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SCREENING OF AQUATIC MICROORGANISMS FOR ANTIMICROBIAL METABOLITE PRODUCTION

By

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DECLARATION

I hereby declare that this submission is my own work toward the M. Phil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of a university, except where due acknowledgement has been made in the text.

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ABSTRACT

The search for novel therapeutic agents for use in the pharmaceutical industry is driven by the need to combat the increase in infections due to antibiotic resistant pathogens. Natural products have been the major source of numerous therapeutic agents producing more than half of the drugs in use today. In this study, microorganisms from Lake Bosomtwe, River Wiwi and the Duakor Sea shore were isolated and screened for antimicrobial metabolite production. Out of 119 isolates subjected to antimicrobial screening, activity against at least one test organism was detected for 27 isolates. Three of the strains, which had the highest inhibitory activity (zones of inhibition > 19mm), were selected for further characterization of their antimicrobial properties and optimization of physical and cultural factors for maximum antibiotic production carried out. The strains, MAI 1, MAI 2 and MAI 3 were identified as *Bacillus species* (MAI 1) and *Pseudomonas species* (MAI 2 and MAI 3). The antimicrobial metabolite produced by MAI 1 was heat stable up to 40°C only whereas MAI 2 and MAI 3 metabolites were thermally stable up to 100°C. The metabolites produced by all the three strains deteriorated gradually with time in solution. Results of the optimization procedures for MAI 1 showed that, an incubation period of 9 days, a pH of 8 and a temperature of 25°C were needed for maximum activity of the antimicrobial metabolite. Also glycerol and asparagine produced the best activity as carbon and nitrogen sources respectively for MAI 1. For MAI 2 and MAI 3, an incubation period of 9 days, a pH of 8 and a temperature of 30°C were needed for maximum antibiotic activity. Starch and asparagines were best utilized as carbon and nitrogen source respectively for MAI 2 and MAI 3. Chloroform extract of MAI 2 showed antimicrobial activities with MIC ranging from 250µg/ml to 4000µg/ml. The Thin layer chromatography bio-autography overlay assay showed seven (7) spots with antimicrobial activity, implying that MAI 2 produced more than one antimicrobial agent.

CHAPTER ONE

1.0 INTRODUCTION

Bioactive natural products are chemical compounds isolated or derived from plants, animals and microorganisms that usually have a pharmacological or biological activity. These compounds are typically secondary metabolites of these organisms. Many of these compounds are employed in agriculture as pesticides and in the pharmaceutical industry for drug discovery and design (Berdy, 2005). Natural products are the most consistently successful source of drug leads, both historically and currently. Throughout the ages, they have afforded a rich source of compounds that have found many applications in the fields of medicine, pharmacy and agriculture and are likely to continue to be the sources of new commercially viable drug leads because the chemical novelty associated with natural products is higher than that of any other source such as combinatorial synthesis. Drug discovery from natural products is particularly important when searching for lead molecules against new targets. Natural products can also be a more economical source of chemical diversity compared with synthesis of equivalent numbers of diverse chemicals (Harvey, 2009). Additionally, many natural products that are found to be biologically active are generally small molecules that are capable of being absorbed and metabolised by the body. Hence, development costs to produce orally active medicines are likely to be much lower than with most compounds produced from combinatorial chemistry (Lahana, 1999; Myers, 1997). Since less than 10% of the world's biodiversity is reckoned to have been tested for biological activity (Harvey, 2009), many more useful lead compounds are waiting to be discovered from natural products.

The World Health Organization (WHO) estimated that 80% of the earth's inhabitants mainly depend on traditional medicines for their health care (Farnsworth *et al*., 1985; Fabricant and Farnsworth, 2001). Plants have been the root of traditional medicine that has existed for many years. Some of these plants are still in use today for the treatment of ailments ranging from coughs and cold to parasitic infections and inflammation (Newman *et al.*, 2000).

Since the discovery of penicillin, the first antibiotic in history in 1928, studies on soil bacteria and fungi have shown that microorganisms are a rich source of structurally unique bioactive substances (Fenical, 1993). After Penicillin, many other drugs including chlortetracycline, chloramphenicol, streptomycin, erythromycin, rifamycin, lincomycin, cephalosporin C, vancomycin, erythromycin, nalidixic acid, amphotericin B, nystatin, and daunorubicin the antitumor agent were derived from microorganisms. A new chemical class of antibacterial agents brought recently onto the market are the carbapenems (such as imipenem), which are synthetic compounds based on the structure of the natural product, thienamycin (Demain, 2009). Currently there are reports of treatment failures (Zetola *et al*., 2005) due to the fact that some pathogens implicated in infectious diseases have developed resistance against antibiotics in current use. This has therefore presented the need to search for new effective antimicrobial drugs and history appears to favour microorganisms as being the key to novel compounds which may be useful in antimicrobial drug development.

1.1 Problems in the management of infectious diseases

Infectious diseases are caused by pathogenic microorganisms such as bacteria, viruses, fungi and parasites. Looking back on the history of human diseases, infectious diseases have

accounted for a very large proportion. It was not until the latter half of the 19th century that microorganisms were found to be responsible for causing these diseases. Accordingly, chemotherapy aimed at the causative organisms was developed as the main therapeutic strategy. The discovery of penicillin by Alexander Fleming, followed by streptomycin, tetracycline and other antimicrobials, provided physicians with effective therapeutic options against a range of bacterial diseases such as tuberculosis and sexually transmitted diseases (such as syphilis and gonorrhoea) and this helped to markedly reduce human morbidity and mortality from most of these diseases. From the time antibiotics were first used, scientists observed the development of resistance; however, the subsequent universal dependence on the curative powers of these agents led the scientific and medical community to underestimate the potential scale of the problem. By the 1970s it was widely believed that infectious diseases were finally under control (Davies, 2007).

While infectious diseases were considered virtually conquered, many pathogenic organisms were becoming resistant to virtually every clinically available drug (Greenberg, 2003). Activities such as the use of antimicrobial drugs for prophylactic or therapeutic purposes in human and veterinary or for agricultural purposes, has provided the selective pressure favouring the survival and spread of these resistant organisms. The resistant bacteria that have developed over the years are generally uninhibited by most commercial antibiotics. *Enterococci* resistant to all antibiotics have arisen (Morris *et al*., 1998). There has also been a dramatic increase, worldwide, in the prevalence of penicillin-resistant *Streptococcus pneumoniae* (PRSP) (Goldstein and Garau, 1997) the most common cause of bacterial pneumonia. Infections due to Methicillin-resistant *Staphylococcus aureus* (MRSA) has also increased to an alarming extent throughout the world (Goldstein and Garau, 1997). The appearance of multi-resistant pathogenic strains of bacteria has caused substantial increase in

morbidity and mortality especially among the elderly and immunocompromised patients. In addition to the problem of antibiotic resistance, toxicity of some of the antibiotics, e.g. ototoxicity and nephrotoxicity caused by aminoglycosides as well as the high cost of some of the antibiotics has made it difficult for these drugs to be used properly (Brumfitt and Hamilton-Miller, 1988). In order to meet the growing challenges of antibiotic resistance among pathogens, the discovery and development of new antibiotics and alternative treatments for infectious diseases, together with tools for rapid diagnosis that will ensure effective and appropriate use of existing antibiotics, are imperative. How the health services, pharmaceutical industry and academia respond in the coming years will determine the future of treating infectious diseases.

1.2 Potential of microorganisms as source of new anti-microbial agents

Microbial natural products have been the source of most of the antibiotics currently in use and still remain the main source of the future antibiotics for the treatment of infectious diseases that society is expecting. Until recently, the majority of antimicrobial compounds have been isolated from terrestrial microorganisms. For example, amphotericin B from *Streptomyces nodosus* (Abu-Salah, 1996), chlortetracycline from *Streptomyces aureofaciens* (Darken *et al*., 1960) and gentamycin from *Micromonospora purpurea* (Abou-Zeid *et al*., 1972)**.** During the last two decades, the rate of discovery of novel compounds from this source has declined significantly, as exemplified by the fact that extracts from soil-derived actinomycetes have yielded high numbers of clinically unacceptable metabolites (Mincer *et al.*, 2002). However, the aquatic environment is becoming increasingly appreciated as a rich untapped reservoir of novel natural products. The marine environment alone is known to contain taxonomically diverse bacterial groups which exhibit unique physiological and

structural characteristics that enable them to survive in extremes of pressure, salinity and temperature, with the potential production of novel secondary metabolites not observed in terrestrial microorganisms (Radajewski *et al*., 2002). In the last decade, the number of reported secondary metabolites from marine bacteria and fungi has steadily increased (Fenical, 1993); In the year 2007 alone 961 new compounds with activities such as anti-infective, anticancer and anti-inflammatory were isolated from marine microorganisms reflecting an increase of 24% compared with the number of compounds, (775) reported for 2006 (Blunt *et al.*, 2009). Most of the research attention is directed on sediment derived microorganisms and those that form highly specific symbiotic associations with aquatic plants and animals in response to the scarcity of nutrients in these environments, and thus produce compounds for defence and competition (Jensen and Fenical, 1994). The aquatic microbial flora appears to serve as a source of structurally diverse and valuable bioactive agents that are providing leads for antimicrobial drug development. Some of these agents such as pestalone, hypoxysordarin and equisetin have shown promising antibacterial, antifungal and antiviral activities respectively and are probable candidates for clinical trials (Bhadury *et al*., 2006).

In Ghana there is a diverse array of aquatic habitats. These water bodies are reservoirs of many different kinds of microorganisms which have not been adequately exploited for bioactive natural products. This research therefore seeks to screen microorganisms isolated from Ghanaian aquatic habitats for antimicrobial metabolites production.

1.3 Aim of study

To investigate the antimicrobial metabolite producing properties of microorganisms isolated from some water bodies in Ghana.

1.4 Specific objectives

- To select some water bodies in Ghana for sampling for aquatic microorganisms.
- To cultivate, isolate and screen aquatic microorganisms, for antimicrobial metabolite producing properties.
- To optimize conditions for maximum activity of antimicrobial compound.
- To ferment and extract compounds from the fermentation medium of selected microorganisms.
- To screen the crude extract for antimicrobial activity
- To identify the antimicrobial metabolite producing microbes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bioactive secondary metabolites

Secondary metabolites are organic compounds produced by organisms which are not directly involved in the normal growth, development, or reproduction of the organism. Studies have however shown that secondary metabolites serve as a competition mechanism for the producing organisms as well as regulators of cellular differentiation processes (Demain and Fang, 2000). Plant, animal and microbial secondary metabolites have been a rich source of drug leads for centuries. Plant secondary metabolites such as morphine, quinine and cocaine have helped mankind, serving both as drugs and as leads for the synthesis of other drugs such as local anaesthetics from cocaine. Clinically useful drugs which have been recently isolated from plants include the anticancer agent paclitaxel (Taxol) from the yew tree (*Taxus brevifolia*), and the antimalarial agent artemisinin from *Artemisia annua* (Prasad and Dhanapal, 2010). Microorganisms such as bacteria and fungi have been invaluable in the discovery of drugs and lead compounds for drug development. The screening of microorganisms became highly popular after the discovery of penicillin and this led to the discovery of many drugs including, the cephalosporins, tetracyclines, aminoglycosides, rifamycins, and chloramphenicol. In recent years, there has been a great interest in finding lead compounds from marine sources (Wang *et al*., 2009). Marine microorganisms represent a promising source for natural products of the future due to the incredible diversity of chemical compounds that have been isolated. A number of these compounds with activities such as antimicrobial, anticancer and anti-inflammatory are currently in clinical trials (Rawat *et al*., 2006).

2.2 Bioactive compounds from marine actinomycetes

Terrestrial actinomycetes are one of the most efficient groups of secondary metabolite producers. They are responsible for the production of about half of the discovered microbial bioactive secondary metabolites (Berdy, 2005), notably antibiotics, antitumor agents, immunosuppressive agents and enzymes. However the marine actinomycetes are also becoming increasingly appreciated as a rich source of novel bioactive agents. Various novel compounds with biological activities including antifungal, antibacterial and antiviral have been isolated from marine actinomycetes genera: *Streptomyces, Saccharopolyspora, Amycolatopsis, Micromonospora* and *Actinoplanes* (Solanki *et al.*, 2008). Some of the recent compounds are:

Abyssomicin C (**1**), a polycyclic polyketide antibiotic produced by a marine Verrucosispora strain. This compound is a potent inhibitor of para-aminobenzoic acid biosynthesis and, therefore, inhibits the folic acid biosynthesis at an earlier stage than the well-known synthetic sulphonamides (Bister *et al.*, 2004). Abyssomicin C is highly active against gram-positive bacteria, including the multi-drug resistant and vancomycin-resistant *Staphylococcus aureus* (Rath *et al*., 2005).

Compounds such as lipoxazolidinone A, B and C (**2-4**) isolated from a bacterium of the genus *Marinispora* (strain NPS008920) showed broad spectrum antimicrobial activities similar to those of the commercial antibiotic linezolid (Barbachyn and Ford, 2003).

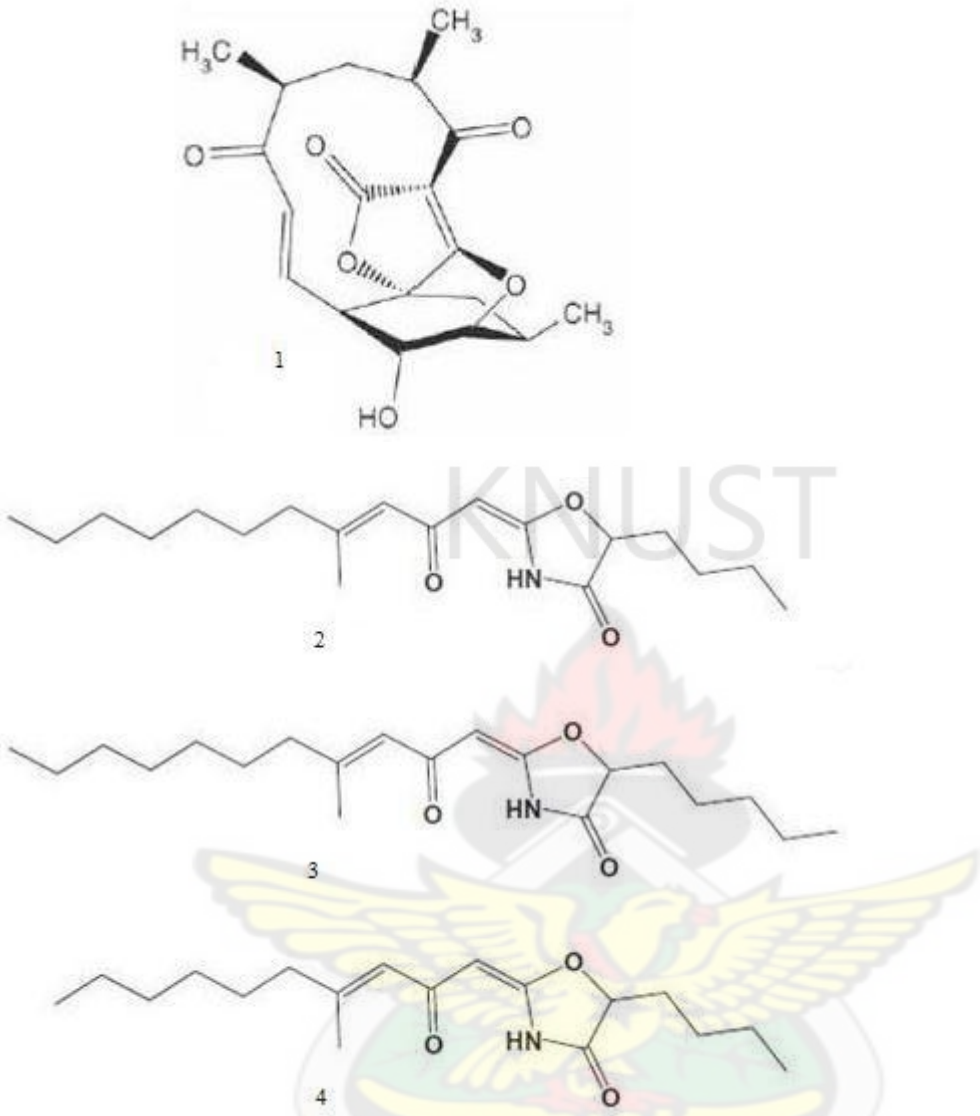


Fig. 2.1 The structures of Abyssomicin C (1) and lipoxazolidinone A (2), B (3) and C (4)

Another marine actinomycete, NPS12745, isolated from sediments off the coast of San Diego, California yielded chlorinated bisindole pyrroles; Lynamicins A and B (**5-6**) and they exhibited activity against *Enterococcus faecalis*, *Staphylococcus epidermidis* and *Staphylococcus aureus* including the methicillin-resistant strains, suggesting potential for treatment of nosocomial infections (McArthur *et al.*, 2008). The extract of *Nocardia* sp. ALAA 2000, isolated from the marine red alga *Laurenica spectabilis* collected from Ras-Gharib coast of the Red Sea in Egypt, was also found to be active against pathogenic

microorganisms with MIC values ranging from 0.1 to 10 µg/ml. Bioassay guided isolation of this crude extract afforded the new bioactive compound ayamycin [1,1-dichloro-4-ethyl-5-(4-nitrophenyl)- hexan-2-one] (**7**) which exhibited activity against both gram-positive and gram-negative bacteria as well as pathogenic fungi such as *Candida albicans, Aspergillus niger* and *Botrytis fabae* (El-Gendy *et al.*, 2008).

The crude extract of the actinomycete strain CNQ-418 isolated from a marine sediment sample collected near La Jolla, California, exhibited strong antibiotic activity. Two products marinopyrroles A and B (**8**-**9**), were isolated which displayed noteworthy activity against methicillin-resistant *Staph. aureus* (Hughes *et al.*, 2008). Other compounds including frigocyclinone (Bruntner *et al.,* 2005), glaciapyrroles (Macherla *et al.,* 2005), gutingimycin (Maskey *et al.*, 2004) and himalomycins (Maskey *et al.*, 2003) isolated from *Streptomyces* species have also been shown to have antibacterial activity.

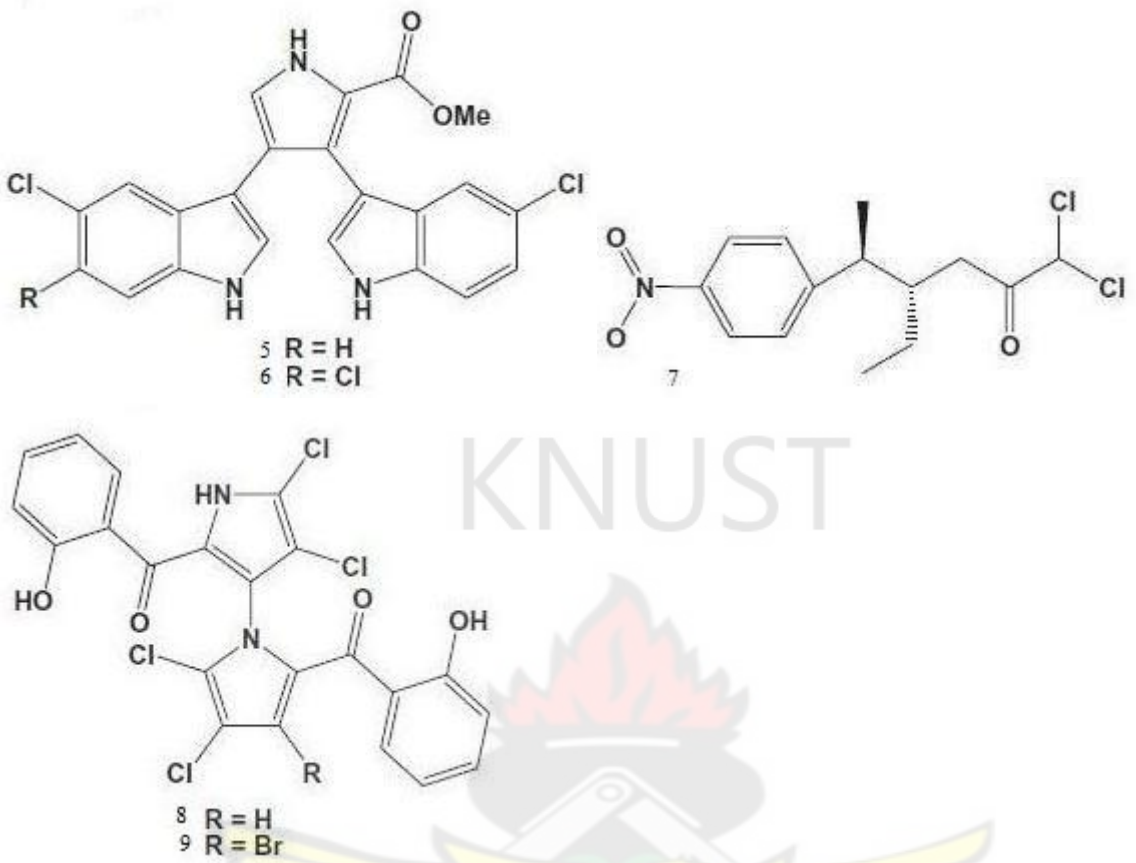


Fig. 2.2 The structures of Lynamicins A (5) and B (6), ayamycin (7) and Marinopyrroles A (8) and B (9)

2.3 Marine Pseudomonas

Pseudomonas is a genus of non-fermentative gram-negative *Gamma proteobacteria* found both on land and in water. Many terrestrial isolates of this genus have been studied extensively for bioactive substances, enzymes, and biosurfactants, whilst only a few marine isolates have been described that produce novel bioactive substances. The first marine isolate of *Pseudomonas* known to produce a bioactive substance was from *Thalassia* (turtle grass) near La Parguera, Puerto Rico (Burkholder *et al.*, 1966). The bacterium, *P. bromoutilis*, produced a pyrrole antibiotic, 2,3,4-tribromo 5(1hydroxy,2,4 dibromophenyl) pyrrole that in

in vitro studies inhibited gram-positive bacteria such as *Staph. aureus*, *Diplococcus pneumoniae*, and *Streptococcus pyogenes* at a concentration of 0.0063µg/ml, and *Mycobacterium tuberculosis* at 0.2µg/ml. This substance was not toxic in mice at 25 or 250 mg/kg by intravenous and subcutaneous injection, respectively. However, subcutaneous injection of 200 mg/kg of this pyrrole antibiotic did not protect mice infected with *Staph. aureus*. This was the first effort to explore the bioactivity of a substance from a marine isolate of *Pseudomonas* (Isnansetyo *et al.*, 2009).

A *Pseudomonas* species 102-3 isolated by Wratten *et al*. (1977) in California, produced three compounds: 4-hydroxybenzaldehyde (**10**), 2-*n*-heptyl-4-quinolinol (**11**), and 2-*n*-pentyl-4-quinolinol (**12**) which exhibited antimicrobial activity against *C. albicans*, *Staph. aureus*, *V. Harveyi* and *V. Anguillarum*.

The marine isolate *P. fluorescens*, from the surface of tunicates, have been reported to produce three unique substances, moiramides A (**13**), B (**14**), and C (**15**), along with a known compound, andrimid (**16**), a pseudopeptide pyrrolidinedione antibiotic (Needham *et al.*, 1994). Compounds 14 and 16 both have broad-spectrum antibacterial activity. Their mode of action is through inhibition of bacterial acetyl-CoA carboxylase (Freiberg *et al.*, 2004). *Pseudomonas* species 1531-E7 isolated from a sponge, *Homophymia* species, produced quinolones (2-undecyl-4-quinolone (**17**), 2-undecen-18-yl-4-quinolone (**18**), 2-nonyl-4-quinolone (**19**), and 2-nonyl-4-hydroxyquinoline *N*-oxide (**20**) (Bultel-Ponce *et al.*, 1999). Compounds 17, 18 and 19 were found to exhibit anti-*Plasmodium falciparum* activity and 17 and 20 showed antimicrobial activity against HIV-1 and *Staph. aureus.*

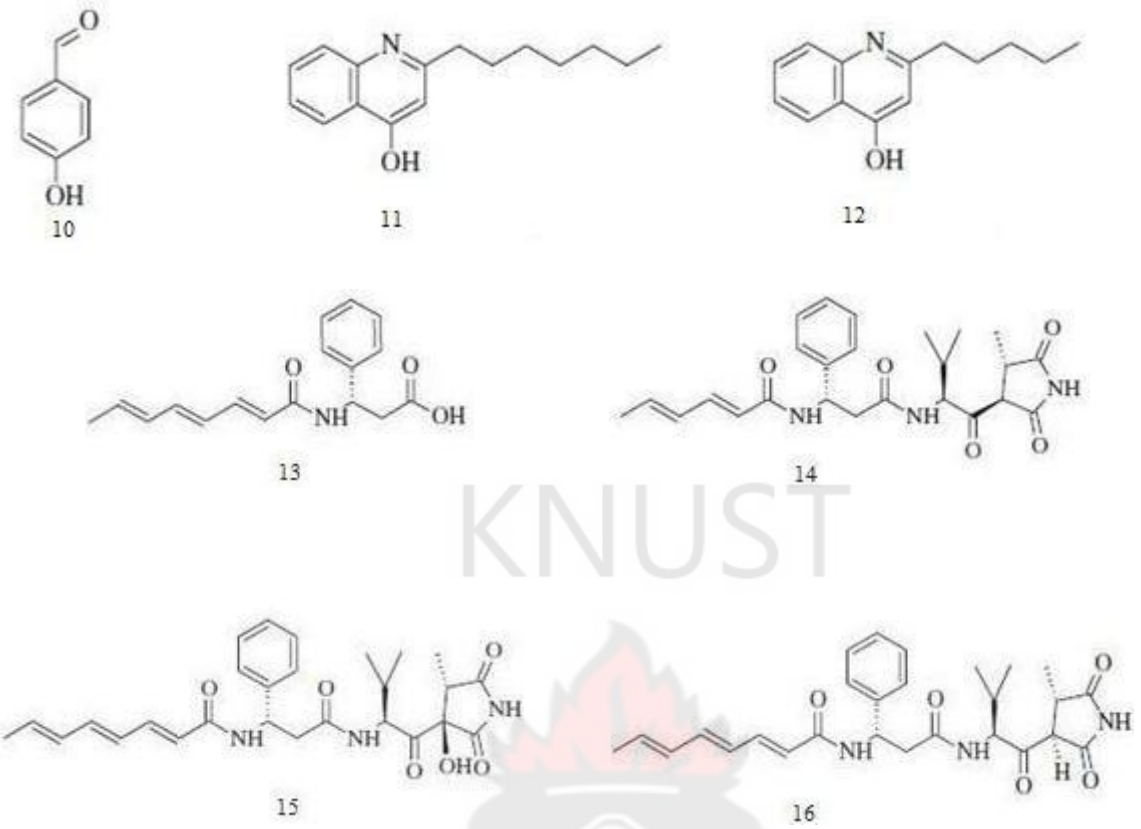


Fig. 2.3 The structures of 4-hydroxybenzaldehyde (10), 2-*n*-heptyl-4-quinolinol (11), 2-*n*-pentyl-4- quinolinol (12), moiramides A (13), B (14) and C (15) and andrimid (16)

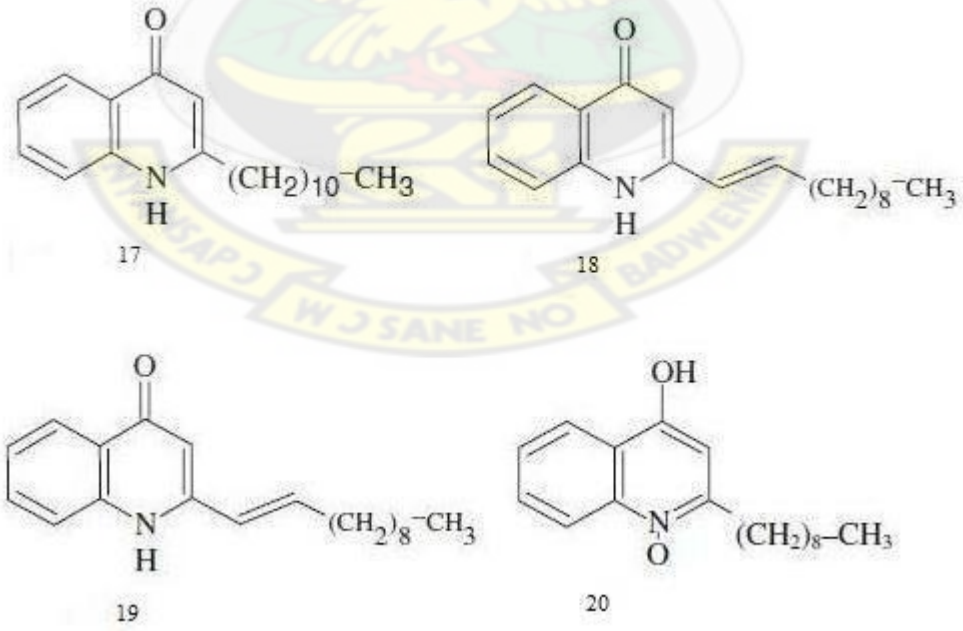


Fig. 2.4 The structures of, 2-undecyl-4-quinolone (17), 2-undecen-18-yl-4-quinolone (18), 2-nonyl-4-quinolone (19) and 2-nonyl-4-hydroxyquinoline *N*-oxide (20).

Isnansetyo *et al*. (2001) purified the antibiotic 2, 4-diacetylphloroglucinol (DAPG) (**21**) from the culture supernatant of *Pseudomonas* species AMSN, which was isolated from a marine alga. This antibiotic exhibited potent activity against methicillin-resistant *Staph. aureus* (MRSA), vancomycin-resistant *Staph. aureus* (VRSA) and vancomycin-resistant *Enterococcus* with MICs ranging between 0.25–8 µg/ml. *Pseudomonas aeruginosa* isolated from an antarctic sponge, *Isodictya setifera*, produced six diketopiperazines and two phenazine alkaloids (Jayatilake *et al.*, 1996). The two phenazine alkaloids were active against *Bacillus cereus, Micrococcus luteus*, and *Staph. aureus*. *Pseudomonas* species WAK-1 isolated from the brown seaweed *Undaria pinnati- fida* also produced a sulfated polysaccharide which was active against herpes simplex virus 1 (HSV-1) (EC_{50} = 1.4 µg/ml) (Matsuda *et al.*, 1999).

Uzair *et al*. (2008) reported that the marine *Pseudomonas* species CMG1030 produced the novel antibacterial compound zafrin [4b-methyl-5,6,7,8 tetrahydro-1(4b-H)-phenanthrenone] (**22**) which has activity against *Staph. aureus* and *S. typhi*, with MICs ranging from 50 to 125µg/ml. The organism also produced another broad spectrum antibiotic bushrin (7-(3-furyl)-3,7-dimethyl-7,8-dihydro-1 naphthalenol) (**23**), with MICs ranging from 50 to 125µg/ml. Studies of the mechanism of action revealed that the substances exert their bactericidal effects by bacterial cell lysis.

There are several marine *Pseudomonas* species with antimicrobial activity that is due to substances that have not yet been isolated or characterized. For example, marine *Pseudomonas* strain I-2, isolated from estuary water, produced a non-proteinaceous substance which has anti-vibrio activity (Chythanya and Karunasagar, 2002). Also the extract of

Pseudomonas species PB2 associated with the sponge, *Suberites domuncula*, exhibited antimicrobial activity against *Staph. aureus*, *S. epidermidis*, *S. lentus*, *E. coli*, and *Candida albicans* (Thakur *et al.*, 2005). Likewise, Radjasa *et al*. (2007) isolated *Pseudomonas* species associated with the soft coral *Sinularia polydactyla*. The bacterium produced a substance that exhibited antibacterial activity against *Streptococcus equi* subsp. zooepidemics, but the substance responsible for the antibacterial activity has not been identified.

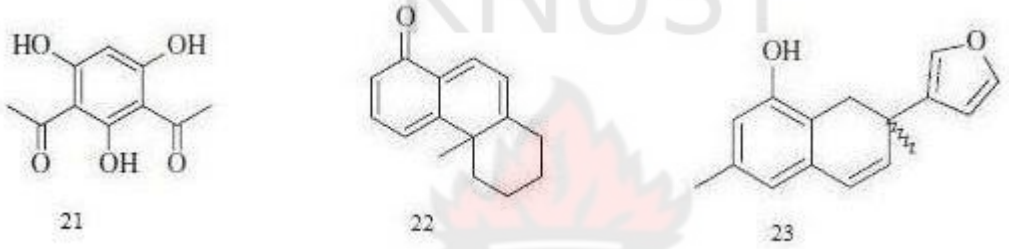


Fig. 2.5 The structures of DAPG (21), zafrin (22) and bushrin (23)

2.4 Marine Bacillus

A number of bioactive compounds have also been isolated from marine *Bacillus* species. An example is tauramamide (**24**), a lipopeptide isolated from the marine bacteria *Brevibacillus laterosporus* PNG276 obtained from Papua New Guinea. This compound was found to be active against *Enterococcus* species and multidrug-resistant *Staph. aureus* (Desjardine *et al.*, 2007).

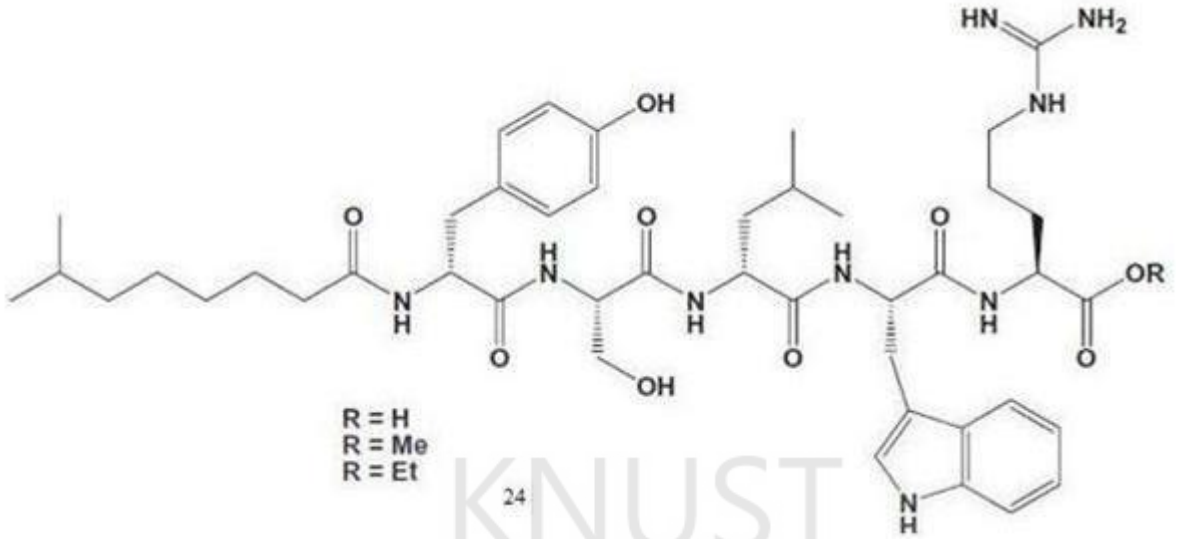


Fig. 2.6 The structure of Tauramamide with its methyl and ethyl esters

2.5 Marine Fungi

Fungi growing in the sea can be grouped into obligate and facultative marine fungi. Obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat. On the other hand, facultative marine fungi are those from fresh water or terrestrial areas that have undergone physiological adaptations that allow them to grow and possibly also sporulate in the marine environment (Kohlmeyer, 1974). Marine fungi have proven to be a rich and promising source of diverse novel antibacterial, antifungal and antiviral agents (Fenical, 1997). Currently, over 272 new compounds have been isolated from marine fungi (Bugni and Ireland, 2004). Pestalone, a chlorinated benzophenone isolated from the marine fungus *Pestalotia* species showed potent antibiotic activity against methicillin- resistant *Staph. aureus* and vancomycin-resistant *Enterococcus faecium*, indicating that it could be evaluated further in advanced models of infectious disease (Cueto *et al.*, 2001).

An *Aspergillus* species (family *Trichocomaceae*) isolated from the surface of the marine brown alga *Sargassum horneri* collected at Gadeok Island, Busan, Korea yielded a

polyoxygenated decalin derivative, dehydroxychlorofusarielin B (**25**) which was found to exhibit antibacterial activity against *Staph. aureus* including methicillin-resistant and multidrug-resistant *Staph. aureus* (Nguyen *et al.*, 2007). Another fungi of the genus *Exophiala* (family *Herpotrichiellaceae*) isolated from the surface of a marine sponge *Halichondria panicea* afforded the new aspyrone derivatives chlorohydroaspyrones A (**26**) and B (**27**) which were active against *Staph. aureus* including the methicillin-resistant and multidrug-resistant strains (Zhang *et al.*, 2008).

Another compound, speradine A, isolated from the marine fungus *Aspergillus tamarii*, showed antibacterial activity against *Mycrococcus luteus* (MIC 16.7 µg/ml) (Tsuda *et al*., 2003a). Two new compounds, xanalteric acids I and II (**28** and **29**), have been isolated from the fungus *Alternaria* species, which was isolated from fresh leaves of the mangrove plant *Sonneratia alba* (*Sonneratiaceae*). The compounds exhibited antibiotic activity against multidrug-resistant *Staph. aureus* (Kjer *et al.*, 2009). Zopfiellamide A, a pyrrolidinone derivative isolated from the facultative marine ascomycete, *Zopfiella latipes*, inhibited gram-positive and gram-negative bacteria, with MICs ranging between 2 and 10 µg/ml (Daferner M *et al.*, 2002).

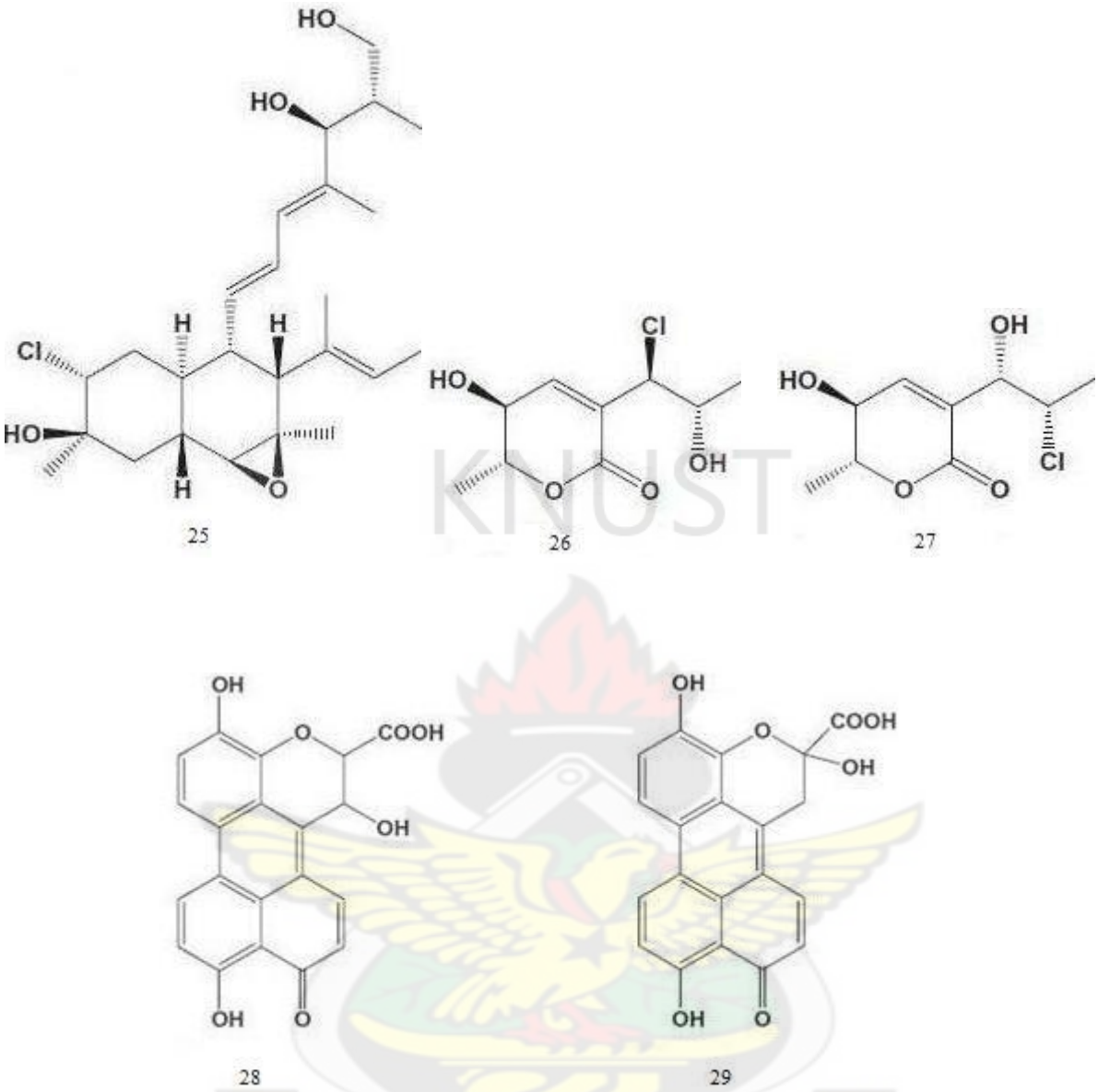


Fig. 2.7 The structures of Dehydroxychlorofusarielin B (25), Chlorohydroaspyrones A (26) and B (27) and Xanalteric acids I (28) and II (29)

Nigrospora species isolated from the Similan Island of Thailand, produced four new metabolites named nigrospoxydons A–C and nigrosporapyrone, together with nine known compounds. The crude ethyl acetate extract showed antibacterial activity against *Staph. aureus* ATCC 25923 and MRSA, with MIC values of 64 and 128 µg/ml respectively (Trisuwan *et al.*, 2008). *Ascochyta* species NGB4, collected at a fishing port in Japan, yielded

a novel compound named ascochytatin which exhibited activity against gram-positive bacteria and *Candida albicans* (Kanoh *et al.*, 2008). The fermentation broth of the fungus *Cladosporium* species F14 also yielded nine compounds including cinnamic acid, cyclo-(Phe-Pro) and cyclo-(Val-Pro). These compounds showed antibacterial activity against *Loktanella hongkongensis* (UST950701-009), *Micrococcus luteus* (UST950701-006) and *Ruegeria* species (UST010723-008) (Qi *et al*., 2009).

Several compounds from marine fungi have also been screened for antifungal activities Hypoxysordarin, isolated from the facultative marine fungus *Hypoxylon croceum* and1-Hydroxy-6-methyl-8-(hydroxymethy) xanthone isolated from *Ulocladium botrytis* have shown potent antifungal activities (Christie *et al.*, 1997). A new antifungal antibiotic, YM-202204, found in the culture broth of marine fungus *Phoma* species Q60596 (Nagai *et al.*, 2002) exhibited potent antifungal activity against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigates* (Nagai *et al.*, 2002). Also another compound, YM-215343 found in the culture extract of *Phoma* species QN04621 showed antifungal activity against *C. albicans*, *C. neoformans* and *Aspergillus fumigates* with MIC values of approximately 2–16 µg/ml (Shibazaki *et al.*, 2004). Keisslone, isolated from the fungus *Keissleriella* species exhibited MICs of 50, 70 and 40 µg/ml against *Candida albicans*, *Trichophyton rubrum* and *Aspergillus niger* respectively (Liu *et al*., 2003b).

Trichodermamide B, a dipeptide based compound isolated from the marine fungus *Trichoderma virens* has also shown antifungal activity against amphotericin resistant *C. albicans* with MIC value of 15 µg/ml (Garo *et al.*, 2003). A marine-derived *Penicillium* species PSU-F44 isolated from the sea fan (*Annella* species) yielded two novel compounds; penicipyrone and penicilactone, together with three known macrolides, (+)-brefeldin A,

(+)-brefeldin C and 7-oxobrefeldin A which were active against methicillin-resistant *Staph. aureus* (Trisuwan *et al.*, 2009). Three new fungal polyketide metabolites, chaetocyclinones A–C, were obtained from cultures of *Chaetomium* species (strain Gö 100/2). Chaetocyclinone A exhibited an inhibitory activity against *Phytophthora infestans* (Lösgen *et al.*, 2007).

The search for antiviral compounds from marine fungi has also yielded some promising results. Compounds like equisetin, phomasetin and integric acid obtained from *Fusarium heterosporum*, *phoma* species and *xylaria* species respectively have shown significant anti-HIV activities in *in vitro* experiments (Tziveleka *et al.*, 2003). Sansalvamide A, a cyclic depsipeptide isolated from the marine fungus *Fusarium* species was found to inhibit the topoisomerase of the poxvirus *Molluscum contagiosum* (MCV) (Hwang *et al.*, 1999). A series of novel linear peptides halovirs A–E isolated from the marine fungus *Scytidium* species have also shown potent antiviral activity against Herpes Simplex Virus 1 and 2 (HSV-1 and HSV-2) (Rowley *et al.*, 2003). The mode of action is still not clear; however it is presumed that halovirs render HSV non-infectious by possible membrane destabilization (Rowley *et al.*, 2003).

Stachyflin, a novel terpenoid isolated from the fungus *Stachybotrys* species RF-7260 showed in vitro antiviral activity against influenza A virus (H1N1) with an IC_{50} value of 0.003 µM, which is significantly better than other antivirals such as amantadine and zanamivir (Minagawa *et al.*, 2002). Its antiviral activity is mediated through the inhibition of fusion between the viral envelope and the host cell membrane (Baker and Alvi, 2004). Two new compounds asperxanthone (difuranxanthone) and asperbiphenyl, obtained from a strain of *Aspergillus* species MF-93 isolated from sea water in China showed inhibitory activity against tobacco mosaic virus of the tobamovirus group (Wu *et al.*, 2009).

2.6 Cytotoxic secondary metabolites

Aside the search for antimicrobials, a number of agents with anticancer properties have also been isolated. Apralactone A and six other curvularin macrolides have been isolated from *Curvularia* species (strain no. 768). The most active metabolite (+)-(10*E*,15*R*)-10,11-dehydrocurvularin has been shown to be selective for nine tumor cell lines including; bladder cancer, glioblastoma, lung adenocarcinoma, mammary cancer, melanoma, ovarian cancer and prostate cancer (Greve *et al.*, 2008). Compounds (*Z*)-6-benzylidene-3-hydroxymethyl-1,4-dimethyl-3-methylsulfanylpiperazine-2,5-dione and (3*S*,3′*R*)-3-(3′-hydroxybutyl)-7-methoxyphthalide, isolated from a fungus of the order Pleosporales (strain CRIF2) exhibited cytotoxic activity against a panel of cancer cell lines (Prachyawarakorn *et al.*, 2008).

Spicellamide A and spicellamide B, new cyclohexadepsipeptides from the marine fungus *Spicellum roseum* was found to be active against rat neuroblastoma B104 cell line (Kralj *et al.*, 2007). The marine-derived fungus *Massarina* species (strain CNT- 016) also yielded two new secondary metabolites named spiromassaritone and massariphenone which were found to be active against human colon carcinoma (Abdel-Wahab *et al.*, 2007). The fungus *Aspergillus carbonarius* afforded two new secondary metabolites, carbonarones A and B. Both compounds exhibited anti-proliferative activity against K562 cell lines with EC_{50} values of 244.54 and 121.39 µM, respectively (Zhang *et al.*, 2007). The crude ethyl acetate extract of the fungus *Aspergillus ustus* isolated from the marine sponge *Suberites domuncula*, displayed cytotoxic activity against the murine lymphoma cell line L5178Y (Liu *et al.*, 2009).

The fungus *Petriella* species isolated from the sponge *S. domuncula* yielded the cyclic tetrapeptide WF-3161 which was strongly cytotoxic against the L5178Y mouse lymphoma cell line (Proksch *et al.*, 2008). The marine-derived fungus *Aspergillus aculeatus* CRI323-

04A, also yielded a new tyrosine derived metabolite, aspergillusol A, which displayed cytotoxic activity towards acute lymphoblastic leukaemia, human lung cholangiocarcinoma, and A549 cell lines with EC_{50} values of 19, 50 and 74 µM respectively (Ingavat *et al*., 2009). Aspergiolide A, an anthraquinone derivative isolated from cultures of the marine-derived fungus *Aspergillus glaucus* selectively inhibited the proliferation of A549, HL-60, BEL-7402 and P388 cancer cell lines (Du *et al*., 2007). Recently, animal tests with mice indicated that aspergiolide A also inhibited tumour growth *in vivo* (Sun *et al*., 2009).

A marine-derived isolate of the common terrestrial fungus, *Aspergillus versicolor* (MST-MF495), recovered from a beach sand sample in Western Australia yielded a new alkaloid, cottoquinazoline A and two new cyclopentapeptides, cotteslosins A and B which exhibited cytotoxic activity against human melanoma and breast cancer (Fremlin *et al.*, 2009). *Pestalotiopsis* species, obtained from the fresh healthy leaf material of *Rhizophora mucronata* (*Rhizophoraceae*) collected in Dong Zhai Gang-Mangrove Garden in China, yielded a number of new compounds including pestalotiopsone F which exhibited cytotoxicity against the murine cancer cell line L5178Y (Xu *et al.*, 2009). Chromanone A, a new chromone derivative isolated from the fungus, *Penicillium* species, showed potent radical scavenging activity against hydroxyl radicals starting from a dose of 45.45 µM (Gamal-Eldeen, 2009).

2.7 Antidiabetic secondary metabolites

Some anti-diabetic agents have also been isolated. An example is aquastatin A obtained from the marine-derived fungus *Cosmospora* species SF-5060, isolated from an inter-tidal sediment collected in Korea. The compound exhibited potent inhibitory activity against protein tyrosine phosphatase 1B (PTP1B) with an EC_{50} value of 0.19 µM. Protein tyrosine

phosphatases (PTPs) constitute a large family of enzymes, which are responsible for modulation of tyrosine phosphorylation-dependent cellular events. Studies demonstrated that PTP1B, an intracellular non-receptor type PTP, negatively regulates insulin- and leptin-receptor mediated signalling pathways. Thus, its inhibition may represent an outstanding, novel therapy for type II diabetes and obesity. In addition, hydrolyzing studies of the compound suggested that the dihydroxypentadecyl benzoic acid moiety present in the molecule was responsible for the inhibitory activity (Seo *et al.*, 2009).

2.8 Bioassay techniques

2.8.1 Antimicrobial bioassays

Antimicrobial activity of extracts and pure compounds can be detected by observing the growth response of various microorganisms to samples that are placed in contact with them. Several methods for detecting activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method. Among the methods considered valuable for antibacterial and antifungal tests are the dilution and diffusion techniques. Many research groups have modified these methods for specific samples, such as essential oils and non-polar extracts and these small modifications make it almost impossible to directly compare results (Cos *et al*., 2006).

2.8.1.1 Dilution methods

In the dilution methods, test compounds are mixed with a suitable medium that has previously been inoculated with the test organism. It can be carried out in liquid as well as solid media. Usually serial dilutions of the test sample are made in the culture media and then inoculated with the test organism. After incubation, growth of the microorganism may be determined by direct visual or turbidimetric comparison of the test culture with a control

culture which did not receive the sample being tested, or by plating out both test and control cultures. In the agar-dilution method, the Minimal Inhibitory Concentration (MIC) is usually the lowest concentration able to inhibit any visible microbial growth (Cos *et al*., 2006). In liquid or broth-dilution methods, turbidity and redox indicators are most frequently used. Turbidity can be estimated visually or obtained more accurately by measuring the optical density at 405 nm. The liquid-dilution method also allows to determine whether a compound or extract has a microbicidal or microbistatic action at a particular concentration. The minimal bactericidal or fungicidal concentration (MBC or MFC) is determined by plating-out samples of completely inhibited dilution cultures and assessing growth (static) or otherwise (cidal) after incubation. At present, the redox indicators, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and resazurin are frequently used to detect bacterial (Gabrielson *et al.*, 2002) and fungal growth (Jahn *et al.*, 1995). In general, dilution methods are appropriate for assaying polar and non-polar extracts or compounds for determination of MIC and MBC/MFC values. Using redox indicators or turbidimetric endpoints, dose–response effects allow calculation of IC_{50}- and IC_{90}-values, which are respectively the concentrations required to produce 50 and 90 % growth inhibition.

2.8.1.2 Agar-diffusion methods

In the agar diffusion technique, a reservoir containing the test compound at a known concentration is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir (inhibition diameter) is measured at the end of the incubation period. The inoculated system is kept at a lower temperature for several hours before incubation to favour compound diffusion over microbial growth, thereby increasing the inhibition diameter. Different types of reservoirs can be used, such as filter paper discs, stainless steel cylinders placed on the surface and holes punched in the medium. To ensure

that the sample does not leak under the agar layer, fixed agar is left on the bottom of the hole (Cole, 1994). Generally, the relative antimicrobial potency of different samples may not always be compared, mainly because of differences in physical properties, such as solubility, volatility and diffusion characteristics in agar (Cos *et al*., 2006).

2.8.1.3 Bio-autographic methods

Bio-autography localizes antimicrobial activity on a chromatogram using three approaches: (a) direct bio-autography, where the microorganism grows directly on the thin-layer chromatographic (TLC) plate, (b) contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and (c) agar overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate. These procedures are based on the agar diffusion technique, whereby the antimicrobial agent is transferred from the thin layer or paper chromatogram to an inoculated agar plate through a diffusion process. Zones of inhibition are then visualized by appropriate vital stains such as p-iodonitrotetrazolium chloride (Hamburger and Cordell, 1987).

2.8.1.4 Antiviral assays

The viral screening bioassays involve the detection of inhibition of the visible cytopathogenic effect (CPE) on monolayer tissue culture cells after inoculation of the cells with a pre-titred virus suspension and incubation in a maintenance medium containing the extract or test component. A bio-autoghraphic assay to screen extracts for antiviral agents involves overlaying *Herpes simplex* virus (HSV)–infected CV-1(monkey kidney) cells with a developed TLC plate. Time is allowed for diffusion of the compounds from the plate into the tissue culture. After incubation, areas of viral growth inhibition caused by the active compounds are looked out for. This assay also gives indication of cytotoxicity by showing

inhibition of the CV-1 cells. During the evaluation of antiviral agents, many test conditions such as cell culture, virus strain, virus challenge dose and time of harvesting can affect the test results (Tan *et al.*, 1991).

2.8.2 Anticancer assays

2.8.2.1 Brine shrimp bioassay

The brine shrimp bioassay is a technique capable of detecting a broad spectrum of bioactivity, including cytotoxicity and pesticidal activity (Evieyer *et al*, 1982). The eggs of *Artemis salina* (crustacean) when placed in brine solution hatch within 48 hours into shrimps. Compounds and extracts are tested in vials containing five millilitres of brine solution and 10 brine shrimps in three replicates initially at 10, 100 and 1000 ppm. Survivors are counted after 24 hours with the aid of a stereoscopic microscope, and LC_{50} (concentration that is lethal to 50% of the shrimps) values at 95% confidence limit are calculated. This bioassay technique has been used in the detection of active antitumor agents and pesticides produced by plants (Alkofahi *et al*., 1989).

2.8.2.2 Crown-Gall Tumour Bioassay

This bioassay is fairly accurate in predicting *in vivo* murine antileukin activity. Crown-gall is a neoplastic disease induced by *Agrobacterium tumefaciens*, a bacterium known to infect crop plants. It is due to the transfer of tumour-inducing plasmid from the bacterium to the plant genome. In this assay, potato tubers are surface sterilized, and a core of the tissue is extracted from each tuber. A 2 cm piece is cut from each end, and the remainder is sectioned into 0.5 cm thick disks that are then placed onto petri dishes containing 1.5 % water agar

which serves to provide a substrate that is sterile and contains enough moisture for optimal growth of the bacterium. A solution of the extract or compound dissolved in a suitable solvent is spread over a disk, and the solvent is allowed to evaporate. The disks are then inoculated with 0.1 ml of the bacterial suspension and the plates are incubated at 27°C. The assay measures the inhibition of tumours induced by the bacterium on potato disc by various plant extracts (Galski *et al*., 1980).

2.9 Specific recommendations on antibacterial and antifungal screening

2.9.1 Panel of test organisms

The choice of test microorganisms depends on the specific purpose of the investigation. In a primary screening, drug-sensitive reference strains are preferable. The panel should consist of at least a gram-positive and a gram-negative bacterium. Extracts with prominent activity against gram-positive cocci should preferentially also be tested against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE), as they represent the greatest current medical need. ATCC strains are well characterized and very popular for that purpose, but clinical field isolates may also be used if fully characterized by antibiogram. Another challenging new area in the microbiological world is biofilms (Mah *et al*., 2001). Although many bacteria grow in a free-living state, it is quite common for them to adhere to surfaces by producing extracellular polymeric substances (EPS), e.g. biofilms. Due to their higher resistance against antimicrobial agents, an interesting option in antibacterial research is to include a bacterial biofilm model (e.g. *Staphylococcus aureus* ATCC6538). A small set of reference fungi is used for primary screening and includes *Trichophyton mentagrophytes* and *Epidermophyton floccosum* as representatives of the dermatophytes. Opportunistic filamentous fungi, *Aspergillus niger* and *Fusarium solani* may also be used

.

2.9.2 Growth medium

Mueller-Hinton (MH) agar or broth and tryptic soy agar or broth (TSA or TSB) are general growth media for bacteria, while Sabouraud (SAB) agar or broth is used for fungi. Growth of fastidious microorganisms, such as *Streptococcus pneumoniae* and *Legionella pneumophila*, may require more complex media, enrichment of the incubation atmosphere with 5 % CO_2 and/or extension of the incubation time. Slight differences in the composition of the growth medium can greatly affect the antibacterial activity of a compound. For example, addition of sheep blood to Mueller-Hinton medium increases the MIC of flavomycin from 0.12 to 256 mg/l (Butaye *et al.*, 2000). Consequently, a definite choice of growth medium is essential to compare different antibacterial compounds or extracts. Mueller- Hinton medium allows good growth of most non-fastidious bacteria and is generally low in antagonists. It also meets the requirements of the NCCLS standard and is recommended as reference medium for agar and broth dilution tests (Anon, 2003).

2.9.3 Inoculum

The level of infection, i.e. inoculum concentration can have a profound influence on the antibacterial and antifungal potency of a sample, therefore there is the need for standardization of inoculates (Anon, 2003). In dilution methods, an inoculum of about 10^5 CFU/ml is adequate for most bacterial species while for yeasts and fungi between 10^3 and 10^4 CFU/ml is sufficient (Hadacek and Greger, 2000). Too low inoculum size (e.g.10^2 CFU/ml) will create many false-positives, while a too high inoculum size (e.g. 10^7 CFU/ml) will hamper endpoint reading and increase the chances for false-negatives. It is recommended to collect from cultures during the logarithmic growth phase and always to take four or five colonies of a pure culture on agar to avoid selecting an atypical variant (Anon., 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Test organisms

The following test organisms were obtained from the Microbiology Laboratory of the Department of Pharmaceutics, KNUST, Kumasi and maintained on agar slants for use in bioassay. Whenever needed, a sample of the stock test microorganism was aseptically transferred into sterile nutrient broth (for bacteria) or Sabouraud broth (for fungus) and incubated for 18-24 hours.

1. *Bacillus thuringiensis* (ATCC 13838)
2. *Staphylococcus aureus* (ATCC 25923)
3. *Bacillus subtilis* (NCTC 10073)
4. *Pseudomonas aeruginosa* (ATCC 27853)
5. *Proteus vulgaris* (NCTC 4175)
6. *Enterococcus faecalis* (ATCC 29212)
7. *Escherichia coli* (clinical isolate)
8. *Salmonella typhi* (clinical isolate)
9. *Candida albicans* (clinical isolate)

3.2 Sample collection

Three field sites, Lake Bosomtwe, a natural lake located in Kumasi, River Wiwi on KNUST campus, Kumasi and the Duakor Sea at Cape Coast were selected for this study. Samples of water, sediments, weeds and stones were collected from each of these sites. A total of thirty four (34) samples were collected; Nine each from lake Bosomtwe and river Wiwi and sixteen from the Duakor Sea of Cape Coast. Water samples were collected by submerging sterile glass bottles in the water to a depth of about 10 cm and then opened to fill after which they

were closed and brought to surface. About five grams (5 g) of sediment materials at the base of the water bodies were collected into sterile plastic containers. The stones and weed samples were also collected into sterile plastic containers. All the samples were appropriately labelled and transported to the laboratory for processing.

3.3 Isolation of antibiotic producing organisms

Twenty (20) ml each of Nutrient agar (Oxoid) and Sabouraud agar were melted in a boiling water bath and stabilized at 45°C for 15 minutes in a thermostatically controlled water bath (Model R76, New Brunswick Scientific, Edison N.J., USA). One millilitre (1 ml) of water samples was inoculated into the molten agar, mixed well, poured into sterile petri dishes and allowed to solidify. The surfaces of the solid samples (stones and weeds) were gently scrubbed with sterile toothbrush into sterile distilled water, it was well shaken and 1 ml added to the molten nutrient and sabouraud agar at 45°C and poured into sterile petri dishes to set. About 10 ml of water was added to one gram of soil, it was well shaken and 1 ml added to the molten agars. The agars were poured into sterile petri dishes and allowed to set. The plates were incubated at 25°C and 37°C up to seven days with daily observation. Colonies on the agar plates with clear zones around them were suspected of showing antagonistic activities. These colonies were picked with the help of a sterile toothpick and purified by streaking on the surface of a set nutrient agar. The pure colonies were then transferred with the help of a sterile platinum loop into tubes containing 10 ml sterile nutrient broth and incubated at 37°C for 72 hours.

3.4 Screening of microbial isolates for antimicrobial metabolite production

The isolates were screened for their ability to inhibit the growth of the test microorganisms using the agar well diffusion method. Twenty tubes containing 20 ml each of Mueller-Hinton agar (Oxoid) were melted in a boiling water bath and stabilized at 45°C for 15 minutes in a thermostatically controlled water bath. The molten agars were aseptically inoculated with 0.1ml of an 18 hour broth culture of *B. subtilis* and rolled in the palm to mix thoroughly. The seeded agars were then transferred into sterile petri dishes under a laminar air flow cabinet, (Model T2 2472 Skan AG, Switzerland). The agars were allowed to set and four wells equidistant from each other were made in the agar plates with the help of a No. 6 cork borer (diameter 10 mm). Each well was then filled with 0.1ml of a broth culture of an isolate from section 3.3. The plates were allowed to stand for one hour to allow diffusion of any metabolite in the broth into the seeded agar. They were then incubated at 37°C for 24 hours and observed for signs of growth and zones of inhibition. The experiment was repeated using *Pseudomonas aeruginosa*, *Bacillus thuringiensis*, *Proteus vulgaris*, *Staphylococcus aureus* and *Enterococcus faecalis* as the test organisms. Three replicates of the screening were done and the diameters of the inhibition zones were measured and recorded (Table 4.1). Isolates showing promising activities were selected for further studies.

3.5 Separation of microbial cells from soluble metabolites produced

About one millilitre (1 ml) broth cultures of the selected isolates were separately inoculated into 10 ml nutrient broths and incubated at 37°C for 72 hours. They were then centrifuged at 3622g for one hour to precipitate the microbial cells from the metabolite solutions. The resulting supernatants were decanted and filtered through Whatman No. 1 filter paper into clean sterile test tubes and tested for antimicrobial activities by the agar well diffusion

method described in section 3.4, using *Bacillus subtilis* as the test organism. The diameters of the zones of inhibition were measured and recorded (Table 4.2).

3.6 Effect of heat on the antimicrobial metabolites produced by the isolates

The metabolite solutions as obtained in section 3.5 were heated in a water bath to temperatures of 40, 50, 60, 70, 80, 90 and 100°C as well as 121°C (in an autoclave) for 15 minutes. After cooling to room temperature (25°C), they were tested for antimicrobial activity by the agar well diffusion method (as in section 3.4) using *Bacillus subtilis* as test organism. The test was done in triplicates and the results obtained recorded.

3.7 Stability of antimicrobial metabolites in solution

The metabolite solutions as obtained in section 3.5 were stored at 4°C for a period of twelve weeks to check how stable the antimicrobial metabolites are in solution. The antimicrobial activities of the solutions were checked every week against *Bacillus subtilis* using the agar well diffusion method described in section 3.4 and the results obtained recorded.

3.8 Effect of some growth factors on antimicrobial metabolite production

Certain parameters of growth such as temperature, period of incubation, pH of the fermentation medium as well as effects of various sources of carbon and nitrogen were evaluated for optimum production of antimicrobial metabolites.

3.8.1 Temperature

The isolates (1 ml each) were inoculated into five of 10 ml nutrient broths and incubated at different temperatures of 25, 30, 32, 34 and 37°C for 72 hours. The cultures were then treated as in section 3.5 to separate the microbial cells from the metabolite solutions. The metabolite solutions obtained were then analysed for antimicrobial activity as described in section 3.4 using *Bacillus subtilis* as the test organism. The exercise was performed in duplicates and the results recorded.

3.8.2 Time

The isolates (1 ml each) were inoculated into three tubes of 10 ml sterile nutrient broth and incubated at 37°C. During the incubation period, samples were taken every 24 hours for twelve days, centrifuged to remove the cells and the supernatant liquid analysed for inhibitory activity as in described in section 3.4. This was done in duplicates and the results recorded.

3.8.3 pH

The isolates (1ml each) were inoculated into six tubes of 10 ml nutrient broth of varying pH values (4, 5, 6, 7, 8 and 9). NaOH (1M) and HCl (1M) were used to adjust the pH in the broths. The tubes were then incubated at 37°C for 72 hours, after which the cultures were treated as in section 3.5 and the supernatant liquid analysed for antimicrobial activity by the agar well diffusion method (section 3.4).

3.8.4 Carbon sources

The effect of carbon sources on antimicrobial metabolite production was verified by fortifying the production medium with different sources of carbon. Sixty milligrams (60mg) of the various carbon compounds (glucose, galactose, xylose, sucrose, mannitol, lactose, starch, fructose and maltose) and 0.05ml of glycerol (Density, 1.261g/ml) were separately added to 10 ml quantities of the fermentation medium in test tubes and sterilized. One millilitre of an isolate suspension was inoculated into each of the media aseptically and incubated at 37°C for 72 hours. The cultures were then centrifuged and treated as in section 3.5. The metabolite solutions obtained were tested for antimicrobial activity. The experiment was replicated for each of the isolates and the results recorded.

3.8.5 Nitrogen sources

The effect of nitrogen sources was also investigated by fortifying the fermentation medium with different nitrogen compounds. Sixty milligrams (60 mg) each of the various nitrogen compounds (sodium nitrate, potassium nitrate, ammonium chloride, ammonium nitrate, asparagine, ammonium phosphate and ammonium sulphate) were separately added to 10 ml quantities of the fermentation medium in test tubes and sterilized. One millilitre of an isolate suspension was inoculated into each of the media aseptically and incubated at 37°C for 72 hours. The cultures were then centrifuged and treated as in section 3.5. The metabolite solutions obtained were tested for antimicrobial activity. The experiment was repeated in duplicates for each of the isolates and the results recorded.

3.9 Extraction and bioassay of the crude antibiotic

3.9.1 Preparation of crude extract.

The crude antibiotic extract was prepared by extracting with organic solvents. Five millilitres (5 ml) of the isolate was inoculated into five (500 ml) flasks containing 300 ml nutrient broth. The flasks were incubated at 37°C for 10 days and then centrifuged at 3622 g for one hour. The supernatant (about 2500 ml) was filtered and transferred into a clean one litre separating funnel (Figure 3.1) in 500 ml quantities. This was extracted five times with 50 ml portions of petroleum ether which were pooled together and evaporated to dryness. The extraction was continued several times with 50 ml portions of chloroform. The chloroform extracts were also pooled together and evaporated to dryness. The chloroform extract was then used as a crude sample for bioactivity assay.

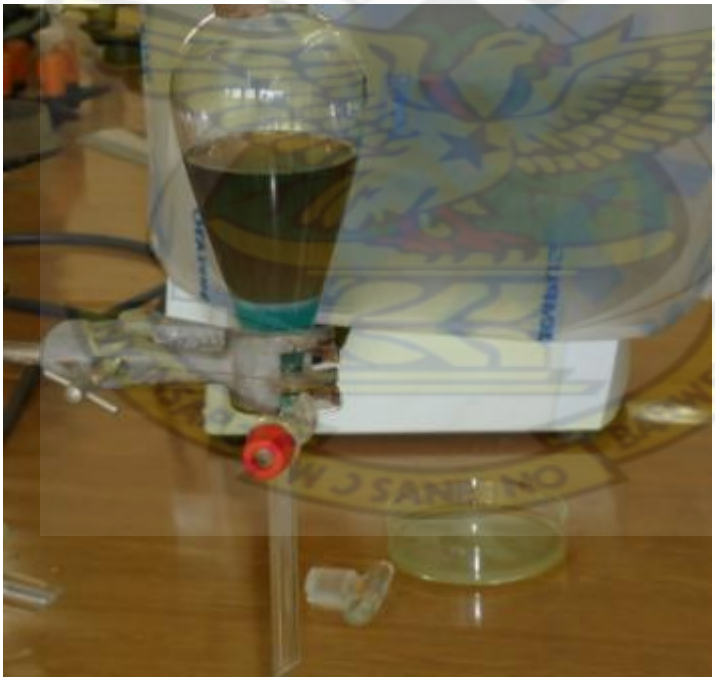


Fig. 3.1 Set up of the extraction process, showing the chloroform layer below and the aqueous layer on top.

3.9.2 Determination of MIC and MBC of the chloroform extract

The Minimum Inhibitory Concentration (MIC) determination was carried out using the 96 micro well broth dilution method. Serial dilutions of the crude extract in the range of 250µg/ml to 4000µg/ml were made. Quantities of 100 µl double strength nutrient broth were dispensed into the micro wells and 100 µl of the test extract was added to each appropriate well. This was followed by the addition of 20 µl of an overnight culture of bacteria (approximately 10^5cfu/ml) to the wells. The plates were incubated at 37°C for 48 hours after which 20 µl of MTT (Sigma, M5655, USA) dye was added to the wells. Bacterial growth inhibition was visible as a yellow well and the presence of growth detected by the presence of a purple colour. Two replicates of the test were performed and the MIC values recorded (table 4.3). The minimum bactericidal or fungicidal concentration (MBC or MFC) was determined by plating out the contents of wells which showed no growth on nutrient agar (for bacteria) and sabouraud agar (for fungi). The plates were incubated at 37°C for three days with daily observations for signs of growth.

3.9.3 Thin Layer Chromatography (TLC)

The crude extract was subjected to TLC analysis on 2.6 × 8 cm silica gel plate (TLC aluminium sheets). 100 mg of the extract was dissolved in 1 ml of ethylacetate and this was heavily spotted onto a TLC plate 2 cm above the base. After thorough drying, the plate was placed in a solvent system (chloroform: ethylacetate; in the ratio 3.5:1.5) in a chromatography tank to develop. The experiment was repeated and the spots were then observed under UV light (wavelengths of 254 nm and 365 nm). The distances travelled by the constituents were measured and used to determine their R_f values.

3.9.4 Bio-autography Assay

Two (20 ml) tubes of Mueller-Hinton agar were melted at 100°C, stabilized at 45°C and seeded with an overnight broth culture of *Proteus vulgaris*. This was then poured in a thin film over the developed TLC plate such that the separated constituents were in contact with the seeded agar. The agar was allowed to set and the plate kept in a refrigerator at a temperature of 4°C for 24 hours to allow diffusion of the various constituents after which it was incubated at 37°C for 24 hours and sprayed with MTT. Zones of bacterial growth inhibition were visible as clear yellow areas against a purple background.

3.10 Characterization of selected antibiotic producers

Morphological features as well as biochemical tests were used to characterise the selected isolates as far as possible.

3.10.1 Macroscopic characterization

The isolates were grown on the surface of nutrient agar plates and their morphological features such as margins, pigmentation, colour and texture were observed.

3.10.2 Microscopic characterization

The microscopic morphological features of the isolates were analyzed by using an Olympus light microscope (Olympus, Tokyo, Japan). Gram stain reaction and motility tests were conducted. All reagents used were obtained from Fisher Scientific (Leicestershire, UK). The gram-positive isolate was subjected to the modified Moeller's spore staining.

3.10.2.1 Gram Staining

A clean grease free slide was obtained by cleaning with hot water and soap. After which it was rinsed and the excess water blotted out with a lint-free cloth. A small drop of distilled water was placed on the slide and with the help of an inoculating needle, the isolate picked from the surface of nutrient agar plate was gently and thoroughly emulsified in the water drop, and spread to make a thin smear. The smear was dried in air and passed through a Bunsen flame to fix. It was then flooded with ammonium oxalate crystal violet for about 30 seconds and rinsed off with water. Iodine solution was then applied for about 30 seconds and washed off. The excess water was blotted off with bibulous paper and 95% ethyl alcohol was applied and allowed to drain off until no more colour was seen leaving the smear. It was again washed with water and the contrast stain, safranin was added and left for about 30 seconds. The contrast stain was then washed off and the smear dried in air. The slide was examined under oil immersion (Hucker and Conn, 1923).

3.10.2.2 Motility test

With a toothpick, a ring of vaseline was spread around the concavity of a depression slide. A platinum loop was then used to introduce a small drop of the bacterial suspension in the centre of a cover slip. The depression slide was lowered onto the cover slip with the concavity of the slide facing down so that the drop protrudes into the centre of the concavity. It was then pressed gently to form a seal. The depression slide with the cover slip on top was placed on the microscope so that the drop is over the light hole. It was then examined under oil immersion. Care was taken to observe true locomotion but not passive drifting and Brownian motion.

3.10.2.3 Spore staining

The smear as prepared in section 3.10.2.1 was flooded with strong carbol-fuchsin, steamed on a boiling water bath for five minutes and washed with water. The smear was then decolourized with ethanol and again washed with water. This was followed by counter staining with Loeffler's methylene blue for about two minutes. After washing, the excess water was blotted with bibulous paper and examined under oil immersion. The bacterial cells were stained blue whilst the spores appeared red (Moeller, 1891).

3.10.3 Biochemical tests

To test the physiological requirements of the isolates, biochemical tests were done which included: indole production, hydrogen sulphide production, citrate utilization and fermentation of sugars. Other biochemical tests such as oxidase test and nitrate reduction were also performed on the isolates. *E. coli* (indole test), *P. aeruginosa* (oxidase test), *S. typhi* (hydrogen sulphide production test) and *B. subtilis* (citrate utilization test) were used as controls.

3.10.3.1 Carbohydrate Fermentation

The various fermentation media (Table 4.5) were inoculated with two loopfuls of a broth culture of the selected isolates. The tubes were then incubated at 37°C and observed 24 hourly for two days. Growth, acid and gas production were observed and recorded.

3.10.3.2 Indole Test

The indole test was performed by growing the isolates in sterile tryptone water for 24 hours. Following overnight incubation, a few drops of Kovacs' reagent (appendix II) was added to the culture broth, using a pipette. Presence of indole was detected by the appearance of a red layer in the medium while its absence was denoted by a yellow layer.

3.10.3.3 Citrate Utilization

Using a straightened wire, the isolates were inoculated into Koser's Citrate medium and incubated at 37°C for 72 hours. Citrate utilization was denoted by turbidity and colour change of the medium from light green to blue.

3.10.3.4 Oxidase test

The oxidase reagent needed was prepared by dissolving 0.1 g of tetramethyl-p-phenylenediamine in 10 ml of water. The isolates were streaked on nutrient agar and incubated at 37°C for 24 hours. A piece of filter paper was placed in a petri dish and three drops of the freshly prepared oxidase reagent added to it. The culture of the isolates was smeared across the impregnated paper with a platinum loop. A positive reaction was indicated by the appearance of a dark purple colour on the paper within 10 seconds.

3.10.3.5 Nitrate reduction

Two loopfuls of the isolates were inoculated into nitrate broth and incubated at 37°C for five days after which a drop of 0.8% sulfanilic acid and 0.6% N, N-dimethyl-α-naphthyylamine

solutions were added. The colour change was recorded after 3 minutes. A red colour indicated a positive test.

3.10.3.6 Hydrogen sulphide production

Two loopfuls of isolate suspension were inoculated into 10 ml peptone water. Lead acetate paper was inserted between the cotton wool plug and the tube. The tubes were incubated at 37°C with daily observations for two days. A positive test was denoted by a blackened lead acetate paper.

3.10.3.7 Growth at 42^{o}C

This test was used to differentiate between certain species of *Pseudomonas* since they react similarly to the various biochemical tests. The test was done by growing the organisms at 42°C in nutrient broth for 24 hours. *Pseudomonas aeruginosa* grows at 42^{o}C whilst *Pseudomonas fluorescens* is not able to do so (Hsueh *et al*., 1998).

3.10.3.8 Cultivation of isolates on selective media.

The organisms were also streaked on different selective media to observe the growth and appearances of colonies. Selective media used include Cetrimide agar, MacConkey agar, Mannitol Salt agar and Bismuth Sulphite agar.

(i) Cetrimide Agar

Four tubes of 20 ml cetrimide agar were melted at 100°C, stabilised at 45°C for 15 minutes and poured into different sterile petri dishes and allowed to set. Two loopfuls of a broth culture of the isolates were separately streaked on the surface of the agar. A 24-hour broth culture of *Pseudomonas aeruginosa* was also streaked on another plate to serve as the control. The plates were incubated inverted at 37°C and observed daily for 48 hours.

(ii) MacConkey agar

Four tubes of 20 ml MacConkey agar were melted at 100°C, stabilised at 45°C for 15 minutes and poured into sterile petri dishes and allowed to set. Two loopfuls of a broth culture of the isolates were separately streaked on the surface of the agar. A 24-hour broth culture of *E. coli* was also streaked on another plate to serve as the control. The plates were incubated inverted at 37°C and observed daily for 48 hours.

(iii) Mannitol Salt agar

Four tubes of 20 ml Mannitol Salt agar were melted at 100°C, stabilised at 45°C for 15 minutes and poured into sterile petri dishes and allowed to set. Two loopfuls of a broth culture of the isolates were streaked separately on the surface of the agar. A 24-hour broth culture of *Staph. aureus* was also streaked on another plate to serve as the control. The plates were incubated inverted at 37°C for 48 hours.

(iv) Bismuth Sulphite agar

Four tubes of 20 ml Bismuth Sulphite agar were melted at 100°C, stabilised at 45°C for 15 minutes and poured into sterile petri dishes and allowed to set. A loopful of the broth cultures of the isolates was separately streaked on the surface of the agar. A 24-hour broth culture of *S. typhi* was streaked on another plate to serve as the control. The plates were incubated inverted at 37°C for 48 hours.

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation and screening of antibiotic producing microorganisms

Samples were obtained from different areas at each of the sampling sites. The temperatures of the sampling sites ranged between 25°C and 37°C. The temperatures were measured to help determine the initial incubation temperatures. Samples collected yielded a total of 119 microbial isolates from the master plates; thirty-two (32) from Duakor Sea shore, seventy-two (72) from River Wiwi and fifteen (15) from Lake Bosomtwe.

All the 119 isolates were screened for their ability to produce antibiotics using the agar diffusion method (Figure 4.1) and 27 of the isolates exhibited antimicrobial properties against at least one of the test bacteria used: *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212), *Bacillus thuringiensis* (ATCC 13838), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (NCTC 10073) and *Proteus vulgaris* (NCTC 4175). Generally the gram-positive bacteria were more susceptible to the inhibitory effects of the isolates than the gram-negative ones. Only two of the isolates showed activity against *Pseudomonas aeruginosa*. Out of the 27 isolates, three exhibited average zones of inhibition greater than 19 mm against at least two of the test organisms. These were the marine isolates 1, 2 and 3. The result of the screening is shown in the following table (table 4.1).

Table 4.1 Results obtained from screening of isolates for antimicrobial activity.

Producers	Mean zones of inhibition (mm)					
	Test organisms					
	PA	BT	BS	EF	SA	PV
SAI 19	14	16	15	11	14	15
SAI 22	0	0	11	14	11	12
SAI 20	0	11	0	11	0	13
SAI 28	0	12	0	13	0	11
SAI 29	0	14	13	14	0	0
SAI 18	0	12	0	12	0	12
SAI 9	0	11	0	0	0	0
SAI 12	0	12	0	0	0	12
SAI 36	0	13	13	14	14	12
SAI 31	0	12	0	11	0	0
SAI 32	0	12	11	12	13	12
SAI 35	0	14	14	14	12	12
SAI 23	0	0	0	0	0	12
SAI 5	0	0	11	0	0	11
WEI 3	0	14	14	15	15	13
WEI 7	0	11	0	12	12	0
WEI 13	0	11	0	13	0	11
WEI 14	0	14	13	16	13	13
WEI 16	0	0	0	11	0	0
WEI 19	0	0	0	11	0	0
BS 1	13	14	15	12	13	14
BS 8	0	0	0	0	0	17
BS 26	0	0	13	15	0	0
MAI 1	0	20	17	22	20	17
MAI 2	0	24	24	23	0	20
MAI 3	0	0	20	22	0	0
MAI 4	0	0	0	15	0	0

PA = *Pseudomonas aeruginosa*, BT = *Bacillus thuringiensis*, BS = *Bacillus subtilis*, EF = *Enterococcus faecalis*, SA = *Staphylococcus aureus*, PV = *Proteus* vulgaris, SAI = Sand isolates from River Wiwi, WEI = weed isolates from River Wiwi, BS = isolates from Lake Bosomtwe, MAI = marine isolates.

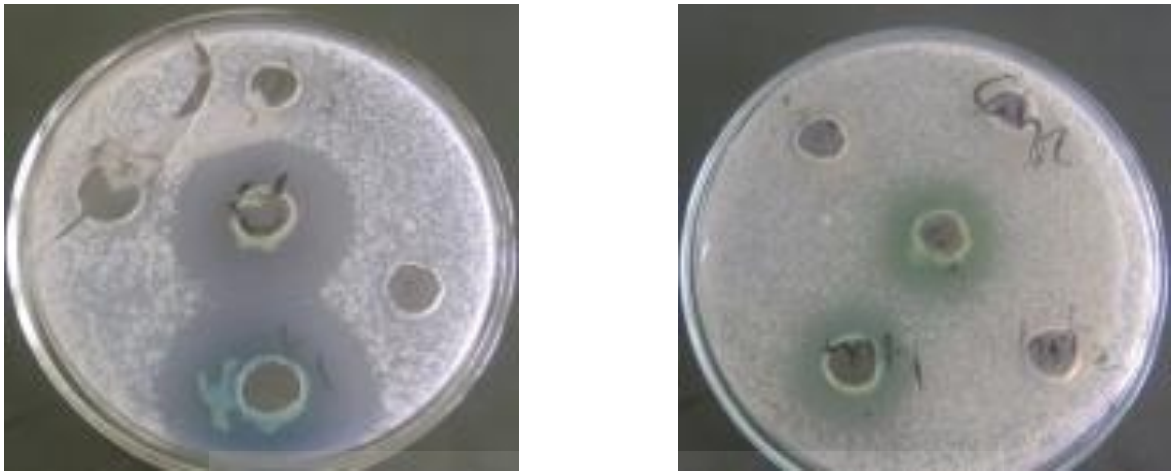


Fig.4.1 Zones of growth inhibition produced by some of the isolates during the screening.

4.1.1 Activity of soluble metabolites from the selected isolates

Isolates MAI 1, MAI 2 and MAI 3 produced zones in the range 20-24 mm against at least one of the test bacteria (Table 4.1). They were therefore selected for further analysis: their soluble extracellular metabolites were tested for antimicrobial activity. The mean zones of growth inhibition against *B. subtilis* are shown in table 4.2. The highest zone of inhibition was recorded for MAI 2 while MAI 1 had the lowest zone of inhibition.

Table 4.2 Antimicrobial activity of the cell-free supernatant of the isolates against *Bacillus subtilis.*

Isolates	Mean zone of inhibition (mm)
MAI 1	20
MAI 2	23
MAI 3	22

4.2 Effect of increasing temperature on antibiotic activity of the metabolites of selected isolates

The antimicrobial metabolite of MAI 1 was active when heated at 40°C. However at higher temperatures, the metabolite lost its activity (figure 4.2). The metabolites of MAI 2 and MAI 3 showed a general decrease in activity with increasing temperature (Figure 4.3 and 4.4). After sterilization (121°C for 15 minutes) however, the metabolites of both isolates completely lost their inhibitory activities.

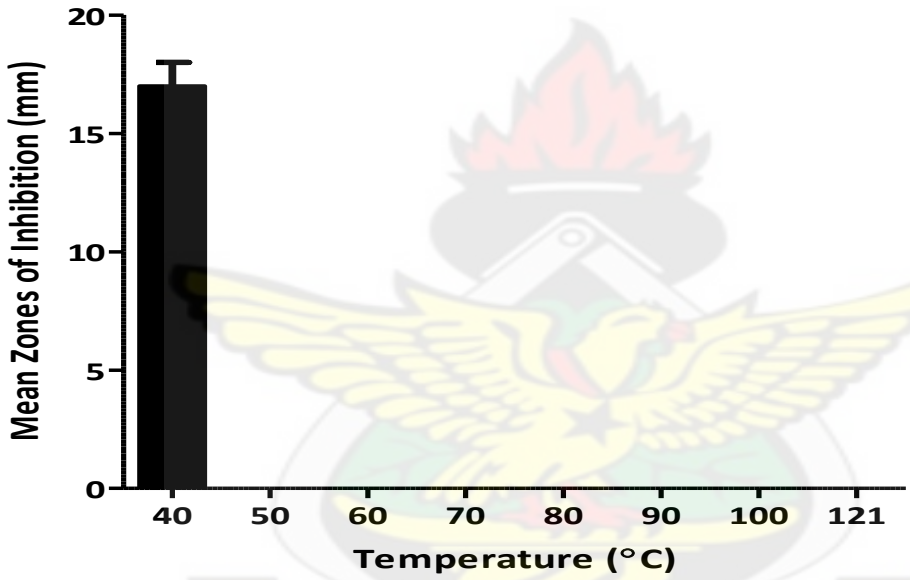


Fig.4.2 Effect of heat treatment on the stability of the antimicrobial metabolites produced by MAI 1

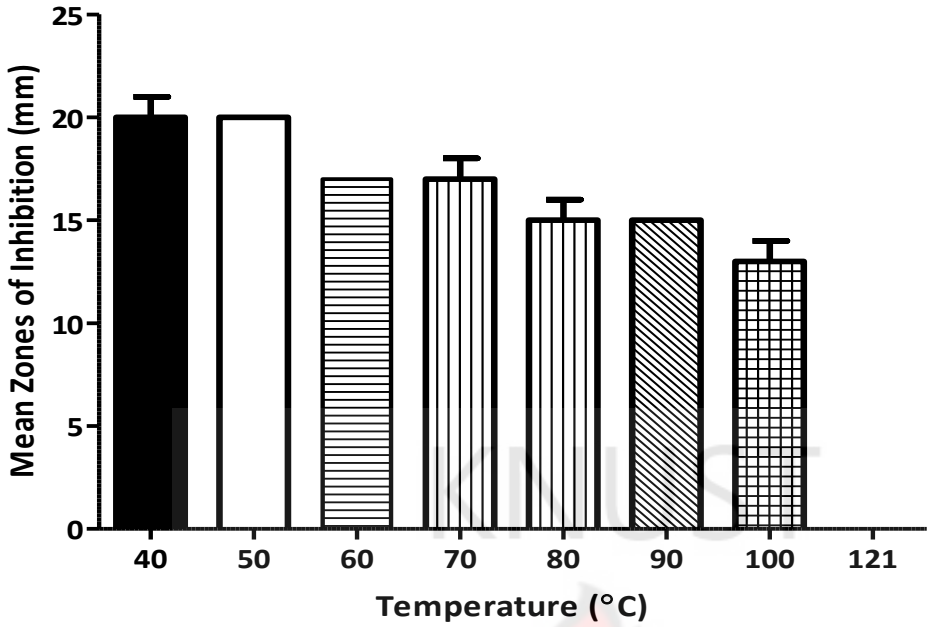


Fig.4.3 Effect of heat treatment on the stability of the antimicrobial metabolites produced by MAI 2

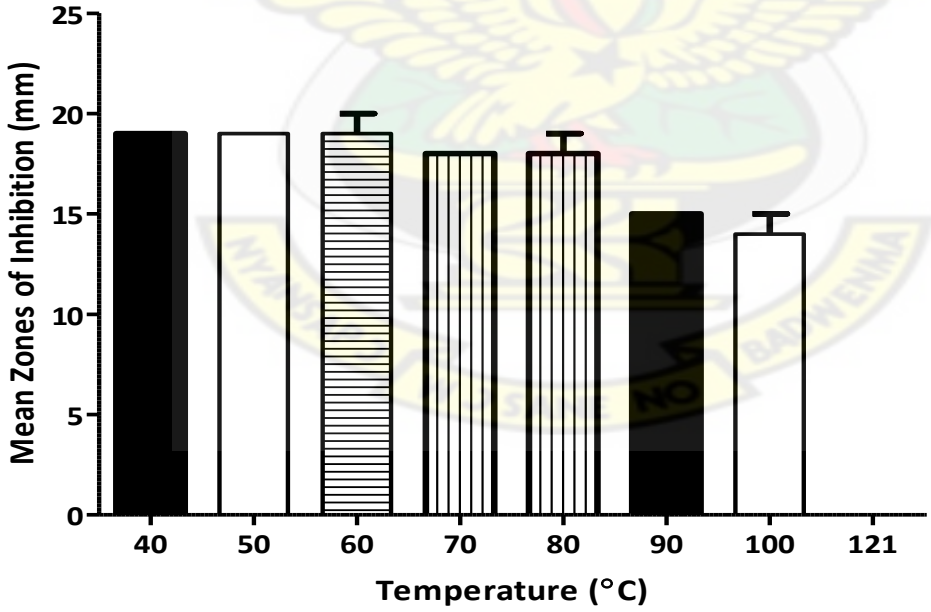


Fig.4.4 Effect of heat treatment on the stability of the antimicrobial metabolites produced by MAI 3.

4.3 Stability of antimicrobial metabolites in solution.

The metabolites of all the three isolates exhibited inhibitory activity for a period of twelve weeks (figure 4.5, 4.6 and 4.7). There was however a general decrease in their activities from the first week to the twelfth week.

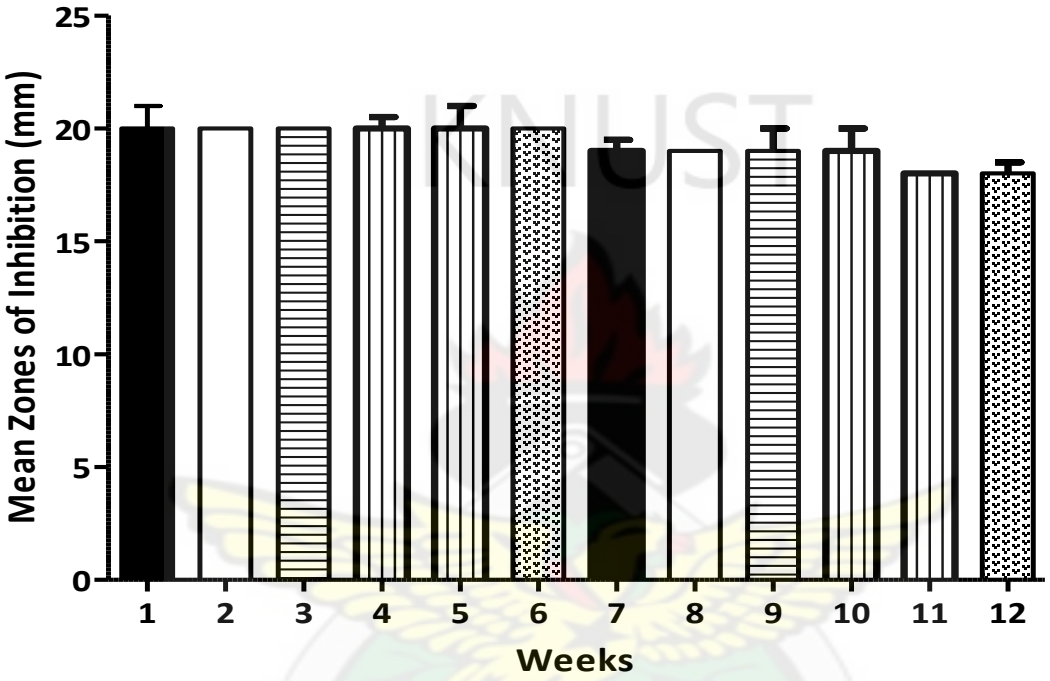


Fig.4.5 Stability of the cell-free supernatants of MAI 1

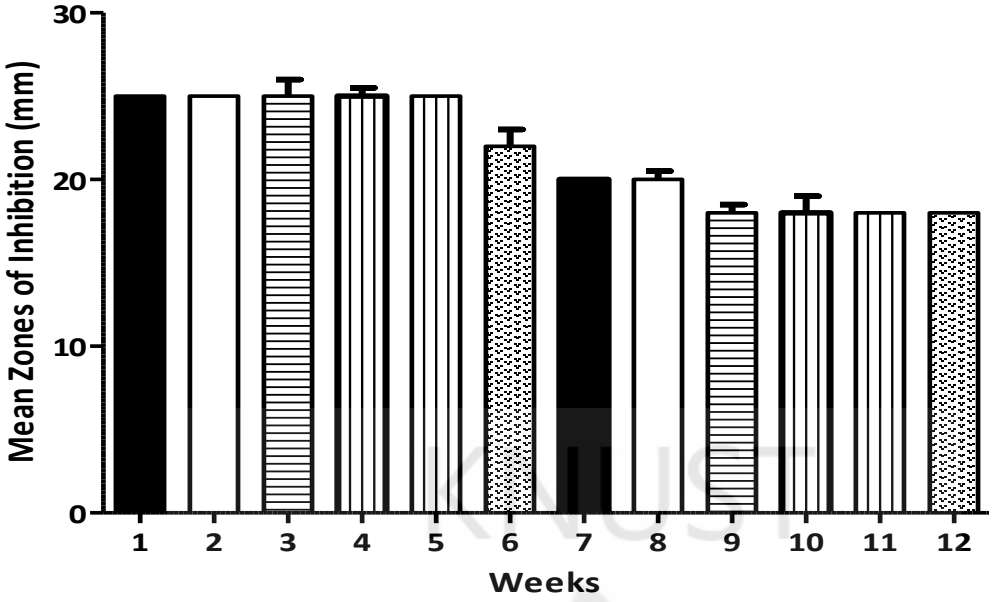


Fig.4.6 Stability of the cell-free supernatants of MAI 2

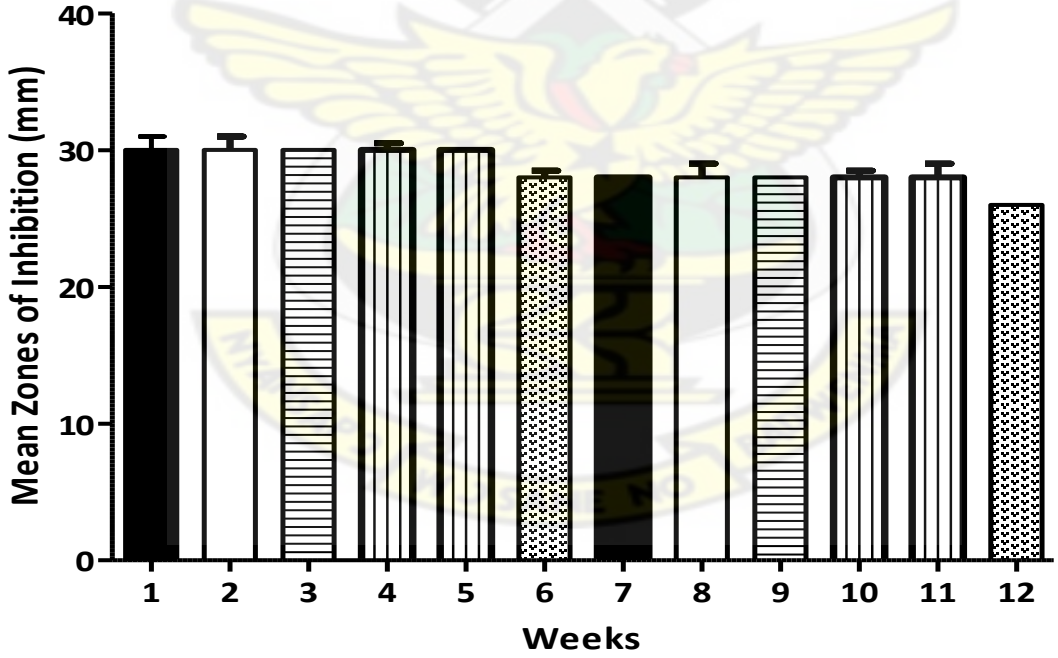


Fig.4.7 Stability of the cell-free supernatants of MAI 3

4.4 Effect of some growth factors on antimicrobial metabolite production of the selected isolates

4.4.1 Growth temperature

Antimicrobial activity of metabolites by MAI 1 was highest at 25°C whilst the lowest activity was observed at 34°C (fig 4.8). With MAI 2 and MAI 3, maximum activity was observed at 30°C whilst the lowest activity was recorded at 25°C (fig 4.9 and 4.10).

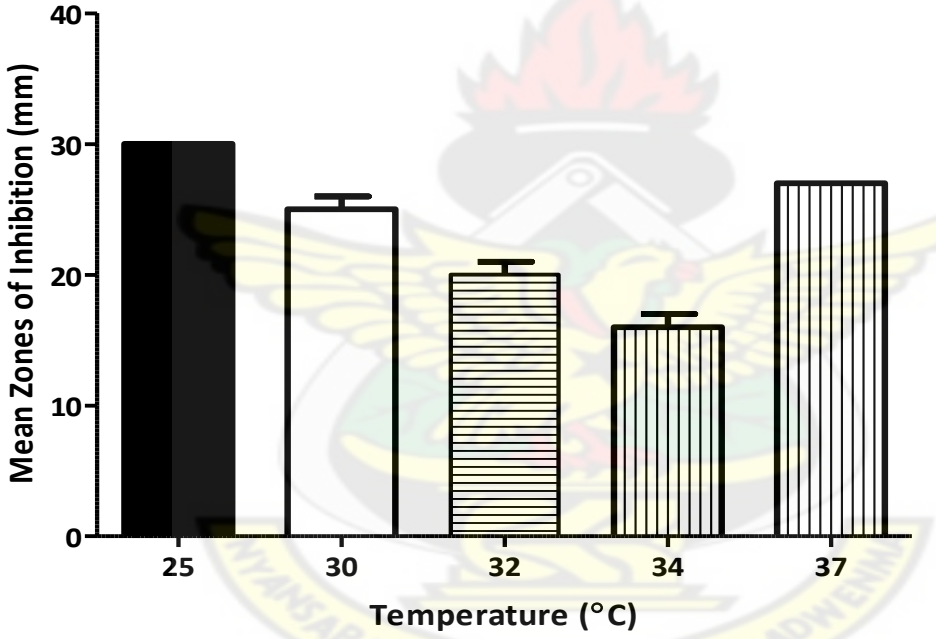


Fig.4.8 Effect of growth temperature on the antimicrobial activity of metabolites of MAI

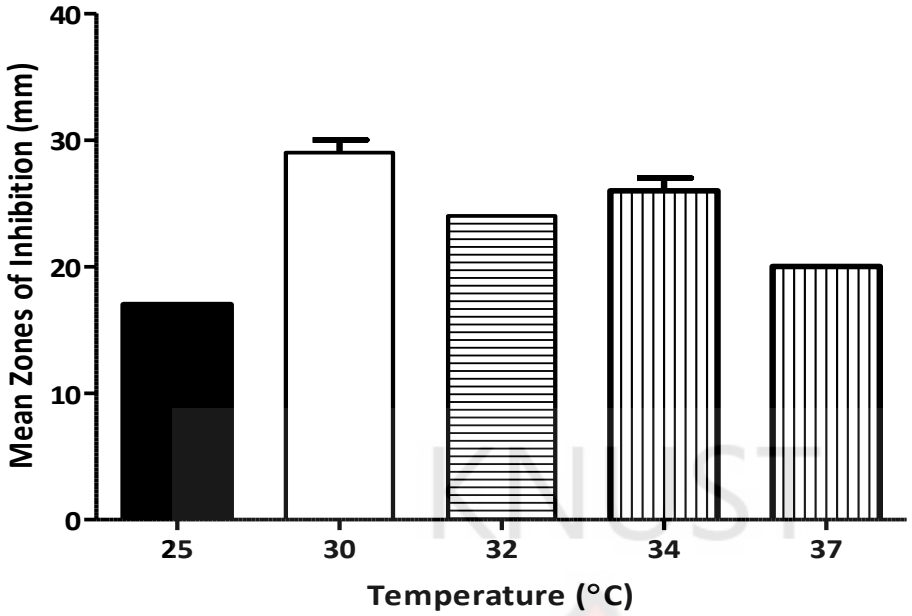


Fig.4.9 Effect of growth temperature on the antimicrobial activity of metabolites of MAI 2

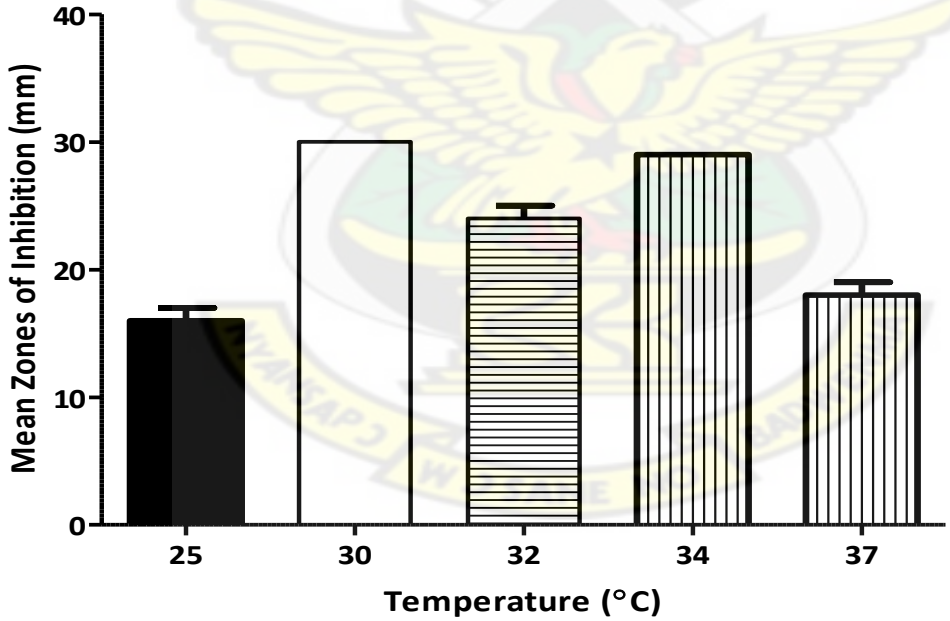


Fig.4.10 Effect of growth temperature on the antimicrobial activity of metabolites of MAI 3.

4.4.2 Time

The antimicrobial activity of the metabolites increased generally from the first to the ninth day of incubation, after which the activity became stable (figure 4.11). This was the case for all the isolates (MAI 1, MAI 2 and MAI 3).

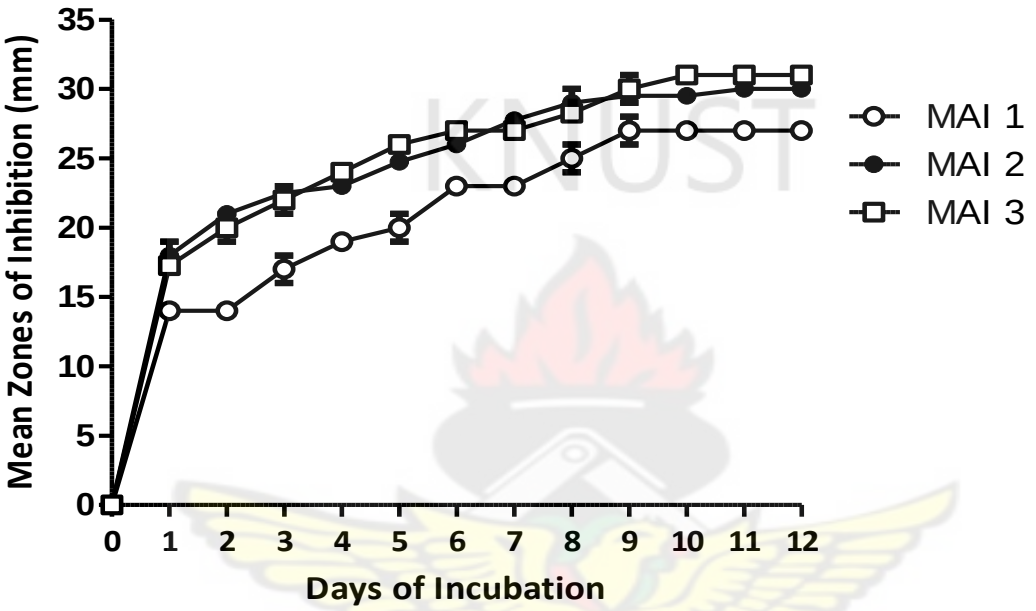


Fig.4.11 Effect of incubation period on antimicrobial metabolite activity

4.4.3 pH of fermentation medium

Maximum activity of antimicrobial metabolite by MAI 1 was observed at pH 8 with no activity at pH 4 (Figure 4.12). Likewise there was no activity of the metabolites of MAI 2 and MAI 3 at pH 4 but maximum activity for both isolates was observed at pH 7 (Figure 4.13 and 4.14).

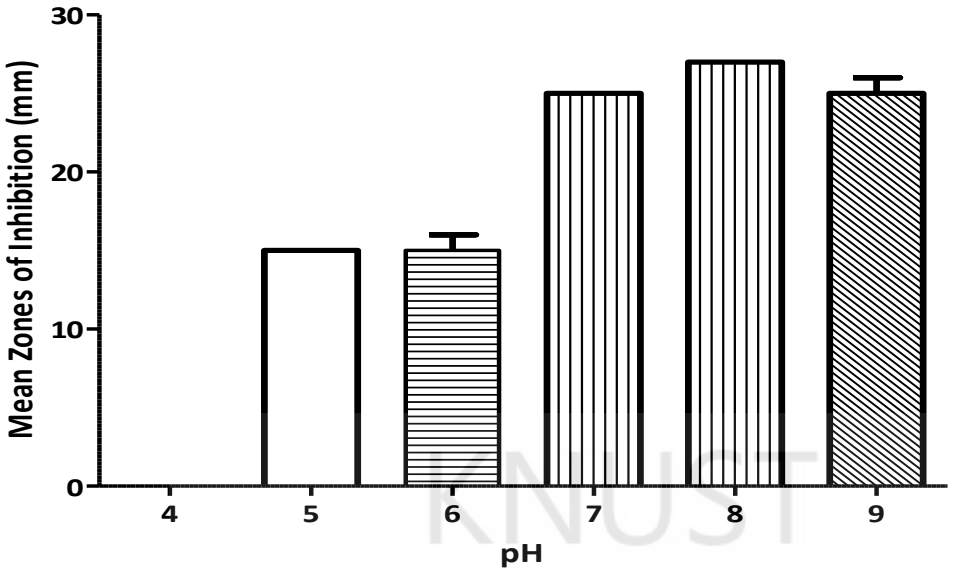


Fig. 4.12 Effect of pH on the antimicrobial activity of metabolites of MAI 1

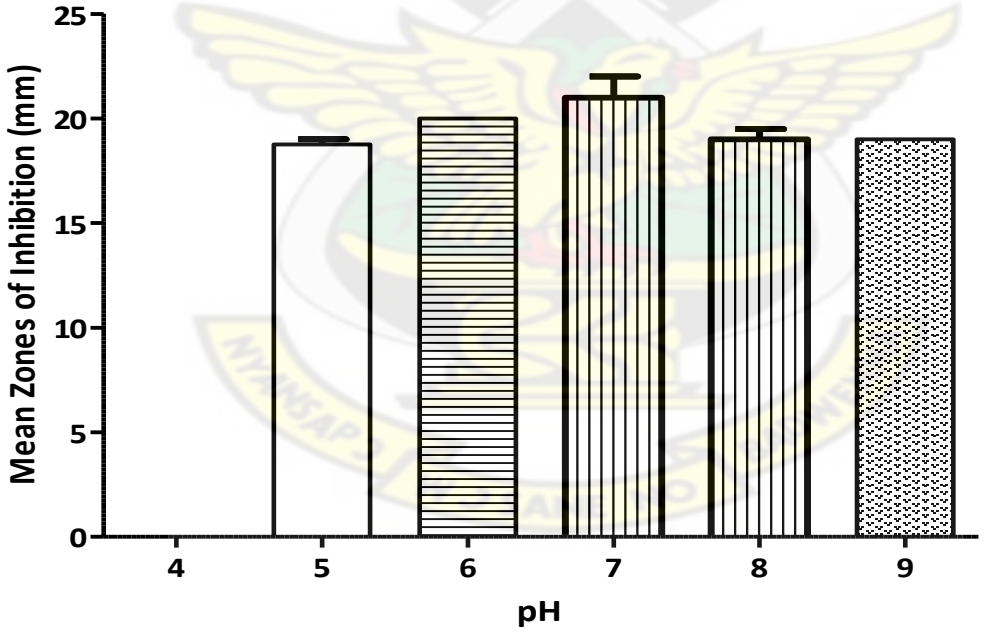


Fig. 4.13 Effect of pH on the antimicrobial activity of metabolites of MAI 2

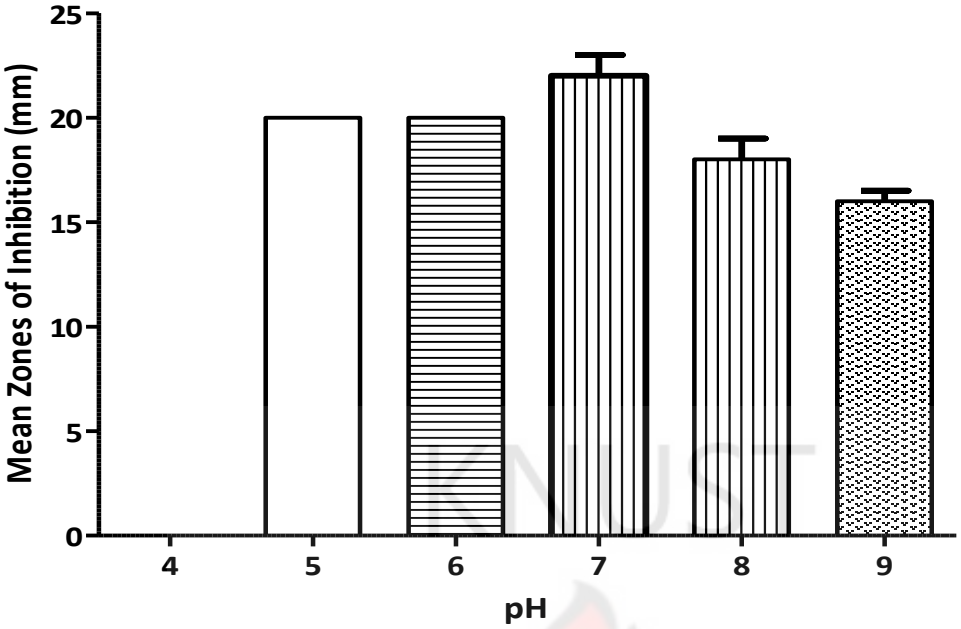


Fig. 4.14 Effect of pH on the antimicrobial activity of metabolites of MAI 3.

4.4.4 Effect of carbon sources.

The antimicrobial activities of MAI 1, MAI 2 and MAI 3, against *E. coli* and *B. subtilis*, when the fermentation medium was supplemented with fructose, galactose, glucose, glycerol, lactose, maltose, xylose, mannitol, starch and sucrose, are presented in the following figures (figure 4.15, 4.16 and 4.17). For MAI 1, the highest activity was observed in the medium supplemented with glycerol. No antimicrobial activity against *E. coli* was detected when the medium was supplemented with glucose, xylose, sucrose, mannitol, fructose and maltose, neither was any activity observed against *B. subtilis* when the medium was supplemented with glucose, mannitol and maltose. Generally antimicrobial activity against *B. subtilis* was greater than *E. coli*. For MAI 2, highest activity was observed with both starch and glycerol whilst for MAI 3, the highest activity was observed when the medium was supplemented

with starch. In contrast to the activity of MAI 1, the antimicrobial activity against *E. coli* was greater than *B. subtilis* for both MAI 2 and MAI 3.

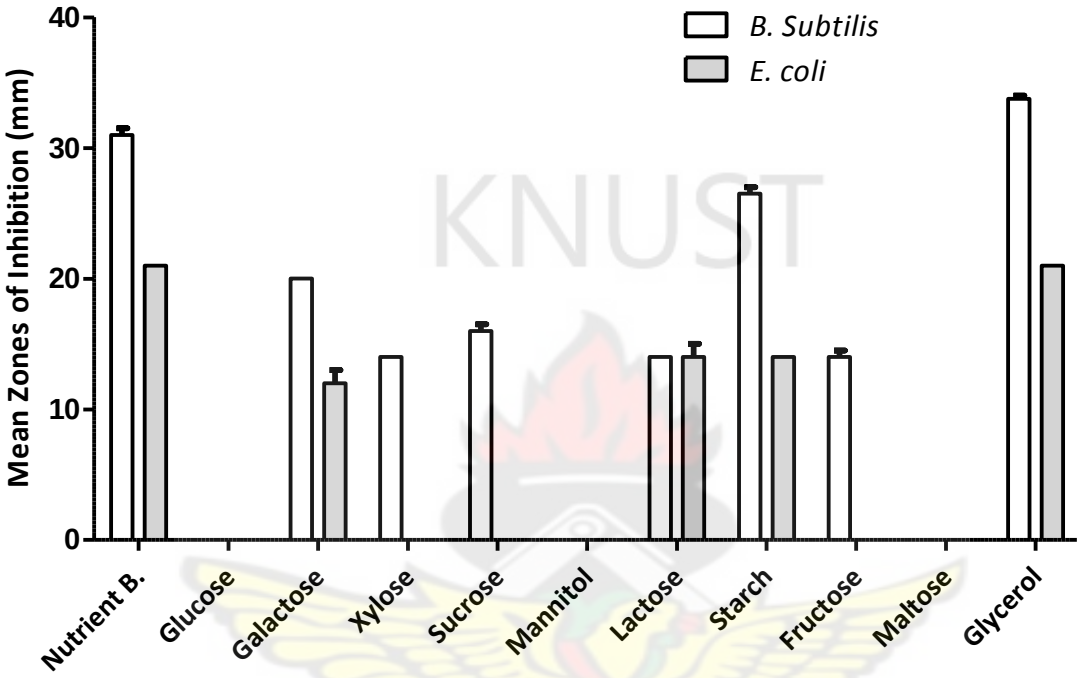


Fig. 4.15 Effect of carbon sources on the antimicrobial activity of metabolites of MAI 1

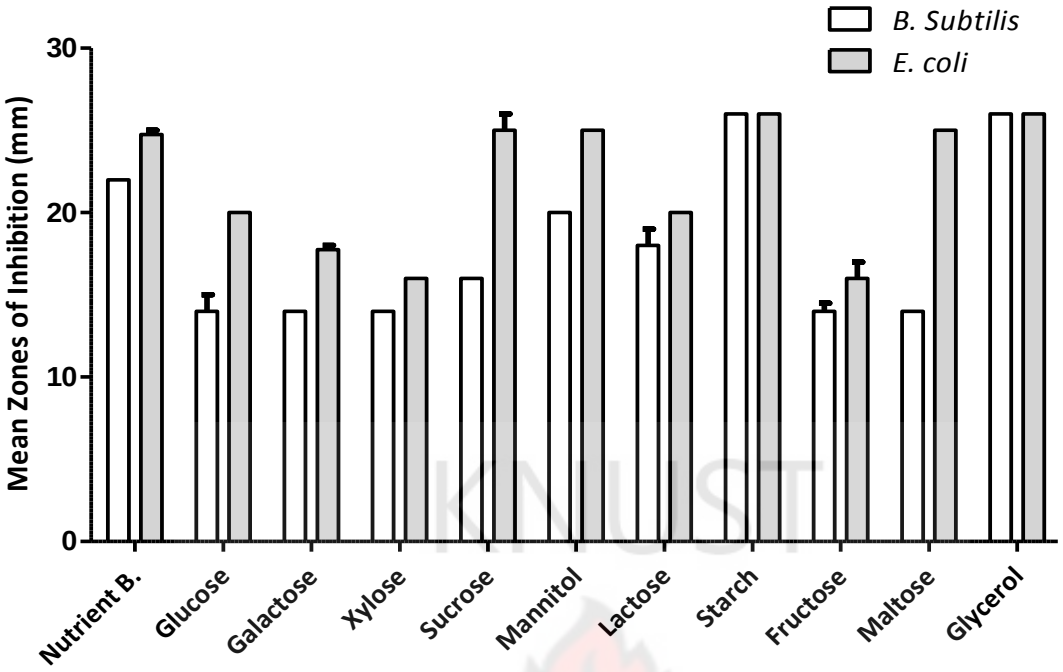


Fig. 4.16 Effect of carbon sources on the antimicrobial activity of metabolites of MAI 2

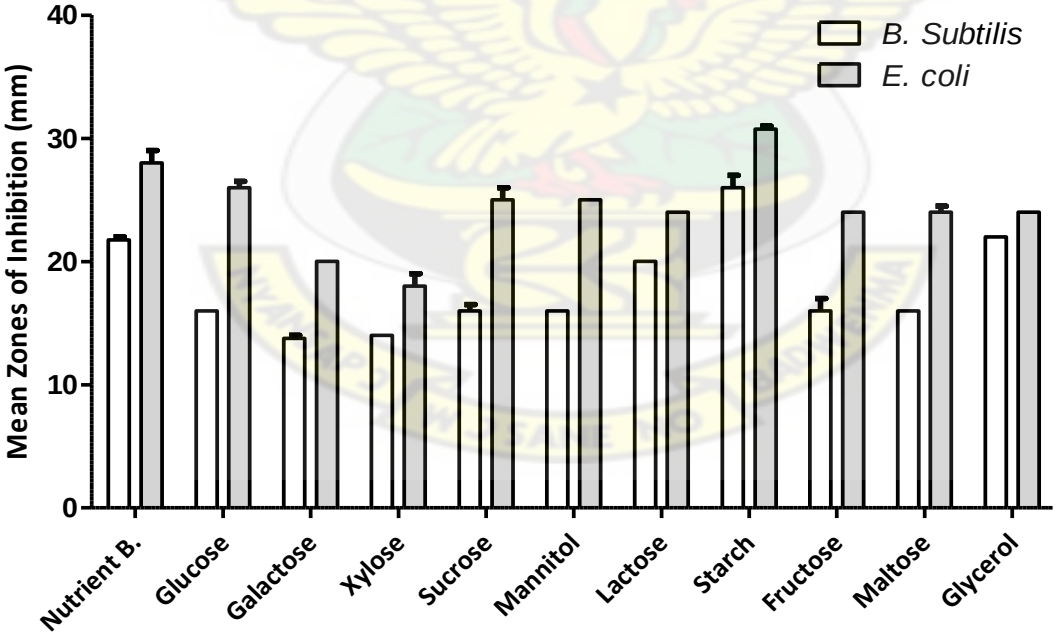


Fig. 4.17 Effect of carbon sources on the antimicrobial activity of metabolites of MAI 3

4.4.5 Effect of Nitrogen Sources.

The antimicrobial activity against *E .coli* and *B. subtilis* by MAI 1, 2 and 3, when the medium was supplemented with sodium nitrate, potassium nitrate, ammonium chloride, ammonium nitrate, asparagine, ammonium phosphate, and ammonium sulphate is presented in the following figure (figure 4.18, 4.19 and 4.20). The highest antimicrobial activity by MAI 1 occurred when the medium was supplemented with asparagine as nitrogen source whilst the least activity was observed with the addition of sodium nitrate. The activity against *B. subtilis* was much greater than *E. coli*. For MAI 2 and MAI 3, the highest activity occurred using asparagine. However, for MAI 2, the least activity was produced by ammonium phosphate whilst for MAI 3 the least activity was produced by sodium nitrate. For both MAI 2 and 3, activity against *E. coli* was generally greater than against *B. subtilis*.

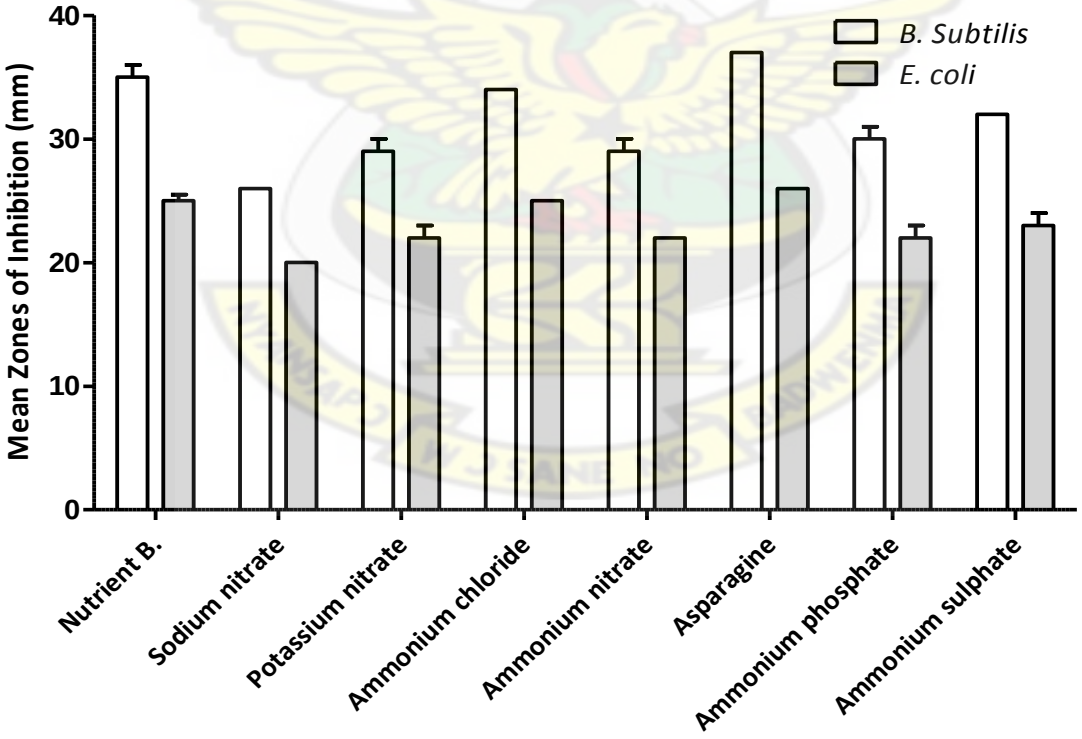


Fig. 4.18 Effect of nitrogen sources on the antimicrobial activity of metabolites of MAI 1

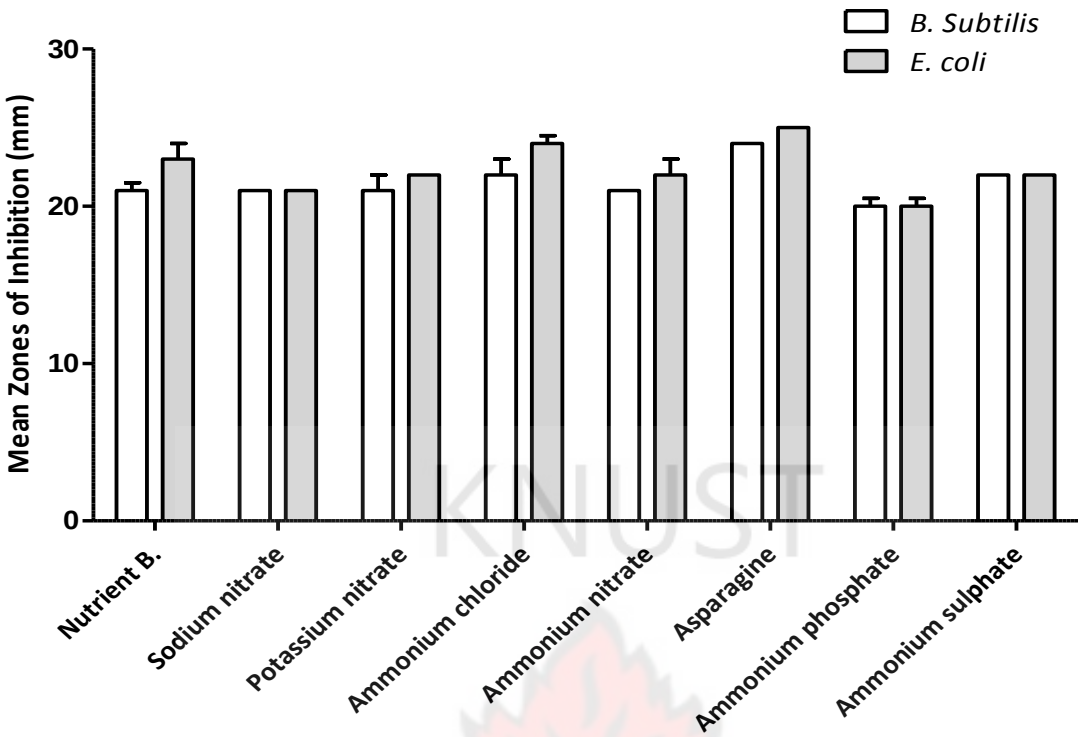


Fig. **4.19** Effect of nitrogen sources on antimicrobial activity of metabolites of MAI 2

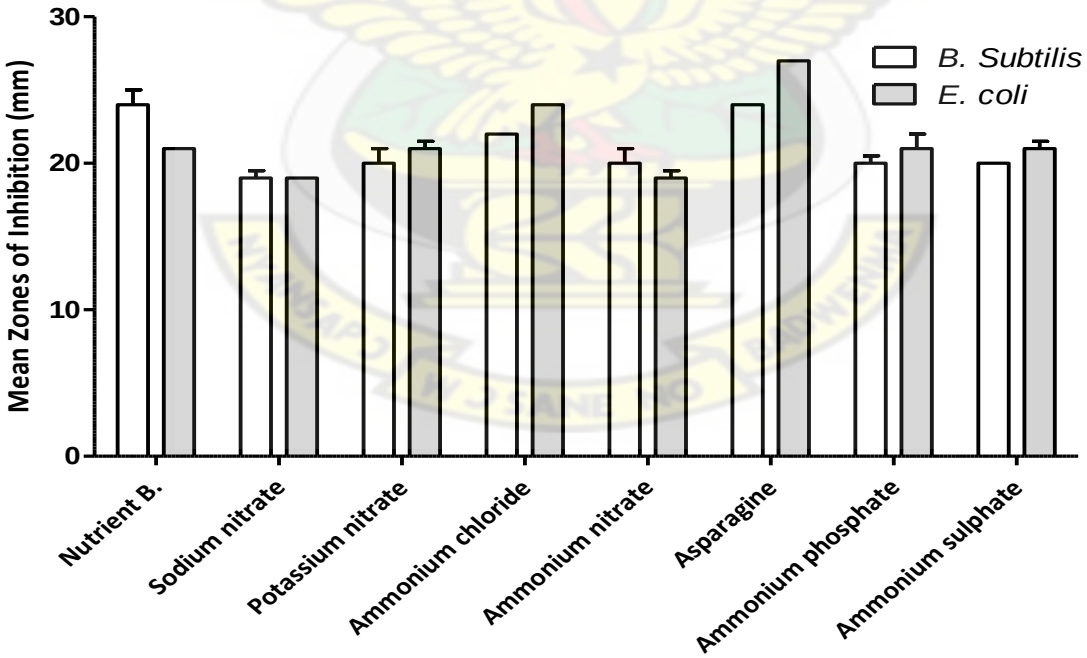


Fig. **4.20** Effect of nitrogen sources on antimicrobial metabolite production by MAI 3

4.5 Extraction and bioassay of antimicrobial metabolite of MAI 2

The chloroform extract appeared as a dark brown precipitate with a yield of 0.0112%w/v. The crude extract tested positive in bioassay against the test strains, *E. coli*, *P. vulgaris* (NCTC 4635), *E. faecalis* (ATCC 29212), *Staph. aureus* (ATCC 25923), *B. subtilis* (NCTC 10073), *B. thuringiensis* (ATCC 13838), *S. typhi* and *C. albicans* with minimum inhibitory concentration (MIC) values ranging from 0.25 mg/ml to 4 mg/ml. *Proteus vulgaris* (NCTC 4635) was the most susceptible organism with MIC of 0.25 mg/ml whilst *B. subtilis* NCTC 10073 was the least susceptible organism with MIC value of 4 mg/ml. There was however no activity against *Pseudomonas aeruginosa* (ATCC 27853). The MICs of the crude extract is shown in the following table (table 4.3). No values were obtained for MBC/MFC. There was growth on the nutrient agar plates when loopfuls from the wells that showed no signs of growth were plated on them. The MBC or MFC may be outside the concentration range used.

Table 4.3 Minimum Inhibitory Concentration (MIC) of crude extract

Test organism	MIC in mg/ml
Escherichia coli	0.50
Proteus vulgaris NCTC 4635	0.25
Enterococcus faecalis ATCC 29212	0.50
Staph aureus ATCC 25923	1.00
Bacillus subtilis NCTC 10073	4.00
Bacillus thuringiensis ATCC 13838	1.00
Salmonella typhi	0.50
Candida albicans	2.00

4.6 Bio-autography assay

The thin layer chromatography of the crude extract of MAI 2 revealed seven (7) spots under UV light at 254nm. Three of the spots fluoresced at 356 nm wavelength. When the developed plates were subjected to bio-autography assay, all the seven spots exhibited zones of growth inhibition against *P. vulgaris*. The R_f values of the spots were 0.86, 0.77, 0.55, 0.52, 0.44, 0.30 and 0.22. The developed TLC plate and the bio-autography assay are shown in the following figure (figure 4.21 and 4.22).

Fig. 4.21 The TLC plate of the crude extract of MAI 2 showing the various separated components of the metabolite.

Fig. 4.22 Bio-atuography of crude extract of MAI 2 showing the zones of inhibition as yellow spots against a purple background.

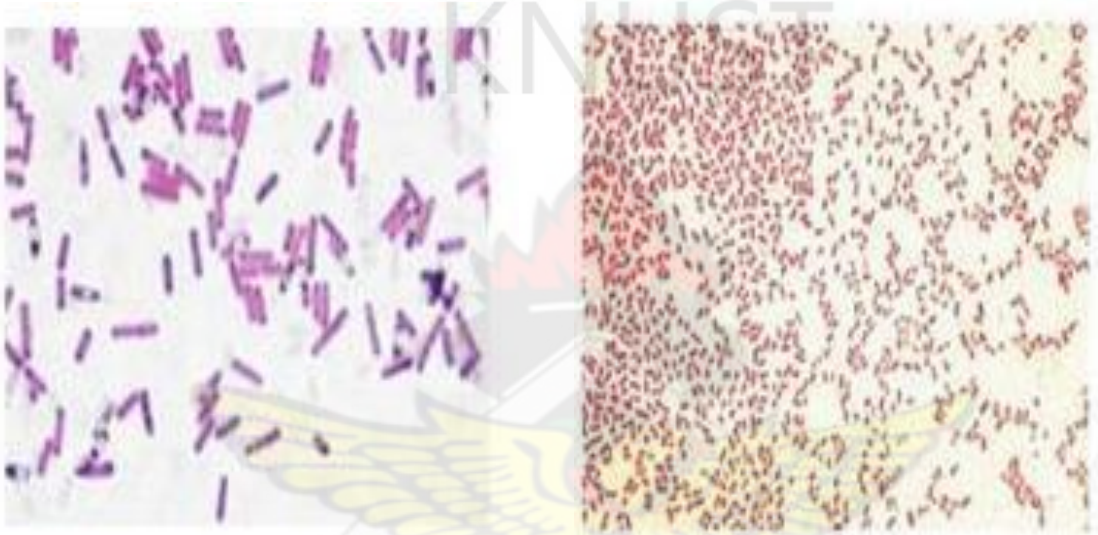
4.7 Characterization of selected isolates with antimicrobial activity.

The morphological features of the isolates are shown in table 4.4 and figure 4.23. Microscopic examination showed that MAI 1 was a motile spore-forming, gram-positive rod (figure 4.23a). MAI 2 and MAI 3 were motile gram-negative rods (figure 4.23b and c). For MAI 1, there was no gas production in the fermentation media but fermentation of glucose and sucrose resulted in acid production (table 4.5). MAI 1 was capable of utilizing citrate for growth and also produced the enzyme oxidase but could not produce indole when cultured in tryptone water. MAI 1 was thus identified as a *Bacillus species*. MAI 2 and MAI 3 both utilized citrate for growth and produced the oxidase enzyme. Both organisms appeared as green colonies on cetrimide agar with a green diffusible pigment. The organisms also grew at 42^{o}C. MAI 2 and MAI 3 were thus identified to be strains of *Pseudomonas aeruginosa*. The following tables, Tables 4.5 and 4.6 show the results of the biochemical reactions.

Table 4.4 Cultural Characteristics of Isolates.

Isolate	Margin	Elevation	Colour	Shape	Texture
MAI1	Undulate	Raised	white	Circular	Wrinkled
MAI2	Filamentous	Flat	green	circular	Smooth
MAI3	Filamentous	Flat	green	circular	Smooth

(a) (b)



(c)

Fig. 4.23 Gram stains showing gram-positive rods of (a) MAI 1 and gram-negative rods of (b) MAI 2 and (c) MAI 3.

Table 4.5 Carbohydrate fermentation reactions of MAI 1, MAI 2 and MAI 3.

Isolates	Lactose			Glucose			Sucrose			Mannitol		
	Gr	Ac	Ga	Gr	Ac	Ga	Gr	Ac	Ga	Gr	Ac	Ga
MAI1	+	-	-	+	+	-	+	+	-	+	-	-
MAI2	+	-	-	+	+	-	+	+	-	+	+	-
MAI3	+	-	-	+	-	-	+	-	-	+	-	-

Gr = growth, Ac = acid, Ga = gas, (+) = present, (-) = absent

Table 4.6 Biochemical tests of MAI 1, MAI 2 and MAI 3

Biochemical tests	MAI 1	MAI 2	MAI 3
Growth on Cetrimide	-	+	+
Growth on MacConkey	+	+	+
Growth on Mannitol Salt	+	+	+
Growth on Bismuth Sulphite	-	+	+
Citrate utilization	+	+	+
H_2S production	+	-	-
Indole production	-	-	-
Nitrate reduction	+	+	+
Motility	+	+	+
Gram reaction	+	-	-
Pigment	-	+	+
Diffusion of pigment	-	+	+
Spores	+	-	-
Shape	Rods	Rods	Rods
Growth at 42^oC	*	+	+
Oxidase test	+	+	+

(+) = positive, (-) = negative, (*) = not applicable

CHAPTER FIVE

5.1 DISCUSSION

Screening for antimicrobial activity of microbes isolated from River Wiwi, Lake Bosomtwe and the Duakor Sea has not been reported yet. Results from this study have however shown the presence of microbes capable of producing antimicrobial metabolites in these habitats. A large number of the colonies (119) exhibited inhibitory actions in the master plates as reported in section 4.1. The high proportion of antibiotic producers isolated from the master plates may be associated with an ecological role, serving as a defensive mechanism to maintain their niche, or enabling the invasion of an established microbial community. Although twenty-seven (27) out of the 119 isolates (23%) showed antimicrobial activity in this study, some inhibitory screening investigations have recorded values closer to what was obtained in this study, whilst others have also recorded very high and very low values. For example a study carried out by Ivanova *et al*. (1998) reported that out of the 491 bacteria isolated from different marine sources, 26% of the isolates were active. Zheng *et al*. (2005) also reported that eight out of twenty-nine (29) strains, representing 28 % of the microbes considered in their study were able to inhibit the growth of at least one of the target microorganisms. Another study carried out by Brandelli *et al*. (2004) recorded 70% of active isolates from the Amazon Basin whilst O'Brien *et al*. (2004) recorded as low as 0.29% (13 out of 4496) of active microbes from soil samples collected at different locations in the Antarctica. The differences among the detection rates reported in literature strongly depend on the isolation and assay procedures, test organisms, type of media used, as well as the sources of bacterial isolates (Giudice *et al*., 2007).

Screening of isolated microorganisms

The isolates inhibited gram-positive bacteria better than the gram-negative bacteria. This could be attributed to the differences in the sensitivities of gram-positive and gram-negative bacteria due to the differences in the structure and composition of their cell walls. Gram-negative bacteria possess a thin peptidoglycan layer and a unique outer membrane which consists of lipopolysaccharide (LPS) components. This outer membrane makes the cell wall impermeable to lipophilic solutes thus blocking certain antibiotics such as penicillin, dyes, and detergents from penetrating the cell. Gram-positive bacteria only possess the peptidoglycan layer which is not a very effective permeability barrier (Scherrer and Gerhardt, 1971).

Some of the isolates exhibited inhibitory activities in the master plates but when they were isolated into pure cultures, they lost this effect. Several reasons could account for the presence of inhibition zones exhibited by isolates in the master plates which later disappeared when the isolates were grown as pure cultures. According to Bushell and Grafe (1989), in the master plates the isolates exhibited a higher degree of competition for space which provoked the cells to secrete diverse compounds to serve as competition mechanisms. However in the absence of competition they were unable to secrete compounds in the liquid media. Also as cells in the master plates were closer to each other, chemical signals could have been emitted which could induce the isolates to secrete inhibitory substances. Also the genes that synthesize antimicrobial agents may have to be activated by diverse signals, for example the presence of a protein or enzyme, so that if the target that was used does not synthesize the same inducers that were synthesized in the master plates then the antagonistic substance will not be produced.

The antimicrobial activity exhibited by the supernatant solutions of isolates MAI 1, MAI 2 and MAI 3 showed that antimicrobial agents are not produced only in the presence of competition. In some organisms, these metabolites are produced perhaps to serve as regulators for cellular differentiation processes and in other instances, as a means of avoiding predation on themselves and also increase predation on other bacteria. The stability study of the cell-free supernatant solutions indicated that the antimicrobial agents of these isolates can gradually deteriorate with time.

Effect of heat treatment on the isolates

The thermal stability of the antimicrobial metabolites of the selected isolates was tested by subjecting them to various heat treatments. Metabolites of MAI 1 showed activity at 40°C only. There was loss of activity at the higher temperatures. The result shows the thermo labile nature of the compound produced by MAI 1. This result is in contrast to heat stability studies carried out on antimicrobial metabolites produced by *Bacillus* species. For example, a study carried out by Tabbene *et al*. (2009) reported that the antimicrobial agent from *Bacillus subtilis* species, strain B38 was still active after autoclaving at 121°C for 20 minutes. Bernal *et al*, (2002) also reported a thermally stable antimicrobial agent from *Bacillus* species. For MAI 2 and MAI 3, the antimicrobial agents they produced were stable up to 100°C but activity was completely lost at 121°C. This result is also in contrast to those reported by Preetha *et al*. (2009) who reported that the antimicrobial agent produced by *Pseudomonas* species MCCB was stable after sterilization even though there was a significant reduction in activity. Uzair *et al*. (2006) also reported the thermal stability of an antimicrobial agent produced by *Pseudomonas aeruginosa* at a temperature of 121°C for 20 minutes. There are many species of the genus *Bacillus* and *Pseudomonas* that can produce a wide variety of antibiotics. Roitman and Janisiewicz (1990) reported that variations in the fermentation

medium often result in changes in the composition of the antibiotics produced. The differences in the thermal stability of the antimicrobial agents produced in this study as compared to literature may therefore be due to differences in some nutritional and physical factors which led to the production of metabolites that were thermo labile.

Optimization of some physical and nutritional parameters of MAI 1, MAI 2 and MAI 3

Several factors influence production of secondary metabolites by microorganisms, the most important one being the composition of the production medium (Price-Whelan *et al*., 2006). Knowledge of the effects of these factors helps to determine the optimum conditions for maximum metabolite production by the organisms. Variations in the fermentation media resulted in differences in the size of zones of inhibition produced which might be due to changes in pH during bacterial growth which in turn affected secondary metabolite production. Roitman and Janisiewicz (1990) reported that by varying the conditions under which *Burkholderia cepacia* is grown, the yields and the composition of the antibiotic could be changed. Glucose which is usually an excellent source of carbon for growth did not produce any activity in MAI 1 and also decreased antibiotic activity in MAI 2 and MAI 3. Sole *et al*. (1997) noted that glucose can be used as a source for bacterial growth while repressing the production of secondary metabolites. The organisms utilised glycerol and starch best for maximum production of the antimicrobial metabolites.

Nitrogen is very vital in the synthesis of enzymes involved in primary and secondary metabolism (Merrick and Edwards, 1995). Therefore depending on the biosynthetic pathways involved, nitrogen sources may affect antibiotic formation. Shapiro (1989) noted that the type of nitrogen source (organic or inorganic) plays a role in the synthesis of secondary metabolites. According to Farid *et al*. (2000) inorganic nitrogen sources such as nitrates,

nitriles and several ammonium salts decrease the production of secondary metabolites by increasing cell biosynthesis. Madhava and Gnanamani (2010) in their study on 'Condition stabilization for *Pseudomonas aeruginosa* MTCC 5210 to yield high titres of extra cellular antimicrobial secondary metabolite using Response Surface Methodology' also reported that *Pseudomonas aeruginosa* MTCC 5210 utilized organic nitrogen source for better yield than the inorganic sources. These observations are consistent with the findings of this study as asparagine was better used for antibiotic production than the inorganic nitrogen sources used.

Effect of pH, temperature and incubation time

In general, the intracellular pH of most microorganisms is maintained near neutrality regardless of the pH in the outside medium (Garland, 1977). However as the proton gradient across the cytoplasmic membrane increases, the cells are forced to commit more of their resources towards maintaining the desired intracellular pH (Riebeling *et al*., 1975), thus changes in external pH affect many cellular processes such as growth and the regulation of the biosynthesis of secondary metabolites (Chang *et al*., 1991). Effect of pH was studied by adjusting the fermentation medium to different pH values. Antimicrobial activity of metabolites of MAI 1 which was identified as a *Bacillus* species was highest at pH 8. Similarly a study carried out by Muaaz *et al*. (2007) showed that *Bacillus subtilis* produced more of an antimicrobial agent at a pH between 7 and 8. Another study carried out by Awais *et al*. (2008) reported that *Bacillus pumilus* produced metabolites with maximum activity at pH 8 against *Micrococcus Luteus*. Similar results have also been reported by Moita *et al*. (2005) in their study of inhibitory activity of *Bacillus subtilis* 355. For MAI 2 and MAI 3 which were both identified as strains of *Pseudomonas aeruginosa*, a pH of 7 was the optimum for maximum activity of their metabolites. This result agrees with a study carried out by Charyulu and Gnanamani (2010) who reported maximum production of metabolite by

Pseudomonas aeruginosa MTCC 5210 at pH 7. Differences in pH requirements by organisms may be due to the fact that different strains of organisms are unique in their own way and therefore may require different conditions for production of antibiotics. All the organisms showed no growth when the pH of the medium was adjusted to pH 4, indicating their intolerance of acidic conditions.

Effect of different temperatures on the activity of the metabolites was also studied. Each of the organisms had different temperatures at which the greatest antimicrobial activity was observed. Maximum antimicrobial activity was obtained at 25°C for MAI 1 and 30°C for MAI 2 and MAI 3. Temperatures higher than the optimum resulted in smaller zones of inhibition. This clearly shows that organisms have specific temperatures or range of temperatures that enhance growth and secondary metabolite production. The effect of extent of incubation on the activity of the antibacterial compounds also showed that the activity of the antibacterial compound increase with increasing culture age up to a point (10 days in this study) where it becomes constant.

Extraction and bioassay of antimicrobial metabolite of MAI 2

The crude antibiotic extract of isolate MAI 2 exhibited activity against all the test organisms except *Pseudomonas aeruginosa*. These results indicated that the antagonistic activity was due to the production of an antimicrobial compound which can be extracted from the growth medium with organic solvents. MIC of the crude extract ranged from 0.25mg/ml to 4mg/ml. Results of the TLC bio-autography assay revealed seven spots with antimicrobial activity. This demonstrates that organisms can produce more than one antimicrobial agent to provide themselves the survival competition superiority.

The extract appears to be bacteriostatic in its mode of action. Bacteriostatic agents (e.g. β-lactams, chloramphenicol, clindamycin, macrolides and linezolid) have been effectively used for treatment of a range of bacterial infections, including endocarditis, meningitis, and osteomyelitis (Pankey and Sabath, 2004). Furthermore, a bacteriostatic agent like clindamycin has been shown to completely inhibit the toxic shock syndrome toxin-1 production by *Staphylococcus aureus* (Van Lagevelde *et al.*, 1997) and toxin production in both *Streptococci* and *Staphylococci* (Russell and Pachorek, 2000). These reports suggest that the active constituents present in this crude extract have the potential of being efficacious in the treatment of various infectious. The extract is therefore a rich source of bioactive compounds which can serve as leads for the development of efficacious antibiotics.

5.2 CONCLUSIONS

It was found out from this study that antibiotic producing microorganisms are present in Lake Bosomtwe, River Wiwi and the Duakor Sea. Out of the 119 antibiotic producers isolated from the study sites, 27 of them were active against at least one of the test organisms of which three (MAI 1, MAI 2 and MAI 3) exhibited zones of inhibition greater than 19 mm. The optimum temperature for maximum activity by MAI 1 was 25°C whilst MAI 2 and MAI 3 achieved maximum activity at 30°C. The optimum pH for maximum activity by MAI 1 was 8 whereas maximum antibiotic activity by MAI 2 and MAI3 was at pH 7. The best carbon and nitrogen sources which supported antimicrobial agent production by MAI 1 were glycerol and asparagine respectively. For MAI 2 and MAI 3, starch and asparagine were the carbon and nitrogen sources respectively, which gave maximum antibiotic activity. The antimicrobial agent produced by MAI 2 was extracted from the production medium using chloroform. The crude extract was active against the test organisms; *Bacillus thuringiensis* (ATCC 13838), *Proteus vulgaris* (NCTC 4175), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (NCTC 10073), *E. coli* and *Candida albicans*. Based on biochemical and microscopic examinations, MAI 1 was identified as *Bacillus* species whilst MAI 2 and MAI 3 were identified as strains of *Pseudomonas aeruginosa*.

5.3 RECOMMENDATIONS

The compounds responsible for the antimicrobial action should be isolated, purified and identified in further studies. As there remain scanty information on antibiotic producing microorganisms from Lake Bosomtwe and River Wiwi, there should be extensive research in these habitats to explore the possibility of obtaining new bioactive compounds.

REFERENCES

Abdel-Wahab, M.A., Asolkar R.N., Inderbitzin, P. and Fenical, W. (2007). Secondary metabolite chemistry of the marine-derived fungus Massarina sp., strain CNT-016. *Phytochem.* **68**(8)**:** 1212-1218.

Abou-Zeid, A.A., Abd El-Wahab, I.E. and Salam, M. (1972). The fermentative production of gentamicin by Micromonospora purpurea. *J. Appl. Chem and biotechnol.* **26**: 316-322

Abu-Salah, K.M. (1996). Amphotericin B: an update. *Br. J. Biomed. Sci.* 53: 122-133.

Alkofahi, A., Rupprecht, J.K., Anderson, J.E., McLaughlin, J.L., Mikolakczak, K.L. and Scott, B.A. (1989). Search for new pesticides from higher plants. *Insecticides of Plant Origin*, ACS Symp. Ser. 387.

Anon. (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin Microbiol Infect.* **9:** 1–7.

Awais, M., Pervez, A., Qayyum, S. and Saleem, M. (2008). Effects of glucose, incubation period and pH on the production of peptide antibiotics by *Bacillus pumilus. Afri. J. Microbiol. Res.* **2**: 114-119.

Baker, D.D. and Alvi, K.A. (2004). Small-molecule natural products: new structures, new activities. *Curr Opin Biotechnol.* **15**(6)**:** 576-583.

Barbachyn, M.R. and Ford, C.W. (2003). Oxazolidinone structure-activity relationships leading to linezolid. *Angew Chem Int Ed Engl* **42**(18)**:** 2010-2023.

Berdy, J. (2005). Bioactive microbial metabolites. *J Antibiot (Tokyo)* **58**(1)**:** 1-26.

Bernal, G., Lilanes, A., and Ciampi, L. (2002). Isolation and partial purification of a metabolite from a mutant strain of *Bacillus sp*. with antibiotic activity against plant pathogenic agents. *EJB Electronic Journal of Biotechnol.* **5:** 12-20.

Bhadury, P., Balsam, T. and Wright, M.P.C (2006). The current status of natural products from marine fungi and their potential as anti-infective agents. *J Ind Microbiol Biotechnol* **33:** 325–337.

Bister, B., Bischoff, D., Strobele, M., Riedlinger, J., Reicke, A., Wolter, F., Bull, A. T., Zahner, H. Fiedler, H. P. and Sussmuth, R. D. (2004). Abyssomicin C-A polycyclic antibiotic from a marine Verrucosispora strain as an inhibitor of the p-aminobenzoic acid/tetrahydrofolate biosynthesis pathway. *Angew Chem Int Ed Engl* **43**(19)**:** 2574-2576.

Blunt, J.W., Copp, B.R., Hu, W.P., Munro, M.H., Northcote, P.T. and Prinsep, M.R. (2009). Marine natural products. *Nat Prod Rep* **26**(2)**:** 170-244.

Brandelli, A., Cladera-Olivera, F. and Motta, S.A. (2004). Screening for antimicrobial activity among bacteria isolated from the Amazon Basin. *Brazilian Journal of Microbiol.* **35:** 307-310.

Brumfitt, W. and Hamilton-Miller, J.M. (1988). The changing face of chemotherapy. *Postgrad Med J* **64**(753)**:** 552-558.

Bruntner, C., Binder, T., Pathom-aree, W., Goodfellow, M., Bull, A.T., Potterat, O., Puder, C., Hörer, S., Schmid, A., Bolek, W., Wagner, K., Mihm, G. and Fiedler, H.P. (2005). Frigocyclinone, a novel angucyclinone antibiotic produced by a Streptomyces griseus strain from Antarctica. *J Antibiot (Tokyo)* **58**(5)**:** 346-349.

Bugni, T.S. and Ireland, C.M. (2004). Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Nat Prod Rep* **21**(1)**:** 143-163.

Bultel-Ponce, V.V., Berge, J.P., Debitus, C., Nicolas, J.L. and Guyot, M. (1999). Metabolites from the Sponge-Associated Bacterium Pseudomonas Species. *Mar Biotechnol (NY)* **1**(4)**:** 384-390.

Burkholder, P.R., Pfister, R.M., Leitz, F.H. (1966). Production of a pyrrole antibiotic by a marine bacterium. *Appl Microbiol* **14**(4)**:** 649-653.

Bushell, M. and Grafe, U. (1989). Bioactive metabolites from microorganisms. *Ind Microbiol* **27**: 402-418.

Butaye, P., Devriese, L.A. and Haesebrouck, F. (2000). Influence of different medium components on the in vitro activity of the growth-promoting antibiotic flavomycin against enterococci. *J Antimicrob Chemother* **46**(5)**:** 713-716.

Charyulu, M.E. and Gnanamani, A. (2010). Condition stabilization for Pseudomonas aeruginosa MTCC 5210 to Yield High Titres of Extra Cellular Antimicrobial Secondary metabolite using Response Surface Methodology. *Curr Res in Bacteriol* **4:** 197-213.

Chang, S.C., Wei, Y.H., Wei, D.L., Chen, Y.Y. and Jong, S.C. (1991). Factors affecting the production of eremofortin C and PR toxin in *Penicillium roqueforti*. *Appl. Environ. Microbiol*., **57**: 2581- 2585.

Christie, S.N., McCaughey, C., McBride, M. and Coyle, P.V. (1997). Herpes simplex type 1 and genital herpes in Northern Ireland. *Int J STD AIDS* **8**(1)**:** 68-69.

Chythanya, R. and Karunasagar I. (2002). Inhibition of shrimp pathogenic vibrios by a marine Pseudomonas I-2 strain. *Aquaculture* **208:** 1–10.

Cole, M.D. (1994). Key antifungal, antibacterial and anti-insect assays—a critical review. *Biochem Syst Ecol* **22:** 837–856.

Cos, P., Vlietinck, A.J., Berghe, V.D. and Maes, L. (2006). Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. *J Ethnopharmacol* **106:** 290–302.

Cueto, M., Jensen, P.R., Kauffman, C., Fenical, W., Lobkovsky, E. and Clardy, J. (2001). Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. *J Nat Prod* **64**(11)**:** 1444-1446.

Daferner, M., Anke, T. And Sterner, O. (2002). Zopfiellamides A and B, antimicrobial pyrrolidinone derivatives from the marine fungus Zopfiella latipes. *Tetrahedron* **58:** 7781–7784.

Darken, M.A., Berenson, H., Shirk, R.J. and Sjolander, N.O. (1960). Production of tetracycline by Streptomyces aureofaciens in synthetic medium. *Appl Microbiol* **8**: 46–51.

Davies, J. (2007). Microbes have the last word. *EMBO Rep* **8**: 616–621.

Demain, A.L. and Fang, A. (2000). The natural functions of secondary metabolites. *Adv Biochem Eng Biotechnol* **69:** 1-39.

Demain, A.L. (2009). Antibiotics: natural products essential to human health. *Med Res Rev* **29**(6)**:** 821-842.

Desjardine, K., Pereira, A., Wright, H., Matainaho, T., Kelly, M. and Andersen, R.J. (2007). Tauramamide, a lipopeptide antibiotic produced in culture by Brevibacillus laterosporus isolated from a marine habitat: structure elucidation and synthesis. *J Nat Prod* **70**(12)**:** 1850-1853.

Du, L., Zhu, T., Fang, Y., Liu, H., Gu, Q., and Zhu, W. (2007). Aspergiolide A, a novel anthraquinone derivative with naphtho[1,2,3-de]chromene-2,7-dione skeleton isolated from a marine-derived fungus Aspergillus glaucus. *Tetrahedron* **63:** 1085–1088.

El-Gendy, M.M.A., Hawas, U.W., and Jaspars, M. (2008). Novel bioactive metabolites from a marine derived bacterium Nocardia sp. ALAA 2000. .*J Antibiot* **61:** 379–386.

Evieyer, N.N., Ferrigni, N.R., Putman, J.E., Jacobsen, L.B., Nicholas, D.E. and McLaughlin, J.L. (1982). Brine shrimps: a convenient general bioassay for active plant constituents. *Planta Medicina,* **45**: 31

Fabricant, D.S. and Farnsworth, N.R. (2001). The value of plants used in traditional medicine for drug discovery. *Environ. Health Perspect* **109**(1): 69–75.

Farid, M.A., Enshasy, H.A.E., Diwany, A.I.E. and Sayed, A.E.E. (2000). Optimization of the cultivation for natamycin production by *Streptomyces natalensis*. *J. Basic Microbiol*. **3**: 157-166.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D. and Guo, Z. (1985). Medicinal plants in therapy. *Bull World Health Organ* **63**(6)**:** 965-981.

Fenical, W. (1993). Chemical studies of marine bacteria: developing a new resource. *Chem. Rev.* **93**(5)**:** 1673-1683.

Fenical, W. (1997). New pharmaceuticals from marine organisms. *Trends Biotechnol* **15**(9)**:** 339-341.

Freiberg, C., Brunner, N.A., Schiffer, G., Lampe, T., Pohlmann, J., Brands, M., Raabe, M., Habich, D., Ziegelbauer, K. (2004). Identification and characterization of the first class of potent bacterial acetyl-CoA carboxylase inhibitors with antibacterial activity. *J Biol Chem* **279**(25)**:** 26066-26073.

Fremlin, L.J., Piggott, A.M., Lacey, E. and Capon, R.J. (2009). Cottoquinazoline A and cotteslosins A and B, metabolites from an Australian marine-derived strain of Aspergillus versicolor. *J Nat Prod* **72**(4)**:** 666-670.

Gabrielson, J., Hart, M., Jarelov, A., Kuhn, I., McKenzie, D. and Mollby, R. (2002). Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates. *J Microbiol Methods* **50**(1)**:** 63-73.

Galski, A.G., Wisley, J.P., and Powell, R.J. (1980). Crown gall tumor disc bioassay: a possible aid in the detection of compounds with antitumor activity. *Plant Physiol*ogy **65**: 184.

Gamal-Eldeen, A.M., Abdel-Lateff, A., and Okinoc, T. (2009). Modulation of carcinogen metabolizing enzymes by chromanone A; a new chromone derivative from algicolous marine fungus Penicillium sp. *Environ Toxicol Pharmacol* **28**(317–322.).

Garland, P.B. (1977). Energy transduction in microbial systems. *Symp. Soc. Gen. Microbiol*. **27**:1-21

Garo, E., Starks, C.M., Jensen, P.R., Fenical, W., Lobkovsky, E. and Clardy, J. (2003). Trichodermamides A and B, cytotoxic modified dipeptides from the marine-derived fungus Trichoderma virens. *J Nat Prod* **66**(3)**:** 423-426.

Giudice A.L., Bruni, V. and Michaud, L. (2007). Characterization of Antarctic psychrotrophic bacteria with antibacterial activities against terrestrial microorganisms. *J. Basic Microbiol* **47:** 496–505.

Goldstein, F.W. and Garau, J. (1997). 30 years of penicillin-resistant S pneumoniae: myth or reality? *Lancet* **350**(9073)**:** 233-234.

Greenberg, E.P. (2003). Bacterial communication and group behaviour. *J Clin Invest* **112**(9): 1288-90.

Greve, H., Schupp, P.J., Eguereva, E., Kehraus, S., Kelter, G.M.A., Maier, A., Fiebig, H. F. and König, G. M. (2008). Apralactone A and a new stereochemical class of curvularins from the marine fungus Curvularia sp. *Eur J Org Chem* **2008:** 5085–5092.

Hadacek, F. and Greger, H. (2000). Testing of antifungal natural products: methodologies, comparability of results and assay choice. *Phytochem Analysis* **11:** 137–147.

Hamburger, M.O. and Cordell, G.A. (1987). A direct bioautographic TLC assay for compounds possessing antibacterial activity. *J Nat Prod* **50:** 19–22.

Harvey, A.L. (2009). The continuing value of natural products for drug discovery. *Rev. salud anim.* **31:** 8-12.

Hsueh, P.R., Teng, L.J., Pan, H.J., Chen,Y.C., Sun, C.C., Ho, S.W. and Luh, K.T. (1998). Outbreak of *Pseudomonas fluorescens* bacteremia among oncology patients. *J.Clin Microbiol*. **36**: 2914-7.

Hucker, G. J. and Conn, H. J. (1923). Methods of Gram staining. *Tech. Bull. N. Y. St. agric. Exp. Sta.* **no. 93**.

Hughes, C.C., Prieto-Davo, A., Jensen, P.R. and Fenical, W. (2008). The marinopyrroles, antibiotics of an unprecedented structure class from a marine Streptomyces sp. *Org Lett* **10**(4)**:** 629-631.

Hwang, Y. Rowley, D., Rhodes, D., Gertsch, J., Fenical, W. and Bushman, F. (1999). Mechanism of inhibition of a poxvirus topoisomerase by the marine natural product sansalvamide A. *Mol Pharmacol* **55**(6)**:** 1049-1053.

Ingavat, N., Dobereiner, J., Wiyakrutta, S., Mahidol, C., Ruchirawat, S. and Kittakoop, P. (2009). Aspergillusol A, an alpha-glucosidase inhibitor from the marine-derived fungus Aspergillus aculeatus. *J Nat Prod* **72**(11)**:** 2049-2052.

Isnansetyo, A. and Kamei, Y. (2009). Bioactive substances produced by marine isolates of Pseudomonas. *J Ind Microbiol Biotechnol* **36**(10)**:** 1239-1248.

Isnansetyo, A., Horikawa, M. and Kamei, Y. (2001). In vitro anti-methicillin-resistant Staphylococcus aureus activity of 2,4-diacetylphloroglucinol produced by Pseudomonas sp. AMSN isolated from a marine alga. *J Antimicrob Chemother* **47**(5)**:** 724-725.

Ivanova, E.P., Nicolau, D.V., Yumoto, N. and Taguchi, T. (1998). Impact of conditions of cultivation and adsorption on antimicrobial activity of marine bacteria. *Mar. Biol* **130:** 545–551.

Jahn, B., Martin, E., Stueben, A. and Bhakdi, S. (1995). Susceptibility testing of Candida albicans and Aspergillus species by a simple microtiter menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. *J Clin Microbiol* **33**(3)**:** 661-667.

Jayatilake, G.S., Thornton, M.P., Leonard, A.C., Grimwade, J.E. and Baker, B.J (1996). Metabolites from an Antarctic sponge-associated bacterium, Pseudomonas aeruginosa. *J Nat Prod* **59**(3)**:** 293-296.

Jensen, P.R. and Fenical, W. *(*1994*)* Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives. *Annu Rev Microbiol* **48***:* 559–584.

Kanoh, K., Okada, A., Adachi, K., Imagawa, H., Nishizawa, M., Matsuda, S., Shizuri, Y. and Utsumi, R. (2008). Ascochytatin, a novel bioactive spirodioxynaphthalene metabolite produced by the marine-derived fungus, Ascochyta sp. NGB4. *J Antibiot (Tokyo)* **61**(3)**:** 142-148.

Kjer, J., Wray, V., Edrada-Ebel, R., Ebel, R., Pretsch, A., Lin, W. and Proksch, P. (2009). Xanalteric acids I and II and related phenolic compounds from an endophytic Alternaria sp. isolated from the mangrove plant Sonneratia alba. *J Nat Prod* **72**(11)**:** 2053-2057.

Kohlmeyer, J. (1974). Veroff Inst. Meeresforsch. *Bremerhaven Suppl* **5:** 263–286.

Kralj, A., Kehraus, S., Krick, A., Van Echten-Deckert, G. and Konig, G.M. (2007). Two new depsipeptides from the marine fungus Spicellum roseum. *Planta Med* **73**(4)**:** 366-371.

Lahana, R. (1999). How many leads from HTS? *Drug Discov Today* **4**(10)**:** 447-448.

Liu, H., Edrada-Ebel, R., Ebel, R., Wang, Y., Schulz, B., Draeger, S., Mueller, W. E. G., Wray, V., Lin, W. and Proksch P. (2009). Drimane sesquiterpenoids from the fungus Aspergillus ustus isolated from the marine sponge Suberites domuncula. *J Nat Prod* **72**(9)**:** 1585-1588.

Liu, Z., Jensen, P.R. and Fenical, W. (2003b). A cyclic carbonate and related polyketides from a marine-derived fungus of the genus Phoma. *Phytochemistry* **64:** 571–574.

Lösgen, S., Schlörke, O., Meindl, K., Herbst-Irmer, R., and, Zeeck, A. (2007). Structure and Biosynthesis of Chaetocyclinones, New polyketides produced by an endosymbiotic fungus. *Eur J Org Chem* **2007:** 2191–2196.

Macherla, V.R., Liu, J., Bellows, C., Teisan, S., Nicholson, B., Lam, K.S. and Potts, B. C. (2005). Glaciapyrroles A, B, and C, pyrrolosesquiterpenes from a Streptomyces sp. isolated from an Alaskan marine sediment. *J Nat Prod* **68**(5)**:** 780-783.

Mah, T.F.C. and O'Toole, G.A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiol* **9:** 34–39.

Maskey, R.P., Helmke, E. and Laatsch, H. (2003). Himalomycin A and B: isolation and structure elucidation of new fridamycin type antibiotics from a marine Streptomyces isolate. *J Antibiot (Tokyo)* **56**(11)**:** 942-949.

Maskey, R.P., Sevvana, M., Uson, I., Helmke, E. and Laatsch, H. (2004). Gutingimycin: a highly complex metabolite from a marine streptomycete. *Angew Chem Int Ed Engl* **43**(10)**:** 1281-1283.

Matsuda, M., Shigeta, S. and Okutani, K.(1999). Antiviral Activities of Marine Pseudomonas Polysaccharides and Their Oversulfated Derivatives. *Mar Biotechnol (NY)* **1**(1)**:** 68-73.

McArthur, K.A., Mitchell, S.S., Tsueng, G., Rheingold, A., White, D.J., Grodberg, J., Lam, K. S. and Potts, B. C. (2008). Lynamicins A-E, chlorinated bisindole pyrrole antibiotics from a novel marine actinomycete. *J Nat Prod* **71**(10)**:** 1732-1737.

Merrick, M.J. and Edwards, R.A. (1995). Nitrogen control in bacteria. *Microbiol. Rev*. **59**: 604-622

Minagawa, K., Kouzuki, S., Yoshimoto, J., Kawamura, Y., Tani, H., Iwata, T., Terui, Y., Nakai, H., Yagi, S., Hattori, N., Fujiwara, T. and Kamigauchi, T. (2002). Stachyflin and acetylstachyflin, novel anti-influenza A virus substances, produced by Stachybotrys sp. RF-7260. I. Isolation, structure elucidation and biological activities. *J Antibiot (Tokyo)* **55**(2)**:** 155-164.

Mincer, T.J., Jensen, P.R., Kauffman, C.A. and Fenical, W. (2002). Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Appl Environ Microbiol* **68**(10)**:** 5005-5011.

Moeller, H. (1891). Ueber eine neue Methode der Sporenfarbung. *Zbl. Bakt., I Abt. Orig.* **10, 273**.

Moita, C., Savluchinske, S.F., Nunes, L., Curto, M.M. and Roseiro, J.C. (2005). Optimisation of physical factors on the production of active metabolites by Bacillus subtilis 355 against wood surface contaminant fungi. *Int Biodeter Biodegrad* **55**: 261–269

Muaaz, M.A., Sheikh, M.A., Ahmad, Z. and Hasnain, S. (2007). Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium. *Microbial Cell Fact.* **10**: 6-17.

Myers, P.L. (1997). Will combinatorial chemistry deliver real medicines? *Curr Opin Biotechnol* **8**(6)**:** 701-707.

Nagai, K., Kamigiri, K., Matsumoto, H., Kawano, Y., Yamaoka, M., Shimoi, H., Watanabe, M. and Suzuki, K. (2002). YM-202204, a new antifungal antibiotic produced by marine fungus Phoma sp. *J Antibiot (Tokyo)* **55**(12)**:** 1036-1041.

Needham, J., Kelly, M.T., Ishige, M. and Andersen, R.J. (1994). Andrimid and moiramides A-C, metabolites produced in culture by a marine isolate of the bacterium Pseudomonas Xuorescens structure elucidation and biosynthesis. *J Org Chem* **59:** 2058–2063.

Newman, D.J., Cragg, G.M. and Snader, K.M. (2000). The influence of natural products upon drug discovery. *Nat Prod Rep* **17**(3)**:** 215-234.

Nguyen, H.P., Zhang, D., Lee, U., Kang, J.S., Choi, H.D. and Son, B.W. (2007). Dehydroxychlorofusarielin B, an antibacterial polyoxygenated decalin derivative from the marine-derived fungus Aspergillus sp. *J Nat Prod* **70**(7)**:** 1188-1190.

O'Brien, A., Sharp, R., Russell, N.J. and Roller, S. (2004). Antarctic bacteria inhibit growth of food-borne microorganisms at low temperatures. *FEMS Microbiol Ecol* **48**(2)**:** 157-167.

Pandey B, Ghimire, P., and Agrawal, V.P. (2004). Studies on the antibacterial activity of actinomycetes isolated from the Khumbu region of Mt. Everest. A paper presented in the International Conference on the Great Himalayas: Climate, Health, Ecology, Management and Conservation, Kathmandu. Organized by Kathmandu University and the Aquatic Ecosystem Health and Management Society, Canada.

Pankey, G.A. and Sabath, L.D. (2004). Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clin Infect Dis*. **38**(6): 864-70.

Prachyawarakorn, V., Mahidol, C., Sureram, S., Sangpetsiripan, S., Wiyakrutta, S., Ruchirawat, S. and Kittakoop, P. (2008). Diketopiperazines and phthalides from a marine derived fungus of the order pleosporales. *Planta Med* **74**(1)**:** 69-72.

Prasad, G.S. and Dhanapal, R. (2010). Antibacterial and antifungal activity of methanolic extract of Argemone mexicana leaves. *Int. J. Phytopharmacol* **1**(2)**:** 64-67.

Preetha, R.S.J., Prathapan, S., Vijayan, K.K., Jayaprakash, S.N., Philip, R. and Singh, B.S. (2010). An inhibitory compound produced by Pseudomonas with effectiveness on Vibrio harveyi. *Aquaculture Res* **41:** 1452-1461.

Price-Whelan, A., Dietrich, L.E.P. and Newman, D.K. (2006). Rethinking secondary metabolism: Physiological roles for phenazine antibiotics. *Nat. Chem. Biol.* **2**: 71-78

Proksch, P., Ebel, R., Edrada, R.A., Riebe, F., Liu, H., Diesel, A., Bayer, M., Li, X., Lin, W.H., Grebenyuk, V., Müller, W.E.G., Draeger, S., Zuccaro, A. and Schulz, B. (2008). Sponge-associated fungi and their bioactive compounds: the Suberites case. . *Bot Mar* **51:** 209–218.

Qi, S.H., Xu, Y., Xiong, H.R., Qian, P.Y., and Zhang, S. (2009). Antifouling and antibacterial compounds from a marine fungus Cladosporium sp. F14. *World J Microbiol Biotechnol* **25:** 399–406.

Radajewski, S., Webster, G., Reay, D.S., Morris, S.A., Ineson, P., Nedwell, D.B., Prosser, J.I. and Murrell, J.C (2002). Identification of active methylotroph populations in an acidic forest soil by stable isotope probing. *Microbiol.* **148:** 2331–2342.

Radjasa, O.K., Salasia, S.I.O., Sabdono, A., Weisei, J., Imho,V.J.F., Lämmler, C. and Risk, M. J. (2007). Antibacterial activity of marine bacterium Pseudomonas sp. associated with soft coral Sinularia polydactyla against Streptococcus equi subsp. Zooepidermicus. *Int J Pharmacol* **3:** 170–174.

Rath, J.P., Kinast, S. and Maier, M.E. (2005). Synthesis of the full functionalized core structure of the antibiotic abyssomicin C. *Org Lett* **7:** 3089-3092.

Rawat, D.S., Joshi, M.C., Joshi, P. and Atheaya, H. (2006). Marine peptides and related compounds in clinical trial. *AntiCancer Agents Med. Chem*. **6**: 33-40.

Riebeling, V., Thauer, R.K. and Jungermann, K. (1975). Internal-alkaline pH gradient, sensitive to uncoupler and atpase inhibitor, in growing *Clostridium pasteurianum*. *Eur. J.biochem*. **55**: 445-453

Roitman, J., Mahoney, N. and Janisiewicz, W. (1990). Production and composition of phenylpyrrole metabolites produced by Pseudomonas cepacia. . *Appl Microbiol and Biotechnol.* **34:** 381-386.

Rowley, D.C., Kelly, S., Kauffman, C.A., Jensen, P.R. and Fenical, W. (2003). Halovirs A-E, new antiviral agents from a marine-derived fungus of the genus Scytalidium. *Bioorg Med Chem* **11**(19)**:** 4263-4274.

Russell, N.E. and Pachorek, R.E. (2000). Clindamycin in the treatment of streptococcal and staphylococcal toxic shock syndromes. *Ann Pharmacother*. **34**(7-8): 936-39.

Seo, C., Sohn, J.H., Oh, H., Kim, B.Y. and Ahn, J.S (2009). Isolation of the protein tyrosine phosphatase 1B inhibitory metabolite from the marine-derived fungus Cosmospora sp. SF-5060. *Bioorg Med Chem Lett* **19**(21)**:** 6095-6097.

Scherrer, R. and Gerhardt, P. (1971). Molecular sieving by the Bacillus megatherium cell wall and protoplast. *J Bacteriol* **107**: 718–735.

Shapiro, S. (1989). Nitrogen Assimilation in Actinomycetes and the Influence of Nitrogen Nutrition on Actinomycetes Secondary Metabolism. In: Regulation of Secondary Metabolism in Actinomycetes, Shapiro, S. (Ed.). CRC Press, Boca Raton, Florida, pp: 135-211

Shibazaki, M., Taniguchi, M., Yokoi, T., Nagai, K., Watanabe, M., Suzuki, K. and Yamamoto, T. (2004). YM-215343, a novel antifungal compound from Phoma sp. QN04621. *J Antibiot (Tokyo)* **57**(6)**:** 379-382.

Solanki, R., Khanna, M. and Lal, R. (2008). Bioactive compounds from marine actinomycetes. *J Ind Microbiol* **48:** 410–431.

Sole, M., Francia, A., Rius, N. and Loren, J.G. (1997). The role of pH in the glucose effet on prodigiosin production by non-proliferating cells of *Serratia marcescens*. *Lett. Applied Microbiol*. **25**:81-84.

Sun, X., Zhou, X., Cai, M., Tao, K. and Zhang, Y. (2009). Identified biosynthetic pathway of aspergiolide A and a novel strategy to increase its production in a marine-derived fungus Aspergillus glaucus by feeding of biosynthetic precursors and inhibitors simultaneously. *Bioresour Technol* **100**(18)**:** 4244-4251.

Tabbene, O., Slimene, I.B., Bouabdallah, F., Mangoni, M., Urdaci, M. and Limam, F. (2009). Production of anti-Methicillin-Resistant Staphylococcus activity from *Bacillus subtilis* sp. strain B38 newly isolated from soil. *Appl Biochem Biotechnol*. **157**:407–419.

Tan, G.T., Pezzuto, J.M., Kinghorn, A.D. and Hughes, S.H (1991). Evaluation of natural products as inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. *J Nat Prod* **54**(1)**:** 143-154.

Thakur, A.N., Thakur, N.L., Indap, M.M., Pandit, R.A., Datar, V.V. and Muller, W.E. (2005). Antiangiogenic, antimicrobial, and cytotoxic potential of sponge-associated bacteria. *Mar Biotechnol (NY)* **7**(3)**:** 245-252.

Trisuwan, K., Rukachaisirikul, V., Sukpondma, Y., Phongpaichit, S., Preedanon, S. and Sakayaroj, J. (2009). Lactone derivatives from the marine-derived fungus Penicillium sp. PSU-F44. *Chem Pharm Bull (Tokyo)* **57**(10)**:** 1100-1102.

Trisuwan, K., Rukachaisirikul, V., Sukpondma, Y., Preedanon, S., Phongpaichit, S., Rungjindamai, N. and Sakayaroj, J. (2008). Epoxydons and a pyrone from the marine-derived fungus Nigrospora sp. PSU-F5. *J Nat Prod* **71**(8)**:** 1323-1326.

Tsuda, M., Mugishima, T., Komatsu, K., Sone, T., Tanaka, M., Mikami, Y., Shiro, M., Hirai, M., Ohizumi, Y. and Kobayashi, J. (2003a). Speradine A, a new pentacyclic oxindole alkaloid from a marine derived fungus Aspergillus tamarii. *Tetrahedron* **59:** 3227–3230

Tziveleka, LA., Vagias, C. and Roussis, V. (2003). Natural products with anti-HIV activity from marine organisms. *Curr Top Med Chem* **3**(13)**:** 1512-1535.

Uzair, B., Ahmed, N., Ahmad, V.U., Mohammad, F.V. and Edwards, D.H. (2008). The isolation, purification and biological activity of a novel antibacterial compound produced by Pseudomonas stutzeri. *FEMS Microbiol Lett* **279**(2)**:** 243-250.

Uzair, B., Ahmed, N., Kousar, F. and Edwards, D. H. (2006). Isolation and Characterization of Pseudomonas Strain That Inhibit Growth of Indigenous and Clinical Isolate. *The Internet Journal of Microbiol.* Volume 2

Van Lagevelde, P., Van Dissel, J.T., Meurs, C.J.C., Renz, J. and Groeneveld, P.H.P. (1997). Combination of flucloxacillin and gentamicin inhibits toxic shock syndrome toxin 1 production by *Staphylococcus aureus* in both logarithmic and stationary phases of growth. *Antimicrob Agents Chemother*. **41**: 1682-85.

Wang, J., Peng, Q. and Li, G. (2009). New compounds of natural resources in 2008. *Afri. J. Biotechnol* **8**(18)**:** 4299-4307.

Wratten, S.J., Wolfe, M.S., Andersen, R.J. and Faulkner, D.J (1977). Antibiotic metabolites from a marine pseudomonad. *Antimicrob Agents Chemother* **11**(3)**:** 411-414.

Wu, Z.J., Ouyang, M.A. and Tan, Q.W. (2009). New asperxanthone and asperbiphenyl from the marine fungus Aspergillus sp. *Pest Manag Sci* **65**(1)**:** 60-65.

Xu, J., Kjer, J., Sendker, J., Wray, V., Guan, H., Edrada, R., Lin, W., Wu, J. and Proksch, P. (2009). Chromones from the endophytic fungus Pestalotiopsis sp. isolated from the chinese mangrove plant Rhizophora mucronata. *J Nat Prod* **72**(4)**:** 662-665.

Zetola, N., Francis, J.S., Nuermberger, E.L. and Bishai, W.R. (2005).Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging threat. *Lancet Infect Dis.* **5**: 275-86.

Zhang, D., Yang, X., Kang, J.S., Choi, H.D., and Son, B.W. (2008). Chlorohydroaspyrones A and B, antibacterial aspyrone derivatives from the marine-derived fungus Exophiala sp. *J Nat Prod* **71**(8)**:** 1458-1460.

Zhang, Y., Zhu, T., Fang, Y., Liu, H., Gu, Q. and Zhu, W. (2007). Carbonarones A and B, new bioactive gamma-Pyrone and alpha-Pyridone derivatives from the marine-derived fungus Aspergillus carbonarius. *J Antibiot (Tokyo)* **60**(2)**:** 153-157.

Zheng, L., Chen, H., Han, X., Lin, W. and Yan, X. (2005). Antimicrobial screening and active compound isolation from marine bacterium NJ6-3-1 associated with the sponge Hymeniacidon perleve. *World J. Microbiol. Biotechnol.* **21:** 201–206

APPENDICES

APPENDIX I - PREPARATION OF CULTURE MEDIA

A. CULTURE MEDIA

1. NUTRIENT AGAR (MERCK)

Formula	Quantity (g/L)
Peptone from meat	3.45
Peptone from casein	3.45
Sodium chloride	5.10
Agar- agar	13.00

Nutrient agar powder (22.5g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20ml, plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

2. NUTRIENT BROTH (OXOID)

Formula	Quantity (g/L)
Lab lemco Powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar no 3	15.5

Nutrient broth powder (25g) was weighed into a conical flask and dissolved in distilled water to 1L. The mixture was distributed into glass tubes in portions of 10 ml, plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

3. MUELLER-HINTON AGAR (OXOID)

Formula	Quantity (g/L)
Beef infusion	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

Mueller-Hinton agar powder (38g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20 ml, plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave

4. MACKONKEY AGAR NO 2 (OXOID)

Formula	Quantity (g/L)
Peptone	20.0
Lactose	10.0
Bile salts No 2	1.50
Sodium chloride	5.00
Neutral red	0.05
Crystal violet	0.001
Agar	5.00

MacConkey agar powder (51.5g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20ml, plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

5. MANNITOL SALT AGAR (OXOID)

Formula	Quantity (g/L)
Lab –lemco Powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol Red	0.025
Agar	15.0
Water to	1000ml

Mannitol salt agar powder (111g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20 ml, plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

6. CETRIMIDE AGAR (OXOID)

Formula	Quantity (g/L)
Gelatin peptone	20.0
Magnesium chloride	1.4
Potassium sulphate	10.0
Cetrimide	0.3
Agar	13.6

Cetrimide agar powder (45.3g) was weighed into a conical flask and dissolved in about 900ml distilled water. Ten (10) ml of glycerol was added and made up to 1L. The mixture was heated in a boiling water bath to dissolve, distributed into glass tubes in portions of 20ml,

plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

7. SABOURAUD 4% DEXTROSE AGAR (MERK)

Formula	Quantity (g/L)
Peptone	10.0
D (+) Glucose	40.0
Agar –Agar	15.0

Sabouraud dextrose agar powder (65g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20 ml, plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

8. BISMUTH SULPHITE AGAR (CM 0201)

Formula	Quantity (g/L)
Peptone	5.0
Lab-Lemco Powder	5.0
Glucose	5.0
Di-sodium phosphate	4.0
Ferrous sulphate	0.3
Bismuth Sulphite indicator	0.8
Brilliant green	0.016

40g of the powder was suspended in 1 litre distilled water and heated till the agar dissolved. It was cooled to 50°C and poured thickly into sterile petri dishes and allowed to solidify.

9. FERMENTATION MEDIA

Formula	Quantity (g/L)
Peptone	20.0
Sodium chloride	5.0
Sugar	5

Peptone water powder (15g) and 5 g of the sugar were weighed into a conical flask and dissolved in distilled water to 1L. The mixture was distributed into glass tubes in portions of 10 ml, plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

10. TRYPTONE WATER

Formula	Quantity (g/L)
Tryptone	10.0
Sodium chloride	5.0

Tryptone water powder (15g) was weighed into a conical flask and dissolved in distilled water to 1L. The mixture was distributed into glass tubes in portions of 10 ml, plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

11. PEPTONE WATER (OXOID)

Formula	Quantity (g/L)
Peptone	10.0
Sodium chloride	5.0

Peptone water powder (15g) was weighed into a conical flask and dissolved in distilled water to 1L. The mixture was distributed into glass tubes in portions of 10 ml, plugged firmly with

cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

12. KOSER CITRATE MEDIUM (OXOID)

Formula	Quantity (g/L)
Sodium Ammonium Phosphate	1.5
Potassium Dihydrogen Phosphate	1.0
Magnesium Sulphate	0.2
Sodium Citrate	2.5
Bromo-thymol Blue	0.016

Koser citrate powder (5.2g) was weighed into a conical flask and dissolved in distilled water to 1L. The mixture was distributed into clean glass tubes in portions of 10 ml, covered with test tube cups and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

13. NITRATE BROTH

Formula	Quantity (g/L)
Beef Extract	3.0
Peptone	4.0
Proteose Peptone No. 3	1.0
Potassium Nitrate	1.0

Nitrate broth powder (9g) was weighed into a conical flask and dissolved in distilled water to 1L. The mixture was distributed into glass tubes containing inverted Durham's tubes in portions of 10 ml, plugged firmly with cotton wool and sterilized at 115°C for 30 minutes in an autoclave (Arnold and sons Ltd, Basildon).

APPENDIX II – PREPARATION OF REAGENTS

1. KOVAC'S REAGENT

Formula	Quantity
p-dimethylaminobenzaldehyde	5g
Amyl alcohol	75ml
Conc. HCl	25ml

The aldehyde was dissolved in the alcohol by gently warming in a water bath (about 50-55°C). It was then cooled and the acid added to it.

2. LUGOL'S IODINE

Formula	Quantity
Iodine	5g
Potassium Iodide	10g
Distilled water	100ml

The potassium iodide and iodine were dissolved in 10 ml of the water. It was mixed well and enough water added to make 100ml.

3. STRONG CARBOL-FUCHSIN

Formula	Quantity
Solution A	
Basic fuchsin	10g
Ethanol (95%)	100ml
Solution B	
Phenol	5g
Distilled water	100ml

Basic fuchsin was dissolved in the alcohol to prepare solution A and phenol was dissolved in distilled water to prepare solution B. For use, 10 ml of solution A was poured into 100 ml of solution B

4. AMMONIUM OXALATE CRYSTAL VIOLET

Formula	Quantity
Solution A	
Crystal violet	10g
Ethanol (95%)	100ml
Solution B	
Ammonium oxalate	1g
Distilled water	100ml

Crystal violet was dissolved in the alcohol to prepare solution A and ammonium oxalate was dissolved in distilled water to prepare solution B. For use, 20 ml of solution was mixed with 80 ml of solution B

5. SAFRANIN

Ten millilitres (10ml) of safranin (2.5% solution in 95% ethyl alcohol) is added to 100 ml of distilled water.

6. NITRATE TEST REAGENTS

Solution A: 0.8g of sulphanilic acid was dissolved by gentle heating in 5 N-acetic acid.

Solution B was prepared by dissolving 0.6 g of dimethyl-α-naphthylamine in 5 N-acetic acid.

APPENDIX III – TABLES OF GRAPHS

Table III A Effect of different heat treatments on antimicrobial substance

Isolates	Mean zones of Inhibition ±SEM (mm)							
	40°C	50°C	60°C	70°C	80°C	90°C	100°C	121°C
MAI 1	17±1.4	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
MAI 2	20±1.4	20±0.2	17±0.0	17±1.4	15±1.1	15±0.2	13±1.4	0±0.0
MAI3	19±0.1	19±0.1	19±1.0	18±0.1	18±1.4	15±0.2	14±1.4	0±0.0

Table III B Stability of antimicrobial agents in solution

Weeks	Mean zones of Inhibition ±SEM (mm)		
	MAI 1	MAI 2	MAI 3
1	20±1.4	25±0.1	30±1.1
2	20±0.5	25±0.0	30±1.1
3	20±0.1	25±1.0	30±0.1
4	20±0.7	25±0.5	30±0.7
5	20±1.0	25±0.2	30±0.2
6	20±0.7	22±1.4	28±0.5
7	19±0.5	20±0.5	28±0.0
8	19±0.0	20±0.7	28±1.0
9	19±1.0	18±0.5	28±0.0
10	19±1.0	18±1.0	28±0.7
11	18±0.0	18±0.0	28±1.0
12	18±0.5	18±0.1	26±0.1

Table III C Effect of Incubation temperature

Isolates	Mean zones of Inhibition ±SEM (mm)				
	Temperature				
	25°C	30°C	32°C	34°C	37°C
MAI 1	30±0.2	25±1.1	20±1.4	16±1.4	27±0.2
MAI 2	17±0.1	29±1.4	24±0.1	26±1.1	20±0.2
MAI 3	16±1.4	30±0.0	24±1.4	29±0.0	18±1.4

Table III D Effect of time on the production of antibacterial metabolite,

Days	Mean zones of Inhibition ±SEM (mm)		
	MAI 1	*MAI 2*	*MAI 3*
0	0±0.0	0±0.0	0±0.0
1	14±0.2	17.5±1.1	17±0.2
2	14±0.2	21±0.3	20±1.4
3	17±1.2	22.5±0.7	22±1.0
4	19±0.1	23±0.2	24±0.0
5	20±1.0	25±0.2	26±0.1
6	23±0.3	26±0.1	27±0.5
7	23±0.1	28±0.7	27±0.1
8	25±1.4	29±1.4	28.5±0.2
9	27±1.4	30±0.5	30±1.4
10	27±0.1	30±0.5	31±0.0
11	27±0.1	30±0.7	31±0.2
12	27±0.5	30±0.1	31±0.2

Table III E Effect of pH on the antimicrobial activity of the metabolites

Isolates	Mean zones of Inhibition ±SEM (mm)					
	pH					
	4	5	6	7	8	9
MAI 1	0±0.0	15±0.1	15±1.1	25±0.1	27±0.3	25±1.4
MAI 2	0±0.0	19±0.4	20±0.2	21±1.4	19±0.7	19±0.1
MAI 3	0±0.0	20±0.2	20±0.2	22±1.4	18±1.4	16±0.7

Table III F Effect of carbon sources on the antimicrobial metabolite production by MAI 1, MAI 2 and MAI 3

Carbon Source	Mean zone of inhibition ±SEM (mm)					
	MAI1		MAI2		MAI3	
	B. subtilis	E. coli	B. subtilis	E. coli	B. subtilis	E. coli
Nutrient Broth	31±0.7	21±0.5	22±0.3	25±0.7	22±0.5	28±1.4
Glucose	0±0.0	0±0.0	14±1.4	20±0.1	16±0.1	26±0.5
Galactose	20±0.1	12±1	14±0.3	18±1.0	14±0.5	20±0.2
Xylose	14±0.0	0±0.0	14±0.5	16±0.0	14±0.1	18±1.0
Sucrose	16±0.7	0±0.0	16±0.2	25±1.4	16±0.7	25±1.4
Mannitol	0±0.0	0±0.0	20±0.5	25±0.1	16±0.1	25±0.2
Lactose	14±0.2	14±1.4	18±1.4	20±0.3	20±0.5	24±0.1
Starch	25±1.0	14±0.3	26±0.1	26±0.2	26±1.4	31±0.5
Fructose	14±0.7	0±0.0	14±0.7	16±1.4	16±1.4	24±0.4
Maltose	0±0.0	0±0.0	14±0.2	25±0.4	16±0.1	24±0.7
Glycerol	34±0.5	21±0.1	26±0.5	26±0.1	22±0.3	24±0.2

Table III G Effect of nitrogen sources on the antimicrobial metabolite production by MAI 1

Nitrogen source	Mean zones of inhibition ±SEM (mm)	
	B. subtilis	E. coli
Nutrient Broth	35±1.4	25±0.7
Sodium nitrate	26±0.4	20±0.2
Potassium nitrate	29±1.4	22±1.4
Ammonium chloride	34±0.2	25±0.2
Ammonium nitrate	29±1.4	22±0.2
Asparagine	37±0.1	26±0.5
Ammonium phosphate	30±1.4	22±1.4
Ammonium sulphate	32±0.2	23±1.4

Table III H Effect of nitrogen sources on the antimicrobial metabolite production by MAI 2

Nitrogen source	Mean zones of inhibition ±SEM (mm)	
	B. subtilis	E. coli
Nutrient broth	21±0.7	23±1.4
Sodium nitrate	21±0.4	21±0.2
Potassium nitrate	21±1.0	22±0.1
Ammonium chloride	22±1.4	24±0.7
Ammonium nitrate	21±0.5	22±0.5
Asparagine	24±0.2	25±0.3
Ammonium phosphate	20±0.7	20±0.7
Ammonium sulphate	22±0.0	22±0.4

Table III I Effect of nitrogen sources on the antimicrobial metabolite production by MAI 3

Nitrogen source	Mean zones of inhibition ±SEM (mm)	
	B. subtilis	E. coli
Nutrient Broth	24±1.4	21±0.1
Sodium nitrate	19±0.7	19±0.3
Potassium nitrate	20±1.7	21±0.7
Ammonium chloride	22±0.4	24±0.3
Ammonium nitrate	20±1.4	19±0.7
Asparagine	24±0.2	27±0.4
Ammonium phosphate	20±0.7	21±0.5
Ammonium sulphate	20±0.4	21±0.7