ANTIMICROBIAL POTENTIAL OF SELECTED ACTINOMYCETES ISOLATED FROM THE MARINE ECOSYSTEM

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ABSTRACT

Actinomycetes are widespread in marine ecosystem. They are of interest in research field as they are capable to produce bioactive secondary metabolites which can be developed into useful pharmaceutical products. An example of the product is antibiotics which can be used to treat infectious diseases. The potential of antibacterial and antifungal activity of eleven marine derived actinomycetes from the genus *Streptomyces* were studied. They were tested against bacteria *Enterobacter faecalis, Staphylococcus aureus*, *Bacillus subtilis*, *Pleisiomonas shigelloides* and *Pseudomonas aeruginosa* and fungi *Candida parapsilosis* and *Candida albicans.* Primary screening by the cross streak method and secondary screening by diffusion using paper disc method with crude extracts were carried out in this study. Media optimization was also carried out to optimize the media for production of bioactive secondary metabolites. Of the eleven strains studied, strain T53 exhibited the strongest antibacterial activity against *E. faecalis.* Gram-positive bacteria were more susceptible than Gram-negative bacteria. Strain T53, T52, T9 and T12 had broad spectrum activities. Strain T53, T16, T15 and T4 exhibited antifungal activity against all tested fungi. Yeast extract-malt extract agar (ISP2) and agitated fermentation were the best media and the best fermentation condition, respectively.

ABSTRAK

Actinomycete boleh ditemui dengan banyak di dalam ekosistem marin. Organisma tersebut digunakan dalam bidang penyelidikan berdasarkan keupayaannya menghasilkan produk bioaktif sekunder. Produk tersebut boleh diproses untuk menghasilkan produk farmaseutikal. Antibiotik adalah antara produk farmaseutikal yang boleh dihasilkan untuk merawat jangkitan penyakit. Sebelas actinomycete daripada ekosistem marin yang telah dikenalpasti sebagai *Streptomyces* sp. telah dipilih untuk ujian aktiviti antibakteria dan antikulat. Aktiviti antibakteria dan antikulat telah di uji terhadap bakteria *Enterobacter faecalis, Staphylococcus aureus, Bacillus subtilis, Pleisiomonas shigelloides* dan *Pseudomonas aeruginosa* serta kulat/yis *Candida parapsilosis* dan *Candida albicans*. Kaedah 'cross streak' dan 'paper disc' telah diaplikasikan dalam ujian ini. Kaedah 'paper disc' turut diaplikasikan dalam ujian kedua untuk mengetahui media yang terbaik dalam penghasilan produk bioaktif sekunder. Daripada sebelas actinomycete yang dikaji, T53 mempamerkan aktiviti penyekatan pertumbuhan yang paling tinggi terhadap *E. faecalis*. Bakteria Gram-positif adalah lebih mudah disekat pertumbuhannya berbanding bakteria Gram-negatif. Aktinomycete T53, T52, T9 dan T12 menyekat pertumbuhan kedua-dua bakteria Gram-positif dan Gram-negatif. Actinomycete T53, T16, T15 dan T4 menyekat pertumbuhan semua kulat/yis. Yis-malta ekstrak agar (ISP2) dan sistem fermentasi 'agitation' adalah media dan sistem fermentasi yang terbaik dalam penghasilan produk bioaktif sekunder.

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%	percentage
^{o}C	degree celcius
et al.	and others
g	gram
µg	microgram
mg	milligram
ml	milliliter
v/v	volume/volume
sp.	specie
spp.	species

1. INTRODUCTION

Healthcare-associated infections are problems that affect human life. The problems have been of concern in our community many years back until today. The infections are usually caused by pathogens which are bacteria and fungi. Pathogenic bacteria can be divided into two, Gram-positive bacteria and Gram-negative bacteria. These pathogenic bacteria can cause diseases in host organisms including human. Examples of Gram-positive pathogenic bacteria are *Enterococcus faecalis*, *Staphylococcus aureus* and *Bacillus subtilis*. Examples of Gram-negative pathogenic bacteria are *Pleisiomonas shigelloides* and *Pseudomonas aeruginosa*.

One of the comparative characteristic that differentiates Gram-positive bacteria from Gram-negative bacteria is their cell walls. Gram-positive bacteria have simpler walls, with a relatively large amount (multilayered) of peptidoglycan (Campbell *et al.*, 1999) without lipid outer membrane. On the other hand, Gram-negative bacteria have complex wall but less peptidoglycan (single-layered). An outer membrane on the Gram-negative bacteria cell wall contains lipopolysaccharides which are carbohydrates bonded to lipids (Campbell *et al.*, 1999). Another comparative characteristic is the difference in the Gram-staining by Gram-staining method. Gram-positive bacteria retain the crystal violet dye and stain dark violet or purple. While Gram-negative bacteria can be decolorized to accept counterstain (safranin) and stain red.

Gram-positive pathogenic bacteria as well as Gram-negative pathogenic bacteria can cause infections in human body. They are usually associated with cancer patients and nosocomial infections. The bacteria are either pathogens or opportunistic pathogens. The bacteria have also been reported to cause opportunistic infections of patients after operations in hospitals. Jones (1996) and Moellering (1998a) reported that the past two decades have seen a dramatic shift in the incidence of certain bacterial infections in both

the community and hospital settings. This shows that infections by pathogenic bacteria are increasing. With the increase in bacteria infection, human health will be affected.

Gram-positive pathogenic bacteria can be found in the gastrointestinal of human body as well as mucous membranes such as mouth, vagina and skin. Appelbaum and Jacobs (2005) reported that Gram-positive bacteria such as staphylococci and streptococci have historically been and still remain, major causes of human morbidity and mortality throughout the world. Other than that, there is a report that staphylococci are the most common pathogens in nosocomial meningitis (Palabiyikoglu, 2003) and these infections can cause serious clinical outcomes (Palabiyikoglu *et al.*, 2006). Archer (1998) reported that in the last decade, the staphylococci have again emerged as the predominant organisms causing infections in the hospital setting. One example is *S. aureus* which can cause infections of the skin and other organs. The infections are common in people with frequent skin injury. It is seen most commonly in pre-pubertal children and certain occupational groups such as healthcare workers. The bacteria are able to invade via broken skin and mucous membranes.

Other than the staphylococci, the enterococci are also among the most common pathogens. Moellering (1998a,b) reported that enterococci have also ascended in importance. Other researchers also reported that the *Enterococcus* spp. are now the third or fourth most common blood stream infection pathogen among hospitalized patients (Pfaller *et al.*, 1998). Jones *et al.* (1999) reported that *E. faecalis* is the dominant species among hospitalized patients and the most prevalent (59.6%) and the most common cause of enterococcal infection. *E. faecalis* can cause endocarditis, as well as bladder, prostate and epididymal infections. The bacteria can cause life-threatening infections in humans especially in the nosocomial (hospital) environment.

On the other hand, *B. subtilis* is normally considered to be non-pathogenic. However, the bacteria has been linked to food borne illness causing diarrhea, nausea, vomiting, and associated with rice dishes served in oriental restaurants. *B.subtilis* can survive the extreme heating that is often used to cook food. The bacteria is also responsible for causing ropiness, a sticky and stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough.

Gram-negative pathogenic bacteria can be found in the gastrointestinal tracts in the human body. The pathogenic bacteria such as *P. aeruginosa* have been reported to cause opportunistic infectious diseases in hospitals. The bacteria can cause nosocomial pneumonia, hospital-acquired urinary tract infections, surgical wound infections as well as bloodstream infections. The bacteria can also cause opportunistic infections in immunocompromised patients such as cancer and bone patients. *P. aeruginosa* can also cause chronic infections in cystic fibrosis patients.

Another example of Gram-negative pathogenic bacteria is *P. shigelloides* which is a common cause of gastrointestinal complications. The bacteria can also cause septicaemia and meningitis in patients with underlying disorders and as well as in immunocompromised patients.

Besides pathogenic bacteria, fungi also cause infections and diseases in human. Fungi are normally present on the skin and also in mucous membranes such as the vagina, mouth or rectum. They can also travel through the blood stream and affect the throat, intestines and heart valves. Fungi including yeasts have also been reported to cause a high rate of infections in human body. Casadevall *et al.* (2002) stated that the prevalence of fungal diseases has increased markedly in hospitalized patients and other individuals with immune impairment. Kuti *et al.* (2002) reported that the fungal infections which constituted a problem exclusively relevant to dermatology can also cause mortality.

One example of human pathogenic fungi is yeast. Yeast is identified as a single-cell organism. Examples of yeasts are *Candida albicans* and *Candida parapsilosis*. Yeasts infections known as candidiasis are very common in human. Pfaller and Wenzel (1992) reported that *Candida* spp. became the third most common present isolates found in hemocultures in the USA. *C. albicans* is the most common cause of candidiasis. The pathogen is normally found in the lower bowel, vagina and skin. Another example of pathogenic fungi is *C. parapsilosis* which can cause major infection in intensive care units, premature infants and immunocompromised adults.

The increase in the infections of pathogenic bacteria and fungi happens because of drug resistance among the pathogens. A number of pathogenic bacteria are multiple drugs resistant. The bacteria are reported to rapidly develop resistance to multiple classes of antimicrobial agents (Kwa *et al*., 2007). Resistance to antibiotics occurs typically as a result of drug inactivation/modification, target alteration and reduced accumulation owing to decreased permeability and/or increased efflux (Poole, 2002).

Amyes and Thomson (1995) reported that some hospital-acquired pathogens are becoming totally resistant to antibiotics. Examples of those pathogens are *E. faecalis*, *S. aureus* and *P. aeruginosa*. Appelbaum and Jacobs (2005) reported that enterococci are largely of interest because of their increasing resistance to vancomycin. *E. faecalis* is resistant to many commonly used antimicrobial agents such as aminoglycosides, cephalosporins, oxacillin and vancomycin.

Kitouni *et al.* (2005) reported that the vancomycin was the antibiotic of choice for the treatment of the infections caused by methicillin-resistant *Staphylococcus aureus*, until the appearance of the first resistance strains to vancomycin. While methicillin resistance in *S. aureus* (MRSA) has been reported to become common (Diekema *et al.*, 2001) and has become a major problem in many countries (Palavecino, 2004). Woo *et al.* (2006) reported

that antibiotic resistant bacteria, such as penicillin resistant *Streptococcus pneumoniae* and vancomycin intermediate-resistant *Staphylococcus aureus*, are often multi-drug resistant, and thus produced numerous problems and obstacles on the clinical management of these infections.

Jones (2001) and Landman *et al.* (2002) reported that the prevalence rates of multidrug resistance (MDR) among Gram-negative bacteria are on the rise. Other than that, the increase in the multidrug resistance of Gram-negative bacteria is reported to be high especially in intensive care units (Flournoy *et al.*, 2000; Obstritch *et al.*, 2004). *P. aeruginosa* is reported to be resistant to carbapenem (Amyes and Thomson, 1995).

According to Walsh and Amyes (2004), antibiotic resistance has increased progressively in the past 20 years and is reaching a crisis because we are running out of options to treat certain pathogenic bacteria, mainly causing hospital-acquired infection but with the potential to occur in the community. Kuti *et al.* (2002) reported that the increasing emergence of multiresistant bacteria throughout the world and the lack of antibiotics to combat such pathogenic agents continue to be the major concern of the medical community. With the increase in drugs resistant among pathogens that happens because of not enough options to treat certain pathogenic bacteria, we can conclude that infections by the pathogens in human will also increase.

Increase in fungal infection happens because the antifungal drugs are not very effective to treat fungal diseases. Casadevall *et al.* (2002) reported that fungal diseases are often difficult to diagnose and treat because antifungal drugs are often not very effective in the setting of impaired immunity. *C. albicans* can develop resistance to antimycotic drugs such as fluconazole which is often used to treat candidiasis. Hitchcock *et al.* (1993) reported that the frequency of multiazole-resistant strains belonging to *Candida* species other than *Candida albicans* is increasing.

This clearly proves that antibiotics or drugs resistance among pathogenic bacteria and fungi are the causes of infections in human. These are the reasons that influence the interest in the search for new antibiotics in research field. Palabiyikoglu *et al.* (2006) stated that although many of the older antibiotics remain effective, new drug development remains crucial owing to the increase in drug resistance among these important pathogens. Search for new antibiotics effective against multidrug resistant pathogenic bacteria is presently an important area of antibiotic research (Sitachitta *et al.*, 1996) and the scope of search for various bioactive microbial products had, however, broadened (Berdy, 2005). New therapeutic agents are urgently needed to treat medical needs that are unmet (Zhang *et al.*, 2005) and to combat the increasing incidences of resistant pathogenic organisms including fungi (Vikineswary *et al.*, 1997). Casadevall *et al.* (2002) stated that there is considerable interest in the development of vaccines to prevent fungal diseases. This shows that older antibiotics can still be used. However, development of new antibiotic is important to improve the treatment of antimicrobial infections.

In researching for new antibiotics, actinomycetes are now of interest because of its ability to produce bioactive compounds which are useful to be developed as new antibiotic. Okami and Hotta (1988) stated that actinomycetes are useful biological tools producing antimicrobials against bacteria. Actinomycetes are now of interest because they are the most economically and biotechnologically valuable prokaryotes (Lam, 2006). Other than that, Omura (1992) stated that actinomycetes are the most important source of bioactive metabolites. Ward and Bora (2006) stated that actinomycetes are the pre-eminent source of bioactive natural products. The bioactive metabolites or bioactive natural products derived from actinomycetes are useful compounds to be developed as new antibiotics to treat pathogenic organism infections.

Actinomycetes produce a large amount of useful bioactive compounds. They are reported to be responsible for the production of about half of the discovered bioactive secondary metabolites (Berdy, 2005). The metabolites are reported as antibiotics (Berdy, 2005; Strohl, 2004). Okami and Hotta (1988) reported that by the 1980, actinomycetes accounted for almost 70% of the world's naturally occurring antibiotics. This clearly shows that actinomycetes have been proved to be the most important producers of a large amount of bioactive compounds which can be used as antibiotics.

Actinomycetes isolated from marine ecosystems are now of interest in antibiotic research field. Marine actinomycetes have been reported to be important producers of a wide range of bioactive compounds useful in the development of new antibiotics. Goodfellow and Haynes (1984) have suggested that actinomycetes found in the marine ecosystem have been relatively neglected and they may be viewed as a selected gene pool possibly containing organisms capable of producing useful metabolic substances.

The bioactive compounds produced by marine actinomycetes are believed to differ from those produced by terrestrial actinomycetes because of the difference in the environmental conditions. Many of these metabolites (bioactive compounds) possess biological activities and have the potential to be developed as therapeutic agents (Lam, 2006).

Actinomycetes have been frequently isolated from marine waters and sediments but they are given relatively little attention. They are largely unexplored as a source of bioactive agents for industrial production. Tan *et al.* (2004) also reported that actinomyetes isolated from marine organisms could possibly provide an alternative source of potential bioactive substances against those fungal pathogens. Jensen and Fenical (1996) reported that the production of useful compounds by actinomycetes from aquatic environments is

just beginning to be studied, but this area looks extremely promising. This obviously shows that the research for new antibiotics from marine actinomycetes is now of interest.

OBJECTIVES OF STUDY

The objectives of this study were to:

1) study the antibacterial and antifungal potentials of selected marine actinomycetes
2) optimize media for the production of bioactive compounds

2 LITERATURE REVIEW

2.1 General overview of actinomycetes

Actinomycetes are Gram-positive bacteria. These bacteria were first regarded as fungi as early as 1878 (Srinivasan *et al*., 1991) because of the superficial similarity in the filaments between actinomycetes and fungi. However, actinomycetes are classified as true bacteria. Srinivasan *et al*. (1991) also reported that it is firmly established that these organisms are prokaryotes with close affinities to the mycobacteria and the coryneforms and have no phylogenetic relationship with fungi. Das *et al*. (2006) reported that because of their well developed morphological and cultural characteristics, actinomycetes have been considered as a group well separated from common bacteria.

This complex group of bacteria is prokaryotic organisms. They have cell wall but have no nuclear membrane. They reproduce by spores in two ways, either by fissions or by conidia. Actinomycetes are called filamentous bacteria because they form branching filaments or hyphae during their growth which resemble the mycelia of the fungi. However, their filaments are in the diameter of 1mikron(μ) which is smaller than those of the fungi. This distinguished the actinomycetes from the fungi.

Actinomycetes belong to the kingdom bacteria (Phylum: Actinobacteria, Class : actinobacteria). They can be divided into five subclasses Acidimicrobidae, Actinobacteridae, Coriobacteridae, Rubrobacteridae and Sphaerobacteridae.

Williams *et al*. (1984) reported that these bacteria are primarily saprophytic. Most of the bacteria are aerobic and mesophilic. They are also heterotrophic organisms. The habitats of actinomycetes are wide spread in our environments.

Zaitlin and Watson (2006) reported that actinomycetes are a complex group of bacteria present in a wide range of environments, either as dormant spores or actively growing. The common habitat of actinomycetes is soils. Imada (2005) reported that

actinomycetes are representative of terrestrial microorganisms and usually are isolated from soils. However, these bacteria can also be found in aquatic environments. Bull *et al*. (2005) reported that actinomycetes are very widely distributed in the world's oceans. They are also reported to be easily isolated from the marine environment (Ward and Bora, 2006).

Other than that, actinomycetes can also be found in marine organisms. Ward and Bora (2006) reported that actinomycetes are also present in many free-swimming marine vertebrates and invertebrates, as well as in sessile ones. They can be found living in marine organisms such as decaying marine algae, seaweed and sponges.

Among the microorganisms, actinomycetes gained special importance due to their capacity to produce bioactive secondary metabolites and enzymes (Das *et al*., 2006). They are the most important producers of bioactive secondary metabolites. They produce vitamins, enzymes, antitumor agents, immunodefying agents and mainly antibiotic compounds (Goodfellow *et al*., 1988; Demain, 1995).

Baltz (2007) reported that most antibiotics in clinical use are direct natural products or semisynthetic derivatives from actinomycetes or fungi. Many of those products, including erythromycin and derivatives, vancomycin and teicoplanin, cephalosporins, rifamicin, tetracyclines and daptomycin were discovered through whole-cell antibacterial screening procedures.

The bioactive secondary metabolites are very useful. The secondary metabolites have the properties of interest in medicine and agriculture (Lechevalier, 1992). Other than that, the bioactive secondary metabolites are said to be of value to humans (Srinivasan *et al*., 1991). Lechevalier (1992) also stated that these metabolites will also continue to be starting points for drug design and chemical modifications. Tens of thousands of such compounds have been isolated and characterized, many of which have been developed into

drugs for treatment of a wide range of human diseases (Bull *et al*., 1992; Franco *et al*., 1991).

Of all the bioactive secondary metabolites produced by actinomycetes, antibiotics are the most important products. Mincer *et al*. (2002) stated that actinomycetes are best known as a source of antibiotics. They produce a very large amount of antibiotics. Takizawa *et al*. (1993) reported that approximately two-third of the thousands of naturally occurring antibiotics have been isolated from actinomycetes. Approximately 7000 of the compounds (antibiotics) reported in the Dictionary of Natural Products were produced by actinomycetes (Jensen *et al*., 2005).

Because of their importance as producers of bioactive secondary metabolites especially antibiotics, actinomycetes have been the focus of many researches. They play an important role in pharmaceutical field as well as in biotechnology. Colquhoun *et* al. (2000) reported that in many areas of microbiology, there is a growing requirement for rapid and unambiguous characterization of organisms. Two situations for which this requirement is especially acute are in clinical diagnosis and biotechnology search and discovery programs due to failure of drug treatment, reinfection or relapse.

2.2 Marine actinomycetes

Marine ecosystem is one of the habitats of actinomycetes. The world's oceans include some of the most biodiverse ecosystems on the planet (Mincer *et al*., 2002), and provide the largest inhabitable space for living organisms, particularly microbes (Das *et al*., 2006). Lam (2006) reported that recent culture-independent studies have shown that marine environments contain a high diversity of actinomycetes that are rarely, if at all, recovered by culture-dependent methods. Zaitlin and Watson (2006) reported that dormant spores of actinomycetes may be isolated from aquatic environments in high concentrations.

Marine actinomycetes are always regarded as wash-in organisms. They were said to be transported from the terrestrial into the oceans. There have been reports of strains isolated from aquatic habitats are usually considered as wash-in organisms and, regarding longer periods, then living there in a dormant state (Bull *et al.*, 2000; Cross, 1981). They are in many respects regarded as boundary bacteria (Weyland, 1986) and are merely of terrestrial origin (Mincer *et al*., 2002).

However, indigenous marine actinomycetes do occur in marine ecosystem. Bull *et al*. (2005) reported that truly indigenous marine actinomycetes have now been described. Recent findings confirm the presence of indigenous marine actinomycetes in the oceans and indicate that marine actinomycetes are widely distributed in different marine environments and habitats (Lam, 2006).

The habitats of actinomycetes in the marine ecosystem are wide spread. Ward and Bora (2006) reported that the actinomycetes, although not all the Actinobacteria, are easy to isolate from the marine environment. Lam (2006) reported that both culture-dependent and culture-independent methods demonstrate that novel actinomycetes can be found everywhere in the oceans – from the deep sea floor to coral reefs, from sediments to invertebrates and plants.

It has long been recognized that actinomycetes can be recovered from the sea (Weyland, 1969). Other than the open ocean, the actinomycetes can also be found in the sea sediments. There have been reports of actinomycetes recovered from the deep sea sediments (Zhang *et al.*, 2005).

Actinomycetes can also be recovered from the mangroves. Zhang *et al*. (2005) reported of the isolation of actinomycetes from the mangroves. Weyland (1986) also reported that the mangroves exhibited the highest density of actinomycetes amongst the areas investigated also within their higher salinity regions.

Other than that, actinomycetes can also be found living in marine organisms. There have been reports of actinomycetes isolated from marine organisms such as coral reefs (Zhang *et al*., 2005), from sponges (Sponga *et al*., 1999) and from seaweeds (Genilloud *et al*., 1994). Baltz (2007) reported that actinomycetes comprised about 10% of bacteria colonizing marine aggregates, and can be isolated from marine sediments, including those obtained at depths of 10898 m from the deepest part of the Marianas Trench. This obviously shows that actinomycetes are widespread in the marine ecosystem. Hence, the marine ecosystem must be explored in order to study the actinomycetes in deeper.

2.3 The genus *Streptomyces*

Streptomyces belong to the order Actinomycetales (Kingdom : Bacteria, Phylum : Actinobacteria, Subclass : Actinobacteridae, Family : *Streptomycetaceae*). Many researchers revealed that this group of actinomycetes species is the majority from many isolation programs. From 439 actinomycetes isolated, Zheng *et al.* (2000) reported that strains belonging to the genera *Streptomyces* and *Micromonospora*, especially the former, represented the majority. Other than that, Kitouni *et al.* (2005) also reported that from their studies 93% of active actinomycetes belong to *Streptomyces* genus. This obviously shows that *Streptomyces* genus is the most prolific group of actinomycetes. More than that, Weyland (1986) also reported that from their studies the bulk of the isolates from sea sediments could be classified only in three taxonomic groups: streptomycetes, *Micromonospora* and nocardioforms.

The species in the genus *Streptomyces*, as with the other actinobacteria, are Gram-positive organisms. They are also aerobic and filamentous bacteria. They are saphrophytic and not highly pathogenic, but are associated with inflammatory disorders of the airways, and possibly other symptoms of an autoimmune character (Huttunen *et al*., 2002).

The *Streptomyces* sp. produces substrate mycelium and aerial mycelium. Francisco and Silvey, (1971) reported that *Streptomyces* substrate mycelium is described as facultatively aerobic while the aerial growth was obligately aerobic. Rintala (2003) reported that the streptomycetes are aerobic, Gram-positive bacteria, which produce extensive branching vegetative (substrate) mycelium and aerial mycelium bearing chains of arthrospores. Specifically, the organism produced abundant gray-white aerial mycelium, which transformed into chains of smooth, cylindrical arthrospores, as shown by scanning-electron microscopy (Sitachitta *et. al*., 1996).

Getha *et al*. (2007) reported that the streptomycete-like group formed substrate mycelium and abundant aerial mycelium with powdery spore mass. The occurrence of distinct aerial mycelium with abundant spore mass formation represents an important macroscopic criterion to identify the genus *Streptomyces*. They can be differentiated from other actinomycetes genera that also produce aerial mycelium by other morphological, biochemical and physiological properties (Goodfellow and Williams, 1983).

The substrate mycelium and spores can be pigmented. However, diffusible pigments are also produced. On agar plates, they form lichenoid, leathery butyrons colonies (Williams *et al*., 1989).

The genus *Streptomyces* are widespread in the marine environment. They can be found in coastal and shelf regions (Weyland, 1986). In previous studies enumerating culturable *Streptomyces* isolates in marine ecosystems, the majority of isolates were recovered from coastal environments, such as shallow seas, mangrove swamps, or sea grass communities (Rehnstam *et al*., 1993).

Among all Actinobacteria, the genus *Streptomyces* is regarded as the most potent producers of bioactive secondary metabolites. After the discovery of streptomycin and later chloramphenicol, tetracyclins and macrolides the attention turned to the *Streptomyces*

species. *Streptomyces* in the family *Streptomycetacae* (Stackebrandt *et al*., 1997) is one of the most prolific producers of secondary metabolites. Sanglier *et al*. (1993) reported between 1988 and 1992, more than a hundred new molecules from actinomycetes were discovered. Approximately 75% of these originated from the *Streptomyces* genus and at least 5000 documented bioactive compounds are known as being produced by this genus (Anderson and Wellington, 2001). In fact, the genus *Streptomyces* also accounts for a remarkable 80% of the actinomycetes natural products reported to date, a biosynthetic capacity that remains without rival in the microbial world (Jensen *et al*., 2005). Wagner *et* al. (2002) reported that most secondary metabolites from marine microorganisms found so far were isolated from *Streptomyces* and *Alteromonas* sp.

The bioactive secondary metabolites are very useful. The metabolites have different functions which is very useful to human life. The bioactive secondary metabolites produced by the *Streptomyces* genus have different biological activities such as antibacterial and antifungal activities (Demain, 1999). Miyadoh (1993) reported that the *Streptomyces* species produce about 75% commercially and medically useful antibiotics. Out of the approximately 10000 known antibiotics, 45-55% are produced by streptomycetes (Demain, 1999; Lazzarini *et al*., 2000). The secondary metabolites produced by them have a broad spectrum of biological activities such as antibacterial (streptomycin, tetracycline, chloramphenicol) and antifungal (nystatin) (Rintala, 2003).

Peela *et al*. (2005) reported that from 88 actinomycete strains isolated from marine sediments collected near nine islands of the Andaman coast of the Bay of Bengal, 64 isolates were identified as belonging to the genus *Streptomyces*, family *Streptomycetaceae* (spore chain with coiling and branching). Other than that, Berdy (2005) reported that obviously various actinomycetales, first of all the *Streptomyces* species and filamentous fungi, and to a lesser extent several bacterial species are the most noteworthy producers

both in respect of numbers, versatility and diversity of structures of the produced metabolites.

This obviously shows that the genus *Streptomyces* are abundant in our ecosystem. They are the majority species isolated by many researchers. They are also regarded as the most important producers of bioactive secondary metabolites especially antibacterial and antifungal. The bioactive secondary metabolites are very useful in human life where they can be developed to treat infectious diseases.

2.4 Marine actinomycetes as a source of bioactive metabolites

Marine actinomycetes produce many useful bioactive metabolites. They have been reported to produce highly active substances and are regarded as the most important source of bioactive metabolites. Mincer *et al*. (2002) reported that despite their importance in soil ecology, actinomycetes are best known as a source of antibiotics. More than that, Osada (1998) and Saadoun and Gharaibeh (2003) also reported that actinomycetes are prolific producers of antibiotics and other industrially useful secondary metabolites such as antibiotics, herbicides, pesticides and anti-parasitic. Actinomyctes have long been tapped by pharmaceutical researchers as a source of novel antibiotics, actinomycin and streptomycin for instance (Rintala, 2003).

Fiedler *et al*. (2005) reported that in contrast to marine invertebrates, marine bacteria seem to be a promising source as producers of drug candidates. They focused their investigations solely on marine microorganisms whose biotechnological production process can easily be scaled-up, as in the case of the bacterial order *Actinomycetales*. Other than that, Jensen *et al*. (2005) reported that marine-adapted actinomycetes produce a relatively high rate of new secondary metabolites and that these bacteria do in fact represent a natural product resource worthy of thorough exploration.

The filamentous actinomycetes account for a significant fraction of microbial metabolite (Busti *et al*., 2006). Microbial extracts could be developed into useful drugs to treat infectious diseases. Microbial extracts have been and continue to be a productive source of new biologically active molecules for drug discovery (Cragg *et al.*, 1997; Shu, 1998). It is estimated that more than 30% of worldwide human pharmaceutical sales have compounds from natural sources as their origin (Schmid *et al*., 1999). More than 70% of antibiotics (including not only antibacterial agents but also bioactive microbial compounds) have been reported to be produced by actinomycetes, which were composed of the genus Streptomyces (68%) and the rare actinomycetes (32%) (Miyadoh, 1993). More than 720 marine metabolites were reported in the literature during 2003, about half of which demonstrated biological activities (Zhang *et al*., 2005).

This shows that marine actinomycetes are the most important producers of bioactive secondary metabolites. The bioactive secondary metabolites have been developed into useful pharmaceutical products. The products could be used to treat infectious diseases in human.

2.4.1 Bioactive metabolites

Bioactive metabolites are secondary metabolites. Berdy (2005) reported that the secondary metabolites are low molecular (MW<3000), chemically and taxonomically extremely diverse compounds with obscure function, characteristic mainly to some specific; distinct types of organisms.

The most characteristic features are their incredible array of unique chemical structures and their very frequent occurrence and versatile bioactivities. The presently known secondary microbial metabolites, exhibit a great numbers of diverse and versatile biological effects, first of all antimicrobial activities. Some 60% of the presently known

bioactive microbial metabolites, about 14000 compounds exhibit antimicrobial (antibacterial, antifungal) (Demain and Fang, 2000). Berdy (2005) reported that the presently known secondary microbial metabolites, exhibit a great numbers of diverse and versatile biological effects, first of all antimicrobial activities.

In a broad sense, antibiotics include any chemical of natural origin, which has the effect on the growth of other types of cells. They are antimicrobial agents secreted by microorganisms that kill or inhibit other microorganisms. They are secondary molecules (produced only when needed) (Dairi *et al.*, 1999). Srinivasan *et al.* (1991) reported that antibiotics are secondary metabolites and, while they have no physiological role in the growth phase of the actinomycete culture, they are produced as idiophase metabolites after the active growth phase is over.

Antibiotics are produced as secondary metabolites by certain groups of microorganisms, especially streptomycetes. Over 6000 of these compounds are produced by *Streptomyces* species and many are commercially important medicinal products used therapeutically as anti-infective (antibiotic, antifungal and antiparasitic), anticancer or immunosuppressant agents (Takahashi and Mura, 2003). Antibiotics may have a bactericidal(killing) effect or a bacteriostatic(inhibitory) effect on a range of microbes.

Zheng *et al.* (2000) reported that as a great promising source for new natural products which have not been observed from terrestrial microorganisms, marine bacteria are being developed for the discovery of bioactive substances with new types of structure, with growing intensive interest. The achievements have been well reviewed (Jensen and Fenical, 1994; Bernan *et al.*, 1997), where many new antibiotics were obtained from actinomycetes. Selvin *et al.* (2004) reported that many bacteria and cyanobacteria associated with sponges were found to be the sources of antibiotics and other bioactive compounds in marine environment.

From this, we can conclude that actinomycetes are wide spread in our environment. They are regarded as potent producers of bioactive secondary metabolites. Recently, the marine actinomycetes have become the focus of studies. Marine actinomycetes are also widespread in the marine ecosystem. Marine actinomycetes are also regarded as the most important producers of bioactive secondary metabolites.

The bioactive secondary metabolites are very important especially in the pharmaceutical fields. The metabolites can be developed into useful pharmaceutical products which can be used to treat emerging and re-emerging infectious diseases in human. This helps to improve the human health as well as extend human life.

3 MATERIALS AND METHODS

3.1 Test actinomycetes

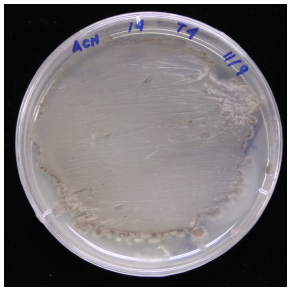
The actinomycetes used in this study were obtained from Prof. Dr. Vikineswary Sabaratnam from Plant Mycology and Pathology Laboratory, Institute of Postgraduate Studies, University of Malaya (IPS, UM). The actinomycetes were identified as putative *Streptomyces* spp. and 11 strains were selected for further studies. Table 3.1 shows the selected putative strains of *Streptomyces* spp. Figures 3.1 a – k shows the cultures of selected putative *Streptomyces* spp.

Table 3.1: Selected putative strains of S*treptomyces* spp. for bioactivity screening on inorganic salts-starch agar (ISP4) for strains T3 and T4 and sporulation agar (SA) for the remaining strains

Media	Code of strains	Colour groups
ISP4	T3	Grey
	T4	
SA	T9	White/Red
	T12	
	T13	
	T15	
	T52	
	T6	White/Brown
	T16	
	T20	
	T53	

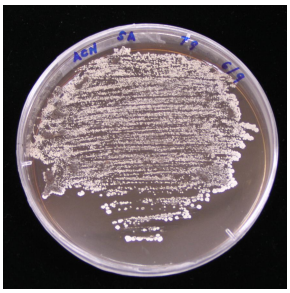
a) Strain T3

b) Strain T4

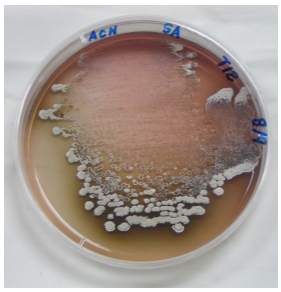


c) Strain T6

d) Strain T9

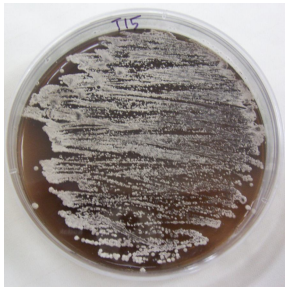


e) Strain T12

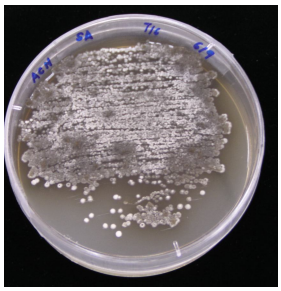


f) Strain T13

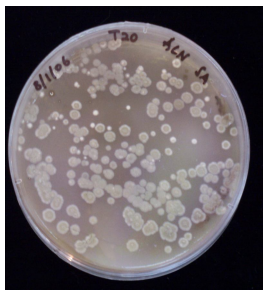
g) Strain T15



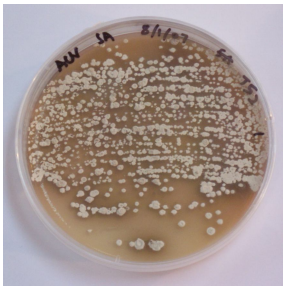
h) Strain T16



i) Strain T20



j) Strain T52



k) Strain T53

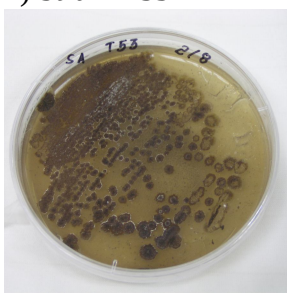


Figure 3.1: Cultures of selected putative strains of S*treptomyces* spp. for bioactivity screening incubated at 28±2°C for 7 – 14 days on ISP4 for strains T3 and T4 and SA for the remaining strains

3.1.1 Preparation of stock cultures

The colonies to be preserved were selected from the agar culture with heavy growth. The agar culture was aseptically cut into small 0.5x0.5mm pieces using sterilized loop. The plugs were then transferred into sterilized Bijou bottles containing 4ml of 30% (v/v) glycerol (Wellington and Williams, 1978) (Appendix A, 1). The stock cultures were then stored at -20±2^{o}C. For each actinomycetes strain, the stock cultures were prepared in triplicates.

3.1.2 Preparation of pure cultures

Pure cultures of actinomycetes strains could be prepared using the stock cultures. The frozen stock cultures were left at 10±2^{o}C overnight and then brought to room temperature before it was used to prepare pure cultures. The plugs were removed from the glycerol stock using a sterilized loop and transferred to growth media on agar plates. The actinomycetes strains were then streaked on the surface of the growth media. Actinomycetes strains T3 and T4 were streaked on inorganic salts-starch agar (ISP4) (Appendix A, 2). The remaining strains were streaked on sporulation agar (SA) (Appendix A, 3). The plates were then incubated at 28±2^{o}C for 7-14 days. The pure cultures were also prepared in triplicates for each actinomycetes strain.

3.2 Test microorganisms

The test microorganisms used in this study were divided into two groups, the pathogenic bacteria and fungi. The pathogenic bacteria, *Enterobacter faecalis, Staphylococcus aureus, Bacillus subtilis*, *Pleisiomonas shigelloides* and *Pseudomonas aeruginosa* were obtained from Prof. Dr. Thong Kwai Lin from the Institute of

Postgraduate Studies, University of Malaya (IPS, UM). The fungi, *Candida parapsilosis* and *Candida albicans* were obtained from Prof. Ng Kee Peng.

The pathogenic bacteria were grown on nutrient agar (NA) (Appendix A, 4) and incubated at 37±2^{o}C for 24 hours. Yeasts were grown on Sabaroud-dextrose agar (SDA) (Appendix A, 5) at 37±2^{o}C for 24 hours.

3.3 Primary screening

Primary screening by the cross streak method was carried out in this study. The actinomycetes strains were streaked on approximately one third of the agar plates, ISP4 for actinomycetes strains T3 and T4 and SA for the remaining strains. The plates were then incubated at 28±2^{o}C for 7-14 days. After the incubation period, single colonies of two days old test microorganisms were cross streaked perpendicular to actinomycetes strains on the test plates. About 4 to 5 streaks of test microorganisms were streaked at about 0.5-1.0cm from the border of the actinomycetes. Figure 1 shows the cross streak method for primary screening. Positive control plates for the test were prepared by streaking only the test microorganisms on the agar plates, without the actinomycetes.

After cross streak, the plates were incubated for 48 hours at 37±2^{o}C. After the incubation period, the inhibition of the test microorganisms' growth was observed. The degrees of the inhibition were recorded.

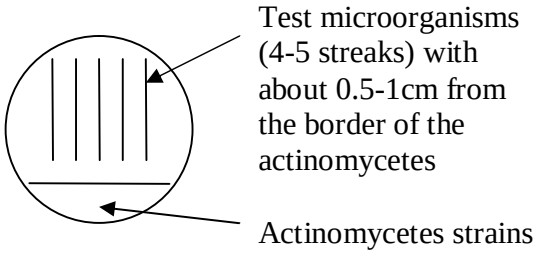


Figure 1. Cross streak method for primary screening

3.4 Secondary screening

Secondary screening by diffusion using paper disc method was carried out in this study. The strains were cultivated in submerged cultures. The fermentation media ISP4 for strains T3 and T4 and SA for the remaining strains were prepared without the agar in the 250ml Erlenmeyer flasks. The surface of the agar plates with dense growth of actinomycetes strains were scraped using sterilized loop and transferred into 10ml sterilized distilled water in the small conical flasks. The mixture was then transferred into 90ml (90%) sterilized fermentation media using sterilized pipette. T3 and T4 were cultivated in ISP4, and the remaining actinomycetes strains were cultivated in SA. The flasks were than incubated on a rotary shaker at 120rpm, 28±2^{o}C for 7-14 days.

After the incubation period, the submerged cultures were transferred into round bottom flasks. The flasks were coated using dry ice. Freeze dry using Beta 1-8 Freeze Dryer was applied in order to get rid of water in the submerged cultures. The freeze dried materials were then transferred into 250ml Erlenmeyer flasks. 50ml of dichloromethane (DCM) and 50ml of methanol with the ratio of 1:1 were added into the flasks. The mixtures were than left overnight on the rotary shaker at 120rpm at 28±2^{o}C.

The mixtures were then sonicated using Ultrasonic Bath at 40 kHz for 30 minutes at room temperature. Sonication was done to break up the actinomycetes cells.

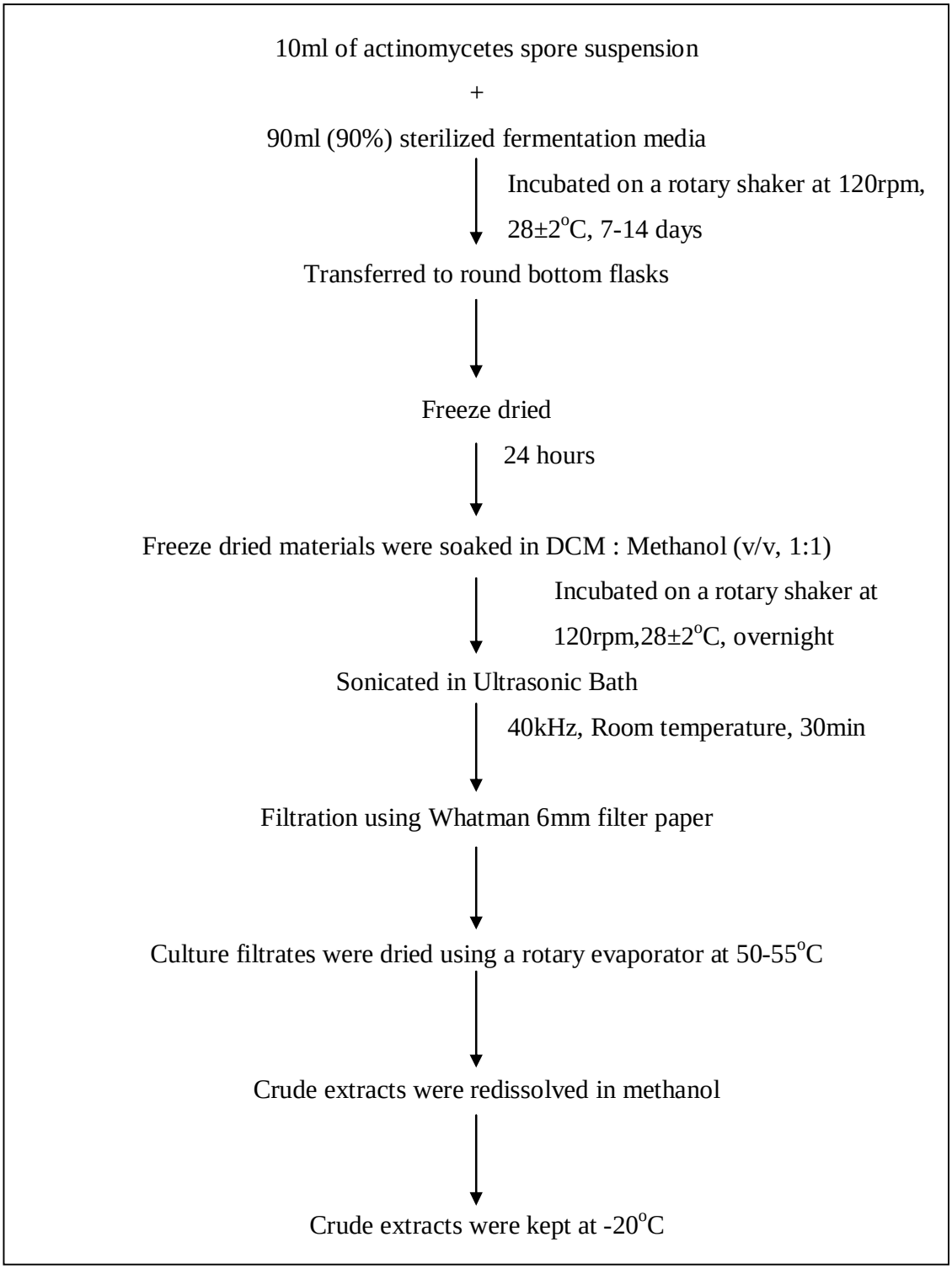
After sonication, the mixtures were filtered using sterilized Whatman filter paper to get the culture filtrates. The culture filtrates were then dried using a rotary evaporator at 50-55^{o}C. This process was done to get the crude extracts of the actinomycetes strains.

The crude extracts were dissolved in methanol. For every 25mg of the crude extracts, 1ml of methanol was added. The crude extracts could be kept at -20^{o}C. They were tested for its bioactivity within one to two weeks (Tan *et al.*, 2004).

Sterilized Whatman 6mm paper discs were used. Test microorganisms were first lawned on the surface of the agar media in the test plates. The pathogenic bacteria were lawned on NA while the pathogenic fungi were lawned on SDA. After that, five sterilized Whatman paper discs were placed on the surface of the seeded plates. 15µl of the dissolved crude extract were loaded on four of the paper discs. The paper discs at the center of the plates were loaded with methanol. Paper discs loaded with methanol were assigned as negative control. Ready made antibiotics discs were used as positive control. Novobiocin and streptomycin discs were used as antibacterial test while nystatin disc was used as antifungal test. The test plates were then incubated for 24 hours 37±2^{o}C.

After 24hours, the diameter of the inhibition zone around the paper discs was observed. The clearance around the paper discs showed that the test microorganisms' growth was inhibited by the actinomycetes strains. The diameter of the inhibition zone was recorded. Figure 2 shows the flow diagram of procedures for secondary screening of actinomycetes strains.

Figure 2: Flow diagram of procedures for secondary screening of actinomycetes strains



3.5 Media optimization

In optimization of media for production of bioactive compounds, only one *Streptomyces* sp. was selected and tested against one selected test microorganism. In this study, the *Streptomyces* sp. selected was strain T53. The strain was tested against pathogenic bacteria, *E. faecalis*.

In media optimization, only secondary screening method was applied. The processes applied were as mentioned earlier (Chapter 3, Section 3.4, page 24 – 25). Howevernthree growth media in liquid form were used. The growth media were yeast extract-malt extract agar (ISP2) (Appendix A, 6), ISP4 and SA. Dimethylsulfoxide (DMSO) was used as a solvent.

In optimization of media for production of bioactive compounds, submerged culture fermentation was carried out in two conditions, static and agitation. For each of the growth medium, twelve flasks of the submerged cultures were prepared in 250ml Erlenmeyer flasks. For submerged culture fermentation in static condition, the cultures were incubated at 28±2^{o}C for twelve days without agitation. On the other hand, submerged cultures fermentation in agitation condition were incubated at 28±2^{o}C for twelve days and agitated at 120rpm.

Every three days, three flasks were taken out. The pH of the cultures was recorded. The submerged cultures were processed as mentioned earlier (Chapter 3, Section 3.4, page 24 – 25) to get crude extracts. The crude extracts were used to test for antibacterial activities of the actinomycetes strain using the paper disc method. Ready made streptomycin disc and novobiocin disc were used as positive control for the test. The diameter of the inhibition zone around the paper discs was observed and recorded.

4 RESULTS AND DISCUSSIONS

4.1 Antagonistic pattern in primary and secondary screening

Table 4.1 shows the antibacterial and antifungal activities of selected *Streptomyces* spp. in primary and secondary screening. The number of active *Streptomyces* spp. against test bacteria was higher in secondary screening compared to primary screening. However, the number of active strains against test fungi was higher in secondary screening compared to primary screening.

Table 4.1: Antibacterial and antifungal activities of selected *Streptomyces* spp. in primary and secondary screening

		Number of active strains[a]
Antibacterial[b]	**Primary screening[d]**	9
	Secondary screening[e]	11
Antifungal[c]	**Primary screening**	0
	Secondary screening	7

[a] Number of active strains antagonistic against at least one test microorganism in primary and secondary screening. Total strains tested for bioactivity were eleven; [b] Antibacterial activity against: *E. faecalis, S. aureus, P. shigelloides, B. subtilis* and *P. aeruginosa;* [c] Antifungal activity against: *C. parapsilosis* and *C. albicans;* [d] Primary screening: Cross streak method was applied on ISP4 for T3 and T4 and SA for the remaining strains. The plates were incubated for 48 hours at 37±2°C; [e] Secondary screening: Paper disc method was applied on NA for test bacteria and on SDA for test fungi. The discs were loaded with 15µl of crude extracts and the plates were incubated for 24 hours at 37±2°C.

In primary screening, nine strains showed antagonistic activity against at least one test pathogenic bacteria. However, no antifungal activity was recorded against the test fungi. In secondary screening, all strains showed antagonistic activity against at least one test pathogenic bacteria. The antifungal activity was also recorded where seven strains showed antagonistic activity against at least one test fungi.

4.1.1 Antibacterial activity

Table 4.2 shows the antibacterial activity of selected *Streptomyces* spp. against test bacteria in primary and secondary screening. Total number of St*reptomyces* spp. tested for antagonistic activity against test bacteria was eleven. Antibacterial activity against test bacteria was recorded in both primary and secondary screening. The antibacterial activity was recorded against both Gram-positive bacteria and Gram-negative bacteria.

Table 4.2: Antibacterial activity of selected *Streptomyces* spp. against test bacteria in primary and secondary screening

Antagonistic activity / Test bacteria		Primary screening[a]	Secondary screening[b]
		Number of active strains	Number of active strains
Gram-positive bacteria	***E. faecalis***	8	11
	S. aureus	6	11
	B. subtilis	6	11
Gram-negative bacteria	***P. shigelloides***	6	11
	P. aeruginosa	7	4

[a] Primary screening: Cross streak method was applied on ISP4 for T3 and T4 and SA for the remaining strains. The plates were incubated for 48 hours at 37±2°C; [b] Secondary screening: Paper disc method was applied on NA agar medium. The discs were loaded with 15µl of crude extracts and the plates were incubated for 24 hours at 37±2°C.

The number of active strains against test bacteria was higher in secondary screening compared to primary screening (Table 4.1). In primary screening, eight strains showed antagonistic activity against *E. faecalis*. Seven strains showed antagonistic activity against *P. aeruginosa* and six strains showed antagonistic activity against *S. aureus*, *B. subtilis* and *P. shigelloides.*

In secondary screening, the number of active strains against all Gram-positive bacteria increased. All strains showed antagonistic activity against the Gram-positive

bacteria. The number of active strains against *P. shigelloides* also increased. All strains showed antagonistic activity against the pathogen. However, the number of active strains against *P. aeruginosa* decreased to only four strains. In this study, the antagonistic pattern of *P. aeruginosa* was not similar with other test bacteria. The antagonistic activity against the pathogen was higher in primary screening compared to secondary screening.

Table 4.3 shows the antibacterial activity of selected *Streptomyces* spp. against test bacteria in secondary screening. The inhibition zones around the paper discs mounted with crude extracts of selected *Streptomyces* spp. was recorded. In this study, the degree of inhibition zones were grouped into weak (<10mm), moderate (10-15mm) and good (>15mm). Most of the active strains exhibited weak inhibition against all test bacteria. A few active strains exhibited moderate inhibition against all test bacteria except *E. faecalis*. Good inhibition was only recorded against *E. faecalis* and *P. shigelloides.*

Ten strains showed weak inhibition against *E. faecalis* and *S. aureus*. Nine strains showed weak inhibition against *B. subtilis*. Seven and three strains showed weak inhibition against *P. shigelloides* and *P. aeruginosa*, respectively. Moderate inhibition by one active strain was recorded against *S. aureus*. One strain also showed moderate inhibition against *P. aeruginosa*. Two strains showed moderate inhibition against *B. subtilis* and *P. shigelloides*. Active strains only showed good inhibition against *E. faecalis* and *P. shigelloides*. One strain showed good inhibition against *E. faecalis*. Two strains showed good inhibition against *P. shigelloides.*

Table 4.3: Antibacterial activity of selected *Streptomyces* spp. against test bacteria in secondary screening on NA for 24 hours at 37±2°C

Antagonistic activity / Test bacteria		Total number of active strains	Weak (<10mm)	Moderate (10 – 15mm)	Good (>15mm)
			Number of active strains	Number of active strains	Number of active strains
Gram-positive bacteria	*E. faecalis*	11	10	-	1
	S. aureus	11	10	1	-
	B. subtilis	11	9	2	-
Gram-negative bacteria	*P. shigelloides*	11	7	2	2
	P. aeruginosa	4	3	1	-

In this study, the percentage of active strains that exhibited antibacterial activity against at least one test bacteria was higher in secondary screening than in primary screening. This is in agreement with the study done by Kavithambigai (2006). The author reported that the antibacterial activity was prominent in secondary screening (54.6%). In her study, the percentage of active strains that exhibited antibacterial activity against at least one test bacteria from root samples collected in Kuantan was higher in secondary screening (58.3%) than in primary screening (43.2%). The author also reported that the percentage of active strains from Port Dickson mangrove was also higher in secondary screening (51.4%) than in primary screening (31.1%).

Peela *et al*. (2005) reported that 44% of marine actinomycetes isolated from the Bay of Bengal showed antibacterial activity. Tan *et al*. (2004) reported that 25 (51%) out of 49 crude extracts of actinomycetes exhibited inhibition against at least one test bacteria. The ability of actinomycetes exhibiting antibacterial activity was once again proved in the present study. Antimicrobial compounds work against bacteria by way of a variety of mechanisms. Antimicrobial agents act by interfering with cell wall synthesis, cell membrane function, nucleic acid synthesis, ribosomal function and folate synthesis. Cell wall synthesis can be affected either by preventing the production of new cell walls, effectively stopping the cell from reproducing, or by providing analogues for the bacteria to include as the new cell is produced, leading to cell lysis and death (Appelbaum and Jacobs, 2005).

Tan *et al*. (2004) reported that seven strains of actinomycetes exhibited broad spectrum inhibition against both Gram-positive and Gram-negative bacteria. Seventeen (68%) out of the 25 strains inhibited Gram-positive test bacteria and one (4%) inhibited Gram-negative test bacteria. Selvin *et al*. (2004) reported that the extracellular products (ECPs) of *Streptomyces* sp. strain BTL7 isolated from marine sponge, *Dendrilla nigra* successfully prevented the growth of the Gram-positive bacteria to the extent of 60% whereas the inhibitory potential was decreased towards the Gram-negative bacteria (40%). Zheng *et al*. (2000) reported that eleven (65%) out of seventeen tested strains showed antibacterial activity against Gram-positive bacteria, eight (47%) against Gram-negative bacteria and four (23.5%) against both Gram-positive and Gram-negative bacteria. Bernan *et al*. (1994) reported that the extracellular proteins (ECPs) of actinomycete strain (LL-31F508) isolated form an intertidal sediment collected in Key West, Florida, showed potent antimicrobial activity against *Staphylococcus* spp. and *Enterococcus* spp. Pisano *et al*. (1992) reported that 68% out of 85 marine actinomycetes capable of degrading chitin

exhibited antibacterial activity against Gram-positive bacteria. Only 11% of the actinomycetes were active against Gram-negative bacteria. This is in agreement with the results obtained in the present study because the antibacterial activity was prominent against Gram-positive bacteria compared to Gram-negative bacteria.

Sujatha *et al*. (2005) reported that streptomycete strain BT-408 showed a broad spectrum against Gram-positive and Gram-negative bacteria, fungi and yeast including the pathogenic methicillin resistant *S. aureus* when tested with crude culture filtrates and also with purified antibiotic SBR-22. Peela *et al.* (2005) reported that *Streptomyces* sp strains BT606 and BT652 showed antimicrobial activites against *S. aureus* and *P. aeruginosa*. Thorne and Alder (2002) reported that *Streptomyces roseosporus* produced Daptomycin which exhibited antibacterial activity against *S. aureus*. Kavithambigai (2006) reported that in secondary screening, the percentage of active strains against Gram-positive bacteria increased to 48.2% and 39.8% for *B. subtilis* and *S. aureus*, respectively. This is again in agreement with the result obtained in the present study because test actinomycetes exhibited antibacterial activity against Gram-positive bacteria including *S. aureus* and *B. subtilis*.

Pisano *et al*. (1986) reported that from their studies, 19 strains of actinomycetes isolated from marine sediments in Sandy Hook Bay, New Jersey displayed antimicrobial activity. The most prominent inhibitory activity noted was directed against Gram-positive bacteria (*S. aureus* and *B. subtilis*). All of the actinomycetes displayed significant inhibition of *B. subtilis* and 12 inhibited the growth of *S. aureus*, whereas only one inhibited *P. aeruginosa*. This is in agreement with the results obtained in the present study because prominent inhibitory activity was also noted against Gram-positive bacteria (*S. aureus* and *B. subtilis*).

Kavithambigai (2006) reported that in secondary screening, the number of active strains against *P. aeruginosa* decreased (4.0%). The author also reported that 50% of active strains showed weak antibiosis (inhibition zone in between 8-10mm) against *P. aeruginosa*. This is in agreement with the result obtained in the present study. In this study, the antagonistic activity against *P. aeruginosa* decreased from 7 strains in primary screening to 4 strains in secondary screening. Present study also showed that active strains exhibited weak inhibition zone against *P. aeruginosa.*

Present study showed that majority of active strains exhibited weak inhibition against *B. subtilis* and *S. aureus*. This result, however, is not in agreement with the results obtained by Kavithambigai (2006) because the majority of active strains showed moderate antibiosis (inhibition zone in between 11-20mm) against *B. subtilis* (54.5%) and *S. aureus* (54.1%). The author also reported that 25% of active strains showed good antibiosis (inhibition zone more than 20mm) against *B. subtilis* and 16.2% against *S. aureus*. This might suggests that the marine actinomycetes might not have the same antibacterial activity with the actinomycetes isolated from root samples. During the screening of the novel secondary metabolite, actinomycetes isolates are often encountered which show antibiotic activity on agar but not in liquid culture. This might explain the higher number of antibacterial activity against *P. aeruginosa* in primary screening compared to secondary screening in this study.

According to Kokare *et al*. (2004) during the screening of the novel secondary metabolite, actinomycetes isolates are often encountered which show more active antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria. Pandey *et al*. (2006) reported that the reason for different sensitivity between Gram-positive 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. On the other hand, the Gram-positive bacteria only have an outer peptidoglycan layer. Brock *et al.* (1994) reported that porins structure that situated in outer membrane of Gram-negative bacteria serve as membrane channels for the entrance and exit of hydrophilic low-molecular weight substances. When the structure comes in contact with high molecular antibiotics, such porins can be closed to prevent antibiotic. This increased the resistance of Gram-negative bacteria to antibiotics. This explains the higher number of active strains against Gram-positive bacteria compared to Gram-negative bacteria.

Pisano *et al*. (1989) reported that 68 (46%) out of 147 strains of actinomycetes isolated from the sediments of the south shore of Brooklyn, the East River and New Jersey exhibited antimicrobial activity. Most of the inhibitory activity was directed against Gram-positive bacteria. *B. subtilis* was the most susceptible, followed closely by *S. aureus*. Activity against Gram-negative bacteria was minimal with only eight or 5% of the isolates proving effective. Only one strain (strain SG-944) inhibited the growth of *P. aeruginosa*. 41(28%) of the isolates was inhibitory to one or more of the fungal test species. Pisano *et al*. (1992) reported that *B. subtilis* was inhibited by 58% of the active marine actinomycetes strains, *S. aureus* by 50%, *P. aeruginosa* by 5% and *C. albicans* by 32%.

According to Kavithambigai (2006), the antibacterial activity might be stimulated by intracellular metabolites, which were bound inside membrane of actinomycetes and released during submerged cultivation. This explains the higher number of antibacterial and antifungal activity in secondary screening compared to primary screening.

4.1.2 Antifungal activity

Table 4.4 shows the antifungal activity of selected *Streptomyces* spp. against test fungi in primary and secondary screening. Total number of St*reptomyces* spp. tested for antagonistic activity against test fungi was eleven. Antifungal activity was only recorded in secondary screening. In primary screening, selected *Streptomyces* spp. did not inhibit the test fungi.

Table 4.4: Antifungal activity of selected *Streptomyces* spp. against test fungi in primary and secondary screening

Antagonistic activity / Test fungi	Primary screening[a]	Secondary screening[b]
	Number of active strains	Number of active strains
C. parapsilosis	-	7
C. albicans	-	4

[a] Primary screening: Cross streak method was applied on SA agar medium and the plates were incubated for 48 hours at 37±2°C; [b] Secondary screening: Paper disc method was applied on SDA agar medium. The discs were loaded with 15µl of crude extracts and the plates were incubated for 24 hours at 37±2°C.

In secondary screening, seven strains showed antagonistic activity against *C. parapsilosis*. Four strains showed antagonistic activity against *C. albicans*. Table 4.5 shows the antifungal activity of selected *Streptomyces* spp. against test fungi in secondary screening. All active strains exhibited weak inhibition against the test fungi.

The number of active strains that showed weak inhibition against *C. parapsilosis* was seven. Four strains showed weak inhibition against *C. albicans*. The most susceptible fungal was *C. parapsilosis*.

Table 4.5: Antifungal activity of selected *Streptomyces* spp. against test fungi in secondary screening on SDA for 24 hours at 37±2°C

Antagonistic activity / Test fungi	Total number of active strains	Weak (<10mm)	Moderate (10 – 15mm)	Good (>15mm)
		Number of active strains	Number of active strains	Number of active strains
C. parapsilosis	7	7	-	-
C. albicans	4	4	-	-

Present study also showed that active strains exhibited higher percentage of antifungal activity against at least one test fungi in secondary screening than in primary screening. This is however not in agreement with the study done by Kavithambigai (2006). The author reported that active strains exhibited higher antifungal activity in primary screening for both root samples from Kuantan (69.7%) and Port Dickson (59.7%). This might suggest that marine actinomycetes might not have the same antifungal substance with the actinomycetes isolated from root samples.

Kavithambigai (2006) reported that antifungal activity was prominent in secondary screening. The antifungal activity was more prominent on phytopathogenic fungi (*F. oxysporum cubense* race 1; 26.1%, *F. oxysporum cubense* race 2; 30.4%, *F. oxysporum cubense* race 4; 30.4%, *Colletotrichum* sp.; 21.7%, *G. boninense* ; 31.5%) compared to *C. albicans* (11.6%) and *C. parapsilosis* (9.8%). Most of the active strains showed moderate antibiosis (inhibition in between 11-20mm) against *C. albicans* (83.3%) and *C. parapsilosis* (58.8%). Atta and Ahmad (2009) reported that an actinomycete culture was isolated from a

soil sample collected from Alam Alroom districted, Marsa Matrouh governorate, Egypt. This isolate AZ-AR-262 was found to be active against unicellular and filamentous fungi.

Peela *et al*. (2005) reported that Streptomyces sp. strains BT606 and BT624 were active against *C. albicans*. Tan *et al*. (2004) reported that 15 (31%) of 49 crude extracts tested exhibited inhibition against the test fungi including *C. albicans* and *C. parapsilosis*. Six out of the fifteen strains exhibited inhibition against *C. parapsilosis* and all strains inhibited *C. albicans.* Zheng *et al*. (2000) reported that nine (52.9%) out of seventeen tested strains exhibited antifungal activity against fungi including *C. albicans.*

Vikineswary *et al*. (1997) reported that eleven (33%) out of 33 actinomycetes strains isolated from a tropical mangrove ecosystem showed activity against all the test fungi including *C. albicans.* Okazaki and Okami (1976) reported that only two strains out of 37 antagonistic actinomycetes exhibited antifungal properties. This is also in agreement with the present study because the percentage of antifungal activity was smaller. Shiomi *et al.* (2005) reported that the antimycins (produced by *Streptomyces* sp. K01-0031) have also other biological properties such as antifungal activity, inhibition of enzymatic activity as well as the ability to induce the death of cancer cells.

Vikineswary *et al*. (1997) reported that 11 strains (33%) out of 33 strains of actinomycetes isolated from marine environment showed antifungal activity by cross-plug method. The authors also reported that ten out of eleven actinomycetes strains exhibited antifungal activity against *C. albicans* by shake flasks studies. This is again in agreement with the results obtained in the present study because antifungal activity was only recorded against fungi including *C. albicans* only in secondary screening. The authors reported that only the culture supernatant was tested for bioactivity. The bioactive substance could be membrane-bound or intracellular. However, in the present study, all the culture mixtures were tested for bioactivity.

4.2 Antagonistic activity of different colour groups of selected *Streptomyces* spp. against test microorganisms

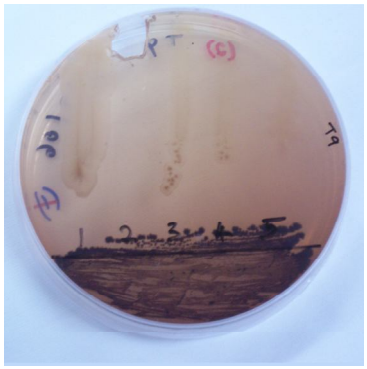
Table 4.6 shows the antagonistic activity of different colour groups of selected *Streptomyces* spp. against test microorganisms in primary and secondary screening. Figure 4.1 a – h shows the antagonistic activity of different colour groups of selected *Streptomyces* spp. against test microorganisms in primary screening.

Table 4.6: Antagonistic activity of different colour groups of selected *Streptomyces* spp. against test microorganisms in primary and secondary screening

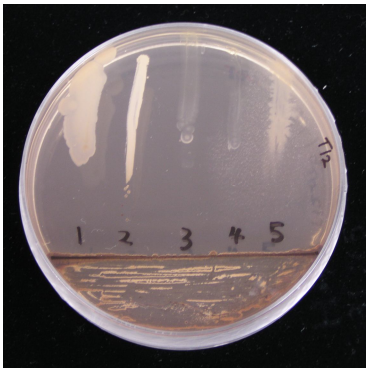
Colour groups	Strains	Test microorganisms[a]													
		Gram-positive bacteria						Gram-negative bacteria				Fungi			
		EF		SA		BS		PS		PA		CP		CA	
		1[ob]	2[oc]	1[o]	2[o]	1[o]	2[o]	1[o]	2[o]	1[o]	2[o]	1[o]	2[o]	1[o]	2[o]
Grey	T3	+	+	+	+	-	+	-	+	-	-	-	-	-	-
	T4	-	+	-	+	-	+	-	+	-	-	-	+	-	+
White/Red	T9	+	+	+	+	+	+	+	+	+	+	-	+	-	-
	T12	+	+	+	+	+	+	+	+	+	+	-	+	-	-
	T13	+	+	+	+	+	+	+	+	+	-	-	+	-	-
	T15	+	+	+	+	+	+	+	+	+	-	-	+	-	+
	T52	+	+	+	+	+	+	+	+	+	+	-	-	-	-
White/Brown	T6	+	+	-	+	-	+	-	+	+	-	-	-	-	-
	T16	-	+	-	+	-	+	-	+	-	-	-	+	-	+
	T20	-	+	-	+	-	+	-	+	-	-	-	-	-	-
	T53	+	+	-	+	+	+	+	+	+	+	-	+	-	+

[a] Test microorganisms: SA: *S.aureus*; BS: *B.subtilis*; EF: *E.faecalis*; PS: *P.shigelloides*; PA: *P.aeruginosa*; CP: *C.parapsilosis* and CA: *C.albicans;* [b] 1^0: Primary screening: Cross streak method was applied on ISP4 for T3 and T4 and SA for the remaining strains. The plates were incubated for 48 hours at 37±2°C; [c] 2^0: Secondary screening: Paper disc method was applied on NA for test bacteria and on SDA for test fungi. The discs were loaded with 15µl of crude extracts and the plates were incubated for 24 hours at 37±2°C; +: Inhibition**;** -: No inhibition

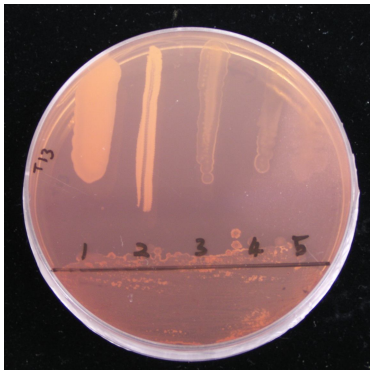
a) Strain T9



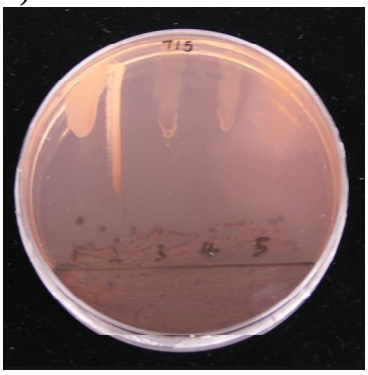
b) Strain T12



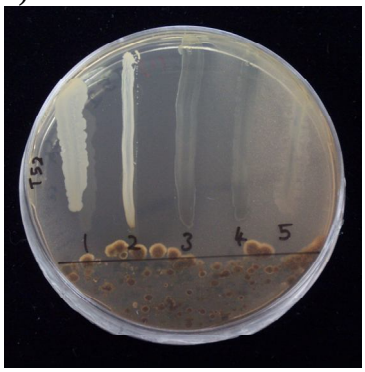
c) Strain T13



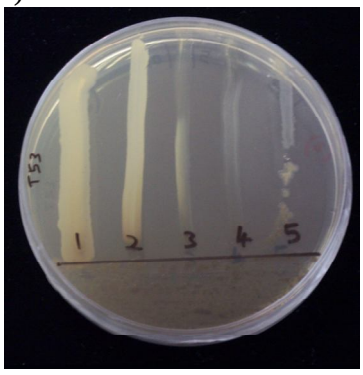
d) Strain T15



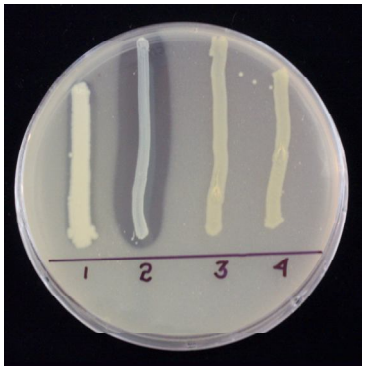
e) Strain T52



f) Strain T53



g) Control



h) Control

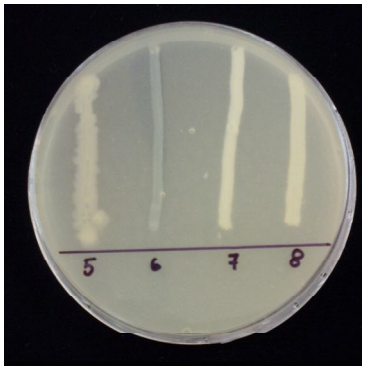


Figure 4.1: Antagonistic activity of different colour groups of selected *Streptomyces* sp strains in primary screening against; 1) *E. faecalis*; 2) *S. aureus*; 3) *P. shigelloides*; 4) *B. subtilis*; 5) *P. aeruginosa* on ISP4 for strains T3 and T4 and SA for the remaining strains after 48 hours incubation at 37±2°C

All strains of grey, white/red and white/brown colour groups inhibited the growth of all Gram-positive bacteria in secondary screening. Inhibition of the growth of *P. shigelloides* by all strains was also recorded in secondary screening. All strains of different colour groups did not inhibit the growth of test fungi in primary screening.

Table 4.7 shows the inhibition zones (mm) of different colour groups of selected *Streptomyces* spp. against test microorganisms in secondary screening. The degree of inhibition zones was grouped into weak (<10mm), moderate (10-15mm) and good (>15mm). Figure 4.2 a – l shows the antagonistic activity of different colour groups of selected *Streptomyces* spp. against test bacteria in secondary screening. The following analyses the anatagonistic activity of different colour groups of selected *Streptomyces* spp. against test microorganisms in primary and secondary screening.

Grey colour group

The two strains in this colour group, strains T3 and T4 exhibited poor antagonistic activity against test microorganisms in primary screening (Table 4.6). The antagonistic activity was only recorded against *E. faecalis* and *S. aureus*. The two test microorganisms were inhibited by strain T3.

In secondary screening, the antagonistic activity was more prominent against Gram-positive bacteria compared to Gram-negative bacteria. All the two strains inhibited the growth of all Gram-positive bacteria. Strains T3 and T4 inhibited the growth of only one test Gram-negative bacteria which was *P. shigelloides*. Antifungal activity was only recorded against *C. parapsilosis* and *C. albicans*. The test fungi were inhibited by strain T4. *E. faecalis, S. aureus, B. subtilis* and *P. shigelloides* were the most susceptible test microorganisms in this screening. The microorganisms were inhibited by all strains of grey colour group.

Table 4.7: Inhibition zones (mm) of selected *Streptomyces* spp. against test microorganisms in secondary screening on NA for test bacteria and on SDA for test fungi for 24 hours at 37±2°C

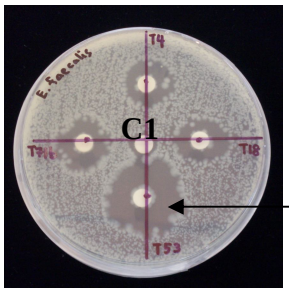
Colour groups	Strains	Gram-positive bacteria			Gram-negative bacteria		Fungi	
		Ef	*Sa*	*Bs*	*Ps*	*Pa*	*Cp*	*Ca*
Grey	T3	1.7±1.2 a	2.3±1.5 a	4.0±3.0 a	4.7±0.6 a	-	-	-
	T4	6.0±1.0 ab	5.3±2.3 ab	4.0±1.0 a	2.7±1.5 ab	-	6.3±2.5 b	5.3±2.9 ab
White/red	T9	4.7±3.2 ab	6.3±2.1 b	1.7±1.2 a	6.0±1.0 b	2.5±0.7 a	1.7±1.2 a	-
	T12	6.3±2.5 b	3.7±1.5 ab	5.0±1.7 ab	5.0±2.6 ab	5.0±4.2 ab	2.3±1.2 a	-
	T13	2.0±1.0 a	4.7±1.5 a	4.0±2.0 a	12.0±1.0 b	-	2.0±1.7 a	-
	T15	7.7±2.1 a	6.3±2.1 a	12.0±1.7 b	16.3±0.6 c	-	5.0±2.0 a	5.7±1.5 a
	T52	9.3±1.5 a	8.3±2.1 a	8.3±0.6 a	8.3±0.6 a	9.0±1.4 a	-	-
White/brown	T6	1.3±0.6 a	2.3±2.1 b	1.3±0.6 a	4.0±1.0 b	-	-	-
	T16	6.3±4.7 ab	5.0±2.0 ab	9.3±1.5 b	15.7±1.5 c	-	4.7±1.2 a	5.0±2.0 ab
	T20	8.3±1.5 b	8.0±2.6 b	8.0±1.0 b	1.3±0.6 a	-	-	-
	T53	16.0±2.0 c	14.0±0 bc	13.7±0.6 bc	12.0±1.7 bc	13.0±1.4 b	5.3±2.5 a	6.7±0.6 a

Table 4.7, Continuation

Streptomycin, 10µg (S10)	13	16	14	14	15	-	-
Novobiocin, 5µg (NB5)	15	13	15	18	16	-	-
Nystatin, 100µg (MY100)	-	-	-	-	-	28	27
Methanol	10.0±1.0	10.3± 1.5	11.3± 0.6	9.0± 1.0	12.5± 0.7	11.7± 0.6	13.3± 0.6

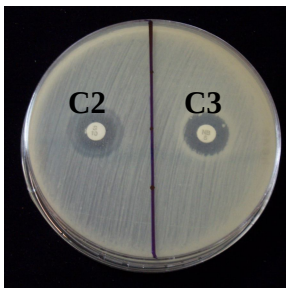
Values expressed are mean and standard deviation of triplicate measurements. Means with different letters in a same row are significantly different ($p<0.05$); *Ef:E faecalis*; *Sa:S. aureus*; *Bs:B. subtilis*; *Ps:P. shigelloides*; *Pa:P.aeruginosa*; *Cp:C. parapsilosis*; *Ca:C. albicans*

a)

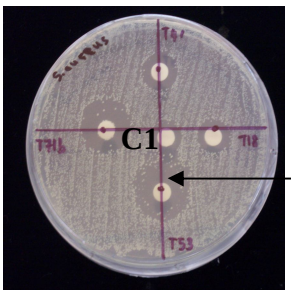


Inhibition zone produced by T53 against *E.faecalis*

b) Control plate for *E. faecalis*

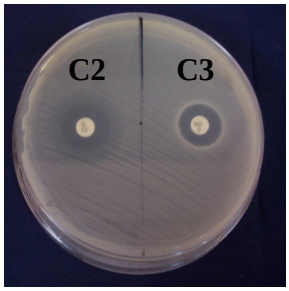


c)

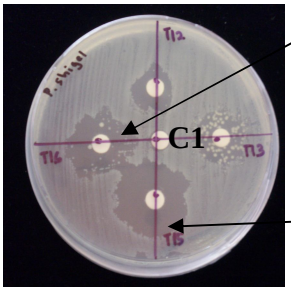


Inhibition zone produced by T53 against *S.aureus*

d) Control plate for *S. aureus*



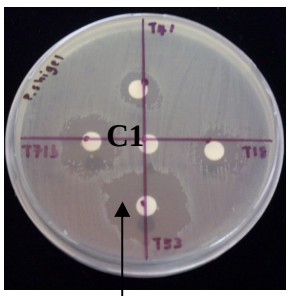
e)



Inhibition zone produced by T16 against *P.shigelloides*

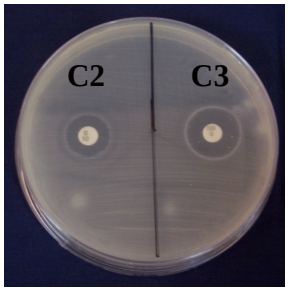
Inhibition zone produced by T15 against *P.shigelloides*

f)



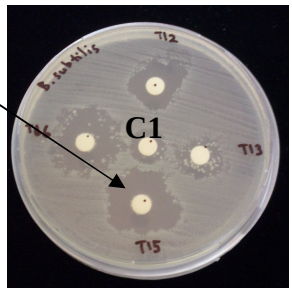
Inhibition zone produced by strain T53 against *P.shigelloides*

g) Control plate for *P.shigelloides*

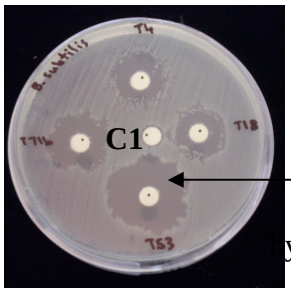


h)

Inhibition zone produced by strain T15 against *B.subtilis*

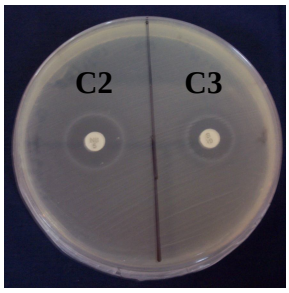


i)

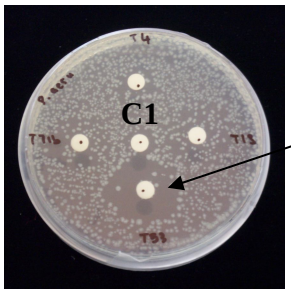


Inhibition zone produced by strain T53 against *B.subtilis*

j) Control plate for *B.subtilis*



k)



Inhibition zone produced by strain T53 against *P.aeruginosa*

l) Control plate for *P.aeruginosa*

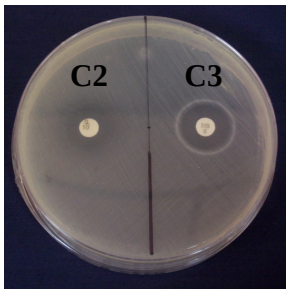


Figure 4.2: Antagonistic activity of selected *Streptomyces* sp strains in secondary screening against test bacteria after 24 hours incubation at 37±2°C.
Negative control: C1 (Methanol); Positive control: C2 (Streptomycin, 10µg) and C3 (Novobiocin, 5µg).

Strains T3 and T4 exhibited weak antagonistic activity (<10mm) against the test microorganisms (Table 4.7). The degree of inhibition zones of strain T3 against test microorganisms followed the order of *P. shigelloides* (4.7±0.6mm) > *B. subtilis* (4.0±3.0mm) > *S. aureus* (2.3±1.5mm) > *E. faecalis* (1.7±1.2mm) (Table 4.8). However,

there were no significant differences ($p>0.05$) (Appendix B, Table 1) of the inhibition zones between the four test microorganisms.

The inhibition zones of strain T4 against test microorganisms followed the order *E. faecalis* (6.0±1.0mm) > *C. parapsilosis* (6.3±2.5mm) > *S. aureus* (5.3±2.3mm) > *B. subtilis* (4.0±1.0mm) > *C. albicans* (5.3±2.9mm) > *P. shigelloides* (2.7± 1.5mm) (Table 4.8). However, there were no significant differences ($p>0.05$) (Appendix B, Table 2). Strain T4 was the most active strain. The strain exhibited antagonistic activity against four test bacteria and all test fungi.

White/Red colour group

Strains of white/red colour group were active in both primary and secondary screening (Table 4.6). All the five strains showed good antagonistic activity against test bacteria in primary screening. All strains inhibited the growth of all test bacteria. The antibacterial activity was more prominent than antifungal activity in primary screening. No antifungal activity was recorded in this screening. *E. faecalis, S. aureus, B. subtilis, P. shigelloides* and *P. aeruginosa* were the most susceptible test bacteria in this screening. The growth of the bacteria was inhibited by all strains of white/red colour group.

In secondary screening, all the five strains exhibited good antagonistic activity against all Gram-positive bacteria. All strains also exhibited antagonistic activity against *P. shigelloides*. However, only three strains inhibited the growth of *P. aeruginosa*. The strains were T9, T12 and T52. Four strains exhibited antagonistic activity against *C. parapsilosis*. The strains were T9, T12, T13 and T15. The growth of *C. albicans* was inhibited by only one strain which was T15. *E. faecalis, S. aureus, B. subtilis* and *P. shigelloides* were the most susceptible test bacteria in this screening. All strains exhibited antagonistic activity against the test bacteria.

Strain T15 showed good antagonistic activity (>15mm) against *P. shigelloides* (16.3±0.6mm) (Table 4.7). This strain also showed moderate antagonistic activity (10-15mm) against *B. subtilis* (12.0±1.7mm) and weak antagonistic activity (<10mm) against other test microorganisms. The inhibition zones of strain T15 against test microorganisms followed the order *P. shigelloides* (16.3±0.6mm) > *B. subtilis* (12.0±1.7mm) > *E. faecalis* (7.7±2.1mm) > *S. aureus* (6.3±2.1mm) > *C. albicans* (5.7±1.5mm) > *C. parapsilosis* (5.0± 2.0). The inhibition zones of strain T15 against *P. shigelloides* was significantly higher than *E. faecalis, S. aureus, B. subtilis, C. parapsilosis* and *C. albicans*, as well as the inhibition zones of strain T15 against *B. subtilis* which was significantly higher than *E. faecalis, S. aureus, C. parapsilosis* and *C. albicans* ($p=0.05$) (Appendix B, Table 10 and 11).

Strain T13 showed moderate antagonistic activity (10-15mm) against *P. shigelloides* (12.0±1.0mm) and weak antagonistic activity (<10mm) against other test microorganisms. The inhibition zones of strain T13 against test microorganisms followed the order *P. shigelloides* (12.0±1.0mm) > *S. aureus* (4.7±1.5mm) > *B. subtilis* (4.0±2.0mm) > *E. faecalis* (2.0±1.0mm) > *C. parapsilosis* (2.0± 1.7) (Table 4.8). The inhibition zones of strain T13 against *P. shigelloides* was significantly higher than *E. faecalis, S. aureus, B. subtilis* and *C. parapsilosis* ($p<0.05$) (Appendix B, Table 8 and 9).

Strain T9 showed weak antagonistic activity (<10mm) against the test microorganisms. The inhibition zones of strain T9 against test microorganisms followed the order *P. shigelloides* (6.0±1.0mm) > *S. aureus* (6.3±2.1mm) > *P. aeruginosa* (2.5±0.7mm) > *E. faecalis* (4.7±3.2mm) > *B. subtilis* (1.7±1.2mm) > *C. parapsilosis* (1.7±1.2mm). The inhibition zones of strain T9 against *S. aureus* and *P. shigelloides* were significantly higher than *B. subtilis, P. aeruginosa* and *C. parapsilosis* ($p<0.05$) (Appendix B, Table 5 and 6).

Strain T12 also showed weak antagonistic activity (<10mm) against the test microorganisms. The inhibition zones of strain T12 against test microorganisms followed

the order *E. faecalis* (6.3±2.5mm) > *B. subtilis* (5.0±1.7mm) > *P. shigelloides* (5.0±2.6mm) > *S. aureus* (3.7±1.5mm) > *C. parapsilosis* (2.3± 1.2mm) > *P. aeruginosa* (5.0±4.2mm). However, there were no significant differences ($p>0.05$) (Appendix B, Table 7).

Strain T52 showed weak antagonistic activity (<10mm) against the test microorganisms. The inhibition zones of strain T52 against test microorganisms followed the order *E. faecalis* (9.3±1.5mm) > *B. subtilis* (8.3±0.6mm) > *P. shigelloides* (8.3±0.6mm) > *P. aeruginosa* (9.0±1.4mm) > *S. aureus* (8.3±2.1mm). There were no significant differences between the inhibition zones of strain T52 against all test microorganisms ($p>0.05$) (Appendix B, Table 16).

White/Brown colour group

Strains of white/brown colour group exhibited poor antagonistic activity in primary screening (Table 4.6). Strain T6 only showed antagonistic activity against *E. faecalis* and *P. aeruginosa* in primary screening. Strain T53 inhibited the growth of all test bacteria except *S. aureus*. No antifungal activity was recorded in this screening.

In secondary screening, antagonistic activity against Gram-positive bacteria was more prominent compared to Gram-negative bacteria. All the four strains of white/brown colour group exhibited antagonistic activity against all Gram-positive bacteria. All strains also showed antagonistic activity against *P. shigelloides*. The growth of *P. aeruginosa* was only inhibited by strain T53. Only two strains exhibited antagonistic activity against *C. parapsilosis* and *C. albicans*. They were strains T16 and T53. *E. faecalis, S. aureus, B. subtilis* and *P. shigelloides* were the most susceptible test microorganisms in this screening. All strains of white/brown colour group exhibited antagonistic activity against the test

bacteria. Strain T53 was the most active strain. The strain showed antagonistic activity against all test microorganisms in secondary screening.

Strain T53 exhibited good antagonistic activity (>15mm) against *E. faecalis* (16.±2.0mm). The strain also showed moderate antagonistic activity (10-15mm) against *S. aureus* (14.0±0.0mm), *B. subtilis* (13.7±0.6mm), *P. shigelloides* (12.0±1.7mm) and *P. aeruginosa* (13.0±1.4mm) and weak antagonistic activity (<10mm) against the remaining strains (Table 4.7). The inhibition zones of strain T53 against test microorganisms followed the order *E. faecalis* (16.0±2.0mm) > *S. aureus* (14.0mm)> *B. subtilis* (13.7±0.6mm) > *P. aeruginosa* (13.0±1.4mm) > *P. shigelloides* (12.0±1.7mm) > *C. albicans* (6.7±0.6mm) > *C. parapsilosis* (5.3±2.5mm). There were significant differences between the inhibition zones of strain T53 against *E. faecalis* with *P. shigelloides*, *P. aeruginosa*, *C. parapsilosis* and *C. albicans*, as well as the inhibition zones between *S. aureus, P. shigelloides, B. subtilis* and *P. aeruginosa* which were significantly higher than *C. parapsilosis* and *C. albicans* (p=0.05) (Appendix B, Table 17 and 18).

Strain T6 exhibited weak antagonistic (<10mm) against the test microorganisms. The inhibition zones of strain T6 against test microorganisms followed the order *P. shigelloides* (4.0±1.0mm) > *E. faecalis* (1.3±0.6mm) > *B. subtilis* (1.3±0.6mm) > *S. aureus* (2.3±2.1mm). The inhibition zones of strain T6 against *P. shigelloides* was significantly higher than *E. faecalis* and against *B. subtilis* as well as the inhibition zone o f *B. subtilis* which was significantly higher than *S. aureus* (p<0.05) (Appendix B, Table 3 and 4).

Strain T16 showed good antagonistic activity (>15mm) against *P. shigelloides* (15.7±1.5mm) and weak antagonistic activity (<10mm) against the remaining test microorganisms. The inhibition zones of strain T16 against test microorganisms followed the order *P. shigelloides* (15.7±1.5mm) > *B. subtilis* (9.3±1.5mm) > *C. parapsilosis* (4.7± 1.2mm) > *S. aureus* (5.0±2.0mm) > *C. albicans* (5.0±2.0mm) > *E. faecalis* (6.3±4.7mm).

The inhibition zones of strain T16 against *P. shigelloides* was significantly higher than *E. faecalis, S. aureus, B. subtilis, C. parapsilosis* and *C. albicans*, as well as the inhibition zones of strain T16 against *B. subtilis* which was significantly higher than *C. parapsilosis* ($p<0.05$) (Appendix B, Table 12 and 13).

Strain T20 exhibited weak antagonistic activity (<10mm) against the test microorganisms. The inhibition zones of strain T20 against test microorganisms followed the order *B. subtilis* (8.0±1.0mm) > *E. faecalis* (8.3±1.5mm) > *S. aurues* (8.0±2.6mm) > *P. shigelloides* (1.3±0.6mm). The inhibition zones of strain T20 against *E. faecalis, S. aureus* and *B. subtilis* was significantly higher than *P. shigelloides* ($p<0.05$) (Appendix B, Table 14 and 15).

The results obtained in present study showed that majority of active strains exhibited weak antagonistic activity against test microorganisms. Shomura *et al.* (1979) reported that the failure of antibiotic productivity during *Streptomyces* spp. growth in submerged culture correlated with their mycelial morphology. In agar cultures, the mycelium of *Streptomyces* spp. was in filamentous form, but once cultivated in submerged culture they formed fragments. This resulted in reduced potential of antibiotics production by *Streptomyces* spp.

In the present study, majority of strains of grey, white/red and white/brown colour groups exhibited prominent antagonistic activity against Gram-positive bacteria. Majority of strains of white/red colour group, strain T3 of grey and two strains of white/brown colour groups were active against both bacteria and fungi. This might be due to the action of more than one antibiotic with different targets (Gonzales *et al.,* 1999). The broad spectrum of the strains might also be due to possible presence of a new antimicrobial substance which was able to cross both bacterial and fungal cell walls (Tsvetanova and Price, 2001)

Kavithambigai (2006) reported that antimicrobial activity of active strains in grey colour group was prominent in primary screening. Active strains inhibited the growth of *B. subtilis* and *S. aureus* in both primary and secondary screening. Majority of active strains showed moderate antibiosis (inhibition between 11-20mm) against *B. subtilis* and *S. aureus* and one strain showed good antibiosis (inhibition more than 20mm) against *B. subtilis*. This is however not in agreement with the results obtained in the present study because strains in grey colour group were more prominent in secondary screening than in primary screening. The strains also showed weak inhibition zone against the test bacteria. This might explain the difference in bioactive substances in actinomycetes from marine ecosystem with root samples.

In this study, strain T53 of white/brown colour group was the most active strain. The strain inhibited the growth of all test microorganisms. Strain T53 produced inhibition zone of 13.0±1.4mm against *P.* aeruginosa, 14.0±0.0mm against *S. aureus* and 13.7±0.6mm against *B. subtilis*. Selvin *et al.* (2004) reported that the extracellular proteins of *Streptomyces* strain BTL7 isolated from marine sponge *Dendrilla nigra* produced inhibition zone of 21mm against *P. aeruginosa*, 16mm against *S. aureus* and 15mm for *B. subtilis*. This is quite similar with the result obtained in the present study. Tan *et al.* (2004) reported that comparable inhibition relative to 10µg streptomycin standard was shown by majority of the strains against *S. aureus*, *B subtilis* and *P. aeruginosa*. Bioactivity of all extracts tested against *P. aeruginosa* was the weakest, followed by *B. subtilis* and strongest against *S. aureus*. This is however not comparable with the present study because active strains produced different degree of inhibition zone against the test bacteria.

Tan *et al.* (2004) reported that comparable or better inhibition compared to nystatin standard (15mm) was observed against *C. albicans*. Strains X13, X46 and X60 produced inhibition zone bigger by 18% each to nystatin standard. Strain Z250 produced inhibition

zone bigger by 29% to nystatin standard. Sacramento *et al.* (2004) reported that the crude supernatants of *Streptomyces* sp. strain 606 showed zones of wide inhibition against yeast (*C. albicans*, 23mm). This is however not in agreement with the result obtained in present study. The test marine actinomycetes produced weak inhibition zone against the test fungi. This study showed that the inhibiton produced by active strains against the test fungi was not comparable to nystatin standard. This might suggest that the actinomycetes strains in this study did not have antifungal substance to inhibit the growth of the test fungi.

Devi *et al*. (2006) reported that out of three selected and identified actinomycetes, *Streptomyces* sp. showed significant antimicrobial activity against human pathogens. The highest zone of inhibition of 57 mm antimicrobial activity was showed by *Streptomyces* sp. against *P. aeruginosa*. Marine *Streptomyces* sp. exhibited the highest antibacterial activity against *P. aeruginosa* followed by *S. aurues*. This is not in agreement with the present result obtained in the present study. The highest inhibition zone was showed against *P. shigelloides,* followed by *E. faecalis.* This might suggest that marine actinomycetes have different antibacterial substances.

Sacramento *et al*. (2004) reported that *P. aeruginosa* was resistant to crude supernatant, extracts and fractions of *Streptomyces* sp. strain 606 isolated from a Brazilian tropical forest soil. This is quite comparable to the result obtained in present study because only a few strains inhibited the growth the test bacteria.

The search for novel metabolites especially from actinomycetes requires a large number ofisolates (over thousands) in order to discover a novel compound of pharmaceutical interest. The search will be more promising if diverse actinomycetes are sampled and screened (Lo *et al*., 2002).

4.3 Media optimization

Figure 4.3 a – c shows the antagonistic activity of crude extracts of strain T53 against *E. faecalis* in ISP2 medium. Table 4.8 shows the inhibition zones of crude extracts of strain T53 against *E. faecalis* in agitation and static fermentation conditions using three different media. Inhibition zones of crude extracts of strain T53 against *E. faecalis* was only observed in SA and ISP2 media in agitation and static fermentation condition. In SA medium, in agitation fermentation condition, the inhibition zone was recorded in day 9 and day 12. In static fermentation condition, the inhibition zone was only observed in day 9. In ISP2 medium, the inhibition zone was only recorded in day 3, day 6, day 9 and day 12 in agitation fermentation condition. Plate 4.3 a – c shows the antagonistic activity of crude extracts of strain T53 against *E. faecalis* in ISP2 medium. Table 4.9 shows the pH value of crude extracts of submerged cultures fermentation of T53 in agitation and static fermentation conditions.

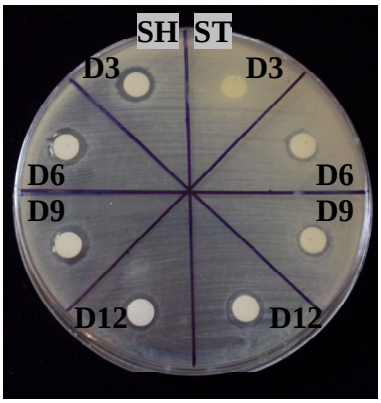
SA medium

In SA medium, antibacterial activity could be observed in both agitation and static fermentation conditions (Table 4.8). In agitation fermentation, the antibacterial activity was recorded in day 9 and day 12. Antibacterial activity in static fermentation condition was only observed in day 9.

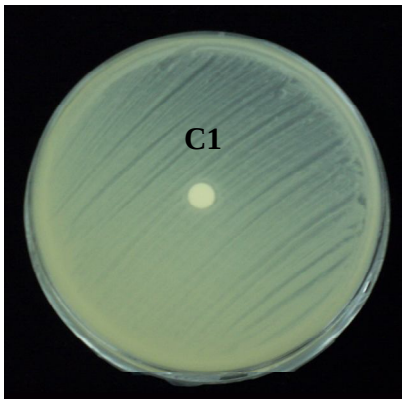
The inhibition zones of strain T53 against *E. faecalis* in SA medium followed the order of day 12 (Agitation) (10.3±1.5mm) > day 9 (Agitation) (6.0±5.2mm) > day 9 (Static) (8.7±0.6mm) (Table 4.8). However, there were no significant differences ($p>0.05$) (Appendix B, Table 19) of the inhibition zones between the three different days.

There were also no significant differences in the pH value pH value of crude extracts of submerged cultures fermentation of T53 in agitation and static fermentation conditions ($p>0.05$) (Appendix B, Table 21).

a)



b)



c)

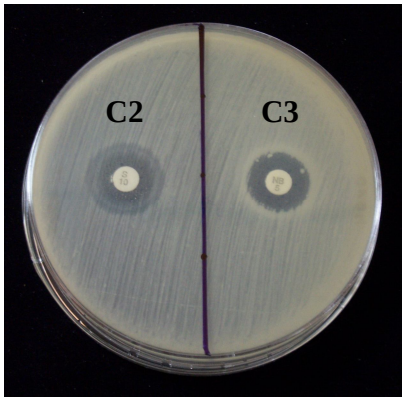


Figure 4.3: Antagonistic activity of crude extracts of T53 against *E. faecalis* in ISP2 medium after 24 hours incubation at 37±2°C. Negative control: C1 (Methanol); Positive control: C2 (Streptomycin, 10µg) and C3 (Novobiocin, 5µg); SH: Shaking fermentation condition; ST: Static fermentation condition

Table 4.8: Inhibition zones (mm) of crude extracts of submerged cultures fermentation of strain T53 against *E. faecalis* in agitation and static fermentation conditions on SA, ISP2 and ISP4 for 24 hours 37±2°C

	Submerged cultures fermentation conditions							
	Agitation				Static			
Days / Media	3	6	9	12	3	6	9	12
SA	-	-	9.3±0.6[a]	10.3±1.5[a]	-	-	8.7±0.6[a]	-
ISP2	8.7±1.2[a]	8.3±0.6[a]	10.7±1.2[b]	8.7±1.2[a]	-	-	-	-
ISP4	-	-	-	-	-	-	-	-

Values expressed are mean and standard deviation of triplicate measurements. Means with different letters in a same row are significantly different ($p<0.05$).

ISP2 medium

In ISP2 medium, the antibacterial activity was only observed in agitation fermentation condition (Table 4.8). In agitation fermentation condition, the antibacterial activity was recorded in days 3, 6, 9 and 12. No antibacterial activity was recorded in static fermentation condition.

The inhibition zones of crude extracts of strain T53 against *E. faecalis* in agitation fermentation condition followed the order of Day 9 (10.7±1.2) > Day 6 (8.3±0.6) > Day 3 (8.7±1.2) > Day 12 (8.7±1.2). However, there were no significant differences ($p>0.05$) (Appendix B, Table 20).

Table 4.9: pH value of crude extracts of submerged cultures fermentation of strain T53 in agitation and static fermentation conditions on SA, I2 and I4 for 24 hours 37±2°C

	Submerged cultures fermentation conditions							
	Agitation				Static			
Days / Media	3	6	9	12	3	6	9	12
SA	7.10±0.51^{ab}	7.86±0.18^{c}	7.12±0.26^{abc}	7.18±0.05^{abc}	6.67±0.38^{a}	6.99±0.39^{ab}	7.18±0.89^{abc}	7.55±0.24^{bc}
ISP2	6.80±0.20^{abc}	6.28±0.25^{a}	6.43±0.20^{a}	6.57±0.03^{ab}	6.78±0.06^{ab}	6.82±0.53^{abc}	7.06±0.27^{bc}	7.39±0.69^{c}
ISP4	7.35±0.24^{c}	6.14±0.03^{ab}	6.08±0.40^{ab}	6.87±0.56^{bc}	6.64±0.66^{abc}	6.69±0.62^{abc}	5.98±0.19^{a}	6.50±0.69^{ab}

Values expressed are mean and standard deviation of triplicate measurements. Means with different letters in a same row are significantly different ($p<0.05$).

The pH value (Table 4.9) shows that there were significant differences between the pH value of submerged cultures of strain T53 in day 6 (Agitation) (6.28±0.25) with day 9 (Static) (7.06±0.27) and day 12 (Static) (7.39±0.69), day 9 (Agitation) (6.43±0.20) with day 9 (Static) (7.06±0.27) and day 12 (Static) (7.39±0.69) as well as day 12 (Static) (6.57±0.03) with day 12 (Agitation) (7.39±0.69) and day 3 (Static) (6.78±0.06) ($p<0.05$) (Appendix B, Table 22 and 23).

ISP4 medium

In ISP4 medium, no antibacterial activity was recorded in both shaking and static fermentation condition (Table 4.8). The pH value (Table 4.9) shows that there were significant differences between day 3 (7.35±0.24) in shaking fermentation condition with day 6 (6.14±0.03) and day 9 (6.08±0.40) in shaking fermentation condition and day 9 (5.98±0.19) and day 12 (6.50±0.69) in static fermentation condition as well as day 12 (6.87±0.56) in shaking fermentation condition with day 9 (5.98±0.19) in static fermentation condition ($p<0.05$) (Appendix B, Table 24 and 25).

From this study, ISP2 was the best medium for bioactive compound production compared to SA and ISP4 media. Shake flask fermentation was the best fermentation system compared to static fermentation.

In media optimization, dimethyl sulfoxide (DMSO) was used as a solvent to dissolve the crude extracts. The usage of methanol in secondary screening (Chapter 3, Section 3.4, page 24 – 26) was replaced with DMSO because of its low toxicity. Vignes (2000) reported that DMSO has very low toxicity to humans and the environment, recyclable after most uses, presents in many foods and plays a significant role in nature's Global Sulfur Cycle, highly polar and aprotic solvent. The low toxicity of the solvent ensured that the inhibition zone of the test pathogens by the actinomycetes strains was not affected.

The production of bioactive secondary metabolites by actinomycetes is influenced by a few factors. Srinivasan *et* al. (1991) reported that antibiotics productivity of strains is profoundly influenced by factors such as the composition of the nutrient medium, temperature and duration of incubation. Other than that, the production is also influenced by fermentation conditions.

Antibiotic production generally takes place after the growth reaches the stationary phase or has passed an optimum level. Bu'lock (1961) reported that the production of secondary metabolites classically exhibits a two-stage process, with a trophophase (or growth phase) and an idiophase (or production phase), usually when growth has slowed or stopped. In the idiophase, productivity depends on maintenance of sub-optimal (cryptic) growth through control of the concentrations of inorganic phosphate, NH_4^+, metal ions, nitrogen or oxygen (Srinivasan *et al.*, 1991).

The nutritional factors strongly influence the production of bioactive secondary metabolites. The factors include the levels of inorganic phosphorus, metal ions, organic nitrogen sources and metabolic carbon, and in several cases substrate precursors are also important for high titres of the desired antibiotics (Higashide, 1984). Pisano *et al*. (1992) reported that the nature of the fermentation medium directly influenced the elaboration of antimicrobial substances.

Carbon sources are one of the most important factors that influence the production of bioactive secondary metabolites. Glucose is generally used in the fermentation as a preferred carbon source but catabolite repression is avoided by feeding glucose during the fermentation. In some cases, carbohydrates, such as lactose, or polysaccharides, such as starch, which are used more slowly, have been found to be better carbon sources for antibiotic production (Srinivasan *et al*., 1991). Tiwaril *et al.* (2008) reported that actinomycetes can use peptone as carbon source too. Addition of glucose as carbon source allows better growth, and up to 0.8% of peptone can be used along with glucose for growth assessment of actinomcyetes. Farid *et al*. (2000) reported that natamycin production by *Streptomyces natalensis* required glucose in the submerged culture medium with a concentration of 20g/l. Sujatha *et al*. (2005) reported that *Streptomyces psammmoticus* strain BT-408 isolated from the sediments of Bay of Bengal Ocean produced maximum

antibiotic in medium supplemented with glucose as a sole carbon source followed by fructose and glycerol. The increased in glucose level in the culture from 10 to 12.5 g/l led to 1.2 fold increase in antibiotic production. Mellouli *et al*. (2003) reported that the highest antibacterial activities were obtained when starch at 1% (w/v) was used as sole carbon source in the presence of traces of mineral oligoelements. In another study reported by Fguira *et al*. (2005) highest antimicrobial activities were obtained in *Streptomyces* spp. when glucose at 1% (w/v) was used as sole carbon source in the presence of magnesium.

Other than carbon sources, nitrogen sources are also important in the production of bioactive secondary metabolites. Iwai and Omura (1982) reported that the use of slowly-consumed antifungal medium carbon sources such as soluble starch and nitrogen sources such as peptone and yeast-extract have been reported to be effective in the production of various types of antibiotics by actinomycetes especially the streptomycetes. Marwick *et al*. (1999) reported that the nitrogen source is understood to regulate secondary metabolism. High nitrogen levels have been noted to repress idiophase production of antibiotics (Marwick *et al*., 1999). Tiwaril *et al*. (2008) reported that actinomycetes can use inorganic nitrogen (KNO_3), however organic nitrogen enhanced growth. Farid *et al*. (2000) reported that among different nitrogen sources tested, only ammonium sulphate, sodium nitrate and beef extract were the suitable nitrogen sources in supporting the antibiotic production. Furthermore, the mixture of beef extract and yeast extract (8 g/l and 2 g/l, respectively) exhibited a synergistic effect in enhancing the natamycin production reaching about 1.5 g/l. Sujatha *et al*. (2005) the highest antibiotic production was obtained in culture of streptomycete strain BT-408 containing ammonium nitrate as nitrogen source, followed by cultures containing sodium nitrate, potassium nitrate and alanine.

More than that, ammonium is also an important factor that influences the production of bioactive secondary metabolites. Zhang *et al*. (1996) found ammonium to stimulate an antibiotic produced by *Streptomyces grisiofuscus*. Srinivasan *et al*. (1991) reported that ammonium ion often exert a negative effect on production of several antibiotics (cephamycin, clavulanic acid and streptomycin) and in commercial fermentations use of a slowly metabolized nitrogen source (releasing ammonia slowly), such as soymeal, partially overcomes this problem.

Phosphate is also important for the production of bioactive secondary metabolites. However, Spizek and Tichy (1995) reported that phosphate, although essential for growth, can at certain concentrations suppress secondary metabolism, inhibiting, for example, phosphatases and oxygenases. Srinivasan *et al*. (1991) also reported that fermentative production of aminoglycoside antibiotics, tetracyclines, polyenes and cephalosporins are sensitive to the phosphate concentration in the fermentation medium. Farid *et al*. (2000) reported that the highest antibiotic production was obtained in a cultivation medium containing 0.05 g/l of potassium dihydrogen phosphate. Further increase in phosphate concentration resulted in a significant increase in biomass concomitant with lower antibiotic production. Sujatha *et al*. (2005) reported that K_2HPO_4 at a concentration of 1.2 g/l gave maximum yield of antibiotic. The addition of 0.5 g/l of magnesium sulfate to the culture medium was optimal for antibiotic production.

Ions might also be important to improve the production of bioactive secondary metabolites. VanPee (1996) reported that in terms of marine bacteria, the concentration of bromide ions would seem to be significant in some cases, probably because of the prevalence of bromide in halogenated marine antibiotics. However, the macrolide antibiotic productivity tended to decrease when metal ion deficient media were used and also when the inocula were incubated for long periods and at high temperatures (Higashide, 1984).

These might be the reasons that are in agreement with the results obtained in the present study where ISP2 were the best medium for the production of bioactive secondary metabolites. Further research should be done to study the compositions of the media.

However, other than nutritional factors, the production of bioactive compounds is also influenced by fermentation conditions. Submerged fermentation is the most common submerged culture system that was used to produce bioactive secondary metabolites. Pelczar and Reid (1972) reported that submerged cultures are widely used to produce many secondary metabolites because they allow filamentous fungi and bacteria to produce freely suspended mycelia and pellets, essential for secondary metabolite production.

Agitation fermentation condition is also the most common submerged culture system that was used to produce bioactive secondary metabolites. Submerged culture fermentations require a stirred nutrient medium, and in the case aerobic microorganisms, a supply of oxygen (Duetz and Witholt, 2004). In small-scale fermentation, shake flasks are usually used. Standard Erlenmeyer flasks is one of the most common types used for small scale microbial fermentations because they are readily available, their conical shape allows vigorous shaking without spillages, the small opening limits evaporation so slow-growing strains can be cultivated without excess moisture loss and the relatively large surface at the bottom allows a high surface-to-volume ratio (Duetz and Witholt, 2004). This is in agreement with the results obtained in the present study where antibacterial activity of selected actinomycetes against test bacteria were recorded in shaking fermentation conditions. Sacramento *et al.* (2004) reported that after two weeks incubation under shaking (agitation) conditions, the supernatants of *Streptomyces* sp. strain 606 showed the largest inhibition zones against pathogenic microorganisms.

Other than that, incubation temperature is also an important factor that influences the production of bioactive secondary metabolites. Sujatha *et al.* (2005) reported that the

increase of the incubation temperature from 20 to 30^{o}C increased the growth of the cells of streptomycete strain BT-408 by 3-fold. The production of the antibiotic had also increased by 4.7-fold. Ouhdouch *et al.* (2001) reported that *Streptomyces* spp. usually produced antibiotics at temperatures near 27^{o}C. Iwai and Omura (1992) reported that it would be interesting to use temperature shifts in antibiotic screening because the cultivation for antibiotic production was usually performed under constant temperature from the beginning until the end, but the adequate temperature for growth was not always the same as that for production.

pH of the submerged cultures was also an important factor that influence the production of bioactive secondary metabolites. Sujatha *et* al. (2005) reported that the maximum antibiotic activity of streptomycete strain BT-408 was obtained at a pH of 7.2 the results also showed that an incubation time of 96 hours as optimal. Optimization of medium components and physical parameters (pH, temperature, time) allowed an improvement over 182% in the concentration of antibiotic. Higashide (1984) reported that attainment of high antibiotic production required optimal concentration of the components in the fermentation medium, optimum pH and viscosity. Antibiotic production was optimum at 3% NaCl with slight decrease at 5%. Antibiotic production was comparable in the pH range of 7-9, whereas at pH10, there was no antibiotic production (Vasavada *et al.*, 2006). Marwick *et al*. (1999) reported that the pH level of the growth medium has a marked effect on secondary metabolism production, with synthesis falling rapidly either side of an optimal level.

Tan *et al.* (2004) reported that the sensitivity of the antibacterial substances *ex-situ* towards light and temperature might influenced the antibacterial activity using crude extracts. The lower antibacterial activity might also due to naturally unstable antibacterial substances after prolonged storage or low amount of bioactive compound present in the crude extracts.

4.4 Conclusion

- The antibacterial activity was higher in secondary screening compared to primary screening. The antifungal activity was only recorded in secondary screening with significantly higher percentage.

- Gram-positive bacteria were more susceptible than Gram-negative bacteria. *E. faecalis* was the most susceptible Gram-positive bacteria. On the other hand, *P. shigelloides* was the most susceptible Gram-negative bacteria. The most susceptible fungi was *C. parapsilosis.*

- Many of the selected *Streptomyces* spp. exhibited weak antagonistic activity against test pathogens. Strains T9, T12, T52 and T53 exhibited broad spectrum activity where they exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. Strains T4, T15, T16 and T53 exhibited antifungal activity against all test fungi.

- Strains of grey, white/red and white brown colour groups exhibited antagonistic activity against all Gram-positive bacteria and against one Gram-negative test bacteria which was *P. shigelloides.*

- Strain T53 of white/brown colour group exhibited antagonistic activity against all test bacteria and test fungi. This strain showed good inhibition against *E. faecalis*. Hence, it was concluded that strain T53 was the most active strain among the test microorganisms and *E. faecalis* was the most susceptible test bacteria.

- In media optimization, ISP2 was identified as the best medium for the production of bioactive secondary metabolites. Agitation fermentation condition was the best submerged culture fermentation condition for the production of bioactive secondary metabolites.

- Further investigation should be done to study the composition as well as to identify the bioactive compounds produced by the selected marine actinomycetes. The composition of optimized media for the production of bioactive secondary metabolites should also be studied in further details.

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APPENDIX A: MEDIA

1 30% (v/v) GLYCEROL

Glycerol	360ml
Yeast extracts	1.2g
Glucose	4.5g
Casein	1.5g
Distilled water	840ml

2 INORGANIC SALTS-STARCH AGAR (ISP4)

ISP_4	18.5g
Sodium chloride (NaCl)	3g
Bacteriological agar	5g
Distilled water	1L
pH 7.2	

3 SPORULATION AGAR (SA)

Yeast extract	4g (gly)
$CaCO_3$	1g
Soluble starch	20g
Glucose	15g (gly)
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.5g
Casein	5g (gly)
Instant ocean 50%	17g

Agar	17g
Distilled water	1L
pH 7.0	

4 NUTRIENT AGAR (NA)

Nutrient agar powder	23g
Distilled water	1L

5 SABAROUD DEXTROSE AGAR (SDA)

Sabaroud liquid broth powder	30g
Agar	20g
Distilled water	1L

6 YEAST EXTRACT-MALT EXTRACT AGAR (ISP2)

Yeast extracts	2g
Malt extracts	5g
Glucose	2g
Sodium chloride (NaCl)	3g
Agar	20g
Distilled water	1L
pH	7.3

APPENDIX B: STATISTICAL TABLES

Table 1: ANOVA: Inhibition zones of strain T3 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	17.6667	3	5.88889	1.81	0.2229*
Within groups	26.0	8	3.25		
Total	43.6667	11			

*p > 0.05

Table 2: ANOVA: Inhibition zones of strain T4 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	28.2778	5	5.65556	1.39	0.2939*
Within groups	48.6667	12	4.05556		
Total	76.9444	17			

*p > 0.05

Table 3: ANOVA: Inhibition zones of strain T6 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	15.5833	3	5.19444	7.79	0.0093*
Within groups	5.33333	8	0.666667		
Total	20.9167	11			

*p < 0.05

Table 4: Multiple range tests: Inhibition zones of strain T6 against test microorganisms

Contrast	Difference	+/- Limits
E. faecalis – S. aureus	*-1.66667	1.53734
E. faecalis – P. shigelloides	*-2.66667	1.53734
E. faecalis – B. subtilis	0.0	1.53734

Table 4, Continuation

Contrast	Difference	+/- Limits
S. aureus – P. shigelloides	-1.0	1.53734
S. aureus – B. subtilis	*1.66667	1.53734
P. shigelloides – B. subtilis	*2.66667	1.53734

* denotes a statistically significant difference.

Table 5: ANOVA: Inhibition zones of strain T9 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	67.1667	5	13.4333	4.32	0.0176*
Within groups	37.3333	12	3.11111		
Total	104.5	17			

*$p < 0.05$

Table 6: Multiple range tests: Inhibition zones of strain T9 against test microorganisms

Contrast	Difference	+/- Limits
E. faecalis – S. aureus	1.66667	3.13786
E. faecalis – P. shigelloides	4.33333	3.13786
E. faecalis – B. subtilis	3.0	3.13786
E. faecalis – P. aeruginosa	2.0	3.13786
E. faecalis – C. parapsilosis	3.0	3.13786
S. aureus – P. shigelloides	0.333333	3.13786
S. aureus – B. subtilis	*4.66667	3.13786
S. aureus – P. aeruginosa	*3.66667	3.13786
S. aureus – C.parapsilosis	*4.66667	3.13786
P. shigelloides – B. subtilis	*4.33333	3.13786
P. shigelloides – P. aeruginosa	*3.33333	3.13786
P. shigelloides – C. parapsilosis	*4.33333	3.13786

Table 6, Continuation

Contrast	Difference	+/- Limits
B. subtilis – P. aeruginosa	-1.0	3.13786
B. subtilis – C. parapsilosis	0.0	3.13786
P. aeruginosa – C. parapsilosis	1.0	3.13786

* denotes a statistically significant difference.

Table 7: ANOVA: Inhibition zones of strain T12 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	29.6111	5	5.92222	1.21	0.3614*
Within groups	58.6667	12	4.88889		
Total	88.2778	17			

*p > 0.05

Table 8: ANOVA: Inhibition zones of strain T13 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	204.267	4	51.0667	22.53	0.0001*
Within groups	22.6667	10	2.26667		
Total	226.933	14			

*p < 0.05

Table 9: Multiple range tests: Inhibition zones of strain T13 against test microorganisms

Contrast	Difference	+/- Limits
E. faecalis – S. aureus	-2.66667	2.739
E. faecalis – P. shigelloides	*-10.0	2.739
E. faecalis – B. subtilis	-2.0	2.739
E. faecalis – C. parapsilosis	0.0	2.739

Table 9, Continuation

Contrast	Difference	+/- Limits
S. aureus – P. shigelloides	*-7.33333	2.739
S. aureus – B. subtilis	0.666667	2.739
S. aureus – C. parapsilosis	2.66667	2.739
P. shigelloides – B. subtilis	*8.0	2.739
P. shigelloides – C. parapsilosis	*10.0	2.739
B. subtilis – C. parapsilosis	2.0	2.739

* denotes a statistically significant difference.

Table 10: ANOVA: Inhibition zones of strain T15 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	295.833	5	59.1667	19.36	0.0000*
Within groups	36.6667	12	3.05556		
Total	332.5	17			

*p = 0

Table 11: Multiple range tests: Inhibition zones of strain T15 against test microorganisms

Contrast	Difference	+/- Limits
E. faecalis – S. aureus	1.33333	3.10971
E. faecalis – P. shigelloides	*-8.66667	3.10971
E. faecalis – B. subtilis	*-4.33333	3.10971
E. faecalis – C. parapsilosis	2.66667	3.10971
E. faecalis – C. albicans	2.0	3.10971
S. aureus – P. shigelloides	*-10.0	3.10971
S. aureus – B. subtilis	*-5.66667	3.10971
S. aureus – C. parapsilosis	1.33333	3.10971
S. aureus – C. albicans	0.666667	3.10971

Table 11, Continuation

Contrast	Difference	+/- Limits
P. shigelloides – B. subtilis	*4.33333	3.10971
P. shigelloides – C. parapsilosis	*11.3333	3.10971
P. shigelloides – C. albicans	*10.6667	3.10971
B. subtilis – C. parapsilosis	*7.0	3.10971
B. subtilis – C. albicans	*6.33333	3.10971
C. parapsilosis – C. albicans	-0.666667	3.10971

* denotes a statistically significant difference.

Table 12: ANOVA: Inhibition zones of strain T16 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	275.333	5	55.0667	9.09	0.0009*
Within groups	72.6667	12	6.05556		
Total	348.0	17			

*$p < 0.05$

Table 13: Multiple range tests: Inhibition zones of strain T16 against test microorganisms

Contrast	Difference	+/- Limits
E. faecalis – S. aureus	1.33333	4.37776
E. faecalis – P. shigelloides	*-9.33333	4.37776
E. faecalis – B. subtilis	-3.0	4.37776
E. faecalis – C. parapsilosis	1.66667	4.37776
E. faecalis – C. albicans	1.33333	4.37776
S. aureus – P. shigelloides	*-10.6667	4.37776
S. aureus – B. subtilis	-4.33333	4.37776
S. aureus – C. parapsilosis	0.333333	4.37776
S. aureus – C. albicans	0.0	4.37776

Table 13, Continuation

Contrast	Difference	+/- Limits
P. shigelloides – B. subtilis	*6.33333	4.37776
P. shigelloides – C. parapsilosis	*11.0	4.37776
P. shigelloides – C. albicans	*10.6667	4.37776
B. subtilis – C. parapsilosis	*4.66667	4.37776
B. subtilis – C. albicans	4.33333	4.37776
C. parapsilosis – C. albicans	-0.333333	4.37776

* denotes a statistically significant difference.

Table 14: ANOVA: Inhibition zones of strain T20 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	103.583	3	34.5278	12.95	0.0019*
Within groups	21.3333	8	2.66667		
Total	124.917	11			

*$p < 0.05$

Table 15: Multiple range tests: Inhibition zones of strain T20 against test microorganisms

Contrast	Difference	+/- Limits
E. faecalis – S. aureus	0.333333	3.07468
E. faecalis – P. shigelloides	*7.0	3.07468
E. faecalis – B. subtilis	0.333333	3.07468
S. aureus – P. shigelloides	*6.66667	3.07468
S. aureus – B. subtilis	0.0	3.07468
P. shigelloides – B. subtilis	*-6.66667	3.07468

* denotes a statistically significant difference.

Table 16: ANOVA: Inhibition zones of strain T52 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2.66667	4	0.666667	0.40	0.8045*
Within groups	16.6667	10	1.66667		
Total	19.3333	14			

*p > 0.05

Table 17: ANOVA: Inhibition zones of strain T53 against test microorganisms

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	280.571	6	46.7619	21.35	0.0000*
Within groups	30.6667	14	2.19048		
Total	311.238	20			

*p = 0

Table 18: Multiple range tests: Inhibition zones of strain T53 against test microorganisms

Contrast	Difference	+/- Limits
E. faecalis – S. aureus	2.33333	2.59184
E. faecalis – P. shigelloides	*4.0	2.59184
E. faecalis – B. subtilis	2.33333	2.59184
E. faecalis – P. aeruginosa	*3.0	2.59184
E. faecalis – C. parapsilosis	*10.6667	2.59184
E. faecalis – C. albicans	*9.33333	2.59184
S. aureus – P. shigelloides	1.66667	2.59184
S. aureus – B. subtilis	0.0	2.59184
S. aureus – P. aeruginosa	0.666667	2.59184
S. aureus – C. parapsilosis	*8.33333	2.59184
S. aureus – C. albicans	*7.0	2.59184
P. shigelloides – B. subtilis	-1.66667	2.59184
P. shigelloides – P. aeruginosa	-1.0	2.59184

Table 18, Continuation

Contrast	Difference	+/- Limits
P. shigelloides – C. parapsilosis	*6.66667	2.59184
P. shigelloides – C. albicans	*5.33333	2.59184
B. subtilis – P. aeruginosa	0.666667	2.59184
B. subtilis – C. parapsilosis	*8.33333	2.59184
B. subtilis – C. albicans	*7.0	2.59184
P. aeruginosa – C. parapsilosis	*7.66667	2.59184
P. aeruginosa – C. albicans	*6.33333	2.59184
C. parapsilosis – C. albicans	-1.33333	2.59184

* denotes a statistically significant difference.

Table 19: ANOVA: SA medium: Inhibition zones of strain T53 against *E. faecalis* (shaking vs static)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4.22222	2	2.11111	2.11	0.2022*
Within groups	6.0	6	1.0		
Total	10.2222	8			

*p > 0.05

Table 20: ANOVA: ISP2 medium: Inhibition zones of strain T53 against *E. faecalis* (shaking vs static)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	10.25	3	3.41667	3.15	0.0862*
Within groups	8.66667	8	1.08333		
Total	18.9167	11			

*p > 0.05

Table 21: ANOVA: pH of submerged cultures of strain T53 in SA

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2.6896	7	0.384229	2.02	0.1156*
Within groups	3.0422	16	0.190137		
Total	5.7318	23			

*p > 0.05

Table 22: ANOVA: pH of submerged cultures of strain T53 in ISP2

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2.58105	7	0.368721	3.05	0.0306*
Within groups	1.93253	16	0.120783		
Total	4.51358	23			

*p < 0.05

Table 23: Multiple range tests: pH of submerged cultures of strain T53 in ISP2

Contrast	Difference	+/- Limits
Day 3 (shaking) – Day 6 (shaking)	0.523333	0.601555
Day 3 (shaking) – Day 9 (shaking)	0.373333	0.601555
Day 3 (shaking) – Day 12 (shaking)	0.23	0.601555
Day 3 (shaking) – Day 3 (static)	0.0266667	0.601555
Day 3 (shaking) – Day 6 (static)	-0.0166667	0.601555
Day 3 (shaking) – Day 9 (static)	-0.253333	0.601555
Day 3 (shaking) – Day 12 (static)	-0.583333	0.601555
Day 6 (shaking) – Day 9 (shaking)	-0.15	0.601555
Day 6 (shaking) – Day 12 (shaking)	-0.293333	0.601555
Day 6 (shaking) – Day 3 (static)	-0.496667	0.601555
Day 6 (shaking) – Day 6 (static)	-0.54	0.601555
Day 6 (shaking) – Day 9 (static)	*-0.776667	0.601555
Day 6 (shaking) – Day 12 (static)	*-1.10667	0.601555
Day 9 (shaking) – Day 12 (shaking)	-0.143333	0.601555

Table 23, Continuation

Contrast	Difference	+/- Limits
Day 9 (shaking) – Day 3 (static)	-0.346667	0.601555
Day 9 (shaking) – Day 6 (static)	-0.39	0.601555
Day 9 (shaking) – Day 9 (static)	*-0.626667	0.601555
Day 9 (shaking) – Day 12 (static)	*-0.956667	0.601555
Day 12 (shaking) – Day 3 (static)	-0.203333	0.601555
Day 12 (shaking) – Day 6 (static)	-0.246667	0.601555
Day 12 (shaking) – Day 9 (static)	-0.483333	0.601555
Day 12 (shaking) – Day 12 (static)	*-0.813333	0.601555
Day 3 (static) – Day 6 (static)	-0.0433333	0.601555
Day 3 (static) – Day 9 (static)	-0.28	0.601555
Day 3 (static) – Day 12 (static)	*-0.61	0.601555
Day 6 (static) – Day 9 (static)	-0.236667	0.601555
Day 6 (static) – Day 12 (static)	-0.566667	0.601555
Day 9 (static) – Day 12 (static)	-0.33	0.601555

* denotes a statistically significant difference.

Table 24: ANOVA: pH of submerged cultures of strain T53 in ISP4

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4.4461	7	0.635157	2.73	0.0454*
Within groups	3.7174	16	0.232337		
Total	8.1635	23			

*$p < 0.05$

Table 25: Multiple range tests: pH of submerged cultures of strain T53 in ISP4

Day 3 (shaking) – Day 6 (shaking)	*1.21	0.834318
Day 3 (shaking) – Day 9 (shaking)	*1.26667	0.834318
Day 3 (shaking) – Day 12 (shaking)	0.483333	0.834318
Day 3 (shaking) – Day 3 (static)	0.706667	0.834318
Day 3 (shaking) – Day 6 (static)	0.663333	0.834318
Day 3 (shaking) – Day 9 (static)	*1.37333	0.834318
Day 3 (shaking) – Day 12 (static)	*0.853333	0.834318
Day 6 (shaking) – Day 9 (shaking)	0.0566667	0.834318
Day 6 (shaking) – Day 12 (shaking)	-0.726667	0.834318
Day 6 (shaking) – Day 3 (static)	-0.503333	0.834318
Day 6 (shaking) – Day 6 (static)	-0.546667	0.834318
Day 6 (shaking) – Day 9 (static)	0.163333	0.834318
Day 6 (shaking) – Day 12 (static)	-0.356667	0.834318
Day 9 (shaking) – Day 12 (shaking)	-0.783333	0.834318
Day 9 (shaking) – Day 3 (static)	-0.56	0.834318
Day 9 (shaking) – Day 6 (static)	-0.603333	0.834318
Day 9 (shaking) – Day 9 (static)	0.106667	0.834318
Day 9 (shaking) – Day 12 (static)	-0.413333	0.834318
Day 12 (shaking) – Day 3 (static)	0.223333	0.834318
Day 12 (shaking) – Day 6 (static)	0.18	0.834318
Day 12 (shaking) – Day 9 (static)	*0.89	0.834318
Day 12 (shaking) – Day 12 (static)	0.37	0.834318
Day 3 (static) – Day 6 (static)	-0.0433333	0.834318
Day 3 (static) – Day 9 (static)	0.666667	0.834318
Day 3 (static) – Day 12 (static)	0.146667	0.834318
Day 6 (static) – Day 9 (static)	0.71	0.834318
Day 6 (static) – Day 12 (static)	0.19	0.834318
Day 9 (static) – Day 12 (static)	-0.52	0.834318

*denotes a statistically significant difference.