ISOLATION AND CHARACTERIZATION OF *Streptomyces* sp. ISOLATED FROM UNEXPLORED ESTUARIN MANGROVE SEDIMENTS FOR ANTIOXIDANTS ACTIVITIES

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by

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PENGASINGAN DAN PENCIRIAN *Streptomyces* sp.YANG DIPENCILKAN DARIPADA SEDIMEN MUARA PAYA BAKAU BELUM DITEROKAI UNTUK AKTIVITI ANTIOXIDAN

ABSTRAK

Dalam kajian ini, potensi lapan puluh aktinomiset, dipencilkan daripada sedimen bakau di negeri Perak dan Kedah Malaysia, telah dinilai untuk penghasilan metabolit antimikrob dan antioksidan. Lima *Streptomyces* sp. menunjuk aktiviti antimikrob terhadap bakteria dalam saringan awal yang telah dieramkan di dalam media ISP1 media (Projek Streptomyces International 1) dan diekstrak menggunakan etil asetat pada nisbah 1: 1 (v / v). Lima pencilan aktif telah dilabel sebagai MBL 159, MBL 161, MBL 169, MBL 185 dan MBL 201. Ekstrak etil asetat MBL169 (KU663071) menyatakan aktiviti antimikrob yang paling tinggi terhadap kaedah cakera penyebaran dan ujian kepekatan perencatan minimum (MIC). Asai memerangkap radikal bebas mendedahkan MBL201 (KU705719) mempamerkan peratusan tertinggi aktiviti memerangkap pada 92.36 ± 6.50 peratus, manakala jumlah kapasiti antioksidan ialah (80.03 ± 7.63 μg AAE / ml), pengurangan ferik pada (0.274 ± 0.007 OD) dan asai pelunturan β-karotena-linoleate pada (zon pengekalan warna: 3.25 ± 0.16 mm). Spesies *Streptomyces* yang diasingkan telah dikenal pasti menggunakan pencirian morfologi dan jujukan 16S rRNA gen analisis. Pokok filogenetik telah dibina untuk memahami pencilan dalam pertalian rapat antara spesies *Streptomyces*. Hasil kajian ini menyatakan bahawa ekstrak etil asetat memiliki memerangkap radikal bebas dan antimikrob aktivit,i yang merupakan sifat wajar penemuan baru untuk farmaseutikal dan aplikasi industri.

ISOLATION AND CHARACTERIZATION OF *Streptomyces* sp. ISOLATED FROM UNEXPLORED ESTUARIN MANGROVE SEDIMENTS FOR ANTIOXIDANTS ACTIVITIES

ABSTRACT

In this study, the potency of eighty actinomycetes, isolated from mangrove sediment in states Perak and Kedah of Malaysia, were evaluated for production of antimicrobial and antioxidant metabolites. Five *Streptomyces* sp. that demonstrated antimicrobial activity against pathogenic bacteria in a preliminary screening were cultivated in ISP1 (International *Streptomyces* Project 1) broth and extracted using ethyl acetate at a ratio of 1:1 (v/v). Five active isolates were titled as MBL 159, MBL 161, MBL 169, MBL 185 and MBL 201. Ethyl acetate extract of MBL169 with accession number (KU663071) expressed the highest antimicrobial activity on the disc diffusion method and minimum inhibitory concentration (MIC) test. Free radical scavenging assay revealed MBL201 (KU705719) exhibited the highest percentage of scavenging activity at 92.36±6.50 percent, total antioxidant capacity (80.03±7.63 µg AAE/ml), ferric reducing (0.274±0.007 OD) and β-carotene-linoleate bleaching (zone of color retention: 3.25±0.16 mm) assays. The newly isolated *Streptomyces* species were identified using morphological characterization and 16S rRNA gene sequence analysis. The phylogenetic tree was constructed to perceive the isolates in close affinity to the Streptomyces species. The outcomes of this study specify that the ethyl acetate extract possessed free-radical scavenging and antimicrobial activities, which are desirable properties of a novel discovery for pharmaceutical and industrial applications.

CHAPTER 1

INTRODUCTION

1.1 Research background

Oxygen is utilised by all living organisms for the production of energy except anaerobic organisms. Oxidative stress is a universal phenomenon arises in the three domains of life, namely the archaea, bacteria and eukaryote (Venturi & Venturi, 2007). This phenomenon takes place once the concentration of oxygen is greater than the normal circumstances, exceeding the thresholds that are toxic to plants, animals, and aerobic bacteria. During the oxidative reactions, excessive reactive oxygen species in the form of activated oxygen is produced, which include free radicals such as superoxide ions, O_2^- and hydroxyl radicals, OH^-, as well as non-free radical species such as hydrogen peroxide, $H_2O_2^-$ (Barros et al., 2007; Townsend, 2007).

A free radical is a species with a mean effective radius of 30 Å and with a half-life of few milliseconds. It is capable of an independent existence, which contains one or more unpaired electrons and is extremely reactive. More free radicals are generated when they react with new compounds whereby this chain reaction leads to thousands of events. Generated from regular cell metabolism and oxidative phosphorylation, free radicals play the dual character, being either toxic or beneficial towards the human body, whereby an excessive of free radicals leads to oxidation stress. In living organisms, various reactive oxygen species are the causative agents for the damage of lipid, enzyme, protein, and nucleic acid leading to cell and tissue injury implicated in the aging processes, as well as degenerative diseases including inflammation, diabetes, liver injury, atherosclerosis, cancer, Parkinson's disease, Alzheimer's disease and coronary heart pathologies (Townsend, 2007; Augustine,

2014). In the plant, the presence of excessive oxygen causes membrane damage and inhibition of chloroplast development (Wroblewska et al., 1994), whereas *in vitro* increase of oxygen tension in *Escherichia coli* inhibits the growth of bacteria (Brown, 1975).

Nevertheless, living organisms are protected from the production of free radicals by a defence mechanism known as antioxidant (Diraviyam et al., 2011). The main characteristics of an antioxidant are the capability to trap free radicals and neutralize possible destructive reactive free radicals in the body cells, which oxidize the nucleic acid, protein, lipid or DNA. Thus, antioxidant may reduce the potential mutation. As a result, antioxidants may be helpful in preventing degenerative diseases and other pathologies. Many antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral properties. A natural antioxidant is commonly found in medicinal plants, vegetables, and fruits. Natural products derived from living organisms such as plants, animals and microorganisms are isolated chemical compounds of secondary metabolites, leading to the discovery of many drugs, which can be used for the treatment of numerous human diseases (Grabley & Thiericke, 1999). Nonetheless, it has been reported that metabolites from endophytes can be a potential source of novel natural antioxidants (Jariwala & Ranjan, 2013).

Actinomycetes are Gram-positive bacteria known as filamentous and sporulating organisms with various metabolic activities. Actinomycetes have been recounted as the main prolific producers of microbial bioactive secondary metabolites for prospective agricultural, pharmaceutical and industrial applications (Dasari et al., 2012; Balachandran et al., 2012). The filamentous actinomycetes produce over 10,000 bioactive compounds with 7,600 derived from Streptomycetes

as the main source of bioactive microbial metabolites. The metabolites are effective as antimicrobial agents on a wide range of pathogenic microorganisms (Ceylan et al., 2008; Ghandin et al., 2008). Generally, therapeutic compounds, which are exploited for curing numerous human diseases, are acquired from plant sources, although these drugs exhibit side effects on human body. Recently, microbes are progressively resistant towards clinical drugs hence encourages new discoveries of natural products from microorganisms (Se-Kwon Kim, 2013).

The ocean covers almost 70% of the earth surface and little is acknowledged about the microbial diversity of marine sediment, which is a vast resource that has not been completely exploited. Marine extremophiles serve as a valuable natural resource for novel products with potent biological activity (Bush & Macielag, 2000). The compounds isolated from marine *Streptomyces*, namely 2-alloxyphenol and streptopyyrolidine, have been reported to possess antioxidant. Between antibiotics carbomycin B and carazostatin, the former antibiotic demonstrated greater antioxidant activity (Kato, 1994). The new metabolites derived from *Streptomyces prunicolor*, such as benthocyanins A, B and C as well as benthophoenin: possess higher antioxidant activity than vitamin E (Karthik et al., 2013). Natural antioxidants are frequently isolated from medicinal plants, vegetables and fruits, although metabolites from endophytes were reported as potential sources as well (Jariwala & Ranjan, 2013). The present study evaluates the antioxidant and antimicrobial activity from mangrove actinomycetes isolates.

1.2 Problem Statement

Microbial secondary metabolites have great positive impact on human health and represent an attractive resource for novel drug discovery. Thus, isolation, characterization and screening of actinomycetes strains, which are capable to produce potential antibiotics and other therapeutics, have been a major part of this research. Modern studies in term of biotechnology are concentrating on the consequences of antioxidant system of bacteria. Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to humans against various infections and degenerative diseases. Previously, there were many scientific studies conducted on secondary metabolites activities from plant extracts. However, bioactive metabolites from microorganisms are still not fully explored in Malaysia. The aim of this study is to focus on the antioxidant activities of the isolated actinomycetes that are capable of producing bioactive metabolites such as antimicrobial. These experiments also try to find new metabolites from microorganism that is capable to function as antioxidant and antimicrobial.

1.3 Objectives of study

The aims of the present study are as follows:

1. To isolate and characterize actinomycetes from the mangrove environment.
2. To determine the antimicrobial activity of extracts prepared from actinomycetes isolates.
3. To determine the antioxidant activity of positive antimicrobial activity extracts prepared.
4. To determine antioxidant activity of partition and fraction prepared from *Streptomyces* sp. MBL 201.
5. To isolate and identify compound of antioxidant activity of fraction prepared from *Streptomyces* sp. MBL 201.

CHAPTER 2

LITERATURE REVIEW

2.1 General overview of actinomycetes

Actinomycetes, also known as Actinobacteria, are a phylum comprising of Gram-positive bacteria, commonly considered as an intermediate group of bacteria and fungi, which were then recognised as prokaryote organisms. These bacteria were regarded as fungi in 1878 (Srinivasan et al., 1991). However, actinomycetes are classified as true bacteria with a filamentous growth similar to fungi. These organisms are firmly established as prokaryotes with close affinities to the mycobacteria and coryneform with no phylogenetic relationship with fungi (Srinivasan et al., 1991). They have a cell wall but no nuclear membrane and their DNA is rich in G+C content percentage of 57 to 75% (Lo et al., 2002). They reproduce from spores either by fission or by conidia. Some actinomycetes produce external spores with the shape of bacillary or coccoid elements. They produce two forms of branching mycelia, which are substrate mycelium and aerial mycelium (Siva, 2001). They grow well from 35 to 37 °C and behave as mesophilic bacteria in the laboratory (Sathiyaseelan & Stella, 2011).

Actinomycetes are primarily reported to be saprophytic, aerobic and mesophilic. They are heterotrophic organisms whereby their habitats are widely spread throughout the environment (Williams et al., 1989). Zaitlin and Watson (2006) have reported the presence of actinomycetes in a wide range of environment, as dormant spores or actively growing. The soil is a common habitat of actinomycetes with the most prominent being alkaline soil hence they are known as representative terrestrial microorganisms (Imada, 2005). Actinomycetes play a

significant role as a cycler of organic matter in the soil ecosystem (Hirsch & Christensen, 1983). However, they are metabolically active in the aquatic environment and have adapted to life in the sea (Jeffrey et al., 2007). Bull and co-workers (2005) have reported that actinomycetes are widely distributed in the ocean and can easily be isolated from marine environment. They can be found living in marine organisms such as decaying marine algae, seaweed and sponges.

2.2 Natural products from actinomycetes

Actinomycetes are known as prolific producers of bioactive secondary metabolites such as vitamin, enzyme inhibitor, antitumor agent, immunosuppressive agent and antibiotic (Sathiyaseelan & Stella, 2011). According to Mincer et al., (2002), actinomycetes are the best source of antibiotics. Based on the previous reports in the Dictionary of Natural Products, approximately 7000 antibiotic compounds were produced by these bacteria (Jensen et al., 2005) which occupied the vast majority of 45% out of 22,500 biologically active compounds identified from microorganisms (Mincer et al., 2002).

2.3 Genus of *Streptomyces*

Streptomyces is the largest genus of actinomycetes, whereby more than 500 species have been reported. *Streptomyces* is well known for its great ability to produce antibiotics as secondary metabolites. Studies have shown that 80% of total antibiotics were produced by this genus (Valli et al., 2012). About 50% of *Streptomyces* that were isolated from the marine environment are able to produce

great bioactive compounds including streptomycin, polyene, macrolide and aminoglycoside (Intra et. al., 2011). *Streptomyces* is a genus of Gram-positive aerobic bacteria belonging to the phylum of Actinobacteria (Stackebrandt et al. 1997). It is acknowledged as a prolific source of useful bioactive metabolites (Berdy, 2005). It has similar cellular structure and life cycle as fungi. The traditional developmental cycle of *Streptomyces* culture on agar surface describes the growth of branched filaments that consist of a network of multinucleoid hyphae known as the "substrate mycelium". After a few days of cultivation, the growth of substrate mycelium produces specialised aerial hyphae, known as the "aerial mycelium", which extend away from the substrate mycelium into the air. Later, the aerial hyphae undergo massive septation to create a series of uninucleoid compartments. These compartments differentiate to create spore chains. The spores are semi dormant hence persist in soil for a long period. Both substrate and aerial mycelia are multinucleated (Kieser et al., 2000).

The industrial processes for the production of bioactive secondary metabolites of most *Streptomyces* strains are carried out with liquid cultures since they do not sporulate in liquid culture. The antibiotic is one of the secondary metabolites produced by the substrate mycelium at the end of the proliferation phase. Four morphological classes are distinguished in *Streptomyces* submerged culture, which are pellet (compact masses of 950 µm in diameter), clump (less compact masses of 600 µm in diameter), branched and non-branched hyphae forms. In addition, *Streptomyces* pellets have been proven to develop in the form of a biofilm containing sticky extracellular polymers and the insoluble substrate. It has been accepted that mycelial morphology is associated with the production of secondary metabolites since cellular aggregation, pellet and clump formation are fundamental

for good production. On the other hand, there is also some dispute stating a lack of clear relationship between mycelial morphology and the production of secondary metabolites. In conclusion, there is no general consensus that relates morphology with production (Kieser et al 2000).

2.4 Antioxidant

2.4.1 Free radical and Oxidative stress

Oxidation is necessary for numerous living organisms to produce energy for biological processes (Yang et al., 2002). Oxidative stress is characterised as an imbalance between the production of free radical species and the activity of antioxidant defence. Free radicals are atoms, molecules or ions with unpaired valence electrons, which are highly active towards chemical reactions with other molecules. In a biological system, free radicals are often derived from oxygen, nitrogen and sulphur known as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulphur species (RSS). Examples of ROS include free radicals such as superoxide anion (O^{2-}), perhydroxyl radical (HO_2), hydroxyl radical (^-OH), nitric oxide, hydrogen peroxide (H_2O_2), singlet oxygen ($1O_2$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$). The RNS is derived from nitric oxide via reaction with O^{2-} to form $ONOO^-$. The RSS are easily formed from thiols by the reaction with ROS (Giles & Jacob, 2002).

Free radicals derived from molecular oxygen represent the most important class of radical species generated in the living system (Miller et al., 1990). ROS is produced during cellular metabolism and functional activities, portraying important roles in cell signalling, apoptosis, gene expression and ion transportation. However,

excessive ROS may lead to the oxidation of lipid, protein or DNA, which are the major causes of aging, degenerative disease, cancer, cardiovascular disease, compromised immune function, inflammation and renal failure (Volko et al., 2006b). Mitochondria are often the first target of a free radical attack known as lipid peroxidation since the lipid membrane is vulnerable to degradation by ROS. The ROS attacks bases in nucleic acids, amino acid side chains in proteins, and double bonds in unsaturated fatty acids, in which ^{-}OH is the strongest oxidant. The ROS attacking macromolecules are often termed as oxidative stress. Cells are normally able to defend themselves against ROS damage by intracellular enzymes to keep the homeostasis of ROS at a low level. However, during the time of the environmental stress and cell dysfunction, ROS level increases dramatically, causing significant cellular damage in the body. Thus, oxidative stress significantly contributes to inflammatory disease, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, autism and aging (Lu et al., 2010).

There are numerous factors which influence the free radical scavenging capacity such as the rate and number of radical molecules scavenged, the fate of antioxidant-derived radicals, interaction with other antioxidants, concentration, mobility in the environment, as well as the adsorption, distribution, retention and metabolism of the antioxidant compounds (Niki & Noguchi, 2000; Niki, 2010). When an active radical is scavenged by an antioxidant compound, a stable non-radical product is formed. At the same time, the antioxidant yields one antioxidant-derived radical. The fate of this radical is also an important consideration of antioxidant efficiency (Niki et al., 2000).

2.4.2 Antioxidant properties

Antioxidants are molecules that inhibit or quench free radical reaction and delay or inhibit cellular damage by accepting or donating electrons to eliminate the unpaired condition of radicals. In order to maintain normal cell function, health and well-being, the human body and other organisms have developed an antioxidant defence system that prevents the initiation or propagation of oxidising chain reaction, which in turn inhibits or delays oxidative damage related to disease and aging. The amount of antioxidant produced under normal conditions is not always sufficient (Lu et al., 2010). Antioxidants can be divided into two classes, the endogenous and the exogenous groups. Antioxidants exist as enzymatic or non-enzymatic form, intra or extracellular to neutralise the oxidative agents. Enzymatic antioxidants break down and remove free radicals. They flush out dangerous oxidative products by converting them into hydrogen peroxide before they become water. This is done through a multi-step process that requires a number of trace mental cofactors, such as zinc, copper, manganese and iron.

Enzymatic antioxidants cannot be found in supplements but are produced in our body instead. The main enzymatic antioxidants in our body are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHpx) and glutathione reductase (GR). Non-enzymatic antioxidants interrupt free-radical chain reactions. Carotenoids, vitamin C, vitamin E, polyphenols and glutathione (GSH) are examples of non-enzymatic antioxidants. Most antioxidants in supplement and food are non-enzymatic, which support enzymatic antioxidants by doing a “first sweep” in disarming the free radicals. This helps to prevent the enzymatic antioxidants from being depleted (Smith, 2014).

The antioxidants may directly react with the reactive radicals and destroy them, or they might become new free radicals that are less active, longer-lived and less dangerous than those radicals they have neutralised. They may be neutralised by other antioxidants or mechanisms to terminate their radical status (Lu et al., 2010). Many antioxidants have aromatic ring structures and are able to delocalise the unpaired electron (Figure 2.1). Vitamin C (AscH-) in the aqueous phase and vitamin E (TOH) in the lipid phase will directly react with or neutralise hydroxyl, alkoxyl and lipid peroxyl (ROO-) radicals to form H_2O, alcohol and lipid hydroperoxides, respectively (Figure 2.1 a-b). Due to its delocalised structure, vitamin E becomes a phenyl radical and vitamin C turns to a very stable radical (Asc-). Besides that, vitamin C also neutralises the radical form of other antioxidants such as glutathione radical and vitamin E radical (Figure 2.1c) (Lu et al., 2010).

The intake of dietary antioxidants helps to maintain an adequate antioxidant status in the body. Although there are several enzyme systems within the body that scavenge free radicals, the principle micronutrient (vitamin) antioxidants are vitamin E, beta-carotene, and vitamin C. The body cannot manufacture these micronutrients so they must be supplied in the diet (Lu et al., 2010). Other than their health promoting effect, antioxidants also prevent oxidation in other essential areas. In the food industry, oxidation affects the nutritional value of the food and causes rancidity or deterioration of colour, flavour and texture. Antioxidants are molecules that safely interact with free radicals and terminate the chain reaction before vital molecules are damaged (Smith, 2014).

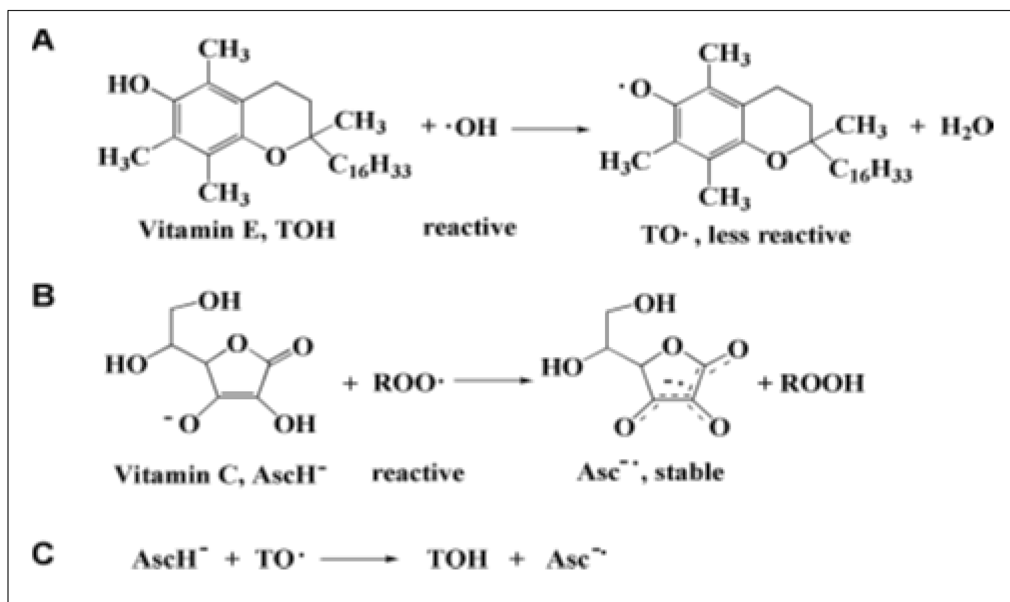

Figure 2.1: (A) Direct reaction of vitamin E (TOH) with –OH, (B) vitamin C (AscH) with ROO- and (C) regeneration of vitamin E from vitamin C (Lu et al., 2010).

2.4.3 Natural antioxidant

Natural compounds such as ascorbic acid, vitamin E, phenolic acid, carotenoids and flavonols possess the ability to scavenge free radicals in the human body, playing a key role in health maintenance and the prevention of chronic and degenerative diseases (Doughari, 2012). The two defence mechanisms of antioxidants are enzymatic and non-enzymatic. The enzymatic antioxidants are copper-zinc and manganese peroxide, whereas the non-enzymatic molecules are glutathione, vitamin A, C and E (Jones et al., 2014).

Antioxidants are characterised based on their mode of action in preventing oxidative damage by scavenging, preventing, repairing or forming de novo antioxidant (Lobo et al., 2010). Antioxidants prevent the formation of ROS/RNS by reducing the hydrogen peroxide and lipid hydroperoxidases, respectively, or by sequestering metal ions such as iron and copper (Niki, 2010). Scavenging antioxidants remove active species rapidly before they attack biologically essential

molecules such as, superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide, while carotenoids scavenge singlet oxygen (Lobo et al., 2010; Niki, 2010). Repair enzymes act as antioxidants by repairing damage, clearing wastes or reconstituting lost function (Niki, 2010).

Phenolic compounds are aromatic hydroxylated compounds possessing at least one aromatic ring with one or more hydroxyl groups. These compounds are commonly found in plants and certain microorganisms, which are significant sources of bioactive substances. A structure-function relationship exists between phenolic compounds and antioxidants, where the latter is influenced by the number and position of the hydroxyl groups and the nature of substitutions on the aromatic rings (Smith, 2014). Phenolic acids are divided into two main groups, namely hydroxybenzoic acid and hydroxycinnamic acids (Ferreira et al., 2009). Hydroxybenzoic acid is a natural component of a complex structure such as lignin and hydrolysable tannins. However, hydroxycinnamic acid is generally linked to the cell wall of the structural components. The *ρ*-hydroxybenzoic, vanillic acid and syringic acid from the group of hydroxybenzoic acids are less active than caffeine, chlorogenic, ferulic, sinapic and *ρ*-coumaric acids (Larson, 1988).

Flavonoids are usually found in high quantities in fruits, vegetables, plants and nuts (Liu, 2004). They represent the most widely distributed sub-group of antioxidants. These groups are highly effective scavengers of free radicals with the ability to prevent cardiovascular disease, DNA destruction and tumour progression (Smith, 2014). Carotenoids are natural pigments synthesised by plants for the colouration of various fruits, flowers and vegetables, and have been associated in the prevention of numerous diseases. Structurally, they possess a 40-carbon poly-isoprenoid skeleton, with an oxygen in the functional groups, responsible for the

colour and antioxidant functions. Carotenoids are influenced by the chain length and the terminal groups of the radicals and are regarded as dietary sources of vitamin A (Ferreira et al., 2009).

Vitamin E is a liposoluble vitamin, which plays a significant role in the prevention of lipid peroxidation in the cell membrane and as a protective scavenger of free radicals against disease. Vitamin E is made up of eight compounds, α, β, γ and δ-tocopherols, including both tocopherol and tocotrienols (Smith, 2014). The α-tocopherol is the most active form of vitamin E in humans with high biological activity (Ferreira et al., 2009). On the other hand, vitamin C (ascorbic acid) has demonstrated a protective role against oxidative stress associated with various diseases (Smith, 2014). It protects lipid membrane from oxidative damage by reacting with peroxyl radicals prior to lipid peroxidation (Davey et al., 2000). Both vitamin E and C form a synergistic relationship. Vitamin E converts into a radical when scavenging active free radicals, which the radical is then being reduced to its antioxidant form by vitamin C before the radical induces lipid peroxidation. Other antioxidants such as glutathione, ubiquinol and some other compounds have also demonstrated a synergistic relationship with vitamin E. The relationship between these nutrients is essential, because one antioxidant compound may not be able to provide sufficient protection against lipid peroxidation and disease prevention (Nagaoka et al., 2007).

2.4.4 Antioxidant activity from *Streptomyces* sp.

Mangrove ecosystem is an enormous source for isolating new actinomycetes with the potential to produce biologically active secondary metabolites. Among several genera, *Streptomyces*, Amycolatopsis, Saccharopolyspora, Micromonospora

and Actinoplanes are the main producer of the secondary metabolites. The metabolites produced by actinomycetes have a broad spectrum of biological activity. *Streptomyces* species is regarded as a source of antibiotics and other important metabolites, which include antifungal agents, antioxidants, antitumor agents, anthelmintic agents and herbicides (Karthik et al., 2013).

The present research focuses on the identification of natural antioxidant-producing microorganisms, serving as safe therapeutics. Ever since free oxygen radicals were discovered to play an important role in various diseases, it provoked investigations for novel and potent antioxidant compounds from microorganisms for therapeutic use. Between the antibiotics of carbazomycin B and carazostatin, the former demonstrated greater antioxidant activity (Kato, 1994). New metabolites derived from *Streptomyces prunicolor* such as benthocyanins A, B and C, as well as benthophoenin, possess higher antioxidant activity than vitamin E (Bhaskara, 2012). They show not only the brain-protecting activity in hippocampal neurone system but also inhibit glutamate activity. Furthermore, three isoflavonoids were isolated from *Streptomyces* sp. OH-1049, in which compounds 4',7,8-trihydroxyisoflavone has shown antitumor activity (Karthik et al., 2013). In addition, 2-alloxyphenol and streptopyyrolidine, isolated from marine *Streptomyces* have also been reported to possess antioxidants (Arumugam et al., 2010). It was reported that melanin which is extensively produced by *Streptomyces* species is a natural antioxidant (Hewedy & Ashour, 2009). Other than melanin, protocatechualdehyde from *Streptomyces lincolnensis* M-20 (Kyoung-Ja et al., 2008) and surugapyrone A from *Streptomyces coelicoflavus* USF-6280 (Yasumasa et al., 2010) were reported as natural antioxidants produced by this species.

2.4.5 Chemical-based assay for antioxidant screening

Antioxidants have different functions as they carry out their protective role through a number of processes. Therefore, there is no universal method to accurately and quantitatively assess antioxidant capacity to date (Lobo et al., 2010; Niki, 2010). Antioxidant activity is frequently established in terms of the degree of antioxidant power based on colorimetric reactions, a measure of scavenging ability following the reduction of metal ions or radical scavenging capacity based on competitive methods. There are different methods to measure the reactivity of natural compounds towards radicals. Reactivity of antioxidant compounds to stable free radicals such as 2,2,-diphenyl-1-picrylhydrazyl (DPPH) is a method which provided a stable colorimetric assay to establish antioxidant capacity (Niki, 2010). The reducing antioxidant power assay is common in evaluating the reduction of metal ions due to a radical-scavenging antioxidant for plant compounds, food and nutrition chemistry, as well as marine natural product research (Niki, 2010). The antioxidative activity of the extract was evaluated by the reductive capacity in terms of the conversion degree from ferricyanide (Fe^{3+}) to ferrous (Fe^{2+}) complex, which involves colour changes. The molar concentration of the reductant can be plotted as a function of colour intensity as measured on a spectrophotometer. The procedure of a reducing assay, which involves checking the strength of an antioxidant compound as a reductant, is relatively faster in gathering preliminary data with a large number of samples (Shahidi & Zhong, 2015).

The antioxidant activity test, which employs beta-carotene-linoleate solid model system, involves the measuring of a yellow to orange zone of the retention, which surrounds the well, in diameters. An earlier study reports that the reactivity towards a natural radical of the microorganism origin, such as fungal, can be

analysed by the bleaching reaction of carotenoid. The strong visible adsorption of a carotenoid is reduced via reaction with radicals, as the suppression of bleaching measures the antioxidant reactivity (Niki et al., 2000). Another chemical evaluation for antioxidant screening is the phosphomolybdate assay, as well as the estimation of total phenolic and total flavonoid contents. Compounds such as flavonoids and phenolic, which contain hydroxyls, are responsible for the radical scavenging activity (Younes, 1981; Karamian & Ghasemlou, 2013). Flavonoids and phenols were reported to be responsible for antibacterial activities (Stampar et. al., 2006). This implied that the membrane is the microbial target whereby hydroxylation induced the antimicrobial activity (Sato et. al., 1996). The reduction of molybdenum complex from Mo (V) to Mo (IV) was used to evaluate the total antioxidant capacity involving the green coloured complex.

2.5 Antimicrobial

2.5.1 Antimicrobial agents

Infectious diseases caused by microorganisms cause about 50 thousand of premature deaths daily. An antimicrobial agent is a substance that kills or inhibits growth, or prevents damage that is due to infectious microorganisms. Since the discovery of antimicrobial activity of penicillin by Alexander Fleming, the field of antimicrobial agent and drug discovery have been largely dominated. Antibiotics are significant biochemicals produced by microorganisms, such as bacteria and fungi, which are widely used in semi-synthetic forms (Sengupta et al., 2013; Smith, 2014). There are varieties of antimicrobial agents but selective toxicity was a concern prior to the consumption of a specific antimicrobial agent against a disease or particular microorganism. It was relatively important to eliminate the bacteria without harming

the host organism. Uncontrolled use of antibiotics, caused by either patients or prescriptions, enhances the bacteria resistance. The increase in bacteria resistance triggered interests in finding new antimicrobial agents from microorganisms. Each class of antimicrobial agents represents a unique mode of action against microorganisms. The mode of action is related to the cellular structure of microorganisms. The difference in the membrane structures of Gram-negative and Gram-positive bacteria affects their antimicrobial resistance mechanisms (Holley & Patel, 2005).

2.5.2 Mode of antimicrobial action

The mode of antimicrobial actions can be categorised into different antimicrobial activities, such as interfering with the cell wall synthesis, inhibiting protein synthesis, inhibiting nucleic acid synthesis or blocking metabolic pathways to inhibit the growth of microorganisms or eliminating them. Antimicrobial agents interrupt the cell wall synthesis by blocking the synthesis of the peptidoglycan layer, which covers the outer surface of the cytoplasmic membrane. On the other hand, the interference of protein synthesis occurs with the inhibition of ribosome function. Bacterial ribosomes contain two subunits of 50S and 30S subunits. Binding to these causes the protein chain termination and protein synthesis termination. However, antimicrobial agents not only interrupt and destabilise the cytoplasmic membrane but also restrict the DNA synthesis by interfering with the purine and pyrimidine syntheses with the interconversion or utilisation of nucleotides. Other agents act as nucleotide analogs that are incorporated into polynucleotides. Antimicrobial agents may also bind to the enzyme gyrase to block the DNA replication (Aytul, 2010).

2.5.3 Antibiotic drugs from *Streptomyces* sp.

At least 7000 different secondary metabolites have been discovered in *Streptomyces* isolates (Berdy, 2005). *Streptomyces* are best known for their synthesis of a vast array of antibiotics, some of which are useful in medicine and agriculture (Watve et al., 2001). They are small molecules between 100 to 3000 Daltons, which are biologically active outside the producer cell. These small molecules of antibiotics inhibit enzymes and cellular processes (Chater et al., 2010). Table 2.1 shows a list of some commercially known antimicrobial compounds produced by *Streptomyces* spp.

Table 2.1 Bioactivity of secondary metabolites of antimicrobial compounds produced from *Streptomyces* (Holley and Patel, 2005).

Antimicrobial compound	Activity	Producer
Cephamycin	Antibacterial	*Streptomyces clavuligerus*
Chloramphenicol	Antibacterial	*Streptomyces venezuelae*
Kanamycin	Antibacterial	*Streptomyces kanamyceticus*
Tetracycline	Antibacterial	*Streptomyces aureofaciens*
Spectinomycin	Antibacterial	*Streptomyces spectablis*
Streptomycin	Antibacterial	*Streptomyces griseus*
Clavulanic acid	Antibacterial	*Streptomyces clavuligerus*
Monensin	Antibacterial / Anticoccidial	*Streptomyces cinnamonensis*
Amphotericin	Antifungal	*Streptomyces nodosus*
Aureofacin	Antifungal	*Streptomyces aureofaciens*
Candicidin	Antifungal	*Streptomyces griseus*
Nystatin	Antifungal	*Streptomyces nourse, Streptomyces aureus*
Oligomycin	Antifungal	*Streptomyces diastachromogenes*
Actinomycin D	Antibacterial / Antitumor	*Streptomyces antibioticus, Streptomyces parvulus*
Mytomycin C	Antibacterial / Antitumor	*Streptomyces lavendulae*

2.6 Application in industries

Actinomycetes are acknowledged for their metabolic versatility that is frequently accompanied by the production of the primary and secondary metabolites of economic importance. The attention given to actinomycetes in biotechnological applications is a natural result of the great metabolic diversity of these organisms and their long association with the environment. This group is a potential producer of antimicrobial substances, enzyme inhibitors, immunomodifiers, enzymes and growth promoting substances for plants and animals. They are not only producers for enzyme inhibitors, which are valuable for cancer treatment and immunomodifiers that enhance immune response but also they have the ability to degrade a wide range of hydrocarbons, pesticides, as well as aliphatic and aromatic compounds (Collins et al., 1995). Members of many genera of actinomycetes have potential in the bioconversion of the under-utilised agricultural and urban wastes into high-value chemical products. Their metabolic potential offers a strong area for researchers in biotechnology and medicine. Actinomycetes have been acknowledged as the greatest source of antibiotics whereby a large fraction of antibiotics in the market was attained from actinomycetes. The antibiotics that are commonly extracted from actinomycetes sources include anthracyclines, β-lactams, aminoglycosides, macrolides, chloramphenicol, tetracyclines, nucleosides, peptides and polyethers (Cross, 1981).

Furthermore, researchers have also discovered the prospects of actinomycetes in biotechnological applications as enzyme inhibitors. More than 60 inhibitors have been reported including leupeptins, which inhibits papain, plasmin and trypsin (Umezawa et al., 1976). An enzyme inhibitor, which is useful in cancer treatment, includes revistin, which inhibits reverse transcriptase produced from *Streptomyces*

species. Additionally, streptonigrin and retrostatin were also synthesised by Streptomycete that inhibit reverse transcriptase. The important enzymes present in actinomycetes include amylase, lipase, gilatinase, chitinase and alkalophilic enzymes. The applications of these enzymes were found to be important in leather industry, photographic industry, medical industry, milk industry, detergent industry and also pharmaceutical industry (Dilip et al., 2013).

CHAPTER 3

METHODOLOGY

3.1 Chemicals and materials

Chemicals purchased include cycloheximide (Sigma-Aldrich, Switzerland); 80 % (v/v) glycerol (QReC® Grade AR); soluble starch (SRL, India); nutrient agar (SRL, India); Muller-Hinton agar (Merck, Germany); tryptone (SRL, India); yeast extract (SRL, India); ISP medium 2 (Difco TM, USA); peptone (SRL, India); NaCl (Merck, Germany); KCl (SRL, India); CaCl (SRL, India); $CaCO_3$ (SRL, India); casein (SRL, India); $MgCl_2.6H_2O$ (Merck, Germany); $MgSO_4.7H_2O$ (Merck, Germany); K_2HPO_4 (R&M, U.K); KNO_3 (Merck, Germany); $MgSO_4.7H_2O$ (SRL, India); $FeSO_4.7H_2O$ (SRL, India); bacteriological agar (Oxoid, U.K); $BaCl_2$ (Merck, Germany); 99.9% concentrated H_2SO_4 (QReC® Grade AR); hydrochloric acid (QReC® Grade AR); ethyl acetate; dimethylsulfoxide; potassium ferricynade (Sigma USA); trichloroacetic acid (Bendozen Chemical); sodium carbonate (Bendosen Chemical); ferric chloride solution (R&M, U.K); linoleic acid solution (Alfa Aesar, US) and Folin-Ciocalteau reagent (FCR) (Sigma, USA).

3.2 Preparation methods

3.2.1 Sterilisation method

All prepared media and reusable apparatus were sterilised using high-pressure steam sterilisation autoclave model SX-500, Japan at 121 °C for 20 minutes. All microbiological procedures were performed aseptically in the Esco Frontier™ laboratory laminar flow cabinet model AVC-401 to prevent contamination.

3.2.2 Media preparation

Artificial seawater, starch casein agar, nutrient agar, Mueller-Hinton agar, tryptone yeast extract media, MI broth and yeast extract starch media were used throughout the experiments. Materials needed for the preparation of medium were listed in Table 3.1 to 3.9 accordingly. Nutrient agar, Yeast extract-starch (ISP2) agar and Mueller-Hinton agar media were ready made agar used for this experiment.

Table 3.1: Artificial seawater (ASW) (Kester et. al., 1967).

Ingredient	Amount (per litre)
NaCl (Merck, Germany)	26.30 g
KCl (SRL, India)	0.70 g
CaCl (SRL, India)	1.00 g
$MgCl_2.6H_2O$ (Merck, Germany)	6.10 g
$MgSO_4.7H_2O$ (Merck, Germany)	4.00 g
pH	7.2 ± 0.2

Table 3.2: Starch casein agar (SCA) (Rao & Tamanam, 2013).

Ingredients	Amount (per litre)
Soluble starch (SRL, India)	10.00 g
K_2HPO_4 (R&M, U.K)	2.00 g
KNO_3 (Merck, Germany)	2.00 g
Casein (SRL, India)	0.3 g
$MgSO_4.7H_2O$ (SRL, India)	0.05 g
$CaCO_3$ (SRL, India)	0.02 g
$FeSO_4.7H_2O$ (SRL, India)	0.01 g
*Bacteriological agar (Oxoid, U.K)	16.00 g
Artificial seawater	0.50 L
Distilled water	0.50 L
pH	7.2 ± 0.2