteria, yeasts, and fungi). Therefore, marine actinomycetes in primary screening observed by Pisano *et al*. (1986) and Pisano *et al*. (1989) had broad antagonistic spectrum compared to the present study. Most of the activity was directed against Gram-positive bacteria, but inhibition of Gram-negative bacteria and yeasts were also evident. This was contrary to the results observed in the present study, where activity against Gram-negative bacteria was prominent compared to Gram-positive bacteria.

In secondary screening, all of the strains were observed to have the ability to produce growth inhibition of one or other pathogens tested. The findings in the present study had exceeded the estimation by Ndonde and Semu (2000), where about 75% of *Streptomyces* spp. were estimated to produce antibiotics of one type or another. This may be an indication of the potential of the marine environment sampled in the present study to harbour antibiotic-producing *Streptomyces* spp. In secondary screening, all the strains were antagonistic against at least one Gram-positive bacteria tested. Meanwhile, 90.9% strains were antagonistic against at least one Gram-negative bacteria tested. In contrast with the results observed in primary screening, number of active strains against all the Gram-positive bacteria tested was higher than that of Gram-negative bacteria tested. In secondary screening, activity against all the Gram-positive bacteria tested increased by 36.3%, while activity against all the Gram-negative bacteria tested

decreased by 18.1%. It could be suggested that higher concentration of bioactive metabolite(s) inhibitory towards Gram-positive bacteria tested was produced in liquid culture, while higher concentration of bioactive metabolite(s) inhibitory towards Gram-negative bacteria tested was produced in solid culture (Tan *et al.*, 2004). Activity against both Gram-positive and Gram-negative bacteria tested in the present study was higher by 3.8% compared to the findings by Zheng *et al.* (2000).

In the present study, growth inhibition of yeasts was evident in secondary screening, but none in primary screening. This result indicated that the diffusible extracellular metabolites in solid medium did not induce the anti-yeast activity. However, the anti-yeast activity was induced by the intracellular metabolites in liquid medium. Kavithambigai (2006) demonstrated that anti-yeast activity was greater in secondary screening compared to primary screening. Bioactive metabolite(s) that was active against *C. parasilopsis* and *C. albicans* was produced by five and four strains, respectively. Anti-yeast assay indicated that *C. parasilopsis* was more susceptible compared to *C. albicans*, which was in contrast with the results observed by Tan (2007), where 10.6% and 25.9% strains inhibited *C. parasilopsis* and *C. albicans*, respectively. Findings by Tan (2007) were corroborated by the findings of Kavithambigai (2006) for other actinomycetes. Strains T3, T6, T9, T12, T20, and T52 did not exhibit anti-yeast activity extracellularly and intracellularly. The solvent used for extraction may not be suitable for the strains or the metabolite(s) may not be properly extracted by the solvent (Pandey *et al.*, 2002).

Extraction with methanol was needed by strains T4, T13, T15, T16, and T53 which were inactive against the yeasts in primary screening, but discovered to be inhibitory towards the yeasts in secondary screening. However, these methanol extracts

had weaker anti-yeast activity compared to that of methanol extracts reported by Remya and Vijayakumar (2008). Harindran *et al.* (1999) reported that a soil-derived *Streptomyces* CDRIL-312 had a promising anti-yeast activity. Furthermore, findings by Augustine *et al*. (2005a) showed that *S. albidoflavus* PU23 posed inhibition zone diameter of 16 mm (strong inhibition) against *C. albicans.* The present results showed that seven strains did not inhibited *C. albicans*; including strain T13 which inhibited only *C. parasilopsis.* Rabeh *et al.* (2007) reported that *S. viridiviolaceus* which was isolated from the lake also did not inhibited *C. albicans.* Therefore, it could be suggested that bioactivity of marine-derived actinomycetes against yeasts was very minimal compared to that of soil-derived actinomycetes.

Activity of strain T53 was minimal in primary screening, where it only posed weak inhibition against the Gram-negative bacteria tested. Interestingly, the activity of strain T53 was very promising in secondary screening, where it strongly inhibited *E. faecalis*, moderately inhibited the remaining test bacteria, but weakly inhibited the yeasts. Another promising strain was strain T15, where in primary screening, it displayed potent activity against all the Gram-positive and Gram-negative bacteria tested except for minimal activity against *S. aureus*. Furthermore, in secondary screening, strain T15 was inhibitory towards all the pathogens tested except for *P. aeruginosa*. These findings indicated that strains T53 and T15 had a broad spectrum activity. The production of high concentration of bioactive metabolite(s) could possibly explain the potent activity by the active strains, and vice versa. Ndonde and Semu (2000) reported a strain of *Streptomyces* which had ability of producing an antibiotic(s) which inhibited a number of pathogens, including *B. subtilis*, *S. aureus*, and *C. albicans*. In addition, Peela *et al*. (2005) reported that S*treptomyces* sp. BT 606 showed

antimicrobial activity against three multi-drug resistant pathogens; *S. aureus*, *P. aeruginosa*, and *C. albicans*.

Strains T4, T13, T15, T16, and T53 displayed a broad spectrum activity against at least one of the yeasts, Gram-positive and Gram-negative bacteria tested in secondary screening. Bioactivity of a single strain of *Streptomyces* against a variety of pathogenic microorganisms indicated that a single strain of *Streptomyces* could possibly produce a variety of antibiotics as demonstrated in previous studies. Du *et al.* (2004) reported that a variety of aminoglycoside antibiotics, mainly apromycin, tobramycin, and kanamycin B were produced by *S. tenebrarius* H6. In addition, Betina (1994) mentioned that asparenomycins A, B, and C were metabolites of *S. tokumonensis*. According to Hobbs *et al*. (1992), *S. coelicolor* A3(2) produced actinorhodin, calcium-dependent antibiotic, and methylenomycin A.

As indicated in Table 4.5, antibacterial potential of *Streptomyces* spp. in primary and secondary screenings was compared. Strains T3, T6, T9, T12, T13, T15, and T53 were active against at least one same bacteria tested in both primary and secondary screenings. This same pattern of activity indicated that the *Streptomyces* spp. produced extracellular and intracellular bioactive metabolite(s) antagonistic towards the same bacteria tested. Interestingly, strains T3, T4, T6, T16, T20, and T53 that were inactive against at least one of the bacteria tested in primary screening had inhibited those particular bacteria in secondary screening. There were few factors that could lead to this pattern of improved activity. Probably, the low concentration of the bioactive metabolite(s) or the intracellularly-bound bioactive metabolite(s) within the *Streptomyces* spp. was the reason why no inhibition was detected in primary screening. In addition, the increased production of the intra- or extracellular bioactive

metabolite(s) in liquid medium and subsequently in the crude extracts might have increased the antibacterial potential of the strains in secondary screening (Tan, 2007).

Strains T4, T16, and T20 might not have any extracellular antagonistic activity; however, they had intracellular activity (Table 4.5). It was possible that the inactive strains produced bioactive metabolite(s) extracellularly that might be effective against other pathogens not tested presently. The extraction of the intracellular or membrane-bound bioactive metabolite(s) needed to be performed on these strains. Fragmentation of mycelia in liquid medium during fermentation might cause inactivation of the bioactive metabolite(s) in the extracts (Shomura *et al.*, 1979; Tan, 2007). Thus, this could explain the non-inhibitory effect of strain T9 against *B. subtilis* and strains T6, T13, and T15 against *P. aeruginosa* in secondary screening, although they were inhibitory towards those particular bacteria tested in primary screening. Insufficient bioactive metabolite(s) in the crude extracts to reach the effective dose could be another possible reason for the non-inhibitory effect (Tan, 2007). Strains T3, T4, T6, T16, T20, and T53 were inactive against at least one bacteria tested in both primary and secondary screenings. *Pseudomonas aeruginosa* was the most insensitive bacteria tested, where five strains (T3, T4, T16, T20, and T53) were inactive against it in both primary and secondary screenings. This result suggested that these strains did not produced intracellular and extracellular bioactive metabolite(s) inhibitory towards *P. aeruginosa*.

Table 4.5 Comparison of antibacterial bioactivity of *Streptomyces* spp. in primary and secondary screenings

Strain	Screening	Test bacteria				
		B. subtilis	***E. faecalis***	***S. aureus***	***P. aeruginosa***	***P. shigelloides***
T3	**Primary**	-	√	√	-	-
	Secondary	√	√	√	-	√
T4	**Primary**	-	-	-	-	-
	Secondary	√	√	√	-	√
T6	**Primary**	-	√	-	√	-
	Secondary	-	√	√	-	√
T9	**Primary**	√	√	√	√	√
	Secondary	-	√	√	√	√
T12	**Primary**	√	√	√	√	√
	Secondary	√	√	√	√	√
T13	**Primary**	√	√	√	√	√
T15	**Primary**	√	√	√	√	√
	Secondary	√	√	√	-	√
T16	**Primary**	-	-	-	-	-
	Secondary	√	√	√	-	√
T20	**Primary**	-	-	-	-	-
	Secondary	√	√	√	-	-
T52	**Primary**	√	√	√	√	√
	Secondary	√	√	√	√	√
T53	**Primary**	-	-	-	√	√
	Secondary	√	√	√	√	√

* Primary screening: Test bacteria were streaked on SA plates and incubated at 37ºC ± 2ºC for 48 h for the cross streak antibacterial assay. Secondary screening: Test bacteria and yeasts were lawned on NA and SDA plates, respectively, and incubated at 37ºC ± 2ºC for 48 h for the disc diffusion assay

4.2.3 Susceptibility of test pathogens in primary and secondary screenings

In primary screening as indicated by Table 4.6, more strains tested displayed strong inhibition against Gram-positive bacteria tested compared to Gram-negative bacteria tested. *Enterococcus faecalis* and *P. aeruginosa* were the most sensitive pathogens tested, where seven out of eleven strains tested were active against them, followed closely by *S. aureus* and *P. shigelloides* that were inhibited by six strains. *Bacillus subtilis* was the less sensitive pathogen tested, where only five strains were active against it. It was strongly inhibited by strain T15 and moderately inhibited by strain T12, but was weakly inhibited by strains T9, T13, and T52.

Enterococcus faecalis was strongly inhibited by strains T3 and T15, moderately inhibited by strains T6 and T12, but weakly inhibited by strains T9, T13, and T52 (Table 4.6). Strains T3 and T9 displayed strong inhibition; while, strain T12 displayed moderate inhibition against *S. aureus*. Furthermore, strains T13, T15, and T52 displayed weak inhibition against it. Both *P. aeruginosa* and *P. shigelloides* were strongly and moderately inhibited by strain T15 and strain T12, respectively. Furthermore, *P. aeruginosa* was weakly inhibited by strains T6, T9, T13, T52, and T53. Meanwhile, the same strains that exhibited weak inhibition towards *P. aeruginosa* except strain T6 also weakly inhibited *P. shigelloides.*

Table 4.6 Sequence of inhibitory levels exhibited by *Streptomyces* spp. against the test bacteria in primary screening

B. subtilis	:	T15	> T12	>	T9, T13, T52
E. faecalis	**:**	T3, T15	> T6, T12	>	T9, T13, T52
S. aureus	**:**	T3, T9	> T12	>	T13, T15, T52
P. aeruginosa **:**		T15	> T12	>	T6, T9, T13, T52, T53
P. shigelloides**:**		T15	> T12	>	T9, T13, T52, T53

* Test bacteria were streaked on SA plates and incubated at 37ºC ± 2ºC for 48 h for the cross streak antibacterial assay

In secondary screening as shown in Table 4.7, Gram-positive bacteria tested were inhibited by at least nine strains; meanwhile, Gram-negative bacteria tested were inhibited by at least four strains. Furthermore, yeasts were inhibited by at least four strains. *Enterococcus faecalis* and *S. aureus* were the most sensitive bacteria tested, where all the strains were active against them, followed closely by *P. shigelloides* that was inhibited by ten strains then *B. subtilis*, inhibited by nine strains. *Pseudomonas aeruginosa* was the less sensitive bacteria tested with only four strains active against it. *Bacillus subtilis* was not susceptible to strains T6 and T9. However, strains T15 and T53 exhibited moderate antagonistic activity against it. *Bacillus subtilis* was weakly inhibited by strains T3, T4, T12, T13, T16, T20, and T52. *Enterococcus faecalis* and *S. aureus* were weakly inhibited by all the strains except for strain T53 that had strong and moderate inhibitions, respectively. *Pseudomonas aeruginosa* was insensitive to seven

strains. Strain T53 moderately inhibited it, while strains T9, T12, and T52 weakly inhibited it. *Plesiomonas shigelloides* was insensitive to strain T20, but was strongly inhibited by strains T15 and T16, moderately inhibited by strains T13 and T53, but weakly inhibited by six other strains. Results of anti-yeast assay showed only weak inhibition, where *C. parasilopsis* was more sensitive than *C. albicans*. Strains T4, T15, T16, and T53 inhibited both yeasts. Furthermore, inhibition by strain T13 against *C. parasilopsis* was detected but no inhibition was displayed against *C. albicans*.

Table 4.7 Sequence of inhibitory levels exhibited by *Streptomyces* spp. against the test pathogens in secondary screening

B. subtilis	:	-	>	T15, T53	>	T3, T4, T12, T13, T16, T20, T52
E. faecalis	**:**	T53	>	-	>	T3, T4, T6, T9, T12, T13, T15, T16, T20, T52
S. aureus	**:**	-	>	T53	>	T3, T4, T6, T9, T12, T13, T15, T16, 20, T52
P. aeruginosa	**:**	-	>	T53	>	T9, T12, T52
P. shigelloides	**:**	T15, T16	>	T13, T53	>	T3, T4, T6, T9, T12, T52
C. albicans	**:**	-	>	-	>	T4, T15, T16, T53
C. parasilopsis	**:**	-	>	-	>	T4, T13, T15, T16, T53

* Test bacteria and yeasts were lawned on NA and SDA plates, respectively, and incubated at 37ºC ± 2ºC for 48 h for the disc diffusion antibacterial and anti-yeast assays

Antagonistic bioactivity of antimicrobial agents towards the pathogens tested produced varying inhibition zones (Table 1, Appendix B). In secondary screening, novobiocin and streptomycin with the potency of 5 µg and 10 µg, respectively were used as the positive controls for antibacterial assay. For novobiocin, the inhibition zone against *B. subtilis* was 15.0 ± 0.0 mm, while the values for *E. faecalis*, *S. aureus*, *P. aeruginosa*, and *P. shigelloides* were 15.0 ± 0.0, 13.0 ± 0.0, 16.0 ± 0.0, and 18.0 ± 0.0 mm, respectively. However, the inhibition zone produced by streptomycin against *B. subtilis* was 14.0 ± 0.0 mm, while the values for *E. faecalis*, *S. aureus*, *P. aeruginosa*, and *P. shigelloides* were 13.0 ± 0.0, 16.0 ± 0.0, 15.0 ± 0.0, and 14.0 ± 0.0 mm, respectively. Nystatin with the potency of 100 units (equivalent to 22.4 µg of pure nystatin) served as the positive control for anti-yeast assay. The inhibition zones produced against *C. albicans* and *C. parasilopsis* were 27.0 ± 0.0 and 28.0 ± 0.0 mm, respectively (Table 2, Appendix B). Methanol (solvent used for preparing and diluting the extracts) served as the negative control. However, methanol itself posed antagonism against the pathogens tested. The antagonistic activity of methanol against *B. subtilis*, *E. faecalis*, *S. aureus*, *P. aeruginosa*, *P. shigelloides*, *C. albicans*, and *C. parasilopsis* were measured as 11.3 ± 0.6, 10.0 ± 1.0, 10.3 ± 1.5, 12.3 ± 0.6, 9.0 ± 1.0, 13.3 ± 0.6, and 11.7 ± 0.6 mm, respectively (Tables 1 and 2, Appendix B).

In the case of *B. subtilis* inhibition, the activity of strains T15 and T53 were comparable to the inhibitory activity of 5 µg novobiocin and 10 µg streptomycin, where inhibition zones of 12.0 ± 1.7 mm and 13.7 ± 0.6 mm were produced, respectively. In *E. faecalis* inhibition, strain T53 had antagonistic activity higher than the inhibitory activity of 5 µg novobiocin and 10 µg streptomycin, where inhibition zone of 16.0 ± 0.7 mm was produced. The activity of strain T53 against *S. aureus*, indicated by inhibition zone of 14.0 ± 0.0 mm, was slightly lower compared to the inhibitory activity of 5 µg

novobiocin, but higher than the inhibitory activity of 10 µg streptomycin. In *P. aeruginosa* inhibition, the activity of strain T53 was comparable to the inhibitory activity of 5 µg novobiocin and 10 µg streptomycin, where the inhibition zone produced was 13.0 ± 0.0 mm. In the case of *P. shigelloides* inhibition, the antagonistic activity of strains T13 and T53 were comparable to the inhibitory activity of 10 µg streptomycin, where equal inhibition zones of 12.0 ± 1.0 mm were produced by both strains. Meanwhile, the antagonistic activity of strains T15 and T16 were comparable to the inhibitory activity of 5 µg novobiocin, where the inhibition zones produced were 16.3 ± 0.4 mm and 15.7 ± 1.1 mm, respectively. The antagonistic activity of the five strains that were found to be active against at least one of the yeasts was very low compared to the inhibitory potential possessed by the positive control nystatin.

In this study, *B. subtilis* and *P. aeruginosa* were the most resistant bacteria tested in primary screening and secondary screening, respectively. Pisano *et al*. (1989) reported that *B. subtilis* was the most susceptible bacteria tested in the previous study. Pandey *et al*. (2002) reported that 31 (86.1%) and 27 out of 36 (75%) actinomycetes were active against *B. subtilis* and *S. aureus*, respectively in primary screening. In secondary screening, 23 out of 36 (63.9%) strains were inhibitory towards both *B. subtilis* and *S. aureus*. The antibacterial activity against *B. subtilis* and *S. aureus* in primary screening, demonstrated by Pandey *et al*. (2002) was greater compared to the present study. However, the antibacterial activity against those particular bacteria in secondary screening was greater in this study. In the present study, *B. subtilis* was inhibited by nine out of eleven strains in which two strains had inhibition zones diameter of greater than 10 mm. Zheng *et al*. (2000) reported that *B. subtilis* was inhibited by nine out of fifteen *Streptomyces* spp. with inhibition zones diameter of less than 10 mm. Hence, the antibacterial activity against *B. subtilis* in the present study was

higher compared to the findings by Zheng *et al*. (2000). According to Kavithambigai (2006), *P. aeruginosa* was mostly resistant against the metabolites produced by actinomycetes. In addition, Drusano (1992) highlighted that *P. aeruginosa* remained a persistent clinical pathogen in which extended-spectrum penicillins, third-generation cephalosporins, penem and carbapenems antibiotics, and the new fluoroquinolones were not always effective clinically. In the present study, number of active strains against *P. aeruginosa* observed in primary screening was greater than the findings by Tan (2007). The antibacterial activity against *E. faecalis* was highest in both primary and secondary screenings; therefore, *E. faecalis* was described as the most susceptible bacteria tested. These results showed that diffusible extracellular metabolites produced on agar plate and the intra- or extracellular metabolites in liquid medium and subsequently in the crude extracts could greatly induce the antibacterial activity against *E. faecalis*.

The cross streak anti-yeast assay might not detect the anti-yeast activity extracellularly. Although none of the strains were active against the yeasts in primary screening, but four and five strains in secondary screening inhibited the growth of *C. albicans* and *C. parasilopsis*, respectively. This was contrary to the results observed by Shomura *et al.* (1979), which reported that bioactive compound(s) was only produced on agar. Unlike the observation in the present study, anti-yeast activity against *C. albicans* and *C. parasilopsis* in primary screening was demonstrated by Kavithambigai (2006). Frequency of activity against *C. albicans* among the marine actinomycetes varied between 23-42% (Bredholt *et al*., 2008). All strains active against *C. parasilopsis* were also active against *C. albicans*, but not vice versa. This result was in good agreement with findings from other antibiotic fermentation (Tan, 2007). Therefore, the bioactive metabolite(s) was assumed to be more selective against *C.*

albicans. The anti-yeast activity might be stimulated by intracellular bioactive metabolite(s), which were bound inside the membrane of the strains and released during submerged cultivation (Kavithambigai, 2006).*Candida parasilopsis* was more susceptible than *C. albicans*. The *Streptomyces* spp. in this study posed poor antagonism against the yeasts. Opportunistic infections have increased gradually, with *Candida* spp. as the major fungal pathogens (Tanaka, 1992).

In accordance to the findings by Kavithambigai (2006), antibacterial activity was prominent in secondary screening. In this study, all the strains had antibacterial activity. The antibacterial activity in secondary screening was higher than the findings by Tan *et al*. (2004), Peela *et al*. (2005), and Tan (2007), which showed 51.0%, 44.0%, and 75.8% active strains, respectively. The highest antibacterial activity on Gram-positive bacteria tested; *B. subtilis, E. faecalis*, and *S. aureus* was displayed by strain T53, where it produced inhibition zone of 13.7 ± 0.6 mm, 16.0 ± 0.7 mm, and 14.0 ± 0.0 mm, respectively. Meanwhile, the highest antibacterial activity on Gram-negative bacteria tested; *P. aeruginosa* and *P. shigelloides* was displayed by strain T53 (13.0 ± 0.0 mm inhibition zone) and strain T15 (16.3 ± 0.4 mm inhibition zone), respectively.

The antibacterial pattern exhibited by the strains in the present investigation, where the antagonism against Gram-positive bacteria was greater than Gram-negative bacteria was similar to the ones reported by Kavithambigai (2006) and Tan *et al*. (2004). According to Pandey *et al*. (2002), the reason for different sensitivity between Gram-positive bacteria and Gram-negative bacteria could be ascribed to the morphological differences between these microorganisms; Gram-negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes. The Gram-

positive bacteria were more susceptible having only an outer peptidoglycan layer, which was not an effective permeability barrier. Three strains in secondary screening were found to inhibit all the Gram-positive and Gram-negative bacteria tested. These strains could possibly produce the same bioactive metabolite(s) inhibitory towards the Gram-positive and Gram-negative bacteria tested. Hobbs *et al.* (1992) reported that Gram-positive and Gram-negative bacteria were susceptible to methylenomycin, which was produced by *S. violaceus* and *S. coelicolor*.

According to Ndonde and Semu (2000), the sensitivity of the pathogens tested to the bioactive metabolite(s) produced by the *Streptomyces* spp. might imply that the pathogens tested might have not been exposed to similar bioactive metabolite(s) previously. As a result, they were still susceptible to such metabolite(s). Greater resistance of the pathogens tested might be due to previous exposure to antibiotics routinely used in disease control which might be similar to those produced by the present *Streptomyces* spp. In addition, the sensitivity of the antimicrobial substances *ex-situ* towards light and temperature, natural instability after prolonged storage, or low amount of the bioactive substances present in the crude extracts were the possible explanations for the low antimicrobial potential (Tan *et al*., 2004).

4.3 Optimisation of Culture Conditions for Bioactive Metabolite(s) Production by *Streptomyces* Strain T15 against *Plesiomonas shigelloides*

Table 4.8 gives the pH profiles during growth of strain T15 in ISP2 medium at 120 rpm and 28ºC ± 2ºC, pH analysis in twelve days showed that it had changed from 6.87 ± 0.14 (day 3) to 7.64 ± 0.62 (day 6) to 6.10 ± 1.70 (day 9) and to 6.20 ± 0.01 (day 12). In ISP2 medium under agitation condition, highest yield of bioactive metabolite(s)

was generated when pH level was at its lowest (6.10 ± 1.70). During cultivation in ISP4 medium under agitation condition, pH levels in twelve days had changed from 6.67 ± 0.09 (day 3) to 8.25 ± 0.12 (day 6) to 7.01 ± 0.71 (day 9) and to 7.16 ± 0.40 (day 12). However, there was no production of bioactive metabolite(s) in ISP4 medium under agitation condition. During cultivation in SA medium under agitation condition, pH changed from 5.87 ± 0.89 (day 3) to 6.69 ± 0.08 (day 6) to 6.80 ± 0.07 (day 9) and to 7.34 ± 0.06 (day 12). Highest yield of bioactive metabolite(s) was generated when pH level was 6.80 ± 0.07 in SA medium under agitation condition.

During cultivation in ISP2 medium under static condition, pH levels were recorded as 6.76 ± 0.00 (day 3), 6.63 ± 0.12 (day 6), 6.96 ± 0.03 (day 9), and 6.40 ± 0.30 (day 12) (Table 4.8). There was no bioactive metabolite(s) produced in ISP2 medium under static condition. During cultivation in ISP4 medium under static condition, pH levels were recorded as 7.18 ± 0.40 (day 3), 7.10 ± 0.49 (day 6), 7.07 ± 0.12 (day 9), and 6.71 ± 0.63 (day 12). In ISP4 medium, highest yield of bioactive metabolite(s) was generated when pH level was 7.07 ± 0.12. During cultivation in SA medium under static condition, changes of pH levels were from 7.10 ± 0.52 (day 3) to 7.60 ± 0.51 (day 6) to 7.46 ± 0.16 (day 9) and to 7.47 ± 0.54 (day 12). The same case with cultivation in ISP2 medium under static condition, there was also no production of bioactive metabolite(s) in SA medium under static condition.

Table 4.8 pH profiles during growth of 3, 6, 9, and 12 day-old *Streptomyces* strain T15 in ISP2, ISP4, and SA media under agitation and static submerged conditions

Day	pH level					
	Agitation			Static		
	ISP2	ISP4	SA	ISP2	ISP4	SA
3	ap 6.87 ± 0.14	ap 6.67 ± 0.09	ap 5.87 ± 0.89	apq 6.76 ± 0.00	ap 7.18 ± 0.40	ap 7.10 ± 0.52
6	ap 7.64 ± 0.62	aq 8.25 ± 0.12	bpq 6.69 ± 0.08	apr 6.63 ± 0.12	abp 7.10 ± 0.49	bp 7.60 ± 0.51
9	ap 6.10 ± 1.70	ap 7.01 ± 0.71	aq 6.80 ± 0.07	aq 6.96 ± 0.03	ap 7.07 ± 0.12	bp 7.46 ± 0.16
12	ap 6.20 ± 0.01	bp 7.16 ± 0.40	bq 7.34 ± 0.06	ar 6.40 ± 0.30	abp 6.71 ± 0.63	bp 7.47 ± 0.54

* Mean of three readings with standard deviation. For the same growth period with different growth media, means with different letters (a-b) in the same row are significantly different ($p < 0.05$). For the same growth media with different growth period, means with different letters (p-r) in the same column are significantly different ($p < 0.05$). *Streptomyces* strain T15 was cultivated in ISP2, ISP4, and SA media under agitation and static submerged conditions. The cultures were incubated at 28ºC ± 2ºC for 3-12 days for static condition; meanwhile, 28ºC ± 2ºC at 120 rpm for 3-12 days for agitation condition

Tables 3 and 4 in Appendix B are referred. Statistically, pH of 3 and 9-day old cultures of strain T15 in terms of cultivation in ISP2, ISP4, and SA media under agitation condition was not significantly different ($p>0.05$). Under agitation condition, pH of 6-day old culture cultivated in SA medium was significantly different ($p<0.05$) compared to that of cultures cultivated in ISP2 and ISP4 media. The lowest pH (6.69; acidic) observed in 6-day old culture was obtained in SA medium. Under agitation condition, pH of 12-day old culture cultivated in ISP2 medium was significantly different ($p<0.05$) compared to that of cultures cultivated in ISP4 and SA media. The

lowest pH (6.20; acidic) observed in 12-day old culture was obtained in ISP2 medium. Tables 5 and 6 in Appendix B are referred. Statistically, pH of 3, 6, and 12-day old cultures in terms of cultivation in ISP2, ISP4, and SA media under static condition was not significantly different ($p>0.05$). Under static condition, pH of 9-day old culture cultivated in SA medium was significantly different ($p<0.05$) compared to that of cultures cultivated in ISP2 and ISP4 media. The highest pH (7.46; alkaline) observed in 9-day old culture was obtained in SA medium.

Tables 7 and 8 in Appendix B are referred. Statistically, pH of cultures of strain T15 cultivated in ISP2 medium under agitation condition in terms of fermentation period of 3, 6, 9, and 12 days was not significantly different ($p>0.05$). During cultivation in ISP4 medium under agitation condition, pH of 6-day old culture was significantly different ($p<0.05$) compared to that of 3, 9, and 12-day old cultures. The highest pH (8.25; alkaline) obtained in ISP4 medium was observed from 6-day old culture. During cultivation in SA medium under agitation condition, pH of 3-day old culture was significantly different ($p<0.05$) compared to that of 9 and 12-day old cultures. The lowest pH (5.87 ± 0.89; acidic) obtained in SA medium was observed from 3-day old culture. Tables 9 and 10 in Appendix B are referred. Statistically, pH of cultures cultivated in ISP2 medium under static condition in terms of fermentation period of 3, 6, 9, and 12 days was significantly different ($p<0.05$). There was a significant difference in the pH of 3-day old and 12-day old cultures, 6-day old and 9-day old cultures, and 9-day old and 12-day old cultures. The highest pH (6.96 ± 0.03; almost neutral) obtained in ISP2 medium was observed from 9-day old culture. However, pH of cultures cultivated in ISP4 and SA media under static condition in terms of fermentation period of 3, 6, 9, and 12 days was not significantly different ($p>0.05$).

Weights of crude extracts that possessed bioactive metabolite(s) inhibitory to *P. shigelloides* are discussed based on the tabulated data in Table 4.9. During cultivation in ISP2 medium under agitation condition, weight analysis in twelve days showed that it had changed from 0.16 ± 0.13 g (day 3) to 0.14 ± 0.03 g (day 6) to 0.08 ± 0.02 g (day 9) and to 0.33 ± 0.07 g (day 12). In ISP2 medium, 0.08 ± 0.02 g (day 9) crude extract had generated the strongest antagonistic activity against *P. shigelloides*. During cultivation in ISP4 medium under agitation condition, weights of crude extracts were recorded as 0.05 ± 0.02 g (day 3), 0.07 ± 0.04 g (day 6), 0.17 ± 0.08 g (day 9), and 0.22 ± 0.06 g (day 12). However, there was no production of bioactive metabolite(s) in ISP4 medium under agitation condition. During cultivation in SA medium under agitation condition, weights of crude extracts were recorded as 0.14 ± 0.06 g (day 3), 0.23 ± 0.12 g (day 6), 0.25 ± 0.02 g (day 9), and 0.11 ± 0.07 g (day 12). In SA medium, the strongest bioactivity against *P. shigelloides* was produced by bioactive metabolite(s) contained in crude extract weighed 0.25 ± 0.02 g (day 9).

During cultivation in ISP2 medium under static condition, weights of crude extracts were recorded as 0.26 ± 0.02 g (day 3), 0.22 ± 0.01 g (day 6), 0.25 ± 0.03 g (day 9), and 0.17 ± 0.06 g (day 12) (Table 4.9). There was no production of bioactive metabolite(s) in ISP2 medium under static condition. During cultivation in ISP4 medium under static condition, weights of crude extracts were recorded as 0.09 ± 0.07 g (day 3), 0.07 ± 0.01 g (day 6), 0.14 ± 0.12 g (day 9), and 0.10 ± 0.03 g (day 12). In ISP4 medium, the bioactive metabolite(s) with highest antagonistic activity against *P. shigelloides* was produced in crude extract weighed 0.14 ± 0.12 g (day 9). During cultivation in SA medium under static condition, weights of crude extracts were recorded as 0.12 ± 0.11 g (day 3), 0.24 ± 0.09 g (day 6), 0.14 ± 0.07 g (day 9), and 0.38

± 0.17 g (day 12). As in ISP2 medium, there was also no production of bioactive metabolite(s) in SA medium under static condition.

Table 4.9 Crude extracts weight (g) profiles during growth of 3, 6, 9, and 12 day-old *Streptomyces* strain T15 in ISP2, ISP4, and SA media under agitation and static submerged conditions

Day	Crude extracts weight (g)					
	Agitation			Static		
	ISP2	ISP4	SA	ISP2	ISP4	SA
3	^{ap} 0.16 ± 0.13	^{ap} 0.05 ± 0.02	^{ap} 0.14 ± 0.06	^{ap} 0.26 ± 0.02	^{bp} 0.09 ± 0.07	^{abp} 0.12 ± 0.11
6	^{abp} 0.14 ± 0.03	^{apq} 0.07 ± 0.04	^{bp} 0.23 ± 0.12	^{apq} 0.22 ± 0.01	^{bp} 0.07 ± 0.01	^{apq} 0.24 ± 0.09
9	^{ap} 0.08 ± 0.02	^{abqr} 0.17 ± 0.08	^{bp} 0.25 ± 0.02	^{ap} 0.25 ± 0.03	^{ap} 0.13 ± 0.12	^{ap} 0.14 ± 0.07
12	^{aq} 0.33 ± 0.07	^{abr} 0.22 ± 0.06	^{bp} 0.11 ± 0.07	^{abq} 0.17 ± 0.06	^{ap} 0.10 ± 0.03	^{bq} 0.38 ± 0.17

* Mean of three readings with standard deviation. For the same growth period with different growth media, means with different letters (a-b) in the same row are significantly different ($p< 0.05$). For the same growth media with different growth period, means with different letters (p-r) in the same column are significantly different ($p< 0.05$). *Streptomyces* strain T15 was cultivated in ISP2, ISP4, and SA media under agitation and static submerged conditions. The cultures were incubated at 28ºC ± 2ºC for 3-12 days for static condition; meanwhile, 28ºC ± 2ºC at 120 rpm for 3-12 days for agitation condition

Tables 11 and 12 in Appendix B are referred. Statistically, weight of crude extracts of 3 and 6-day old cultures of strain T15 in terms of cultivation in ISP2, ISP4, and SA media under agitation condition was not significantly different ($p>0.05$). Under agitation condition, weight of crude extracts of 9-day old cultures cultivated in ISP2 and SA media was significantly different ($p<0.05$). Although the crude extract

production in ISP2 medium under agitation condition in nine days period was low (0.08 ± 0.02 g); the most potent bioactive metabolite(s) inhibitory to *P. shigelloides* was produced in it compared to other crude extracts from different growth conditions, fermentation media, and fermentation period. Under agitation condition, weight of crude extracts of 12-day old cultures cultivated in ISP2 and SA media was significantly different ($p<0.05$). The lowest weight of crude extracts (0.11 ± 0.07 g) observed in 12-day old culture was obtained in SA medium. Tables 13 and 14 in Appendix B are referred. Statistically, weight of crude extracts of 3 and 9-day old cultures in terms of cultivation in ISP2, ISP4, and SA media under static condition was not significantly different ($p>0.05$). Under static condition, weight of crude extracts of 6-day old culture cultivated in ISP4 medium was significantly different ($p<0.05$) compared to that of cultures cultivated in ISP2 and SA media. The lowest weight of crude extracts (0.07 ± 0.01 g) observed in 6-day old culture was obtained in ISP4 medium. Under static condition, weight of crude extracts of 12-day old cultures cultivated in ISP4 and SA media was significantly different ($p<0.05$). The lowest weight of crude extracts (0.10 ± 0.03 g) observed in 12-day old culture was obtained in ISP4 medium.

Tables 15 and 16 in Appendix B are referred. Statistically, weight of crude extracts of 12-day old strain T15 cultivated in ISP2 medium under agitation condition was significantly different ($p<0.05$) compared to that of 3, 6, and 9-day old cultures. The highest weight of crude extracts (0.33 ± 0.07 g) obtained in ISP2 medium was observed from 12-day old culture. During cultivation in ISP4 medium under agitation condition, there was a significant difference ($p<0.05$) in weight of crude extracts of 3 and 9-day old cultures, 3 and 12-day old cultures, and 6 and 12-day old cultures. The highest weight of crude extracts (0.22 ± 0.06 g) obtained in ISP4 medium was observed from 12-day old culture. However, weight of crude extracts of cultures cultivated in SA

medium under agitation condition in terms of fermentation period of 3, 6, 9, and 12 days was not significantly different ($p>0.05$). Table 17 in Appendix B is referred. Statistically, weight of crude extracts of cultures cultivated in ISP2, ISP4, and SA media under static condition in terms of fermentation period of 3, 6, 9, and 12 days was not significantly different ($p>0.05$).

As shown in Table 4.10 and Plate 4.5, there was no bioactivity against *P. shigelloides* by strain T15 grown in ISP2 and SA media under static growth condition, and in ISP4 medium under agitation growth condition. In agitation condition, however, antagonistic activity was detected from crude extracts produced in ISP2 and SA media. The largest inhibition zone (11.7 ± 0.6 mm) was produced by bioactive metabolite(s) in crude extract grown in ISP2 medium for nine days, followed by bioactive metabolite(s) in crude extract grown in SA medium for nine days (11.3 ± 1.2 mm), and bioactive metabolite(s) in crude extract grown in ISP2 medium for three days (11.0 ± 0.0 mm). Bioactive metabolite(s) in crude extracts from ISP2 medium produced inhibition zones in the range of 10.0 ± 0.0 mm (day 12) to 11.7 ± 0.6 mm (day 9). There was no inhibitory activity displayed by bioactive metabolite(s) in crude extract produced in day 3. The inhibitory potential of bioactive metabolite(s) in crude extracts produced in ISP2 medium was expressed in sequence of decreasing values as 11.7 ± 0.6 mm (day 9), 11.0 ± 0.0 mm (day 6), and 10.0 ± 0.0 mm (day 12). In contrast to ISP2 medium, there was bioactive metabolite(s) production in SA medium from day 3. Bioactive metabolite(s) in crude extracts from SA medium produced inhibition zones in the range of 9.3 ± 1.2 mm (day 6) to 11.3 ± 1.2 mm (day 9). The inhibitory potential of crude extracts produced in SA medium was expressed in sequence of decreasing values as 11.3 ± 1.2 mm (day 9), 10.3 ± 0.6 mm (day 12), 10.0 ± 0.0 mm (day 3), and 9.3 ± 1.2 mm (day 6). In static condition, antagonistic activity was only observed from bioactive metabolite(s)

in crude extracts produced in ISP4 medium. Bioactive metabolite(s) in crude extracts from ISP4 medium produced inhibition zones in the range of 8.0 ± 0.0 mm (day 12) to 9.7 ± 0.6 mm (day 9). The inhibitory potential of bioactive metabolite(s) in crude extracts produced in ISP4 medium was expressed in sequence of decreasing values as 9.7 ± 0.6 mm (day 9), 9.0 ± 0.0 mm (day 6), and 8.0 ± 0.0 mm (day 12). In ISP4 medium, there was no inhibitory activity displayed by bioactive metabolite(s) in crude extract produced in day 3. Plate 4.6 shows the control plates.

Table 4.10 Inhibition spectrum (mm) profiles during growth of 3, 6, 9, and 12-day old *Streptomyces* strain T15 (cultivated in ISP2, ISP4, and SA media under agitation and static submerged conditions) against *P. shigelloides* assessed via disc diffusion method

Day	**Diameter of inhibition zone (mm)**		
	Agitation		**Static**
	ISP2	**SA**	**ISP4**
3	- ^{ap}	10.0 ± 0.0 ^{bpq}	-
6	11.0 ± 0.0 ^{aq}	9.3 ± 1.2 ^{bp}	9.0 ± 0.0
9	11.7 ± 0.6 ^{ar}	11.3 ± 1.2 ^{aq}	9.7 ± 0.6
12	10.0 ± 0.0 ^{as}	10.3 ± 0.6 ^{apq}	8.0 ± 0.0

* Mean of three readings with standard deviation. For the same growth period with different growth media, means with different letters (a-b) in the same row are significantly different ($p < 0.05$). For the same growth media with different growth period, means with different letters (p-s) in the same column are significantly different ($p < 0.05$). *Streptomyces* strain T15 was cultivated in ISP2, ISP4, and SA media under agitation and static submerged conditions. The cultures were incubated at 28ºC ± 2ºC for 3-12 days for static condition; meanwhile, 28ºC ± 2ºC on a rotary shaker at 120 rpm for 3-12 days for agitation condition. Extracts were prepared by extraction with methanol: dichloromethane (1:1). *Plesiomonas shigelloides* was lawned on NA plates for the disc diffusion assay, and incubated at 37ºC ± 2ºC for 48 h. Growth inhibition was defined as mm

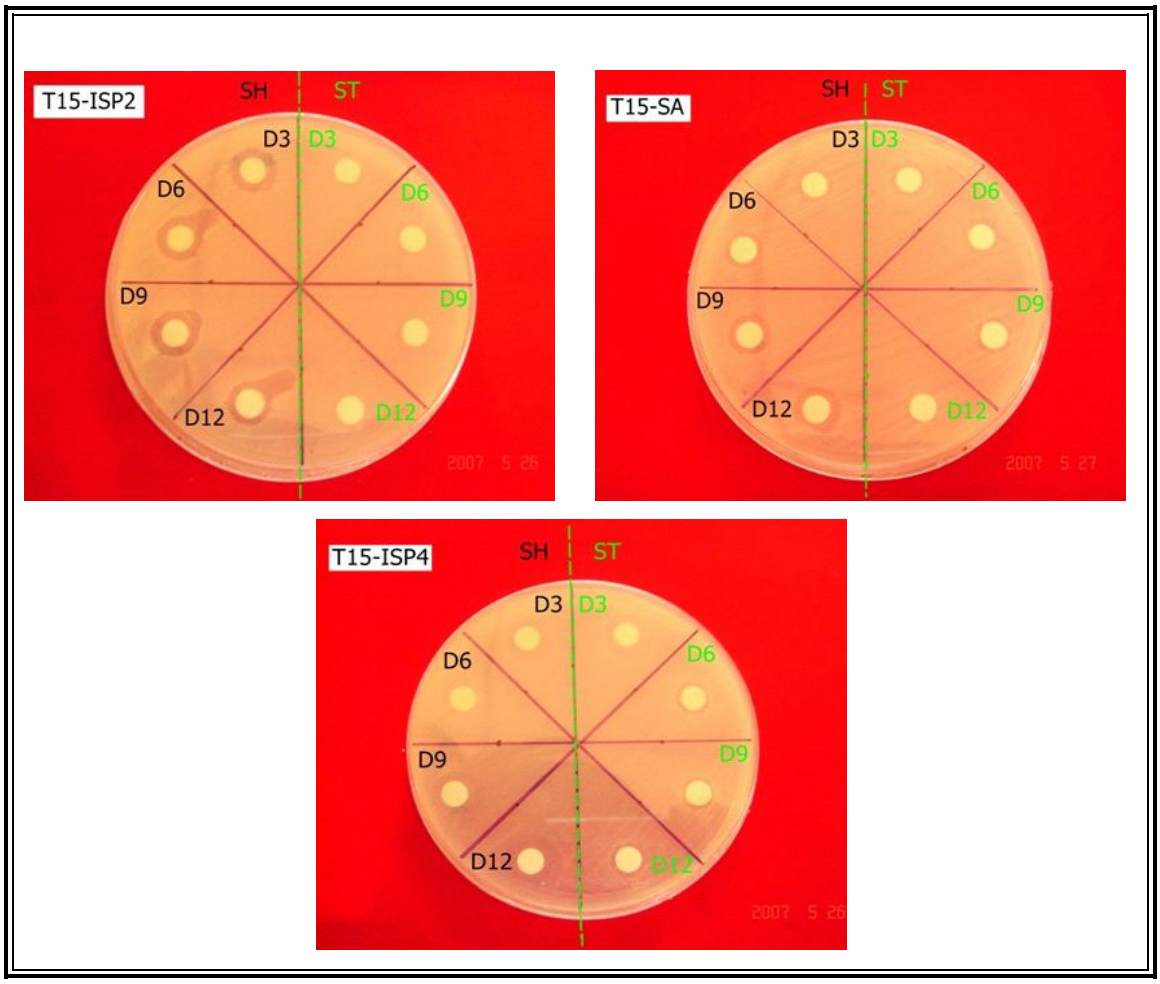
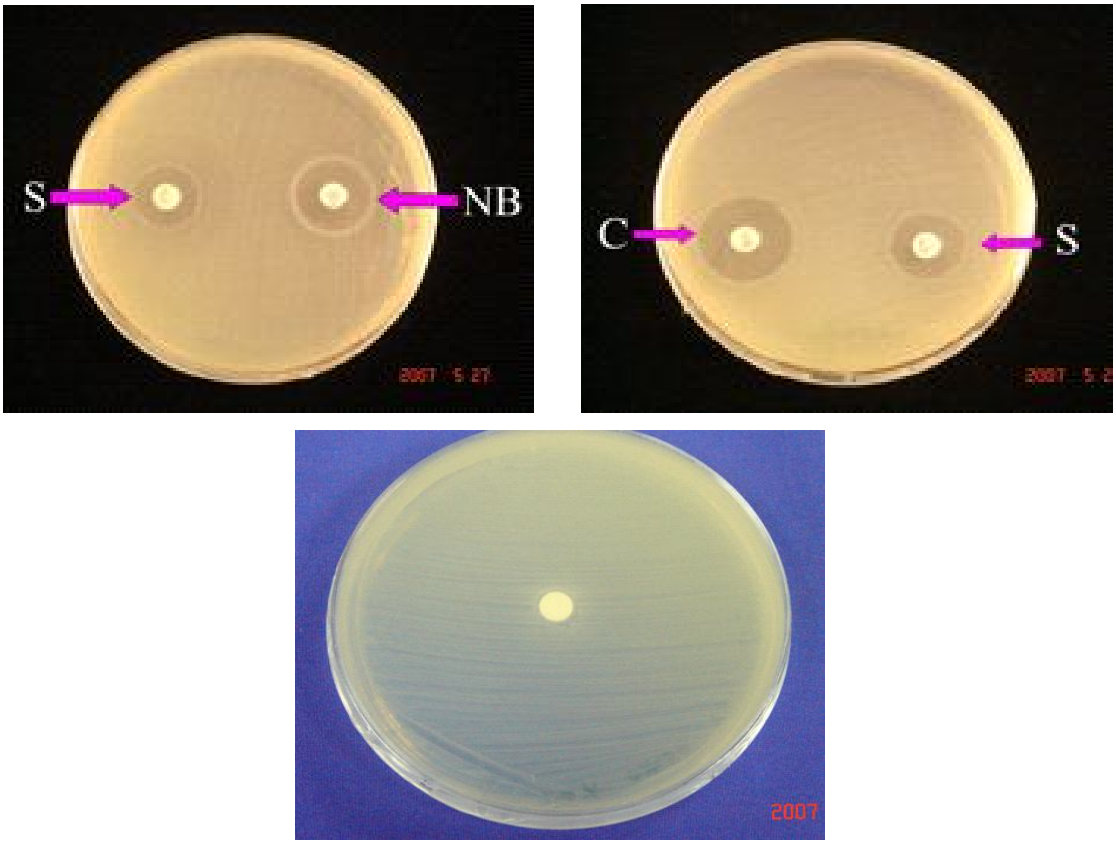


Plate 4.5 Antagonistic activity of crude extracts of 3-day old (D3), 6-day old (D6), 9-day old (D9), and 12-day old (D12) *Streptomyces* strain T15 against *P. shigelloides* (*Streptomyces* strain T15 was cultivated in ISP2 (T15-ISP2), ISP4 (T15-ISP4), and SA (T15-SA) liquid media in agitation (SH) and static (ST) growth conditions. The cultures were incubated at 28ºC ± 2ºC for 3-12 days for static condition; meanwhile, 28ºC ± 2ºC on a rotary shaker at 120 rpm for 3-12 days for agitation condition)



DMSO

Plate 4.6 Inhibition zones on control plates of NA lawned with *P. shigelloides* assessed via disc diffusion method. Standard antibiotic discs of 10 µg streptomycin (S), 5 µg novobiocin (NB), and 30 µg chloramphenicol (C) served as positive control; and DMSO served as negative control

Tables 18 and 19 in Appendix B are referred. Under agitation condition, bioactive metabolite(s) production in day 3 was not comparable because bioactive metabolite(s) was only produced in SA medium. Therefore, SA medium was considered as the best medium for the production of bioactive metabolite(s) in day 3. Statistically, in day 6, bioactive metabolite(s) produced through agitation condition in ISP2 and SA media (ISP4 medium was not taken into consideration due to the absence of antagonistic activity from the crude extracts produced in it) was significantly different ($p<0.05$). Between these media, the highest yield of bioactive metabolite(s) was generated in ISP2 medium. Thus, ISP2 was assumed as the best medium for supporting the production of bioactive metabolite(s) inhibitory towards *P. shigelloides* in day 6. The bioactive metabolite(s) of crude extract produced in ISP2 medium was significantly higher than that of SA medium ($p<0.05$). The crude extracts produced in ISP2 and SA media in day 9 and day 12 had no significant difference in production of bioactive metabolite(s) ($p>0.05$).

Tables 20 and 21 in Appendix B are referred. In cultivation in ISP2 medium under agitation condition, bioactive metabolite(s) production in terms of fermentation period of 3, 6, 9, and 12 days was significantly different ($p<0.05$). Fermentation period of 9 days was observed as the optimum period for maximum yield of bioactive metabolite(s), followed by 6-day period, and 12-day period. The bioactive metabolite(s) production varied with different antagonistic levels ($p<0.05$). Under agitation condition, bioactive metabolite(s) production in SA medium in terms of fermentation period of 3, 6, 9, and 12 days was not significantly different ($p>0.05$).

Fermentation conditions are optimized to express larger quantities of compound when desirable activity is discovered (Gullo *et al.*, 2006). According to Ndonde and

Semu (2000), probably all streptomycetes possessed some antimicrobial properties if suitable conditions were provided during culturing of these organisms for purposes of evaluating antibiotic production. Antibiotic productivity in the genus *Streptomyces* is profoundly influenced by factors such as the composition of the nutrient medium, temperature, and duration of incubation (Srinivasan *et al*., 1991; Shikura *et al*., 2002; Yu *et al*., 2008). Nutrition is important for the onset and intensity of secondary metabolism, not only because limiting the supply of an essential nutrient is an effective means of restricting growth but also because the choice of limiting nutrient can have specific metabolic and regulatory effects. There is usually a relationship between the media composition and the biosynthesis of antibiotics, and the role of the medium is usually considered in terms of the nutrients and precursors it provides to the culture (Elibol, 2004). Growth and antibiotic production is dependent on the medium composition; carbon and nitrogen sources are the most important impact factors (Yu *et al*., 2008), and on the fermentation conditions (Theobald *et al*., 2000). It is desirable that the fermentation medium allows the formation of a large biomass in the shortest possible time, and sustains the productive phase for as long as possible (Lancini and Lorenzetti, 1993).

To select the best medium for maximum bioactive metabolite(s) production, different carbon and nitrogen sources in different combinations were used. *Streptomyces* strain T15 was cultivated in three different fermentation media; ISP2, ISP4, and SA, under agitation and static submerged conditions. ISP2, ISP4, and SA media are also referred to as yeast extract-malt extract agar, inorganic salts-starch agar, and sporulation agar, respectively. Agar, the solidifying agent was omitted prior to the preparation of the liquid fermentation media. The concentrations of carbon and nitrogen sources added to ISP2, ISP4, and SA media were varied. ISP2 medium in which the

highest yield of bioactive metabolite(s) by strain T15 was obtained had the following composition: 4.0 g yeast extract, 10.0 g malt extract, 4.0 g glucose, 6.0 g sodium chloride, 20.0 g agar, and 1.0 L distilled water. In ISP2 medium, glucose was the carbon source, while yeast extract and malt extract were sources of nitrogen, vitamins, and growth factors. *Jiangella gansuensis*, an actinomycete that is placed in the family *Nocardioidaceae*, grows very well on ISP2 medium (Song *et al.*, 2005). Balagurunathan and Subramanian (1994) demonstrated that glucose and soybean meal were the best sources for the maximum production of antibiotic from *S. griseobrunneus* (P-33). ISP4 medium is composed of many inorganic salts and soluble starch. Meanwhile, SA medium contained soluble starch and glucose as the carbon sources, and yeast extract and amino acids as sources of nitrogen, B vitamins, and growth factors.

Sujatha *et al*. (2005) found that carbohydrates, ammonium nitrate, phosphate, and magnesium positively affected the antibiotic production by *S. psammoticus* BT-408. Iron, zinc, and magnesium ions are the most critical in secondary metabolism (Adinarayana *et al.*, 2003). According to Vasavada *et al*. (2006), starch agar was the preferred medium for antibiotic production. The results in this study were in accordance to findings by Sujatha *et al*. (2005), where strain T15 was able to grow in all the tested carbon sources. The findings by the authors showed that antibiotic production was higher in glucose-grown cells. Glucose is generally used in the fermentation as a preferred carbon sources for antibiotic production (Srinivasan *et al*., 1991). In this study, bioactive metabolite(s) production was higher in ISP2 and SA media in which glucose was the carbon source. However, soluble starch was the carbon source in ISP4 medium. Glucose was not supplemented in ISP4 medium, therefore

explained the low bioactive metabolite(s) production compared to that of SA and ISP2 media that were supplemented with 15.0 g and 4.0 g glucose, respectively.

However, in the presence of excess carbon sources especially glucose, many bioactive metabolite(s) are not produced. Elibol (2004) demonstrated that actinorhodin production was highly sensitive to the concentration of glucose. Marwick *et al*. (1999) reported that the enzyme that catalyzed the formation of the phenoxazinone ring of actinomycin was inhibited by glucose. Cruz *et al*. (1999) had explained the typical diauxic effect of the consumption of two carbohydrates in the process of cephalosporin C production. In the first phase, an easily metabolisable carbon source like glucose was quickly consumed and most of the growth took place. Meanwhile, a slowly metabolisable carbon source like sucrose was assimilated and the antibiotic synthesis commenced in the second phase. Two carbohydrates; soluble starch and glucose were the carbon sources in SA medium. Based on reviews by Cruz *et al*. (1999) and Ismet *et al*. (2004), presumably glucose acted as the easily metabolised carbon source, while soluble starch acted as the slowly metabolised carbon source. Rapid catabolism of glucose and other carbohydrates has been shown to cause a decrease in the rate of antibiotic biosynthesis (Large *et al*., 1998).

In this study, yeast extract and malt extract contained in ISP2 medium were shown to be excellent sources of nitrogen for the production of bioactive metabolite(s) antagonistic towards *P. shigelloides*. As reported by Voelker and Altaba (2001), there was experimental evidence for repression of antibiotic production exerted by some nitrogen sources especially ammonium. Thus, explained the low production of bioactive metabolite(s) by strain T15 cultivated in ammonium-containing ISP4 medium. However, this was contrary to the findings by Marwick *et al*. (1999) who

reported that ammonium stimulated an antibiotic produced by *S. griseofuscus*. Yeast extract was the sole nitrogen source in SA medium. The consumption of yeast extract in SA medium must be driven primarily by the need of nitrogen because it represented the sole source of nitrogen for the cell until its exhaustion in the medium. Thus, the nitrogen source in SA medium did not sustained until the end of productive phase compared to that of ISP2 medium. Marwick *et al*. (1999) mentioned that high nitrogen levels repressed iodophase production of antibiotics. In addition, the use of unsuitable amino acids as a nitrogen source could inhibit good synthesis of secondary metabolites. Conversely, specific amino acids could also enhance antibiotic production.

Phosphate is a major factor in the synthesis of a wide range of antibiotics in streptomycetes. The production of actinorhodin by *S. coelicolor* grown on minimal medium containing an inorganic nitrogen source was subject to phosphate regulation (Hobbs *et al*., 1992). However, an excessive amount of inorganic phosphate suppresses the production of antibiotics such as tetracycline, actinomycin, and candicidin (Sujatha *et al*., 2005). Both ISP4 and SA media were supplemented with 1.0 g and 0.5 dipotassium phosphate (K_2HPO_4), and 2.0 g and 1.0 g $CaCO_3$, respectively. Buffering agent was not supplemented in ISP2 medium. According to Tanaka (1992), buffering agents to avoid overly acidic conditions such as phosphate salts and calcium carbonate occasionally had negative effects on antibiotic production. Hence, the low bioactive metabolite(s) production of strain T15 grown in ISP4 medium might have resulted from the inhibitory effects of buffering agents; phosphate salts and $CaCO_3$. Many antibiotic-producing organisms grow adequately in the presence of 0.3 to 500 mM of inorganic phosphate but the ranges of phosphate for good antibiotic production are generally quite narrow (Srinivasan *et al*., 1991). According to Marwick *et al.* (1999), the level of adenosine triphosphate (ATP) decreased significantly before secondary metabolism

began, being attributed to the rise in phosphatase activity after the cessation of growth. Inorganic phosphate repressed the synthesis of phosphatase, allowing the ATP level to remain high for repressing secondary metabolism. The stimulation of growth phase might give rise to feedback inhibition of secondary metabolism if phosphate was added. Moreover, phosphate limitation stimulated phystigimine production by *S. griseofuscus*.

In this study, the greatest yield of antibiotic was obtained in antibiotic submerged condition because agitation was an ideal growth condition to provide uniform conditions for every cell, as emphasized by Penn (1991). Efficient mixing of the liquid, solid, and gaseous phases of which a fermentation system is composed must be provided. The solid nutrients and the mycelium must be kept in a homogeneous suspension, and the temperature and dissolved nutrient concentration must be equally distributed throughout the fermenter (Lancini and Lorenzetti, 1993). In this study, cotton plugs were used to seal the flasks in such a way as to permit gaseous exchange, but prevent the entry of contaminating microorganisms. The flasks were indented to increase the turbulence that enabled dispersion of air in the liquid phase and thus the oxygen transfer. According to Smith (1956), the cells of filamentous organisms often were broken by striking against the baffle in the flask. Although the baffle increased the effective aeration rate to the medium within the flask, it might cause extensive disruption of filamentous cells. The aeration efficiency was increased by decreasing the volume of medium in the flask to circumvent injury to the cells from striking the baffle.

During the agitation condition experiments, the agitation speed was kept constant at 120 rpm. Agitation had promoted the bioactive metabolite(s) production of strain T15 cultivated in ISP2 and SA media. Contrarily, agitation had suppressed the bioactive metabolite(s) production of strain T15 cultivated in ISP4 medium. According

to Augustine *et al*. (2005b), agitation affected aeration and mixing of the nutrients in the fermentation medium. The yield of cephalosporin C is known to increase with increased agitation. In general, vigorous agitation is beneficial for mycelial growth in a submerged culture, as it increases the uptake rate of oxygen and nutrients (Kim *et al*., 2006). The oxygenation of cultures is critical for optimal growth in aerobic fermentations. The increase of partial pressure oxygen was found to induce new secondary metabolites by *S. parvulus*. The oxygen transfer from sparged air to the bacterial cell is partially dependent on the media composition in which viscous media being harder to oxygenate (Marwick *et al*., 1999).

Interestingly, bioactive metabolite(s) were produced by strain T15 cultivated in ISP4 medium only in static condition. In static condition, the highest yield of bioactive metabolite(s) was generated in ISP4 medium (day 9) in which the growth rate was at its maximum, indicated by the high production of crude extract. According to Tanaka (1992), dissolved oxygen tension decreased to its lowest level when the growth rate was at its maximum. Therefore, bioactive metabolite(s) in ISP4 medium was produced when oxygen level decreased. This result was in good agreement with the findings by Keulen *et al*. (2003) and Keulen *et al*. (2004), where shaken liquid cultures of *S. coelicolor* did not differentiate, but differentiation occurred in standing (static) liquid minimal medium. Furthermore, liquid standing cultures rapidly became anoxic, implying the existence of metabolic pathways supporting anaerobic growth or enabling the organism to survive long periods of low oxygen conditions. Standing liquid cultures might resemble flooded soils; a condition *S. coelicolor* might escape from by forming floating sporulating colonies. Growing standing liquid cultures were expected to develop oxygen gradients. It was shown that 7-day old liquid cultures were anoxic at a depth of 0.7 and 2 mm, respectively. Yet, biomass still increased as measured by total

protein determinations, indicating an active metabolism. *Streptomyces coelicolor* did not form colonies at the air interface in anoxic nutrient-rich standing cultures. Thus, oxygen availability may not be the signal for their formation. However, the bioactive metabolite(s) biosynthesis was hindered in some organisms if the minimum level of oxygen reached zero, and bioactive metabolite(s) production would not start until the normal production phase was entered. This was obviously true in the case of strain T15 grown in ISP2 and SA media in static (anoxic) condition. Carbon dioxide dissolved together with oxygen when air was used, and both of these gases affected bioactive metabolite(s) production. Tetracycline production by *S. aureofaciens* was susceptible to low oxygen tension, but was not to carbon dioxide. It was found that the antibacterial activity of fosfomycin is more potent under anaerobic condition than under aerobic condition (Oiwa, 1992). Theobald *et al*. (2000) demonstrated that an additional drop in aeration (beneath 0.5 vvm) caused a decrease in biomass production by *S. antibioticus* Tü 6040 due to oxygen limitation.

In this study, the pH value of medium was adjusted to 7.0 at the beginning of cultivation. The pH level of the growth medium has a marked effect on secondary metabolites production, with synthesis falling rapidly either side of an optimal level (Marwick *et al*., 1999). In ISP2 medium, highest yield of bioactive metabolite(s) was generated when pH level was at its lowest (6.10 ± 1.70), which was in accordance with the results obtained by Basak and Majumdar (1973). According to the authors, there was a lowering of pH of the broth during the phase of antibiotic synthesis in the case of glucose as carbon source. Contrary to the findings by Balagurunathan and Subramanian (1994), pH levels were decreasing throughout the static condition fermentation in ISP4 medium.

In a study by Balagurunathan and Subramanian (1994), the changes in the acidity of the fermentation medium had a significant effect on the yield of the end products, which was presumably connected with increase in ammonium and nitrogen due to the decomposition of soy proteins and autolysis of the mycelium. However, for fermentation in ISP2 medium in the present study, the changes of acidity were presumed to be connected with increase in ammonium and nitrogen caused by decomposition of yeast and malt and autolysis of the mycelium. Meanwhile, the decomposition of yeasts and mycelium autolysis might cause the acidity reduction in SA medium. Overall, pH variation was minimal in the present study.

Basak and Majumdar (1973) highlighted that antibiotic formation was not solely dependent on cellular growth. This observation was in good agreement with the findings in this study where it could be used to explain the unpredictable relationship between the productions of crude extracts in ISP2 and SA media in agitation growth condition as measured in weight with the potency of antibacterial metabolite(s) contained in the crude extracts. The greatest inhibitory potential of nine days old strain T15 grown in ISP2 and SA media against *P. shigelloides* were measured as 11.5 ± 0.7 mm and 11.0 ± 1.2 mm, respectively. It was apt to assume that the amount of inhibitory substance(s) produced by strain T15 grown in ISP2 medium and that of SA medium were not of much difference. However, this assumption was proven wrong. In terms of crude extract, nine days old strain T15 grown in SA medium produced about three times more crude extract than nine days old strain T15 grown in ISP2 medium. Because of the almost equal inhibitory potential that they both possessed, this finding suggested that the crude extracts of strain T15 grown in ISP2 medium contained inhibitory substance(s) that was about three times greater than the crude extracts of strain T15 grown in SA medium.

The present results were contrary to the results observed by Basak and Majumdar (1973). Previous findings by the authors indicated that D-galactose proved to be an excellent carbon source for kanamycin production by *S. kanamyceticus*, although soluble starch, potato starch, and maltose allowed greater amounts of growth of the organism. It was possible that rapidly utilized carbon sources such as glucose, mannose, and arabinose supported abundant growth of the organism, but these sugars were poor carbon sources for kanamycin production. Galactose might be utilized less rapidly, and thus it was available during the phase of kanamycin production. However, ISP2 medium in which glucose was consumed rapidly supported good bioactive metabolite(s) production. ISP4 medium, where the amount of crude extract produced was the lowest compared to that of ISP2 and SA media had generated low bioactive metabolite(s) production. This observation was in good agreement with the concept that increasing amount of crude extract (biomass) gives higher bioactivity (Ismet *et al.*, 2004). It was concluded that strain T15 was a slow growing *Streptomyces* strain, and thus needed a long fermentation time for the bioactive metabolite(s) production compared to other producer *Streptomyces* spp. (Hobbs *et al*., 1992). Findings tally with study by Kavithambigai (2006) in which longer incubation was needed to enhance the productivity of bioactive metabolite(s).

According to Lancini and Lorenzetti (1993), the secondary metabolites were produced in two phases: a vegetative phase, or trophophase in which there was a vigorous growth and a negligible antibiotic production; and a fermentative phase, or idiophase in which the culture was stationary and the antibiotic production was initiated. As described by Balagurunathan and Subramanian (1994), production of bioactive metabolites was activated in the late logarithmic to stationary stage of fermentation, after cell division and biomass accumulation had largely ceased. This

statement was corroborated by the findings in present study where the highest yield of bioactive metabolite(s) was produced in day 9, when strain T15 was grown in ISP2 medium, and in which the biomass of crude extracts produced was at its lowest.

The majority of antibiotics are the products of complex biosynthetic pathways that are activated in a growth phase dependent manner; upon entry into stationary phase, or following a reduction in growth rate in liquid-grown (Aigle *et al.*, 2005), or has passed an optimum level (Srinivasan *et al.*, 1991). During fermentation, the highest bioactive metabolite(s) production of strain T15 was noted on day 9 for all the fermentation media used in this study. In accordance to findings by Augustine *et al*. (2005b), the production of bioactive metabolite(s) in ISP2, SA, and ISP4 media took place during the late log phase of growth in the fermentation media indicating that the metabolite production was directly proportional to the growth rate. In this study, contaminations had occurred and hindered the initial stage of study, which was the sub-culturing of actinomycete strains from stock cultures. Hence, the antimicrobial tests on fully-grown cultures could not be conducted in the same batch. The slightest differences in growth conditions could possibly affect the production of secondary metabolites.

CHAPTER 5

5.0 FUTURE RECOMMENDATIONS AND CONCLUSIONS

Further test to identify the bioactive metabolite(s) of promising strains in the primary and secondary screenings should be conducted in order to detect the presence of novel metabolites, if any. The promising strains, which exhibited strong growth inhibition against their respective clinical pathogens, are listed in Table 5.1 below.

Table 5.1 *Streptomyces* spp. with promising antibacterial activities against their respective clinical pathogens

Screening	Strain	Pathogen
Primary	**T3**	*Enterococcus faecalis* and *Staphylococcus aureus*
	T9	*Staphylococcus aureus*
	T15	*Enterococcus faecalis, Plesiomonas shigelloides, Bacillus subtilis*, and *Pseudomonas aeruginosa*
Secondary	**T53**	*Enterococcus faecalis*
	T15 and T16	*Plesiomonas shigelloides*

Streptomyces spp. with weak and moderate bioactivities should be subjected to media optimisation in order to optimise the growth and to increase the production of bioactive metabolite(s). There is an obvious need to develop media, which utilize natural nutrients and growth factors derived directly from marine sources in pursue of optimum bioactive metabolite(s) production. Unconventional culture methods should be employed to enhance the growth of these actinomycetes, and extremely sensitive assays should be used to detect low concentrations of bioactive metabolite(s),

particularly on strains that posed weak or no inhibition at all. In near future, the present study should be conducted through immobilisation method, and the biological material to be examined should be expanded in which rare actinomycetes and 'uncultivable' marine actinomycetes could possibly be the source of novel metabolites.

From this investigation, the following conclusions were made based on the bioactivity of the selected marine *Streptomyces* spp. The actinomycetes tested were good antibacterial agent, but weak anti-yeast agent. *Streptomyces* strain T15 was selected for media optimisation to promote bioactive metabolite(s) production due to the findings that it was the best strain in inhibiting the growth of *P. shigelloides* in both primary and secondary screenings. It was found that agitation significantly affected the production of bioactive metabolite(s) from *Streptomyces* strain T15, thereby resulting in different levels of antagonistic activities. Batch fermentation in ISP2 medium at an incubation period of nine days at 28ºC ± 2ºC, pH 6.10 ± 1.70, and shaking at a speed of 120 rpm was the most ideal for growth and bioactive metabolite(s) production of *Streptomyces* strain T15 at laboratory conditions. The findings in the present study showed that the selected marine-derived *Streptomyces* spp. had great potential to produce bioactive metabolites against multidrug-resistant test bacteria; *B. subtilis*, *E. faecalis*, *S. aureus*, *P. aeruginosa*, and *P. shigelloides*. The large spectrum of inhibitions displayed by the marine *Streptomyces* spp. provided evidence that marine ecosystem harbour species that can produce useful secondary metabolites for discovery of novel bioactive metabolites. Furthermore, the optimisation of liquid culture conditions had enhanced the yield of metabolites.

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APPENDIX A

MEDIA USED

International Streptomyces Project (ISP) Media (Shirling & Gottlieb, 1966)**:**

A1. Yeast Extract – Malt Extract Agar (ISP2)

Yeast extract	4.0 g
Malt extract	10.0 g
D(+)-glucose	4.0 g
Sodium chloride	6.0 g
Agar	20.0 g
Distilled water	1.0 L

* pH adjusted to 7.3 with 1 M NaOH prior autoclaving at 121ºC at 15 psi for 20 min

A2. Inorganic Salts – Starch Agar (ISP4)

Soluble starch	10.0 g
Potassium phosphate dibasic	1.0 g
Magnesium sulphate	1.0 g
Sodium chloride	4.0 g
Ammonium sulphate	2.0 g
Calcium carbonate	2.0 g
Ferrous sulphate	1.0 mg
Manganous chloride	1.0 mg
Zinc sulphate	1.0 mg
Agar	25.0 g
Distilled water	1.0 L

* pH adjusted to 7.2 with 1 M NaOH prior autoclaving at 121ºC at 15 psi for 20 min

General Laboratory Media:

A3. Sporulation Agar (SA)

Yeast extract	4.0 g
Soluble starch	20.0 g
D(+)-glucose	15.0 g
Casein	5.0 g
Instant ocean	17.0 g
Calcium carbonate	1.0 g
Potassium phosphate dibasic	0.5 g
Magnesium sulphate	0.5 g
Agar	17.0 g
Distilled water	1.0 L

* pH adjusted to 7.0 with 1 M NaOH prior autoclaving at 121ºC at 15 psi for 20 min

A4. Sabouraud Dextrose Agar (SDA)

Casein	10.0 g
Dextrose	20.0 g
Agar	15.0 g
Distilled water	1.0 L

* pH adjusted to 7.0 with 1 M NaOH prior autoclaving at 121ºC at 15 psi for 20 min

A5. Nutrient Agar (NA)

Nutrient agar powder	23.0 g
Bacteriological agar	5.0 g
Distilled water	1.0 L

* pH adjusted to 7.2 with 1 M NaOH prior autoclaving at 121ºC at 15 psi for 20 min

A6. Glycerol Stock Solution (30%) (Wellington & Williams, 1978)

Glycerol	30.0 ml
Yeast extract	0.1 g
D(+)-glucose	0.375 g
Casein	0.125 g
Distilled water	70.0 ml

* pH adjusted to 7.0 with 1 M NaOH prior autoclaving at 121ºC at 15 psi for 20 min

APPENDIX B

Table 1 Inhibition spectrum (mm) of positive and negative controls against the test bacteria assessed via disc diffusion method

Test bacteria		Diameter of inhibition zone (mm)		
		Positive control		Negative control
		Novobiocin (5 µg/disc)	Streptomycin (10 µg/disc)	Methanol
Gram-positive	*Bacillus subtilis*	15.0 ± 0.0	14.0 ± 0.0	11.3 ± 0.6
	Enterococcus faecalis	15.0 ± 0.0	13.0 ± 0.0	10.0 ± 1.0
	Staphylococcus aureus	13.0 ± 0.0	16.0 ± 0.0	10.3 ± 1.5
Gram-negative	*Pseudomonas aeruginosa*	16.0 ± 0.0	15.0 ± 0.0	12.3 ± 0.6
	Plesiomonas shigelloides	18.0 ± 0.0	14.0 ± 0.0	9.0 ± 1.0

* Mean of three readings with standard deviation, test bacteria were lawned on nutrient agar (NA) plates, and incubated at 37ºC ± 2ºC for 48 h. Growth inhibition was defined as mm

Table 2 Inhibition spectrum (mm) of positive and negative controls against the test yeasts assessed via disc diffusion method

Test yeast	Diameter of inhibition zone (mm)	
	Positive control	Negative control
	Nystatin (100 unit/disc)	Methanol
Candida albicans	27.0 ± 0.0	13.3 ± 0.6
Candida parasilopsis	28.0 ± 0.0	11.7 ± 0.6

* Mean of three readings with standard deviation, test yeasts were lawned on Sabouraud dextrose agar (SDA) plates, and incubated at 37ºC ± 2ºC for 48 h. Growth inhibition was defined as mm

Table 3 ANOVA: pH of cultures of *Streptomyces* strain T15 cultivated under agitation submerged condition (SH)

(between fermentation period and media)

Between fermentation period of 3 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	1.68802	2	0.844011	3.05	0.1216 ^{ns}
Within groups	1.65773	6	0.276289		
Total	3.34576	8			

Between fermentation period of 6 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	3.72047	2	1.86023	13.77	0.0057 **
Within groups	0.810333	6	0.135056		
Total	4.5308	8			

Between fermentation period of 9 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	1.37627	2	0.688133	0.61	0.5757 ^{ns}
Within groups	6.81033	6			
Total	8.1866	8			

Table 3, continued

Between fermentation period of 12 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	2.25007	2	1.12503	21.15	0.0019 **
Within groups	0.319133	6	0.0531889		
Total	2.5692	8			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 4 Multiple range tests: pH of cultures of *Streptomyces* strain T15 cultivated under agitation submerged condition (SH)

(between fermentation period and media)

Contrast (fermentation period of 6 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	-0.616667	0.734226
ISP2-SA	0.946667	0.734226 *
ISP4-SA	1.56333	0.734226 *

Contrast (fermentation period of 12 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	-0.963333	0.46077 *
ISP2-SA	-1.13667	0.46077 *
ISP4-SA	-0.173333	0.46077

* denotes a statistically significant difference

Table 5 ANOVA: pH of cultures of *Streptomyces* strain T15 cultivated under static submerged condition (ST)

(between fermentation period and media)

Between fermentation period of 3 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.296867	2	0.148433	1.02	0.4157 ^{ns}
Within groups	0.873333	6	0.145556		
Total	1.1702	8			

Between fermentation period of 6 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	1.41162	2	0.705811	4.07	0.0764 ^{ns}
Within groups	1.04073	6	0.173456		
Total	2.45236	8			

Between fermentation period of 9 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.410556	2	0.205278	15.43	0.0043 **
Within groups	0.0798	6	0.0133		
Total	0.490356	8			

Table 5, continued

Between fermentation period of 12 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	1.83387	2	0.916933	3.55	0.0961 ^{ns}
Within groups	1.54953	6	0.258256		
Total	3.3834	8			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 6 Multiple range tests: pH of cultures of *Streptomyces* strain T15 cultivated under static submerged condition (ST)

(between fermentation period and media)

Contrast (fermentation period of 9 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	-0.116667	0.230409
ISP2-SA	-0.5	0.230409 *
ISP4-SA	-0.383333	0.230409 *

* denotes a statistically significant difference

Table 7 ANOVA: pH of cultures of *Streptomyces* strain T15 cultivated under agitation submerged condition (SH)

(between fermentation media and fermentation period)

Between fermentation medium ISP2 and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	4.56076	3	1.52025	1.85	0.2172 ^{ns}
Within groups	6.59133	8	0.823917		
Total	11.1521	11			

Between fermentation medium ISP4 and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	4.22967	3	1.40989	8.19	0.0080 **
Within groups	1.378	8	0.17225		
Total	5.60767	11			

Between fermentation medium SA and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	3.32369	3	1.1079	5.44	0.0247 **
Within groups	1.6282	8	0.203525		
Total	4.95189	11			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 8 Multiple range tests: pH of cultures of *Streptomyces* strain T15 cultivated under agitation submerged condition (SH)

(between fermentation media and fermentation period)

Contrast (fermentation medium ISP4 and fermentation period)	**Difference +/-**	**Limits**
3-6	-1.58667	0.781439 *
3-9	-0.343333	0.781439
3-12	-0.496667	0.781439
6-9	1.24333	0.781439 *
6-12	1.09	0.781439 *
9-12	-0.153333	0.781439

Contrast (fermentation medium SA and fermentation period)	**Difference +/-**	**Limits**
3-6	-0.823333	0.849424
3-9	-0.936667	0.849424 *
3-12	-1.47	0.849424 *
6-9	-0.113333	0.849424
6-12	-0.646667	0.849424
9-12	-0.533333	0.849424

* denotes a statistically significant difference

Table 9 ANOVA: pH of cultures of *Streptomyces* strain T15 cultivated under static submerged condition (ST)

(between fermentation media and fermentation period)

Between fermentation medium ISP2 and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.492158	3	0.164053	6.16	0.0178 **
Within groups	0.212933	8	0.0266167		
Total	0.705092	11			

Between fermentation medium ISP4 and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.395492	3	0.131831	0.65	0.6056 ^{ns}
Within groups	1.626	8	0.20325		
Total	2.02149	11			

Between fermentation medium SA and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.405158	3	0.135053	0.63	0.6137 ^{ns}
Within groups	1.70447	8	0.213058		
Total	2.10963	11			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 10 Multiple range tests: pH of cultures of *Streptomyces* strain T15 cultivated under static submerged condition (ST)

(between fermentation media and fermentation period)

Contrast (fermentation medium ISP2 and fermentation period)	**Difference +/-**	**Limits**
3-6	0.133333	0.30718
3-9	-0.196667	0.30718
3-12	0.36	0.30718 *
6-9	-0.33	0.30718 *
6-12	0.226667	0.30718
9-12	0.556667	0.30718 *

Contrast (fermentation medium ISP4 and fermentation period)	**Difference +/-**	**Limits**
3-6	0.0766667	0.84885
3-9	0.103333	0.84885
3-12	0.47	0.84885
6-9	0.0266667	0.84885
6-12	0.393333	0.84885
9-12	0.366667	0.84885

* denotes a statistically significant difference

Table 11 ANOVA: Weight of crude extracts of *Streptomyces* strain T15 cultivated under agitation submerged condition (SH)

(between fermentation period and media)

Between fermentation period of 3 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0222087	2	0.0111043	1.56	0.2852 ^{ns}
Within groups	0.0427739	6	0.00712899		
Total	0.0649826	8			

Between fermentation period of 6 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0398547	2	0.0199274	3.64	0.0923 ^{ns}
Within groups	0.032873	6	0.00547883		
Total	0.0727277	8			

Between fermentation period of 9 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0406652	2	0.0203326	8.06	0.0199 **
Within groups	0.0151286	6	0.00252143		
Total	0.0557938	8			

Table 11, continued

Between fermentation period of 12 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0707666	2	0.0353833	8.95	0.0158 **
Within groups	0.0237211	6	0.00395351		
Total	0.0944877	8			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 12 Multiple range tests: Weight of crude extracts of *Streptomyces* strain T15 cultivated under agitation submerged condition (SH)

(between fermentation period and media)

Contrast (fermentation period of 9 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	-0.0844333	0.100322
ISP2-SA	-0.164633	0.100322 *
ISP4-SA	-0.0802	0.100322

Contrast (fermentation period of 12 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	0.1074	0.125622
ISP2-SA	0.2172	0.125622 *
ISP4-SA	0.1098	0.125622

* denotes a statistically significant difference

Table 13 ANOVA: Weight of crude extracts of *Streptomyces* strain T15 cultivated under static submerged condition (ST)

(between fermentation period and media)

Between fermentation period of 3 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0485	2	0.02425	4.21	0.0722 ^{ns}
Within groups	0.0345985	6	0.00576641		
Total	0.0830985	8			

Between fermentation period of 6 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0547431	2	0.0273715	10.68	0.0105 **
Within groups	0.0153756	6	0.00256261		
Total	0.0701187	8			

Between fermentation period of 9 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0247978	2	0.0123989	1.87	0.2340 ^{ns}
Within groups	0.0398216	6	0.00663693		
Total	0.0646194	8			

Table 13, continued

Between fermentation period of 12 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.125209	2	0.0626045	5.49	0.0442 **
Within groups	0.0684702	6	0.0114117		
Total	0.193679	8			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 14 Multiple range tests: Weight of crude extracts of *Streptomyces* strain T15 cultivated under static submerged condition (ST)

(between fermentation period and media)

Contrast (fermentation period of 6 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	0.1573	0.101138 *
ISP2-SA	-0.0152333	0.101138
ISP4-SA	-0.172533	0.101138 *

Contrast (fermentation period of 12 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	0.0795	0.213427
ISP2-SA	-0.2008	0.213427
ISP4-SA	-0.2803	0.213427 *

* denotes a statistically significant difference

Table 15 ANOVA: Weight of crude extracts of *Streptomyces* strain T15 cultivated under agitation submerged condition (SH)

(between fermentation media and fermentation period)

Between fermentation medium ISP2 and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.101001	3	0.033667	5.96	0.0195 **
Within groups	0.045174	8	0.00564675		
Total	0.146175	11			

Between fermentation medium ISP4 and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0586781	3	0.0195594	6.42	0.0159 **
Within groups	0.0243585	8	0.00304481		
Total	0.0830366	11			

Between fermentation medium SA and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0404348	3	0.0134783	2.40	0.1435 ^{ns}
Within groups	0.0449641	8	0.00562052		
Total	0.0853989	11			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 16 Multiple range tests: Weight of crude extracts of *Streptomyces* strain T15 cultivated under agitation submerged condition (SH)

(between fermentation media and fermentation period)

Contrast (fermentation medium ISP2 and fermentation period)	**Difference +/-**	**Limits**
3-6	0.0236	0.141486
3-9	0.0776	0.141486
3-12	-0.167933	0.141486 *
6-9	0.054	0.141486
6-12	-0.191533	0.141486 *
9-12	-0.245533	0.141486 *

Contrast (fermentation medium ISP4 and fermentation period)	**Difference +/-**	**Limits**
3-6	-0.0252	0.103895
3-9	-0.119167	0.103895 *
3-12	-0.172867	0.103895 *
6-9	-0.0939667	0.103895
6-12	-0.147667	0.103895 *
9-12	-0.0537	0.103895

* denotes a statistically significant difference

Table 17 ANOVA: Weight of crude extracts of *Streptomyces* strain T15 cultivated under static submerged condition (ST)

(between fermentation media and fermentation period)

Between fermentation medium ISP2 and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.0125026	3	0.00416752	3.30	0.0785 ^{ns}
Within groups	0.0100987	8	0.00126234		
Total	0.0226013	11			

Between fermentation medium ISP4 and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.00742072	3	0.00247357	0.47	0.7125 ^{ns}
Within groups	0.0422518	8	0.00528147		
Total	0.0496725	11			

Between fermentation medium SA and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	0.12378	3	0.04126	3.12	0.0882 ^{ns}
Within groups	0.105915	8	0.0132394		
Total	0.229695	11			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 18 ANOVA: Bioactive metabolite(s) production by *Streptomyces* strain T15 cultivated under agitation submerged condition (SH)

(between fermentation period and media)

Between fermentation period of 6 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	210.889	2	105.444	237.25	0.0000 *
Within groups	2.66667	6	0.444444		
Total	213.556	8			

Between fermentation period of 9 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	264.667	2	132.333	238.20	0.0000 *
Within groups	3.33333	6	0.555556		
Total	268.0	8			

Between fermentation period of 12 days and media					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	206.889	2	103.444	931.00	0.0000 *
Within groups	0.666667	6	0.111111		
Total	207.556	8			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 19 Multiple range tests: Bioactive metabolite(s) production by *Streptomyces* strain T15 cultivated under agitation submerged condition (SH) (between fermentation period and media)

Contrast (fermentation period of 3 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	0.0	0.0
ISP2-SA	-10.0	0.0 *
ISP4-SA	-10.0	0.0 *

Contrast (fermentation period of 6 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	11.0	1.33193 *
ISP2-SA	1.66667	1.33193 *
ISP4-SA	-9.33333	1.33193 *

Contrast (fermentation period of 9 days and media)	**Difference +/-**	**Limits**
ISP2-ISP4	11.6667	1.48915 *
ISP2-SA	0.333333	1.48915
ISP4-SA	-11.3333	1.48915 *

Table 19, continued

Contrast (fermentation period of 12 days and media)	Difference +/-	Limits
ISP2-ISP4	10.0	0.665967 *
ISP2-SA	-0.333333	0.665967
ISP4-SA	-10.3333	0.665967 *

* denotes a statistically significant difference

Table 20 ANOVA: Bioactive metabolite(s) production by *Streptomyces* strain T15 cultivated under agitation submerged condition (SH) (between fermentation media and fermentation period)

Between fermentation medium ISP2 and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	271.0	3	90.3333	1084.00	0.0000 *
Within groups	0.666667	8	0.0833333		
Total	271.667	11			

Between fermentation medium SA and fermentation period					
Source of variation	**Sum of squares**	**d.f**	**Mean square**	**F-ratio**	**Significance level**
Between groups	6.25	3	2.08333	2.78	0.1102 ^{ns}
Within groups	6.0	8	0.75		
Total	12.25	11			

*: (p= 0); **: (p< 0.05); ^{ns}: not significant (p> 0.05)

Table 21 Multiple range tests: Bioactive metabolite(s) production by *Streptomyces* strain T15 cultivated under agitation submerged condition (SH) (between fermentation media and fermentation period)

Contrast (fermentation medium ISP2 and fermentation period)	**Difference +/-**	**Limits**
3-6	-11.0	0.543532 *
3-9	-11.6667	0.543532 *
3-12	-10.0	0.543532 *
6-9	-0.666667	0.543532 *
6-12	1.0	0.543532 *
9-12	1.66667	0.543532 *

* denotes a statistically significant difference

APPENDIX C

Table 1 Raw data for antibacterial bioactivity of *Streptomyces* spp. in primary screening assessed via cross streak method (Chapter 4)

***Streptomyces* strain**		**Test Bacteria**				
		Gram-positive			**Gram-negative**	
		B. subtilis	***E. faecalis***	***S. aureus***	***P. aeruginosa***	***P. shigelloides***
T3	**R1**	-	+++	+++	-	-
	R2	-	+++	+++	-	-
	R3	-	+++	+++	-	-
T4	**R1**	-	-	-	-	-
	R2	-	-	-	-	-
	R3	-	-	-	-	-
T6	**R1**	-	++	-	+	-
	R2	-	++	-	+	-
	R3	-	+	-	+	-
T9	**R1**	+	+	+++	+	+
	R2	+	+	+++	+	+
	R3	+	+	+++	+	+
T12	**R1**	++	++	++	++	++
	R2	++	++	++	++	++
	R3	++	++	+	++	++
T13	**R1**	+	+	+	+	+
	R2	+	+	+	+	+
	R3	+	+	+	+	+

Table 1, continued

***Streptomyces* strain**		**Test Bacteria**				
		Gram-positive			**Gram-negative**	
		B. subtilis	***E. faecalis***	***S. aureus***	***P. aeruginosa***	***P. shigelloides***
T15	**R1**	+++	+++	+	+++	+++
	R2	+++	+++	+	+++	+++
	R3	+++	+++	+	+++	+++
T16	**R1**	-	-	-	-	-
	R2	-	-	-	-	-
	R3	-	-	-	-	-
T20	**R1**	-	-	-	-	-
	R2	-	-	-	-	-
	R3	-	-	-	-	-
T52	**R1**	+	+	+	+	+
	R2	+	+	+	+	+
	R3	+	+	-	+	+
T53	**R1**	+	+	-	+	+
	R2	-	-	-	+	+
	R3	-	-	-	+	+

***** R1, R2, and R3: Replicates

Table 2 Raw data for inhibition spectrum (mm) of *Streptomyces* spp. against the test pathogens in secondary screening assessed via disc diffusion method (Chapter 4)

Streptomyces strain		Diameter of inhibition zone (mm)						
		Gram-positive bacteria			Gram-negative bacteria		Yeast	
		E. faecalis	*S. aureus*	*B. subtilis*	*P. shigelloides*	*P. aeruginosa*	*C. parasilopsis*	*C. albicans*
T3	R1	10	13	18	13	-	11	10
	R2	13	12	15	14	-	11	11
	R3	12	11	13	14	-	11	10
T4	R1	16	13	15	12	-	20	20
	R2	16	18	16	12	-	16	20
	R3	16	16	15	11	-	18	16
T6	R1	10	12	11	13	-	10	10
	R2	11	14	12	13	-	11	11
	R3	12	12	12	13	-	12	11
T9	R1	15	17	10	15	15	14	11
	R2	17	17	9	15	15	11	11
	R3	10	16	15	15	15	11	11
T12	R1	18	14	17	14	18	11	11
	R2	16	14	9	16	20	15	13
	R3	15	10	11	12	15	11	12
T13	R1	12	15	15	21	-	12	-
	R2	12	15	13	21	-	12	-

	R3	12	15	18	21	-	16	-

Table 2, continued

Streptomyces strain		Diameter of inhibition zone (mm)						
		Gram-positive bacteria			Gram-negative bacteria		Yeast	
		E. faecalis	*S. aureus*	*B. subtilis*	*P. shigelloides*	*P. aeruginosa*	*C. parasilopsis*	*C. albicans*
T15	R1	19	17	22	25	-	18	20
	R2	16	17	22	25	-	15	17
	R3	18	16	26	26	-	17	20
T16	R1	19	16	20	24	-	17	20
	R2	18	15	22	23	-	16	18
	R3	12	15	20	27	-	16	17
T20	R1	19	18	19	9	-	10	12
	R2	18	20	20	11	-	11	12
	R3	18	17	19	8	-	11	13
T52	R1	20	19	19	17	21	11	13
	R2	19	19	20	17	20	11	12
	R3	19	18	20	18	23	12	13
T53	R1	25	23	25	19	25	19	20
	R2	28	24	25	23	26	15	20
	R3	25	26	25	21	25	17	20

***** R1, R2, and R3: Replicates

Table 3 Raw data for inhibition spectrum (mm) of methanol (negative control) against the test pathogens in secondary screening assessed via disc diffusion method (Chapter 4)

Test pathogen		Diameter of inhibition zone (mm)		
		R1	**R2**	**R3**
Gram-positive bacteria	***Bacillus subtilis***	11	11	12
	Enterococcus faecalis	9	10	11
	Staphylococcus aureus	9	10	12
Gram-negative bacteria	***Pseudomonas aeruginosa***	12	12	13
	Plesiomonas shigelloides	8	9	10
Yeast	***Candida albicans***	13	13	14
	Candida parasilopsis	11	12	12

* R1, R2, and R3: Replicates

Table 4 Raw data for pH level of 3, 6, 9, and 12-day old *Streptomyces* strain T15 cultivated in ISP2, ISP4, and SA media under agitation and static submerged conditions (Chapter 4)

Day		pH level					
		Agitation			Static		
		ISP2	ISP4	SA	ISP2	ISP4	SA
3	R1	6.94	6.64	5.36	6.76	6.71	6.50
	R2	6.71	6.77	5.34	6.76	7.40	7.39
	R3	6.96	6.59	6.90	6.76	7.42	7.42
6	R1	8.00	8.20	6.61	6.58	7.42	7.95
	R2	7.99	8.39	6.70	6.54	6.53	7.01
	R3	6.92	8.17	6.76	6.76	7.35	7.83
9	R1	8.06	6.24	6.76	6.94	7.15	7.62
	R2	5.12	7.65	6.77	6.94	7.13	7.30
	R3	5.11	7.14	6.88	6.99	6.94	7.45
12	R1	6.20	6.94	7.40	6.22	6.89	7.80
	R2	6.21	7.62	7.31	6.23	6.01	6.85
	R3	6.19	6.93	7.30	6.75	7.22	7.77

* R1, R2, and R3: Replicates

Table 5 Raw data for weight of crude extracts (g) of 3, 6, 9, and 12-day old *Streptomyces* strain T15 cultivated in ISP2, ISP4, and SA media under agitation and static submerged conditions (Chapter 4)

Day		Crude extracts weight (g)					
		Agitation			Static		
		ISP2	ISP4	SA	ISP2	ISP4	SA
3	R1	0.24	0.03	0.07	0.27	0.17	0.24
	R2	0.23	0.04	0.19	0.26	0.06	0.09
	R3	0.01	0.06	0.17	0.24	0.04	0.02
6	R1	0.15	0.12	0.35	0.23	0.08	0.14
	R2	0.15	0.03	0.12	0.24	0.05	0.27
	R3	0.10	0.06	0.23	0.21	0.07	0.30
9	R1	0.08	0.25	0.24	0.25	0.26	0.07
	R2	0.10	0.17	0.23	0.22	0.13	0.20
	R3	0.06	0.08	0.27	0.28	0.02	0.15
12	R1	0.25	0.20	0.16	0.20	0.13	0.52
	R2	0.36	0.28	0.03	0.10	0.06	0.19
	R3	0.37	0.17	0.14	0.22	0.10	0.42

* R1, R2, and R3: Replicates

Table 6 Raw data for inhibition spectrum (mm) of 3, 6, 9, and 12-day old *Streptomyces* strain T15 (cultivated in ISP2, ISP4, and SA media under agitation and static submerged conditions) against *Plesiomonas shigelloides* assessed via disc diffusion method (Chapter 4)

Day		**Diameter of inhibition zone (mm)**					
		Agitation			**Static**		
		ISP2	**ISP4**	**SA**	**ISP2**	**ISP4**	**SA**
3	**R1**	-	-	10	-	-	-
	R2	-	-	10	-	-	-
	R3	-	-	10	-	-	-
6	**R1**	11	-	8	-	9	-
	R2	11	-	10	-	9	-
	R3	11	-	10	-	9	-
9	**R1**	11	-	12	-	9	-
	R2	12	-	12	-	10	-
	R3	12	-	10	-	10	-
12	**R1**	10	-	10	-	8	-
	R2	10	-	11	-	8	-
	R3	10	-	10	-	8	-

* R1, R2, and R3: Replicates